The UNC-6/Netrin receptors UNC-40/DCC and UNC-5 inhibit growth cone filopodial protrusion via UNC-73/Trio, Rac-like GTPases and UNC-33/CRMP

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ABSTRACT

UNC-6/Netrin is a conserved axon guidance cue that can mediate both attraction and repulsion. We previously discovered that attractive UNC-40/DCC receptor signaling stimulates growth cone filopodial protrusion and that repulsive UNC-40–UNC-5 heterodimers inhibit filopodial protrusion in C. elegans. Here, we identify cytoplasmic signaling molecules required for UNC-6-mediated inhibition of filopodial protrusion involved in axon repulsion. We show that the Rac-like GTPases CED-10 and MIG-2, the Rac GTP exchange factor UNC-73/Trio, UNC-44/Ankyrin and UNC-33/CRMP act in inhibitory UNC-6 signaling. These molecules were required for the normal limitation of filopodial protrusion in developing growth cones and for inhibition of growth cone filopodial protrusion caused by activated MYR::UNC-40 and MYR::UNC-5 receptor signaling. Epistasis studies using activated CED-10 and MIG-2 indicated that UNC-44 and UNC-33 act downstream of the Rac-like GTPases in filopodial inhibition. UNC-73, UNC-33 and UNC-44 did not affect the accumulation of full-length UNC-5::GFP and UNC-40::GFP in growth cones, consistent with a model in which UNC-73, UNC-33 and UNC-44 influence cytoskeletal function during growth cone filopodial inhibition.

KEY WORDS: UNC-40/DCC, UNC-5, UNC-6, Axon repulsion, Filopodia, Growth cone, Caenorhabditis elegans

INTRODUCTION

Extracellular guidance cues are detected by receptors on the growth cone and guide growth cone migration. The guidance cue UNC-6/Netrin and its receptors UNC-5 and UNC-40/DCC control both attraction and repulsion in the dorsal-ventral axis (Chan et al., 1996; Leonardo et al., 1997; Hong et al., 1999; Montell, 1999; Shekarabi and Kennedy, 2002; Moore et al., 2007). UNC-40/DCC homodimers mediate attraction to Netrin, and UNC-5–UNC-40 heterodimers mediate repulsion from Netrin (Hong et al., 1999; MacNeil et al., 2009). In C. elegans, UNC-6, UNC-40 and UNC-5 mediate the dorsal-ventral circumferential migrations of growth cones and their axons (Hedgecock et al., 1990; Ishii et al., 1992; Leung-Hagesteijn, 1992; Chan et al., 1996). The VD motor neurons extend axons dorsally in a circumferential manner (Fig. 1A) and are repelled from a ventral source of UNC-6 (Hedgecock et al., 1990; Norris and Lundquist, 2011). As growth cones migrate, they extend dynamic lamellipodial and filopodial protrusions in the direction of migration. The repelled VD growth cones display dorsally directed dynamic lamellipodial and filopodial protrusions, with fewer protrusions directed ventrally (Fig. 1B) (Knobel et al., 1999; Norris and Lundquist, 2011). The roles of UNC-6/Netrin and its receptors in attractive and repulsive axon guidance are well documented. However, less is known about cell biological mechanisms of axon guidance and the regulation of growth cone protrusion by axon guidance signaling pathways such as UNC-6/Netrin.

Previously, we discovered a link between axon guidance and the regulation of growth cone protrusion by UNC-6, UNC-40 and UNC-5 (Norris and Lundquist, 2011). Genes involved in the attraction to UNC-6 were required for growth cone protrusion, including filopodia, and those involved in repulsion were required to inhibit growth cone filopodial protrusion (Norris and Lundquist, 2011). VD growth cones, repelled from UNC-6, are highly dynamic and display dorsally directed filopodial protrusions with an average maximal length of 1 µm and average duration of 5 min (Norris and Lundquist, 2011) (Fig. 1A,B; supplementary material Movie 1). Loss of UNC-6 and UNC-5 resulted in VD growth cones that were larger and more protrusive (Fig. 1C; supplementary material Movie 2), and activation of UNC-5 and UNC-40 using a myristoylated version of the UNC-40 cytoplasmic domain (Gitai et al., 2003) resulted in small growth cones with very little filopodial protrusion (Norris and Lundquist 2011) (Fig. 1D,E; supplementary material Movie 3). UNC-40 was required for the excess protrusion in unc-5 mutants, indicating that, in repelled VD growth cones, UNC-6 and UNC-40 control both pro- and anti-protrusive activity. Furthermore, UNC-6 and UNC-5 were also required to bias protrusion asymmetrically to the dorsal side of the growth cone (i.e. in unc-5 mutants protrusions were observed both dorsally and ventrally as opposed to mainly dorsally in wild type) (Fig. 1B,C) (Norris and Lundquist, 2011). These data suggest a mechanism of axon repulsion by a balance of UNC-6-mediated pro- and anti-protrusive forces in the growth cone, with pro-protrusive forces (UNC-40 homodimers) predominating dorsally, distant from the UNC-6 source, and anti-protrusive forces (UNC-5–UNC-40 heterodimers) predominating ventrally, adjacent to the UNC-6 source.

Signaling pathways required for UNC-40/DCC-mediated attractive axon guidance have been extensively described (reviewed by Lai Wing Sun et al., 2011). In C. elegans, these pathways drive neuronal lamellipodial and filopodial protrusion. For example, the Rac-like GTPases CED-10 and MIG-2, CDC-42, UNC-34/Enabled, the Arp2/3 complex, the Rac-specific guanine nucleotide exchange factor (GEF) TIAM-1, and the actin-interacting protein UNC-115/abl/LIM stimulate neuronal protrusion and mediate attractive axon guidance in...
response to UNC-40 (Gitai et al., 2003; Struckhoff and Lundquist, 2003; Shakir et al., 2008; Norris et al., 2009; Demarco et al., 2012; Alan et al., 2013). The Arp2/3 complex, UNC-34 and UNC-115 are required for growth cone filopodia formation (Norris et al., 2009). However, the roles of these molecules differ in different growth cones. For example, in the longitudinally migrating PLM touch sensory growth cones, VAB-1/EphR signaling inhibits growth cone filopodia and outgrowth by activating Arp2/3 and inhibiting UNC-34 (Mohamed et al., 2012).

Less is known about mechanisms of UNC-6/Netrin-based repulsion, although Src and Fak kinases, the PAK-like molecule MAX-2, the PH/MyTH4/FERM adaptor protein MAX-1, and the SHP2 tyrosine phosphatase are important (Tong et al., 2001; Huang et al., 2002; Killeen et al., 2002; Li et al., 2006; Lucanic et al., 2006). In this work we use the activated MYR::UNC-40-encoding transgene expressed in repelled VD neurons to decipher mechanisms of growth cone filopodial inhibition by UNC-6 receptor signaling in repulsive axon guidance.

Rac GTPases and Trio GEFs have central roles in axon guidance (Steven et al., 1998; Bateman et al., 2000; Blangy et al., 2000; Lundquist et al., 2001; Kishore and Sundaram, 2002; Lundquist, 2006). UNC-73/Trio acts as a GEF for Rac GTPases and is required for proper neuronal migration and axon guidance, including the VD commissural axons. In Drosophila and vertebrates, Trio interacts with the Netrin receptor DCC and activates Rac in response to Netrin (Forsthoefel et al., 2005; Briancon-Marjollet et al., 2008; DeGeer et al., 2013). In these cases, Trio apparently acts in attractive axon guidance mediated by Netrin, suggesting that it might stimulate protrusion. However, in C. elegans, unc-73 is not required for UNC-40-stimulated neuronal protrusion (Gitai et al., 2003), which requires the Rac GEF TIAM-1 (Demarco et al., 2012). Here, we show that the Rac-like GTPases CED-10 and MIG-2 and UNC-73 inhibit growth cone filopodial protrusion. Our results suggest that CED-10 and MIG-2 are involved in both pro- and anti-protrusive functions in the growth cone and that their roles in each are controlled by distinct GEFs: UNC-73 in inhibition (this work) and TIAM-1 in stimulation (Demarco et al., 2012) of protrusion.}

unc-33 encodes a protein that is similar to the Collapsin response mediator protein (CRMP) (Li et al., 1992), which mediates growth cone collapse in response to the Semaphorin/Collapsin family of repulsive axon guidance cues (Goshima et al., 1995; Takahashi et al., 1999; Alabed et al., 2007, 2010). unc-33 is required for axon guidance and for regulating axonal versus dendritic sorting of trafficked molecules (Li et al., 1992; Maniar et al., 2012). unc-44 encodes an Ankyrin-like molecule that is involved in the axonal localization of UNC-33 (Otsuka et al., 1995; Maniar et al., 2012). Here, we show that UNC-33/CRMP and UNC-44/Ankyrin are required by MYR::UNC-40 to limit growth cone filopodial protrusion, and that they act downstream of Rac GTPases in this process.
Previous studies found that UNC-73/Trio and MIG-2/RhoG affected the accumulation and distribution of the SAX-3/Robo and UNC-40 receptors in neurons (Levy-Strumpf and Culotti, 2007; Watari-Goshima et al., 2007), and that UNC-33 and UNC-44 affect axon-dendrite trafficking (Maniar et al., 2012). We show that full-length UNC-40::GFP and UNC-5::GFP localization to growth cones is unaffected by unc-73, unc-44 and unc-33, consistent with these molecules acting downstream of UNC-6 receptor signaling.

RESULTS
The Rac GEF UNC-73 is required to inhibit VD growth cone filopodial protrusion
The unc-73(rh40) mutation eliminates the Rac GEF activity of UNC-73 but does not affect other activities (e.g. Rho GEF activity) (Steven et al., 1998; Lundquist et al., 2001; Demarco et al., 2012). We found that in unc-73(rh40) mutants VD growth cones had significant increases in filopodial protrusiveness, exhibiting on average longer filopodia (e.g. 0.96 µm in wild type compared with 1.44 µm in unc-73(rh40); P<0.01) and a longer duration of the filopodia once formed (4.9 min in wild type compared with 8.2 min in unc-73(rh40); P<0.01) (Fig. 2A,B; supplementary material Movie 4). Indeed, some filopodia endured throughout the length of the experiment (greater than 20 min). In some cases, the exceptionally long filopodia consolidated into neurites, resulting in a terminated axon with extensive branching. Indeed, unc-73 mutant adults exhibit extensive branching of the PDE neurons (Struckhoff and Lundquist, 2003) and of the VD and DD axons (Fig. 3), suggesting that failure to retract growth cone filopodia can result in the formation of ectopic neurites and axon branching. unc-5 mutants also displayed persistent growth cone filopodial extensions as well as axon branching (Norris and Lundquist, 2011).

The Rac-like GTPases CED-10 and MIG-2 inhibit growth cone filopodial protrusion
CED-10 is similar to the Rho GTPase Rac1 (Reddien and Horvitz, 2000) and MIG-2 is an Mtl GTPase (Zipkin et al., 1997), which is an invertebrate-specific Rho GTPase family with similarity to both Rac and Cdc42. MIG-2 might be the functional equivalent of the vertebrate GTPase RhoG (deBakker et al., 2004). We refer to MIG-2 and CED-10 collectively as the C. elegans Rac GTPases.

Fig. 2. Mutations in Rac GTPases, UNC-73, UNC-44 and UNC-33 cause increased growth cone filopodial protrusion. (A) The average VD growth cone filopodial duration in different mutant backgrounds in early L2 juIs76 animals. More than 50 filopodia per genotype were scored. (B) Maximal filopodial length in different mutants as described in A. (C) A time-lapse series of a wild-type VD growth cone in early L2. Numbers indicate minutes after imaging began. (D) An unc-44(e362) mutant growth cone showing increased protrusion in the form of longer and more persistent filopodia. Dorsal is up, and anterior is left. Scale bar: 5 µm. (E) Average filopodial length in different genotypes. At least 15 filopodia were scored from at least 15 different growth cones. M+ indicates that the animals had wild-type maternal ced-10(+). Error bars represent s.e.m. Two-sided t-tests with unequal variance were used to determine statistical significance.
double mutant does not represent a complete loss of ced-10 and mig-2 function, which might have a more severe filopodial phenotype than mig-2(mu28); ced-10(n1993).

**UNC-44 and UNC-33 inhibit VD growth cone filopodial protrusion**

unc-33 and unc-44 mutants display branched and prematurely terminated axons, including the VDs (Fig. 3), indicative of a role in filopodial inhibition. VD growth cone time-lapse analysis indicated that unc-33 and unc-44 mutant growth cones display excessive filopodial protrusion similar to unc-73, mig-2; ced-10, and unc-5 (e.g. increased filopodial length and duration) (Fig. 2; supplementary material Movies 6 and 7). unc-33 and unc-44 also displayed persistent filopodia that resolved into stable neurite-like structures (supplementary material Movies 6 and 7), resulting in axon branching (Fig. 3).

**UNC-73, UNC-33 and UNC-44 are required for activated MYR::UNC-40 inhibition of VD growth cone protrusion**

We next determined whether UNC-73, UNC-33 and UNC-44 are required for filopodial inhibition driven by activated MYR::UNC-40 (see Fig. 1). VD growth cones in loss-of-function mutants of unc-73, unc-33 and unc-44 harboring myr::unc-40 resembled the loss-of-function mutants alone (i.e. increased growth cone filopodial protrusiveness as indicated by length and duration) (Fig. 4; supplementary material Movie 8). Additional alleles unc-33(e1197) and unc-44(e1193) showed the same effect (data not shown). Thus, UNC-73, UNC-33 and UNC-44 are required for inhibition of growth cone protrusion mediated by MYR::UNC-40. These results are in line with previous studies that identified unc-44 mutations in a screen for suppressors of UNC-5 axon repulsion activity (Colavita and Culotti, 1998).

**ced-10(n1993)** weakly but significantly suppressed the filopodial duration of myr::unc-40, and mig-2(mu28) weakly but significantly suppressed maximal length (Fig. 4A,B). This weak suppression is likely to be due to the demonstrated redundancy of MIG-2 and CED-10 in filopodial inhibition.

While unc-73, unc-33 and unc-44 were epistatic to myr::unc-40, the duration of filopodia in unc-33(e204); unc-40 and unc-73(rh40); myr::unc-40 resembles the loss-of-function mutants alone, and the maximal length of unc-33; myr::unc-40 was significantly reduced compared with unc-33(e204) alone. This suggests that myr::unc-40 can slightly suppress unc-33 and unc-73, indicating that it might engage effectors in parallel to unc-33 and unc-73 to inhibit protrusion.

**CED-10 and MIG-2 require UNC-33 and UNC-44 to limit growth cone filopodial protrusion**

Our results indicate that the Rac GTPases CED-10 and MIG-2 are required to limit VD growth cone filopodial protrusion. We generated activated ced-10(G12V) and mig-2(G16V) expressed in the VD neurons by the unc-25 promoter (see Materials and Methods). In the PDE axons that are attracted to UNC-6, activated CED-10(G12V) and MIG-2(G16V) result in excess protrusion (Struckhoff and Lundquist, 2003). However, in the repelled VD axons, CED-10(G12V) and MIG-2(G16V) resulted in growth cones that displayed reduced filopodial protrusion compared with wild type (Fig. 5; supplementary material Movie 9), with a reduction in average filopodia duration [e.g. 4.9 min in wild type compared with 3.6 min in mig-2(G16V); P<0.01] and length [0.96 µm in wild type compared with 0.68 µm in mig-2(G16V); P<0.01]. This phenotype is the opposite of that observed in unc-73 and mig-2; ced-10 loss-of-function mutants, and...
resembled inhibition of growth cone protrusion caused by activated myr::unc-40 (Norris and Lundquist, 2011). CED-10 and MIG-2 have pro-protrusive roles in other neurons with axons attracted to UNC-6 (Struckhoff and Lundquist, 2003; Demarco et al., 2012). In the VD growth cones repelled from UNC-6, MIG-2 and CED-10 have an anti-protrusive role.

When ced-10(G12V) and mig-2(G16V) were introduced into an unc-73(rh40) loss-of-function background, the growth cones resembled those upon activated Rac GTPase expression alone, including significant reduction in filopodia duration and length (Fig. 5; supplementary material Movie 10). That activated CED-10 and MIG-2 were epistatic to unc-73 could mean that CED-10 and MIG-2 act downstream of UNC-73 in the same pathway, or that they act independently of UNC-73 in a parallel pathway, consistent with the double-mutant analysis in Fig. 2E.

By contrast, double mutants of unc-33 and unc-44 with ced-10(G12V) and mig-2(G16V) resembled unc-33 and unc-44 mutants (unc-33 and unc-44 were epistatic to activated Rac GTPases), with excessive growth cone filopodial protrusion as evidenced by increased filopodial length and duration (Fig. 5). Additional alleles unc-33(e1197) and unc-44(e1193) showed the same effect (data not shown). These data indicate that UNC-33 and UNC-44 are required for Rac GTPases to inhibit growth cone protrusion and suggest that they act downstream of Rac GTPases in the process.

Mutants with increased filopodial protrusion exhibit increased axon branches

Our results suggest a correlation between increased growth cone filopodial protrusion and ectopic axon branches in the adult animal (Fig. 3). The stable and long filopodia in mutant growth cones might be the precursors to these ectopic axon branches. To test this idea, we studied the effects of activated Rac GTPases on ectopic axon branching. Activated Rac GTPases suppressed excess growth cone filopodial protrusion in unc-73 but not unc-33 and unc-44 (Fig. 5). In adults, unc-73(rh40); mig-2(G16V) and unc-73(rh40); ced-10(G12V) mutants displayed reduced ectopic VD axon branching compared with unc-73(rh40) alone (Fig. 6), similar to the effect on growth cone filopodial protrusion. This effect was specific to axon branching defects, as overall pathfinding defects (axons that wander, or that stop short of the dorsal nerve cord) remained unchanged. By contrast, mig-2(G16V) or ced-10(G12V) did not reduce ectopic axon branches of unc-33 and unc-44 mutants (Fig. 6), which is also similar to their effects on growth cone filopodial protrusion. These data support the idea that axon branches in adult axons can result from failure to inhibit the extent of filopodia protrusion in the developing growth cone.

MYR::UNC-5 inhibits growth cone protrusion in a manner dependent on UNC-73 and UNC-33

Functional UNC-5 was required for the anti-protrusive effects of MYR::UNC-40, suggesting that an UNC-5–MYR::UNC-40 complex is involved in inhibiting growth cone protrusion (Norris and Lundquist, 2011). To test the effects of activated UNC-5 signaling, we constructed a transgene with a myristoylated version of the UNC-5 cytoplasmic domain (MYR::UNC-5) expressed in the VD neurons using the unc-25 promoter (Fig. 1E). Expression of MYR::UNC-5 caused reduced VD growth cone filopodial

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**Fig. 4.** UNC-73, UNC-33 and UNC-44 are required for myr::unc-40 filopodial inhibition. (A,B) Quantification of filopodia dynamics in VD growth cones as described in Fig. 2A. Error bars represent s.e.m. Two-sided t-tests with unequal variance were used to determine statistical significance. (C) Time-lapse series of an unc-73(rh40); myr::unc-40 growth cone, taken at 2 min per frame. The arrow points to a long, stable filopodium of a type that was never observed in myr::unc-40 alone but often observed in unc-73(rh40). Dorsal is up, and anterior is left. Scale bar: 5 µm.
protrusion that resembled the effect of MYR::UNC-40, including decreased filopodial length and duration (Fig. 7A-C; supplementary material Movie 11). This effect was not dependent upon functional UNC-6, but was dependent upon functional UNC-40, suggesting that a MYR::UNC-5–UNC-40 heterodimeric complex was involved (Fig. 7A,B). Surprisingly, functional endogenous UNC-5 was also required (Fig. 7A,B). Functional endogenous UNC-40 was not required for the effect of MYR::UNC-40 (Norris and Lundquist, 2011). This result suggests the involvement of UNC-5–UNC-40 heterodimers as well as UNC-5 homodimers that require at least one full-length UNC-5 molecule. *unc-73(rh40); mig-2(G16V) growth cone resembled that of mig-2(G16V) alone and did not exhibit the excess protrusion seen in *unc-73(rh40) mutants. Scale bar: 5 µm.

Fig. 5. UNC-33 and UNC-44, but not UNC-73, are required for constitutively active MIG-2 and CED-10 filopodial inhibition. (A,B) Quantification of filopodia dynamics in VD growth cones as described in Fig. 2A. Error bars represent s.e.m. Two-sided t-tests with unequal variance were used to determine statistical significance. NS, not significant. (C,D) Time-lapse series of live growth cones (arrowheads) in early L2 animals, taken at 2 min per frame. Dorsal is up, and anterior is left. The *unc-73(rh40); mig-2(G16V) growth cone resembled that of mig-2(G16V) alone and did not exhibit the excess protrusion seen in *unc-73(rh40) mutants. Scale bar: 5 µm.

Fig. 6. Excessive filopodial protrusion correlates with axon branching defects in adult animals. Quantification of VD/DD axon pathfinding defects and ectopic axon branches as described in Fig. 3. Activated ced-10(G12V) and mig-2(G16V), which suppress excessive growth cone filopodial protrusion of *unc-73(rh40), also suppress ectopic axon branching but not other guidance defects. ced-10(G12V) and mig-2(G16V) did not suppress excessive growth cone filopodial protrusion of *unc-33 and *unc-44, nor did they suppress ectopic axon branching of these mutants. M+ indicates that the animals had wild-type maternal ced-10(+*) contribution. Error bars represent 2× standard error of proportion. Fisher’s exact tests were used to determine statistical significance.

that MYR::UNC-5 might engage effectors in parallel to UNC-33 to inhibit protrusion.

UNC-40::GFP and UNC-5::GFP accumulation in growth cones is unaffected by *unc-73, *unc-33 and *unc-44

In wild type, MYR::UNC-40::GFP accumulated uniformly at the edges of the VD growth cones. Although MYR::UNC-40 is likely to be trafficked to the growth cone by a mechanism distinct from endogenous UNC-5 and UNC-40, its retention or stability in the growth cone could depend upon endogenous UNC-5. MYR::UNC-40::GFP displayed a grossly similar growth cone accumulation in *unc-73, *unc-33 and *unc-44 mutants (Fig. 8A,B) despite increased growth cone protrusion. Indeed, levels of MYR::UNC-40::GFP were often increased in these mutants (Fig. 8B), possibly owing to their increased growth cone size and protrusions.

We constructed a full-length unc-40::gfp transgene (Levy-Strumpf and Culotti, 2007; Sundararajan and Lundquist, 2012) driven by the unc-25 promoter in the VD neurons. Full-length UNC-40::GFP accumulated in puncta that were located in the growth cone body and at the growth cone edges, as well as in the axon (Fig. 8A). A grossly similar distribution of UNC-5::GFP was observed in growth cones and axons in *unc-33(e1193), *unc-44(e1197) and
unc-73 mutants (Fig. 8). unc-33(e204) and unc-44(e362) alleles were also analyzed with similar results (data not shown).

Wild-type growth cones expressing full-length UNC-5::GFP were small and had significantly fewer filopodial protrusions compared with wild type (Fig. 8A,C), suggesting that full-length UNC-5 transgenic expression can inhibit filopodial protrusion. In unc-33, unc-44 and unc-73 mutants, the growth cones were still small and displayed significantly reduced numbers of filopodial protrusions that were the same as those associated with full-length UNC-5::GFP alone (Fig. 8C). This is contrast to MYR::UNC-5 and MYR::UNC-40, which were suppressed by unc-73, unc-44 and unc-33. Possibly, transgenic expression of full-length UNC-5::GFP has a stronger gain-of-function effect than MYR::UNC-5 that cannot be overcome by loss of UNC-73, UNC-33 and UNC-44. This also suggests the possibility of redundant downstream mechanisms in growth cone inhibition by UNC-5, i.e. full-length UNC-5::GFP might engage multiple downstream mechanisms more robustly than MYR::UNC-5, and loss of one pathway does suppress this effect.

These data indicate that localization of functional UNC-40::GFP and UNC-5::GFP to growth cones is grossly normal in unc-73, unc-33 and unc-44 mutants, suggesting that these molecules are likely to act downstream of UNC-40 and UNC-5. By contrast, a previous study in C. elegans sensory neurons described evidence that UNC-73 can also act upstream to alter trafficking of UNC-40::GFP (Levy-Strumpf and Culotti, 2007). Although UNC-73 could potentially exert a similar effect in VD growth cones that our assays did not detect, the results reported here are consistent with the idea that UNC-73, UNC-33 and UNC-44 do not affect UNC-5 and UNC-40 accumulation in VD growth cones but rather act downstream to mediate changes in growth cone protrusion.

**DISCUSSION**

Previous results suggested that UNC-6/Netrin and the receptor UNC-40/DCC can both stimulate and inhibit growth cone protrusion in the same repelled growth cone, which might result in directed protrusion and migration away from UNC-6/Netrin (Norris and Lundquist, 2011). Our results here show that the Rac GEF UNC-73/Trio, the Rac-like GTPases CED-10/Rac and MIG-2/RhoG, and the cytoskeleton-associated molecules UNC-33/CRMP and UNC-44/Ankyrin mediate inhibition of growth cone filopodial protrusion via UNC-5 and UNC-40 Netrin receptors in repulsive axon guidance (Fig. 9). UNC-33 and UNC-44 were required for...
filopodial inhibition by activated MIG-2 and CED-10, suggesting that they act in a common pathway. UNC-73 is also likely to act in this pathway, as it is a GEF specific for MIG-2 and CED-10 (Wu et al., 2002), and the Rac GEF activity of UNC-73 is abolished by unc-73(rh40) (Steven et al., 1998). However, UNC-73 might not be the only GEF that regulates MIG-2 and CED-10 in this process, as unc-73(rh40) double mutants with mig-2 and ced-10 display increased filopodial protrusion compared with both unc-73(rh40) and the ced-10(n1993); mig-2(mu28) double mutant. Activated ced-10 and mig-2 were epistatic to unc-73 loss of function (i.e. growth cones in the double mutants displayed inhibited protrusion similar to activated mig-2 and ced-10 alone), consistent with the known role of UNC-73/Trio as an upstream Rac regulator.

**UNC-33 is required to inhibit growth cone filopodial protrusion mediated by UNC-6 receptor and Rac signaling**

Collapsin response mediator proteins (CRMPs) are required for semaphorin-3A-mediated growth cone collapse through a receptor complex that includes Plexin-A and Neuropilin-1 (Goshima et al., 1995; Takahashi et al., 1999). Here, we demonstrate that UNC-33, a *C. elegans* CRMP-like molecule, is required for the inhibition of growth cone filopodial protrusion caused by the UNC-6/Netrin receptors UNC-40 and UNC-5. In cultured mammalian neurons, CRMP4 (DPYSL3) knockdown results in longer filopodial protrusions and more axon branches on myelin-derived substrates (Alabed et al., 2007), consistent with our results with UNC-33 in *C. elegans*. Rather than acting as a specialized effector of semaphorin signaling, CRMPs might provide a more general mechanism to inhibit growth cone protrusion in response to multiple signals including semaphorins and netrins. Although both growth cone collapse and filopodial inhibition result in reduced growth cone protrusion, it is unclear whether the mechanisms used by CRMPs in these processes are distinct.

unc-33 and unc-44 mutations were epistatic to activated MIG-2/RhoG and CED-10/Rac, as the double-mutant growth cones displayed excess filopodial protrusions similar to unc-33 and unc-44 mutants alone. This suggests that UNC-33/CRMP and UNC-44/Ankyrin are required for the effects of activated Rac GTPases and that they act downstream of them to mediate filopodial inhibition. CRMPs interact with both the actin and microtubule cytoskeletons. CRMP4 interacts with F-actin in vitro (Rosslenbroich et al., 2005), and CRMP1 colocalizes to the actin cytoskeleton of dorsal root ganglion neurons grown in culture (Higurashi et al., 2012). Furthermore, CRMP2 (DPYSL2) physically interacts with tubulin dimers and promotes microtubule assembly (Fukata et al., 2002). Therefore, UNC-33 might directly modulate the actin and/or microtubule cytoskeletons of growth cones in response to UNC-6 to inhibit protrusion.

**Rac signaling and UNC-33 are likely to act downstream of MYR::UNC-40 and MYR::UNC-5**

Previous studies suggest that UNC-73/Trio and MIG-2/RhoG act upstream of guidance receptors and affect their localization. Activated MIG-2 caused redistribution of UNC-40::GFP to submembrane structures in axons of the ALM touch neurons (Levy-Strumpf and Culotti, 2007), and UNC-73/Trio acts with the kinesin-like protein VAB-8L to cause increased cell surface localization of the Slit receptor SAX-3/Robo (Watari-Goshima et al., 2007). Furthermore, UNC-33 and UNC-44 affect axondendrite trafficking (Maniar et al., 2012). Growth cones were not...
Trio of guidance receptors (B). An unidentified GEF might act in parallel to UNC-73/Trio, suggesting that these molecules are involved in some aspect of UNC-5 signaling, possibly on the growth cone cytoskeleton.

In summary, these results show that UNC-73/Trio, the Rac-like GTPases MIG-2 and CED-10 are required to both stimulate and inhibit growth cone protrusion, and that distinct GEFs regulate their activities in each role: TIAM-1 to stimulate protrusion and UNC-73 to inhibit protrusion.

Conclusions

In summary, these results show that UNC-73/Trio, the Rac-like GTPases MIG-2 and CED-10, UNC-44/Ankyrin and UNC-33/CRMP inhibit growth cone filopodial protrusion and are required for inhibition of filopodial protrusion by UNC-6/Netrin receptor signaling. UNC-33 is required to inhibit protrusion by activated MIG-2 and CED-10, suggesting that these molecules act in a common pathway. UNC-73, UNC-33 and UNC-44 are not involved in the accumulation of UNC-40::GFP or UNC-5::GFP to growth cones, suggesting that they might mediate downstream effects of UNC-40 and UNC-5 signaling, possibly on the growth cone cytoskeleton.

MATERIALS AND METHODS

Genetic methods

Experiments were performed at 20°C using standard C. elegans techniques (Brenner, 1974). Mutations used were: X: mig-2(mu28); I: unc-73(h40); II: juls76 [Punc-25::gfp]; IV: unc-5(e53 and e152), unc-33(e204 and e1197), unc-44(e362, e1260 and e1193), ced-10(e1193). Chromosomal locations not determined: lqIs128 [Punc-25::myr::unc-40::gfp], lqIs242 [Punc-25::myr::unc-5::gfp], lqIs204 [Punc-25::ced-10(G12V)] and lqIs182 [Punc-25::myr::unc-40::gfp].
**Analysis of axon guidance defects**

VD growth cones were visualized with a *Punc-25::gfp* transgene, *jul(s)* (Jin et al., 1999), which is expressed in all GABAergic neurons, including the 13 VDs. VD axon defects scored include axon guidance (termination before reaching the dorsal nerve cord or wandering at an angle greater than 45° before reaching the dorsal nerve cord) and ectopic branching. Fisher’s exact test was used to determine statistical significance between proportions of defective axons.

**Growth cone time-lapse imaging**

VD growth cones were imaged as previously described (Norris et al., 2009). Briefly, animals harboring the indicated transgenes were selected 16 h post-hatching at 20°C and placed on a 2% agarose pad with a drop of 10 mM 3-isonitropropionic acid (3-IPPA; Sigma-Aldrich) in M9 (Weinkove et al., 2008), which was allowed to evaporate for 4 min before placing a coverslip over the sample. Growth cones were imaged with a Qimaging Retiga mGI camera on a Leica DMR microscope. Images were acquired at intervals of 120 s, with total duration of time-lapse ranging from 20 to 60 min.

Dynamic projections less than 0.5 µm in width emanating from the growth cone were scored as filopodia. Maximal filopodia length was measured using ImageJ software, and filopodial duration was determined by persistence of the protrusion through time-lapse images. All filopodia on multiple growth cones were analyzed, and at least seven growth cones of each genotype were included in the analysis (at least 25 filopodia). In Fig. 2E, the average length of filopodia was determined from images of growth cones (at least ten growth cones; at least 25 filopodia). The significance of differences was determined by a two-sided *t*-test with unequal variance.

**UNC-5::GFP, UNC-40::GFP and MYR::UNC-40::GFP growth cone analysis**

Images of VD growth cones with *MYR::UNC-40::GFP* and full-length UNC-40::GFP and UNC-5::GFP were taken as described above. Using ImageJ, the perimeters of the growth cones were traced, and the average pixel intensity in the defined growth cone area was reported. At least ten growth cones for each genotype were analyzed, except for *unc-73(rh40); unc-5::gfp*, which were subiviable and sterile. *unc-73(rh40); unc-5::gfp* growth cones were not quantified, but those observed showed no gross change in UNC-5::GFP growth cone localization.

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**Competing interests**

The authors declare no competing financial interests.

Author contributions

A.D.N. developed the concepts and approach, performed experiments and data analysis, and prepared and edited the manuscript prior to submission. L.S. performed experiments and data analysis, and edited the manuscript prior to submission. D.E.M. performed experiments and data analysis, and edited the manuscript prior to submission. J.Z. performed experiments and data analysis, and prepared and edited the manuscript prior to submission.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.110437/-/DC1

**References**


RESEARCH ARTICLE


