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Germ-line tumor formation caused by activation of *glp-1*, a *Caenorhabditis elegans* member of the *Notch* family of receptors

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SUMMARY

Caenorhabditis elegans **germ-line proliferation is controlled by an inductive interaction between the somatic distal tip cell and the germ line. GLP-1, a member of the Notch family of transmembrane receptors, is required continuously in the germ line to transduce the proliferative signal. In the absence of GLP-1, all proliferative germ cells exit the mitotic cell cycle and enter meiotic prophase. We have characterized an activating mutation in *glp-1, oz112gf*, that has the opposite phenotype. Homozygous *glp-1(oz112gf)* hermaphrodites and males have a completely tumorous germ line in which germ cells never leave the mitotic cycle. In *glp-1(oz112gf)* heterozygotes, germ-line polarity is established correctly, but as adults age, the distal proliferative population expands leading to a late-onset tumorous phenotype. The mutant receptor is constitutively active, promoting proliferation in the absence of ligand. The normal distal-proximal spatial restriction of GLP-1 expression is lost in tumorous and late-onset tumorous animals; ectopically proliferating germ cells contain membrane-associated GLP-1. The correlation between proliferation and expression, both in wild-type where *glp-1* signalling is limited by localized ligand and in *glp-1(oz112gf)* where signalling is ligand-independent, suggests that *glp-1* signalling positively regulates GLP-1 expression. In addition to germ-line defects, *glp-1(oz112gf)* causes inappropriate vulval cell fate specification. A missense mutation in a conserved extracellular residue, Ser642, adjacent to the transmembrane domain, is sufficient to confer the *glp-1(oz112gf)* mutant phenotypes. Two mammalian Notch family members, TAN-1 and *int-3*, are proto-oncogenes. Thus, activating mutations in both invertebrate and vertebrate Notch family members can lead to tumor formation.**

Key words: germ line, tumor, proliferation, meiosis, cell fate, oncogene, *glp-1*, Notch

INTRODUCTION

During metazoan development, tissues are formed by the coordination of proliferative and differentiative decisions. Such cell fate decisions are often controlled by signalling between nearby cells and, in many cases, the signal transduction pathways employed are evolutionarily conserved (Greenwald and Rubin, 1992). A number of vertebrate proto-oncogenes are components of conserved signal transduction pathways (Bishop, 1991). During tumor formation, the activity of signalling pathways can be deregulated, leading to inappropriate proliferative and differentiative decisions.

The Notch family of transmembrane receptors function in cell signalling during vertebrate and invertebrate development (Artavanis-Tsakonas et al., 1995). Certain mammalian Notch family members are proto-oncogenes; activating mutations in the human TAN-1 and mouse *int-3* loci are associated with T-cell leukemia and mammary epithelial tumors, respectively (Ellisen et al., 1991; Robbins et al., 1992; Jhappan et al., 1992). The *Drosophila Notch* gene and the *C. elegans* Notch family members *glp-1* and *lin-12* function in cell fate decisions; loss-of-function (lf) mutations cause inappropriate cell fate specification resulting in abnormal differentiation (Lambie and Kimble, 1991a; Greenwald and Rubin, 1992). Previously described gain-of-function (gf) activating mutations/transgenes for *Notch*, *glp-1* and *lin-12* can cause cell fate alterations that are opposite those of loss-of-function mutations but do not lead to tumor formation (Greenwald, 1994). In this paper, we describe an activating gain-of-function mutation in *glp-1* that causes both germ-line tumor formation and a somatic multivulva phenotype.

GLP-1 functions in several cell fate decisions during *C. elegans* development (Bowerman, 1995; Lambie and Kimble, 1991a). Here we focus on the function of GLP-1 in the decision between germ-line proliferation versus entry into the meiotic pathway.

GLP-1 is the germ-line receptor for an inductive signal from the somatic gonadal distal tip cell (DTC). The signalling molecule produced by the DTC, LAG-2, is related to the transmembrane protein Delta, a ligand for Notch (Tax et al., 1994; Henderson et al., 1994). Killing the DTC with a laser microbeam (Kimble and White, 1981) and loss-of-function mutations in *glp-1* (Austin and Kimble, 1987) and *lag-2* (Lambie and Kimble, 1991b) result in essentially identical phenotypes: all proliferative germ cells enter meiosis. The DTC-GLP-1 signalling pathway thus acts to specify the pro-

liferative germ cell fate (or inhibit entry into meiotic prophase).

Germ-line polarity observed in wild-type late larval and adult worms (Fig. 1A,C) is generated by the DTC-GLP-1 signalling pathway. At the distal end of the gonad, capped by the DTC, is a population of proliferating germ-line stem cells that extends ~24 cell diameters from the distal tip (Crittenden et al., 1994). As germ cells move proximally, they exit the mitotic cycle, enter meiotic prophase and progress through pachytene. Gametogenesis and further meiotic progression occur as germ cells move through the proximal germ line. By localizing the source of ligand to the DTC (Henderson et al., 1994), GLP-1 signalling, and thus proliferation, is spatially restricted. Although the exact mechanism is unclear (see Crittenden et al., 1994; Fitzgerald and Greenwald, 1995), it appears that at increasing distance from the DTC, the level of ligand falls, GLP-1 signalling decreases and germ cells exit the mitotic cycle and enter meiosis. Consistent with this model, transgenic animals that produce a secreted form of ligand, which presumably can diffuse to more proximal regions of the gonad, display ectopic germ-line proliferation (Fitzgerald and Greenwald, 1995).

In this paper, we present characterization of a novel activating mutation in *glp-1*. The *glp-1(oz112gf)* mutation causes inappropriate specification of the proliferative germ-line stem cell fate, leading to tumor formation, and inappropriate specification of the vulval secondary cell fate, leading to abnormal differentiation in the ventral epidermis. A positive feedback model is presented to explain the observed correlation between proliferation and GLP-1 expression in wild-type and *oz112gf* mutants.

MATERIALS AND METHODS

General methods and strains

Standard methods were used for maintaining and manipulating *Caenorhabditis elegans* (Brenner, 1974). All strains were derived from the wild-type *C. elegans var. Bristol* strain N2 (Brenner, 1974), except for *glp-1(oz112gf)* which was derived from CB4855 (natural-isolate) (Hodgkin and Barnes, 1991). Mutations used in this study are listed in Tables 1-6 and described in Hodgkin et al. (1988) unless indicated. Experiments were done at 20°C unless otherwise indicated.

Isolation of *glp-1(oz112gf)* and intragenic revertants

glp-1(oz112gf) was isolated as a tumorous mutant in a screen for dominant hermaphrodite steriles following ethyl methanesulfonate (EMS) mutagenesis of CB4855. *oz112gf* was placed in the Bristol background by repeated backcrossing to N2-derived strains followed by recombining linked markers (*unc-36* and *dpy-18*) on and off the *oz112gf* chromosome.

To determine the loss-of-function phenotype of the gene identified by the *oz112gf* mutation, we screened for *cis*-intragenic revertants. *unc-32(e189) oz112gf/ eT1 let-500(s2165)* (Rosenbluth et al., 1983) hermaphrodites were mutagenized with EMS, shifted to 25°C as adults and nontumorous F_1 hermaphrodites with eggs were cloned. F_2 progeny were analyzed for phenotypes such as lethality or sterility. From 1500 mutagenized genomes, three revertants, *oz112oz120*, *oz112oz132* and *oz112oz134* were obtained with a recessive *glp-1(lf)* phenotype. Both *glp-1(oz112oz132)* and *glp-1(oz112oz134)* mutants have a strong Glp phenotype similar to *glp-1(q172)* (Kodoyianni et al., 1993). The *oz120* lesion is identical to that of *glp-1(q231ts)* (Fig. 3; Kodoyianni et al., 1992) and both have similar temperature-sensitive Glp phenotypes (Austin and Kimble, 1987).

Identification of *glp-1* missense mutations

Both strands of the *glp-1* coding region, 5′ and 3′ untranslated regions were sequenced from N2, CB4855, *glp-1(oz112gf)*, *glp-1(oz112oz120)* and *glp-1(oz112oz132)* as described in Jones and Schedl (1995) using primers kindly provided by S. Crittenden, J. Kimble and E. Maine (Kodoyianni et al., 1992; Lissemore et al., 1993). In addition to the *oz112gf* missense mutation, two silent polymorphisms were found in the ~5000 bases of CB4855 *glp-1* DNA that was sequenced.

Transgenic studies

Plasmid pPD25.47 (provided by Andy Fire) contains *glp-1(+)* in pBluescript.KS (numbering refers to this plasmid). A 300 bp fragment that spans the *oz112gf* point mutation at bp 7061 was PCR amplified from two *unc-32glp-1(oz112gf)* homozygotes (Williams et al., 1992) using the primers em4 and vk1 (Lissemore et al., 1993). The fragment was ligated into a pGLP1(+) plasmid lacking this region to form pGLP1.S642N. Individual clones of this plasmid were sequenced through the *oz112gf* region to confirm the presence of the point mutation and three independent clones were selected for microinjection.

Either pPD25.47 or pGLP1.S642N (50-100 μg/ml) was coinjected into adult N2 hermaphrodites along with pRF4 (75-200 μg/ml) as a dominant roller transformation marker (Mello et al., 1991). 55 F_1 Rol hermaphrodites, including seven heritable lines, were generated from the pPD25.47 injections. Four F_1s were Glp sterile and three lines segregated Glp steriles, but no progeny with Muv or tumorous germ-line phenotypes were observed. It is not uncommon to obtain Glp sterile phenotypes from injection of pPD25.47, but Muv and tumorous phenotypes are never observed (K. Fitzgerald, personal communication). The pGLP1.S642N injections yielded 89 F_1 Rol hermaphrodites and six heritable lines. Two F_1s were Glp sterile, three were Muv and one had a tumorous germ line. Three of the six lines segregated Glp steriles, one of these also segregated Muvs and that same line gave rise to a single animal with one tumorous gonad arm.

Characterization of *glp-1(oz112gf)* tumorous phenotype

Synchronization of animals, dissection, fixation and staining with DAPI, nuclear counts, and laser ablations are basically the same as described in Francis et al. (1995a,b). For staining with α-GLP-1 antibodies (kindly provided by S. Crittenden and J. Kimble), dissected animals were fixed and stained essentially as described by Crittenden et al. (1994) except Chemicon alkaline phosphatase goat anti-rat IgG secondary antibody was used to visualize GLP-1 staining.

RESULTS

Identification of *glp-1(oz112gf)*, a tumorous germ-line mutant

We have isolated a mutation, *oz112gf*, with a tumorous germ-line phenotype. The term ‘tumorous’ describes germ lines with three characteristics: (1) a vast excess of germ cells compared to wild-type, (2) ectopic proliferation and (3) little or no germ cell differentiation (Figs 1B,D, 2). The *oz112gf* phenotype is temperature sensitive (ts) and dose sensitive in both hermaphrodites and males (Tables 1, 3; see below).

Genetic and phenotypic characterization indicates that *oz112gf* is a novel allele of *glp-1*. First, *oz112gf* maps genetically to a two centimorgan interval containing *glp-1* (data not shown). Second, the dominant *oz112gf* tumorous phenotype is opposite that of the recessive Glp-1(lf) phenotype (premature entry of germ cells into the meiotic pathway). Third, three tightly linked (intragenic) revertants of *oz112gf* were identified

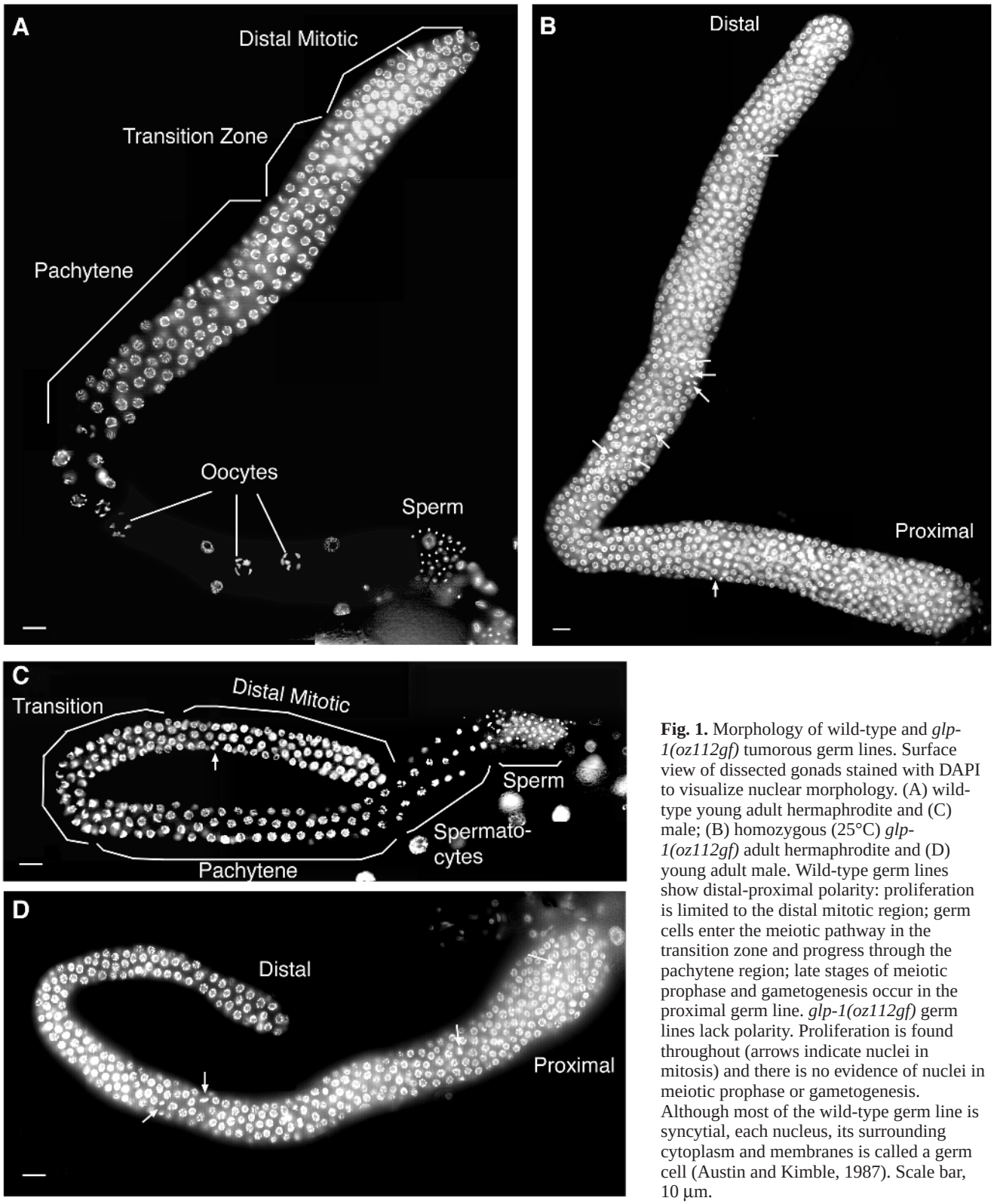


Fig. 1. Morphology of wild-type and *glp-1(oz112gf)* tumorous germ lines. Surface view of dissected gonads stained with DAPI to visualize nuclear morphology. (A) wild-type young adult hermaphrodite and (C) male; (B) homozygous (25°C) *glp-1(oz112gf)* adult hermaphrodite and (D) young adult male. Wild-type germ lines show distal-proximal polarity: proliferation is limited to the distal mitotic region; germ cells enter the meiotic pathway in the transition zone and progress through the pachytene region; late stages of meiotic prophase and gametogenesis occur in the proximal germ line. *glp-1(oz112gf)* germ lines lack polarity. Proliferation is found throughout (arrows indicate nuclei in mitosis) and there is no evidence of nuclei in meiotic prophase or gametogenesis. Although most of the wild-type germ line is syncytial, each nucleus, its surrounding cytoplasm and membranes is called a germ cell (Austin and Kimble, 1987). Scale bar, 10 μm.

(Materials and Methods) which have a recessive Glp-1(lf) phenotype and fail to complement canonical *glp-1(lf)* alleles.

Identification of the *glp-1(oz112gf)* molecular lesion

To define the molecular lesion, the *glp-1* coding region (Yochem and Greenwald, 1989) was sequenced in *oz112gf* and the parental strain, CB4855 (Hodgkin and Barnes, 1991). CB4855-derived *glp-1(+)* was sequenced to identify any polymorphisms differing from the reference strain N2. Sequence analysis revealed a single missense mutation (S642N) located

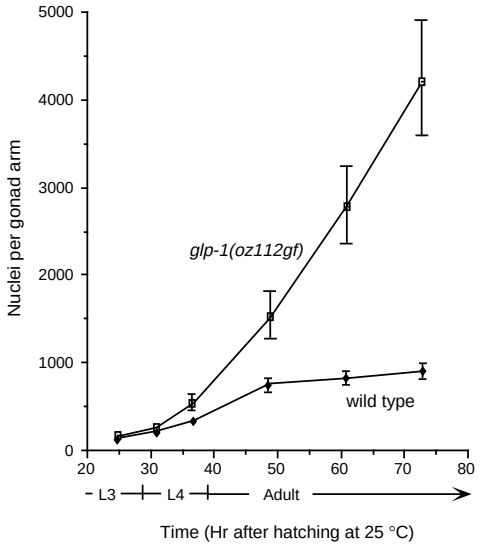


Fig. 2. Comparison of germ-line proliferation in wild-type and *glp-1(oz112gf)* hermaphrodites. The number of germ-line nuclei per gonad arm is plotted versus developmental time for wild-type (*unc-32*) and *unc-32 glp-1(oz112gf)*. *oz112gf* germ lines produce a significantly greater number of germ cells than wild-type. In older adults, extragonadal germ cells are found in the pseudocoelom, presumably because of rupture of the somatic gonad/basement membrane, and in the spermatheca and uterus. Nuclei were counted from four or more gonad arms per time point. Error bar represents ± one standard deviation.

extracellularly in a region between the third LNG repeat and the transmembrane domain (Fig. 3). An alignment of this region (data not shown) with invertebrate Notch homologs and mammalian Notch 1 homologs (Maine et al., 1995) indicates that this serine residue is conserved in these Notch family members. Gain-of-function mutations in both *lin-12* and *Notch* are also located in this general area (Greenwald and Seydoux, 1990; Lyman and Young, 1993), although the *oz112gf* molecular lesion is novel.

To verify that the identified *oz112gf* molecular lesion was responsible for the observed *glp-1(gf)* mutant phenotypes, the S642N change was introduced into a *glp-1(+)* clone, pPD25.47 (provided by A. Fire). Following microinjection into wild type, transgenic animals were observed with somatic multivulvae and low penetrance tumorous phenotypes, indicating the S642N lesion is sufficient to confer the *glp-1(oz112gf)* mutant phenotypes (see Materials and Methods).

glp-1(oz112gf) hermaphrodites and males have germ-line tumors

Two tumorous germ-line phenotypes are observed in *glp-1(oz112gf)* animals. Expressivity of the defects varies with gene dosage and temperature. At higher temperatures, homozygous *oz112gf* germ lines are completely tumorous (contain only proliferating germ cells); at lower temperatures, heterozygous and hemizygous *oz112gf* animals exhibit a late-onset tumorous phenotype. Table 1 summarizes a systematic analysis of the dose and temperature dependence of the tumorous phenotypes.

All *glp-1(oz112gf/oz112gf)* and *glp-1(oz112gf/oz112gf/+)* hermaphrodites at 25°C are sterile, with a completely tumorous germ line (Fig. 1B). Proliferation occurs throughout the gonad and there is no evidence of entry into the meiotic pathway (see below). Thus, the distal-to-proximal germ-line polarity that is normally observed in late larvae and adults is completely absent (Fig. 1), a novel *C. elegans* phenotype.

Hemizygous and heterozygous *oz112gf* hermaphrodites grown at 15°C and 20°C are initially fertile, but develop a late-onset tumorous phenotype. Normal germ-line polarity is initially established, but over time the distal mitotic region expands and eventually fills the entire gonad arm. The late-onset tumorous phenotype results in an ~4-fold reduction in brood size compared to wild type (from ~300 to ~75), probably because proliferation is occurring at the expense of oocyte development. Additionally, less than a quarter of the progeny of *oz112gf* hemizygous or heterozygous hermaphrodites are viable (Table 2). Crosses using *oz112gf/+* males indicate this lethality is due to a dominant maternal effect. Maternally derived GLP-1 is necessary for specifying cell fates in the embryo (reviewed by Bowerman, 1995). We have not investigated the basis of the embryonic lethality.

Hemizygous and heterozygous *oz112gf* hermaphrodites grown at 25°C and homozygotes grown at 15°C and 20°C are sterile. Some gonad arms are filled with proliferating cells while the remaining display limited germ cell differentiation prior to the appearance of the late-onset tumorous phenotype. Elevated *glp-1* dose and temperature causes both the late-onset tumorous phenotype to appear at a younger age and the per-

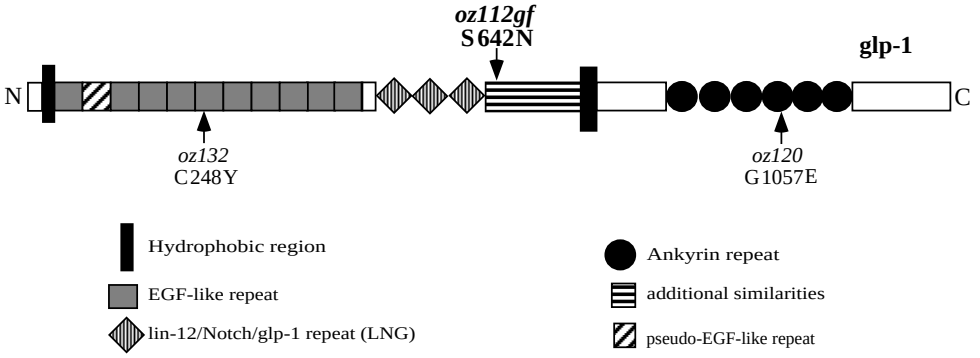


Fig. 3. Molecular lesions for *glp-1(oz112gf)* and intragenic revertants. The positions and amino acid changes are shown on a schematic diagram of GLP-1. The extracellular domain contains two cysteine-rich regions, ten EGF-like repeats and three LNG repeats. The cytoplasmic domain contains six ankyrin (cdc10/SWI6) motifs. The *glp-1(oz112gf)* missense mutation (S642N) is located extracellularly, between LNG-3 and the transmembrane domain. The *oz132* lesion in the intragenic revertant *oz112oz132* is C248T, a conserved Cys residue in the fifth EGF-like repeat. *oz120* in *oz112oz120* is G1057E in the fourth ankyrin repeat, indicating that at least one ankyrin repeat is required for *oz112gf* activity.

Table 1. *glp-1(oz112gf)* hermaphrodites and males exhibit a dosage-sensitive and temperature-sensitive tumorous germ-line phenotype

		Hermaphrodite germ-line phenotype[a]				Male germ-line phenotype[a]		
Genotype	Temperature	Self-fertile	% Late-onset tumorous[b]	% Tumorous[c]	(*n*=)[d]	% Late-onset tumorous[b]	% Tumorous[c]	(*n*=)[d]
Control[e]	15, 20, 25°C	Yes	0%	0%	(>100)	0%	0%	(>100)
$\frac{oz112}{null}$[f]	15°C	Yes	100%	0%	(>200)	ND[g]	ND	
$\frac{oz112}{oz112}$[h]	15°C	No[m]	71%	29%	(>100)	ND	ND	
$\frac{oz112}{null}$[f,i]	20°C	Yes	100%[k]	0%	(>300)	58%	42%	(>100)
$\frac{oz112}{oz112}$[h]	20°C	No[m]	39%	61%	(>100)	2%	98%	(50)
$\frac{oz112}{oz112}$[j] +	20°C	No[m]	26%	74%	(>100)	ND	ND	
$\frac{oz112}{null}$[f,i]	25°C	No[m]	16%	84%	(>100)	24%	76%	(50)
$\frac{oz112}{oz112}$[h]	25°C	No	0%	100%[l]	(>200)	0%	100%	(20)
$\frac{oz112}{oz112}$[j] +	25°C	No	0%	100%	(>100)	ND	ND	

[a]Germ-line phenotypes were analyzed using Nomarski microscopy and DAPI staining (Francis et al., 1995a,b). 100% of *glp-1(oz112gf)* gonad arms (for any of the indicated genotypes or temperatures) show overproliferation defects that have been classified as tumorous or late-onset tumorous defects.

[b]The percentage of gonad arms that are not completely tumorous. Gonad arms initially have normal distal-to-proximal polarity of proliferation, meiotic prophase progression and gametogenesis. Over time the distal mitotic zone expands proximally, often filling the entire gonad with proliferating cells and thus abolishing germ-line polarity (see text). See Fig. 5A.

[c]Gonad arms contain only mitotically cycling nuclei. See Fig. 1B,D.

[d]Number of gonad arms examined.

[e]Non-Unc hermaphrodite self-progeny from *unc-32(e189)/unc-36(e251) glp-1(q175)* and *him-8(e1489)* male self-progeny.

[f]Non-Unc self-progeny from *unc-32(e189) glp-1(oz112gf)/unc-36(e251) glp-1(q175).*

[g]Not determined.

[h]Unc-32 hermaphrodite self-progeny from *unc-32(e189) glp-1(oz112gf)/unc-36(e251) glp-1(q175)* and Unc-32 male self-progeny from *unc-32(e189) glp-1(oz112gf)/unc-36(e251) glp-1(q231); him-8(e1489).*

[i]Non-Unc male self-progeny from *unc-32(e189) glp-1(oz112gf)/unc-36(e251) glp-1(q231); him-8(e1489).*

[j]Unc non-Dpy hermaphrodites from *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/dpy-19(e1259) glp-1(q172);qDp3*. *qDp3* contains *unc-32(e189)* and wild-type alleles of *dpy-19* and *glp-1* (Austin and Kimble, 1987).

[k]Proximal proliferation observed in 1% of examined gonad arms.

[l]<1% of observed gonad arms have 1-2 germs cells in the meiotic pathway (pachytene).

[m]Sterility is the result of limited gametogenesis and excess proliferation.

centage of gonad arms containing only proliferating germ cells to increase.

glp-1(oz112gf) males also display tumorous (Fig. 1D) and late-onset tumorous phenotypes that are *glp-1* dose and temperature dependent (Table 1).

glp-1(oz112gf) acts as a genetic hypermorph

The dose dependence of the tumorous phenotype (Table 1) suggests that *oz112gf* acts as a genetic hypermorph (Muller, 1932). To quantitatively test this idea, the number of germ cells in the meiotic pathway was determined in strains with differing copies of *glp-1* (Table 3). If *glp-1(oz112gf)* is hypermorphic, the addition of *glp-1(+)* should result in a more tumorous phenotype (i.e. smaller number of germ cells in the meiotic pathway).

Wild-type young adult hermaphrodites (56 hours after hatching, 20°C) have approximately 400 germ cells in the meiotic pathway/gonad arm. Homozygous *oz112gf* hermaphrodites have 105 meiotic germ cells. In contrast, *oz112gf/oz112gf/+* have only ~23 germ cells (Table 3). Thus, the number of germ cells in the meiotic cell cycle decreases as *glp-1(+)* activity is added, consistent with *glp-1(oz112gf)* acting as a hypermorphic lesion.

Tumors arise from a failure of germ cells to exit from the mitotic cell cycle

Since *glp-1(oz112gf/oz112gf/+)* germ lines lack polarity and *glp-1(+)* functions to promote proliferation, we hypothesized that tumors arose from a failure of germ cells to leave the mitotic cycle and enter meiosis. To test this possibility, germ lines were examined for entry into the meiotic pathway at different times during late larval development in *glp-1(oz112gf/oz112gf/+)* and control hermaphrodites at 25°C (Fig. 4). In wild-type animals 32 hours after hatching (L4), 100% of the gonad arms have proximal germ cells in pachytene. By contrast, proximal pachytene nuclei are never observed in *glp-1(oz112gf/oz112gf/+)* animals, even 48 hours after hatching (adult). Instead, proximal germ cells continue

Table 2. *glp-1(oz112gf)* exhibits semi-dominant maternal effect lethality

Parental genotype	% Embryonic lethality[a,b]	% L1 larval lethality[a,c]	% Viable adults[a,d]
$\frac{oz112gf}{null}$ ♀[e]	83%	3%	14%
$\frac{oz112gf}{+}$ ♀[f]	72%	5%	23%
$\frac{+}{+}$ ♀ × $\frac{oz112gf}{+}$ ♂[g]	3%	5%	92%

[a]Single L4 or young adult hermaphrodites (genotype: *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/unc-32(e189) glp-1(q175)* or *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/unc-32(e189) mua-3(rh169)*) were placed on agar plates at 20°C. Animals were transferred every 12 hours to fresh plates. Embryonic viability was determined 24-36 hours after embryos were laid. Larvae were counted every 24 hours after hatching. The number of viable adults was determined after progeny had achieved at least the fourth larval stage. *mua-3(rh169)* was provided by J. Plenefisch.

[b]The percentage of embryonic lethality was calculated for each brood as the number of dead embryos/total number of laid embryos (brood size). Percentages are averages of >10 broods. See Table 3 for brood sizes.

[c]The percentage of larval lethality was calculated for each brood as the number of dead L1 larvae/total number of laid embryos. Percentages are averages of >10 broods. *mua-3* displays a larval lethal phenotype distinct from that observed in *oz112gf* and is not included in this column.

[d]The percentage of viable adults was determined as the number of viable adults/total number of laid embryos and is the average of >10 broods. Expected percentages of F_1 genotypes were observed in each brood.

[e]Self-progeny from *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/unc-32(e189) glp-1(q175)* (*n*=15).

[f]Self-progeny from *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/unc-32(e189) mua-3(rh169)* (*n*=13).

[g]Cross progeny generated from mating single *oz112gf/+* males to *fog-3(q443)* females (*n*=13) (Ellis and Kimble, 1995). Brood sizes were determined as described in footnote 'a' except single L4 *unc-32(e189) glp-1(oz112gf)/+* males (generated from outcrossing *unc-32(e189) glp-1(oz112gf)/unc-32(e189) glp-1(q175)* hermaphrodites to N2 males) were mated to single adult *fog-3* females. The *oz112gf/+* male tumorous phenotype can be distinguished by dissecting scope. Embryos were picked to fresh plates every 12 hours. Embryonic, larval and adult viabilities were determined as described in 'a'.

Table 3. *glp-1(oz112gf)* acts as a genetic hypermorph

Genotype	No. of germ cells in meiotic pathway[a]	Range	*n*[b]	Brood size[c]
$\frac{+}{+}$[d]	396±12$^{\alpha}$	384-408	7	ND
$\frac{oz112}{null}$[e]	206±19$^{\alpha}$	187-238	7	77±26$^{\phi}$
$\frac{oz112}{+}$[f]	180±28$^{\sigma}$	142-210	6	48±30$^{\phi}$
$\frac{oz112}{oz112}$[g]	105±11$^{\sigma,\delta}$	92-121	6	Sterile
$\frac{oz112}{oz112}$[h]	23±10$^{\delta}$	10-47	7	Sterile

[a]Young adults (56 hours after hatching at 20°C) were fixed and stained with DAPI as described by Francis et al. (1995a). The number of nuclei in the meiotic pathway were calculated as the total number of sperm (divided by four), primary spermatocytes, oocytes and nuclei in pachytene of meiosis. Each arm was counted two times and the numbers averaged.

[b]Number of gonad arms examined. One gonad arm/animal was counted.

[c]Brood size is the number of laid eggs and is the average of 15 broods. L4 hermaphrodites (genotype: *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/unc-32(e189) glp-1(q175)* or *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/unc-32(e189) mua-3(rh169)*) were cloned on Petri plates at 20°C. Hermaphrodites were transferred every 12 hours and the number of laid eggs were counted. Brood size was used as another measure of the tumorous phenotype, since ectopic proliferation occurs at the expense of gamete formation. For *oz112gf/null* and *oz112gf/+* strains, there was a significant difference in brood sizes supporting the hypothesis *glp-1(oz112gf)* acts as a genetic hypermorph.

[d]Unc-32 self-progeny from *unc-32(e189)/unc-36(e251) glp-1(q175)*.

[e]Unc-32 non-Dpy, non-Glp self-progeny from *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/unc-32(e189) glp-1(q175)*.

[f]Unc non-Dpy self-progeny from *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/unc-32(e189) mua-3(rh169)*.

[g]Unc-32 self-progeny from *unc-32(e189) glp-1(oz112gf)/unc-36(e251) glp-1(q175)*.

[h]Unc non-Dpy self-progeny from *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/dpy-19(e1259) glp-1(q172); qDp3*.

$^{\alpha,\sigma,\delta,\phi}$For the indicated pairs, values are significantly different (>95%, *t*-test).

to proliferate as evidenced by mitotic figures. This result supports the idea that *glp-1(oz112gf)* tumors arise from a failure of germ cells to exit from the mitotic cycle.

Progressive expansion of the distal mitotic region results in a late-onset tumorous phenotype

In *glp-1(oz112gf)* hemizygous hermaphrodites grown at 15°C and 20°C, 100% of gonad arms (*n*>500) develop a late-onset tumorous germ-line phenotype (Fig. 5A). We suspected that the late-onset tumorous phenotype might arise from a progressive enlargement of the distal mitotic region caused by a failure of germ cells in the adult to exit the mitotic cell cycle. We therefore determined the size of the distal mitotic region as a function of time in both wild-type and *glp-1(oz112gf)* hemizygotes. In wild-type adult hermaphrodites, the distal mitotic zone is maintained at ~24 cell diameters in length (Crittenden et al., 1994). Normal distal/proximal germ-line polarity is initially established in *glp-1(oz112gf/null)* hermaphrodites (data not shown) with the distal mitotic zone averaging ~26 cell diameters in length at 45 hours after hatching (late L4, 20°C). However, the distal mitotic zone expands to ~37 cell diameters by 50 hours (young adult), and to more than 65 cell diameters by 70 hours after hatching (Fig. 5B). In older adults,

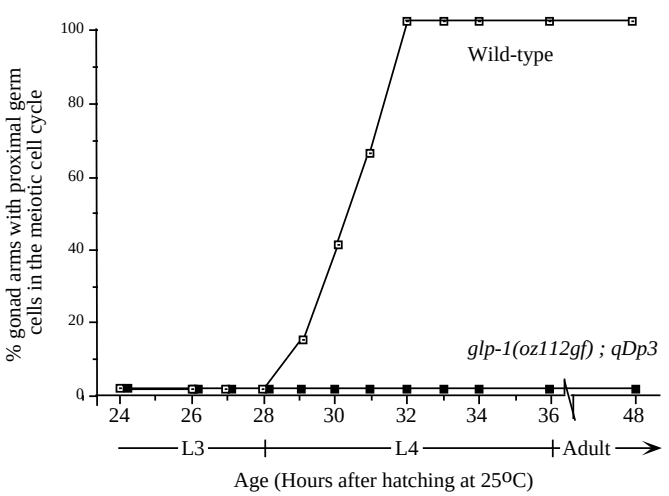
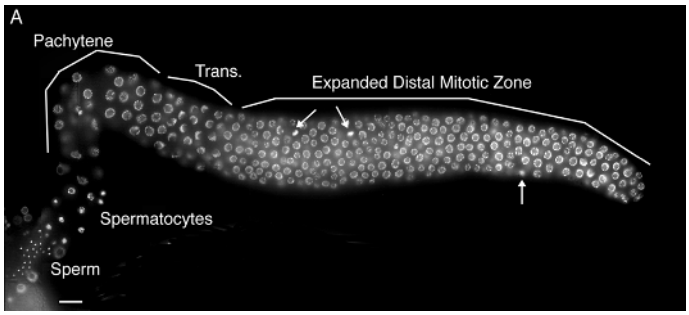


Fig. 4. *glp-1(oz112gf/oz112gf/+)* germ cells never leave the mitotic cell cycle. Synchronized wild-type (*unc-32*) and *dpy-19 unc-32 glp-1(oz112gf); qDp3* hermaphrodite larvae were examined at 1-2 hour intervals by DAPI staining for the presence of proximal pachytene nuclei. The ordinate represents the fraction of gonad arms at each time point with pachytene nuclei (*n*>8).



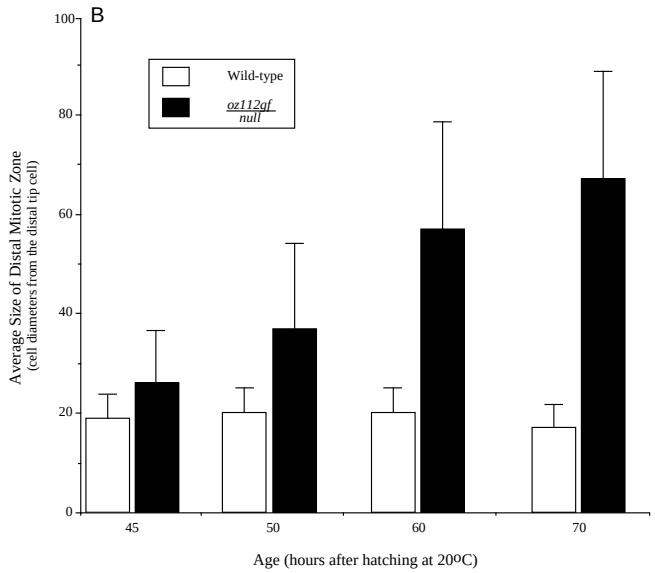


Fig. 5. The late-onset tumorous phenotype arises by a progressive expansion of the distal mitotic region. (A) Dissected gonad stained with DAPI shows the late-onset tumorous phenotype (surface view). Note the distal mitotic zone is expanded to approximately 50 cell diameters (arrows indicate mitotic nuclei) which is followed by the transition zone (Trans), the pachytene region and germ cells undergoing spermatogenesis. Scale bar, 10 μm. (B) Plot of the size of the distal mitotic region as a function of time. Gonad arms (n>20) from synchronized wild-type [*unc-32/unc-36 glp-1(q175)*] and hemizygotes [*unc-32 glp-1(oz112gf)/unc-36 glp-1(q175)*] were dissected and stained with DAPI at the indicated time. The relative distal-proximal position of mitotic figures, the transition zone and the pachytene region was determined. The size of the distal mitotic zone was the average position (cell diameters from the DTC) of the most proximal mitotic figure (only gonad arms with continuous mitotic regions are included). Error bar represents ± one standard deviation.

the germ line can consist solely of proliferating cells. The expansion of the distal mitotic zone in adult *oz112gf* hemizygotes is consistent with a progressive failure of germ cells to exit the mitotic cycle after distal-mitotic/proximal-meiotic germ-line polarity is initially established.

While 100% of *glp-1(oz112gf/null)* animals have the late-onset tumorous phenotype, only 1% display proximal proliferation [where the most proximal germ cells fail to enter meiotic prophase during larval development while more distal germ cells enter meiosis normally (Table 1; Seydoux et al., 1990)]. This preferential disruption of the decision to proliferate versus enter meiotic prophase during adulthood rather than during larval development suggests that downregulation of *glp-1* signalling in larval and adult germ cells may differ qualitatively.

Table 4. *glp-1(oz112gf)* receptor acts independent of distal tip cell ligand

Cell(s) ablated[a]	Genotype	Germline phenotype[b]	No. of animals[c]
Distal tip cell precursors[d]	+[e]	Glp[g]	4
	oz112gf[f]	Tumorous[h]	6
Distal tip cell[i]	+	Glp[g]	25
	oz112gf	Tumorous[h]	>100
Anchor cell precursor[j]	+	Wild-type[k]	4
	oz112gf	Tumorous[l]	4

[a]Ablations were performed as described by Francis et al. (1995b). Somatic cells were identified based on their positions and morphology (Kimble and Hirsh, 1979) and ablated using 20-50 laser pulses.

[b]Germ-line phenotypes were determined using Nomarski optics and DAPI staining as described by Francis et al. (1995a,b).

[c]Animals were staged at 25°C as described by Francis et al. (1995a).

[d]Z1.a and Z4.p are distal tip cell precursors (Kimble and Hirsh, 1979). Cells were ablated during L1 when the gonad consisted of 7-8 cells (4 somatic cells and 3-4 germ cells). Animals were recovered and raised at 25°C. Germ-line phenotypes were analyzed 24-36 hours after ablation. No gonadal arms were formed since the DTC is required for gonadal arm migration (Kimble and White, 1981).

[e]Unc-32(e189) self-progeny.

[f]Unc-32 self-progeny from *unc-32(e189) glp-1(oz112gf)/unc-36(e251) glp-1(q175)*.

[g]As described in Kimble and White (1981).

[h]Excess proliferation.

[i]One distal tip cell/animal was ablated during early-mid L3 (approximately 21-22 hours after hatching at 25°C). Animals were recovered and raised at 25°C. Germ-line phenotypes were analyzed 12-36 hours after ablation. Each unablated gonad arm was used as an internal control. L3 DTC ablated arms are not reflexed due to loss of DTC leader function.

[j]Z1.pp and Z4.aa are anchor cell precursors (Kimble and Hirsh, 1979). Cells were ablated during L1 when the gonad consisted of 15-17 cells. Animals were recovered and raised at 25°C. Vulval and germ-line phenotypes were analyzed 24-36 hours after ablation. This experiment was used to verify independence of the *glp-1(oz112gf)* tumorous germ-line phenotype from any somatic signal (LAG-2) produced by the anchor cell precursors (Wilkinson et al., 1994).

[k]Vulvaless (Kimble, 1981).

[l]Ablated *oz112gf* animals displayed ectopic pseudovulvae indicating the multivulval phenotype is anchor cell independent.

GLP-1*(oz112gf)* receptor signals constitutively

Two methods were used to examine whether the GLP-1*(oz112gf)* receptor requires ligand to promote proliferation. First, a laser microbeam was used to ablate the DTC, the somatic cell that produces the ligand (Kimble and White, 1981). Second, double mutants were constructed with *lag-2*, the gene that encodes the DTC proliferative signal (Tax et al., 1994; Henderson et al., 1994). If the *oz112gf* receptor acts independently of the DTC ligand, then removal of the signal will not affect the tumorous phenotype. In contrast, if the *oz112gf* receptor requires ligand, then removal of the signal should eliminate the tumorous phenotype.

Germ cells continue to proliferate in *glp-1(oz112gf)*, but not wild-type, following ablation of either the DTC during the L3 stage (Fig. 6) or the DTC precursors during L1 (Table 4). *glp-1(oz112gf)* is epistatic to *lag-2(q420)* [a germ-line proliferation defective, *glp-1(lf)*-like phenotype] and the putative null *lag-2(q411)* [L1 lethal phenotype] (Lambie and Kimble, 1991b; Henderson et al., 1994); both double mutants are tumorous

Table 5. Constitutively active *glp-1(oz112gf)* suppresses mutations in *lag-2*, but not *lag-1*

Genotype	Phenotype[a]
lag-2(q420)[b]	Glp(lf)-like sterile[c]
glp-1(oz112gf); lag-2(q420)[d]	Tumorous
lag-2(q411)[e]	L1 lethal[e]
glp-1(oz112gf); lag-2(q411)[f]	Tumorous[g]
lag-1(q416 or *q426)*[h]	Glp(lf)-like sterile[c]
glp-1(oz112gf); lag-1(q416 or *q426)*[i]	Glp(lf)-like sterile[c]
lag-1(q385)[j]	L1 lethal[c]
glp-1(oz112gf); lag-1(q385)[k]	L1 lethal[l]

[a]Phenotypes were analyzed at 25°C where the *glp-1(oz112gf)* tumorous phenotype is most severe. Phenotypes were determined using a dissecting scope and verified by Nomarski optics. Genotypes of the parental strains were confirmed by outcrossing and recovering the appropriate phenotypes.

[b]Unc-32 self-progeny from *unc-32(e189)/eT1; lag-2(q420)/eT1*.

[c]Described by Lambie and Kimble (1991).

[d]Unc-32 self-progeny from *unc-32(e189) glp-1(oz112gf)/eT1; lag-2(q420)/eT1*. 100% of examined *unc-32 glp-1(oz112gf); lag-2(q420)* animals were tumorous (n>25).

[e]Described by Henderson et al. (1994) as putative *lag-2(null)*.

[f]Dpy-Unc self-progeny from *dpy-19(e1259) unc-32(e189) glp-1(oz112gf)/glp-1(q50)eT1; lag-2(q411)/glp-1(q50)eT1*. 100% of examined Dpy-Unc animals [genotype: *dpy-19 unc-32 glp-1(oz112gf); lag-2(q411)*] were tumorous (n>25).

[g]Germline phenotype was analyzed at 20°C.

[h]Unc-32;Unc-24 self-progeny from *unc-32(e189)/unc-36(e251); unc-24(e138) lag-1(q416* or *q426)/bli-6(sc16)*.

[i]Unc-32;Unc-24 self-progeny from *unc-32(e189) glp-1(oz112gf)/unc-36(e251); unc-24(e138) lag-1(q416* or *q426)/bli-6(sc16)*. The double Unc-32;Unc-24 phenotype was difficult to distinguish from Unc-32 alone. Thus germline phenotypes of all Unc-32-like progeny were examined with 25% expected to have the desired genotype: *unc-32(e189) glp-1(oz112gf); unc-24(e138) lag-1(q416* or *q426)*. Of the 92 examined animals, 27% had a *glp-1(lf)*-like sterile phenotype while 73% were tumorous.

[j]Self-progeny from *unc-44(e1260) lag-1(q385)/deb-1(st385)* (Williams et al., 1992).

[k]Self-progeny from *glp-1(oz112gf)/unc-36(e251); unc-44(e1260) lag-1(q385)/bli-6(sc16)*.

[l]Presumed lethal since no viable Unc-44 [*glp-1(oz112gf); unc-44(e1260) lag-1(q385)*] animals were observed in >100 broods.

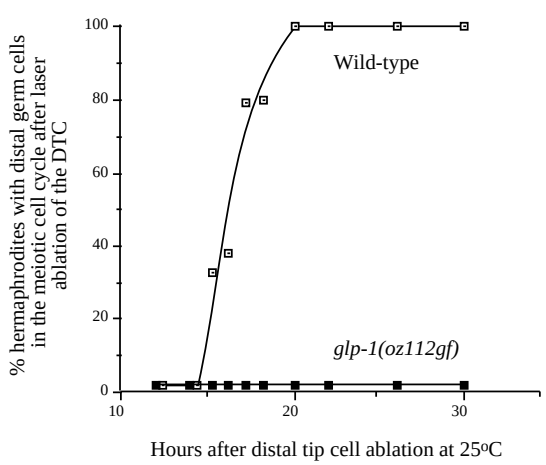


Fig. 6. DTC (ligand) independence of germ-line proliferation in *glp-1(oz112gf)*. One DTC per wild-type (*unc-32*) or *unc-32 glp-1(oz112gf)* hermaphrodite was ablated with a laser microbeam at 21-22 hours post-hatching (mid-L3). The ablated gonad arms were examined at the indicated times by DAPI staining for the presence of distal pachytene nuclei. The ordinate represents the fraction of ablated gonad arms at each time point with distal pachytene nuclei (n>7).

(Table 5). These data indicate that the GLP-1*(oz112gf)* receptor signals in the absence of zygotically supplied ligand and that *glp-1* acts downstream of *lag-2* in controlling germ-line proliferation. Thus, GLP-1*(oz112gf)* functions as a constitutively active (ligand-independent) receptor. Additionally, suppression of the L1 lethal phenotype of *lag-2(q411)* indicates that the GLP-1*(oz112gf)* receptor is activated in tissues other than the germ line (also see below).

lag-1 is epistatic to *glp-1(oz112gf)*

The *lag-1* gene also functions in the GLP-1 signalling pathway to promote germ-line proliferation (Lambie and Kimble, 1991b) and encodes a homolog of *Suppressor of Hairless* (Christensen et al., 1996), a putative DNA-binding protein thought to function downstream of *Drosophila Notch*. We found that three alleles of *lag-1*, *q416* and *q426* [both *glp-1(lf)*-like phenotype], and *q385* [L1 lethal phenotype], are epistatic to *glp-1(oz112gf)* (Table 5), suggesting that *lag-1* acts downstream of *glp-1* in controlling germ-line proliferation. This result is consistent with *glp-1(oz112gf)* acting as a genetic hypermorph that, like *glp-1(+)*, signals through LAG-1.

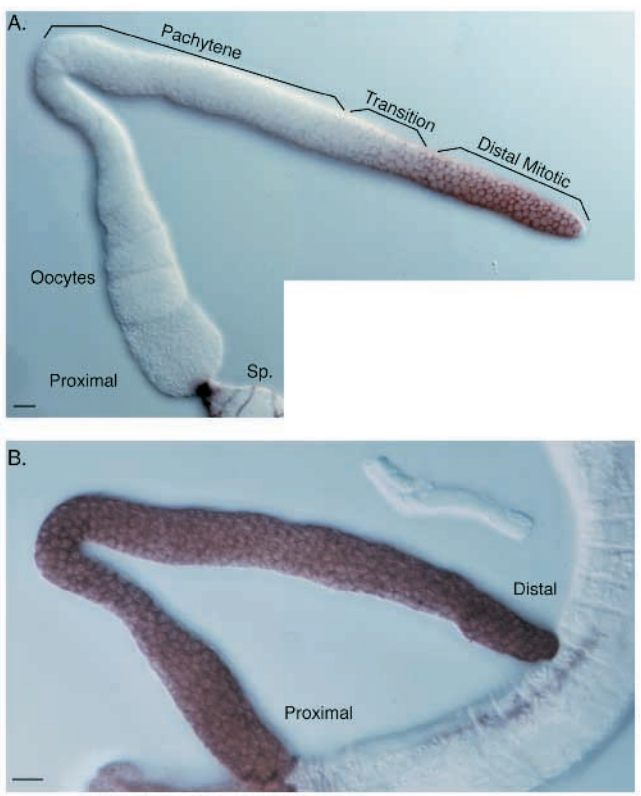


Fig. 7. Ectopic expression of GLP-1 in *glp-1(oz112gf)* germ lines. Dissected gonad arms from (A) a wild-type adult (*unc-32*) and (B) a homozygous *unc-32 glp-1(oz112gf)* young adult (grown at 25°C) were stained with anti-GLP-1 antibody (Crittenden et al., 1994). Alkaline-phosphatase-conjugated secondary antibody was used to visualize GLP-1 staining. GLP-1 is spatially restricted to the distal mitotic region of wild type, but found throughout the tumorous germ line in *glp-1(oz112gf)*. The spermathecal (Sp) staining, which outlines cell boundaries, is also seen in control animals incubated with the alkaline-phosphatase-conjugated secondary antibody alone. Scale bar, 10 μm.

GLP-1 is ectopically expressed in *glp-1(oz112gf)* hermaphrodites

In wild-type animals, Crittenden et al. (1994) demonstrated GLP-1 accumulation correlates with proliferation (Fig. 7A). Consistent with this observation, we find that GLP-1 accumulates throughout the tumorous germ lines of *glp-1(oz112gf)* animals (Fig. 7B). In addition, hemizygous *glp-1(oz112gf)* hermaphrodites were also stained with anti-GLP-1 antibody to determine whether GLP-1 accumulates in an expanded region in animals with enlarged distal mitotic zones. GLP-1 accumulation is expanded but remains correlated with proliferation in the late-onset tumors (data not shown).

glp-1(oz112gf) hermaphrodites have a somatic multivulva phenotype

In addition to a tumorous germ line, *glp-1(oz112gf)* hermaphrodites and males exhibit a semi-dominant multivulva (Muv) phenotype (Greenwald et al., 1983). The Muv phenotype is incompletely penetrant; 71% of homozygous and 3% of heterozygous *glp-1(oz112gf)* hermaphrodites have at least one pseudovulvae, while 44% of homozygous males have at least one pseudovulvae (20°C). To establish the cellular basis of the Muv phenotype, we determined the vulval lineage of three *glp-1(oz112gf)* hermaphrodites.

Briefly, six vulval precursor cells (VPCs, P3.p-P8.p) all have the potential to give rise to vulval structures. However, due to a complex set of cell-cell interactions that culminate in the specification of the 1°, 2° and 3° cell fates, a vulva is normally formed from the descendants of just three VPCs (P5.p, P6.p and P7.p; Sternberg and Horvitz, 1986). By observing the pattern of cell divisions for each of the VPCs, we found that ectopic 2° lineages were specified in *glp-1(oz112gf)* hermaphrodites (Table 6).

glp-1 has no essential function in vulval development (Austin and Kimble, 1987), although it appears to be expressed at a low level in the VPCs (Mango et al., 1991). In contrast, the related LIN-12 receptor is required for specification of the 2° cell fate. Moreover, ligand-independent *lin-12(gf)* mutations cause inappropriate specification of secondary cell fates and a multivulva phenotype (Greenwald et al., 1983) similar to *glp-1(oz112gf)*. The GLP-1 and LIN-12 receptors are interchangeable in all developmental decisions that have been analyzed to date (Mango et al., 1991; Fitzgerald et al., 1993; Roehl and Kimble, 1993). Therefore, ligand-independent hyperactive *glp-1(oz112gf)* is likely to be activating signal transduction machinery in the VPCs that would normally respond to LIN-12.

Table 6. *glp-1(oz112gf)* vulval cell lineages[a,b]

Genotype	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p
Wild-type	S S	S S	LLTN	TTTT	NTLL	S S
glp-1(oz112gf)[c]	LLLN	S S	LLTN	TTTT	NTLL	S S
	NLLL	LLTN	TTLL	TTTT	NTLL	S S
	S S	NTLL	LLTN	TTTT	NTLL	S S

[a]The complete vulval lineage (Sulston and Horvitz, 1977) of three homozygous *unc-32(e189) glp-1(oz112gf)* hermaphrodites grown at 25°C was determined. Animals were recovered after lineaging and maintained at 25°C to verify that P5.p, P6.p and P7.p differentiated normally. Similar results were obtained from partial lineages of additional animals. Specification of the P8.p lineage as secondary was also observed, but less frequently (data not shown).

[b]Nomenclature adapted from Sternberg and Horvitz (1986, 1989). S (joins hypodermal syncytium), L (longitudinal division), T (transverse division) and N (no division) refer to vulval cell types. Vulval cells are generated by both the 1° lineage ([TTTT]) and the 2° lineage ([LLTN] or [NTLL]; sometimes [LLTT] and [TTLL] are generated and are equivalent). LL adherence to the ventral cuticle is an important criterion for distinguishing 2° vulval cell types. [S S] differentiates as 3°.

[c]In some animals, development of the normal vulva (by P5.p, P6.p and P7.p) was aberrant: protruding vulval (10%, n>175) or vulvaless (13%, n>125) phenotypes were observed.

DISCUSSION

We have characterized a novel *glp-1* gain-of-function mutation, *oz112gf*, that produces tumorous germ-line and multivulval phenotypes. The *oz112gf* mutation is temperature and dose sensitive and appears to result in constitutive activation of the GLP-1 receptor; germ-line proliferation still occurs in *oz112gf* mutants following elimination of its ligand. Genetic experiments indicate that the *glp-1(oz112gf)* mutation results in hyperactive signalling. Increasing the gene dose of *glp-1(+)* increases the penetrance of the tumorous phenotype, consistent with the hypothesis that the *oz112gf* mutation causes an elevation of essentially wild-type GLP-1 signalling. Our analysis thus provides strong support for the proposal that the GLP-1 receptor acts as a binary switch (Austin and Kimble, 1987) for the decision between germ-line proliferation versus entry into meiotic prophase: loss of *glp-1* activity eliminates germ-line proliferation, while a mutation that activates *glp-1* causes proliferation to occur independent of the somatic inductive signal.

Mutational activation of *Notch* family receptors

The molecular lesion responsible for the *glp-1(oz112gf)* phenotypes is a missense mutation (Ser642Asp) located in the extracellular domain, between the third LNG repeat and the transmembrane domain (Fig. 3). This Ser residue is conserved in a number of Notch family members suggesting that it may have an important structural/regulatory function. Missense activating gain-of-function mutations have also been identified in the region from the third LNG repeat to the transmembrane domain for *lin-12* and *Notch* (Greenwald and Seydoux, 1990; Lyman and Young, 1993). While many aspects of Notch family receptor biochemistry are not known (for example, is dimerization part of the signalling mechanism?), this region appears to have a negative regulatory function, possibly by structurally constraining the intracellular domain so that signalling is responsive to ligand. Certain mutations in this region could relieve the structural constraint, allowing the intracellular domain to signal in the absence of ligand.

glp-1(oz112) is a gain-of-function mutation that leads to uncontrolled germ-line proliferation and therefore can be considered as an activated proto-oncogene. Oncogenically activated forms of mammalian Notch family genes *int-3* and *TAN-1* have also been identified. Breast cancer in mice is associated with mouse mammary tumor virus insertion into the *int-3* locus which appears to result in the expression of a truncated molecule containing only the intracellular domain (Robbins et al., 1992; Jhappan et al., 1992). Acute T lymphoblastic leukemia in humans is associated with translocation break-

points in the *TAN-1* gene which appear to result in the expression of truncated molecules that lack much of the extracellular domain (Ellisen et al., 1991). By analogy to the *glp-1(oz112gf)* germ-line phenotype, activated forms of *int-3* and *TAN-1* may disrupt the choice between a proliferative cell fate and a more differentiated cell fate. It is noteworthy that the Notch receptor family is the first evolutionarily conserved gene family in which oncogenic activating mutations occur in both vertebrates and invertebrates.

The cellular origin of the *glp-1(oz112gf)* tumors

The *glp-1(oz112gf)* tumorous phenotype appears to arise from a defect in the proliferative germ cell population. In animals showing a completely tumorous phenotype, proximal germ cells fail to enter the meiotic pathway at the normal time during larval development and distal germ cells greater than 24 cell diameters from the DTC fail to exit the mitotic cell cycle in late larvae and adults. Furthermore, the late-onset tumorous phenotype (hemizygotes and heterozygotes at 20°C) is characterized by a failure of distal germ cells to exit the mitotic cycle in adults after distal-mitotic/proximal-meiotic polarity is established.

The cellular origin of tumors in *glp-1(oz112gf)* is distinct from that of tumors in the *C. elegans gld-1* mutant. Germ cells enter the meiotic pathway normally in *gld-1(null)* hermaphrodites. However, *gld-1(null)* germ cells exit pachytene and return to the mitotic cycle resulting in ectopic proliferation (Francis et al., 1995a,b). The difference in cellular origin is reflected in the adult germ-line phenotype: *glp-1(oz112gf)* homozygotes have germ lines that lack polarity, while *gld-1(null)* mutants display normal polarity in the distal germ line. Additionally, the *glp-1(oz112gf)* mutation causes both hermaphrodite and male germline tumors, while the *gld-1(null)* mutation only causes tumors when the germ line is undergoing female development. The sex specificity is consistent with *glp-1* functioning to control proliferation in both sexes (Austin and Kimble, 1987) and *gld-1* functioning in oocyte development.

Analogous to *glp-1(oz112gf)*, certain germ cell tumors in humans may arise from a defect in the proliferative (premeiotic) germ cell population. Most testicular germ cell tumors are thought to arise from proliferative germ cells (gonocytes), while a low percentage of ovarian teratomas may arise from proliferative germ cells (Skakkebaek et al., 1987; Surti et al., 1990). Currently, the genetic/molecular mechanisms for germline tumorigenesis in humans are unknown.

GLP-1 signalling and the control of GLP-1 expression

Expression studies have revealed that GLP-1 is spatially restricted to the distal proliferative region in wild-type adult hermaphrodites (Crittenden et al., 1994). Membrane-associated GLP-1 is found at high levels in the proliferating germ cell population (1 to 20 cell diameters from the DTC) then decreases as germ cells stop proliferating and enter meiotic prophase (transition zone) and is virtually absent in the pachytene region. Localization of GLP-1 to the proliferative germ cell population in wild-type hermaphrodites appears to be due in large part to translational control; *glp-1* mRNA is found throughout the germ line and reporter RNAs carrying the *glp-1* 3′UTR are not translated in the proximal germ line (Crittenden et al., 1994; Evans et al., 1994).

GLP-1 protein is expressed ectopically, although still concordant with proliferation, in *glp-1(oz112gf)* mutants. In fully tumorous homozygotes at 25°C, membrane-associated GLP-1 and proliferating cells are found throughout the germ line while, in adult hemizygotes at 20°C, membrane-associated GLP-1 is found in an extended region that corresponds to the expanded mitotic zone. How does the *oz112gf* mutation override the normal spatial control of GLP-1 expression? We do not think that the residue altered in the *oz112gf* missense mutation has a direct effect on *glp-1* transcription or translation. Rather, we believe ectopic GLP-1 accumulation is a secondary effect of ligand-independent signalling and reveals a positive feedback mechanism between GLP-1 signalling (or proliferation) and *glp-1* expression.

We propose that the distal proliferative region is maintained in wild-type hermaphrodites by a positive feedback of GLP-1 signalling on *glp-1* expression (Fig. 8). Since the spatial restriction of GLP-1 accumulation appears to result from a translational control mechanism, the feedback loop may function at this level. Initially, ligand binding activates GLP-1 signalling, leading to proliferation and increased *glp-1* mRNA translation. Increased *glp-1* mRNA translation provides more receptor which can respond to ligand. As germ cells move away (proximally) from the localized source of ligand, GLP-1 signalling decreases, leading to reduced receptor production. When signalling by GLP-1 falls below a certain threshold, germ cells exit the mitotic cycle to enter meiotic prophase and *glp-1* mRNA translation ceases. Previously, a feedback loop that maintains *glp-1* in an active state has been proposed to explain the all-or-none phenotype of certain *glp-1(lf)* alleles (Kodoyianni et al., 1992).

A feedback mechanism provides a simple explanation for the correlation of proliferation with ectopic GLP-1 accumulation in both tumorous and late-onset tumorous *oz112gf* mutants (Fig. 8). In *oz112gf* mutants, receptor signalling is no longer limited by ligand. In fully tumorous homozygous larvae and adults (25°C), GLP-1 signalling is never down regulated, so proliferation continues and *glp-1* mRNA is always translated. As continued translation produces more of the mutationally activated receptor, proliferation is stimulated further.

Since *glp-1* mRNA is found throughout the hermaphrodite germ line (Crittenden et al., 1994), the positive feedback loop would appear to act post-transcriptionally. However, it is formally possible that germ-line *glp-1* transcription is controlled by two distinct mechanisms, one in proliferative germ cells and a second in meiotic prophase germ cells undergoing oogenesis. A positive transcriptional feedback loop could then function in proliferative germ cells, where *glp-1* mRNA translation would be unregulated. In meiotic prophase germ cells, feedback (signalling) independent *glp-1* transcription would presumably supply maternal *glp-1* mRNA necessary for embryogenesis, while repression of *glp-1* translation would be initiated immediately following entry into the meiotic pathway. Ectopic GLP-1 accumulation in tumorous germ lines would occur because germ cells never enter meiotic prophase and would continue to transcribe and translate *glp-1* mRNA. Transcriptional feedback loops that amplify the activity of the LIN-12 and GLP-1 pathways have been proposed (Wilkinson et al., 1994; Christensen et al., 1996).

We can not rule out the possibility that the positive feedback loop functions at the level of receptor stability or subcellular

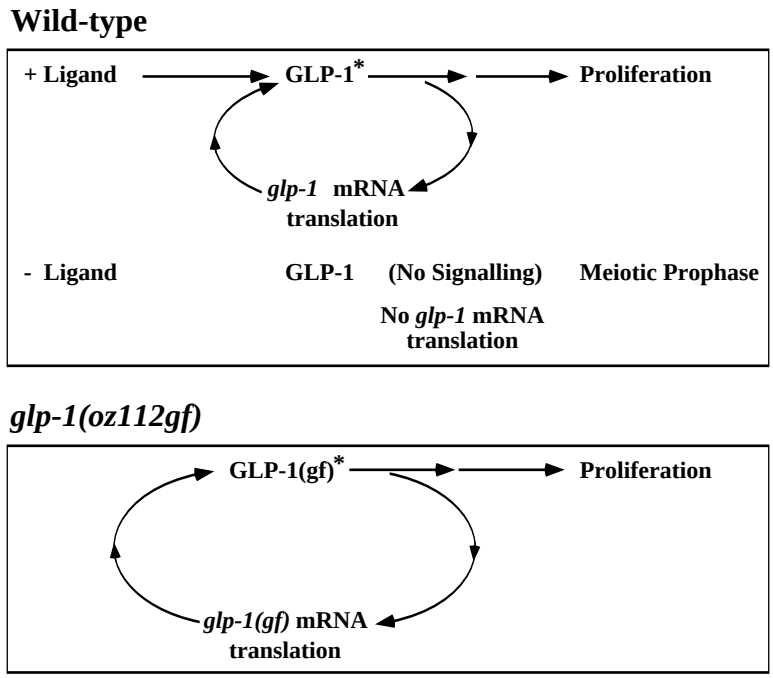


Fig. 8. Model for positive feedback of GLP-1 signalling on *glp-1* expression in the hermaphrodite germ line. In wild type, germ-line proliferation is controlled by a localized source of ligand. Ligand binding activates (*) GLP-1, promoting receptor signalling and proliferation. GLP-1 signalling then initiates a positive feedback loop that promotes translation (as shown) or transcription (not shown; see text) of *glp-1* mRNA, leading to increased GLP-1 expression. As germ cells move away from the source of ligand, signalling decreases and the positive feedback loop is down regulated leading to reduced GLP-1 levels. Eventually, germ cells enter the meiotic pathway and *glp-1* translation ceases. In *glp-1(oz112gf)* mutants, the GLP-1 receptor is constitutively active; signalling is never down regulated and both proliferation and GLP-1 synthesis are maintained. GLP-1 immunolocalization data for wild type are consistent with the model (Crittenden et al., 1994), but did not establish whether the levels of membrane-associated GLP-1 begin to fall before or after germ cells enter the meiotic pathway. Although the syncytial nature of the germ line complicates interpretation of the *glp-1* RNA/protein accumulation patterns, additional studies may resolve this question.

localization. However, if the feedback loop did not involve new protein synthesis, one would expect that proximal germ cells in *glp-1(oz112gf)* animals, which are continuing to proliferate, should contain less GLP-1 than distal mitotic cells. In contrast to this prediction, we see uniform high levels of GLP-1 staining throughout the tumorous germ line.

Implications of cell-type-specific *glp-1(oz112gf)* phenotypes for tumorigenesis in mammals

In the germ line, mutationally activated GLP-1 causes tumor formation by inappropriate specification of a proliferative stem cell and/or preventing entry into the meiotic pathway. In the ventral epidermis, activated GLP-1 causes a multivulval phenotype by inappropriate specification of a differentiative fate (2° VPC; see Results). Signalling by the GLP-1(*oz112gf*) receptor is likely to be identical in both cell types since GLP-1 and LIN-12 are interchangeable between cell types (Fitzgerald et al., 1993; Roehl and Kimble, 1993). Thus, other cell-type-specific factors must exist to account for the different phenotypic outcomes.

Certain proto-oncogenes and tumor suppressor genes in mammals are implicated in a somewhat restricted set of tumor types (Bishop, 1991). *glp-1(oz112gf)* may provide a useful model for one of the possible explanations for tissue/cell-type specificity of oncogenic mutations. During multistep tumor formation in mammals, a mutationally activated proto-oncogene may, in one cell type, cause inappropriate specification of a stem cell fate or prevent stem cell differentiation. Such an excess of proliferative stem cells would likely contribute to tumor formation (Sawyers et al., 1991), and is analogous to the excess germ-line proliferation in *glp-1(oz112gf)*. In another cell type, the same activating mutation may cause inappropriate specification of a differentiative fate, analogous to *glp-1(oz112gf)* in VPCs. However, such a change in cell fate would lead to inappropriate differentiation and thus be less likely to have a predisposing effect for tumor formation.

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