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# Genetic regulation of entry into meiosis in *Caenorhabditis elegans*

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## SUMMARY

**The *Caenorhabditis elegans* germline is composed of mitotically dividing cells at the distal end that give rise to meiotic cells more proximally. Specification of the distal region as mitotic relies on induction by the somatic distal tip cell and the *glp-1* signal transduction pathway. However, the genetic control over the transition from mitosis to meiosis is not understood. In this paper, we report the identification of a gene, *gld-2*, that has at least two functions in germline development. First, *gld-2* is required for normal progression through meiotic prophase. Second, *gld-2* promotes entry into meiosis from the mitotic cell cycle. With respect to this second function, *gld-2* appears to be functionally redundant with a previously described gene, *gld-1* (Francis, R., Barton, M. K., Kimble, J. and Schedl, T. (1995) *Genetics* 139, 579-606). Germline cells in *gld-1(ø)* and *gld-2* single mutants enter meiosis at the normal time, but germ cells in *gld-2 gld-1(ø)* double mutants do not enter meiosis. Instead, the double mutant germline is mitotic throughout and forms a large tumor. We suggest that *gld-1* and *gld-2* define two independent regulatory pathways, each of which can be sufficient for entry into meiosis. Epistasis analyses show that *gld-1* and *gld-2* work downstream of the *glp-1* signal transduction pathway. Therefore, we hypothesize that *glp-1* promotes proliferation by inhibiting the meiosis-promoting functions of *gld-1* and *gld-2*.**

Key words: *Caenorhabditis elegans*, Germline, Mitosis, Meiosis, Cell cycle, *gld-1*, *gld-2*

## INTRODUCTION

The generation of animal tissues relies on the coordinated regulation of proliferation and differentation. Defects in that regulation can lead to excess proliferation and tumor formation on the one hand or to premature differentiation and small, nonfunctional tissues on the other. Despite tremendous progress in understanding controls governing mitosis and differentation, much less is known about how these two processes are balanced during development to achieve tissues with a typical size, shape and pattern of cell types.

The *C. elegans* germline provides an excellent model for analysis of the regulation of proliferation and differentiation. In the wild-type adult germline, mitotic cells in the distal-most region give rise to meiotic cells more proximally (Fig. 1). Mitotic divisions in the *C. elegans* germline are induced by a signal from the distal tip cell (DTC), a somatic cell that caps the distal end of the gonadal arm (Kimble and White, 1981). This induction is mediated by a highly conserved signal transduction pathway, the LIN-12/Notch pathway (for review, see Kimble and Simpson, 1997). For induction of germline mitoses, the DTC signal is LAG-2 (Henderson et al., 1994; Tax et al., 1994), the germline receptor is GLP-1 (Austin and Kimble, 1987; Crittenden et al., 1994; Yochem and Greenwald, 1989) and the downstream transcription factor is LAG-1 (Christensen et al., 1996). In the absence of induction, germ cells leave the mitotic cell cycle, enter meiosis and complete gametogenesis (Austin and Kimble, 1987; Lambie and Kimble, 1991). In contrast, excess or unregulated induction leads to ectopic germline mitoses (Berry et al., 1997; Fitzgerald and Greenwald, 1995; Henderson et al., 1997). Therefore, regulation of the inductive pathway is essential for achieving the balance of proliferation and differentiation observed in the adult germline.

What genes promote the transition of germ cells from mitosis to meiosis? Just as loss-of-function mutations in *glp-1* result in meiosis throughout the germline, it is expected that loss-of-function mutations in genes required to promote meiosis might result in proliferation throughout the germline. However, large-scale mutageneses have revealed no such mutations. Instead, genes that affect other aspects of germline development have been found. For example, several genes have been found that may be required for the germline cell cycle (*glp-2*: E. Lambie, L. Mathies, A. Petcherski, and J. Kimble, unpublished; *glp-3*: Kadyk et al., 1997; *glp-4*: Beanan and Strome, 1992). In addition, genes that govern progression through meiotic prophase have been identified. The *gld-1* gene is required for progression through pachytene during oogenesis: oogenic nuclei in *gld-1(ø)* mutants exit pachytene and return to the mitotic cell cycle, resulting in ectopic germline proliferation (Francis et al., 1995a). Furthermore, three genes encoding members of a MAP kinase signal transduction pathway are required for exit from pachytene during spermatogenesis and oogenesis (Church et al., 1995).

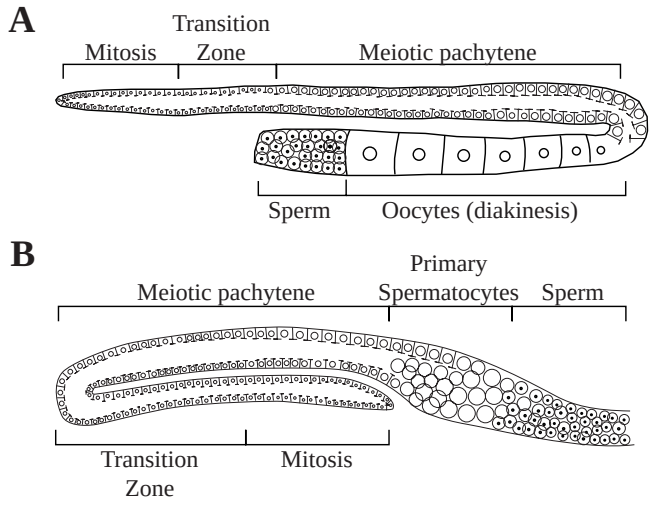


**Fig. 1.** Anatomy of wild-type hermaphrodite and male germlines. Anterior is to the left and dorsal is up. The germline is syncytial: each nucleus is only partially enclosed by cell membranes. For simplicity, we define each partially enclosed nucleus as a germ 'cell'. (A) Adult hermaphrodite germline. The distal-most germ cells are in the mitotic cell cycle. Germ cells leave the mitotic cell cycle and enter the meiotic cell cycle in the transition zone. More proximally, they enter the pachytene stage of meiotic prophase. As oogenesis begins, they exit pachytene and progress to the diakinesis stage of meiotic prophase, concomitant with a further enlargement of cell volume. Mature haploid sperm, which were formed in L4, are stored proximal to the germline, in the spermatheca. (B) Adult male germline. Progression from the mitotic cell cycle into the meiotic cell cycle occurs from distal to proximal, much as in hermaphrodites. Primary spermatocytes have not yet undergone meiosis I, and have a distinctive shape. Mature haploid sperm occupy the most proximal region. More detail on germline anatomy can be found in Kimble and Ward (1988).

In this paper, we identify a gene, *gld-2*, that affects two processes in germline development. First, *gld-2* is required for progression through meiotic prophase during both oogenesis and spermatogenesis. Second, *gld-2* is functionally redundant with *gld-1* for promotion of entry into meiosis. Furthermore, we show that the *glp-1* pathway promotes mitosis in the germline by negatively regulating the expression or activities of *gld-2* and *gld-1*.

## MATERIALS AND METHODS

### Nematode strains and maintenance

Worm strains used are derivatives of the wild-type Bristol strain N2 and were maintained as described (Brenner, 1974). Growth was at 20°C unless otherwise indicated.

We used the following mutations, described in Hodgkin et al. (1988) or cited references:

LGI: *fog-1(q253 ts)* (Barton and Kimble, 1990), *ces-1(n703 gf)* (Ellis and Horvitz, 1991), *dpy-5(e61) bli-4(e937), gld-2(q497, h292, q535, q540, oz188, dx32,* and *dx40*) (this paper), *dpy-14(e188 ts), unc-87(e843 and e1216 ts), unc-13(e51), unc-15(e1402 ts), gld-1(q485),* Enh*(q525)*; LGII: *tra-2(e1095)*; LGIII: *glp-1(q46)*; LGIV: *unc-44(e326), lag-1(q426)*; LGV: *egl-1(n2164 d)*; LGX: *xol-1(y9)*. Rearrangements: *hDf8* (McKim et al., 1992), *qDf16* (this paper), *hT2 (I:III)* (Edgley et al., 1995), *mnC1(II)* (Edgley et al., 1995).

*gld-2(q497)* is a putative null allele (see Results); it is therefore the canonical *gld-2* allele and is designated *gld-2(lf)*.

### Genetic mapping

Each *gld-2* mutation maps to chromosome I between *dpy-5* and *unc-13*, and fails to complement the allele *q497*; hence these new mutations define a single gene. Detailed three-factor mapping with *q497* places *gld-2* about 0.05 map units to the right of *bli-4*, at position 0.89 (Fig. 2). Three factor mapping data has been submitted to ACeDB. All *gld-2* mutations were outcrossed to the N2 strain at least five times.

### Mutagenesis

Five *gld-2* alleles were isolated as recessive sterile mutations. Of these, *q497* was induced with 50 mM EMS according to Brenner (1974), *h292* was induced with 17 mM EMS by Jennifer McDowall and Ann Rose, and is also called *let-606* (McDowall and Rose, 1997), *oz188* with 50 mM EMS (R. Clifford and T. Schedl, personal communication); and *dx32* and *dx40* with 250 J/m$^2$ of 310 nm UV light (E. Lambie, personal communication).

### Isolation of deficiency *qDf16*

Three previously existing deficiencies, *sDf4*, *hDf8* and *nDf25*, all complemented *gld-2(q497)*. To generate a deficiency that removed the *gld-2* locus, young adult *ces-1(n703 gf)* males were treated with 10 krads of gamma-irradiation and mated to *fog-1(q253 ts) bli-4(e937) unc-87(e1216)* females raised at 25°C. Cross-progeny were screened for animals that were both Bli and Unc, as expected for animals carrying a deficiency that removes both *bli-4* and *unc-87*. Of 5725 gamma-irradiated chromosomes screened, one carried such a deficiency. This deficiency, *qDf16*, was backcrossed and failed to complement mutations in *bli-4*, *gld-2*, *unc-37*, *unc-87*, *dpy-14* and *let-86*. PCR deficiency mapping was used to map the left breakpoint of *qDf16* between exon 3 and exon 10 of *bli-4* (primers generously provided by Colin Thacker).

### Screening for *gld-2* alleles by non-complementation

We first determined that *gld-2(q497)/qDf16* animals were viable and sterile (data not shown), so null alleles of *gld-2* could be isolated by non-complementation. Strain RE219 [*ces-1(n703 gf) I; tra-2(e1095)/mnC1 II; egl-1(n2164 d) V; xol-1(y9) X*] (Ellis and Kimble, 1995) was mutagenized with 40 mM EMS. L4 hermaphrodites were picked and allowed to self-fertilize, giving rise to $F_1$ XX males that were *ces-1, tra-2; egl-1; xol-1*. These males were mated singly or in pairs to two L4 heterozygotes from a strain of genotype *gld-2(q497) unc-13(e51)/dpy-5(e61) unc-13(e51)*. From plates that segregated approximately 1/8 to 1/4 non-Unc sterile progeny, all non-Unc siblings were placed individually on plates to rescue the non-complementing mutation from siblings of genotype *gld-2 (new)/dpy-5 unc-13*. From 4258 chromosomes screened, two new *gld-2* alleles were isolated: *q535* and *q540*.

### Identification of double and triple mutants

*gld-2; unc-32 glp-1* homozygotes were identified as Unc self-progeny of *gld-2(q497)/hT2; unc-32 glp-1(q46)/hT2* animals. Similarly, *gld-2 gld-1(ø); unc-32* animals were identified as Unc self-progeny of *gld-2(q497) gld-1(q485)/hT2; unc-32/hT2* animals, and *gld-2 gld-1(ø); unc-32 glp-1(ø)* as Unc self-progeny of *gld-2(q497) gld-1(q485)/hT2; unc-32 glp-1(q46)/hT2*. *gld-2 gld-1(q485); unc-44 lag-1* animals were self-progeny of *gld-2(q497) gld-1(q485)/++; unc-44(e326) lag-1(q426)/++* animals. 25% of the Unc-44 progeny were expected to be *gld-2 gld-1* homozygotes. We found that 15/59 (25%) of Unc-44 progeny from 9 broods had tumorous germlines; the remaining 44 Unc-44 animals had Glp-1-like germlines.

### Scoring penetrance of the Pro phenotype

For each allele, L4 animals from strain *gld-2(x)/dpy-5 unc-13* were

picked onto a single plate. 3 days later, sterile animals were scored by DIC optics for the Pro phenotype of proximal proliferation. To examine hemizygous animals, males of genotype *gld-2(x)*/+ were crossed to *qDf16/dpy-5 unc-13* hermaphrodites, and sterile progeny scored as above.

### Brood analysis

The phenotypes of all progeny of a single animal of genotype *gld-2(x)/dpy-5(e61) unc-13(e51)* were scored (as sterile, wild type, DpyUnc or dead eggs). Alleles tested were: *q497, q535, q540, h292, oz188* and *dx40*. Occasional dead eggs were seen, but never constituted more than 1% of total progeny. The sterile phenotype was found to be recessive for all alleles tested. All brood sizes were normal except that of *oz188*, which was about half the normal size.

### Synchronization of animals for DAPI-staining

About 30 gravid hermaphrodites were transferred to a single plate for 1-2 hours, then removed. The plate was incubated at 20°C for the indicated length of time, then animals of the appropriate genotype were picked and stained. To stain, animals were washed once in M9, fixed 5 minutes in –20°C methanol, washed twice with M9, stained with 0.1 μg/ml DAPI in M9 for 20-30 minutes, washed twice again in M9 and mounted for fluorescence microscopy.

### Antibody staining

Antibody staining was essentially as described in Crittenden et al. (1994). Briefly, adult germlines were dissected and fixed with 1% paraformaldehyde in PBS for 10 minutes, followed by 0.5% BSA, 0.1% Triton-X-100 in TBS for 3 minutes, blocked for 30 minutes in 0.5% BSA in TBS and then stained. The anti-GLP-1 antibodies used were a mixture of three different polyclonal rat antibodies directed against the EGFL, LNG and ANK domains of GLP-1 (Crittenden et al., 1994). The anti-phosphohistone H3 antibodies were rabbit polyclonals purchased from Upstate Biotechnology (Cat. no. 06-570). The SP56 mouse monoclonal antibody was from the laboratory of Sam Ward (Ward et al., 1986).

## RESULTS

### *gld-2* mutants are defective in gametogenesis and can have ectopic mitosis

To identify new genes required for regulating the germline decision between mitosis and meiosis, we sought mutants with ectopic germline proliferation. Initially, one mutant strain was identified with a vast excess of mitotic germ cells and no visible gamete differentiation. We found, however, that this striking phenotype was the result of two mutations in closely linked, but separate genes: *gld-2(q497)* (for germ-line development defective) and Enh*(q525)*. Upon separating these two mutations, we found that *gld-2(q497)* single mutants have a defect in progression through meiotic prophase, accompanied infrequently by ectopic mitosis (see below). We were unable to detect a phenotype of Enh*(q525)* on its own (see Materials and Methods). Therefore, Enh*(q525)* will not be discussed further in this paper.

Seven *gld-2* alleles have now been isolated and characterized. Each maps to chromosome I between *dpy-5* and *unc-13* (Fig. 2), and fails to complement *gld-2(q497)*. We isolated two alleles (*q535* and *q540*) in a non-complementation screen that could potentially detect null alleles (see Materials and Methods). Four alleles were isolated in other labs (thanks to Jennifer McDowall and Ann Rose for *h292*, to Bob Clifford and Tim Schedl for *oz188*, and to Eric Lambie for *dx32* and *dx40*). The *h292* allele was originally identified as *let-606* (McDowall and Rose, 1997).

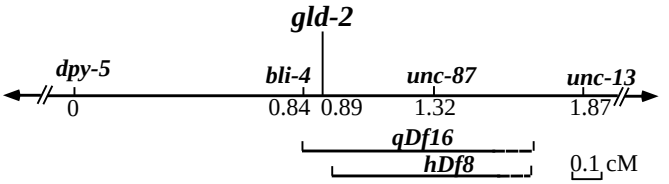


**Fig. 2.** Genetic map of the *gld-2* region. Map positions are based on three-factor mapping data and on the *C. elegans* genetic map (Hodgkin, 1997).

All seven *gld-2* mutants are sterile with two defects in germline development. The first defect, which is 100% penetrant, is failure to form normal gametes during both spermatogenesis and oogenesis. In wild-type adult hermaphrodites, mature sperm are found in the spermatheca and the proximal germline consists of maturing oocytes (Fig. 3A). In contrast, adult *gld-2* mutants possess neither mature sperm nor oocytes; instead, defective gametes are observed proximally (Fig. 3B). Most proximal are defective spermatocytes: their size is similar to that of a normal primary spermatocyte and their cytoplasm lacks the granular material characteristic of oocytes. Slightly more distal are defective oocytes: these cells have a granular cytoplasm, but are smaller in size than mature oocytes and abnormal in shape. To further examine the abnormal *gld-2* gametes, germlines were stained with the sperm-specific antibody SP56 (Fig. 4). Staining was observed only in the most proximal cells, corresponding to those that lack a granular cytoplasm. Therefore, spermatogenesis and oogenesis appear to be initiated in proper sequence, but neither is completed in *gld-2* mutants. *gld-2* mutations also affect germline development in males: defective spermatocytes occur proximally, where mature sperm are normally found (compare Fig. 3D,E). No oocyte-like cells are observed. We conclude that *gld-2* is required to complete both spermatogenesis and oogenesis.

The second germline defect of *gld-2* mutants is the presence of ectopic proliferation in the proximal germline, (a 'Pro' phenotype, for proximal proliferation). In *gld-2* Pro adults, distally proliferating cells move proximally, enter meiosis and give rise to defective gametes. However, proximal to these

**Table 1. Penetrance of Pro phenotype in *gld-2* mutants**

| Genotype | % Pro (*n*) |
|---|---|
| *gld-2(q497)* | 2 (62) |
| *gld-2(h292)* | 65 (57) |
| *gld-2(q535)* | 15 (61) |
| *gld-2(q540)* | 3 (39) |
| *gld-2(oz188)* | 1 (84) |
| *gld-2(dx32)* | 2 (49) |
| *gld-2(dx40)* | 2 (45) |
| *gld-2(q497)/qDf16** | 2 (53) |
| *gld-2(h292)/qDf16)** | 5 (38) |
| *gld-2(h292)/gld-2(q497)** | 3 (87) |

*n* = number of germ-line arms scored. Scoring was 3 days after L4, except as noted.
*Scored 1-2 days after L4.

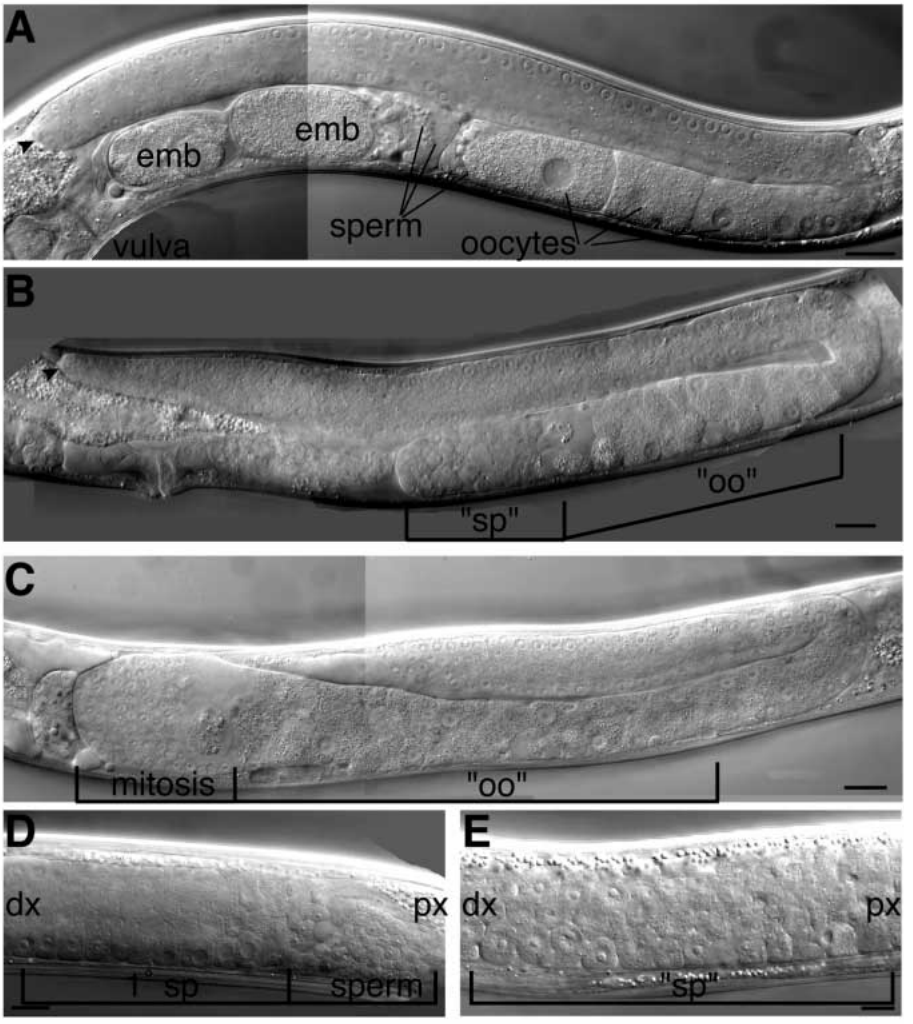


**Fig. 3.** Wild-type and *gld-2* germlines, as viewed with DIC microscopy. One gonadal arm is shown for each hermaphrodite; the proximal germline is shown for each male. (A) Wild-type adult hermaphrodite. Arrowhead, distal tip. A few sperm remain in the proximal germline, the rest are stored in the spermatheca. Developing embryos (emb) occupy the uterus. (B) *gld-2(q497)* adult hermaphrodite. Arrowhead, distal tip. Defective gametes occupy the proximal germline. Defective oogenic cells (oo) are enlarged, with granular cytoplasm; defective spermatogenic cells ("sp") are smaller, with non-granular cytoplasm. The spermatogenic cells are about the size of wild-type primary spermatocytes (not shown). (C) *gld-2(h292)* adult hermaphrodite. In addition to a region of defective oocytes ("oo"), the most proximal germline contains proliferating cells. No spermatogenic cells are seen by DIC optics or by staining with the sperm-specific antibody SP56 (not shown). (D) Wild-type adult male germline, proximal region. Distal (dx)-proximal (px) axis extends from left to right. Areas containing primary spermatocytes (1° sp) and mature sperm (sp) are bracketed. (E) *gld-2(q497)* adult male germline, proximal region. Note absence of mature sperm. Defective spermatogenic cells ("sp") are about the size of primary spermatocytes. Scale bars, 10 μm.

defective gametes, a second group of proliferating cells is observed (Fig. 3C). We make a distinction between this Pro phenotype and the previously defined 'tumorous' germline phenotype, which is typified by a vast excess of proliferating germ cells, but no overt sign of gametogenesis (Berry et al., 1997; Francis et al., 1995a). In all seven alleles of *gld-2*, the Pro phenotype is incompletely penetrant (Table 1). The *h292* allele has an unusually high penetrance of this phenotype and is probably not a simple loss-of-function allele (described below). We find that *h292* males also exhibit the Pro phenotype (males carrying other *gld-2* alleles have not been examined for the Pro phenotype).

Mutants homozygous for all alleles of *gld-2* appear to be normal in size, shape and movement, and to have normal somatic structures. Since the canonical allele *q497* is likely to be a strong loss-of-function and possibly a null allele (see below), it appears that *gld-2* does not have an essential function in the development of somatic tissues such as nerve, muscle, hypodermis, intestine or somatic gonad.

### *gld-2* is required for progression through meiotic prophase

To assess the state of meiotic cell cycle progression in *gld-2*

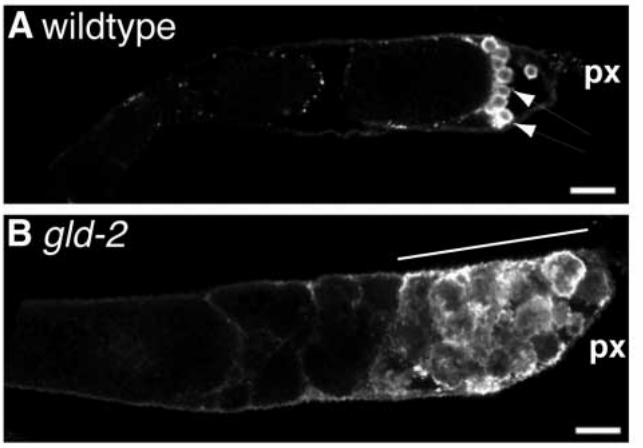


**Fig. 4.** Sperm differentiation in wild-type and *gld-2(q497)* germlines. Staining with spermatogenic-specific antibody SP56. Shown is the proximal germline with proximal (px) to right. (A) Wild-type sperm are compact and stain brightly (arrowheads). Note absence of staining from the oogenic region of germline. (B) In *gld-2(q497)* adult germline, defective spermatogenic cells stain with the SP56 antibody (line); no mature sperm are observed. More distal oogenic cells fail to stain. Scale bars, 10 μm.

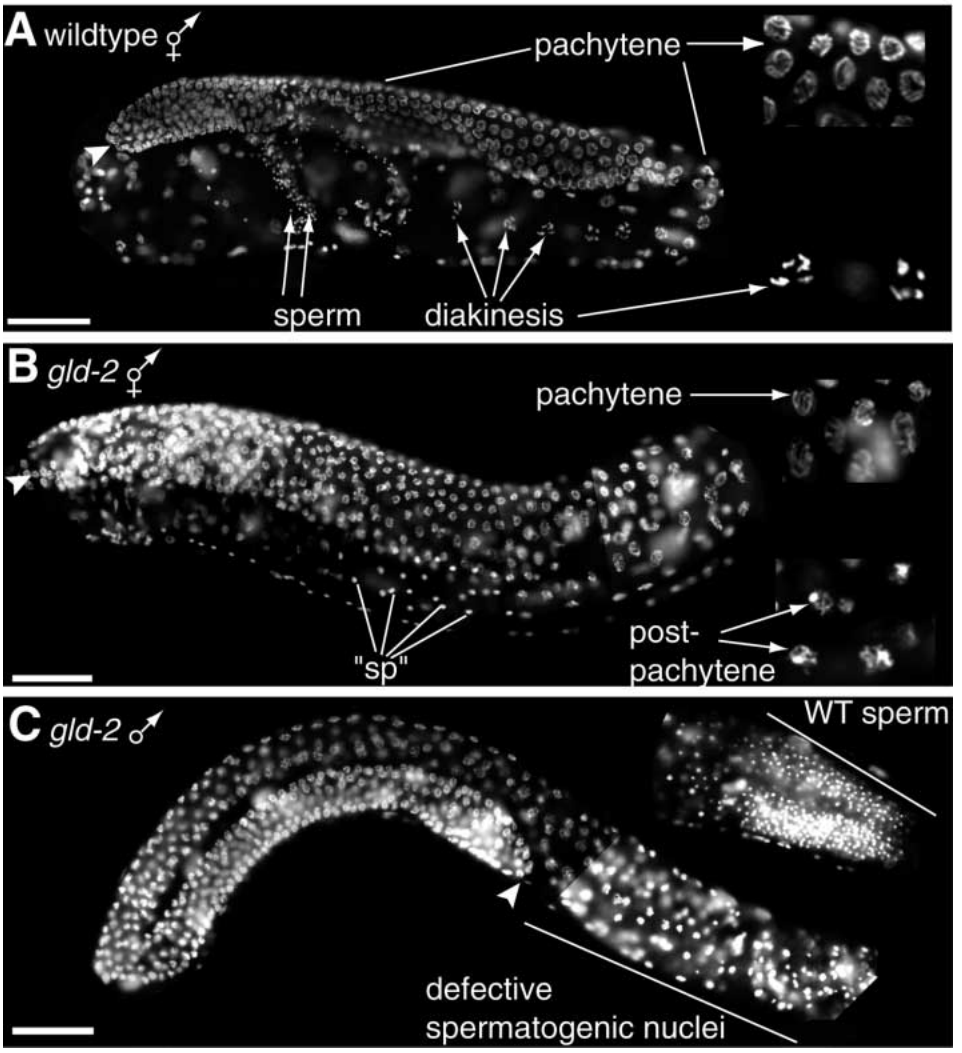


**Fig. 5.** Morphology of germline nuclei as observed by DAPI staining. Arrowhead, distal tip. (A) Wild-type adult hermaphrodite germline. Small, densely packed mitotic nuclei at the distal end give rise to slightly larger, more widely spaced pachytene nuclei more proximally. Pachytene chromosomes have a characteristic thread-like morphology (top inset, ×2.5). Proximal to the bend are oocytes arrested in diakinesis (bottom inset, ×2.5). Mature sperm, located in the spermatheca, have tiny, highly condensed nuclei. (B) *gld-2(q497)* adult hermaphrodite germline. Nuclei typical of mitotic region, transition zone, and pachytene region are found distal to the bend (top inset, ×2.5). However, no nuclei typical of diakinesis or mature sperm are observed proximal to the bend. Instead, these nuclei have often lost the distinct thread-like appearance of pachytene chromosomes (bottom inset, ×2.5). The partially condensed nuclei seen in the most proximal region (sp) correspond to cells that stain with the sperm-specific antibody SP56 (Fig. 4). (C) *gld-2(q497)* adult male germline. The proximal-most germline (extent indicated by a white line) contains defective spermatogenic nuclei with partially condensed chromosomes, similar to those seen in spermatogenic cells of the hermaphrodite. In contrast, the proximal wild-type male germline is filled with highly condensed nuclei typical of mature sperm (see inset, same magnification). Scale bars, 20 μm.

mutant germlines, adults were DAPI-stained and the morphologies of the germ nuclei compared to those of the wild type. Two stages of meiotic prophase are particularly prominent in the wild type: (1) nuclei in pachytene are present in an extended region distal to the bend and contain distinctive thread-like chromosomes and (2) nuclei in diakinesis are found in oocytes and contain dramatically condensed chromosomes (Fig. 5A). In addition, the nuclei of mature sperm appear as tiny DAPI-staining dots in the spermatheca (Fig. 5A).

In *gld-2* hermaphrodites, pachytene nuclei are observed as in the wild type (Fig. 5B top inset). However, no typical oocyte or sperm nuclei are found. Presumptive spermatogenic nuclei are smaller than pachytene nuclei, but larger than wild-type haploid sperm (Fig. 5B, 'sp'). Furthermore, in *gld-2* mutants, the number of defective spermatogenic nuclei is roughly the same as the number of primary spermatocytes in the wild type. Therefore, it seems likely that most of these spermatogenic nuclei have not yet undergone the first meiotic division and so are defective in meiotic prophase. Similarly, in *gld-2* males, the region that normally is spermatogenic contains only partially condensed nuclei that are larger than wild-type mature sperm nuclei (Fig. 5C). Nuclei in the oogenic region of hermaphrodites remain about the size of pachytene nuclei; they lose the thread-like morphology of pachytene chromosomes but do not acquire the condensed morphology typical of chromosomes in diakinesis (Fig. 5B, bottom inset). We conclude that meiotic prophase is defective during both spermatogenesis and oogenesis and that the defective step is likely to occur at or after pachytene and before metaphase I.

### The nature of the *gld-2* alleles

All *gld-2* mutations tested (*q497*, *q535*, *q540*, *h292*, *oz188* and *dx40)* are fully recessive and fully penetrant for sterility (see Materials and Methods). However, the seven alleles vary with respect to penetrance of the Pro phenotype (Table 1). Five alleles, *q497*, *q540*, *oz188*, *dx32* and *dx40*, have a low-penetrance Pro phenotype (1-3% Pro gonadal arms); *q535* has an intermediate penetrance (15% of gonad arms) and the *h292* allele is highly penetrant (60% of gonad arms). To assess the nature of these alleles, we placed either *gld-2(q497)* (representing the class with low Pro penetrance) or *gld-2(h292)* (the high Pro penetrance allele) in *trans* to *qDf16*, a deficiency that removes the *gld-2* locus (see Materials and Methods). For *gld-2(q497),* the hemizygote has essentially the same

phenotype as that of the homozygote: both exhibit defective gametogenesis with rare proximal proliferation. In contrast, for *gld-2(h292)*, the penetrance of the Pro phenotype was much lower in hemizygotes than in homozygotes (Table 1). Therefore, *gld-2(q497)* is likely to be a simple loss-of-function mutation, but *h292* is not.

To determine whether *gld-2(q497)* might be a null allele, we compared the penetrance of the Pro phenotype in *h292/q497 trans*-heterozygotes and *h292/qDf16* hemizygotes. These two were similar (Table 1), suggesting that *gld-2(q497)* is a strong loss-of-function and, perhaps, null allele. This interpretation is supported by the finding that *gld-2(q497)* bears a nonsense mutation about halfway through the *gld-2* coding region (L. K., J. Benson and J. K., unpublished data). Therefore, subsequent genetic experiments were done using *gld-2(q497)*, which we abbreviate as *gld-2(lf)*.

### *gld-2 gld-1* double mutants fail to enter meiosis

The defects in *gld-2(lf)* mutants resemble those in *gld-1(ø)* mutants in two ways (Francis et al., 1995a). First, both *gld-1(ø)* and *gld-2(lf)* mutants fail to progress through meiotic prophase during oogenesis. Second, both can contain ectopic mitoses in the proximal hermaphrodite germline. Despite these similarities, the *gld-1(ø)* and *gld-2(lf)* phenotypes are not identical. One major difference is that *gld-1(ø)* males are cross-fertile and phenotypically wild type, whereas *gld-2(lf)* males are sterile with defective meiosis and gametogenesis. Hence, *gld-2* but not *gld-1* is required for male spermatogenesis. A second difference is that virtually all *gld-2(Pro)* animals have overt signs of gametogenesis (i.e. enlarged cells, distinguishable from mitotic cells by DIC optics), whereas *gld-1(ø)* animals do not (Francis et al., 1995a).

To investigate the relationship between *gld-1* and *gld-2*, we examined *gld-2(lf) gld-1(q485 ø)* double mutants. Like *gld-1(ø)* single mutants, the *gld-2(lf) gld-1(ø)* double mutants generated a vast excess of germ cells with no signs of gametogenesis, a ‘tumorous’ phenotype (Berry et al., 1997). However, *gld-1(ø)* single mutants and *gld-2(lf) gld-1(ø)* double mutants differ in two important respects. First, in *gld-1(ø)* single mutants, the excess germline proliferation is limited to hermaphrodites (Francis et al., 1995a) whereas, in *gld-2 gld-1(ø)* double mutants, it occurs in both hermaphrodites and males (Fig. 6A,B). Second, *gld-2* and *gld-1(ø)* single mutants enter meiosis normally, whereas *gld-2 gld-1(ø)* double mutants show no sign of entering meiosis (Fig. 6A,B and see below). Therefore, although a dramatic overproliferation of germ cells is observed in both *gld-1(ø)* single and *gld-2 gld-1(ø)* double mutants, only the double mutant fails to enter meiosis.

To examine this overproliferation phenotype in more detail, we stained wild-type and *gld-2 gld-1(ø)* double mutant germlines with two antibodies that serve as markers for proliferating cells. Anti-GLP-1 antibodies stain the membranes of proliferating germ cells (Crittenden et al., 1994) and anti-phosphohistone H3 antibodies stain nuclei in mitosis (Ajiro et al., 1996). Whereas GLP-1 is localized to the distal mitotic germline in the wild type (Fig. 6C; also Crittenden et al, 1994), GLP-1 protein extends throughout the germline in the double mutant (Fig. 6D). Similarly, nuclei in mitosis are restricted to the distal region in the wild type (Fig. 6C), but are scattered throughout the germline in the double mutant (Fig. 6D). Therefore, evidence from anti-GLP-1 and anti-phosphohistone H3 antibody staining further shows that the mitotic region in the double mutant extends throughout the germline. In contrast, *gld-2(lf)* single mutants that lack proximal proliferation have GLP-1 staining only in the distal region (data not shown) and *gld-1(ø)* single mutants have strong GLP-1 staining in the distal and proximal regions of the germline, but only weak and patchy GLP-1 staining in the region where meiotic nuclei can be found (Crittenden et al., 1994). The altered pattern of GLP-

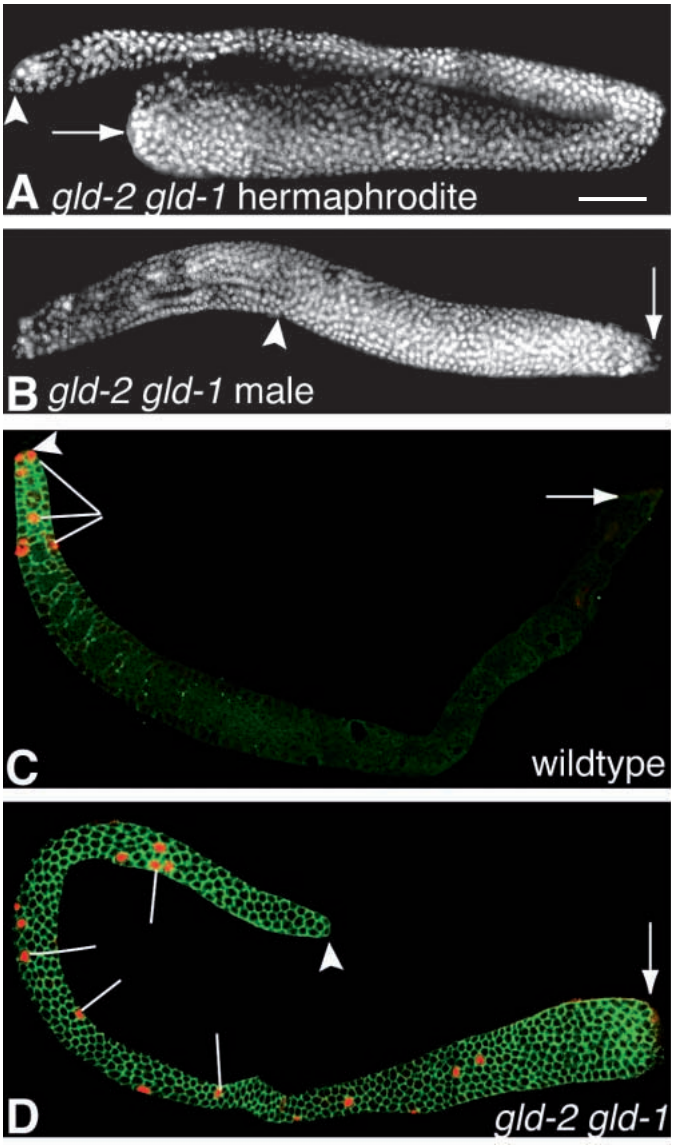


**Fig. 6.** *gld-2 gld-1* double mutants have tumorous germlines. Arrowhead, distal tip; arrows, proximal end. (A,B) Dissected germlines stained with DAPI. Note uniform size and morphology of all germ nuclei. No pachytene nuclei are seen in either the *gld-2(q497) gld-1(q485)* adult hermaphrodite (A) or in the *gld-2(q497) gld-1(q485)* adult male (B). (C,D) Dissected germlines stained with anti-GLP-1 antibodies (green) and anti-phosphohistone H3 antibodies (red). H3 antibodies detect nuclei that are in mitosis (Ajiro et al., 1996), see lines for examples. GLP-1 localizes to membranes in the mitotic region of the wild-type germline (C), but extends throughout the *gld-2(q497) gld-1(q485)* hermaphrodite germline (D). Scale bars, 20 μm.

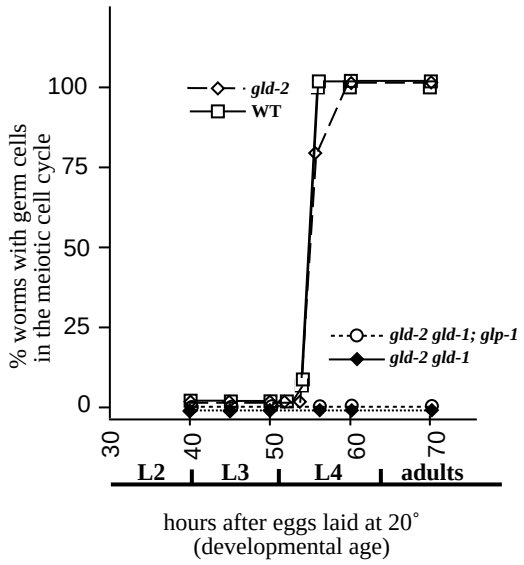


**Fig. 7.** *gld-2(q497) gld-1(q485)* and *gld-2(q497) gld-1(q485); glp-1(q46)* germ cells never leave the mitotic cell cycle. Synchronized wild-type, *gld-1(q497) gld-1(q485)*, and *gld-2(q497) gld-1(q485); glp-1(q46)* hermaphrodite larvae were DAPI-stained and examined at indicated times for pachytene nuclei. All animals also carry *unc-32(e189)*. *gld-1(q485)* mutants also enter meiosis at the normal time (Francis et al., 1995a).

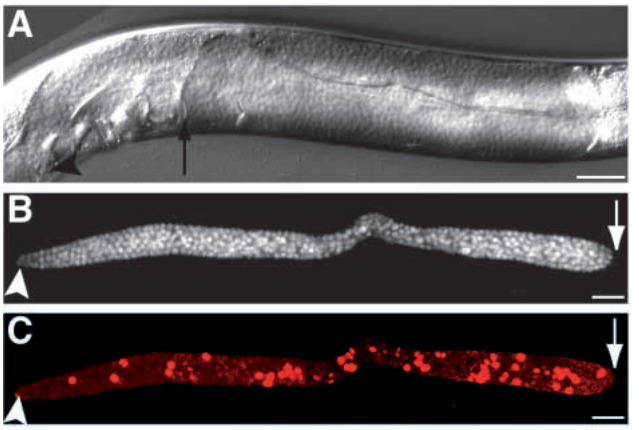


**Fig. 8.** *gld-2 gld-1(ø); glp-1(ø)* triple mutants have tumorous germlines. Arrowhead, distal tip; arrow, proximal (A) DIC photograph of a *gld-2(q497) gld-1(q485); glp-1(q46)* triple mutant hermaphrodite. Note uniformly small nuclei throughout the germline. (B,C) *gld-2(q497) gld-1(q485); glp-1(q46)* hermaphrodite germline stained with DAPI (B) and anti-phosphohistone H3 antibodies (C). Confocal image in C represents the sum of all mitotic nuclei in this germline; it is a projection of all focal planes onto a single image. Scale bars, 20 μm.

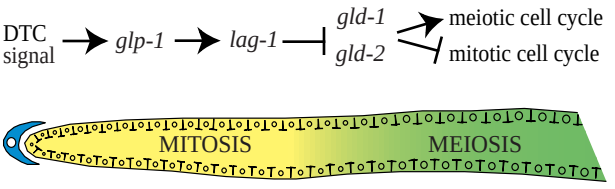


**Fig. 9.** Model for genetic control of the transition from mitosis to meiosis in the *C. elegans* germline. Diagram depicts the distal region of a germline arm. Light grey, mitotic region; dark grey, meiotic region. The role of *glp-1* in promoting mitosis in the germline is to downregulate (directly or indirectly) the synthesis or activities of *gld-2* and *gld-1*. *gld-2* and *gld-1* define separate pathways, each of which is sufficient for entry into meiosis. *gld-1* and *gld-2* may function by inhibition of premeiotic mitosis, promotion of meiosis, or both.

1 expression in the *gld-2 gld-1* double mutant germline relative to either single mutant shows that *gld-1* and *gld-2* have overlapping roles in downregulating GLP-1 expression in the region of the germline where germ cells enter meiosis.

Although we saw no sign of pachytene nuclei in *gld-2 gld-1(ø)* double mutant adults, it remained possible that germ nuclei had entered meiosis earlier during larval development. Therefore, double mutants were stained with DAPI at intervals starting at L2/L3 lethargus and germlines examined for pachytene nuclei. In wild-type, *gld-2(lf)* and *gld-1(ø)* germlines, pachytene nuclei were first observed in early L4 (Fig. 7, □, ◊, Francis et al., 1995a, Kimble and White, 1981) However, in *gld-2(lf) gld-1(ø)* double mutants, no pachytene nuclei were seen at any stage (Fig. 7, ♦). Similarly, no pachytene nuclei were present in double mutant males at ages ranging from L3 to adulthood (data not shown). These data support the idea that *gld-2(lf) gld-1(ø)* double mutants never enter meiosis. Since both *gld-1(ø)* and *gld-2(lf)* single mutants enter meiosis normally, these two genes are functionally redundant for this process. Hence, *gld-2* and *gld-1* appear to define two independent pathways, each of which is sufficient to promote entry into meiosis.

Further evidence for a role of *gld-2* in controlling entry into meiosis comes from analysis of a *gld-2(lf); glp-1(ø)* double mutant. *glp-1(ø)* single mutants generate only 5-7 germ cells that enter meiosis and differentiate as sperm (Austin and Kimble, 1987). However, in *gld-2(lf); glp-1(ø)* double mutants, 10-16 undifferentiated germ cells are made, indicating that an extra cycle of mitosis occurs relative to the *glp-1(ø)* single mutant (Table 2). This observation is consistent with the idea that *gld-2* plays a role in entry into meiosis or inhibition of germline mitosis. Interestingly, a similar effect was found for *gld-1(ø); glp-1(ø)* double mutants, suggesting a non-essential role for *gld-1* in the same process (Francis et al., 1995b).

## Germline mitosis in *gld-2(lf) gld-1(ø)* double mutants is independent of *glp-1* and *lag-1*

In *gld-2 gld-1(ø)* double mutants, GLP-1 is not restricted to the distal region, but is found throughout the germline. This observation suggested that *gld-2* and *gld-1* might negatively regulate *glp-1*. Consistent with this idea, the germline phenotype of *gld-2 gld-1(ø)* double mutants resembles that of the *glp-1* gain-of-function mutation, *glp-1(oz112)* (Berry et al., 1997). In both cases, germ cells fail to enter meiosis and, as a result, become tumorous. Hence, we considered the possibility that the *gld-2 gld-1(ø)* double mutant phenotype results from failure to downregulate the activity of *glp-1*.

To test the regulatory relationship between *glp-1* and *gld-1/gld-2*, we examined *gld-2(lf) gld-1(ø); glp-1(ø)* triple mutants. Whereas adult *glp-1(ø)* germlines contain 16-32 mature sperm (Austin and Kimble, 1987), the triple-mutants

**Table 2. Double and triple mutant phenotypes with *gld-1*, *gld-2* and *glp-1***

| Genotype* | Germline phenotype† | |
|---|---|---|
| | XX hermaphrodite | XO male |
| wild-type | mitosis → meiosis → gametogenesis | Mitosis → meiosis → gametogenesis |
| *gld-2* | mitosis → abn. meiosis → abn. gametogenesis | mitosis → abn. meiosis → abn. gametogenesis |
| *gld-1*‡ | mitosis → abn. meiosis → mitosis | Mitosis → meiosis → gametogenesis |
| *unc-32 glp-1*§ | mitosis (1-2 cycles) → meiosis → gametogenesis | mitosis (1-2 rounds) → meiosis → gametogenesis |
| *gld-2; unc-32 glp-1* | mitosis (3-4 cycle) → abn. meiosis → abn. gam.¶ | N.D. |
| *gld-2 gld-1; unc-32* | mitosis only | mitosis only |
| *gld-2 gld-1; unc-32 glp-1* | mitosis only | mitosis only |
| *gld-2 gld-1; unc-44 lag-1* | mitosis only | N.D. |

*Alleles used: *gld-2(q497)* is a strong loss-of-function, probably null (see text)*; gld-1(q485)* is a null (Francis et al., 1995a); *unc-32(e189)* is a marker, *glp-1(q46)* is a null (Kodoyianni et al., 1992), *unc-44(e326)* is a marker, and *lag-1(q426)* is a partial loss-of-function allele (see text).
†abn., abnormal; gam., gametogenesis.
‡Data from Francis et al., 1995(a).
§Data from Austin and Kimble, 1987.
¶Late L2 to mid-L3 larvae examined by DIC optics, *n*=13.

possess germline tumors resembling those of the *gld-2 gld-1* double mutant (Fig. 8A, Table 2). In addition, the triple mutants show no sign of entry into meiosis in either hermaphrodites (Figs 7, 8B) or males (data not shown). Staining with the anti-phosphohistone H3 antibody demonstrates that mitotic nuclei are scattered throughout the triple mutant germline (Fig. 8C). We conclude that the failure of *gld-2 gld-1* double mutants to enter meiosis is not due to the constitutive activation of *glp-1*. Instead, *glp-1* signalling is no longer essential for mitosis in the absence of *gld-1* and *gld-2*. Therefore, it is more likely that *glp-1* signalling downregulates *gld-1* and *gld-2* (Fig. 9).

We also asked if *gld-2 gld-1(ø)* double mutants might constitutively activate the signalling pathway downstream of *glp-1*. The only gene known to be downstream of *glp-1* is *lag-1*, which encodes a transcription factor (Christensen et al., 1996; Lambie and Kimble, 1991). Although the null phenotype of *lag-1* is larval lethality, 80% of animals homozygous for a partial loss-of-function allele, *lag-1(q426)*, escape that lethality and grow up as sterile adults that resemble a *glp-1(ø)* mutant. To ask if *lag-1* is activated in *gld-2 gld-1(ø)* double mutants, we examined *gld-2(lf) gld-1(ø); lag-1(q426)* triple mutants and found that they, too, had tumorous germlines with no sign of entry into meiosis (Table 2 and Material and Methods). Therefore, *gld-2* and *gld-1* appear to function downstream of both *glp-1* and *lag-1* in the decision between mitosis and meiosis (Fig. 9). Although this experiment suffers from the caveat that *lag-1(q426)* is a non-null allele, *q426* appears to have little or no germline activity: not only does *lag-1(q426)* resemble a *glp-1(ø)* mutation in the germline, but it is epistatic to the *glp-1(gf)* mutation with respect to germline proliferation (Berry et al., 1997).

## DISCUSSION

We have identified a new *C. elegans* gene, *gld-2*, that plays two roles in germline development. First, *gld-2* is required for progression through meiotic prophase during both spermatogenesis and oogenesis. Second, *gld-2* promotes the transition from mitosis to meiosis. This second role is shared with another gene, *gld-1*. Strikingly, in the absence of *gld-1* and *gld-2*, the *glp-1* signal transduction pathway is no longer essential for germline mitosis. We conclude that a major role of the *glp-1* pathway in controlling the germline decision between mitosis and meiosis is the inhibition of *gld-1* and *gld-2*.

### The role of *gld-2* in meiotic prophase and gametogenesis

The *gld-2* mutants exhibit defects in meiotic prophase during both spermatogenesis and oogenesis. In both cases, germ nuclei reach pachytene, but then appear abnormal in more proximal regions of the germline. Hence, the *gld-2* defect appears to occur at or after pachytene. The lack of meiotic progression in *gld-2* mutants bears some resemblance to the germline phenotype of three members of the MAP kinase signal transduction pathway, *let-60 (Ras)*, *mpk-1* and *mek-2*. Mutations in these three genes cause an arrest in pachytene during both spermatogenesis and oogenesis so that pachytene nuclei accumulate in the proximal germline (Church et al., 1995). However, the phenotypes of *gld-2* and of the MAP kinase pathway mutants are distinct. First, *gld-2* germline nuclei appear to reach pachytene, but then become abnormal: either a defect during pachytene results in degeneration of the pachytene nuclei or else progression through subsequent stages of meiosis is abnormal. In contrast, the germline nuclei of MAP kinase pathway mutants arrest with a normal pachytene morphology. Second, *gld-2* mutants sometimes exhibit proximal proliferation, whereas the MAP kinase pathway mutants never display this second defect (E. Lambie, personal communication). We conclude that *gld-2* is likely to have a separate role in progression through meiosis than that of the MAP kinase pathway.

The *gld-2* defect in progression through meiotic prophase is also distinct from that of *gld-1*. First, *gld-2* is required during both oogenesis and spermatogenesis, whereas *gld-1* is required only during oogenesis (Francis et al., 1995a). Therefore, *gld-2* may play a more fundamental role in meiotic progression than does *gld-1*. Second, *gld-2* germ cells usually remain in meiosis (albeit in an abnormal state), whereas *gld-1(ø)* germ cells return to the mitotic cycle, forming a tumorous germline. Hence, *gld-1* and *gld-2* have distinct roles in progression through meiotic prophase.

*gld-2* mutants have defects in gametogenesis as well as in meiotic cell cycle progression, suggesting that the regulation of these two processes is coupled. One possibility is that *gld-*

*2* may regulate both cell cycle progression and proper gametogenesis. Alternatively, the failure in gametogenesis may simply be a secondary effect of failure to progress through the meiotic cycle.

### Either *gld-1* or *gld-2* can promote entry into meiosis

Although *gld-1* and *gld-2* have distinct roles in progression through meiotic prophase, they appear to have overlapping roles in entry into the meiotic cell cycle. Germ cells in either single mutant appear to leave the mitotic cell cycle and enter meiosis normally, but have distinct defects later in meiotic prophase (Francis et al., 1995a; this paper). In contrast, germ cells in the double mutant apparently do not leave the mitotic cell cycle. The earliest signs of meiosis that are detectable in wild-type germlines are never observed in the double mutant and mitotically dividing cells are scattered throughout the double mutant germline. The simplest interpretation of these results is that germline cells in the double mutant never leave the mitotic cell cycle. An alternative explanation is that germline cells transiently leave the mitotic cell cycle, but never reach the pachytene stage of meiotic prophase before reverting to mitosis. In either case, the single and double mutant germlines are strikingly different. We suggest that *gld-1* and *gld-2* represent two independent pathways, each of which is sufficient to promote entry into meiosis. Hence, only in the double mutant do germ cells fail to make the transition between mitosis and meiosis.

A second line of evidence also supports a role for *gld-1* and *gld-2* in regulating entry into the meiotic cell cycle: germline precursors in either *gld-1(ø); glp-1(ø)* double mutants (Francis et al., 1995b) or *gld-2(lf); glp-1(ø)* double mutants (Table 2) generate more germ cells before entering meiosis than are observed in *glp-1(ø)* single mutants. Therefore, both *gld-1* and *gld-2* may play a non-essential role in promoting entry into meiosis or inhibiting mitosis.

### The origin of the Pro phenotype in *gld-2* mutants

The incompletely penetrant Pro phenotype of *gld-2* mutants may result from occasional germ cells that enter the early stages of meiosis but then return to the mitotic cell cycle. For example, a return from meiotic pachytene to the mitotic cell cycle is thought to account for the ectopic proliferation phenotype of *gld-1(ø)* mutants (Francis et al., 1995a). Alternatively, the Pro phenotype of *gld-2* mutants may result from the occasional failure of germ cells to enter meiosis. This model would provide an explanation for the fact that the Pro phenotype is so rare: since *gld-1* has an overlapping role with *gld-2* in promoting entry into meiosis, only occasionally would germ cells in either single mutant fail to enter meiosis. Because of the low penetrance of the Pro phenotype, it is difficult to distinguish between these two models.

### Germline mitosis is independent of the *glp-1* signal transduction pathway in *gld-2 gld-1* double mutants

In the wild-type germline, *glp-1* signalling promotes mitosis at the expense of meiosis (Austin and Kimble, 1987; reviewed in Kimble and Simpson, 1997). Here, we show that either *gld-1* or *gld-2* can promote entry into meiosis (or inhibit mitosis). What is the interaction between the mitosis-promoting activity of *glp-1* and the meiosis-promoting activities of *gld-1* and *gld-2*? Epistasis analyses using *gld-2 gld-1(ø); glp-1(ø)* and *gld-2 gld-1(ø); lag-1(lf)* triple mutants suggest that *gld-1* and *gld-2* function downstream of the *glp-1* pathway. In either triple mutant, the germline remains mitotic throughout; no sign of meiosis is detected. This result indicates that the primary role of *glp-1* is to inhibit *gld-1* and *gld-2*, and that *glp-1* plays no other essential role in promoting mitosis. Hence, a simple model to describe the interactions of these genes is that the *glp-1* signalling pathway inhibits the meiosis-promoting activities of *gld-1* and *gld-2* (Fig. 9). This model suggests that the decision between mitosis and meiosis is controlled by the relative activities of the *glp-1* pathway and the *gld-1* and *gld-2* pathways.

### Control of *gld-1* and *gld-2* by the *glp-1* pathway

How might the *glp-1* pathway affect expression or activity of *gld-1* and *gld-2*? One idea is that LAG-1 represses *gld-1* or *gld-2* transcription. Consistent with this possibility is the pattern of *gld-1* mRNA expression detected by in situ hybridization (Jones et al., 1996): expression is low in the distal-most end of the adult hermaphrodite germline (where the *glp-1* pathway is activated), but appears at higher levels prior to the transition into meiosis. Strong expression is seen throughout the meiotic region (Jones et al., 1996). An easy, though not rigorous test of the idea that LAG-1 directly regulates *gld-1* or *gld-2* transcription is to examine the *gld-1* and *gld-2* genomic sequences for putative LAG-1-binding sites in the 5' flanking region or in introns (Christensen et al., 1996). However, a computer search revealed no such sites in the *gld-1* gene, and only four widely scattered sites in the *gld-2* gene, including one in an exon (data not shown); these sites occurred at a frequency no higher than expected at random. Hence, sequence examination is not sufficient to suggest a direct regulation of *gld-1* and *gld-2* by LAG-1.

### The effect of *gld-1* and *gld-2* on GLP-1 expression

It is important to emphasize that *glp-1* is not required for germline proliferation in *gld-2 gld-1(ø)* double mutants, even though GLP-1 protein is expressed ectopically throughout these germlines (Fig. 6). Why is GLP-1 expressed so broadly, if it need not be active? The simplest possibility is that GLP-1 expression is a secondary effect of proliferation. In this model, proliferation in *gld-2 gld-1* mutants may result directly from failure to enter meiosis; the increase in GLP-1 expression might merely be due to a positive feedback between proliferation and GLP-1 expression. Such a positive feedback between proliferation and GLP-1 expression has been suggested previously (Berry et al., 1997; Kodoyianni et al., 1992). An alternative possibility is that *gld-1* and *gld-2* have two overlapping functions: (1) promotion of meiosis and (2) negative regulation of GLP-1 expression. In this model, the first function would be required for entry into meiosis, whereas the second function would not be required for that transition. Hence, even in *gld-2 gld-1; glp-1* triple mutants, germ cells remain mitotic because of the requirement for either *gld-1* or *gld-2* in the transition to meiosis.

The second model presented above suggests that *gld-1* and *gld-2* may negatively regulate GLP-1 expression in addition to their role in promoting meiosis. Why might *gld-1* and *gld-2* downregulate GLP-1 expression? We envision two possible reasons. First, removal of GLP-1 from meiotic cells may be critical for early embryogenesis, when GLP-1 expression is

spatially regulated (Evans et al., 1994). Second, downregulation of GLP-1 expression may help tip the balance between the mitosis-promoting activity of *glp-1* and the meiosis-promoting activities of *gld-1* and *gld-2*, allowing a transition in cell fates. Hence, an increase in *gld-1* and *gld-2* activity at a distance from the distal tip cell may itself lead to a decrease in GLP-1 expression, a negative feedback that would reinforce the decision to enter meiosis.

Regulation of GLP-1 levels by *gld-1* and *gld-2* would be expected to be at the level of translation or protein stability: the GLP-1 protein is localized, whereas *glp-1* mRNA extends throughout the germline (Crittenden et al., 1994). One possibility is that *gld-1*, which encodes an RNA-binding protein (Jones et al., 1996), could inhibit translation of *glp-1* mRNA. However, since GLP-1 is downregulated at the transition from mitosis to meiosis in either *gld-1(ø)* or *gld-2(lf)* single mutant, either gene must be sufficient for that regulation.

### Spatial regulation of the mitosis/meiosis decision in the *C. elegans* germline

The genetic pathway presented in Fig. 9 provides a starting point to think further about the spatial regulation that drives proliferation distally and meiosis more proximally. Genes that affect the relative activity of these competing pathways must be expressed differentially either in the germline or in cells that are in contact with the germline. At the distal end, *lag-2* expressed in the distal tip cell activates *glp-1* signalling (Henderson et al., 1994); therefore, the *gld-1* and *gld-2* pathways are inhibited and the mitotic cell cycle occurs in the distal germline. More proximally, germ cells escape from the influence of the distal tip cell, *gld-1* and *gld-2* are relieved from inhibition and meiosis ensues. The details of how and where germ cells escape *glp-1* signalling are not yet understood. Although the extent of membrane-bound GLP-1 correlates remarkably well with the extent of germline mitoses (Crittenden et al., 1994), no good molecular marker is available to indicate active signalling. Given the proposal that *gld-1* and *gld-2* are inhibited by *glp-1* signalling, perhaps the expression pattern of one of these genes will serve as a molecular marker for *glp-1* signalling.

### Control of the mitosis/meiosis cell fate decision in other organisms

How conserved is the regulation of the choice between mitosis and meiosis? In the yeast *S. cerevisiae*, regulatory pathways that control the choice between the mitotic and meiotic fates respond to nutritional signals and genotype at the mating loci. Many genes in the regulatory pathways that respond to these conditions have been identified (for review, see Honigberg et al., 1993). However, our computer searches indicate that there are no clear homologs of the GLD-1 and GLD-2 proteins in the *S. cerevisiae* genome (data not shown). In contrast to yeast, the transition from mitosis to meiosis in *C. elegans* responds to developmental signals. Hence, regulation of this process is more likely to be conserved with other multicellular organisms. Two genes in Drosophila, *bam* and *bgcn*, play a role in the transition from germline stem cell proliferation to meiotic differentiation during spermatogenesis and oogenesis (Gonczy et al., 1997; McKearin and Ohlstein, 1995; Ohlstein and McKearin, 1997). Although there appear to be no homologies between these proteins and the GLD-1 and GLD-2 proteins, it remains to be seen whether they have any functional similarities.

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