p24 Proteins and Quality Control of LIN-12 and GLP-1 Trafficking in Caenorhabditis elegans
Chenhui Wen* and Iva Greenwald*

Abstract. Mutations in the Caenorhabditis elegans sel-9 gene elevate the activity of lin-12 and glp-1, which encode members of the LIN-12/NOTCH family of receptors. Sequence analysis indicates SEL-9 is one of several C. elegans p24 proteins. Allele-specific genetic interactions suggest that reducing sel-9 activity increases the activity of mutations altering the extracellular domains of LIN-12 or GLP-1. Reducing sel-9 activity restores the trafficking to the plasma membrane of a mutant GLP-1 protein that would otherwise accumulate within the cell. Our results suggest a role for SEL-9 and other p24 proteins in the negative regulation of transport of LIN-12 and GLP-1 to the cell surface, and favor a role for p24 proteins in a quality control mechanism for endoplasmic reticulum–Golgi transport.

Key words: LIN-12 • SEL-9 • Notch • p24 • Emp24p

The lin-12 and glp-1 genes of Caenorhabditis elegans encode members of the LIN-12/NOTCH family of receptor proteins. LIN-12/NOTCH proteins mediate cell–cell interactions to specify cell fate during animal development (Greenwald, 1998). The extracellular domains of LIN-12/NOTCH proteins contain multiple tandem EGF-like motifs and three LIN-12/NOTCH repeat (LNR) motifs. The intracellular domains of LIN-12/NOTCH proteins contain tandem cdc10/SWI16 (also known as ankyrin) motifs. LIN-12/NOTCH proteins are activated by ligands of the Delta/Serrate/LAG-2 family (Weinmaster, 1997). Binding of ligand is believed to induce a cleavage event in or near the transmembrane domain; this apparent cleavage event enables the intracellular domain to translocate to the nucleus, where it participates directly in regulating downstream gene expression (Schroeter et al., 1998; Struhl and A dachi, 1998).

Genetic screens in C. elegans and Drosophila have identified factors that influence the activity of lin-12/Notch activity. Many genes have been identified in sensitized genetic backgrounds, by suppressing or enhancing mutations in lin-12 (Sundaram and Greenwald, 1993b; Tax et al., 1997). Most of these genes have been named sel genes, for suppressor/enhancer of lin-12. The suppressor/enhancer approach mitigates potential difficulties arising from possible functional redundancy of mechanisms that control receptor activity as well as gene redundancy in each step. Several sel genes that have been characterized are involved in basic cell biological processes. Two sel genes, sel-12 (presenillin) and sup-17 (ADAM10/Kuzbanian), appear to affect processing of LIN-12 and GLP-1 (Wen et al., 1997; Levitan and Greenwald, 1998). Two other sel genes, sel-1 and sel-10, are likely to affect LIN-12 and GLP-1 turnover (Grant and Greenwald, 1997; Hubbard et al., 1997). The sel genes, and their interactions with lin-12 and glp-1, therefore may illuminate connections between basic cell biological processes and signaling.

In this study, we show that sel-9 functions by affecting LIN-12 and GLP-1 trafficking. SEL-9 is a member of the p24 family of proteins, and reducing sel-9 activity increases the level of lin-12 and glp-1 activity. We have identified other genes encoding C. elegans p24 proteins, and shown that reducing the activity of one of these genes also increases the level of lin-12 and glp-1 activity. Members of the p24 family have been implicated in cargo selectivity of ER to Golgi transport. The genetic interactions of sel-9 with lin-12 and glp-1, and the effect of sel-9 on the subcellular localization of mutant GLP-1, are consistent with a role for SEL-9 and other p24 proteins in cargo selection during trafficking to the cell surface.

Materials and Methods

General Methods and Strains

General methods are described by Brenner (1974). The wild-type parent for all strains was C. elegans var. Bristol strain N2. Strains were grown at 20°C unless otherwise noted. Mutations used were: LG I: art12 (lin-12 [intral]) (Struhl et al., 1993); LG III: unc-36(e251) (Brenner, 1974), unc-
32(e189) (Brenner, 1974), lin-12(n676n930) (Sundaram and Greenwald, 1993b), lin-12(n302) (Greenwald et al., 1983), lin-12(ar170) (Hubbard et al., 1986), glp-1(e2141s, e2174, e2125s, e2311) (Austin and Kimble, 1987), glp-1(q415) (Koodyan et al., 1992); LG V: dpy-11(e224) (Brenner, 1974), rol-3(e754) (Brenner, 1974), unc-23(e25) (Brenner, 1974), sel-9(ar22, ar26) (Sundaram and Greenwald, 1993b), mom-2(ne141) (Rocheleau et al., 1997); and extrachromosomal array arf x29(in-lin-12 (+)) (Fitzgerald et al., 1993).

**Mutagenesis and Screen for New sel-9 Alleles**

At 25°C, glp-1(e2142) hermaphrodites produced inviable progeny; this phenotype is suppressed by sel-9(ar22) (Sundaram and Greenwald, 1993b). Furthermore, glp-1(e2142); sel-9(ar22)/DF1 hermaphrodites also produce viable progeny (data not shown), suggesting that null alleles in principle may be obtained by complementation screening.

EMS mutagenesis was performed as described by Brenner (1974), glp-1(e2142); him-8(e1489); rol-3(e754) sel-9(ar26)/sel-9(ar22) unc-23(e25) males were mated to EMS mutagenized glp-1(e2142); dpy-11(e224) hermaphrodites at 15°C. The parents were transferred to fresh plates daily for 5 d. First progeny were scored at the L4 stage. Non-Dpy cross progeny were picked to fresh plates and transferred to 25°C. 10 F1 animals were put on each plate and the total number of F1 cross progeny was counted while picking. A flter 4 d, plates at 25°C were screened for live F2 progeny. Eventually only one animal from each plate was kept as a candidate. Dpy animals were backcrossed at least twice before further analysis.

**Geneic Mapping of sel-9**

sel-9 was previously mapped between rol-3 and unc-42 (Sundaram and Greenwald, 1993b). W e mapped sel-9 between rol-3 and unc-23: 2/10 Rol non-Unc recombinants from heterozygotes of the genotype rol-3 unc-23/rol-3 unc-23; sel-9(ar26) between rol-3 and unc-23: 2/10 Rol non-Unc recombinants from heterozygotes of the genotype rol-3 sel-9 unc-23; mom-2; 1232 Rol non-Unc recombinants harbored a rol-3 sel-9 mom-2 recombinant chromosome.

**sel-9 Cloning by an Antisuppression Assay**

Transgenic lines were generated by microinjected lin-12(n676n930); sel-9(ar22) hermaphrodites with cosmid or plasmid DNA at a concentration of 10 µg/ml, along with the dominant rol-6 marker pRF4 at a concentration of 100 µg/ml (Mello el al., 1991). Stable Rol animals were reared at 25°C, and individual Rol hermaphrodites from each line were analyzed for the Egl defect. A line is considered rescued if >50% of the Rol hermaphrodites were Egl. Initial rescue was obtained with each of two overlapping cosmids, F21F8 and W02D7. The 20-kb overlapping region was further mapped between rol-3 and unc-23: 2/10 Rol non-Unc recombinants harbored a rol-3 sel-9 mom-2 recombinant chromosome.

**Sequence Analysis of sel-9 and lin-12**

Standard molecular biology protocols were performed as described in Sambrook et al. (1989). The DNA sequences of F21F8 and W02D7 were obtained from the C. elegans genome sequencing project (Waterston et al., 1997). The exons of sel-9 were predicted by GENEFINDER (Edgley et al., 1997; we confirmed this prediction by sequencing a C. elegans clone, yk371h2 (generously provided by Dr. Y uji K ohar a, National Institute of Genetics, Japan).

The lesions associated with all sel-9 mutations were found by sequencing the sel-9 coding region of the mutants. We amplified the sel-9 genomic region by PCR reactions from individual sel-9 mutant hermaphrodites. For each mutation, two independent PCR products were cloned into Bluescript(SK -) (Stratagene). A lesion was considered confirmed if it appeared in two independent clones.

Lesions associated with lin-12(n941), lin-12(n676n930), lin-12(ar170), and lin-12(e2311) were found by sequencing most of the coding region of the mutants, as for sel-9. lin-12(n941) corresponds to W00GSTOF. The n930 lesion corresponds to C13BT. The ar170 lesion corresponds to G270R. The e2311 lesion corresponds to G449R.

**SEL-9:GFP**

A PCR product containing the coding sequence of GFP565T was cloned into a PMel site at the end of the sel-9 coding region in pSX 2.8 (see above). A s a result, the stop codon of sel-9 was changed to Ser. The resulting SEL-9:GFP hybrid protein is nonfunctional in the antisuppression assay, but, as it is expressed under the control of sel-9 regulatory sequences, it was useful for determining that all somatic cells express SEL-9 (data not shown).

**RNA-mediated Interference**

Double-stranded RNA (dsRNA) was prepared using the RNA transcription kit (Stratagene) and injected without dilution according to Fire et al. (1998).

**Laser Microsurgery**

lin-12(n676n930); sel-9(ar26) hermaphrodites, along with unoperated control animals, were kept at 15°C except for the period of laser microsurgery (20°C for ~5 min). The nucleus of Z 4 was ablated in newly hatched L1 larvae. The presence of an anchor cell (A C) was scored during the late L3 stage.

**Immunofluorescence**

An nti--GLP-1 staining of dissected hermaphrodite gonads was performed as described in Crittenden et al. (1994). Phalloidin staining was performed as described in Strome (1986). W orms were mounted on a 2% agarose pad with 5% N-pyrryl-galact and viewed with a Zeiss LSM 410 laser scanning confocal attachment on a Zeiss A xiovert 100 microscope.

**Results**

**Relevant Properties of lin-12 and glp-1 Mutations**

Our analysis of sel-9 depends on genetic interactions between sel-9 and the lin-12 or glp-1 genes. H ere, before we describe the genetic analysis of sel-9, we summarize the relevant properties of mutations in lin-12 and glp-1, which both encode receptors of the LIN-12/Notch family (Y ochem et al., 1988; Y ochem and G reenwald, 1989). W e note that lin-12 and glp-1 are functionally redundant in some cell fate decisions (Lambie and K imble, 1991), and that GLP-1 can fully substitute for LIN-12 when expressed under the control of lin-12 regulatory sequences (Fitzgerald et al., 1993). In our analysis, we have made consistent observations using both genes. T hree morphological characteristics influenced by lin-12 were used for our analysis of sel-9 (Table I): the number of A Cs, vulval morphology, and egg laying. W ild-type hermaphrodites have one A C and a normal vulva, and are able to lay eggs. Red cuced lin-12 activity causes extra A C to be produced (the 2 AC defect), a variably abnormal vulva, and a defect in egg laying (Egl) (Greenwald et al., 1983; Sundaram and G reenwald, 1993a). I n this study, we focus on genetic interactions between sel-9 and two alleles that reduce, but do not eliminate, lin-12 activity: lin-12(n676n930) and lin-12(ar170).

Constitutive, elevated lin-12 activity causes the A C to be missing (the 0 A C defect) and, as a consequence, an egg laying defect (the 0 A C-Egl defect) which is different from the Egl defect associated with reduced lin-12 activity (Greenwald et al., 1983). A greater elevation of lin-12 activity also causes extra vulval cells to be made (the M ulitivulva defect). Constitutive activity may result from mis-
A II sel-9 alleles show genetic interactions with several different missense mutations affecting the LIN-12 extracellular domain (see below). However, none suppress defects caused by lin-12(n941), a lin-12 null allele associated with a stop codon at position 400 in the extracellular domain (Materials and Methods).

Genetic analysis suggests that all sel-9 mutations are antimorphic (dominant-negative) (Sundaram and Greenwald, 1993b). A heterozygous deficiency is unable to suppress the 2 AC defect of lin-12(n676n930) or the maternal effect lethality of glp-1(e2142). In contrast, sel-9/+ heterozygosity suppresses these defects, implying that sel-9 alleles have gain-of-function character. The gain-of-function character appears to be antimorphic, because addition of wild-type alleles can reverse the suppression of lin-12(n676n930) and glp-1(e2142) by mutations in sel-9 (Sundaram and Greenwald, 1993b; Wen, C., and I. Greenwald, unpublished observations). In addition, we have evidence that all sel-9 mutations cause reduction of sel-9(+) activity (see below).

### Cell Autonomy of sel-9 Function in the AC/Ventral Uterine (VU) Decision

To examine the cell autonomy of sel-9 function, we examined its effect on the decision of two cells, Z1.ppp and Z4.aaa, between the A C and VU precursor cell fates. Normally, lin-12-mediated interactions between Z1.ppp and Z4.aaa causes one to become the A C (see Greenwald, 1993b; and data not shown).

### Table II. sel-9 Combinations with lin-12 Hypomorphs

<table>
<thead>
<tr>
<th>Relevant genotype*</th>
<th>sel-9 group†</th>
<th>n</th>
<th>% 0 AC‡</th>
<th>Multivulva defect</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>lin-12(n676n930)</td>
<td>+</td>
<td>40</td>
<td>0</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>lin-12(n676n930); sel-9(ar22)</td>
<td>A</td>
<td>37</td>
<td>0</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>lin-12(n676n930); sel-9(ar26)</td>
<td>A</td>
<td>Many (None)</td>
<td>0</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>lin-12(n676n930); sel-9(ar178)</td>
<td>A</td>
<td>Many (None)</td>
<td>0</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>lin-12(n676n930); sel-9(ar173)</td>
<td>B</td>
<td>16</td>
<td>75 ± 10.8</td>
<td>Muv</td>
<td></td>
</tr>
<tr>
<td>lin-12(n676n930); sel-9(ar174)</td>
<td>B</td>
<td>17</td>
<td>100</td>
<td>Muv</td>
<td></td>
</tr>
<tr>
<td>lin-12(n676n930); sel-9(ar175)</td>
<td>B</td>
<td>Many (All)</td>
<td>Muv</td>
<td>Muv</td>
<td></td>
</tr>
<tr>
<td>lin-12(n676n930); sel-9(ar176)</td>
<td>B</td>
<td>Many (All)</td>
<td>Muv</td>
<td>Muv</td>
<td></td>
</tr>
</tbody>
</table>

All experiments were done at 25°C. Sequence analysis subsequently established that sel-9(ar22) is the same alteration as sel-9(ar178), and that sel-9(ar175) the same alteration as sel-9(ar174). Standard variance is shown for the AC data. sel-9 alleles also suppress the hypomorphic allele lin-12(c648) (data not shown).

### Table I. Relevant Features of lin-12 Mutations Used in This Study

<table>
<thead>
<tr>
<th>Allele</th>
<th>Nature of mutation</th>
<th>No. of AC</th>
<th>Multivulva</th>
<th>Egg-laying defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>Wild-type</td>
<td>1 AC</td>
<td>No</td>
<td>None</td>
</tr>
<tr>
<td>n941</td>
<td>Null</td>
<td>2 AC</td>
<td>No</td>
<td>N/A*</td>
</tr>
<tr>
<td>ar170</td>
<td>Hypomorph</td>
<td>2 AC</td>
<td>No</td>
<td>None †</td>
</tr>
<tr>
<td>n676n930</td>
<td>25°C homomorph</td>
<td>2 AC</td>
<td>No</td>
<td>Egl</td>
</tr>
<tr>
<td>n302</td>
<td>Constitute, hypermorph</td>
<td>0 AC ‡</td>
<td>No</td>
<td>0 AC-Egl ¤</td>
</tr>
<tr>
<td>lin-12(n930)</td>
<td>15°C constitutive, hypermorph</td>
<td>0 AC ‡</td>
<td>No</td>
<td>0 AC-Egl ¤</td>
</tr>
</tbody>
</table>

The n930 lesion [C138T] appears to be a heat-sensitive partial loss-of-function mutation. At 25°C, lin-12(n676n930) behaves like a hypomorph (partial loss-of-function allele), and displays variably penetrant defects associated with loss of lin-12 activity, as shown; at 15°C, this allele behaves like a very weak hypomorph.

† Low penetrance Egl defect.
‡ Incompletely penetrant gain-of-function defect.
¶ lin-12(n676n930) was derived by reverting the lin-12(d) allele lin-12(n676) (G884D).
† lin-12(n930) also has an egg-laying defect that may be unrelated to lin-12 activity, possibly due to the insertion site.

### Isolation and Classification of New sel-9 Alleles

Two sel-9 mutations were isolated as suppressors of the egg laying defect (Egl) of the partial loss-of-function (hypomorphic) allele lin-12(n676n930) (Sundaram and Greenwald, 1993b). Genetic analysis suggested that these two sel-9 alleles, sel-9(ar22) and sel-9(ar26), are not null alleles (Sundaram and Greenwald, 1993b).

We isolated new sel-9 mutations in noncomplementation screens that in principle could have yielded null alleles (see Materials and Methods). Five sel-9 alleles (ar173, ar174, ar175, ar176, and ar178) were isolated after screening 12,000 mutagenized haploid genomes. We classified the existing sel-9 mutations into two groups, group A (weaker) and group B (stronger), based on their interactions with lin-12 hypomorphic alleles (Table I). Mutations in group B appear to increase lin-12 activity to a greater extent than mutations in group A. This inference is based on two observations: in combination with lin-12(n676n930), alleles of group B result in a multivulva phenotype (Table I), and in combination with lin-12(ar170), alleles of group B suppress the 2 AC defect (Table I). We note that the alleles in group B were all isolated in noncomplementation screens and could not have been isolated as suppressors of lin-12(n676n930), since they cause lin-12(n676n930) to lack an AC, which is necessary for vulval development and egg laying.
When lin-12 is constitutively active, hermaphrodites lack an AC, because both Z1.ppp and Z4.aaa become VUs. The penetrance of the 0 AC phenotype reflects the degree of elevated activity (Greenwald et al., 1983). For example, lin-12(n676n930) behaves like a weakly activated allele at 15°C, in that ~5% of hermaphrodites lack an AC. In the presence of sel-9(ar26), the proportion of lin-12(n676n930) hermaphrodites lacking an AC is increased to 78%, suggesting that lin-12 activity is elevated by sel-9(ar26).

When either Z1 or Z4 (the precursors to Z1.ppp or Z4.aaa) is ablated with a laser microbeam, the fate of the remaining cell reflects its intrinsic level of lin-12 activity in the absence of signaling. In wild-type hermaphrodites, if Z1 (or Z1.ppp) is ablated, Z4.aaa always becomes an AC (Kimble, 1981) because lin-12 is not activated in the absence of ligand. However, in lin-12(d) hermaphrodites, if Z1 (or Z1.ppp) is ablated, Z4.aaa becomes a VU because of the ligand-independent activation of LIN-12(d) (Greenwald and Seydoux, 1990; Grant and Greenwald, 1997). If sel-9(+) functions in the receiving end of cell–cell interactions, we would expect to see that sel-9(ar26) increases the intrinsic lin-12 activity in the absence of the signaling cell.

The results of ablation experiments suggest that sel-9 can affect lin-12 activity cell autonomously (Table I). As mentioned above, 78% of lin-12(n676n930); sel-9(ar26) hermaphrodites lack an AC. Similarly, in operated lin-12(n676n930); sel-9(ar26) hermaphrodites, when Z4 was removed at early L1 stage, Z1.ppp became a VU in 77% of the cases. These results demonstrate that the effect of sel-9 on lin-12 activity does not depend on the signaling cell, since sel-9(ar26) can increase the intrinsic level of lin-12 activity in the absence of the signaling cell. A though this experiment does not rule out an additional role for sel-9 in the signaling cell, the extent of enhancement of lin-12(n676n930) can be completely accounted for by sel-9 function in the receiving cell.

Molecular Cloning of sel-9

sel-9(ar22) suppresses the Egl phenotype caused by lin-12(n676n930ts) at 25°C. A dding a copy of sel-9(+) allele [lin-12(n676n930); sel-9(ar22)/sel-9(ar22)/sel-9(+)]) can partially reverse the suppression (Sundaram and Greenwald, 1993b). Therefore, we were able to use reversal of suppression to assess the sel-9(+ ) activity of microinjected DNA s (Fig. 1).

We mapped sel-9 between rol-3 and the cloned gene mom-2, an interval of <0.1 map unit. The mom-2 gene resides on cosmid clone F38E1 (Rocheleau et al., 1997; Thorpe et al., 1997). We tested cosmid clones to the left of F38E1 for their ability to complement sel-9(ar22) in a lin-12(n676n930); sel-9(ar22) background (see Materials and Methods). Cosmid clones containing either one of two overlapping cosmid clones, F21F8 and W02D7, gave rescue in the antisuppression assay. The overlapping region was further subcloned and the 2.8-kb fragment in pSX 2.8 was determined to contain sel-9(+) activity. The sequence of the 2.8-kb fragment is predicted to encode a single gene (by GENEFINDER; see Edgley et al., 1997). A cDNA clone, yk371h2 (generously provided by Dr. Y. Kohara), contains the DNA sequence of all predicted exons contained in pSX 2.8. This predicted gene was confirmed to be sel-9 by finding that all sel-9 mutations contain molecular lesions in the coding region (Fig. 2).

sel-9 Encodes a Member of the p24 Family of Proteins

The predicted SEL-9 protein sequence reveals that SEL-9 belongs to the p24 family of proteins (Figs. 2 and 3). Multiple members of the p24 family are found in all eukaryotes, from yeast to mammals. Members of the p24 family are type I membrane proteins with a signal peptide at the amino terminus, a luminal (extracytosolic) domain, a single transmembrane domain, and a short cytoplasmic tail. P24 proteins have a predicted luminal coiled-coil domain, conserved amino acids in the transmembrane domain and cytoplasmic tail, and similar overall size and organization (Blum et al., 1996; Fiedler and Rothman, 1997). They may be grouped into at least three subfamilies based on primary sequence (Blum et al., 1996). One subfamily comprises yeast Emmp24p and mammalian p24A; SEL-9 appears to be a member of this subfamily. Another subfamily comprises yeast Erv25p and mammalian Tmp21, and the third subfamily comprises mammalian gp25L proteins.

We searched the C. elegans genomic sequence database for additional p24 proteins and identified at least four...
other proteins with the hallmarks of p24 proteins (Fig. 3). As described below, we have also performed a functional test of one of these, F47G9.1, a member of the Erv25/Tmp21 subfamily (Fig. 3).

p24 proteins are major membrane components of COPI- and COPII-coated vesicles (Schimmoller et al., 1995; Stamnes et al., 1995; Sohn et al., 1996). Genetic studies in yeast and biochemical studies in yeast and mammalian cells have led to proposals for p24 protein function in cargo selectivity of ER to Golgi transport (Schimmoller

Figure 2. Schematic depiction of SEL-9. All p24 proteins are predicted to be type I membrane proteins with the amino terminus in the luminal region of the ER and the carboxy terminus in the cytoplasm; in some cases, this prediction has been confirmed (see Blum et al., 1996). Sequence alterations associated with all sel-9 mutations are shown. We cannot guarantee that sel-9(ar178) and sel-9(ar175) are independent isolates of sel-9(ar22) and sel-9(ar174), respectively. SS, signal sequence; CC, heptad repeats predicted to form a coiled-coil structure; TM, transmembrane domain.

Figure 3. p24 protein subfamilies. EMP24, yeast Emp-24p; P24A, human p24A; ERV25, yeast Erv25p; Tmp21, human Tmp21. Sequences of other members of the p24 family can be found in Blum et al. (1996) and Stamnes et al. (1995). The transmembrane (TM) and predicted coiled-coil (CC) domains are overlined. A signal sequence is present at the amino terminus of each protein but is not indicated. Residues that are identical in at least two of the three proteins within a subfamily are shown in reverse contrast letters. Conserved cysteines in the luminal domain are indicated with asterisks and residues identified as functionally important for coat protein binding in the transmembrane/cytosolic domain are indicated with open triangles. The genomic DNA sequences of sel-9 (accession number AF014940) and of F47G9.1 (accession number Z74035) are in GenBank. Two other C. elegans sequences, F57B10.5 (accession number AF039713) and K08E4.6 (accession number Z68316), also display hallmarks of p24 proteins, as does another C. elegans sequence present in the unfinished sequence database at the time of submission of this manuscript (data not shown).
et al., 1995; Belden and Barlowe, 1996; E Irod-Erickson and K aiser, 1996). O ur analysis of the interaction of sel-9 with lin-12 and glp-1 is consistent with a role in cargo se lectivity, as described further below and in the Discussion.

We constructed a SEL-9::GFP hybrid protein in which green fluorescent protein (GFP) was fused in frame to the car boxy terminus of SEL-9 (see Materials and Methods). The hybrid protein appeared to accumulate intracellularly in all somatic cells and at all developmental stages (data not shown). This observation implies that SEL-9 is present in cells undergoing lin-12-mediated cell fate decisions. U nfortunately, this SEL-9::GFP hybrid protein does not appear to function normally, as it was unable to reverse the suppression of lin-12(n676n930) by sel-9 at 25°C (data not shown), so we cannot meaningfully analyze its subcellular distribution.

**Sequence Analysis and the Nature of sel-9 Mutations**

We sequenced all existing sel-9 mutations and found that none creates an early stop codon or deletion that would be a clear molecular null allele (Fig. 2). sel-9(ar173) creates a stop codon at the beginning of the predicted transmembrane domain, and is predicted to result in a truncated SEL-9 protein lacking most of the TM domain and the entire cytoplasmic tail. All other sel-9 mutations contain missense changes in the predicted luminal region of SEL-9.

Six of the seven sel-9 alleles, corresponding to four different missense mutations, do not cause any obvious defects in an otherwise wild-type background. In contrast, sel-9(ar173) homozygous hermaphrodites are dumpy(Dpy), uncoordinated(unc), and slightly roller(rol) and egg-laying defective (but have normal vulval lineages; data not shown). These phenotypes are all complemented by an extrachromosomal array containing multiple copies of the sel-9(+) gene, and hence appear to result from the sel-9(ar173) mutation. However, sel-9(ar173)/sel-9(ar173)/mdp26[sel-9(+)] hermaphrodites still display a similar spectrum of phenotypes as sel-9(ar173), although they are less abnormal than sel-9(ar173)/sel-9(ar173), suggesting that sel-9(ar173) is an antimorph (data not shown), perhaps interfering with the secretion of proteins other than LIN-12 and GLP-1. We postulate that the presence of the luminal portion of SEL-9 interferes with secretion when an absent or nonfunctional carboxy terminus prevents association with vesicle coat proteins. In support of this interpretation is the observation that fusion of GFP in frame at the carboxy terminus causes the same spectrum of phenotypes as sel-9(ar173) (data not shown).

Although we do not have demonstrable sel-9 null alleles, we note that deletion of either emp24 or erv25, or of both genes, has measurable effects on specific aspects of secretion without any significant deleterious effects (Schimmoller et al., 1995; Belden and Barlowe, 1996; E Irod-Erickson and K aiser, 1996). Thus, it is conceivable that the sel-9 null phenotype may, at a gross level, be wild-type. Bellow, we provide evidence suggesting that available sel-9 alleles, in addition to being antimorphs, also reduce or eliminate sel-9 activity.

**Reducing sel-9 and F47G9.1 Activity Increases lin-12 and glp-1 Activity**

The technique of RNA-mediated interference (RNAi) may be used to investigate the effects of reducing gene activity (Rocheleau et al., 1997). RNAi is based on the observation that injection of RNA, and, in particular, dsRNA (Fire et al., 1998), can produce specific phenotypes similar to loss or reduction of function of the target gene. We have used this method to investigate the effects of reducing sel-9 and F47G9.1 activity.

The assay we used depends on the observation that at 15°C, lin-12(n676n930) behaves like a weak gain-of-function allele, and a small proportion of lin-12(n676n930) hermaphrodites lacks an AC and displays the 0 A C-Egl phenotype. We examined the ability of dsRNA to enhance the 0 A C-Egl phenotype of lin-12(n676n930) at 15°C. Double-stranded sel-9 or F47G9.1 RNA was injected into lin-12(n676n930) homozygous L4 hermaphrodites grown at 15°C, and injected hermaphrodites and mock-injected control hermaphrodites were maintained at 15°C. All hermaphrodites injected with sel-9 dsRNA or F47G9.1 dsRNA produced a markedly greater proportion of 0 A C-Egl progeny than did control hermaphrodites (see Table IV).

We also examined the ability of dsRNA to suppress the maternal effect lethal phenotype of glp-1(e2142) at 25°C, since maternal gene activity seems to be particularly sensitive to inhibition by this method. Double-stranded sel-9 or F47G9.1 RNA was injected into glp-1(e2142) dsRNA homozygous L4 hermaphrodites grown at the permissive temperature. The injected hermaphrodites and uninjected control hermaphrodites were shifted to 25°C. Control hermaphrodites laid only dead eggs. In contrast, all hermaphrodites injected with sel-9 dsRNA or F47G9.1 dsRNA produced live progeny (see Table IV). The suppression of glp-1(e2142) by sel-9 or F47G9.1 RNAi appears to be incompletely heritable for at least one additional generation, as has been observed in RNAi experiments for certain other genes.

The sel-9 and F47G9.1 RNAi effects on lin-12(n676n930) and glp-1(e2142) indicate that a reduction in p24 activity appears to elevate lin-12 and glp-1 activity. These results also suggest that available sel-9 alleles reduce sel-9 activity, since all sel-9 mutations enhance lin-12(n676n930) and suppress the maternal effect lethality of glp-1(e2142) (Sundaram and Greenwald, 1993b; see Table VI).

We also injected F47G9.1 dsRNA into sel-9(ar174), and did not observe any reduced viability or obvious effects on the egg-laying ability or vulval morphology of adult progeny (see Table IV). This result is consistent with the observation that the phenotype of Δemp24 Δerv25 strain is no more severe than either single mutant (Belden and Barlowe, 1996).

**Allele Specificity of sel-9 Suppression/Enhancement**

.sel-9 mutations do not suppress/enhance all lin-12 or glp-1 mutations. Rather, they appear to be specific for alleles of lin-12 and glp-1 that cause alterations in the extracellular domain (Fig. 4 and Tables II–VI).

sel-9 appears to increase the activity of the partial loss-of-function mutations lin-12(n676n930), lin-12(ar170), and lin-12(oz48), all of which have missense mutations in the extracellular domain (Table II, Fig. 4, and data not shown). sel-9 also appears to increase the activity of gain-of-function lin-12(d) mutations such as lin-12(n302): the
penetrance of the M. multivulva phenotype is increased in the presence of sel-9 mutations (Table V). In contrast, sel-9 does not appear to increase the activity of arl912[lin-12(intra)] (Table V): the penetrance of the M. multivulva phenotype is not increased in the presence of sel-9(ar174). These results suggest that the interaction between sel-9 and lin-12 requires the extracellular domain of LIN-12 or that LIN-12 be a transmembrane protein. The ability of sel-9 to enhance the weak gain-of-function phenotype caused by a multicopy lin-12(+/-) transgene (Table V) is consistent with this inference.

We further investigated the possibility that sel-9 suppression is specific for mutations in the extracellular domain by taking advantage of a variety of available, sequenced alleles of glp-1 (Kodoyianni et al., 1992). All sel-9 alleles can suppress the maternal effect lethality caused by glp-1(e2142), a missense mutation in the first EGF-like motif of the extracellular domain, but cannot suppress either the germline defect or the embryonic lethality caused by glp-1(e2141) and glp-1(q23), missense mutations in the cdc10/SWI6 domain of GLP-1 (Kodoyianni et al., 1992) (Table VI). Since glp-1(e2141) and glp-1(e231) appear to lower glp-1 activity more than glp-1(e2142) (Kodoyianni et al., 1992), the ability of sel-9 alleles to suppress glp-1(e2142) but not glp-1(e2141) and glp-1(q231) may be explained in three different ways: sel-9 does not function in the germline; sel-9 mutations can only suppress mild loss of glp-1 activity; or sel-9 mutations interact only with specific glp-1 alleles.

Table III. Cell Autonomy of sel-9 Function in the AC/VU Decision

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cells ablated</th>
<th>0 AC</th>
<th>1 AC</th>
</tr>
</thead>
<tbody>
<tr>
<td>lin-12(n676n930)</td>
<td>None</td>
<td>1/15 (7%)</td>
<td>14/15 (93%)</td>
</tr>
<tr>
<td>lin-12(n676n930)</td>
<td>Z4</td>
<td>1/12 (8%)</td>
<td>11/12 (92%)</td>
</tr>
<tr>
<td>lin-12(n676n930); sel-9(ar26)</td>
<td>None</td>
<td>55/70 (78%)</td>
<td>15/70 (22%)</td>
</tr>
<tr>
<td>lin-12(n676n930); sel-9(ar26)</td>
<td>Z4</td>
<td>10/13 (77%)</td>
<td>3/13 (23%)</td>
</tr>
</tbody>
</table>

Hermaphrodites were grown at 15°C except for the short period of laser microsurgery (see Materials and Methods).

To distinguish among these possibilities, we examined the effect of sel-9 on glp-1(q415) (Table V). glp-1(q415) contains a missense mutation (GLy226⇒Glu) in the fourth EGF-like repeat (Kodoyianni et al., 1992). At 15°C, the germline of glp-1(q415) proliferates normally but all embryos are dead; at 25°C, the germline proliferation of glp-1(q415) is defective (Kodoyianni et al., 1992). We found that sel-9(ar26), a weaker sel-9 allele, can suppress the germline proliferation defect but not the maternal effect lethality of glp-1(q415) at 25°C. However, sel-9(ar174), a stronger sel-9 allele, can suppress both the germline defect and the maternal effect lethality caused by glp-1(q415) at 25°C. Thus, sel-9 does function in the germline. In the germline, glp-1(q415) probably has a glp-1 activity level similar to that of glp-1(e2141) and glp-1(q231), since all three mutants produce similar numbers of germ cells at the restrictive temperature (Austin and Kimble, 1987; Priess et al., 1987; Kodoyianni et al., 1992). Thus, the ability of sel-9(ar26) to suppress glp-1(q415) but not glp-1(e2141) and glp-1(q231) suggests that the interaction between sel-9 and glp-1 depends on alterations in the extracellular domain of GLP-1.

sel-9 Affects the Subcellular Localization of GLP-1(q415)

To determine if the effect of sel-9 is on LIN-12/GLP-1 trafficking in C. elegans, we examined the level or subcellular localization of wild-type and mutant GLP-1 proteins in a sel-9(+/-) and sel-9 mutant background using an antibody cocktail that recognizes GLP-1 in dissected gonads (Crittenden et al., 1994; Fig. 5). In the germline of wild-type hermaphrodites, GLP-1 is visible mainly in the plasma membrane in the distal region in a honeycomb pattern corresponding to the membranes surrounding each germ-cell nucleus (Crittenden et al., 1994). We saw no evidence for a change in the level or subcellular localization of GLP-1(+/-) in a sel-9(ar174) mutant background (data not shown). In the germline of glp-1(q415) hermaphrodites, the membranes are present in their typical honeycomb pattern, as shown by phallloidin staining; however, GLP-
1(q415) does not display the honeycomb pattern, and instead accumulates within the cell, consistent with a defect in its transport to the plasma membrane. In contrast, in the germline of glp-1(q415); sel-9(q174) hermaphrodites, plasma membrane accumulation of GLP-1(q415) is at least partially restored, as a honeycomb pattern is evident. These results suggest that the absence of sel-9 activity relieves the block on trafficking of mutant GLP-1(q415) protein to the plasma membrane.

### Discussion

In this study, we have shown that SEL-9 is a member of the Emp24/p24A subfamily of p24 proteins. Reducing the activity of sel-9 and F47G9.1, which encodes a member of the Erv25/Tmp21 subfamily of p24 proteins, can increase the activity of certain mutations in lin-12 or glp-1. The interaction between sel-9 and lin-12 appears to be cell autonomous. The common feature of the alleles that are affected by sel-9 is that they are missense mutations in the extracellular domain of LIN-12 or GLP-1; in contrast, the mutations that are not affected include an activated form of LIN-12 caused by truncation of the extracellular domain and transmembrane domain, and point mutations of GLP-1 that alter the intracellular domain. We examined the subcellular localization of the protein encoded by glp-1(q415), one of the suppressed mutations, and found that it accumulates within the cell; however, a mutation in sel-9 enables GLP-1(q415) to accumulate in the plasma membrane. We discuss specific aspects of these results in the previous section. Here, we discuss how these results are consistent with a function for SEL-9 in cargo selection during transport of LIN-12 and GLP-1 to the cell surface.

In eukaryotic cells, secretory protein trafficking is mediated by transport vesicles, which bud from a donor membrane of one compartment and fuse with a recipient membrane of a different compartment. Distinct vesicle coat protein complexes mediate different budding/fusion events. A retrograde transport from ER to Golgi is mediated by COPII-coated vesicles (Bednarik et al., 1996; Schekman and Orci, 1996). Bidirectional transport between the ER and Golgi, and intra-Golgi transport, is me-
mediated by COPI-coated vesicles (Rothman and Wieland, 1996; Orci et al., 1997). Endocytic trafficking is mediated by clathrin-coated vesicles (Robinson, 1994).

A key feature of vesicle-mediated trafficking is the net transfer of cargo from one compartment to another, while components of the donor compartment are selectively excluded from vesicles and/or recycled. Furthermore, there appears to be a quality control mechanism, so that misfolded or mutant proteins cargo proteins are not transferred (Hammond and Helenius, 1995). Little is known about how selective packaging or quality control occurs. Signals on cargo and the coat proteins appear to influence assembly of the COPI coat complex (Springer and Schekman, 1998). However, other factors appear to influence selectivity: for example, null mutations which bypass the anterograde secretion block associated with the absence of Sec13p (one component of the COPII coat complex) also cause leakage of ER resident proteins and mutant invertase (Elrod-Erickson and Kaiser, 1996).

One gene identified as a bypass suppressor of Δsec13 was Emp24 (Elrod-Erickson and Kaiser, 1996), a defining member of the p24 subfamily to which SEL-9 belongs. p24 proteins are transmembrane protein components of COPI- and COPII-coated vesicles (Schimmoller et al., 1995; Belden and Barlowe, 1996; Elrod-Erickson and Kaiser, 1996; Sohn et al., 1996), and interact with coat proteins via their transmembrane/carboxy-terminal domains (Fiedler et al., 1996; Sohn et al., 1996; Dominguez et al., 1998). Strains lacking the p24 proteins Emp24p or Erv25p have similar secretion defects: there is reduced ER to Golgi transport of a subset of secretory proteins and leakage of ER resident proteins (Schimmoller et al., 1995; Belden and Barlowe, 1996; Elrod-Erickson and Kaiser, 1996). Two different roles for p24 proteins have been proposed. One possibility is that p24 proteins are receptors/adaptors for lumenal cargo (Kirchhausen et al., 1997). Alternatively, Elrod-Erickson and Kaiser (1996) have proposed that the proteins encoded by EMP24 and other Δsec13 bypass suppressors are part of a quality control mechanism that prevents the premature formation of vesicles that have not properly segregated cargo from ER-resident proteins.

The genetic interactions between sel-9 and lin-12 or glp-1 are consistent with an Emp24p-like role for SEL-9 in the transport of LIN-12 and GLP-1. SEL-9 and F47G9.1 may act during the sorting process to keep misfolded or mutant LIN-12 and GLP-1 proteins from transport vesicles, or, as proposed by Elrod-Erickson and Kaiser (1996), as a general block to the progress of vesicles containing aberrant proteins. Our genetic data are more consistent with a role for p24 proteins in a quality control mechanism as opposed to a role in cargo reception. In our functional interactions, sel-9 behaves as a negative regulator. If sel-9 were principally to function as a LIN-12/GLP-1 cargo receptor, we might have expected it to behave as a positive factor: loss or reduction of a cargo receptor should reduce the amount of LIN-12 or GLP-1 at the cell surface. Instead, loss or reduction of sel-9 activity increases the amount of LIN-12 or GLP-1 at the cell surface, enhancing the weak gain-of-function activity associated with overexpression of an essentially wild-type LIN-12 protein, and demonstrably increases the amount of a mutant GLP-1 protein at the cell surface. Our results, like those of Elrod-Erickson and Kaiser (1996), are therefore more consistent with a major role for p24 proteins in quality control as opposed to cargo reception.

All of the mutations that were affected by reducing sel-9 activity alter the extracellular domain of LIN-12 or GLP-1. These mutations may lead to general structural defects in the extracellular domain, since the mutations affect different subregions (Fig. 4) and have different effects (some elevate and some reduce activity). SEL-9(+) may directly or indirectly recognize the abnormal extracellular domains of the mutant LIN-12 or GLP-1 proteins and block their transport, thus effectively functioning to negatively regulate the amount of LIN-12/GLP-1 in the plasma membrane. In sel-9 mutants, however, abnormal LIN-12/GLP-1 proteins may instead be transported to cell surface, where they may be able to function. This inference is supported...
by our examination of the cell biology underlying these genetic interactions. When sel-9 activity is normal, the mutant GLP-1(q415) protein appears to be retained within the cell, and the hermaphrodites display a glp-1 mutant phenotype. In contrast, when sel-9 is mutant, the GLP-1(q415) mutant protein is found in the plasma membrane, and the hermaphrodites display a wild-type phenotype.

We postulate that the effect of sel-9 on mutant LIN-12 or GLP-1 reflects a role for SEL-9(+), or GLP-1 in the transport of LIN-12(+) and GLP-1(+) proteins, which may occur at some frequency due to misfolding or misprocessing. The finding that sel-9 mutations enhance the weak gain-of-function defect associated with overexpression of a tagged LIN-12 protein with a wild-type extracellular domain is consistent with this postulated role.

One issue that deserves comment is the lack of a severe phenotype associated with reduced sel-9 activity. In yeast, Empl24 causes only a moderate reduction in secretion of a select group of proteins and does not cause a marked visible phenotype (Schimmoller et al., 1995; Belden and Barlowe, 1996; E. Irod-Erickson and K. Kaiser, 1996). This lack of a visible phenotype might be attributable to functional redundancy among the multiple p24 proteins in yeast; however, particularly if p24 proteins depend on each other for stability (Belden and Barlowe, 1996; Dominguez et al., 1998), then perhaps elimination of all p24 protein activity might not result in a deleterious phenotype. In C. elegans, there also appear to be multiple p24 proteins. The sel-9 alleles we isolated appear to reduce sel-9 activity, but we do not know the sel-9 null phenotype with certainty: none of the existing mutations cause early stop codons or deletions of the coding region. Like Empl24, SEL-9 may be involved in the transport of a select group of proteins, including LIN-12 and GIP-1. We note that if sel-9 activity were essential for all secretory protein transport, we might reasonably have expected to see some evidence for a phenotype caused by RNAi. The definitive answer to the question of the phenotype caused by a lack of p24 proteins will be most readily addressed in yeast, where it will be feasible to construct strains lacking multiple genes for p24 proteins.

Our characterization of sel-9 emphasizes a link between the secretory apparatus and cell signaling during development. The characterization of other developmental genes is providing other linkages between secretion and cell signaling processes. For example, the establishment of dorsal-ventral polarity occurs during oogenesis and involves a signal from the oocyte to the follicle cells and a second signal from follicle cells back to the oocyte (Ray and Schupbach, 1996). The gene windbeutel, which is required for proper dorsal-ventral polarity, acts in the follicle cells and encodes an E3 ubiquitin ligase that has been proposed to chaperone a secreted signal produced in the follicle cells (K onsoiaki and Schupbach, 1998). Whether the linkages that have been found between the secretory apparatus and cell signaling processes reflect constitutive secretory functions or serve as points of regulation will be an issue for future study.

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