Distinct Cell Guidance Pathways Controlled by the Rac and Rho GEF Domains of UNC-73/TRIO in *Caenorhabditis elegans*

Nancy Marcus-Gueret,*,† Kristopher L. Schmidt,*,† and Eve G. Stringham*,†,1

*Department of Biology, Trinity Western University, Langley, BC V2Y 1Y1, Canada and †Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC V5A 1S6, Canada

ABSTRACT The cytoskeleton regulator UNC-53/NAV2 is required for both the anterior and posterior outgrowth of several neurons as well as that of the excretory cell while the kinesin-like motor VAB-8 is essential for most posteriorly directed migrations in *Caenorhabditis elegans*. Null mutations in either *unc-53* or *vab-8* result in reduced posterior excretory canal outgrowth, while double null mutants display an enhanced canal extension defect, suggesting the genes act in separate pathways to control this posteriorly directed outgrowth. Genetic analysis of putative interactors of UNC-53 or VAB-8, and cell-specific rescue experiments suggest that VAB-8, SAX-3/ROBO, SLT-1/Slit, and EVA-1 are functioning together in the outgrowth of the excretory canals, while UNC-53 appears to function in a parallel pathway with UNC-71/ADAM. The known VAB-8 interactor, the Rac/Rho GEF UNC-73/TRIO operates in both pathways, as isoform specific alleles exhibit enhancement of the phenotype in double-mutant combination with either *unc-53* or *vab-8*. On the basis of these results, we propose a bipartite model for UNC-73/TRIO activity in excretory canal extension: a cell autonomous function that is mediated by the Rho-specific GEF domain of the UNC-73E isoform in conjunction with UNC-53 and UNC-71 and a cell nonautonomous function that is mediated by the Rac-specific GEF domain of the UNC-73B isoform, through partnering with VAB-8 and the receptors SAX-3 and EVA-1.

THE migration of cells and cellular processes to their final positions requires the integration of a multitude of attractive and repulsive cues and, in response, the coordinated reorganization of the cytoskeleton to direct cell shape changes. In *Caenorhabditis elegans* and other organisms, studies of the global guidance mechanisms controlling migration have revealed that positioning decisions occur along both the anterior–posterior (AP) and the dorsoventral (DV) axes (Levy-Strumpf and Culotti 2007) and that many of these guidance molecules and their downstream effectors have been conserved in evolution (Dickson 2002). For example, UNC-6/Netrin is a laminin-like protein expressed in a variety of guidepost cells on the ventral side that guides ventral extensions of axons through its receptor UNC-40/DCC (Wadsworth *et al.* 1996). At the same time, UNC-6/Netrin also repels axons and cells expressing both UNC-40 and UNC-5 receptors toward the dorsal side (Hedgecock *et al.* 1990). Similarly, vertebrate Netrin-1 and -2 promote attraction of the commissural neurons while repelling the trochlear motor neurons (Serafini *et al.* 1994, 1996; Colamarino and Tessier-Lavigne 1995). Concomitantly, dorsally expressed SLT-1 functions as a repellent to direct SAX-3/ROBO and EVA-1 expressing axons toward the ventral side (Zallen *et al.* 1998; Fujisawa *et al.* 2007).

In contrast to DV migrations, we are only beginning to unravel the cell guidance pathways that regulate the long-range migrations along the AP axis in *C. elegans*. Some of the key signaling molecules that have been implicated in longitudinal guidance include the fibroblast growth factor EGL-17/FGF (Burdine *et al.* 1997) and its receptor EGL-15/FGFR (Devore *et al.* 1995; Bulow *et al.* 2004; Birnbaum *et al.* 2005), LIN-17/Frizzled (Hilliard and Bargmann 2006), the Wnts LIN-44, CWN-1, CWN-2, and EGL-20 (Maloof *et al.* 1999; Zinovyeva *et al.* 2008), and UNC-53/NAV2, a cytoskeletal binding protein related to the mammalian neuronal navigators (NAVs) (Stringham and Schmidt 2009). Loss-of-function mutations in *unc-53* cause both anterior and posterior extension and guidance defects in several cell types including the axons of the mechanosensory neurons (Hekimi

doi: 10.1534/genetics.111.134429
Manuscript received September 4, 2011; accepted for publication September 27, 2011
[1]Corresponding author: Department of Biology, Trinity Western University, 7600 Glover Rd., Langley, BC V2Y 1Y1, Canada. E-mail: stringha@twu.ca

and Kershaw 1993), the excretory canals (Hedgecock *et al.* 1990; Stringham *et al.* 2002), and the sex myoblasts (Stringham *et al.* 2002). Conversely, overexpression of *unc-53* leads to exaggerated growth cone extension during embryogenesis (Stringham *et al.* 2002). UNC-53 and the NAVs contain several cytoskeletal binding domains including an actin-binding calponin-homology domain, and putative microtubule binding domains (Stringham *et al.* 2002). UNC-53 localizes to the cytoskeleton, binds F-actin *in vitro*, and binds ABI-1(Abelson kinase interactor 1), a regulator of ARP2/3-mediated actin filament assembly (Stringham *et al.* 2002; Schmidt *et al.* 2009). UNC-53 may also function in signal transduction as it is known to genetically and physically interact with the SH2–SH3 adapter protein SEM-5/GRB-2, a mediator of EGL-15/FGFR signaling in sex myoblast migration in *C. elegans* (Chen *et al.* 1997; Stringham *et al.* 2002). The three human homologs of *unc-53* (*NAV-1*, *NAV-2*, and *NAV-3*), are expressed in a range of tissues including the developing brain (Maes *et al.* 2002; Peeters *et al.* 2004). UNC-53 is most closely related to NAV2/RAINB1, a gene discovered in a study that identified molecules upregulated in response to all *trans*-retinoic acid (atRA), which is required for patterning of the nervous system in mammals (Merrill *et al.* 2002). Expression of NAV-2 in the PLM mechanosensory neurons rescued the axon outgrowth defects of *unc-53* mutants, suggesting NAV-2 is a true ortholog of UNC-53 (Muley *et al.* 2008).

The gene *vab-8* has been proposed as a component of a global directional guidance system that steers cell and growth cone migrations posteriorly in the AP axis (Wightman *et al.* 1996; Wolf *et al.* 1998). The largest transcript (VAB-8L) contains six 5′ exons that are not shared with the five smaller transcripts (collectively referred to as VAB-8S) and encodes a protein that contains an N-terminal domain similar in sequence to kinesin motors (Wolf *et al.* 1998). VAB-8L is necessary and sufficient for all *vab-8*–dependent growth cone extensions but not all cell migrations, while VAB-S is necessary only for certain cell body migrations but not for axon outgrowth (Wolf *et al.* 1998). Recent evidence from Levy-Strumpf and Culotti (2007) and Watari-Goshima *et al.* (2007) shows that VAB-8L promotes the posterior migration of cells and growth cones by regulating the activity of guidance receptors that also function in DV guidance. VAB-8 localizes UNC-40/DCC and SAX-3/ROBO in the growth cone of the ALM axons and these effects require the activity of the Rho and Rac guanine nucleotide exchange factor (GEF), UNC-73/Trio (Levy-Strumpf and Culotti 2007; Watari-Goshima *et al.* 2007).

UNC-73 is related to mammalian Trio and Kalirin, and Drosophila *Trio* has been implicated as a key regulator of axon development by signaling through the RacGEF to regulate cytoskeletal rearrangements necessary for growth cone migrations (Debant *et al.* 1996; Newsome *et al.* 2000; Lundquist 2003). In *C. elegans*, three Rac GTPases (CED-10, MIG-2, and RAC-2) have redundant roles in the migration of growth cones, embryonic cells during gastrulation, and the cells that comprise the vulva (Lundquist *et al.* 2001; Kishore and Sundaram 2002; Soto *et al.* 2002). *Drosophila Trio* also affects axon pathfinding and acts as a GEF for Racs and MIG-2–related proteins (Awasaki *et al.* 2000; Bateman *et al.* 2000; Newsome *et al.* 2000). *unc-73* encodes several protein isoforms containing various recognizable motifs, including two GEF domains: the N-terminal UNC-73 RacGEF domain specifically activates the Rac family GTPases CED-10 and MIG-2 *in vitro* (Steven *et al.* 1998; Wu *et al.* 2002; Kubiseski *et al.* 2003), while the C-terminal RhoGEF domain is specific to Rho (Spencer *et al.* 2001).

Here we report that UNC-53 and VAB-8 act in separate pathways to control the outgrowth of the excretory canals. Genetic analysis directed at putative interactors of UNC-53 or VAB-8 suggests that VAB-8, SAX-3/ROBO, SLT-1/Slit, and EVA-1 are functioning together in the outgrowth of the excretory canals, while UNC-53 appears to function in a parallel pathway with UNC-71/ADAM. The known VAB-8 interactor, UNC-73/Trio operates in both pathways, as isoform-specific alleles exhibit enhancement of the canal defects in double-mutant combination with either *unc-53* or *vab-8*. We show that together with UNC-53/NAV2 and UNC-71/ADAM, UNC-73/TRIO functions cell autonomously within the excretory cell to promote outgrowth, while also functioning in a cell nonautonomous manner through partnering with VAB-8 and the receptors SAX-3 and EVA-1.

Materials and Methods

C. elegans strains

Standard methods of culturing and handling worms were used (Brenner 1974). All genetic crosses were carried out at 20°. All strains were cultured at 20° for scoring phenotypes, with the exception of *eva-1(ev751)*, which is temperature sensitive and was scored at 25° (Fujisawa *et al.* 2007). Double-mutant strains were constructed using standard genetic methods without additional marker mutations. The presence of the mutations was confirmed either by visual inspection of phenotypes or outcrossing to *him-8* males and examining the F_2 progeny. *C. elegans* strains used in this study were:

N2 Bristol (wild type)
BC06288 *ppgp12::gfp (sIs10089)*
VA71 *unc-53(n166); sIs10089*
VA106 *unc-53(n152); sIs10089*
VA396 *unc-73(e936); sIs10089*
VA397 *unc-73(rh40); sIs10089*
VA398 *unc-71(e541); sIs10089*
VA399 *unc-73(ev802); sIs10089*
DR1785 *mIn1[dpy-10(e128)]/unc-4(e120)II*
MT1000 *unc-5(e53)/nT1 IV; dpy-11(e224)/nT1 V*
VA415 *dpy-5(e907)/dpy-5(e907)*; *pmEx107 [ppgp-12::VAB-8L::GFP + pCeh361]*
VA400 *unc-53(n166);vab-8(e1017); sIs10089*
VA401 *unc-73(ev802); unc-53(n166); sIs10089*
VA402 *unc-73(ok936); unc-53(n166); sIs10089*
VA403 *unc-53(n166);sax-3(ky123); sIs10089*

VA404 *eva-1(ev751); unc-53(n166); sIs10089*
VA405 *unc-53(n166);slt-1(eh15); sIs10089*
VA406 *unc-53(n166);slt-1(ev740); sIs10089*
VA407 *unc-73(ev802); vab-8(e1017); sIs10089*
VA408 *unc-73(ok936); vab-8(e1017); sIs10089*
VA409 *vab-8(e1017);sax-3(ky123); sIs10089*
VA410 *eva-1(ev751); vab-8(e1017); sIs10089*
VA411 *vab-8(e1017);slt-1(eh15); sIs10089*
VA412 *vab-8(e1017);slt-1(ev740); sIs10089*
XS82 *uxEx44 [punc-73::unc-73E::gfp]*
VA413 *unc-73(ev802); sIs10089; punc-73::unc-73E::gfp*
VA414 *unc-53(n166); sIs10089; punc-73::unc-73E::gfp*
VA415 *rho-1(ok2418)/nT1[qIs51]; sIs10089*
VA416 *unc-73(ev802); dpy-5(e907)/dpy-5(e907); pmEx108 [rCes pmyo-2::GFP + pCeh361 + WRM0628aD12]*
IC699 *sax-3(ky123); quEx168 [psax-3::sax-3::gfp; odr-1::RFP]*.

Scoring and analysis of excretory canal extension defects

To score the excretory canal processes, adult hermaphrodites carrying the *ppgp-12*::*gfp* reporter, which is expressed specifically in the excretory cell beginning in the threefold embryonic stage (Zhao *et al.* 2005), were immobilized in 30 mM sodium azide and immediately viewed with epifluorescence to determine canal length and morphology. Locations of excretory cell bodies were scored relative to the terminal pharyngeal bulb in young adults. Animals were scored for excretory canal outgrowth with respect to the position of the gonad arms, the vulva, and the anus.

Chi-squared analysis was used to establish statistical significance between mutants using Graphpad Prism 5 (Sigma Stat). In cases where single mutants were compared to wild-type N2 animals, posterior canal extension was scored as described, and statistical comparisons were made between groups by comparing the number of animals exhibiting normal canal extension (scored as a 5) and reduced canal extension (<5). In cases where double null mutant alleles were examined, the worse of the two null alleles was set as the baseline for comparison (as indicated on the right side of each figure) while phenotypes displaying a further reduction in canal extension were grouped together.

RNA interference

RNAi experiments were performed by feeding (Kamath *et al.* 2001) using RNAi clones obtained from Geneservice (Cambridge, UK). The F_1 progeny of the young adults fed the RNAi were scored for excretory canal migration defects as described previously.

Rescue experiments

To create the excretory cell-specific VAB-8L construct pVA705, a *Hind*III/*Pst*I genomic fragment containing the *pgp-12* promoter was first cloned into pPD95.77 (gift of Andrew Fire, Stanford University School of Medicine, Stanford, CA). An amplified product comprising the VAB8L genomic sequence from exons 1–5, fused to the remainder of the VAB8L cDNA at the *Sal*I (+1018) site within exon 5 from pFWV8LG (Wolf *et al.* 1998), was digested with *Xba*I/*Xma*I and then inserted into the *Xba*I/*Xma*I-cut pPD95.77 construct and confirmed by sequencing. The primers used for this reaction were GCGGCGCTCTAGAATGGAGGCATGCAGCAGT (left) and GCGCCGCCCCGG GATAATGAAAGTGGTAACCA (right). Transgenic strains were generated by injecting 10 ng/μl of pVA705 and 100 ng/μl of pCeh361 construct [*dpy-5*(+)], into the *dpy-5(e907)* strain. Transmitting wild-type lines carrying the extrachromosomal array *pmEx107* were crossed into *vab-8(e1017)* mutants. Excretory canal morphology was scored in young adult animals expressing GFP for general defects and for posterior canal extension position, as described above.

Rescue of the *unc-73(ev802)* posterior canal defects was tested by crossing the previously constructed XS82 *punc-73E*::*unc-73E*::*gfp* transgenic strain (Steven *et al.* 2005) into the strain VA399 *unc-73(ev802)*; *sIs10089* to create VA413 *unc-73(ev802)*; *sIs10089*; *punc-73E*::*unc-73E*::*gfp* and scoring transgenic homozygous animals as described above. Similarly, we tested the ability of a strain containing the *unc-53* fosmid to rescue the *unc-73(ev802)* allele. The *unc-53* fosmid expressing array *pmEx108* was generated by co-injecting *pmyo-2::GFP, WRM0628aD12*, along with the plasmid *pCeh361* [*dpy-5*(+)], into the *dpy-5(e907)* strain. Transmitting wild-type lines carrying the extrachromosomal array *pmEx108* were crossed into *unc-73(ev802)* mutants and transgenic homozygous animals were scored as described above. Rescue of *sax-3(ky123)* posterior canal defect was tested by crossing the previously constructed IC699 *sax-3 (ky123)*; *quEx168 [psax-3*::*sax-3*::*gfp*; *odr-1*::*RFP]* into *unc-53(n166)*; *sax-3(ky123)* double mutants as *sax-3(ky123)* mutant animals did not display excretory cell (EC) defects alone.

Results

UNC-53 and VAB-8 function independently to control excretory cell development

UNC-53 and VAB-8 are required for the longitudinal migration of several cells and cellular processes along the longitudinal axis in *C. elegans* including the excretory cell and the axons of both the PDE neuron and the ALA neuron (Wightman *et al.* 1996; Wolf *et al.* 1998; Stringham *et al.* 2002). The EC is the largest single cell in the worm and the leading edges of the growing canals resemble growth cones in that they must be able to sense and integrate directional cues in both the dorsoventral and anteroposterior axes. Genes affecting dorsal and posterior outgrowth in neurons frequently affect the outgrowth of the excretory canals as well, including *unc-5*, *unc-34*, *unc-71*, and *unc-73* (Hedgecock *et al.* 1987). In wild-type animals, the EC body sends out two processes dorsolaterally from the ventral side of the terminal pharyngeal bulb toward the lateral midline (Buechner 2002). Once these processes reach the lateral hypodermis, they bifurcate to extend an anterior branch that extends to the

very anterior region of the head and a posterior branch that extends all the way to the anus (Figure 1A; Hedgecock *et al.* 1987; Buechner 2002).

Using the EC-specific reporter *ppgp-12*::*gfp*, which is expressed exclusively in the excretory cell from the threefold embryonic stage onwards (Zhao *et al.* 2005), we examined the defects seen in *unc-53* and *vab-8* null animals. In *unc-53 (n166)* mutants, a null allele, the EC body is positioned normally with respect to the posterior pharyngeal bulb, but both the anterior and posterior canals are severely truncated (Figure 1B; Schmidt *et al.* 2009). The anterior processes terminate close to the EC body and do not extend further than the anterior pharyngeal bulb, while the posterior canals grow out to the midbody, terminating at the level of the vulva (Figure 1B; Schmidt *et al.* 2009). Examination of the null mutant *vab-8(e1017)* revealed a failure of the majority of the posterior canals to exit the gonad region (Figure 1C). Unlike *unc-53 (n166)* animals, *vab-8(e1017)* mutants also have displaced EC bodies, positioned posteriorly to the terminal pharyngeal bulb of the pharynx compared to wild type (Figure 1C). Additionally, *vab-8(e1017)* mutants alone did not display truncated anterior canals as observed in *unc-53(n166)* (Figure 1C). Together, these results suggest that *unc-53* and *vab-8* both function in the posterior outgrowth of the excretory canals, and that *vab-8* but not *unc-53* is required for determining the anterior position of the EC body.

To determine whether *vab-8* and *unc-53* function in the same or distinct genetic pathways to control excretory cell development we examined the EC in *unc-53(n166)*; *vab-8 (e1017)* double mutants. The posterior excretory canal defects were observed to be more severe in *unc-53(n166)*; *vab-8(e1017)* than in either *unc-53(n166)* or *vab-8(e1017)* single mutants (Figure 1D). As *vab-8(e1017)* and *unc-53 (n166)* are null alleles (Figure 1, E and F), these findings suggest the genes act in separate pathways, with each exerting their influence independently, on the posterior outgrowth of the excretory canals.

UNC-53 is required cell autonomously to control the outgrowth of the excretory canals as full-length *unc-53* cDNA driven by the *ppgp-12* excretory cell-specific promoter is sufficient to rescue the canal outgrowth defects of *unc-53 (n166)* mutants (Figure 1G; Stringham *et al.* 2002; Schmidt *et al.* 2009). To determine whether *vab-8* also functions cell autonomously we expressed the long isoform of VAB-8 (VAB-8L) specifically in the excretory cell under the control of the *ppgp-12* EC-specific promoter. As the long isoform of VAB-8 is known to drive growth cone extension while the small isoform (VAB-8S) is required for cell body migrations (Wolf *et al.* 1998), we questioned specifically whether VAB-8L could rescue excretory cell outgrowth if deliberately expressed in this cell. Expression of VAB-8L in the EC was unable to rescue defects seen in the excretory cell body, or the posterior excretory canals of *vab-8(e1017)* mutants, suggesting that, unlike UNC-53, VAB-8 functions cell nonautonomously in excretory cell development and posterior canal outgrowth (Figure 1G).

Examination of the anterior canals in the double mutants revealed that they were severely shortened or even absent compared to wild type or either single mutant (Figure 2, A–D). As *vab-8(e1017)* mutants have normal anterior canals, these results suggest that the presence of wild-type *unc-53* masks a previously unidentified anterior guidance function for *vab-8*, at least for the EC canals. To address the possibility of a secondary effect on the anterior canals as a result of defects in a pioneering posteriorly migrating process in the head, we examined the phenotype of neighboring cell migrations. The ALA cell body sends processes laterally adjacent to excretory canal and also along the dorsal nerve cord. Both *vab-8(e1017)* and *unc-53(n166)* have outgrowth defects in the ALA posteriorly migrating processes, so we reasoned that this migration may be so severely disrupted in double mutants that if the outgrowth of the canals relied on these pioneers, they would be unable to extend. However, defects seen in the ALA processes of *unc-53(n166)*; *vab-8(e1017)* double mutants were no more severe than in each null allele alone (data not shown), eliminating the possibility that the enhanced canal phenotype was secondary to the ALA pioneer.

Molecules functioning in the gonad-independent pathway for sex myoblast migration also function in EC outgrowth

During the second larval stage, two precursor cells, the sex myoblasts (SMs) migrate anteriorly to flank the center of the gonad. The gonad attracts the SMs, but if the gonad is ablated, the SMs are still able to migrate anteriorly but to a variable position, revealing the presence of a second gonad independent pathway (Chen *et al.* 1997). The attractive cue from the gonad is EGL-17/FGF, which mediates its effect through the EGL-15/FGFR receptor and a downstream signaling pathway that passes through the GRB2 adapter homolog SEM-5 (Chen *et al.* 1997). Mutations in *unc-53*, *unc-73* (GNEF similar to the Trio protein), and *unc-71* (ADAM) disrupt sex myoblast positioning in the absence of the gonad, while they do not affect positioning in the presence of the gonad. Thus, these genes appear to be part of a gonad-independent pathway that controls sex myoblast migration (Chen *et al.* 1997). Interestingly, like *unc-53*, *unc-71* and *unc-73* are expressed in the excretory cell and vulval cells, two cell types affected in *unc-53* mutants (Stringham *et al.* 2002; Huang *et al.* 2003; Steven *et al.* 2005).

To test whether these proteins postulated to function with UNC-53 in SM migration are also involved in EC outgrowth, we analyzed the excretory canal phenotypes of mutant and RNAi-treated animals. We reasoned that if two molecules act in the same pathway, animals with null mutations in both genes should not be more severely affected than single null mutants. If, by contrast, the molecules act in parallel pathways, the double mutants should be more severely affected. Of the genes tested, *unc-71(e541)*, and *unc-73(ev802)* produced excretory canal extension phenotypes reminiscent of *unc-53* mutants, while *sem-5 (RNAi)* did not (Figure 3). Notably, none of these alleles tested had

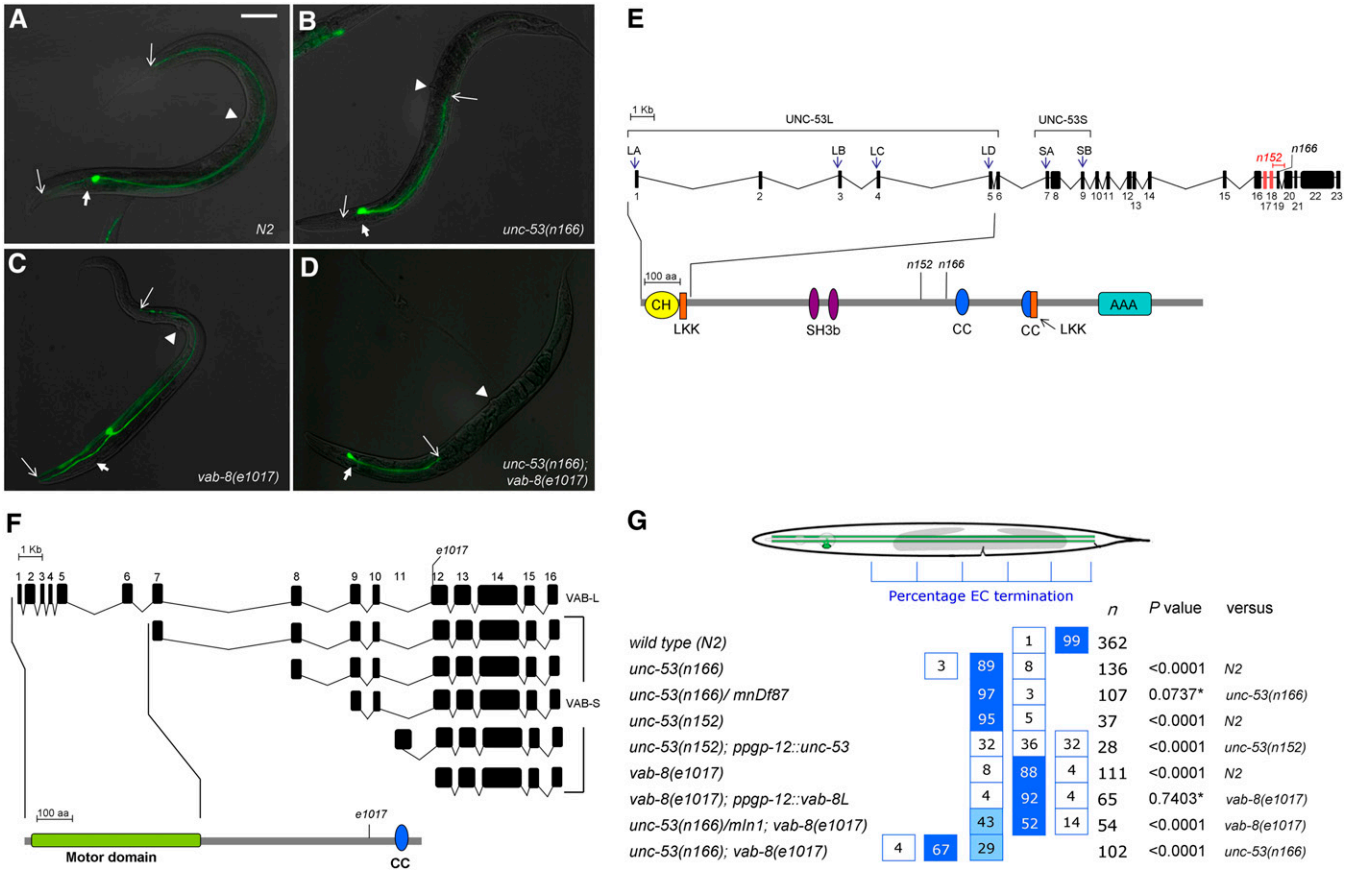
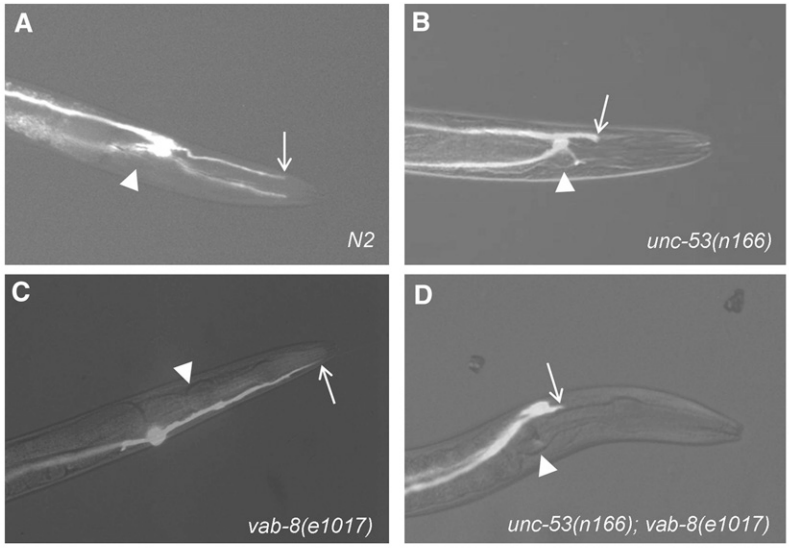


Figure 1 Excretory canal morphology in *unc-53*, *vab-8*, and *unc-53*; *vab-8* animals and genomic characterization of *unc-53* and *vab-8*. (A–D) Fluorescence micrographs of animals carrying the *ppgp-12*::*gfp* transgene, allowing for the visualization of the excretory cell body and canals (anterior and posterior termini are marked by long thin arrows, vulva is marked by an arrowhead, terminal bulb of the pharynx is marked by a short arrow, C–F.) Bar, 100 μm. Anterior is to the left and animals are displayed laterally. (A) Morphology of the wild-type excretory cell body and processes. The excretory cell body is positioned on the ventral side of the posterior pharyngeal bulb and extends two canals toward the anterior of the animal to the tip of the head and two canals posteriorly to the tail. (B) Excretory canal outgrowth phenotype of *unc-53 (n166)*. The posterior canals terminate midway at the vulva. (C) In *vab-8(e1017)* mutants, the posterior canals terminate beyond the vulva within the posterior gonad arm. (D) The *unc-53(n166)*; *vab-8(e1017)* double mutants exhibit enhanced canal defects where termination occurs before reaching the vulva, and often before reaching the anterior gonad arm. (E) Structure of the *unc-53* gene. The start of the various UNC-53L and UNC-53S isoforms are indicated by arrows. The promoter for UNC-53SA is between exons 5 and 8, and the promoter for UNC-53SB is located between exons 8 and 13 (Stringham *et al.* 2002). Alternatively spliced exons are shown in pink. *unc-53(n152)* is a 319-bp deletion removing parts of exons 18 and 19, producing a stop codon in exon 20 (Stringham *et al.* 2002), and *n166* is a single nucleotide C-to-T transition in exon 19 that introduces a premature stop codon (Schmidt *et al.* 2009). The longest polypeptide, UNC-53LA, is 1654 amino acids and contains a calponin homology domain (CH, red; amino acids 11–109), two LKK motifs (LKK, purple; 114–133 and 1097–1116), two proline-rich SH3-binding motifs (SH3b, green; 487–495 and 537–545), two coiled-coil regions (CC, blue; 890–923 and 1078–1113), and an AAA domain (yellow; 1292–1425). *n166* introduces a premature stop codon at amino acid 949. Both *n152* and *n166* remove the coiled-coil, LKK, and AAA domains from all isoforms. (F) Structures of the six characterized *vab-8* transcripts (Wolf *et al.* 1998). Exons are numbered above. The first six exons encode the kinesin-like motor domain. The position of the *vab-8(e1017)* null allele is indicated, where a C-to-T transition at bp 10,647 results in premature stop codon (Wolf *et al.* 1998). The VAB-8L protein contains a kinesin-like motor domain at its N terminus, and a domain predicted to form a coiled-coil is shared with all isoforms of VAB-8. (G) Quantification of posterior excretory canal outgrowth defects. The outgrowth of the posterior canals was divided into five regions (1–5) before the anterior gonad arm to the tail as shown. The stop point of canals was determined by fluorescence microscopy. Chi-squared analysis was used to establish statistical significance between mutants using GraphPad Prism 5 (Sigma Stat). For this comparison, phenotypes were grouped into two categories and the mutant compared to a baseline of either wild type, or the most severe single allele in the case of double mutants, as indicated. **P* value is not statistically significant.

more severe phenotypes either alone or in the background of the null *unc-53* allele *(n166)* when compared to *unc-53 (n166)* single mutants (Figure 3). This suggests that the initial trajectory of the posterior excretory canals to the anterior gonad arm is unaffected by loss of *unc-53* or other members of the gonad-independent SM migration pathway. Like the *unc-53*; *vab-8* double mutants, *unc-71*; *vab-8* double mutants displayed an enhanced phenotype, suggesting *unc-71* operates solely within the *unc-53* pathway.

Excretory canal extension requires DV guidance cues and their receptors

We were inspired to test the role of the guidance receptor SAX-3 in the outgrowth of the excretory canals because of



E

Genotype	Full length anterior canals (%)	Truncated anterior canals (%)	Absence of anterior canals (%)	*n*
wild type (N2)	100	0	0	52
unc-53(n166)	4	93	3	72
vab-8(e1017)	98	2	0	111
unc-53(n166); vab-8(e1017)	0	16	84	95

Figure 2 Anterior excretory canal morphology in wild-type, *unc-53(n166)*, *vab-8(e1017)*, *and unc-53(n166)*; *vab-8(e1017)* animals. (A–D) Fluorescence micrographs showing a lateral view of hermaphrodites carrying the *ppgp-12*::*gfp* transgene. The stop point of the anterior canals was scored with respect to the wild-type position near the head (arrows mark the final positions of the anterior excretory canals). Anterior is to the right. (A) Wild-type canals extend to the anterior end of the animal. (B) The anterior canals terminate prematurely in the strong allele *unc-53(n166)*. (C) The anterior canals were considered wild type in *vab-8 (e1017)* mutants, though the excretory cell body was displaced posteriorly with respect to the static terminal bulb of the pharynx (arrowhead). (D) *unc-53(n166)*; *vab-8(e1017)* animal showing the anterior canals are severely truncated and often absent. (E) Quantification of anterior longitudinal extension defects. *unc-53 (n166)* ($n = 72$), *vab-8(e1017)* ($n = 111$), and *unc-53(n166)*; *vab-8(e1017)* ($n = 95$).

evidence that VAB-8 and UNC-73 regulate SAX-3 to direct cell and growth cone migrations (Watari-Goshima *et al.* 2007). These investigators found that VAB-8L promotes the posterior migration of cells and growth cones by regulating the activity of guidance receptors that also function in DV guidance (Watari-Goshima *et al.* 2007). A null mutation in *sax-3* on its own did not exhibit significant defects in EC extension; however, *unc-53(n166)/mIn1*; *sax-3(ky123)* heterozygous animals exhibited excretory canal truncation similar to that of *unc-53* homozygous null mutants, and homozygous double mutants gave an enhanced phenotype (Figure 4A). SLT-1 encodes the sole *C. elegans* homolog of *Drosophila* Slit, a secreted extracellular protein that functions as a ligand for the SAX-3/Robo receptor to direct ventral axon guidance and guidance at the midline (Hao *et al.* 2001). Loss of SLT-1 caused posterior canal truncation similar to *vab-8(e1017)* mutants (Figure 4B). Interestingly, *slt-1* mutants also displayed reduced anterior canal extension (truncated in 71%, absent in 16%, $n = 89$), a phenotype not seen with any other single mutant examined except for *unc-53*. EVA-1 is predicted to be a receptor for SLT-1 that acts as a coreceptor with SAX-3 to provide cell specificity for the activation of SAX-3 signaling by SLT-1 (Fujisawa *et al.* 2007). Loss of EVA-1 also caused truncation reminiscent of that of *vab-8(e1017)* mutants (Figure 4C).

Parallel pathways mediate the outgrowth of the excretory canals

Our examination of *unc-53*; *vab-8* mutants suggested that these genes function in separate pathways. As noted previously, the *unc-53*; *unc-71* double mutants exhibited canal truncation no more severe than the single mutants, while *unc-71*; *vab-8* double mutants displayed an enhanced phenotype, consistent with these genes also operating in separate pathways. Therefore we tested whether known VAB-8 interactors could be restricted to a VAB-8 pathway. The *vab-8*; *sax-3* double mutants showed the same level of truncation as the *vab-8* single mutant, suggesting that VAB-8 acts through SAX-3 to promote excretory canal extension (Figure 4A). The same results were found with *vab-8* in double-mutant combination with either *eva-1* or *slt-1* (Figure 4, B and C). In contrast, the *unc-53*; *sax-3* double mutants showed more severe excretory canal truncation, as was the case for *unc-53*; *vab-8* doubles. This suggests *sax-3* may be operating exclusively in a parallel pathway with *vab-8* (Figure 4A). Similarly, *unc-53* in double-mutant combination with either *slt-1* or *eva-1* resulted in an enhancement of the *unc-53(n166)* mutant phenotype.

We chose to further examine SAX-3 and obtained the strain *sax-3(ky123)*; *quEx168 [psax-3*::*sax-3*::*gfp*; *odr-1*::*RFP]* (Ian Chin-Sang, Queen's University, Kingston, ON, Canada), which has the ability to rescue *sax-3(ky123)* and resembles the true endogenous expression pattern (Ghenea *et al.* 2005). To determine whether SAX-3 can rescue canal extension defects under control of its endogenous promoter, this strain was crossed into *unc-53(n166)*; *sax-3(ky123)* double null mutants, as *sax-3* single mutants do not display posterior canal truncation. The *sax-3(ky123)*; *quEx168 [psax-3*::*sax-3*::*gfp*; *odr-1*::*RFP]* transgenic strain was sufficient to partially rescue the posterior canal defects seen in *unc-53(n166)*; *sax-3*

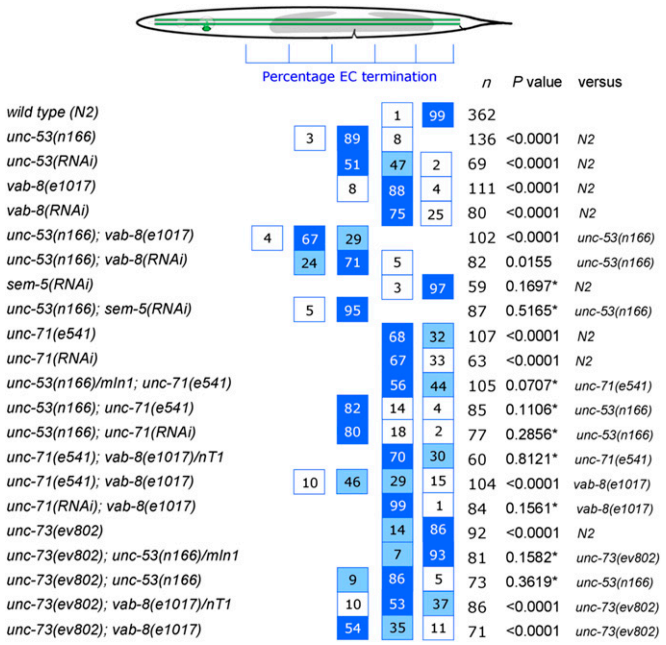


Figure 3 Loss of molecules involved in the gonad-independent pathway for SM migration disrupt excretory canal extension. The outgrowth of the posterior canals was divided into five regions (1–5) before the anterior gonad arm to the tail as shown. The stop point of canals was determined by fluorescence microscopy. Loss of UNC-53, VAB-8, UNC-71, and UNC-73 perturb posterior canal outgrowth. *unc-71* and *unc-73(ev802)* mutants do not enhance the migration phenotype seen in *unc-53 (n166)*. By contrast, *vab-8(e1017)* mutants were enhanced in double-mutant combination with *unc-71* or *unc-73(ev802)*. Chi-squared analysis was used to establish statistical significance between mutants using GraphPad Prism 5 (Sigma Stat). For this comparison, phenotypes were grouped into two categories and the mutant compared to a baseline of either wild type or the most severe single allele in the case of double mutants, as indicated. **P* value is not statistically significant.

(ky123), and rescued animals resembled *unc-53(n166)* single mutants (Figure 4A). This partial rescue supports the idea that SAX-3 is indeed in a VAB-8 pathway parallel to UNC-53, as we observed no animals with canal extension rescued beyond the level of *unc-53* mutants. Also, because SAX-3 is not expressed in the excretory canals, this suggests that another member of the pathway that acts genetically with VAB-8 also acts nonautonomously. Collectively, these data suggest that VAB-8, SAX-3/Robo, SLT-1/Slit, and EVA-1 are functioning together in the outgrowth of the excretory canals, while UNC-53 appears to function in a parallel pathway with UNC-71/ADAM. These observations are consistent with the canonical view that SAX-3/Robo receptors mediate the effects of SLT-1/Slit cues and that UNC-53 functions together with UNC-71 in the gonad-independent pathway for SM migration (Chen *et al.* 1997; Hao *et al.* 2001).

UNC-73 functions in two distinct genetic pathways

We chose to further examine *unc-73* because VAB-8 and UNC-73 physically interact and together regulate SAX-3/Robo to direct cell outgrowth (Watari-Goshima *et al.* 2007), and *unc-73* functions in SM migration together with

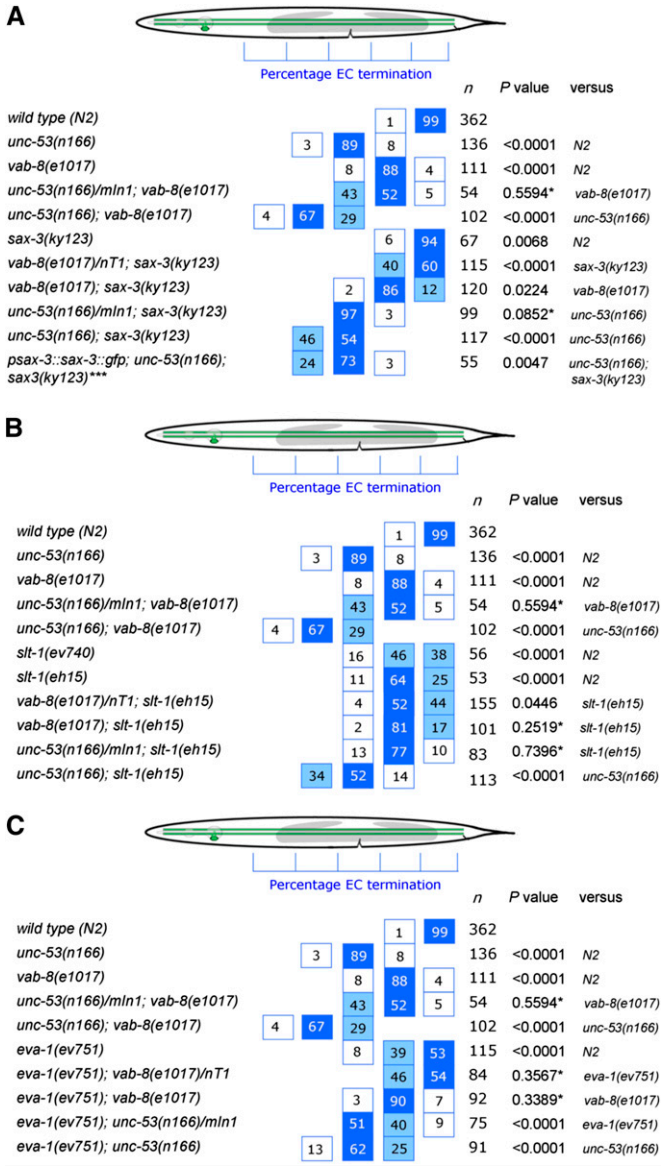


Figure 4 Loss of molecules known to function in dorsoventral guidance disrupt excretory canal migration. (A–C) The outgrowth of the posterior canals was divided into five regions (1–5) before the anterior gonad arm to the tail as shown. The stop point of canals was determined by fluorescence microscopy. Loss of UNC-53, VAB-8, EVA-1, and SLT-1 perturb posterior canal outgrowth. (A) *sax-3*, (B) *slt-1*, and (C) *eva-1* mutants do not enhance the extension phenotype seen in *vab-8(e1017)*. By contrast, *unc-53(n166)* mutants were enhanced in double-mutant combination with *sax-3*, *slt-1*, or *eva-1*. (A) *sax-3(ky123)*; *quEx168 [psax-3*::*sax-3*::*gfp*; *odr-1*::*RFP]* rescue strain was able to partially rescue defects seen in *unc-53(n166)*; *sax-3(ky123)* double mutants, and rescued animals resembled *unc-53* mutants, which is consistent with *unc-53* and *sax-3* functioning in parallel pathways. Chi-squared analysis was used to establish statistical significance between mutants using GraphPad Prism 5 (Sigma Stat). For this comparison, phenotypes were grouped into two categories and the mutant compared to a baseline of either wild type or the most severe single allele in the case of double mutants, as indicated. **P* value is not statistically significant. ****psax-3*::*sax-3*::*gfp*; *unc-53(n166)*; *sax-3(ky123)* is the *sax-3(ky123)*; *quEx168 [psax-3*::*sax-3*::*gfp*; *odr-1*::*RFP]* rescue strain in *unc-53(n166)*; *sax-3(ky123)* double mutants.

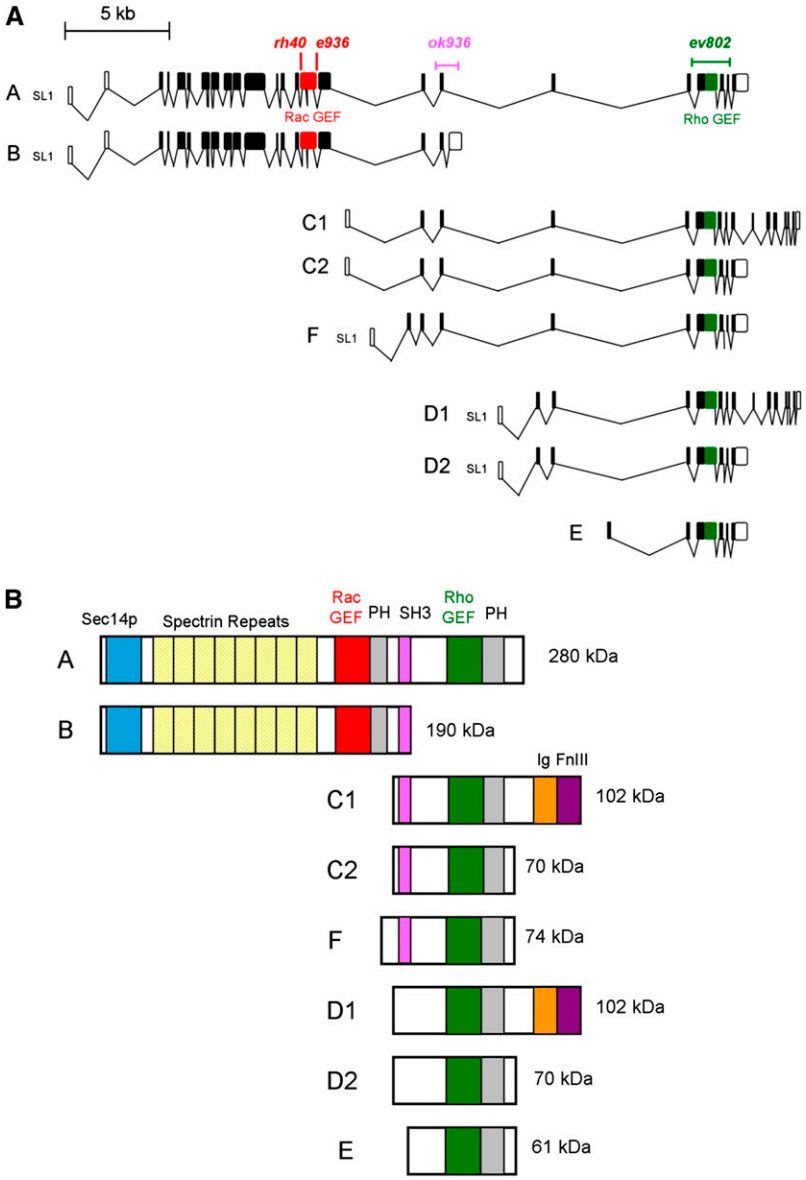


Figure 5 Genomic organization of the predicted *unc-73* transcripts and corresponding UNC-73 isoforms. (A) The locations of mutations of the *unc-73* gene are indicated above the locus (modified from Steven *et al.* 2005). The mutation *ok936* (in pink) affects the SH3 domain and results in an Unc phenotype, the mutations *rh40* and *e936* (in red) affect the RacGEF domain, and *ev802* (in green) eliminates the RhoGEF domain and is associated with L1 lethality (Steven *et al.* 2005). Exons of the predicted *unc-73* transcripts (A, B, C1, C2, D1, D2, E, and F) are shown. Only isoform E shows expression within the excretory cell (Steven *et al.* 2005). (B) The predicted UNC-73 isoforms are shown. *unc-73* encodes proteins with several domains, including the two tandem RhoGEF and PH domain (gray) combinations (RacGEF (red) and RhoGEF (green), a Sec14p motif (blue), eight spectrin-like repeats (yellow), a variant SH3 domain (pink), an immunoglobulin domain (Ig, orange), and a fibronectin type III (FnIII) domain (brown).

unc-53 and *unc-71* (Chen *et al.* 1997), yet our results suggested that *unc-53* and *vab-8* function in parallel pathways to control excretory cell outgrowth. To resolve this paradox, we examined multiple isoform-specific alleles of *unc-73*. The *unc-73* gene is complex in that it encodes at least eight differentially expressed UNC-73 intracellular protein isoforms (Figure 5; Steven *et al.* 2005). *unc-73* encodes proteins with several domains, including two GEF and PH domain combinations, a Sec14p motif, eight spectrin-like repeats, a variant SH3 domain, an immunoglobulin domain (Ig), and a fibronectin type III (FnIII) domain. The N-terminal UNC-73 GEF domain specifically activates the Rac family GTPases CED-10 and MIG-2 *in vitro* (Steven *et al.* 1998; Wu *et al.* 2002; Kubiseski *et al.* 2003), while the C-terminal GEF domain is specific to Rho (Figure 5; Spencer *et al.* 2001). Moreover, Watari-Goshima *et al.* (2007) found that UNC-73B, an isoform containing the RacGEF but not the RhoGEF domain, interacted with VAB-8L in a yeast two-hybrid assay and that this was mediated through a region containing the spectrin repeats of UNC-73B (Watari-Goshima *et al.* 2007).

To determine the impact of the RacGEF domain on EC migration, we chose to examine the alleles *unc-73(rh40)* and *unc-73(e936)*. In the case of *unc-73(rh40)*, which contains a missense mutation (amino acid 1216 S to F), within the RacGEF, severe posterior canal truncation was observed (Figure 6). For *unc-73(e936)*, a splice site gt to tt substitution allele, the level of posterior canal extension phenotype was highly variable, ranging from wild-type length canals to canals that were as short as *unc-53*; *vab-8* double mutants (Figure 6). This mutation potentially affects the frequency and accuracy of splicing and may allow for a low level of wild-type transcripts of both the A and B isoforms. This may explain why several animals displayed wild-type canal outgrowth. Both *unc-73(rh40)* and *unc-73(e936)* alleles affect the RacGEF domain of *unc-73* and therefore are predicted to disrupt the RacGEF-containing A and B isoforms. Neither

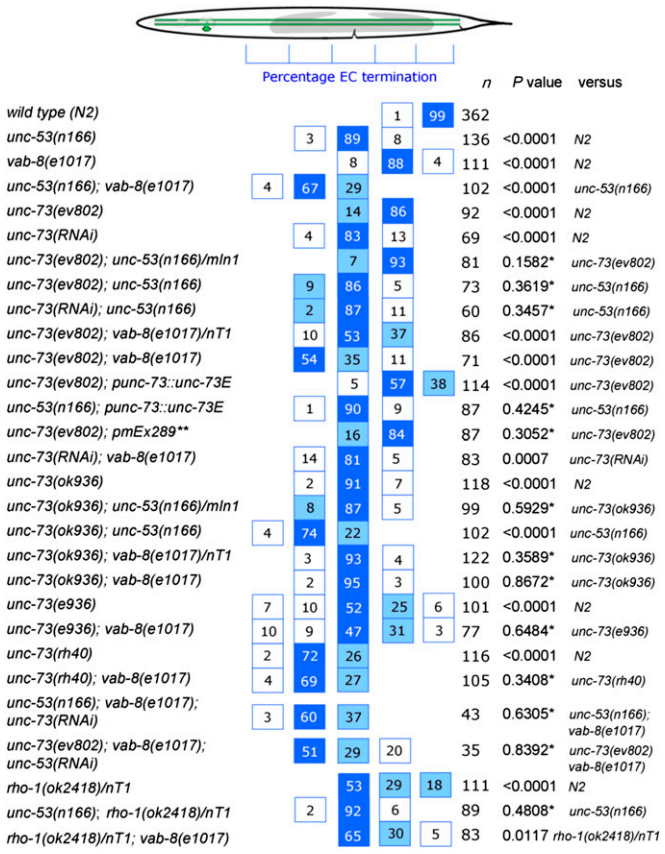


Figure 6 UNC-73 may function in both the UNC-53 and the VAB-8 pathways for excretory canal outgrowth. Alleles affecting the RacGEF domain of UNC-73 [*unc-73(rh40*) and *unc-73(e936)*] showed an enhancement in excretory canal truncation in double-mutant combination with *unc-53(n166)* but not with *vab-8(e1017)*, suggesting that the RacGEF domain of UNC-73 may be required solely for mediating the VAB-8 pathway. In contrast, *unc-73(ev802)*; *unc-53(n166)* double mutants are no more severely affected than the *unc-53* single mutant alone, while *unc-73(ev802)*; *vab-8(e1017)* animals exhibited severe enhancement of defects when compared to *vab-8* null mutants. This suggests that one or more of the UNC-73 isoforms other than isoform B may be *unc-53* specific. Supporting this hypothesis, transgenic analysis showed that the *punc-73E*::*unc-73E*::*gfp* transgenic strain was sufficient to partially rescue the posterior canal defects seen in *unc-73(ev802)*, suggesting that UNC-73E, which contains the RhoGEF domain, is required cell autonomously together with UNC-53 for the proper migration of the excretory canals. RNAi data showing *unc-73* RNAi in the background of *unc-53*; *vab-8* double mutants or *unc-53* RNAi in the background of *vab-8*; *unc-73* double mutants exhibits no more severe canal defects than either double mutant alone, suggesting that *unc-73* is likely not functioning in a third, separate pathway. The outgrowth of the posterior canals was divided into five regions (1–5) before the anterior gonad arm to the tail as shown. Also, the *rho-1(ok2418)/nT1* heterogyzous strain was able to enhance defects seen in *vab-8* null mutants but not in *unc-53* null mutants. Chi-squared analysis was used to establish statistical significance between mutants using GraphPad Prism 5 (Sigma Stat). For this comparison, phenotypes were grouped into two categories, and the mutant compared to a baseline of either wild type or the most severe single allele in the case of double mutants, as indicated. **P* value is not statistically significant. ***pmEx289* is: *dpy-5(e907)/dpy-5(e907)*; *pmEx289* [rCes pmyo-2::GFP + pCeh361 + WRM0628aD12].

allele showed an enhancement in excretory canal truncation in double-mutant combination with *vab-8(e1017)*, suggesting that the RacGEF domain of UNC-73 may be required solely for mediating the VAB-8 pathway. In support of this, we found that *ok936*, an allele affecting the SH3 domain of *unc-73* and consequently isoforms A, B, C1, C2, and F, showed enhancement of the excretory canal truncation in double-mutant combination with *unc-53* but not with *vab-8* (Figure 6).

In contrast, the *unc-73(ev802)* deletion allele eliminates the RhoGEF but not the RacGEF genomic region, disrupting isoforms A, C1, C2, D1, D2, F, and E (Figure 5, Steven *et al.* 2005). Interestingly *unc-73(ev802)*; *unc-53(n166)* double mutants are no more severely affected than the *unc-53* single mutant alone, while *unc-73(ev802)*; *vab-8(e1017)* animals exhibited severe enhancement of defects when compared to *vab-8* null mutants (Figure 6). This suggests that one or more of the UNC-73 isoforms other than isoform B may be *unc-53* specific. The UNC-73 isoform E is the smallest isoform and the only one known to be expressed in the excretory canals, as is *unc-53* (Steven *et al.* 2005). Indeed, the *punc-73E*::*unc-73E*::*gfp* transgenic strain was sufficient to partially rescue the posterior canal defects seen in *unc-73 (ev802)* (Figure 6), suggesting that UNC-73E, which contains the RhoGEF domain, is required cell autonomously together with UNC-53 for the proper migration of the excretory canals. Placing *punc-73E*::*unc-73E*::*gfp* in the background of *unc-53(n166)* mutants was insufficient to rescue the *unc-53(n166)* excretory canal truncation. Similarly, expressing *unc-53* was insufficient to rescue *unc-73(ev802)*, suggesting these genes are mutually interdependent on each other. Consistent with the view that the RhoGEF domain of UNC-73E is important for UNC-53 activity, *rho-1* mutants enhanced the *vab-8* null phenotype but not that of *unc-53* null mutants (Figure 6).

RNA interference experiments revealed that *unc-73* RNAi in the background of *unc-53(n166)*; *vab-8(e1017)* double mutants or *unc-53* RNAi in the background of *vab-8 (e1017)*; *unc-73(ev802)* double mutants exhibits no more severe canal defects than either double mutant alone, suggesting that *unc-73* is likely not functioning in a third, separate pathway (Figure 6). This data are also consistent with the isoform-specific model as the *unc-73* RNAi clone targets isoforms A and B but not the six smaller isoforms.

Discussion

UNC-53 and VAB-8 function in parallel pathways to control cell migration

The enhanced reduction of excretory canal outgrowth observed in *vab-8*; *unc-53* double null mutants suggest that these genes act in separate pathways on the posterior outgrowth of the excretory canals, with each exerting their influence independently. Interestingly, the anterior excretory canal outgrowth is more severely disrupted in *unc-53 (n166)*; *vab-8(e1017)* double mutants, suggesting a role for *vab-8* in anterior guidance processes not previously

reported. Moreover, the misplaced EC body in *vab-8* null mutants suggests that VAB-8 is required for both the anterior migration of the excretory cell body as well as the outgrowth of the anterior canals. In contrast, UNC-53 is apparently required for only anterior canal outgrowth.

This study confirmed a role for the guidance cue SLT-1 in the longitudinal guidance of the excretory cell. In *Drosophila*, it has been shown that migrating mesodermal cells *in vivo* respond to Slit as both an attractant and a repellent and ROBO receptors are required for both functions (Kramer *et al.* 2001). During early embryogensis in *C. elegans*, SLT-1 is expressed at high levels in the anterior of the embryo and moderate levels in the dorsal tail muscles, while lower levels are seen in cells in the center of the body (Hao *et al.* 2001). By the first larval stage, SLT-1 is predominantly expressed from the dorsal body wall muscle (Hao *et al.* 2001). The EC processes begin migration during embryogensis and have completed outgrowth at the L1 stage, suggesting that early SLT-1 expression in the anterior and posterior ends of the animal may act as an attractant both for the VAB-8–mediated anterior migration of the EC body, as well as for posterior canal outgrowth. This model is supported by the observation that *slt-1* mutants were the only single mutant animals besides *unc-53* mutants to display anterior canal truncation.

DV guidance cues and their receptors are also utilized in AP migrations

Several global guidance molecules functioning in DV migrations have been identified; however, less is known about an equivalent global guidance system in the AP axis. Why have ligands and receptors functioning in AP guidance been more difficult to uncover? One possibility is that there are several redundant signaling pathways guiding long-range AP migrations. Disrupting a single pathway would therefore be insufficient to cause generalized migration defects. Another possibility is that AP guidance cues and receptors function in essential processes during development, and thus disrupting a guidance pathway might result in lethality.

The data collected in this study demonstrate a role for SAX-3/ROBO and SLT-1, two known DV guidance molecules, in the AP migration of the excretory canals. There is other evidence of these molecules functioning in other AP migrations. For example, *sax-3* and *slt-1* mutants show defects in the posterior migration of the CAN and ALM cell bodies (Hao *et al.* 2001). In addition, mutations in *sax-3* and *slt-1* have been shown to suppress the ALM axon rerouting phenotype caused by VAB-8L misexpression (Watari-Goshima *et al.* 2007). Isoform specificity, as seen in this study with UNC-73B and UNC-73E, as well as modulation of receptor function may partially explain how a small number of conserved guidance cues and receptors regulate such a large number of trajectories along both the DV and AP axes. For example, Fujisawa *et al.* (2007) predict that there are two classes of cells that express and respond to SAX-3. One class coexpresses EVA-1, allowing the SAX-3 receptor to respond to SLT-1, while the other class expresses SAX-3 but not EVA-1 and does not respond to SLT-1. In this case, it would appear that the excretory cell belongs to the first class, since both *eva-1* and *slt-1* mutants exhibited a reduction in posterior canal outgrowth. The discovery that several cues and receptors function in both DV and AP migration has contributed to our understanding of how a rather limited repertoire of guidance molecules can establish migration of several growth cones in *C. elegans*.

It is possible that *vab-8* may change SAX-3′s response to SLT-1 from repulsive to attractive. Kramer *et al.* (2001) found that migrating mesoderm cells expressing Robo receptors respond to Slit first as a repellent, and a few hours after migration the same cells require Robo to extend toward Slit-expressing muscle attachment sites. Individual cells *in vivo* may switch their response to Slit from repulsion to attraction, and this may reflect a change in another receptor subunit or a change in the internal state of the cell.

One possibility is that VAB-8L, through its kinesin-like motor, is responsible for shuttling SAX-3 receptors to the membrane surface (Watari-Goshima *et al.* 2007). Another possibility is that SAX-3 may function with a coreceptor to specify attraction to Slit within the excretory cell. For example, SAX-3 is known to physically interact with the VAB-1 Eph receptor during embryogensis, while EVA-1 physically interacts with SAX-3 to provide cell specificity to the activation of SAX-3 signaling by SLT-1 in the AVM and PVM neurons (Ghenea *et al.* 2005; Fujisawa *et al.* 2007).

The Rac and Rho GEF domains of UNC-73/TRIO mediate two genetically distinct pathways to regulate EC outgrowth

In this study we found that alleles affecting the RacGEF domain of UNC-73 (*rh40* and *e936*), thereby disrupting the RacGEF-containing UNC-73B isoform, did not show enhancement of the excretory canal truncation in double-mutant combination with *vab-8*, suggesting that the UNC-73B isoform may operate solely through VAB-8. This is consistent with data showing the full-length UNC-73B interacted with a VAB-8L fragment in a yeast two-hybrid assay (Watari-Goshima *et al.* 2007). On the other hand, the *unc-73(ev802)* deletion allele that eliminates the RhoGEF but not the RacGEF genomic region, disrupting all isoforms except for UNC-73B (Steven *et al.* 2005), was no more severe in the background of an *unc-53* mutant than either mutant alone, while *unc-73 (ev802)*; *vab-8(e1017)* animals exhibited severe enhancement. As the UNC-73 isoform E is expressed in the excretory canals (Steven *et al.* 2005), we predict that UNC-73E is required cell autonomously together with UNC-53 for the proper extension of the excretory canals. Collectively, these observations point to a model that the RacGEF domain of UNC-73 is specific to the VAB-8 pathway while the RhoGEF domain is specific to the UNC-53 pathway. Notably, *C. elegans unc-73* is the only gene, aside from its orthologs Trio and Kalirin, that encodes two GEF domains (Steven *et al.* 2005).

Interestingly, the first part of the posteriorly directed migration of the excretory canals to the anterior gonad arm was intact for all genes tested, suggesting that another mechanism independent of *unc-73*, *unc-53*, *vab-8*, and their putative interactors might be guiding the initial posterior outgrowth of the canals. The molecules in this study may be required to coordinate actin filament assembly with microtubule capture only as an outgrowing growth cone becomes more distant from the cell's synthetic machinery in the cell body.

A model for excretory cell outgrowth

Genetic analysis suggests that VAB-8, SAX-3/ROBO, SLT-1/Slit, and EVA-1 are functioning together in the migration and outgrowth of the excretory cell, and it appears that the Rac-specific GEF domain of UNC-73 may mediate this pathway (Figure 7A). These molecules are known to function together elsewhere as *sax-3* null mutants suppress rerouting of the ALM caused by *vab-8* misexpression (Watari-Goshima *et al.* 2007). The authors found that there is higher SAX-3/ROBO receptor in animals with increased amounts of VAB-8 and they proposed that VAB-8, together with the UNC-73/TRIO, promotes localization of the SAX-3/ROBO receptor to the cell surface or inhibits its removal by endocytosis. In addition, *unc-73* mutants disrupting the B isoform can also suppress rerouting of the ALM caused by *vab-8* misexpression (Watari-Goshima *et al.* 2007). UNC-73B interacts physically with VAB-8L and the cytoplasmic domain of SAX-3/ROBO, which suggests that these protein interactions mediate VAB-8L's regulation of the SAX-3/ROBO receptor. We predict that VAB-8L may work through SAX-3 and a second coreceptor, as we found SAX-3 to have no excretory canal phenotype on its own. SAX-3 may function with a coreceptor to specify attraction to Slit within the excretory cell, and our data are consistent with EVA-1 being the potential SAX-3 coreceptor. There are other examples of SAX-3 functioning with a coreceptor to specify receptor function: SAX-3 is known to physically interact with the VAB-1 Eph receptor during embryogensis, while EVA-1 physically interacts with SAX-3 to provide cell specificity to the activation of SAX-3 signaling by SLT-1 in the AVM and PVM neurons (Ghenea *et al.* 2005; Fujisawa *et al.* 2007). Already known for being involved in DV guidance, our findings confirm a role for SLT-1, SAX-3/ROBO, and EVA-1 in AP migration of the excretory cell.

VAB-8, SAX-3/ROBO, SLT-1/Slit, and EVA-1 are not expressed in the excretory canals (Wolf *et al.* 1998; Zallen *et al.* 1998; Hao *et al.* 2001; Fujisawa *et al.* 2007), and deliberate expression of VAB-8 specifically in the excretory cell failed to restore canal outgrowth. In addition, a *sax-3*::*gfp* rescuing strain driven by its endogenous promoter was able to partially rescue the extension of the posterior excretory canals in *unc-53(n166)*; *sax-3(ky123)* double mutants, supporting a nonautonomous role for both SAX-3 and VAB-8. Thus we predict this pathway is functioning cell nonautonomously in this extension. Further rescue experiments expressing either VAB-8 or EVA-1 panneuronally or within the body wall muscles or hypodermis may help to determine the cell types involved in this signaling pathway.

UNC-53 on the other hand appears to function in a parallel pathway with the E isoform of UNC-73/TRIO and UNC-71/ADAM to promote extension of the excretory canals (Figure 7B). This is consistent with the fact that *unc-71*, *unc-73*, and *unc-53* function together in SM migration and the observation that UNC-53, UNC-73E, and UNC-71 are all expressed in the excretory canals (Stringham *et al.* 2002; Huang *et al.* 2003; Steven *et al.* 2005). Moreover, expression of *unc-53* only in the excretory cell is sufficient to rescue the canal outgrowth phenotype of *unc-53* null mutants (Schmidt *et al.* 2009). Likewise, in this study we found that expression of the UNC73E isoform alone was sufficient to rescue the outgrowth phenotype of *unc-73* mutants where the RhoGEF domain was disrupted. Collectively, these data suggest that UNC-53, UNC-71, and UNC-73E function cell autonomously in excretory canal extension, and that this pathway is mediated via the Rho-specific GEF domain of UNC-73. Consistent with this model, reduction of *rho-1* activity was sufficient to enhance the phenotype of *vab-8* null mutants, suggesting the RhoGEF domain of UNC-73 mediates cell outgrowth independently of VAB-8.

This study demonstrates that VAB-8 and UNC-53 function in parallel genetic pathways to control excretory cell outgrowth, and that the dual GEF domains of UNC-73 allows it to modulate both pathways. While UNC-73 and UNC-53 were previously shown to function together in the gonad-independent pathway for sex myoblast migration (Chen *et al.* 1997), the results from this study further suggests that the RhoGEF domain of UNC-73 is specific to the UNC-53 pathway. Conversely, UNC-73 and VAB-8 had been shown to function together in neuron outgrowth, with the RacGEF domain mediating this pathway (Levy-Strumpf and Culotti 2007; Watari-Goshima *et al.* 2007). Here, we observed that pathway specificity of the two GEF domains of UNC-73 apparently allows this molecule to control the outgrowth of a single cell via two distinct mechanisms, one occurring within the excretory cell and another outside the cell.

Guanine nucleotide exchange factors stimulate the Rho family GTPases (Lundquist *et al.* 2001). The three key members of this family of GTPases include Rho, Rac, and Cdc42 (Steven *et al.* 2005). Each Rho GTPase has a different effect on the actin cytoskeleton: activation of the Rho pathway results in the formation of stress fibers, activation of Rac results in extension of lamellipodia, and Cdc42 activation results in the formation of filopodia (Lundquist *et al.* 2001). In addition, the different Rho GTPases can stimulate different types of actin polymerization. For example, Rho is known to activate formins, leading to the formation of longer, less branched actin filaments. Cdc42 binds to and activates WASP, stimulating actin polymerization by the Arp2/3 complex, while Rac can activate WAVE, another WASP family protein (Higgs and Pollard 2000; Evangelista *et al.* 2003; Takenawa and Suetsugu 2007).

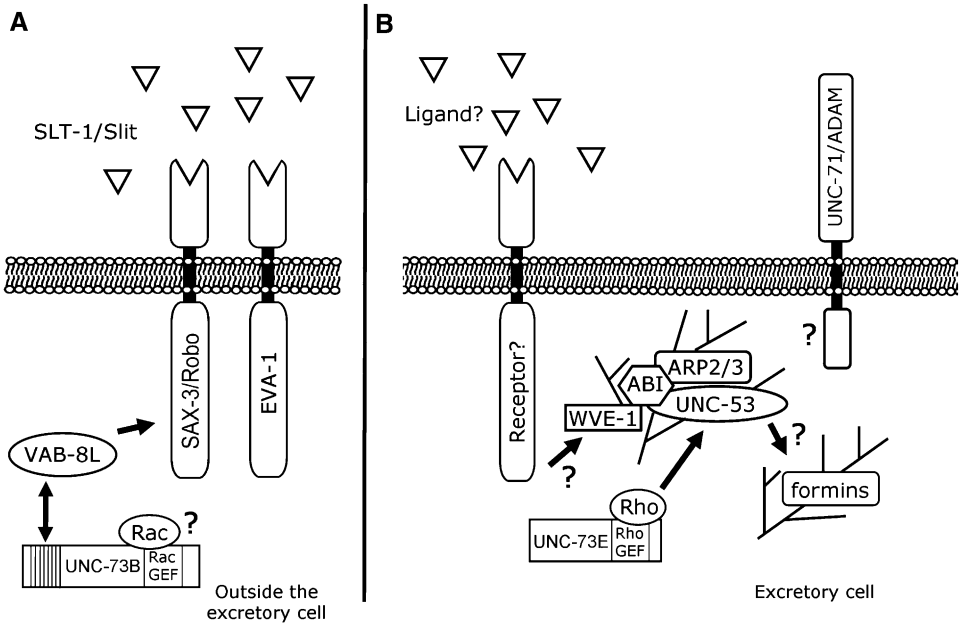


Figure 7 A model for two parallel pathways controlling posterior extension of the excretory canals. Triangles represent a proposed guidance cue. (A) Genetic analysis suggests that VAB-8, SAX-3/ROBO, SLT-1/Slit, and EVA-1 are functioning together in the migration of the canals. VAB-8L may regulate the SAX-3 receptor via the RacGEF activity of UNC-73B by promoting localization of SAX-3/ROBO to the cell surface or inhibiting its removal by endocytosis (Watari-Goshima *et al.* 2007). (B) UNC-53 functions in a cell autonomous pathway with UNC-73E and UNC-71/ADAM to promote migration of the excretory canals. In this pathway the RhoGEF domain of UNC-73E activates UNC-53 (either through direct binding or indirectly) to promote formin-mediated assembly of actin filaments. In addition, UNC-53 binds ABI that forms part of the WAVE complex, which has been shown to mediate the actin nucleation activity of ARP2/3. UNC-53 therefore may be a scaffold that coordinates multiple signals to the actin cytoskeleton machinery.

The Rac-specific GEF domain of UNC-73 is known to activate Rac GTPases, which in turn mediates actin filament assembly by Arp2/3 by activating WAVE (Steven *et al.* 1998). We have shown previously that UNC-53 binds ABI-1 (Schmidt *et al.* 2009), a member of the WAVE complex, which has been shown to mediate the actin nucleation activity of ARP2/3 (Bompard and Caron, 2004; Takenawa and Suetsugu 2007). We predict that UNC-53 may be a scaffold that coordinates upstream signals to ABI-1 and the actin cytoskeleton (Schmidt *et al.* 2009). Interestingly however, we found that it is the Rho-specific GEF domain of UNC-73 that may mediate UNC-53 activity, while the Rac-specific GEF domain appears to mediate VAB-8 activity. The duality of the UNC-73 Rac/Rho GEF raises the possibility that UNC-53 could be modulating actin cytoskeleton dynamics via both Rho and Rac signaling. UNC-53 may mediate actin cytoskeletal rearrangement through Rac activation of the WAVE/ABI-1 and ARP2/3 complex, while also functioning with the RhoGEF domain of UNC-73 to activate formins.

Currently, it is unclear which upstream receptors and ligands might act with UNC-53 and UNC-73 RhoGEF or what cells present these potential guidance molecules. UNC-53 is known to interact genetically and physically with the SH2–SH3 adaptor protein SEM-5 (GRB-2), a mediator of EGL-15/FGFR signaling in sex myoblast migration in *C. elegans* (Chen *et al.* 1997; Stringham *et al.* 2002). However, although *unc-53* and *egl-15/FGFR* are both expressed in migrating sex myoblasts (Goodman *et al.* 2003), *egl-15* is not expressed in axons but instead regulates outgrowth through the underlying hypodermis on which they migrate (Bulow *et al.* 2004). Because UNC-53 is a cytoplasmic protein that functions cell autonomously, and because *egl-15* is not expressed in the excretory canals, this suggests that it does not act directly downstream of EGL-15/FGFR signaling in neuronal cell migrations and extension of the excretory canals. In addition, UNC-53, UNC-71/ADAM, and UNC-73/TRIO are thought to function in an EGL-17/FGF independent signaling pathway controlling sex myoblast migration (Chen *et al.* 1997), suggesting non-FGFR signaling is involved in the SM pathway and probably here as well.

The UNC-53 human homolog NAV1 associates with microtubule plus ends on developing neuronal growth cones and is required for netrin-induced directionality in pontine neurons (Martinez-Lopez *et al.* 2005). We hypothesized that UNC-53 may function with the ligand UNC-6/Netrin, and its receptors UNC-40/DCC and UNC-5, however none of these proteins displayed defects in the extension of the anterior or posterior excretory canals (data not shown). Indeed the *unc-5* receptor, as well as the receptors *npr-1* and *nhr-67*, show expression within the excretory canals and would be worthwhile to investigate in the future. Therefore the identity of ligands and receptors upstream of the UNC-53/UNC-71/UNC-73 pathway in excretory canal migration remains elusive.

Acknowledgments

We thank Domena Tu, Laura Ramsay, and Caitlyn Grypma for technical assistance. We are grateful to Rob Steven (Toledo, OH) and Joseph Culotti (Toronto, ON, Canada) for *unc-73* constructs and strains, Ian Chin-Sang for *sax-3* strains, and Gian Garriga (Berkeley, CA) for *vab-8* constructs and strains. We also thank the *Caenorhabitis* Genetics Center, Monica Driscoll, and Shohei Mitani for nematode strains. We are grateful to David Baillie, Nancy Hawkins,

Ester Verheyen, and members of the Stringham and Baillie labs for helpful discussions. This work was supported by an National Sciences and Engineering Research Council of Canada Discovery grant and by the Canada Research Chairs.

Literature Cited

Awasaki, T., M. Saito, M. Sone, E. Suzuki, R. Sakai *et al.*, 2000 The *Drosophila* trio plays an essential role in patterning of axons by regulating their directional extension. Neuron 26: 119–131.

Bateman, J., H. Shu, and D. Van Vactor, 2000 The guanine nucleotide exchange factor trio mediates axonal development in the *Drosophila* embryo. Neuron 26: 93–106.

Birnbaum, D., C. Popovici, and R. Roubin, 2005 A pair as a minimum: the two fibroblast growth factors of the nematode *Caenorhabditis elegans*. Dev. Dyn. 232: 247–255.

Bompard, G., and E. Caron, 2004 Regulation of WASP/WAVE proteins: making a long story short. J. Cell Biol. 166: 957–962.

Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics 77: 71–94.

Buechner, M., 2002 Tubes and the single *C. elegans* excretory cell. Trends Cell Biol. 12: 479–484.

Bulow, H. E., T. Boulin, and O. Hobert, 2004 Differential functions of the *C. elegans* FGF receptor in axon outgrowth and maintenance of axon position. Neuron 42: 367–374.

Burdine, R. D., E. B. Chen, S. F. Kwok, and M. J. Stern, 1997 *egl-17* encodes an invertebrate fibroblast growth factor family member required specifically for sex myoblast migration in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA 94: 2433–2437.

Chen, E. B., C. S. Branda, and M. J. Stern, 1997 Genetic enhancers of *sem-5* define components of the gonad-independent guidance mechanism controlling sex myoblast migration in *Caenorhabditis elegans* hermaphrodites. Dev. Biol. 182: 88–100.

Colamarino, S. A., and M. Tessier-Lavigne, 1995 The axonal chemoattractant Netrin-1 is also a chemorepellent for trochlear motor axons. Cell 81: 621–629.

Debant, A., C. Serra-Pages, K. Seipel, S. O'Brien, M. Tang *et al.*, 1996 The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac- specific and rho- specific guanine nucleotide exchange factor domains. Proc. Natl. Acad. Sci. USA 93: 5466–5471.

DeVore, D. L., H. R. Horvitz, and M. J. Stern, 1995 An FGF receptor signalling pathway is required for the normal cell migrations of the sex myoblasts in *C. elegans* hermaphrodites. Cell 83: 611–620.

Dickson, B. J., 2002 Molecular mechanisms of axon guidance. Science 298: 1959–1964.

Evangelista, M., S. Zigmond, and C. Boone, 2003 Formins: signaling effectors for assembly and polarization of actin filaments. J. Cell Sci. 116: 2603–2611.

Fujisawa, K., J. L. Wrana, and J. G. Culotti, 2007 The Slit receptor EVA-1 coactivates a SAX-3/Robo–mediated guidance signal in *C. elegans*. Science 317: 1934–1938.

Ghenea, S., J. R. Boudreau, N. P. Lague, and I. D. Chin-Sang, 2005 The VAB-1 Eph receptor tyrosine kinase and SAX-3/Robo neuronal receptors function together during *C. elegans* embryonic morphogenesis. Development 132: 3679–3690.

Goodman, S. J., C. S. Branda, M. K. Robinson, R. D. Burdine, and M. J. Stern, 2003 Alternative splicing affecting a novel domain in the C. elegans EGL-15 FGF receptor confers functional specificity. Development 130: 3757–3766.

Hao, J., T. W. Yu, K. Fujisawa, J. G. Culotti, K. Gengyo-Ando *et al.*, 2001 *C. elegans* Slit acts in midline, dorsal-ventral, and anterior-posterior guidance via the SAX-3/Robo receptor. Neuron 32: 25–38.

Hedgecock, E., J. G. Culotti, D. Hall, and B. Stern, 1987 Genetics of cell and axon migrations in *Caenorhabditis elegans*. Development 100: 365–382.

Hedgecock, E. M., J. G. Culotti, and D. H. Hall, 1990 The *unc-5*, *unc-6*, and *unc-40* genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. Neuron 4: 61–85.

Hekimi, S., and D. Kershaw, 1993 Axonal guidance defects in a *Caenorhabditis elegans* mutant reveal cell-extrinsic determinants of neuronal morphology. J. Neurosci. 13: 4254–4271.

Higgs, H. N., and T. D. Pollard, 2000 Activation by Cdc42 and PIP (2) of Wiskott-Aldrich syndrome protein (WASp) stimulates actin nucleation by Arp2/3 complex. J. Cell Biol. 150: 1311–1320.

Hilliard, M. A., and C. I. Bargmann, 2006 Wnt signals and frizzled activity orient anterior–posterior axon outgrowth in *C. elegans*. Dev. Cell 10: 379–390.

Huang, X., P. Huang, M. K. Robinson, M. J. Stern, and Y. Jin, 2003 UNC-71, a disintegrin and metalloprotease (ADAM) protein, regulates motor axon guidance and sex myoblast migration in *C. elegans*. Development 130: 3147–3161.

Kamath, R. S., M. Martinez-Campos, P. Zipperlen, A. G. Fraser, and J. Ahringer, 2001 Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. Genome Biol. 2: research0002.1-research0002.10.

Kishore, R. S., and M. V. Sundaram, 2002 *ced-10* Rac and *mig-2* function redundantly and act with *unc-73* trio to control the orientation of vulval cell divisions and migrations in *Caenorhabditis elegans*. Dev. Biol. 241: 339–348.

Kramer, S. G., T. Kidd, J. H. Simpson, and C. S. Goodman, 2001 Switching repulsion to attraction: changing responses to slit during transition in mesoderm migration. Science. 292: 737–740.

Kubiseski, T. J., J. G. Culotti, and T. Pawson, 2003 Functional analysis of the *Caenorhabditis elegans* UNC-73B PH domain demonstrates a role in activation of the Rac GTPase in vitro and axon guidance *in vivo*. Mol. Cell. Biol. 23: 6823–6835.

Levy-Strumpf, N., and J. G. Culotti, 2007 VAB-8, UNC-73 and MIG-2 regulate axon polarity and cell migration functions of UNC-40 in *C. elegans*. Nat. Neurosci. 10: 161–168.

Lundquist, E. A., 2003 Rac proteins and the control of axon development. Curr. Opin. Neurobiol. 13: 384–390.

Lundquist, E. A., P. W. Reddien, E. Hartwieg, H. R. Horvitz, and C. I. Bargmann, 2001 Three *C. elegans* Rac proteins and several alternative Rac regulators control axon guidance, cell migration and apoptotic cell phagocytosis. Development 128: 4475–4488.

Maes, T., A. Barcelo, and C. Buesa, 2002 Neuron navigator: a human gene family with homology to *unc-53*, a cell guidance gene from *Caenorhabditis elegans*. Genomics 80: 21–30.

Maloof, J. N., J. Whangbo, J. M. Harris, G. D. Jongeward, and C. Kenyon, 1999 A Wnt signaling pathway controls hox gene expression and neuroblast migration in *C. elegans*. Development 126: 37–49.

Martinez-Lopez, M. J., S. Alcantara, C. Mascaro, F. Perez-Branguli, P. Ruiz-Lozano *et al.*, 2005 Mouse neuron navigator 1, a novel microtubule-associated protein involved in neuronal migration. Mol. Cell. Neurosci. 28: 599–612.

Merrill, R. A., L. A. Plum, M. E. Kaiser, and M. Clagett-Dame, 2002 A mammalian homolog of *unc-53* is regulated by all-trans retinoic acid in neuroblastoma cells and embryos. Proc. Natl. Acad. Sci. USA 99: 3422–3427.

Muley, P. D., E. M. McNeil, M. A. Marzinke, K. M. Knobel, M. M. Barr *et al.*, 2008 The atRA-responsive gene neuron navigator 2 functions in neurite outgrowth and axonal elongation. Dev. Neurobiol. 68: 1441–1453.

Newsome, T. P., S. Schmidt, G. Dietzl, K. Keleman, B. Asling *et al.*, 2000 Trio combines with dock to regulate Pak activity during photoreceptor axon pathfinding in *Drosophila*. Cell 101: 283–294.

Peeters, P. J., A. Baker, I. Goris, G. Daneels, P. Verhasselt *et al.*, 2004 Sensory deficits in mice hypomorphic for a mammalian homologue of *unc-53*. Brain Res. Dev. Brain Res. 150: 89–101.

Schmidt, K. L., N. Marcus-Gueret, A. Adeleye, J. Webber, D. Baillie *et al.*, 2009 The cell migration molecule UNC-53/NAV2 is linked to the Arp 2/3 complex by ABI-1. Development 136: 563–574.

Serafini, T., T. E. Kennedy, M. J. Galko, C. Mirzayan, T. M. Jessell *et al.*, 1994 The Netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans*. Cell 78: 409–424.

Serafini, T., S. A. Colamarino, E. D. Leonardo, H. Wang, R. Beddington *et al.*, 1996 Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell 87: 1001–1014.

Soto, M. C., H. Qadota, K. Kasuya, M. Inoue, D. Tsuboi *et al.*, 2002 The GEX-2 and GEX-3 proteins are required for tissue morphogenesis and cell migrations in *C. elegans*. Genes Dev. 16: 620–632.

Spencer, A. G., S. Orita, C. J. Malone, and M. Han, 2001 A RHO GTPase-mediated pathway is required during P cell migration in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA 98: 13132–13137.

Steven, R., T. J. Kubiseski, H. Zheng, S. Kulkarni, J. Mancillas *et al.*, 1998 UNC-73 activates the Rac GTPase and is required for cell and growth cone migrations in *C. elegans.* Cell 92: 785–795.

Steven, R., L. Zhang, J. G. Culotti, and T. Pawson, 2005 The UNC-73/Trio RhoGEF-2 domain is required in separate isoforms for the regulation of pharynx pumping and normal neurotransmission in *C. elegans*. Genes Dev. 19: 2016–2029.

Stringham, E. G., and K. L. Schmidt, 2009 Navigating the Cell: UNC-53 and the navigators, a family of cytoskeletal regulators with multiple roles in cell migration, outgrowth and trafficking. Cell Adh. Migr. 3: 1–5.

Stringham, E., N. Pujol, J. Vandekerckhove, and T. Bogaert, 2002 *unc-53* controls longitudinal migration in *C. elegans*. Development 129: 3367–3379.

Takenawa, T., and S. Suetsugu, 2007 The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. Nat. Rev. Mol. Cell Biol. 8: 37–48.

Wadsworth, W. G., H. Bhatt, and E. M. Hedgecock, 1996 Neuroglia and pioneer neurons express UNC-6 to provide global and local Netrin cues for guiding migrations in *C. elegans*. Neuron 16: 35–46.

Watari-Goshima, N., K. Ogura, F. W. Wolf, Y. Goshima, and G. Garriga, 2007 *C. elegans* VAB-8 and UNC-73 regulate the SAX-3 receptor to direct cell and growth-cone migrations. Nat. Neurosci. 10: 169–176.

Wightman, B., S. Clark, A. Taskar, W. Forrester, A. Maricq *et al.*, 1996 The *C. elegans* gene *vab-8* guides posteriorly directed axon outgrowth and cell migration. Development 122: 671–682.

Wolf, F., M. S. Hung, B. Wightman, J. Way, and G. Garriga, 1998 *vab-8* is a key regulator of posteriorly directed migrations in *C. elegans* and encodes a novel protein with kinesin motor similarity. Neuron 20: 655–666.

Wu, Y. C., T. W. Cheng, M. C. Lee, and N. Y. Weng, 2002 Distinct rac activation pathways control *Caenorhabditis elegans* cell migration and axon outgrowth. Dev. Biol. 250: 145–155.

Zallen, J. A., B. A. Yi, and C. I. Bargmann, 1998 The conserved immunoglobulin superfamily member SAX-3/Robo directs multiple aspects of axon guidance in *C. elegans*. Cell 92: 217–227.

Zhao, Z., L. Fang, N. Chen, R. C. Johnsen, L. Stein *et al.*, 2005 Distinct regulatory elements mediate similar expression patterns in the excretory cell of *Caenorhabditis elegans*. J. Biol. Chem. 280: 38787–38794.

Zinovyeva, A. Y., Y. Yamamoto, H. Sawa, and W. C. Forrester, 2008 Complex network of Wnt signaling regulates neuronal migrations during *Caenorhabditis elegans* development. Genetics 179: 1357–1371.

Communicating editor: K. Kemphues