Genetic Analysis of *Caenorhabditis elegans glp-1* Mutants Suggests Receptor Interaction or Competition

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ABSTRACT

glp-1 encodes a member of the highly conserved LIN-12/Notch family of receptors that mediates the mitosis/meiosis decision in the *C. elegans* germline. We have characterized three mutations that represent a new genetic and phenotypic class of *glp-1* mutants, *glp-1(Pro)*. The *glp-1(Pro)* mutants display gain-of-function germline pattern defects, most notably a proximal proliferation (Pro) phenotype. Each of three *glp-1(Pro)* alleles encodes a single amino acid change in the extracellular part of the receptor: two in the LIN-12/Notch repeats (LNRs) and one between the LNRs and the transmembrane domain. Unlike other previously described gain-of-function mutations that affect this region of LIN-12/Notch family receptors, the genetic behavior of *glp-1(Pro)* alleles is not consistent with simple hypermorphic activity. Instead, the mutant phenotype is suppressed by wild-type doses of *glp-1*. Moreover, a *trans*-heterozygous combination of two highly penetrant *glp-1(Pro)* mutations is mutually suppressing. These results lend support to a model for a higher-order receptor complex and/or competition among receptor proteins for limiting factors that are required for proper regulation of receptor activity. Double-mutant analysis with suppressors and enhancers of *lin-12* and *glp-1* further suggests that the functional defect in *glp-1(Pro)* mutants occurs prior to or at the level of ligand interaction.

THE GLP-1 protein is a member of the conserved LIN-12/Notch family of receptors. In addition to receptor conservation, many components of the pathway including ligands, proteases, and nuclear components are also highly conserved among metazoans (Greenwald 1998; Mumm and Kopan 2000; Baron *et al.* 2002). The role of LIN-12/Notch-mediated signaling has been analyzed in many systems, and aberrant activity of the receptors and other pathway components has been linked to human disease (Joutel and Tournier-Lasserve 1998). While much has been learned in recent years regarding the mechanism of LIN-12/Notch-mediated signaling, questions still remain regarding the precise form that the receptor takes at the membrane, how the receptor is modified and processed prior to membrane localization, and how receptor activity is regulated in specific developmental contexts (Baron *et al.* 2002).

Caenorhabditis elegans has two well-characterized receptors in the LIN-12/Notch family that act in binary cell fate decisions: LIN-12 acts in somatic development (Greenwald *et al.* 1983), and GLP-1 acts in both somatic and germline cell fate decisions (Austin and Kimble 1987; Priess *et al.* 1987; Table 1). These two receptors are functionally interchangeable (Fitzgerald *et al.* 1993) and can utilize the same ligands and effectors (Lambie and Kimble 1991; Henderson *et al.* 1994; Fitzgerald and Greenwald 1995; Gao and Kimble 1995; Christensen *et al.* 1996; Doyle *et al.* 2000; Petcherski and Kimble 2000).

A general picture for signaling by LIN-12/Notch family receptors has emerged from studies in both vertebrate and invertebrate systems (Greenwald 1998; Mumm and Kopan 2000; Weinmaster 2000; Baron *et al.* 2002). A brief overview follows, with an emphasis on *C. elegans* (Figure 1). After translation, receptors are transported from the endoplasmic reticulum (ER) to the Golgi, a process that involves the p24 family of proteins (Kaiser 2000). SEL-9 is a *C. elegans* p24 protein that is likely involved in quality control at this step (Wen and Greenwald 1999). Once in the Golgi, the receptor is glycosylated; the glycosyltransferase Fringe modifies Notch (Hicks *et al.* 2000; Ju *et al.* 2000; Moloney *et al.* 2000). The receptor is then cleaved, appearing on the membrane in the form of a heterodimer. This S1 cleavage is ligand independent and is mediated by the furin class of proteases (Blaumueller *et al.* 1997; Logeat *et al.* 1998). However, furin-mediated cleavage is not necessary for signaling in all cases examined (Bush *et al.* 2001; Kidd and Lieber 2002). Although roles for *C. elegans* Fringe and furin homologs have not been reported, evidence exists for cleavage and glycosylation in this system (Crittenden *et al.* 1994).

Ligand activation by DSL (*D*elta, *S*errate, *L*AG-2) family ligands leads to a metalloprotease-dependent S2 cleavage, just N-terminal to the transmembrane domain. Specifically, a TNF-α-converting enzyme metalloprotease

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TABLE 1

Summary of *glp-1* mutant germline phenotypic classes and associated alleles used or mentioned in this study

Class[a]	*glp-1* allele(s)	Reference(s)
Putative null	*q46, q175*	Austin and Kimble (1987) Kodoyianni *et al.* (1992)
Strong loss of function	*q158, q172*	Kodoyianni *et al.* (1992)
Partial loss of function	*e2141*	Austin and Kimble (1987) Priess *et al.* (1987); Kodoyianni *et al.* (1992)
Gain of function		
Pro	*ar218, ar202*[b], *ar224*[b]	This work
Tum	*oz112*[c]	Berry *et al.* (1997)

[a] See Figure 2 for schematic representation of phenotypic classes.
[b] Mutants homozygous for these alleles display a highly penetrant Pro phenotype in the early adult and a highly penetrant late-onset expansion of the distal mitotic zone (late-onset Tum). *glp-1(+)* dosage interferes with the mutant phenotypes in the Pro class (see text for details).
[c] Mutants homozygous for this allele are highly penetrant for the Tum phenotype at the restrictive temperature and otherwise display a highly penetrant late-onset Tum phenotype (see text for details); *glp-1(+)* dosage exacerbates the mutant phenotype in the Tum class (Berry *et al.* 1997).

is thought to mediate S2 cleavage in mammalian systems, while the ADAM-family metalloprotease Kuzbanian is required for S2 cleavage in Drosophila (Pan and Rubin 1997; Brou *et al.* 2000; Mumm *et al.* 2000; Lieber *et al.* 2002). In *C. elegans*, genetic evidence is consistent with the Kuzbanian-related SUP-17 playing a similar role (Wen *et al.* 1997). Subsequent to S2 cleavage, a presenilin-dependent cleavage (S3) occurs within the transmembrane domain of the receptor, detaching the intracellular domain from the membrane and allowing translocation to the nucleus (Struhl and Greenwald 1999). Two *C. elegans* presenilins, SEL-12 and HOP-1, are functionally redundant (Levitan and Greenwald 1995; Li and Greenwald 1997; Westlund *et al.* 1999). Once the intracellular domain of the receptor reaches the nucleus, target gene activity is altered by the action of the intracellular domain in a complex with other proteins, including a CSL family member (*C*BF1, *S*uppressor of Hairless, *L*AG-1). All LIN-12 and GLP-1 signaling analyzed to date is dependent on LAG-1 (Christensen *et al.* 1996), although there are reports of CSL-independent Notch signaling in other systems (Brennan and Gardner 2002). Finally, receptor signaling can be terminated by ubiquitination. *sel-10* encodes a conserved F-box-containing protein that interacts with and facilitates ubiquitination of the intracellular do-

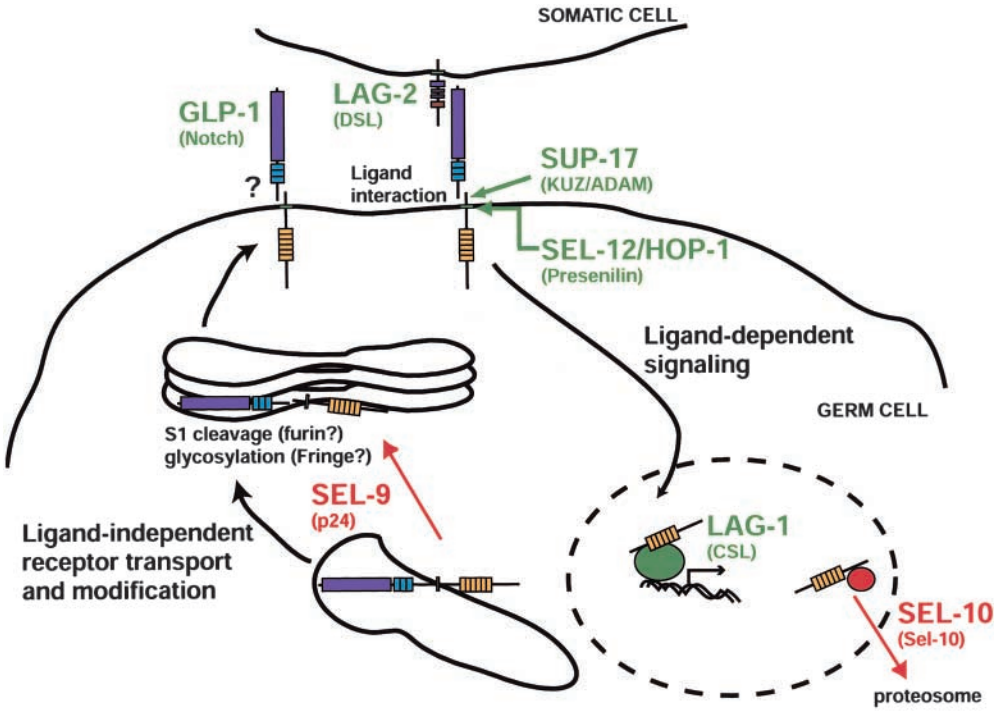

Figure 1.—Composite schematic representation of GLP-1 signaling in the *C. elegans* germline. Major events of GLP-1 receptor maturation and signaling are represented, including events inferred from work on other organisms. See text for details. Genes used in this study that encode positively acting factors are depicted in green and those that encode negatively acting factors are depicted in red. SEL-10 is depicted in the nucleus on the basis of results obtained with vertebrate Sel-10 (Gupta-Rossi *et al.* 2001). The layout of the figure was adapted from Baron *et al.* (2002).

main of LIN-12/Notch proteins in both *C. elegans* and mammals (Hubbard *et al.* 1997; Gupta-Rossi *et al.* 2001; Oberg *et al.* 2001; Wu *et al.* 2001).

In the *C. elegans* germline, GLP-1 signaling mediates the mitosis/meiosis decision. GLP-1 activity is associated with mitosis and/or inhibition of meiosis. Loss of *glp-1* causes a severe germline proliferation defect and premature entry into meiosis (Austin and Kimble 1987). One gain-of-function allele, *glp-1(oz112gf)*, causes the opposite germline phenotype: persistent mitosis and failure to enter meiosis (Berry *et al.* 1997). This gain-of-function phenotype is referred to as tumorous (Tum), and this allele behaves as a genetic hypermorph. For *glp-1(oz112gf)*, lowering the temperature or lowering the dosage of *glp-1* does not alter the initial larval pattern of germline development but results in a highly penetrant late-onset Tum phenotype in which the distal mitotic region expands as the animals age (Berry *et al.* 1997).

We have characterized three gain-of-function *glp-1* mutations that cause an array of germline defects consistent with elevated GLP-1 activity but that are phenotypically and genetically distinct from the previously characterized *glp-1(Tum)* allele. These three alleles display a proximal proliferation (Pro) phenotype that is characterized by ectopic germline proliferation in the proximalmost region of the adult *C. elegans* germline (Seydoux *et al.* 1990; Westlund *et al.* 1997). Since all three alleles display similar genetic behavior and each displays the Pro phenotype, we refer to them collectively as *glp-1(Pro)* alleles. Each *glp-1(Pro)* allele encodes a single amino acid substitution near proposed sites of extracellular proteolytic cleavage and protein-protein interactions. Although these *glp-1(Pro)* alleles cause gain-of-function phenotypes, they are genetically distinct from other mutations in LIN-12/Notch family genes that affect the same region of the protein. The alleles are dose dependent for germline pattern defects and, surprisingly, are suppressed by wild-type doses of *glp-1*. Moreover, the mutant phenotype of a *trans*-heterozygous combination of the two highly penetrant *glp-1(Pro)* alleles is suppressed compared to either homozygous mutant. These results support a model in which GLP-1 receptors normally form a dimer or other higher-order multimer and/or they compete for localized interacting factors. Finally, interactions with characterized GLP-1/LIN-12/Notch pathway components and modifiers suggest that these mutant receptors act within the canonical signaling pathway and that the phenotypes they cause are due to defects prior to or at the level of ligand interaction.

MATERIALS AND METHODS

Strains and genetic manipulations: Strains were maintained and constructed using standard genetic techniques (Brenner 1974). All strains were raised at 15° and shifted to 25° for analysis unless otherwise noted. All strains were derived from *C. elegans var. Bristol* strain N2 and described in Brenner (1974) or as cited:

LG I: *dpy-5(e61), sup-17(n1258)* (Tax *et al.* 1997).

LG III: *dpy-17(e164), ncl-1(e1865)* (Hedgecock and White 1985), *unc-36(e251), unc-32(e189), lin-12(n676n930)* (Sundaram and Greenwald 1993a), *glp-1(ar202), glp-1(ar218), glp-1(ar224), glp-1(q175, q46, e2141)* (Austin and Kimble 1987), *mog-1(q223)* (Graham and Kimble 1993), *unc-69(e587)* (Siddiqui 1990).

LG IV: *dpy-13(e184), unc-5(e53)* (Hedgecock *et al.* 1990), *lag-1(q385, q426)* (Lambie and Kimble 1991), *unc-24(e138)* (Waterston *et al.* 1980).

LG V: *lag-2(q411, q420)* (Lambie and Kimble 1991), *dpy-11(e224), sel-9(ar22)* (Sundaram and Greenwald 1993b), *sel-10(ar41)* (Sundaram and Greenwald 1993b), *him-5(e1490)* (Hodgkin *et al.* 1979), *ego-3(om40)* (Qiao *et al.* 1995), *unc-76(e911)* (Hedgecock *et al.* 1987).

LG X: *sel-12(ar131, ar171)* (Levitan and Greenwald 1995), *unc-1(e719)* (Park and Horvitz 1986).

Rearrangements and duplications: *eT1(III;V)* (Rosenbluth and Baillie 1981), *nT1[unc-?(n754) let-?] (IV;V)* (Ferguson and Horvitz 1985) [in the derivative of *nT1*, *unc-?(n754)* confers a dominant Unc phenotype and *let-?* confers a recessive lethal phenotype, referred to as "*DnT1*" (E. Ferguson, unpublished data)], *qDp3 (III;f)* (Austin and Kimble 1987), *mnDp68 (X;f)* (Herman and Kari 1989).

Special notes regarding *glp-1(Pro)* mutants: All three *glp-1(Pro)* alleles show extreme sensitivity to marker mutations (*e.g.*, enhancement, suppression, or variation in phenotype). Also, the original *glp-1(ar224)* strain was markedly slow growing, sickly, and exhibited an incompletely penetrant "small germline" phenotype. After multiple backcrosses, the mutation was recombined onto several marked chromosomes and the health of the strain markedly improved. The *ar224* mutation was subsequently reisolated from a healthier marked strain several times independently and the original slow-growing, sickly, and small germline phenotypes reappeared. In heterozygous combinations with marked chromosomes, the phenotypes do not appear. These results suggest that these phenotypes are a property of either *ar224* or a closely linked mutation and are dominantly suppressed by (otherwise recessive) markers. We have scored the phenotype of this homozygous mutant in a strain that is *m ar224/n ar224*, where *m* and *n* refer to linked recessive marker mutations.

Special considerations for *glp-1(Pro)* strain constructions: In all cases where the resulting strain was expected to be sterile at the restrictive temperature due to a *glp-1(Pro)* mutation, presumptive homozygous animals were cultured individually at 15°. Some of their progeny were reared at 15° while others were shifted to 25°. Therefore, these strains were verified by their phenotype at 25° and were established from the siblings at 15°. Low penetrance of *glp-1(ar218)* (Table 2) and the maternal rescue of the *glp-1(ar202)* and *glp-1(ar224)* defects (Table 4) ensured unambiguous identification of the homozygous strains carrying these alleles since self-progeny of homozygous mothers display the mutant phenotype whereas homozygous self-progeny of heterozygous mothers do not. Where identical strategies were employed in the construction of strains bearing different *glp-1(Pro)* mutant alleles, they are described below as *glp-1(Pro)*. Allele designations are as noted above and are omitted from the strain constructions except when necessary to avoid ambiguity. Progeny analysis was used in all cases where self-progeny and cross-progeny were not immediately distinguishable by phenotype. Strains for which construction details are not provided were built using standard methods (Brenner 1974).

Strains relevant to dosage analysis: *dpy-17 ncl-1 unc-36 glp-1 (Pro); qDp3:* Three steps were employed to construct these strains. First, *ncl-1 unc-36 glp-1(q46); qDp3* hermaphrodites were mated with *glp-1(Pro)* males. Non-Unc cross-progeny hermaphrodites were picked to individual plates and their Ncl, Unc, non-Glp recombinant progeny were picked onto individual plates [presumed genotype: *ncl-1 unc-36 glp-1(Pro)/ncl-1 unc-36 glp-1(q46)*]. Animals homozygous for the recombinant chromosome were identified in the next generation, and the strain was verified by temperature shift. Second, these *ncl-1 unc-36 glp-1(Pro)* hermaphrodites were mated with *dpy-17unc-32/++* males and the progeny of their non-Unc F_1 hermaphrodites were analyzed to establish a *ncl-1 unc-36 glp-1(Pro)/dpy-17 unc-32* strain. Dpy non-Unc recombinant progeny [presumed genotype: *dpy-17 ncl-1 unc-36 glp-1(Pro)/dpy-17 unc-32*] were picked onto individual plates. Animals homozygous for the recombinant chromosome were identified, and the strain was verified by temperature shift and by Nomarski analysis for the Ncl phenotype. Finally, *glp-1(Pro)* males were mated with *dpy-17 ncl-1 unc-36 glp-1(Pro)* hermaphrodites. F_1 males were mated with *ncl-1 unc-36 glp-1(q46); qDp3* hermaphrodites. Non-Unc hermaphrodite progeny [presumed genotype: *dpy-17 ncl-1 unc-36 glp-1(Pro)/ncl-1 unc-36 glp-1(q46); qDp3* or *ncl-1 unc-36 glp-1(q46)/glp-1(Pro); ±qDp3*] were picked onto individual plates. Dpy non-Unc hermaphrodite progeny [presumed genotype: *dpy-17 ncl-1 unc-36 glp-1(Pro); qDp3*] were picked onto individual plates and tested by temperature shift and progeny analysis to establish the strain.

glp-1(Pro) unc-x/unc-y or *glp-1(Pro) unc-x/eT1 III; +/eT1 V: unc-y /+* or *+/eT1 III; +/eT1 V* males were mated with *glp-1 (Pro) unc-x* hermaphrodites and non-Unc hermaphrodite cross-progeny were picked onto individual plates. F_1 animals of the desired genotype were identified by progeny analysis. Continued linkage of *glp-1(Pro)* to *unc-x* was verified by shifting progeny of Unc-x animals.

glp-1(Pro) unc-69/unc-32 glp-1(q175) and glp-1(Pro) unc-69/eT1 III; +/eT1 V: glp-1(Pro) unc-69 hermaphrodites were mated with *unc-32 glp-1(q175)/eT1 III; him-5/eT1[him-5] V* males. Hermaphrodite cross-progeny [presumed genotype: *glp-1(Pro) unc-69/unc-32 glp-1(q175); him-5/+* or *glp-1(Pro) unc-69/eT1 III; +/eT1[him-5] V*] were picked onto individual plates and the two strains were identified by progeny analysis. To eliminate *him-5* from the first strain, 20 non-Unc hermaphrodites were picked from plates segregating Glp, Unc-32, and Unc-69 animals. From each hermaphrodite mother that did not segregate males (presumed genotype +/+ or *him-5*/+), 12 hermaphrodite progeny were picked onto individual plates, and the self-progeny were inspected for the absence of males from all 12 broods.

Strains relevant to *glp-1(Pro)/glp-1(Pro) trans*-heterozygote analysis: *glp-1(Pro) unc-x/glp-1(Pro) unc-y: glp-1(Pro) unc-x/++* males were mated with *glp-1(Pro) unc-y* hermaphrodites and non-Unc F_1 progeny were picked onto separate plates to establish the strain. The germline phenotypes of all three classes (Unc-x, Unc-y, and non-Unc) of F_1 progeny of heterozygous mothers were scored.

Strains relevant to *glp-1(Pro); lag* double mutants: *glp-1(ar202); lag-1(q385)/DnT1:* First, *glp-1(ar202); dpy-13/+* males were mated with *ego-3 unc-76/DnT1* hermaphrodites. Unc F_1 hermaphrodite progeny were picked onto individual plates and the *glp-1(ar202); dpy-13/DnT1* strain was established by progeny analysis for *glp-1(ar202)* homozygotes that segregated only Dpy non-Unc and Unc non-Dpy animals. Unc hermaphrodites from this strain were then mated with *lag-1 (q385)/dpy-13 unc-24* males. Unc non-Dpy hermaphrodites were picked onto individual plates and the final strain was established by progeny analysis for Unc *glp-1(ar202)* homozygotes.

glp-1(ar202); lag-1(q426)/DnT1: glp-1(ar202); dpy-13/DnT1 hermaphrodites were mated with *lag-1(q426)/unc-5* males. Unc non-Dpy F_1 hermaphrodites were picked onto individual plates and the strain was established by progeny analysis for *glp-1 (ar202)* homozygotes that segregated Unc non-Glp and Glp non-Unc animals.

glp-1(ar202); lag-2(q411)/DnT1: glp-1(ar202); dpy-11/+ males were mated with *lag-2(q411)/DnT1* hermaphrodites. Non-Unc male F_1 progeny (*glp-1/+; dpy-11/lag-2* or *glp-1/+; +/lag-2*) were mated with *glp-1(ar202); +/DnT1* hermaphrodites. Individual Unc hermaphrodite progeny were picked onto separate plates and selfed. Progeny of individuals that segregated only Unc worms [presumed genotype: *glp-1(ar202); lag-2 (q411)/DnT1, glp-1(ar202)/+; lag-2 (q411)/DnT1* or *+/+; lag-2 (q411)/DnT1*] were picked onto separate plates and their progeny were shifted to the restrictive temperature to identify *glp-1(ar202)* homozygotes.

unc-32 glp-1(ar202); lag-2(q420): lag-2(q420) males were mated with *unc-32 glp-1(ar202)* hermaphrodites. Non-Unc hermaphrodite cross-progeny were picked onto individual plates [presumed genotype: *unc-32 glp-1(ar202)/+; lag-2(q420)/+*]. F_2 animals homozygous for *lag-2(q420)* were identified by temperature shift. Homozygous *glp-1(ar202)* animals were identified in the next generation. The resulting strain was sequenced at the *lag-2* locus to verify the presence of *lag-2(q420)*.

Double-mutant strains carrying *glp-1(Pro)* with *sel* genes and *sup-17*: *sel-12 double mutants and associated control strains: dpy-17/+* males were mated with *sel-12 unc-1; mnDp68 [sel-12(+) unc-1(+)]* and *unc-1* hermaphrodites. Non-Unc hermaphrodite F_1 progeny were picked onto individual plates. From plates segregating Dpy, Unc, and Dpy Unc hermaphrodites, Dpy Unc hermaphrodites were picked onto individual plates to establish the strains *dpy-17; sel-12 unc-1* and *dpy-17; unc-1*. These hermaphrodites were mated with *glp-1(Pro)* males. Individual non-Dpy, non-Unc F_1 hermaphrodites were picked and the *glp-1(Pro); sel-12 unc-1* and *glp-1(Pro); unc-1* strains were established from their F_2 progeny by progeny testing and temperature shift.

sel-10 double-mutant strain and associated control: ncl-1 unc-36 glp-1(ar202) or *ncl-1 unc-36 glp-1(ar202); him-5* hermaphrodites were mated with *glp-1(ar202)* males. F_1 males were mated with *unc-32; sel-10 him-5* or *unc-32*. Non-Unc F_1 hermaphrodites were picked onto individual plates. From plates that segregated both Unc-32 and Unc-36, non-Unc F_2 individuals were picked and their progeny inspected for the presence of males to establish the strains *ncl-1 unc-36 glp-1(ar202)/unc-32; sel-10 him-5* and *ncl-1 unc-36 glp-1(ar202)/unc-32; him-5*.

sup-17; glp-1(ar202): glp-1(ar202) males were mated with *dpy-5; glp-1(ar202)* hermaphrodites. Non-Dpy cross-progeny males were mated with *sup-17; unc-32* hermaphrodites. Non-Unc hermaphrodite cross-progeny were picked onto individual plates and from plates that segregated Dpy animals, non-Dpy non-Unc animals were picked onto individual plates and the strain was established from F_2 progeny that segregated neither Dpy nor Unc.

glp-1(Pro); sel-9: glp-1(Pro); dpy-11/+ males were mated with *unc-32 lin-12; sel-9* hermaphrodites and non-Unc F_1 progeny were picked onto individual plates. F_1 progeny that segregated Dpy, Unc, Dpy Unc, and non-Dpy non-Unc progeny were retained. Homozygous *sel-9* mutants were identified first by selecting non-Dpy animals that segregated no Dpy progeny but segregated Unc and non-Unc progeny. From their self-progeny, homozygous *glp-1* animals were identified by absence of Unc-32 self-progeny.

Isolation and identification of *glp-1(Pro)* alleles: Mutageneses were carried out as described (Brenner 1974) except that 25 mM EMS was used to reduce the level of additional second-site mutations that cause fertility defects (in one mutagenesis 50 mM was used). Mutagenized hermaphrodites and

their F_1 progeny were raised at 15°, and the self-progeny of individual F_1 hermaphrodites were transferred to 25° as late embryos or L1 larvae while siblings were kept at 15°. F_2 animals were screened first under low magnification for the absence of embryos in the uterus and then under high magnification for a more specific assessment of the fertility defect. Three of the six Pro mutants that displayed an apparently normal somatic gonad mapped to the same genetic interval on linkage group III. For each allele, linkage to III was determined by standard genetic techniques (Brenner 1974) and/or by mapping using polymorphic sequence tagged sites (STS; Williams *et al.* 1992). STS mapping was also used in some cases to give an indication of position within LG III. Recombinants were isolated from a *dpy-17 unc-32/glp-1(Pro)* strain; for each allele (n = 9–19 recombinants in each direction) all Dpy non-Unc recombinants were *glp-1(Pro)*/+ and all Unc non-Dpy recombinants were +/+. Unc non-Mog recombinants were also selected from *mog-1 unc-69/glp-1(Pro)* and each recombinant was also *glp-1(Pro)*/+. All three alleles mapped within the same interval on LG III—between *unc-32* and *mog-1*.

The entire *glp-1* coding region and all splice junctions were sequenced on ABI 377 and ABI 3700 genetic analyzers. Primers were designed to amplify six large segments of *glp-1* genomic DNA from worm lysates. Direct sequencing was performed on the forward strand of these PCR products with nested primers. Where changes were found, the reverse strand was also sequenced to verify the change. A single missense base-pair change was found in each mutant as follows: *ar202* G529E (codon change GGG to GAG), *ar218* R499Q (codon change CGA to CAA), and *ar224* A729T (codon change GCT to ACT).

Synchronization of worms for scoring germline phenotypes: For phenotypic analysis of all strains the following protocol was used unless noted. Animals were grown at 15° and synchronized using a hatch-off protocol adapted from Francis *et al.* (1995). All larvae and adults were washed off mixed-stage nematode growth medium (NGM) plates with M9 buffer (Wood 1988). Plates were inspected under low-power magnification to confirm that only embryos remained on the bacterial lawn. Embryos were then allowed to hatch for 2 hr at 15°. All newly hatched L1 larvae were washed (with M9) into 15-ml Falcon tubes and centrifuged at ~2000 rpm for 4 min. Supernatant was removed, and larvae were collected in a glass Pasteur pipette and distributed to 60-mm plates (~50 larvae/plate) that had been seeded with OP50 bacteria (Wood 1988). Plates of synchronous larvae were placed at 25°, and the animals were harvested 48 hr later (young adult stage) for fixation and scoring. All data presented here were obtained by examining worms under ×400–1000 magnification after fixation and staining with 4′,6-diamidino-2-phenylindole (DAPI). In all cases, n is the number of gonad arms scored. In cases where F_1 progeny of a heterozygous strain were scored, 20–30 heterozygous adults were placed on one plate and allowed to lay eggs overnight. F_1 progeny were then synchronized by hatch-off. When necessary, worms of different genotypes were separated as L4s.

Fixation and staining protocols: Worms were washed off of growth plates with M9 buffer into siliconized 1.5-ml Eppendorf tubes and briefly centrifuged and the supernatant was removed. Animals were then resuspended in 95% ethanol and fixed for 10 min, by which time animals had settled to the bottom of the tube. Ethanol was then removed and a drop of Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) was dropped onto the worms. Worms were then transferred to 5% agar pads using a glass Pasteur pipette and covered for microscopy. These preparations could be kept in the dark at room temperature for up to 4 days without losing resolution.

RNAi methods: RNAi feeding experiments were performed using the system developed by Timmons and Fire (Timmons *et al.* 2001) that employs the HT115(DE3) bacterial strain on NGM plates containing 100 μm/ml ampicillin and 1 mm isopropyl thiogalactoside. Bacteria were grown overnight on LB plates containing 100 μg ampicillin and 50 μg/ml tetracycline. Single colonies were picked and grown overnight in LB with 100 μg/ml ampicillin. Plates were seeded immediately from the overnight culture and were kept at room temperature in the dark for 1–2 days before worms were added. pGC11 contains 720 bp of *hop-1* genomic DNA inserted into the L4440 vector. The insert was prepared by PCR (primer 1, TCCCATT CCTAACCGAATTG; primer 2, GAAACTCAGCCAGCCAG AAC) from N2 worms. The PCR product was subsequently digested with *Xba*I and *Acc*I and ligated into the same sites in the vector. pGC2, a feeding construct containing the *lag-1* cDNA, was used in parallel as a positive control for the RNAi conditions. Three L4 worms were placed on each HT115-seeded plate and then transferred after a short time to remove any OP50 bacteria that remained on the worms. Worms were maintained at 15° and then transferred onto fresh RNAi plates each day for 3 days. After each transfer, the plates containing eggs and larvae were placed at 25° while the adults were maintained at 15°. Progeny from the third-day transfer were scored after 2 days at 25°.

RESULTS

Identification of Pro mutants: We performed a genetic screen for mutations that affect development of the germline. A temperature shift was incorporated into the screening strategy (see materials and methods) to permit the isolation of mutations in essential genes that also affect gonadogenesis. One class of mutants we identified in this screen displays a Pro phenotype in which proliferative germ nuclei are present in the proximal gonad—between mature gametes and the proximal somatic gonad of the adult (Figure 2; Seydoux *et al.* 1990). Normally, the adult proximal germline contains germ cells undergoing gametogenesis and proliferation is restricted to the distalmost part of the adult gonad. Within the first ~10,000 haploid genomes screened, >30 mutants were found that appeared to exhibit a Pro phenotype. Of these, 6 Pro mutants exhibited grossly normal somatic gonad development (the somatic gonad in the others appeared abnormal), and these 6 were characterized further. Of the 6, genetic mapping data indicated that three alleles mapped within the same interval (between *unc-32* and *mog-1*) on LG III (see materials and methods). These three proved to carry mutations in the *glp-1 locus* (other non-*glp-1* Pro mutants will be described elsewhere).

***glp-1(Pro)* alleles are missense mutations that alter the extracellular domain of the receptor:** Direct sequence analysis revealed that each *glp-1(Pro)* strain harbored a missense mutation in the *glp-1* coding region, just N-terminal to the transmembrane domain (Figure 3). Two of the mutations, *ar218* (R499Q) and *ar202* (G529E), change a single residue within the LIN-12/Notch repeats (LNR), while the third changes a residue C-terminal to a pair of conserved cysteine residues, *ar224* (A729T). The first two mutations affect residues that are con-

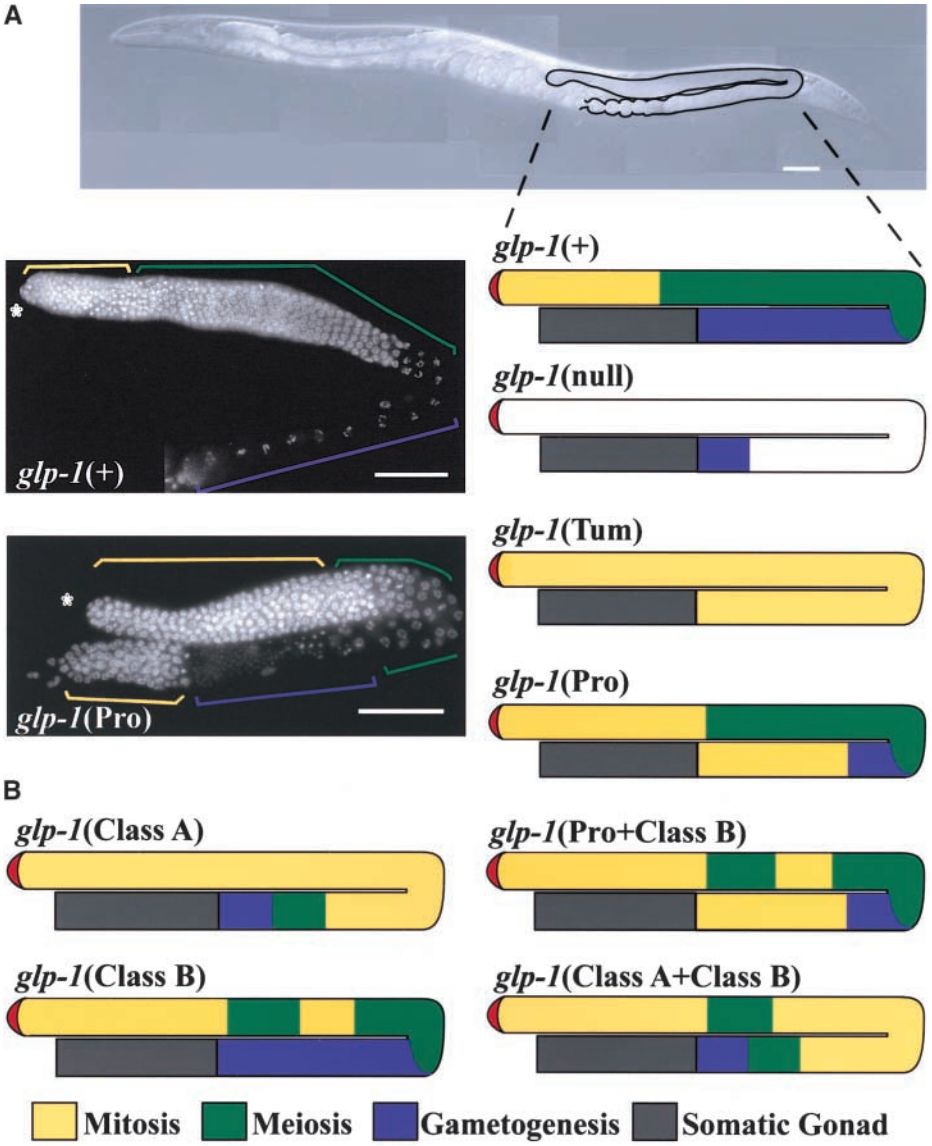

FIGURE 2.—Summary of *glp-1* mutant germline pattern defects. Yellow indicates zone of mitosis, green indicates transition (leptotene and zygotene) and pachytene of meiosis I, blue denotes late prophase of meiosis I and gametogenesis, gray shows the position of the proximal somatic gonad, and the distal tip cell is drawn in red. (A, top) Nomarski image of a live adult wild-type hermaphrodite. Anterior is to the left and ventral is down. The posterior arm of the gonad is visible in this orientation and is outlined in black. Bar, 50 μm. (A, right) Schematic representation of the four major adult *glp-1* germline phenotypes. The white space in the *glp-1(null)* represents a gonad containing few germ cells, all of which are sperm. (A, left) Photomicrographs of dissected DAPI-stained wild-type and *glp-1(Pro)* gonads. The asterisk indicates the distal end of each gonad arm. The colored brackets correspond to the stages of germline development depicted in the diagrams on the right. Bar, 50 μm. (B) Schematic representation of minor adult germline pattern defects observed in *glp-1(Pro)* mutant animals. The minor phenotypes are variable with respect to the size and position of each cluster of nuclei; diagrams depict only overall patterns.

served among nematode GLP-1 sequences, but not between phyla (RUDEL and KIMBLE 2001). Nonetheless, both changes are in regions that are quite well conserved and both are nonconservative substitutions. In particular, *ar202* (G529E) may disrupt the structure of the loop between the first two LNRs (ASTER *et al.* 1999). The third mutation, *ar224* (A729T), alters a well-conserved amino acid near the putative site for ligand-dependent S2 cleavage (Figure 3; BROU *et al.* 2000; MUMM *et al.* 2000). All of these mutations affect a region of the protein that is thought to negatively regulate receptor activity in the absence of ligand binding (GREENWALD and SEYDOUX 1990; LIEBER *et al.* 1993; GREENWALD 1994; BRENNAN *et al.* 1997).

The *glp-1(Pro)* mutants display temperature-sensitive germline pattern defects: GLP-1 activity in the *C. elegans* germline promotes mitosis and/or inhibits meiosis. *glp-1* mutant germline phenotypes are summarized in Figure 2. Null, strong, and partial loss-of-function alleles cause a defect in germline proliferation (Glp) and early entry into meiosis (AUSTIN and KIMBLE 1987). A hypermorphic gain-of-function allele, *glp-1(oz112gf)*, can cause a Tum phenotype in which all germ cells remain mitotic and never enter meiosis (BERRY *et al.* 1997).

The early adult Pro phenotype in our *glp-1* mutants is characterized by a conspicuous ectopic mass of proliferative germ nuclei in the proximal ovary between gametes and the spermatheca. Excess germline mitosis is consistent with an elevation of GLP-1 activity. In contrast to the Tum phenotype of *glp-1(oz112gf)*, however, the ectopic mitosis of the early adult Pro animals is anatomically limited to the proximal part of the germline, and the distal-to-proximal pattern of germline development appears quite normal (Figure 2). The proximal disruption of germline development in Pro animals is apparent in the late-larval germline, and further studies indicate that this Pro phenotype is due to a delay and mis-positioning of the earliest onset of meiosis in larvae (A.

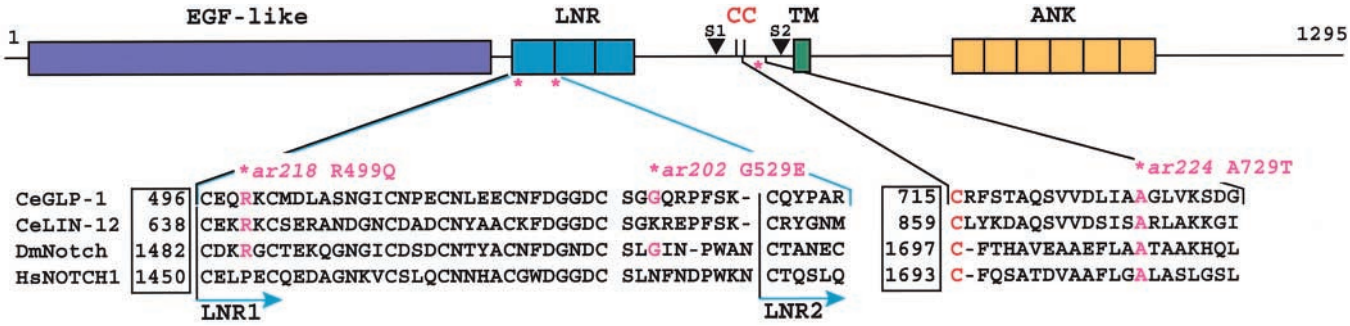

FIGURE 3.—Schematic diagram of GLP-1 receptor and positions of missense changes encoded by *glp-1(Pro)* alleles. The positions of conserved motifs are indicated for the EGF-like repeats, LIN-12/Notch repeats (LNR), two cysteine residues (CC), the transmembrane domain (TM), and the cdc10/SWI6/ankyrin repeats (ANK). The corresponding positions of cleavage sites S1 and S2 are also indicated (MUMM and KOPAN 2000). Local alignments in the regions affected by the three *glp-1(Pro)* mutations are given for three related proteins: LIN-12 from *C. elegans*, Notch from *Drosophila melanogaster*, and Notch1 from *Homo sapiens*. Alignment of the LNRs is based on ASTER *et al.* (1999). The second alignment begins with the C-terminal cysteine of the conserved pair of cysteines depicted in red.

PEPPER, T.-W. LO and E. J. A. HUBBARD, unpublished observations). The *glp-1(Pro)* mutants are qualitatively different from the Tum mutant *glp-1(oz112gf)* in several respects. Lowering the temperature or dosage of *glp-1 (oz112gf)* results in a completely penetrant late-onset Tum phenotype (a phenotype shared by *glp-1(Pro)* mutants; see below), but <1% Pro. Another phenotypic difference between *glp-1(Pro)* mutants and *glp-1(oz112gf)* is that the latter display a Multivulva phenotype in addition to the Tum phenotype, whereas the vulva of *glp-1(Pro)* mutant animals appears normal.

All three new *glp-1* alleles, *ar202*, *ar218*, and *ar224*, are temperature sensitive, but they behave differently with respect to temperature dependence and penetrance of the Pro phenotype. In our analysis, we strictly defined the Pro phenotype as the presence of mitotic germ nuclei proximalmost in the early adult germline, proximal to mature gametes, and distal to the spermatheca. Experiments presented here were designed to maximize our ability to score the Pro phenotype since a highly penetrant Pro phenotype has not been previously observed for *glp-1*. The Pro phenotype is spatially invariant and distinct. A time-course analysis was undertaken to determine the best time to score the Pro phenotype. Early time points underestimated the penetrance of the Pro phenotype since meiotic entry is delayed in animals that display the Pro phenotype (A. PEPPER, T.-W. LO and E. J. A. HUBBARD, unpublished observations). Later time points also underestimated and possibly confounded the scoring of the Pro phenotype since older animals sometimes expelled the proximal mitotic cells. Therefore, we chose 48 hr (that is, 48 hr after synchronized early L1 larvae were shifted to the restrictive temperature) for our analysis. Scoring animals at this time point in the early adult, we observed a very low penetrance in *glp-1(ar218)*, while the other two alleles were highly penetrant for the Pro phenotype at the restrictive temperature (Table 2).

In addition to the Pro phenotype, we observed several non-Pro germline pattern defects in early adult *glp-1(Pro)* mutants. A slight but highly penetrant extension of the distal mitotic zone beyond the usual 20–25 cell diameters is found in early adults; this extension progresses during adulthood (see below). Two additional classes of germline pattern defects were observed sporadically in early adults and were designated Class A and Class B (Figure 2). A dramatic extension of the distal mitotic zone, together with gametes in the proximalmost part of the germline, defines Class A. Discrete (but variable) patches of meiotic nuclei in the distal germline define Class B. Combinations of "Class A + Class B" and "Pro + Class B" were also observed and are noted in the tables and depicted in Figure 2. The first-described Pro mutants, *lin-12(loss-of-function)*, did not display additional germline pattern defects (SEYDOUX *et al.* 1990). Unlike the *glp-1(Pro)* mutant phenotype, however, the *lin-12(loss-of-function)* Pro phenotype is a secondary consequence of defects in the somatic gonad rather than of the germline-autonomous effects of *glp-1* mutants, and the activity of *glp-1* in the germline likely accounts for the different observations.

Like the Pro phenotype, both classes of non-Pro phenotypes are consistent with elevated *glp-1* activity since both exhibit an excess of mitotic nuclei. The genotypes of strains in which non-Pro germline pattern defects appear do not offer a simple interpretation regarding the level of *glp-1* activity compared to that of the Pro phenotype. The non-Pro phenotypes appear in the presence of specific marker mutations, in certain genetic combinations, and under certain temperature-shift conditions. They may reflect subtle differences in maternal and zygotic levels of *glp-1* activity, as well as distinct late-onset defects (see below). Thus, although the Pro phenotype is the primary and most prevalent phenotype, due to the presence of other defects, the degree to which *glp-1* activity is normal or elevated is best as-

TABLE 2

Temperature dependence and penetrance of *glp-1(Pro)* phenotypes

Genotype	Temperature	Germline pattern phenotype[a]			
		% wild type	% Pro	% other	(*n*)
ar218	15°	100	0	0	(129)
	25°[b]	98	2	0	(315)
ar202	15°	98	2	0	(151)
	25°	0	97	3[c]	(419)
ar224[d]	15°	99	1	0	(150)
	25°	0	92[e]	8[f]	(127)

[a] The Pro phenotype is strictly defined as the presence of mitotic germ nuclei proximalmost in the germ line, proximal to mature gametes, and distal to the spermatheca. Other phenotypes include Class A and Class B: Class A is defined as an extended distal mitotic zone with gametes proximalmost and Class B is defined as discrete patches of mitotic and meiotic nuclei in the distal arm. A combination of Class A and Class B can occur in the same gonad arm ("Class A + Class B"), as can a combination of Pro and Class B ("Pro + Class B"). See text for details.

[b] Strain was grown at 25°.

[c] Other: Class A.

[d] Actual genotype: *dpy-17(e164) glp-1(ar224)/unc-32(e189) glp-1(ar224). dpy-17(e164)* and *unc-32(e189)* are recessive mutations (see MATERIALS AND METHODS).

[e] % Pro includes 78% Pro + Class B.

[f] Other includes 6% Class A + Class B and 2% Tum.

sessed by the penetrance of the wild-type germline pattern.

To investigate the possibility of late-onset germline pattern defects, we examined the germlines of late-adult *glp-1(ar202)* animals. We determined the penetrance of Pro and non-Pro defects and the extent of distal mitotic zone expansion at 60 and 72 hr post-shift at 25°. Our results indicate that the penetrance of the Class B phenotype and the size of the distal mitotic zone increase over time. At 48 hr, the Class B phenotype was not observed ($n = 419$; Table 2). Of 47 gonad arms scored at 60 hr, 37 displayed a Pro phenotype and 13 of these were Pro + Class B. In addition, 10/47 displayed an apparent Class A phenotype, 4 of which were Class A + Class B. Similar results were obtained at 72 hr. While it is difficult to make firm conclusions regarding the scoring of the Class A phenotype at the later time points (since an early adult Pro animal that later expelled proximal mitotic cells might be indistinguishable from Class A), the increased Class B penetrance suggests that the Class B phenotype is fundamentally a late-onset phenotype that is separable from the Pro phenotype. This possibility may also explain why the two phenotypes are affected differently by genetic marker mutations. Data obtained from the later time points also indicate that the distal mitotic zone expands significantly in the background of all observed pattern defects (Pro and non-Pro) from 48 to 60 hr. We quantitated the extent of the distal mitotic zone by counting the distance in cell diameters from the distal tip to the first full ring of transition nuclei. Whereas the distal mitotic zone of *glp-1 (ar202)* extends an average of 25.8 ± 3.1 (standard deviation) cell diameters from the distal tip at 48 hr ($n = 11$), the zone extends an average of 102.6 ± 61.0 cell diameters at 60 hr ($n = 10$). By 72 hr ($n = 63$ arms), 17% of *glp-1(ar202)* gonad arms display a completely Tum phenotype, likely the result of further extension of the distal mitotic zone. It is likely that the Class B phenotype is a variant of the distal zone extension phenotype in which mitosis is not maintained in the Class B animals. Thus, our *glp-1(Pro)* alleles are phenotypically distinct from *glp-1(oz112gf)* in that they display a highly penetrant Pro phenotype, but are similar to *glp-1(oz112gf)* in that they display a more or less continuous late-onset extension of the distal mitotic zone (late-onset Tum).

***glp-1(Pro)* alleles display unusual genetic behavior:** Genetically, the *glp-1(oz112gf)* mutation behaves in a classic hypermorphic fashion: addition of a wild-type dose of *glp-1* to the homozygote (*oz112/oz112/+*) increases the penetrance of the Tum phenotype. For *glp-1 (oz112gf)*, lowering *glp-1* dosage or lowering the temperature results in a highly penetrant late-onset Tum phenotype, not in a Pro phenotype. *glp-1(oz112gf)/glp-1 (null)*, however, does cause a very low penetrance (1%) Pro phenotype at 20° (BERRY *et al.* 1997). Thus, one hypothesis is that our *glp-1(Pro)* mutations are simply weak hypermorphs and that the consequence of increasing GLP-1 activity in the germline is either Pro or Tum, depending on the level of receptor activity. In this case, we would expect the Pro mutant phenotype to be enhanced with increasing doses of *glp-1(+)* and suppressed by lower doses of *glp-1(+)*.

To test this hypothesis, we performed a dosage analysis, examining the penetrance of mutant germline pattern phenotypes at the 48-hr time point at the restrictive temperature in strains with varying doses of *glp-1(Pro)* and *glp-1(+)* (Table 3). We used the *glp-1(q175)* null allele that encodes a severely truncated product; similar results were obtained with a temperature-sensitive partial loss-of-function allele, *glp-1(e2141)* (data not shown).

The results of the dosage analysis indicate that the Pro phenotype is highly dosage sensitive and that the alleles are not hypermorphic. A comparison of the penetrance of the wild-type and mutant phenotypes of *glp-1(Pro)/glp-1(null)* relative to the *glp-1(Pro)* homozygote reveals that the highly penetrant alleles, *ar202* and *ar224*, are extremely sensitive to dosage (Table 3). Interestingly, the *glp-1(Pro)/glp-1(null)* animals still displayed a low penetrance of the Pro phenotype while *glp-1(Pro)/glp-1(+)* animals were completely wild type (Table 3, lines 6, 7, 13, 14). These results suggest that the presence of *glp-1(+)* interferes with the expression of the Pro phenotype. Since both *Pro/null* and *Pro/+* have one copy of the mutant allele in both the mother and the scored progeny, dosage sensitivity alone does not account for these results. Further analysis with strains bearing one extra copy of *glp-1(+)* indicates that, rather than enhancing mutant phenotypes, *glp-1(+)* suppressed *glp-1(Pro)* mutant phenotypes (Table 3, lines 3, 4, 8, 9, 15, 16). The non-Pro germline pattern defects appeared to be more sensitive than the Pro phenotype to suppression by an extra copy of *glp-1(+)* in these experiments. Even at lower temperatures, one additional copy of *glp-1(+)* did not enhance the Pro mutant phenotype (Table 3, lines 10, 11, 17, 18). Taken together, we infer that the antagonism of *glp-1(Pro)* mutant phenotypes by *glp-1(+)* is a general property of these alleles. This behavior is not allele specific, since we observed a similar trend in all three alleles.

In summary, (i) for the highly penetrant alleles, the penetrance of the wild-type phenotype was higher in *Pro/null* than in *Pro/Pro*, but, surprisingly, lower than in *Pro/+*, and (ii) *Pro/Pro/+* exhibited a higher percentage of wild type than *Pro/Pro*, but a lower percentage than *Pro/+*. Thus, although the Pro mutants display a gain-of-function phenotype as indicated by increased and ectopic germline mitosis, the *glp-1(Pro)* alleles are neither hypermorphic nor neomorphic in the classic sense. Rather, the *glp-1(Pro)* alleles are dosage sensitive and *glp-1(+)* appears to compete with *glp-1(Pro)*, lowering or correcting the apparent elevation of *glp-1* activity as the dosage of *glp-1(+)* is increased. This genetic behavior is consistent with competition between *glp-1(+)* and *glp-1(Pro)* and is inconsistent with the hypothesis that *glp-1(Pro)* alleles are simply weak hypermorphs.

Maternal effects of *glp-1(Pro)*: Previous analysis of *glp-1* function indicated that maternal *glp-1* is required for embryogenesis and zygotic *glp-1* is required for germline development (Austin and Kimble 1987; Priess *et al.* 1987). Our results indicate a role for maternal *glp-1* in the germline. Consistent with our observation that the mutant phenotype of *glp-1(Pro)* alleles is highly sensitive to dosage and is antagonized by *glp-1(+)*, we observed a strong maternal rescue of the *glp-1(Pro)* mutant phenotype of *glp-1(Pro)* homozygotes coming from heterozygous mothers (Table 4). The results for *glp-1(ar202)* indicate that the penetrance of the wild-type germline pattern was higher in *ar202/ar202* animals derived from heterozygous (*ar202/+*) mothers than in animals derived from homozygous (*ar202/ar202*) mothers (Table 4, lines 1 and 2). We observed a similar phenomenon with *glp-1(ar224)* (data not shown). We infer that the maternal rescue is likely due to lower maternal dosage of the mutant allele rather than to antagonism by *glp-1(+)* since the penetrance of the wild-type germline pattern of *ar202/ar202* animals derived from *ar202/ar202/+* mothers was similar to that of *ar202/ar202* animals derived from *ar202/ar202* mothers (Table 4, lines 3 and 4). A minor caveat to this interpretation is that we cannot score strains with exactly equivalent *cis* markers in both mothers and progeny of strains with and without the duplication (Table 4). We also observe a maternal effect based on the maternal presence of *glp-1(ar202)*. Heterozygous animals derived from *glp-1(ar202)* mothers displayed a mutant phenotype more highly penetrant than that displayed by heterozygous progeny derived from wild-type mothers (Table 4, lines 5 and 6).

***Trans*-heterozygous combinations of *glp-1(Pro)* mutants:** Given that the *glp-1(Pro)* mutants encode receptors with elevated activity, one possibility is that *in trans* to each other, they would behave as in an allelic series, reflecting their level of activity as homozygotes. Alternatively, they might mutually suppress or enhance *in trans*, consistent with mutant receptor interaction and/or independent function. To distinguish between these possibilities, we compared the phenotypes of the three possible *trans*-heterozygous combinations of our *glp-1(Pro)* mutants with those of three individual homozygous strains (Table 5). Both strong alleles (*ar202* or *ar224*) *in trans* to *ar218* displayed an increased penetrance of the wild-type phenotype, as expected given previous dosage analysis and the nearly wild-type activity of *ar218*. In contrast, the *ar202/ar224* animals exhibited a striking deviation from the expected result based on the behavior of these homozygous mutants. Rather than exhibiting a phenotype at least as strong as the weakest homozygous strain, the *ar202/ar224 trans*-heterozygote exhibited a dramatic increase in the percentage of wild-type animals, indicating mutual suppression (Table 5). These results are inconsistent with a simple allelic series and are consistent with a physical interaction between receptors encoded by these alleles. Given that each allele is highly dosage sensitive, these data are also consistent with a model of independent mutant receptor activity. Finally, the results suggest that qualitative differences exist be-

TABLE 3

Dosage analysis of *glp-1(Pro)* mutants

glp-1 genotype[a]	Temperature	Germline pattern phenotype			(*n*)
		% wild type	% Pro	% other	
ar218/ar218	25°	98**	2	0	(315)
ar218/+[b]	25°	100	0	0	(363)
ar218/ar218[c]	25°	96*	4	0	(129)
ar218/ar218/+[d]	25°	100	0	0	(190)
ar202/ar202[e]	25°	0	97	3	(419)
ar202/(null)[f]	25°	94***	6	0	(93)
ar202/+[g]	25°	100	0	0	(215)
ar202/ar202[h]	25°	16	52	31	(61)
ar202/ar202/+[i]	25°	51	45	4	(53)
ar202/ar202[j]	15°	89	11	0	(64)
ar202/ar202/+[k]	15°	89	11	0	(35)
ar224/ar224[l]	25°	0	92	8	(127)
ar224/(null)[m]	25°	94***	6	0	(154)
ar224/+[n]	25°	100	0	0	(332)
ar224/ar224[o]	25°	40**	26	34	(104)
ar224/ar224/+[p]	25°	59	41	0	(90)
ar224/ar224[q]	15°	90	10	0	(80)
ar224/ar224/+[r]	15°	92	8	0	(57)

A Fisher exact test (one-sided) was used for data marked with asterisks to test the null hypothesis that there is no difference between the penetrance of wild type (*vs.* mutant) in the indicated genotype compared to that in the genotype directly below it. **P* value < 0.05; ***P* value < 0.01; ****P* value < 0.001.

[a] Results are grouped according to comparable experiments given marker mutations. All strains containing *glp-1(ar218)* were raised at 25° (rather than shifted) and were synchronized and scored as indicated in MATERIALS AND METHODS.

[b] Actual genotype: *glp-1(ar218) unc-69(e587)/eT1*.

[c] Actual genotype: *dpy-17(e164) glp-1(ar218)*. *dpy-17(e164)* is included in this and other appropriate strains in this table for comparison to strains bearing *qDp3*.

[d] Actual genotype: *dpy-17(e164) ncl-1(e1865) unc-36(e251) glp-1(ar218); qDp3*. *qDp3* carries wild-type alleles of *ncl-1*, *unc-36*, and *glp-1* (AUSTIN and KIMBLE 1987).

[e] "% other": Class A.

[f] Actual genotype: *glp-1(ar202) unc-69(e587)/unc-32(e189) glp-1(q175)* .

[g] Actual genotypes: *unc-32(e189) glp-1(ar202)/unc-36(e251)* ($n = 142$) and *unc-32(e189) glp-1(ar202)/eT1* ($n = 73$).

[h] Actual genotype: *dpy-17(e164) glp-1(ar202)*. % Pro includes 3% Pro + Class B. All "% other" display Class A; half of these display Class A + Class B.

[i] Actual genotype: *dpy-17(e164) ncl-1(e1865) unc-36(e251) glp-1(ar202); qDp3*. "% Pro" includes 11% Pro + Class B. % other: Class A.

[j] Actual genotype: *dpy-17(e164) glp-1(ar202)*.

[k] Actual genotype: *dpy-17(e164) ncl-1(e1865) unc-36(e251) glp-1(ar202); qDp3*.

[l] Actual genotype: *dpy-17(e164) glp-1(ar224)/unc-32(e189) glp-1(ar224)*. "% Pro" includes 78% Pro + Class B. "% other" includes 6% Class A + Class B and 2% Tum.

[m] Actual genotype: *glp-1(ar224) unc-69(e587)/unc-32(e189) glp-1(q175)*.

[n] Actual genotype: *unc-32(e189) glp-1(ar224)/unc-36(e251)*.

[o] Actual genotype: *dpy-17(e164) glp-1(ar224)*. "% other" includes 28% Class A and 6% Class A + Class B.

[p] Actual genotype: *dpy-17(e164) ncl-1(e1865) unc-36(e251) glp-1(ar224); qDp3*.

[q] Actual genotype: *dpy-17(e164) glp-1(ar224)*.

[r] Actual genotype: *dpy-17(e164) ncl-1(e1865) unc-36(e251) glp-1(ar224); qDp3*.

tween *glp-1(ar202)* and *glp-1(ar224)* despite their similar phenotypic and genetic properties.

Genetic interactions of *glp-1(ar202)* with *lag-1* and *lag-2*: The *glp-1(Pro)* phenotype could be the result of locally elevated GLP-1 receptor activity that acts within the known conserved signaling pathway or the result of aberrant activation via some other noncanonical mechanism. In all organisms in which the signaling pathway

TABLE 4

***glp-1(ar202)* maternal effects**

Genotype			Progeny germline pattern			
Maternal	Paternal	Progeny	% wild type	% Pro	% other	(*n*)
ar202/ar202[a]	(Self)	*ar202/ar202*	10	90	0	(129)
ar202/+[b]	(Self)	*ar202/ar202*[c]	76	21	3	(63)
ar202/ar202[d]	(Self)	*ar202/ar202*[d]	16	52	31	(61)
ar202/ar202/+[e]	(Self)	*ar202/ar202*[f]	13	84	3	(75)
ar202/ar202[g]	+/+	*ar202/+*[h]	43	14	42	(65)
+/+[i]	*ar202/ar202*	*ar202/+*[j]	97	1	2	(76)

[a] Actual genotype: *glp-1(ar202) unc-69(e587)*.

[b] Actual genotype: *glp-1(ar202) unc-69(e587)/eT1*.

[c] Actual genotype: *glp-1(ar202) unc-69(e587)*. "% other": Class A.

[d] Actual genotype: *dpy-17(e164) glp-1(ar202)*. "% Pro" includes 3% Pro + Class B. All "% other" display Class A; half of these display Class A + Class B. As an additional control for the other markers present in the duplication-bearing strain, self-progeny from mothers of the genotype *dpy-17(e164) ncl-1(e1865) unc-36(e251) glp-1(ar202)* were also scored ($n = 65$) and they displayed penetrance of 34% wild type and 66% Pro (the "% other" classes were not determined in this experiment).

[e] Actual genotype: *dpy-17(e164) ncl-1(e1865) unc-36(e251) glp-1(ar202); qDp3*.

[f] Actual genotype: *dpy-17(e164) ncl-1(e1865) unc-36(e251) glp-1(ar202)*. "% Pro" includes 27% Pro + Class B. "% other": Class A.

[g] Actual genotype: *unc-32(e189) glp-1(ar202)*.

[h] Actual genotype: *unc-32(e189) glp-1(ar202)/+ +* [identified as non-Unc F_1 cross-progeny of wild-type males mated with *unc-32(e189) glp-1(ar202)* hermaphrodites]. "% Pro" includes 12% Pro + Class B. "% other" includes 2% Class A, 35% Class A + Class B, and 5% Class B alone. Cross-progeny were synchronized by removing the parents after an overnight egg laying. Eggs and L1s were shifted to the restrictive temperature and scored as described in MATERIALS AND METHODS.

[i] Actual genotype: *unc-32(e189)*.

[j] Actual genotype: *glp-1(ar202)/unc-32(e189)* [identified as non-Unc F_1 cross-progeny of *glp-1(ar202)* males mated with *unc-32(e189)* hermaphrodites]. The Pro animal was Pro + Class B. "% other": Class A. Temperature-shift protocol was used as in note [h] above.

has been analyzed, LIN-12/Notch receptors are activated by ligands of the DSL family and act in concert with CSL family effectors to alter the transcription of target genes in response to signaling. Notch-mediated signaling that is independent of CSL family effectors has also been reported (SHAWBER *et al.* 1996; NOFZIGER *et al.* 1999; RAMAIN *et al.* 2001; YAMAMOTO *et al.* 2001).

We examined the dependence of the *glp-1(ar202)* Pro phenotype on the conserved CSL family member, LAG-1. We tested two alleles of *lag-1*: one apparent null allele, *lag-1(q385)*, which displays a larval lethal "Lin and Glp" (Lag) phenotype, and a second allele, *lag-1(q426)*, which causes a highly penetrant *glp-1(loss-of-function)*-like germline phenotype (LAMBIE and KIMBLE 1991). In both cases, the *lag-1* mutant phenotypes were completely epistatic to the Pro phenotype, indicating that expression of the Pro phenotype depends on functional LAG-1 (Table 6).

The LAG-2 ligand is expressed in the distal tip cells and activates GLP-1 in the germline (HENDERSON *et al.* 1994; TAX *et al.* 1994). In addition, LAG-2 is expressed at a very low level during the second larval stage (L2) in two cells in the hermaphrodite proximal somatic gonad, Z1.ppp and Z4.aaa (WILKINSON *et al.* 1994). Here, in the late L2, LAG-2 participates in the anchor cell/ventral uterine precursor cell decision as a ligand for LIN-12, and eventually the cell destined to become the anchor cell expresses a high level of LAG-2. Between the time Z1.ppp and Z4.aaa are born in the late L1 until the very end of the L2, however, these cells are in contact with germ cells. Therefore, one possible explanation for the Pro phenotype is that GLP-1 activity is locally elevated by an inappropriately strong response (hypersensitive receptor) to the low level of LAG-2 ligand produced in the proximal somatic gonad during the L2 stage. Alternatively, the Pro phenotype could be responding to an alternate ligand or it could be ligand independent, consistent with constitutive receptor activity.

To determine whether the *glp-1(Pro)* mutant phenotype is dependent on LAG-2, we examined the phenotypes of two different *glp-1(ar202); lag-2* double-mutant strains (Table 7). First, we tested a null allele, *lag-2(q411)*, that confers a Lag phenotype (LAMBIE and KIMBLE 1991). Several individual non-Lag *glp-1(ar202); lag-2(q411)* double-mutant animals were recovered from *glp-1(ar202); lag-2 (q411)/+* mothers. These *glp-1(ar202); lag-2(q411)* animals developed with a Pro germline. This result suggests that

TABLE 5

***trans*-Heterozygous combinations of *glp-1(Pro)* mutants**

Genotype	Germline pattern phenotype			
	% wild type	% Pro	% other	(*n*)
glp-1(ar218)[a]	98	2	0	(315)
glp-1(ar202)[b]	0	97	3	(419)
glp-1(ar224)[c]	0	92	8	(127)
glp-1(ar218)/glp-1(ar202)[d]	91	5	4	(74)
glp-1(ar218)/glp-1(ar224)[e]	99	1	0	(96)
glp-1(ar202)/glp-1(ar224)[f]	78	8	14	(155)

[a] Strain was grown at 25°.

[b] "% other": Class A.

[c] Actual genotype: *dpy-17(e164) glp-1(ar224)/unc-32(e189) glp-1(ar224). dpy-17(e164)* and *unc-32(e189)* are recessive mutations. "% Pro" includes 78% Pro + Class B. "% other" includes 6% Class A + Class B and 2% Tum.

[d] Actual genotype: *unc-32(e189) glp-1(ar218)/ncl-1(e1865) unc-36(e251) glp-1(ar202)*. "% other" includes 3% Class A and 1% Class A + Class B.

[e] Actual genotype: *ncl-1(e1865) unc-36(e251) glp-1(ar218)/unc-32(e189) glp-1(ar224)*.

[f] Actual genotype: *ncl-1(e1865) unc-36(e251) glp-1(ar202)/unc-32(e189) glp-1(ar224)*. "% other" includes 7% Class A, 1% Class B, and 6% Class A + Class B. Unc-32 and Unc-36 self-progeny of these hermaphrodites were also scored [for *unc-32 ar224*, $n = 51$, 70% wild type, 20% Pro, and 10% Class A and for *ncl-1(e1865) unc-36(e251) glp-1(ar202)*, $n = 37$, 59% wild type, 41% Pro]. Consistent with Table 4 results, the phenotypically wild-type *ar202/ar224 trans*-heterozygote mothers confer some maternal rescue since 26% of *unc-32 ar224* self-progeny from *unc-32 ar224* mothers are wild type ($n = 31$), whereas 70% of *unc-32 ar224* self-progeny from *unc-32 ar224/ncl-1(e1865) unc-36(e251) glp-1(ar202)* mothers are wild type. A second *ar202/ar224 trans*-heterozygous strain with different *cis* markers was also scored [actual genotype: *dpy-17(e164) glp-1(ar224)/unc-32(e189) glp-1(ar202)*, $n = 59$], and 90% were fertile, 2% displayed Pro, and 8% displayed Class A + Class B.

the receptor encoded by *glp-1(ar202)* retains function in the absence of LAG-2. Rescue of the Lag lethal phenotype by *glp-1(ar202)* was not very efficient (6 animals out of an expected 190), suggesting that the level of constitutive activity of the receptor encoded by *glp-1(ar202)* is relatively low or that the effect of *glp-1(ar202)* is tissue specific.

We also examined homozygous double mutants carrying a weaker allele of *lag-2, lag-2(q420)*. *lag-2(q420)* is the only available *lag-2* allele that confers a *glp-1(loss-of-function)*-like Glp phenotype. Although the single *lag-2(q420)* mutant displays an incompletely penetrant Glp phenotype, in combination with *glp-1(ar202)* the Glp phenotype is not observed. This result indicates that the activity of the receptor encoded by *glp-1(ar202)* is sufficient to bypass a reduction in LAG-2 activity that would normally prevent germline proliferation in 50% of the animals (Table 7) and that both proximal and distal *glp-1(ar202)* activity is ligand independent. While *glp-1(ar202); lag-2(q420)* animals did not exhibit a Glp phenotype, the percentage of wild-type animals was elevated in the double mutant compared to *glp-1(ar202)* alone (Table 7). In summary, *glp-1(ar202)* can cause a Pro phenotype in the absence of the LAG-2 ligand although the mutant receptor may still be sensitive to the presence of the ligand.

Genetic interactions with other components and modifiers of GLP-1/LIN-12/Notch-mediated signaling: To better understand the effect of *glp-1(Pro)* mutations on receptor activity, we examined the phenotype of double-mutant strains carrying the *glp-1(Pro)* alleles together with mutations previously identified as suppressors or enhancers of *lin-12* and *glp-1* (Sundaram and Greenwald 1993b; Levitan and Greenwald 1995; Tax *et al.* 1997). The results are presented in Table 8. Interactions with products that affect cleavage or stability of the intracellular domain (*sel-12*, *hop-1*, and *sel-10*; see Figure 1) behaved as expected on the basis of previous results (Sundaram and Greenwald 1993b; Levitan and Greenwald 1995; Li and Greenwald 1997). Specifically, the *glp-1(Pro)* mutant phenotypes are partially suppressed by loss of function of the presenilins *sel-12* and *hop-1* and enhanced by *sel-10(ar41)*. Enhancement by *sel-10(ar41)* overcomes both maternal and zygotic rescue of *glp-1(Pro)* by reduced dosage and/or *glp-1(+)* (Table 8).

Modifiers of the pathway that act on the extracellular part of the receptor were of particular interest since *glp-1(Pro)* mutations alter this part of the protein. One possibility is that the Pro mutations render the receptor susceptible to a low level of constitutive S2 cleavage. If this hypothesis were correct, mutations that reduce the level of S2 cleavage would be effective suppressors. As yet, no *C. elegans* enzyme is known to be responsible for the S2 cleavage, but in other organisms this cleavage is dependent on metalloproteases. The ADAM-family metalloprotease SUP-17 in *C. elegans* is homologous with Drosophila Kuzbanian and both are required for signaling (Wen *et al.* 1997). In Drosophila, Kuzbanian is re-

TABLE 6

glp-1(ar202)* in combination with mutations in *lag-1

Genotype	Phenotype
lag-1(q385)[a]	Lag
glp-1(ar202)[b]	Pro
glp-1(ar202); lag-1(q385)[c]	Lag

	Germline pattern phenotype				
Genotype	% wild type	% Pro	% Glp	% other	(*n*)[d]
lag-1(q426)[e]	2	0	98	0	(85)
glp-1(ar202)[f]	0	100	0	0	(33)
glp-1(ar202); lag-1(q426)[g]	0	2	98	0	(113)

[a] No late-larval or adult non-Unc progeny were observed among self-progeny of *lag-1(q385)/nT1([unc-?(n754)let-?]IV;V); +/nT1([unc-?(n754)let-?]IV;V)* mothers; Lag (L1 lethal) progeny were observed.

[b] Scored non-Unc self-progeny from *glp-1(ar202); +/nT1([unc-?(n754) let-?]IV;V)* mothers; $n = 33$.

[c] No late-larval or adult non-Unc progeny were observed among self-progeny of *glp-1(ar202); lag-1(q385)/nT1([unc-?(n754) let-?]IV;V); +/nT1([unc-?(n754) let-?]IV;V)* mothers; Lag (L1 lethal) progeny were observed.

[d] Gonad arms were scored in non-Lag animals only.

[e] Scored non-Unc self-progeny of *lag-1(q426)/nT1([unc-?(n754)let-?]IV;V); +/nT1([unc-?(n754)let-?]IV;V)* mothers.

[f] Scored non-Unc self-progeny of *glp-1(ar202); +/nT1([unc-?(n754)let-?]IV;V; +/nT1([unc-?(n754)let-?]IV;V)* mothers.

[g] Scored non-Unc self-progeny of *glp-1(ar202); lag-1(q426)/nT1([unc-?(n754)let-?]IV;V); +/nT1([unc-?(n754)let-?]IV;V)* mothers.

quired for ligand-mediated cleavage of the extracellular portion of the receptor (Lieber *et al.* 2002). We tested interactions between *glp-1(ar202)* and a reduction-of-function mutation in *sup-17, sup-17(n1258)* (Tax *et al.* 1997). This mutant displays temperature-sensitive maternal-effect lethality and was originally isolated as a suppressor of a hypermorphic allele of *lin-12*. We observed dramatic suppression of the *glp-1(ar202)* Pro phenotype in the double-mutant *sup-17(n1258); glp-1(ar202)* (Table 8). These results suggest that although genetic interactions with *lag-2* indicate little ligand dependence, expression of the *glp-1(Pro)* phenotype is dependent on SUP-17 function. Moreover, this genetic interaction suggests that the structure/function defect in *glp-1(ar202)* occurs at or before the level of ligand-dependent cleavage.

Prior to ligand-dependent cleavage, the receptor is transported from the ER to the Golgi, processed and modified in the Golgi, and directed to the extracellular compartment (Figure 1). We investigated the effect of the mutation *sel-9(ar22)*, which likely interferes with proper selection for transport between the ER and Golgi. *sel-9* was identified by mutations that suppress phenotypes caused by reduction-of-function mutations in *lin-12* and *glp-1*. In addition, *sel-9* mutations enhance phenotypes caused by gain-of-function alleles of *lin-12* (Sundaram and Greenwald 1993b; Wen and Greenwald 1999). We investigated the consequence of a reduction-of-function mutation in *sel-9* in combination with *glp-1(ar202)* and *glp-1(ar224)*. To our surprise, rather than enhancing the *glp-1(Pro)* phenotype, *sel-9(ar22)* partially suppressed the Pro phenotype (Table 7). Therefore, the genetic interaction among the Pro phenotypes of *glp-1(Pro)* mutants differs from *lin-12* mutants in their genetic interaction with *sel-9*. A slight elevation of the penetrance of the non-Pro mutant phenotypes was also observed in the *glp-1(Pro); sel-9* double-mutant animals, further supporting the notion that the Pro and non-Pro germline pattern defects exhibited by *glp-1(Pro)* mutants are distinct.

DISCUSSION

We have isolated three new alleles of *glp-1* that differ from previously described *glp-1* alleles in several ways. First, they present a Pro phenotype. Second, although the alleles are clearly gain-of-function in character, they are not simple genetic hypermorphs, as indicated by both interactions with *glp-1(+)* and with each other. The mutant phenotypes are dosage sensitive, *glp-1(+)* interferes with expression of the mutant phenotype, and *trans*-heterozygous combinations of highly penetrant alleles mutually suppress. Genetic interactions with the conserved core GLP-1 signaling pathway components LAG-1 and LAG-2 support the idea that the *glp-1(Pro)* alleles act through the canonical signaling pathway. Interactions between *glp-1(Pro)* and mutations in genes encoding other components and modifiers of the GLP-1/LIN-12/Notch-mediated signaling pathway indicate that the mutant receptor acts as expected for a ligand-independent, hyperactive receptor. One exception is the interaction with a mutation that likely affects quality control during ER-to-Golgi transport.

TABLE 7

glp-1(ar202)* in combination with mutations in *lag-2

Genotype	Phenotype
lag-2(q411)[a]	Lag
glp-1(ar202)	Pro
glp-1(ar202); lag-2(q411)[b]	Lag (many), Pro (few)

	Germline pattern phenotype				
Genotype	% wild type	% Pro	% Glp	% other	(*n*)[c]
lag-2(q420)[d,e]	53	0	47	0	(62)
glp-1(ar202)[e,f]	16	46	0	38	(195)
glp-1(ar202); lag-2(q420)[e,g]	25*	60	0	15	(116)

*P value < 0.05 using a one-sided Fisher exact test [null hypothesis: percentage of wild type (*vs.* mutant) is different from that of *glp-1(ar202)* alone in the line directly above].

[a] No late-larval or adult non-Unc progeny were observed from self-progeny of *+/nT1([unc-?(n754)let-?]IV;V); lag-2(q411)/nT1([unc-?(n754)let-?]IV;V)* mothers; Lag (L1 lethal) progeny were observed.

[b] Rarely, non-Unc (non-Lag) adult progeny were observed from self-progeny of *glp-1(ar202); +/nT1([unc-?(n754) let-?]IV;V); lag-2(q411)/nT1([unc-?(n754) let-?]IV;V)* mothers.

[c] Gonad arms were scored in non-Lag animals only. These progeny ($n = 6$) represented 3% of the expected number of live progeny and 100% of these were Pro as adults. Many Lag (L1 lethal) progeny were also observed.

[d] Actual genotype: *unc-32(e189); lag-2(q420)*. Approximately 10% additional Lag (dead) larvae were also observed.

[e] Mothers were raised at 20°, and progeny were synchronized and scored as described in MATERIALS AND METHODS.

[f] Actual genotype: *unc-32(e189) glp-1(ar202)*. "% other" includes 37% Class A and 1% Class A + Class B.

[g] Actual genotype: *unc-32(e189) glp-1(ar202); lag-2(q420)*. Approximately 10% additional Lag (dead) larvae also observed. "% Pro" includes 1% Pro + Class B. "% other" includes 3% Class A and 12% Class A + Class B.

Comparison of *glp-1(Pro)* and *glp-1(Tum)* gain-of-function alleles: The *glp-1(Pro)* mutants described in this article share some similarities with the previously described Tum allele *glp-1(oz112gf)*. Both *glp-1(Pro)* and *glp-1(Tum)* mutants (i) are temperature sensitive, exhibiting a more penetrant phenotype at higher temperature; (ii) exhibit a ligand-independent increase in mitotic activity in the germline, including a late-onset extension of the distal germline; and (iii) carry changes in amino acids within the extracellular domain between the epidermal growth factor (EGF)-like repeats and the transmembrane domain of the receptor.

Mutations that cause *glp-1* Pro and Tum phenotypes also differ in several important ways. The most obvious difference is the Pro phenotype itself. One particularly puzzling aspect of the Pro phenotype exhibited by *glp-1(Pro)* alleles is that although *glp-1* activity is elevated and largely LAG-2 independent, the activity does not appear to be elevated equally in all germ cells. The general effect of lower dosage of *glp-1(Tum)* (as in *oz112*/null) or of more permissive temperature is a highly penetrant late-onset Tum phenotype, not a Pro phenotype. Therefore, meiotic entry from the distal stem-cell population appears to be most sensitive in the Tum class of mutants, whereas an earlier proximal defect results in a Pro phenotype (A. PEPPER, T.-W. LO and E. J. A. HUBBARD, unpublished observations). Genetic analysis shows that the Tum allele *glp-1(oz112gf)* behaves as a hypermorphic allele, increasing in penetrance with increasing doses of *glp-1(+)* (BERRY *et al.* 1997), whereas *glp-1(Pro)* alleles do not. Rather, *glp-1(+)* competes with *glp-1(Pro)*. Even the phenotypes displayed by the *glp-1(Pro)* mutants that have a late-onset character may not be hypermorphic since *glp-1(Pro)/glp-1(Pro)/+* is not more penetrant for the Class B phenotype than the corresponding *glp-1(Pro)/glp-1(Pro)* strain (Table 3). We also looked for evidence of distal mitotic zone extension at 48 hr in strains carrying an extra copy of *glp-1(+)*, but no obvious extension was observed. The Pro and Tum *glp-1* phenotypic classes are, therefore, genetically separable. This genetic separability suggests that the GLP-1 receptor may undergo different (although overlapping) regulatory interactions within the germline in its role in the meiosis/mitosis decision. In addition, *glp-1(Pro)* mutants do not display a Multivulva phenotype as does *glp-1 (oz112gf)*. This difference may be due to different levels of GLP-1 activity in the somatic cells of the two mutant classes.

Genetic behavior of *glp-1(Pro)* alleles suggests multimerization of or competition between GLP-1 receptors: The *glp-1(Pro)* lesions cause amino acid changes in the same region as that of other alleles that confer elevated receptor activity. Two *glp-1(Pro)* mutations map to the LNRs and the third maps just C-terminal to a pair of conserved extracellular cysteine residues. These changes are near putative cleavage sites S1 and S2 (Figure 3). Ge-

TABLE 8

Genetic interactions between *glp-1(ar202)* and suppressors and enhancers of *lin-12* and *glp-1*

Genotype	Germline pattern phenotype			
	% wild type	% Pro	% other	(*n*)
glp-1(ar202)[a,b]	0	96	4	(44)
glp-1(ar202); sel-12(ar131)[c]	5	95	0	(43)
glp-1(ar202); sel-12(ar171)[d]	9	91	0	(32)
glp-1(ar202); hop-1(RNAi)[a,e]	19	79	1	(89)
glp-1(ar202); sel-12(ar171); hop-1(RNAi)[d,f,g]	36	69	1	(122)
glp-1(ar202)/+[h]	100	0	0	(56)
glp-1(ar202)/+; sel-10(ar41)[i,e]	56	41	3	(98)
glp-1(ar202)[g]	0	97	3	(419)
sup-17(n1258); glp-1(ar202)	99	1	0	(242)
glp-1(ar202)[g]	0	94	6*	(68)
glp-1(ar202); sel-9(ar22)[g]	23	59	18	(90)
glp-1(ar224)[j]	0	92	8**	(127)
glp-1(ar224); sel-9(ar22)[e]	56	20	24	(54)

A two-sided Fisher exact test was performed on data marked with an asterisk(s) to test the null hypothesis that the indicated value ("other" *vs.* Pro + wild type) is different (in either direction) from the corresponding value in the line directly below it. **P* value < 0.05; ***P* value < 0.01.

[a] Actual genotype: *glp-1(ar202); unc-1(e719)*.

[b] "% Pro" includes 5% Pro + Class B. "% other" includes 2% Class A and 2% Class A + Class B.

[c] Actual genotype: *glp-1(ar202); sel-12(ar131) unc-1(e719)*.

[d] Actual genotype: *glp-1(ar202); sel-12(ar171) unc-1(e719)*.

[e] "% other": Class A + Class B.

[f] The observed level of suppression of *glp-1(ar202)* by *sel-12(ar171); hop-1(RNAi)* may be an underestimate since we did not observe a Glp phenotype in *sel-12(ar171); hop-1(RNAi)* animals scored in a parallel experiment ($n = 136$). A Glp phenotype was previously observed in *sel-12 (ar171); hop-1 (RNAi)* animals (Li and Greenwald 1997); however, our RNAi construct differed from that used by Li and Greenwald (1997); see materials and methods.

[g] "% other": Class A.

[h] Actual genotype: *ncl-1(e1865) unc-36(e251) glp-1(ar202)/unc-32(e189); him-5 (e1490)*. Non-Unc self-progeny were scored from hermaphrodite mothers of the same genotype.

[i] Actual genotype: *ncl-1(e1865) unc-36(e251) glp-1(ar202)/unc-32(e189); sel-10(ar41) him-5(e1490)*. "% Pro" includes 6% Pro + Class B. Non-Unc self-progeny were scored from hermaphrodite mothers of the same genotype. The double-mutant *glp-1(ar202); sel-10(ar41)* could not be scored due to synthetic lethality.

[j] Actual genotype: *dpy-17(e164) glp-1(ar224)/unc-32(e189) glp-1(ar224)*. "% Pro" includes 78% Pro + Class B. "% other" includes 6% Class A + Class B and 2% Tum.

netic evidence from many systems indicates that these regions of the protein have a negative regulatory influence on receptor activity, probably preventing receptor activity in the absence of interaction with a ligand (Greenwald 1994). One possibility is that the *glp-1(Pro)* lesions lead to an alteration in LNR conformation that renders the receptors more susceptible to S2 or S3 cleavage in the absence of ligand activation. Alternatively, the mutant receptors could be less sensitive to a negative regulatory factor. While models invoking an alteration of general susceptibility to activation can account for receptor hyperactivity evident from the *glp-1(Pro)* mutant phenotypes, they cannot account for the unusual genetic behavior of these alleles.

No other characterized LIN-12/Notch gene family mutations that map within the LNRs or between the LNRs and the transmembrane domain exhibit the same genetic behavior as the *glp-1(Pro)* mutations [suppression by *glp-1(+)* dosage and mutual suppression in a *trans*-heterozygous configuration]. Other alleles that map to this region are hypermorphic (Greenwald and Seydoux 1990; Lieber *et al.* 1993; Greenwald 1994; Berry *et al.* 1997; Brennan *et al.* 1997). Exceptions are loss-of-function alleles *glp-1(q158)*, a point mutation, and *glp-1(q172)*, an in-frame deletion that deletes most of the LNR region (Kodoyianni *et al.* 1992). Surprisingly, *lin-12(n950)* encodes the identical amino acid change as *glp-1(ar224)* (Figure 3), and yet *lin-12(n950)* behaves genetically as a hypermorph (Greenwald *et al.* 1983; Greenwald and Seydoux 1990). Given that LIN-12 and GLP-1 are functionally interchangeable (Fitzgerald *et al.* 1993), this observation suggests that regulation or local molecular environments differ between LIN-12 and GLP-1 such that the same amino acid change in a conserved residue has different genetic outcomes.

The observation that increasing doses of *glp-1(+)* can

interfere with the *glp-1(Pro)* mutant phenotypes suggests that either the wild-type receptor binds to and reduces the susceptibility to constitutive activation or the receptors compete for an additional factor or factors that modulate signaling. This, in turn, suggests that similar interactions may normally occur among wild-type receptors. While these are only two of several possible models that could account for our data, they are worth considering in more detail.

The possibility that LIN-12/Notch receptors form a dimeric or a higher multimeric complex that affects signaling has been suggested by previous genetic and biochemical data (Greenwald and Seydoux 1990; Kopan *et al.* 1996; Struhl and Adachi 2000; Sakamoto *et al.* 2002). In the case of *lin-12*, the data are consistent with receptor dimerization promoting activation (Greenwald and Seydoux 1990). Since *glp-1(+)* dosage suppresses gain-of-function phenotypes of *glp-1(Pro)* mutants, our results are consistent with a model in which a heterodimeric or heteromultimeric form is resistant to non-ligand-induced activation, whereas the homodimeric (or monomeric) form is more readily activated.

The dosage sensitivity of these alleles suggests the presence of a threshold effect for GLP-1 signaling. At levels under the threshold, little to no signaling occurred, while at levels above the threshold, a high level of signaling occurred. Threshold effects are consistent with the formation of homomultimers in the receptor interaction model. A threshold effect is also consistent with the presence of a feedback mechanism that amplifies the signal once a critical level of signaling is reached. Positive feedback in GLP-1 signaling has been suggested previously (Berry *et al.* 1997). Feedback and receptor interaction models are not mutually exclusive.

Competition among receptors for a limiting nonreceptor protein could also account for our genetic dosage results. In one such scenario, a positive factor required for non-ligand-mediated activation of *glp-1(Pro)* receptors would bind to both mutant and wild-type receptors but would bind with greater affinity to wild-type receptors. Thus, insufficient levels of mutant receptor may not allow efficient activation and increasing wild-type dosage would sequester the positive factor from the mutant receptor. The competition model is more difficult to reconcile with mutual suppression observed in animals *trans*-heterozygous for the highly penetrant *glp-1(Pro)* alleles. To accommodate a competition model, the two alleles would have to sequester the positive factor from each other without themselves becoming activated to a high level. This possibility, while unlikely, is not completely implausible given the dosage sensitivity of the alleles. A lower level of each mutant receptor in *trans*-heterozygous animals may be bound to a positive factor but activated below a threshold required for expression of the mutant phenotype.

***glp-1(Pro)* mutations could alter receptor signaling, trafficking, or both:** The proposed interactions between receptor proteins, either multimerization or competition, may occur at the cell surface as part of the signaling mechanism or, alternatively, they may occur within the cell during receptor maturation. Dimerization or multimerization of G-protein-coupled receptors during receptor transport is well documented and can be required for signaling, quality control, and trafficking (Bouvier 2001).

Genetic interactions between *glp-1(Pro)* and a *sel-9* mutant were unexpected and raise the possibility of an important regulation point at the level of SEL-9-dependent quality control or trafficking of GLP-1. SEL-9 is a member of the p24 family of proteins. p24 proteins exist in a large heteromeric complex, and disruption of this complex leads to several defects in the early secretory pathway. On the basis of these phenotypes and of *in vitro* experiments, several models for p24 function have been proposed, but their exact function remains controversial. The p24 complex has been implicated in quality control or trafficking from the ER to Golgi (Kaiser 2000; Springer *et al.* 2000), in suppression of the unfolded protein response (Belden and Barlowe 2001), and in cargo reception (Muniz *et al.* 2000). Elegant experiments by Wen and Greenwald (1999) suggested that SEL-9 normally acts in a quality-control capacity, preventing mutant receptors from gaining access to the plasma membrane.

Previous models for SEL-9 function cannot easily accommodate our results. *sel-9* mutations suppress and enhance phenotypes caused by loss-of-function and hypermorphic mutations, respectively, of *lin-12* and *glp-1* (Sundaram and Greenwald 1993b). In contrast, a *sel-9* mutation did not enhance the Pro phenotype of *glp-1 (Pro)* mutants, but rather partially suppressed this phenotype (Table 8).

One speculative model to address the discrepancies between the genetic interactions we observed between *sel-9* and the Pro phenotype and previous observations with *lin-12* and *glp-1* (Sundaram and Greenwald 1993b; Wen and Greenwald 1999), along with our other observations, is that receptors encoded by *glp-1(Pro)* mutants undergo some unregulated (but SUP-17-dependent) S2 cleavage and subsequent S3 cleavage while they are in the ER. Reduced SEL-9 levels would allow mutant receptors to transit to the plasma membrane, rather than being trapped in the ER. If the level of spontaneous activation were lower (or more properly regulated) once the receptor reaches the plasma membrane, this scenario could result in suppression of the Pro phenotype by reduced *sel-9*. This model is appealing since it accounts for suppression by *sel-9* and for the dependence of the Pro phenotype on S2 cleavage. The possibility that aberrant cleavage could occur before the mutant receptor reaches the plasma membrane is supported by the perinuclear localization of SEL-12, consistent with accumulation in the ER/Golgi (Levitan and Greenwald 1998). Interestingly, *sel-9(ar22)* en-

hances the non-Pro defects of *glp-1(Pro)* mutants (Table 8). The non-Pro phenotypes are also more similar to the late-onset Tum phenotype. Taken together, these results support the view that the Pro phenotype reflects a regulation of *glp-1* in the early proximal germline that is qualitatively different from that in the distal end.

Regardless of the precise molecular mechanisms underlying germline defects caused by *glp-1(Pro)* mutants, our results indicate that these mutant receptors act within the canonical LAG-1-mediated pathway for signaling. Moreover, *glp-1(Pro)* defects are dependent upon SUP-17 and presenilin-mediated cleavage. Hence, these alleles may offer new insights into GLP-1-mediated signaling in the *C. elegans* germline and into LIN-12/Notch-mediated signaling in general.

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