Semaphorin 1a and semaphorin 1b are required for correct epidermal cell positioning and adhesion during morphogenesis in *C. elegans*

Val E. Ginzburg¹,², Peter J. Roy¹,²,* and Joseph G. Culotti¹,²,†

¹Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada
²Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada

*Present address: Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305-5427, USA

†Author for correspondence (e-mail: culotti@mshri.on.ca)

Accepted 6 February 2002

**SUMMARY**

The semaphorin family comprises secreted and transmembrane proteins involved in axon guidance and cell migration. We have isolated and characterized deletion mutants of *C. elegans* semaphorin 1a (*Ce-sema-1a* or *smp-1*) and semaphorin 1b (*Ce-sema-1b* or *smp-2*) genes. Both mutants exhibit defects in epidermal functions. For example, the R1.a-derived ray precursor cells frequently fail to change anterior/posterior positions relative to their sister tail lateral epidermal precursor cell R1.p, causing ray 1 to be formed anterior to its normal position next to ray 2. The ray cells, which normally separate from the lateral tail seam cell (SET) at the end of L4 stage, remains connected to the SET cell even in adult mutant males. The ray 1 defects are partially penetrant in each single *Ce-sema-1* mutant at 20°C, but are greatly enhanced in *Ce-sema-1* double mutants, suggesting that *Ce-Sema-1a* and *Ce-Sema-1b* function in parallel to regulate ray 1 position. Both mutants also have defects in other aspects of epidermal functions, including head and tail epidermal morphogenesis and touch cell axon migration, whereas, *smp-1* mutants alone have defects in defecation and brood size. A feature of *smp-1* mutants that is shared with mutants of *mab-20* (which encodes Sema-2a) is the abnormal perdurance of contacts between epidermal cells.

Key words: *C. elegans*, Semaphorins, Morphogenesis, Cell positioning

**INTRODUCTION**

The precise migration of motile cells is achieved by cycles of cytoskeletal-induced membrane extension, and retraction, which must be coordinated with making and breaking contacts with the underlying extracellular matrix (ECM) and neighboring cells. Axon growth occurs by a specialized form of cell movement involving transient contacts made by a motile organelle at the tip of the axon, the growth cone. The motility of cells and growth cones is modulated by changes in the cytoskeleton in response to the cues in its environment. These molecular cues come in two categories: attractive, i.e. those that promote, and repulsive, i.e. those that inhibit, migration (Hedgecock et al., 1990; Kolodkin, 1996).

Semaphorins from evolutionary disparate animals have been shown to cause growth cone collapse and repulsion in vitro (Kolodkin et al., 1992; Luo et al., 1993; Matthes et al., 1995; Messersmith et al., 1995; Puschel et al., 1995; Winberg et al., 1998a). However, several studies have found that semaphorins can also mediate growth cone attraction (Bagnard et al., 1998; Polleux et al., 2000; Wong et al., 1999). Cellular responses to certain molecular guidance cues can be switched from repulsion to attraction, and vice versa, via alterations in the composition of the corresponding receptor complexes or in the activity of downstream signaling components (Chan et al., 1996; Hamelin et al., 1993; Su et al., 2000). One relevant example of this is Sema-3A-induced growth cone collapse, which can be reverted to attraction by increasing the concentration of cyclic nucleotide (i.e. cGMP) within the growth cone (Song et al., 1998). In spite of these insights, relatively little is known about the cellular and molecular mechanisms that in some cases cause a given cue to be used as an attractant and in other cases to be used as a repellant.

In addition to functions in cell migration and axon guidance, other functions have been attributed to semaphorin proteins based on phenotypic analysis of mutants in combination with in vitro studies. Thus, in addition to neuronal defects, mouse *Sema3a* mutants also exhibit cardiac malformations, cardiovascular defects, fused vertebrae and fused ribs suggesting that *Sema3a* has functions outside the nervous system that may be essential for survival (Behar et al., 1996). Consistent with this idea, *Sema-2a* is essential for viability in *Drosophila* (Kolodkin et al., 1993). In addition, semaphorin proteins have been shown to be involved in lung cancer, immune response and neuronal apoptosis (Furuyama et al., 1996; Sekido et al., 1996; Shirvan et al., 1999).

Two families of transmembrane proteins, plexins (Comeau et al., 1998; Takahashi et al., 1999; Tamagnone et al., 1999;
Winberg et al., 1998b) and neuropilins (Chen et al., 1997; Feiner et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), are known to bind semaphorins and to mediate semaphorin signaling. Notably, small GTP-binding proteins have been shown to mediate the Sema-3A stimulated collapse of growth cones (Jin and Strittmatter, 1997; Kuhn et al., 1999; Vastrik et al., 1999), otherwise little is known about the signal transduction mechanisms that mediate semaphorin functions except in Drosophila where inhibition of Rac and simultaneous activation of RhoA appear to be involved in growth cone guidance (Hu et al., 2001).

Precisely how extracellular cues like semaphorins modulate cytoskeletal components that are required for cell shape changes, including ones associated with guided movements and other changes in cell associations, remains unclear. In order to address this, we have begun a genetic analysis of semaphorins using C. elegans as a model (Roy et al., 2000). One goal of our genetic approach is to identify additional functions of semaphorin signaling in development and to reveal previously unknown components of semaphorin signaling pathways in C. elegans and in other animals.

Possible redundancy among the many semaphorin family members in the mouse would complicate a genetic approach to elucidating semaphorin function in this animal. By contrast, the C. elegans genome has only three semaphorin genes, two putative plexin receptor genes and no obvious neuropilin genes. The limited number of semaphorins and their known receptors in C. elegans enables a thorough description of their in vivo functions through genetic analysis in this animal.

Indeed, we have mutated all three known semaphorin genes and both known plexin genes in C. elegans (Roy et al., 2000) (G. Dalpe and J. G. C., unpublished; R. Ikegami and J. G. C., unpublished). We now report the isolation and phenotypic description of their in vivo functions through genetic analysis (Roy et al., 2000; Zwaal et al., 1993). Once a deletion sample was identified, sib selections were performed to isolate the homozygous deletion strains NW1358 smp-1(ev715), NW1334 smp-1(ev708) and NW1335 smp-2(ev709). AmpliTaq GOLD™ (Perkin-Elmer) was used in all PCR reactions. Primer sequences can be provided upon request. The isolated deletion alleles in both smp-1 and smp-2 were outcrossed with N2 Bristol strain or him-5(e1490) Bristol at least ten times before further analysis.

Defining the first nucleotide of the initiation codon as the first nucleotide in a DNA sequence, the smp-1(ev708) allele is deleted for nucleotide pairs 219 to 678, while smp-1(ev715) is deleted for nucleotides 5535 to 5844, and the smp-2(ev709) allele is deleted for nucleotides 2981 to 3562.

Gene specific mRNA and cDNA analysis
Total RNA was isolated using the standard Trizol© (GIBCO-BRL) protocol. A standard reverse transcription (RT) protocol (Krause et al., 1989) was used to amplify gene specific products either using oligo dT or random primers to identify all RNA populations. RT-PCR products comprising wild-type or mutant cDNAs were cloned into pBSK+ or pGEM Teasy vectors and sequenced to confirm the ORFs. RT-PCR was further used to detect mutant mRNAs in smp-1(ev715) and smp-2 (ev709). To identify RNA products of the wild-type smp-1 gene in N2, we performed northern blot analysis on 5 µg of C. elegans poly(A)+ RNA from a mixed-stage population of N2 animals. The blot was probed with a 32P-labeled 1.2 kb Ce-sema-1a cDNA isolated from a library provided by S. Kim (Stanford U.).

lacZ and gfp reporter constructs
The 3.8 kb 5′ putative regulatory region of smp-1 5′ to the initiation codon was amplified by PCR to generate an XbaI site immediately after the initiation codon and cloned into pPD95.75 to generate smp-1::lacZ (evpXSN.LZ). The same 3.8 kb sequence was cloned into pPD16.51 to generate cep-1::lacZ (evp5226) with a nuclear localization signal, and into pPD16.51 containing the nuclear localization signal, and into pPD16.51 (evpXSN.LZ) to generate smp-1::lacZ with no nuclear localization signal. Constructs evpDNTMI (a putative dominant-negative construct of smp-1 that is deleted for the semaphorin and MRS encoding sequences, retaining a signal sequence, plus transmembrane and cytoplasmic domains), evpMGS (a mini-gene that has 1.8 kb of coding sequence and is missing the cytoplasmic domain) and evpAnti-MGS (a putative antisense construct equivalent to evpMGS, but with a reversed coding sequence) were made and used for transformation.

The 2.4 kb putative regulatory region of smp-2 (5′ to the initiation codon) was amplified with gene-specific primers introducing two unique BamHI sites, one at the 5′ end and one immediately after the initiation codon. The fragment was cloned into BamHI pretreated pPD95.75 gfp reporter vector to construct the smp-2::gfp transcriptional reporter (evpVGS1B.75). All gfp and lacZ vectors were provided by A. Fire (Carnegie Mellon Institute).

smp-1 Rescuing construct
A 12.7 kb XbaI fragment carrying smp-1(+) was generated by cloning two smaller contiguous XbaI-Xmal fragments (isolated from cosmid K14B11) into pBSK+ in two steps. The construct contains the full-length smp-1(+) gene with 3.8 kb of sequence immediately 5′ to the initiation codon and 906 bp 3′ to the stop codon.
A partial 1.2 kb *smp-1* cDNA was isolated and a near full-length cDNA was constructed by RT-PCR amplification of RNA using gene-specific primers (Materials and Methods). The nucleotide sequence of RT-PCR products confirmed that the mRNA corresponds to the approximately 2.2 kb ORF for this gene predicted by the Sanger Center *C. elegans* sequencing consortium (Cambridge, UK). Northern blot analysis revealed a single *smp-1* mRNA species of approximately 2.6 kb. The ORF encodes a 713 amino acid semaphorin protein (*SMP-1* or *Ce-Sema-1a*), which comprises an N-terminal hydrophobic signal sequence for secretion, followed by a semaphorin domain, a single MRS (Met-related sequence) domain, a hydrophobic transmembrane (TM) domain and an intracellular domain of 43 residues (Fig. 1). The Sema and MRS domains together contain 14 of the 17 cysteines conserved in the semaphorin superfamily. The *SMP-1* protein is 40% and 36% identical to grasshopper and *Drosophila* Sema-1a, respectively (Fig. 1).

Cloning of *smp-2*, which encodes *C. elegans* semaphorin 1b

The sequence of linkage group I determined by the Sanger Center *C. elegans* consortium (Wilson et al., 1994) revealed the *smp-2* gene D1037.2, which encodes another semaphorin 1 homolog, *Ce-Sema-1b* (Roy et al., 2000). A near full-length cDNA of 2 kb was constructed by RT-PCR-amplification of RNA using predicted gene-specific primers (Materials and Methods) and sequence analysis of RT-PCR products confirmed that the mRNA sequence corresponds to the approximately 2 kb ORF predicted by the *C. elegans* consortium (Cambridge, UK). The ORF encodes a 676 amino acid protein (*SMP-2* or *Ce-Sema-1b*), which, like *SMP-1*, comprises an N-terminal hydrophobic signal sequence for secretion, followed by a semaphorin domain, a MRS domain, a hydrophobic transmembrane (TM) domain and an intracellular domain of 69 residues. *Ce-Sema-1b* has 26% identity to *Ce-Sema-1a* within the semaphorin domain. Both *Ce-Sema-1a* and *Ce-Sema-1b* have a consensus Furin protease cleavage site within the semaphorin domain and a putative PDZ binding domain at the C terminus (Fig. 1). These features are common to all known class 1 semaphorins.

The isolation and molecular analysis of *smp-1* and *smp-2* deletion mutants

A library of frozen worms representing 1.7 million mutagenized haploid genomes was constructed and screened using a nested PCR method (Materials and Methods). Two deletion alleles of the *smp-1* gene (*ev708* and *ev715* – formaldehyde and EMS induced, respectively) and one allele of the *smp-2* gene (*ev709*, EMS induced) were isolated. Sequence analysis showed that *ev708* comprises a deletion of 530 bp within the first intron and *ev715* comprises a deletion of 313 bp, which removes intron 8 and the exon 9 boundary, and puts the next two exons (10–11) out of frame (Fig. 2). Thus, we focused on the *ev715* deletion because it is predicted to produce a semaphorin that lacks a conserved region of the semaphorin domain of SMP-1, plus the entire MRS and TM domains. The mutant protein might be secreted, but should be severely enfeebled. In contrast to a wild-type strain, *smp-1(ev715)* mRNA was not detected by RT-PCR, indicating that
Fig. 1. Multiple sequence alignment of invertebrate semaphorins. Multiple sequence alignment of members of the class I semaphorin family using the ClustalX program. The alignment includes *Tribolium* semaphorin 1a (T-Sema-1, Accession Number L26080), Grasshopper semaphorin 1a (G-Sema-1, Accession Number L00709), *Drosophila melanogaster* semaphorin 1a and semaphorin 1b (D-Sema-1a and D-Sema-1b, Accession Numbers AAF52696 and AAF57816, respectively), *C. elegans* semaphorin 1a and semaphorin 1b (Ce-Sema-1a and Ce-Sema-1b, Accession Number Q17330 and Accession Number unpublished, respectively). Completely conserved residues are highlighted in black, similar residues in gray. *C. elegans* Ce-Sema-1a and Ce-Sema-1b predicted domains and motifs are as indicated: signal sequence for secretion (purple), semaphorin domain (yellow), Furin protease consensus cleavage site (green), Met related sequence (MRS) (blue), transmembrane domain (black) and a predicted PDZ binding domain (red).
smp-1(ev715) is likely to be a strong loss-of-function mutation (data not shown).

PCR analyses and sequencing revealed that smp-2(ev709) is deleted for a stretch of 565 bp spanning from intron 6 to exon 8 and produces a corresponding mRNA with exon 6 spliced in frame to exon 9 (Fig. 2). The missing exons encode part of the semaphorin domain and the entire MRS, predicting formation of a non-functional TM protein.

Ray 1 is displaced anterior to its normal position in smp-1 and smp-2 mutant males

smp-1(ev715) and smp-2(ev709) share a novel recessive phenotype. In both mutants, sensory ray 1 is frequently displaced to a more anterior or anterior dorsal position relative to its position in the wild type (Fig. 3, Table 1). Ray 1 is almost always shorter than normal in these mutants. These phenotypes do not affect male mating and are largely rescued by a wild-type smp-1 minigene construct (Table 1).

The adult male tail possesses nine bilaterally symmetric sensory rays protruding within a spade-shaped fan made of cuticle. In males, starting as early as the third larval stage, the posterior seam cells (V5, V6 and T) undergo additional rounds of division producing nine bilaterally symmetric ray/SET precursor cells [R(n) cells where n=1-9]. V5 generates the most anterior precursor, R1, while V6 and T generate the other ray precursors. Each ray precursor divides to produce an anterior (Rn.a) and a posterior cell (Rn.p). For ray lineages 1-6, the Rn.p cells ultimately fuse to one another to form the lateral epidermal syncytium in the tail called the SET cell. For all nine ray lineages, the Rn.a cell divides to produce four cells, one of which undergoes a programmed cell death and the other three of which ultimately form a ray sensillum.

Examination of cell shapes using jam-1::gfp as a marker for adherens junctions at cell boundaries (Simske and Hardin, 2001) reveals that the three cells derived from each Rn.a ray precursor (especially R1.a to R4.a) change positions relative to their cognate Rn.p SET precursor cell. During this process, which occurs during the late third larval stage, the smaller Rn.a progeny cells, born anterior to Rn.p, first become aligned along the ventral surface of their larger cognate SET precursor cell. As the positioning process progresses, the most posterior of the precursor cells for each ray contacts the most anterior of the precursor cells of each posterior neighboring ray (Baird et al., 1991; Roy et al., 2000). During the L4 stage, the three Rn.a progeny appear smaller and cluster together. They lose contact with cells destined to form neighboring rays, but retain contact

Table 1. Penetrance of male tail sensory ray 1 phenotype

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Temperature (°C)</th>
<th>Displaced ray</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16</td>
<td>1 (~1%)</td>
<td>214</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>3 (1%)</td>
<td>218</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>2 (1%)</td>
<td>202</td>
</tr>
<tr>
<td>smp-1(ev715)</td>
<td>16</td>
<td>88 (43%)</td>
<td>205</td>
</tr>
<tr>
<td>smp-1(ev715)</td>
<td>20</td>
<td>82 (41%)</td>
<td>201</td>
</tr>
<tr>
<td>smp-1(ev715)</td>
<td>25</td>
<td>188 (88%)</td>
<td>214</td>
</tr>
<tr>
<td>smp-2(ev709)</td>
<td>16</td>
<td>14 (7%)</td>
<td>205</td>
</tr>
<tr>
<td>smp-2(ev709)</td>
<td>20</td>
<td>42 (15%)</td>
<td>279</td>
</tr>
<tr>
<td>smp-2(ev709)</td>
<td>25</td>
<td>45 (18%)</td>
<td>250</td>
</tr>
<tr>
<td>smp-2(ev709)smp-1(ev715)</td>
<td>16</td>
<td>199 (88%)</td>
<td>227</td>
</tr>
<tr>
<td>smp-2(ev709)smp-1(ev715)</td>
<td>20</td>
<td>184 (91%)</td>
<td>202</td>
</tr>
<tr>
<td>smp-2(ev709)smp-1(ev715)</td>
<td>25</td>
<td>183 (90%)</td>
<td>203</td>
</tr>
<tr>
<td>smp-1(ev715)smp-1(ev715)evEx138[smp-1(+)]</td>
<td>25</td>
<td>9 (11%)</td>
<td>83</td>
</tr>
<tr>
<td>smp-2(ev709)/sDf4</td>
<td>20</td>
<td>19 (15%)</td>
<td>125</td>
</tr>
</tbody>
</table>

*All strains contain him-5(e1940).
with their cognate Rn.p cell (SET cell precursor). Each of the ray 1 to ray 4 clusters normally becomes situated at the ventral end of the junction between its cognate SET precursor (Rn.p) and the next most posterior SET precursor (Rn+1.p). The clusters are destined to form the individual rays of the male tail and comprise two sensory neurons and a support cell that aids in attachment of the sensory dendrites to the cuticle (Nguyen et al., 1999; Sulston et al., 1980). At some point during the positioning process, the three cells that ultimately form each ray attach their distal (dendritic) ends to the cuticle and their cell somata sink below the epidermis pulling out the sensory processes of the ray sensillae.

As the three-cell ray clusters separate from each other, the SET precursors elongate towards the anterior and begin to fuse with their neighbors. Later in L4 stage, the entire male tail retracts towards the anterior, leaving behind the ray sensillae surrounded at their ends by epidermis and cuticle. These are visible as papillae protruding from the lateral surface of the cuticular spade-shaped fan. During this retraction, the ray processes, which are in contact with the lateral SET cell, lose contact with the SET and become surrounded by hyp 7. Ray 1 is normally the last of the rays to lose contact with the SET cell (Nguyen et al., 1999; Sulston et al., 1980).

In the smp-1 and smp-2 mutants, the three-cell cluster that will comprise ray 1 is often displaced well anterior to its normal position soon after the ray 1 cluster normally separates from the ray 2 cluster (Fig. 4). In the most extreme case, the three-cell cluster representing ray 1 may be found at the anterior end of its clonally related R1.p cell, even before the latter cell fuses with R2.p to begin to form the syncytial SET cell. Interestingly, ray 1 frequently fails to lose contact with the SET cell, and remains attached to the SET cell during male tail retraction. In adult mutant males, every displaced ray 1 is associated with a SET cell extension (Fig. 4D), which probably forms because of its continued attachment to the ray 1 cells during anterior retraction. Thus, the semaphorin 1 mutants in C. elegans clearly cause a persistent adhesion between the SET cell and cells of ray 1, in addition to causing a failure to change completely positions relative to their cognate Rn.p cell. It is likely that there is a causal connection between perdurant ray 1 adhesion to the SET cell, which manifests later in development, and the displacement of the ray 1 cluster, which manifests earlier (see Discussion).

smp-1 and smp-2 act in parallel to determine ray 1 position

In smp-1(ev715) and smp-2(ev709), the ray 1 defects are 41% and 15% penetrant, respectively, at 20°C. In the double mutant, the penetrance is enhanced to 91% at 20°C (Table 1). In addition, at 20°C smp-2(ev709)/sDf4 heterozygotes have the same penetrance of displaced ray 1 defects as smp-2(ev709) homozygotes, showing that smp-2(ev709) behaves as a genetic null mutation (Table 1). smp-1(ev715)/eDf14 deficiency heterozygotes were also made; however, misplaced rays were more difficult to identify because the deficiency heterozygotes also harbored ray fusions (not shown). Preliminary data indicate that the ev715 mutation causes a strong loss of function (data not shown). The double mutant results can therefore be safely interpreted as demonstrating that at 20°C SMP-1 and SMP-2 function in parallel to regulate placement of ray 1 and detachment of the ray 1 cluster from the SET cell. At 25°C SMP-2 may play a minor role in ray 1 positioning because at this temperature smp-1(ev715), but not smp-2(ev709), exhibits increased penetrance of displaced R1 rays to 88% (Table 1).

smp-1 is partially required for correct morphogenesis of hyp 3 and hyp 4 in the C. elegans head

In approximately 3-4% of smp-1(ev715) larvae, the buccal opening is positioned to one side (anywhere from ventral to lateral) of the head (displaced mouth phenotype; Table 2). We analyzed head epidermal cell shapes and positions using jam-1::gfp to mark junctions between epidermal cells. As described previously (Sulston et al., 1983), during embryogenesis, head epidermal precursors arrange into six dorsally located bands of cells that extend along the dorsoventral axis. Each band is a precursor for a head epidermal cell. Later in embryogenesis, the
most ventral of the cells in each band extend ventrally and meet their contralateral counterpart at the ventral midline. All of the cells in each band ultimately fuse to form toroidal-shaped epidermal cells (hyp 1 to hyp 6) that encircle the head of the embryo. These cells are visible by jam-1::gfp immediately after hatching (Fig. 5). Ventral enclosure of the head, which is completed by the fusion of equivalent contralateral cells at the ventral midline occurs before enclosure of the body and involves visibly different, but possible molecularly related mechanisms (see Discussion).

In smp-1(ev715) animals with misplaced buccal openings, hyp 3 and hyp 4 are abnormally narrow at their ventral sides compared with their dorsal sides (Fig. 5). As a result, the anterior faces of hyp 3 and hyp 4 are tilted relative to the anterior/posterior axis, and cells anterior to hyp 4 are also tilted. This causes the pharynx, which is attached to arcade cells anterior to hyp 2 (as in the wild type) to be similarly tilted, hence the formation of a displaced mouth.

**smp-1 and smp-2 are required for correct morphogenesis of hyp10**

Both mutants show defects in the morphogenesis of the tail tip,

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**Fig. 4.** Perdurant SET cell attachment to ray 1 in the sema-1 mutant males. Male tail epidermal cells and ray precursors clusters are visualized using jam-1::gfp to mark adherens junctions (A-E). Male tail ray 1 and 2 precursor clusters are indicated (arrows). In the wild type (A), the clusters are closer together than in the smp-1 and smp-2 mutants (B). In the smp-1 and smp-2 mutants, the displaced ray 1 always remains connected to the seam syncytium (Set cell) in the adult male tail (D,E), which differs from the wild type (C).

**Fig. 5.** Morphological defect in hyp4 of smp-1 mutant animals results in a displaced mouth phenotype. DIC microscopy reveals a mouth displaced to the ventral side in the mutant (B, arrow) compared with the wild type (A, arrow). (C,D) jam-1::gfp is used to visualize adherens junctions between hypodermal cells. Arrows indicate buccal opening (mouth). (E,F) Analysis of contacts between the mutant (F) and wild-type (E) head epidermal cells are summarized. Instead of forming a cylindrical shaped hyp 4 cell and cylindrical head as in the wild type (A,C,E), in smp-1 (ev715) (B,D,F) hyp 4 forms an abnormal contact with itself (failure to fuse) and probably with hyp 3 and hyp 5 that results in torrid-shaped cells that are thinner on the ventral side. The oddly shaped hyp 4 presumably causes tilting of the pharynx toward the ventral side and consequent formation of a displaced mouth.
the structure that is formed by epidermal cell 10 (hyp 10), which is the only binucleate epidermal cell in the tail. The typical split tail defect is shown in Fig. 6, but it can range from unequally split tails to a finger-like pair of thick protrusions (not shown). Based on our analysis of epidermal cell shapes (determined with jam-1::gfp to mark epidermal cell junctions), hyp 10 appears to form wild-type connections with surrounding cells, hyp 9 and hyp 11, in spite of the defect in tail tip morphogenesis.

The penetrance of the split tail phenotype is approximately 56% in smp-2(ev709) and 25% in smp-1(ev715) at 22°C (Table 2). Interestingly, this defect can be observed only in early larval stages and is corrected as animals develop into adults (data not shown). The tip of the C. elegans tail in the first larval stage consists of hyp 10; however, during early post-embryonic development, hyp 9 elongates towards the posterior and together with hyp 10 forms the tip of the tail. The disappearance of the split tail phenotype as mutant animals mature suggests that as hyp 9 rearranges relative to hyp 10, it corrects the split tail phenotype. A similar defect and correction has been observed in cdh-3 cadherin mutants of C. elegans (Pettitt et al., 1996).

smp-1 and smp-2 mutants have pleiotropic defects in body morphology

Immediately after hatching, smp-1(ev715) and smp-2(ev709) mutant larvae exhibit various defects in body morphology, such as a swollen head or other deformities (variable abnormal or Vab defects) (Fig. 6). These defects are initially observed in less than 10% of mutant animals (Table 2). Interestingly, mab-20(ev574) (Ce-sema-2a) mutant animals exhibit more severe body morphology defects than do smp-1 and smp-2 mutants (Roy et al., 2000). However, in mab-20 mutants, the lateral epidermal body seam cells are often positioned abnormally and make ectopic contacts with several lateral seam cells causing severe bulges in the body during elongation (Roy et al., 2000).

In smp-1 and smp-2 mutants, the lateral epidermal body seam cells do not make ectopic contacts with other seam cells, but they can have an altered orientation or position, aligning themselves perpendicular or parallel to neighboring cells, but usually still contacting both neighbors on either side (Fig. 6). Bumps on the body observed in less than 10% of young larvae are always associated with incorrectly oriented or positioned seam cells (Fig. 6). Animals exhibiting this phenotype assume a normal shape as development proceeds (data not shown). The lateral seam cells are known to fuse into a single large lateral syncytium during the third larval stage, so this fusion may correct the body bulges caused by abnormally oriented individual seam cells. Taken together, these results suggest that smp-1 and smp-2 are required for correct orientation and positioning of the lateral body epidermal cells during embryogenesis, but these defects fail to manifest as a bump in the adult because the larval lateral seam cells later fuse to form a normal adult lateral seam.

Defecation defect in smp-1 mutants

The defecation cycle in C. elegans comprises a well-characterized series of reproducible events resulting in expulsion of contents from the posterior intestine through the anus. smp-1(ev715) and smp-2(ev709) animals were examined for defecation cycle defects using a standard procedure that was described previously (Thomas, 1990). smp-2(ev709) animals have neither a defect in the expulsion frequency nor in the duration of the defecation cycle (Table 3). smp-1(ev715) mutants, however, exhibit a severe reduction in the expulsion frequency with only one in ten defecation cycles resulting in expulsion. Thus, smp-1(ev715) animals become constipated, sluggish and sick. These results correlate with the defecation phenotype observed in transgenic animals expressing antisense, putative dominant-negative and wild-type forms of smp-1 under control of smp-1 5′ regulatory sequence (Materials and Methods) (Roy, 1999).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Displaced mouth*</th>
<th>Split tail*</th>
<th>VAB*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0/221 (0%)</td>
<td>4/221 (1.8%)</td>
<td>0/221 (0%)</td>
</tr>
<tr>
<td>smp-1(ev715)</td>
<td>13/370 (3.5%)</td>
<td>94/370 (25.4%)</td>
<td>42/370 (7.5%)</td>
</tr>
<tr>
<td>smp-2(ev709)</td>
<td>0/331 (0%)</td>
<td>259/462 (56%)</td>
<td>18/331 (5.4%)</td>
</tr>
<tr>
<td>smp-1(ev715) smp-2(ev709)</td>
<td>6/309 (1.9%)</td>
<td>25/309 (8.1%)</td>
<td>22/309 (7.1%)</td>
</tr>
</tbody>
</table>

*Experiments were conducted at 22°C.
†Apparent suppression may result from mutual antagonism.
**smp-1(ev715) is required for fecundity and survival**

Six *smp-1(ev715)* mutant hermaphrodites laid a total of 344 fertilized eggs of which 179 or 52% hatched into viable progeny. The brood size varied from 15 to 125 with an average of 57.3 (s.d.=41.7). On average, 29.8 (s.d.=22.37) of the fertilized eggs hatched into viable progeny. The *smp-1(ev715)* worms do not have an egg-laying defect and gonad morphology is normal. When individual *smp-1(ev715)* hermaphrodites were mated to *him-5(e1490) mec-7::gfp* males in an attempt to rescue the low brood size with normal sperm, the total brood size from five crossed hermaphrodites was 187 (average of 37.4%, s.d.=20.8), of which 172 (average of 34.4, s.d.=17.2) or 92% hatched. Thus, no significant increase in the number of fertilized eggs was observed; however, a significantly higher percentage of fertilized eggs were viable (92% versus 52%). These results exclude sperm defects as the cause of the low brood size of *smp-1(ev715)* self-fertilization progeny.

In order to examine a possible parental effect on viability further, we cloned 6 heterozygous *smp-1(ev715)/+* hermaphrodite larvae and assayed the genotypes of their progeny by PCR. These animals produced an average of 164 (s.d.=39) viable progeny with normal Mendelian ratios (of 142 viable progeny examined by PCR, 32 were homozygous wild type, 40 were homozygous mutant and were 70 heterozygous) for *smp-1(ev715)* segregation. The brood size was also partially rescued to an average of 123 (s.d.=30) progeny (n=4) when a wild-type *smp-1(+)* gene was introduced into *smp-1(ev715)* by transgenesis (Materials and Methods). Taken together, these results indicate that expression of *smp-1* in the maternal parent appears to be both necessary and sufficient for embryonic survival (Wood et al., 1980).

**smp-1 and smp-2 mutants exhibit mild defects in guidance of PLML and PLMR axons**

As the semaphorins are known to affect *Drosophila* and vertebrate axon growth cone migrations in vivo and in vitro (Culotti and Kolodkin, 1996; Van Vactor and Lorenz, 1999), we decided to look for axon guidance defects in *smp-1* and *smp-2* mutants. DA and DB motor axons were examined by expression of neuron-specific *unc-129::gfp* (Colavita et al., 1998), amphid and phasmid axons were examined by Dil staining (Perkins et al., 1986) (E. Hedgecock, personal communication), and touch receptor axons (ALMs, PLMs, A VM and PVM) were examined with a *mec-7::gfp* reporter (gift of M. Driscoll and C. Kenyon). Motor, phasmid and amphid axons were normal; however, the PLM axons were misguided in 15 out of 137 *smp-1(ev715)* animals and in four out of 116 *smp-2(ev709)* animals. All PLM axons were normal in 100 wild-type animals (Table 4, Fig. 7). Most of the defective migratory paths of PLM axons (Table 4, Fig. 7).

### Table 3. Expulsion frequency defect in *smp-1*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Expulsion frequency (%) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td><em>smp-1(ev715)</em></td>
<td>7</td>
</tr>
<tr>
<td><em>smp-2(ev709)</em></td>
<td>100</td>
</tr>
<tr>
<td><em>smp-1(ev715)</em>/smp-2(ev709)</td>
<td>5</td>
</tr>
</tbody>
</table>

*In each experiment, 10 worms were assayed for at least 11 sequential defection cycles.

### Table 4. Axon guidance defects of Ce-Sema-1a and Ce-Sema-1b

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Temperature (°C)</th>
<th>Misguided PLML/PLMR axons</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>0 (0%)</td>
<td>100</td>
</tr>
<tr>
<td><em>smp-1(ev715)</em></td>
<td>25</td>
<td>4 (3.2%)</td>
<td>125</td>
</tr>
<tr>
<td><em>smp-1(ev715)</em></td>
<td>20</td>
<td>15 (10.9%)</td>
<td>137</td>
</tr>
<tr>
<td><em>smp-2(ev709)</em></td>
<td>25</td>
<td>15 (12%)</td>
<td>125</td>
</tr>
<tr>
<td><em>smp-2(ev709)</em></td>
<td>20</td>
<td>4 (3.4%)</td>
<td>116</td>
</tr>
<tr>
<td><em>smp-2(ev709)</em></td>
<td>25</td>
<td>14 (13%)</td>
<td>105</td>
</tr>
<tr>
<td><em>smp-1(ev715)</em></td>
<td>20</td>
<td>11 (10.4%)</td>
<td>105</td>
</tr>
<tr>
<td><em>smp-2(ev709)</em>/smp-1(ev715)</td>
<td>25</td>
<td>23 (21%)</td>
<td>109</td>
</tr>
</tbody>
</table>

*All strains analyzed contain *mec-7::gfp* integrated transcriptional reporter and *him-5(e1490)*.
axons in the mutants can be described as an initial anterior-ventral migration followed by an abrupt dorsal turn to a dorsolateral position. In addition, premature cessation of migration and unusual branching of the axons were observed. Based on these results, we suggest *smp-1* and *smp-2* are partially required, directly or indirectly, for precise guidance of the PLML and PLMR axons.

### Expression patterns of Ce-Sema-1a (*smp-1*) and Ce-Sema-1b (*smp-2*) transcriptional reporters

One *smp-2* and three *smp-1* expression vectors were constructed using a gene-specific 5’ regulatory region to drive expression of *lacZ* or *gfp* reporters (see Materials and Methods). Transcriptional reporters of this kind often reflect the expression pattern determined by transgenes encoding GFP-tagged proteins capable of rescuing the mutant phenotype (Chan et al., 1996; Nash et al., 2000; Steven et al., 1998; Su et al., 2000). However, this not always the case, so the following descriptions of transcriptional reporter expression are meant only to reveal what are likely to be some aspects of the expression patterns in need of further validation.

In animals transgenic for the *smp-1::lacZ* reporter constructs (Materials and Methods), β-galactosidase is first detected in epidermal cells at the beginning of morphogenesis 200 minutes after fertilization. GFP fluorescence from *smp-1::gfp* expression is initially observed at approximately the 50 cell stage in the E lineage (Fig. 8). Later, in larvae and adults, GFP can be seen in all body wall, vulval, uterine and enteric muscles, as well as male-specific muscles of the tail and copulatory system. In adults, *smp-1::gfp* is expressed in the tail tip (hyp 10), in ray 6 and in the spicules of the adult male. Approximately 10 sensory neuron support cells in the head with dendrites extending to the tip of the head, also express the *smp-1* GFP and β-galactosidase transcriptional reporters (not shown). GFP fluorescence is observed in several individual cells, including an interneuron (tentatively identified as AVL; Fig. 9D), the excretory channel, the distal tip cells (DTCs) throughout their migration, somatic cells of the gonad (data not shown), and epidermal cells hyp 4 (and possibly hyp 3) and hyp 10 (Fig. 9A,B). In the adult, we observed expression in the fused seam cell syncytium comprising the lateral epidermis. Although ray 6 expresses in the adult male tail, it is difficult to determine whether the ray 6 precursors or any other ray or SET precursors or progeny express *smp-1::gfp* during the L3 and L4 stages of development when the male tail is forming. This is because GFP fluorescence in the male sex-specific muscles is so bright at this stage as to obscure what may be faint expression of other cells in the male tail.

Expression of the *smp-2::gfp* transcriptional reporter is detected initially in twofold embryos, in one mononucleate pharyngeal muscle cell and in intestinal cells (Fig. 8G,H). In the L1 stage, GFP fluorescence is observed in one mononucleate pharyngeal muscle cell m6, in all intestinal cells, in the head epidermal cells, in a restricted number of body wall muscles (Fig. 9C), in inner labial sensory neurons, and tentatively in URA VL/R and URADL/R motorneurons. In L4 larvae and adults, GFP fluorescence is restricted to intestinal cells and pharyngeal muscle cell m6. During larval development of the male tail, GFP expression is observed only in the hook. In adult males, rays 8 and 9, and the tail bursa express *smp-2::gfp*. *smp-2::gfp* is not seen in hyp 9 or hyp 10.

## DISCUSSION

Semaphorins in vertebrates and invertebrates have been shown to function in regulating growth cone migration during development and also to play a role in other processes such as malignant metastasis, immune responses and apoptosis.
C. elegans morphogenesis

(Furuyama et al., 1996; Sekido et al., 1996; Shirvan et al., 1999). Although the semaphorins play a role in axon guidance in C. elegans, their major role in this animal appears to be in regulating cell shape changes and cell associations (Roy et al., 2000) (this paper). We have shown that smp-1 and smp-2 mutants share defects in male tail ray positioning, defects in apparently different aspects of head, body and tail epidermal cell morphogenesis, and defects in axon guidance. In addition, smp-1(ev715) has specific defects in defecation and fecundity. Taken together, these results suggest that smp-1 and smp-2 have multiple functions in C. elegans development.

The developmental functions of semaphorins in C. elegans may be more closely related to the non-neuronal functions of semaphorins that have been revealed in mice. For example, mice mutant for Sema3a have rib and vertebrae fusions plus cardiac malformations and cardiovascular defects (Behar et al., 1996; Taniguchi et al., 1997), and mice that overexpress the neuropilin 1 receptor have cardiovascular defects (He and Tessier-Lavigne, 1997; Kitsukawa et al., 1995). In C. elegans, all of the non-neuronal functions of semaphorins could reflect different manifestations of a similar effect of semaphorin signaling on the cytoskeletal machinery of interacting cells. Whether the same is true of the non-neuronal effects of semaphorins deduced from mouse mutants or from transgenic mice with alterations in semaphorin signaling remains to be examined.

smp-1 and smp-2 affect the movement of the precursor cells of the ray 1 relative to the cognate lateral epidermal seam cell

Each male ray that protrudes from the tail in the L4 and adult stages of development comprises two neurons and a support cell. In mutants of smp-1 and smp-2, the ray 1 sensillum is frequently located well anterior to its normal position next to ray 2. As the best understood role of semaphorins is in regulating cell movements, especially axon growth cone migrations, it is tempting to speculate that the R1 cells are displaced because they or their common precursor migrate abnormally when many cells are rearranging to form the male tail (Nguyen et al., 1999). Using jam-1::gfp to examine mutant ray precursor cells when they form three-cell clusters in the L4 stage, the ray 1 cluster is frequently further anterior to the ray 2 cluster than in the wild type (Fig. 4A,B). This anterior displacement is most likely caused by a defect in the posterior movement of the three R1.a-derived ray precursors relative to their cognate SET precursor R1.p.

Fig. 9. Post-embryonic stage expression patterns of smp-1 and smp-2 visualized by gfp transcriptional reporters. smp-1::gfp expression was observed in all four quadrants of body wall muscle, all four enteric muscles, all sex-specific muscles of hermaphrodite and male, in distal tip cells throughout their migration and in cells of the head tentatively identified as sensory neuron support cells (not shown). We also observed smp-1::gfp expression in epidermal cells hyp 4, in the head (A, arrowhead), and hyp 10, in the tail whip (B, arrowhead), gonadal sheath cell, sperm and excretory system (data not shown). (D) We have also tentatively identified inner labial neurons (data not shown) and the AVL neuron cell body (arrowhead) and axon (arrow), which is located in the nerve ring and sends its axon along the ventral nerve cord into the tail. (C) smp-2::gfp expression was observed in a mononuclear pharyngeal cell (m6) (open arrow), in tentatively identified URAV/D motoneurons and inner labial neurons, in intestinal cells and in the sphincter muscle (data not shown). smp-2::gfp is also expressed in the body wall and head muscle quadrants (C, white arrow and white arrowhead, respectively).
with its cognate SET cell precursor, but not with its posterior neighboring SET cell precursor. In this model, growth of ventral SET cell membrane in the region between these two proposed points of association with the ray cluster could displace the more anterior ray even further anterior in the mutants. This is a more complex model, but can not be ruled in or out without a more thorough analysis of the timing of ray dendrite attachment to the epidermis (which probably fixes the relative distances between neighboring rays), relative to the establishment of contacts with SET cell precursors. Unfortunately, the expression patterns of the \textit{smp-1} and \textit{smp-2} transcriptional reporters described here do not provide any obvious insights into the cellular and molecular mechanisms involved in the ray 1 displacement, but are consistent with head and tail tip defects.

\textbf{\textit{smp-2} functions in parallel to \textit{smp-1} in male tail morphogenesis}

Our observation of an increased penetrance of the male tail ray 1 trait in \textit{smp-2(ev709) smp-1(ev715)} double mutants relative to each single mutant, coupled with the finding that \textit{smp-1(ev715)} and \textit{smp-2(ev709)} behave as strong loss-of-function or genetic null alleles, respectively, indicates that \textit{smp-2} acts in parallel to \textit{smp-1} at 20°C. Thus, the transient connection between ray 1 and the lateral epidermis of the tail (SET cell) that normally dissociates at the L4 stage, instead becomes stabilized in the \textit{smp-1} and \textit{smp-2} single mutants and more so in the double mutant at 20°C. This indicates that \textit{smp-1} and \textit{smp-2} act in parallel to regulate ray attachment to the SET cell. However, analysis of the \textit{smp-1(ev715)} ray 1 defect at various temperatures indicate that \textit{smp-1} plays a much larger role than \textit{smp-2} in regulating the connection between ray 1 and the SET cell at 25°C (Table 1).

\textbf{Do the Sema-1 proteins have a role in destabilizing transient contacts between ectodermal cells?}

In contrast to MAB-20 (Ce-Sema-2a), which primarily regulates transient contacts between three-cell male tail ray clusters during morphogenesis (Roy et al., 2000), \textit{smp-1} and \textit{smp-2} may regulate transient contacts of individual rays with the lateral epidermal SET cell (Fig. 4D) [see Baird et al. (Baird et al., 1991) and Roy et al. (Roy et al., 2000) for examples of abnormally perdurant contacts of fused rays with SET cells in \textit{mab-20} mutants]. The proposed ability to regulate transient contacts between ectodermal cells observed in \textit{C. elegans} correlates with the more studied ability of semaphorins to regulate axon guidance in vertebrates, as growth cone migrations require the ability to make and break contacts with other cells or with the extracellular matrix. The ability to break a transient contact during growth cone migration may be equivalent (on a shorter time and smaller spatial scale) to the ability of semaphorins to regulate transient contacts between epidermal cells during development. It may not be surprising, therefore, that \textit{smp-1} and \textit{smp-2} mutants revealed mild but specific effects on PLMR and PLML axon growth cone migrations in \textit{C. elegans} (Table 4; Fig. 7).

\textbf{\textit{smp-1} functions in epidermal cell interactions at the ventral midline involved in enclosure of the head}

\textit{smp-1} has a cell specific function in morphogenesis of hyp 3 and hyp 4 that results in a tilted pharynx and displaced buccal opening. Use of the adherens junction marker \textit{jam-1::gfp} to mark cell boundaries, indicates hyp 3 and hyp 4 are narrower on one side (usually the ventral side, but varies to lateral) than on the opposite side in \textit{smp-1(ev715)} animals with a displaced buccal opening. Thus, \textit{smp-1} is partially required for morphogenesis of one region of the hyp 3 and hyp 4 cells.

During embryogenesis, hyp 4, and other head epidermal cell bodies align in bands around both sides of the head. The most ventral of these cells on each side extends to the ventral midline, where it meets its contralateral homolog, forms an adherens junction then fuses with it. Fusions of all the cells within each band forms a toroidal-shaped cell encircling the head. The fact that the ventral side of hyp 3 and hyp 4 is narrower than the dorsal side in the mutants suggests that there is something wrong with contacts that normally form on the ventral side. The defects may be in hyp 4 to hyp 4 or in hyp 3 to hyp 3 contacts or in contacts between hyp 4 and its flanking neighbors, hyp 3 and hyp 5. Indeed, among the animals with an displaced buccal opening, hyp 4 always fails to fuse to itself as evidenced by an adherens junction evident on the narrow side of the cell (Fig. 5). In at least some of these cases, hyp 3, hyp 4 and hyp 5 frequently appear to be in direct contact with one another (Fig. 5F). Thus, \textit{smp-1} could be required to break a three-way contact between hyp 3, hyp 4 and hyp 5. The failure to break this contact may prevent hyp 3 and hyp 4 from growing locally in girth. A defect in the shape of hyp 4 and its attachment to flanking hyp 3 and hyp 5 cells is consistent with the expression pattern of \textit{smp-1::gfp} reporter in head epidermal cells, which include hyp 4 (and possibly hyp 3) (Fig. 9) and suggests that \textit{smp-1} may have a cell-autonomous function in hyp 4 morphogenesis.

The low penetrance of the displaced mouth defect suggests that \textit{smp-1} is redundant with other molecules that modulate transient cell contacts during \textit{C. elegans} head enclosure. In some respects, the defect in ventral midline interactions of the head epidermal cells in \textit{smp-1(ev715)} are reminiscent of the lateral enclosure defects in the body epidermis observed in mutants of \textit{Ce-sema-2a} (Roy et al., 2000). In the wild type, the leading epidermal P cells on left and right sides migrate ventrally during epibole and meet their counterparts from the other side at the ventral midline, before intercalating into a single row of cells. In \textit{mab-20} mutants, equivalent cells from both sides not only contact one another at the ventral midline, but they appear to form normal stable contacts with other P cells even before they attempt to intercalate. Abnormal contacts between head epidermal cells at the ventral midline may also account for the displaced buccal openings observed in \textit{smp-1} mutants. Some redundancy in the ventral enclosure of head epidermal cells of \textit{smp-1} with Sema-2a is suggested by the enhancement of \textit{smp-1} mutant displaced mouth defects by \textit{mab-20(bx61)} (V. E. G., unpublished). We have not attempted to quantify ventral enclosure by body epidermis (i.e. migrating P cells and followers) in \textit{smp-1 mab-20} double mutants to determine if \textit{smp-1} plays a redundant role with Sema-2a in this process. However, our initial results at the very least suggest that both Sema-1a and Sema-2a may be involved in epidermal cell associations at the ventral midline that lead to proper positioning of the buccal opening.

Another defect shared by mutants of \textit{sema-2a}, \textit{sema-1a} and \textit{sema-1b} is the split tail phenotype. We have not examined this phenotype in double or triple mutants that include alleles of
Ce-sema-2a (also known as mab-20), so we do not know if the secreted and TM semaphorins act in the same or in parallel pathways. The expression of smp-1::gfp in the posterior tail whip as early as the L2 stage (Fig. 9D) is consistent with a role for SMP-1 in regulating tail tip morphology.

**Defecation defect**

The smp-1 mutant defecation defect correlates with the expression of the smp-1::gfp transcriptional reporter in all enteric muscles and in the single AVL AVL interneuron (Fig. 9D). AVL synapses onto the DVB neuron, which is the only neuron that innervates the enteric muscles. This raises the interesting possibility that Sema-1a is involved in determining synapse formation in *C. elegans*, perhaps similar to the demonstrated role of *Drosophila* Sema-2a in formation of neuromuscular junctions (Culotti and Kolodkin, 1996; Winberg et al., 1998a). Further examination of this exciting possibility will require ultrastructural analysis of the synapses between AVL and DVB or between enteric muscle.

**Conclusion**

The phenotypes of semaphorin mutants of *C. elegans* indicate that the semaphorins are largely required either for preventing initiation of inappropriate contacts between cells or for breaking normally transient contacts that are made between cells during wild-type development. For certain cell interaction defects observed in the semaphorin mutants (e.g. interactions at the ventral midline), it is difficult to distinguish which, if either, of these proposed roles is correct. However, the perdurance of an initially normal contact between ray 1 and the male tail lateral epidermis (SET cell) in *sema*phorin 1a and semaphorin 1b mutants is more consistent with a role in breaking normally transient contacts between cells. It is tempting to generalize that the semaphorins provide cells with the ability to break contacts with other cells, particularly as this would fit the known role of semaphorins in cell and growth cone migrations, which are known to involve cycles of contact formation and breakage.

We thank Gratien Dalpe for independently discovering and helping to interpret the ray 1 phenotype described in this paper. We also thank R. Ikegami, A. Spence and J. Rossant for many helpful discussions, to interpret the ray 1 phenotype described in this paper. We also thank the laboratory of Caenorhabditis Genetic Center (which is funded by the NIH National Center for Research Resources) for providing strains, and A. Fire for providing the GFP reporter vectors. We also thank the laboratory of Dr A. Rose for developing the Genetic Toolkit, which was used in these studies. This work was supported by a grant from the C.I.H.R. to J. C. and a Canadian OGSST postgraduate scholarship to V. E. G.

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