The Lateral Signal for LIN-12/Notch in C. elegans: Vulval Development Comprises Redundant Secreted and Transmembrane DSL Proteins

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Summary

The vulval precursor cells (VPCs) are spatially patterned by a LET-23/EGF receptor-mediated inductive signal and a LIN-12/Notch-mediated lateral signal. The lateral signal has eluded identification, so the mechanism by which lateral signaling is activated has not been known. Here, we computationally identify ten genes that encode potential ligands for LIN-12, and show that three of these genes, apx-1, dsl-1, and lag-2, are functionally redundant components of the lateral signal. We also show that transcription of all three genes is initiated or upregulated in VPCs in response to inductive signaling, suggesting that direct transcriptional control of the lateral signal by the inductive signal is part of the mechanism by which these cell signaling events are coordinated. In addition, we show that DSL-1, which lacks a predicted transmembrane domain, is a natural secreted ligand and can substitute for the transmembrane ligand LAG-2 in different functional assays.

Introduction

Vulval precursor cell (VPC) specification in C. elegans has been a valuable paradigm for elucidating cell signaling pathways. There are six vulval precursor cells (VPCs), consecutively numbered P3.p–P8.p (see Figure 1). Each has the potential to adopt one of three fates, called 1*, 2*, or 3* (Figure 1; reviewed in Greenwald [1997] and Kornfeld [1997]). In wild-type hermaphrodites, P6.p adopts the 1* fate, and P5.p and P7.p adopt the 2* fate; their descendants form the vulva. The three other VPCs adopt the 3* fate and generate daughters that join the hypodermal syncytium. The VPCs express LET-23, a receptor tyrosine kinase, and LIN-12, a Notch protein. LIN-3, an EGF-like ligand produced by the anchor cell (AC) of the somatic gonad (Hill and Sternberg, 1992), activates LET-23 and a canonical Ras-MAP kinase (MAPK) cascade to promote the 1* fate (reviewed in Greenwald [1997] and Kornfeld [1997]). The LET-23-Ras pathway modulates the activity of several different transcription factors or cofactors (Miller et al., 1993; Singh and Han, 1995; Tuck and Greenwald, 1995; Beitel et al., 1995; Howard and Sundaram, 2002), at least some via direct phosphorylation by MAPK (Jacobs et al., 1998; Tan et al., 1998). An important role of inductive signaling may be to antagonize inhibitory effects from the hypodermis that promote the 3* fate (Herman and Hedgecock, 1990).

Laser ablation experiments led to the suggestion that there is a lateral signal that prevents adjacent VPCs from adopting the 1* fate (Sternberg, 1988). Genetic evidence suggested that LIN-12 is the receptor for the lateral signal, as alleles of lin-12 that cause constitutive activity cause all six VPCs to adopt the 2* fate, whereas no VPCs adopt the 2* fate in lin-12 null mutants (Greenwald et al., 1985). However, the lateral signal itself has been elusive.

In order to understand the nature of the lateral signaling event, it is important to address three outstanding issues. One issue is whether this event truly involves a lateral signal, i.e., whether the signal originates as alleles of lin-12 that cause constitutive activity cause all six VPCs to adopt the 2* fate. A second issue is whether the lateral signal is controlled by the inductive signal, as is implicit in the lateral signal hypothesis. A third issue is whether the lateral signal is regulated at the transcriptional or post-transcriptional level. The Ras pathway can regulate the activity of downstream genes by many different mechanisms (e.g., Engelberg et al., 1994). In the case of the lateral signal, transcriptional control seems plausible, given that known transcription factors are modulated by the inductive signaling pathway. However, a possible mechanism for posttranslational control is suggested by the finding that LIN-12 protein must be downregulated in P6.p for lateral signaling to occur (Shaye and Greenwald, 2002).

Genes encoding the lateral signal have not been identified despite extensive genetic screens for mutations that might in principle have identified it (e.g., Ferguson and Horvitz, 1985; Eisenmann and Kim, 2000). The failure to identify the lateral signal genetically suggests that it may be encoded by more than one member of a gene family or that mutations in the gene for the lateral signal may cause pleiotropic effects that have precluded their recovery in screens for vulval mutants.

In this study, we have used a computational approach to identify genes encoding the lateral signal, predicated on the assumption that the lateral signal would resemble characterized ligands for LIN-12/Notch proteins. Most known ligands for LIN-12/Notch proteins are transmembrane proteins of the Delta/Serrate/Lag-2 (DSL) family, with an amino-terminal DSL motif followed by one or more EGF motifs. Three ligands with this predicted structure were previously known in C. elegans: lag-2, apx-1, and arg-1. lag-2 and apx-1 were identified genetically and shown to function in several distinct cell fate decisions (Lambie and Kimble, 1991; Tax et al., 1994; Mango et al., 1994; Mello et al., 1994), and arg-1 had been identified by cross-hybridization to a probe from apx-1 (Mello et al., 1994). Our computational analysis also identified seven additional potential ligands of LIN-12/Notch based on the presence of the hallmark...
motifs. Surprisingly, while all contain the hallmark motifs, at least five of them lack predicted transmembrane domains.

The available genetic and expression information argued against roles for lag-2 and apx-1 in VPC specification, but here we provide evidence that these two genes are components of the lateral signal. We also provide evidence that one of the computationally identified genes, dsl-1, encodes a secreted ligand and is a component of the lateral signal. In addition to defining the lateral signal, we demonstrate that the transcription of all three genes in P6.p is controlled by activity of the LET-23-Ras pathway.

Results

Ten Potential Ligands for LIN-12/Notch in C. elegans

To identify genes encoding candidate ligands for LIN-12/Notch, we used the DSL motif of the known ligands Lag-2 and APX-1 to search the complete C. elegans genome sequence database, and analyzed the highest-scoring proteins by SMART (Letunic et al., 2002). This analysis revealed ten predicted proteins of the DSL family (Figure 2).

Sequence analysis (see Figure 3A) indicates that three DSL proteins have highly probable predicted transmembrane domains; these were encoded by the three genes that had been identified previously, lag-2, apx-1, and arg-1. Two of the proteins identified by our computational analysis, DSL-2 and DSL-6, may also have transmembrane domains, although the potential transmembrane domains were assessed as lower probability in prediction programs. Surprisingly, the five remaining genes (dsl-1, dsl-3, dsl-4, dsl-5, and dsl-7) encode DSL proteins that are predicted to lack transmembrane domains and hence are likely to be secreted. We verified that the predicted ORFs encoding these proteins are correct by analyzing 5' and 3' RACE products (see Experimental Procedures). These five proteins also lack predicted GPI (glycosyl-phosphatidyl-inositol)-linkages (Eisenhaber et al., 2003). Analysis of available C. briggsae orthologs supports our inferences about the C. elegans proteins (see supplemental data [http://www.developmentalcell.com/cgi/content/full/6/2/183/DC1]).

The SMART database (http://smart.embl-heidelberg.de) (Letunic et al., 2002) includes potential secreted ligands in several different vertebrate and insect species; these predictions must be considered provisional until the transcripts are confirmed by RACE analysis. However, the sequence of the two spider DSL proteins included in this database had been confirmed by RACE (Stollewerk, 2002). When we analyzed their sequences, we found that they, too, appear to lack transmembrane domains (Figure 3B), suggesting that naturally secreted DSL proteins exist in other phyla.

Functional Identification of Components of the Lateral Signal

We assessed lateral signal function by reducing the activity of individual genes using available mutations or using “feeding RNAi” in order to bypass embryonic requirements (e.g., lag-2 and apx-1 are required for viability). We assessed VPC fates by looking at the expression of egl-17::gfp, a marker of the 1' fate (Burdine et al., 1998). Reduced lateral signaling may be manifested as expression of egl-17::gfp in adjacent VPCs. We saw no effect of reducing dsl gene activity in a wild-type or rrf-3 (Simmer et al., 2002) background; however, lin-12(RNAi) also has little effect on lateral signaling in a wild-type background, suggesting that it may be difficult to inhibit lateral signaling sufficiently by RNAi to see a strong phenotype (data not shown).

As a potential way to sensitize the system, we attempted to reduce lateral signaling in genetic backgrounds in which inductive signaling is constitutively activated in all six VPCs. In such backgrounds, it appears that the activation of lateral signaling superimposed upon the constitutive Ras pathway activity results in an alternating pattern of 1' and 2' fates (Stemberg,
188. We first used a lin-15(n309) background, the same background that was used to infer the existence of a lateral signal (Stemmerb, 1988). In a lin-15(n309) background (unlike in wild-type), depletion of lin-12 activity results in a highly penetrant lateral signaling defect (Figure 4A). When we performed RNAi for each candidate gene in the lin-15(n309) background, we observed a significant increase in the proportion of pairs of adjacent VPCs expressing egl-17::gfp when the activity of apx-1, dsl-1, or lag-2 was reduced (Figure 4A). To test whether this observation is specific to lin-15, we performed RNAi in a kuls14 background (Sundaram et al., 1996), in which constitutively activated LET-60 Ras is expressed from the transgene, and obtained similar results (Figure 4B).

We tried various combinations of existing mutations and RNAi to examine the effect of depleting the activity of all three putative components of the lateral signal (see Experimental Procedures). The most informative experiments involved the dsl-1(ok810) null allele (see Experimental Procedures). dsl-1(ok810) hermaphrodites are viable and fertile (data not shown), and display a 4% penetrant defect in lateral signaling (Figure 4D). In a lin-15(+/-) background, dsl-1(ok810) combined with feeding RNAI for apx-1 + lag-2 caused about 13% of hermaphrodites to display a defect in lateral signaling, which is comparable to the effect of lin-12 RNAI in the same background (Figure 4D). When we combined apx-1 + lag-2 RNAI with dsl-1(ok810) in a lin-15 mutant background, over 90% of hermaphrodites contained at least one pair of adjacent VPCs that adopted the 1 fate, and almost 70% of all pairs of adjacent VPCs adopted the 1 fate (Figure 4C).

These genetic data indicate that apx-1, dsl-1, and lag-2 are functionally redundant components of the lateral signal. Given the limitations of feeding RNAI, the data suggest that apx-1, dsl-1, and lag-2 are the main components of the lateral signal, although we cannot rule out the possibility that there is another component that we have not yet identified. The identification of these three genes has enabled us to explore the cellular origin of the lateral signal and the level at which its production is regulated.

Transcriptional Reporters and Analysis of VPC Expression
We made transcriptional reporters to assess the expression of apx-1, lag-2, and dsl-1 (see Experimental Procedures and Figure 5A). In the early L3 stage, as assessed by the extent of gonad extension, apx-1 and dsl-1 are not expressed. Later, when the gonad is more extended and beginning to reflex, apx-1 and dsl-1 begin to be expressed in P6.p (Figure 5C), the cell in which LET-23...
Figure 3. Some DSL Proteins Lack Predicted Transmembrane Domains

(A) Assessment of potential transmembrane domains for C. elegans DSL proteins. We used several different programs to analyze the DSL protein sequences. The plots shown here were generated using TMAP (Persson and Argos, 1997), which gives an indication of the hydrophobicity throughout the protein sequence. Peaks represent the predicted signal sequence (S) and potential transmembrane domains, both indicated in black. The probability values calculated by TMHMM (Krogh et al., 2001) are indicated for potential transmembrane domains; 1.0 is the highest value, and where probability values are not shown, no potential membrane spanning domain was found by this program. We note that TMHMM has been evaluated as the best-performing program for this purpose of many available (Moller et al., 2001; Melen et al., 2003). The presence or absence of predicted transmembrane domains is conserved in C. briggsae (see supplemental figures).

(B) Two spider DSL proteins also lack predicted transmembrane domains.

activity is maximally activated by the inductive signal. Expression of the transcriptional reporter for lag-2 is evident in all six VPCs in the early L3 stage (Figure 5B), but then appears to be much more highly expressed in P6.p while the expression in the other VPCs diminishes or becomes undetectable (Figure 5C). Expression of all three reporters continues in P6.p descendants (Figure 5C). T. Vellai et al. have seen a similar expression pattern using an independent transcriptional reporter for lag-2 (personal communication). The restricted expression or upregulation of apx-1, dsl-1, and lag-2 in P6.p strongly supports the hypothesis that the lateral signal originates in the presumptive 1° VPC and directly activates LIN-12 in the neighboring VPCs. We note that transcriptional reporters for other dsl genes are not expressed in the VPCs, further supporting the inference from RNAi experiments that they are not part of the lateral signal (Experimental Procedures and data not shown).

apx-1, dsl-1, and lag-2 Expression Is Responsive to Inductive Signaling and Mediator Complex Activity

The expression patterns observed for apx-1, dsl-1, and lag-2 suggest that their transcription is initiated or upregulated in response to the inductive signal. Indeed, loss of lin-3 activity prevents expression of these reporter genes (Figure 6A), and in a lin-15 mutant, the reporter genes display a striking pattern concordant with the alternating 1° (reporter on) and 2° (reporter off) fates (Figure 6A). However, in lin-3 and lin-15 mutants, the VPCs also undergo complete cell fate transformations, making interpretation of these observations somewhat problematic.

Another mutant background, sur-2, is more informative. sur-2 encodes a component of the Mediator transcription complex, and acts downstream of Ras (Singh and Han, 1995; Boyer et al., 1999). In sur-2 mutants, P6.p usually adopts a recognizable 1° fate, but P5.p and P7.p usually adopt the 3° fate, suggesting that lateral signaling is lost (Singh and Han, 1995; see also Figure 6B). Expression of transcriptional reporters for all three genes is dramatically reduced in a sur-2 mutant background (Figure 6B), suggesting that they are regulated by sur-2 as targets of the inductive signaling pathway.

We note that there is substantial but variable expression of all three ligand genes in ventral cord motor neurons in the vicinity of the VPCs (see Figure 5). The functional relevance of this neuronal expression of DSL ligands is unknown. However, the nerve cord expression of the lag-2 reporter is similar in wild-type (27/41 hermaphrodites display detectable nerve cord staining), lin-3 (31/54), or sur-2 (31/53) backgrounds, suggesting that it is not likely to be relevant to VPC specification.

DSL-1 Is Secreted and Can Function as an Activating Ligand in Other Cell Fate Decisions

The predicted structure of DSL-1 as a secreted protein is unusual for a LIN-12/Notch ligand. In the VPCs, DSL-1 functions in concert with the transmembrane ligands LAG-2 and APX-1. We therefore asked whether DSL-1 can functionally replace LAG-2. When lag-2(q420ts) eggs are grown at 25°C, larvae arrest development with a characteristic Lag phenotype; when lag-2(q420) L1 larvae are shifted to 25°C, hermaphrodites have 2 anchor cells, as in lin-12 null mutants, instead of 1 AC, as in wild-type (Lambie and Kimble, 1991). These highly penetrant
defects indicate that lag-2 is the only functionally relevant ligand for LIN-12 during these cell fate decisions. When we expressed DSL-1 using lag-2 regulatory sequences in the background of the temperature-sensitive lag-2(q420) allele, both larval lethality and the 2 AC defect could be rescued (Figure 7A). In addition, expression of DSL-1 under the control of myo-3 regulatory sequences, which drive expression in body wall muscles and the gonadal sheath cells (Okkema et al., 1993), causes a low-penetrance tumorous germline phenotype (data not shown), suggesting that GLP-1 has been ectopically activated (Berry et al., 1997; Pepper et al., 2003). These observations suggest that DSL-1 can substitute for transmembrane ligands and can activate both LIN-12 and GLP-1.

We used the tumorous germline phenotype to develop a functional assay for DSL ligand secretion from coelomocytes. Each coelomocyte is surrounded by a basement membrane, and the germline is ensheathed in somatic gonadal cells, which are enclosed by a basement membrane (Schedl, 1997). The cell membranes of the coelomocytes and germline are therefore not in direct contact, but the germline can take up yolk and perhaps other proteins from the pseudocoelom. Expression of DSL-1 or a truncated form of APX-1 that lacks the transmembrane domain under the control of a coelomocyte-specific promoter (Loria et al., 2004; see Experimental Procedures) causes a tumorous germline phenotype, whereas expression of full-length, transmembrane APX-1 does not (Figures 7B and 7C). These observations support the inference based on sequence analysis that DSL-1 is indeed secreted.

Discussion

Stemberg (1988) proposed that a LIN-12-mediated lateral signal contributes to VPC patterning. Since then, however, the lateral signal has eluded identification, so its cellular source and how it is regulated has not been
known. We have provided evidence that three DSL proteins, encoded by apx-1, lag-2, and dsl-1, function redundantly as components of the lateral signal. All three genes are transcribed in P6.p, the presumptive 1° VPC, consistent with function as a hypothesized lateral signal between P6.p and the neighboring VPCs. Furthermore, transcriptional initiation or upregulation requires the activity of the transcriptional Mediator complex, which acts downstream of the LET-23-Ras pathway to promote the 2° fate. These observations suggest that lateral signaling appears to involve the transcription of apx-1, lag-2, and dsl-1 as a direct response to activation of LET-23.

Understanding how inductive and lateral signaling are coordinated is the key to understanding how the precise spatial pattern of VPC fates is specified. Our finding that the inductive signaling pathway appears to regulate the transcription of genes encoding the lateral signal is consistent with the “sequential induction” model for the patterning events that operate during VPC specification (see Koga and Ohshima, 1995; Simske and Kim, 1995). However, a simple, linear transcriptional cascade does not adequately describe the complex molecular events underlying VPC patterning. The inductive signal appears to be spatially graded (Stemberg and Horvitz, 1986; Yoo et al., in press), and its effects become limited to P6.p in part through the transcription of multiple negative regulators of the LET-23-Ras pathway in P5.p and P7.p (Berset et al., 2001; Yoo et al., in press). In addition, cell-cycle-dependent gating of the inductive and lateral signaling pathways appears to contribute to a temporal sequence of commitment, to the 1° fate in G1 and to the 2° fate after S phase (Ambros, 1999). Finally, down-regulation of LIN-12 protein in response to inductive signaling in P6.p is necessary for lateral signaling to occur (Shaye and Greenwald, 2002).

The Mediator complex appears to be dispensable for some aspects of the 1° fate, but is required for lateral signaling (Singh and Han, 1995; Tuck and Greenwald, 1995). The requirement for the Mediator complex for lateral signal gene transcription suggests that the impairment of lateral signaling in Mediator complex mutants may be a direct consequence of failure to express components of the lateral signal. However, while transcription of the lateral signal must be a prerequisite for lateral signaling to occur, it is not sufficient, as removal of LIN-12 protein from the presumptive 1° VPC is also
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Figure 6. Transcriptional Regulation of apx-1 by Inductive Signaling
(A) Expression of apx-1::lacZ depends on inductive signaling. arIs89 is an integrated transgene carrying the apx-1::lacZ transcriptional reporter (see Experimental Procedures). Relevant Pn.px cells are indicated by arrows. MH27 was stained with Cy5-conjugated secondary antibody (blue), and lacZ was stained with Cy3-conjugated secondary antibody (red). apx-1 reporter expression is not seen in lin-3(e1417) hermaphrodites; ectopic expression is seen in alternating VPCs in lin-15(n309) hermaphrodites. Similar results were also obtained for transcriptional reporters for dsl-1 and lag-2 (data not shown).

(B) Expression of transcriptional reporters for apx-1, dsl-1, and lag-2 is specifically reduced in a sur-2 null mutant background. Note that most sur-2(0) hermaphrodites express the marker egl-17::cfp-lacZ, indicating that by this criterion, they have adopted the 1/H11034 fate. described in Experimental Procedures. For each genotype, the number of hermaphrodites scored at the Pn.px stage is indicated above the corresponding bar.

necessary for lateral signal activity (Shaye and Greenwald, 2002). The Mediator complex also controls down-regulation of LIN-12 in P6.p, presumably through the transcription of a factor that promotes LIN-12 endocytosis and degradation (Shaye and Greenwald, 2002). Potential interplay between the regulation of lateral signal gene expression and LIN-12 downregulation will be an interesting possibility for future exploration.

In contrast to canonical transmembrane LIN-12/Notch ligands, DSL-1 and four other C. elegans DSL proteins lack predicted transmembrane domains and instead appear likely to be secreted or peripheral membrane proteins. We have also demonstrated that DSL-1 can activate LIN-12/Notch at a distance from its cellular source, indicating that it is secreted. When we analyzed available DSL proteins of confirmed sequence by the same criteria, we found that two DSL proteins recently identified in spiders (Stollewerk, 2002) also lack predicted transmembrane domains. These spider DSL proteins appear to be required for Notch-mediated signaling (Stollewerk et al., 2003), supporting the view that secreted DSL proteins can function as ligands for LIN-12/Notch in other phyla. Perhaps additional sequence analysis will identify secreted ligands in vertebrates.

In addition to functioning as a component of the lateral signal, DSL-1 can substitute for the transmembrane ligand LAG-2 in different cell fate decisions. Whether the putative secreted ligands must undergo endocytosis for full activity, as transmembrane ligands apparently do
Parks et al., 2000; see Le Borgne and Schweig Guth, 2003), is not known. Perhaps secreted ligands have special modifications that promote oligomerization or association with membranes, bypassing the need for a transmembrane domain or endocytosis. In this context, however, we note that engineered secreted forms of APX-1 and LAG-2 are functional (Fitzgerald and Greenwald, 1995; this study), suggesting that natural secreted ligands may not need any special carboxy-terminal modifications to function.

In terms of VPC specification, the cell biology of lateral signaling offers a rationale for why a secreted or peripheral membrane protein ligand might be a component of the lateral signal. The VPCs are polarized epithelial cells: they have an apical region and a basolateral domain, separated by adherens junctions (Kaech et al., 1998). As the apical regions of adjacent VPCs appear to be in contact only in the vicinity of the cell junctions (Kaech et al., 1998), and LIN-12 is distributed over the whole apical surface (e.g., Shaye and Greenwald, 2002), a transmembrane ligand on the surface of one VPC might have access to LIN-12 on the apical surface of its neighbor only in a relatively limited area. A ligand that can diffuse may be available to activate LIN-12 over a greater region of the apical domain, affording one solution to such a topographical problem.

Experimental Procedures

Confirmation of the Predicted dsl Coding Regions
The 5′ and 3′ ends of the predicted dsl messages were determined by comparing the predicted ends (from Wormbase [http://www.wormbase.org] and Intronerator [http://www.cse.ucsc.edu/~kent/intronerator/index.html]) to the sequences obtained by PCR amplification from three cDNA libraries and RACE (rapid amplification of cDNA ends) products. Details of our analysis are available upon request. The information in Wormbase now reflects our analysis.

We verified 5′ ends that are consistent with the Wormbase predictions for all dsl genes except for dsl-5 and dsl-6. For dsl-5, we were unable to verify the 5′ end; we used the Wormbase prediction as the basis for our analysis, as none of the 5′ RACE products and the PCR fragments we obtained from the cDNA libraries for dsl-5 contained a spliced leader sequence. The 5′ end of the longest clone was 77 bp downstream of the predicted start codon and there is no EST available for dsl-5. For dsl-6, the 5′ RACE products showed a predicted start codon that was consistent with Intronerator but not Wormbase. The start codon is 94 bp downstream of the predicted ATG (Wormbase), resulting in protein that is 31 amino acids shorter but contains a predicted signal sequence.

The 3′ ends are consistent with the Wormbase predictions for all dsl genes except for dsl-7, which matches to the prediction from Intronerator.

Genetics and RNAI
All RNAi data shown are the sum of at least two independent trials; in all cases, the results were consistent from trial to trial.

The lin-3(e1417) and lin-15(n309) alleles (Ferguson and Horvitz, 1985) and sur-2(ku9) (Singh and Han, 1995) were used to manipulate the activity of the inducive signaling pathway. Transgenes used as cell fate markers were: ayls4[egf1-17;gfp] (Burdine et al., 1998), jcs1[ajm-1::gfp] (Koppen et al., 2001), and arIs92[egf1-17;ctp:lacZ] (Yoo et al., in press). Routine manipulations were generally performed at 20°C, but strains containing lacZ reporters were grown at 25°C prior to fixation for antibody staining.


All available alleles of apx-1 are not null; they cause maternal-effect lethality due to loss of a gfp-1 maternal function (Mango et al., 1994; Mello et al., 1994). We used apx-1(zu183) (Mello et al., 1994) for genetic studies, but as this allele, and presumably others with the same phenotype, affects translation of maternal mRNA, it may not offer a sensitized background for a study of the function of apx-1 in vulval development.

The Caenorhabditis elegans Knockout Consortium (http://eleganskoconsortium.omrf.org/) generated dsl-1(ok810). We sequenced dsl-1(ok810) and found that it contains a deletion starting at 1038 bp 5′ of the start codon and removing the entire coding region, ending at 364 bp 3′ of the stop codon. RNAi was performed by “feeding” (Timmons and Fire, 1998), using a cDNA fragment for each dsl gene subcloned into the feeding vector pPD129.36 and transformed into bacterial strain HT115. Synchronized L1 larvae were placed on a lawn of such bacteria and allowed to grow for 19 hr at 25°C, and egf-17::gfp or ajm-1::gfp expression was analyzed with a Zeiss Axioplan 2 microscope.

In addition to the experiments described in the Results, we performed additional RNAi experiments in the following genetic backgrounds (data not shown): (1) rrf-3(pk1420); ayls4 and (2) lin-15(n309); rrf-3(pk1420); ayls4. The rrf-3 mutant is hypersensitive to RNAi (Simmer et al., 2002), but RNAi performed for individual dsl genes gave similar results as in the rrf-3(1) background. (3) lag-2 (q420ts); jcs1[ajm-1::gfp] and (4) lag-2(zu183); lin-15(n309); ayls4. Her-maphrodites grown in the presence of bacteria expressing dsRNA at 15°C until the L2 molt so that they would have one AC transferred to 25°C and grown to the late L3/early L4 stage. We performed apx-1 + dsl-1 RNAi in this background. No obvious loss of 2′ fate was observed.

Transcriptional Reporter Genes
Transcriptional reporters were based on gene structures diagrammed in Figure 5A. The 5′ flanking regions of apx-1 (9 kb upstream), lag-2 (6.2 kb upstream), and dsl-1 (8 kb upstream) were inserted into vector pPD16.51. The 5′ flanking regions were designed to extend to the next upstream gene, based on the Wormbase predictions and RACE (see above). The data shown in the Figures are for transgenes formed from these constructs, listed below. Previous reporter genes for lag-2 (Henderson et al., 1994; Fitzgerald and Greenwald, 1995) that do not show upregulation in P6.p used only 3.4 kb of 5′ flanking region.

We made other transcriptional reporters for each of the dsl genes except for lag-2 and apx-1 using a design originally developed for expression analysis of lin-12 (see Wilkinson et al., 1994). lacZ was inserted at the position of the predicted ATG into a large genomic region encompassing the entire dsl coding region as well as the 5′ and 3′ flanking regions, extending to the neighboring predicted genes. Such constructs only express lacZ in a smg-1 background. None of the reporters for the other dsl genes were expressed in the VPCs (data not shown). For apx-1, the very large genomic region (26 kb) precluded the use of such a construct.

Transgenic Lines for Expression Studies
The transcriptional reporters described above were injected at 100 μg/ml, together with plasmids pH85 [dpy-20(1·)] (50 μg/ml; Han and Sternberg, 1991), and pCW2.1 [cej-22::gfp] (20 μg/ml; Okkema et al., 1997), Mixes containing the apx-1, dsl-1, or lag-2 reporters were injected into dpy-20(e1282) animals. Mixes containing the smg-1-dependent reporters were injected into unc-54 smg-1; dpy-20(e1282) animals. F1 progeny that expressed GFP in the phar, indicating the presence of cej-22::gfp, were picked individually to establish independent extrachromosomal lines.

Extrachromosomal arrays carrying apx-1::lacZ or dsl-1::lacZ constructs were integrated using standard methods (Mello and Fire, 1999). All integrated lines were backcrossed five times to N2 prior to further analysis. We analyzed two independent integrants for apx-1::lacZ [arls98, derived from arEx479, and arls99, derived from arEx480], two independent integrants for dsl-1::lacZ [arls94, derived
from arEx481, and arls95, derived from arEx482), and eight extra-chromosomal arrays for lag-2::lacZ (arEx471-arEx478). Different transgenes show the same expression pattern for each individual gene.

Constructs and Transgenic Lines for DSL-1 Rescue and Secretion Studies

pNC21.1 [lag-2p::DSL-1] contains a DSL-1 cDNA extending from the start to the stop codon in the lag-2 expression vector p226S (described in Fitzgerald and Greenwald, 1995) at the SpeI site. The expression of DSL-1 is under the control of the lag-2 regulatory region previously shown to be sufficient to rescue the viability and 2 AC defects of lag-2 mutants (Tax et al., 1994). This regulatory region includes 3.4 kb of 5’ flanking region. lag-2 [324020b]; smg-1 1 animals were cojected with cdh-3::gfp (25 µg/ml), pBlueScript (75 µg/ml), and without or with pNC21.1 (2 µg/ml), arEx486-arEx487 and arEx488-arEx491, respectively, were obtained at 15°C. cdh-3::gfp expression was used to identify and follow transgenic worms, and also served as an AC marker (Pettitt et al., 1996).

To assess rescue, synchronized eggs or L1 larvae were shifted to 25°C. Worms with one or more AC expressing GFP were analyzed with a Zeiss Axioplan 2 microscope. arEx489 rescued both the viability and 2 AC phenotypes; arEx490 rescued the viability but not 2 AC phenotype (data not shown).

pNC31 [unc-122p::apx-1(-1::)] contains the full-length DSL-1 cDNA and a fragment of the unc-122 promoter that drives coelomocyte-specific expression (Loria et al., 2004). pNC32 [unc-122p::apx-1(-1::)] and pNC33 [unc-122p::apx-1(-1::)] contain either the “APX-1 (extra)” cDNA, a truncated APX-1 cDNA that lacks the TM domain (Fitzgerald and Greenwald 1995), or the full-length APX-1 cDNA and the unc-122 promoter. pma-1 [2(e2123)] animals were cojected with ceh-22::gfp (20 µg/ml), pBluex [pha-1(-1::)] (50 µg/ml), and pNC31, pNC32, or pNC33 (each at 100 µg/ml), and grown at 15°C for 4 days. Three extrachromosomal arrays were obtained for each DNA mixture by switching the plates to 25°C to select for pha-1 rescue (Granato et al., 1994). ceh-22::gfp expression was also used here to confirm the presence of the arrays.

Worms with GFP expression in the pharynx were picked and stained with DAPI, and analyzed for tumorous germline phenotype protein sequences: big-Pi, NMT and PTS1. Nucleic Acids Res. 31, 3631–3634.

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