Flavin monooxygenases regulate *Caenorhabditis elegans* axon guidance and growth cone protrusion with UNC-6/Netrin signaling and Rac GTPases

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Abstract

The guidance cue UNC-6/Netrin regulates both attractive and repulsive axon guidance. Our previous work showed that in *C. elegans*, the attractive UNC-6/Netrin receptor UNC-40/DCC stimulates growth cone protrusion, and that the repulsive receptor, an UNC-5:UNC-40 heterodimer, inhibits growth cone protrusion. We have also shown that inhibition of growth cone protrusion downstream of the UNC-5:UNC-40 repulsive receptor involves Rac GTPases, the Rac GTP exchange factor UNC-73/Trio, and the cytoskeletal regulator UNC-33/CRMP, which mediates Semaphorin-induced growth cone collapse in other systems. The multidomain flavoprotein monooxygenase (FMO) MICAL (Molecule Interacting with CasL) also mediates growth cone collapse in response to Semaphorin by directly oxidizing F-actin, resulting in depolymerization. The *C. elegans* genome does not encode a multidomain MICAL-like molecule, but does encode five flavin monooxygenases (FMO-1, -2, -3, -4, and 5) and another molecule, EHBP-1, similar to the non-FMO portion of MICAL. Here we show that FMO-1, FMO-4, FMO-5, and EHBP-1 may play a role in UNC-6/Netrin directed repulsive guidance mediated through UNC-40 and UNC-5 receptors. Mutations in *fmo-1*, *fmo-4*, *fmo-5*, and *ehbp-1* showed VD/DD axon guidance and branching defects, and variably enhanced *unc-40* and *unc-5* VD/DD axon guidance defects. Developing growth cones in vivo of *fmo-1*, *fmo-4*, *fmo-5*, and *ehbp-1* mutants displayed excessive filopodial protrusion, and transgenic expression of FMO-5 inhibited growth cone protrusion. Mutations suppressed growth cone inhibition caused by activated UNC-40 and UNC-5 signaling, and activated Rac GTPase CED-10 and MIG-2, suggesting that these molecules are required downstream of UNC-6/Netrin receptors and Rac GTPases. From these studies we conclude that FMO-1, FMO-4, FMO-5, and EHBP-1 represent new players downstream of UNC-6/Netrin receptors and Rac GTPases that inhibit growth cone filopodial protrusion in repulsive axon guidance.
Author summary

Mechanisms that guide axons to their targets in the developing nervous system have been elucidated, but how these pathways affect behavior of the growth cone of the axon during outgrowth remains poorly understood. We previously showed that the guidance cue UNC-6/Netrin and its receptors UNC-40/DCC and UNC-5 inhibit lamellipodial and filopodial growth cone protrusion to mediate repulsion from UNC-6/Netrin in *C. elegans*. Here we report a new mechanism downstream of UNC-6/Netrin involving flavin monoxygenase redox enzymes (FMOs). We show that FMOs are normally required for axon guidance and to inhibit growth cone protrusion. Furthermore, we show that they are required for the anti-protrusive effects of activated UNC-40 and UNC-5 receptors, and that they can partially compensate for loss of molecules in the pathway, indicating that they act downstream of UNC-6/Netrin signaling. Based on the function of the FMO-containing MICAL molecules in *Drosophila* and vertebrates, we speculate that the FMOs might directly oxidize actin, leading to filament disassembly and collapse, and/or lead to the phosphorylation of UNC-33/CRMP, which we show also genetically interacts with the FMOs downstream of UNC-6/Netrin. In conclusion, this is the first evidence that FMOs might act downstream of UNC-6/Netrin signaling in growth cone protrusion and axon repulsion.

Introduction

The formation of neural circuits during development depends on the guidance of growing axons to their proper synaptic targets. This process relies on the growth cone, a dynamic actin based structure present at the tip of a growing axon. Growth cones contain a dynamic lamellipodial body ringed by filopodial protrusions, both important in guiding the axon to its target destination [1–4]. Guidance receptors present on the leading edge of the growth cone sense and respond to various extracellular guidance cues, which attract or repel axons enabling them to reach their proper target destination [5, 6].

The secreted laminin-like guidance molecule UNC-6/Netrin mediates both axon attraction and axon repulsion and defines a dorsal-ventral guidance mechanism conserved from invertebrates to vertebrates [7–9]. Attractive or repulsive responses to UNC-6/Netrin depend on the receptors expressed on the growth cone. Homodimers of the UNC-6/Netrin receptor UNC-40/DCC mediate attraction, and UNC-5:UNC-40 heterodimers or UNC-5 homodimers mediate repulsion [10–12].

In *C. elegans*, UNC-6/Netrin is secreted by the ventral cells and along with its receptors UNC-40 and UNC-5 is required for the dorsal ventral guidance of circumferential neurons and axons [8, 13, 14]. Previous studies of repelled VD growth cones in Netrin signaling mutants revealed a correlation between attractive axon guidance and stimulation of growth cone protrusion, and repulsive axon guidance and inhibition of growth cone protrusion [15]. For example, in *unc-5* mutants, growth cones were larger and more protrusive, and often displayed little or no directed movement. This is consistent with observation that increased growth cone size was associated with decreased neurite growth length [16]. Conversely, constitutive activation of UNC-5:UNC-40 signaling in repelled VD growth cones led to smaller growth cones with severely reduced filopodial protrusion [15, 17]. Thus, directed growth cone repulsion away from UNC-6/Netrin requires a balance of pro- and anti-protrusive activities of the receptors UNC-40 and UNC-40:UNC-5, respectively, in the same growth cone [15].
Genetic analysis has identified a cytoskeletal signaling pathway involved in stimulation of growth cone protrusion in response to the attractive UNC-40 signaling that includes CDC-42, the Rac-specific guanine nucleotide exchange factor TIAM-1, the Rac-like GTPases CED-10 and MIG-2, as well as the cytoskeletal regulators Arp2/3 and activators WAVE-1 and WASP-1, UNC-34/Enabled, and UNC-115/abLIM [18–23], consistent with findings in other systems [7]. Mechanisms downstream of UNC-5 in axon repulsion are less well described, but the PH/MyTH4/FERM molecule MAX-1 and the SRC-1 tyrosine kinase have been implicated [24, 25]. We delineated a new pathway downstream of UNC-5 required for its inhibitory effects on growth cone protrusion, involving the Rac GEF UNC-73/Trio, the Rac GTPases CED-10 and MIG-2, and the cytoskeletal-interacting molecule UNC-33/CRMP [17].

Collapsin response mediating proteins (CRMPs) were first identified as mediators of growth cone collapse in response to the Semaphorin family of guidance cues [26], and we have shown that UNC-33/CRMP inhibits growth cone protrusion in response to Netrin signaling [17]. This motivated us to consider other mediators of Semaphorin-induced growth cone collapse in Netrin signaling. In *Drosophila*, the large multidomain cytosolic protein MICAL (Molecule Interacting with CasL) is required for the repulsive motor axon guidance mediated by interaction of Semaphorin 1a and Plexin A [27, 28]. MICAL proteins are a class of flavoprotein monoxygenase enzymes that bind flavin adenine dinucleotide (FAD) and use the cofactor nicotinamide dinucleotide phosphate (NADPH) to facilitate oxidation-reduction (Redox) reactions [27]. MICAL regulates actin disassembly and growth cone collapse in response to semaphorin via direct redox interaction with F-actin [29, 30]. MICAL molecules from *Drosophila* to vertebrates have a conserved domain organization: an N-terminal flavin-adenine dinucleotide (FAD)-binding monoxygenase domain, followed by a calponin homology (CH) domain, a LIM domain, a proline-rich domain, and a coiled-coil ERM α-like motif [27, 31].

The *C. elegans* genome does not encode a MICAL-like molecule with the conserved domain organization described above. However, it does encode five flavin monoxygenase (*fmo*) genes similar to the Flavin monoxygenase domain of MICAL: *fmo-1, fmo-2, fmo-3, fmo-4* and *fmo-5* [32]. Like MICAL, the *C. elegans* FMO molecules contain an N-terminal FAD binding domain and a C-terminal NADP or NADPH binding domain [27, 32]. The *C. elegans* gene most similar to the non-FMO portion of MICAL is the Eps-15 homology domain binding protein EHBP-1 [33], which contains a CH domain as does MICAL.

In this work, we test the roles of the *C. elegans* FMOs and EHBP-1 in Netrin-mediated axon guidance and growth cone protrusion. We find that *fmo-1, fmo-4, fmo-5* and *ehbp-1* mutants display pathfinding defects of the dorsally-directed VD/DD motor neuron axons that are repelled by UNC-6/Netrin, and that they interact genetically with *unc-40* and *unc-5*. We also find that VD growth cones in these mutants display increased filopodial protrusion, similar to mutants in repulsive UNC-6/Netrin signaling (e.g. *unc-5* mutants), and that transgenic expression of FMO-5 inhibits growth cone protrusion, similar to constitutively-activated UNC-40 and UNC-5. We also show that FMO-1, FMO-4, FMO-5 and EHBP-1 are required for the growth cone inhibitory effects of activated UNC-5, UNC-40, and the Rac GTPases CED-10 and MIG-2. Together, these genetic analyses suggest that FMO-1, FMO-4, FMO-5, and EHBP-1 normally restrict growth cone protrusion, and that they might do so in UNC-6/Netrin-mediated growth cone repulsion.

**Materials and methods**

**Genetic methods**

Experiments were performed at 20°C using standard *C. elegans* techniques [34]. Mutations used were LGI: *unc-40(n324), unc-73(rh40)*; LGII: *juls76[Punc-25::gfp]*; LGIII: *fmo-3*
as previously described [36].

The expression data of homozygous animals from a heterozygous mother were scored. The predicted additive effect of the mutants was calculated by the formula $P_1 + P_2 - (P_1 \times P_2)$, where $P_1$ and $P_2$ are the phenotypic proportions of the single mutants. The predicted additive effect of single mutants was used in statistical comparison to the observed double mutant effect.

P1 and P2 are the phenotypic proportions of the single mutants. The predicted additive effect of the mutants was calculated by the formula $P_1 + P_2 - (P_1 \times P_2)$, where $P_1$ and $P_2$ are the phenotypic proportions of the single mutants. The predicted additive effect of single mutants was used in statistical comparison to the observed double mutant effect.

Transgene construction

Details about transgene construction are available by request. Punc-25::fmo-1, Punc-25::fmo-4 and Punc-25::fmo-5 were made using the entire genomic regions of fmo-1, fmo-4 and fmo-5 respectively. Expression analysis for fmo-5 was done by amplifying the entire genomic region of fmo-5 along with its endogenous promoter (1.2kb upstream) and fusing it to gfp followed by the 3' UTR of fmo-5.

Analysis of axon guidance defects

VD neurons were visualized with a Punc-25::gfp transgene, julS76 [37], which is expressed in GABAergic neurons including the six DDs and 13 VDs, 18 of which extend commissures on the right side of the animal. The commissure on the left side (VD1) was not scored. In wildtype, an average of 16 of these 18 VD/DD commissures are apparent on the right side, due to fasciculation of some of the commissural processes. In some mutant backgrounds, fewer than 16 commissures were observed (e.g. unc-5). In these cases, only observable axons emanating from the ventral nerve cord were scored for axon guidance defects. VD/DD 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 (ectopic neurite branches present on the commissural processes). In the case of double mutant analysis with unc-40 and unc-5 only lateral midline crossing (axons that fail to extend dorsally past the lateral midline) were considered. Fisher’s exact test was used to determine statistical significance between proportions of defective axons. In double mutant comparisons, the predicted additive effect of the mutants was calculated by the formula $P_1 + P_2 - (P_1^* P_2)$, where $P_1$ and $P_2$ are the phenotypic proportions of the single mutants. The predicted additive effect of single mutants was used in statistical comparison to the observed double mutant effect.

Growth cone imaging

VD growth cones were imaged as previously described [15, 22]. Briefly, animals at 16 h post-hatching at 20°C were placed on a 2% agarose pad and paralyzed with 5mM sodium azide in M9 buffer, which was allowed to evaporate for 4 min before placing a coverslip over the...
sample. Some genotypes were slower to develop than others, so the 16 h time point was adjusted for each genotype. Growth cones were imaged with a Qimaging Rolera mGi camera on a Leica DM5500 microscope. Projections less than 0.5 µm in width emanating from the growth cone were scored as filopodia. Filopodia length and growth cone area were measured using ImageJ software. Filopodia length was determined by drawing a line from the base where the filopodium originates on the edge of the peripheral membrane to the tip of the filopodium. Growth cone area was determined by tracing the periphery of the growth cone, not including filopodial projections. Significance of difference was determined a two-sided t-test with unequal variance.

**Results**

*fmo-1, 4, 5* and *ehbp-1* affect VD/DD axon pathfinding

The *C. elegans* genome lacks an apparent homolog of MICAL. However, it contains five flavin monooxygenase genes (*fmo-1,2,3,4,5*) (Fig 1A) [32]. The *C. elegans* molecule most similar to the non-FMO portion of MICAL is EHBP-1, the homolog of the mammalian EH domain binding protein 1 (Ehbp1) protein [33]. We analyzed existing mutations in *fmo* genes and *ehbp-1* (Fig 1B) for VD/DD axon guidance defects. *fmo-1(ok405)* was a 1,301-bp deletion that removed part of exon 3 and all of exons 4, 5 and 6. *fmo-2(ok2147)* was a 1070-bp deletion that removed part of exon 4 and 5. *fmo-4(ok294)* was a 1490-bp deletion that removed all of exons 2, 3, 4 and 5. *fmo-5(tm2438)* is a 296-bp deletion which removes part of intron 3 and exon 4. These deletions all affected one or both predicted enzymatic domains of the FMO molecules. *fmo-3(gk184651)* was a G to A substitution in the 3’ splice site of intron 6 and may not be a null mutation. *ehbp-1(ok2140)* is a 1,369-bp deletion that removed all of exon 5 and 6. Genotypes involving *ehbp-1(ok2140)* have wild-type maternal *ehbp-1* activity, as homozygotes are sterile and must be maintained as heterozygotes balanced by *nT1*.

The 19 D-class motor neurons cell bodies reside in the ventral nerve cord. They extend axons anteriorly and then dorsally to form a commissure, which normally extend straight dorsally to the dorsal nerve cord (Fig 2 and Fig 3B). On the right side of wild-type animals, an average of 16 commissures were observed, due to the fasciculation of some processes as a single commissure (Fig 2C and Materials and Methods). *fmo-1, 4, and 5* and *ehbp-1* mutants showed significant defects in VD/DD axon pathfinding, including ectopic axon branching and wandering (~3–5%; see Materials and Methods and Fig 3A, 3C and 3D), although most crossed the lateral midline despite wandering. *fmo-2* and *fmo-3* mutations showed no significant defects compared to wild-type (Fig 3A). We used RNAi directed against *ehbp-1*, which has the potential to eliminate any maternally-supplied mRNAs, but not translated proteins. *ehbp-1* (RNAi) resembled *ehbp-1 M+* mutants (Fig 3A), suggesting that maternal mRNAs are not involved in VD/DD axon guidance.

Most double mutants showed no strong synergistic defects compared to the predicted additive effects of the single mutants (Fig 3A). However, the *fmo-2; fmo-3* and the *fmo-2; fmo-4* double mutants showed significantly more defects compared to the predicted additive effects of the single mutants. The *fmo-4; ehbp-1* double mutant displayed significantly reduced defects than either mutation alone. The *fmo-1; fmo-4 fmo-5* triple mutant also showed no synergistic defects as compared to single mutants alone (Fig 3A). Lack of extensive phenotypic synergy suggests that the FMOs do not act redundantly, but rather that they might have discrete and complex roles in axon guidance, as evidenced by *fmo-4; ehbp-1* mutual suppression.
Axon pathfinding defects of *unc-40* and *unc-5* are increased by *fmo-1*, *fmo-4* and *fmo-5* mutations

In *unc-40*(n324) strong loss-of-function mutants, most axons (92%) extended past the lateral midline despite wandering (see Materials and Methods and Fig 4A and 4B). *fmo-1*, *fmo-4*, *fmo-5*, and *ehbp-1* displayed < 1% failing to extend past the lateral midline (Fig 4A). *fmo-1*, *fmo-4*, and *fmo-5* mutations significantly enhanced the VD/DD lateral midline crossing defects of *unc-40*(n324) (Fig 4A and 4C). *ehbp-1* did not enhance *unc-40* (Fig 4A).

*unc-5*(e53) strong loss-of-function mutants display a nearly complete failure of VD axons to reach the dorsal nerve cord [13, 15]. *unc-5*(e152) is a hypomorphic allele [38] and displayed 22% failure of axons to cross the lateral midline (Fig 5A). The *unc-5*(op468) allele [39] also displayed a weaker lateral midline crossing phenotype (10%), indicating that it is also a
A hypomorphic allele (Fig 5B). *fmo-1*, *fmo-4* and *fmo-5* significantly enhanced the VD/DD axon guidance defects of both *unc-5(e152)* and *unc-5(op468)*, but *ehbp-1* did not (Fig 5). While *fmo* mutations alone caused few midline crossing defects compared to *unc-40* and *unc-5*, they enhanced the midline crossing defects of *unc-40* and *unc-5* hypomorphic mutants. These results indicate that FMO-1, 4, and 5 might act with UNC-40 and UNC-5 in VD/DD axon pathfinding.

B *fmo-1*, *fmo-4* and *fmo-5* act cell-autonomously in the VD/DD neurons

Expression of the *fmo-1*, *fmo-4*, and *fmo-5* coding regions were driven in VD/DD motor neurons using the *unc-25* promoter. *Punc-25::fmo* transgenes significantly rescued lateral midline...
Fig 3. Mutations in fmo-1, fmo-4, fmo-5 and ehbp-1 cause axon pathfinding defects. (A) Percentage of VD/DD axons with pathfinding defects (see Materials and Methods) in single mutants, double mutants and triple mutant harboring the jul76[Punc-25:gfp] transgene. Single asterisks (*) indicate the significant difference between wild-type and the mutant phenotype ($p < 0.01$); Double asterisks (**) indicate significant difference between double mutants and the predicted additive effect of single mutants ($p < 0.01$) determined by Fischer’s exact test. Error bars represent 2x standard error of proportion. (B-D) Representative fluorescent micrograph of L4 VD/DD axons. Anterior is to the left, and dorsal is up. The scale bar represents 5μm. DNC, dorsal nerve cord; and VNC, ventral nerve cord. (B) A wild-type commissure is indicated by an arrow. (C) An fmo-1(ok405) commissure branched and failed to reach to dorsal nerve cord (arrow). (D) fmo-5(tm2438) VD/DD axons branched and wandered (arrows). A gap in the dorsal nerve cord (asterisk) indicates that commissural processes failed to reach the dorsal nerve cord. determined by Fischer’s exact test. At least 1500 axons were scored per genotype. M+ indicates that the animal has wild-type maternal ehbp-1(+) activity.

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crossing defects in \( fmo; unc-5(op468) \) and \( fmo; unc-5(e152) \) (Fig 6). These data suggest that the axon defects observed in \( fmo \) mutants are due to mutation of the \( fmo \) genes themselves, and that \( fmo-1 \), \( fmo-4 \), and \( fmo-5 \) can act cell-autonomously in the VD/DD neurons in axon guidance.

Previous studies showed that \( fmo-1 \) and \( fmo-5 \) promoter regions were active in intestinal cells and the excretory gland cell, whereas the \( fmo-4 \) promoter was active in hypodermal cells, duct and pore cells [32, 40]. \( ehbp-1 \) is expressed in all somatic cells including neurons [33]. Furthermore, cell-specific transcriptome profiling indicated that \( fmo-1 \), \( fmo-4 \) and \( fmo-5 \) were expressed in embryonic and adult neurons, including motor neurons [41–43]. We fused the upstream promoter regions of \( fmo-1 \), \( fmo-4 \), and \( fmo-5 \) to \( gfp \). We could observe no \( fmo-1::gfp \) expression in transgenic animals, in contrast to previous studies using a \( LacZ \) reporter [32].
However, transcriptome profiling indicates neuronal expression of fmo-1 [43]. Our fmo-1::gfp transgene might be missing regulatory regions required for expression. fmo-4::gfp was expressed strongly in hypodermal cells, excluding the seam cells and vulval cells, consistent with previous studies [32] (Fig 6D). We also observed fmo-4::gfp expression in cells in the ventral nerve cord (Fig 6D and 6D’). Pfmo-5::fmo-5::gfp was expressed strongly in the intestine as previously reported [32] (Fig 6E). We also observed expression along the ventral nerve cord (Fig 6E and 6E’). In sum, previous expression studies combined with those described here suggest that fmo-1, 4, and ehhp-1 are expressed in neurons, and that fmo-1, 4, and 5 can act cell-autonomously in the VD/DD motor neurons in axon guidance.
A

Flavin monooxygenases in repulsive axon guidance and growth cone

B

C

N = > 1000
** = p < 0.001 compared to each transgenic line

D

D'

Pfmo-4::GFP

E

E'

Pfmo-5::GFP
Fig 6. Expression of *fmo-1, fmo-4 and fmo-5* in VD/DD neurons rescues axon pathfinding defects. (A) The percentages of VD/DD axons failing to cross the lateral mid-line are as described in Fig 5A. unc-5 double mutant genotypes are indicated, and the *Punc-25::fmo-1, Punc-25::fmo-4,* and *Punc-25::fmo-5* transgenes are bracketed. Data for transgenic strains are the combined results from three independently-derived transgenes with similar effects. Double asterisks (**) indicate a significant difference between the double mutant and the transgenic strain (p<0.001; Fisher’s exact test). Error bars represent 2x standard error of the proportion. (B) *fmo-1(ok405) unc-5(e152)* axons often fail to cross the lateral midline (arrow). (C) *fmo-1(ok405) unc-5(e152); Ex(Punc-25::fmo-1)* axons crossed the lateral midline (arrows). (D-E and D’-E’) Images are micrographs of L2 animals with transgenic expression of *Plmo-4::gfp* and *Plmo-5::fmo-5::gfp*. Dorsal is up and anterior is left. Scale bar: 5 µm. The lateral midline of the animal is indicated by the dashed white line. The dorsal nerve cord and ventral nerve cord are indicated by dotted white lines. (B) *fmo-1(ok405) unc-5(e152)* axons often fail to cross the lateral midline (arrow). (C) *fmo-1(ok405) unc-5(e152); Ex(Punc-25::fmo-1)* axons crossed the lateral midline (arrows). (D-E and D’-E’) Images are micrographs of L2 animals with transgenic expression of *Plmo-4::gfp* and *Plmo-5::fmo-5::gfp*. Dorsal is up and anterior is left. Scale bar: 5 µm. (D) *fmo-4::gfp* is broadly expressed, including in hypodermis and in cells along the ventral nerve cord that resemble motor neurons. Expression is not evident in the lateral hypodermal seam cells (asterisks). (D’) Enlarged image of *fmo-5::gfp* expression in ventral nerve cord cells (arrows). (E) *fmo-5::gfp* is expressed strongly in the gut (asterisks), as well as in cells along the ventral nerve cord. (arrow) (E’) Enlarged image of *fmo-5::gfp* expression in ventral nerve cord cells (arrows).

*fmo-1, fmo-4 and fmo-5* mutants display increased growth cone filopodial protrusion

The growth cones of dorsally-directed VD commissural axons are apparent in early L2 larvae (Fig 2B). We imaged VD growth cones at 16 hours post-hatching, when the VD growth cones have begun their dorsal migrations, as described previously [15]. *fmo-1, fmo-4,* and *fmo-5* mutant growth cones displayed longer filopodial protrusions compared to wild type (e.g. 0.96 µm in wild type compared with 1.55 µm in *fmo-5(tm2438); p < 0.001) (Fig 7). This effect was not significant in *ehbp-1(ok2140)* (Fig 7). Growth cone area was not significantly different in any mutant. These results suggest that *fmo-1, fmo-4* and *fmo-5* normally limit growth cone filopodial protrusion length. This is consistent with ectopic axon branches observed in post-development VD/DD neurons in these mutants (Fig 3), as other mutants with increased growth cone filopodial protrusions (e.g. *unc-5, unc-73,* unc-33) also display ectopic branches, likely due to failure of filopodial retraction and subsequent consolidation into a neurite [15, 17].

Expression of the *fmo-5* coding region driven in VD/DD motor neurons using the *unc-25* promoter also significantly rescued axon guidance defects as well as the long filopodial protrusions seen in *fmo-5(tm2438)* (Fig 8). We expressed the *fmo-5* coding region in the hypodermis using the *dpy-7* promoter [44] and observed no significant rescue of axon guidance defects or filopodial protrusions (Fig 8). These data confirm that *fmo-5* can act cell-autonomously in the VD/DD neurons in axon guidance and growth cone filopodial protrusion.

*fmo-1, fmo-4, fmo-5* and *ehbp-1* mutations suppress activated *myr::unc-40* and *myr::unc-5* and activated Rac GTPases

Previous studies showed that UNC-6/netrin signaling via the heterodimeric UNC-5:UNC-40 receptor leads inhibition of growth cone protrusion important in UNC-6/Netrin’s role in repulsive axon guidance [15, 17]. Constitutive activation of this pathway using expression of myristoylated versions of the cytoplasmic domains of UNC-40 and UNC-5 (myr::unc-40 and myr::unc-5) results in small growth cones with few if any filopodial protrusions (i.e. protrusion is constitutively inhibited by MYR::UNC-40 and MYR::UNC-5) [15, 17, 18]. Loss of *fmo-1, fmo-4,* *fmo-5* and *ehbp-1* significantly suppressed inhibition of filopodial protrusion and growth cone size caused by *myr::unc-40* (Fig 9) and *myr::unc-5* (Fig 10). Notably, *ehbp-1* did not enhance loss-of-function mutations in *unc-5 or unc-40* (Fig 4), suggesting that *myr::unc-5* and *myr::unc-40* are sensitized backgrounds in which interactions can be determined that are not apparent in loss-of-function backgrounds.

Expression of activated CED-10(G12V) and MIG-2(G16V) in the VD neurons results in reduced growth cone protrusion similar to MYR::UNC-40 and MYR::UNC-5 [17]. We found that *fmo-1, fmo-4* and *fmo-5* suppressed filopodial protrusion deficits caused by *ced-10(G12V)* and *mig-2(G16V)* (Fig 11). *ehbp-1* suppressed *mig-2(G16V), but ehbp-1(ok2140M+); ced-10*
Double mutants were inviable and could not be scored. Furthermore, fmo-4 and fmo-5, but not fmo-1, significantly suppressed growth cone size reduction caused by CED-10(G12V) and MIG-2(G16V). ehbp-1 also suppressed growth cone size reduction of MIG-2(G16V). Taken together, these data indicate that functional FMO-1, FMO-4, FMO-5, and EHBP-1 are required for the full effect of MYR::UNC-40, MYR::UNC-5, CED-10(G12V), and MIG-2 (G16V) on growth cone protrusion inhibition, including filopodial protrusion and growth cone size.

**FMO-5 can inhibit growth cone protrusion**

fmo-5 loss-of-function mutant growth cones displayed excessively-protrusive filopodia (Fig 7) and suppressed activated UNC-5:UNC-40 and Rac signaling (Figs 9–11). Transgenic expression of wild-type FMO-5 driven by its endogenous promoter rescued the axon guidance.
defects and long filopodial protrusions seen in fmo-5(tm2438) mutant VD growth cones (Fig 12A–12E). In a wild-type background, fmo-5 transgenic expression resulted in growth cones with smaller area and shortened filopodia (Fig 13A, 13B and 13E), indicating that wild-type FMO-5 activity can inhibit growth cone protrusion. This inhibition was not observed in the fmo-5(tm2438) background, possibly due to the decreased levels of FMO-5 compared to the wild-type background.

Mutations in unc-5, unc-73, and unc-33 result in excessively large growth cones with increased filopodial length (Fig 13A and 13B) [15, 17]. Transgenic fmo-5 expression significantly reduced growth cone size and filopodial protrusion in unc-5(e152), unc-73(rh40), and unc-33(e204) (Fig 13A, 13B, 13F, 13G and 13H). However, fmo-5 expression only partially inhibited filopodial protrusion in unc-5 and unc-33 (i.e. to wild-type levels, higher than fmo-5 transgenic expression alone) (Fig 13A). These data indicate that FMO-5 activity does not rely
on UNC-5, UNC-73, or UNC-33 and that it might act downstream of them. However, the hybrid interaction of fmo-5 transgenic expression with unc-33(e204) could also indicate that FMO-5 and UNC-33 represent distinct, compensatory pathways downstream of UNC-5 and the Rac GTPases to inhibit filopodial protrusion. unc-33; fmo-5 double mutants did not show any significant increase in filopodial length (Fig 13A and 13B), which would be expected if they act in parallel pathways.

In contrast to unc-5 mutants, unc-40 single mutants display shortened filopodial protrusions and a relatively normal growth cone size (Fig 13A and 13B) [15]. This is likely due to the dual role of UNC-40 in both stimulating protrusion as a homodimer and inhibiting protrusion as a heterodimer with UNC-5. fmo-5 transgenic expression had no effect on filopodial...
protrusions in unc-40, but did reduce growth cone size, consistent with a role of FMO-5 in inhibiting protrusion.

Finally, we also found that transgenic fmo-5 expression in fmo-1(ok405) suppressed growth cone area and filopodial length of fmo-1(ok405) mutants (Fig 13A, 13B and 13H), indicating that fmo-5 activity can partially compensate for loss of fmo-1.

Discussion

Results here implicate the C. elegans flavoprotein monooxygenase molecules FMO-1, FMO-4 and FMO-5 in inhibition of growth cone protrusion via UNC-6/Netrin receptor signaling in repulsive axon guidance (Fig 14). The MICAL molecule found in vertebrates and Drosophila is a flavoprotein monooxygenase required for semaphorin-plexin mediated repulsive motor
Here we focus on UNC-6/Netrin signaling and have not analyzed the role of the FMOs in semaphorin signaling in *C. elegans*. MICAL is a multi-domain molecule that also includes a calponin homology (CH) domain, a LIM domain and multiple CC domains. No molecule encoded in the *C. elegans* genome has a similar multi-domain organization. However, the Eps-15 homology (EH) domain binding protein EHBP-1 is similar to the non-FMO portion of MICAL and contains a CH domain [33]. We show here that EHBP-1 also is involved in inhibition of growth cone protrusion and axon guidance. Thus, while *C. elegans* does not have a multidomain MICAL-like molecule, it is possible that the functional equivalents are the FMOs and EHBP-1.

**Fig 11.** FMO-1, FMO-4 and EHBP-1 are required for Rac GTPase-mediated inhibition of VD growth cone protrusion. (A,B) Quantification of VD growth cone filopodial length and growth cone area in wild-type, activated *ced-12*(*G12V*) and *mig-2*(*G16V*), and double mutants. (A) Average filopodial length, in μm. (B) Growth cone area in μm². Error bars represent 2x standard error of the mean. Asterisks indicate significant difference between *ced-10*(*G12V*) and their respective double mutants (*p < 0.05, **p < 0.001*) determined by two-sided *t*-test with unequal variance. n.s., not significant. (C-F) Representative fluorescent micrographs of mutant VD growth cones. (C,D) Images of *ced-10*(*G12V*) and *fmo-4*(ok294); *ced-10*(*G12V*) growth cones. The arrowhead in (C) points to a growth cone with limited protrusion, and the arrow in (D) indicates a filopodial protrusion. (E,F) Images of *mig-2*(*G16V*) and *fmo-5*(tm2438); *mig-2*(*G16V*) growth cones. The arrowhead in (E) points to a growth cone with limited protrusion, and the arrow in (F) indicates a filopodial protrusion. Scale bar: 5μm.

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FMO-1, FMO-4, FMO-5 and EHBP-1 regulate axon guidance and growth cone filopodial protrusion

*fmo-1, fmo-4, fmo-5, and ehbp-1* mutants display defects in dorsal guidance of the VD/DD motor axons that are repelled from UNC-6/Netrin (Fig 3). Double and triple mutant analysis did not uncover significant redundancy, suggesting that these molecules might have discrete and complex roles in axon guidance. Consistent with this idea, *fmo-4* and *ehbp-1* mutually suppress VD/DD axon guidance defects. Furthermore, transgenic expression of FMO-5 rescued excess growth cone and filopodial protrusions of *fmo-1* mutants. This suggests that FMO-5 can partially compensate for loss of FMO-1, and that the function of FMO-5 does not depend on FMO-1. Combined with lack of phenotypic synergy, these data suggest that the FMOs act in a common pathway, where loss of one abolishes pathway function, and that FMO-5 might...
act downstream of FMO-1 in this pathway. fmo-2 and fmo-3 mutations displayed no significant defects alone, suggesting that they are not involved in axon guidance. fmo-2 did significantly enhance fmo-4. Possibly, fmo-2 and fmo-3 might have roles in axon guidance that were not revealed by the mutations used.

*Flies* and vertebrate MICAL regulate actin cytoskeletal dynamics in both neuronal and non-neuronal processes through direct redox activity of the monooxygenase domain [27, 30, 46–50]. In *Drosophila*, loss of MICAL showed abnormally shaped bristles with disorganized and larger F-actin bundles, whereas, overexpression of MICAL caused a rearrangement of F-actin into a complex meshwork of short actin filaments [29]. Here we show that loss of fmo-1, fmo-4, and fmo-5 resulted in longer filopodial protrusions in the VD motor neurons (Fig 7), suggesting that their normal role is to limit growth cone filopodial protrusion. Indeed, transgenic expression of wild-type FMO-5 resulted in VD growth cones with a marked decrease in growth cone filopodial protrusion (Fig 13). Growth cone size was not affected in any loss-of-function mutation, but growth cone size was reduced by transgenic expression of wild-type FMO-5 (Fig 13), suggesting a role of the FMO-5 in both filopodial protrusion and growth cone lamellipodial protrusion.

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**Fig 13.** FMO-5 activity can partially compensate for UNC-5, UNC-73/Trio, and UNC-33/CRMP. (A,B) Quantification of VD growth cone filopodial length and growth cone area in indicated genotypes. Error bars represent 2x standard error of the mean. Asterisks indicate significant difference between wild-type and mutants (**p < 0.001) and *** indicate a significant difference between each single mutant compared to the double mutant. Pound signs (#) indicate a significant difference between [fmo-5 genomic] and double mutant (#p < 0.001) determined by two-sided t-test with unequal variance. n.s., not significant. (C-H) Fluorescence micrographs of VD growth cones from wild-type, fmo-5(tm2438), [fmo-5 genomic], unc-5; [fmo-5 genomic], unc-33; [fmo-5 genomic] and fmo-1; [fmo-5 genomic]. The arrowhead points to a growth cone with limited protrusion. Arrows indicate representative filopodia. Scale bar: 5μm.

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**Fig 14.** Genetic model of inhibition of growth cone protrusion. UNC-5 homodimers and/or UNC-5:UNC-40 heterodimers act through the Rac GTP exchange factor UNC-73/Trio and the Rac GTPases, which then utilize the flavin monooxygenases and UNC-33/CRMP to inhibit protrusion. The FMOs might inhibit protrusion directly, by possibly directly oxidizing F-actin, or by promoting phosphorylation of UNC-33/CRMP.

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Previous studies have shown that *Drosophila* MICAL may require both its FMO and CH domain to induce cell morphological changes; however, mammalian MICAL in non-neuronal cell lines requires only its FAD domain suggesting a difference in the mechanism of action in these MICALs [29, 51]. These data suggest that in some cases, the FMO domain is sufficient for the function of MICAL. Thus, single domain FMOs as in *C. elegans* could function despite lacking the multi-domain structure of MICAL. Loss of the *C. elegans* MICAL-like molecule EHBP-1, which contains a CH domain and is similar to the non-FMO portion of MICAL (Fig 1), also resulted in VD/DD axon guidance defects, but did not significantly affect growth cone filopodial protrusion. EHBP-1 might act with the FMOs in axon guidance. Phenotypic differences could be due to EHBP-1-dependent and independent events, or to the wild-type maternal contribution in *ehbp-1* homozygous mutants derived from a heterozygous mother. It is also possible that EHBP-1 affects axon guidance independently of the FMOs. EHBP-1 is involved in Rab-dependent endosomal vesicle trafficking by bridging interaction of endosomal Rabs with the actin cytoskeleton [33, 52]. MICAL has also been implicated in Rab-dependent endosomal biogenesis and trafficking [53–55], suggesting that FMO/EHBP-1 and MICALs might share common functions, although it remains to be determined if FMOs in *C. elegans* regulate endosomal trafficking.

MICAL has been shown to directly oxidize cysteine residues in F-actin, leading to actin depolymerization and growth cone collapse [29, 30, 56, 57]. We speculate that FMO-1, FMO-4, and FMO-5 might act by a similar mechanism to inhibit growth cone filopodial protrusion. Previous studies have shown that the single calponin homology (CH) domain containing protein CHDP-1 promotes the formation of cell protrusions in *C. elegans* by directly binding to Rac1/CED-10 through its CH domain [58]. The role of EHBP-1 however, is less clear, but previous studies have shown that *Drosophila* MICAL might require both its FMO and CH domain to induce cell morphological changes [29]. Thus, in axon guidance, FMO-1, FMO-4, and FMO-5 might require the CH domain provided by EHBP-1 in some instances. Mammalian MICAL requires only the FMO domain [51], suggesting that in some cases the CH domain is not required and the FMO domain can act alone. Future studies will be directed at answering these questions.

**FMOs can act autonomously in the VD/DD neurons**

Expression of full length *fmo-1, fmo-4* and *fmo-5* coding regions under the control of the *unc-25* promoter specific for GABA-ergic neuron expression (including the VD/DD neurons) rescued VD/DD axon guidance defects (Figs 6 and 8). Furthermore, the promoters of *fmo-4* and *fmo-5* were active in ventral nerve cord cells (Fig 6). Expression of full length *fmo-5* coding region under the control of the *unc-25* promoter rescued axon guidance defects as well as the long filopodial protrusions seen in *fmo-5(tm2438)*, whereas expression from the hypodermal *dpy-7* promoter did not (Fig 8). Cell-specific transcriptome profiling indicated that *fmo-1, fmo-4* and *fmo-5* were expressed in embryonic and adult neurons, including motor neurons [41–43]. Together, these results suggest that the FMOs can act cell-autonomously in the VD/DD neurons in axon guidance and growth cone filopodial protrusion.

**FMO-1, FMO-4 and FMO-5 mediate UNC-6/Netrin receptor signaling in growth cone inhibition of protrusion**

Our findings suggest that the FMOs act with the UNC-40 and UNC-5 receptors to mediate UNC-6/netrin repulsive axon guidance and inhibition of growth cone protrusion. *fmo-1, fmo-4*, and *fmo-5* mutations enhanced axon pathfinding defects in *unc-40* and hypomorphic *unc-5* mutants (Figs 4 and 5). The axon guidance defects of the *fmos* were weaker than those of *unc-
40 and unc-5 mutants (e.g. the fmos displayed few lateral midline crossing defects despite axon wandering). We speculate that the FMOs are but one of several pathways mediating the effects of UNC-40 and UNC-5 in axon pathfinding. ehbp-1 did not enhance unc-40 or unc-5, suggesting discrete roles of these molecules or wild-type maternal ehbp-1 contribution. fmo-1, fmo-4, fmo-5, and ehbp-1 mutations each suppressed the effects of activated MYR::UNC-40 and MYR::UNC-5 on inhibition of growth cone protrusion (Fig 10). In this case, both filopodial protrusion and growth cone area was restored, consistent with a role of these molecules in inhibiting both growth cone filopodial and lamellipodial protrusion. We also find the fmo-5 transgenic expression suppressed unc-5(e152) growth cone area and filopodial protrusions (Fig 13). That the FMOs and EHBP-1 were required for the effects of the constitutively active MYR::UNC-40 and MYR::UNC-5 suggest that they act downstream of these molecules in growth cone inhibition of protrusion. While the loss-of-function and gain-of-function data are consistent with acting downstream of UNC-40 and UNC-5, it is possible that the FMOs define a parallel pathway in growth cone protrusion.

Interestingly, mutations in these genes have very distinct penetrances (e.g. the axon guidance and protrusion defects of unc-5 are much stronger than those of the fmo mutants). One explanation for this is that these molecules act in networks rather than simple linear pathways. The FMOs might be one of many mechanisms acting downstream of UNC-5, and multiple pathways might converge on UNC-33 (e.g. UNC-5 and UNC-33 are major “nodes” in this network). Further loss- and gain-of-function studies will be required to understand this signaling network.

FMOs and EHBP-1 act downstream of Rac GTPase signaling in inhibition of growth cone protrusion

Similar to activated MYR::UNC-40 and MYR::UNC-5, constitutively-activated Rac GTPases CED-10(G12V) and MIG-2(G16V) inhibit VD growth cone protrusion. We show that fmo-1, fmo-4, fmo-5 and ehbp-1 mutations suppressed activated CED-10(G12V) and MIG-2(G16V) (e.g. double mutant growth cones displayed longer filopodial protrusions similar to fmo-1, fmo-4, fmo-5 and ehbp-1 single mutants) (Fig 11). Furthermore, loss of the Rac GTP exchange factor UNC-73/Trio had no effect on the inhibited growth cone phenotype of FMO-5 transgenic expression (i.e. the growth cones resembled those of fmo-5 over expression alone) (Fig 13). UNC-73/Trio acts with the Rac GTPases CED-10 and MIG-2 in growth cone protrusion inhibition, and unc-73 mutants display excessive growth cone protrusion [17]. That FMO-5 transgenic expression could inhibit protrusion in the absence of the Rac activator UNC-73/Trio suggests that FMO-5 acts downstream of UNC-73/Trio, consistent with the FMOs and EHBP-1 acting downstream of the Rac GTPases.

FMO-5 interacts with UNC-33/CRMP

Previous studies have shown that the C. elegans CRMP-like molecule UNC-33 is required in a pathway downstream of Rac GTPases for inhibition of growth cone protrusion in response to UNC-6/Netrin [17]. unc-33 loss-of-function mutants with FMO-5 transgenic expression displayed a mutually-suppressed phenotype. The excessively-long filopodial protrusions of unc-33 mutants were reduced to wild-type levels, but were significantly longer than in animals with FMO-5 transgenic expression, and the growth cone area was reduced to resemble FMO-5 transgenic expression alone (Fig 13). This hybrid phenotype makes it difficult to determine if FMO-5 and UNC-33 act in the same pathway, in parallel pathways, or both.

One proposed mechanism of cytoskeletal regulation by MICAL is the production of the reactive oxygen species (ROS) H₂O₂ by the FAD domain in the presence of NADPH [59].
Upon activation by Sema3A, MICALs generate $\text{H}_2\text{O}_2$, which can, via thioredoxin, promote phosphorylation of CRMP2 via glycogen synthase kinase-3, leading to growth cone collapse \[60\]. Thus, the FMOs have the potential to inhibit growth cone protrusion through direct oxidation of F-actin resulting in depolymerization, and through redox regulation of the activity of UNC-33/CRMP (i.e. to act both in the UNC-33 pathway and in parallel to it).

**Conclusion**

In summary, we present evidence of a novel role of the *C. elegans* flavin-containing monooxygenase molecules (FMOs) in inhibition of growth cone protrusion downstream of UNC-6/Netrin signaling. The FMOs acted downstream of the UNC-6/Netrin receptors UNC-5 and UNC-40, and downstream of the Rac GTPases CED-10 and MIG-2 (Fig 14). Future studies will determine if the FMOs regulate UNC-33/CRMP, if they cause actin depolymerization, or both, to inhibit growth cone protrusion.

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