Sensitized genetic backgrounds reveal a role for \textit{C. elegans} FGF EGL-17 as a repellent for migrating CAN neurons

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Summary
Although many molecules are necessary for neuronal cell migrations in \textit{C. elegans}, no guidance cues are known to be essential for any of these cells to migrate along the anteroposterior (AP) axis. We demonstrate that the fibroblast growth factor (FGF) EGL-17, an attractant for the migrating sex myoblasts (SMs), repels the CANs, a pair of neurons that migrate posteriorly from the head to the center of the embryo. Although mutations in genes encoding EGL-17/FGF and a specific isoform of its receptor EGL-15/FGFR had little effect on CAN migration, they enhanced the CAN migration defects caused by mutations in other genes. Two cells at the anterior end of the embryo express EGL-17/FGF, raising the possibility that EGL-17/FGF functions as a repellent for migrating CANs. Consistent with this hypothesis, ectopic expression of EGL-17/FGF shifted the final CAN cell positions away from these novel sites of expression. Cell-specific rescue experiments demonstrated that EGL-15/FGFR acts in the CANs to promote their migration. We also found that the tyrosine phosphatase receptor CLR-1 regulates CAN migration by inhibiting EGL-15/FGFR signaling, and that the FGFR adaptor protein SEM-5/GRB2 mediates EGL-15/FGFR signaling in CAN migration. Thus, EGL-17/FGF signaling through an EGL-15/FGFR isoform and possibly SEM-5/GRB2 mediates both attraction of the SMs and repulsion of the CANs. This study also raises the possibility that several guidance cues regulate cell migrations along the \textit{C. elegans} AP axis, and their role in these migrations may only be revealed in sensitized genetic backgrounds.

Key words: \textit{Caenorhabditis elegans}, Cell migration, FGF, FGFR, Repellent

Introduction
Many steps in development depend upon the coordinated migrations of cells, and their paths are often defined by gradients of attractants and repellents (Guan and Rao, 2003; Tessier-Lavigne and Goodman, 1996). The proteins UNC-6/Netrin and Slit, for example, guide the migrations of both cells and axonal growth cones along the metazoan dorsoventral (DV) axis and are conserved in organisms as diverse as nematodes and vertebrates (Brose et al., 1999; Hao et al., 2001; Ishii et al., 1992; Serafini et al., 1994; Yuan et al., 1999). In \textit{C. elegans}, ventrally expressed UNC-6 attracts growth cones, while dorsally expressed SLT-1 functions as a repellent to direct axons to the ventral nerve cord (Chan et al., 1996; Hao et al., 2001; Wadsworth et al., 1996). UNC-6 also repels a different set of growth cones dorsally to the dorsal nerve cord (Chan et al., 1996; Colamarino and Tessier-Lavigne, 1995; Hamelin et al., 1993).

In vertebrates, homologs of UNC-6 and SLT-1 proteins collaborate in different ways to coordinate growth cone migrations along the neuroaxis. As with \textit{C. elegans} UNC-6, vertebrate Netrins are expressed ventrally and attract commissural axons to the vertebrate floorplate (Kennedy et al., 1994; Serafini et al., 1994). Sonic hedgehog, which is expressed in the floorplate and functions as a morphogen to pattern the ventral spinal cord, also attracts commissural axons ventrally (Charron et al., 2003). Slit coordinates commissural axon guidance at the floorplate, but it does not collaborate with Netrin and Sonic Hedgehog to guide migrations ventrally. Instead, Slit expression in the floorplate inhibits commissural axons expressing its receptor Robo from recrossing the midline (Brose et al., 1999; Long et al., 2004; Yuan et al., 1999; Zou et al., 2000). Robo also forms a complex with the Netrin receptor DCC, inhibiting the ability of DCC to respond to Netrin and facilitating the extension of these growth cones across the floorplate (Stein and Tessier-Lavigne, 2001).

Although molecules that guide migrations along the DV axis have been identified, the mechanisms that guide migrations along the AP axis are poorly understood. Wnt proteins, which are conserved secreted glycoproteins that act as graded morphogens, have recently been shown to guide commissural axons along the AP axis of the mammalian spinal cord. After crossing the midline, commissural axons turn anteriorly and extend towards the brain. Several Wnt proteins have been shown to stimulate commissural growth cones after they have crossed the midline. These Wnt proteins are expressed in the developing spinal cord. In particular, Wnt4 RNA is present in a gradient decreasing from anterior to posterior, placing Wnt4 in a key position to guide commissural axons anteriorly (Lyuksyutova et al., 2003).

In \textit{C. elegans}, the gene \textit{vab-8} is both necessary and sufficient
for posteriorly directed migrations of cells and growth cones. Most posterior migrations require vab-8 (Wightman et al., 1996), and ectopic expression of vab-8 can reroute anteriorly projected axons towards the posterior (Wolf et al., 1998) (N. Watari-Goshima and G.G., unpublished). The vab-8 locus encodes at least two novel intracellular proteins that act in the cells to promote their migration (Wolf et al., 1998). How these proteins regulate these migrations, however, remains unknown.

Unlike VAB-8, the C. elegans transmembrane protein MIG-13 plays a nonautonomous role in guiding cell migrations along the AP axis (Sym et al., 1999). mig-13 loss-of-function alleles display more specific defects than vab-8 mutants, disrupting the anterior directed migrations of only the BDU neurons and descendants of the right Q neuroblast. Ectopic expression of mig-13 from a heat shock promoter, however, induces an anterior shift in the final positions of neurons that migrate in either direction along the AP axis, indicating that MIG-13 plays a broader role than was suggested by the effects of mig-13 mutants. As with VAB-8, the role of MIG-13 in these migrations remains unclear (Sym et al., 1999).

In C. elegans, the fibroblast growth factor (FGF) homolog EGL-17 functions as an attractant for the precise positioning of the anteriorly directed migrations of the sex myoblasts (SMs) (Burdine et al., 1998; Burdine et al., 1997). In early larval stages, the SMs migrate from the posterior midbody to positions flanking the center of the gonad (Sulston and Horvitz, 1977). During SM migration, EGL-17 is expressed in the primary vulval precursor cells (VPCs) and the dorsal uterine (DU) cells of the somatic gonad, which define the final destination of the SMs (Branda and Stern, 2000; Burdine et al., 1998). EGL-17 signals through the FGF receptor (FGFR) EGL-15 to attract SMs (Burdine et al., 1998; DeVore et al., 1995).

In an effort to understand AP guidance in C. elegans, we have focused on the posterior migrations of the CANs, a pair of bilaterally symmetric neurons that are born in the head and migrate to the middle of the embryo (Sulston et al., 1983). Although a previous screen for CAN migration mutations identified a number of genes, none of them encoded guidance cues (Forrester and Garriga, 1997; Forrester et al., 1998). One explanation for this outcome is that multiple cues contribute to CAN migration, and therefore removing one might result in only subtle CAN migration defects. To test this hypothesis, we used sensitized genetic backgrounds to re-evaluate the potential role of secreted molecules in CAN migration. The use of these sensitized backgrounds revealed a role for FGF in CAN migration.

Although mutations in genes encoding EGL-17/FGF and EGL-15/FGFR had negligible effects on CAN migration, they enhanced the CAN migration defects caused by other mutations. Further supporting a role for FGF signaling in CAN migration, the tyrosine phosphatase receptor CLR-1, which inhibits EGL-15/FGFR signaling to regulate fluid homeostasis (Huang and Stern, 2004; Kokel et al., 1998), also inhibited EGL-15/FGFR activity in CAN migration. We also show that EGL-17/FGF functions as a guidance cue for CAN migration. Two cells at the anterior end of the embryo express EGL-17/FGF when the CANs migrate, suggesting that EGL-17/FGF may function as a repellent. Consistent with this hypothesis, ectopic expression of EGL-17/FGF could repel the CANs from the new sites of expression. Finally, we provide evidence that EGL-15/FGFR acts in the CANs to promote their posteriorly directed migrations. Our results raise the possibility that multiple cues might promote guidance of cells and growth cones along the C. elegans AP axis.

Materials and methods

Strains and genetics

Standard methods for maintaining strains and performing genetics were employed as described by Brenner (Brenner, 1974). Worms were raised at 20°C unless stated otherwise. The standard wild-type strain N2 and strains with the following mutations were used in this work.


LGIV: soc-2(n1774) (Selfors et al., 1998), let-60(sy101sy127), let-60(n2021), let-60(n1046) (Beitel et al., 1990; Ferguson and Horvitz, 1985; Han and Sternberg, 1991), mls11[myo-2::gfp, pes-10::gfp, F22879::gfp].

LGV: unc-34(gm104) (Forrester and Garriga, 1997), unc-34(e951) (Brenner, 1974), vab-8(e1017) (Manser and Wood, 1990), vab-8(gm84) (Wolf et al., 1998).

LGV: egl-17(n1377) (Burdine et al., 1997), sax-3(ky200ts) (Zallen et al., 1998), unc-6(ev400) (Hedgecock et al., 1990), sem-5(n2019) (Clark et al., 1992), egl-15(n1477), egl-15(n454), egl-15(n1454) (DeVore et al., 1995), elt-1(eh15) (Hao et al., 2001).

The extrachromosomal array otEx1270 with the dpy-7::egl-15(5A)cDNA transgene, as well as the egl-15 rescuing arrays otEx1254 and otEx1267, and let-765 rescuing arrays otEx1467 and otEx1468, were kindly provided by O. Hobert (Bulow et al., 2004). Transgenes generated in this study are described below.

egl-15 and egl-17 constructs and germline transformations

In the egl-15(new*) construct, the transmembrane domain of EGL-15 was replaced with that of the Neu oncoprotein, which results in constitutive activity of the EGL-15 receptor (Kokel et al., 1998). The plasmid NH4420, which contains the egl-15(new*) construct, was injected into a soc-2(n1774) mutant background at a concentration of 50 ng/µl. As co-transformation markers, the prRF4 plasmid containing the dominant rol-6(su1006) mutation (Mello et al., 1991) and a ttx-3::gfp transgene (Hobert et al., 1997) were co-injected at 50 ng/µl and at 20 ng/µl, respectively. Germline transformation was conducted using standard procedures described by Mello et al. (Mello et al., 1991) to generate the extrachromosomal array gmEx224. The presence of the soc-2 mutation was necessary to prevent the lethality caused by constitutive EGL-15 activity (Kokel et al., 1998). As a control, the genomic egl-15 construct NH150 (Huang and Stern, 2004) was injected into a soc-2(n1774); vab-8(gm84) mutant background at a concentration of 50 ng/µl with prRF4 at 50 ng/µl and a ttx-3::gfp transgene at 20 ng/µl.

Global EGL-17 expression was induced using the plasmid NH4402, which contains hsp::egl-17::gfp (provided by Michael Stern). An extrachromosomal array was generated by injecting into wild-type hermaphrodites NH402 at 50 ng/µl along with prRF4 (Mello et al., 1991) at 20 ng/µl and ttx-3::gfp (Hobert et al., 1997) at 20 ng/µl. The resulting extrachromosomal array gmEx197 was then crossed into a vab-8(gm84) mutant background. Strains containing the hsp::egl-17::gfp array were scored at 25°C, a temperature that induced detectable Egl-17::GFP expression.

To express EGL-17 ectopically, we generated a Pmba-5::egl-17cDNA::gfp translational fusion. The primers 5'-AACCGTTGATC-AGGGTGTTGCAAGT-3' and 5'-AACCGTTAATTTTAGTTA-TAAGGATCCAGT-3' were used to amplify by PCR an 8.8 kb mbab-5 promoter fragment with flanking HindIII sites. The mbab-5 promoter was cloned into the HindIII site of the plasmid pPD95.85.
(provided by Andy Fire). The egl-17 cDNA was amplified by PCR from the plasmid NH#315 (Burdine et al., 1997) using the primers 5'-TCCCCCGGGGCTATGCTCAAGGCTCACAGT-3', which contains an XmaI site, and 5'-GGACCCGGTGTTTGGTGTGATC-3', which contains an AgeI site. The egl-17 cDNA was then inserted into plasmid pPD95.85 cut with XmaI and AgeI. This construct was then cut with Xmal and XhoI, and the insert was cloned into the mab-5(pPD95.85) plasmid cut with the Xmal and XhoI. The resulting plasmid pTF2, which contains Pmab-5::egl-17::gfp, was injected into an egl-17(n1377) mutant background at a concentration of 50 ng/µl with the co-transformation markers pRF4 (50 ng/µl) and ttx-3::gfp (20 ng/µl). Animals carrying the resulting extrachromosomal array gmEx256 were then scored for CAN migration defects.

We also constructed a Plim-4::egl-17::gfp transgene by PCR amplification of a 3.6 kb lim-4 promoter using the primers 5'-AAACTGACCGAGTTGAATAGGAGGC-3', which contains a PsrI site, and 5'-TCCCCCGGGGGTCAACTTGTGACAGC-3', which contains an XmaI site. The lim-4 promoter was cut with PsrI and XmaI, and inserted into pPD95.85 plasmid cut with PsrI and XmaI. The egl-17 cDNA in pPD95.85, described previously, was cloned into the Plim-4::gfp construct using XmaI and AgeI sites. The extrachromosomal array gmEx244 was generated by injecting egl-17(n1377) mutants with 50 ng/µl of pTF3, which contains Plim-4::egl-17::gfp, and 50 ng/µl of pRF4.

In order to assess whether EGL-15 functions cell autonomously, we used the plasmid NH#1078 (provided by Michael Stern), which carries a 1.2 kb ceh-10 promoter that drives egl-15 genomic DNA. The resulting Pceh-10::egl-15 transgene was injected at 50 ng/µl with the co-transformation markers pRF4 (50 ng/µl) and ttx-3::gfp (20 ng/µl) into a vab-8(gm84); egl-15(n484) mutant background. Animals expressing the extrachromosomal array gmEx280 [ceh-10::egl-15] were then scored for CAN migration defects.

Scoring CAN positions
To determine the extent of CAN migration in wild-type and mutant animals, the CAN positions were scored relative to the positions of hypodermal nuclei (V1, P1/2, V2, P3/4, V3, P4/5 and V4) using Nomarski optics. Only newly hatched first larval stage (L1) hermaphrodites were scored. We only scored the positions of the CANs that had migrated posterior of the V1 cell, because we could not distinguish CANs located anterior to V1 from other neurons in the head. When we could not detect a CAN neuron posterior to V1, we scored it as anterior to V1.

To score the CAN migration defects of strains carrying the larval lethal alleles egl-17(n1456) and let-756(s2887), we observed the progeny of hermaphrodites that were homozygous for these alleles and carried a rescuing egl-15 or let-756 extrachromosomal transgenic array, respectively. The CANs of LIs not expressing ceh-22::gfp, which was used as a co-injection marker for the egl-15 and let-756 rescuing arrays were scored. To ascertain the CAN position of strains with the larval lethal let-60(sy1013sy127) allele, we balanced let-60(sy1013sy127) with the integrated transgene mls11 which expresses a gfp cDNA from the myo-2 promoter. We then scored LIs not expressing GFP as let-60(sy1013sy127) homozygotes. We mapped mls11 to within 1 m.u. of let-60.

A two-sample z-test comparing the proportion of CANs in specific positions was used to determine whether two strains showed statistical differences in CAN migration defects. The statistics program StatCrunch was used for the analysis and can be found online at http://www.statcrunch.com/. A P-value of less than 0.01 was considered to be statistically significant. Unless otherwise stated, the P-values shown were determined using a two-sample z-test on the proportion of CANs found in the most anterior position.

Embryonic expression pattern of EGL-17
The transgene ayIs9 contains an integrated array that expresses a gfp cDNA from a 10.5 kb egl-17 promoter region (Branda and Stern, 2000). We also generated a translational fusion that expresses egl-17::gfp from the 10.5kb egl-17 promoter of ayIs9 (pTF4). This construct and the co-transformation marker pRF4 were injected at 50 ng/µl each into wild-type hermaphrodites to create the transgenic array gmEx283. The embryonic expression pattern of EGL-17 was assessed for both arrays by visualizing GFP fluorescence on the Zeiss Axioskop2 microscope, and pictures were obtained using a Hamamatsu ORCA-ER digital camera with Openlab software.

Results
Genetically sensitized backgrounds reveal roles for SLT-1/Slit and FGF signaling in CAN migration
As Netrin, Slit and FGF proteins can act as guidance cues, we asked whether UNC-6/Netrin, SLT-1/Slit, EGL-17/FGF or LET-756/FGF were involved in the migration of the CANs, a pair of bilaterally symmetric neurons that migrate posteriorly from their birthplace in the head to the middle of the embryo (Sulston et al., 1983). Single mutations in any of these genes did not significantly alter CAN cell migration (Fig. 1). However, in the sensitized background of a vab-8(gm84) mutation, we found that the slt-1(eh15) and egl-17(n1377) mutations enhanced the Vab-8 CAN migration defects (Fig. 1). vab-8 encodes novel proteins that are required for posteriorly directed cell and growth cone migrations (Wightman et al., 1996; Wolf et al., 1998). The slt-1 enhancement was not surprising as Hao et al. (Hao et al., 2001) showed that slt-1 mutants carrying the ceh-23::gfp transgene displayed CAN migrations defects, and we had previously shown that the presence of this transgene sensitized the background to CAN defects (Forrester and Garriga, 1997). As expected, a mutation in sax-3, the gene encoding the SLT-1 receptor homolog Robo, also enhanced vab-8(gm84) CAN migration defects (Fig. 1), and a mutation in the unc-6 receptor unc-40 did not show significant enhancement (Fig. 1).

Although a role for SLT-1 in CAN migration was predicted, EGL-17 had not been shown previously to play a role in CAN migration. FGF/egl-17 and its receptor EGL-15 are essential for the normal migrations of the SMs (Burdine et al., 1998). Neither molecule, however, has been shown to function in any additional, long-range cell migration. Like the egl-17 mutation, mutations in egl-15 had negligible effects on the CAN migrations. Mutations in both genes resulted in a similar enhancement of the CAN defects of vab-8(gm84) mutants (Fig. 1). Enhancement of CAN migration defects by egl-17 and egl-15 mutations is also observed in the sensitized background of unc-34(e951) and unc-34(gm104) null alleles (Fig. 1; data not shown). unc-34 encodes a member of the Ena/VASP family of proteins and functions in many cell and growth cone migrations (Colavita and Culotti, 1998; Forrester and Garriga, 1997; McIntire et al., 1992; Yu et al., 2002). Enhancement of unc-34 null alleles indicates that egl-17 and egl-15 function, at least partially, in parallel to the unc-34 pathway. Elimination of vab-8 function results in an almost complete failure of CAN cell migration, precluding us from asking whether egl-17 or egl-15 mutations enhanced the CAN defects of mutants lacking all vab-8 activity.

EGL-15/FGFR possesses two genetically distinct functions that are mediated by different isoforms. EGL-17/FGF attracts the SMs through a specific isoform of the FGF receptor known as EGL-15(5A), and a second FGF known as LET-756 acts through a different isoform of the receptor known as EGL-
To regulate fluid homeostasis and axon outgrowth (Bulow et al., 2004; Goodman et al., 2003; Huang and Stern, 2004). The let-756(s2887) null allele neither displayed CAN migration defects nor significantly altered the CAN defects of vab-8(gm84) mutants (Fig. 1). The mutation egl-15(n484) specifically eliminates the 5A isoform, the egl-15(n1477ts) mutation reduces the activity of both isoforms, and the egl-15(n1456) mutation should eliminate all egl-15 function (DeVore et al., 1995). All three mutations showed similar enhancement of the CAN migration defects caused by vab-8(gm84) (Fig. 1). vab-8(gm84); egl-17(n1377), egl-15(n484) showed CAN migration defects similar to vab-8(gm84); egl-17(n1377) and vab-8(gm84); egl-15(n484) mutants (Fig. 1). Taken together, these observations indicate that EGL-17/FGFR acts through the 5A isoform of EGL-15/FGFR to promote CAN migration.

The CLR-1 tyrosine phosphatase negatively regulates FGF signaling in CAN migration

The receptor tyrosine phosphatase CLR-1 negatively regulates EGL-15/FGFR activity in fluid homeostasis. Although CLR-1 plays an important role in this essential function of EGL-15, it does not appear to play a prominent role in SM migration (Huang and Stern, 2004; Kokel et al., 1998). To determine whether CLR-1 inhibits EGL-15 function in CAN migration, we asked whether the temperature-sensitive clr-1 allele e1745 could affect CAN migration. In the clr-1 mutant, all of the CANs were positioned normally. However, the clr-1 mutation...
suppressed the CAN migration defects of both unc-34 and vab-8 null mutants, as well as those of a hypomorphic vab-8(gm84) mutant (Fig. 2). A null allele of egl-15 eliminated the Clr-1 suppression of vab-8 and unc-34 mutants, consistent with the hypothesis that reducing CLR-1 function promotes CAN migration by increasing the activity of EGL-15/FGFR (Fig. 2).

The ability of the clr-1 mutation to partially bypass the requirement for unc-34 and vab-8 suggests that FGF signaling acts, at least in part, in parallel to these two genes (Fig. 2). Consistent with this hypothesis, egl-17 and egl-15 mutations enhanced the CAN migration defects of the two unc-34 null mutants e951 and gm104 (Fig. 1; data not shown).

**SEM-5/GRB2 is involved in CAN migration**

The Src homology 2 (SH2)/SH3 adaptor protein SEM-5/GRB2 (Clark et al., 1992), the leucine-rich repeat protein SOC-2 (Selfors et al., 1998) and the Ras homolog LET-60 (DeVore et al., 1995) can all function downstream of EGL-15. Genetic data implicate SEM-5 but not the other molecules in FGF-mediated SM migration (DeVore et al., 1995; Schutzman et al., 2001). sem-5(n2019), soc-2(n1774), let-60(n2021), let-60(sy101sy127) and let-60(n1046) single mutants showed no CAN defects (Figs 3, 4). We then tested whether the mutations in sem-5, soc-2 and let-60 could enhance the CAN defects of vab-8(gm84) mutants. As with the egl-17 and egl-15 mutations, sem-5(n2019) enhanced the CAN defects of vab-8(gm84) mutants (Fig. 3). This observation is consistent with but does not prove that SEM-5 acts as a downstream mediator of EGL-15 in CAN migration.

By contrast, neither the soc-2 nor the let-60 mutations significantly affected CAN migration in a vab-8(gm84) mutant background, suggesting that neither soc-2 nor let-60 function in CAN migration (Figs 3, 4). However, as soc-2(n1774) is a hypomorphic allele we cannot completely rule out soc-2 involvement in CAN migration. Furthermore, let-60(sy101sy127) null mutants arrest as larvae and were scored as progeny of heterozygous hermaphrodites, raising the possibility that a maternal contribution of let-60 may mask its role in CAN migration. Nevertheless, the lack of an effect for either hypomorphic (n2021) or gain-of-function (n1046) alleles that reduce or increase let-60 activity zygotically and maternally supports the hypothesis that let-60 is not involved in CAN migration (Fig. 3).
FGF gradient and enhance the CAN defects of the vab-8(gm84) mutant. (B) A transgene expressing a constitutively activated EGL-15(neu) enhanced vab-8(gm84) CAN migration defects, while overexpression of wild-type egl-15 did not (P<0.01). Strains containing the transgenes were scored in a soc-2(n1774) background to avoid the lethality caused by constitutive EGL-15 activity.

EGL-17 and EGL-15 act instructionally to promote CAN migration

FGF has been shown to function both as a permissive and as an instructive cue for different cell migrations. To determine whether EGL-17 promotes CAN migration by providing permissive or instructive information, we expressed EGL-17 throughout the embryo using a hsp::egl-17::gfp construct. If EGL-17 functions as a permissive cue, its distribution should not matter, and global EGL-17 expression, like the clr-1 mutation, should partially rescue the CAN migration defects of vab-8 mutants. If, however, EGL-17 functions as an instructive cue, then its distribution should be important, and global EGL-17 expression should enhance the CAN migration defects of vab-8 mutants. Consistent with EGL-17 acting as an instructive cue, ectopic expression of egl-17 from the heat-shock promoter enhanced the CAN migration defects of vab-8(gm84) mutants (Fig. 4A).

As an additional test to differentiate between permissive and instructive signaling, we asked whether a constitutively active EGL-15 could rescue or enhance the CAN migration defects of the vab-8 mutant. If EGL-17 functions permissively, then unregulated FGFR signaling should partially bypass the requirement for vab-8. If EGL-17 functions instructively to guide the CANs, then unregulated FGFR signaling should disrupt the asymmetric signaling normally conferred by an FGF gradient and enhance the CAN defects of the vab-8 mutant.

To constitutively activate EGL-15, we used the egl-15(neu) transgene (Kokel et al., 1998). Because expression of the egl-15(neu) construct causes lethality, all strains were scored in a soc-2(n1774) mutant, which suppresses the lethality caused by the egl-15(neu) transgene. soc-2(n1774) had no effect on CAN migration either alone or in combination with a vab-8 mutation (Fig. 4B). Expression of egl-15(neu) enhanced the vab-8(gm84) CAN migration defects, while expression of wild-type egl-15 did not (Fig. 4B).

Increasing EGL-15 activity in a clr-1 mutant suppressed the CAN defects of the vab-8 mutants, but increasing EGL-15 with an activating mutation enhanced the defects. Although these results may appear inconsistent, they support a model where asymmetric activation of EGL-15 by EGL-17 guides the migrating CANs. In this model, EGL-15 is normally activated asymmetrically in the CAN by an EGL-17 gradient. When CLR-1 function is reduced, we propose that normal asymmetric activity of EGL-15 is increased, resulting in the suppression of CAN migration defects. We propose that the activating mutation increases all EGL-15 activity, masking the asymmetric activation by EGL-15 and leading to an enhancement of CAN migration defects. The effects of the activated EGL-15 receptor and global EGL-17 expression on CAN migration suggest that both the asymmetric activation of EGL-15 and the distribution of EGL-17 are important for CAN guidance.

EGL-17 is a CAN repellent

The distribution of endogenous egl-17 expression during the time of CAN migration may address whether EGL-17 attracts or repels the CANs. We analyzed the GFP expression pattern of animals carrying ayIs9, an egl-17::gfp transcriptional reporter transgene (Branda and Stern, 2000), and gmEx283, a functional egl-17::gfp translational reporter transgene that can rescue the enhancement of vab-8(gm84) CAN migration defects caused by egl-17(n1377) (Fig. 5A). At the time when the CANs are migrating, we detected GFP in only two hyp5 hypodermal cells at the anterior tip of the embryo (Fig. 5B-D). This expression pattern suggests that EGL-17 repels the CANs. To test this hypothesis, we expressed egl-17 ectopically both at the anterior end of the embryo using the lim-4 promoter (Plim-4::egl-17::gfp) and in the posterior midbody using a mab-5 promoter (Pmab-5::egl-17::gfp) (Fig. 6A). The LIM homeodomain LIM-4 functions in the cell-fate specification of olfactory neurons. lim-4 is expressed in the AWB neurons as well as a few other head neurons (Sagasti et al., 1999). mab-5 encodes a homolog of Drosophila Antennapedia, and its expression in the posterior midbody region of the worm is required for proper epidermal, neuronal and mesodermal cell differentiation (Costa et al., 1988; Wang et al., 1993). If our hypothesis that EGL-17 acts as a CAN repellent is correct, then the Plim-4::egl-17::gfp transgene should promote normal CAN migration, while the Pmab-5::egl-17::gfp transgene should hinder migration. As predicted by the repellent model, the Plim-4::egl-17::gfp transgene partially rescued the CAN
FGF repellent for cell migration

migration defects of vab-8(gm84); egl-17(n1377) double mutants, while the Pmab-5::egl-17::gfp transgene enhanced the CAN migration defects of vab-8(gm84) mutants (Fig. 6B). These experiments suggest that EGL-17 can repel the CANs.

**EGL-15 functions cell-autonomously in CAN migration**

The EGL-17 repellent model predicts that the EGL-17 receptor, EGL-15, acts cell autonomously in the CANs to promote their migration. To test this prediction, we expressed egl-15 from a ceh-10 promoter. The homeobox gene ceh-10 is expressed in the CANs and a few sensory neurons in the head and tail (Hawkins and McGhee, 1990; Svendsen and McGhee, 1995). As predicted, the Pceh-10::egl-15 transgene rescued the CAN migration defects of vab-8(gm84); egl-17(n1477) double mutants (Fig. 7). We also tested whether expression of EGL-15 in the hypodermal cells could rescue the CAN defects of vab-8(gm84); egl-17(n1377) double mutants, while the Pmab-5::egl-17::gfp transgene partially rescued the CAN migration defects of vab-8(gm84); egl-17(n1377) CAN migration defects. By contrast, ectopic expression of EGL-17 in the head from a Pceh-10::egl-17::gfp transgene partially rescued vab-8(gm84); egl-17(n1377) CAN migration defects. By contrast, ectopic expression of EGL-17 in the mid-posterior region from a Pmab-5::egl-17::gfp transgene enhanced the CAN migration defects of a vab-8(gm84) background.
Fig. 7. EGL-15 functions cell autonomously in CAN migration. The CAN positions were scored and are displayed as described in Fig. 1. Expression of a Pceh-10::egl-15 transgene rescued the enhancement of the CAN migration defects caused by egl-17(n484) in a vab-8(gm84) background. The ceh-10 promoter drives expression in a small subset of sensory neurons and in the migrating CANs. By contrast, the expression of EGL-15 in hypodermal cells from a Pdp7::egl-15 transgene failed to rescue the CAN migration defects of vab-8(gm84); egl-15(n484) (P=0.52).

egl-15 mutants. EGL-15 acts in hypodermal cells to regulate fluid balance, axon outgrowth and maintenance of axon position (Bulow et al., 2004; Huang and Stern, 2004). Expression of EGL-15 from the dpy-7 promoter, which drives expression in hypodermal cells (Bulow et al., 2004; Gilleard et al., 1997), failed to rescue the CAN migration defects of vab-8(gm84); egl-15(n484) mutants. These results indicate that EGL-15 functions cell autonomously in the CANs to promote their migrations.

Discussion

We show here that the C. elegans EGL-17/FGF can act as a repellent to direct CAN cell migration in sensitized backgrounds. EGL-15/FGFR was found to function in the CAN neurons as the receptor for EGL-17/FGF, and its signaling was inhibited by the receptor tyrosine phosphatase CLR-1. Our results also suggest that the SH2/SH3 adaptor protein SEM-5 mediates EGL-15/FGFR signaling in CAN cell migration.

FGFs play multiple roles in development

FGFs and their receptors are highly conserved proteins involved in a wide variety of developmental events that include patterning, differentiation and morphogenetic movements. In Drosophila, the FGF Branchless and its receptor Breathless can provide different functions for tracheal cell migrations. Branchless can function through Breathless as an attractant for primary branching in tracheal development and then as a permissive cue for secondary branches. Later, Branchless is an inducer and chemoattractant for terminal branches (Jarecki et al., 1999; Lee et al., 1996).

Two different FGFs act as guidance cues during chick gastrulation. FGFR expression along the primitive streak repels cells away from the streak, whereas FGF4 expression in the head process and notochord attracts cells emerging from the anterior streak (Yang et al., 2002). Cells expressing a dominant negative FGFR1 fail to respond to FGFR4 attraction, suggesting that FGFR1 may function as a receptor for FGFR4 (Yang et al., 2002).

C. elegans has a single FGFR, EGL-15, and two FGFs, EGL-17 and LET-756. EGL-15/FGFR has at least five functions: an essential function in fluid balance regulation revealed by the larval lethality of egl-15 null mutants; a guidance function in attracting the migrating SMs; a role in axon outgrowth; a role in maintenance of axon bundles; a guidance function in repelling the migrating CANs (Bulow et al., 2004; DeVore et al., 1995; Stern and Horvitz, 1991). EGL-15/FGFR acts in the hypodermis, an epithelial cell type that encases the worm, to regulate fluid homeostasis, axon outgrowth and the maintenance of axon bundle integrity (Bulow et al., 2004; Huang and Stern, 2004).

FGF signaling in CAN migration

Our results and the studies on SM migrations demonstrate that a single FGF and a single FGFR can mediate both attraction and repulsion (Burdine et al., 1998; DeVore et al., 1995; Stern and Horvitz, 1991). It is not unusual for the same guidance cue to function both as an attractant and a repellent. UNC-6/Netrin, for example, functions as a repellent for cells and growth cones that migrate dorsally and as an attractant for cells and growth cones that migrate ventrally (Chan et al., 1996; Colamarino and Tessier-Lavigne, 1995). Even the same cells can respond differently to the same cue at different times during development. Migrating mesodermal cells in Drosophila are first repelled by Slit expression at the midline and later attracted towards Slit expression at muscle attachment sites (Kramer et al., 2001).

Two mechanisms appear to regulate how migrating cells and growth cones respond to a guidance cue. UNC-6/Netrin can attract or repel migrating cells and growth cones depending on the types of receptors that are expressed. Expression of the UNC-40 homolog DCC, for example, results in an attractive response, while expression of the UNC5 receptor and DCC results in repulsion (Hong et al., 1999). Consistent with a conserved role for this mechanism, repulsion can be mediated in Xenopus spinal axons by Netrin-induced UNC5/DCC heterodimers. Alternatively, intracellular molecules can alter the response to a guidance cue. Netrin, for example, can either attract or repel the growth cones of cultured embryonic Xenopus spinal neurons depending on the levels of cAMP within the neuron (Ming et al., 1997). cGMP levels play a similar role for other guidance cues (Xiang et al., 2002).

Alternate splicing produces two distinct EGL-15 isoforms (5A and 5B) that contain distinct extracellular domains (Goodman et al., 2003). LET-756 signals through the EGL-15(5B) to regulate fluid homeostasis and axon outgrowth, while EGL-17 signals through EGL-15(5A) to attract the SMs (Bulow et al., 2004; Goodman et al., 2003). Although each receptor isoform mediates the response to a unique FGF, one isoform is capable of responding to either FGF: egl-17 driven from the let-756 promoter can rescue the larval lethality of a let-756 mutant and, conversely, let-756 driven from the egl-17 promoter can rescue the SM migration defects of an egl-17 mutant (Goodman et al., 2003). We found that the 5A isoform mutant egl-15(n484) enhanced CAN migration defects of a vab-8 mutant to a similar extent as egl-15(n1456) null mutants, which affect both 5A and 5B isoforms. In
addition, a let-756 null allele failed to significantly enhance \textit{vab-8}(gm84) CAN migration defects. Therefore, EGL-17 appears to function through the 5A isofrom as both an attractant and as a repellent. However, it is unclear whether different co-receptors, the levels of cyclic nucleotides or yet another mechanism determines the attractive or repulsive functions of EGL-17.

We have shown that similarities and differences exist in downstream components of EGL-15/FGFR signaling when comparing CAN migration and the other processes mediated by this pathway. In addition to acting downstream of EGL-15 in SM migration and fluid balance regulation (Clark et al., 1992), the adaptor protein SEM-5 is also involved in CAN migration. SOC-2, a leucine-rich repeat protein, and LET-60/Ras functions downstream of EGL-15 in axon outgrowth (DeVore et al., 1995; Selfors et al., 1998). However, neither SOC-2 nor LET-60/Ras appears to be involved in directing SM or CAN migration (DeVore et al., 1995) (this study).

Defining additional downstream components of EGL-17/FGF will be important to determine how EGL-17 can act both as an attractant and a repellent. Phospholipase C-\(\gamma\) (PLC-\(\gamma\)) and phosphoinositide 3-kinase (PI3-kinase) could potentially function downstream of EGL-15/FGFR. Coactivation of PLC-\(\gamma\) and PI3-kinase are required for the chemoaatraction towards nerve growth factor (NGF) by \textit{Xenopus} spinal neurons expressing exogenous rat TrkA, a receptor tyrosine kinase (Ming et al., 1999). Perhaps PLC-\(\gamma\) and PI3-kinase also contribute to the signaling of EGL-15 to promote attraction of the SMs or repulsion of the CANs.

**The receptor tyrosine phosphatase CLR-1 inhibits EGL-15/FGFR signaling**

Although CLR-1 does not have any obvious role in SM migration, we have found that, as with fluid homeostasis, CLR-1 inhibits EGL-15/FGFR signaling in CAN migration (Kokel et al., 1998). CLR-1 also inhibits UNC-6/Netrin signaling of the \textit{C. elegans} AVM growth cones (Chang et al., 2004). UNC-34/ENA and the Rac CED-10 function in parallel downstream of the UNC-40/DCC receptor to mediate UNC-6/Netrin attraction (Gitai et al., 2003). Additionally, CLR-1 mediated inhibition of UNC-40/DCC signaling requires UNC-34/Ena (Chang et al., 2004). This observation differs from our finding that in CAN migration CLR-1 and UNC-34/Ena can act independently.

**EGL-17 may be one of several guidance cues for CAN migration**

Although our results show that EGL-17 and its receptor EGL-15 function in CAN migration, the role of each gene is only revealed in sensitized genetic backgrounds. Why does FGF signaling play such a subtle role in CAN migration? One observation that may be relevant to this question is that no other guidance cue or receptor has been shown to be necessary for CAN cell migration. Although two receptors, the INA-1/PAT-3 integrin and the RTK CAM-1, function in the CAN to promote its migration, these molecules are unlikely to act as guidance receptors. The ligand for INA-1/PAT-3 appears to be the permissive substrate laminin (Baum and Garriga, 1997), and the kinase domain of CAM-1 is not involved in its migration functions (Forrester et al., 1999). In fact, the extracellular CRD domain of CAM-1 is both necessary and sufficient for its role in CAN migration, and genetic experiments suggest that CAM-1 functions in cell migration by inhibiting Wnt function (Forrester et al., 2005; Kim and Forrester, 2003).

The CANs migrate normally in \textit{C. elegans} that are mutant for the homologs of the guidance cues Netrin and Slit, and in worms that are mutant for the guidance receptor UNC-40. SLT-1/Slit and its receptor SAX-3/Robo, by contrast, play a subtle role in CAN migration. Like egl-17 and egl-15 mutations, \textit{slt-1} and \textit{sax-3} mutations have little effect on CAN migration in an otherwise wild-type background, but can alter CAN migration in a sensitized background that contains a \textit{ceh-23}::\textit{gfp} transgene or a \textit{vab-8} mutation (Hao et al., 2001) (this study). At the time when the CANs migrate, SLT-1 is expressed at the anterior end of the embryo, consistent with it acting as a repellent. Taken together, the FGF and SLT-1 results are consistent with a model in which several different cues guide the CANs to their final destinations. Removing any specific cue has little effect on CAN migration because the remaining cues are able to provide adequate guidance information. This model predicts that genetically sensitized backgrounds are needed to identify the molecules that guide the CANs and perhaps other neurons that migrate along the anteroposterior axis.

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**References**


