DOI: 10.1534/genetics.105.048041

New Positive Regulators of *lin-12* Activity in *Caenorhabditis elegans* Include the BRE-5/Brainiac Glycosphingolipid Biosynthesis Enzyme

Iskra Katic,*,† Laura G. Vallier†,‡,[1] and Iva Greenwald†,‡,[2]

**Department of Genetics and Development, †Howard Hughes Medical Institute, ‡Department of Biochemistry and Molecular Biophysics, Columbia University College of Physicians and Surgeons, New York, New York 10032*

Manuscript received July 11, 2005
Accepted for publication August 26, 2005

ABSTRACT

Screens for suppressors of *lin-12* hypermorphic alleles in *C. elegans* have identified core components and modulators of the LIN-12/Notch signaling pathway. Here we describe the recovery of alleles of six new genes from a screen for suppressors of the egg-laying defect associated with elevated *lin-12* activity. The molecular identification of one of the new suppressor genes revealed it as *bre-5*, which had previously been identified in screens for mutations that confer resistance to Bt toxin in *C. elegans*. *bre-5* is the homolog of *D. melanogaster brainiac*. BRE-5/Brainiac catalyzes a step in the synthesis of glycosphingolipids, components of lipid rafts that are thought to act as platforms for association among certain kinds of membrane-bound proteins. Reducing the activity of several other genes involved in glycosphingolipid biosynthesis also suppresses the effects of constitutive *lin-12* activity. Genetic analysis and cell ablation experiments suggest that *bre-5* functions prior to ligand-induced ectodomain shedding that activates LIN-12 for signal transduction.

RECEPTORS of the LIN-12/Notch family mediate many cell-cell interactions that cause cells with equivalent potentials to adopt distinct fates. These receptors undergo three proteolytic processing events (reviewed in Greenwald 2005). They undergo a proteolytic cleavage at site 1 during their transit to the cell surface in mammals and reside on the surface as a heterodimer between the N- and C-terminal fragments. After activation by binding to transmembrane ligands of the Delta/Serrate/LAG-2 (DSL) family, proteolytic cleavage at site 2 in the extracellular domain results in ectodomain shedding. The remaining transmembrane portion of the receptor is then cleaved at site 3 in the transmembrane domain, which releases the intracellular domain. The released intracellular domain translocates to the nucleus where it forms a transcriptional activation complex with a sequence-specific DNA-binding protein LAG-1 (Suppressor of Hairless in Drosophila and CBF1/RBP-J in mammals) and other cofactors to promote transcription of target genes.

In *Caenorhabditis elegans*, three different classes of constitutively active forms of LIN-12 have been described. One class is encoded by hypermorphic alleles with missense mutations that alter the extracellular domain (Greenwald and Seydoux 1990). These alleles are termed *lin-12(d)*, and recently, similar alleles have been found to be commonly associated with acute T lymphoblastic leukemia (Weng *et al.* 2004). The two other classes are engineered transgenic forms that have also been used extensively in Drosophila and mammalian studies. These truncated forms mimic the products formed from cleavage at site 2 (ectodomain shedding) or site 3 (transmembrane cleavage). In *C. elegans*, the site 2 cleavage mimic is called *lin-12(ΔE)* (Shaye and Greenwald 2005; described below) and the site 3 cleavage mimic is called *lin-12(intra)* (Struhl *et al.* 1993).

Constitutively active forms of LIN-12 affect many different cell fate decisions, leading to phenotypes that are amenable to genetic analysis. The most widely used basis for genetic screens has been a cell fate decision in the developing gonad, the anchor cell (AC)/ventral uterine (VU) precursor cell decision (Greenwald 1998). In wild-type hermaphrodites, two gonadal cells interact so that one becomes the AC and the other becomes the VU. *lin-12* activity mediates this interaction, so that in animals homozygous for null alleles of *lin-12* both cells become ACs and, conversely, in mutants with elevated *lin-12* activity, both cells become VUs (Greenwald *et al.* 1983). The absence of an AC leads to the absence of a functional vulva and hence an egg-laying (Egl)-defective phenotype. The 0 AC-Egl phenotype of *lin-12(d)* mutants has been exploited in large-scale screens for suppressors, yielding intragenic revertants and extragenic suppressors defining seven *suppressor/enhancer of lin-12* (*sel*) genes that modulate *lin-12* activity (Greenwald *et al.* 1983; Ferguson and Horvitz 1985; Tax *et al.* 1997).

[1]*Present address:* Biology Department, Hofstra University, Hempstead, NY 11550.

[2]*Corresponding author:* 701 W. 168th St., Room 720, College of Physicians and Surgeons, Columbia University, New York, NY 10032. E-mail: greenwald@cancercenter.columbia.edu

Tax *et al.* (1997) focused on extragenic suppressors and identified seven *sel* genes, five of which have been characterized to date. Three essential genes were identified by non-null alleles: *lag-2*, which encodes the DSL ligand that mediates the AC/VU decision (Tax *et al.* 1994); *sup-17*, which encodes an ADAM family protease that may cleave LIN-12 at site 2 in its extracellular domain and mediate ectodomain shedding (Tax *et al.* 1994, 1997; Wen *et al.* 1997); and *sel-8*, which encodes a component of the nuclear complex and appears to bridge LIN-12 and LAG-1 (Doyle *et al.* 2000; Petcherski and Kimble 2000). Tax *et al.* (1997) also recovered null alleles of *sel-5*, which encodes a cytoplasmic serine/threonine kinase that may influence LIN-12 trafficking (Fares and Greenwald 1999) and of *sel-7*, which encodes a novel nuclear protein (Chen *et al.* 2004). The screen performed by Tax *et al.* (1997) was not saturated and was understandably biased toward highly penetrant suppressors, which are easier to work with and to recognize among the background of intragenic revertants (see also results). The lack of saturation implied that there would be new genes to discover through this approach. In addition, the bias toward high penetrance and the requirement for robust egg laying would have precluded the identification of genes such as *sel-12* presenilin, for which null alleles, because of functional redundancy and pleiotropic effects, do not restore normal egg laying at high penetrance.

To identify more factors that affect LIN-12/Notch signaling, we performed a screen for suppressors of the 0 AC-Egl defect of a *lin-12(d)* allele and characterized some of the low-penetrance suppressors that we obtained. We identified alleles of six apparent new *sel* genes, as well as intragenic revertants and alleles of previously identified *sel* genes. We cloned one of the new *sel* genes and found that it corresponds to *bre-5*, which codes for a β1,3 *N*-acetylglucosaminyltransferase and is orthologous to Drosophila *brainiac* (Goode *et al.* 1996b; Griffitts *et al.* 2001). We extend this observation to show that two other genes encoding glycosyltransferases thought to function in the synthesis of glycosphingolipids, structural components of lipid rafts, are positive regulators of LIN-12/Notch signaling and discuss how glycosphingolipids might affect LIN-12/Notch signaling.

MATERIALS AND METHODS

General *C. elegans* methods and strains: Standard methods as described in Brenner (1974) were used for handling, maintenance, mutagenesis, and genetic analysis of *C. elegans*. Experiments were performed at 20° unless indicated otherwise. The wild-type parent for all strains was *C. elegans* var. Bristol N2 (Brenner 1974), except for mapping experiments using the Bergerac strains DP13 (Williams *et al.* 1992) and GS3063, in which *lin-12(n302)* had been placed into the Hawaiian strain CB4856 background by repeated backcrossing (data not shown).

The main alleles used in this study are: LG I—*sup-17(n1258)* (Tax *et al.* 1997), *bre-4(ye27)* (Marroquin *et al.* 2000); LG II—*sel-4(n1259)* (Tax *et al.* 1997); LG III—*sel-8(sa54)*, *sel-5(n1254)* (Tax *et al.* 1997; Fares and Greenwald 1999; Doyle *et al.* 2000), *lin-12(n302, n950, n941)* (Greenwald *et al.* 1983; Greenwald and Seydoux 1990), *lin-12(ar170)* (Hubbard *et al.* 1996), *glp-1(ar202)* (Pepper *et al.* 2003), *glp-1(e2141, e2142)* (Priess *et al.* 1987), *bre-3(ye26)*, *bre-2(ye31)* (Marroquin *et al.* 2000); LG IV—*bre-1(ye4)* (Marroquin *et al.* 2000), *bre-5(ye17, ar560)* (Marroquin *et al.* 2000; this study); LG X—*sel-7(n1253)* (Tax *et al.* 1997; Chen *et al.* 2004).

Additional information about the alleles listed above and about the markers used for mapping and for facilitating genetic analyses in this work can be found via Wormbase at http://www.wormbase.org. The transgene *arIs53 [lin-12(ΔE)::gfp]* expresses a GFP-tagged version of LIN-12 lacking most of its extracellular domain under the *sel-12* promoter (Shaye and Greenwald 2005).

Identification of extragenic suppressor mutations: All suppressors discussed in this work were isolated after EMS mutagenesis of *unc-36(e251) lin-12(n302)* hermaphrodites. Three mutagenized L4 P_o hermaphrodites were picked per 10-cm plate and allowed to self-fertilize for two generations. Animals carrying a mutation in a suppressor gene form a vulva and are egg laying competent, so any F_2 eggs were transferred together to a new plate and adults were examined for egg-laying competence. A single Egl^+ individual was used to found a revertant stock from a single P_o plate to ensure independence of all mutations.

A major class of suppressors encompasses intragenic revertants in *lin-12* that reduce or eliminate *lin-12* activity (Greenwald *et al.* 1983; Tax *et al.* 1997). Since loss of *lin-12* activity acts dominantly, we tested all suppressors for dominance as a triage step. *sel; unc-36(e251) lin-12(n302)* hermaphrodites were crossed with *dpy-17(e164) lin-12(n302); him-5(e1467)* males and 30 non-Unc F_1's were scored for egg-laying ability. Suppressors that conferred egg-laying ability on >10% of heterozygous hermaphrodites in this test were discarded. As a reference point, ~75% of hermaphrodites carrying *lin-12(n302) in trans* to a null allele are egg laying competent, and ~62% of hermaphrodites carrying *lin-12(n302) in trans* to the hypomorphic allele *lin-12(n676n930)* are egg laying competent (Sundaram and Greenwald 1993a). Revertants that remained after this triage step were first tested for linkage to *lin-12* and unlinked suppressors were subjected to sequence-tagged site mapping (Williams *et al.* 1992; data not shown).

Complementation tests with mutations in known *sel* genes: Complementation tests were performed between new suppressor mutations and recessive alleles of known *sel* genes mapping on the same chromosome. *unc-36(e251) lin-12(n302); sel(unknown)* hermaphrodites were mated with *lin-12(n302); sel(known); him-5(e1490)* males, where "*sel(known)*" corresponds to *sup-17(n1258)*, *sel-4(n1259)*, *sel-8(sa54)*, *sel-5(n1254)*, or *sel-7(n1253)*, and 30 non-Unc progeny were scored for egg-laying ability. Mutations were scored as failing to complement when the egg-laying ability of *trans*-heterozygous animals resembled that exhibited by animals homozygous for the canonical allele of the tested *sel* gene. Many suppressors that failed to complement a known *sel* gene were sequenced for the presence of a mutation within the coding region of that gene.

Ten alleles of *sup-17* (*ar528*, *ar537*, *ar540*, *ar542*, *ar543*, *ar546*, *ar552*, *ar553*, *ar569*, and *ar585*), 1 putative allele of *sel-4* (*ar555*), 13 new alleles of *sel-5* (*ar518*, *ar520*, *ar521*, *ar529*, *ar541*, *ar551*, *ar556*, *ar559*, *ar568*, *ar571*, *ar574*, *ar579*, and *ar588*), and 6 alleles of *sel-7* (*ar516*, *ar517*, *ar523*, *ar539*, *ar558*, and *ar586*) were identified by these tests. The *sel-7* alleles are described in Chen *et al.* (2004).

ar519 mapped to chromosome IV and was found to be an allele of *lag-1* on the basis of complementation tests, rescue experiments, and sequencing of the *lag-1*-coding region.

Atypical intragenic revertant alleles: Other alleles linked to chromosome III either failed to complement both *sel-5* and *sel-8* alleles to some extent or complemented both *sel-5* and *sel-8* alleles. Three of these alleles were found to map to an ~3-MU region between *stP120* and *stP127* that includes *lin-12*. We then sequenced the coding region of *lin-12* in the two mutants that failed to complement both *sel-5* and *sel-8* alleles and in one mutant that complemented both *sel-5* and *sel-8* alleles and found missense changes in *lin-12* (Table 1) in all of them. We therefore assume that a majority of mutations that were linked to *lin-12* but were not *sel-5* alleles encode second-site mutations in *lin-12* itself and we did not analyze these further.

One mutation, *ar562*, failed to complement *sel-8* but complemented *sel-5*. No sequence changes were present in the coding region of *sel-8*, which was also excluded as a candidate gene because *ar562* maps to the right of *unc-36*. To confirm that *ar562* was not one of the majority class of *lin-12* intragenic mutation, we sequenced the coding region of *lin-12* and found no changes. This mutant was also sequenced for changes in the coding region of *bre-3*, which can suppress the egg-laying defect of *lin-12(n302)/+* animals (see below) and which maps to the right of *lin-12* and close to it. No sequence changes were found.

Additional mapping of new *sel* genes: *sel(ar578) V* homozygotes segregating from Bristol/DP13 heterozygotes were used to map *sel(ar578)* to the left of *stP18* (data not shown). This position excludes *sel(ar578)* as a candidate *sel-6* allele. Furthermore, there were no changes in the coding region of *lag-2* or in the coding region of *skp-1*, the *C. elegans* homolog of the Notch interactor Skip (Zhou *et al.* 2000), which both map on chromosome V.

sel(ar570) IV homozygotes segregating from Bristol/GS3063 heterozygotes were used to map *sel(ar570)* to the right of SNP *pkP4046* on clone Y105C5 on the right arm of chromosome IV (data not shown).

Identification of *sel(ar560)* as an allele of *bre-5*: *sel(ar560) IV* homozygotes segregating from Bristol/GS3063 heterozygotes were used to map *sel(ar560)* to the right of the SNP *pkP4079* on cosmid C28C12. We then constructed *lin-12(n302); dpy-20(e1282) sel(ar560)* and proceeded as described above. Further SNP mapping indicated that *sel(ar560)* mapped to the right of *pkP4084* on cosmid M117. Finally, we constructed a *lin-12(n302); dpy-20(e1282) sel(ar560) unc-26(e205)* mapping strain. We mated these hermaphrodites with GS3063 males and picked non-Dpy, non-Unc F_1's and from those obtained Dpy non-Unc recombinants and assessed the presence of *sel(ar560)*, evidenced by segregating some non-Egl animals. Fourteen of 87 recombinants lost *sel(ar560)*, while 73 of 87 recombinants kept it. SNP analysis of these recombinants delineated *sel(ar560)* between a new A → G SNP at position 17,458 of cosmid T12G3 and SNP F35G2.1 on cosmid F35G2. This region contains 30 predicted genes. A mutation in *bre-5* was confirmed by sequencing the PCR product using one pair of primers: bre-5(CF) (5′-GGCTTAAGATCCACAAACACAG-3′) and bre-5(CR) (5′-GGAAGAATGCTTCTGGGAAG-3′).

***bre* mutant suppression of *lin-12(n302)/+*:** In the case of *bre-1(ye4)*, *bre-4(ye27)*, and *bre-5(ye17)*, strains of *unc-32(e189); bre* hermaphrodites were mated with males of the genotype *lin-12(n302); bre; him-5(e1490)*. Their non-Unc cross progeny were scored for the ability to lay eggs.

Complementation between *bre-5(ar560)* and *bre-5(ye17)* was assessed similarly: *unc-32(e189); bre-5(ye17)* hermaphrodites were mated with *lin-12(n302); bre-5(ar560); him-5(e1490)* males and their non-Unc progeny were scored for egg-laying ability.

For *bre-2(ye31)*, Egl *lin-12(n302) bre-2(ye31)* hermaphrodites were fed bacteria expressing an RNA-mediated interference (RNAi) construct against *sel-7* to induce an AC, and non-Egl progeny of such worms were mated to *bre-2(ye31)* males. Cross progeny (verified by segregation of some non-Egl animals) were then scored for egg laying.

***bre-5* interaction with other *lin-12* and *glp-1* alleles:** To assess the number of ACs, L3 larvae were scored by Nomarski optics and ACs were identified by morphology. For the Multivulva phenotype, L4 larvae were generally picked onto separate plates and checked for the number of pseudovulvae the next day. For germline proliferation, L1 larvae of hermaphrodites carrying *glp-1(ar202)* were transferred to individual plates at 25° and scored for progeny production. For embryonic lethality, individuals carrying *glp-1(e2141)* and *glp-1(e2142)* were picked as L4 larvae and transferred to fresh plates for 3 consecutive days and the eggs on plates were scored for hatching. This assay was conducted at 15°.

Complementation tests among mutations that map to chromosome IV: Males of the genotype *lin-12(n302); sel(ar522); him-5(e1490)* were mated with non-Egl hermaphrodites homozygous for *unc-36(e251) lin-12(n302); sel(ar570)* or *sel(ar560)*. F_1 hermaphrodites were scored for egg laying. *sel(ar522)* failed to complement *sel(ar570)* but complemented *sel(ar560)*.

sel(ar584) was tested for complementation with *bre-5(ar560)* and *bre-5(ye17)* as follows: *unc-36(e251) lin-12(n302); sel(ar584)* hermaphrodites were mated with *bre-5(ar560)* or *bre-5(ye17)* males (or N2 males as a negative control). Their non-Unc progeny were scored for the ability to lay eggs. The coding region of *bre-5* was sequenced in the *unc-36(e251) lin-12(n302); sel(ar584)* background.

RNAi: For RNAi feeding against *bre-3*, serine palmitoyltransferase subunit C23H3.4, and the putative glucosylceramide synthases F59G1.1, F20B4.6, and T06C12.10, we used bacterial strains from the *C. elegans* RNAi library (Kamath *et al.* 2003). L4 stage *unc-32(e189)/lin-12(n302); him-5(e1490)/+* parents were placed onto lawns of such bacteria. Their non-Unc progeny were singly picked to separate plates, and their egg-laying ability and *unc-32(e189)* segregation were scored so that the effects of RNAi on *unc-32(e189)/lin-12(n302)* and *lin-12(n302)* worms could be separately analyzed. An empty feeding vector was used as a negative control.

Laser ablations of vulval precursor cells: P3.p and P5.p-P8.p or P3.p-P7.p were killed with a laser microbeam in early L2 hermaphrodites before vulval precursor cell (VPC) fates were specified, as described in Levitan and Greenwald (1995), to isolate either P4.p or P8.p. Success of ablations was confirmed. A vulval fate was inferred if a VPC divided and an invagination was formed.

RESULTS

Identification of extragenic suppressors of the Egl defect of *lin-12(n302)* hermaphrodites: Animals homozygous for the dominant hypermorphic *lin-12(d)* allele *lin-12(n302)* display a 0 AC-Egl defect but are otherwise normal and fertile. We screened 203,000 mutagenized haploid genomes by examining the F_2 and F_3 progeny of mutagenized *unc-36(e251) lin-12(n302)* hermaphrodites and identified 284 independent revertants of *lin-12(n302)* (see materials and methods). On the basis of the outcome of the previous screens, we anticipated that a large proportion of suppressors would result from intragenic *lin-12* mutations, as loss or reduction of *lin-12* activity behaves as a dominant suppressor of the hypermorphic *lin-12(d)* alleles (Greenwald *et al.* 1983;

TABLE 1

Suppressor alleles identified by Tax *et al.* (1997) and our screen

Gene	Tax *et al.* (1997)	Our study	% suppressed[a]
lin-12[b]	109	247	ND
sup-17	5	10	52 ($n = 353$)[c]
lag-2	2	0[d]	94 ($n = 189$)[c]
sel-6	2	0	90[c]
sel-5[e]	2	13	51[c]
sel-4	1	1	31[c]
sel-8	1	0	25[c]
sel-7	1	6	53[c]
sel(ar526)	0	1	10 ($n = 68$)[f]
lag-1[g]	0	1	73 ($n = 19$)[f]
bre-5(ar560)	0	1	4 ($n = 193$)[f]
sel(ar562)	0	1	37 ($n = 49$)[f]
sel(ar584)	0	1	17 ($n = 51$)[f]
sel(ar522, ar570)	0	2	17 ($n = 93$)[f]
sel(ar578)	0	1	5 ($n = 40$)[f]

[a] Suppression of the *lin-12(n302)* egg-laying defect by the most penetrant allele of each *sel* gene.

[b] Sequence changes associated with new alleles of *lin-12*: *ar563*, E492K; *ar564*, R721Q; and *ar575*, K1127E.

[c] Suppression of egg laying by the most penetrant allele. Data are from Tax *et al.* (1997).

[d] *lag-2* alleles recovered by Tax *et al.* (1997) were dominant and would have been eliminated by our triage step.

[e] Sequence changes associated with new alleles of *sel-5*: *ar568*, Q551stop; *ar571*, g → a acceptor splice-site mutation; *ar574*, R91stop; *ar579*, 525-bp deletion within the coding region; and *ar588*, g → a acceptor splice-site mutation.

[f] Animals were also homozygous for *unc-36(e251)*.

[g] *lag-1(ar519)* encodes the L302F change.

TABLE 2

New extragenic suppressors of the *lin-12(d)* 0 AC-Egl defect

Alleles of new *sel* genes	Relevant mapping and sequencing data	Penetrance[a] (% egg laying competent/total)
LG I		
ar526	Complements *sup-17(n1258)*	10 ($n = 68$)
LG III		
ar562	Maps to the right of *unc-36*; no sequence changes in the *lin-12* or *bre-3* coding regions.	37 ($n = 49$)
LG IV		
ar560		4 ($n = 193$)
ar584		17 ($n = 51$)
ar522, ar570	Single complementation group	15 ($n = 40$), 17 ($n = 93$)
LG V		
ar578	No sequence changes in *lag-2* and *skp-1* coding regions; *ar578* maps to the left of *sel-6*.	5 ($n = 40$)

[a] Penetrance of the suppression of the egg-laying defect (after at least two backcrosses) was determined at 20° in animals homozygous for the indicated mutation and *unc-36(e251) lin-12(n302)* from Table 1.

Sundaram and Greenwald 1993b; Tax *et al.* 1997). Therefore, we discarded all mutants that exceeded a threshold value of 10% semidominance, which is substantially lower than the dominance observed with *lin-12* null intragenic revertants, the major class of revertant observed after mutagenesis of *lin-12(d)* alleles (Greenwald *et al.* 1983; see also materials and methods). A total of 74 revertants remained after this triage step.

Linkage analysis indicated that 50 of the revertant strains contained suppressor mutations that were tightly linked to *lin-12*, while 24 were unlinked. Our detailed analysis will be presented below and is summarized in Tables 1 and 2. Thirteen of 50 linked suppressors were found to be alleles of the known gene *sel-5*, and 36/50 appeared to be intragenic revertants; one linked suppressor appears likely to define a new *sel* gene. The 24 unlinked mutations were clearly extragenic suppressors, and these were mapped and assigned to complementation groups; 17/24 unlinked mutations appeared to be alleles of *sup-17*, *sel-4*, and *sel-7*, and 1/24 proved to be a non-null allele of *lag-1*. Six of 24 unlinked mutations defined five new potential *sel* genes. A total of six potential new *sel* genes were therefore identified in this screen.

Linked suppressors: *sel-5* and *sel-8*, as well as *lin-12*, map to chromosome III. We tested those mutations that showed linkage to *lin-12* for complementation with canonical alleles of *sel-5* and *sel-8*. Thirteen suppressors failed to complement *sel-5(n1254)* and complemented *sel-8(sa54)* and were therefore concluded to be alleles of the *sel-5* gene. We have confirmed the presence of a mutation in the *sel-5* coding region for five of them (Table 1).

The other alleles on chromosome III fall into two major categories with respect to *sel-5* and *sel-8* complementation, either complementing or, surprisingly, failing to complement reference mutations in both genes. We used STS mapping and discovered that the mutations in either category that were tested by this method map to a region that includes *lin-12*. We then sequenced the coding region of *lin-12* in three mutants and saw specific missense changes (see Table 1). On the basis of the mapping and sequencing results with these alleles, we believe that the majority of other mutations showing linkage to *lin-12* on chromosome III are likely to be atypical, very weakly semidominant second-site mutations in *lin-12* itself that were not discarded after the triage step. Our observations suggest that the nonallelic noncomplementation observed between certain weak *lin-12* alleles and *sel-5* or *sel-8* mutations can be explained by a synergistic interaction that lowers *lin-12* activity sufficiently.

One mutation, *sel(ar562)*, failed to complement *sel-8(sa54)* but sequence analysis of the *sel-8* coding region failed to detect any alterations. Mapping data placing the *sel(ar562)* mutation to the right of the gene *unc-36* indicated that *sel(ar562)* is not an allele of *sel-8*, which maps to the left of *unc-36*. We sequenced the *lin-12* coding region of *sel(ar562)* and found no sequence

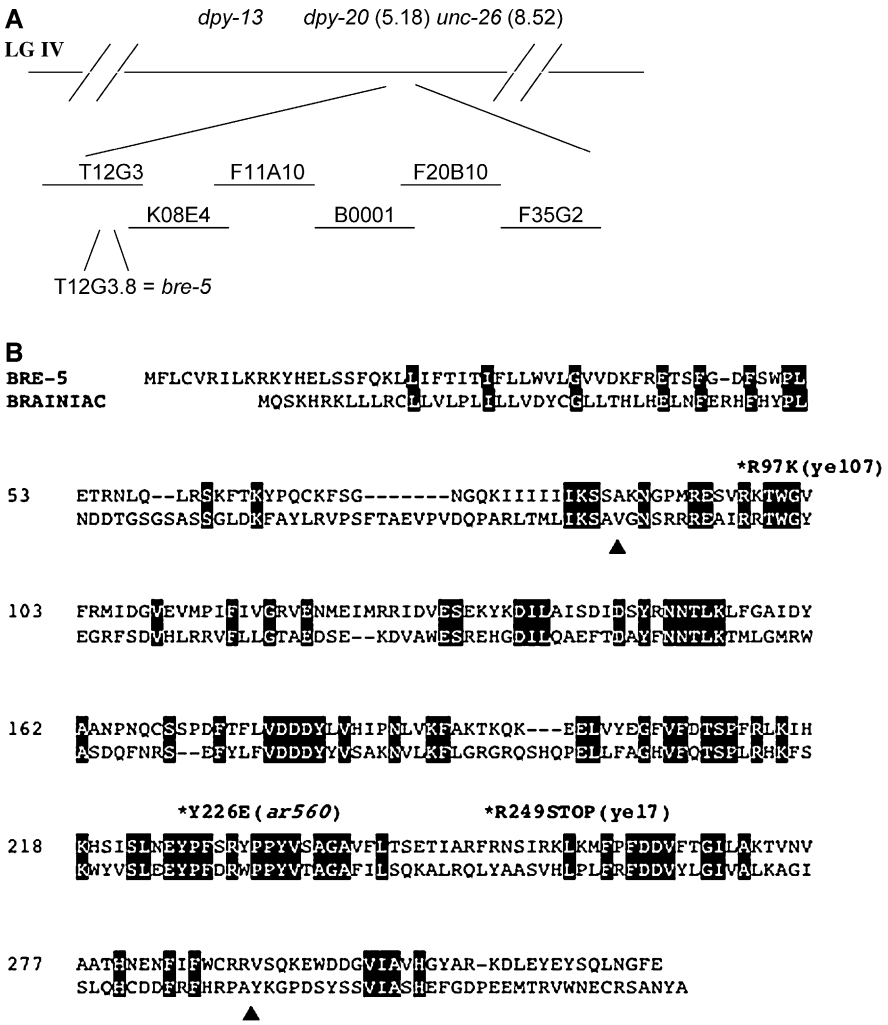

FIGURE 1.—Molecular cloning and DNA sequence analysis of *bre-5(ar560)*. (A) Mapping and cloning of *bre-5(ar560)*. Only the relevant genes and cosmids are shown. (B) Alignment of BRE-5 and *D. melanogaster* homolog Brainiac. Proteins were aligned using ClustalW and their alignments are superimposed here. Asterisks denote the amino acids mutated in *bre-5* alleles *ye107*, *ye17*, and *ar560*. Shaded letters designate amino acid identity. Galactosyltransferase domain homology is in the region between the triangle symbols.

alterations, suggesting that *sel(ar562)* is not one of the majority class of atypical *lin-12* intragenic revertant recovered in our screen. These observations together suggest that *sel(ar562)* may define a novel *sel* gene on LG III, although we cannot exclude the possibility that it contains a mutation in a noncoding region that affects the expression of *lin-12*.

Unlinked suppressors: To assign the mutations that did not show linkage to *lin-12* to chromosomes, we used the STS mapping method (WILLIAMS *et al.* 1992) and tested the new mutations for complementation with the known recessive suppressors on their respective chromosomes, reasoning that these were likely candidates. Complementation tests suggest that we identified 10 new *sup-17* alleles and 6 new *sel-7* alleles. The new allele *sel(ar555)* appears likely to be an allele of *sel-4*, with the caveat that *sel(ar555)* acts semidominantly; *sel(ar555)* maps to the *sel-4* region (data not shown), but as the molecular identity of *sel-4* is not known, allelism could not be confirmed by sequencing.

Our genetic and molecular analysis indicates that one of the suppressors is a weak hypomorph of *lag-1*, which encodes a core component of the pathway that had not been previously recovered in suppressor screens (Table 1). The recovery of such an allele underscores the usefulness of this screen for identifying core components of the LIN-12 signaling pathway, even when their null phenotypes are lethal.

Seven mutations define six new *sel* genes: Seven mutations do not appear to be alleles of known extragenic suppressors of *lin-12* nor of *lin-12* itself (Table 2). One of these is the linked mutation *sel(ar562)*, described above.

We assigned the remaining six mutations to chromosomes by using STS mapping (see MATERIALS AND METHODS). One mutation each mapped to chromosomes I and V, and four mutations mapped to chromosome IV. *Inter se* complementation tests among the mutations on chromosome IV showed that *sel(ar570)* and *sel(ar522)* form a single complementation group and that *sel(ar584)* and *sel(ar560)* are distinct complementation groups. We focused our analysis of *sel(ar560)*, which mapped to the LG IV cluster.

***sel(ar560)* corresponds to *bre-5*, the *C. elegans* homolog of Drosophila *brainiac*:** We mapped *sel(ar560)* to a small region containing 30 predicted genes (see MATERIALS AND METHODS and Figure 1) and observed

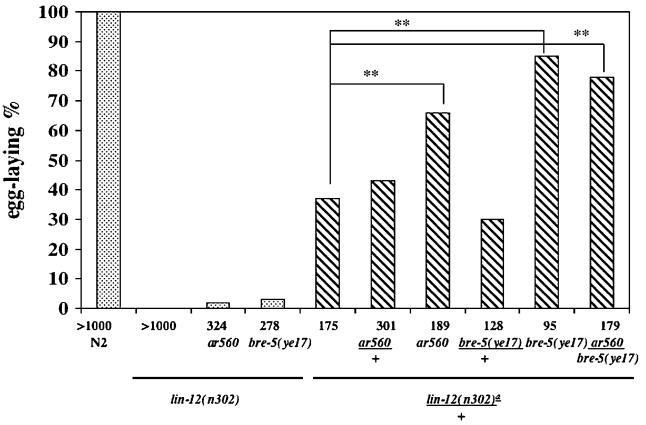

FIGURE 2.—*sel(ar560)* reduces the activity of a *lin-12(d)* allele and fails to complement *bre-5(ye17)*. A chi-square test was performed to assess whether there is a significant difference between indicated strains. Asterisks denote differences significant at the 99% level. Numbers of animals scored are below corresponding bars. We note that, for *lin-12(d)* alleles, the ability to lay eggs correlates absolutely with the presence of an anchor cell; thus, the percentage of hermaphrodites that lay eggs is equivalent to the percentage of hermaphrodites that have an anchor cell (GREENWALD *et al.* 1983). (*a*) Actual genotype on chromosome III: *lin-12(n302)/unc-32(e189)*.

TABLE 3

***bre-5* affects *lin-12* activity in VPC specification**

Genotype	No. of Muv[a] animals/total (%)	Average no. of pseudovulvae
lin-12(n950)	77/77 (100)	4.8 +/− 0.1
lin-12(n950); bre-5(ar560)	62/62 (100)	4.6 +/− 0.1
lin-12(n950); bre-5(ye17)	93/93 (100)	4.7 +/− 0.1
lin-12(ΔE)::gfp	115/116 (99)	2.7 +/− 0.1
bre-5(ar560); lin-12(ΔE)::gfp	95/96 (99)	1.7 +/− 0.1
bre-5(ye17); lin-12(ΔE)::gfp	52/94 (55)	0.7 +/− 0.1
lin-12(0); lin-12(ΔE)::gfp[b]	44/92 (48)	0.7 +/− 0.1
lin-12(0); bre-5(ye17); lin-12(ΔE)::gfp[b]	36/113 (32)	0.5 +/− 0.1

These strains were grown and scored in parallel. All animals containing *arIs53[lin-12(ΔE)::gfp]* were able to lay eggs.

[a] Muv is defined as the presence of one or more pseudovulva.

[b] *lin-12(0) = unc-36(e251) lin-12(n941).*

that one of the genes within this region is *bre-5*, which encodes a β1,3-GlcNAc-transferase and is orthologous to the Drosophila gene *brainiac* (MARROQUIN *et al.* 2000; GRIFFITTS *et al.* 2001). The phenotype of *brainiac* null mutants and genetic interactions with *Notch* suggest that Brainiac modulates Notch signaling in Drosophila (GOODE *et al.* 1992, 1996a). Indeed, sequence analysis of the coding region of *bre-5* in the *ar560* background revealed a missense mutation in a conserved residue of the glycosyltransferase domain (Figure 1). Alleles of *bre-5* in *C. elegans* had previously been identified in a screen for animals resistant to a *Bacillus thuringiensis* (Bt) toxin, a process that has not been linked to LIN-12/Notch signaling. The *bre-5(ye17)* allele appears likely to be a strong loss of function or null for *bre-5* function, as it encodes truncated mutant protein that has no enzymatic activity *in vitro* (MARROQUIN *et al.* 2000; GRIFFITTS *et al.* 2003).

We used *bre-5(ye17)* to obtain further support for the conclusion that *sel(ar560)* is an allele of *bre-5*. First, we found that *bre-5(ye17)*, like *sel(ar560)*, is a low-penetrance but reproducible suppressor of *lin-12(n302)* (Figure 2). Second, *bre-5(ye17)*, like *sel(ar560)*, is a strong suppressor of *lin-12(n302)/+* (Figure 2). Third, *lin-12(n302)/+; sel(ar560)/bre-5(ye17)* is strongly suppressed, indicating that *sel(ar560)* fails to complement *bre-5(ye17)* for suppression. We therefore will refer to *sel(ar560)* as *bre-5(ar560)* for the remainder of this work.

Characterization of *bre-5* interactions with *lin-12* and *glp-1*: Neither of the *bre-5* alleles shows any obvious phenotypes associated with reduction of *lin-12* or *glp-1* activity in a wild-type background. For example, the AC/VU decision proceeds normally in *bre-5* mutant animals: 50/50 *bre-5(ar560)* and 74/74 *bre-5(ye17)* mutant animals have 1 AC. However, *bre-5* appears to be a positive regulator of *lin-12* activity in the AC/VU decision, as loss of *bre-5* activity suppresses the 0 AC-Egl phenotype associated with elevating *lin-12* activity. This suppression is the basis for concluding that *bre-5* acts in the AC/VU decision, as we have not observed any synergy between *bre-5* and null alleles of either *sel-7* or *sel-12* [83/83 *bre-5(ye17); sel-7(n1253)* and 80/80 *bre-5(ye17); sel-12(ar171)* double mutants have a single AC].

To test whether *bre-5* positively regulates *lin-12* activity in other cell fate decisions, we looked for suppression of the ectopic vulval induction caused by *lin-12(n950)* and did not observe any effect. However, *bre-5* is able to suppress ectopic vulval induction caused by *lin-12(ΔE)*, the engineered transgenic form of LIN-12 missing most of the extracellular domain that mimics the product formed from cleavage at site 2 (Table 3), suggesting that *bre-5* also positively regulates LIN-12 activity in vulval precursor cell specification.

We also investigated whether *bre-5* can affect the signaling of the LIN-12 homolog, GLP-1. We combined *bre-5(ye17)* with the partial loss-of-function alleles *glp-1(e2141)* or *glp-1(e2142)* (PRIESS *et al.* 1987). We saw no effect on total brood size or maternal-effect lethality (data not shown). In addition, *bre-5(ye17)* did not suppress the temperature-sensitive sterility caused by the constitutive *glp-1* allele *ar202* (PEPPER *et al.* 2003): 119/119 *glp-1(ar202); bre-5(ye17)* were sterile at 25°, similar to *glp-1(ar202)* control animals (115/115 animals were

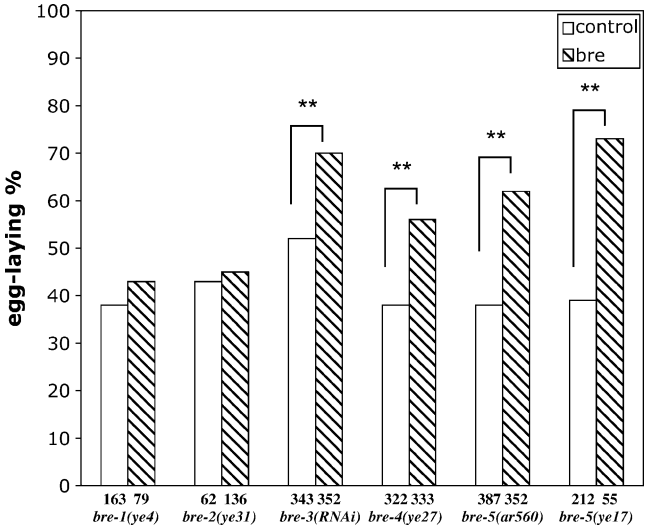

FIGURE 3.—Suppression of the 0 AC Egl defect of *lin-12(n302)/+* animals by *bre-4* and *bre-5* mutations and *bre-3* RNAi. *lin-12(n302)/unc-32(e189); bre* hermaphrodites were compared to control *lin-12(n302)/unc-32(e189)* hermaphrodites. For *bre-2*, both strains contained *unc-32(+)* in lieu of *unc-32(e189)*. *lin-12(n302)/unc-32(e189); bre* hermaphrodites were compared to control *lin-12(n302)/unc-32(e189)* hermaphrodites. For *bre-2*, both strains contained + in lieu of *unc-32(e189)*. We note that the higher percentage of egg-laying-competent hermaphrodites observed for *lin-12(n302)/unc-32(e189)* grown on "feeding vector" bacteria as opposed to OP50 may be attributed to the RNAi conditions (I. KATIC, unpublished observations). A chi-square test was performed to assess whether there is a significant difference between *bre* mutants and a negative control in the percentage of egg-laying animals. Asterisks indicate differences significant at the 99% level. Numbers of animals scored are below corresponding bars.

sterile at 25°). Also, *bre-5* mutant larvae do not exhibit embryonic lethality or the characteristic Lag phenotype associated with abrogating both LIN-12 and GLP-1 signaling (LAMBIE and KIMBLE 1991): 398/398 eggs laid by *bre-5(ar560)* animals hatched, while 158/160 eggs laid by *bre-5(ye17)* animals hatched. All of those larvae grew to adulthood and were able to lay eggs.

Suppression of elevated *lin-12* activity by reducing the activity of other genes important for glycosphingolipid synthesis: There are five *bre* genes that are necessary for Bt toxin susceptibility. Four of them—*bre-2*, *bre-3*, *bre-4*, and *bre-5*—encode glycosyltransferases that are believed to act in a single pathway (GRIFFITTS *et al.* 2003, 2005). Drosophila *egghead* (*egh*), whose mutant phenotype is very similar to that of *brn*, is homologous to *bre-3*, so it appears that this pathway has been conserved between *Drosophila melanogaster* and *C. elegans* (GOODE *et al.* 1996a; GRIFFITTS *et al.* 2003). Drosophila BRN and EGH have been shown to catalyze successive steps in the synthesis of glycosphingolipids (MULLER *et al.* 2002; SCHWIENTEK *et al.* 2002; WANDALL *et al.* 2003, 2005).

These observations prompted us to test whether loss of *bre* gene activity suppresses the 0 AC-Egl phenotype associated with *lin-12(n302)* (data not shown) or *lin-12(n302)/+* (Figure 3) and the Muv phenotype associated with *lin-12(ΔE)* (Table 4). We used mutant alleles of *bre-1*, *bre-2*, and *bre-4* and *bre-3(RNAi)* to circumvent the difficulty of constructing a double mutant with the tightly linked *lin-12* gene.

TABLE 4

***lin-12* activity in VPC specification is affected by certain other *bre* genes**

Genotype	Muv[a]/*n* (%)	Average no. of pseudovulvae
lin-12(ΔE)::gfp	164/164 (100)	2.4 +/− 0.1
bre-1(ye4); lin-12(ΔE)::gfp	98/100 (98)	2.2 +/− 0.1
bre-2(ye31); lin-12(ΔE)::gfp	132/134 (99)	2.3 +/− 0.1
bre-3(ye26); lin-12(ΔE)::gfp	93/115 (81)	1.5 +/− 0.1
bre-4(ye27); lin-12(ΔE)::gfp	72/92 (78)	1.3 +/− 0.1

These strains were grown and scored in parallel. All animals containing *lin-12(ΔE)::gfp* were able to lay eggs.

[a] Muv is defined as the presence of one or more pseudovulva.

bre-4(ye27) and *bre-3(RNAi)* or *bre-3(ye26)* significantly suppressed the 0 AC-Egl phenotype of *lin-12(n302)/+* and the Muv phenotype associated with *lin-12(ΔE)*, suggesting that *bre-3* and *bre-4*, like *bre-5*, are also positive regulators of *lin-12* activity. *bre-2(ye31)* did not suppress the 0 AC-Egl phenotype of *lin-12(n302)/+* or the Muv phenotype associated with *lin-12(ΔE)*. As *bre-2(ye31)* is a missense mutation, it may not reduce *bre-2* activity sufficiently to see suppression. *bre-1(ye4)* also does not suppress *lin-12(n302)/+*. The *bre-1* gene has not been molecularly characterized, so this allele may be a hypomorph; however, it has a different Bt toxin resistance phenotype and appears sicker than the others (MARROQUIN *et al.* 2000) so the absence of suppression may be further evidence that it acts by a mechanism different from other *bre* genes.

Since *bre-5* and other cloned *bre* genes affect glycosphingolipid synthesis (KAWAR *et al.* 2002; GRIFFITTS *et al.* 2005), we tested some other enzymes known or hypothesized to be involved in glycosphingolipid synthesis for influence on *lin-12* activity. Serine palmitoyltransferases catalyze the first step in the biosynthesis of sphingolipids by condensation of serine and palmitoyl CoA (HANADA 2003). RNAi against the *C. elegans* ortholog of the LCB1 subunit of serine palmitoyltransferase, C23H3.4, causes *lin-12(n302)/+* worms to arrest prior to adulthood, so its effect on egg laying could not be assessed. Glucosylceramide synthases are enzymes that add UDP-glucose to ceramide in the first step of glycosphingolipid synthetic pathway (LEIPELT *et al.* 2001). RNAi against two genes encoding proteins that exhibit glucosylceramide synthase activity *in vitro* (F59G1.1 and F20B4.6) and one predicted (T06C12.10) glucosylceramide synthase does not compromise viability. *lin-12(n302)/+* hermaphrodites subjected to RNAi against each of these genes singly survived to adulthood, but their egg-laying ability was unchanged as compared to an empty-vector control (data

not shown). BRE-3 and BRE-5 catalyze biosynthetic steps subsequent to the one catalyzed by glucosylceramide synthases, so this lack of effect might be due to functional redundancy between different glucosylceramide synthases.

Genetic evidence that *bre-5* acts prior to LIN-12 activation by ligand-induced ectodomain shedding: *lin-12(ΔE)*, as a putative S2 cleavage mimic, would be expected to be constitutively active; ablation experiments described in the next section support this inference. However, we identified a surprising genetic property of *lin-12(ΔE)*: it depends on the presence of *lin-12(+)* for full activity; *i.e.*, *lin-12(ΔE)* has a more highly penetrant Muv phenotype in a *lin-12(+)* background than in a *lin-12(0)* background (Table 3, lines 4 and 7). As a short extracellular domain is believed to be sufficient to mark a transmembrane protein as a substrate for presenilin-mediated transmembrane cleavage (Struhl and Adachi 2000), our observation suggests that the LIN-12(+) protein may be playing a role in the trafficking, processing, or stability of LIN-12(ΔE). To our knowledge, the possibility that equivalent truncated forms in other systems also depend on endogenous wild-type activity has not been examined.

The dependence of *lin-12(ΔE)* on *lin-12(+)* for its constitutive activity enabled us to ask whether *bre-5* suppresses the activity of the site 2 cleavage mimic or the intact LIN-12(+) form by testing the ability of *bre-5(ye17)* to suppress *lin-12(ΔE)* in the presence or the absence of *lin-12(+)*. Suppression was observed in the presence of *lin-12(+)*, but was not observed in the absence of *lin-12(+)*, even though the presence of *lin-12(+)* makes *lin-12(ΔE)* "stronger" (Table 3). This observation indicates that *bre-5* reduces the activity of *lin-12(+)*, rather than the constitutive activity of *lin-12(ΔE)*, and therefore suggests a role for *bre-5* prior to ligand-induced ectodomain shedding. Ablation experiments described in the next section support this interpretation.

We note that hermaphrodites carrying transgenes that express LIN-12(ΔE) execute a normal AC/VU decision, so we could not assess the effect of *bre-5* on *lin-12* activity in that context. There are recurring problems with transgene expression in the AC/VU pair, so we believe that transgene expression, rather than an additional unusual property of *lin-12(ΔE)*, accounts for the lack of a mutant phenotype in the AC/VU decision.

VPC isolation experiments also suggest that *bre-5* acts prior to LIN-12 activation by ligand-induced ectodomain shedding. We performed cell ablation experiments in which all VPCs except P4.p or P8.p were killed by a laser microbeam in hermaphrodites carrying the *lin-12(ΔE)* transgene in a *lin-12(+)* background [referred to here as *lin-12(+); lin-12(ΔE)* for convenience]. The fate of the isolated VPC should reflect its intrinsic level of *lin-12* activity, as the source of any potential lateral signal has been eliminated. In wild-type hermaphrodites, an isolated P4.p or P8.p generally adopts a nonvulval fate (Sulston and White 1980; Sternberg and Horvitz 1986). In contrast, in *lin-12(+); lin-12(ΔE)* hermaphrodites, an isolated P4.p or P8.p often adopts a vulval fate (Table 5, lines 1 and 5), indicating that it has elevated intrinsic *lin-12* activity. Similarly, an isolated *lin-12(+); bre-5(ye17); lin-12(ΔE)* VPC also appears to have elevated intrinsic activity, as it always adopts a vulval fate (Table 5, lines 3 and 7). If loss of *bre-5* were to affect the trafficking, processing, or stability of LIN-12(+) cell-autonomously, then we would have expected the intrinsic activity of *lin-12(ΔE)* in an isolated VPC to be reduced in the *bre-5(ye17)* background, and a nonvulval fate adopted, since the activity of *lin-12(ΔE)* depends on *lin-12(+)*. The observation that intrinsic activity of *lin-12(ΔE)* is higher after the ablation is consistent with a nonautonomous function of *bre-5*. Alternatively, *bre-5* function may be cell-autonomous to allow LIN-12(+) to be activated by ligand, if such activation is necessary for LIN-12(+) to potentiate *lin-12(ΔE)* activity.

TABLE 5

VPC isolation experiments

Genotype[a]	VPC	Vulval fate	Nonvulval fate
lin-12(ΔE)::gfp	P4.p (isolated)	6	0
lin-12(ΔE)::gfp	P4.p (unoperated)	7	0
bre-5(ye17); lin-12(ΔE)::gfp	P4.p (isolated)	11	0
bre-5(ye17); lin-12(ΔE)::gfp	P4.p (mock operated)[b]	4	12
lin-12(ΔE)::gfp	P8.p (isolated)	12	0
lin-12(ΔE)::gfp	P8.p (unoperated)	7	0
bre-5(ye17); lin-12(ΔE)::gfp	P8.p (isolated)	9	0
bre-5(ye17); lin-12(ΔE)::gfp	P8.p (mock operated)[b]	6	10

[a] These animals are also *lin-12(+)*. Each unoperated animal of either genotype has a normal anchor cell and forms a functional vulva.

[b] Mock-operated animals were treated in exactly the same way as laser operated ones, but no VPCs were ablated. Each VPC was scored as adopting either a vulval or a nonvulval fate; all pseudovulvae that were observed had the characteristic morphology associated with the 2° fate.

DISCUSSION

We reverted the 0 AC-Egl phenotype caused by a hypermorphic *lin-12(d)* allele and identified six new potential positive regulators of *lin-12* activity. We molecularly characterized one of the new loci and found that it corresponded to *bre-5*, which encodes an enzyme of the glycosphingolipid biosynthetic pathway. We discuss first some general issues raised by this suppressor screen and then we focus on possible roles for glycosphingolipids in LIN-12/Notch signaling.

General issues raised by the suppressor screen: Suppression of the 0 AC-Egl phenotype caused by *lin-12(d)* mutations has proven to be a powerful way to identify positive regulators of *lin-12* activity, including core components and modulators. The characterization of *sup-17* (Wen *et al.* 1997), *lag-2* (originally known as *sel-3*; Tax *et al.* 1994), and *sel-8* (Doyle *et al.* 2000; Petcherski and Kimble 2000) indicated that these genes encode core components of the LIN-12/Notch pathway.

Screens for suppressors of the 0 AC-Egl phenotype caused by *lin-12(d)* mutations have been carried out on a large scale, with >350,000 mutagenized haploid genomes scored and 14 extragenic suppressor loci identified (Tax *et al.* 1997; this work). Despite the large scale, the screen is far from saturation: about half of all genes identified in these screens are defined by single alleles. Furthermore, there is a remarkable lack of overlap between the set of genes defined by Tax *et al.* (1997) and by us (see Table 1).

Why has saturation been so difficult to achieve? We believe there are several contributing factors. One is that revertants must be viable and fertile to be recovered. Thus, only non-null alleles of genes required for *lin-12* and/or *glp-1* activity, or of genes having other pleiotropies, would be recovered. This problem is exemplified by the recovery of only single alleles of *sel-8* (Tax *et al.* 1997; Doyle *et al.* 2000) and *lag-1* (this work) in suppressor screens. Nevertheless, the suppressor screen is able to detect such genes: although *lag-1* was also identified on the basis of phenotypic criteria, *sel-8* was not (Lambie and Kimble 1991). Another factor is that the large number of intragenic revertants means that there is a large background that is tedious to sort through; the triage step that we designed to minimize the recovery of intragenic revertants, on the basis of past experience, was only partially effective.

Some of the technical issues that have limited conventional genetic suppressor screens may in principle be circumvented by RNAi, as genes essential for embryonic development may be identified by "feeding" L1 larvae (Timmons *et al.* 2001), and the variability inherent in reducing gene activity by RNAi may offer a wider range of reduced *lin-12* activity and would bypass the problem of *lin-12* intragenic revertants. The existence of libraries containing feeding constructs corresponding to a large percentage of *C. elegans* genes makes this approach practical (Kamath *et al.* 2003). However, pilot experiments have suggested that there is a high rate of false positives when RNAi is used in a 0 AC-Egl suppressor screen; so in this case, it is probably not a useful adjunct to the conventional suppressor screen (I. Katic, unpublished observations).

Glycosphingolipids and LIN-12/Notch signaling: We have found that *bre-3*, *bre-4*, and *bre-5*, genes encoding three enzymes involved in the glycosphingolipid biosynthetic pathway, are positive regulators of *lin-12* function. The Drosophila homologs of two of these genes, *egghead* (*bre-3*) and *brainiac* (*bre-5*), have also been studied in relation to Notch signaling. We discuss here the findings in *C. elegans* and Drosophila and potential ways in which glycosphingolipids may influence LIN-12/Notch activity.

Goode *et al.* (1996a) studied the roles of *brn* and *egh* during Drosophila oogenesis. They reported that *brn* and *egh* appear to be essential for the organization, but not the specification, of stalk and polar cells, whereas Notch is involved in specification of a stalk/polar cell fate decision as well as the polarity of these cell types. Thus, *egh* and *brn* do not appear to be involved in a Notch-mediated lateral interaction during oogenesis, but instead appear to play a role in the development and maintenance of epithelial cells. On the basis of their observations, they proposed that *brn* and *egh* regulate follicular morphogenesis by mediating germline-follicle cell adhesion.

In studying modulators of *lin-12* activity, we have found that *bre-3*, *bre-5*, and *bre-4*, another gene involved in glycosphingolipid biosynthesis, influence two *lin-12*-mediated cell fate decisions. One, VPC specification, involves cell-cell interactions between polarized epithelial cells. The other, the AC/VU decision, does not; instead, it involves two mesodermally derived cells that do not have the characteristics of polarized epithelial cells. These observations contrast with those of Goode *et al.* (1996a) and suggest a broader role for glycosphingolipids in influencing LIN-12/Notch activity in conventional signaling. However, we did not find a role for *bre* genes in other *lin-12*- or *glp-1*-mediated decisions. Negative results do not necessarily prove that the *bre* genes do not contribute to other decisions, but they do raise the possibility that glycosphingolipids are involved only in a subset of *lin-12/Notch*-mediated decisions.

Glycosphingolipids are components of lipid rafts, which are thought to partition proteins into specific membrane microdomains and to provide platforms for association between certain kinds of proteins (Simons and Toomre 2000). Signaling molecules such as G proteins, Ras, and receptor tyrosine kinases have been found to be associated with lipid rafts (Waugh *et al.* 1999; Simons and Toomre 2000). It is not known whether rafts can include LIN-12/Notch proteins or their DSL family ligands, but it has been hypothesized that *brn* might affect *Notch* signaling through its effects on raft composition (Schwientek *et al.* 2002). Alternatively, a glycosphingolipid may act directly to modify LIN-12 or another factor in the LIN-12 signaling pathway, analogously to EGF receptor binding to a ganglioside (Miljan *et al.* 2002).

In assessing potential roles for *bre-5/brainiac* and *bre-3/egghead* in LIN-12/Notch signaling, it is important to account for the evidence that these genes function cell-nonautonomously. Goode *et al.* (1996a) have shown that while *Notch* appears to be necessary in the somatic follicular cells, *egh* and *brn* are required in the germline.

We have shown that, in *lin-12(ΔE); bre-5(ye17)* animals, an isolated VPC has an elevated activity associated with *lin-12(ΔE)* and adopts a vulval fate. Thus, the function of *bre-5/brainiac* is more consistent with a role for glycosphingolipids in the signaling side of the LIN-12-mediated cell-cell interactions that specify cell fate. In view of this result, it does not appear likely that the effect of *bre-5* on *lin-12* activity is mediated through the γ-secretase complex, which has been shown to associate with lipid rafts in cell culture (Vetrivel *et al.* 2004; Urano *et al.* 2005), as γ-secretase activity is cell-autonomous for LIN-12/Notch signaling (Levitan and Greenwald 1995).

The activity of LIN-12(ΔE), the putative site 2 mimic, is higher in the presence of LIN-12(+), suggesting that it needs LIN-12(+) for its trafficking, processing, or stability. As *bre-5* also requires *lin-12(+)* activity to suppress *lin-12(ΔE)*, we infer that *bre-5* affects a process that acts on or requires the extracellular domain of LIN-12.

The results of VPC isolation experiments, together with the inferred action of *bre-5* prior to ectodomain shedding, are consistent with a role in signaling by DSL ligands. In Drosophila, endocytosis of DSL proteins is required for their signaling activity (Wang and Struhl 2004) and there may be a role for endocytosis of DSL proteins in certain situations in *C. elegans* as well (Tian *et al.* 2004). Although *lin-12(d)* alleles are constitutively active in the absence of ligand (Greenwald and Seydoux 1990), they remain sensitive to ligand, as can be seen when their activity or expression is low (Sundaram and Greenwald 1993b; C. Wen and I. Greenwald, unpublished observations). Furthermore, there are mutant ligand alleles that reduce *lin-12* activity, although it is not known whether their function is cell-autonomous or cell-nonautonomous (Tax *et al.* 1994); nevertheless, in principle, it is possible that certain ligands would be able to engage the receptor in a nonproductive way. Thus, one model is that glycosphingolipids influence ligand conformation or activity, so that in the absence of the *bre* genes, the ligands are "worse" at engaging the receptor in a productive, signal-transducing event. Alternatively, glycosphingolipids may regulate the activity of a factor that modifies the extracellular milieu so as to influence the proper folding and activity of the extracellular portion of LIN-12, perhaps influencing its receptivity to ligand or influencing the productivity of the ligand-receptor interaction in some other way.

Phenotypic similarities and genetic interactions point to the importance of glycosphingolipids for proper LIN-12/Notch signaling in *D. melanogaster* and *C. elegans* (Goode *et al.* 1996a; this study). In *C. elegans*, the lack of overt phenotypic abnormalities and the low level of suppression of the *lin-12(n302)* egg-laying defect suggest that the contribution of *bre* gene function to LIN-12/Notch activity is modest. Even in Drosophila, where *egh* and *brn* mutations cause lethality, many Notch signaling processes do not appear to be affected (Goode *et al.* 1992, 1996a). Perhaps other mechanisms are redundant with glycosphingolipid function with respect to LIN-12/Notch signaling, or the small contribution is significant under conditions in nature.

It remains to be established whether glycosphingolipids affect Notch signaling in vertebrates, but there is no reason to believe that this is exclusively an invertebrate phenomenon. While the structures of glycosphingolipids are markedly different in invertebrates and vertebrates, most biosynthetic steps are catalyzed by homologous glycosyltransferases, and a mammalian glycosphingolipid precursor with a core structure different from those found in Drosophila is indeed functional in Drosophila (Wandall *et al.* 2005). Thus, these complex molecules appear to be functionally conserved through evolution, making their potential conservation in LIN-12/Notch signaling more plausible.

We gratefully acknowledge Jiabin Chen and Abigail Druck-Shudofsky for their contributions to this work. We also thank Richard Ruiz for excellent technical assistance, Sophie Jarriault, Natalie de Souza, and Oliver Hobert for comments on the manuscript, and past and present members of the Greenwald laboratory for useful discussions. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health (NIH) National Center for Research Resources. This work was supported by NIH grants NS35556 and CA095389 (to I.G.). I.K. is currently a Postdoctoral Associate, and I.G. is an Investigator, of the Howard Hughes Medical Institute.

LITERATURE CITED

Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics **77:** 71–94.

Chen, J., X. Li and I. Greenwald, 2004 sel-7, a positive regulator of lin-12 activity, encodes a novel nuclear protein in *Caenorhabditis elegans*. Genetics **166:** 151–160.

Doyle, T. G., C. Wen and I. Greenwald, 2000 SEL-8, a nuclear protein required for LIN-12 and GLP-1 signaling in Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA **97:** 7877–7881.

Fares, H., and I. Greenwald, 1999 SEL-5, a serine/threonine kinase that facilitates *lin-12* activity in *Caenorhabditis elegans*. Genetics **153:** 1641–1654.

Ferguson, E. L., and H. R. Horvitz, 1985 Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode *Caenorhabditis elegans*. Genetics **110:** 17–72.

Goode, S., D. Wright and A. P. Mahowald, 1992 The neurogenic locus brainiac cooperates with the Drosophila EGF receptor to establish the ovarian follicle and to determine its dorsal-ventral polarity. Development **116:** 177–192.

Goode, S., M. Melnick, T. B. Chou and N. Perrimon, 1996a The neurogenic genes egghead and brainiac define a novel signaling pathway essential for epithelial morphogenesis during Drosophila oogenesis. Development **122:** 3863–3879.

Goode, S., M. Morgan, Y. P. Liang and A. P. Mahowald, 1996b Brainiac encodes a novel, putative secreted protein that cooperates with Grk TGF alpha in the genesis of the follicular epithelium. Dev. Biol. **178:** 35–50.

Greenwald, I., 1998 LIN-12/Notch signaling: lessons from worms and flies. Genes Dev. **12:** 1751–1762.

Greenwald, I., 2005 LIN-12/Notch signaling in *C. elegans*, in *WormBook*, edited by The *C. elegans* Research Community (doi/10.1895/wormbook.1.10.1; http://www.wormbook.org).

Greenwald, I., and G. Seydoux, 1990 Analysis of gain-of-function mutations of the lin-12 gene of Caenorhabditis elegans. Nature **346:** 197–199.

Greenwald, I. S., P. W. Sternberg and H. R. Horvitz, 1983 The lin-12 locus specifies cell fates in Caenorhabditis elegans. Cell **34:** 435–444.

Griffitts, J. S., J. L. Whitacre, D. E. Stevens and R. V. Aroian, 2001 Bt toxin resistance from loss of a putative carbohydrate-modifying enzyme. Science **293:** 860–864.

Griffitts, J. S., D. L. Huffman, J. L. Whitacre, B. D. Barrows, L. D. Marroquin *et al.*, 2003 Resistance to a bacterial toxin is mediated by removal of a conserved glycosylation pathway required for toxin-host interactions. J. Biol. Chem. **278:** 45594–45602.

Griffitts, J. S., S. M. Haslam, T. Yang, S. F. Garczynski, B. Mulloy *et al.*, 2005 Glycolipids as receptors for Bacillus thuringiensis crystal toxin. Science **307:** 922–925.

Hanada, K., 2003 Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. Biochim. Biophys. Acta **1632:** 16–30.

Hubbard, E. J., Q. Dong and I. Greenwald, 1996 Evidence for physical and functional association between EMB-5 and LIN-12 in Caenorhabditis elegans. Science **273:** 112–115.

Kamath, R. S., A. G. Fraser, Y. Dong, G. Poulin, R. Durbin *et al.*, 2003 Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature **421:** 231–237.

Kawar, Z. S., I. Van Die and R. D. Cummings, 2002 Molecular cloning and enzymatic characterization of a UDP-GalNAc: GlcNAc(beta)-R beta1,4-N-acetylgalactosaminyltransferase from Caenorhabditis elegans. J. Biol. Chem. **277:** 34924–34932.

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

Leipelt, M., D. Warnecke, U. Zahringer, C. Ott, F. Muller *et al.*, 2001 Glucosylceramide synthases, a gene family responsible for the biosynthesis of glucosphingolipids in animals, plants, and fungi. J. Biol. Chem. **276:** 33621–33629.

Levitan, D., and I. Greenwald, 1995 Facilitation of lin-12-mediated signalling by sel-12, a Caenorhabditis elegans S182 Alzheimer's disease gene. Nature **377:** 351–354.

Marroquin, L. D., D. Elyassnia, J. S. Griffitts, J. S. Feitelson and R. V. Aroian, 2000 *Bacillus thuringiensis* (Bt) toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. Genetics **155:** 1693–1699.

Miljan, E. A., E. J. Meuillet, B. Mania-Farnell, D. George, H. Yamamoto *et al.*, 2002 Interaction of the extracellular domain of the epidermal growth factor receptor with gangliosides. J. Biol. Chem. **277:** 10108–10113.

Muller, R., F. Altmann, D. Zhou and T. Hennet, 2002 The Drosophila melanogaster brainiac protein is a glycolipid-specific beta 1,3N-acetylglucosaminyltransferase. J. Biol. Chem. **277:** 32417–32420.

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

Petcherski, A. G., and J. Kimble, 2000 LAG-3 is a putative transcriptional activator in the C. elegans Notch pathway. Nature **405:** 364–368.

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

Schwientek, T., B. Keck, S. B. Levery, M. A. Jensen, J. W. Pedersen *et al.*, 2002 The Drosophila gene brainiac encodes a glycosyltransferase putatively involved in glycosphingolipid synthesis. J. Biol. Chem. **277:** 32421–32429.

Shaye, D. D., and I. Greenwald, 2005 LIN-12/Notch trafficking and regulation of DSL ligand activity during vulval induction in Caenorhabditis elegans. Development **132:** 5081–5092.

Simons, K., and D. Toomre, 2000 Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. **1:** 31–39 (erratum: Nat. Rev. Mol. Cell Biol. **2:** 216).

Sternberg, P. W., and H. R. Horvitz, 1986 Pattern formation during vulval development in C. elegans. Cell **44:** 761–772.

Struhl, G., and A. Adachi, 2000 Requirements for presenilin-dependent cleavage of notch and other transmembrane proteins. Mol. Cell **6:** 625–636.

Struhl, G., K. Fitzgerald and I. Greenwald, 1993 Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. Cell **74:** 331–345.

Sulston, J. E., and J. G. White, 1980 Regulation and cell autonomy during postembryonic development of Caenorhabditis elegans. Dev. Biol. **78:** 577–597.

Sundaram, M., and I. Greenwald, 1993a Genetic and phenotypic studies of hypomorphic *lin-12* mutants in *Caenorhabditis elegans*. Genetics **135:** 755–763.

Sundaram, M., and I. Greenwald, 1993b Suppressors of a *lin-12* hypomorph define genes that interact with both *lin-12* and *glp-1* in *Caenorhabditis elegans*. Genetics **135:** 765–783.

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

Tax, F. E., J. H. Thomas, E. L. Ferguson and H. R. Horvitz, 1997 Identification and characterization of genes that interact with *lin-12* in *Caenorhabditis elegans*. Genetics **147:** 1675–1695.

Tian, X., D. Hansen, T. Schedl and J. B. Skeath, 2004 Epsin potentiates Notch pathway activity in Drosophila and C. elegans. Development **131:** 5807–5815.

Timmons, L., D. L. Court and A. Fire, 2001 Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. Gene **263:** 103–112.

Urano, Y., I. Hayashi, N. Isoo, P. C. Reid, Y. Shibasaki *et al.*, 2005 Association of active gamma-secretase complex with lipid rafts. J. Lipid Res. **46:** 904–912.

Vetrivel, K. S., H. Cheng, W. Lin, T. Sakurai, T. Li *et al.*, 2004 Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. J. Biol. Chem. **279:** 44945–44954.

Wandall, H. H., J. W. Pedersen, C. Park, S. B. Levery, S. Pizette *et al.*, 2003 Drosophila egghead encodes a beta 1,4-mannosyltransferase predicted to form the immediate precursor glycosphingolipid substrate for brainiac. J. Biol. Chem. **278:** 1411–1414.

Wandall, H. H., S. Pizette, J. W. Pedersen, H. Eichert, S. B. Levery *et al.*, 2005 Egghead and brainiac are essential for glycosphingolipid biosynthesis in vivo. J. Biol. Chem. **280:** 4858–4863.

Wang, W., and G. Struhl, 2004 Drosophila Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. Development **131:** 5367–5380.

Waugh, M. G., D. Lawson and J. J. Hsuan, 1999 Epidermal growth factor receptor activation is localized within low-buoyant density, non-caveolar membrane domains. Biochem. J. **337:** 591–597.

Wen, C., M. M. Metzstein and I. Greenwald, 1997 SUP-17, a Caenorhabditis elegans ADAM protein related to Drosophila KUZBANIAN, and its role in LIN-12/NOTCH signalling. Development **124:** 4759–4767.

Weng, A. P., A. A. Ferrando, W. Lee, J. P. t. Morris, L. B. Silverman *et al.*, 2004 Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science **306:** 269–271.

Williams, B. D., B. Schrank, C. Huynh, R. Shownkeen and R. H. Waterston, 1992 A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. Genetics **131:** 609–624.

Zhou, S., M. Fujimuro, J. J. Hsieh, L. Chen, A. Miyamoto *et al.*, 2000 SKIP, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC to facilitate NotchIC function. Mol. Cell. Biol. **20:** 2400–2410.

Communicating editor: B. J. Meyer