SRC-1, a non-receptor type of protein tyrosine kinase, controls the direction of cell and growth cone migration in C. elegans

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Summary
Src family tyrosine kinase (SFK) has been implicated in the regulation of cell adhesion and migration during animal development. We show that SRC-1, an ortholog of SFK, plays an essential role in directing cell migration in Caenorhabditis elegans. The mutation in the src-1 gene results in defective distal tip cell (DTC)-directed gonad morphogenesis in an activity-dependent and DTC cell-autonomous manners. In the src-1 mutants, DTCs fail to turn and continue their centrifugal migration along the ventral muscles. The effect of the src-1 mutation is suppressed by mutations in genes that function in the CED/Rac pathway, suggesting that SRC-1 in DTCs is an upstream regulator of a Rac pathway that controls cytoskeletal remodeling. In the src-1 mutant, the expression of unc-5/netrin receptor is normally regulated, and neither the precocious expression of UNC-5 nor the mutation in the unc-5 gene significantly affects the DTC migration defect. These data suggest that SRC-1 acts in the netrin signaling in DTCs. The src-1 mutant also exhibits cell-autonomous defects in the migration and growth cone path-finding of Q neuroblast descendants A VM and PVM. However, these roles of SRC-1 do not appear to involve the CED/Rac pathway. These findings show that SRC-1 functions in responding to various extracellular guidance cues that direct the cell migration via disparate signaling pathways in different cell types.

Key words: Src family tyrosine kinase, SRC-1, Cell migration, Axon guidance, C. elegans

Introduction
Cell migration is crucial for the development of multicellular animals (Hedgecock et al., 1987; Antebi et al., 1997). Concerted cell movements are required to establish the tissue layers during gastrulation, individual cell migration contributes to tissue organization and the establishment of the body pattern, and growth cone migrations help construct the neural network. Such cell and growth cone migrations are guided by extracellular cues that provide polarity information to the cell in the form of substratum-bound or diffusible molecules (Tessier-Lavigne and Goodman, 1996; Varela-Echavarria and Guthrie, 1997). However, it remains unclear how these extracellular cues are transduced by the cell into the cytoskeletal and molecular motor activities that result in cell migration.

The gonad of a Caenorhabditis elegans hermaphrodite is located in the anterior-right and posterior-left areas of the body cavity (Fig. 1A). This bilobed gonad develops during larval development from a 4-cell primordium positioned in the ventral midbody. The shape of the two gonad arms is determined by the migratory path of a distal tip cell (DTC) at the leading edge of each arm (Kimble and Hirsh, 1979; Hedgecock et al., 1987). DTC migration proceeds through three sequential linear phases (Fig. 1B). Phase I is the centrifugal migration along the ventral bands of the body wall muscles away from the midbody. Phase II begins with a right-angled turn of DTC, after which it migrates along the inner surface of the epidermis from the ventral to the dorsal muscle bands. Finally, in phase III, DTC makes another right-angled turn and then migrates centripetally along the dorsal muscle bands back towards the midbody (Fig. 1B) (Kimble and Hirsh, 1979; Hedgecock et al., 1987).

DTC migration is regulated by several extracellular guidance cues (Hedgecock et al., 1987; Leung-Hagesteijn et al., 1992; Blelloch et al., 1999; Montell, 1999; Lehmann, 2001). The ventral-to-dorsal migration of DTC during phase II is mediated in part by the netrin family protein UNC-5 and its receptors UNC-5 and UNC-40 (Hedgecock et al., 1990; Culotti and Merz, 1998). UNC-5 and UNC-40 are expressed by DTC, whereas UNC-6 is expressed by the ventral body wall muscles. These observations suggest that UNC-5 and UNC-40 mediate the chemorepulsion of DTC away from the ventrally expressed UNC-6 (Wadsworth et al., 1996; Su et al., 2000). It has also been suggested that UNC-129/TGFβ is involved in DTC migration during phase II (Colavita et al., 1998; Nash et al., 2000).
Development

The extracellular cues directing the migration of neuronal cells and growth cones in *C. elegans* (Zipkin et al., 1997; Steven et al., 1998; Lundquist et al., 2001; Lundquist, 2003). Furthermore, the regulation of the actin cytoskeleton by UNC-34/Enabled and UNC-115/abLIM, which act downstream of UNC-40/DCC, is implicated in the axon guidance of the AVM and DA/DB motoneurons and the axon pathfinding of the CAN and PDE neurons (Yu et al., 2002; Giti et al., 2003; Struckhoff and Lundquist, 2003; Chang et al., 2004). Although these molecules, together with small G proteins, are potential effectors that drive cell migration, the signaling pathways that directly relay the extracellular guidance cues to these effectors of cell migration are still unclear.

Tyrosine phosphorylation is required for crucial functions in multicellular animals such as cell differentiation, cell adhesion and migration, axon guidance and cell-cell communication (Hunter, 2000). The Src family of non-receptor protein tyrosine kinases (SFKs) serves as a crucial molecular switch that transmits extracellular cues into the intracellular tyrosine phosphorylation events that lead to the cellular responses (Brown and Cooper, 1996; Sicheri and Kuriyan, 1997; Thomas and Brugge, 1997). In *C. elegans*, there are two SFK orthologs, *src-1* and *src-2kin-2* (Bei et al., 2002; Hirose et al., 2003). A deletion allele of *src-1*, *cj293*, was originally isolated by imprecise transposon excision (Bei et al., 2002). The *src-1*(*cj293*) allele lacks the SH2 and kinase domains and is potentially a null allele. Homozygous *src-1*(*cj293*) hermaphrodites are themselves viable but produce inviable embryos, suggesting SRC-1 plays several essential roles in early development. It has been shown that SRC-1 is required for the accumulation of tyrosine-phosphorylated proteins at the membrane boundary between the P2 and EMS cells, and functions in parallel with Wnt/Wg signaling to specify the endoderm and to orient the division axis of EMS in the early embryo (Bei et al., 2002). However, the functions of SRC-1 in organogenesis and the development of nervous system, where SRC-1 is abundantly expressed, remain unknown (Hirose et al., 2003).

To address the roles SRC-1 plays in the later stages of *C. elegans* development, we have characterized homozygous *src-1*(*cj293*) hermaphrodites. We found that SRC-1 is essential for directing the migration of DTCs and a subset of neuronal cell bodies, and the growth cone path findings, which are regulated by different guidance cues including UNC-6/netrin. Furthermore, analyses of the genetic interactions between SRC-1 and potential downstream factors revealed that SRC-1 transduces the various extracellular guidance cue that direct cell migration via different pathways that depend on the cell type.

**Materials and methods**

**Strains and genetics**

*C. elegans* strains were cultured at 20°C as described by Brenner (Brenner, 1974). N2 Bristol served as the wild-type strain. The alleles used are listed by linkage groups. The strains used are as follows.

- **LGI:** *src-1*(*cj293*), *ced-12*(*k149*), *src-2*(*ok819*), *hT2*(*qIs48*)(*I;III*)
- **LHIL:** *muIs32*[mec-7::*gfp, lin-15(+)]
- **LHILL:**
  - *ced-2*(*n1994*), *ced-10*(*n1993*), *unc-5*(*e53*), *ced-5*(*n1812*), *rac-2*(*ok326*), *kyIs179*[unc-86::*gfp, lin-15(+)]
  - *kyIs179*[unc-86::*gfp, lin-15(+)]

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**Fig. 1.** (A) Schematic representation of the gonad structure of a wild-type hermaphrodite. The U-shaped gonadal lobes are rotationally symmetrical around the dorsoventral axis at the center of the body. DTC, distal tip cell. The dorsal side is upwards. (B) Schematic illustration of the time course and pattern of gonadal lobe extensions in wild-type hermaphrodites (lateral view). The DTCs lead the gonad lobes near the vulva, gonad and anus. (A) and (B) are modified from S. Chalfie, G. Bloom, W.4. Millar, E. W. Boyer and W. 5. C. The cell biology of Caenorhabditis elegans. Science 234, 400-406 (1982).
Phenotypic analysis

Gonad morphology was observed on a 5% agar pad by Nomarski differential interference contrast microscopy. The DTC migration pattern was inferred from the gonadal morphology of young adults at 20°C except as indicated. At the L1 stage, the positions of the AVM, ALM and PVM neurons were determined by using mec-7::gfp, mtlIs32 (Ch'ng et al., 2003), the position of CAN was determined by using che-23::gfp, kyIs4 (Forrester and Garriga, 1997), and the position of HSN was determined by using unc-86::gfp, kyIs179 (Shen and Bargmann, 2003). The images of the neuronal cells were captured by a LSM-510 confocal laser-scanning microscope (Zeiss). The positions of AVM, ALM, CAN, HSN and PVM were scored on the basis of their positions relative to the V-cell daughters, as these are stationary landmarks. A defect in the AVM axon morphology was scored when it failed to extend in the anterior direction after the nerve ring branch. A defect in the PVM axon morphology was scored when it failed to turn in the anterior direction after reaching the ventral nerve cord. Defects in AVM, CAN, HSN and PLM migration were determined by comparisons with their wild-type morphology. This morphology was defined by electron microscopic reconstitution of the *C. elegans* nervous system (White et al., 1986).

Feeding RNAi

RNAi was performed essentially as described by Timmons and Fire (Timmons and Fire, 1998). After preparing the src-1 RNAi feeding plate, some parental worms were allowed to lay eggs on the plates for 3-4 hours and were then removed. The remaining eggs were cultured into young adults and then assessed for gonad morphology.

Plasmid construction

To express src-1, the src-1 cDNA was amplified by PCR from the plasmid yk11712 and cloned into pPD49.26 carrying the src-1 promoter (4.9 kb) to generate Psrc-1::src-1. pPD49.26 carrying the lag-2 promoter (3.0 kb) was kindly provided by J. Kimble and was used to generate Plag-2::src-1. pPD95.86 and pPD96.41 were used to generate Plag-2::src-1 and Pmec-7::src-1, respectively. To generate the kinase-negative form of src-1, the lysine 290 residue was substituted with methionine to produce src-1K290M. src-1K290M was then inserted into the vectors described above instead of src-1.

Transgenic strains

Transgenic lines were generated using standard techniques (Mello et al., 1991). For rescue experiments, a lin-44::gfp construct was co-injected as a marker (50 μg/ml). To rescue the defect in DTC migration, the expression construct was injected at 1 μg/ml into the src-1/hT2[qIs48] heterozygote with an injection marker. The defect in DTC migration was then scored in the src-1(cj293) homozygote carrying the extrachromosomal array. To rescue the defects in the positioning of AVM cell body, src-1 or src-1K290M in pPD96.41 was injected at 50 μg/ml into the src-1/hT2[qIs48] heterozygote with an injection marker. To rescue the aberrant growth cone migration of PVM, src-1 or src-1K290M in pPD96.41 was injected at 50 μg/ml into the src-1/hT2[qIs48] heterozygote with an injection marker. The axon trajectories of PVM were observed by fluorescence microscopy and were compared with those in the src-1(cj293) homozygote. For the precocious expression of unc-5, the plasmid pSU16 (emb-9::unc-5) construct (a gift from J. G. Culotti and L. Brown) was injected at 1 μg/ml into the src-1/hT2[qIs48] worms with an injection marker. The gonad morphology of the resulting heterozygotes (src-1/+ and homozygotes src-1/src-1) was then analyzed.

Tissue staining

Adult worms were dissected and fixed essentially as described by Francis et al. (Francis et al., 1995). Briefly, the dissected gonads were fixed with 3.7% formaldehyde for 1 hour and postfixed with 100% methanol for 5 minutes. The specimens were then blocked with 3% TBS, and stained with anti-phosphotyrosine monoclonal antibody, 4G10 (Upstate), followed by detection with secondary antibodies conjugated with Texas Red. The images were captured by confocal laser-scanning microscopy on a Fluoview microscope.

Fig. 2. Defective DTC migration in the src-1(cj293) mutant. The position of DTC was monitored by analyzing the expression of lag-2::gfp in wild-type (A,C,E,G) and src-1(cj293) mutant (B,D,F,H) worms. The DTCs migrate in opposite directions along the ventral body wall muscle during the L2 stage in both the wild-type and mutant (A,B). However, the DTCs fail to turn dorsally in the src-1(cj293) mutant (D) during the L3 stage and continue their centrifugal migration in the anterior and posterior directions (F). By contrast, in the wild-type worm at the adult stage, the DTCs migrate centripetally along the dorsal muscle bands back toward the midbody (E). In the adult src-1(cj293) mutant, the extended gonads are randomly bent and accordionated (H). The arrowheads indicate the DTCs. Arrows show the migration pattern of the DTCs. Scale bar: 100 μm.
Results

Defects in DTC migration caused by the src-1 mutation

To examine the roles of SRC-1 in the development of postembryonic stages, we analyzed a homozygous src-1(cj293) mutant produced from the balanced heterozygote src-1(cj293)/+, as the src-1(cj293) mutant showed a maternal embryonic lethal phenotype (Bei et al., 2002). The src-1(cj293) mutants grew into adults but showed an apparent defect in gonad morphogenesis (Fig. 2). DTCs in the src-1(cj293) mutant migrated normally during the first phase but frequently failed to turn at subsequent phases of gonad development. Consequently, DTCs continued their centrifugal migration along the ventral muscles and the tips of the anterior and posterior lobes eventually reached the pharynx and anus, respectively (Fig. 2F). We here term this defect as ‘no-turn’ phenotype. Perhaps because of space limitations, these extended gonads in the adult worms became randomly bent and accordionated (Fig. 2H).

We also analyzed worms that had developed on src-1 RNAi feeding plates. The src-1(RNAi) worms had a similar phenotype to the src-1(cj293) mutants. Observed DTC migrations were classified into three categories (Table 1). The first is the normal migration path [100% in N2, 19% in src-1(cj293), 28% in src-1(RNAi)]. The second type represents the migration path that turns in the opposite direction during the third phase [0% in N2, 16% in src-1(cj293), 8% in src-1(RNAi)]. The third type is the no-turn phenotype, which was the most frequently exhibited phenotype for the src-1(cj293) mutant and the src-1(RNAi) worms [0% in N2, 65% in src-1(cj293), 64% in src-1(RNAi)]. These results raise the possibility that DTCs in the src-1(cj293) mutants are not being controlled by the guidance cues that direct their turning at the appropriate time and position.

As indicated above, the defect in DTC migration in the src-1(cj293) mutant was phenocopied in the src-1(RNAi), but neither the src-2 mutation nor the src-2 RNAi impairs DTC migration. (A-H) Shown are Nomarski images of the posterior gonad lobe at the young adult stage in the wild-type N2 (A), the control gfp (RNAi) worm (B), the src-1 (cj293) mutant (C), the src-1(RNAi) worm (D), the src-2(ok819) mutant (E) and the src-2(RNAi) worm (F). Also shown are the male gonads of the wild-type N2 (G) and the src-1(cj293) mutant (H). Arrows show the migration pattern of the DTCs. Scale bar: 20 μm. (I) Percentages of the various worm types that have a DTC migration defect in their anterior or posterior lobe. The total numbers of worms observed (n) are also indicated.
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migration of the male linker cells is different from that guiding DTCs.

To determine whether the defects in the src-1(cj293) mutant are due to the loss of SRC-1 function in DTCs, SRC-1 was expressed in the mutant under the src-1 promoter or the DTC-specific lag-2 promoter (Fig. 4). The expression of SRC-1 under either promoter rescued the defects, with the defect frequency being decreased to 12%/33% in the anteroposterior lobes by the src-1 promoter and to 10%/41% by the lag-2 promoter (Fig. 4G). However, the defect was not rescued by expression of a kinase-negative form of SRC-1 (SRC-1K290M) or by the expression of SRC-1 under the body wall muscle-specific myo-3 promoter. These results demonstrate that SRC-1 acts in an activity-dependent and DTC cell-autonomous manner.

To further examine the contribution of SRC-1 to DTC migration, the expression of SRC-1 was determined by detecting the expression of a reporter gene (GFP) expressed under the src-1 promoter. The expression of src-1 gene was clearly detected in DTCs (Fig. 5A,B) as described previously (Hirose et al., 2003). Furthermore, immunostaining with an anti-phosphotyrosine antibody (4G10), which specifically recognizes phosphorylated tyrosine residues of various proteins, revealed high levels of tyrosine phosphorylated proteins in DTCs (Fig. 5C). By contrast, DTCs in the src-1(cj293) mutant gave only faint signals (Fig. 5D), showing that SRC-1 activity is a major source of tyrosine phosphorylation in DTCs.

Genetic interaction of the src-1 gene with other genes involved in DTC migration

To position SRC-1 in a cell signaling pathway involved in DTC migration, we first analyzed the genetic interaction of src-1 with components of the Rac pathway, including ced-2/CrkII, ced-5/Dock180, ced-12/Elmo and ced-10/Rac. Mutations in these genes induce unregulated turns of DTC migration (Wu and Horvitz, 1998; Reddien and Horvitz, 2000; Gumienny et al., 2001; Wu et al., 2001; Zhou et al., 2001; Reddien and Horvitz, 2004). We analyzed DTC migration in the following double mutants: src-1;ced-2, src-1;ced-5, src-1;ced-10 and src-1;ced-12. In these experiments, the phenotypes of these mutants were evaluated by assessing the occurrence of typical no-turn phenotype of the src-1(cj293) mutant.

Table 1. Classification of abnormal DTC migration in src-1(cj293) mutant

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N2</th>
<th>src-1(cj293)</th>
<th>src-1(RNAi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>100%</td>
<td>19%</td>
<td>28%</td>
</tr>
<tr>
<td>Posterior</td>
<td>0%</td>
<td>16%</td>
<td>8%</td>
</tr>
<tr>
<td>n</td>
<td>112</td>
<td>102</td>
<td>285</td>
</tr>
</tbody>
</table>

The schematic representations of DTC migration routes of posterior lobe of gonad are shown. The ventral midbody is marked with an asterisk. Percentages of each type of DTC migration at L4 stage of wild-type worm (N2), src-1(cj293) and src-1(RNAi) are shown. The total numbers of worms observed (n) are also shown.
It is well established that the chemorepulsive mechanism mediated by UNC-6/netrin and its receptors UNC-5 and UNC-40 guide DTC migration, particularly during phase II (Hedgecock et al., 1990). The programmed expression of unc-5 in DTCs is shown to time the turning of DTC migration (Su et al., 2000). Mutations in unc-5(e53) and unc-6(ev400), which are putative null alleles, cause specific defects in the ventral-to-dorsal migration of DTCs. In either src-1(cj293);unc-5(e53) mutants or src-1(cj293);unc-6(ev400) mutants, the no-turn defect characteristic for src-1(cj293) mutant was not significantly affected (Table 2). These observations demonstrate that src-1(cj293) mutation is epistatic to unc-5(e53) or unc-6(ev400) mutations.

As the timing of DTC turning is affected by regulation of unc-5 expression (Su et al., 2000), we examined the effect of the src-1(cj293) mutation on the expression of unc-5 by detecting the unc-5 promoter activity (unc-5B::lacZ). As shown in Fig. 6B, the src-1(cj293) mutant at the L3 stage expressed unc-5 at the appropriate time, while still showing the no-turn phenotype. Furthermore, the precocious turning of DTCs that is induced by the precocious expression of unc-5 under the emb-9 promoter (emb-9::unc-5) was substantially decreased from 30%/70% (anteroposterior lobes) to 2%/13%, 2%/5% and 2%/7% in the src-1;ced-2, src-1;ced-5 and src-1;ced-12 double mutants, respectively. These results suggest that SRC-1 potentially acts as a suppressor of the Rac signaling pathway. Furthermore, in these double mutants, DTCs turned twice as much as in wild-type worm, raising the possibility that an alternative pathway exists that functions in parallel to the Rac signaling pathway. Hence, the relationship between src-1 and these Rac relatives in src-1;ced-2 mutants, the no-turn phenotype was suppressed in the posterior lobes to the same extent as in src-1;ced-10 mutants, while it was significantly enhanced in src-1;rac-2 mutants (98%) (Table 2). These results suggest that RAC-2 functions in parallel with SRC-1, but do not rule out the possibility that it also functions in the same signaling pathway as SRC-1.

Table 2. Genetic interaction of src-1(cj293) with rac-related and unc-5/-6 genes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Anterior</th>
<th>Posterior</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0%</td>
<td>0%</td>
<td>112</td>
</tr>
<tr>
<td>src-1(cj293)</td>
<td>13%</td>
<td>70%</td>
<td>120</td>
</tr>
<tr>
<td>ced-2(n1994)</td>
<td>0%</td>
<td>0%</td>
<td>60</td>
</tr>
<tr>
<td>ced-5(n1812)</td>
<td>2%</td>
<td>2%</td>
<td>60</td>
</tr>
<tr>
<td>ced-10(n1993)</td>
<td>0%</td>
<td>2%</td>
<td>60</td>
</tr>
<tr>
<td>ced-12(k149)</td>
<td>0%</td>
<td>0%</td>
<td>60</td>
</tr>
<tr>
<td>src-1(cj293); ced-2(n1994)</td>
<td>2%</td>
<td>5%</td>
<td>60</td>
</tr>
<tr>
<td>src-1(cj293); ced-10(n1993)</td>
<td>20%</td>
<td>45%</td>
<td>60</td>
</tr>
<tr>
<td>src-1(cj293); ced-12(k149)</td>
<td>2%</td>
<td>7%</td>
<td>60</td>
</tr>
<tr>
<td>mig-2(mu28)</td>
<td>0%</td>
<td>3%</td>
<td>60</td>
</tr>
<tr>
<td>rac-2(ok326)</td>
<td>0%</td>
<td>0%</td>
<td>60</td>
</tr>
<tr>
<td>src-1(cj293); mig-2(mu28)</td>
<td>23%</td>
<td>42%</td>
<td>60</td>
</tr>
<tr>
<td>src-1(cj293); rac-2(ok326)</td>
<td>60%</td>
<td>98%</td>
<td>60</td>
</tr>
<tr>
<td>unc-5(e53)</td>
<td>0%</td>
<td>0%</td>
<td>111</td>
</tr>
<tr>
<td>unc-6(ev400)</td>
<td>7%</td>
<td>4%</td>
<td>106</td>
</tr>
<tr>
<td>src-1(cj293); unc-5(e53)</td>
<td>10%</td>
<td>61%</td>
<td>164</td>
</tr>
<tr>
<td>src-1(cj293); unc-6(ev400)</td>
<td>13%</td>
<td>51%</td>
<td>100</td>
</tr>
</tbody>
</table>

Percentage of no-turn DTC migration defects at the L4 stage in the anterior and posterior lobes of the gonad are shown. The total numbers of worms observed (n) are also shown.

Table 3. Effect of precocious unc-5 expression on DTC migration in the src-1(cj293) mutant

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Precocious turning</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior</td>
</tr>
<tr>
<td>Wild type</td>
<td>0%</td>
</tr>
<tr>
<td>src-1(cj293)</td>
<td>0%</td>
</tr>
<tr>
<td>src-1(cj293); Ex[emb-9::unc-5]</td>
<td>30%</td>
</tr>
<tr>
<td>src-1(cj293); Ex[emb-9::unc-5]</td>
<td>7%</td>
</tr>
</tbody>
</table>

Percentage of precocious turning phenotypes in the anterior and posterior lobes of the gonad are shown. The total numbers of worms observed (n) are also shown.
but also normal DTC turning induced by UNC-5, and that SRC-1 is required for UNC-5 mediated signaling pathway that directs DTC migration.

Defects in neuronal cell migration caused by the src-1(cj293) mutation

To examine the roles SRC-1 plays in the migration of other cell types, we analyzed the migration of the QR and QL neuroblasts in the src-1(cj293) mutant. In the wild-type worm, the QR neuroblast and its descendants migrate over long distances. As a result, the AVM neuron, a QR descendant, eventually becomes positioned anterior to the ALM neuron (Fig. 7A-C). In the src-1(cj293) mutant, however, the QR neuroblast and its descendants migrate over short distances and AVM is located posteriorly to ALM (Fig. 7D,E). When SRC-1 was expressed in the src-1(cj293) mutant under the control of the mec-7 promoter (emb-9::unc-5) was injected into the src-1(hT2) worm, and the gonad morphology of the resulting heterozygote (src-1/+; C) and homozygote (src-1/src-1; D) was analyzed. The arrows show the migration pattern of the DTCs. Scale bar: 40 μm.

Table 4. SRC-1 rescues the AVM position defect in a cell-autonomous manner

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Abnormal AVM position</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1%</td>
<td>159</td>
</tr>
<tr>
<td>src-1(cj293)</td>
<td>99%</td>
<td>156</td>
</tr>
<tr>
<td>src-1; Ex[pmec-7::src-1]</td>
<td>42%</td>
<td>101</td>
</tr>
<tr>
<td>src-1; Ex[pmec-7::src-1KM]</td>
<td>98%</td>
<td>152</td>
</tr>
</tbody>
</table>

Defects in the positioning of AVM was scored at the larval stage in muIs32 animals in the src-1(cj293) or wild-type background. For each transgene, two independent lines were scored; similar results were obtained. The total numbers of worms observed (n) are also shown.
neurons make a right-angled turn anteriorly after reaching the ventral nerve cord (Fig. 8H). In the src-1(cj293) mutant, however, about half of the PVM growth cones (46%) turn in the opposite direction (Fig. 8I-K). During this inappropriate migration, a substantial number of the PVM axons (26%) make a reverse turn in the anterior direction, thus allowing the growth cones to reach the anterior body (Fig. 8J). These observations suggest that the PVM growth cones in the src-1(cj293) mutant fail to respond to the guidance cues that direct their migration along the anteroposterior axis. This defect was completely rescued by the expression of SRC-1 but not the kinase-defective form of SRC-1 under the mec-7 promoter (Table 5), indicating again the cell-autonomous and kinase-dependent role of SRC-1 in cell migration. However, as in the case of A VM cell body migration, the ced-5/-12 mutations did not significantly alter the normal PVM growth cone migration, nor did they modify the src-1(cj293) phenotype (Table 5). These observations suggest that SRC-1 uses different downstream pathway in PVM from the one it uses in DTCs.

Discussion

DTC migration in the src-1(cj293) mutants

In this study, we first showed that the kinase activity of SRC-1 is required for the patterned DTC migration that occurs during gonad morphogenesis. In the src-1(cj293) mutant, the motility of DTCs seems to be normal because these cells migrate normally during phase I. In subsequent phases, however, DTCs fail to change direction, eventually traveling to the pharynx and anus (Fig. 2). These observations suggest that SRC-1 is required for DTC response to the guidance cues that direct its migration, including UNC-6/netrin and UNC-129/TGFβ. A probable candidate upstream regulator of SRC-1 is UNC-5/netrin receptor, which is known to regulate the ventral-to-dorsal phase II migration of DTCs (Hedgecock et al., 1990; Culotti and Merz, 1998). The genetic interactions of src-1 with unc-6 or unc-5 suggest that SRC-1 acts in the UNC-6/netrin signaling pathway through UNC-5, which is supported by the observations that the src-1(cj293) mutation does not affect the timing of the unc-5 expression and suppresses DTC turning induced by not only precocious but also normal expression of unc-5. Previously, it has been shown that UNC-5 functions require phosphorylation of a cytoplasmic tyrosine residue, suggesting a crucial role for a

Fig. 7. Defects in the migration of the QR neuroblast and its descendants in the src-1(cj293) mutant. (A) Schematic representation of the A VM and ALM neuronal cells that originate from the QR neuroblast and the PVM descendant of the QL neuroblast. (B-E) The GFP signals produced from mec-7::gfp in wild-type worms (B) and the src-1(cj293) mutant (D) were visualized by epifluorescence. The merged GFP and DIC images are shown in C and E. Scale bar: 20 μm. (F) The genetic interaction of the aberrant A VM phenotype in the src-1(cj293) mutant with the ced-5/-12 mutations. The final positions of A VM and PVM in the wild-type and indicated mutant worms were scored according to their relative distance from the stationary Vn.a and Vn.p cells shown on the x-axis.
tyrosine kinase(s) in the UNC-6/netrin signaling pathway (Killean et al., 2002). It has also been reported that the activation of a vertebrate Src family kinase is required for the phosphorylation of the netrin receptor and its subsequent cell signaling (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004), and that SRC-1 could mediate UNC-5 signaling in *C. elegans* by directly interacting with UNC-5 (Lee et al., 2005). These findings together with our observations support a model in which SRC-1 acts as a component of a netrin signaling pathway, at least during a particular time period in gonad development. However, it is also notable that the precocious expression of UNC-5 is still able to induce the precocious turning of DTC in the *src-1(cj293)* mutants, albeit with low frequency (7%/14% in anterior/posterior lobes). Thus, it remains possible that alternative SRC-1-independent pathways can be involved in the guidance of DTC migration. Furthermore, the fact that the *src-1(cj293)* mutants fail to make the second turn that usually occurs during phase II reveals that SRC-1 may also be required for the DTC to respond to some other signaling mechanism that directs the secondary turning of DTCs.

In *C. elegans*, defects in DTC migration have been observed in the *ced-2/CrII, ced-5/Dock180, ced-10/Rac and ced-12/Elmo* mutants (Wu and Horvitz, 1998; Reddien and Horvitz, 2000; Gumienny et al., 2001; Wu et al., 2001; Zhou et al., 2001; Reddien and Horvitz, 2004). CED-2, CED-5 and CED-12 form a ternary complex that can trigger the localized remodeling of the actin cytoskeleton through CED-10. It has also been shown that the Dock180-Elmo complex functions as a guanine nucleotide-exchanging factor for Rac, and that CrkII binding enhances the functions of this complex (Brugnera et al., 2002). Src may act upstream of this pathway to regulate cell migration through the actin cytoskeleton as the binding of CrkII to Dock180 is enhanced in v-Src transformed 3Y1 cells (Kiyokawa et al., 1998). Furthermore, Hck, a Src family member, binds to Elmo1 through its SH3 domain in vitro and phosphorylates Elmo1 in cultured cells (Scott et al., 2002). These lines of evidence strongly support our proposal that SRC-1 functionally acts in the Rac pathway in DTCs. Of the three *rac*-related genes in *C. elegans* (*ced-10, mig-2* and *rac-2*), CED-10 and MIG-2 have been shown to redundantly control the migration of some cells, including DTCs (Lundquist et al., 2001; Wu et al., 2002). Consistent with this is our observation that a mutation in either *mig-2* or *ced-10* only partially suppressed the no-turn phenotype of DTC migration in the *src-1(cj293)* mutant, whereas mutations in *ced-2, ced-5* or *ced-12* robustly suppressed this phenotype. These results suggest that CED-10 and MIG-2 function redundantly downstream of the CED-2 complex under the control of SRC-1. By contrast, a mutation in the *rac-2* gene enhanced the DTC migration defect in the *src-1(cj293)* phenotype, suggesting that RAC-2 is involved in DTC migration but acts in an alternative pathway independently of SRC-1.

### Table 5. Effect of the *src-1(cj293)* and *ced-5/-12* mutations on the axon trajectory of PVM

<table>
<thead>
<tr>
<th>Genotype</th>
<th></th>
<th></th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td><em>src-1(cj293)</em></td>
<td>54%</td>
<td>26%</td>
<td>20%</td>
</tr>
<tr>
<td><em>src-1(cj293); Ex[pm16::src-1]</em></td>
<td>92%</td>
<td>5%</td>
<td>3%</td>
</tr>
<tr>
<td><em>src-1(cj293); Ex[pm17::src-1KM]</em></td>
<td>55%</td>
<td>24%</td>
<td>22%</td>
</tr>
<tr>
<td><em>ced-5(n1812)</em></td>
<td>99%</td>
<td>1%</td>
<td>0%</td>
</tr>
<tr>
<td><em>ced-12(k149)</em></td>
<td>98%</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td><em>src-1(cj293); ced-5(n1812)</em></td>
<td>62%</td>
<td>18%</td>
<td>21%</td>
</tr>
<tr>
<td><em>src-1(cj293); ced-12(k149)</em></td>
<td>63%</td>
<td>14%</td>
<td>23%</td>
</tr>
</tbody>
</table>

Percentages of the worms with the indicated axon trajectory are shown. The total numbers of worms observed (*n*) are also shown.

### Fig. 8. Defective axon guidance in the *src-1(cj293)* mutant.

(A-F) The typical axon trajectories of the AVM (A,B), ALM (C,D) and CAN (E,F) neurons in the wild-type (A,C,E) and *src-1(cj293)* (B,D,F) mutant were visualized as described in the materials and methods. Defects in the nerve ring branch of the AVM trajectory (B), the ALM cell body and its trajectory (D) and the CAN cell body and posterior axon trajectory (D) were observed. The percentages of worms with defective axon guidance and the total numbers of worm observed (*n*) are indicated in each panel. Scale bar: 20 μm.

(G) Schematic representation of the PVM axon trajectory in a wild-type worm.

(H-K) The defective PVM axon trajectory in the *src-1(cj293)* mutant. The defects were classified into two patterns as indicated schematically at the bottom of the panels. Some of the PVM axons in the *src-1(cj293)* mutant turn in the posterior direction on the ventral cord and then makes a reverse turn in the anterior direction (J), while others turn in the posterior direction on the ventral cord and continues in that direction (K). The percentages of worms with the various PVM axon trajectory patterns are shown. Scale bar: 20 μm.
Our analysis of the genetic interaction between src-1 and the genes in the Rac pathway revealed that the DTC migration defect of src-1(cj293) mutants was suppressed in all the double mutants. This raised the possibility that SRC-1 may act as an upstream suppressor of the Rac signaling pathway. If this is the case, it may be that the Rac pathway is constitutively active in migrating DTCs, allowing a continuous remodeling of the cytoskeleton. Upon exposure to some guidance cues that activate SRC-1 (potentially netrin), the Rac pathway is suppressed, thereby inhibiting actin remodeling; this may be required for the DTC to change cell polarity and turn. This is consistent with the previous observation that loss-of-function of the Rac pathway induces an extra-turn phenotype (Wu and Horvitz, 1998). To verify this hypothesis, the mechanism by which SRC-1 suppresses Rac activity should be elucidated. Notably, it is generally accepted that Src positively regulates the Rac signaling pathway in mammals. This inconsistency may be resolved by identifying downstream effectors of SRC-1 that inactivate the Rac pathway in C. elegans.

Neuronal cell and growth cone migration in the src-1(cj293) mutants

In the src-1(cj293) mutant, we also observed apparent defects in the migration of neuronal cells. In particular, the positioning of AVM, a descendant of the QR neuroblast, is strongly affected by the src-1(cj293) mutation. As with DTC migration, the kinase activity of SRC-1 is required for its role in the directional migration of QR and its descendants. Although the cues that guide Q cell migration along the anteroposterior axis are still unknown, it has been reported that the transmembrane protein MIG-13 is a key determinant in the final positioning of AVM (Sym et al., 1999). MIG-13 expression is restricted to the anterior and central body regions and functions in a non-cell-autonomous manner to promote migration in the anterior direction. Like the src-1(cj293) mutant, a mig-13 mutant shows defects in the migration of QR and its descendants, but not in the migration of QL and its descendants (ALM, CAN and HSN). Furthermore, the final position of AVM in the mig-13 mutant is very similar to that in the src-1(cj293) mutant. These observations raise the interesting possibility that SRC-1 mediates the cell signaling induced by extracellular MIG-13 cues. Mutations in ced-5/-12 genes that affected DTC turning did not alter the migration defects of QR and its descendants in the src-1(cj293) mutants. This shows that the signaling pathways that are dependent on SRC-1 vary depending on cell type.

The src-1(cj293) mutant also showed defects in the migration of the growth cones of some neuronal cells, namely, AVM, ALM, CAN and PVC. For example, whereas the growth cone of PVC normally makes a right-angled turn in the anterior direction after reaching the ventral nerve cord, in the src-1(cj293) mutant it frequently makes a turn in the opposite direction (Fig. 8). It appears that the axon of PVC in the src-1(cj293) mutant may randomly determine the direction in which it turns. SRC-1 may be involved in the regulation of cell signaling evoked by the guidance cues that direct the migration of a growth cone, suggesting that the migration of a neural growth cone uses the same mechanisms employed by migrating cell bodies like DTCs. However, identification of the guidance cues involved in the attraction or repulsion of the PVC growth cone within the ventral nerve cord will be required to confirm the role of SRC-1 in this process.

In this study, we showed initially that SRC-1 plays a potential role in transducing the netrin signal to the Rac pathway at a particular time of DTC migration. However, our subsequent observations of neuronal cells and growth cones suggest that SRC-1 plays a more general role in directing cell migration, and that it does so via different pathways depending on the cell types. Thus, in response to various extracellular guidance cues, activated SRC-1 may transduce the signals into an appropriate intracellular pathway that probably regulates the cytoskeletal remodeling required for providing polarity information to the cells. In vertebrates, Src family kinases have been shown to respond to a wide variety of extracellular cues, including guidance cues, extracellular matrices and growth factors, and to play roles in regulating a wide variety of cellular functions, including cell adhesion, migration, secretion, endocytosis, proliferation and differentiation. These multifunctional aspects of Src family kinases have hampered the unraveling of their most crucial function(s). Further analysis of the functions of SRC-1, particularly by focusing on the process of cell migration, may help elucidate its basal role, which may be conserved during animal evolution.

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