

The Netrin Receptor UNC-40/DCC Stimulates Axon Attraction and Outgrowth through Enabled and, in Parallel, Rac and UNC-115/AbLIM

Zemer Gitai,^{1,3} Timothy W. Yu,¹ Erik A. Lundquist,² Marc Tessier-Lavigne,^{3,*} and Cornelia I. Bargmann^{1,*}

¹Howard Hughes Medical Institute
Department of Anatomy
Department of Biochemistry and Biophysics
University of California, San Francisco
San Francisco, California 94143

²Department of Molecular Biosciences
University of Kansas
Lawrence, Kansas 66045

³Howard Hughes Medical Institute
Department of Biological Sciences
Stanford University
Stanford, California 94305

Summary

Netrins promote axon outgrowth and turning through DCC/UNC-40 receptors. To characterize Netrin signaling, we generated a gain-of-function UNC-40 molecule, MYR::UNC-40. MYR::UNC-40 causes axon guidance defects, excess axon branching, and excessive axon and cell body outgrowth. These defects are suppressed by loss-of-function mutations in *ced-10* (a Rac GTPase), *unc-34* (an Enabled homolog), and *unc-115* (a putative actin binding protein). *ced-10*, *unc-34*, and *unc-115* also function in endogenous *unc-40* signaling. Our results indicate that Enabled functions in axonal attraction as well as axon repulsion. UNC-40 has two conserved cytoplasmic motifs that mediate distinct downstream pathways: CED-10, UNC-115, and the UNC-40 P2 motif act in one pathway, and UNC-34 and the UNC-40 P1 motif act in the other. Thus, UNC-40 might act as a scaffold to deliver several independent signals to the actin cytoskeleton.

Introduction

Axons navigate through complex and varied environments by responding to attractants and repellents (Tessier-Lavigne and Goodman, 1996). Axons sense, interpret, and integrate these spatial cues through a structure at their leading edge known as the growth cone (Bray and Hollenbeck, 1988). The growth cone is rich in actin-based structures, and guidance cues are thought to exert their effects by inducing rearrangements in the actin cytoskeleton (Korey and Van Vactor, 2000). Several families of axon guidance cues including Netrins, Semaphorins, Slits, and Ephrins are known to act in the developing nervous system, but the signaling pathways linking these receptors to the cytoskeleton are not fully understood.

The conserved UNC-6/Netrin family of secreted proteins attract axons, promote axon outgrowth, and repel axons in vivo and in vitro (Ishii et al., 1992; Serafini et

al., 1994; Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995; Serafini et al., 1996). Netrins act through two families of receptors, the UNC-40/DCC family and the UNC-5 family (Leung-Hagesteijn et al., 1992; Chan et al., 1996; Keino-Masu et al., 1996; Leonardo et al., 1997). The UNC-40/DCC family is responsible for Netrin-induced axon attraction and outgrowth (Chan et al., 1996; Keino-Masu et al., 1996), whereas the UNC-40/DCC and UNC-5 families both contribute to axon repulsion from Netrin (Hamelin et al., 1993; Hong et al., 1999; Merz et al., 2001). Second messengers such as Ca²⁺ and cAMP can modulate Netrin signaling (Ming et al., 1997; Hong et al., 2000), but the targets of DCC signaling that elicit axon outgrowth and turning are unknown.

In the nematode *Caenorhabditis elegans*, UNC-6 is expressed ventrally, and UNC-40 is expressed in several neurons whose axons migrate ventrally, including the AVM sensory neuron (Chan et al., 1996; Wadsworth et al., 1996). Ventral axon migration is disrupted in *unc-6* and *unc-40* mutants (Hedgecock et al., 1990) and requires UNC-40 to function cell autonomously within AVM (Hedgecock et al., 1990; Chan et al., 1996).

Cytoplasmic signaling molecules that influence the actin cytoskeleton during axon pathfinding have been identified by genetic, molecular, and biochemical studies. The Rho family GTPases (Rho, Rac, and Cdc42) and the molecules that regulate their activity (GTP exchange factors [GEFs] and GTPase-activating proteins [GAPs]) influence the actin cytoskeleton in vitro and play roles in numerous cell and axon migrations in vivo (Luo et al., 1994; Nobes and Hall, 1995; Lundquist et al., 2001; Hakeda-Suzuki et al., 2002; Ng et al., 2002). Genetic evidence indicates a role for both Rac and the Rho family GEF UNC-73/Trio in axon guidance in *C. elegans* and *Drosophila* (Steven et al., 1998; Awasaki et al., 2000). Furthermore, Plexin-B guidance receptors bind to Rac proteins directly (Vikis et al., 2000); Eph guidance receptors interact with the Rho family GEF ephexin (Shamah et al., 2001); and Robo guidance receptors interact with a Cdc42 GAP (Wong et al., 2001). These results link Rho family members to signaling by Semaphorin, Ephrin, and Slit ligands, respectively.

Analyses of axon guidance mutants in *C. elegans* and *Drosophila* have yielded many additional cytoplasmic molecules that affect axonal outgrowth and pathfinding, including Enabled, Abl, Disabled, UNC-44/Ankyrin, UNC-51, UNC-76, and UNC-115 (Branda and Stern, 1999; Korey and Van Vactor, 2000). Together with Rho family GTPases, these molecules could be considered candidate effectors for axon guidance. However, the phenotypes resulting from mutations in these genes do not correspond to the phenotypes of specific guidance receptors in an obvious way. It is thus unclear how different guidance receptors utilize these cytoplasmic molecules to achieve specific axon pathfinding events.

Of the candidate axon guidance effectors, a great deal of work has been done on Enabled (Ena). Ena and its family members have striking effects on axon guidance in *Drosophila* and *C. elegans* and can influence actin dynamics in vitro (Gertler et al., 1995, 1996; M. Dell and

*Correspondence: marctl@stanford.edu (M.T.-L.), cori@itsa.ucsf.edu (C.I.B.)

G. Garriga, personal communication). Enabled proteins nucleate actin polymerization *in vitro* (Huttelmaier et al., 1999; Lambrechts et al., 2000) and enhance the actin-dependent motility of the intracellular pathogen *Listeria monocytogenes* (Laurent et al., 1999), suggesting that they might function to enhance cellular outgrowth. However, in *Drosophila* and *C. elegans*, genetic and physical interactions demonstrate that UNC-34/Enabled and the SAX-3/Robo repulsive guidance receptor cooperate in mediating axon repulsion (Bashaw et al., 2000; Yu et al., 2002). Furthermore, leading edge depletion and enrichment of an Ena homolog, Mena, demonstrate that it functions to inhibit fibroblast motility by modulating actin filament length and branching to promote unstable cellular protrusions (Bear et al., 2000, 2002). These results raise the possibility that Ena might be dedicated to repelling axons and inhibiting outgrowth.

Here we present genetic evidence that Ena can function in an attractive axon guidance pathway *in vivo*. We arrived at this conclusion through analysis of the UNC-40 signaling pathway, which was dissected by generating a gain-of-function UNC-40 molecule that produces a strong axon guidance and outgrowth defect in *C. elegans*. Candidate genes were assigned to the UNC-40 pathway by the ability of loss-of-function mutations to suppress this activated *unc-40(gf)* allele. This approach defined a bifurcated UNC-40 signaling pathway, mediated by two separate motifs in the UNC-40 cytoplasmic domain. One branch involves the Rac GTPase CED-10 (Reddien and Horvitz, 2000) and the putative actin binding protein UNC-115/abLIM (Lundquist et al., 1998), and the other branch involves UNC-34, a *C. elegans* Enabled homolog (M. Dell and G. Garriga, personal communication).

Results

MYR::UNC-40 Is a Gain-of-Function Form of the UNC-40 Receptor

UNC-40/DCC is a type I single-pass transmembrane receptor. The UNC-40 cytoplasmic domain has no obvious catalytic motifs; however, it plays an essential role in UNC-40 signaling, as demonstrated by chimeras of UNC-40/DCC with the Met receptor tyrosine kinase and the Robo axon guidance receptor (Bashaw and Goodman, 1999; Stein and Tessier-Lavigne, 2001). By analogy with many receptor tyrosine kinases, which are rendered constitutively active by the deletion of their extracellular domain (Ullrich and Schlessinger, 1990), we asked whether a myristoylated form of the UNC-40 cytoplasmic domain could activate the UNC-40 signaling pathway. We therefore generated an UNC-40 fusion protein in which the extracellular and transmembrane domains were deleted and replaced by sequences encoding a membrane-targeting myristoylation signal. We refer to this fusion protein as MYR::UNC-40.

To determine the effects of MYR::UNC-40 on axon development *in vivo*, we placed MYR::UNC-40 under the control of the mechanosensory neuron-specific *mec-7* promoter and examined axonal morphology in transgenic animals. The *mec-7* promoter drives expression in six touch-sensitive neurons, including AVM (Hamelin et al., 1992). AVM has a small, round cell body positioned

along the lateral body of the worm, with one axon that grows ventrally to the ventral nerve cord and then extends anteriorly (Figures 1A–1C). In *unc-6* and *unc-40* mutants, AVM cell body morphology is unaffected but the axon fails to migrate ventrally, resulting in anterior migration in a lateral position (Figures 1A and 1D; Chan et al., 1996). *unc-40* is thought to act cell autonomously in AVM: an *unc-40::gfp* promoter fusion is expressed in AVM, and expression of full-length *unc-40* under the control of the *mec-7* promoter rescues AVM ventral guidance in *unc-40* mutants (Chan et al., 1996). *mec-7::unc-40* expression in wild-type animals did not cause significant defects on its own (data not shown).

myr::unc-40 expression under the control of the *mec-7* promoter caused dominant AVM defects that were more severe than those seen in *unc-40* loss-of-function mutants. AVM neurons displayed enlarged and deformed cell bodies, additional axons, misguided axons, and additional axon branches (Figure 1E, Table 1). The excess outgrowth phenotype caused by MYR::UNC-40 is consistent with the known role of UNC-40 family members in promoting axon outgrowth as well as turning (Keino-Masu et al., 1996; Fazeli et al., 1997; Ming et al., 1997), suggesting that MYR::UNC-40 is a constitutively active UNC-40 molecule. This interpretation is further supported by the suppressor analysis presented below. Expression of a myristoylated GFP construct (*myr::gfp*) under the *mec-7* promoter did not produce severe defects in AVM (Table 1), demonstrating that the effects of MYR::UNC-40 were not caused by either the *mec-7* promoter or the myristoylation signal.

The AVM axon extends during the L1 larval stage and maintains a stable shape thereafter. In MYR::UNC-40 animals, additional outgrowths appeared as the animals developed, as indicated by the increased fraction of animals displaying excess outgrowths in each subsequent stage of development (Figure 2A). Additional outgrowths were also observed in individual adult animals imaged at several hour intervals (Figures 2B and 2C). These results demonstrate the ability of MYR::UNC-40 to act throughout development and into adulthood. Consistent with this result, an *unc-40::gfp* promoter fusion is expressed in AVM throughout the life of the animal (Chan et al., 1996).

The MYR::UNC-40 effects were not dependent on the endogenous *unc-6* and *unc-40* genes, as null mutations in either *unc-6(ev400)* or *unc-40(e271)* failed to suppress the excess outgrowth (Figure 3A). This result supports the interpretation that MYR::UNC-40 is a constitutively active molecule and not a dominant-negative molecule or a modulator of normal UNC-40 activity.

To determine if *myr::unc-40* affects axon development outside of AVM, we targeted *myr::unc-40* to HSN neurons using the *unc-86* promoter (Baumeister et al., 1996). HSN ventral axon guidance is mediated by UNC-6 and UNC-40 (Hedgecock et al., 1990; McIntire et al., 1992). When expressed under the *unc-86* promoter, *myr::unc-40* but not *myr::gfp* caused excess outgrowth in HSN (Table 1). The HSN axons were severely affected by MYR::UNC-40 but, in contrast to AVM, HSN cell body morphology was unaffected (Table 1). Interestingly, *myr::unc-40* also caused defects in the ALM and PLM neurons when expressed there under the control of the *mec-7* promoter (data not shown). ALM and PLM ex-

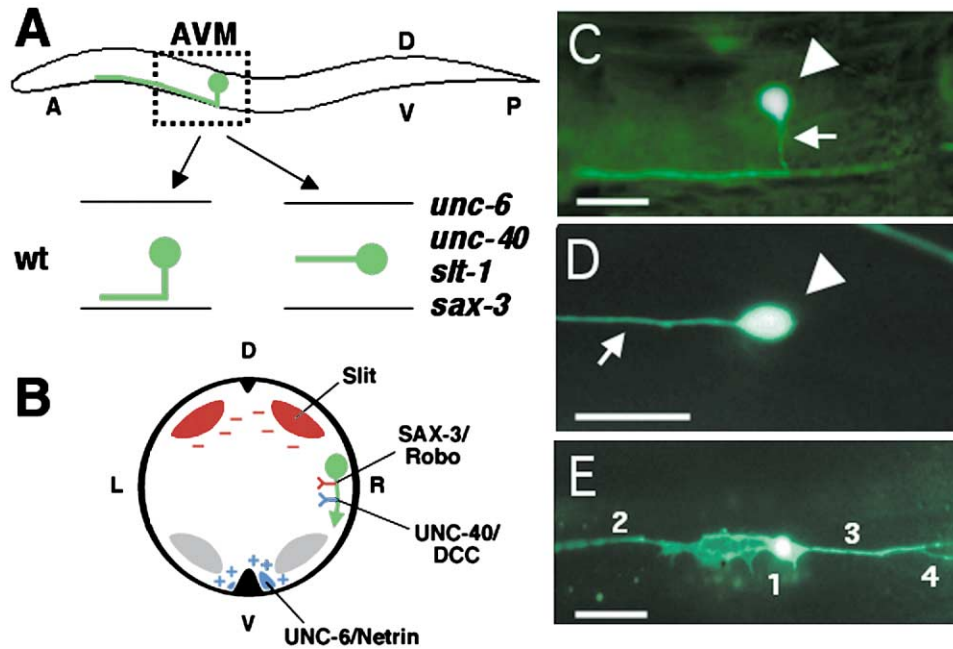


Figure 1. MYR::UNC-40 Causes Excess Outgrowth in the AVM Neuron

(A and B) Organization of the guidance pathways important for AVM ventral guidance.

(A) Lateral view. The AVM neuron (green) has a cell body located in the anterior third of the animal, and extends its axon ventrally in wild-type animals and laterally in *unc-6*, *unc-40*, *slit-1*, and *sax-3* mutants.

(B) Crosssection view. The expression of both SAX-3/Robo and UNC-40/DCC guidance receptors in AVM (green) allows its axon to grow toward the ventral UNC-6/Netrin cue made by neurons (blue) and away from the dorsal SLT-1 cue made by muscles (red). Ventral muscles are shown in gray.

(C-E) AVM morphology in wild-type (C), *unc-40(e271)* (D), and *mec-7::myr::unc-40* (E) animals. Arrowheads point to the cell body and arrows point to the axons. Numbers show: (1) enlarged and deformed cell body; (2) lateral misguided axon; (3) additional axon; and (4) axon branch. In (C)–(E), ventral is down and anterior is left. AVM is visualized with a *zlds4[mec-4::gfp]* reporter. MYR::UNC-40 animals are *kyEx456[mec-7::myr::unc-40, str-1::gfp]*. Scale bar equals 10 μ m.

press UNC-40 but do not appear to respond to UNC-6 during normal development (Chan et al., 1996). Thus, the MYR::UNC-40 excess outgrowth phenotype is observed in several cell types, including both Netrin-responsive and Netrin-nonresponsive neurons.

To assess its ability to carry out any native UNC-40 functions, we examined the ability of MYR::UNC-40 to mediate repulsion from UNC-6. UNC-5/UNC5H represents a family of UNC-6/Netrin receptors whose cytoplasmic domains interact with UNC-40/DCC, converting UNC-6/Netrin attraction into repulsion (Leung-Hages-

teijn et al., 1992; Hamelin et al., 1993; Leonardo et al., 1997; Hong et al., 1999). Since the extracellular domain of UNC-5/UNC5H interacts with UNC-6/Netrin, a complex of UNC-5 and MYR::UNC-40 could sense an UNC-6 gradient and transduce the appropriate repulsive signal to the cytoplasm (Hong et al., 1999). We used the *unc-25* promoter (Jin et al., 1999) to target *myr::unc-40* to the VD and DD motoneurons. These neurons have ventrally located cell bodies and extend axons dorsally, away from the ventral source of UNC-6. The dorsal guidance of these axons is dependent on *unc-6*, *unc-5*, and *unc-*

Table 1. Effects of MYR::UNC-40 and MYR::GFP in AVM and HSN

	WT	Misguided or Additional Axons	Deformed Cell Body	Mutant Axon and Cell Body	n
AVM					
<i>mec-7::MYR::UNC-40</i>	11%	22%	25%	42%	110
<i>mec-7::MYR::GFP</i>	94%	6%			103
HSN					
<i>unc-86::MYR::UNC-40</i>	23%	74%		2%	127
<i>unc-86::MYR::GFP</i>	99%	1%			112

AVM and HSN morphologies were characterized using *zlds4[mec-4::gfp, lin-15(+)]* and *kyls179[unc-86::gfp, lin-15(+)]*, respectively. In schematics, ventral is down and anterior is left. n = number of neurons scored.

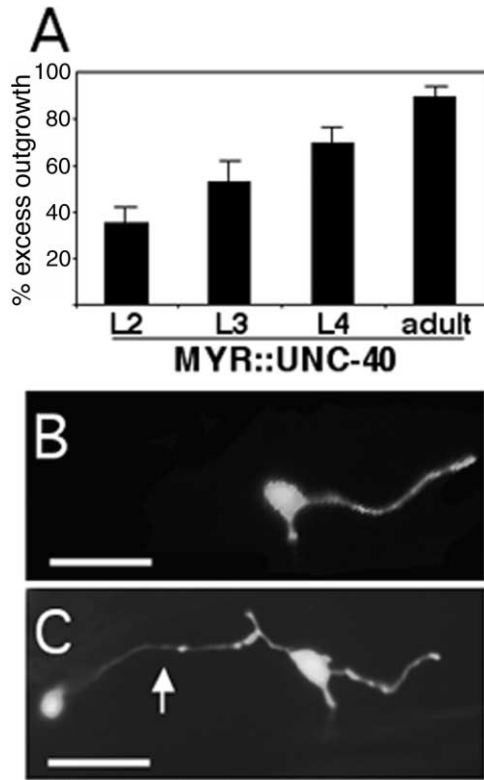


Figure 2. MYR::UNC-40 Causes Defects throughout Development and Adulthood

(A) The percentage of MYR::UNC-40 animals with any excess outgrowth in AVM increases in the L2, L3, and L4 larval stages and in first-day adult animals.

(B and C) AVM in the same MYR::UNC-40 day-1 adult animal at time 0 (B) and 6 hr (C). The arrow points to a new process in (C) that grew out between the two time points. In all images, dorsal is down and anterior is left. AVM is visualized with a *zdfs4[mec-4::gfp]* reporter. MYR::UNC-40 animals are *kyEx456[mec-7::myr::unc-40, str-1::gfp]*. Scale bar equals 10 μ m.

40 (McIntire et al., 1992). In an *unc-40* mutant background, only $23\% \pm 3\%$ of the axons properly reach the dorsal nerve cord. When expressed under the *unc-25* promoter, both full-length *unc-40* ($41\% \pm 2\%$, $p < .01$) and *myr::unc-40* ($45\% \pm 3\%$, $p < .01$) were capable of partially rescuing the *unc-40* mutant VD and DD dorsal axon guidance defects. This result suggests that MYR::UNC-40 retains the ability to carry out some endogenous UNC-40 functions, consistent with the model that the novel phenotypes caused by MYR::UNC-40 expression in AVM result from hyperactivation of the endogenous UNC-40 pathway.

ced-10, *unc-34*, and *unc-115* Suppress the *myr::unc-40* Phenotype

To identify other genes that act in the *unc-40* pathway, we analyzed genetic interactions between known axon pathfinding mutations and *myr::unc-40*. If MYR::UNC-40 represents a constitutively active UNC-40 molecule, mutations in genes that act downstream of UNC-40 should suppress the excessive and misguided outgrowth phenotype caused by *myr::unc-40* expression. For this analysis, we focused on the AVM neuron, as

it utilizes *unc-40* cell autonomously during its normal ventral guidance (Chan et al., 1996).

We first considered the possibility that MYR::UNC-40 acts by interacting with other guidance receptors, potentially via heterodimerization. As described above, *unc-40* mutants failed to suppress *myr::unc-40* (Figure 3A), suggesting that MYR::UNC-40 does not require an intact UNC-40 receptor. We next examined animals mutant for *sax-3/Robo*, the *C. elegans* receptor for Slit (SLT-1) (Zallen et al., 1998). *unc-6* and *slt-1* act in parallel in AVM ventral guidance (Figure 1; Hao et al., 2001), but both vertebrate and *C. elegans* UNC-40/DCC and SAX-3/Robo can modulate each others' function and bind each other in vitro (Stein and Tessier-Lavigne, 2001; Yu et al., 2002). However, strong *sax-3(ky123)* mutations did not suppress *myr::unc-40*, indicating that SAX-3 is not required for MYR::UNC-40 activity (Figure 3A). UNC-5 receptors bind UNC-40/DCC and mediate repulsion from UNC-6 (Leung-Hagesteijn et al., 1992; Hong et al., 1999), but *unc-5(e53)* mutants failed to suppress MYR::UNC-40 (Figure 3A), indicating that MYR::UNC-40 acts independently of UNC-5. This result is consistent with the observation that an *unc-5::gfp* fusion gene is not expressed at detectable levels in AVM (Su et al., 2000). The VAB-1/Eph receptor has been implicated in multiple axon guidance events (George et al., 1998; Zallen et al., 1999), but *vab-1(e2)* mutations failed to suppress *myr::unc-40* (Figure 3A). Thus, MYR::UNC-40 acts independently of the endogenous UNC-40, SAX-3, UNC-5, and VAB-1 axon guidance receptors.

We next initiated a screen of other *C. elegans* genes with known roles in axon guidance and identified three that suppressed the AVM cell and axon defects caused by MYR::UNC-40. The strong loss-of-function mutants *ced-10(n1993)* and *ced-10(n3246)* suppressed approximately half the excessive outgrowth of MYR::UNC-40 (Figure 3B). *ced-10* encodes a Rac member of the Rho family of small G proteins (Reddien and Horvitz, 2000), and CED-10 affects axon guidance together with the Rac-like protein MIG-2 (Lundquist et al., 2001). The excessive outgrowth caused by MYR::UNC-40 was also partially suppressed in *unc-34(gm104)* and *unc-34(e951)* backgrounds (Figure 3B). *unc-34* encodes the only *C. elegans* Enabled protein (M. Dell and G. Garriga, personal communication), and *unc-34* mutants have defects in axon extension (McIntire et al., 1992; Wightman et al., 1997). *unc-115(ky275)* and *unc-115(ky274)* also partially suppressed the excessive outgrowth caused by MYR::UNC-40. *unc-115* encodes a putative actin binding protein similar to vertebrate abLIM (Roof et al., 1997), and *unc-115* mutants have widespread defects in axon outgrowth and guidance (Lundquist et al., 1998). Interestingly, the *ced-10*, *unc-34*, and *unc-115* genes each suppressed all classes of axon and cell body phenotypes caused by MYR::UNC-40 in AVM (Figure 3D). This result suggests that the axon guidance, outgrowth, branching, and cell shape defects are all manifestations of the same enhanced outgrowth activity of MYR::UNC-40.

mig-2 encodes a Rho family GTPase most similar to Rac (Zipkin et al., 1997), and *unc-73* encodes a Trio protein that acts as a GEF for Rho family small G proteins (Steven et al., 1998). Neither of the strong loss-of-function mutations *mig-2(mu28)* nor *unc-73(e936)* suppressed the excess outgrowth of MYR::UNC-40 (Figure

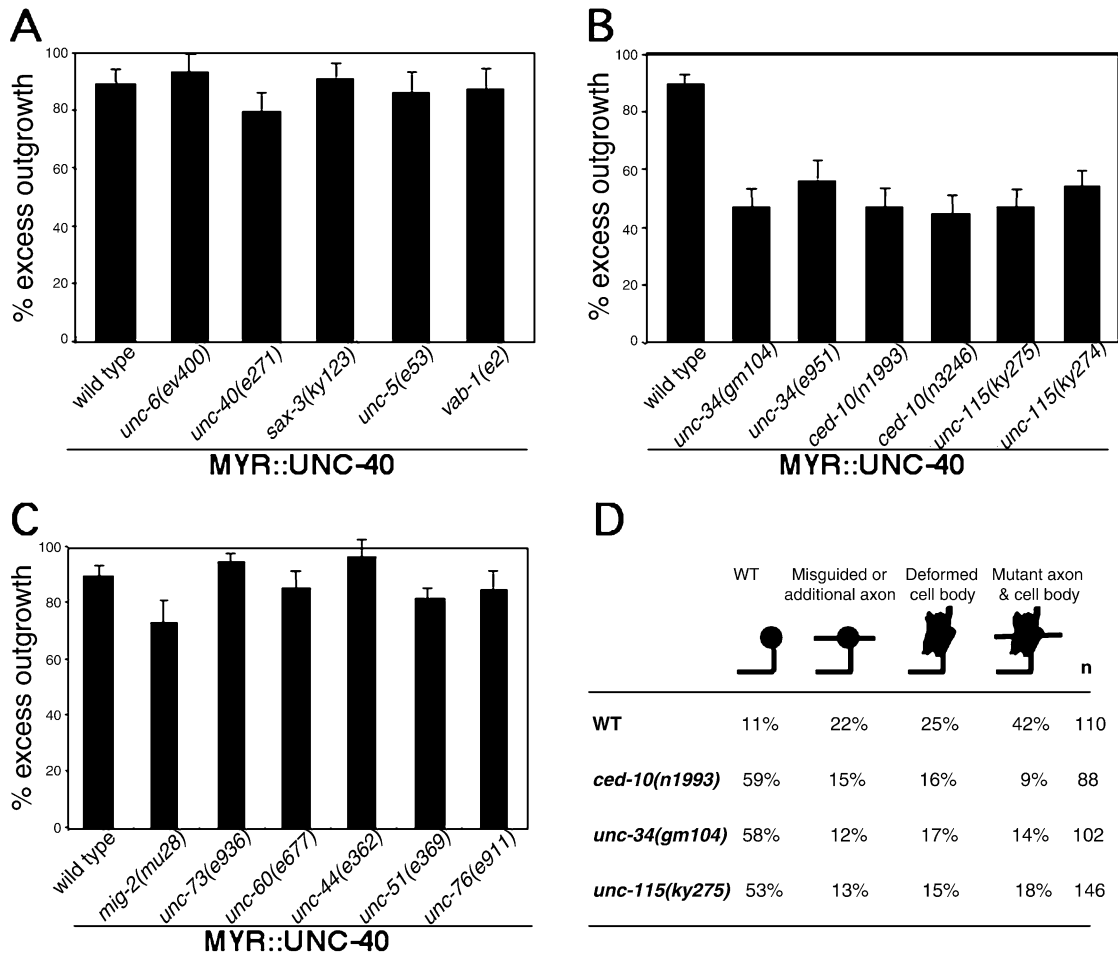


Figure 3. *unc-34*, *ced-10*, and *unc-115* Suppress MYR::UNC-40 in AVM

In all panels, “% excess outgrowth” refers to the percentage of animals with any defect in AVM (see Table 1). Animals shown express *kyEx456[mec-7::myr::unc-40, str-1::gfp]; zdl4[mec-4::gfp]*. Qualitatively similar results were observed with another array, *kyIs192[mec-7::myr::unc-40, str-1::gfp]*.

(A) The axon guidance ligands and receptors *unc-6(ev400)*, *unc-40(e271)*, *sax-3(ky123)*, *unc-5(e53)*, and *vab-1(e2)* do not suppress MYR::UNC-40. (B) Multiple alleles of the axon guidance and actin cytoskeleton signaling molecules *ced-10*, *unc-34*, and *unc-115* suppress MYR::UNC-40. (C) The cytoplasmic signaling molecules *mig-2(mu28)*, *unc-73(e936)*, *unc-60(e677)*, *unc-44(e362)*, *unc-51(e369)*, and *unc-76(e911)* do not suppress MYR::UNC-40. All mutations represent the strongest available loss-of-function alleles.

(D) *unc-34*, *ced-10*, and *unc-115* similarly suppress the cell body and axon defects caused by MYR::UNC-40. In schematics, ventral is down and anterior is left; n = number of animals scored.

3C). In addition, *ced-10*; *mig-2* double mutants did not show any more suppression than *ced-10* alone (41% ± 5%, n = 93). The specific involvement of CED-10, but not MIG-2 or UNC-73, in MYR::UNC-40 signaling suggests that the reported redundancy of CED-10 and MIG-2 Rac-like proteins (Lundquist et al., 2001; Kishore and Sundaram, 2002) may actually mask specific functions in individual guidance decisions.

Several other mutations that did not suppress MYR::UNC-40 included *unc-60(e677)*, a cofilin protein capable of actin filament disassembly (McKim et al., 1994); *unc-44(e362)*, an ankyrin protein whose vertebrate homologs link integral membrane proteins to the spectrin cytoskeleton (Otsuka et al., 1995); *unc-51(e369)*, a kinase required for normal axon guidance and outgrowth (Ogura et al., 1994); and *unc-76(e911)*, a novel cytoplasmic protein involved in axon guidance and outgrowth (Bloom and Horvitz, 1997) (Figure 3C).

mec-7::myr::unc-40 is expressed in three types of

neurons in addition to AVM: PVM, ALM, and PLM. MYR::UNC-40 caused excessive outgrowth phenotypes in all of these classes of cells. Surprisingly, *ced-10*, *unc-34*, and *unc-115* did not suppress the *mec-7::myr::unc-40* excessive outgrowth in any touch cell other than AVM (data not shown). However, *ced-10*, *unc-34*, and *unc-115* did suppress the *unc-86::myr::unc-40* defect in the HSN motor neuron (data not shown). The reason for this cell type specificity is unclear, but it is interesting that suppression was specific to AVM and HSN, which are the two pioneer neurons whose normal ventral guidance is strongly dependent on UNC-6 and UNC-40.

unc-34 and *unc-115* Act Cell Autonomously for MYR::UNC-40 Suppression

If CED-10, UNC-34, and UNC-115 act directly downstream of MYR::UNC-40, these molecules should function in the same cell as MYR::UNC-40. All of these genes affect many neurons, and GFP fusions to the promoters

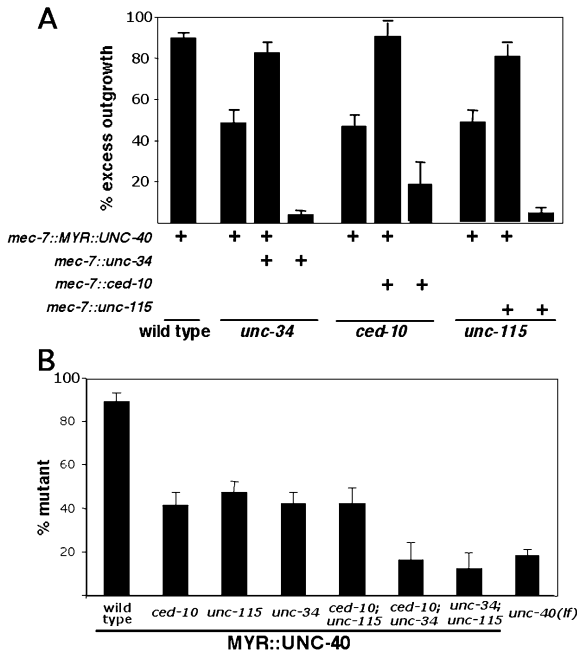


Figure 4. Cell-Autonomous and Parallel Functions of *ced-10*, *unc-115*, and *unc-34*

(A) *unc-34*, *ced-10*, and *unc-115* act cell autonomously for MYR::UNC-40 suppression. “% excess outgrowth” refers to the percentage of animals with any excess outgrowth in AVM (see Table 1). Transgenes were introduced into wild-type, *unc-34(gm104)*, *ced-10(n1993)*, or *unc-115(ky275)* genetic backgrounds. All animals also express *zdfs4[mec-4::gfp]*. The effects of expressing *kyEx456[mec-7::myr::unc-40, str-1::gfp]*, *kyEx652[mec-7::unc-34, odr-1::rfp]*, *kyEx653[mec-7::unc-115, odr-1::rfp]*, and *kyEx681[mec-7::ced-10, odr-1::rfp]* in various combinations are shown.

(B) CED-10 and UNC-115 function together, in parallel to UNC-34, downstream of MYR::UNC-40. “% mutant” refers to the percentage of animals with any defect in AVM morphology (see Table 1 and Figure 1). All animals express *zdfs4[mec-4::gfp]*, and all animals other than *unc-40(lf)* also express *kyEx456[mec-7::myr::unc-40, str-1::gfp]*.

of *ced-10* and *unc-115* indicate that they are broadly expressed (Lundquist et al., 1998, 2001). We used cell type-specific expression of transgenes to ask whether the *myr::unc-40* suppression is due to cell-autonomous activity of *unc-34*, *ced-10*, and *unc-115*.

For *unc-34*, we introduced two transgenes into an *unc-34* mutant background: *mec-7::myr::unc-40* and *mec-7::unc-34*. Each of these transgenes expresses either MYR::UNC-40 or UNC-34 only in AVM, ALM, PVM, and PLM. As discussed above, *unc-34; mec-7::myr::unc-40* animals exhibited partial suppression of the excessive outgrowth phenotype caused by *mec-7::myr::unc-40* (Figures 3B and 4A). *unc-34* animals bearing both the *mec-7::myr::unc-40* and the *mec-7::unc-34* transgenes exhibited excessive outgrowth at a level comparable to *mec-7::myr::unc-40* in a wild-type background, indicating that *unc-34* and *myr::unc-40* act in the same cell. The *mec-7::unc-34* transgene did not cause significant AVM defects in the absence of *mec-7::myr::unc-40* (Figure 4A).

The same strategy was employed for *unc-115* and *ced-10*, by expressing *mec-7::myr::unc-40* and *mec-*

7::unc-115 or *mec-7::ced-10* transgenes in *unc-115* or *ced-10* mutants, respectively. The partial suppression of *mec-7::myr::unc-40* in an *unc-115* mutant background was eliminated in animals expressing *mec-7::unc-115*. *mec-7::unc-115* did not cause significant AVM defects on its own (Figure 4A). Similarly, the partial suppression of *mec-7::myr::unc-40* in a *ced-10* mutant background was eliminated in animals expressing *mec-7::ced-10*. *mec-7::ced-10* caused only a mild AVM defect on its own (Figure 4A). These results suggest that the requirement of *unc-34*, *unc-115*, and *ced-10* for the suppression of the excess outgrowth caused by *myr::unc-40* in AVM is cell autonomous.

ced-10 and *unc-115* Function Together for *myr::unc-40* Suppression

The finding that *unc-34*, *ced-10*, and *unc-115* all function to suppress the *myr::unc-40* phenotype raised the question whether they all function in the same signaling pathway or in parallel pathways. For two gene products acting in the same pathway, removing both genes should cause a phenotype similar to that of the strongest single null mutant; for products acting in parallel pathways, additive or synergistic effects might be observed when both genes are removed. We therefore examined the consequences of removing pairwise combinations of *unc-34*, *ced-10*, and *unc-115* in a *myr::unc-40* background. Strong loss-of-function alleles representing presumed null phenotypes were used in all cases. As shown in Figure 4B, removing both *ced-10* and *unc-115* function did not lead to any additional suppression of the *myr::unc-40* phenotype compared to removing either one alone, strongly suggesting that *unc-115* and *ced-10* act in the same pathway. By contrast, double mutants of either *unc-115* or *ced-10* with *unc-34* caused further suppression of the *myr::unc-40* phenotype (Figure 4B), indicating that *unc-34* functions in a pathway parallel to *unc-115* and *ced-10*. Interestingly, the extent of defects in these double mutants in the *myr::unc-40* background was similar to that in *unc-40* loss-of-function mutants (Figure 4B). These observations are consistent with the model that there are two pathways for signaling downstream of MYR::UNC-40: one pathway involves CED-10 and UNC-115, the other involves UNC-34, and inactivation of both pathways abolishes MYR::UNC-40 signaling.

ced-10 and *unc-115* Function Together in Parallel to *unc-34* in the Endogenous *unc-40* Pathway

We next wished to ask whether *ced-10*, *unc-115*, and *unc-34* function in endogenous UNC-40-mediated axon guidance, not just MYR::UNC-40 signaling, and if so, whether *ced-10*, *unc-115*, and *unc-34* act in the same or parallel pathways.

ced-10(n1993) and *unc-115(ky275)* null mutants have qualitatively similar defects in AVM axon guidance to those observed in *unc-40(e271)* mutants, but with significantly lower penetrances (Figure 5A). Double mutants of *ced-10* or *unc-115* with *unc-40* showed no enhancement over *unc-40* alone, consistent with CED-10 and UNC-115 functioning downstream of UNC-40 (Figure 5A). Furthermore, a *ced-10; unc-115* double mutant showed no enhancement over the phenotype seen in either single

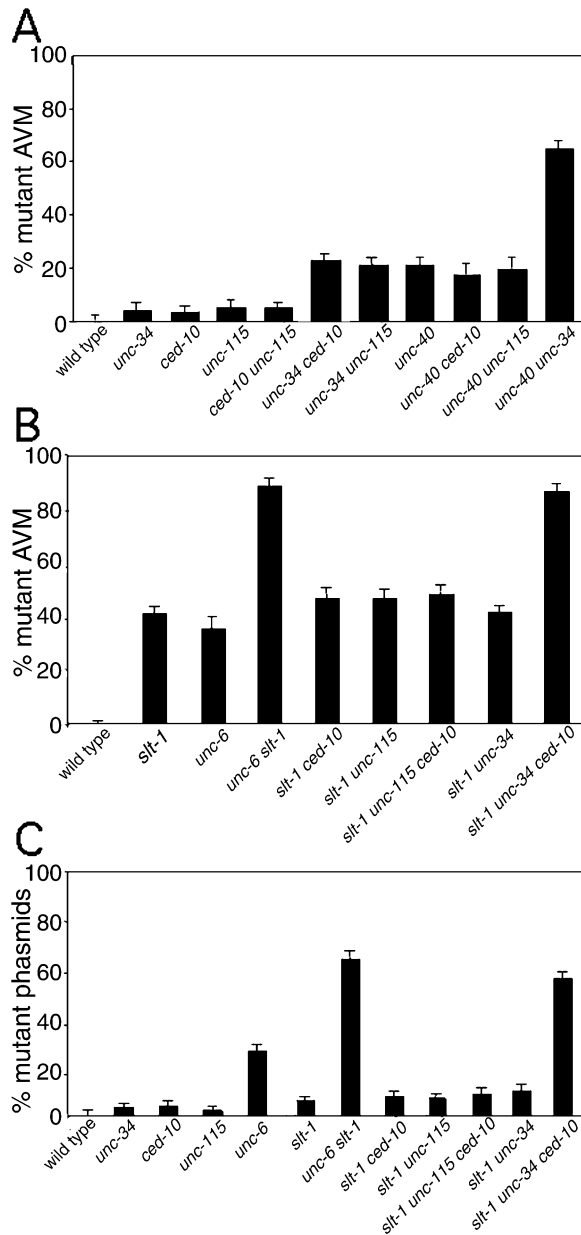


Figure 5. *ced-10* and *unc-115* Function Together, in Parallel to *unc-34*, in the Endogenous *unc-40* Pathway
 “% mutant” refers to the percentage of animals with lateral axons. (A) AVM axon phenotypes are shown for *ced-10*, *unc-34*, *unc-115*, and *unc-40* single and double mutants. (B) AVM axon phenotypes are shown for *unc-6*, *slt-1*, and double and triple mutants of *slt-1* with *unc-6*, *ced-10*, *unc-115*, and *unc-34*. (C) Phasmid axon phenotypes, as assessed by dye-filling with DiD, are shown for *ced-10*, *unc-34*, *unc-115*, *unc-6*, and *slt-1* single, double, and triple mutants. In (A)–(C), all animals express *zdfs5[mec-4::gfp]*.

mutant (Figure 5A), consistent with the model that *ced-10* and *unc-115* function in the same pathway and mediate a portion of the *unc-40* signal.

We next asked whether UNC-34 functions in a pathway parallel to CED-10 and UNC-115 and mediates the remainder of the UNC-40 signal. *unc-34(gm104)* null mu-

tants again have qualitatively similar defects in AVM axon guidance to those observed in *unc-40(e271)* mutants but with a much lower penetrance (Figure 5A). Double mutants of *unc-34* with either *ced-10* or *unc-115* showed an enhanced phenotype compared to each of the three single mutants (Figure 5A), consistent with *unc-34* being in a pathway parallel to *ced-10* and *unc-115*. This result is in principle consistent with the possibility that UNC-34 is downstream of UNC-40. A simple interpretation of the latter findings would be that *unc-40* signaling is mediated by two pathways: one involving both *ced-10* and *unc-115*, and the other *unc-34*. Before drawing this conclusion, however, it is necessary to consider an alternative explanation for the double mutant results involving *unc-34*: that *unc-34* does not function downstream of *unc-40* and instead functions in a pathway parallel to *unc-40*. Indeed, *unc-34* must function at least partly in a pathway parallel to *unc-40* because *unc-34*; *unc-40* double mutants had a significantly enhanced phenotype compared to *unc-40* mutants alone (Figure 5A). This enhancement reflects the fact that AVM is guided not only by UNC-6/Netrin attraction mediated by UNC-40, but also by SLT-1 repulsion mediated by SAX-3/Robo (Figures 1A and 1B; Hao et al., 2001). *unc-34* functions downstream of *sax-3* (Yu et al., 2002), just as Enabled functions downstream of Robo in *Drosophila* (Bashaw et al., 2000).

Thus, in AVM *unc-34* acts with *sax-3/Robo*, but the question remains whether *unc-34* also functions in the endogenous *unc-40* pathway, as suggested by the ability of *unc-34* to suppress the MYR::UNC-40 phenotype. To address this possibility, we first eliminated SAX-3/Robo signaling by removing the SAX-3 ligand SLT-1. As previously described, in *slt-1* or *unc-6* mutants alone, the penetrance of the AVM guidance defects is about 40%; however, removing *unc-6* in a *slt-1* mutant background increases the penetrance of the defects to about 90% (Figure 5B), reflecting the fact that UNC-6 attraction and SLT-1 repulsion account for virtually all of AVM ventral guidance (Figure 1B; Hao et al., 2001). Thus, a test for the involvement of a gene’s product in mediating UNC-6 attraction via UNC-40 is to ask whether removing the gene will enhance the *slt-1* mutant phenotype. Removing either *ced-10*, *unc-115*, or *unc-34* singly, or both *ced-10* and *unc-115* together, did not significantly enhance the *slt-1* phenotype (Figure 5B). However, when both *ced-10* and *unc-34* were removed together in a *slt-1* mutant background, the penetrance of the defect was as high (~90%) as when *unc-6* (Figure 5B) or *unc-40* (data not shown) were removed in a *slt-1* background. In other words, the residual guidance after removal of *slt-1*, which is mediated almost entirely by UNC-6 activating UNC-40, is abolished by simultaneous removal of *ced-10* and *unc-34*.

To confirm these results, we examined the ventral axon guidance of additional neurons. For this purpose we focused on the PHA and PHB phasmid neurons, whose axons can be visualized by filling with fluorescent dyes (Hedgecock et al., 1985). Like AVM, phasmid axon guidance is defective in both *unc-6* and *slt-1* single mutants, and is significantly enhanced in the *unc-6 slt-1* double mutant (Figure 5C). As was seen in AVM, removing any one of *ced-10*, *unc-115*, or *unc-34* failed to significantly enhance the *slt-1* phenotype, whereas removing

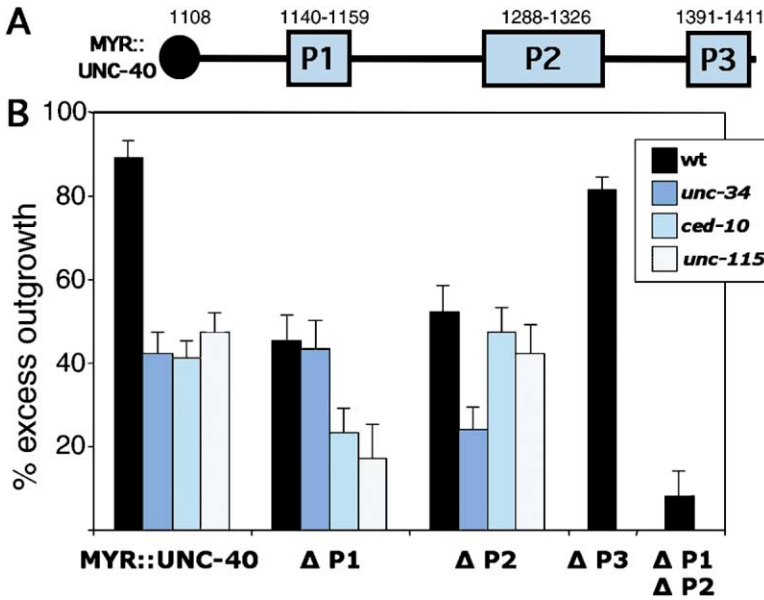


Figure 6. Two Motifs in MYR::UNC-40 Have Independent and Additive Effects on Outgrowth

(A) Schematic of MYR::UNC-40. The black circle represents the myristoylation sequence, and the numbers represent the amino acid positions in the full-length UNC-40 protein. (B) “% excess outgrowth” refers to the percentage of animals with any defect in AVM. All animals express the transgene listed below the bar graphs (MYR::UNC-40, ΔP1, ΔP2, ΔP3, or ΔP1ΔP2). The effect of the transgenes is shown in wild-type (black), *unc-34* (dark blue), *ced-10* (medium blue), and *unc-115* (light blue) backgrounds. All animals express *zdl4[mec-4::gfp]*. Qualitatively similar results were obtained with two independently derived transgenic lines for each MYR::UNC-40 deletion. Results shown are from one representative line.

both *ced-10* and *unc-34* together resulted in a great enhancement of the *slt-1* phasmid guidance defect to a level comparable to that of the *unc-6 slt-1* double mutant (Figure 5C). Once again, removal of *ced-10* and *unc-115* together was indistinguishable from removal of either gene alone (Figure 5C). Combined, these genetic results provide strong support for the model that endogenous *unc-40* signaling requires two pathways: one involving *unc-34*, and the other involving *ced-10*. The double mutant analysis of *ced-10* and *unc-115* indicates that *unc-115* functions in the same pathway as *ced-10*.

The CED-10/UNC-115 and UNC-34 Activities Correspond to Distinct Motifs in the UNC-40 Cytoplasmic Domain

The UNC-40 cytoplasmic domain does not contain any known enzymatic activities or motifs. However, it does contain three conserved motifs, P1, P2, and P3, that are present in vertebrate, fly, and worm UNC-40/DCC proteins (Kolodziej et al., 1996). In vitro, the P3 motif of DCC acts as both a homodimerization domain and a Robo-interacting domain (Stein and Tessier-Lavigne, 2001; Stein et al., 2001). While Robo can bind Enabled (Bashaw et al., 2000; Yu et al., 2002), the possibility that SAX-3/Robo provides the molecular link between MYR::UNC-40 and UNC-34/Enabled was ruled out by the inability of *sax-3* mutants to suppress *myr::unc-40* (Figure 3A). The UNC-40/DCC P1 motif is required for the binding of DCC to UNC5H2 and for repulsion from Netrin-1 (Hong et al., 1999). The function of P1 and P2 in the attractive response to Netrin is unknown.

To assess the importance of the conserved UNC-40 cytoplasmic motifs, these domains were deleted in MYR::UNC-40. Both the P1 motif (amino acids 1140–1159) and the P2 motif (amino acids 1288–1326) (Figure 6A) were required for full MYR::UNC-40 excess outgrowth (Figure 6B). Each of the MYR::UNC-40 P1 and P2 deletions yielded defects that were roughly half as strong as MYR::UNC-40. Deletion of the P3 motif did not significantly affect the MYR::UNC-40 phenotype. Si-

multaneous deletion of both P1 and P2 resulted in a MYR::UNC-40 protein that caused very little excess outgrowth (Figure 6B). These results suggest that the P1 and P2 motifs of UNC-40 have independent and additive activities in UNC-40 signaling.

Since deletion of the P1 and P2 motifs partially suppressed *myr::unc-40* activity in a manner similar to *unc-34*, *ced-10*, and *unc-115*, we used double mutant analysis to order these genes’ activities relative to the P1 and P2 motifs of UNC-40. The excess outgrowth caused by *myr::unc-40(ΔP1)* was not further suppressed by *unc-34*, indicating that deletion of P1 and loss of *unc-34* affect the same *unc-40* activity. However, excess outgrowth caused by *myr::unc-40(ΔP1)* was significantly suppressed by both *unc-115* and *ced-10* (Figure 6B), indicating that *unc-115* and *ced-10* act in parallel to the P1 motif.

Unlike *myr::unc-40(ΔP1)*, the excess outgrowth of *myr::unc-40(ΔP2)* was suppressed in an *unc-34* background but was not suppressed by either *ced-10* or *unc-115* (Figure 6B). Since *ced-10* and *unc-115* were not required for MYR::UNC-40(ΔP2) activity, they are likely to function downstream of the P2 motif. The observation that *ced-10* and *unc-115* genetically interact with the same domain of UNC-40 agrees with the results suggesting that *ced-10* and *unc-115* act in the same signaling pathway.

Discussion

In recent years, various effectors of axon guidance signaling have been identified. However, assigning effectors to specific axon guidance receptor signaling pathways has proven difficult, which may reflect both the sharing of signaling molecules between guidance pathways and redundancy within guidance pathways. This study addresses these problems by employing a sensitized genetic background, a constitutively activated UNC-40 molecule, to analyze UNC-40 signaling. This approach identified two partially redundant effector

pathways, with CED-10/Rac and UNC-115/abLIM defining one pathway and UNC-34/Enabled defining the second. Rho family proteins have previously been implicated in both axon attraction and repulsion but have not been demonstrated to affect Netrin signaling *in vivo*. A role for Enabled in axon repulsion has been established, but it has not been known whether Enabled might be bifunctional. Our results provide evidence that Enabled can also be an effector of an attractive axon guidance pathway.

MYR::UNC-40 Is an Activated Form of the UNC-40 Receptor

Expression of MYR::UNC-40 in specific neurons produced excessive outgrowth including additional axons, misguided axons, additional axon branches, and deformed cell bodies. Netrins have previously been shown to promote outgrowth and guidance: vertebrate Netrin-1 was originally identified based on its ability to enhance axon outgrowth into a collagen matrix, and Netrin-1 knockout mice have defects in axon outgrowth in addition to axon guidance (Serafini et al., 1994, 1996). Netrin can also orient axon outgrowth (Kennedy et al., 1994; de la Torre et al., 1997). Both of these effects of Netrin are dependent on the DCC receptor (Keino-Masu et al., 1996; Fazeli et al., 1997). Our results suggest that MYR::UNC-40 activates cytoplasmic signaling of the UNC-40 pathway in a constitutive, ligand-independent manner. The *in vivo* activation of signaling by the deletion of the extracellular and transmembrane domains suggests that these domains normally function to prevent UNC-40 activation but are disinhibited when UNC-6 binds to UNC-40. A similar disinhibition model has been proposed for the role of Netrin in activating the DCC-UNC-5 complex for axon repulsion (Hong et al., 1999).

Double and triple mutant analysis indicates that all of the *myr::unc-40* suppressors, *unc-34*, *ced-10*, and *unc-115* are likely to participate in the endogenous *unc-40* signaling pathway. These results suggest that *myr::unc-40* activates the endogenous *unc-40* signaling pathway, consistent with its acting as a constitutively active form of *unc-40*. *unc-34*, *ced-10*, and *unc-115* were found to signal downstream of *unc-40* in two parallel, partially redundant pathways. *unc-34/Enabled* also plays a partially redundant role in the *sax-3/Robo* pathway (Bashaw et al., 2000; Yu et al., 2002). The activation of parallel signaling modules with some functional overlap or redundancy may be a general feature of axon guidance signaling. It is worth noting that this apparent genetic redundancy could result from disrupting cell biological processes that are actually distinct. The activation of multiple pathways for cytoskeletal remodeling by guidance receptors may contribute to accurate guidance through various physical environments.

MYR::UNC-40 is capable of inducing axon outgrowth, misguidance, branching, and cell body deformation. All of these phenotypes can be suppressed by *unc-34*, *ced-10*, and *unc-115* or by deletions in the P1 and P2 motifs. These results suggest that distinct effects on cell morphology can be induced by the same signaling pathways, consistent with the observation that Netrin can signal through DCC to regulate cell migration, axon outgrowth, axon attraction, and axon repulsion.

MYR::UNC-40 activity generates new outgrowths even in the adult stage, well past the normal period of neuronal development. It thus seems likely that downstream effectors of UNC-40 persist and remain functional into adulthood. Indeed, reporter gene fusions to *unc-115* and *ced-10* are expressed throughout the life of *C. elegans* (Lundquist et al., 1998, 2001). One possibility is that these genes function later in development to increase the size of the neuron as the size of the animal increases.

Two of our findings are seemingly different from previously observed results for the UNC-40 homolog, DCC, in *Xenopus* spinal cord neurons: expression of a MYR::DCC construct does not by itself produce a gain-of-function phenotype in *Xenopus* neurons, and the P3 motif in the DCC cytoplasmic domain is required for multimerization of the cytoplasmic domain and for DCC function in axon attraction (Stein et al., 2001). Potential explanations for these differences may lie in species differences or a likely difference in expression level: in the experiments reported here, MYR::UNC-40 expression was driven from the strong *mec-7* promoter, whereas in *Xenopus* expression was driven by injection of mRNA at the 2-cell stage, which might result in significant degradation and dilution by cell division. It is possible that high-level expression may result in multimerization of MYR::UNC-40 without need for the P3 homomultimerization domain, and may trigger a gain-of-function phenotype.

UNC-34/Enabled Promotes Axon Attraction by the UNC-40 Pathway

Enabled was initially identified as a dosage-sensitive suppressor of Abl tyrosine kinase mutations in *Drosophila* (Gertler et al., 1995). Enabled and its family members UNC-34, Mena, VASP, and EVL share a conserved domain structure that includes an N-terminal EVH1 domain and a C-terminal EVH2 domain (Gertler et al., 1996). The EVH1 domain binds to proteins containing a FPPPP consensus sequence, found in actin-associated molecules such as zyxin and vinculin (Niebuhr et al., 1997), whereas the EVH2 domain has been implicated in oligomerization as well as G and F actin binding (Bachmann et al., 1999).

Enabled proteins can nucleate actin polymerization *in vitro* (Huttelmaier et al., 1999; Lambrechts et al., 2000). *In vivo*, Ena proteins are important for a number of actin-based cellular processes including axon guidance, platelet shape change, and Jurkat T cell polarization (Bear et al., 2000). Ena proteins were initially thought to promote cellular outgrowth, since VASP enhances the actin-based motility of the intracellular pathogen *Listeria monocytogenes*, and overexpression of Mena in fibroblasts produces actin-based outgrowths (Gertler et al., 1996; Laurent et al., 1999). However, this view was reversed when enrichment of Mena at the leading edge of fibroblasts was found to decrease motility, while depletion of Mena from the leading edge enhanced motility (Bear et al., 2000). These observations led to the idea that Ena proteins negatively affect outgrowth. This idea was reinforced when in *Drosophila* and *C. elegans*, UNC-34/Enabled was found to interact physically and genetically with the SAX-3/Robo guidance receptor to

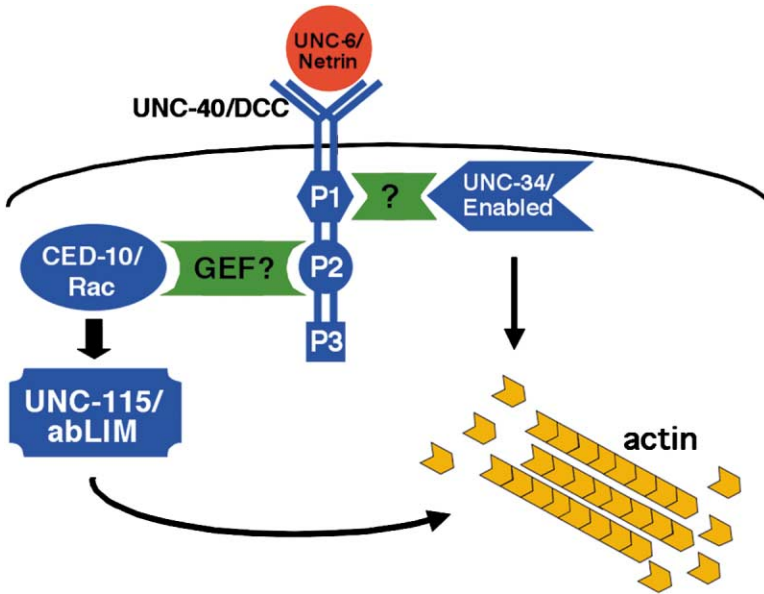


Figure 7. Model for UNC-40 Signaling

Shown here is a model for UNC-40 signaling in which UNC-6 induces UNC-40 receptor dimerization, activating it in a manner analogous to receptor tyrosine kinases (Stein et al., 2001). The P1 domain may recruit UNC-34 to promote actin rearrangements, though this interaction would likely be indirect, since P1 lacks sequences known to bind Enabled family members. The P2 domain may recruit CED-10 and UNC-115 to promote actin rearrangements. This interaction is also likely to be indirect, and we postulate that it might occur through a specific GEF. We speculate that UNC-115 may act downstream of CED-10 due to its ability to directly bind actin, the ultimate target of CED-10 activity.

mediate axon repulsion (Bashaw et al., 2000; Yu et al., 2002). Furthermore, *unc-34* mutants suppress the axon repulsion induced by ectopic expression of *unc-5*, suggesting a role for UNC-34 in mediating repulsion from UNC-6/Netrin (Colavita and Culotti, 1998). These results created a paradox between the observed role for Enabled in promoting actin-based activities generally associated with stimulation of outgrowth in vitro and its clear roles in axon repulsion and inhibition of cell motility in vivo.

A recent paper provided a potential resolution for this paradox by examining the mechanism by which Mena inhibits fibroblast motility (Bear et al., 2002). Mena enrichment at the leading edge was actually found to enhance the dynamics of lamellipodial protrusion; the paradoxical decreased net motility resulted from the fact that these additional protrusions were not stabilized. These observations led Gertler and colleagues to propose that Ena proteins function to stimulate the dynamics of protrusions at the leading edge. Whether the presence of additional protrusions promotes or inhibits cell migration or axon outgrowth may depend on whether the protrusions are stabilized or destabilized. It is thus possible that Mena-induced fibroblast protrusions were not stabilized because the actin filaments within them were isolated and unstable. We propose that actin filament bundling, observed in the filopodia of axonal growth cones (Gordon-Weeks, 1987), could provide a cellular context in which Mena-induced protrusions are stabilized. Thus, this new view of the mechanism of Enabled protein function is potentially consistent with a role for Enabled not just in axon repulsion and outgrowth inhibition, but also in axon attraction.

Our results provide direct evidence that UNC-34 can indeed function in an attractive axon guidance pathway: the endogenous UNC-6/UNC-40 pathway. These data establish the idea that Enabled proteins can promote outgrowth and attraction in vivo. In AVM we have a remarkable example of Enabled's duality, as this single cell uses UNC-34/Ena downstream of both UNC-40 and

SAX-3 to promote axon attraction and repulsion, respectively. The mild effect of *unc-34* mutations on AVM axon guidance suggests that UNC-34 is not essential for either UNC-40 or SAX-3 function. This finding is consistent with the above model wherein Ena proteins promote outgrowth dynamics but are not dedicated factors required for a specific outgrowth response.

UNC-34/Enabled Collaborates with CED-10/Rac and UNC-115/abLIM Downstream of Specific Motifs in the UNC-40 Receptor

Our results identified two distinct pathways that mediate UNC-40 signaling: UNC-34/Enabled acts in one and CED-10/Rac and UNC-115/abLIM act in another. Rac proteins have previously been shown to play roles in axon guidance (Luo et al., 1994; Lundquist et al., 2001; Hakeda-Suzuki et al., 2002; Ng et al., 2002), and Rac function is essential for repulsive axon guidance signaling by the Semaphorin receptor, Plexin (Jin and Strittmatter, 1997; Hu et al., 2001). The involvement of a Rac protein in Netrin attraction is consistent with the observation that Rac promotes lamellipodial extension, as growth cones have a flattened area with some similarities to lamellipodia (Aletta and Greene, 1988). Indeed, recent reports demonstrate that Netrin stimulation can activate Rac in vitro (Shekarabi and Kennedy, 2002). It is interesting that *ced-10* is important in the *unc-40* pathway, but both *mig-2*, which encodes another *C. elegans* Rac-like protein, and *unc-73*, which encodes a Guanine Nucleotide Exchange Factor (GEF), are not. In preliminary studies, a mutation in *rac-2(ok326)* (kindly provided by the *C. elegans* Knockout Consortium), the third Rac-like gene in *C. elegans* (Lundquist et al., 2001), appears to partially suppress the excess outgrowth of MYR::UNC-40 (data not shown), suggesting that UNC-40 may signal to several, but not all, Rac proteins.

The mechanisms by which Rac proteins cause changes in the actin cytoskeleton during axon guidance are largely unknown. Our results suggest that UNC-115 acts as an element in the Rac signaling pathway. The

UNC-115 protein contains three LIM domains and a villin headpiece domain (Lundquist et al., 1998). UNC-115 has been proposed to bind actin through its villin headpiece domain; thus, UNC-115 may provide a link between Rac and actin. A different LIM domain-containing protein, LIM-kinase, acts downstream of Rac through a PAK intermediate (Edwards et al., 1999). The role of UNC-115 in axon guidance is not specific to *C. elegans*; a dominant-negative form of a vertebrate UNC-115 homolog, abLIM, can cause axon defects in retinal ganglion cells (Erkman et al., 2000).

Directed turning toward an axonal attractant requires propagation of spatial information about the source of the attractant to downstream signaling events. Localized signaling might be achieved by localized nucleation of a signaling complex around the activated receptor. The activation of the UNC-34- and CED-10/UNC-115-dependent pathways by UNC-40 correspond to the specific conserved P1 and P2 motifs within the UNC-40 cytoplasmic domain (Figure 7). We suggest that these actin-regulatory activities may remain closely associated with the activated receptor. UNC-40 may thus function as a scaffold for assembling several independent activities that regulate the cytoskeleton.

Experimental Procedures

Strains

Nematodes were cultured by standard techniques (Brenner, 1974). All experiments were performed at 20°C. The following mutations were used: LGI, *unc-40(e271)*, *unc-73(e936)*, *zcls5[mec-4::gfp, lin-15(+)]*; LGII, *vab-1(e2)*, *kyls192[mec-7::myr::unc-40, str-1::gfp]*; LGIV, *ced-10(n1993, n3246)*, *unc-44(e362)*, *unc-5(e53)*, *rac-2(ok326)*, *zcls4[mec-4::gfp, lin-15(+)]*, *kyls179[unc-86::gfp, lin-15(+)]*; LGV, *unc-34(gm104, e951)*, *unc-60(e677)*, *unc-76(e911)*; and LGX, *unc-6(ev400)*, *sax-3(ky123)*, *slt-1(eh15)*, *unc-115(ky275, ky274)*, *mig-2(mu28)*. The *zcls4* and *zcls5* strains were kindly provided by Scott Clark. Some strains were provided by the *Caenorhabditis* Genetics Center.

Molecular Biology

Standard molecular biology techniques were used. A myristoylated UNC-40 was generated by PCR, placing a KpnI site upstream of the human *c-src* myristoylation sequence (MGSSKS) (Kamps et al., 1985) in-frame with the UNC-40 cytoplasmic domain (aa 1108–1415). Myristoylated GFP was generated by the same method. *mec-7* promoter fusions were generated by cloning MYR::UNC-40 or MYR::GFP into pPD96.41. The *unc-86* promoter was isolated by PCR (Baumeister et al., 1996) and cloned into pPD95.75 to generate *unc-86::gfp*. The *unc-86* promoter was also cloned into pPD49.26, and MYR::UNC-40 and MYR::GFP were cloned into the resulting construct to generate *unc-86::myr::unc-40* and *unc-86::myr::gfp*. *mec-7::unc-34*, *mec-7::unc-115*, and *mec-7::ced-10* were generated by cloning the *unc-34* cDNA (Yu et al., 2002), *unc-115* cDNA (Lundquist et al., 1998), and *ced-10* cDNA (Lundquist et al., 2001) into pPD96.41. To generate *unc-25::myr::unc-40* and *unc-25::unc-40*, the *unc-25* promoter was isolated by PCR (Jin et al., 1999) and cloned into pPD49.26, into which were then cloned the *unc-40* cDNA and *myr::unc-40*. pPD96.41, pPD95.75, and pPD49.26 were gifts of Andrew Fire (Carnegie Institute of Washington). All MYR::UNC-40 motif deletion clones were generated using the Quikchange Site-Directed Mutagenesis System (Stratagene, La Jolla, CA). Specific oligonucleotide primer sequences used are available upon request.

Transgenic Animals

Germline transformation of *C. elegans* was performed using standard techniques (Mello and Fire, 1995). *mec-7* and *unc-25* promoter fusions were injected at 50 ng/μl. *unc-86* promoter fusions were

injected at 1 ng/μl. *str-1::gfp* (50 ng/μl) or *odr-1::rfp* (50 ng/μl) were used as coinjection markers.

For the *unc-34* and *unc-115* cell autonomy experiments, *mec-7::unc-34* or *mec-7::unc-115* were injected with *odr-1::rfp*, while *mec-7::myr::unc-40* was separately injected with *str-1::gfp*. The resulting lines were then crossed to each other to generate animals bearing two independently segregating arrays, each containing a different marker. For each clone injected, at least two independently isolated lines were analyzed. The data shown are from one representative line for each experiment.

Microscopy

Animals were mounted on 2% agarose pads in M9 buffer containing 5 mM sodium azide and examined by fluorescence microscopy. Images were captured using a Zeiss AxioCam.

Acknowledgments

We thank Megan Dell and Gian Garriga for sharing results prior to publication; Scott Clark and the *Caenorhabditis* Genetics Center for nematode strains; Andrew Fire for vectors; Hai Nguyen and Joe Hill for technical support; and Carrie Adler, Andy Chang, Jesse Gray, Maria Gallegos, Joe Hao, Amanda Kahn, Steve McCarroll, Coleen Murphy, Kang Shen, Miri VanHoven, and Jen Zallen for helpful discussions and comments on the manuscript. Z.G. was a Howard Hughes Medical Institute predoctoral fellow; T.W.Y. was a MINDS predoctoral fellow and a UCSF MSTP student; and M.T.-L. and C.I.B. are investigators with the Howard Hughes Medical Institute. This work was funded by the Howard Hughes Medical Institute.

Received: July 2, 2002

Revised: November 5, 2002

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