

Structure, function, and expression of SEL-1, a negative regulator of LIN-12 and GLP-1 in *C. elegans*

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SUMMARY

Previous work indicated that *sel-1* functions as a negative regulator of *lin-12* activity, and predicted that SEL-1 is a secreted or membrane associated protein. In this study, we describe cell ablation experiments that suggest *sel-1* mutations elevate *lin-12* activity cell autonomously. We also use transgenic approaches to demonstrate that the predicted signal sequence of SEL-1 can direct secretion and is important for function, while a C-terminal hydrophobic region is not required for SEL-1 function. In addition, by

analyzing SEL-1 localization using specific antisera we find that SEL-1 is localized intracellularly, with a punctate staining pattern suggestive of membrane bound vesicles. We incorporate these observations, and new information about a related yeast gene, into a proposal for a possible mechanism for SEL-1 function in LIN-12 turnover.

Key words: LIN-12, Notch, *C. elegans*, degradation, SEL-1

INTRODUCTION

The *lin-12* and *glp-1* genes of *C. elegans* encode members of the LIN-12/Notch family of proteins that specify cell fate decisions in animals. All LIN-12/Notch proteins are transmembrane proteins with intracellular domains containing tandem 'cdc10/SWI6' (also called 'ankyrin') motifs and extracellular domains containing epidermal growth factor (EGF)-like motifs and 'LIN-12/Notch' repeat (LNR) motifs. It is now generally accepted that LIN-12/Notch proteins function as receptors that are activated by binding ligands of the Delta/Serrate/LAG-2 family (reviewed by Greenwald and Rubin, 1992; Artavanis-Tsakonas et al., 1995).

The role of LIN-12/Notch proteins in development is exemplified by a decision of two somatic gonadal cells in *C. elegans* hermaphrodites, Z1.ppp and Z4.aaa, between the anchor cell (AC) and ventral uterine precursor cell (VU) fates. Z1.ppp and Z4.aaa are equivalent: each has an equal chance of becoming the AC, but in any given hermaphrodite, these cells interact with each other so that only one becomes the AC, while the other becomes a VU (Kimble and Hirsh, 1979; Kimble, 1981; Seydoux and Greenwald, 1989). These interactions are mediated by *lin-12* (Greenwald et al., 1983; Seydoux and Greenwald, 1989) and *lag-2* (Lambie and Kimble, 1991). Studies of genetic mosaics and reporter gene expression patterns have indicated that both Z1.ppp and Z4.aaa initially express *lin-12* and *lag-2*, but that a stochastic variation in ligand (*lag-2*) and/or receptor (*lin-12*) activity is subsequently amplified by a feedback mechanism involving differential transcription of *lin-12* and *lag-2* (Seydoux and Greenwald, 1989; Wilkinson et al., 1994).

There are a number of processes, involving other gene products, that may influence *lin-12/Notch* signaling. These

products include proteins that could influence the activity of the extracellular domain, perhaps by affecting ligand-receptor and/or receptor-receptor interactions. There may also be factors that specifically influence the stability or turnover of activated receptor complexes, so that cells may respond to the relative levels of signaling activity (as in the feedback mechanism described above) or so that successive cell-cell interactions may occur. Finally, there may be factors involved in specific aspects of the secretion, transport or processing of LIN-12/Notch proteins or their ligands. These potential regulatory processes, and others, may be identified by characterizing genes that influence *lin-12/Notch* activity.

Genetic screens based on phenotype or suppressor/enhancer activity have already identified a number of genes that influence *lin-12/Notch* activity in *C. elegans* and *Drosophila*. Here, we report further characterization of the *sel-1* gene of *C. elegans*. *sel-1* was identified as an extragenic suppressor of mutations that reduce *lin-12* activity (Sundaram and Greenwald, 1993b). Genetic analysis indicates that *sel-1* behaves as a negative regulator of *lin-12* and *glp-1* activity (Sundaram and Greenwald, 1993b; Grant and Greenwald, 1996). The predicted SEL-1 protein has a potential signal sequence (Grant and Greenwald, 1996), suggesting that it is more likely to modulate LIN-12 activity than to function as a downstream effector. In this paper, we describe experiments concerning the structure, function, and expression of SEL-1, and propose a model for SEL-1 function in LIN-12 turnover.

MATERIALS AND METHODS

General methods and strains

Methods for the handling and culturing of *C. elegans* were essentially

as described by Brenner (1974). The wild-type parent for all strains used was *C. elegans* var. Bristol strain N2 (Brenner, 1974). All strains were grown at 20°C unless otherwise noted. LGIII: *lin-12(n676n930)* (Sundaram and Greenwald, 1993a), *unc-32(e189)* (Brenner, 1974), *unc-36(e251)* (Brenner, 1974). LGV: *sel-1(ar23)*, *sel-1(ar29)*, *sel-1(ar75)*, *sel-1(ar77)*, *sel-1(e1948)*, *sel-1(ar166)*, *sel-1(ar167)* (Sundaram and Greenwald, 1993b; Grant and Greenwald, 1996), *sqt-3(sc63ts)* (van der Keyl et al., 1994).

Laser ablation

Laser ablations were done essentially as described by Sulston and White (1980) and Sternberg (1988). *unc-32(e189) lin-12(n676n930)* or *unc-32(e189) lin-12(n676n930); sel-1(ar75)* animals were allowed to lay eggs at 20°C. Newly hatched L1s were mounted in 5 mM sodium azide/M9 on 5% agar pads for ablation, and subsequently removed to individual Petri dishes at 15°C. Each animal was re-examined by Nomarski optics for the presence of an anchor cell at the L3 stage and for egg laying at the adult stage. Ablation of Z1 or Z4 was confirmed by the absence of one gonad arm. Mock ablated animals were taken through the same procedure except that no cells were ablated.

Plasmids

pB1/NLSLacZ: a *SmaI* to *SpeI* (overhang removed) fragment from plasmid pPD22.11 (Fire et al., 1990), encoding β -galactosidase with the SV40 nuclear localization signal (NLS), was cloned into the *StyI* site (overhang removed) of pB1 (Grant and Greenwald, 1996) in frame with the *sel-1* second exon.

pB1/TMLacZ: an *MscI* to *SpeI* (overhang removed) fragment from pPD34.110 (Fire et al., 1990), encoding β -galactosidase with a synthetic transmembrane sequence, was cloned into the *StyI* site (overhang removed) of pB1 in frame with the *sel-1* second exon.

MTL-2/S65T: an *SphI* to *KpnI* fragment containing the *mtl-2* promoter from pPD54.01 (A. Fire, personal communication) was cloned into the *SphI* and *KpnI* sites of vector pPD95.85 (A. Fire, S. Xu, J. Ahnn and G. Seydoux, personal communication).

MTL-2/SIG/S65T: an *EcoRV* to *ScaI* fragment from the *sel-1* cDNA clone BB1C (Grant and Greenwald, 1996) encoding the first 79 amino acids of SEL-1, cloned into the unique *SmaI* site of MTL-2/S65T, between the promoter and GFP. This plasmid encodes a fusion of the first 79 amino acids of SEL-1 to GFP (Chalfie et al., 1994).

GSTOP: a modified form of the rescuing plasmid FSTOP (Grant and Greenwald, 1996) in which one copy of an *NheI* linker (NEB) containing stop codons in all frames was inserted into the unique *Ecl136II* site (position +2197) leading to a truncated form of the predicted *sel-1* product called SEL-1(Δ C-TERM).

FGPI: a modified form of the rescuing plasmid FSTOP (Grant and Greenwald, 1996) in which an *EcoRV* to *AccI* (overhang removed) fragment from pGPI α (gift from D. Lublin), encoding the C-terminal 43 amino acids of Decay Accelerating Factor (DAF) with its own stop codon, was inserted into the unique *Ecl136II* site (position +2197). This plasmid encodes a SEL-1::DAF fusion in which the C-terminal 42 amino acids of SEL-1 are replaced by the C-terminal 43 amino acids from DAF.

INFRAMETT: a modified form of the rescuing plasmid FSTOP (Grant and Greenwald, 1996) in which a PCR product encoding GFP (variant S65T, I167T; Y. Jin and H.R. Horvitz; personal communication), without its own stop codon, is cloned in frame within the last exon of *sel-1* at the unique *Ecl136II* site (position +2197).

KPX: a form of the rescuing plasmid pB1RS (Grant and Greenwald, 1996) modified by PCR to delete bases +877 to +924 and introduce a unique *SmaI* site (CCCGGG). This plasmid encodes modified SEL-1 with Pro Gly substituted for most of the predicted signal sequence (amino acids 5-20).

FTM: a modified form of FSTOP (Grant and Greenwald, 1996) in which GFP (S65T, I167T) containing its own stop codon and a synthetic transmembrane sequence (constructed from pPD34.110 and

pPD95.85; Fire et al., 1990; A. Fire, S. Xu, J. Ahnn and G. Seydoux, personal communication) was inserted at the unique *Ecl136II* site.

Y20: the complete *sel-1* cDNA from BB1C (Grant and Greenwald, 1996) cloned as an *XbaI* fragment into the *NheI* site of *myo-3* expression vector pPD95.86 (A. Fire, personal communication).

M8: the complete *sel-1* cDNA from BB1C (Grant and Greenwald, 1996) cloned as an *XbaI* fragment into the *NheI* site of *mtl-2* expression vector pPD54.01 (Freedman et al., 1993; A. Fire, personal communication).

HB1: a PCR product corresponding to *sel-1* cDNA sequences +77 to +2027, encoding SEL-1 amino acids 19-642, cloned to the *BamHI* and *HindIII* sites of *E. coli* expression plasmid pQE30 (Qiagen). This construct produces SEL-1 protein with a 6-HIS tag at the amino terminus.

BX6: a PCR product corresponding to *sel-1* cDNA sequences +77 to +2027, encoding SEL-1 amino acids 19-642, cloned to the *BamHI* site of *E. coli* expression plasmid pQE70 (Qiagen). This construct produces SEL-1 protein with a 6-HIS tag at the carboxy terminus.

DH1: a modified form of HB1, in which the *SacI* to *XbaI* fragment from HB1 has been replaced with the equivalent fragment from BX6. This construct produces SEL-1 protein with 6-HIS tags at the amino and carboxy termini.

F1: a PCR product corresponding to *sel-1* cDNA sequences +77 to +2078, encoding SEL-1 amino acids 19-685, cloned to the *BamHI* and *XhoI* sites of *E. coli* expression plasmid pET24b (Novagen). This construct produces SEL-1 protein with a 6-HIS tag at the carboxy terminus.

Transgenic *C. elegans*

Microinjection of DNA into the germ line of *C. elegans* hermaphrodites for transformation was essentially as described by Fire (1986) and modified by Mello et al. (1991). DNAs for injection were prepared by CsCl banding or Qiagen Midi columns. Co-injection makers used in these experiments were not found to substantially affect *C. elegans* egg laying.

sel-1 complementation was done in one of two ways: (1) *unc-32(e189) lin-12(n676n930) / qC1; dpy-20(e1282); sel-1(e1948)* animals were injected with *sel-1* plasmids at 5 μ g/ml, and pMH86 (*dpy-20(+)*; Han and Sternberg, 1991) at 10 μ g/ml and pBS(KS)- (Stratagene) at 85 μ g/ml. Stable non-Dpy lines were generated and homozygous Unc non-Dpy animals were scored for egg laying in assays done by picking L4 animals grown at 25°C to new plates. These animals were then re-scored over the next 2 days for the presence of many eggs laid by more than 80% of Unc mothers assayed (no rescue), or severe bloating and/or internal hatching in more than 40% of Unc mothers assayed (rescue). GSTOP rescued 8/8 lines. FGPI rescued 3/4 lines. KPX rescued 0/4 lines. INFRAMETT rescued 2/4 lines. FTM rescued 0/4 lines. FTM re-injected at 50 μ g/ml rescued 0/3 lines. (2) *unc-36(e251) lin-12(n676n930) / qC1; sel-1(ar75)* animals were injected with *sel-1* plasmids and the pRF4 *rol-6(d)* marker plasmid at 100 μ g/ml (Mello et al., 1991). Stable Rol lines were generated and homozygous Rol Unc animals were scored for egg laying as above. KPX injected at 50 μ g/ml rescued 0/3 lines. Y20 injected at 1 μ g/ml rescued 0/5 lines. M8 injected at 50 μ g/ml rescued 0/5 lines. Four Y20 lines and five M8 lines showed strong SEL-1 immunoreactivity when assayed using α -SEL-1 antisera described below. All staining was restricted to the body wall muscle (Y20) or intestine (M8).

MTL-2/S65T and MTL-2/SIG/S65T were injected at 50 μ g/ml with 10 μ g/ml pMH86 and 40 μ g/ml pBS(KS)- into a *dpy-20(e1282)* strain. Two non-Dpy lines were examined. Each showed accumulation of GFP in the pseudocoelomic and uterine spaces as well as punctate staining within the coelomocytes.

In addition to rescue experiments above, INFRAMETT was injected at 50 μ g/ml into a *dpy-20(e1282); sel-1(e1948)* strain. One integrated line (shown in Fig. 3D) was generated by X-irradiation with 1800 rads.

Production of α -SEL-1 Abs

6HIS::SEL-1 fusion protein was produced by growing *E. coli* (M15) cells carrying plasmid BX6 to an OD₆₀₀ of 0.7, adding 1M isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 2 mM, and incubating for 4 hours at 37°C with vigorous shaking. Fusion protein was purified under denaturing conditions by nickel-nitrilo-triacetic acid (Ni-NTA) chromatography according to the manufacturer's instructions (Qiagen). 2 mg of purified protein was isolated by preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and excised as a gel slice after very light Coomassie blue staining (Harlow and Lane, 1988). Injection of protein antigen and collection of sera was performed by East Acres Biologicals. The gel slice was homogenized with adjuvant and injected subcutaneously into two rabbits, R4281 and R4282. Initial injections were composed of 250 μ g BX6 protein mixed with Freund's complete adjuvant. Boosts were performed with 100 μ g protein and Freund's incomplete adjuvant every 21 days. Test bleeds were performed 10 days after boosts. The animals were exsanguinated after the fifth boost. Antibodies were affinity purified after the third and fifth bleeds according to published methods (Gu et al., 1994). Purified Ab preparation DH1 was produced by affinity chromatography of a mixture of serum from the third bleed of rabbits 4281 and 4282 to a 6HIS::SEL-1::6HIS affinity column, made from plasmid DH1 expressing *E. coli* M15 cells. Purified Ab preparations MG1 and MG2 were produced by affinity chromatography of serum from the final bleed of either rabbit 4281 or 4282 respectively, to protein expressed from plasmid F1, in *E. coli* BL21(DE3). We found identical staining patterns for all α -SEL-1 Ab preparations except that bleeds from rabbit 4282 displayed higher titers.

Western analysis, immunostaining and photography

Total protein extracts were made from mixed populations of *C. elegans*. Worm pellets were often stored at -70°C prior to processing. An equal volume of worm boil buffer (100 mM Tris pH 6.8, 8% SDS, 20 mM β -mercaptoethanol) was added to the frozen pellet. The mixture was boiled for 30-60 minutes with frequent vortexing. Solubilization of protein was considered complete when all worm carcasses were dissolved when examined under a dissecting microscope. Proteins were size separated by SDS-PAGE and transferred to nitrocellulose. Probing and visualization of SEL-1 was performed using the ECL detection system (Amersham) essentially as described by the manufacturer except that α -SEL-1 Abs (1:100 dilution of DH1) were incubated with the blot overnight at 4°C. Secondary Ab (Amersham; HRP-conjugated goat α -rabbit) was used at a 1:3000 dilution. Detection reagents were sometimes diluted 1:2 – 1:10 with distilled water prior to use.

Immunostaining of fixed preparations of *C. elegans* was performed essentially as described by Finney and Ruvkun (1990). Primary antibodies (MG2 preparations are shown) were used at a dilution of 1:500. Cy3 or FITC conjugated goat α -rabbit secondary Abs (Jackson Labs) were used at 1:500 and 1:100 respectively. Primary and secondary Abs were pre-absorbed with *sqt-3(sc63) sel-1(ar167)* *C. elegans* acetone powder to remove remaining background (Epstein and Shakes, 1995). Samples were sometimes counter-stained for DNA by adding a 1:1000 dilution of YO-PRO-1 (Molecular Probes) or 4,6-diamidino-2-phenylindole (DAPI) to the final wash (Ellis and Horvitz, 1986). Double staining was identical to the above except for the following modifications. Mouse monoclonal MH27 (Priess and Hirsh, 1986) was included in the primary incubation at a 1:1000 dilution, and a 1:100 dilution of FITC-conjugated goat α -mouse Ab (Jackson Labs) was added to the second incubation. Immunostained animals were mounted in 90% glycerol/PBS with 2% N-propyl gallate or the SlowFade reagent (Molecular Probes). Samples were examined by laser confocal microscopy on a BIORAD MRC6000 system. Images were processed with Adobe Photoshop version 3.05 software.

INFRAMETT animals (Fig. 3D) were prepared for confocal microscopy by fixation for 2 hours in 3.7% formaldehyde/PBS at

room temperature. These samples were freeze cracked in a dry ice/ethanol bath after the first hour. Samples were further processed by one wash in 100 mM Tris, pH 8, 1% Triton X-100 followed by one wash in 25 mM sodium borate pH 9.2 and mounted for confocal microscopy as above in SlowFade Light reagent (Molecular Probes). This processing preserved most GFP fluorescence visible in living animals and reduced photobleaching while removing all gut granule autofluorescence.

Living animals expressing GFP (Fig. 1) were mounted in 1 mM levamisole and photographed with Kodak Ektachrome 400 film on a Zeiss Axiophot microscope with epifluorescent illumination and an FITC filter set.

RESULTS

Cell autonomy of *sel-1* function

Genetic studies suggested that *sel-1* is a negative regulator of *lin-12* activity (Sundaram and Greenwald, 1993b; Grant and Greenwald, 1996). In principle, loss of a negative regulator could increase the activity of components of the signaling

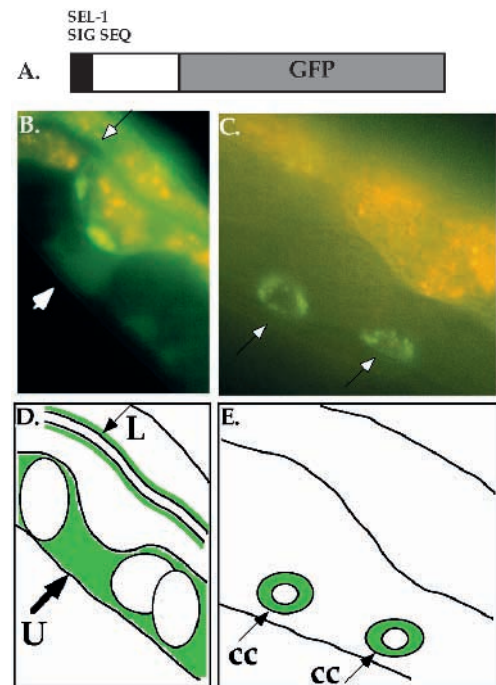


Fig. 1. A SEL-1 signal sequence :: green fluorescent protein (GFP) fusion (plasmid MTL-2/SIG/S65T) was expressed in *C. elegans* using an intestine-specific promoter, *mtl-2*. (Freedman et al., 1993). Live animals immobilized with levamisole are shown (see Materials and Methods). GFP fluoresces green. Background autofluorescence of the intestine is yellow. (A) Diagram of the SEL-1::GFP fusion protein. The first 79 amino acids of SEL-1 are fused to the entire green fluorescent protein. The SEL-1 predicted signal sequence is shown as a black box. GFP is shown as a gray box. (B) An adult hermaphrodite expressing the SEL-1::GFP fusion. Strong green fluorescence is seen at the apical (small arrow) and basal surfaces of the intestinal membranes, and in pseudocoelomic spaces. The empty uterine spaces around unlaied embryos (dark structures) is filled with green fluorescent protein in the hermaphrodite shown (see large arrow). (C) An adult hermaphrodite expressing SEL-1 signal sequence :: GFP, in which fusion protein secreted into the pseudocoelomic space has been scavenged by a pair of coelomocytes (arrows) just anterior of the vulva. (D,E) Camera lucida drawings of B and C respectively, GFP fluorescence shown in green. L, lumen of intestine; U, uterine space; cc, coelomocyte.

mechanism or the receiving mechanism (or both). We have tested whether *sel-1* functions in the receiving end of *lin-12*-mediated cell-cell interactions by performing cell ablation experiments. When either Z1 or Z4 (the precursors to Z1.ppp and 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. Normally, LIN-12 is not active in the absence of ligand; thus, for example, in wild-type hermaphrodites, if Z1 (or Z1.ppp) is ablated, Z4.aaa always becomes an AC (Kimble, 1981). However, mutations such as *lin-12(n676)* result in apparent ligand-independent activation; thus, in a *lin-12(n676)* background, if Z1 (or Z1.ppp) is ablated, Z4.aaa becomes a VU (Greenwald and Seydoux, 1990).

To examine possible cell autonomy of *sel-1* function, we compared the effect of ablating Z1 or Z4 in *lin-12(n676n930)* versus *lin-12(n676n930); sel-1(ar75)* hermaphrodites. When grown at 15°C, 7% of *lin-12(n676n930)* hermaphrodites display the 0 AC defect associated with *lin-12* hyperactivation; this phenotype probably reflects the residual activation by the *n676* lesion after an overall reduction in activity caused by the *n930* lesion (Sundaram and Greenwald, 1993a,b). In contrast, 69% of *lin-12(n676n930); sel-1(0)* hermaphrodites grown at 15°C display the 0 AC defect (Sundaram and Greenwald, 1993b; Table 1). If *sel-1* were to function in the signaling cell, then in operated *lin-12(n676n930); sel-1(ar75)* hermaphrodites, Z4.aaa (or Z1.ppp) should become an AC, unlike unoperated controls. However, if *sel-1* were to function within the receiving cell, then in operated *lin-12(n676n930); sel-1(ar75)* hermaphrodites, Z4.aaa (or Z1.ppp) should become a VU, like unoperated controls. We observed that ablation of Z1 or Z4 does not change the proportion of hermaphrodites displaying the 0 AC defect: 7% of operated *lin-12(n676n930)* hermaphrodites and 82% of operated *lin-12(n676n930); sel-1(ar75)* hermaphrodites display the 0 AC defect, similar to unoperated controls (Table 1). These results suggest that *sel-1(+)* functions cell autonomously to reduce *lin-12* activity. Although this experiment cannot rule out an additional role for *sel-1* in the signaling cell, the extent of enhancement of *lin-12(n676n930)* by *sel-1(ar75)* can be completely accounted for by *sel-1* function in the receiving cell.

Structure/function analysis of SEL-1

From sequence analysis, SEL-1 is predicted to enter the secretory pathway (Grant and Greenwald, 1996). Its structure is curious in light of the cell autonomy of *sel-1* function inferred from the laser ablation experiments. We have analyzed SEL-1 to understand better the relationship between SEL-1 structure and its function. The predicted SEL-1 sequence

Fig. 2. SEL-1 structure/function analysis. Diagrams of modified versions of SEL-1 are shown. Rescue data for wild-type SEL-1 is from Grant and Greenwald (1996). Rescue assays and data for all other constructs are shown in Materials and Methods. Δ SIG SEQ is encoded by plasmid KPX. Δ C-TERM is encoded by plasmid GSTOP. SEL-1::DAF is encoded by plasmid FGPI. SEL-1::TM::GFP is encoded by plasmid FTM. SEL-1::GFP::SEL-1 is encoded by plasmid INFRAMETT.

Table 1. Ablation experiments testing cell autonomy of *sel-1* function in the presumptive VU cell

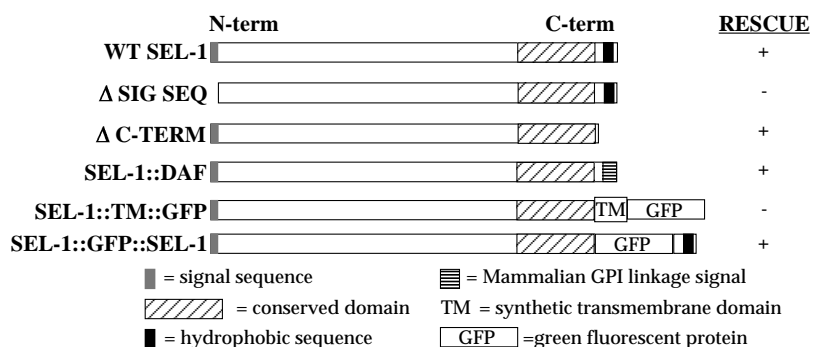
Genotype	Z1 or Z4 ablated	#0AC/total (%)
<i>lin-12(n676n930)</i>	Yes	1/14 (8)
<i>lin-12(n676n930)</i>	No	0/13 (0)
<i>lin-12(n676n930); sel-1(ar75)</i>	Yes	10/12 (82)
<i>lin-12(n676n930); sel-1(ar75)</i>	No	9/13 (69)

Hermaphrodites were grown at 15°C after laser microsurgery and scored for the number of anchor cells (ACs) (see Materials and Methods). In about half of the animals, Z1 was ablated; in the other half, Z4 was ablated. No apparent difference was seen between Z1 and Z4 ablations (data not shown).

indicated at least three potential functional domains: (1) an amino terminal hydrophobic sequence (2-16) with features of a secretory signal sequence (von Heijne, 1986); (2) a carboxy terminal hydrophobic sequence (661-678) with features of a glycosyl-phosphatidylinositol linkage signal (Ferguson and Williams, 1988); (3) a 70 amino acid sequence (563-635) that is highly similar to regions of the human IBD2 and yeast L8167.5 predicted proteins. We explored the function of these regions by testing mutated *sel-1* transgenes for their ability to complement a *sel-1* mutant (see Materials and Methods).

The amino-terminal hydrophobic region

The amino-terminal hydrophobic region is necessary for SEL-1 function: if this domain is deleted, the ability to rescue *sel-1* mutants is abolished (Fig. 2). This domain appears to be a functional signal sequence, since it can direct secretion of β -GAL and GFP. Transgenic lines expressing a fusion protein containing the putative signal sequence fused to β -GAL (SIG:: β -GAL) do not display β -GAL activity, in accordance with the prediction that extracellular β -GAL is non-functional (Silhavy and Beckwith, 1985), although fusion protein is produced as assayed by α - β GAL immunostaining (data not shown). In contrast, transgenic lines expressing a fusion protein in which the putative signal sequence is fused to a synthetic transmembrane domain fused to β -GAL (SIG::TM:: β -GAL) does display β -GAL activity, in accordance with the prediction that SIG::TM:: β -GAL is a transmembrane protein in which the β -GAL portion remains cytoplasmic and functional (Silhavy and Beckwith, 1985; Fire et al., 1990). In addition, when we fused GFP (Chalfie et al., 1994) to the first 79 amino acids of SEL-1 and expressed this fusion protein from the gut-specific *mtl-2* promoter (Freedman et al., 1993) we observed GFP outside of the intestine in the pseudocoelomic space (Fig. 1B), some of which was



scavenged by coelomocytes (Fig. 1C), which are known to take up secreted GFP (Fitzgerald and Greenwald, 1995; A. Fire and J. Fares, personal communication). Control animals expressing unmodified GFP from the *mtl-2* promoter showed very strong green fluorescence within intestinal cells, but no green fluorescence in the pseudocoelomic space, nor did they show any fluorescence in coelomocytes (data not shown).

The carboxy-terminal hydrophobic region

The C-terminal hydrophobic region is not essential for function, since deletion of this region does not affect the ability to rescue a *sel-1* mutant (Fig. 2). This region has characteristics of a potential glycosyl-phosphatidylinositol (GPI) linkage signal and can be replaced by a known GPI linkage signal from the human decay accelerating factor (DAF) protein (Shenoy-Scaria et al., 1992) without affecting SEL-1 function (Fig. 2). However, *sel-1* activity is abolished when this region is replaced by a known functional transmembrane domain (Fire et al., 1990). The latter hybrid protein was produced, although at somewhat reduced levels, as assessed by fluorescence from an intracellular GFP tag (data not shown). The elimination of rescuing activity by the addition of a transmembrane domain to SEL-1 suggests that integral attachment of SEL-1 to a membrane prevents function. It is also possible that other fusion sequences such as those between SEL-1 and the transmembrane domain, or the GFP tag, interfere with SEL-1 function.

The conserved region

The *sel-1(ar77)* mutation introduces a stop codon at position 646, and is therefore predicted to encode a protein that truncates the C-terminal region near the end of the conserved domain (Grant and Greenwald, 1996). This truncated SEL-1 lacks function. However, transgenic animals expressing SEL-1 Δ C-TERM, another truncated version of SEL-1 containing only 27 more amino acids, display wild-type SEL-1 activity. Taken together these two results indicate that: (1) amino acids 673-685 (C terminus) are not required for SEL-1 activity and (2) at least some of the amino acids in between, 646 to 673, are required for SEL-1 activity. Therefore, SEL-1(646-673) defines the right hand border of the conserved functional domain, where homology extends through amino acid 665. To investigate the importance of the conserved domain to SEL-1 function, we examined the distribution of mutant SEL-1 in the missense mutants *sel-1(ar23)* [G(594)->E] and *sel-1(ar29)* [G(594)->R], using the antibody described below. The level, expression pattern, and subcellular localization of these mutant SEL-1 proteins was indistinguishable from wild-type SEL-1 (data not shown). Since these missense mutants behave genetically like null alleles (Sundaram and Greenwald, 1993b; Grant and Greenwald, 1996), these staining results suggest that the conserved region is not required for subcellular localization or stability and thus is necessary for SEL-1 function.

SEL-1 localization

We have used two different methods to visualize SEL-1 protein localization with the light microscope: antibody staining and green fluorescent protein (GFP) tagging (Chalfie et al., 1994). The results from these experiments suggest that SEL-1 is present in all larval and adult tissues, except for the pharynx. These experiments also suggest that SEL-1 is located in intra-

cellular vesicles, and may not be found in the plasma membrane.

We generated affinity-purified polyclonal antisera against SEL-1 (see Materials and Methods) to use for immuno-staining in fixed preparations of mixed-stage wild-type hermaphrodites. The affinity-purified antisera recognized a single band of about $75 \times 10^3 M_r$ in western blots of protein extracts from wild-type hermaphrodites, which was absent from protein extracts from a *sel-1* nonsense mutant (Fig. 3B). Using this antibody to stain animals prepared for whole-mount immunofluorescence (see Materials and Methods), we found that SEL-1 could be detected in all larval and adult tissues, but not in the pharynx (data not shown). The *sel-1(ar167)* nonsense mutant remained unstained when processed in parallel, indicating that staining seen in wild-type animals is specific for SEL-1 (data not shown). We confirmed that SEL-1 is indeed present in cells undergoing *lin-12* mediated decisions by focusing on P5.p and P7.p, the two vulval precursor cells that require *lin-12* to adopt their proper fates (Greenwald et al., 1983). Co-immune localization with α -SEL-1 and the MH27 α -desmosomal antibody (Priess and Hirsh, 1986), which outlines the tight junctions of vulval precursor cells (Kenyon, 1986), showed that SEL-1 is present in P5.p and P7.p (and other vulval precursor cells) at the time they are deciding between alternative cell fates (Fig. 3E). No changes in SEL-1 distribution were detected at this time.

When wild-type hermaphrodites stained with α -SEL-1 Abs were examined by confocal microscopy, punctate staining was observed in the cytoplasm, but appeared strongest near the apical surface and cell-cell boundaries of intestinal cells (see Fig. 3). We also expressed SEL-1 exclusively in the intestine (using the *mtl-2* promoter; Freedman et al., 1993) or body wall muscle (using the *myo-3* promoter; Okkema et al., 1993) in a *sel-1* null mutant. In these hermaphrodites, punctate SEL-1 staining was restricted to intestinal and body wall muscle cells respectively (data not shown). We did not observe rescue of *sel-1* suppression of *lin-12* defects in animals expressing SEL-1 in only intestine or muscle, consistent with the cell-autonomy of *sel-1* function inferred from the laser ablation experiments described above.

To demonstrate that our SEL-1 cellular and subcellular localization data was not an artifact of the staining procedure, we examined SEL-1 localization in living worms. To do this, we generated a hybrid protein, in which GFP is inserted into SEL-1. This SEL-1::GFP::SEL-1 hybrid protein can complement a *sel-1* mutant (Fig. 2; Materials and Methods), and displays widely distributed punctate intracellular fluorescence, similar to the staining detected with α -SEL-1 antibodies (Fig. 3D).

DISCUSSION

We have investigated properties of SEL-1 that bear on its potential mechanism of function as a negative regulator of *lin-12* activity. We have found that SEL-1 enters the secretory pathway, but appears to remain within the cells that produce it and to function in a cell autonomous manner. SEL-1 detected by antibody staining or GFP tagging appears punctate throughout the cytoplasm, except in the polarized cells of the intestine, where higher concentrations of SEL-1 accumulate near the

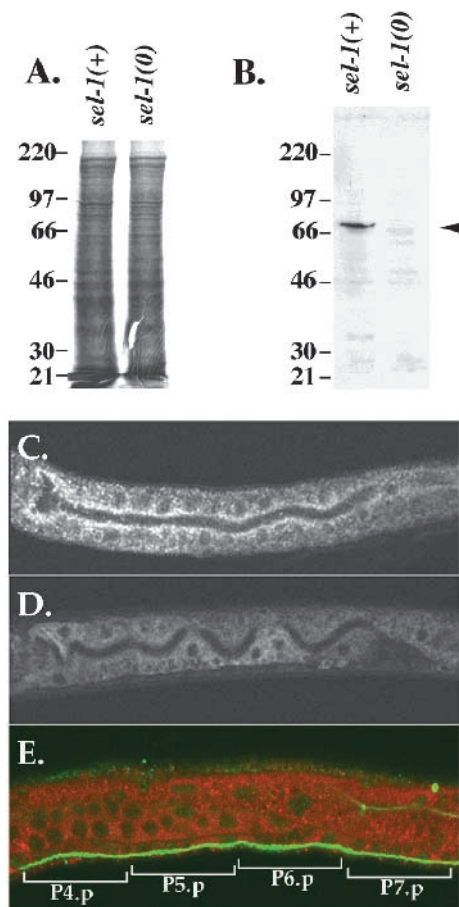


Fig. 3. SEL-1 localization. (A) Total *C. elegans* proteins size separated by SDS-PAGE and visualized by Coomassie blue staining. (B) shows a duplicate gel in which the proteins were transferred to nitrocellulose and probed with α -SEL-1 Abs (see Materials and Methods). Full genotypes: *sel-1(+)* = *unc-32(e189)*, *sel-1(0)* = *unc-32(e189)*; *sqt-3(sc63)* *sel-1(ar167)*. (C) Confocal micrograph of a wild-type L2 larva immunostained with α -SEL-1 Abs: Note the punctate staining pattern and intense staining near the lumen of the intestine, corresponding to the apical surface of these cells. *sel-1* null animals prepared in parallel showed no staining (data not shown). (D) Confocal micrograph of SEL-1::GFP::SEL-1 fusion protein expressed in an L2 larva (see Materials and Methods). (E) Confocal micrograph of wild-type L3 larva immunostained with α -SEL-1 and MH27 antibodies. α -SEL-1 antibodies, shown in red, stain all cells of the central body region (shown). MH27 antibodies, shown in green, stain the tight junctions of all six vulval precursor cells. This double labeling shows that P5.p and P7.p express SEL-1 at the time of a *lin-12*-mediated cell fate decision.

apical plasma membrane. These findings suggest that SEL-1 is found in intracellular vesicles, and therefore may be involved in the production, trafficking, or degradation of LIN-12.

Recent studies have suggested that a yeast protein similar to SEL-1 may be involved in regulated protein degradation. SEL-1 is similar to the *Saccharomyces cerevisiae* hypothetical protein L8167.5, deduced from yeast genomic sequencing: both proteins contain a highly conserved region (which is important for SEL-1 function), a signal sequence, a hydrophobic C terminus, and similar amino acid compositions (Grant and Greenwald, 1996). Recently, L8167.5 was shown to be a product of the *HRD3* gene, which was identified in a screen

for suppressor mutations that stabilize HMG-CoA reductase (Hampton et al., 1996b). The regulated degradation of HMG-CoA reductase, a key enzyme in the cholesterol biosynthetic pathway, is an important mechanism for controlling the synthesis of cholesterol in yeast and mammals (Goldstein and Brown, 1990; Hampton and Rine, 1994; Hampton et al., 1996a). Mutations in *HRD3* result in deficiencies in the degradation of HMG-CoA reductase, implying that HRD-3 is a component of the mechanism for degrading HMG-CoA reductase. Furthermore, *HRD2*, another suppressor identified in the same screen, is a component of the 26S proteasome (Hampton et al., 1996b). The sequence similarity between Hrd3p and SEL-1 suggests that SEL-1 may be involved in the regulated turnover of LIN-12 and/or another component of the LIN-12 signal transduction pathway.

We note also another parallel between *HRD-3* and *sel-1*. Mutants lacking *HRD3* activity efficiently stabilize an isozyme of HMG-CoA reductase, and mutants lacking *sel-1* activity efficiently suppress *lin-12* hypomorphic alleles. However, in neither case do the mutations in the suppressor genes confer an obvious visible phenotype (Sundaram and Greenwald, 1993b; Grant and Greenwald, 1996; Hampton et al., 1996b).

SEL-1 appears likely to influence LIN-12 activity per se, as opposed to influencing the activity of other components of the pathway, for the following reasons. (1) While reducing *sel-1* activity can suppress *lin-12* mutations that greatly reduce *lin-12* activity, reducing *sel-1* activity cannot suppress *lin-12* null mutations (Sundaram and Greenwald, 1993b). These observations suggest that reducing *sel-1* activity does not elevate the activity of downstream signaling components. (2) Laser ablation experiments indicate a role for *sel-1* in the receiving cell (this paper), suggesting that reducing *sel-1* activity does not elevate *lin-12* activity by elevating the activity of upstream signaling components. (3) We have not observed an effect of *sel-1* mutations on sensitized developmental interactions involving mutated receptors other than *lin-12* or *glp-1* (Grant and Greenwald, 1996), and *sel-1* has not been identified in screens for suppressors of other mutants. These observations suggest that loss of *sel-1* activity does not generally elevate or stabilize mutant proteins, but rather suggests that its effects are more limited, perhaps just to LIN-12 and GLP-1.

Based on our studies of *sel-1* and the studies of *HRD3* (Hampton et al., 1996b), we propose that SEL-1 is involved in the regulation of LIN-12 turnover, possibly targeting activated receptor/ligand complexes for degradation. For instance, SEL-1 might be important for targeting vesicular LIN-12 to the lysosome or other proteolytic compartments. Without SEL-1, LIN-12 might recycle to the plasma membrane eventually leading to abnormally high levels of activated receptor at the cell surface.

Specific down-regulation of activated receptors by endocytosis and degradation has been observed in a number of different systems including several growth factor receptors (reviewed by Sorkin and Waters, 1993). Genetic studies have suggested that down-regulation of activated LIN-12/Notch proteins may be important for cell fate decisions to occur normally. First, an important feature of lateral specification that is mediated by LIN-12/Notch proteins is the assessment of the relative levels of signaling between two or more cells (Seydoux and Greenwald, 1989; Heitzler and Simpson, 1991). Thus, rapid turnover may be important for creating a relative differ-

ence in signaling intensity that can be amplified by transcription-based feedback mechanisms (Wilkinson et al., 1994; Heitzler et al., 1996). Second, in *Drosophila* eye development, Notch appears to be utilized for sequential cell fate decisions (Cagan and Ready, 1989); in this case, and no doubt in other cell fate decisions, it may be important to clear activated Notch proteins in order to prepare for successive rounds of signaling. Finally, the presence of a C-terminal PEST sequence is conserved in all LIN-12/Notch proteins, and mutations that truncate LIN-12/Notch proteins prior to the PEST sequence have some characteristics of activated receptors (Mango et al., 1991; A. Melendez and I. G., unpublished observations), suggesting that protein turnover is important for regulating signal intensity. Moreover, in unrelated signaling systems, it has been suggested that different amounts of signal can evoke distinct cell fates (Katz et al., 1995; Nellen et al., 1996). Such systems would likely depend on a rapid turnover of activated receptor complexes in the receiving cell.

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REFERENCES

- Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. *Science* **268**, 225-235.
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.
- Cagan, R. L. and Ready, D. F. (1989). Notch is required for successive cell decisions in the developing *Drosophila* retina. *Genes Dev.* **3**, 1099-1112.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802-804.
- Ellis, H. M. and Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* **44**, 817-829.
- Ferguson, M. A. and Williams, A. F. (1988). Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. *Ann. Rev. Biochem.* **57**, 285-320.
- Finney, M. and Ruvkun, G. (1990). The *unc-86* gene product couples cell lineage and cell identity in *C. elegans*. *Cell* **63**, 895-905.
- Fire, A. (1986). Integrative transformation of *Caenorhabditis elegans*. *EMBO J.* **5**, 2673-2680.
- Fire, A., Harrison, S. W. and Dixon, D. (1990). A modular set of *lacZ* fusion vectors for studying gene expression in *Caenorhabditis elegans*. *Gene* **93**, 189-198.
- Fitzgerald, K. and Greenwald, I. (1995). Interchangeability of *C. elegans* DSL proteins and intrinsic signaling activity of their extracellular domains in vivo. *Development* **121**, 4275-4282.
- Freedman, J. H., Slice, L. W., Dixon, D., Fire, A. and Rubin, C. S. (1993). The novel metallothionein genes of *Caenorhabditis elegans* - structural organization and inducible, cell-specific expression. *J. Biol. Chem.* **268**, 2554-2564.
- Goldstein, J. and Brown, M. (1990). Regulation of the mevalonate pathway. *Nature* **343**, 425-430.
- Grant, B. and Greenwald, I. (1996). The *Caenorhabditis elegans sel-1* gene, a negative regulator of *lin-12* and *glp-1*, encodes a predicted extracellular protein. *Genetics* **143**, 237-247.
- Greenwald, I. and Rubin, G. M. (1992). Making a difference: the role of cell-cell interactions in establishing separate identities for equivalent cells. *Cell* **68**, 271-281.
- Greenwald, I. and Seydoux, G. (1990). Analysis of gain-of-function mutations of the *lin-12* gene of *Caenorhabditis elegans*. *Nature* **346**, 197-199.
- Greenwald, I. S., Sternberg, P. W. and Horvitz, H. R. (1983). The *lin-12* locus specifies cell fates in *Caenorhabditis elegans*. *Cell* **34**, 435-444.
- Gu, J., Stephenson, C. G. and Iadarola, M. J. (1994). Recombinant proteins attached to a nickel-NTA column: Use in affinity purification of antibodies. *Biotechniques* **17**, 257-262.
- Hampton, R. and Rine, J. (1994). Regulated degradation of HMG-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. *J. Cell Biol.* **125**, 299-312.
- Hampton, R., Koning, A., Wright, R., and Rine, J. (1996a). In vivo examination of membrane protein localization and degradation with green fluorescent protein. *Proc. Natl. Acad. Sci. USA* **93**, 828-833.
- Hampton, R., Gardner, R., and Rine J. (1996b). Role of 26S proteasome and HRD genes in the degradation of 3-Hydroxyl-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol. Biol. Cell* in press.
- Han, M. and Sternberg, P. W. (1991). Analysis of dominant-negative mutations of the *Caenorhabditis elegans let-60 ras* gene. *Genes Dev* **5**, 2188-2198.
- Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. and Simpson, P. (1996). Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signaling in *Drosophila*. *Development* **122**, 161-171.
- Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of *Drosophila*. *Cell* **64**, 1083-1092.
- Katz, W. S., Hill, R. J., Clandinin, T. R. and Sternberg, P. W. (1995). Different levels of the *C. elegans* growth factor LIN-3 promote distinct vulval precursor fates. *Cell* **82**, 297-307.
- Kenyon, C. (1986). A gene involved in the development of the posterior body region of *C. elegans*. *Cell* **46**, 477-487.
- Kimble, J. (1981). Alteration in cell lineage following laser ablation of cells in the somatic gonad of *Caenorhabditis elegans*. *Dev. Biol.* **87**, 286-300.
- Kimble, J. and Hirsh, D. (1979). The post-embryonic cell lineages of the hermaphrodite and male gonads in *Caenorhabditis elegans*. *Dev. Biol.* **87**, 396-417.
- Lambie, E. J. and Kimble, J. (1991). Two homologous regulatory genes, *lin-12* and *glp-1*, have overlapping functions. *Development* **112**, 231-239.
- Mango, S. E., Maine, E. M. and Kimble, J. (1991). Carboxy-terminal truncation activates *glp-1* protein to specify vulval fates in *Caenorhabditis elegans*. *Nature* **352**, 811-815.
- Mello, C. C., Kramer, J. M., Stinchcomb, D. and Ambros, V. (1991). Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**, 3959-3970.
- Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long range action of a DPP morphogen gradient. *Cell* **85**, 357-368.
- Okkema, P. G., Harrison, S. W., Plunger, V., Aryana, A. and Fire, A. (1993). Sequence requirements for myosin gene-expression and regulation in *Caenorhabditis elegans*. *Genetics* **135**, 385-404.
- Priess, J. R. and Hirsh, D. I. (1986). *C. elegans* morphogenesis: The role of the cytoskeleton in elongation of the embryo. *Dev. Biol.* **117**, 156-173.
- Seydoux, G. and Greenwald, I. (1989). Cell autonomy of *lin-12* function in a cell fate decision in *C. elegans*. *Cell* **57**, 1237-1245.
- Shenoy-Scaria, A. M., Kwong, J., Fujita, T., Olszowy, M. W., Shaw, A. S. and Lublin, D. M. (1992). Signal transduction through decay-accelerating factor. *J. Immunol.* **149**, 3535-3541.
- Silhavy, T. J. and Beckwith, J. R. (1985). Uses of *lac* fusions for the study of biological problems. *Microbiol. Rev.* **49**, 398-418.
- Sorkin, A. and Waters, C. M. (1993). Endocytosis of growth factor receptors. *BioEssays* **15**, 375-382.
- Sternberg, P. W. (1988). Lateral inhibition during vulval induction in *Caenorhabditis elegans*. *Nature* **335**, 551-554.
- Sulston, J. E. and White, J. G. (1980). Regulation and cell autonomy during

- postembryonic development of *Caenorhabditis elegans*. *Dev. Biol.* **78**, 577-597.
- Sundaram, M. and Greenwald, I.** (1993a). Genetic and phenotypic analysis of hypomorphic *lin-12* mutants in *Caenorhabditis elegans*. *Genetics* **135**, 755-763.
- Sundaram, M. and Greenwald, I.** (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.
- van der Keyl, H., Kim, H., Espey, R., Oke, C. V. and Edwards, M. K.** (1994). *Caenorhabditis elegans* *sqt-3* mutants have mutations in the *col-1* collagen gene. *Dev. Dynam.* **201**, 86-94.
- von Heijne, G.** (1986). A new method for predicting signal sequence cleavage sites. *Nucl. Acids Res.* **14**, 4683-4690.
- Wilkinson, H. A., Fitzgerald, K. and Greenwald, I.** (1994). Reciprocal changes in expression of the receptor *lin-12* and its ligand *lag-2* prior to commitment in a *C. elegans* cell fate decision. *Cell* **79**, 1187-1198.

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