Development 121, 4275-4282 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995
DEV5031

Interchangeability of *Caenorhabditis elegans* DSL proteins and intrinsic signalling activity of their extracellular domains in vivo

Kevin Fitzgerald[1] and Iva Greenwald[2]

[1]Dept. of Biochemistry and Molecular Biophysics, Columbia University College Physicians and Surgeons, New York, NY 10032, USA
[2]Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Columbia University, College of Physicians and Surgeons, New York, New York 10032, USA

*Author for correspondence (e-mail: greenwald@cuccfa.ccc.columbia.edu)

SUMMARY

Ligands of the *Delta/Serrate/lag-2* (DSL) family and their receptors, members of the *lin-12/Notch* family, mediate cell-cell interactions that specify cell fate in invertebrates and vertebrates. In *C. elegans*, two DSL genes, *lag-2* and *apx-1*, influence different cell fate decisions during development. Here we show that APX-1 can fully substitute for LAG-2 when expressed under the control of *lag-2* regulatory sequences. In addition, we demonstrate that truncated forms lacking the transmembrane and intracellular domains of both LAG-2 and APX-1 can also substitute for endogenous *lag-2* activity. Moreover, we provide evidence that these truncated forms are secreted and able to activate LIN-12 and GLP-1 ectopically. Finally, we show that expression of a secreted DSL domain alone may enhance endogenous LAG-2 signalling. Our data suggest ways that activated forms of DSL ligands in other systems may be created.

Key words: Delta, lag-2, lin-12, Notch, glp-1, ligand, *Caenorhabditis*

INTRODUCTION

During animal development, intercellular communication, mediated by ligand-receptor interactions, plays critical roles in specifying cell fates. Ligands of the DSL family (for *Delta*, *Serrate*, *lag-2*) are important mediators of intercellular communication. DSL proteins are transmembrane proteins with a 'DSL' domain and epidermal growth factor (EGF)-like motifs in their extracellular domains, and relatively short intracellular domains (reviewed in Simpson, 1995). Receptors for DSL proteins are members of the *lin-12/Notch* family. These receptors are transmembrane proteins with multiple EGF-like motifs and three LIN-12/Notch repeat (LNR) motifs in their extracellular domains and six cdc10/SWI6 (also termed 'ankyrin') motifs in their intracellular domains (reviewed in Greenwald, 1994; Artavanis-Tsakonas et al., 1995).

In *C. elegans*, two ligands of the DSL family, *lag-2* and *apx-1*, have been described. LAG-2 and APX-1 are only 25% identical, and have different numbers of epidermal growth factor (EGF)-like motifs. LAG-2 appears to be the ligand for the *lin-12*-mediated anchor cell/ventral uterine precursor cell (AC/VU) decision in the hermaphrodite, the *glp-1*-mediated mitosis/meiosis decision in the germline, and *lin-12* or *glp-1*-mediated decisions affecting viability (Lambie and Kimble, 1991; Henderson et al., 1994; Tax et al., 1994; Wilkinson et al., 1994). APX-1 appears to be a maternally provided ligand for a *glp-1*-mediated decision in the early embryo (Mango et al., 1994; Mello et al., 1994). *apx-1* mutants do not have any apparent defects in later embryonic or postembryonic development, although this issue remains open until null mutants of *apx-1* are examined.

Genetic studies have not provided any evidence for functional redundancy of *lag-2* and *apx-1* (Mello et al., 1994). Because *lag-2* and *apx-1* mutants have different phenotypes, and LAG-2 and APX-1 have a relatively low level of homology, they might be biochemically distinctive and hence not functionally interchangeable. Furthermore, the fact that the DSL family have conserved amino acid sequence motifs raises the issue of the function of different structural domains. In this paper, we address these issues by expressing wild-type and mutant proteins under the control of *lag-2* regulatory sequences and examining their ability to replace *lag-2* in defined cell fate decisions and to activate LIN-12 and GLP-1 ectopically. We provide evidence that APX-1 and LAG-2 are biochemically interchangeable. We also show that secreted extracellular domains of DSL ligands have intrinsic signalling activity and that the DSL domain alone appears to enhance the activity of the endogenous LAG-2 protein.

MATERIALS AND METHODS

General methods and strains

Methods for handling and culturing *C. elegans* have been described by Brenner (1974). The wild-type parent for all strains used was *C.*

elegans var. Bristol strain N2 (Brenner, 1974). The key mutations used in this study, and their relevant features, were the following:

LGI: *smg-1(r861)* and *unc-54 (r293)* (Hodgkin et al., 1989). *smg-1* mutations stabilize aberrant mRNAs (Pulak and Anderson, 1993), and *unc-54(r293)* is suppressed by *smg-1(r293)*.

LGIII: *unc-32(e189)*, *dpy-18(e364)* (Brenner, 1974), and *glp-1(e2141ts)* (Priess et al., 1987).

LGV: *lag-2(s1486)* (Johnsen and Baillie, 1991), *lag-2(q411)* (Henderson et al., 1994), and *unc-46(e177)* (Brenner, 1974).

eT1 is a reciprocal translocation between LGIII and LGV whose breakpoint appears to be in *unc-36* (Rosenbluth and Baillie, 1981).

Plasmids

pLGX

pLGX was constructed from p226. p226 contains a 4.0 kb genomic *lag-2* fragment capable of complementing *lag-2(0)* mutations and was derived from JT225 and JT280 as described by Wilkinson et al., (1994). The genomic fragment contains 3.1 kb of 5′ flanking region as well as the *lag-2* coding and 3′ untranslated regions. pLGX, a *lag-2* expression vector, was constructed by using the polymerase chain reaction to insert a *Spe*I linker at position +1 of the genomic fragment.

pLGX derivatives

pLGX::apx-1 expresses the full length *apx-1* cDNA (Mello et al., 1994) under *lag-2* promoter control. *pLGX::GFP* was constructed using the polymerase chain reaction to create *Spe*I sites both 5′ of the GFP ATG and 3′ of the GFP TAA. This PCR product was digested with *Spe*I and ligated to pLGX which had also been digested with *Spe*I. *pLGX::apx-1* was constructed by creating *Xba*I restriction sites both 5′ of the *apx-1* ATG and 3′ of the *apx-1* TAA . This *apx-1* cDNA PCR product, which is flanked by *Xba*I sites, was cloned into pLGX digested with *Spe*I, resulting in *pLGX::apx-1*. Truncated derivatives of *apx-1* and *lag-2* expressed in the pLGX vector were constructed using PCR in a similar fashion to that used to construct *pLGX::apx-1* with the following modifications: the 3′ and 5′ primers both contained *Spe*I sites instead of *Xba*I sites, and the 3′ primers contained an in-frame stop codon. The 3′ stop codon containing primers were at the following positions: for *pLGX::apx-1(extra)*, amino acid 402; for *pLGX::apx-1(DSL)*, amino acid 188; for *pLGX::lag-2(extra)*, amino acid 281. *pLGX::apx-1(1/2DSL)* was created by placing a stop codon into a unique *Nru*I site at amino acid 159 in *pLGX::apx-1*. *pLGX::apx-1(extra)+GFP* is the same as *pLGX::apx-1(extra)* except the 3′ stop codon is now derived from Green Fluorescent Protein (Chalfie et al., 1994). GFP was inserted, in frame, on the 3′ end of *apx-1(extra)*, thereby creating an *apx-1(extra)::gfp* gene fusion. *pLGX::apx-1(extra) + TM-lacZ* was created in a similar fashion to *pLGX::apx-1(extra) + gfp* with *lacZ* inserted with its own stop codon in frame to *apx-1(extra)* at amino acid 402. The inserted modified *lacZ* gene was derived from a combination of the plasmids 16.43 and 34.110 (Fire et al., 1990).

Transgenic lines

Transgenic lines were established by microinjection of plasmid mixtures into the hermaphrodite germline to create extrachromosomal arrays (Fire, 1986; Mello et al., 1991). By accepted convention, 'Ex' is used to represent extrachromosomal arrays. pLGX and derivatives were injected at 1 μg/ml into a recipient strain of genotype *smg-1(r861); dpy-18(e364)/eT1; lag-2(s1486) unc-46(e177)/eT1* and at 50 μg/ml into a recipient strain of genotype *smg-1(r861) unc-54(r293)*. pRF4, a plasmid containing the cloned dominant *rol-6(su1006)* gene (Mello et al., 1991) was used as a cotransformation marker and injected at a concentration of 100 μg/ml. F_1 Roller progeny were picked, and F_2 Roller progeny used to establish lines.

To assess rescue of *lag-2(0)* phenotypes, all Rol progeny from at least three heterozygous *smg-1(r861); dpy-18(e364)/eT1; lag-2(s1486) unc-46(e177)/eT1; Ex* hermaphrodites were picked individually and scored in the next generation for Dpy-18 Unc-46 Rol-6 phenotypes. Rescued homozygous lines segregated only viable Dpy Unc Rol animals and Lag arrested larvae, indicating that the extrachromosomal arrays were necessary for viability. At 1 μg/ml, 4/4 independent lines of pLGX::*apx-1(+)*, 2/2 lines of pLGX::*apx-1(extra)*, 2/3 of pLGX::*apx-1(extra-GFP)*, and 2/2 of pLGX::*apx-1(extra+TM-BGAL)* displayed rescue; transgenic lines could be maintained indefinitely at 25°C. Living hermaphrodites segregating from these strains were scored under Nomarski optics for the number of anchor cells and germline anatomy. At 1 μg/ml, 0/4 lines of pLGX, 0/2 of pLGX::*apx-1(DSL)*, and 0/2 of pLGX::*apx-1(1/2 DSL)* rescued the lethality of *lag-2(s1486)*. When the concentration of pLGX or pLGX::*apx-1(DSL)* was increased, rescue was still not obtained: for pLGX::*apx-1(DSL)*, 0/2 lines made at 25 μg/ml rescued *lag-2(s1486)*, and 0/2 lines of pLGX made at 50 μg/ml rescued *lag-2(s1486)*. In addition, 0/1 lines of pLGX::*apx-1(DSL)* made at 50 μg/ml rescued *lag-2(q411)*.

Rescued hermaphrodites [relevant genotype: *lag-2(s1486); Ex*] were also examined for phenotypes associated with ectopic *glp-1* or *lin-12* activation. Rescued hermaphrodites containing pLGX::*apx-1(extra)* and pLGX::*apx-1(extra-GFP)* exhibited a low penetrance (2-20%) tumorous germline.

Hermaphrodites carrying extrachromosomal arrays made at 50 μg/ml [relevant genotype: *lag-2(+); Ex*] displayed phenotypes associated with ectopic *glp-1* or *lin-12* activation, with penetrances ranging from 10 to 80%. All transgenic lines containing pLGX::*apx-1(extra)*, pLGX::*apx-1(extra-GFP)*; pLGX::*lag-2(extra)*, or pLGX::*apx-1(DSL)* segregated hermaphrodites with a tumorous germline and/or a Multivulva phenotype. For pLGX::*apx-1(DSL)*, these phenotypes were seen in transgenic lines carrying extrachromosomal arrays made at 25 μg/ml and 50 μg/ml, but not at 1 μg/ml. The tumorous germline or Multivulva phenotype was never observed in lines carrying extrachromosomal arrays made from pLGX::*apx-1(1/2 DSL)*, pLGX::*apx-1(extra-TM-BGAL)*, pLGX::*apx-1(+)*, and pLGX. Greater than 50 Rol-6 animals were observed for each line carrying these constructs.

RESULTS

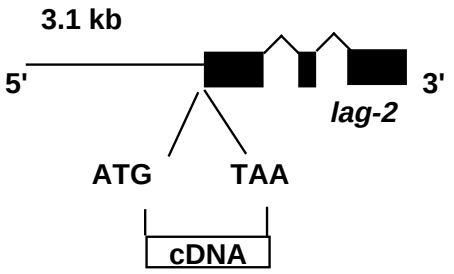
Expression of proteins under the control of *lag-2* regulatory sequences

In the experiments described here, we expressed *lag-2* or *apx-1* coding sequences under the control of *lag-2* regulatory sequences, using the pLGX *lag-2* expression vector (Fig. 1 and Materials and Methods). The pLGX vector is based on a genomic clone that can rescue a *lag-2(0)* mutant (Tax et al., 1994; Tax, 1994). When the inserted cDNA encodes β-galactosidase or green fluorescent protein (GFP), reporter protein activity is detectable in a spatially and temporally restricted pattern consistent with genetic analysis of *lag-2*, including in Z1.ppp and Z4.aaa (Wilkinson et al., 1994) and the distal tip cells (Fig. 1). Since the mRNA expressed from such a construct is predicted to contain an abnormal, long 3′ untranslated region, efficient expression is only obtained in the presence of *smg-1* mutations, which stabilize mRNAs with long 3′ untranslated regions (Pulak and Anderson, 1993). The *smg-1* mutations do not appear to affect hermaphrodite gonadal anatomy or development, the vulval precursor cell lineages, or viability (Hodgkin et al., 1989; Pulak and Anderson, 1993; Wilkinson et al., 1994), and do not suppress the *lag-2(s1486)* and *lag-2(q411)* alleles used in this study.

Assays for *lag-2* activity

Several independent extrachromosomal arrays were generated

A.



B.

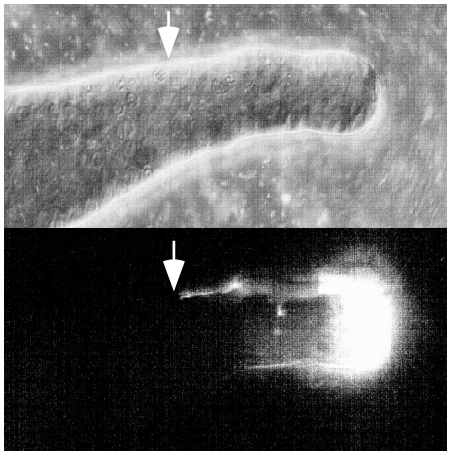


Fig. 1. (A) Expression of wild-type and altered LAG-2 and APX-1 proteins under the control of *lag-2* regulatory sequences. cDNAs with their own start and stop codons but lacking polyadenylation signals are inserted into the pLGX expression vector. Inserted cDNAs are placed in the location and orientation of the first exon with respect to all of the potential regulatory sequences required for rescue. (B) Expression of *lag-2::GFP* in distal tip cells. The top panel shows a dissected gonad viewed with Nomarski optics to visualize mitotic and meiotic nuclei. The bottom panel shows the same dissected gonad viewed with fluorescence to visualize GFP. The arrow indicates 20 nuclear diameters from the distal tip of the gonad, a position consistent with the mitotic/meiotic border (Hirsh et al., 1976; Kimble and White, 1981). Fluorescent distal tip cell processes are clearly seen to extend along the germline, up to 20 nuclear diameters away from the distal end. Fluorescent distal tip cell processes look identical in dissected gonads as well as intact hermaphrodites.

for each construct. Transgenic lines were examined in the presence of endogenous *lag-2* activity for phenotypes associated with *lin-12* and *glp-1* activation, and in the absence of *lag-2* activity for rescue of individual cell fate decisions requiring *lag-2* activity. Details for individual constructs are given in Materials and Methods. However, we note that, for constructs that rescue *lag-2(0)* mutants, rescue was observed even when constructs were injected at a concentration of 1 μg/ml. At this concentration, extrachromosomal arrays contain a low copy number of construct (Mello et al., 1994) and should not be grossly overexpressed with respect to the normal level of *lag-2* gene expression.

Here, we summarize what is known about the roles of *lag-2* and *lin-12* or *glp-1* relevant to the phenotypes we scored.

Viability

lag-2 null mutants arrest as larvae with numerous cell fate transformations (Lambie and Kimble, 1991). The Lag phenotype is distinctive and resembles that of *lin-12(-) glp-1(-)* double mutants (Lambie and Kimble, 1991). Together with the finding that GLP-1 can fully substitute for LIN-12 (Fitzgerald et al., 1993), the available evidence suggests that LAG-2 normally activates LIN-12 and GLP-1 in certain cells so that the cell fate decisions required for viability occur properly.

The AC/VU decision

The *lin-12*-mediated AC/VU decision involves an interaction between Z1.ppp and Z4.aaa, two initially equivalent cells of the somatic gonad. During wild-type development, Z1.ppp and Z4.aaa interact with each other, so that in each hermaphrodite one becomes the AC while the other becomes a VU (Kimble and Hirsh, 1979; Kimble, 1981; Seydoux and Greenwald, 1989). When *lin-12* or *lag-2* activity is eliminated, both Z1.ppp and Z4.aaa become ACs (Greenwald et al., 1993; Lambie and Kimble, 1991). Mutations that activate LIN-12 cause both cells to become VUs (Greenwald et al., 1983; Greenwald and Seydoux, 1990; Struhl et al., 1993).

Germline development

A *glp-1*-mediated interaction between a somatic gonadal cell, the distal tip cell, and the germline promotes germline mitosis (and/or inhibits meiosis) (Kimble and White, 1981). The adult hermaphrodite gonad is a two-armed structure, with a distal tip cell at the end of each arm. Normally, each arm contains mitotic cells in the most distal region of the germline, followed consecutively by a region of meiosis, a region of maturing oocytes and a region of sperm (Hirsh et al., 1976; Kimble and Hirsh, 1979). When *lag-2* or *glp-1* activity is decreased, all germ cells enter meiosis (Austin and Kimble, 1987; Priess et al., 1987; Lambie and Kimble, 1991; Crittenden et al., 1994). A mutation that causes GLP-1 to be active independent of ligand causes germ cells to continue to divide mitotically (L. Wilson Berry and T. Schedl, personal communication).

APX-1 can substitute for LAG-2

Genetic analysis had indicated that LAG-2 can interact with both LIN-12 and GLP-1 (Lambie and Kimble, 1991; Tax et al., 1994), but *apx-1* was only known to interact with *glp-1* (Mango et al., 1994; Mello et al., 1994). LAG-2 and APX-1 are only 25% identical, and have different numbers of epidermal growth factor (EGF)-like motifs. By our criteria (Fig. 2 legend), there are two EGF-like motifs in LAG-2 and four in APX-1. We placed *apx-1* under the control of *lag-2* regulatory sequences and showed that expression of APX-1 in a *lag-2(0)* mutant can rescue the defects causing lethality, as well as defects in the AC/VU decision and germline development. Thus, APX-1 can fully substitute for LAG-2 (Figs 2,3).

In addition, C. Mello and J. Priess identified another DSL gene, *arg-1*, which encodes a DSL ligand of unknown function. ARG-1 has two EGF-like motifs and is only 25% identical to LAG-2. They kindly made *arg-1* available to us, and we found that ARG-1 expressed under the control of *lag-*

2 regulatory sequences can also substitute for LAG-2 (data not shown).

The transmembrane and intracellular domains are dispensable for LAG-2 function

We completely replaced the transmembrane and intracellular domains of LAG-2 by a synthetic transmembrane domain and a modified β-galactosidase protein. The resulting chimeric protein can rescue the defects causing lethality, and defects in the AC/VU decision and germline development, and therefore is able to substitute fully for endogenous *lag-2* activity (Fig. 3). This result implies that the transmembrane and intracellular domains are not necessary for LAG-2 activity (see also Henderson et al., 1994).

Intrinsic signalling activity of secreted extracellular domains of LAG-2 and APX-1

In order to test the dependence of the activity of the extracellular domain on membrane association, we completely deleted the transmembrane and intracellular domains of LAG-2 and APX-1. We found that just the extracellular domains of LAG-2 or APX-1 were able to rescue the defects causing lethality, and defects in the AC/VU decision and germline development of *lag-2(0)* mutants (Fig. 3). These observations suggest that the extracellular domains of DSL ligands have intrinsic signalling activity.

To demonstrate that these truncated proteins are secreted, we tagged the APX-1 extracellular domain with the Green Fluorescent Protein (Chalfie et al., 1994). The resulting protein is fully functional and is found in coelomocytes, suggesting it is secreted into the pseudocoelom (Fig. 4). Non-secreted forms of GFP expressed under the control of *lag-2* regulatory sequences, as in pLGX*::GFP* (Fig. 1), are not found in coelomocytes (K. F., unpublished observations). Furthermore, we also see evidence that secreted extracellular domains can activate LIN-12 in the VPCs, causing a characteristic Multivulva phenotype (Fig. 4). This Multivulva phenotype implies that the secreted extracellular domains of DSL proteins can diffuse to and function at an ectopic location, since *lag-2* does not appear to be expressed in the vulval precursor cells (K. F. and I. G., unpublished observations), and does not appear to function in the vulval precursor cells (Tax et al., 1994; S. Kaech and S. Kim, personal communication). A Multivulva phenotype was not observed in hermaphrodites containing transgenes encoding transmembrane DSL ligands.

Ectopic activation of GLP-1 by secreted extracellular domains of LAG-2 and APX-1

While cell fate decisions often occur normally in animals expressing secreted LAG-2 or APX-1 extracellular domains, some hermaphrodites display germline abnormalities suggestive of GLP-1 ectopic activation or hyperactivation (Fig. 5). As described above, each gonadal arm of wild-type adult hermaphrodites shows an orderly progression of germline maturation, with mitotic cells limited to the most distal region of the germline. In contrast, in hermaphrodites expressing secreted LAG-2 or APX-1 extracellular domains, there is often mitosis in the proximal region of the germline (the 'proximal mitosis' phenotype; Seydoux et al., 1990) or mitosis throughout the germline (the 'tumorous germline' phenotype; Francis et al., 1995 and L. W. Berry and T. Schedl, personal communication).

These germline abnormalities appear to be caused by GLP-1 activation, since *glp-1(e2141ts)* hermaphrodites expressing the APX-1 extracellular domains may have tumorous germlines at the permissive temperature but, after shifting to the restrictive temperature, the germ cells enter meiosis (Fig. 6). Furthermore, the proximal mitosis and tumorous germline phenotypes caused by expression of the APX-1 extracellular

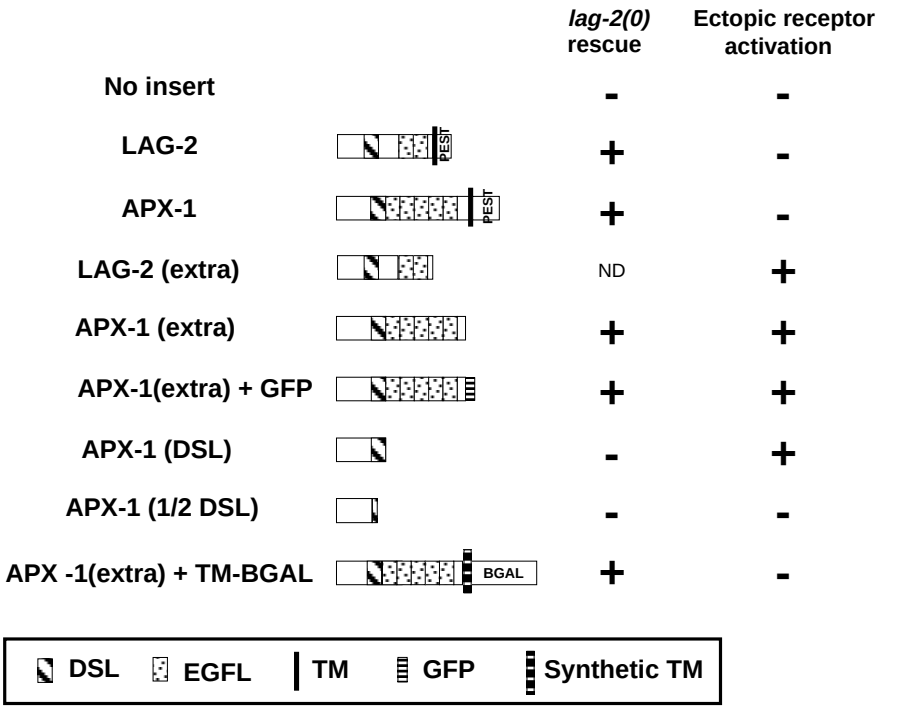


Fig. 2. Wild-type and altered LAG-2 and APX-1 proteins expressed using pLGX. The DSL motifs are as described (Mello et al., 1994; Tax et al., 1994). An EGF-like motif is considered to be a sequence conforming to the consensus C-X_n-C-X_n-C-X_n-CXCXXG(F/Y)XXGXXC (Blomquist et al., 1984; Suedhof et al., 1985). Rescue: *lag-2(0)* hermaphrodites arrest as larvae with a characteristic Lag phenotype (Lambie and Kimble, 1991). Rare viable *lag-2* hypomorphs or *lag-2(ts)* hermaphrodites shifted to the restrictive temperature after the L1 stage display the 2 anchor cell (2 AC) phenotype associated with the absence of *lin-12* activity and the germline proliferation (Glp) defect associated with the absence of *glp-1* activity (Lambie and Kimble, 1991; Henderson et al., 1994). A '+' indicates that the construct rescued the lethal, 2 AC and Glp phenotypes associated with reduced *lag-2* activity. Ectopic activation: Ectopic activation of *lin-12* causes a Multivulva phenotype (Greenwald et al., 1983), and ectopic activation of *glp-1* causes a 'tumorous' germline phenotype (L.W. Berry and T. Schedl, personal communication). A '+' indicates that the construct caused some hermaphrodites to display Multivulva and/or tumorous germline phenotypes. Details about the number of lines and number of individuals scored are given in Materials and Methods.

domain occur when the activity of the endogenous *lag-2* gene is eliminated, indicating that these phenotypes reflect the intrinsic activity of the secreted ligand (Fig. 5). Abnormal germline development was never observed in animals carrying multiple copies of transgenes encoding intact APX-1 or LAG-2 proteins, or the APX-TM-βGAL protein, suggesting that the secreted extracellular domain or DSL domain may be more intrinsically active, more stable, or able to diffuse further than transmembrane protein ligands.

The secreted DSL domain may activate endogenous LAG-2 protein

The DSL domain is found only in DSL proteins and its role is not known. To examine this question, we expressed a secreted DSL domain of LAG-2 or APX-1. The secreted DSL domain is not sufficient to rescue the lethality of a *lag-2(0)* mutant. However, it is able to cause the proximal germline mitosis or tumorous germline phenotype in the presence of *lag-2(+)* activity. Thus, a secreted DSL domain alone may enhance endogenous LAG-2 signalling. Alternatively, a secreted DSL domain may have intrinsic signalling activity that is insufficient to rescue the lethality of *lag-2(0)* but is sufficient to activate GLP-1 in the germline.

DISCUSSION

Interchangeability of *C. elegans* DSL proteins

We have shown that two different *C. elegans* DSL proteins, APX-1 and ARG-1, can substitute for LAG-2 when expressed under control of *lag-2* regulatory sequences. All phenotypes known to be associated with eliminating *lag-2* activity, including defects leading to lethality and defects in the AC/VU decision and germline development, were rescued by APX-1 or ARG-1. The fact that APX-1 and LAG-2 appear to be interchangeable despite their considerable differences in amino acid sequence implies that they share conserved structural features. A similar observation has been made in *Drosophila*, where expression of Serrate under control of a heat shock promoter is able to suppress neuroblast segregation in the absence of Delta (Gu et al., 1995). The interchangeability of APX-1 and LAG-2 implies that differential gene expression rather than different biochemical activity underlies the distinct mutant phenotypes of *lag-2* and *apx-1*.

Intrinsic activity of the extracellular domains of LAG-2 and APX-1

We have shown that the transmembrane domain and the intracellular domain of LAG-2 can be completely replaced by a synthetic transmembrane domain and β-galactosidase without affecting the ability of the protein to mediate cell fate decisions. This result implies that the transmembrane and intracellular domains are not necessary for LAG-2 activity. Furthermore, when the transmembrane domain and intracellular domain of APX-1 is deleted, the resulting secreted protein can replace endogenous *lag-2* activity in all cell fate decisions examined. These results imply that the extracellular domains of LAG-2 and APX-1 have intrinsic signalling activity.

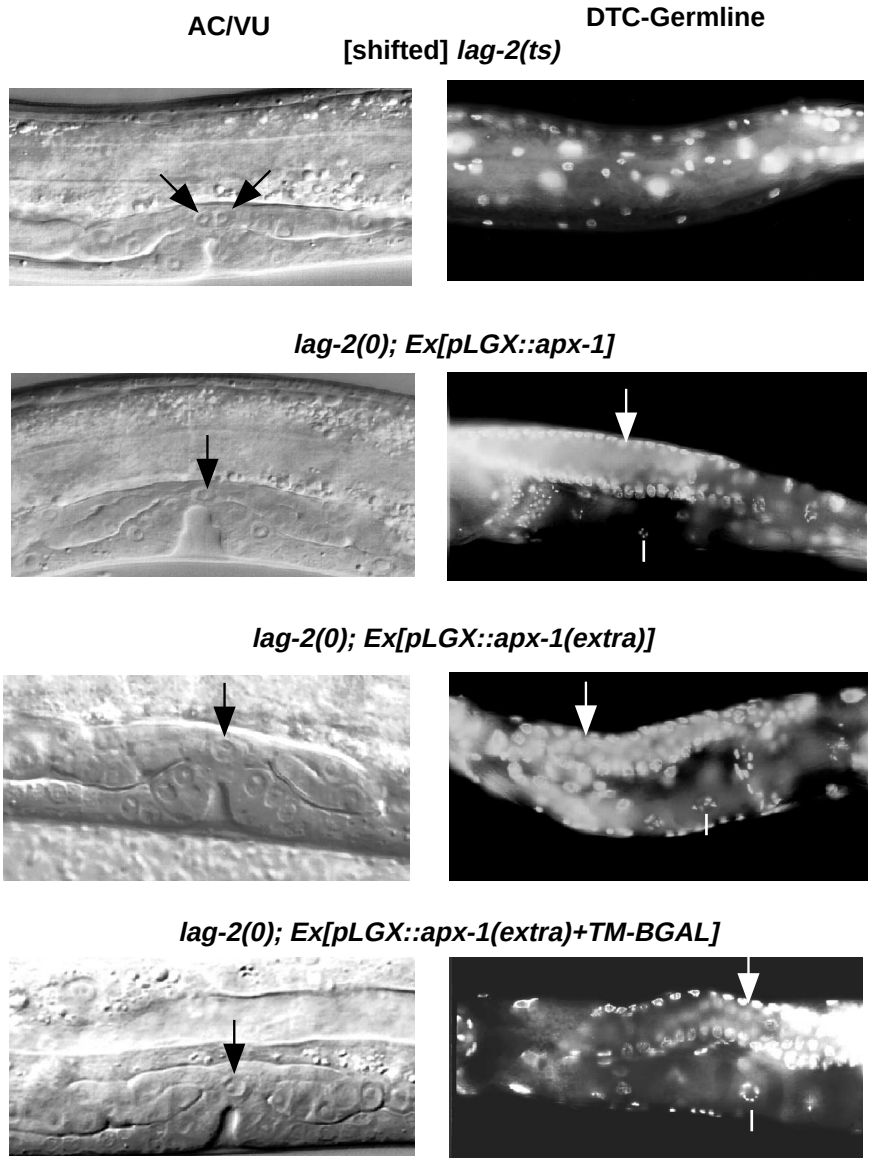


Fig. 3. Rescue of specific *lag-2*-mediated cell fate decisions by wild-type and altered APX-1 proteins. The first column (AC/VU) shows rescue of the 2 AC defect, viewed by Nomarski microscopy. A black arrow indicates an AC. The second column (DTC-germline) shows rescue of the germline proliferation defect, viewed by staining with DAPI. White arrows indicate mitotic nuclei and white lines indicate oocyte nuclei. The complete genotypes are as follows: *lag-2(ts) = lag-2(q420). lag-2(0); Ex[pLGX::apx-1] = smg-1(r861) unc-54(r293); lag-2(s1486); Ex[pLGX::apx-1]. lag-2(0); Ex[pLGX::apx-1(extra) = smg-1(r861) unc-54(r293); lag-2(s1486); Ex[pLGX::apx-1(extra)]. lag-2(0); Ex[pLGX::apx-1(extra) + TM-BGAL] = smg-1(r861) unc-54(r293); lag-2(s1486); Ex[pLGX::apx-1(extra) + TM-BGAL].*

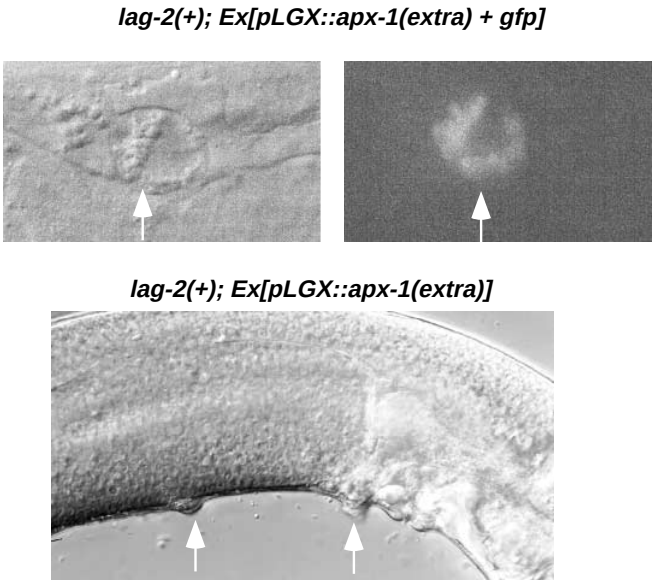


Fig. 4. Top two photos show accumulation of *pLGX::apx-1(extra) + GFP* in the coelomocytes. Left panel shows a Nomarski photo of a single posterior coelomocyte (white arrow). Right panel shows the same coelomocyte which fluoresces in FITC due to an accumulation of APX-1(extra) + GFP. The complete genotype of this animal is *smg-1(r861) unc-54(r293); Ex[pLGX::apx-1(extra) + GFP]*. Bottom panel shows multiple pseudovulvae (white arrows) on an animal carrying a *pLGX::apx-1(extra)* array. The complete genotype of this animal is *smg-1(r861) unc-54(r293); Ex[pLGX::apx-1(extra)]*.

The extracellular domains of DSL proteins have two conserved features: EGF-like motifs and the DSL domain. EGF-like motifs are found in the extracellular domains of many proteins. In DSL proteins, the EGF-like motifs are thought to bind to the EGF-like motifs of LIN-12/Notch proteins and are thought to mediate the ligand-receptor interaction (Rebay et al., 1991, 1993; Lieber et al., 1992, 1993; Rao et al., 1995). The DSL domain thus far appears to be unique to DSL proteins (Tax et al., 1994). We have found that a secreted DSL domain from LAG-2 or APX-1 can cause ectopic activation of GLP-1 in the germline in the presence of endogenous *lag-2* activity, but is unable to rescue the lethality of a *lag-2(0)* mutant, even at a high concentration of injected transgene. One possibility is that the secreted DSL domain has a low level of intrinsic activity that is insufficient to rescue the lethality of *lag-2(0)* but is sufficient to activate GLP-1 ectopically in the germline. However, rescue of defects causing lethality seems to require a lower level of *lag-2* activity than rescue of defects in the AC/VU decision or germline development (Lambie and Kimble, 1991; K.F., unpublished observations). We therefore favor the possibility that the secreted DSL domain enhances the activity of the intact LAG-2 protein. The relevance of this observation for normal ligand activity is not clear, but one possibility is that the DSL domain mediates oligomerization of DSL proteins to form an active ligand.

The role of the transmembrane and intracellular domains of LAG-2 and APX-1

The secreted extracellular domains of LAG-2 and APX-1 can promote normal cell fate decisions. However, some hermaphrodites expressing these mutant proteins displayed phenotypes associated with ectopic activation of LIN-12 or GLP-1, even in the absence of endogenous *lag-2* activity. Greater stability or mobility of secreted extracellular domains may underlie this apparent increase in intrinsic activity.

Ligand activity of DSL proteins may be regulated at the level of ligand stability. All DSL proteins contain PEST

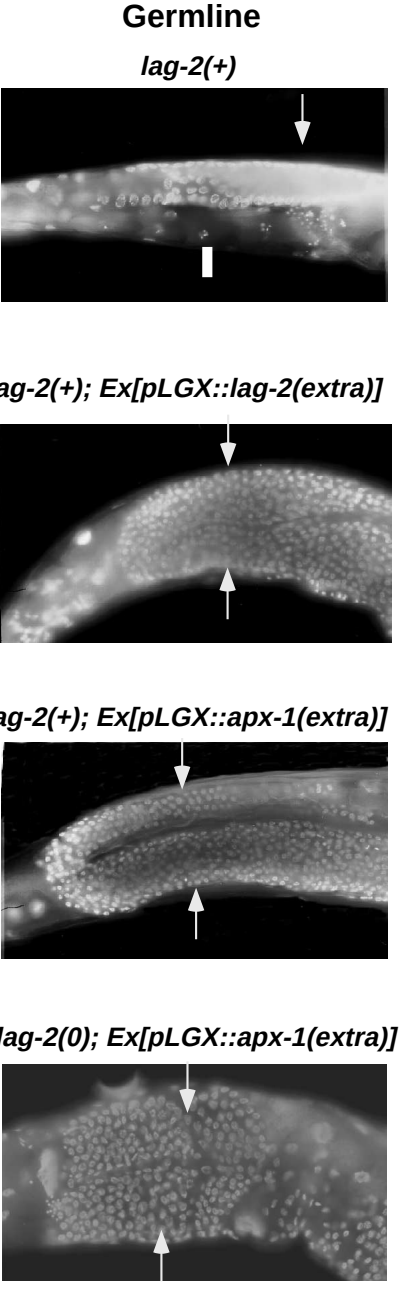


Fig. 5. Tumorous germline phenotype caused by LAG-2(extra) and APX-1(extra). Hermaphrodites are stained with DAPI. White arrows indicates mitotic nuclei and white lines indicate oocyte nuclei. The complete genotypes are as follows: *lag-2(+) = smg-1(r861) unc-54(r293). lag-2(+); Ex[pLGX::lag-2(extra)] = smg-1(r861) unc-54(r293); Ex[pLGX::lag-2(extra)]. lag-2(+); Ex[pLGX::apx-1(extra)] = smg-1(r861) unc-54(r293); Ex[pLGX::apx-1(extra)]. lag-2(0); Ex[pLGX::apx-1(extra)] = smg-1(r861) unc-54(r293);lag-2(s1486); Ex[pLGX::apx-1(extra)]*.

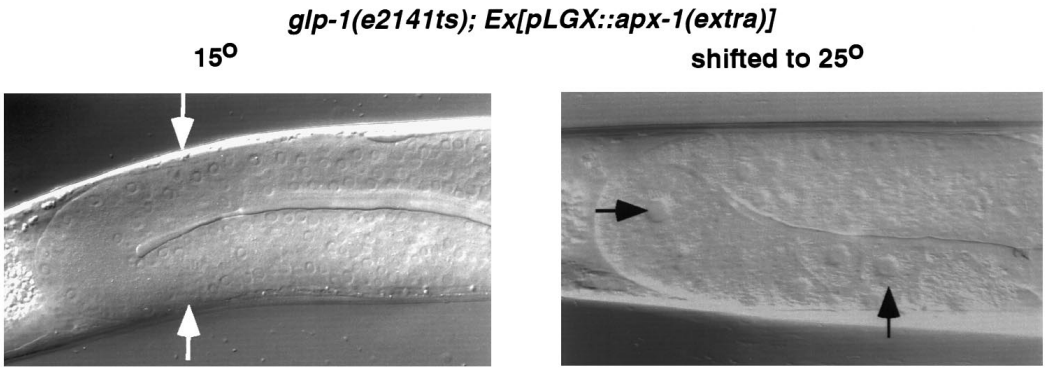


Fig. 6. Dependence of the tumorous germline phenotype caused by pLGX::*apx-1(extra)* on *glp-1* activity. *smg-1(r861); Ex*[pLGX::*apx-1(extra)*] hermaphrodites exhibit a high penetrance tumorous germline phenotype in a *glp-1(e2141ts)* background at 15°C, the permissive temperature for *glp-1(e2141ts)* (Priess et al., 1987). When these adult hermaphrodites are shifted to 25°C, the non-permissive temperature, germ cells cease mitosis and enter meiosis. Hermaphrodites were examined under Nomarski optics before the temperature shift. By 15 hours after the shift, many hermaphrodites had cells resembling oocytes both distally and proximally where formerly there had been only mitotic cells. Left panel shows the tumorous germline of an individual of genotype *smg-1(r861) unc-54(r293); glp-1(e2141ts); Ex[pLGX::apx-1(extra)]*, at 15°C, while the right panel shows the same hermaphrodite 15 hours after being shifted to 25°C. White arrows indicate mitotic nuclei, and black arrows indicate putative oocyte nuclei.

protein turnover sequences (Rogers et al., 1986) in their intracellular domains. An indication that protein stability influences APX-1 activity is that some hermaphrodites expressing a mutant protein containing a partial deletion of the PEST sequence of APX-1 have a tumorous germline phenotype (K. F., unpublished observations). In contrast, ectopic activity is not observed when a modified β-galactosidase that is highly unstable in *C. elegans* (Way and Chalfie, 1989; Fire et al., 1990; Wilkinson et al., 1994) replaces the entire intracellular domain of APX-1 (this work) or part of the intracellular domain of LAG-2 (Henderson et al., 1994; K. F., unpublished observations).

The prevention of inappropriate cell-cell interactions appears to be important during development (e.g., Henry and Grainger, 1987; Seydoux et al., 1990; Waring and Kenyon, 1990). Membrane-association of DSL proteins, which potentially mediate many different cell-cell interactions, may be one mechanism to prevent inappropriate interactions. Our observation that the secreted extracellular domain of LAG-2 or APX-1 appears to activate LIN-12 in the vulval precursor cells, even though LAG-2 does not appear to be the normal ligand for LIN-12 during vulval precursor cell specification (Tax et al., 1994; S. Kaech and S. Kim, personal communication; K. F., unpublished observations), suggests that inappropriate cell-cell interaction may occur when a DSL protein is able to dissociate from the membrane.

If DSL proteins normally remain associated with the membrane, it implies that cell-cell contact is necessary for ligand-receptor interactions. In *lin-12*, *glp-1* and *Notch*-mediated decisions, signalling and receiving cells are in contact. Furthermore, if cell contact between Z1.ppp and Z4.aaa is disrupted in the forming gonad primordium, both cells differentiate as anchor cells (Hedgecock et al., 1987). Our observation that the distal tip cells appear to have processes that in some cases can be seen to extend as far as the zone of germline mitosis (see Fig. 1) may be relevant to this issue. The distal tip cell influences the decision (mitosis/meiosis) of germline nuclei that are at least twenty nuclear diameters away from the distal end of the gonad (Kimble and White, 1981). The distal tip cell has been thought to contact only the distal end of the germline syncytium, so that its influence was mediated by a diffusible factor (Kimble and White, 1981), such as an intracellular second messenger produced upon GLP-1 activation (Yochem and Greenwald, 1989; Henderson et al., 1994). However, our observation that distal tip cells extend long processes suggests that direct contact between distal tip cells and membranes associated with germline nuclei throughout the mitotic zone may be important.

Relevance to other systems

The interchangeability of *C. elegans* DSL proteins and of *Drosophila* DSL proteins suggests that the multiple DSL proteins found in vertebrate systems may also be interchangeable. In addition, our observation that DSL proteins are active, and indeed may be hyperactive, when the transmembrane and intracellular domains are removed will be useful for investigating cell-cell interactions in mammals that are mediated by DSL proteins and LIN-12/Notch proteins (e.g., Chitnis et al., 1995; Lindsell et al., 1995). Secreted extracellular domains will be useful reagents for tissue culture and biochemical studies. Furthermore, since activated forms of LIN-12/Notch family members have been associated with cancer in mammals (Ellisen et al., 1991; Robbins et al., 1992), it is conceivable that truncated forms of DSL proteins could also function as oncogenes.

We thank Craig Mello and Jim Priess, and Frans Tax and Jim Thomas, for their generosity with unpublished as well as published clones and information; David Baillie, Sam Henderson and Judith Kimble for *lag-2* alleles; Andy Fire and Marty Chalfie for reporter genes; Tim Schedl for helpful discussion; Richard Ruiz and Denise Brousseau for valuable assistance; and Mike Beck, Tim Doyle, Jane Albert Hubbard, Gary Struhl and Simon Tuck for critical comments on the manuscript. This work was supported by National Institutes of Health grant GM37602. I. G. is an Associate Investigator of the Howard Hughes Medical Institute.

REFERENCES

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. *Science* **268**, 225-232.

Austin, J. and Kimble, J. (1987) *glp-1* is required in the germ line for

regulation of the decision between mitosis and meiosis in *C. elegans*. *Cell* **51,** 589-599.

Blomquist, M. C., Hunt, L. T. and Barker, W. C. (1984). Vaccinia virus 19-kilodalton protein: relationship to several mammalian proteins, including two growth factors. *Proc. Natl. Acad. Sci. (USA)* **81**, 7363-7367.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

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.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homolog of the *Drosophila* neurogenic gene *Delta*. *Nature* **375**, 761-766.

Crittenden, S. L., Troemel, E. R., Evans, T. C., and Kimble, J. (1994) GLP-1 is localized to the mitotic region of the *C. elegans* germ line. *Development* **120**, 2901-2911.

Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D. and Sklar, J. (1991). *TAN-1*, the human homolog of the Drosophila *Notch* gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66,** 649-661.

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., Wilkinson, H.A., and Greenwald, I. (1993). *glp-1* can substitute for *lin-12* in specifying cell fate decisions in *Caenorhabditis elegans. Development* **119**, 1019-1027.

Francis, R., Barton, M. K., Kimble, J. and Schedl, T. (1995). *gld-1*, a tumor suppressor gene required for oocyte development in *Caenorhabditis elegans*. *Genetics* **139**, 579-606.

Greenwald, I. (1994). Structure/function studies of lin-12/Notch proteins. *Curr. Opin. Gen. Dev.* **4,** 556-562.

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.

Greenwald, I. and Seydoux, G. (1990). Analysis of gain-of-function mutations of the *lin-12* gene of *Caenorhabditis elegans*. *Cell* **34,** 435-444.

Gu, Y., Hukriede, N. A. and Fleming, R. J. (1995). *Serrate* expression can functionally replace *Delta* activity during neuroblast segregation in the *Drosophila* embryo. *Development* **121,** 855-865.

Hedgecock, E. M., Culotti, J. G., Hall, D. H. and Stern, B. D. (1987) Genetics of cell and axon migrations in *Caenorhabditis elegans*. *Development* **100,** 365-382.

Henderson, S. T., Gao, D., Lambie, E. J. and Kimble, J. (1994). *lag-2* may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*. *Development* **120,** 2913-2924.

Henry, J. J. and Grainger, R. M. (1987). Inductive interactions in the spatial and temporal restriction of lens forming potential in embryonic ectoderm of *Xenopus laevis*. *Dev. Biol.* **124**, 200-214.

Hirsh, D., Oppenheim, D. and Klass, M. (1976). Development of the reproductive system of *Caenorhabditis elegans*. *Dev. Biol.* **49,** 200-219.

Hodgkin, J., Papp, A., Pulak, R., Ambros, V. and Anderson, P. (1989). A new kind of informational suppression in the nematode *Caenorhabditis elegans*. *Genetics* **123,** 301-313.

Johnsen, R. C. and Baillie, D. L. (1991). Genetic analysis of a major segment [LGV(left)] of the genome of *Caenorhabditis elegans. Genetics* **129,** 735-752.

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 hermaphrodites and male gonads in *Caenorhabditis elegans. Dev. Biol.* **87,** 396-417.

Kimble, J. E. and White, J. G. (1981). On the control of germ cell development in *Caenorhabditis elegans*. *Dev. Biol.* **81,** 208-219.

Lambie, E. J. and Kimble, J. (1991). Two homologous regulatory genes, lin-12 and glp-1, have overlapping functions. *Development* **112,** 231-239.

Lieber, T., Kidd, S., Alcamo, E., Corbin, V. and Young, M. W. (1993). Antineurogenic phenotypes induced by truncated *Notch* proteins indicate a role in signal transduction and may point to a novel function for *Notch* in nuclei. *Genes Dev.* **7,** 1949-1965.

Lieber, T., Wesley, C. S., Alcamo, A., Hassel, B., Krane, J. F., Campos-Ortega, J. A. and Young, M. W. (1992). Single amino acid substitutions in EGF-like elements of *Notch* and *Delta* modify *Drosophila* development and affect cell adhesion *in vitro*. *Neuron* **9,** 847-859.

Lindsell, C. E., Shawber, C. J., Boulter, J. and Weinmaster, G. (1995) Jagged: a mammalian ligand that activates Notch1. *Cell* **80,** 909-917.

Mango, S. E., Thorpe, C. J., Martin, P. R., Chamberlain, S. H. and Bowerman, B. (1994). Two maternal genes, *apx-1*and *pie-1*, are required to distinguish the fates of equivalent blastomeres in the early *Caenorhabditis elegans* embryo. *Development* **120,** 2305-2315.

Mello, C. C., Draper, B. W. and Priess, J. R. (1994). The maternal genes *apx-1*and *glp-1* and establishment of dorsal-ventral polarity in the early *C. elegans* embryo. *Cell* **77,** 95-106.

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.

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

Pulak, R. and Anderson, R. P. (1993). mRNA surveillance by the *Caenorhabditis elegans smg* genes. *Genes Dev.* **7,** 1885-1897.

Rao, Z., Handford, P., Mayhew, M., Knott, V., Brownlee, G. G. and Stuart, D. (1995). The structure of a Ca^{2+} epidermal growth factor-like domain: its role in protein-protein interactions. *Cell* **82,** 131-141.

Rebay, I., Fehon, R. G. and Artavanis-Tsakonas, S. (1993). Specific truncations of Drosophila *Notch* define dominant activated and dominant negative forms of the receptor. *Cell* **74,** 319-329.

Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonas, S. (1991). Specific EGF repeats of *Notch* mediate interactions with *Delta*and *Serrate*: implications for *Notch* as a multifunctional receptor. *Cell* **67,** 687-699.

Robbins, J., Blondel, B. J., Gallahan, D. and Callahan, R. (1992). Mouse mammary tumor gene *int-3*: a member of the *Notch* gene family transforms mammary epithelial cells. *J. Virol.* **66,** 2594-2599.

Rogers, S., Wells, R. and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234,** 364-368.

Rosenbluth, R. E. and Baillie, D. L. (1981). The genetic analysis of a reciprocal translocation, *eT1(III;V)*, in *Caenorhabditis elegans*. *Genetics* **99,** 415-428.

Seydoux, G. and Greenwald, I. (1989). Cell autonomy of *lin-12* function in a cell fate decision in C. elegans. *Cell* **57**, 1237-1245.

Seydoux, G., Schedl, T. and Greenwald, I. (1990). Cell-cell interactions prevent a potential inductive interaction between soma and germline in C. elegans. *Cell* **61,** 939-51.

Simpson, P. (1995). Developmental genetics: the Notch connection. *Nature* **375**, 736-737.

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

Suedhof, T. C., Goldstein, J. L., Brown, M. S. and Russell, D. W. (1985). The LDL receptor gene: a mosaic of exons shared with different proteins. *Science* **228,** 815-822.

Tax, F. E. (1994). *Analysis of* lag-2 *and other genes that mediate cell interactions in* C. elegans. PhD thesis, University of Washington, Seattle, Washington.

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

Waring, D.A. and Kenyon, C. (1990) Selective silencing of cell communication influences anteroposterior pattern formation in C. elegans. *Cell* **60,** 123-131.

Way, J. and Chalfie, M. (1989) The *mec-3* gene of *Caenorhabditis elegans* requires its own product for maintained expression and is expressed in three neuronal cell types. *Genes Dev.* **3,** 1823-1833.

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.

Wilkinson, H. A. and Greenwald, I. (1995). Spatial and temporal patterns of *lin-12* expression during *C. elegans* hermaphrodite development. *Genetics*, in press.

Yochem, J. and Greenwald, I. (1989). *glp-1* and *lin-12*, genes implicated in cell-cell interactions in *C. elegans* encode similar transmembrane proteins. *Cell* **58**, 553-563.

(Accepted 13 September 1995)