C. elegans PVF-1 inhibits permissive UNC-40 signalling through CED-10 GTPase to position the male ray 1 sensillum

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SUMMARY
Graded distributions of netrin and semaphorin guidance cues convey instructive polarity information to migrating cells and growth cones, but also have permissive (i.e. non-polarity determining) functions in mammalian development and repair. The permissive functions of these cues are largely characterised at a molecular level. We found previously that UNC-6 (netrin) signals permissively through UNC-40 (DCC) and UNC-5 receptors to prevent anterior displacement of the ray 1 sensillum in the C. elegans male tail. UNC-6/UNC-40 signalling functions in parallel with SMP-1 (semaporin 1)/PLX-1 (plexin) signalling to prevent this defect. Here, we report that a deletion allele of pvf-1, which encodes a VEGF-related protein, causes no ray 1 defects, but enhances ray 1 defects of a plx-1 mutant, and unexpectedly also suppresses unc-6(ev400)-null mutant ray 1 defects. These mutant ray 1 inductive and suppressive effects are mimicked by the ability of unc-40(n449) and ced-10(gain-of-function) multi-copy transgene arrays to induce ray 1 defects or suppress unc-6 mutant ray 1 defects, depending on their dosage, suggesting the pvf-1 mutation causes UNC-40 overactivity that interferes with signalling but is partially sensitive to UNC-6. Additional data suggest PVF-1 functions through four VEGF receptor-related proteins and inhibits only CED-10 (a GTPase), but not MIG-2-dependent UNC-40 activity, even though UNC-40 functions through both GTPases to position ray 1. pvf-1 and receptor mutant ray 1 defects are rescued by transgenes expressing mouse VEGF164 and human VEGF receptors, respectively. These data report the first case of VEGF-induced inhibition of the netrin signalling and a molecular conservation of VEGF function from worms to humans.

KEY WORDS: PVF-1/VEGF, UNC-40/DCC, GTPase, Crosstalk

INTRODUCTION
Netrins and semaphorins were discovered as axon guidance cues that instruct the direction of cell movement and axon extension by forming an extracellular gradient that provides polarity information to the migrating cell or axon growth cone. For example, UNC-6 (netrin) is expressed ventrally in C. elegans and is graded along the D/V axis of the body wall (Ishii et al., 1992; Wadsworth et al., 1996). Attractive cell and growth cone responses to ventral sources of UNC-6 are mediated by the UNC-40 (DCC) receptor, whereas repulsive responses to UNC-6 are mediated by UNC-40-independent and UNC-40-dependent UNC-5 receptors (Hedgecock et al., 1990; Leung-Hagestedt et al., 1992; Chan et al., 1996; Merz et al., 2001). Since these discoveries, homologues of these C. elegans genes were found to be required instructively (i.e. act as part of a spatially distributed guidance cue gradient) for guiding growth cone migrations in the vertebrate spinal cord and elsewhere in the vertebrate nervous system (Keino-Masu et al., 1996; Serafini et al., 1996; Ackerman et al., 1997; Leonardo et al., 1997).

The semaphorins and their plexin receptors also function as instructive axon guidance cues in a variety of animals (Kolodkin et al., 1992; Kolodkin et al., 1993; Luo et al., 1993). The C. elegans genome contains two semaphorin 1 genes, smp-1 and smp-2, and a single semaphorin 2a gene, mab-20/smp-3 (Wormbase release WR 237, http://www.wormbase.org). The C. elegans genome also encodes two plexins: PLX-1 mediates responses to SMP-1 and SMP-2, whereas PLX-2 mediates responses to MAB-20/SMP-3. There are no neuropilin genes in C. elegans (Wormbase release WR 237, http://www.wormbase.org).

Although the netrin and semaphorin families of secreted proteins were originally characterised in the nervous system where they function instructively to guide axons (Tessier-Lavigne et al., 1988), they are also expressed outside the nervous system where they orchestrate the development of several vertebrate organs and tissues, including the vasculature, by regulating cell adhesion, motility, differentiation and survival. Netrins and semaphorins mediate processes that do not obviously involve polarised cell movements and are therefore believed to be purely permissive (i.e. not required to provide polarity information to guide cell movements) (Hinck, 2004; Lai Wing Sun et al., 2011). As observed for axon guidance in the nervous system, netrins and semaphorins may act as bifunctional agents in angiogenesis, based on separate reports that they can have pro-angiogenic (including attractive) (Park et al., 2004; Nguyen and Cai, 2006; Wilson et al., 2006) or anti-angiogenic (including repulsive) (Lu et al., 2004; Larrivée et al., 2007; Bouvrière et al., 2008; Lejmi et al., 2008) actions on endothelial cells.

The finding that netrins can have dual angiogenic and anti-angiogenic properties, whereas VEGFs have pro-angiogenic properties raises the possibility that Netrins are regulated by VEGFs or vice versa. Although VEGF is reported to regulate an HLX transcription factor required for induction of UNC-5B, PLX-A1 and SEMA3G (Testori et al., 2011), all of which inhibit blood vessel sprouting – the first via its activation by netrin 1 (Lu et al., 2004; Larrivée et al., 2007; Bouvrière et al., 2008; Lejmi et al., 2008), we are unaware of any other published regulation of Netrin signalling by VEGF.
To investigate the use of *C. elegans* as a model genetic system for revealing interactions between netrin, semaphorin and VEGF signalling in a permissive context, we decided to examine the effects of these cues on the stereotypic patterning of the ray sensillae in the male tail (Dalpé et al., 2004; Dalpé et al., 2012). The nine rays on each side of the male tail are used to sense the hermaphrodite for copulation ( Sulston et al., 1980) (Fig. 1A). Each ray sensillum comprises sensory endings from two neurons encircled by the expanded tip of a structural support cell. The support cell and neuron endings are embedded in the fan (a lateral cuticular specialisation made by the hypodermis/epidermis), such that most ray neuron endings protrude through the support cell and the edges of the fan in a characteristic position within a roughly linear A/P oriented array of these sensillae, with ray 1 most anterior and ray 9 most posterior on each side (Fig. 1A,D).

The two neurons and single support cell of each ray derive from a common Rn blast cell and cluster together sublaterally to form a ray precursor (Fig. 1C,E) (Emmons, 2005). Initially, there is contact between neighbouring three-cell clusters. They then separate into distinct ray precursors. For ray 1, this involves an apparent limited movement of the ray 1 cluster towards the anterior relative to ray 2.

We reported previously that two signalling pathways, Smp-1/PLX-1 and UNC-6/UNC-40, function together to prevent the anterior displacement of ray 1 (Fig. 1B) and that together these two pathways can account for all of this function (Dalpé et al., 2004; Dalpé et al., 2012). Although during ray positioning, fluorescently tagged functional versions of Smp-1 (semaphorin 1a) and PLX-1 (plexin) appear expressed in all ray cells, whereas UNC-40 and UNC-6 are expressed and localised, respectively, to ray structural cells, we previously found that both PLX-1 and UNC-40 function in the ray structural cells to prevent ray 1 displacement (Dalpé et al., 2004; Dalpé et al., 2012).

Several lines of evidence suggested that Smp-1 and UNC-6 signal permissively and not instructively in this context (Dalpé et al., 2012). For example, *unc-40(+)* multi-copy arrays, which putatively overexpress UNC-40, can rescue the ray 1 positioning defects of an *unc-6* null mutant, as can heat shock-induced expression of UNC-6 in all cells of *unc-6* mutant animals (Dalpé et al., 2012). Here, we present data suggesting that UNC-40 signalling through the CED-10 Rho family GTpase in this permissive context is normally inhibited by VEGF signalling. Furthermore, mammalian VEGF164 and human VEGF receptors can functionally substitute for their *C. elegans* homologues to position ray 1, demonstrating a high conservation of function at a molecular level. These findings represent the first indication of a VEGF-mediated inhibition of netrin signalling in any animal and raise the possibility that similar crosstalk may be involved in permissive activities of netrins in mammals – activities that could include one or more functions in blood vessel formation. The characterisation of permissive UNC-6, semaphorin and VEGF signalling in a genetically amenable context could shed light on a number of vertebrate processes that are important to development, regeneration and health.

### MATERIALS AND METHODS

#### Nematode culture

General procedures were used for culture, maintenance and storage of *C. elegans* (Wood, 1988). Strains used in this study were: Linkage Group X (LGX), *unc-6(e400)* (Hedgpeth et al., 1990); *ver-4(ok1079);ver-3(gk227)* (Popovic et al., 2002); *mi-g-2(mu28)* (Zipkin et al., 1997); LGI, *unc-40(e1430)* (Hedgpeth et al., 1990); LGIII, *ver-1(ok1738);ver-2(ok897)* (Popovic et al., 2002); *pvf-1(e763)* (this paper); IV, *ced-10(n1993)* (Ellis et al., 1991); *plx-1(ev724)* (Dalpé et al., 2004); *unc-5(e53)* (Hedgpeth et al., 1990); and LGV, *him-5(e1490)* (Hodgkin and Brenner, 1977). Strains not isolated in our laboratory were obtained from the *C. elegans* knockout consortium courtesy of Dr Y. Goshima (Yokohama City University, Japan), from the *C. elegans* Genetics Center courtesy of T. Stiernagle (University of Minnesota, MN, USA) or from Dr Regine Roubin (Institute of Cancer Research, Marseilles, France).

#### Immunohistochemistry

*him-5(e1490)* mutant animals were co-transformed with plasmid DNA from vector L4687 (*pPD133.97* – encoding *myo-3p::yfp*) and with *pvf-1p::pvf-
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1(+)) construct PVL95 (which rescues the pvf-1 mutant) were permeabilised,
fixed and stained using a modified Finney-Ruvkun procedure (Bettinger et al., 1996). The worms were then incubated overnight with 300 ng/µl of a
rabbit anti-PVF-1 antibody, then for 3 hours each with Texas Red-conjugated goat anti-rabbit secondary antibody (diluted 1:200, Invitrogen)
followed by fluorescein-conjugated mouse anti-GFP antibody (diluted 1:100, Creative Diagnostics). A solution of 1× PBS with 0.1% BSA, 0.5%
Triton X-100, 0.05% sodium azide and 1 mM EDTA was used for antibody
dilution, while washings used 1× PBS with 0.1% BSA, 0.5% Triton X-100,
0.05% sodium azide and 1 mM EDTA.

Microscopy
Animals were viewed by DIC optics using published procedures (Sulston and Horvitz, 1977). All strains carried him-5(e1490) to increase the
frequency of males. Some strains carried the ajm-1::GFP translational reporter (Simske and Hardin, 2001) visualised using a Leica DMRXA
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The previously described

Transgene constructs
The following transgene arrays were established in C. elegans lin-32 promoter (Portman and Emmons, 2000) and the entire 3198 bp C. elegans ver-1 cDNA cloned in
tandem upstream of 735 bp of the unc-54 3′UTR in HindIII-Nco1 cut
pPD96.52 vector; (8) ram-

Germline transformation
Transgenic strains were generated by microinjection of a DNA mix into the
distal gonad arms of N2 or

Transgene arrays
The following transgene arrays were established in him-5(e1490) hermaphrodites (Mello and Fire, 1995). DNA mixtures consisted of a test construct at a concentration of 50 mg/ml
or 30 mg/ml and a co-injection reporter sur-5::gfp (Portman and Emmons, 2000)
or myo-3p::yfp to create a final DNA concentration of 100 mg/ml.

Heat shock
Synchronised strains were grown on NGM plates coated with OP50 bacteria at 20°C until the L2 stage, incubated at 33°C for 2 hours (Stringham et al., 1992), reared at 20°C until the adult stage, then scored for ray 1
displacement defects.

Statistics
Standard errors for percentages of the anterior ray 1 phenotypes were
calculated assuming a binomial distribution with the observed percentage
value and the actual sample size. Statistical tests were carried out using a
standard (two-tailed) comparison of two proportions (Moore, 1998). All P
values represent the probability that the measured penetrance of the
phenotype is significantly different between two strains. A P<0.05 is
considered significant.

RESULTS
PVF-1 expression pattern and genetic characterisation
The C. elegans pvf-1 gene encodes a small secreted protein (PVF-1) with a VEGF-related sequence that includes the highly conserved

Fig. 2. The nature of the pvf-1(ev763) mutation and constructs used to
probe PVF-1 function. (A) The organisation of the pvf-1 locus, including coding (black rectangles) and non-coding regions (adjoining lines). The extent of the pvf-1(ev763) deletion is indicated in red.
(B) Coding and non-coding sequences of the constructs used to probe
pvf-1 function (see Materials and methods) are shown as in A.

plasmid pPD133.82 (previously deleted for the myo-3 promoter) upstream of
cfr and 734 bp of unc-54 3′UTR sequence; (2) pvf-1p::pvf-1(+)-construct (also known as PVL95) – a pvf-1 genomic fragment of 4.9 kb comprising 1.9 kb of
the 5′UTR and the entire 2.86 kb of coding sequence with introns plus 390
bp of 3′UTR PCR amplified and cloned into a BamHI-Apal cut pPD90.575
vector; (3) myo-3p::pvf-1 construct (also known as PVL97) – the 2.86 kb
coding sequence with introns plus 390 bp of 3′UTR of pvf-1(+) present in
PVL95 (see above) PCR amplified and cloned into Not1-Apal cut pPD133.97
(downstream of the 2.4 kb myo-3 promoter); (4) hsp16.41p::pvf-1 construct
(also known as PVL83) – the 2.86 kb coding sequence with introns plus 390
bp of 3′UTR of pvf-1 present in PVL95 (see above) PCR amplified and
cloned into BamHI-Apal cut pPD94.83 (downstream of the hsp16.41 promoter); 
(5) myo-3p::mVEGF164 construct (also known as pZH234) – the
and 734 bp of 3′UTR sequence; (2)
pvp-1(+)

The organisation of the

PVF-1 expression pattern and genetic characterisation
The C. elegans pvf-1 gene encodes a small secreted protein (PVF-1) with a VEGF-related sequence that includes the highly conserved
we isolated a (Fig. 3B). These results are supported by immunofluorescence data and other genetic interactions described below suggest that PVF-1 normally inhibits UNC-6 and UNC-40 signalling. The ability of PVF-1 to position ray 1. We previously showed that semaphorins, UNC-6 and their receptors promote the anterior displacement of the most anterior ray sensillum (ray 1) in the male tail of C. elegans (Dalpé et al., 2004; Dalpé et al., 2012). That neuropilin, the vertebrate semaphorin 3 receptor, also binds VEGF164 prompted us to investigate whether PVF-1 has a function in ray 1 positioning, even though C. elegans does not produce a neuropilin homologue. In these studies, him-5(e1490) was used to generate a high frequency of males in all of the mutant strains examined, thus him-5(e1490) serves as a ‘wild-type’ control (Fig. 1A; Figs 4-6, line 1). Homozygous pvf-1(ev763) males do not display any visible phenotypes or ray displacements (Fig. 4, line 2); however, we found that it significantly enhances a plx-1(ev724)/plexin null mutant for ray 1 severe anterior displacements (i.e. displacements anterior to the cuticular fan in which the rays are normally embedded) (Fig. 1A,B,E; Fig. 4, lines 2-4).

Mouse VEGF164 or muscle-specific or heat shock-induced expression of PVF-1 rescues pvf-1 mutant ray 1 defects

We investigated the cell autonomy of pvf-1 function by cell type-specific rescue experiments. We found that a pvf-1p::pvf-1(+) transgene array (Materials and methods) largely rescues the enhancement of the plx-1 mutant ray 1 defects caused by pvf-1(ev763) (Fig. 4, lines 4,5), as does expressing pvf-1(+) under the control of the muscle-specific myo-3 promoter (Okkema et al., 1993) (Fig. 4, lines 4,6). In principle, PVF-1 secreted from any source could rescue the pvf-1(ev763)-mediated enhancement of plx-1 mutant ray 1 defects if PVF-1 functions permissively. We examined this hypothesis by driving pvf-1(+) expression with the heat shock promoter hsp16.41p (Okkema et al., 1993). We found that heat shock induced hsp16.41p::pvf-1(+) can rescue the pvf-1(ev763)-mediated enhancement of plx-1(ev724) ray 1 defects (Fig. 4, lines 4,7,8). These data and other genetic interactions described below suggest that PVF-1 is normally secreted by body wall muscles and acts to position ray 1; however, secretion by several tissues may be sufficient for PVF-1 function, suggesting that this function of PVF-1 is permissive.

It was established previously that PVF-1 can substitute for VEGF in vertebrate angiogenesis (Tarstano et al., 2006). To determine whether the converse is true, we introduced into the pvf-1(ev763); plx-1(ev724) double mutant a transgene array in which the myo-3 promoter drives body wall muscle expression of mouse VEGF-164. We found this array can rescue the pvf-1(ev763)-enhanced ray 1 defects of plx-1(ev724) (Fig. 4, lines 3,4,9). These results suggest that mouse VEGF164 can functionally substitute for C. elegans PVF-1 to position ray 1.

PVF-1 normally inhibits UNC-6 and UNC-40 signalling

The ability of pvf-1(ev763), which fails to cause ray 1 defects on its own, to enhance plx-1(ev724) suggests that PVF-1 is required for a

The plx-1(ev724) ray 1 anterior displacement phenotype is enhanced by the pvf-1(ev763)

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redundant signal that acts in parallel to PLX-1. We therefore looked for genetic interactions between pvf-1(ev763) and mutations in genes that encode components of the UNC-6 signalling pathway. We expected pvf-1(ev763) to fail to enhance or suppress unc-6(ev400) if PVF-1 is required solely in the UNC-6 pathway for ray 1 positioning. However, we found instead that the penetrance of ray 1 severe displacements in unc-6(ev400) males was significantly suppressed by pvf-1(ev763), and that this suppression was reversed by pvf-1(+) driven by the 1900 nucleotide pvf-1 5’ UTR/promoter (Fig. 4, lines 10-12). Suppression in this case suggests that pvf-1(ev763) causes an effective gain of function in some mechanism required for normal ray 1 positioning that compensates for the unc-6 mutant deficit. As we know of only two signalling mechanisms involved in preventing this ray 1 phenotype (UNC-6/UNC-40 and SMP-1/PLX-1), and we have found that PVF-1 functions in parallel with PLX-1 to prevent ray 1 displacement, it is likely that pvf-1(ev763) causes an effective gain of function downstream of UNC-6 in the UNC-6/UNC-40 signal transduction mechanism involved in ray 1 positioning. Consistent with this idea, pvf-1(ev763) did not significantly enhance or suppress unc-40(ev1430), a predicted null mutant (Chan et al., 1996) for ray 1 defects (Fig. 4, lines 14,15), suggesting that PVF-1 functions in the same pathway as UNC-40. Furthermore, pvf-1(ev763) suppression of the unc-6(ev400) defects was largely reversed by unc-40(ev1430) (Fig. 4, lines 11,13) to about the same penetrance as unc-6(ev400) (Fig. 4, line 10) and the unc-40(ev1430); unc-5(e53); unc-6(ev400) triple (see Dalpé et al., 2012), as expected if the putative gain of function is downstream of UNC-6 and depends on UNC-40. We conclude that pvf-1(ev763) is likely to cause an effective gain of function in UNC-40 or an UNC-40-dependent effector – a gain of function that can enhance a plx-1 deficit for ray 1 defects, yet can bypass the need for UNC-6 function in this permissive context. As the suppression requires the presence of UNC-40, these data suggest that PVF-1 normally inhibits rather than promotes UNC-6/UNC-40 signalling to prevent the ray 1 displacement. No obvious visible effects on the levels of an unc-40::gfp translational reporter were observed (G.D., unpublished results), but these observations do not rule out regulation of UNC-40 levels by PVF-1.

The seemingly contradictory ability of pvf-1(ev673) to mimic both an unc-40 loss of function in one context (e.g. enhancement of the plx-1 mutant ray 1 defects, see Fig. 4, lines 2-4,14,16) and an effective unc-40 gain of function in another context (suppression of the unc-6 mutant ray 1 defects, see Fig. 4, lines 10,11) can be reconciled by data showing that unc-40(+):gfp multicyclop arrays such as evIs103 (Fig. 4, line 18) also enhance plx-1(ev724) (Fig. 4, lines 17,19) and suppress unc-6(ev400) (Fig. 4, lines 10,20,21) (for more
is a possible target for inhibition by PVF-1 [although there is no evidence (Fig. 4, lines 25,26), suggesting that UNC-5, like UNC-40, functions redundantly with PLX-1 (see Dalpé et al., 2012); however, there was no apparent suppression of the strong ray 1 defect (Fig. 4, lines 23,24). This could result from a minor role for UNC-5 in ray 1 positioning because it functions redundantly with PLX-1 (see Dalpé et al., 2012); however, there was no apparent suppression of the double mutant ray 1 defects by pvf-1(ev763) alone (Fig. 4, lines 25,26), suggesting that UNC-5, like UNC-40, is a possible target for inhibition by PVF-1 [although there is no precedent for believing enhanced UNC-5 activity can suppress unc-6(ev400), which is for UNC-40 activity (Dalpé et al., 2012)].

**PVF-1 inhibits CED-10-dependent, but not MIG-2-dependent UNC-40 signalling, to prevent anterior displacement of ray 1**

We previously identified the Rho/Rac small GTPase homologues MIG-2 and CED-10 as probable mediators of the semaphorin 1/PLX-1 signal transduction cascade involved in positioning ray 1 (Dalpé et al., 2004). As shown previously, mig-2(mu28) and ced-10(n1993) mutants have virtually no ray 1 defects (Fig. 5, lines 1-3) but can significantly enhance the ray 1 defects of pvf-1(ev724) mutants (Fig. 5, lines 4-6). This suggests they each affect a signal transduction pathway that functions redundantly (and therefore in parallel) with the PLX-1 pathway to prevent ray 1 defects, the prime candidate being the UNC-6/UNC-40 pathway (Dalpé et al., 2012). We have also found that pvf-1(ev763), which alone does not cause ray 1 defects and does not significantly enhance ced-10(n1993) (Fig. 5, lines 3,7,8), nevertheless does enhance mig-2(mu28), causing synthetic ray 1 anterior displacements in about half of the double mutant animals (Fig. 5, lines 2,7,9). Furthermore, we showed previously that the severe hypomorph unc-73(rh40) induced 84% ray 1 severe anterior defects, suggesting that UNC-73/Rho family GEF functions to a large extent in both PLX-1 and UNC-40 signalling in this context. Considered together, the above results are consistent with a model in which (1) MIG-2 and CED-10 function redundantly with one another and with PLX-1, (2) both largely require UNC-73/GEF for their function, and (3) PVF-1 inhibits CED-10-dependent but not MIG-2-dependent UNC-40 signalling to prevent ray 1 displacement in certain genetic contexts (such as a plex-1 mutant).

**Fig. 5. Genetic interactions between unc-6, pvf-1, plex-1, ced-10, mig-2, unc-73 and transgene arrays expressing a ced-10 gain of function [ced-10(G12V)].** All strains carry the him-5(e1490) mutation, which serves as the wild-type control. Corresponding raw data are presented in supplementary material Table S2. The frequencies and standard errors for severe (solid bar) anterior ray 1 displacements (left and right sides of the male tail considered independently) are shown for a variety of mutant strains. Standard errors for the proportion of animal sides manifesting the ray 1 severe anterior phenotype were calculated assuming a binomial distribution of the same sample size and the observed proportion as mean. Horizontal bars connected by a vertical bar indicate statistical comparisons that were made between the asterisked strain in the group with each of the other strains of the group. The null hypothesis is that the asterisked strain has the same penetrance as each of the strains to which it was compared. All P values, except the three shown, were <0.0002.
A CED-10 gain of function suppresses unc-6 mutant ray 1 defects

The finding that pvf-1(ev763) enhances a mig-2 deficit but not a ced-10 deficit raises the possibility that the effective gain of function in UNC-40 activity caused by pvf-1(ev763) requires CED-10 but not MIG-2 to induce ray 1 defects. The suppression of unc-6(ev400) ray 1 defects by pvf-1(ev763) does at least partially depend on CED-10, as shown by the relatively higher penetrance of ray 1 defects in the unc-6(ev400);pvf-1(ev763);ced-10(n1993)/+ triple mutant compared with the unc-6(ev400);pvf-1(ev763) double (Fig. 5, lines 11,12). We could not test the unc-6(ev400);pvf-1(ev763);ced-10(n1993) triple mutant because it is lethal; however, an extra-chromosomal transgene array [evEx434[ram-5p::ced-10(G12V)] carrying a gain-of-function mutant ced-10 cDNA driven by a ram-5 ray structural cell-specific promoter (Yu et al., 2000) does not display any ray 1 anterior displacement defects in a control him-5(e1490) genetic background, but does enhance plx-1(ev724) (Fig. 5, lines 4,13,15) and largely rescues unc-6(ev400) 1 defects (Fig. 5, lines 10,14) somewhat better than does the evIs103[unc-40(+/+)] multi-copy array (Fig. 4, lines 10,18,20,21). These results suggest that CED-10 normally functions downstream of UNC-40 in the ray 1 structural cell to transduce a permissive signal from UNC-6 to UNC-40 required to prevent the anterior displacement of ray 1, and that a CED-10 gain of function can bypass the need for UNC-6 signalling, at least as well as pvf-1(ev763) or an unc-40(+/+) multi-copy array can. These rescue results support a permissive role for UNC-6 in ray 1 positioning and identify CED-10 and not MIG-2 as a target of enhanced UNC-40 activity caused by pvf-1(ev763) (see also Dalpé et al., 2012).

C. elegans VERs (VEGF-related receptors) and vertebrate FLT and KDR VEGF receptors function in the PVF-1 signalling pathway for ray 1 positioning

Because the vertebrate VEGF receptors are able to mediate VEGF-like signalling by PVF-1 in vertebrates (Tarsitano et al., 2006), we wondered whether putative vascular endothelial growth factor receptors (VERs) of C. elegans could also mediate PVF-1 signalling to position ray 1 in C. elegans males. Four ver genes that encode a family of four RTKs structurally related to VEGF receptors (VEGFRs) were identified previously (Popovici et al., 2002) and reported to express in specialised cells of neural origin such as glial support cells of amphid and phasmid neurons (VER-1), in the chemosensory ADL neurons (VER-2), and in the ALA neurons (VER-3) of C. elegans (Popovici et al., 2002). None of the putative null deletion alleles: ver-1(ok1738), ver-2(ok897), ver-3(gk227) or ver-4(ok1079) (supplementary material Fig. S1) caused a significant male phenotype (Fig. 6, lines 1-5); however, each ver mutation significantly enhanced plx-1(ev724) defects to roughly the same extent that pvf-1(ev763) did (Fig. 6, lines 6-12). Furthermore, ver-1, ver-2 and ver-4 mutations each suppressed unc-6(ev400) ray 1 defects to approximately the same extent as did pvf-1(ev763) (Fig. 6, lines 13-17). We could not test ver-3(gk227) for this phenotype because the unc-6(ev400) ver-3(gk227) double mutant is lethal.

The similar penetrance of the ver mutant defects in two functional assays for ray 1 positioning [enhancement of plx-1(ev724) and suppression of unc-6(ev400)] raises the possibility that the VERs function interdependently (possibly as a higher-order heteromultimer, see Discussion) and in the same signalling pathway.

![Genetic interactions between pvf-1, ver-1, ver-2, ver-3, ver-4, plx-1, unc-6 and transgene arrays expressing VER-1 or human VEGF receptors (hKDR and hFLT). All strains carry the him-5(e1490) mutation, which serves as the wild-type control. The frequencies and standard errors for severe (solid bar) anterior ray 1 displacements (left and right sides of the male tail considered independently) are shown for a variety of mutant strains. Corresponding raw data are presented in supplementary material Table S3. Standard errors for the proportion of animal sides manifesting the ray 1 severe anterior phenotype were calculated assuming a binomial distribution of the same sample size and the observed proportion as mean. Horizontal bars connected by a vertical bar indicate statistical comparisons that were made between the asterisked strain in the group with each of the other strains of the group. The null hypothesis is that the asterisked strain has the same penetrance as each of the strains to which it was compared. All P values, except the three shown, were <0.0002.](image-url)
as PVF-1 to position ray 1. To examine the latter possibility, we made a ver-1(ok1738) pvf-1(ev763); plx-1(ev724) triple mutant and found ver-1 and pvf-1 mutations together enhance the plx-1 mutant defects to the same extent as the individual ver-1 and pvf-1 mutations (Fig. 6, lines 6-9, 18). We conclude that VER-1 functions in the same pathway as PVF-1 to prevent ray 1 defects.

UNC-40 and PLX-1 function in the ray support cell to position ray 1, as determined by rescue of the ray 1 defect using the ram-3 UTR/promoter (Yu et al., 2000) to drive expression of unc-40(+) and plex-1(+) (Dalpé et al., 2012). We have now found that the ver-1(ok1738); plex-1(ev724) double mutant was rescued for the ver-1 mutant enhancement of plex-1(ev724) ray 1 defects by lin-32 promoter-driven ver-1(+) when compared with controls (Fig. 6, lines 2,6,9,18,19), suggesting that VER-1 could function in the same cells as UNC-40 and PLX-1 for ray 1 positioning. This is supported by the support cell focus of CED-10(G12V) activity reported above.

As PVF-1 can bind human VEGFR1 (FLT) and VEGFR2 (KDR) (Tarstianato et al., 2006), we wondered whether human FLT and KDR cDNAs could substitute for CeVER-1 in rescue of the ray 1 defect. We found that plex-1(ev724); ver-1(ok1738) double mutant animals carrying lin-32p::hKDR (Fig. 6, lines 18,20) or lin-32p::hFLT (Fig. 6, lines 18,21) transgene arrays can rescue to nearly the same extent as the lin-32p::ver-1(+) array (Fig. 6, lines 18,19). These results demonstrate an evolutionary conservation of VEGF signalling core components in positioning ectodermally derived cells in an animal devoid of a cardiovascular system.

**DISCUSSION**

The VEGF/PDGF growth factors function in cardiovascular development across many vertebrate species, including humans. These factors induce endothelial cell differentiation and guide their migrations as they undergo morphological changes to form blood vessels (Rousseau et al., 2000; Poole et al., 2001; Zachary et al., 2005; Lamalice et al., 2006; Koch and Claesson-Welsh, 2012). Their functions outside angiogenesis are not as well characterised; however, *Drosophila* PVF-1 has a role in border cell migration in *Drosophila* ovaries (McDonald et al., 2003; Mackenzie and Ruhrberg, 2012) and VEGF can act as a guidance cue by attracting commissural axons to the floor plate of the spinal cord (Ruiz de Almodovar et al., 2011) and by guiding migration of nerve cell soma commissural axons to the floor plate of the spinal cord (Ruiz de Ruhrberg, 2012) and VEGF can act as a guidance cue by attracting mutant enhancement of *pvf-1(ev763)* double mutant was rescued for the multiple mutant combinations and the Rho family GTPase CED-10, but not MIG-2. The logic context. Here, we present strong evidence that PVF-1 (acting thus PVF-1 likely regulates UNC-40 and UNC-5 signalling in this PLX-1 to prevent anterior displacement of ray 1 (Dalpé et al., 2012), so that mirror VEGF roles in vertebrates. Therefore appears to be an excellent candidate VEGF homologue whose developmental role is still undefined, but could act in ways that PVF-1 mimics an effective UNC-40 gain of function roughly equivalent to that caused by a single dose of the multi-copy unc-40(+) array.

We hypothesised previously that the *evlsI03*unc-40(+) multi-copy array creates a partially UNC-6 dependent UNC-40 over-activity that interferes with UNC-6-UNC-40 signal transduction – an activity that is reduced below some threshold for interference by eliminating the activation of UNC-40 by UNC-6 (or by halving the dose of *evlsI03*), while retaining enough UNC-40 overactivity to partially bypass the need for UNC-6. *pvf-1(ev763)* is predicted to cause some UNC-40-interfering overactivation of UNC-40 signal transduction in this context – enough to enhance a plex-1 mutant, but not enough to cause a ray 1 defect on its own. Substantial support for this hypothesis is provided by the finding that the ability of *pvf-1(ev763)* to rescue *unc-6(ev400)* for ray 1 defects depends on UNC-40 activity.

Double mutant analyses demonstrate that, like PVF-1, UNC-5 and CED-10 function redundantly with PLX-1 and in the same pathway with one another. The *ced-10(gf)* array mimics *pvf-1(ev763)* in its abilities to enhance *plex-1(ev724)* and to suppress *unc-6(ev400)* for ray 1 defects, suggesting that CED-10 acts downstream of UNC-6 and by inference UNC-40 in this context. The finding that *unc-40(e1430)*, a putative null allele (Chan et al., 1996), causes significantly more penetrant ray 1 defects than *unc-5(e53)*, suggests that UNC-40 function in ray 1 positioning is at least partially UNC-5 independent. Moreover, the ability of *unc-5(e53)* to revert the suppression of *unc-6(ev400)* by *pvf-1(ev763)*, but less so than the putative *unc-40(e1430)* null allele (Chan et al., 1996), suggests that UNC-5 activity or UNC-40-dependent UNC-5 activity is also regulated by PVF-1 in the same way but possibly not to the same extent that UNC-5-independent UNC-40 activity is.

Taken together, the above results suggest the following molecular model for the way PLX-1, UNC-40 and PVF-1 function to prevent the anterior displacement of ray 1 (Fig. 7). PVF-1 is made and secreted by body wall muscles and binds to one or more VER receptors on the ray 1 glia-like structural cell. The VER receptors transduce a signal to the UNC-40 signalling pathway (possibly including some UNC-5-dependent UNC-40 signalling) that inhibits CED-10 (known to mediate UNC-40 axon guidance signalling) (see Gita et al., 2003), possibly at the level of UNC-40 or CED-10 GTPase function, but not MIG-2-dependent UNC-40 signalling. This inhibition helps set a level of UNC-6 signalling that is necessary for proper ray 1 positioning in certain genetic contexts (e.g. in a plex-1 mutant), but not in the wild-type laboratory strain. It remains to be determined therefore what selective pressures caused the evolution of the inhibitory interaction between PVF-1 and UNC-40 signalling revealed here.

While these studies were in progress, it was reported that ver-1 deletions prevent the dauer-induced structural remodelling of the anterior tip of the glia-like amphid socket cell (Procko et al., 2011),
which encircles the sensory endings of the amphid sensory neurons (Perkins et al., 1986), just as the ray structural cells are glia-like cells that encircle the sensory endings of the ray neurons. As the ray 1 structural cell is most intimately involved in determining the positioning of ray 1 in the male tail.

It is somewhat surprising that, in combination with plx-1(ev724), the ver mutations each cause roughly the same penetrance of ray 1 defects as pvf-1(ev763). This finding does not readily fit with the known function of mammalian VEGF receptors as homodimers and heterodimers (Huang et al., 2001; Nilsson et al., 2010). Our observations suggest that either the pvf-1 mutation is not null, or there is PVF-1-independent VER function in the context of ray 1 positioning, or the VER receptor comprises a higher order multimer, requiring all four VER subunits for function. Further study of the ver genes should help distinguish between these possibilities.

The ability of mammalian VEGF and VEGF receptors to rescue corresponding mutants of C. elegans in the context of ray 1 positioning demonstrates that the molecular function of these proteins is conserved from worms to humans. Although the overall logic of ray 1 positioning in C. elegans and wiring of the vertebrate vascular system are clearly different, steps in these processes could be mechanically related. The place to look for relevance of our findings would be netrin-mediated angiogenic functions involving DCC, the vertebrate homologue of UNC-40 (Keino-Masu et al., 1996). Most data regarding netrin function in angiogenesis show that UNC-5B (and not DCC) activation by netrin 1 regulates angiogenesis by preventing filopodial extension of endothelial cells to limit branching morphogenesis (Suchting et al., 2006), whereas there are limited data suggesting that DCC is expressed by endothelial cells (Nguyen and Cai, 2006) and only correlative evidence that netrin 4 might function through DCC to enhance angiogenesis in a mouse cerebral ischemia model (Hoang et al., 2009). However, there is reasonable evidence that netrin 4, which has anti-angiogenic properties, could function in this context by binding the DCC paralogue neogenin, which then recruits UNC-5B (Wilson et al., 2006; Qin et al., 2007; Lejmi et al., 2008). As our results leave open the possibility that UNC-5-dependent UNC-40 signalling is negatively regulated by VEGF signalling, it is possible that the proposed anti-angiogenic effects of netrin 4 signalling could be regulated by VEGF.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
G.D. performed all of the genetic experiments reported in this manuscript. M.T. and G.P. began this project and provided expression data for pvf-1. H.Z. made constructs and transgenic animals for this project. J.C. co-wrote the manuscript with G.D.

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