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Ross Francis  
*Washington University School of Medicine*

Eleanor M. Maine  
*Syracuse University*

Tim Schedl  
*Washington University School of Medicine*

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Analysis of the Multiple Roles of gld-1 in Germline Development: Interactions With the Sex Determination Cascade and the glp-1 Signaling Pathway

Ross Francis,* Eleanor Maine† and Tim Schedl*

*Department of Genetics, Washington University School of Medicine, St. Louis Missouri 63110, and †Department of Biology, Syracuse University, Syracuse, New York 13244

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ABSTRACT

The Caenorhabditis elegans gene gld-1 is essential for oocyte development; in gld-1 (null) hermaphrodites, a tumor forms where oogenesis would normally occur. We use genetic epistasis analysis to demonstrate that tumor formation is dependent on the sexual fate of the germ-line. When the germline sex determination pathway is set in the female mode (terminal fem/fog genes inactive), gld-1 (null) germ cells exit meiotic prophase and proliferate to form a tumor, but when the pathway is set in the male mode, they develop into sperm. We conclude that the gld-1 (null) phenotype is cell-type specific and that gld-1 (+) acts at the end of the cascade to direct oogenesis. We also use cell ablation and epistasis analysis to examine the dependence of tumor formation on the glp-1 signaling pathway. Although glp-1 activity promotes tumor growth, it is not essential for tumor formation by gld-1 (null) germ cells. These data also reveal that gld-1 (+) plays a nonessential (and sex nonspecific) role in regulating germ cell proliferation before their entry into meiosis. Thus gld-1 (+) may negatively regulate proliferation at two distinct points in germ cell development: before entry into meiotic prophase in both sexes (nonessential premeiotic gld-1 function) and during meiotic prophase when the sex determination pathway is set in the female mode (essential meiotic gld-1 function).

OOGENESIS in multicellular animals represents a complex developmental program in which the meiotic nuclear cycle and gametogenesis are coordinated to produce a functional oocyte. In the preceding paper, we describe the Caenorhabditis elegans gene gld-1 (FRANCIS et al. 1995). The genetic and phenotypic properties of gld-1 argue that it is a tumor suppressor gene that regulates oocyte development. Mutations that eliminate gld-1 function abolish oogenesis in the hermaphrodite and result in the formation of a germline tumor. Germ cells that give rise to the tumor complete the early stages of meiotic prophase but then exit pachytene and return to a mitotic cycle. These cells subsequently undergo repeated rounds of ectopic proliferation, giving rise to a tumor that fills much of the germ-line. Tumor formation is a sex-specific phenotype, as gld-1 (null) males show no abnormalities in germline development. Further, gld-1 has no essential function in the soma.

The sex specificity of the gld-1 tumorous phenotype has led to the proposal that gld-1 (+) may act as an important regulator of oocyte development (FRANCIS et al. 1995). Tumor formation would then result from a failure of oocyte determination and/or female meiotic prophase progression that allows germ cells to return to mitosis. Data available at present, however, do not exclude the possibility that tumors result from an intersexual mode of differentiation. Ovarian tumors in certain Drosophila mutants are comprised of germ cells with intersexual traits (BAE et al. 1994; J. HORABIN, personal communication). In this report, we investigate the basis of the sex-specific tumorous phenotype using genetic epistasis analysis. By constructing mutant combinations with gld-1 (null) and the sex determination genes, we ask whether tumor formation is specifically correlated with the female sexual fate in the germline. The cumulative results suggest that gld-1 (+) acts downstream of all known sex determination genes to either specify the oocyte fate or direct oocyte differentiation.

We also use genetic epistasis to investigate a second previously inferred role of gld-1 in germline development (FRANCIS et al. 1995). Although gld-1 has no essential role in male germline development, it acts to promote spermatogenesis in the hermaphrodite germ-line. For several reasons, we are unable to investigate this aspect of gld-1 function using a null gld-1 allele. Therefore epistasis tests are done with two classes of gain-of-function alleles that cause transformations in sexual fate in the hermaphrodite germ-line. The data obtained are consistent with the proposal that gld-1 (+) promotes spermatogenesis by assisting a set of sex determination loci that specify the male fate.

Finally, we examine whether germ cell proliferation in gld-1 (null) hermaphrodites is under the same controls that regulate proliferation in the wild-type germ-line. Previous work has shown that germ cell prolifera-
tion depends on the gene gld-1, which encodes a transmembrane protein belonging to the lin-12/Notch family of receptor molecules (AUSTIN and KIMBLE 1987, 1989; VOECHM and GREENWALD 1989). gld-1 protein acts as the likely receptor for a somatically derived signal that induces germ cell proliferation (AUSTIN and KIMBLE 1987; CRITTENDEN et al. 1994). We examine the dependence of germ cell proliferation in gld-1(null) mutants on the gld-1-mediated signaling pathway in two types of experiments: by ablating certain sets of somatic cells to eliminate the somatic signals and by analysis of gld-1(null);gld-1(lf) mutant combinations to eliminate the germline receptor. These experiments confirm the meiotic prophase origin of the tumorous phenotype and indicate that gld-1(+) has an additional, nonessential and/or redundant function in negatively regulating proliferation of germ cells before their entry into the meiotic pathway.

**MATERIALS AND METHODS**

**Nematode culture and strains**

General methods for *C. elegans* culture and genetic manipulation were as described (BRENNER 1974; SULSTON and HODGKIN 1988). Experiments were done at 20° unless otherwise noted. *C. elegans* nomenclature follows HORVITZ et al. (1979). *lf* is used for loss-of-function, *0* is used for a null allele that has been shown to fail to produce a gene product, *g* is used for gain-of-function, gld-1(Tum) is used to identify alleles with a tumorous X Germ line (Tum) phenotype, gld-1(Fog) is used to identify alleles with a feminization of the Germ line (Fog) phenotype and gld-1(Mog) is used to identify alleles with a masculinization of the Germ line (Mog) phenotype. Maternal and zygotic genetic and molecular markers are indicated using the notation m(−) or + and z(−) or +, where m represents the maternal genotype and z the zygotic genotype. All nematode strains used in this work are derived from the wild-type *C. elegans* var. Bristol isolate N2. The following general methods, and as described in HODGKIN et al. (1988), the *C. elegans* genetic map (J. HODGKIN, R. DURBIN and M. O’CALLAGHAN, personal communication) or the cited references, were used:

**LGI:** fog-1(q180 and q187) (BARTON and KIMBLE 1990), unc-11(e17), dpy-5(e61), dpy-14(e188), unc-13(e191 and e1091), gld-1(all alleles) (FRANCIS et al. 1995), fog-3(q443) (ELLIS and KIMBLE 1995).

**LGG:** dpy-10(e128), tra-2(e1095), tra-2(q122d) (SCHEDL and KIMBLE 1988), unc-4(e120).

**LGH:** fem-2(e2105), mog-1(q370) (GRAHAM and KIMBLE 1998), unc-69(e587), tra-1(e1834) and tra-1(e1527f) (HODGKIN 1987), unc-32(e189), gld-1 [see text and Table 1, all as described in AUSTIN and KIMBLE (1987) and/or KODOSZYNNI et al. (1992)].

**iG1:** dpy-13(e585d), fem-1(e1991, e2003ts), unc-5(e53), unc-24(e138), fem-3(e1996), fem-3(q20gf and q59gf) (BARTON et al. 1987), dpy-20(e1282), tra-3(e107), tra-3(bn75) (E. CAPOWSKI, B. BRENZDA and S. STROME, personal communication).

**LGIV:** her-1(y101h1), unc-42(e270), fog-2(q71).

**LGX:** len-2(e678).

**Rearrangements:** hT2(1LII-4);AK72(III) [dpy-18] (MCKIM et al. 1992), nDpy(IV);V (MCKIM et al. 1992).

**Construction of double mutants between gld-1 alleles and mutations in the sex determination pathway**

Tables 1 and 2 describe the phenotypes of double or triple mutants made between gld-1(Tum, Fog or Mog) alleles and mutations in the major sex determination loci. Except where noted below, all doubles requiring a gld-1(Tum) allele were made both with the null allele q485 and with the strong *lf* allele q268; identical phenotypes were observed in double mutants containing these gld-1(Tum) alleles. In many constructions, gld-1 alleles were marked with unc-13, which has ~0.2 map units to the left of gld-1 (FRANCIS et al. 1995). In cases where the progeny segregating from a balanced strain were analyzed, the genotype of the balanced strain was verified by complementation tests. For mutations in the sex determination genes, we use *zl* to indicate that the allele is putative null or strong loss-of-function. Other types of alleles are indicated. At least 50 animals of the appropriate genotype were examined for each double or triple mutant constructed.

**fog-1(0f)** gld-1(Tum or Mog) Double mutants of fog-1(0f) allele q180 were made using each of three gld-1(Tum) alleles (q268, q365 and q485), as well as each of three gld-1(Mog) alleles (q268, q365 and q485). To make recombinant fog-1(0f) unc-13 gld-1 chromosomes, we picked female Unc-13 nonDpy-5 recombinants segregating from fog-1(q180) dpy-5 unc-13 unic-13 gld-1(Tum or Mog) hermaphrodites. The resulting fog-1 unc-13 gld-1 chromosomes were maintained balanced over hT2. Double mutants between a second fog-1 allele (q187) and two gld-1(Tum) alleles (q268 and q365) and the Mog allele q93 were constructed by picking nonUnc-11 female progeny segregating from fog-1 unic-13 gld-1 hermaphrodites; the recombinant chromosome was balanced over hT2; fog-1 unc-13 gld-1 X0 males were generated by crossing fog-1 unc-13 gld-1/hT2 or + males to fog-1 unic-13 gld-1/hT2 hermaphrodites.

**gld-1(Tum or Mog) fog-3(0f)** Double mutants with fog-3(0f) were constructed using fog-3(q443) and the gld-1 tumorous allele q485 and the Mog allele q93. Recombinant unc-13 gld-1 fog-3 chromosomes were generated by picking recombinant nonUnc nonDpy females segregating from unc-13 gld-1(dpy-14) fog-3 hermaphrodites. Balanced unc-13 gld-1 fog-3/hT2 strains were used as the source of homozygous unc-13 gld-1 fog-3 animals. Males of the same genotype were generated by crossing.

**fog-1(0f)** gld-1(Tum) fog-3(0f): A balanced triply mutant strain of the genotype fog-1(q180) unc-13 gld-1(q485) fog-3(q443)/hT2 was constructed as follows. Self-progeny of heterozygous fog-1(q180) dpy-5/unc-13 gld-1 fog-3 hermaphrodites were screened for female recombinants that were nonDpy-5 and nonUnc-13. To identify recombinant females of the desired genotype (fog-1 unc-13 gld-1 fog-3/dpy-5, resulting from recombination in the fog-1 dpy-5 interval), single females were crossed with unc-13/hT2 males. Only animals heterozygous for a recombinant fog-1 unc-13 gld-1 fog-3 chromosome produced X0 Unc-13 male progeny (genotype fog-1 unc-13 gld-1 fog-3/unc-13) that display the wildominant fog-1(+) male germine phenotype (production of sperm and then oocytes) (BARTON and KIMBLE 1990). For three females that segregated such males, single XX L4 cross-progeny were picked onto individual plates to identify candidate fog-1 gld-1 unic-13 fog-3/hT2 strains. Genotypes of these balanced strains were confirmed by using complementation tests to show that each was heterozygous for fog-1(0f), gld-1(Tum) and fog-3(0f).

**fog-1(0f)** gld-1(Tum) fog-3(0f): X Animals of the genotype fog-1(q180) unc-13 fog-3(q443) were obtained from a balanced fog-1 unc-13 fog-3/hT2 hermaphrodite strain constructed as follows. NonUnc nonDpy female recombinants segregating from fog-1 dpy-5/unc-13 fog-3 hermaphrodites were picked and crossed individually to hT2/unc-13 males. Recombinant females of
the desired genotype (fog-1 unc-13 fog-3/fog-1 dpy-5) were identified based on their genotype of X0 Unc-13 male cross-progeny (genotype fog-1 unc-13 fog-3/unc-13) that display the semidominant fog-1/+ male phenotype (production of sperm and then oocytes). From such crosses, single non-Unc-13 XX cross-progeny were picked onto separate plates to identify candidate fog-1 unc-13 fog-3/HT2 animals. The genotypes of three independently isolated strains were then confirmed by showing that each carried mutations that fail to complement fog-1 and fog-3 mutations. Like fog-1 and fog-3 single mutants, XX and XO fog-1 unc-13 fog-3 animals have germ lines that make only oocytes and show no obvious defects in proliferation or meiotic development.

gld-1 (Tum or Mog): fem-1 (gf); fem-1 (e1991), an amber allele (Donbach and Hodgkin 1984), was used for the construction of double mutants with gld-1 (Tum) alleles (q485 and q268) and with gld-1 (q93). Approximately 25% of the Unc-24 self-progeny of gld-1 (Tum)/+; fem-1 unc-24/+ hermaphrodites were Tum, indicating gld-1 (Tum) is epistatic to fem-1. Construction of unc-13 gld-1 (Mog); fem-1 unc-24 animals was facilitated by the property that fem-1 XX animals produced by fem-1/+ mothers are often self-fertile hermaphrodites owing to maternal rescue. Therefore, Unc-24 hermaphrodites segregating from unc-13 gld-1 (Mog)/+; fem-1 unc-24/+ mothers were picked onto separate plates. Some hermaphrodites segregated broods consisting of three fourths female Unc-24 and one fourth sterile Unc-13 animals (unc-13 is epistatic to unc-24) of the genotypes unc-13 gld-1 (q93); fem-1 unc-24. Heterozygosity for gld-1 (q93) appears to increase the fraction of XX fem-1 (gf) [m (+/-) z (-/-)] animals that make sperm, but this effect was not quantitated.

gld-1 (Tum or Mog): fem-2 (lf); gld-1 (q485) or gld-1 (q93); fem-2 double mutants were constructed at 25° using fem-2(c2105), a strong fem-2 (lf) allele that is maternally rescued (Hodgkin 1986). Hermaphrodites segregating from unc-13 gld-1 (Tum or Mog)/+; fem-2/+/ mothers were picked onto separate plates. Maternally rescued unc-13 gld-1/+; fem-2 hermaphrodites segregated a 3:1 ratio of nonUnc females and sterile Unc-13 progeny of the genotype unc-13 gld-1 (Tum or Mog)/+; fem-2/+-.


gld-1 (Tum): fem-3 (lf); Doubles of gld-1 (q268 or q485) with fem-3 (lf) were made using e1996, a fem-3 ochre nonsense allele (Hodgkin 1986; Ahhringer et al. 1992). Approximately one fourth of the Unc-24 progeny of gld-1 (Tum)/+; unc-24 fem-3 XX animals had a Tum germline. To generate XO gld-1 (Tum)/+; fem-3 (lf) animals, single gld-1 (q485)/+; unc-24 fem-3(e1996)/+ X0 males were mated with gld-1 (q485)/+; unc-24 fem-3(e1996); fem-3(zf); tra-1/+ females. These crosses yielded Unc-24 Lon-2 X0 hermaphrodites. About one half of these had a Tum germline.

gld-1 (Tum): tra-2 (lf); fem-3 (lf); XX animals of the genotype gld-1 (q485); dpy-10 tra-2(e1095); unc-24 fem-3(e1996) were identified among the self-progeny of gld-1 (Tum)/+; dpy-10/+; unc-24 fem-3/+- hermaphrodites. Approximately one fourth of the Dpy-10 Unc-24 self-progeny were tumorous, showing that the gld-1 (Tum) phenotype is expressed in a tra-2 (lf); fem-3 (lf) background.

gld-1 (Mog): fem-3 (lf); Double mutants of fem-3(e1996) were constructed with gld-1 (Mog) alleles representing each gld-1 (Mog) subclass (C1, q93; C2, o20; C3, a20). In constructions with the C3 allele a20, it was initially found that a20 can suppress the XX Fog genotype of fem-3 (lf) [m (+/-) z (-/-)] animals [XX fem-3 (lf) is not normally rescued by maternal fog-3 (+) activity (Hodgkin 1986)]. Thus gld-1 (a20); unc-24 fem-3(e1996) dpy-20/+/ mothers segregated many self-fertile Unc-24 Dpy-20 progeny that were subsequently shown to be homozygous for both a20 and fem-3 (lf). Heterozygosity for fem-3 (lf) was indicated by their segregation of only female progeny and homozygosity for a20 was determined by testing females for failure to complement gld-1 (q268). These results indicate that a20 can restore spermatogenesis in fem-3 (lf) [m (+/-) z (-/-)] but not fem-3 [m (-/-) z (-/-)] animals. Suppression by a20 was ~40% penetrant. The C1 and C2 gld-1 (Mog) alleles q93 and a20 were also found to sometimes restore spermatogenesis in fem-3 (lf) [m (+/-) z (-/-)] XX animals. However, although sperm are often made in C1 or C2 XX gld-1 (Mog); unc-24 fem-3(e1996) dpy-20 animals produced by heterozygous mothers, these animals are always sterile because oocytes are never made (see RESULTS). The C1 allele, q93, restored spermatogenesis in 56% of fem-3 (lf) [m (+/-) z (-/-)] gonad arms and the C2 allele restored spermatogenesis in 44% of gonad arms.

To generate gld-1 (q93 or a20); fem-3 (lf) animals that lack maternal fem-3 activity, we crossed unc-13 gld-1 (Mog)/+; fem-3 (lf) dpy-20/+ males with unc-13 gld-1 (Mog)/+; unc-24 fem-3 (lf) dpy-20 females. From these crosses >100 Unc-13 Dpy-20 progeny were picked and their germline phenotype scored. Almost all (>97%) animals failed to make sperm and had germ lines that contained only undifferentiated germ cells. The remaining animals made excess sperm in both gonad arms, indicating they were gld-1 (Mog); unc-24 fem-3 dpy-20/ dpy-20 recombinants.

gld-1 (Tum): her-1 (lf); Double mutants with her-1 were constructed using her-1(y1011hs1), a deletion allele that removes most of the her-1 coding region (Trent et al. 1991). To generate XX gld-1 (q485); her-1 (lf) animals, heterozygous gld-1 (Tum)/+; her-1 (lf) unc-42/+ hermaphrodites were made and allowed to self. Approximately one fourth of the Unc-42 self-progeny had a Tum germline. To make XO gld-1 (q485); her-1 (lf) hermaphrodites, X0 males of the genotype gld-1 (Tum)/+; her-1 (lf) unc-42/+ were crossed to gld-1 (Tum)/ +; dpy-11 her-1 (lf) unc-42;lon-2 hermaphrodites. NonDpy-11 Unc-42 Lon-2 animals, which must be X0 cross-progeny, were picked en masse and their germline phenotype scored. About one fourth of the Unc-42 Lon-2 animals were Tum.

gld-1 (Tum): tra-1 (lf); tra-1(e1834), a deletion allele that removes most of the tra-1 coding region (Zarkower and Hodgkin 1992), was used to construct a gld-1(q485); tra-1 (lf) double mutant. unc-13 gld-1 (Tum)/+; tra-1 (lf)/+; XX heterozygotes were constructed and their Unc-13 self-progeny with a male soma [of the genotype unc-13 gld-1 (Tum); tra-1 (lf)] were examined. Like the tra-1(e1834) single mutant, these animals have a completely male nongonadal soma and a gonadal soma that is sometimes male and sometimes morphologically abnormal. Germline phenotypes were scored only in animals whose somatic gonad showed no obvious morphological abnormalities.

gld-1 (Tum): tra-1 (gf); The tra-1 (gf) allele e1575gf transforms both XX and X0 tra-1 (gf)/+ animals into females (Hodgkin 1987). However, whereas XX tra-1 (gf)/+ females are morphologically normal females, X0 tra-1 (gf)/+ females have an abnormal, truncated tail. Therefore XX and X0 tra-1 (gf)/+ females can be distinguished by tail morphology. To generate double mutants, unc-13 gld-1(q485 or q268)/+ males were crossed with single unc-13 gld-1(q485 or q268)/+; tra-1 (gf)/+ and XX females. All Unc-13 cross-progeny were somatic females and had a Tum germline. The Tum animals included X0 somatic females with a truncated tail [X0 unc-13 gld-1; tra-1 (gf)/+ and X0 somatic females with a normal tail [50% unc-13 gld-1; tra-1 (gf)/+ and 50% unc-13 gld-1].

gld-1 (Tum): tra-2 (lf); The tra-2 allele e1095, an ochre nonsense mutation (Kuwabara et al. 1992), was used to make XX gld-1(q268 or q485); tra-2 (lf) animals. From the self-progeny of unc-13 gld-1 (Tum)/+; tra-2 (lf)/+ mothers, we exam-
ined X Unc-13 pseudomales, 100% of which should be unc-13 gld-1 (Tum); tra-2 (gf) in genotype.

gld-1 (Tum); tra-2 (gf)): The tra-2 (gf) allele q122gf dominantly eliminates spermagogenesis in X X but not X0 animals (Schedl and Kimble 1988). To generate X X gld-1 (q485); tra-2 (gf) animals, single gld-1 (Tum)/+; tra-2 (gf) unc-4/+ males were crossed with single females of the same genotype. From crosses that gave nonUnc tumorous progeny, Unc-4 cross-progeny were scored. Of the X unc-4 tra-2 (gf) cross-progeny with a female soma, one fourth had a Tum germline. In contrast, all X0 unc-4 tra-2 (gf) animals with a male soma had a normal male germline.

gld-1 (Tum); tra-3 (gf)): Double mutants of gld-1 (q485) were constructed with two tra-3 alleles, e1107 and bm75. e1107 is an amber allele that transforms X tra-3 [m (−/−) z (−/−)] animals grown at 25° into pseudomales that have a partially masculinized soma and germline (Hodgkin 1980). bm75 is an unusual temperature-sensitive tra-3 allele; it has no major effect on the XX female soma but masculinizes the germline more strongly than do tra-3 amber alleles (E. Capowski, B. Brendza and S. Strom, personal communication). The phenotype of double mutants with both tra-3 alleles were analyzed at 25°. We first generated X X unc-13 gld-1 (q485)+; tra-3 [m (−/−) z (−/−)] animals that, owing to maternal rescue by tra-3 (+) activity, are self-fertile hermaphrodites. These segregated one fourth Unc-13 animals that are genotypically unc-13 gld-1 (Tum); tra-3.

gld-1 (Tum); mog-1 (If)): The X X gld-1 (q485); mog-1 (q270) double mutant was examined at 25°, the temperature at which the mog-1 (If) phenotype is strongest (Graham and Kimble 1998). Starting with gld-1 (Tum)/+; mog-1 unc-69/+ hermaphrodites, we scored all X mog-1 unc-69 self-progeny, one fourth of which should be homozygous for gld-1 (Tum). For the data in Table 1, the percentage of total mog-1 unc-69 animals that showed ectopic germline proliferation was multiplied by four to obtain an estimate (7%) of the fraction of gld-1 (Tum); mog-1 unc-69 animals with a Mog phenotype like that of the mog-1 single mutant (i.e., excess sperm, no ectopic proliferation).

gld-1 (Tum); fem-3 (gf)): The fem-3 (gf) allele q95gf and q95gf confer a temperature-sensitive Mog phenotype in which animals grown at 25° produce only sperm, whereas animals grown at 15° usually (q20gf) or sometimes (q95gf) make both sperm and oocytes (Barton et al. 1987). Double mutants of fem-3 (q20gf and q95gf) with gld-1 (q485 and q365) were obtained from strains of the genotype unc-13 gld-1 (Tum); fem-3 (gf); ndp4/+ that were maintained at 15°. These strains were made by first constructing unc-13 gld-1 (Tum)/+; fem-3 (gf); dpy-20; ndp4/+ hermaphrodites based on their segregation of ~55% Tum animals. Animals homozygous for fem-3 (gf) were identified by following elimination of dpy-20. For the data in Figure 1 and Table 1, unc-13 gld-1 (Tum); fem-3 (gf) XX animals were picked as newly hatched L1 larvae and grown for 58 hr at 25°.

Dominant suppression of fog-2 (gf) and tra-2 (gf) mutations by a gld-1 (Mog) allele

The gld-1 (Mog) allele q93 was shown to dominantly suppress the female self-sterile phenotypes of XX fog-2 (gf) and XX tra-2 (q122gf) animals. For fog-2 (gf), X X unc-13 gld-1 (q93) gld-1 (Tum); fog-2 (gf) rol-9 are hermaphrodites in contrast to fog-2 (gf) animals, which are always female. However, XX unc-13 gld-1 (Tum); fog-2 rol-9 hermaphrodites have a Mog phenotype like that of XX unc-13 (q93) animals, showing that gld-1 (q93) is epistatic to fog-2 (gf). Therefore gld-1 (q93) cannot masculinize the XX germline by acting through fog-2 (gf).

Dominant suppression of tra-2 (gf) by gld-1 (q93) was demonstrated by showing that X X unc-13 gld-1 (q93); tra-2 (gf) animals are self-fertile hermaphrodites instead of females. These hermaphrodites segregate sterile unc-13 gld-1 (Mog); tra-2 (gf) progeny that first make sperm and then undifferentiated germ cells.

Double mutants with the gld-1 fog allele q126

XX double mutants of the genotypes gld-1 (q126); tra-1 (e1832) and gld-1 (q126); tra-2 (e1095) were obtained as XX self-progeny from unc-13 gld-1 (q126)/+; tra-1/+ or gld-1 (q126)/+; tra-2/+ hermaphrodites. X Unc-13 Tra-2 pseudomales and XX Unc-13 Tra-1 males were picked and their germline and somatic phenotypes examined. The double mutant unc-13 gld-1 (q126); tra-3 (e1107) was examined at 25°. Unc-13 pseudomales segregating from unc-13 gld-1 (q126)/+; tra-3 (m−/+; z−/+ ) mothegs were examined for germline and somatic abnormalities. gld-1 (q126) has no obvious effect on the male somatic phenotypes of XX tra-1, tra-2 or tra-3 mutants. Finally, a homozygous gld-1 (q126); unc-24 fem-3 (q20gf) dpy-20 hermaphrodite strain was constructed by virtue of the ability of gld-1 (q126) to suppress the sterile Mog phenotype of fem-3 (q20gf) animals grown at 25°. Specifically, XX gld-1 (q126)/+; unc-24 fem-3 (q20gf) dpy-20/+ heterozygotes grown at 25° segregated many self-fertile Unc-24 Dpy-20 hermaphrodites. Several of these hermaphrodites were shown to contain both gld-1 (q126) and fem-3 (gf) by the independent segregation of both types of mutations after outcrossing with wild-type males.

Construction of strains mutant for gld-1 and gpl-1

gld-1 (Tum); gpl-1 double mutants: Table 4 and Figure 8 describe the different gld-1 (Tum); gpl-1 (0 or if) genotypes examined in this work. For each, we constructed balanced heterozygous strains of the general genotype gld-1 (Tum)/HT2(1); unc-32 gpl-1/HT2(III). Owing to hT2(1) [hT2(1)]; hT2(III) [dpy-18] induced pseudolinkage, these heterozygotes segregate Unc-32 self-progeny that are essentially always of the genotype gld-1 (Tum); unc-32 gpl-1. The gld-1 (+); gpl-1 (+) control animals used for comparison in all experiments were the Unc-32 self-progeny of unc-32 hT2 hermaphrodites. During passage, the hT2 chromosome I-III pseudolinkage was found to occasionally break down: if a single nonUnc tumorous animal or a nonDpy Bli-4 animal was observed, the plate was discarded.

To construct balanced strains heterozygous for gld-1 (Tum) and gpl-1 alleles, gld-1/HT2 males were crossed with unc-32 gpl-1/HT2 hermaphrodites. Of the resulting male progeny, one half were heterozygous for hT2, whereas one quarter had the desired genotype of gld-1/+; unc-32 gpl-1/+ . Single males were crossed with hT2 [hT2(1)]; dpy-18] hermaphrodites; sires that produced no Dpy-18 (hT2) male cross-progeny were identified as being gld-1+/+; unc-32 gpl-1/. From these crosses, 25 or more LA XX cross-progeny were picked onto separate plates and their self-progeny inspected in the following generation. In most cases, balanced gld-1 (Tum)/hT2; unc-32 gpl-1 (0) /HT2 strains were easily identified because their gld-1 (Tum); unc-32 gpl-1 (0 or if) progeny had a germline phenotype distinct from those of both gld-1 (Tum) and gpl-1 single mutants. The genotypes of many of these strains were confirmed by out-crossing or by complementation testing. In addition, because gld-1 (Tum); unc-32 gpl-1 (j722) and gld-1 (Tum); unc-32 animals have identical Tum germline phenotypes, we verified the presence of the gld-1 (q172) allele in double mutants by PCR analysis. PCR analysis was done using
primers that bracket the 260 bp of glp-1 sequence that is deleted by q172 (Kodoyanni et al. 1992). The glp-1 DNA fragment amplified from gld-1(Tum);unc-32 glp-1(q172) homozygotes showed the expected size reduction (compared with gld-1(Tum);unc-32 control animals), confirming that these animals were homozygous for q172.

gld-1(Tum);glp-1(0 or lf) males and masculinized XX animals: Several balanced glld-1(q485)/KT2;unc-32 glp-1(0 or lf)/KT2 strains were maintained as X0 male/XX hermaphrodite strains to provide a source of X0 double mutants. The male germ line phenotypes of different glld-1(q485);glp-1(0 or lf) double mutants varied depending on the allele, ranging from a Gip phenotype [for the glp-1(0) alleles q46 and q175 and the strong if allele q224] to essentially wild type [for the strong if allele q172]. In addition, we examined XX gld-1(q485);glp-1(q172 or q224) animals that were homozygous for fem-3(q20gf), a mutation that masculinizes the XX germ line (Barton et al. 1987). To construct these strains, gld-1(q485)/KT2;unc-32 glp-1(q172 or q224)/KT2 males were crossed with KT2;fem-3(q20gf) hermaphrodites at 15°C. Single nonDpy-18 (non-hT2) cross-progeny were picked onto separate plates and the line proliferated, the above described balanced strains were maintained as X0 male/XX hermaphrodite strains to provide a source of X0 double mutants. Hermaphrodites that were identified based on the unique germ line phenotype (Barton et al. 1987). The glp-1 alleles q172 and q224 behaved differently in the XX glld-1(Tum);unc-32 glp-1;fem-3(qf) triple mutants; the triple mutant with q172 had a Mop phenotype (see RESULTS), whereas with q224 had a Glp phenotype.

**gld-1(Tum);glp-1(0) triple mutants bearing f mutations in fog-1, fem-1 or fem-3:** Table 4 lists the triple mutants that were constructed using a glld-1(Tum) allele, a glp-1(0) allele (q175 or q46) and one of the following f mutations: fog-1(q180), fem-1(z2003ts) or fem-3(e1996). In all cases, triple mutants were obtained from balanced heterozygous strains. To make triple mutants carrying fog-1(If), we first constructed a balanced fog-1 glld-1(KT2;unc-32 glp-1(KT2) strain (as described above for the construction for gld-1(KT2;unc-32 glp-1(KT2). A cross of fog-1 glld-1(KT2;unc-32 glp-1(KT2 hermaphrodites with unc-32 glp-1(0)/KT2 males was then used to generate hermaphrodites of the genotype fog-1 glld-1(Tum)/++;unc-32 glp-1(0)/unc-32. These were then crossed with KT2;unc-32 males, and wild-type hermaphroditic cross-progeny were picked onto individual plates. Hermaphrodites that were fog-1 glld-1(KT2;unc-32 glp-1/KT2 were initially identified based on the unique germ line phenotype of fog-1 glld-1(Tum);unc-32 glp-1(0) segregants (see RESULTS). These strains were outcrossed to confirm that they carried both glld-1(Tum) and glp-1(0).

Triple mutants containing fem-1(z2003ts) were obtained from a balanced strain of the genotype glld-1(Tum);KT2;unc-32 glp-1(0)/KT2 fem-1(0);dpy-13(e538ad) unc-5. Heterozygous glld-1(Tum);KT2;unc-32 glp-1(0)/KT2 males were first crossed to KT2;dpy-13 unc-5 hermaphrodites to generate males that were glld-1(Tum);KT2;unc-32 glp-1(0)/KT2 dpy-13 unc-5/+.

These males were then crossed to KT2;fem-1 females. Cross-progeny that were partially Dpy-13, and hence dpy-13 unc-5/fem-1 in chromosome IV genotype, were picked onto separate plates. Self-progeny were inspected in the following generation to ensure segregation of all phenotypes expected for the balanced strain. Triplicate mutant glld-1(Tum);unc-32 glp-1(0)/fem-1 animals were identified as Unc-32 animals that failed to show the semidominant dpy-13 phenotype.

Triple mutants bearing fem-3(e1996) were obtained as segregants from a balanced strain with the genotype glld-1(Tum);KT2;unc-32 glp-1(0)/KT2 fem-3(e1996)/unc-24 fem-3(q20gf) dpy-20. These strains were constructed by a method analogous to that used for construction of a balanced triple mutant containing fem-1. Approximately one third of Unc-32 nonnc-24 nonDpy-20 segregants are homoyzygous for fem-3(e1996).

**Characterization of glld-1(Tum);glp-1 double mutants and feminized triple mutants**

Quantitation of germ line proliferation: For assays of germ line proliferation, the above described balanced strains were used as a source of hermaphrodites with the general genotype glld-1(q485);unc-32 glp-1(+ or 0 or lf). The feminized triple mutant fog-1(If) glld-1(Tum);glp-1(0) and glld-1(Tum);unc-32 glp-1(0);fem-1 were also examined (see Figure 6). To obtain tightly staged animals, newly hatched L1 larvae were picked as described (Francis et al. 1995) and grown to the L4 stage or adulthood. At the times indicated in Figures 6 and 8, animals were fixed and stained with diamidinophenolindole (DAPI) (Francis et al. 1995). For each timepoint, the number of germ cells per gonad arm was counted twice in >10 gonad arms, and the mean number of germ cells per gonad arm was determined. In experiments with nonconditional glld-1 alleles (Figures 6 and 8A), animals were grown at 20°C. Experiments with temperature-sensitive glp-1 alleles (q224, bni18, q415) were done using animals grown from hatching at 25°C.

**Determination of when germ cells first enter meiotic prophase during larval growth:** Data presented in the accompanying paper (Francis et al. 1995) showed that glld-1(q485) does not affect the timing with which germ cells first enter meiotic prophase in a glld-1(+) background. For the present work, it was important to determine when glld-1(Tum) germ cells in various fog-1(+) or if) glld-1(0 or if) backgrounds first reach pachytene of meiotic prophase. This was done as described (Francis et al. 1995) by examining DAPI-stained preparations of tightly staged larvae for the presence or absence of pachytene-stage meiotic germ cells. For each genotype, the mean age (in hours after hatching) at which pachytene nuclei were first observed was determined using 10 animals. Results were as follows: unc-32 glp-1(0) (23 hr, L2 stage), fog-1(unc-32 glp-1(0) (23 hr), glld-1(unc-32 glp-1(0) (31 hr, L3); fog-1 glld-1(unc-32 glp-1(0 (32 hr, L3); unc-32 glp-1(0 (46 hr, L4 (wild-type control)); fog-1(If);unc-32 glp-1(47 hr), glld-1(Tum);unc-32 glp-1 (46 hr); fog-1 glld-1;unc-32 glp-1(46 hr) and glld-1;unc-32 glp-1 (q172) (46 hr). Entry into meiotic prophase in fog-1(+) or if) glld-1(Tum);unc-32 glp-1(0) animals is delayed relative to a glld-1(0 single mutant, suggesting that germ cells undergo the additional rounds of cell division before entering meiosis. Note that fog-1(If) has no effect on premeiotic proliferation by glld-1(Tum) germ cells nor does it increase the rate of tumor growth in glld-1(Tum) animals (Francis et al. 1995) (data not shown).

**Ablation of somatic gonad cells:** Ablations of specific cells of the hermaphroditic somatic gonad were performed using a nitrogen pulse laser (Laser Sciences Inc.) set up as described by Avery and Horvitz (1987). The laser was coupled to a Zeiss Axiosplan microscope and beam intensity was adjusted by the use of neutral density filters. Somatic cells in L1 and L2 gonad primordia were identified by their position and morphology (Kimble and Hirsh 1979) and ablated using 30–80 pulses from the laser. Operated animals were reexamined within 2 hr to confirm killing of the target cell(s) and to assess collateral damage.

Previous results have shown that ablation of the distal tip cell (DTC) precursors (Z1.a and Z4.p) does not produce a perfect phenocopy of the glp-1(0) phenotype. In the ablated animals, all germ cells enter meiotic prophase at the normal time (Kimble and White 1981). By contrast, germ cells in unc-32 glp-1(0), glld-1(Tum);unc-32 glp-1(0) and the feminized glld-1(Tum);unc-32 glp-1(0) triple mutants enter meiotic pro-
phase prematurely (see previous section) (Austin and Kimble 1987). Furthermore, although ablation of the DTC precursors has no effect on sex determination (Kimble and White 1981), glp-1(?)-induced premature entry into meiotic prophase can cause masculinization of the germline in genetic backgrounds that are partially feminized. For example, fem-2(ts) mutants make only oocytes, whereas fem-2(ts) glp-1(?) mutants make sperm at the restrictive temperature (E. Maine and J. Kimble, unpublished observations). These differences may, at least in part, explain why DTC precursor ablations in gld-1(null) animals and gld-1(null); glp-1(?) mutant combinations result in nonidentical phenotypes (see results).

A time-course study to evaluate the effects on killing the somatic DTC in gld-1(Tum) hermaphrodites was done as follows. unc-32 and gld-1(Tum); unc-32 XX larvae were grown to the late L3 or young L4 stage and the DTC was killed in one or both gonad arms. Operated animals were grown for a further 24–40 hr and then prepared for dissection, fixation and staining with DAPI (Francis et al. 1995). At least 12 operated animals were examined for each 2 hr interval between 24 and 40 hr after ablation of the DTC. Distal germ cells at the pachytene stage of meiotic prophase were generally first observed in the 28–32 hr intervals. Distal mitotic figures were first observed at ~32 hr after DTC ablation but were more common 34–38 hr after ablation.

Morphological evaluation of germline phenotypes: Observations of living animals by Nomarski differential interference contrast (DIC) microscopy were made as described (Sulston and Hodgkin 1988). Generally, worms were picked as L4 larvae and examined as adults between 18 and 48 hr later. For staining with DAPI and antibodies, males and hermaphrodites were dissected, fixed and stained as described (Francis et al. 1995). The monoclonal antibody SP56, which is directed against an epitope expressed only by sperm and primary spermatocytes (Ward et al. 1986), was used at a 1:20 dilution of culture supernatant.

RESULTS

In the first half of this section we describe experiments designed to investigate the sex specificity of the gld-1 tumorous phenotype and the role of gld-1 in promoting spermatogenesis in the hermaphrodite germline. As background for these experiments, we briefly review germline sex determination in C. elegans.

In C. elegans there are two sexes: XX hermaphrodites, which are considered to have a female soma and a hermaphrodite germline that makes first sperm and then oocytes, and XO males, which have a male soma and germline. Sexual identity in the germline is specified by a regulatory pathway of ~14 genes whose activity is set in response to the ratio of X chromosomes to autosomes (refer to Figure 9) (reviewed by Kuwabara and Kimble 1992; Clifford et al. 1994). The known terminal regulators of the pathway are a group of five genes (fem-1, -2 and -3 and fog-1 and -3) that act together to specify the male identity in both XX and XO germ cells. In the XO male germline, activation of these terminal fem/fog genes is achieved indirectly via the her-1 gene, her-1, which is active only in XO animals, negatively regulates the genes tra-2 and tra-3, whose normal function is to negatively regulate one or more of the terminal fem/fog genes. As a result, the terminal fem/fog genes are active continuously in males, and XO germ cells form sperm throughout adulthood. In the hermaphrodite, the production of first sperm and then oocytes is achieved through a different mechanism. In place of her-1, which is not active in XO animals (Trent et al. 1991), the fog-2 gene (Schedl and Kimble 1988) may transiently repress tra-2 and/or tra-3 and thereby free the terminal fem/fog genes to direct a brief period of spermatogenesis. Later, increased tra-2 activity (Doniach 1986; Schedl and Kimble 1988; Goodwin et al. 1993) and/or activation of the mog-1 gene (Graham and Kimble 1993) leads to negative regulation of one or more terminal fem/fog genes, and the germline switches to oogenesis.

Mutations in the sex determination genes allow the manipulation of germline and somatic sex independent of each other and of chromosomal sex. Therefore we have used epistasis analysis with gld-1(Tum) alleles and sex determination gene mutations to examine the effects of chromosomal, somatic and germline sex on tumor formation and to infer the relationship between the gld-1(+) function that directs oogenesis and the sex determination pathway. These data are summarized in Table 1 and detailed below. All experiments were done with the null gld-1(Tum) allele q485, and many were also performed with q268. Identical results were obtained with both alleles.

The gld-1 tumorous phenotype depends on germline sexual identity

Tumor formation in XX germlines is independent of the male fate: Tumor formation in XX hermaphrodites that lack gld-1 activity might result from a failure to specify the female germ cell fate or a defect in an early step in oocyte differentiation (Francis et al. 1995). A prediction of either hypothesis is that inactivation of any of the five genes required for spermatogenesis (fem-1, -2 and -3 and fog-1 and -3) should have no effect on tumor formation in XX gld-1(Tum) germlines. Analysis of XX double mutants (Table 1) shows that tumors still form when any one of the terminal fem/fog genes is inactivated by a null or strong if allele or when both fog-1 and fog-3 are inactivated in the same animal. Further, quantitation of germline proliferation in a fog-1(?) gld-1(Tum) double mutant indicates that fog-1(?) has no effect on tumor growth (Francis et al. 1995) (see below). These results are consistent with gld-1 being essential for oogenesis and also argue that tumor formation does not result from an intersexual mode of development that is dependent on the terminal fem/fog genes.

We also examined whether gld-1(Tum) is similarly epistatic to several other mutations that eliminate hermaphrodite spermatogenesis. These included (1) a fog-2(?) mutation (Schedl and Kimble 1988), (2) a tra-2(gf) allele (Schedl and Kimble 1988) and (3) a tra-
### TABLE 1

**Effect of sexual fate on the gld-I tumorous phenotype**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Somatic phenotype</th>
<th>Germline phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gld-I(+) or gld-I(−)</td>
<td>gld-I(+)</td>
</tr>
<tr>
<td>Wild type</td>
<td>XX Female</td>
<td>Sperm, then oocytes</td>
</tr>
<tr>
<td></td>
<td>X0 Male</td>
<td>Sperm</td>
</tr>
<tr>
<td>Feminizing mutations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fem-1(If), fem-2(If) or fem-3(If)</td>
<td>XX Female</td>
<td>Oocytes</td>
</tr>
<tr>
<td>fem-3(If)</td>
<td>X0 Female</td>
<td>Oocytes</td>
</tr>
<tr>
<td>her-1(If)</td>
<td>XX Female</td>
<td>Sperm, then oocytes</td>
</tr>
<tr>
<td></td>
<td>X0 Female</td>
<td>Sperm, then oocytes</td>
</tr>
<tr>
<td>fog-1(If)</td>
<td>XX Female</td>
<td>Oocytes</td>
</tr>
<tr>
<td></td>
<td>X0 Male</td>
<td>Oocytes</td>
</tr>
<tr>
<td>fog-2(If)</td>
<td>XX Female</td>
<td>Sperm</td>
</tr>
<tr>
<td></td>
<td>X0 Male</td>
<td>Sperm</td>
</tr>
<tr>
<td>fog-3(If)</td>
<td>XX Female</td>
<td>Oocytes</td>
</tr>
<tr>
<td></td>
<td>X0 Male</td>
<td>Oocytes</td>
</tr>
<tr>
<td>fog-1(If) fog-3(If)</td>
<td>XX Female</td>
<td>Oocytes</td>
</tr>
<tr>
<td></td>
<td>X0 Male</td>
<td>Oocytes</td>
</tr>
<tr>
<td>tra-1(gf)/+</td>
<td>XX Female</td>
<td>Oocytes</td>
</tr>
<tr>
<td></td>
<td>X0 Male</td>
<td>Oocytes</td>
</tr>
<tr>
<td>tra-2(gf)</td>
<td>XX Female</td>
<td>Oocytes</td>
</tr>
<tr>
<td></td>
<td>X0 Male</td>
<td>Sperm</td>
</tr>
<tr>
<td>tra-2(If); fem-3(If)</td>
<td>XX Female</td>
<td>Oocytes</td>
</tr>
</tbody>
</table>

**Masculinizing mutations**

<table>
<thead>
<tr>
<th>Tra</th>
<th>XX Complete Male</th>
<th>Sperm</th>
<th>Sperm (43%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tra-2(If)</td>
<td>XX Complete Male</td>
<td>Sperm (46%)</td>
<td>Sperm plus ectopic proliferation (57%)</td>
</tr>
<tr>
<td>tra-3(If)</td>
<td>XX Complete Male</td>
<td>Sperm (54%)</td>
<td>Sperm plus ectopic proliferation (71%)</td>
</tr>
<tr>
<td>tra-1(If)</td>
<td>XX Complete Male</td>
<td>Sperm (63%)</td>
<td>Sperm plus ectopic proliferation (71%)</td>
</tr>
<tr>
<td></td>
<td>XX Complete Male</td>
<td>Sperm (37%)</td>
<td>Sperm plus ectopic proliferation (71%)</td>
</tr>
<tr>
<td>Mog</td>
<td>XX Complete Male</td>
<td>Sperm</td>
<td>Sperm (92%)</td>
</tr>
<tr>
<td>fem-3(gf)</td>
<td>XX Complete Male</td>
<td>Sperm</td>
<td>Sperm plus ectopic proliferation (8%)</td>
</tr>
<tr>
<td>mog-1(If)</td>
<td>XX Complete Male</td>
<td>Sperm</td>
<td>Sperm plus ectopic proliferation (4%)</td>
</tr>
<tr>
<td>tra-3(Mog)</td>
<td>XX Complete Male</td>
<td>Sperm (99%)</td>
<td>Sperm plus ectopic proliferation (7%)</td>
</tr>
</tbody>
</table>

*See MATERIALS AND METHODS for details. The phenotype is completely penetrant unless indicated. The genotypes of fem-1, fem-2, fem-3 and tra-3 were m(−/−) z(−/−) to eliminate maternal rescue effects associated with these genes.

For fog-1(q180) gld-I(q485), 80% of tumorous germ lines make some sperm (see text). The percent of X0 males making sperm was not quantitated for fog-1(gf) gld-I(Tum) double mutants using different alleles.

The amber allele e1107 was used as the putative tra-3 null mutation. Only animals with a normal male somatic gonad were scored. Not included were the ~20% of animals with abnormal/intersexual somatic gonads.

Only animals with a normal male somatic gonad were scored. Not included were the ~50% of total tra-1(e1834) animals with abnormal gonads (HODGKIN 1987; SCHEDL et al. 1989).

tra-3(m73) was used. m73 has a Mog phenotype at 25°C (E. CAPOWSKI, B. BRENDZA and S. STROME, personal communication).

1(gf) allele that results in constitutive tra-1 activity (HODGKIN 1987). None of these mutations have any obvious effect on tumor formation in X X gld-I(Tum) germelines. This result was expected for tra-2(gf) and fog-2(If) because these mutations may lead to increased negative regulation of the terminal fem/fog genes in the XX germline (DONIACH 1986; SCHEDL and KIMBLE 1988; GOODWIN et al. 1993). The role(s) of tra-1 in germline sex determination are uncertain (HODGKIN 1987; SCHEDL et al. 1989).

**Tumor formation is independent of the X chromosome dosage:** gld-I(Tum) alleles disrupt germline de-
velopment in XX hermaphrodites but not in X0 males. To determine whether sex specificity is correlated with either the sexual phenotype of the animal or its chromosome dosage, we examined XX and X0 gld-1 (Tum) animals whose sexual phenotype had been reversed by a mutation in the sex determination pathway. For this purpose her-1 (If), fem-3 (lf) and tra-1 (gf) mutations were each used to transform X0 animals into either hermaphrodites [her-1 (Ilf)] or females [fem-3 (lf) and tra-1 (gf)/+]. When also homozygous for a gld-1 (Tum) allele, these X0 somatic females develop germline tumors identical to those of XX gld-1 (Tum) animals (Table 1). Thus tumor formation is not dependent on X chromosome dosage but is instead correlated with a female sexual phenotype. [Although not tested, we presume that similar results would be obtained using fem-1 (lf) and fem-2 (lf) mutations.] Because of these results, we have not examined mutant combinations with the upstream genes that regulate both sex determination and dosage compensation (Villeneuve and Meyer 1990).

The complementary experiments in which XX animals are transformed into phenotypic males were done using mutant alleles of tra-1, tra-2 and tra-3. Null and strong If mutations in each of the tra genes cause masculinization of both the soma and germline of XX animals. However, none of these mutations completely masculinize all XX tissues (Table 1). For example, although tra-2 (lf) XX animals make only sperm, the soma is incompletely male. For tra-1 (lf), the soma is fully male, but the germline often makes oocytes. Nonetheless, mutations in each of the tra genes were found to be fully [tra-2 (lf)] or partially [tra-1 (lf) and tra-3 (lf)] epistatic to gld-1 (Tum) alleles (Table 1). In the case of tra-2, both tra-2 (lf) and gld-1 (Tum);tra-2 (lf) XX animals have a male germline that never forms a tumor. As XX tra-2 (lf) animals have a completely normal male germline and somatic gonad, this result is consistent with the observation that gld-1 (null) X0 males are unaffected (Frances et al. 1995). Because the normal function of tra-2 is to negatively regulate the fem genes, the suppression of the gld-1 (Tum) phenotype in a tra-2 (lf) background should be dependent on fem gene activity. This expectation was confirmed by constructing a XX gld-1 (Tum);tra-2 (lf);fem-3 (lf) triple mutant. XX animals of this genotype have a female soma and a tumorous germline, showing that fem-3 activity is required for suppression of tumor formation in a tra-2 (lf) background.

Although tra-1 (lf) and tra-3 (lf) alleles can also suppress tumor formation in XX gld-1 (Tum) animals, these mutations are only partially epistatic to a gld-1 (Tum) allele. A male germline is found in 43% of gld-1 (Tum);tra-3 (lf) and in 29% of gld-1 (Tum);tra-1 (lf) XX animals. The remaining animals make sperm but also have ectopically proliferating germ cells (Table 1). This phenotype most likely reflects the fact that tra-1 (lf) and tra-3 (lf) do not completely eliminate oogenesis in the XX germline (Hodgkin 1987; Schedl et al. 1989).

Because of some, gld-1 (Tum);tra-1 (lf) or tra-3 (lf) germ cells may fail to adopt the male fate and so proliferate ectopically.

Suppression of tumor formation by germline masculinizing mutations: The above described results show that tumor formation is correlated with female development but do not address whether it is germline or somatic sex that is important. We therefore examined whether certain mutations that masculinize only the germline (Mog phenotype) are epistatic to gld-1 (Tum) alleles. The Mog mutations used (Table 1) were (1) a fem-3 (gf) allele that makes germline fem-3 activity insensitive to negative regulation (Barton et al. 1987; Arringer and Kimble 1991); (2) a If mutation in mog-1, a gene that may function in repressing the terminal fem/fog genes (Graham and Kimble 1993) and (3) a germline-specific tra-3 (lf) allele bn75 [designated tra-3 (Mog)] (B. Capowski, B. Brendza and S. Strom, personal communication). The corresponding double mutants with gld-1 (Tum) display a masculinized germ line phenotype [shown for gld-1 (Tum);fem-3 (gf) in Figure 1a] similar to that of each Mog single mutant. The germline makes excess sperm and usually displays no evidence of ectopic proliferation. Thus gld-1 (Tum) XX germ cells that are forced to adopt the male identity differentiate as sperm rather than forming tumors. The tumorous phenotype is therefore correlated with germline but not somatic sex. For all three double mutants, however, a small fraction of gonad arms (<10%) have ectopically proliferating germ cells in the most proximal region of the germline. As gld-1 (Tum) alleles cause a partial feminization of the XX germline (Frances et al. 1995), we believe that the ectopic proliferation that sometimes occurs in these double mutants arises because some fem-3 (gf), mog-1 (lf) and tra-3 (Mog) germ cells fail to adopt a male fate in a gld-1 (Tum) background. Consistent with this idea, we found that gld-1 (q485 or q365) can dominantly suppress the Mog phenotype of fem-3 (gf). In particular, unlike fem-3 (gf) animals, which make only sperm at 25°, some gld-1 (Tum)/+;fem-3 (gf) XX animals make both sperm and oocytes and are self-fertile (data not shown). Partial suppression of fem-3 (gf) in gld-1 (Tum) homozygotes may account for why some germ cells in a low percentage of animals proliferate instead of differentiate as sperm.

Tumor formation is independent of somatic sexual environment: As a final test of the sex and tissue specificity of tumor formation, we asked whether tumors form in X0 animals that have a female germline and a male soma. Mutations in the genes fog-1 and fog-3 feminize only the germline (Barton and Kimble 1990; Ellis and Kimble 1995). As a result, fog-1 (lf) and fog-3 (lf) X0 animals have a normal male soma but possess a germline that only makes oocytes. As described above, fog-1 (lf) gld-1 (null), gld-1 (null) fog-3 (lf) and fog-1 (lf) gld-1 (null) fog-3 (lf) XX animals are somatic females that form germline tumors. The corresponding X0 double
and triple mutants have a male soma, but these animals also develop germline tumors [Table 1, and shown in Figure 1 for fog-1(If) gld-1(Tum) X0 males]. Examination of DAPI-stained animals indicates that tumors form in these males by the same mechanism as in XX gld-1(Tum) germlines. In all cases, germ cells in meiotic prophase are first observed proximally in the L4 stage; these cells then appear to exit meiotic prophase and return to mitotic proliferation. In adults, more distal germ cells continue to enter meiotic prophase and may contribute to tumor growth by later returning to mitotic proliferation. Similar germline phenotypes were observed by Ellis and Kimble (1995) for X0 fog-1 gld-1(Tum) and gld-1(Tum) fog-3(If) double mutants. Based on these results, we conclude that tumor formation occurs independently of somatic sexual environment as long as the germline sex determination pathway is set in the female mode.

In the course of these experiments, we observed a surprising result: fog-1(If) gld-1(Tum) X0 males usually make some sperm. As shown in Figure 2, these males have a tumorous germline containing sperm that can be recognized by their compact nuclei and their expression of a sperm-specific antigen (Ward et al. 1986). This is not an allele-specific interaction, as spermatogenesis was observed in mutant combinations of two fog-1 alleles (q180 or q187) with three different gld-1
alleles (q485, q268 or q365) (see MATERIALS AND METHODS). Although the two fog-I alleles examined are not known to be null, both are strong If mutations (BARTON and KIMBLE 1990; ELLIS and KIMBLE 1995). Therefore it appears that in the absence of gld-I function, X0 germ cells can develop as sperm by a pathway that requires little or no fog-I activity. In contrast, sperm were never observed in XX fog-I(If) gld-I(Tum) worms or in gld-I(Tum) fog-3(If) and fog-1(If) gld-I(Tum) fog-3(If) animals of either chromosomal sex.

Summary of interactions between the sex determination genes and gld-I(Tum) mutations: These experiments demonstrate that the gld-I(null) tumorous phenotype is dependent on germline sexual identity but is not dependent on the chromosomal sex of the animal or on somatic sexual identity. For the germline, the ultimate effect of the sex determination gene mutations is to alter the activity of the terminal fem/fog genes. Because the gld-I(null) tumorous phenotype is responsive to the activity state of the terminal fem/fog genes, then gld-I(+) must act downstream of these genes on the branch that directs oogenesis (see DISCUSSION and Figure 9).

Investigation of the role of gld-I in promoting hermaphrodite spermatogenesis using two types of gld-I gf mutations

The existence of a gld-I(+) activity that promotes hermaphrodite spermatogenesis was previously inferred from a gld-I haplo-insufficiency phenotype: XX gld-I(null)/+ and Df(gld-I)/+ germlines make fewer sperm than normal (FRANCIS et al. 1995). This gld-I function may also account for the existence of two classes of gld-I(gf) alleles that cause transformations in germline sexual fates. Alleles of the first type, the gld-I(Fog) mutations, feminize the germline of both sexes, so that X0 animals usually make only oocytes and X0 males make both sperm and oocytes. Alleles of the second type, the gld-I(Mog) mutations, masculinize the XX germline, so that hermaphrodites make an excess of sperm. To learn how these gf alleles affect the sex determination pathway, we constructed double mutants between gld-I(Fog) or gld-I(Mog) and mutations in the major sex determination loci (results summarized in Table 2).

gld-I(Fog) alleles suppress germline masculinizing mutations: Genetic criteria indicate the gld-I(Fog) allele q126 produces a poisonous gld-I product that acts to partially feminize both XX and X0 germlines. Because gld-I(null) males are unaffected, the q126 mutant product must interfere with another gene product involved in germline sex determination (FRANCIS et al. 1995). As indicated in Table 2, q126 is able to partially suppress the masculinized germline phenotypes conferred by a fem-3(gf) allele or by If alleles of each of the tra genes. Whereas each of these mutants makes only sperm [or some animals make sperm and then oocytes in the case of tra-1(If) and tra-3(If) mutants], all XX double mutants with q126 make sperm and then oocytes. The suppression of tra-2(If) and tra-3(If) indicates that the q126 product cannot feminize the germline by affecting tra-2(+) and tra-3(+) activity. Instead gld-I(q126) is likely to interfere with a gene product that acts downstream or independently of tra-2 and tra-3.

gld-I(q126);fem-3(gf) XX animals are always self-fertile hermaphrodites, demonstrating that gld-I(q126) and fem-3(gf) mutually suppress one another. Mutual suppression has previously been reported for other combinations of gf mutations that affect germline sex determination and most likely represents a balance between the opposite effects of masculinizing and feminizing mutations (BARTON et al. 1987; SCHEDL and KIMBLE 1988).

Effects of gld-I(Mog) alleles on sex determination: gld-I(Mog) alleles that fall into three subclasses (C1, C2 and C3) were originally isolated as dominant suppressors of certain mutations that feminize the XX germline (FRANCIS et al. 1995). The C3 allele, oz10, retains gld-I functions that are required for oogenesis. Homozygous oz10 XX animals make excess sperm but also can make oocytes late in adulthood and become self-fertile. In contrast, C1 and C2 gld-I(Mog) homozygotes never make oocytes and, as indicated by complementation data, these alleles disrupt gld-I functions required for oogenesis (FRANCIS et al. 1995).

To determine whether gld-I(Mog) alleles can bypass the need for the fem genes or fog-I and -3 in directing spermatogenesis, we constructed the XX double mutants described in Table 2. XX animals homozygous for the C3 allele oz10 and either a fem-3(If) or fog-1(If) mutation only make oocytes; fem-3(If) and fog-1(If) are thus epistatic to oz10 with regard to germline sex determination. Similarly, no sperm are made in double mutants between C1 or C2 gld-I(Mog) and mutations in the terminal fem/fog genes. Thus, like oz10, the C1 and C2 alleles do not bypass the need for the terminal fem/fog genes in directing spermatogenesis. However, unlike oz10, XX double mutants containing a C1 or C2 allele show no cytological signs of oogenesis. Instead, the proximal germline of each of the C1 and C2 gld-I(Mog) double mutants described in Table 2 fills with undifferentiated germ cells that have a nuclear morphology characteristic of germ cells at the pachytene stage of meiotic prophase (Figure 8). In all the double mutants analyzed, germ cells in adult animals usually remain arrested in meiotic prophase. In double mutants of certain genotypes, however, the germline occasionally becomes tumorous, probably because germ cells exit meiotic prophase and return to mitotic proliferation. These phenotypes are similar to the undifferentiated pachytene arrest phenotype conferred by class B gld-I alleles (FRANCIS et al. 1995).

The absence of spermatogenesis in C1 and C2 double mutants indicates that mutations in the fem genes and
Multiple gld-1 Functions

**TABLE 2**

Interactions of gld-1 Fog and Mog alleles with sex determination gene mutations

<table>
<thead>
<tr>
<th>Genotype(^a)</th>
<th>Soma</th>
<th>Germline</th>
</tr>
</thead>
<tbody>
<tr>
<td>gld-1(Fog) gld-1(q126)</td>
<td>XX</td>
<td>Female</td>
</tr>
<tr>
<td>gld-1(q126)</td>
<td>X0</td>
<td>Male</td>
</tr>
<tr>
<td>q126; tra-2(lf)</td>
<td>XX</td>
<td>Incomplete male</td>
</tr>
<tr>
<td>q126; tra-3(lf)</td>
<td>XX</td>
<td>Incomplete male</td>
</tr>
<tr>
<td>q126; tra-1(lf)</td>
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<td>Male</td>
</tr>
<tr>
<td>q126; fem-3(gf)</td>
<td>XX</td>
<td>Female</td>
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</table>

<table>
<thead>
<tr>
<th>Phenotype(^b)</th>
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<tbody>
<tr>
<td>Soma</td>
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<td>Sperm</td>
</tr>
<tr>
<td>Male</td>
<td>Germ cells arrested in meiotic prophase</td>
</tr>
<tr>
<td>Incomplete male</td>
<td>Germ cells arrested in meiotic prophase</td>
</tr>
<tr>
<td>Incomplete male</td>
<td>Germ cells arrested in meiotic prophase</td>
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\(\text{gld-1(Mog)}\)

<table>
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<tr>
<td>(\text{gld-1(q93)})</td>
<td>XX</td>
</tr>
<tr>
<td>(\text{q93; fem-1(lf)})</td>
<td>XX</td>
</tr>
<tr>
<td>(\text{q93; fem-2(lf)})</td>
<td>XX</td>
</tr>
<tr>
<td>(\text{q93; fem-3(lf)})</td>
<td>XX</td>
</tr>
<tr>
<td>(\text{q93; fog-1(lf)})</td>
<td>X0</td>
</tr>
<tr>
<td>(\text{q93; fog-2(lf)})</td>
<td>XX</td>
</tr>
<tr>
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<td>XX</td>
</tr>
<tr>
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<tr>
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</table>

<table>
<thead>
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<tbody>
<tr>
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</tr>
<tr>
<td>(\text{oz30; fem-3(lf)})</td>
<td>XX</td>
</tr>
<tr>
<td>(\text{oz30; fog-1(lf)})</td>
<td>XX</td>
</tr>
<tr>
<td>(\text{Tumorous (2%)})</td>
<td></td>
</tr>
<tr>
<td>(\text{Tumorous (1%)})</td>
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<table>
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<td>(\text{gld-1(oz10)})</td>
<td>XX</td>
</tr>
<tr>
<td>(\text{oz10; fem-3(lf)})</td>
<td>XX</td>
</tr>
<tr>
<td>(\text{oz10; fog-1(lf)})</td>
<td>XX</td>
</tr>
<tr>
<td>(\text{oz10; fog-1(lf)})</td>
<td>X0</td>
</tr>
</tbody>
</table>

\(^a\) See MATERIALS AND METHODS for details. Refer to Table 1 for the phenotype of sex determination single mutants. Animals were grown at 20\(^{\circ}\) and scored 1 and 2 days after L4 by Nomarski optics and in some cases also by DAPI staining. For \(\text{tra-3, fem-1, -2 and -3, genotype was m(-/-) z(-/-)}\).

\(^b\) Phenotype is completely penetrant unless indicated. \(n > 100\).

\(^c\) Tumorous germ lines were observed in <1% of animals with these genotypes.

\(^d\) Oogenesis continues as in wild type (see FRANCIS et al. 1995).

\(\text{fog-1 and -3 are epistatic to C1 and C2 alleles with regard to promotion of hermaphrodite spermatogenesis. This suggests that the germ cells in the double mutant develop along the female pathway. If so, germ cell arrest at the pachytene stage of meiotic prophase would simply reflect a disruption by C1 and C2 alleles of gld-1 functions that are required for oogenesis and progression through meiotic prophase (FRANCIS et al. 1995). We favor this hypothesis based on several findings. First, the C3 allele \(\text{oz10} \) does not have a defect in oogenesis, and the \(\text{fog-1(lf); gld-1(oz10)} \) and \(\text{gld-1(oz10); fem-3(lf)} \) double mutants make functional oocytes. Second, complementation data show that C1 and C2 alleles are defective for oogenesis and that these alleles can cause germ cells to arrest at pachytene in certain combinations of \(\text{gld-1 trans-heterozygotes (FRANCIS et al. 1995)}\). Third, the pachytene arrested germ cells are not overtly intersexual as they do not express the sperm-specific antigens detected by the SP56 monoclonal antibody (data not shown) (WARD et al. 1986). Fourth, there is a parallel between the pachytene arrest phenotype of the C1 and C2 \(\text{gld-1(Mog)}\) double mutants and what}
occurs in gld-1(Tum) germlines. In either genetic background, germ cells that do not develop as male are unable to progress beyond pachytene to later stages of female meiotic prophase. However, although germ cells return to mitosis in gld-1(Tum) single mutants, germ cells in the C1 and C2 double mutants for the most part remain arrested in meiotic prophase. This difference suggests C1 and C2 gld-1(Mog) alleles retain gld-1(+) functions that prevent germ cells from exiting meiotic prophase but cannot supply the gld-1(+) functions required for further meiotic progression and oogenesis.

Dependence of tumor formation and premeiotic proliferation on the glp-1-mediated signaling pathway

In wild type, proliferating germ cells are found throughout the gonad of early larvae but are limited to the distal region in late larvae and adults as more proximal germ cells have entered the meiotic pathway. Proliferation of these germline stem cells is promoted by the glp-1-mediated signaling pathway. When the distal tip cell (DTC) of the somatic gonad is ablated with a laser microbeam, distal germ cells divide a few times, then enter meiosis and subsequently produce gametes (KIMBLE and WHITE 1981). The germline receptor for the DTC signal is a transmembrane protein, encoded by the glp-1 gene, that belongs to the lin-12/Notch family of receptor molecules (AUSTIN and KIMBLE 1987, 1989; YOCHEN and GREENWALD 1989; CRITTENDEN et al. 1994).

In gld-1(Tum) hermaphrodites, germline proliferation in early larvae is apparently identical to wild type, and the proximal germ cells enter meiotic prophase at the normal time (FRANCIS et al. 1995). However, gld-1(Tum) germ cells exit meiotic prophase, return to the mitotic cell cycle and proliferate ectopically. The distal region of the late larval and adult gld-1(Tum) germline appears similar to wild type; a mitotic stem cell population is followed more proximally by a transition zone where germ cells enter meiotic prophase, which is in turn followed by a region of pachytene germ cells. In the mutant, the germline proximal to the pachytene zone is made up of ectopically proliferating cells that have exited meiotic prophase and returned to mitosis. As a result, gld-1(Tum) hermaphrodite gonads have two distinct populations of mitotically active germ cells: a distal premeiotic population, corresponding to the stem cell population in wild type, and a proximal population comprised of germ cells undergoing ectopic proliferation (Figure 4A). To distinguish between the two populations, we refer to mitotic germ cells in early larvae and in the distal region of late larvae and adults as "premeiotic germ cells" and to the ectopically proliferating germ cells that are the result of exit from meiotic prophase as a "germline tumor" (Figure 4A). In the second half of this section, we investigate the role of the glp-1 signaling pathway in the control of premeiotic germ cell proliferation and tumor formation in gld-1(Tum) mutants.

Premeiotic proliferation is dependent on the...
DTC: If the distal premiotie germ cells in XX gld-1(Tum) mutants are similar to their wild-type counterparts, then their proliferation should depend on DTC signaling. To test this idea, we ablated the DTC in wild-type and gld-1(Tum) larvae (late L3/young L4 stage). Operated animals were allowed to develop for 24–48 hr, after which germlines were released by dissection and stained with DAPI to visualize nuclear morphology.

In ablated wild-type gonads, all distal germ cells enter meiotic prophase and reach the pachytene stage within 28–32 hr after DTC elimination; these cells later differentiate as oocytes (KIMBLE and WHITE 1981) (data not shown). Similarly, distal germ cells in operated gld-1(Tum) gonads also enter meiosis; by 28–32 hr all distal nuclei display a pachytene morphology (Figure 5, a and b). Therefore distal premeiotic proliferation in gld-1(Tum) hermaphrodites is signal dependent, indicating that it is under the same controls as in wild type. In contrast, proximal germ cells in the ablated gonads still form a tumor (Figure 5a), indicating that ectopic proliferation is not dependent on the DTC.

Further analysis of operated gld-1(Tum) gonads revealed that the distal meiotic germ cells fail to complete meiotic prophase and instead appear to return to mitosis. This is indicated by the later appearance (within 32–35 hr of DTC elimination) of dividing cells intermixed among the distal pachytene-stage germ cells (Table 3, Figure 5c). Because dividing cells display both a spindle pole morphology (data not shown) and metaphase plate configuration typical of mitotic germ cells, they appear to have returned to a mitotic cell cycle. Based on these results, we conclude the gld-1(Tum) germ cells enter the meiotic pathway in response to DTC ablation but then return to mitosis. This behavior is analogous to the gld-1(Tum) return to mitosis phenotype that occurs more proximally in the unoperated hermaphrodite gonad (FRANCIS et al. 1995).

Dependence of tumor formation on the somatic gonad: To further examine the dependence of premeiotic proliferation in gld-1(Tum) germlines on the somatic gonad, several additional ablation experiments were performed. As shown in Figure 4B, XX early L1 larvae contain two somatic precursor cells, Z1 and Z4, which give rise to the entire hermaphrodite somatic gonad (KIMBLE and HIRSH 1979). When Z1 and Z4 are ablated in wild type, the germ cell precursors, Z2 and Z3, divide one or two times but then fail to divide further or to form gametes (KIMBLE and WHITE 1981). The same result is observed when the daughters of Z1 and Z4 (Z1.a, Z1.p, Z4.a and Z4.p) are killed in wild type (data not shown). Similarly, when Z1 or Z4 (or their four daughters) are ablated in XX gld-1(Tum) L1 larvae, germ cells undergo one or two divisions but then cease dividing (data not shown). In a few cases, some germ cells in ablated gld-1(Tum) animals appeared to enter meiotic prophase, as judged from the appearance of pachytene-stage nuclei in DAPI-stained preparations.

![Figure 5](image-url)

**Figure 5.** Distal mitotic cells in gld-1(Tum) germlines enter the meiotic pathway after ablation of the somatic DTC but then return to mitosis. The DTC was ablated in gld-1(Tum); unc-22 larvae at the late L3 larval stage and operated animals were grown for an additional 28 (a and b) or 32 (c) hr. Animals were then dissected, fixed and stained with DAPI. By 28 hr (a and b), all distal cells display a pachytene morphology (representative nuclei in the plane of focus are marked with long arrows), indicating entry into meiotic prophase in response to DTC ablation. Proximal germ cells in the same gonads proliferate ectopically (open arrow in a). (Proximal germ cells entered meiotic prophase normally, at about the time of DTC ablation, whereas distal germ cells divided about two times before entering meiosis.) By 32 hr (c), mitotic figures (open arrow) begin to appear among the pachytene nuclei in the distal region, indicating a return to mitosis by the distal meiotic germ cells. Scale bar, 10 μm.

but a tumor was never formed. These results indicate that, as in wild-type, the initial proliferation in gld-1(Tum) early larvae is dependent on the somatic gonad.

We next attempted to determine whether specific lineages giving rise to different parts of the somatic gonad are important for promoting tumor formation by gld-1(Tum) XX germ cells. In wild-type L1 larvae, ablation of Z1.a and Z4.p—the precursor cells that give rise to two DTCs and parts of the sheath and spermatheca (refer to Figure 4B)—results in germ cells undergoing several rounds of division and then differentiating as sperm at the normal time during L4 (KIMBLE and WHITE 1981). When the same ablation is done in gld-1(Tum) hermaphrodites, some germ cells differentiate as sperm but most go on to form a tumor (Table
3, also see MATERIALS AND METHODS). Tumors also form after ablation of Z1.p and Z4.a, the precursor cells that give rise to the anchor cell, uterus and part of the sheath/spermatheca lineage (Table 3). Finally, ablation of the two distal tip cells (Z1.aa and Z4.pp) and the precursors to the anchor cell (Z1.pp and Z4.aa) does not prevent tumor formation. Taken together, these results indicate that somatic factors that support ectopic proliferation (tumor formation) can be supplied both by descendants of Z1.a and Z4.p and by descendants of Z1.p and Z4.a.

**Dependence of the gld-1 (Tum) phenotype on glp-1 function**

Genetic and cytological evidence indicate that the glp-1 protein acts as the germline receptor for signaling by the DTC (AUSTIN and KIMBLE 1987; CRITTENDEN et al. 1994) and potentially by other somatic gonad cells (SEYDOUX et al. 1990). Because somatic signaling is required for continued premeiotic proliferation in gld-1 (Tum) germlines, it appeared likely that premeiotic proliferation would also be dependent on glp-1 (+) activity. This idea was tested by constructing a series of double mutants between gld-1 (Tum) alleles and two glp-1 molecular null alleles (KODOMANNI et al. 1992) [designated glp-1 (0)].

**Continued premeiotic proliferation is dependent on glp-1 (+) function:** The glp-1 (0) molecular null alleles q46 and q175 are each associated with a nonsense mutation in the amino-terminal half of the glp-1 coding region (KODOMANNI et al. 1992) that abolishes accumulation of stable glp-1 protein (CRITTENDEN et al. 1994). In each single mutant, the germline precursors Z2 and Z3 divide about two times to produce two to four germ cells in each hermaphrodite gonad arm and four to eight germ cells in the single male gonad arm (AUSTIN and KIMBLE 1987). In both sexes, these germ cells enter meiosis prematurely and differentiate as sperm by the L3 stage. Table 4 lists the double mutants constructed with glp-1 (q46 or q175) and each of three gld-1 (Tum) alleles (q485, q268 or q365). All double mutants, both as XX hermaphrodites and X0 males, have a glp-1 (0f) phenotype; germ cells divide several times but then enter meiotic prophase prematurely and form sperm. This suggests that premeiotic proliferation by gld-1 (Tum) germ cells remains largely dependent on glp-1 (+) activity (also see below).

**Tumor formation is independent of glp-1 (+) function:** Because the results presented above indicate that

### Table 3

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Larval stage</th>
<th>Precursor cell(s) ablated</th>
<th>Descendants of ablated precursors cell(s)</th>
<th>No. of animals</th>
<th>Adult germline phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>gld-1(q268 or q485); unc-32</td>
<td>L3/L4</td>
<td>Z1.aa or Z4.pp</td>
<td>DTC</td>
<td>&gt;100</td>
<td>Enter meiosis, then return to mitosis*</td>
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<tr>
<td>gld-1(q268); unc-32</td>
<td>L1</td>
<td>Z1.a and Z4.p</td>
<td>DTC, one half of SH/SP</td>
<td>12</td>
<td>Ectopic proliferation*</td>
</tr>
<tr>
<td>gld-1(q172); unc-32</td>
<td>L1</td>
<td>Z1.p and Z4.a</td>
<td>AC, uterus, one half of SH/SP</td>
<td>9</td>
<td>Ectopic proliferation*</td>
</tr>
<tr>
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<td>Z1.aa and Z4.pp</td>
<td>DTC</td>
<td>11</td>
<td>Ectopic proliferation*</td>
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<td>Z1.pp and Z4.aa</td>
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<td>Ectopic proliferation*</td>
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<tr>
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<td>L2</td>
<td>Z1.aaa and Z4.aaa</td>
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<td>10</td>
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<td>gld-1(q485); unc-32</td>
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<td>Z1.aa, Z1.pp, Z4.pp and Z4.aa</td>
<td>DTC, AC, ventral uterus</td>
<td>3</td>
<td>Ectopic proliferation*</td>
</tr>
</tbody>
</table>

DTC, distal tip cell; SH/SP, sheath and spermatheca lineage; AC, anchor cell.

* Distal premeiotic germ cells enter meiotic prophase after DTC ablation but then return to mitosis (see Figure 7, b and c and text). There is no obvious effect on ectopic proliferation.

* Proximal ectopic proliferation was unaffected by the ablation. Distal premeiotic proliferation was affected only when the DTC was eliminated; however, these distal germ cells were not examined for the return to mitosis phenotype.
Multiple gld-1 Functions

Germline phenotypes of gld-1(Tum); glp-1(null) animals

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype and number of germ cells/gonad arm</th>
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<tbody>
<tr>
<td>XX unc-32 glp-1(q175 or 946)*</td>
<td>~2-4 Pachytene germ cells = ~16 sperm^*</td>
</tr>
<tr>
<td>XO unc-32 glp-1(q175 or 946)</td>
<td>~4-8 Pachytene germ cells = ~32 sperm^*</td>
</tr>
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Double mutants

| XX gld-1(q485);unc-32 glp-1(q175 or 946)| ~16 Pachytene germ cells^* = ~64 sperm^* |
| XX gld-1(q268);unc-32 glp-1(q175 or 946)| ~16 Pachytene germ cells^* = ~64 sperm^* |
| XX gld-1(q365);unc-32 glp-1(q175 or 946)| ~16 Pachytene germ cells^* = ~64 sperm^* |
| XO gld-1(q268 or q365);unc-32 glp-1(q46)| ~32 Pachytene germ cells^* = ~128 sperm^* |

Triple mutants (feminized double mutants)

| XX gld-1(q485);unc-32 glp-1(q175);fem-1^- | ~16 Pachytene germ cells^* = ectopic proliferation |
| XX gld-1(q485);unc-32 glp-1(q175);fem-1^* | ~16 Pachytene germ cells^* = ectopic proliferation |
| XX fog-1 gld-1(q485);unc-32 glp-1(q175 or 946)* | ~16 Pachytene germ cells^* = ectopic proliferation |
| XX gld-1(q268);unc-32 glp-1(q175);fem-1^- | ~16 pachytene germ cells^* = ectopic proliferation |
| XX gld-1(q268 or q365);unc-32 glp-1(q175);fem-1^- | ~16 pachytene germ cells^* = ectopic proliferation |
| XX fog-1 gld-1(q268);unc-32 glp-1(q175 or 946)* | ~16 pachytene germ cells^* = ectopic proliferation |

^* Self-progeny of unc-32 glp-1(q175 or 946)/hT2 hermaphrodites.
^* Self-progeny of gld-1(Tum)/hT2;unc-32 glp-1(q175 or 946)/hT2 hermaphrodites.
^* Number of pachytene-stage germ cells/gonad arm counted in DAPI-stained XX hermaphrodites at the mid-L3 larval stage. Most gonad arms contained 15–18 pachytene germ cells (average 16), but a few contained as many as 20.
^ Determined by counting sperm in DAPI-stained L4 animals.
^* Self-progeny of gld-1(Tum)/hT2;unc-32 glp-1(q175)/hT2;fem-3(e1996)/dpy-20 fem-3(q20gf) unc-24 hermaphrodites.
^* Self-progeny of gld-1(Tum)/hT2;unc-32 glp-1(q175)/hT2;fem-3(e2003ts)/dpy-13 unc-5 hermaphrodites.
^ Self-progeny of fog-1(q180) gld-1(Tum)/hT2;unc-32 glp-1(q175 or 946)/hT2 hermaphrodites.

Table 4 describes nine different feminized gld-1(Tum); glp-1(0) triple mutants that were constructed. These triple mutants differ in their gld-1(q485, q268 or q365) and glp-1(q175 or 946) genotypes, and in the particular feminizing mutation they carry, but all display the same tumorous germline phenotype. Although the tumors are generally smaller than those in gld-1(Tum) single mutants, germ cells continue to proliferate throughout adulthood (Figures 6 and 7a). More detailed examination of one feminized triple mutant [fog-1(q180) gld-1(q485); glp-1(q175)] revealed that all germ cells in these animals enter meiotic prophase at the same time as do germ cells in gld-1(Tum); glp-1(0) double mutants (data not shown—see MATERIALS AND METHODS). However, instead of forming sperm as in unfeminized gld-1(Tum); glp-1(0) double mutants, many of these germ cells appear to exit meiotic prophase and return to mitosis. These results indicate that neither exit from meiotic prophase nor ectopic proliferation are absolutely dependent on glp-1(+ ) activity. Therefore tumor formation cannot result solely from a failure to downregulate glp-1 activity as germ cells progress through meiotic prophase. The finding that only feminized gld-1(Tum); glp-1(0) animals form tumors also reinforces the idea that the sex determination pathway must be set in the female mode for tumors to form.

Several properties of feminized gld-1(Tum); glp-1(0) triple mutants indicate that glp-1(+ ) activity, although not essential for tumor formation, is important for maximal tumor growth. First, as shown in Figure 6, tumors grow more slowly and variably in the triple mutants than in the corresponding gld-1(Tum) strains that are glp-1(+ ). Second, unlike tumors formed in a glp-1(+) background, which are comprised mainly of small germ cells with compact nuclei of relatively homogeneous morphology, those formed in feminized gld-1(Tum); glp-1(0) triple mutants display a wide range of nuclear morphologies. Some germ cells display a pachytene morphology, others stain intensely with DAPI and may...
be polyploid and still others have 12 condensed chromosomes (possibly representing early mitotic prophase). These features suggest that an absence of glp-1 activity in gld-1(Tum) germlines sometimes results in aberrant cell cycles and occasional arrest of germ cells in pachytene. Third, the germline in these strains lacks any evident distal/proximal polarity, as all germ cells appear to enter the meiotic pathway at about the same time during larval growth (data not shown). These results indicating that glp-1 (+) activity helps promote tumor formation are consistent with the finding of Crittenden et al. (1994) that gld-1 tumors express glp-1 protein at high levels.

**gld-1 (Tum) mutations also affect premeiotic germline proliferation:** Because germ cells in X X gld-1(Tum) animals enter the meiotic pathway normally (Francis et al. 1995), there was no a priori reason to suspect that gld-1 regulates premeiotic germline proliferation. However, close examination of gld-1(Tum);glp-1(0) double and feminized triple mutants revealed a weak effect of gld-1(Tum) alleles on the proliferation of premeiotic germ cells. In glp-1(0) single mutants, the germline precursor cells Z2 and Z3 divide about two times and then differentiate as sperm (Austin and Kimble 1987). In double mutants and feminized triple mutants, however, Z2 and Z3 undergo four premeiotic divisions to produce ~16 germ cells per hermaphrodite gonad arm and 32 cells in the single male gonad arm (Table 4). In both double and feminized triple mutants, germ cells enter meiosis in relative synchrony, so that all nuclei exhibit pachytene morphology by the mid-L3 stage (Figure 7, b and c). Increased premeiotic proliferation, in contrast to tumor formation, is not dependent on germline sexual fate; it occurs in animals that produce sperm [gld-1(Tum);glp-1(0) XX or X0] and in feminized triple mutants where the male fate is inactivated.

The small increase in premeiotic proliferation in the double and feminized triple mutants argues that gld-1 (+) inhibits this aspect of germline development. However, because gld-1(null) mutations do not noticeably increase premeiotic germ cell proliferation in glp-1 (+) animals (Francis et al. 1995), the role of gld-1 in inhibiting premeiotic germline cell division must be relatively small. Because early larval germ cells do not appear to contain maternally supplied glp-1 (+) RNA or protein (Evans et al. 1994; Crittenden et al. 1994; Seydoux and Fire 1994), the elimination of gld-1 function in glp-1(0) mutants cannot affect glp-1 activity. Therefore gld-1 may repress germline mitosis (or promote entry into the meiotic pathway) by a mechanism that is at least partially independent of glp-1 activity.

**gld-1 (Tum) alleles are epistatic to certain glp-1(0) alleles:** If gld-1 negatively regulates premeiotic proliferation (either by regulating a molecule other than glp-1 RNA or protein), then elimination of gld-1 (+) activity might render germ cells more sensitive to the glp-1-dependent mitotic signal. To test this idea, we examined whether gld-1(null) can fully or partially suppress nonnull glp-1(0) mutations. Double mutants of gld-1(q485) were constructed with two classes of glp-1(0) alleles. The first class includes three strong nonconditional glp-1(0) mutations that affect the extracellular domain of the glp-1 protein (GLP-1) and the second class includes three
temperature-sensitive glp-1 alleles (diagrammed in Figure 8C).

Each nonconditional glp-1 mutation confers a strong glp-1(+/+) phenotype similar to that of glp-1(0) alleles (Austin and Kimble 1987). q172 is an in-frame deletion that removes most of the three extracellular lin-12/Notch (LN) repeats of GLP-1, q158 is a missense mutation in the second LN repeat and oz25 is a missense mutation in the seventh epidermal growth factor-like motif (Kodovanni et al. 1992). Germline tumors form in all three XX gld-1(q485);glp-1(+/+) double mutants. Germ cells in gld-1(q485);glp-1(q172) mutants proliferate at essentially the same rate as those in the gld-1(q485) single mutant, whereas proliferation in double mutants with glp-1(q158 or oz25) is somewhat reduced and shows more variability between animals (Figure 8A). Thus gld-1(q485) partially or completely suppresses the germline proliferation defects of these glp-1(+/+) alleles. The gld-1(q485);glp-1(q172) double mutant was chosen for more detailed analysis because robust proliferation was observed in every animal.

As was previously shown, glp-1(+/+) activity is required for continuous premeiotic proliferation in gld-1(Tum) germlines but is dispensable for ectopic tumorous germ cell proliferation. However, because gld-1(q485) is completely epistatic to glp-1(q172), we suspected that premeiotic proliferation in the double mutant may be close to normal. If correct, this idea would suggest that glp-1(q172) is able to function essentially like a glp-1(+/+) allele when in a gld-1(Tum) background. Several observations indicate that this is the case. First, proximal germ cells in XX gld-1(q485);glp-1(q172) mutants enter meiotic prophase at the same time as those in gld-1(null);glp-1(+/+) and wild-type hermaphrodites (data not shown). Second, the premeiotic glp-1(q172) proliferative defect can be suppressed in germlines that show no ectopic tumorous proliferation. XX gld-1(q485);glp-1(q172) males, for example, have a male germline that appears normal in all respects; in contrast, glp-1(q172) X0 males have a Glp germline that makes only 20–32 sperm (Austin and Kimble 1987). gld-1(Tum) mutations also suppress the glp-1(q172) proliferative defect in XX germlines that are exclusively undergoing meiotic development. XX gld-1(q485);glp-1(q172);fem-3(gf) hermaphrodites, like the XX gld-1(q485);fem-3(gf) double mutant (shown in Figure 1), exhibit a Mog phenotype. Third, we used ablation experiments to show that the premeiotic proliferation occurring in gld-1(q485);glp-1(q172) germlines depends on signaling by the DTC. When the DTC is ablated in the XX gld-1(null);glp-1(q172) double mutant or the gld-1(null);glp-1(q172);fem-3(gf) triple mutant, distal germ cells stop proliferating and enter meiosis (Table 3). This indicates that the glp-1(q172) product supports signal-dependent proliferation in gld-1(Tum) germlines and therefore must retain some glp-1(+/+) activity. The premeiotic proliferation behavior of glp-1(q172) in the gld-1(null) background does not result from a novel property of the glp-1(q172) allele; glp-1(q415) is also strongly suppressed by gld-1(+/+) (Table 3; Figure 8B). That glp-1(q172) can support continued premeiotic proliferation in a gld-1(null) but not a gld-1(+/+) background is consistent with the proposal that gld-1(+/+) has a role in the negative regulation of premeiotic proliferation. This function of gld-1, unlike that which prevents exit from meiotic prophase and a return to mitosis, is not sex-specific.

A second set of experiments was aimed at examining the role of the intracellular domain of the glp-1 protein in expression of the gld-1(Tum) phenotype. Recent work indicates that the GLP-1 intracellular domain, which contains six ankyrin repeats, is necessary and possibly sufficient for glp-1-mediated signaling (Kodovanni et al. 1992; Roehl and Kimble 1993). Double mu-
tants were constructed with two temperature sensitive (ts) glp-1(0f) mutations that affect an ankyrin repeat: glp-1(q224), a missense mutation in ankyrin repeat 4 that results in a strong Glp-1 phenotype (AUSTIN and KIMBLE 1987), and glp-1(bn18), a missense mutation at a different site in repeat 4 that results in a weak Glp-1 phenotype (KODOYIANNI et al. 1992). For comparison, we also constructed a double mutant with glp-1(q415), a ts mutation in the extracellular domain of GLP-1 that alters a conserved cysteine residue (Figure 8C). Of the three gld-1(null);glp-1(ts) double mutants, the greatest effect on tumor growth was observed with glp-1(q224), the ankyrin repeat mutant with a strong Glp-1 phenotype (Figure 8B). In gld-1(null);glp-1(q224) hermaphrodites, some gonad arms fail to form a tumor (all germ cells differentiated as sperm by late L4), whereas others form tumors that grow more slowly and variably than in the other two double mutants. Thus glp-1(q224) has the strongest effect on tumor formation and growth of any nonnull glp-1(0f) allele examined. This suggests that normal functioning of the intracellular ankyrin repeats is important for supporting both premeiotic proliferation in gld-1(Tum) germlines and subsequent tumor growth. Consistent with this idea, Figure 8C also shows that the other ankyrin repeat mutant, glp-1(bn18), promotes tumor growth to a lower level than does the extracellular domain mutant glp-1(q415). Because glp-1(bn18) confers a weaker Glp-1 phenotype than glp-1(q415) in a gld-1(+0 background (KODOYIANNI et al. 1992), this difference suggests the intracellular ankyrin repeats play an important role in promoting ectopic proliferation by gld-1(null) germ cells.

In summary, the analysis of gld-1(null);glp-1(0 or 0f) double mutants shows that glp-1(+) activity is important for two aspects of the gld-1(Tum) phenotype. First, it is required to maintain continued premeiotic

![Figure 8](image-url)

**FIGURE 8.**—Germline proliferation in selected gld-1(q485);glp-1(0f) hermaphrodites. The number of germ cells was determined in 10 or more gonad arms from animals grown for the indicated times after hatching. The mean number of germ cells per hermaphrodite gonad arm is shown with plus or minus one standard deviation represented by an error bar. (A) Comparison of proliferation in double mutants with a nonconditional glp-1(0f) allele. glp-1(q172) supports tumor growth as well as glp-1(0+), whereas q158 and o25 result in decreased proliferation. In a gld-1(+) background, these glp-1(0f) alleles confer a germline phenotype similar to a glp-1(00) allele, about two to four germ cells per gonad arm (AUSTIN and KIMBLE 1987). (B) Comparison of proliferation in gld-1(q485);glp-1(ts) double mutants grown at 25°C (the restrictive temperature for these glp-1 alleles). The two glp-1 alleles (q224 and bn18), which contain substitutions in an intracellular ankyrin repeat (KODOYIANNI et al. 1992), have a much stronger effect on proliferation than does q415, a missense mutation in a conserved extracellular residue of GLP-1. (C) The positions of 8 glp-1(0f) mutations examined in double mutants are shown on a schematic representation of the glp-1 protein. The extracellular GLP-1 domain contains nine EGF-like repeats (open circles), one pseudo-EGF like sequence (hatched circle) and three LN repeats. The intracellular portion contains six ankyrin repeats (also called CDC10/SWI6 repeats). See KODOYIANNI et al. (1992) for discussion of each of the glp-1(0f) mutations shown. All are point mutations except gld-1(q172), which is an in-frame deletion of 87 residues. Adapted from KODOYIANNI et al. (1992) and YOCHEM and GREENWALD (1989).
proliferation by \textit{gld-1 (Tum)} germ cells and second, it promotes a maximal level of ectopic proliferation among germ cells that have returned to mitosis. Non-null \textit{gfp-1 (if)} alleles can differ significantly in their ability to promote ectopic proliferation. In general, ectopic proliferation is promoted more strongly by mutant \textit{gfp-1} receptors with an altered extracellular domain than by receptors with an altered intracellular domain.

**DISCUSSION**

The present work extends our understanding of the roles of \textit{gld-1} in germline development. We used epistasis analysis with \textit{gld-1 (Tum)} alleles to show that the \textit{gld-1} function necessary for oocyte development acts downstream of (and in response to) the germline sex determination pathway. Therefore tumors are likely to result from a defect in oogenesis. These results do not distinguish between whether \textit{gld-1} functions to specify the oocyte fate or instead act to execute an early essential step in oocyte differentiation. We also address the role of \textit{gld-1} in promoting hermaphrodite spermatogenesis. Results from epistasis analysis using \textit{gld-1 (Fog)} and \textit{gld-1 (Mog)} alleles are consistent with the proposal that \textit{gld-1 ( + )} promotes hermaphrodite spermatogenesis by assisting the activities of the terminal \textit{fem/fog} genes. Below, we consider a model for how \textit{gld-1} activity might accomplish the seemingly contradictory tasks of directing oogenesis and promoting hermaphrodite spermatogenesis. Finally, we address the role of the somatic gonad and the \textit{gfp-1} signaling pathway in promoting tumor formation in \textit{gld-1} mutants.

The germline sex determination pathway controls the \textit{gld-1} function that directs oocyte development: Sexual fates in the \textit{C. elegans} germline are determined through the control of five terminal regulators: \textit{fem-1}, \textit{fem-2}, \textit{fem-3}, \textit{fog-1} and \textit{fog-3} (Figure 9) (for recent reviews see Kuwabara and Kimble 1992; Clifford et al. 1994). When the sex determination pathway is set in the male mode (terminal \textit{fem/fog} genes active), oogenesis is repressed and germ cells are directed to adopt the male fate and differentiate as sperm. When the pathway is set in the female mode (by inactivation of at least one of the terminal \textit{fem/fog} genes), germ cells adopt the female fate and differentiate as oocytes. The male mode is thought to be initially set by the \textit{fog-2 (XX hermaphrodite larvae)} and \textit{her-1 (X0 males)} genes being active, whereas the female mode is initially set by both genes being inactive (X X adults). Previous work has not identified a gene (s) that acts downstream of the \textit{fem/fog} genes to specify the female germline fate.

Based on the epistasis results with a null \textit{gld-1 (Tum)} allele, Figure 9 shows \textit{gld-1 (+)} acting downstream of the sex determination loci as part of the pathway for oogenesis. As this position implies, the behavior of \textit{gld-1} tumorous germ cells was found to depend on the activity state of the terminal \textit{fem/fog} genes. When the terminal \textit{fem/fog} genes are active, as occurs in the germ-lines of \textit{X0} males and certain \textit{XX} mutants (\textit{tra-2 (f)}, \textit{tra-3 (f)}, \textit{tra-3 (Mog)}, \textit{mog-1 (if)} and \textit{fem-3 (gf)}), \textit{gld-1 (Tum)} germ cells differentiate as sperm just as wild-type germ cells do. Conversely, when any one of the terminal \textit{fem/fog} genes is inactivated by mutation, \textit{gld-1 (null)} germ cells form a tumor regardless of either somatic sexual phenotype or \textit{X} chromosome dosage. Feminized \textit{X0} \textit{gld-1 (null)} germ cells, for example, form tumors when the soma is female, as in a \textit{fem-3 (if)} background, and when the soma is male, as in a \textit{fog-1 (if)} or \textit{fog-3 (if)} background. Because \textit{X0} \textit{gld-1} tumors are observed in multiple mutant backgrounds, it appears unlikely that they arise from gene- or allele-specific interactions between \textit{gld-1} and genes in the sex determination pathway. Therefore tumor formation in \textit{gld-1 (Tum)} animals appears to only require that the germline sex determination pathway is set in the female mode via inactivation of the terminal \textit{fem/fog} genes. We place \textit{gld-1 (+)} on a branch that directs oocyte development because it does not negatively regulate spermatogenesis.

Functioning at the end of the sex determination pathway, does \textit{gld-1 (+)} direct oocyte development by acting to specify the oocyte (female) fate or by acting at an early step in the differentiation of a cell that is already specified as an oocyte (also see Francis et al. 1995)? Knowledge of the sexual identity of \textit{gld-1} tumorous germ cells is key to distinguishing between these two models; if the tumorous germ cells are sexually uncommitted, then \textit{gld-1 (+)} is likely to specify the oocyte fate, but if they are already specified as female, \textit{gld-1 (+)} would function in oocyte differentiation. A third possibility, that \textit{gld-1} tumorous germ cells are intersexual, is unlikely. As elimination of any one of the terminal \textit{fem/fog} genes has no effect on the \textit{XX} tumorous phenotype, intersexuality of \textit{gld-1 (Tum)} germ cells would have to result from the inappropriate activation of an as yet unidentified gene that acts downstream of the terminal \textit{fem/fog} genes to promote male germ cell development. The genetic epistasis experiments presented here do not distinguish between the first two models as they only show that \textit{gld-1}'s role in oocyte development depends on the upstream sex determination genes being set in the female mode (terminal \textit{fem/fog} genes inactive). The uncertainty of the role of \textit{gld-1} in oocyte development is indicated in Figure 9 by placing \textit{gld-1} in a shaded box that represents both specification of the oocyte fate and oocyte differentiation.

**Analysis of the \textit{gld-1} function that promotes hermaphrodite spermatogenesis:** Experiments discussed in the accompanying paper indicate that \textit{gld-1 (+)} has a function in promoting hermaphrodite spermatogenesis. To determine the likely position of this activity in the germline sex determination pathway, we have investigated epistatic relationships between \textit{gf} Fog and Mog \textit{gld-1} alleles and mutations in sex determination loci. Interpretation of epistasis data using \textit{gf} mutations is less
**FIGURE 9.** — *gld-1* acts at the end of the sex determination cascade, on the branch that directs oogenesis. Genetic pathway for germline sex determination in *C. elegans*. Negative regulation is indicated by a bar and positive regulation by an arrow. Not shown are *tra-1*, whose role in germline sex determination is uncertain, and several upstream genes that act to transduce the X:A ratio and control the activity of the *her-1* gene (reviewed by Villeneuve and Meyer 1990). In hermaphrodite (XX) larvae, the terminal *fem*/*fog* genes are free to direct spermatogenesis because *tra-2* (and possibly *tra-3* and *mog-1*) is negatively regulated by the *fog-2* gene. In the XX adult, *tra-2* is active and functions with *tra-3* and *mog-1* to negatively regulate the *fem*/*fog* genes, resulting in the switch to oogenesis. In males (X0), *her-1* is transcriptionally active, and *her-1* protein acts to negatively regulate *tra-2*. This relieves repression of the terminal *fem*/*fog* genes, which act together to inhibit oogenesis and direct spermatogenesis (reviewed by Kunifuji and Kimble 1992; Cliftord et al. 1994). In the absence of terminal *fem*/*fog* activity, *gld-1 (+)* directs oocyte development. *gld-1* is shown in a shaded box to indicate that it may specify the oocyte fate or act at an early step in oocyte differentiation. See text for details.

straightforward than with null mutations because of uncertainties about the exact relationship between the *gf* activity and wild-type activity. However, we were not able to use gld-1 (*null*) mutations because the tumorous phenotype precludes us from determining the sexual identity of the germ cells.

Based on genetic studies, the *gf* gld-1 (*Fog*) alleles are likely to produce an abnormal gene product that interferes with specification of the male germline fate (Francis et al. 1995). In double mutants, gld-1 (*Fog*) was found to be epistatic to putative null alleles of *tra-2* and *tra-3*. Because the *gf* gld-1 (*Fog*) mutant product interferes with hermaphrodite spermatogenesis even in the absence of the *tra-2* and *tra-3* products, it must act on or poison the product of another gene that functions downstream or independently of *tra-2* and *tra-3*. By extension, gld-1 (+) is likely to act downstream or independently of *tra-2* and *tra-3*. Any of the terminal *fem*/*fog* gene products are possible candidates for activities that might be poisoned by *gf* gld-1 (*Fog*) mutant products; poisoning of terminal *fem*/*fog* gene activity would explain why gld-1 (*Fog*) alleles feminize the X0 male germline whereas the gld-1 (*null*) mutation does not.

*gf* gld-1 (*Mog*) alleles masculinize the hermaphrodite germline by disrupting the switch from spermatogenesis to oogenesis (Francis et al. 1995). The *gf* gld-1 (*Mog*) mutations may masculinize the germline by interfering with negative regulation of the terminal *fem*/*fog* genes. This hypothesis predicts that spermatogenesis in gld-1 (*Mog*) mutants should be dependent on the terminal
fem/fog genes. Double mutant analysis shows that male germ line development in gld-1(M0g) mutants does require the activity of the terminal fem/fog genes (Table 2; also see RESULTS).

How does gld-1 both promote spermatogenesis and provide functions essential for oogenesis? One speculative model to accommodate both functions is based on the idea that the gld-1 product may exist in two forms, one that promotes spermatogenesis and another that is essential for oogenesis. When the terminal fem/fog genes are active, gld-1 product/activity assumes a state that promotes the haphrodiode spermatogenesis. The altered glp-I product/activity might assist or enhance the terminal fem/fog genes, for example, and thereby form part of a positive feedback loop that promotes spermatogenesis. However, because spermatogenesis can occur in gld-1(mut) hermaphrodites and males (Table 1) (FRANCIS et al. 1995), the altered gld-1 product/activity is not essential for male germ line development. When the terminal fem/fog genes are inactive, gld-1 product exists in a state that directs oogenesis. The central feature of this model is that the activity state of terminal fem/fog genes determines whether gld-1 functions to promote haphrodiode spermatogenesis or to direct oocyte development. There is ample precedent from other systems for regulatory molecules that exist in distinct forms to produce different functions. For example, protein-protein interactions allow the yeast alpha2-alpha1 complex to repress haploid-specific genes, whereas alpha2 alone represses only a cell type specific genes (HERSKOWITZ et al. 1992). Similarly, chemical modification (phosphorylation) converts the Echerichia coli OmpR protein from a transcriptional activator to a transcriptional repressor (RAMPERSAUD et al. 1994).

Although it may seem paradoxical that gld-1 has distinct functions in female and male germ line development, a precedent is provided by the C. elegans sex determination gene xol-1, which has distinct functions in the two sexes (MILLER et al. 1988). xol-1 has a major function in directing the male modes of sex determination and dosage compensation in X0 animals and a minor function in promoting female development in XX animals.

Etiology of tumor formation and role of the glp-I gene: The gld-1(Tum) adult hermaphrodite contains two distinct populations of mitotic germline cells. The first, located at the distal end of the gonad, corresponds to the stem cell population in wild type; the second, which fills the proximal gonad, consists of ectopically dividing tumorous cells. Germ cells that have entered meiotic prophase separate the two populations of proliferative cells (Figure 4A). To distinguish the two groups of mitotically active cells, we refer to the normal mitotic population as "premeiotic germ cells" and the ectopically proliferating population as a "germ line tumor" (see RESULTS).

Our previous studies of gld-1(Tum) hermaphrodites suggested that tumors arise from germ cells that exit meiotic prophase and return to mitosis (FRANCIS et al. 1995). Several results presented here further support this idea, while also demonstrating that gld-1(Tum) alleles have no major effect on the controls that govern premeiotic proliferation. Two lines of investigation indicate that premeiotic proliferation in gld-1(Tum) hermaphrodites is, for the most part, dependent on the glp-I-mediated signaling pathway. First, when the DTC is killed in XX gld-1(Tum) larvae, distal germ cells stop proliferating and enter meiotic prophase (Figure 5). Therefore, as in wild type (KIMBLE and WHITE 1980), premeiotic proliferation by gld-1(Tum) germ cells is dependent on the signaling molecule produced by the DTC. Second, the glp-I gene, which encodes the likely receptor for the DTC signal, is also required for premeiotic proliferation in gld-1(Tum) mutants. In XX gld-1(Tum) larvae homozygous for a glp-I molecular null mutation [designated glp-I(0)] (KODIYVINNI et al. 1992; CRITTENDEN et al. 1994), germ cells divide several times but then prematurely enter meiosis and form sperm (Table 4 and Figure 7B). Taken together, these results demonstrate that gld-1(Tum) alleles do not override the normal controls that make premeiotic proliferation dependent on the glp-I signaling pathway.

In contrast to its role in premeiotic proliferation, the glp-I signaling pathway is not required for the ectopic proliferation that accounts for tumor formation in gld-1(Tum) hermaphrodites. After DTC ablation in L5/L4 larvae, distal germ cells enter meiotic prophase but then return to mitosis (Table 3 and Figure 4). Thus, in the absence of the DTC signal, germ cells can return to mitosis. Investigation of the role of glp-I activity in the return to mitosis phenotype is complicated by the fact that all germ cells in gld-1(Tum);glp-I(0) animals differentiate as sperm during larval growth. Therefore we examined XX gld-1(Tum);glp-I(0) triple mutants whose germline had been feminized by a /l mutation in one of three genes (fem-I, fem-3 or fog-I). As in the double mutants, all germ cells in the feminized triple mutants enter meiosis prematurely: by mid-L3, each hermaphrodite gonad arm contains only 15–18 germ cells, all of which reach pachytene at approximately the same time (Table 4 and Figure 7C). Because the germline sex determination pathway is set in the female mode, triple mutant germ cells do not form sperm but instead return to mitosis and give rise to a tumor (Figure 7A). The observation that all germ cells in the feminized triple mutants enter pachytene provides further evidence that tumors derive from meiotic prophase germ cells that return to mitosis. As tumor formation occurs in the absence of glp-I(+) activity, neither the initial exit from pachytene nor the subsequent ectopic proliferation can result solely from a failure to negatively regulate glp-I(+) activity during meiotic prophase.

Although glp-I activity is not essential for tumor for-
mation in gld-1 (Tum) animals, its elimination has quantitative and qualitative effects on tumor growth. Tumors grow more slowly and variably in the absence of glp-1 activity (Figure 6), and many tumorous germ cells exhibit abnormal nuclear morphologies that are not seen in a glp-1(+) background. These differences suggest glp-1 protein may be important for promoting the maximal level of cell cycling by germ cells undergoing ectopic proliferation. Indeed, CRITTENDEN et al. (1994) show that ectopically proliferating cells in gld-1 (Tum) germlines express glp-1 protein at high levels. Because glp-1 protein is not required for tumors to form, its expression by tumorous germ cells is likely to represent a secondary consequence of proliferation; in particular, ectopic proliferation may lead to a general upregulation of factors that drive mitosis, including gld-1. Because it is unclear whether proximal germ cells are exposed to gld-1 ligand, it is possible that ligand is not necessary for the promotion of ectopic proliferation by gld-1. The intracellular ankyrin repeats of glp-1, which mediate signaling (KODOMANNI et al. 1992; ROEHl and KIMBLE 1993), do seem to play a critical role in promoting gld-1 (Tum) ectopic proliferation, as glp-1 mutations affecting these repeats more severely diminish tumor growth than do mutations affecting the extracellular domain of the protein (see RESULTS).

**gld-1 functions as a sex nonspecific negative regulator of premeiotic proliferation:** An unexpected finding to emerge from these studies is that gld-1 (Tum) alleles can have a small effect on premeiotic proliferation. Elimination of gld-1(+) function clearly does not override the need for the glp-1 signaling pathway because all germ cells in gld-1 (Tum); glp-1(0) double mutants (both XX and XO) and feminized triple mutants enter meiosis prematurely. However, compared with glp-1(0) single mutants, germ cells in the double and triple mutants undergo two extra rounds of division before entering meiosis (Table 4). Although slight, this effect suggests that gld-1 may play a nonessential role in either negatively regulating premeiotic proliferation or promoting entry into meiotic prophase. Because added premeiotic divisions occur in both double mutants (which produce sperm) and feminized triple mutants (which produce tumors), this effect of gld-1 (Tum) alleles is not dependent on germline sexual fate. This suggests that the gld-1 function regulating premeiotic proliferation is distinct from the sex-specific gld-1 function required for oogenesis.

Because gld-1 (Tum) and glp-1(0) mutations have opposite effects on premeiotic proliferation, it is possible that gld-1(+) might promote entry into meiotic prophase by acting as a negative regulator of glp-1 (+) activity. However, if this is the case, gld-1 cannot be the only negative regulator of glp-1, as germ cells enter meiotic prophase normally in the gld-1(null); glp-1 (+) single mutant. Moreover, negative regulation of a molecule distinct from glp-1 must play a role in the inhibition of mitosis by gld-1. The two extra rounds of proliferation and entry into meiotic prophase observed in the gld-1(null); glp-1(0) double and feminized triple mutants are not dependent on glp-1 activity. Two lines of evidence argue that germlines of these animals completely lack glp-1 product. First, the two glp-1(0) alleles used in this study make no detectable glp-1 protein (KODOMANNI et al. 1992; CRITTENDEN et al. 1994). Second, antibody staining and RNA in situ hybridization data (EVANS et al. 1993; CRITTENDEN et al. 1994; SEYDOUX and FIRE 1994) argue that glp-1(0) mutant larval germ cells are unlikely to contain maternally derived glp-1(+) RNA (or protein made from maternal RNA).

If gld-1(+) inhibits premeiotic germ cell proliferation (either by negatively regulating a factor distinct from glp-1 or by negatively regulating glp-1 and a second molecule), we reasoned that an absence of gld-1(+) function might lead to suppression of the proliferation defects found in nonnull glp-1(0f) mutants. In fact, gld-1(null) is partially or completely epistatic to several glp-1 mutations that alter residues in the extracellular gld-1 domain (Figure 8). Because full epistasis was observed with glp-1(q172), a strong If mutation, the combination of gld-1(Tum) and glp-1(q172) was examined in most detail. The strong epistasis of gld-1(null) to glp-1(q172) is the result of two effects. First, even though q172 is a strong glp-1(If) allele, it appears to promote ectopic proliferation in tumors to approximately the same extent as does a glp-1(+) allele (Figure 8A). Second, premeiotic proliferation in this double mutant also appears normal. This was initially indicated by examination of X0 gld-1(Tum); glp-1(q172) animals, which have a normal male germline, and confirmed by ablation results that show that the DTC is essential for premeiotic proliferation in the double mutant (Table 3). The DTC ablations indicate that glp-1(q172) product must retain some residual glp-1(+) receptor function. Apparently, gld-1(null) mutants either increase residual glp-1 activity in the glp-1(0f) mutants or allow premeiotic proliferation to occur at a level of glp-1 activity that is not sufficient in a gld-1(+) background.

Certain other glp-1(0f) mutations (e.g., q115 and q158; Figure 8) that affect the extracellular domain of glp-1 also show substantial suppression of the glp-1(If) proliferation defect in a gld-1(null) background. Like q172, these alleles probably retain some glp-1(+) function. In contrast, glp-1(q224), a mutation in one of the intracellular ankyrin repeats of glp-1, is only weakly suppressed by gld-1(null).

Taken together, the various combinations of gld-1 and glp-1 mutations suggest that gld-1(+) acts to negatively regulate germ cell proliferation before entry into the meiotic pathway. Because this gld-1(+) function is not dependent on sexual fate, it must be distinct from the gld-1 function required for oogenesis. Because premeiotic gld-1 function is clearly not essential for the negative regulation of proliferation required for entry
into meiotic prophase, it is possible that this gld-1 (+) function is redundant with that of another gene(s). These results thus suggest that gld-1 acts to negatively regulate mitosis at two distinct stages of germline development: premeiotically, in both sexes, and during the pachytene stage of meiotic prophase, when the upstream sex determination cascade is set in the female mode.

**gld-1** is a cell-type specific tumor suppressor gene: Tumor formation in animals lacking gld-1 activity is not only restricted to a single tissue (the germline) but is also dependent on sexual identity. A consequence of this sex specificity is that expression of the tumorous phenotype can be manipulated by changes in cell identity. In gld-1(Tum) hermaphrodites, X0 germ cells that would normally form a tumor can instead differentiate as sperm when the germline is masculinized by a mutation in a second gene (e.g., fem-3(gf)). Thus differentiation along an alternative pathway (spermatogenesis) has the effect of suppressing tumor formation. X0 gld-1(Tum) males are unaffected, as mutant germ cells adopt the male fate and execute spermatogenesis normally. However, when the male germline fate is inactivated by a mutation in a second gene [fog-1(ly) or fog-3(ly)], X0 gld-1(Tum) germ cells form a tumor by the same mechanism as do mutant X0 germ cells. Because fog-1 and fog-3 mutations by themselves cause X0 germ cells to develop as oocytes, the likely role of fog-1 and fog-3 mutations in generating male germ-line tumors is that they set the germline sex determination pathway in the female mode. This change then generates a cell type that responds to the absence of gld-1 activity by exiting meiotic prophase and proliferating ectopically.

Could changes in cell identity play a role in multistep tumorigenesis in mammals? One example where an alteration in cell type may be important for tumor formation is the acute lymphoblastic leukemia caused by the t(10;14) (q24q11) translocation (Hatano et al. 1991). The translocation causes T cells to ectopically express the homeobox gene SPX (also called Hox11), a locus required for spleen development (Hatano et al. 1992; Roberts et al. 1994). Ectopic expression of SPX presumably results in the expression of genes that are inappropriate for T cell development and may potentially generate a hybrid cell type that is susceptible to additional events leading to tumor formation.

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