OSM-11 Facilitates LIN-12 Notch Signaling during Caenorhabditis elegans Vulval Development

Hidetoshi Komatsu1,2,*, Michael Y. Chao3, Jonah Larkins-Ford1, Mark E. Corkins1, Gerard A. Somers1, Tim Tucey1,*, Heather M. Dionne1,*, Jamie Q. White1,2, Khursheed Wani1,*, Mike Boxem4,5, Anne C. Hart1,2

1 Massachusetts General Hospital, Center for Cancer Research, Charlestown, Massachusetts, United States of America, 2 Department of Pathology, Harvard Medical School, Boston, Massachusetts, United States of America, 3 Department of Biology, California State University San Bernardino, San Bernardino, California, United States of America, 4 Center for Cancer Systems Biology (CCSB) and Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, Massachusetts, United States of America, 5 Department of Genetics, Harvard Medical School, Boston, Massachusetts, United States of America

Notch signaling is critical for cell fate decisions during development. Caenorhabditis elegans and vertebrate Notch ligands are more diverse than classical Drosophila Notch ligands, suggesting possible functional complexities. Here, we describe a developmental role in Notch signaling for OSM-11, which has been previously implicated in defecation and osmotic resistance in C. elegans. We find that complete loss of OSM-11 causes defects in vulval precursor cell (VPC) fate specification during vulval development consistent with decreased Notch signaling. OSM-11 is a secreted, diffusible protein that, like previously described C. elegans Delta, Serrate, and LAG-2 (DSL) ligands, can interact with the lineage defective-12 (LIN-12) Notch receptor extracellular domain. Additionally, OSM-11 and similar C. elegans proteins share a common motif with Notch ligands from other species in a sequence defined here as the Delta and OSM-11 (DOS) motif. Osm-11 loss-of-function defects in vulval development are exacerbated by loss of other DOS-motif genes or by loss of the Notch ligand DSL-1, suggesting that DOS-motif and DSL proteins act together to activate Notch signaling in vivo. The mammalian DOS-motif protein Deltalike1 (DLK1) can substitute for OSM-11 in C. elegans development, suggesting that DOS-motif function is conserved across species. We hypothesize that C. elegans OSM-11 and homologous proteins act as coactivators for Notch receptors, allowing precise regulation of Notch signaling in developmental programs in both vertebrates and invertebrates.

Introduction

The Notch signaling pathway is essential for cell fate determination during embryogenesis and postembryonic development in multicellular organisms. Classical Notch signaling begins with activation of the Notch receptor by transmembrane DSL ligands (Delta and Serrate in Drosophila or LAG-2 [Lin and Glp-2] in C. elegans [1–3]) expressed on adjacent cells, resulting in proteolytic cleavage of the Notch receptor, internalization of the ligand-receptor complex, and nuclear translocation of the Notch IC (intracellular) domain [4–8]. In the nucleus, the Notch IC domain acts as a transcriptional regulator together with a conserved transcription factor called Su(H) (Suppressor of Hairless) in Drosophila and LAG-1 [Lin and Glp-1] in C. elegans [9,10]. The molecular mechanisms of Notch signaling are highly conserved. Vertebrate homologs exist for each of these components in the Notch signaling pathway, and mutations in Notch signaling have been implicated in various developmental disorders, including Alagille and CADASIL [11–14].

In C. elegans, the Notch receptor LIN-12 (Lineage defective-12) plays critical roles in cell fate specification in multiple tissues. The roles of LIN-12 in two steps of vulval development have been particularly well studied. First, LIN-12 is required for cell fate specification of an anchor cell (AC) and a vulval uterine (VU) cell from the descendents of equipotent precursor cells Z1 and Z4 during the L1 larval stage [15–18].
Loss of lin-12 signaling generally results in the specification of two ACs, whereas increased lin-12 signaling results in two VU cells. The AC produces a diffusible epidermal growth factor (EGF) signal that induces the primary (1⁸) cell fate in P6.p, one of six equipotent vulval precursor cells (VPCs) (reviewed in [19]). Additionally, LIN-12 specifies secondary (2⁸) cell fates of P5.p and P7.p. two VPCs adjacent to P6.p, by antagonizing EGF signaling via lateral inhibition [17,20]. Loss of lin-12 signaling generally causes VPCs to take on 1⁸ and tertiary (3⁸) fates, whereas strong lin-12 gain-of-function alleles cause VPCs to take on 2⁸ fates with consequent changes in the fates of descendant cells that contribute to the adult vulva.

Canonical Notch receptor ligands are exemplified by Drosophila Delta, which contains a conserved N-terminal DSL domain originally found in Delta, Serrate, and LAG-2 proteins [2,3,7,21,22]. The DSL domain is followed by a series of EGF repeats and a transmembrane domain. The DSL domain is critical for Notch receptor activation based on tissue culture studies and genetic analysis [23,24], but Notch ligand EGF repeats are also required for Notch receptor activation [25,26]. Numerous Notch ligands containing DSL domains have been identified in various organisms [23,27–32]. C. elegans LAG-2 is a classical Notch ligand containing a canonical DSL domain and transmembrane domain and is essential for LIN-12 activation in vivo in many contexts [21,22].

Although key components in the Notch pathway were identified decades ago in classical genetic studies in Drosophila and C. elegans [33,34], additional proteins that play important or redundant roles in Notch signaling have been identified more recently. C. elegans anterior pharynx defective-1 (APX-1) and DSL-1 are DSL domain–containing soluble proteins that function redundantly with LAG-2 during vulval development [35]. Noncanonical ligands for vertebrate Notch receptors have been identified, including Delta/notch-like EGF repeat containing protein (DNER), F3/contactin, and MAG proteins [36–40], but functional C. elegans homologs of these noncanonical ligands have not been identified. Deltalike 1 (a.k.a., DLK1, fetal antigen 1 [FA1], ZOG, Preadipocyte Factor 1 [PREF1]) also encodes a putative soluble Notch ligand that lacks a DSL domain [41–43,44]. DLK1 is a paternally imprinted gene with diverse developmental roles. DLK1 knockout mice are growth retarded and obese with eye and skeletal defects [45]. Overexpression of DLK1 due to polar overdominance results in callipyge sheep with muscle overproliferation and decreased adipogenesis [45–47]. Although Drosophila lacks a DLK1 homolog, ectopic expression of mammalian DLK1 in Drosophila inhibits Notch signaling [48]. DLK1 has multiple mRNA isoforms; some transcripts are translated as membrane-bound proteins with subsequent proteolytic release of the EGF-repeat–containing extracellular domain, while others encode soluble secreted proteins [42,43,49]. DLK1 EGF repeats bind Notch1 EGF repeats in bacterial two-hybrid assays and inhibit activity of a Notch-dependent reporter gene. However, DLK1 inhibits Notch activation by previously described DSL Notch ligands in these same studies [50]. Therefore, a role for DLK1 as a Notch ligand is controversial, given the lack of a canonical DSL domain and the inability of DLK1 to activate vertebrate Notch receptors.

Here, we examine the secreted C. elegans protein, OSM-11. A role for OSM-11 in osmotic sensitivity and defecation was recently described, but the molecular function of these genes was not elucidated in previous studies, and no homologous proteins outside of nematodes were identified [51,52]. We found that OSM-11 and related C. elegans proteins contain a motif found only in known and putative Notch ligands, including Serrate and DLK1. We examined the functional role of osm-11 in development. We find that osm-11 increases lin-12 Notch receptor signaling during vulval cell fate specification. Our results suggest a model in which OSM-11 normally acts with C. elegans DSL ligands to activate Notch receptor signaling in vivo.

Results

OSM-11 Is Required for Cell Fate Specification during Vulval Development

We identified a deletion allele of osm-11 that removes all of the predicted mature protein, osm-11(rt142). The majority of animals lacking osm-11 had visibly misshapen vulva or defective vulva based on retention of eggs (Figure 1A–1C). A smaller fraction had an additional protrusion near the normal position of the vulva. Vulval development was also modestly perturbed by RNA interference (RNAi) knockdown of osm-11 (16% defective, n = 82), suggesting that osm-11 defects in vulval developmental were caused by loss of osm-11 function. Consistent with this hypothesis, osm-11 defects were rescued by reintroduction of either genomic DNA containing the entire osm-11 gene or the osm-11 cDNA expressed under the control of 3.4 kb of upstream genomic DNA sequences 5’ to the predicted osm-11 initiator methionine (described below) and the unc-54 5’ UTR. osm-11 loss of function also caused non-vulval developmental defects, including misshapen heads and anal protrusions (Figure 1D and 1E) reminiscent of animals with decreased Notch signaling or increased EGF signaling [9]. To determine the biochemical role of OSM-11, a molecular and cellular analysis was first undertaken.
and K02F3.7 proteins were aligned, which revealed conserved sequences of previously described Notch ligands. First, the predicted analysis, which revealed similarity between OSM-11 and outside of helminthes, we undertook further bioinformatic failed to identify additional proteins similar to OSM-11 including Notch receptors and ligands.

cysteine residues with characteristic spacing that forms three 2A). cEGF-1 domains contain a small amino acid and six [53] that is part of a conserved motif described below (Figure 2A). cEGF-1 domains contain a small amino acid and six cysteine residues with characteristic spacing that forms three disulfide bonds, and are found in extracellular proteins including Notch receptors and ligands.

As standard similarity searching programs (i.e., BLAST) failed to identify additional proteins similar to OSM-11 outside of helminthes, we undertook further bioinformatic analysis, which revealed similarity between OSM-11 and previously described Notch ligands. First, the predicted sequences of C. elegans OSM-11, OSM-7, K10G6.2, ZK507.4, and K02F3.7 proteins were aligned, which revealed conserved

amino acids in a common motif containing the putative cEGF-1 domain and additional amino acids: C-X(3)-C-X(3,8)-C-X(2,5)-C-(KVER)-C-X(10,12)-C-X(1,3)-P-X(6,9)-C-X(1,4)-W-X(1,4)-C. Motif-based database searches revealed that all proteins containing the new motif in Drosophila, zebrafish, mouse, and humans are either DSL-containing Notch ligands or suspected Notch ligands. We named the motif DOS because it is found in Delta and OSM-11-like proteins (shaded in Figure 2) and designated the C. elegans genes ZK507.4, K10G6.2, and K02F3.7 as dos-1, dos-2, and dos-3, respectively. All five C. elegans DOS-motif proteins are likely secreted based on the presence of a predicted N-terminal signal peptide. However, OSM-11 and DOS-3 also have a consensus proprotein convertase protease cleavage site and a C-terminal transmembrane domain, suggesting that they may be translated as transmembrane preproproteins prior to proteolytic processing and release of a soluble DOS protein.

In known Notch ligands from Drosophila and vertebrates, the DOS motif is always located immediately following the DSL domain and overlapping the first two EGF repeats. The first two EGF repeats of most Notch ligands differ from the remaining EGF repeats [27] (this study, Figure 2C). The role of these EGF repeats remains unclear, but several previous studies suggest that these EGF repeats play roles in Notch activation: they are required for the DSL domain of Jagged1 to bind to the mammalian Notch2 receptor in biochemical studies [54]; perturbation of the second EGF repeat interferes with Notch signaling in Drosophila [25]; and mutations in these EGF repeats of human Jagged1 are associated with Alagille syndrome [55]. The DOS motif may define a unique group of EGF repeats and EGF-like repeats that have a distinct functional role in Notch signaling.

Outside of helminthes, only three proteins were identified with DOS motifs that are not canonical Notch ligands: C901, DLK1, and EGFL9 (DLK2). These proteins have a signal peptide sequence, and the DOS motif is located in the first two EGF repeats (Figure 2B). C901 is a predicted Drosophila protein of unknown function containing a DSL domain and multiple EGF repeats [56]; it is unclear whether C901 is a transmembrane DSL domain protein. DLK1 and EGFLike 9 (EGFL9) are vertebrate proteins that contain EGF domains, but lack DSL domains. EGFL9 is poorly characterized [57]. DLK1 has membrane-bound and secreted isoforms, and plays diverse roles in normal development. Altered DLK1 expression causes developmental defects in mammals [42,45–47]. DLK1 EGF repeats containing the DOS motif bind to specific Notch1 receptor EGF repeats in two-hybrid studies and in tissue culture [50], but the role of DLK1 in Notch signaling remains controversial because DLK1 lacks a DSL domain and does not activate mammalian Notch receptors [42,45–47].

Given this controversy and given the limited homology observed between OSM-11 and previously described canonical Notch ligands, we turned to cellular, genetic, and molecular tools in C. elegans to elucidate the role of OSM-11 in developmental signaling pathways.

Loss of osm-11 Perturbs Cell Fate Specification during Vulval Development

We first examined the role of osm-11 in specification of the AC. LIN-12 Notch function is required for cell fate specification of an AC and a VU cell from the equipotent precursor cells Z1 and Z4 during the L1 larval stage [15–18].
A

OSM-11 and LIN-12 Notch in Development

B

C

DSL domain
EGF repeat
DOS motif
Transmembrane domain

C. elegans
Drosophila
vertebrates

CeOM-11
CeDOS-1
CeDOS-2
Figure 2. osm-11 Encodes a Protein with a Conserved Motif Found in Notch Ligands

(A) Top: OSM-11 genomic structure. The signal peptide is shaded black, and putative O-linked glycosylation sites are indicated by vertical lines. The DOS motif is shaded blue; it overlaps the previously defined osmotic stress resistant (OSR) motif [52], osm-11(rt142) removes all coding sequence after the signal peptide; osm-11(rt168) converts W177 to a premature stop codon. Bottom: the DOS motif-containing sequences from C. elegans OSM-11, OS-7, DOS-1, DOS-2, and DOS-3 are aligned above the DOS motif consensus and the cEGF-1 consensus [53]. DOS-motif regions from mouse proteins and known Drosophila Notch ligands are aligned under the cEGF-1 consensus. DOS-motif amino acids are shaded blue and previously described EGF repeats are boxed. Asterisks (*) indicate cysteines in the conserved EGF-motif that are not found in the C. elegans DOS proteins. The DOS motif consensus is: C-X(3)-C-X(3)-C-X(2,5)-C-[KVER]-C-X(10,12)-C-X(1,4)-W-X(1,4)-C. In the DOS motif consensus, b represents K, V, E, or R, and the dash (-) indicates possible positions for proline in the DOS motif. In the cEGF-1 consensus, s represents a small amino acid [53].

(B) The position of the DOS motif within or predicted C. elegans, Drosophila, and vertebrate Notch ligands. The DOS motif overlaps with the first two EGF repeats of canonical Notch ligands and may define a unique subset of EGF repeats. The noncanonical Notch ligands DNER [40], F3/contactin [95], and MAGP (36–40) do not contain a DOS motif (unpublished data).

(C) Similarity between DOS motifs, the first and second EGF repeats, and the third and fourth EGF repeats of Notch ligands. As noted by Lissemore and Starner [27], the first and second EGF repeats differ from the third and fourth EGF repeats. DOS-3 was not included in this alignment. Green indicates the DOS motif of proteins that lack canonical EGF repeats; blue indicates the first and second EGF repeats of Notch ligands; red indicates the third and fourth EGF repeats of Notch ligands; and magenta represents the C. elegans Notch ligands that lack DSL domains. See Materials and Methods for accession numbers and other details.

Loss of lin-12 signaling results in the specification of two ACs, whereas increased lin-12 signaling results in two VU cells. AC cells are readily quantified by expression of a lip-1p::gfp reporter construct [58]. No alterations in lip-1p::gfp were observed in osm-11(+) animals compared to osm-11(+) animals (unpublished data; n = 92), suggesting that loss of osm-11 does not alter AC cell fate specification in otherwise normal animals.

We next examined VPC specification. After AC specification, the AC produces the diffusible EGF protein LIN-3 that is required for induction of the 1° cell fate in P6.p, one of six equipotent VPCs (reviewed in [19]). LIN-3 EGF acts via the well-characterized Ras/MAPK (mitogen activated protein kinase) pathway in VPCs. LIN-12 Notch function is required to specify 2° cell fates of P5.p and P7.p, two VPCs adjacent to P6.p, by antagonizing EGF signaling via lateral inhibition [17,59]. Loss of EGF signaling eliminates 1° and 2° cell fates, whereas aberrantly increased EGF/Ras/MAPK kinase signaling can cause all VPCs to adopt 1° cell fate. By contrast, loss of lin-12 Notch signaling causes all VPCs to take on 1° or 3° fates, whereas strong Notch gain-of-function alleles cause all six VPCs to take on 2° fates (Figure 3A). These VPC fate decisions were assessed in osm-11(+) animals and control animals at specific larval stages using the previously described green fluorescent protein (GFP) reporter constructsg. In L3 animals, egl-17p::gfp, lin-11p::gfp, and lip-1p::gfp [60].

In L3 animals, egl-17p::gfp expression in P6.p is directly dependent on EGF/Ras signaling, and egl-17 expression is repressed in P5.p or P7.p by lateral inhibition via LIN-12 Notch signaling [60]. At the Pn.p stage, when cell fates are first established, egl-17p::gfp is only expressed in P6.p in wild-type animals. We found appropriate egl-17p::gfp expression in the P6.p cell (Figure 3B and 3C) of animals lacking osm-11, but ectopic egl-17p::gfp expression in P5.p or P7.p in approximately 10% of osm-11(+) L3 animals. This ectopic egl-17p::gfp expression suggests that in osm-11(+) animals, P5.p and P7.p secondary cell fates are not correctly established whereas the 1° cell fate choice of P6.p is unaffected. Later, at the L4 larval stage, egl-17 expression normally is lost in wild-type animals from P6.p descendants and observed only in 2° cell lineages, i.e., in P5.p and P7.p descendants. In 71% of osm-11(+) L4 animals, egl-17p::gfp expression in P5.p and/or P7.p descendants was lost, consistent with loss of 2° cell fates (unpublished data; n = 63). The aberrant egl-17p::gfp expression observed in osm-11(+) animals suggests that 1° and 2° cell fates are not correctly specified in a fraction of osm-11(+) animals, consistent with decreased Notch signaling.

To determine whether secondary cell fates are lost in osm-11(+) animals, cell fate specification was examined using lip-1p::gfp and lip-1p::gfp reporter genes. lip-1p::gfp is expressed exclusively in P5.p and P7.p vulval secondary lineages during development [61,62] (98% of control wild-type late-L3 larvae), but lin-11p::gfp expression is lost in P5.p and/or P7.p descendants in 67% of osm-11(+) animals (Figure 3B and 3D). Strikingly, 69% of osm-11(+) adult animals had an overtly defective vulva or retained eggs (Figure 1), which correlates quantitatively with the loss of secondary cell fates observed with altered lin-11p::gfp expression. Loss of lin-12 expression at this stage suggests that secondary cell fates are either not properly specified or not maintained in osm-11(+) animals.

Secondary cell fate specification can be more directly assessed using lip-1p::gfp. In normal L3 animals, lip-1p::gfp expression is up-regulated in P5.p and P7.p upon assumption of secondary cell fate [63]. This up-regulation is directly dependent on lin-12 Notch receptor signaling. However, in 35% of osm-11(+) L3 animals, lip-1p::gfp was not up-regulated in P5.p and/or P7.p (Figure 3B and 3E; vs. up-regulation in 98% of control animals). The loss of lip-1p::gfp and lin-11p::gfp expression observed in osm-11(+) animals is reminiscent of changes observed when LIN-12 Notch signaling is decreased and is not consistent with decreased EGF/Ras signaling.

osm-11 Is Expressed in VPCs and Hypodermal Cells

The functional significance of the similarity of OSM-11 to classic Notch ligands was unclear, particularly as OSM-11 lacks a DSL domain. To address the role of osm-11 in development, the cellular and temporal pattern of osm-11 expression was examined to delineate its potential roles in VPC fate specification. A transcriptional GFP reporter (osm-11p::gfp) was generated using the same upstream sequences used for osm-11 cDNA rescue. In animals harboring this transgene, GFP expression was observed in numerous unidentified cells during embryonic development from the comma stage onward (unpublished data). GFP expression was observed in the VPCs during larval development, as well as various hypodermal cells during larval stages (Figure 4). Using polyclonal antisera raised against OSM-11 to stain wild-type animals, we found that OSM-11 was expressed in the VPCs of L3 larvae prior to and during cell fate specification (Figure 4B). OSM-11 immunoreactivity was also observed in the seam cells of L1 larvae and adult animals (Figure 4A and 4D).
adult animals, osm-11p::gfp was expressed only in hypodermal seam cells in adult animals; hypodermal seam cell expression in adult animals was also confirmed with staining with OSM-11 antisera (Figure 4D). The larval hypodermal expression pattern of osm-11p::gfp is reminiscent of the osm-7p::gfp expression pattern described previously, but osm-7p::gfp expression in seam cells was not reported [52]. OSM-11 protein was also expressed in the developing uterus of L4 larvae (Figure 4B) and in the spermatheca (Figure 4D); the LIN-12 Notch receptor plays a developmental role in these tissues as well [64], but only OSM-11 expression in VPCs was characterized further.

Initially, OSM-11 protein is detected at uniform levels in all six equivalent VPCs. OSM-11 disappears from P5.p, P6.p, and P7.p after 1½ and 2½ vulval cell fates are specified (based on up-regulation of lip-1p::gfp; Figure 4B). OSM-11 was not detected in VPC descendents. Previously described C. elegans DSL-containing Notch ligands also have temporally regulated expression patterns in the VPCs [35]. For example, based on reporter construct analysis, soluble DSL-1 is only expressed...
Figure 4. *osm-11* Is Expressed in VPCs and Other Tissues

(A) OSM-11 expression in seam cells of L1 larvae detected using α-OSM-11 antisera. The seam cells on the right side of an L1 animal are in focus; the seam cells on the left side are visible and slightly out of focus. OSM-11 was not expressed in seam cells or hypoderm at other larval stages.

(B) OSM-11 expression in the developing uterus of L4 larvae. Left, α-OSM-11 antisera staining; right, visible light image.

(C) OSM-11 expression in vulval precursor cells (VPCs; arrowheads) in L3 larvae. The top panels show α-OSM-11 antisera staining of VPCs prior (top left) and immediately after (top right) cell fate specification as assessed by *lip-1p::gfp* expression. An overlay of α-OSM-11 staining and *lip-1p::gfp* expression shows that OSM-11 is concentrated on the apical surface of the VPCs (bottom right); this was confirmed using an *ajm-1::gfp* fusion (unpublished data).

(D) OSM-11 expression in seam cells and spermatheca in adult animals. An *osm-11p::gfp* reporter gene containing unc-54 3' UTR sequences is expressed.
in P6.p and its descendants. OSM-11 expression in Pn.p cells is consistent with a role for OSM-11 in initial cell fate specification.

Like LIN-12 Notch receptors, OSM-11 is primarily localized to the apical side of VPCs (Figure 4B, inset). VPCs are polarized epithelial cells; EGF and Notch signaling normally occurs in separate cellular compartments. Lethal-23 (LET-23) EGF receptors are localized to the basolateral surface of the VPCs in close proximity to the AC [65], which is the source of LIN-3 EGF. In contrast, LIN-12 receptors are primarily localized to the apical surface of the VPCs. The apical localization of OSM-11 in VPCs during cell fate specification suggests that OSM-11 is available to bind to LIN-12 receptors in VPCs at the time of cell fate specification.

Osmotic Stress Response Does Not Alter Vulval Cell Fate Specification

_osm-11_ and _osm-7_ were previously implicated in osmotic stress resistance [51,52]. Pre-exposure of wild-type _C. elegans_ to high external osmolality is sufficient to induce osmotic resistance. Loss of either _osm-7_ or _osm-11_ allows animals to survive high external osmolality without pre-exposure. The cellular and molecular mechanisms underlying osmotic stress resistance in either scenario are poorly understood, but up-regulation of _gdh-1_ and increased levels of the osmolyte glycerol have been implicated [51,52]. As loss of _osm-11_ increases glycerol levels and increased osmolyte levels can alter protein folding, _osm-11_ could act indirectly to decrease Notch receptor signaling in VPC fate specification. Alternatively, OSM-11 might act directly upon Notch receptors involved in VPC fate specification. Our experimental results below favor the latter model; the role of OSM-11 in vulval cell fate specification is distinct from the role of OSM-11 in osmotic stress.

If osmotic stress indirectly decreases Notch receptor signaling, then vulval development should be altered by osmotic stress and altered by genetic backgrounds with increased osmotic stress resistance. We first tested this hypothesis by raising wild-type animals under previously defined osmotic stress conditions: 200 and 400 mM NaCl. Rearing under osmotic stress conditions did not alter vulval morphology, and the cellular expression patterns of vulval cell lineage markers (_lip-1p::gfp, egl-17p::gfp_, or _lin-11p::gfp_) in VPCs were unchanged (unpublished data). We also examined genetic backgrounds previously implicated in osmotic stress resistance; neither _osr-1_ nor _daf-2_ animals have altered vulval morphology [66–68]. In addition, we considered the possibility that OSM-11 expression in the vulval cell precursors might be altered by osmotic stress. We found that rearing under osmotic stress conditions (400 mM NaCl) did not alter OSM-11 protein levels in VPCs (unpublished data). Combined, all of these data suggest that osmotic stress does not itself regulate vulval development. Instead, these data suggest that the roles of _osm-11_ in vulval development and osmotic stress resistance are independent.

OSM-11 Is a Secreted Protein

Because the predicted peptide sequence of OSM-11 contains a signal peptide, we tested whether OSM-11 is a secreted protein. When an _osm-11_ cDNA was expressed in _Drosophila_ S2 tissue culture cells, OSM-11 protein accumulates in the media and not in cells (Figure 5A), consistent with OSM-11 acting in vivo as a soluble protein in the extracellular milieu. The ability of OSM-11 to diffuse and act as a soluble factor in vivo was tested by ectopically expressing OSM-11 in non-VPC cells in _osm-11(lf) _animals. _osm-11_ cDNA was fused to _osm-10_ or _glr-1_ promoter fragments that drive expression in nonoverlapping subsets of neurons throughout larval development. The _osm-10_ promoter drives expression in four classes of sensory neurons located exclusively in the head and tail [69]. The _glr-1_ promoter drives expression in 17 other classes of neurons (distinct from _osm-10_-expressing neurons)

**Figure 5. osm-11 Encodes a Secreted Protein Required for Vulval Development**

(A) Western blot of conditioned media from _Drosophila_ S2 cells containing an _osm-11_ cDNA expression construct or empty vector. _Osm-11_ was not detected in cell lysates (unpublished data). The molecular weight of mature _Osm-11_ was predicted at 18.9 kDa; the detected protein migrated at 20.7 kDa (arrowhead). _Osm-11_ may be O-linked glycosylated (see Figure 2).

(B) Transgenic rescue of _osm-11(lf)_ vulval defects. _osm-11(lf)_ animals harboring transgenes with empty expression vectors were indistinguishable from nontransgenic _osm-11(lf)_ animals (n = 129 animals, 5 transgenic lines) and were used as controls. Multiple transgenic lines were scored for all rescue experiments; data are reported as mean ± standard error of the mean (S.E.M.). In addition to a genomic _osm-11_ construct, expression of the _osm-11_ cDNA using the following promoters also significantly rescued _osm-11(lf)_ vulval defects: _osm-11p_, _hsp-16p_ (ubiquitous expression, 79% normal vulval; unpublished data), _wnt-6p_ (hypodermal), _osm-10p_ (sensory neurons), and _glr-1p_ (nonoverlapping set of neurons vs. _osm-10p_). In addition, heterologous expression of mammalian DLK1 driven by the _hsp-16_ promoter also significantly rescued _osm-11(lf)_ vulval phenotypes. n > 52 animals for each transgene, p < 0.05 by χ².

doi:10.1371/journal.pbio.0060196.g004
located in the head and tail [70,71]. Some of the gtr-1-expressing neurons have processes in the ventral nerve cord near the VPCs. We found that neuronal expression of the osm-11 cDNA significantly rescued osm-11 vulval defects to levels comparable with osm-11 promoter-driven cDNA rescue (Figure 5B). Consistent with these results, hypodermal expression of osm-11 cDNA using the wrt-6 promoter [72] also rescued osm-11 defects, albeit at a lower level. We conclude that osm-11 can act nonautonomously and that soluble OSM-11 can diffuse in vivo. Although OSM-11 expressed in VPCs may be sufficient for normal vulval development, OSM-11 can probably function at a distance in some contexts like soluble DSL ligands in C. elegans [35].

osm-11 Acts Upstream of lin-12 Notch Receptor Activation to Increase Signaling

The phenotypic defects caused by loss of osm-11 might be due to OSM-11 action upon previously identified molecular pathways that regulate cell fate specification in vulval development. We tested the sensitivity of the EGF, Notch, and synthetic multivulva (SynMuv) VPC fate specification pathways to osm-11 levels by RNAi knockdown of osm-11 in mutants that have been previously used as sensitized backgrounds for each pathway: lin-12(n137n460)csgf Notch (see below), let-23(sa62gf) EGF receptor, let-60(n1046gf) Ras, or lin-15(n765tsd) SynMuv [73–78]. osm-11RNAi had the most effect in animals with compromised lin-12 Notch signaling (27% change in multivulva (Muv) of lin-12(n137n460);osm-11(RNAi) at 20 °C versus less than 9% change in other backgrounds, p < 0.05, n > 50 each). Although it is difficult to assess the relative sensitivity of these various genetic backgrounds, these results suggested that Notch signaling might be particularly sensitive to OSM-11 levels and that osm-11 might modulate lin-12 signaling during vulval development.

To more accurately assess the possible role of osm-11 in lin-12 Notch signaling in vivo, we undertook genetic studies using the osm-11(lf) null allele and previously described lin-12 alleles. lin-12(n137) is a ligand-independent dominant gain of function (gf) allele, whereas lin-12(n137n460) is a recessive, cold-sensitive gain-of-function allele (csgf). Both cause multiple pseudovulvae (Muv) due to secondary cell specification defects [79,80]. If OSM-11 normally functions to increase Notch signaling, then loss of OSM-11 should decrease LIN-12 Notch signaling. We found that osm-11(lf) partially suppressed the Muv defect of lin-12(csgf) at the restrictive temperature, consistent with OSM-11 normally increasing lin-12 signaling (Figure 6C and 6D). However, osm-11(lf) did not suppress the stronger lin-12(gf) allele (Figure 6E and 6F). Since lin-12(n137gf) is thought to activate lin-12 signaling in a ligand-independent manner, the inability of osm-11(lf) to suppress lin-12(gf) is consistent with osm-11 acting before or during ligand activation of LIN-12.

If osm-11 normally acts before or during ligand activation of LIN-12, then lin-12(gf) should be epistatic to osm-11(lf). lin-12(gf) animals are sterile and have a single large protruding vulva [80], a phenotype that is easily distinguishable from the misshapen vulva of osm-11(lf) animals (compare Figure 1B and 1C with Figure 6B). lin-12(gf);osm-11(lf) double-mutant animals were indistinguishable from lin-12(gf) animals, suggesting that osm-11 acts upstream of lin-12 Notch (Figure 6A and 6B). Combined, these results suggest that OSM-11 normally increases LIN-12 Notch signaling in vivo and acts before or during receptor activation.

OSM-11 Functions with Other DOS Proteins in Development

Five C. elegans genes encode putative secreted DOS-motif proteins: osm-11, osm-7, dos-1, dos-2 [51,52], and dos-3. Loss-of-function alleles are not currently available for dos-2 and dos-3, but osm-7(tm2256) and dos-1(ok2398) are deletion alleles generated by the C. elegans gene knockout consortia and are likely strong loss-of-function (lf) or null alleles. osm-7 and
Figure 7. OSM-11 Acts Synergistically with DSL Ligands and Other DOS Proteins

(A) Genetic interactions between osm-11 and DOS-motif genes osm-7 and dos-1 (ZK507.4). dos-1(lf) and osm-7(lf) are both presumptive null alleles, and animals harboring these alleles had normal vulvas. dos-1(lf);osm-11(lf) and osm-7(lf);osm-11(lf) animals had significantly more severe defects than osm-11(lf) animals (p < 0.005, χ² test). Mutant alleles of dos-2 (K10G6.2) and dos-3 (K02F3.7) are not currently available.

(B) Genetic interactions between osm-11 and DSL-domain genes lag-2 and dsl-1. lag-2(dn) is the dominant negative allele sa37; dsl-1(lf) is ok810 and is a presumptive null allele. lag-2(dn) and dsl-1(lf) animals had few or no vulval defects. lag-2(dn);osm-11(lf) and dsl-1(lf);osm-11(lf) animals had significantly more-severe defects that osm-11(lf) animals (p < 0.005, χ² test).

(C) Vulval precursor cell (VPC) fate analysis for osm-11 and dsl-1. Arrowheads indicate the positions of P5.p, P6.p, and P7.p. Secondary (2°) cell fates were scored as in Figure 3 using lip-1p::GFP as illustrated (right). dsl-1;osm-11 double-mutant animals had significantly more severe 2° fate specification defects compared to either single mutant alone (p < 0.005 by χ²). n ≥ 48 for each genotype in all panels.

doi:10.1371/journal.pbio.0060196.g007
OSM-11 and LIN-12 Notch in Development

Figure 8. OSM-11 and C. elegans DSL Ligands Interact with LIN-12 Notch Extracellular Domain EGF Repeats in the Two-Hybrid System

DSL-1, OSM-11, LAG-2 extracellular domain (LAG-2Ex), EGL-17, or LIN-3 was fused to the GAL4 DNA binding domain (DB); the first six LIN-12 EGF repeats were fused to the GAL4 activation domain (AD). Pairwise interactions were tested with the yeast two-hybrid assay; positive interactions are indicated by blue staining. Both Notch DSL ligands and OSM-11 interacted with LIN-12 EGF repeats, whereas no interaction of LIN-3 EGF or EGL-17 EGF with LIN-12 Notch receptor EGF repeats was detected. LIN-12::DB fusion proteins exhibited strong self-activation (unpublished data); therefore, reciprocal fusions were not tested. Interaction controls are: (1) empty vectors; (2) DB-pRB and AD-E2F; (3) DB-Fos and AD-Jun; (4) Gal4p and pPC86; and (5) DB-DP1 and AD-E2F1. doi:10.1371/journal.pbio.0060196.g008

7(tm2256lf) animals are resistant to osmotic stress and fail to avoid high osmolarity, similar to previously published animals resistant to osmotic stress and fail to develop [51,52]. To determine whether DOS-motif proteins have overlapping functions, we tested whether mutants defective in more than one DOS-motif protein had stronger vulval defects. Loss of either osm-7 or dos-1 alone had little or no overt effect on vulval morphology. However, loss of dos-1 or osm-7 increased the percentage of osm-11(lf) animals with multiple vulval protrusions (Figure 7A). This result is consistent with multiple DOS-motif proteins acting in vulval development.

OSM-11 Functions with DSL Ligands to Increase Notch Signaling

Classical C. elegans Notch DSL ligands are expressed in VPCs and function redundantly during cell specification [35]. Accordingly, DOS-motif proteins may also function redundantly in VPC specification. One might expect that DSL-domain proteins and DOS-motif proteins would act together to activate Notch signaling. Therefore, loss of a DSL protein should exacerbate osm-11 developmental defects. dsl-1 encodes a DSL domain-containing ligand which acts redundantly with two other DSL proteins to activate LIN-12 Notch signaling during vulval development [35]. Because of this redundancy, the dsl-1(okb810lf) allele does not itself cause vulval defects [35]. However, dsl-1(lf)osm-11(lf) double mutants had modestly increased phenotypic defects in vulval morphology compared to osm-11 single mutants. osm-11(lf) vulval defects were similarly enhanced by lag-2(lf), which encodes a DSL ligand (Figure 7B). To more precisely assess interactions between osm-11 and dsl-1, the expression of lip-1p::gfp in dsl-1(lf)osm-11(lf) animals was assessed during VPC fate specification. Eighty-six percent of the double-mutant animals lacked lip-1p::gfp up-regulation in either one or both presumptive secondary VPCs, indicating a substantial synergistic loss of secondary fate specification (Figure 7C). This result is consistent with DOS-motif (i.e., OSM-11) and DSL-domain proteins working together to increase LIN-12 Notch signaling.

OSM-11 Interacts with LIN-12 Extracellular EGF Repeats in a Yeast Two-Hybrid Assay

The cellular nonautonomy of osm-11, the similarity of OSM-11 to Notch ligands, the expression pattern of OSM-11, and the genetic implication that osm-11 functions before or during LIN-12 Notch activation in VPC fate specification collectively suggest that OSM-11 may function as a LIN-12 Notch ligand. We tested the hypothesis that OSM-11 directly interacts with LIN-12 extracellular domain. Previous studies demonstrated that Drosophila and vertebrate DSL ligands bind to the extracellular EGF repeats of Notch receptors. In preliminary studies, we were unable to demonstrate direct binding between OSM-11 and LIN-12 biochemically using a heterologous expression system (unpublished data). Therefore, we turned to the yeast two-hybrid assay to test whether OSM-11 can interact with LIN-12 extracellular EGF repeats. Conventional wisdom suggests that the yeast two-hybrid system is not suitable for testing extracellular protein–protein interactions, especially for domains rich in disulfide bridges (e.g., EGF repeats). However, two-hybrid interactions have been demonstrated between Notch receptors and ligand pairs in other species for which biochemical interactions have been previously validated [38,39,50], as well as for numerous other extracellular proteins [81–86]. To validate our yeast two-hybrid approach, we first confirmed that the extracellular domain of LAG-2 [23] and the soluble DSL-domain LIN-12 ligand DSL-1 [35] interact with LIN-12 extracellular EGF repeats 1 through 6 in the two-hybrid assay (Figure 8). To the best of our knowledge, this is the first in vitro evidence that C. elegans DSL ligands may bind directly to LIN-12 Notch. As a negative control and to confirm specificity of the two-hybrid assay, we showed that the unrelated C. elegans ligands LIN-3 (an EGF homolog) and egg laying defective-17 (EGL-17) (an FGF homolog) do not interact with LIN-12 extracellular EGF repeats (Figure 8). The LAG-2 and DSL-1 interactions with LIN-12 in the two-hybrid assay are consistent with previous genetic studies in C. elegans.
Model: DSL + DOS activates Notch

![Diagram](https://example.com/diagram.png)

**Figure 9.** Model: *C. elegans* DSL and DOS Proteins May Act as Ligands for Notch Receptors

Canonical Notch ligands in *Drosophila* contain both DSL domains and DOS motifs as do some vertebrate Notch ligands (e.g., Delta). However, classical Notch ligands from *C. elegans* and several vertebrate Notch ligands contain a DSL domain, but lack DOS-motif EGF repeats (e.g., LAG-2 or DLL3). The *C. elegans* proteins characterized in this study (e.g., OSM-11) and the two presumptive vertebrate ligands DLK1 and EGFL9/DLK2 lack DSL domains, but contain DOS motifs. In the simplest model, both a DOS motif and DSL domain are required for coordinated Notch receptor activation. These could act in cis in canonical Notch receptors like *Drosophila* Delta or in trans in the case of LAG-2 and OSM-11. Overexpression of a “DOS-only” or a “DSL-only” ligand may inhibit Notch receptor activation by competition with canonical ligands containing both a DSL domain and a DOS motif, such as Jagged1 or Delta. This model is consistent with osm-11(lf) animals having phenotypic defects usually associated with Notch loss of function. We do not exclude other possible scenarios; see Discussion for details.

doi:10.1371/journal.pbio.0060196.g009

and with biochemical analyses of Notch ligand/receptor interactions in other systems. Ligand-receptor interactions were only assayed using LIN-12 fused to the GAL4 activation domain (AD) as LIN-12 EGF fusion to the DNA-binding domain resulted in strong self-activation in the presence of AD empty vector (unpublished data).

We found that OSM-11 also interacted with LIN-12 extracellular EGF repeats 1 through 6 (Figure 8); OSM-11 did not interact with DSL-1 or LAG-2 ligands. We also confirmed previous studies [50] in which murine DLK1 EGF repeats 1 and 2 containing the DOS motif interacted specifically with murine Notch1 EGF repeats 12 and 13 in the same two-hybrid assay format (unpublished data). The two-hybrid interaction does not necessarily demonstrate that OSM-11 and LIN-12 interact in vivo; however, combined with the genetic interactions, the apical expression pattern of OSM-11 in VPCs, and previous studies of DLK1/Notch interactions, we favor a simple model in which OSM-11 binds directly to LIN-12 Notch EGF repeats. Further biochemical studies will be required to demonstrate DOS-motif protein direct interactions with Notch receptors.

The Mammalian DOS-Motif Protein DLK1 Can Substitute for OSM-11

Our results suggest that the DOS-motif protein OSM-11 may act as a soluble LIN-12 ligand in *C. elegans*. This raises the issue of whether other DOS-motif proteins such as DLK1, which has been implicated in Notch signaling in mammalian cells, also acts as soluble Notch ligands. The precise role of DLK1 in mammalian Notch signaling is controversial. To address the function of mammalian DLK1 in Notch signaling, we tested the ability of DLK1 to functionally substitute for OSM-11 in *C. elegans*. We found that expression of a soluble, mature DLK1 protein isoform named FA1 [43] in osm-11(lf) animals significantly rescued vulval development, consistent with DLK1 protein increasing LIN-12 signaling (Figure 5D). This result suggests that the function of *C. elegans* DOS-motif proteins is to increase Notch receptor signaling and that the molecular mechanism may be conserved across species.

**Discussion**

The data presented herein demonstrate that osm-11 is required for normal vulval development in *C. elegans*. *osm-11* encodes a novel cEGF-1 protein that is similar to, but distinct from, previously characterized Notch ligands in vertebrates [53]. OSM-11 contains a previously unidentified protein motif that we have named DOS (Delta and OSM-11) overlapping the EGF motifs. The DOS motif is conserved across species and found in canonical Notch ligands. OSM-11 is a secreted protein that is expressed in VPCs during cell fate specification. Genetic analysis suggests that OSM-11 acts upstream of LIN-12 and that OSM-11 normally increases LIN-12 Notch signaling in vivo. Two-hybrid data and expression on the VPC apical surfaces suggest that OSM-11 may directly bind to the LIN-12 extracellular domain, although additional biochemical studies will be required to further confirm this. Finally, we demonstrated that the mammalian DOS-motif protein DLK1 can partially substitute for OSM-11 in *C. elegans* vulval development, suggesting that DOS-motif protein function is conserved across species.

Our data suggest a model wherein OSM-11 and *C. elegans* DSL ligands act together to activate Notch receptors, potentially as a *C. elegans* bipartite ligand that is functionally equivalent to *Drosophila* Delta or mammalian Jagged1 (Figure 9). Previously described *C. elegans* DSL ligands such as LAG-2 lack a DOS motif; *C. elegans* DSL ligands, such as LAG-2, and
DOS-motif proteins, such as OSM-11, may both be required to activate LIN-12 Notch receptor signaling in vivo. Classical studies in C. elegans have shown that expression of the APX-1 N-terminus (which contains the DSL domain) is sufficient to activate Notch signaling; however, this is not inconsistent with our model because endogenous DOS-motif proteins were present [23]. Our model is also consistent with previous biochemical and genetic studies that showed the first two EGF repeats of Jagged1 and Delta are critical for high-affinity DSL-domain binding to mammalian Notch receptors and Notch receptor activation [25,54].

Bipartite or heteromeric ligands are relatively rare compared to heteromeric receptors. To our knowledge, bipartite ligands have only been described previously in the immune system. The binding of antigen to complement fragment creates, in effect, a bipartite ligand for antigen receptor as does the binding of an antigenic peptide to a compatible major histocompatibility complex (MHC) subunit. Additionally, and perhaps more pertinent, heterodimeric cytokines have been described in the immune system that bind to cytokine receptors [87]; for example, the interleukin-12 (IL-12) cytokine is composed of p40 and p35, whereas the IL-23 is composed of p40 and p19. Although bipartite ligands are unusual, they are not unprecedented.

Previous studies have shown that the mammalian DOS-motif protein DLK1 acts as a competitive antagonist of ligand Jagged1, a canonical ligand that contains both a DSL domain and DOS motif [50]. Therefore, a plausible alternative model (which takes into account DLK1 antagonism of Jagged1) is that DOS-motif proteins bind to Notch receptors, but function as antagonists of DSL-domain Notch ligands in all species. DOS proteins such as OSM-11 might play a role in maintaining C. elegans Notch receptor levels or localization, although LIN-12 Notch expression is unaltered in animals lacking osm-11. Based on our data, we instead favor the simpler model of DOS-motif proteins as activators of Notch receptors acting with DSL proteins. In an independent behavioral analysis (M. Chao, J. Larkins-Ford, T. Tucey, H. Komatsu, and H. Dionne, et al., unpublished data), we also found that OSM-11 activates both LIN-12 and germline proliferation defective-1 (GLP-1) in the adult nervous system to regulate behavior. We speculate that if DLK1 was coexpressed in mammalian systems with a C. elegans DSL-only ligand, then Notch signaling might be increased. Mammalian Delta like 3 (DLL3) and DLL4 ligands contain DSL domains, but not DOS motifs. Biochemical studies have shown that DLL3 inhibits Notch signaling and DLL4 increases Notch signaling in various contexts. It would be useful to examine Notch activation when DLK1 and DLL3 are coexpressed. Clearly, biochemical analyses addressing the role of DOS motifs and DSL domains in Notch receptor activation will be required to discriminate between these two models and to determine the relative contributions of DSL and DOS-motif proteins to Notch signaling.

C. elegans DSL ligands function redundantly, activating LIN-12 Notch during vulval development; loss of any one DSL ligand gene causes mild or no overt defects [35]. Similarly, loss of osm-11 alone caused only mild defects in vulval morphogenesis, whereas loss of more than one DOS-motif gene resulted in more-severe vulval defects. Like DSL ligands, DOS-motif proteins function semiredundantly to increase Notch signaling in vivo. In addition, genetic analysis suggests that DOS-motif proteins and DSL proteins may act together to regulate Notch receptors. It is possible that Notch receptor activation by ligands during VPC development is robust due to this redundancy. This multifactorial system for regulation of Notch receptors might allow use of individual soluble DOS or DSL proteins in other cell-cell signaling events in other tissues simultaneously.

Defining a role herein for osm-7 and osm-11 in Notch signaling suggests that this pathway also plays a previously unsuspected role in osmotic stress response. C. elegans can adapt to increased environmental osmolarity; animals exposed to moderate osmotic stress increase internal osmolyte levels and have altered behavior reminiscent of animals lacking osm-11 or osm-7 [51,52]. A role for Notch signaling in osmotic stress has not been reported in any species. The developmental role of Notch signaling in vulval cell fate specification is distinct from the role in osmotic stress response based on data presented here. Further studies will be required to determine whether diffusible DOS proteins act as humoral factors to regulate Notch signaling in multiple tissues to coordinate physiological and behavioral adaptation to osmotic stress.

The diversity of Notch receptors and ligands is remarkable. C. elegans has two Notch receptors (lin-12 and glp-1), ten DSL domain proteins that lack DOS motifs [35] and five DOS-motif proteins without DSL domains (this study). Mammals have four Notch receptors, multiple DSL ligands, and two presumptive DOS-motif-only ligands: DLK1 and EGF/Like/ DLK2. Additional proteins have been suggested to act as Notch ligands in vertebrates [36–40], but invertebrate homologs have not been identified. At least one DSL domain Notch ligand in each vertebrate species we examined (zebrafish, humans, and mice) lacks the conserved DOS motif; these proteins are potentially analogous to C. elegans DSL domain ligands (e.g., LAG-2) that also lack DOS motifs. Soluble Notch ligands are now predicted in all of these species based on this and previous studies. In contrast, Drosophila has only one Notch receptor, and the two previously characterized transmembrane Drosophila Notch ligands contain both DSL domains and DOS motifs. This heterogeneity of Notch ligands and receptors indicates that the functional relationship between Notch receptors and ligands is highly complex, allowing precise regulation of signaling.

Materials and Methods

Characterization of osm-11. The osm-11(r68) mutant allele was identified in a classical genetic screen based on defective chemosensory response and temporarily designated sel-14 (suppressor/enhancer of lin-12-14). The r68 mutation was mapped to the predicted C. elegans gene F11C7.5 and mutates W177 to a premature stop codon, resulting in premature truncation of translation near the end of the DOS motif. Recent published studies and our analysis herein confirmed that sel-14(r68) is an allele of osm-11 and has the same amino acid change as the previously identified allele osm-11(r1604) [52,88]; therefore, we refer to this gene as osm-11. The deletion allele osm-11(r142) was identified by PCR-based screening of a frozen ethyl-methane sulfonate (EMS)-mutagenized library of C. elegans strains [89]. The r68 and r142 alleles had similar phenotypic defects, but the r142 deletion allele was more severe. Both osm-11 alleles are recessive. osm-11(r142) is likely a complete loss-of-function (fl) allele and was used exclusively herein. RNAi of osm-11 was performed by raising N2 animals on a lawn of bacteria expressing osm-11 double-stranded (dsRNA). Other than morphological defects, vulva perturbations, and consequent egg-laying defects, osm-11(r142fl) animals are overly normal in locomotion, male mating, and reproduction, although
their growth rate is slightly slower and they are slightly smaller than wild-type animals. *osm-11* animals frequently had ventral protrusions posterior to the anus. Postnatal swelling is frequently associated with bacterial infections, but swelling occurs in untransformed *osm-11* animals raised on standard OP50 bacteria. Gonad morphology was subtly altered in *osm-11* animals but was not further characterized here. The detection of *lin-12* mRNA localization defects (M. Chao, J. Larkins-Ford, T. Tucey, H. Komatsu, and H. Dionne, et al, unpublished data). *osm-11(r142)* animals are osmotically stress resistant and are motile on 500 mM NaCl NGM plates, consistent with previously published phenotypes of *osm-11(n1604)* [52].

**Strains and genetics.** Gain-of-function *lin-12* alleles used herein included the constitutive dominant allele *lin-12(n137)* and the cold-sensitive recessive gain-of-function allele *lin-12(n137n460gfcs)*. Results from homozygous *lin-12(n137)* and heterozygote *lin-12(n137)* animals were scored for each transgenic experiment; results were maintained by obtaining females containing either over GFP containing *gfcs* [60-61, lag-2::gfp] or over *unc-2(e189)*. Genetic epistasis of *osm-11* with *lin-12(n941)* was assessed using homozygous *lin-12(n941)* progeny of *lin-12(n941)/ unc-32(e189)* animals. A fraction of animals were singled as larvae and subsequently scored for vulval morphology and genotype. *lin-12(n941)* animals were always sterile regardless of *osm-11* status; *lin-12(n941)/ unc-32 animals lacked protruding vulva and yielded *unc-32* progeny regardless of *osm-11* status.

The deletion alleles *osm-7(m2256)* and *dos-1(r2398)* were generated by the *C. elegans* gene knockout consortia. The *osm-7(m2256)* deletion removes the first part of the DOS motif and eliminates an exon splice site, resulting in a predicted frame shift after amino acid 200 with premature truncation after translation of 21 amino acids. The *dos-1(r2398)* allele is a 1.7-kb deletion that removes the initiator site, resulting in a predicted frame shift after amino acid 200 with premature truncation after translation of 21 amino acids. The *dos-1(r2398)* deletion alleles were backcrossed at least four times prior to analysis. Double-mutant analysis in Figure 7 was performed in an *elt-2::gfp* genetic background. Other alleles used in this study included lag-2(a37) and *dsl-1(lf)*.

**Analysis of VPC fate specification.** Pn.p cells and descendants were identified by differential interference contrast (DIC) imaging on a Zeiss Axioskop2. The transgenic arrays used for VPC fate analysis were: *ayk-1 [egf-2::gfp], syk-107 [lin-3-gfp], *osm-31 [lin-11-gfp]*, and *zhd-4 [tip-1-gfp]]* [58,60,83,90]. Animals were scored on the Pn.p stages for *gfp-17-gfp* and *tip-1-gfp*, but only on the Pn.p stage for *lin-11-gfp*. Rearing on 400 mM NaCl NGM plates dramatically slows growth and results in partially penetrant embryonic and larval lethality. In less than 10% of all animals raised under these conditions, Pn.p cell descendants could not be identified by DIC; these animals were excluded from the analysis. *osm-31* animals were nonviable on 400 mM NaCl NGM plates.

**Immunohistochemistry.** Polyclonal antiserum specific to *OSM-11* were raised in rabbits using the C-terminal peptide YSKICTMFV-PQ (Sigma-Genosys) and was used as a 1:200 dilution of unpurified antiserum. Immunoreactivity of *osm-11* was detected in larval and adult animals in paraformaldehyde-fixed wild-type animals, but not in *osm-11(r142f)* animals (unpublished data). Eggs were not examined, and no immunoreactivity in germ cells was observed. *OSM-11* was detected at the junction of the presumptive vulva and uterine L4 larval and in the spermatheca of late-larval and adult animals. *OSM-11* mRNA localization by in situ hybridization is consistent with previously published phenotypes of *unc-32(e189)* and *unc-54(e281)* animals raised on standard OP50 bacteria. Gonad morphology was subtly altered in *osm-11* animals but was not further characterized here. The detection and scoring of vulval morphology and genotype. *unc-32(e189)* animals were frequently had ventral protrusions posterior to the anus. Postnatal swelling is frequently associated with bacterial infections, but swelling occurs in untransformed *osm-11* animals raised on standard OP50 bacteria. Gonad morphology was subtly altered in *osm-11* animals but was not further characterized here. The detection of *lin-12* mRNA localization defects (M. Chao, J. Larkins-Ford, T. Tucey, H. Komatsu, and H. Dionne, et al, unpublished data). *osm-11(r142)* animals are osmotically stress resistant and are motile on 500 mM NaCl NGM plates, consistent with previously published phenotypes of *osm-11(n1604)* [52].

**Bioinformatics.** *C. elegans* and *C. briggsae* homologs of *OSM-11* were identified by BLAST analysis against genomic sequences and predicted genes at NCBI. The characterized *OSM-11* motif was identified manually and used to search for similar proteins using Pattern Search at the Swiss Institute for Experimental Cancer Research (ISREC) [http://myhits.isb-sib.ch/cgi-bin/pattern_search]. A subset of Notch ligands was identified, *DLK1* and *Drosophila Delta* proteins are CBG18238 for *T05D4.4*, CBG18440 for *K10G6.2*, CBG06995 for *ZK567.4*, and CBG15929 for F11C7.3. The new prediction for K10F5.7/DOS-5 has been submitted to WormBase, and a *DSL* domain, and EGF repeats, but has not been well characterized [56]. *DLK1* and EGF-like do not contain DSL domains, but do contain signal peptides and EGF repeats. Given that all previously identified DSL domains are located between the signal peptide sequence and the EGF repeats, we conclude that *DLK1* and EGF-Like do not contain DSL domains. It is interesting to note that many classical Notch ligand genes contain an intron immediately after the DSL domain.

**OSM-11 mRNA localization by in situ hybridization is consistent with previously published phenotypes of *unc-32(e189)* and *unc-54(e281)* animals raised on standard OP50 bacteria. Gonad morphology was subtly altered in *osm-11* animals but was not further characterized here. The detection and scoring of vulval morphology and genotype. *unc-32(e189)* animals were frequently had ventral protrusions posterior to the anus. Postnatal swelling is frequently associated with bacterial infections, but swelling occurs in untransformed *osm-11* animals raised on standard OP50 bacteria. Gonad morphology was subtly altered in *osm-11* animals but was not further characterized here. The detection of *lin-12* mRNA localization defects (M. Chao, J. Larkins-Ford, T. Tucey, H. Komatsu, and H. Dionne, et al, unpublished data). *osm-11(r142)* animals are osmotically stress resistant and are motile on 500 mM NaCl NGM plates, consistent with previously published phenotypes of *osm-11(n1604)* [52].
References

1. Fleming RJ, Scottgate TN, Diederich RJ, Artavasian-Tsakonas S (1999) The gene egg-serrate encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in Drosophila melanogaster. Genes Dev 4: 2188–2201.

2. Kopczynski CC, Alton AK, Fechtel K, Kooj PJ, Mushavit MA (1988) Delta, a Drosophila neurogenic gene, is transcriptionally complex and encodes a protein related to blood coagulation factors and epidermal growth factor of vertebrates. Genes Dev 2: 1725–1735.

3. Thomas U, Speicher SA, Knust E (1991) The Drosophila gene Serrate encodes an EGF-like transmembrane protein with a complex expression pattern in embryos and wing discs. Development 111: 749–761.

4. Eastman DS, Slee R, Skoufos E, Bangalore L, Bray S, et al. (1997) Synergy of Hairless and Notch in regulation of enhancer of split tamanina and delta expression. Mol Cell Biol 17: 5629–5638.

5. Hsieh JJ, Henkel T, Salmon P, Robey E, Peterson MG, et al. (1996) Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. Mol Cell Biol 16: 952–960.

6. Kidd S, Lieber T, Young MW (1998) Ligand-induced cleavage and regulation of nuclear entry of Notch in Drosophila melanogaster embryos. Genes Dev 12: 3728–3740.

7. Rebay I, Fleming RJ, Fechen RG, Cherbas L, Cherbas P, et al. (1991) Specific EGF repeats of Notch mediate interactions with Delta and Serrate implications for Notch as a multifunctional receptor. Cell 67: 687–699.

8. Tamura K, Taniguchi Y, Minoguchi S, Sakai T, Tun T, et al. (1995) Physical interaction between a novel domain of the receptor Notch and the extracellular matrix protein MAGP-2 interacts with Jagged1 and induces its dissociation and receptor binding in Drosophila. Genetics 174: 1947–1961.

9. Shimizu K, Chiha S, Saito T, Kumanono H, Hirai H (2000) Physical interaction of Delta1, Jagged1, and Jagged2 with Notch1 and Notch3 receptors. Biochem Biophys Res Commun 276: 385–388.

10. Lissemore JL, Starmer WT (1999) Phylogenetic analysis of vertebrate and invertebrate DeltaSerrate/LAG-2 (DSL) proteins. Mol Phylogenet Evol 11: 308–319.

11. DeVries GR, Patel K, Lewis J, Tickle C (1998) Expression patterns of Notch1, Serrate1, Serrate2 and Delta in tissues of the developing chick limb. Mech Dev 77: 197–199.

12. Jarriault S, Le Bail O, Hirsinger F, Pourquié O, Logeat F, et al. (1998) Delta-1 activation of notch-1 signaling results in HES-1 transactivation. Mol Cell Biol 18: 7423–7431.

13. Dunwoodie SL, Henrique D, Harrison SM, Beddington RS (1997) Mouse Delta1: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. Development 124: 3065–3075.

14. Parks AL, Turner FR, Muskavitch MA (1995) Relationships between complex Delta expression and the specification of retinal cell fates during Drosophila eye development. Mech Dev 50: 201–214.

15. Muskavitch MA, Hoffmann FM (1999) Homology of vertebrate growth factors in Drosophila melanogaster and other invertebrates. Curr Top Dev Biol 24: 289–328.

16. Greenwald I (1998) LIN-12Notch signaling: lessons from worms and flies. Genetics 147: 1751–1768.

17. Artavasian-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and integration in development. Science 284: 770–776.

18. Chen N, Greenwald I (2004) The lateral signal for LIN-12Notch in C. elegans vulval development comprises redundant secreted and transmembrane DSL proteins. Dev Cell 6: 183–192.

19. Lai EC, Bodner R, Posakony JW (2000) The enhancer of split complex of Drosophila includes four Notch-regulated members of the bearded gene family. Development 127: 3441–3455.

20. Brennan K, Gardner P (2002) Notching up another pathway. Bioessays 24: 405–410.

21. Miyamoto A, Lau R, Hein PW, Shipley JM, Weinmaster G (2006) Microfibrillar proteins MAGP-1 and MAGP-2 induce Notch1 extracellular domain dissociation and receptor activation. J Biol Chem 281: 10089–10097.

22. Nehring LC, Miyamoto A, Hein PW, Weinmaster G, Shipley JM (2005) The extracellular matrix protein MAGP-2 interacts with Jagged1 and induces its shedding from the cell surface. J Biol Chem 280: 20349–20355.

23. Enari M, Tohgo A, Ono K, Kaneko M, Fujishima K, et al. (2005) DNER acts as a neuron-specific Notch ligand during Bergmann glial development. Nat Neurosci 8: 873–880.

24. Jensen CH, Schroder HD, Teineer B, Laursen L, Brandrup F, et al. (1999) Fetal antigen 1, a member of the epidermal growth factor superfamily, in neuralroblastoma and serum from patients with neuroblastomatisosis type 1. Br J Dermatol 140: 1054–1059.

25. Lee Y, Belman L, Humason T, Laborda J (1995) dlk, pg2 and Pref-1 mRNAs encode similar proteins belonging to the EGF-like superfamily. Identification of polymorphic variants of this RNA. Biochim Biophys Acta 1261: 223–232.
