Netrin/UNC-6 triggers actin assembly and non-muscle myosin activity to drive dendrite retraction in the self-avoidance response.

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SUMMARY

Dendrite growth is constrained by the self-avoidance response but the downstream pathways that balance these opposing mechanisms are unknown. We have proposed that the diffusible cue UNC-6(Netrin) is captured by UNC-40 (DCC) for a short-range interaction with UNC-5 to trigger self-avoidance in the *C. elegans* PVD neuron. Here we report that the actin-polymerizing proteins UNC-34(Ena/VASP), WSP-1(WASP), UNC-73(Trio), MIG-10(Lamellipodin) and the Arp2/3 complex effect dendrite retraction in the self-avoidance response mediated by UNC-6(Netrin). The paradoxical idea that actin polymerization results in shorter rather than longer dendrites is explained by our finding that NMY-1 (non-muscle myosin II) is necessary for retraction and could therefore mediate this effect in a contractile mechanism. Our results also show that dendrite length is determined by the antagonistic effects on the actin cytoskeleton of separate sets of effectors for retraction mediated by UNC-6(Netrin) versus outgrowth promoted by the DMA-1 receptor. Thus, our findings suggest that the dendrite length depends on an intrinsic mechanism that balances distinct modes of actin assembly for growth versus retraction.
INTRODUCTION

Dendritic arbors are defined by the balanced effects of outgrowth which expands the structure versus retraction which constrains the size of the receptive field. Microtubules and filamentous actin (F-actin) are prominent dendritic components and have been implicated as key drivers of dendritic growth and maintenance by the finding that treatments that perturb cytoskeletal dynamics may also disrupt dendritic structure [1-3]. For many neurons, dendritic growth is highly exuberant with multiple tiers of branches projecting outward from the cell soma. Ultimately, growth may be terminated by external cues. For example, neurons with similar functions are typically limited to separate domains by tiling mechanisms in which mutual contact induces dendrite retraction. The related phenomenon of self-avoidance is widely employed to prevent overlaps among sister dendrites arising from a single neuron [4]. Homotypic interactions between the membrane components Dscam and protocaderins can mediate the self-avoidance response [5-7]. Surprisingly, in some instances, soluble axon guidance cues and their canonical receptors are also required. For example, self-avoidance for the highly-branched Purkinje neuron depends on both protocadherins and also repulsive interactions between sister dendrites decorated with the Robo receptor and its diffusible ligand, Slit [8]. In another example, we have shown that UNC-6/Netrin and its cognate receptors, UNC-5 and UNC-40/DCC, mediate self-avoidance for PVD nociceptive neurons in C. elegans [9]. Although multiple cell-surface interactions are now known to trigger self-avoidance, little is known of the downstream effectors that drive dendrite retraction in this mechanism.

The PVD neurons, one on each side of the body, build complex dendritic arbors through a series of successive 1°, 2°, 3° and 4° orthogonal branching events [10][11,12]. Self-avoidance ensures that adjacent 3° branches do not overgrow one another[9,11] (Figure 1). Our previous results
suggested a novel mechanism of self-avoidance in which UNC-40/DCC captures UNC-6/Netrin at the PVD cell surface and then triggers retraction by interacting with UNC-5 on the neighboring 3° branch. UNC-40/DCC may also effect self-avoidance by acting in a separate pathway that does not involve UNC-6/Netrin[9]. Here we describe a cell biological model of dendrite retraction in the PVD self-avoidance response that depends on actin polymerization.

The structure of the actin cytoskeleton is controlled by a wide array of effector proteins that regulate specific modes of actin polymerization. For example, Ena/VASP enhances F-actin elongation at the plus-end [13] and the Arp2/3 complex functions with WASP (Wiskott-Aldrich syndrome protein) and the Wave Regulatory Complex (WRC) to promote F-actin branching[14].

Upstream regulators of WASP and the WRC include Rho family GTPases[15] and their activators, the GEFs (Guanine nucleotide Exchange Factors) UNC-73/Trio[16,17] and TIAM (T-cell Lymphoma Invasion and Metastasis Factor) [18,19]. Members of the Lamellipodin/Lpd family recruit Ena/VASP to localize F-actin assembly at the leading edge of migrating cells[20].

The fact that all of these components (UNC-34/Ena/VASP, Arp2/3 complex, WSP-1/WASP, WRC, UNC-73/Trio, TIAM/TIAM-1, MIG-10/lamellipodin) have been previously shown to function in C. elegans to mediate axon guidance underscores the key role of the actin cytoskeleton in growth cone steering [16,19,21-24].

Our results show that Ena/VASP functions downstream of UNC-5 to mediate PVD self-avoidance. Genetic evidence detecting roles for the Arp2/3 complex and its upstream regulator, WASP/WSP-1, indicates that the formation of branched actin networks may contribute to dendrite retraction. A necessary role for actin polymerization is also indicated by the defective PVD self-avoidance
response of mutants with disabled UNC-73/Trio or MIG-10/lamellipodin. We show that PVD self-avoidance also requires NMY-1/non-muscle myosin II which we propose effects dendrite retraction in a contractile mechanism that drives the reorganization of the nascent actin cytoskeleton. The necessary role for myosin could explain how actin polymerization can result in shorter rather than longer dendrites in the self-avoidance response. Thus, we propose that UNC-6/Netrin triggers self-avoidance by simultaneously stimulating actin assembly and non-muscle myosin activity in $3^o$ dendrites.

Because dendrite growth depends on actin polymerization, our finding that the actin cytoskeleton is also necessary for dendrite self-avoidance points to different modes of actin polymerization for growth vs retraction. Recent work has shown that a multicomponent complex involving the PVD membrane proteins DMA-1 and HPO-30 drives dendritic growth by linking the PVD actin cytoskeleton to adhesive cues on the adjacent epidermis[25]. Dendrite elongation, in this case, depends on the WRC and TIAM-1, both of which are known to promote actin polymerization. In contrast, our work has revealed that effectors of actin polymerization that are required for $3^o$ branch self-avoidance (e.g., UNC-34/Ena/VASP, MIG-10/lamellipodin, UNC-73/Trio, Arp2/3 complex, WSP-1/WASP) are not necessary for $3^o$ dendrite outgrowth. Additionally, we show that UNC-6/Netrin signaling antagonizes the DMA-1-dependent mechanism of dendritic growth. These findings are significant because they suggest that overall dendrite length is defined by the relative strengths of opposing pathways that utilize separate sets of effectors to differentially regulate actin polymerization for either growth or retraction.
RESULTS

UNC-6/Netrin mediates PVD sister dendrite self-avoidance.

To visualize PVD morphology, we utilized a previously characterized GFP marker driven by a PVD-specific promoter ($PVD$:GFP) (Figure 1A-B)[11]. Each PVD neuron adopts a stereotypical morphology characterized by a series of orthogonal junctions between adjacent branches: $1^\circ$ dendrites extend laterally from the PVD cell soma; each $2^\circ$ dendrite projects along the dorsal-ventral axis to generate a T-shaped junction comprised of two $3^\circ$ dendrites each with either an anterior or posterior trajectory; terminal $4^\circ$ dendrites occupy interstitial locations between the epidermis and body muscles. The net result of this branching pattern is a series of tree-like structures or menorahs distributed along the $1^\circ$ dendrite. In contrast to the highly branched dendritic architecture, a single axon projects from the PVD cell soma to join the ventral nerve cord[10-12]. Despite the complexity of this network, dendrites rarely overlap (~2%) in the mature PVD neuron (Figure 1A). Self-avoidance depends on an active process in which sister PVD dendrites retract upon physical contact with one another (Figure 1C). In the wild type, $3^\circ$ branches arising from adjacent menorahs initially grow outward toward one another but then retract after mutual contact. The net result is a characteristic gap between the tips of $3^\circ$ dendrites in neighboring menorahs[11]. We have previously shown that this outcome depends in part on the diffusible cue UNC-6/Netrin and its canonical receptors, UNC-40/DCC and UNC-5[9]. Mutations that disable unc-6, for example, result in a significant increase in the fraction of overlapping $3^\circ$ dendrites (arrowheads, Figure 1B, Figure 2D). Similar self-avoidance defects were observed for mutants of either unc-40 or unc-5. Our results are consistent with a model in which UNC-40 captures UNC-6 for contact with UNC-5 at the tips of adjacent $3^\circ$ dendrites (Figure 1D)[9]. Here we address the
question of how the activated UNC-5 receptor then modifies the actin cytoskeleton to drive dendrite retraction.

**Constitutively active UNC-5 drives dendrite retraction:**

We have proposed that UNC-6 triggers dendrite retraction by activating UNC-5[9]. This model predicts that a constitutively active form of UNC-5 should result in a ‘hyper’-retraction phenotype. To test this idea, we attached a myristoylation sequence to the cytoplasmic domain of UNC-5[26]. The resultant MYR::UNC-5 protein is effectively tethered to the cytoplasmic membrane thus inducing activation of the UNC-5 intracellular domain. Expression of MYR::UNC-5 in PVD results in substantially shorter 3° dendrites in comparison to wild type at the late L3 stage (Figure 2A-C). This phenotype suggests that constitutive activation of UNC-5 prevents normal outgrowth of 3° dendrites. We tested this idea by scoring the dynamic status of 3° dendrites. To quantify movement, we measured the distance from a fiduciary point to the tip of a 3° dendrite at intervals over time (delta t = 240 sec) (Figure 2E) (Movies S1-S2). Examples of these measurements are shown in Figure 2E. We observed that wild-type 3° dendrites typically display saltatory movement with periodic bouts of extension and retraction that result in net elongation. In contrast, 3° dendrites of PVD neurons expressing MYR::UNC-5 grow more slowly and rarely show periods of active extension and retraction (Figure 2E and 2F). The higher overall motility of 3° dendrites in wild-type vs MYR::UNC-5 is evident from a comparison of the standard deviation of the net movement of 3° dendrites from a fiduciary point in each strain (Figure 2F). Because MYR::UNC-5 lacks the UNC-5 extracellular ligand-binding domain, it should function independently of *unc-6*. We confirmed this prediction by demonstrating that MYR::UNC-5 rescues the self-avoidance defects observed in an *unc-6* mutant background (Figure 2G). Together, these results are consistent with
the hypothesis that UNC-6/Netrin triggers the self-avoidance response by activating the
downstream UNC-5 receptor to drive dendrite retraction.

**UNC-34/Ena/VASP mediates self-avoidance in actin-containing PVD dendrites.**

To explore potential modifications of the PVD cytoskeleton as effectors of the UNC-6/Netrin-dependent self-avoidance response, we used live cell markers to visualize actin (PVD::ACT-1::GFP) and microtubules (pdes-2::TBA-1::mCherry) in PVD[27]. We observed a robust ACT-1::GFP signal throughout the PVD neuron including the axon and all dendrites (Figure 3A). In contrast, the TBA-1::mCherry signal was strongest in the PVD axon and 1° dendrite. This result confirms an earlier report suggesting that dendritic microtubules are most abundant in the 1° branch (Figure 3A) [27]. We thus considered the possibility that the self-avoidance response could depend on the regulation of actin dynamics. To test this idea, we examined the PVD self-avoidance phenotype for a mutation that disables the actin binding protein, Ena/VASP. Ena/VASP promotes the elongation of actin filaments and the *C. elegans* homolog UNC-34[28] is enriched in a gene expression profile of PVD neurons[11]. A strong loss-of-function allele of unc-34/Ena/VASP [29] alters overall PVD morphology (e.g., truncated or misplaced 1° dendrite, fewer 2° branches) (Figure 3B-C, Figure S1) but does not produce the severe lateral branching defects arising from mutations in other regulators of actin polymerization (e.g, *tiam-1*) that promote dendrite outgrowth[25]. Notably, PVD neurons in unc-34 mutant animals showed robust self-avoidance defects (Figure 3B-C). PVD-specific expression of mCherry-tagged UNC-34 protein rescued this unc-34 mutant phenotype suggesting that unc-34 function is cell-autonomous for the self-avoidance response (Figure 3C). unc-34 mutant 1° and 2° branch defects were not rescued by PVD expression of UNC-34, however, which is indicative of UNC-34 function in other nearby cell types.
that influence PVD morphogenesis (Figure 3B, Figure S1). A downstream role for unc-34 in the
UNC-6/Netrin pathway is consistent with our finding that the Unc-34 self-avoidance defect is
epistatic to the hyper-retraction phenotype of MYR::UNC-5 (Figure 3C).

For visualizing UNC-34/Ena/VASP in developing dendrites, we labeled UNC-34 with mCherry
and co-expressed it in PVD (PVD::mCherry::UNC-34) with LifeAct::GFP to detect actin-
containing structures. mCherry::UNC-34 is functional because it rescues the unc-34 self-
avoidance defect (Fig 3C). iSIM super resolution imaging detected punctate mCherry::UNC-34
throughout the PVD dendritic architecture. Notably, mCherry::UNC-34 puncta are frequently
detected at the tips of 3° dendrites (Figure 4A-C), a finding consistent with the observed distal
location of Ena/VASP in F-actin containing filopodial structures [30,31]. We used time-lapse
imaging to detect potential trafficking of mCherry::UNC-34 (Movie S6). Kymographs detected
anterograde as well as retrograde movements of mCherry::UNC-34 in 3° dendrites near the tip
(green arrowheads) whereas stationary puncta were characteristically located in the shaft region
(red arrowheads) (Figure 4C-D). These results are consistent with the idea that UNC-
34/Ena/VASP is delivered to 3° dendrites where it mediates actin assembly for branch retraction
in the self-avoidance response.

Mutations that disable actin-polymerizing proteins disrupt dendrite self-avoidance.
Genetic tests of additional regulators of actin polymerization also revealed PVD self-avoidance
phenotypes. Lamellipodin (Lpd) interacts with ENA/VASP at the tips of filopodia and
lamellopodia to extend actin filaments. Mutants of mig-10/Lpd display robust PVD self-avoidance
defects (Figure 3B, E). Similarly, self-avoidance is disrupted by a mutation that eliminates the Rac
GEF activity of UNC-73/Trio, a potent regulator of actin dynamics[16]. We note that both mig-10/Lpd and unc-73/Trio mutants also show other defects in PVD morphology (e.g., displaced 1° dendrite, fewer 2° branches) (Figure S1) but do not impair outgrowth of higher order dendrites as was also observed for unc-34 (Fig. 3). To test for potential roles for branched actin polymerization, we examined a null allele of wsp-1/WASP (Wiskott-Aldrich Syndrome protein) and observed defective PVD self-avoidance. If wsp-1/WASP functions in a common pathway with unc-6, then the self-avoidance defect of the double mutant, wsp-1; unc-6, should be no more severe than that of either wsp-1 or unc-6 single mutants. We confirmed this prediction by scoring PVD self-avoidance in wsp-1; unc-6 mutant animals (Figure 3D). Because WASP is known to activate the Arp2/3 complex[15,32], we also performed RNAi knock down of the conserved Arp2/3 component ARX-5/p21 and detected significant PVD self-avoidance defects (Figure 3E). The canonical roles of WASP and the Arp2/3 complex suggest that self-avoidance depends on the formation of branched F-actin networks. Recent studies have shown that actin is required for PVD dendrite outgrowth[25]. Here our genetic results point to the paradoxical idea that elongation and branching of F-actin filaments are also necessary for dendrite retraction and that this mechanism is activated by UNC-6/Netrin in the self-avoidance response.

The actin cytoskeleton is highly dynamic in growing and retracting PVD dendrites.

To test the idea that the actin cytoskeleton is differentially modulated for both outgrowth and retraction we expressed LifeAct::GFP in PVD neurons to monitor actin dynamics during dendrite morphogenesis. Although LifeAct::GFP binds both filamentous actin (F-actin) and monomeric G-actin with high affinity, bright LifeAct::GFP-labeled foci typically correspond to F-actin-containing structures[33]. We observed that the LifeAct::GFP signal was consistently brightest
nearest the tips of growing dendrites in comparison to internal dendritic regions (Fig. 5B, C) as also reported in other studies [25,34]. Time-lapse imaging of PVD::LifeAct::GFP by spinning disk confocal microscopy during the late L3/early L4 stage detected striking, dynamic fluctuations in the LifeAct::GFP signal (Movie S3). We quantified this effect by comparing time-dependent changes in LifeAct::GFP fluorescence versus that of a PVD::mCherry cytosolic marker. The relatively constant intensity of PVD::mCherry signal in these experiments favors the idea that the observed changes in LifeAct::GFP fluorescence could be due to actin dynamics in growing dendrites (Figure 5A, D-E).

Because genetic knockdown of actin polymerizing proteins (UNC-34/Ena/VASP, MIG-10/Lpd, UNC-73/Trio, WSP-1/WASP, ARX-5/P21) (Figure 3) disrupts self-avoidance and thus suggests that F-actin is required, we monitored LifeAct::GFP in 3° dendrites during the period (L3-L4 transition) in which UNC-6/Netrin mediates dendrite retraction[9]. Time-lapse imaging detected dynamic fluctuations in LifeAct::GFP fluorescence in these distal regions (Figure 6) (Movie S3). For these measurements, the LifeAct::GFP signal near the tips of 3° dendrites was quantified at 1-1.25 minute intervals over a 10 minute period spanning at least one contact event. A cytoplasmic mCherry marker was used to detect contact events as instances in which the measured gap between left and right 3° dendrites approached zero. In some cases, a burst of LifeAct::GFP signal was detected in at least one of the adjacent dendrites during the contact period suggesting a role for actin polymerization in retraction (Figure 6 A, B) (Movies S4 and S5). In other instances, however, we did not observe an increase in LifeAct::GFP fluorescence with contact (Figure 6 C, D) and no correlation was detected in quantitative comparisons (n = 8) between the LifeAct::GFP signal before versus after contact (Figure 6F). An alternative approach for quantifying LifeAct::GFP in
3° dendrites at shorter time intervals (35 s) [34] also did not detect a consistent correlation between LifeAct::GFP fluorescence intensity and dendrite retraction (Figure S2). The simplest explanation of these results is that actin polymerization drives both outgrowth and retraction which are tightly coupled over time and thus difficult to resolve as separate events.

NMY-1/Non-muscle myosin II drives dendrite retraction in the self-avoidance mechanism.

Our results indicate that F-actin is the dominant cytoskeletal structure in growing PVD dendrites and that F-actin assembly is also likely required for dendrite retraction. In considering a mechanism to account for dendrite shortening, we hypothesized that a newly synthesized actin cytoskeleton could mediate retraction by providing a substrate for non-muscle myosin II to drive contraction. To test this idea, we examined loss-of-function alleles that disable the C. elegans non-muscle myosins, NMY-1 and NMY-2. This experiment determined that mutations in nmy-1 but not nmy-2 (data not shown) show PVD self-avoidance defects (Figure 7A, D). This effect is cell autonomous for nmy-1 because PVD-specific RNAi of nmy-1 also results in overlapping 3° dendrites and PVD-specific expression of GFP-tagged NMY-1 is sufficient to rescue the nmy-1 self-avoidance defect (Figure 7 B-D). We conclude that a specific non-muscle myosin, NMY-1, promotes dendrite retraction in the self-avoidance mechanism and could act by driving the translocation of F-actin bundles at the tips of 3° dendrites.

Phosphomimetic activation of MLC-4 shortens PVD 3° dendrites.

Non-muscle myosin II motor proteins are composed of myosin heavy chain (MHC) and light chains, the essential light chain (ELC) and regulatory light chain (RLC) [35,36]. Myosin motor activity is triggered by RLC phosphorylation that induces a conformational change to allow...
assembly of the myosin complex into active bipolar filaments (Figure 8A) [37]. Having shown that NMY-1/non-muscle myosin is required for self-avoidance, we next performed an additional experiment to ask if constitutive non-muscle myosin activity is sufficient to induce dendrite retraction. For this test, we expressed a phosphomimetic mutant of the MLC-4, the C. elegans RLC homolog. MLC-4/myosin regulatory light chain contains serine/S and threonine/T phosphorylation sites in a highly-conserved domain. Both S and T residues were mutated to Aspartate/D and the resultant phosphomimetic construct MLC-4DD was fused to GFP for transgenic expression in PVD (Figure 8B-C) [38] PVD neurons that expressed MLC-4DD showed significantly shorter 3° dendrites in comparison to wild type as well as a concurrent increase in the width of gaps between 3° dendrites from adjacent menorahs (Figure 8D-G). These phenotypic traits are consistent with the proposed role of non-muscle myosin in dendrite retraction and closely resemble that of PVD neurons that express MYR::UNC-5 which functions downstream of UNC-6/Netrin to trigger the self-avoidance response. Our additional finding that MLC-4DD rescues the self-avoidance defect of unc-6 mutants suggests that NMY-1/non-muscle myosin II also functions downstream of unc-6 for dendrite retraction (Figure 8H).

Antagonistic pathways regulate dendrite outgrowth vs self-avoidance.

Our time lapse imaging studies detected dynamic actin polymerization in growing PVD dendrites (Movie S3). This observation and the recent finding that PVD dendritic architecture is significantly perturbed by mutations in either a specific actin structural gene, act-4, or that disable the F-actin nucleating WAVE complex or TIAM-1/GEF, suggest that dendritic outgrowth depends on actin assembly[25]. Actin assembly is also likely required for dendrite retraction given our results showing that genetic knockdown of a separate set of actin-binding proteins (i.e., UNC-
34/Ena/VASP, MIG-10/Lpd, UNC-73/Trio, WSP-1/WASP, ARX-5/p21) (Fig. 3), blocks self-avoidance but not dendrite outgrowth [34]. A shared role for actin polymerization in both outgrowth and retraction argues that the net length of PVD dendrites must depend on an intrinsic mechanism that regulates actin assembly to balance these competing effects.

Our results suggest that UNC-6/Netrin signaling promotes dendrite retraction in a mechanism involving F-actin assembly. PVD dendritic outgrowth is driven by a separate pathway in which DMA-1, a membrane protein expressed in PVD, interacts with a multicomponent receptor anchored in the adjacent epidermis[39-43]. Dendritic growth depends on binding of DMA-1 and the claudin-like protein, HPO-30, to specific regulators of actin assembly (i.e., WAVE complex, TIAM-1/GEF) in the PVD cytoplasm[25,44]. These interactions may be modulated by the Furin KPC-1 which antagonizes DMA-1 surface expression in PVD [45-47]. kpc-1 activity is proposed to temporally weaken adhesion with the epidermis to facilitate changes in trajectory at specific stages in dendritic outgrowth [41,47]. For example, the tips of 3° branches typically execute a right-angle turn after the self-avoidance response to produce a 4° dendrite[11]. This morphological transition largely fails in kpc-1 mutants in which 3° dendrites elongate abnormally and show severe self-avoidance defects due to over-expression of DMA-1 [41,47]. Thus, we considered the idea that self-avoidance is achieved by the combined effects of kpc-1-dependent downregulation of DMA-1 and a separate mechanism that antagonizes outgrowth and requires UNC-6/Netrin. We performed a series of genetic experiments to test this model.

The hypomorphic loss-of-function allele, kpc-1(xr58), results in over-expression of DMA-1 and a consequent, robust self-avoidance defect with ~42% of sister 3° branches overlapping one another
This defect is not observed, however, in \textit{kpc-1/+} heterozygotes (Figure 9D). Similarly, self-avoidance in \textit{unc-6/+} heterozygous animals is indistinguishable from wild type (Figure 9D). Thus, a 2-fold reduction in gene dosage for either \textit{kpc-1} or \textit{unc-6} does not perturb self-avoidance. We reasoned, however, that if expression of UNC-6/Netrin and KPC-1 were simultaneously reduced by half, then self-avoidance should be at least partially disabled since both \textit{unc-6} and \textit{kpc-1} normally function to antagonize dendrite outgrowth. To test this prediction, we constructed double heterozygous mutants of \textit{kpc-1} and \textit{unc-6} (e.g., \textit{kpc-1/+; unc-6/+}) and quantified the self-avoidance phenotype. This experiment revealed that \textasciitilde30\% of adjacent sister $3^\circ$ dendrites fail to self-avoid in \textit{kpc-1/+; unc-6/+} animals which is significantly greater than that of either \textit{kpc-1/+} or \textit{unc-6/+} single mutants (Figure 9B, D). This genetic “enhancer” effect is consistent with the idea that both KPC-1 and UNC-6 function to limit overgrowth of $3^\circ$ dendrites. If this effect is due to elevated levels of DMA-1 arising from reduced KPC-1 activity in the \textit{kpc-1/+; unc-6/+} background, then a concomitant diminution of DMA-1 activity could restore normal self-avoidance. We tested this idea by constructing the triple heterozygote, \textit{kpc-1 +/+ dma-1; unc-6/+}, and detected robust suppression of the self-avoidance defect observed in \textit{kpc-1/+; unc-6/+} animals (Figure 9C, D). Thus, these results suggest that DMA-1-mediated adhesion to the epidermis antagonizes UNC-6/Netrin-driven dendrite retraction and that the relative strengths of these opposing pathways must be finely tuned to achieve the self-avoidance effect (Figure 9E). The key role of actin polymerization in both UNC-6/Netrin-mediated retraction and DMA-1-dependent outgrowth argues that this balancing mechanism likely regulates both modes of actin assembly.
Dendrites arising from the same neuron typically do not overlap. This phenomenon of dendrite self-avoidance is universally observed and derives from an active mechanism in which contact between sister dendrites triggers mutual retraction[4] As might be predicted for an event that depends on physical proximity, cell-surface associated proteins have been shown to mediate dendrite self-avoidance[34,48]. These include Dscam and protocadherins which are expressed in multiple alternative forms to distinguish self from non-self among contacting dendrites [5-7]. Self-avoidance for certain neurons can also depend on the interaction of diffusible cues with their cognate receptors. For example, we have shown that the axon guidance signal, UNC-6/Netrin, and its receptors, UNC-5 and UNC-40/DCC, function together to mediate self-avoidance for PVD sensory neurons in *C. elegans*. We have proposed that UNC-40/DCC captures UNC-6/Netrin on the membrane surface to facilitate repulsion through physical interaction with UNC-5 on PVD dendrites[9] (Figure 1D). Little is known, however of the downstream effectors of dendrite retraction in the self-avoidance response. Here we report that UNC-6/Netrin promotes actin-polymerization to drive dendrite retraction. Our finding that non-muscle myosin is also involved resolves the paradoxical observation that UNC-6/Netrin dependent actin polymerization shortens rather than lengthens PVD 3° dendrites. We propose that non-muscle myosin engages the F-actin cytoskeleton at the tips of PVD dendrites to drive retraction.

**Regulators of actin polymerization are required for dendrite self-avoidance.**

The proposed role for actin polymerization in the self-avoidance response derives from our finding that multiple regulators of F-actin assembly are necessary for efficient dendrite retraction. For
example, a mutation that disables UNC-34, the *C. elegans* homolog of Ena/VASP, disrupts PVD self-avoidance. Ena/VASP promotes actin polymerization by preventing capping proteins from blocking the addition of actin monomers to the plus-end of elongating actin filaments. In axon growth cones, Ena/VASP localizes to the tips of actin bundles in growing filopodia[13,31]. We observed punctate Ena/VASP localization in PVD dendrites and active trafficking from the cell soma (Figure 4). Our results showing that an *unc-34* mutant blocks the hyper-retraction phenotype of a constitutively active UNC-5 receptor (Figure 3) argues that *unc-34* functions downstream to mediate UNC-6/Netrin-dependent self-avoidance. Additional genetic evidence indicates that WSP-1/WASP also acts in a common pathway with UNC-6/Netrin (Figure 3). The established role for WASP of activating the Arp2/3 complex [49] is consistent with our finding that RNAi knockdown of *arx-5/p21*, a conserved Arp2/3 component, partially disables the self-avoidance mechanism (Figure 3). Dual roles for UNC-34 and WSP-1 in actin polymerization, as suggested here, are consistent with previous work indicating that Ena/VASP can activate Arp2/3 through direct interaction with WASP proteins[28,50-52]. Thus, taken together, our results suggest that Ena/VASP and WSP-1 coordinate the creation of branched actin networks at the tips of retracting PVD dendrites in the UNC-6/Netrin dependent self-avoidance response (Figure 9E).

A role for Ena/VASP in dendrite retraction, as have we have proposed here, parallels earlier findings of a necessary function for Ena/VASP in growth cone repulsion. For example, axon guidance defects arising from ectopic expression of UNC-5 in *C. elegans* touch neurons requires UNC-34/Ena/VASP[53], a finding confirmed by our observation that the dendritic shortening phenotype induced by MYR::UNC-5 is prevented by a loss-of-function mutation in *unc-34*. Ena/VASP is also required for axon repulsion mediated by Slit and its receptor Robo[21,54,55].
Although Ena/VASP function can be readily integrated into models of axonal attraction, the well-established role of Ena/VASP in F-actin assembly has been more difficult to rationalize in mechanisms of axonal repulsion which are presumed to involve actin depolymerization[13,56]. A partial explanation for this paradox is provided by a recent study showing that Slit, acting through Robo, induces transient filopodial outgrowth in migrating axonal growth cones and that this effect requires Ena/VASP[55]. The functional necessity for filopodial growth in this mechanism is unclear, but the central role of Ena/VASP-induced actin assembly in the overall repulsive response to Slit mirrors our finding that dendrite retraction in PVD neurons requires UNC-34/Ena/VASP and F-actin assembly.

Our studies also detected necessary roles in self-avoidance for MIG-10/LPD which has been previously shown to function with UNC-34/Ena/VASP to promote F-actin polymerization and UNC-73/Trio, a Rho/Rac GEF that acts in UNC-6/Netrin pathways to regulate the actin cytoskeleton[16,20,21,26]. Altogether, our genetic analysis identified five known effectors of actin polymerization (UNC-34/Ena/VASP, WSP-1/WASP, Arp2/3 complex, MIG-10/Lpd, UNC-73/Trio) that drive retraction of 3° dendrites in the self-avoidance response but which are not required for 3° branch extension during outgrowth.

**Non-muscle myosin II drives dendrite retraction in the self-avoidance response.**

Our genetic analysis detected a cell-autonomous role for NMY-1/non-muscle myosin II in dendrite self-avoidance (Figure 7). Because our results revealed F-actin at the tips of PVD dendrites (Figure 5, 6), we suggest that NMY-1 mediates retraction by interacting with F-actin to drive a contractile mechanism. For example, NMY-1 could accelerate retrograde flow, a non-muscle myosin-
dependent effect that also drives filopodial retraction in growth cones and at the leading edge of migrating cells[57,58]. Alternatively, NMY-1 could shorten PVD dendrites by inducing the reorganization of the nascent actin cytoskeleton into a more compact structure[59]. Phosphorylation of myosin regulatory light chain (RLC) activates non-muscle myosin[37] and we showed that MLC-4DD, a phosphomimetic, and, thus, constitutively active form of the C. elegans homolog of RLC, results in hyper-retraction of PVD 3° dendrites (Figure 8). The excessive retraction induced by chronic activation of MLC-4 suggests a model in which NMY-1 function is selectively triggered by contact between sister dendrites and subsequent UNC-6/Netrin signaling.

A downstream function for non-muscle myosin II in the UNC-6/Netrin pathway is consistent with our finding that the Unc-6 self-avoidance defect is rescued by MLC-4DD (Figure 8). Parallel roles for these components in axon guidance are suggested by recent results showing that non-muscle myosin II is required for Slit and Netrin-dependent midline avoidance in the developing vertebrate spinal cord[60]. Similarly, non-muscle myosin II mediates Semaphorin-induced axonal repulsion. The mechanism of Semaphorin action in this case also involves the assembly of actin bundles in the axon shaft presumably to facilitate non-muscle myosin-driven retraction[61-63]. Thus, our findings suggest that key drivers of axonal repulsion including nascent actin assembly and non-muscle myosin may also mediate dendrite retraction in the self-avoidance response.

RLC phosphorylation activates non-muscle myosin II by releasing myosin monomers for assembly into bipolar structures or myosin “stacks” that interact with actin filaments to induce translocation[37,64]. Although our results are consistent with a necessary role for non-muscle myosin in dendrite retraction, the tips of PVD 3° dendrites are likely too narrow (~50 nm) [65] to accommodate myosin stacks (~300 nm) [64,66]. We suggest that the actin-branching function of
Arp2/3, which we have shown mediates PVD dendrite retraction, could induce transient expansion of the dendritic tip to allow assembly of myosin stacks. In this model, actin-polymerization involving Arp2/3 could effectively limit ectopic NMY-1 activation in the dendritic shaft by restricting the assembly of myosin stacks to the tips of contacting sister dendrites. Alternatively, recent evidence indicates that myosin can also induce contraction in its monomeric or unpolymerized form[67] which should have ready access to the dendrite tip. This idea is consistent with our observation that GFP-tagged NMY is active (e.g., rescues the self-avoidance defect of nmy-1) and did not display the punctate appearance characteristic of myosin stacks (Fenix et al., 2016) but is diffusely localized throughout the PVD cytoplasm (Figure 7B).

**Parallel-acting pathways drive dendrite self-avoidance.**

Although self-avoidance is perturbed in UNC-6/Netrin pathway mutants, a significant fraction (~70%) (Figure 2D) of PVD 3° dendrites show apparently normal self-avoidance behavior[9]. One explanation for this observation is provided by the recent discovery of an independent pathway, mediated by MIG/14/Wntless, that is necessary for self-avoidance in an additional fraction of PVD 3° dendrites[34]. Parallel acting pathways also mediate self-avoidance in mammalian Purkinje neurons where slit-robo signaling acts in concert with protocadherins (Gibson et al., 2014; Lefebvre et al., 2012). The likelihood of additional effectors of self-avoidance is also suggested by the incompletely penetrant effects of Dscam mutants in Drosophila sensory neuron self-avoidance (Matthews and Grueber, 2011). Thus, our results in *C. elegans* mirror findings in other organisms which together suggest that multiple mechanisms have evolved to insure dendrite self-avoidance.
The actin cytoskeleton is highly dynamic in growing and retracting PVD dendrites.

We used time-lapse imaging with LifeAct::GFP, a live-cell marker for actin \cite{Riedl:2008gw}, to monitor actin PVD dendrites. The LifeAct::GFP signal is typically brighter near the distal ends of growing branches in comparison to interstitial regions (Figure 5) and is highly dynamic (Movie S3). The distal localization and rapidly fluctuating LifeAct::GFP signal in PVD dendrites resembles recently reported “actin blobs” that actively migrate in Drosophila sensory neurons with complex dendrite arbors and that appear to presage points of branch initiation \cite{68}.

Antagonistic pathways regulate dendrite outgrowth vs retraction.

Dendritic architecture is defined by the combined effects of outgrowth which expands the arbor vs retraction which limits the size of the receptive field. This interaction of positive and negative effects is readily observed in the development of 3° PVD dendrites. Initially, 3° dendrites emanating from adjacent menorahs grow out along the body axis until contacting one another and then retract to avoid overlap\cite{9,11}. Outgrowth in this case is promoted by a multicomponent receptor-ligand complex that mediates adhesive interactions with the adjacent epidermis (Díaz-Balzac et al., 2016; Dong et al., 2013; Salzberg et al., 2013; Zou et al., 2016) (Figure 9E). When this pathway is dysregulated by over-expression of the DMA-1 receptor in PVD neurons, for example, 3° dendrites continue to adhere to the epidermis and overgrow one another\cite{47,69}. 3° branch self-avoidance also fails in mutants that disable UNC-6/Netrin signaling which we have shown promotes dendrite retraction\cite{9}. Thus, these results suggest that the net length and placement of each 3° branch depends on the balanced effects of locally acting signals that either extend (e.g., DMA-1) or shorten (e.g., UNC-6/Netrin) the dendrite. We confirmed this idea in genetic experiments that detected strong dose-sensitive interactions between these opposing
pathways. For example, a 50% reduction of UNC-6/Netrin expression in a heterozygous \textit{unc-6/+} mutant does not perturb self-avoidance. Similarly, a genetic mutant (\textit{kpc-1/+}) that partially elevates DMA-1 also shows normal 3\degree branch outgrowth. The combination of both mutations in the double heterozygote, \textit{unc-6/+; kpc-1/+}, however, produces a strong self-avoidance defect. Our genetic results identified multiple regulators of actin polymerization (UNC-34/Ena/VASP, WSP-1/WASP, ARX-5/p21, MIG-10/Lamelipodin, UNC-73/Trio) that are required for self-avoidance. We thus propose that UNC-6/Netrin promotes actin polymerization to drive dendrite retraction and that non-muscle myosin II interacts with a nascent actin cytoskeleton to translocate each 3\degree dendrite away from its neighbor. F-actin is also abundant and highly dynamic in growing PVD dendrites. Notably, a different set of F-actin promoting factors (WRC, TIAM/GEF) interacts with the DMA-1 receptor complex to drive PVD dendritic growth (Figure 9E) [25]. Thus, the challenge for future studies is to elucidate the cell biological machinery through which opposing pathways manipulate the actin cytoskeleton to effect either dendrite growth or retraction.
MATERIALS AND METHODS

Strains and genetics

All the strains used were maintained at 20°C and cultured as previously described (Brenner, 1974). We used the N2 Bristol strain as wild-type.

The previously described strains used in this study include: NC1404 [wdIs52 (pF49H12.4::GFP)], NW434 [unc-6(ev400)X;wdIs52(pF49H12.4::GFP)], TV17,200 [kpc-1(xr58); wyIs592(pser2prom3::GFP)], TV9656 [dma-1(wy686); unc-119(ed3)III; wyEx3355(pser2prom3::GFP+odr-1::RFP)], CX1248 [KyEx3482 (pdes-2::TBA-1::mCherry+coel::RFP)] [9], CLP928 [twnEx382(Pser2.3::LifeAct::EGFP, Pser2.3::mCherry, Pgcy-8::gfp)].

Additional strains generated for this study: NC2580 unc-34(gm104)V; wdIs52, NG324 [wsp-l(gm324); wdIs52], +/-szT1 lon-2(e678) I; HR1184 [nmy-1(sb115) dpy-8(e130)/szT1 X], NC2726 [nmy-1(sb113);wdIs52], unc-73(rh40)]; wdIs52.

NC3284 [wdEx1011 (pF49H12.4::myrUNC-5::GFP)], was produced by microinjection of plasmids pLSR18 (pF49H12.4::myrUNC-5::GFP), pmyo-2::mCherry and pCJS04 (pF49H12.4::mCherry).

NC3048 [wdIs98 (pF49H12.4::ACT-1::GFP)] was produced by microinjection of plasmids pLSR03 (pF49H12.4::ACT-1::GFP) and pceh-22::GFP and integrated as previously described (Miller and Niemeyer, 1995).
NC3085 [wdEx967 (pF49H12.4::mCherry::UNC-34)] was obtained by microinjection of plasmids pCJS78(pF49H12.4::mCherry::UNC-34) and pmyo-2::mCherry.

NC3090 [wdEx972 (pF49H12.4::LifeAct::GFP)] was acquired by microinjecting plasmids pLSR06 (pF49H12.4::LifeAct::GFP) and pmyo-2::mCherry.

NC3029 [(pF49H12.4::nmy-1(+)+ pF49H12.4::nmy-1(-))] was obtained by microinjection of pLSR09 (pF49H12.4::nmy-1(+)), pLSR10 (pF49H12.4::nmy-1(-)), pmyo-2::mCherry and pCJS04 (pF49H12.4::mCherry)

NC3087 [wdEx969 (pF49H12.4::GFP::Linker::NMY-1)] was produced by microinjecting plasmids pLSR11 (pF49H12.4::GFP::Linker::NMY-1), pCJS04 (pF49H12.4::mCherry) and pmyo-2::mCherry.

wdEx1009 (pF49H12.4::GFP::Linker::MLC-4DD) [NC3283] was obtained by microinjection of plasmids pLSR17 (pF49H12.4::GFP::Linker::MLC-4), pCJS04 (pF49H12.4::mCherry) and pmyo-2::mCherry.

**Molecular Biology:**

**Building pLSR11 (pF49H12.4::GFP::Linker::NMY-1::unc-10 3'UTR):** We used the Clonetech in-fusion HD cloning kit (Cat # 638910) to build this plasmid. The nmy-1 genomic region was amplified from pACP01 (pF49H12.4::NMY-1::mCherry) with overlapping regions corresponding to pCJS95 (pF49H12.4::GFP::CED-10::unc-10 3'UTR). We also PCR amplified pCJS95 with primers overlapping the nmy-1 genomic sequences to swap CED-10 with NMY-1. We then used the NEB site-directed mutagenesis kit to insert a 24-nucleotide glycine rich linker (see Table 1). The resultant pLSR11 plasmid was sequenced to confirm that the linker was inserted between the GFP sequence and nmy-1. See table 1 for primer sequences.
Constructing pLSR17 (pF49H12.4::GFP::Linker::MLC-4DD): We used the Clonetech infusion HD cloning kit (Cat # 638910) to construct this plasmid. The mlc-4 genomic region was amplified from N2 DNA using primers with overlapping adapter complementary to pLSR11 (pF49H12.4::GFP::Linker::NMY-1). pLSR11 was amplified with primers containing adapter sequences complementary to mlc-4. The amplified fragments were combined for the infusion cloning to replace nmy-1 with mlc-4. We then used the NEB site directed mutagenesis kit to change MLC-4 codons for Serine 18 and Threonine 17 to nucleotide sequences that encode Aspartate acid. The resultant pLSR17 plasmid was confirmed by sequencing.

Confocal microscopy and image analysis: Animals were immobilized with 15 mM levamisole/0.05% tricaine and mounted on 2% agarose pads in M9 buffer as previously described [11]. Z stack images were collected using a Nikon TiE inverted fluorescent microscope equipped with an A1R point scanning confocal and a Plan Apo 40X 1.3NA oil immersion objective or on a Leica TCS SP5 confocal microscope with a 40X or 63X objective. Z stacks were collected at either 0.5 or 1 μm depth from the ventral nerve cord to the top of PVD cell body. Maximum projection images were collated with either LAS-AF (Leica) or with NIS elements software (Nikon). Dendrite length was measured using the line tool in Fiji/image J. Co-localization images were assembled using image J. Z stack images that were collected using the LAS-AF or NIS elements software was imported into ImageJ/Fiji for further analysis.

TIRF microscopy and analysis. Animals were immobilized with 15mM levamisole/0.05% tricane and mounted on 2% agarose pads in M9 buffer. The coverslip placed on top of the agarose
pad encasing the immobilized worms is 1mm in thickness. Time-lapse datasets were acquired using a Nikon TiE inverted fluorescence microscope outfitted with a TIRF illuminator, Apo TIRF 100x, 1.49NA oil immersion objective, and NIS-Elements software (Nikon Instruments, Inc.). The images for the time lapse video were captured in a single plane and the TIRF angle was adjusted for oblique illumination of 3° PVD dendrites. Datasets were imported into FIJI/ImageJ for further analysis, including intensity quantification. The rectangle tool was used to encompass left and right pairs of adjacent 3° dendrites with separate ROI (Regions of Interest) for image frame and the intensities measured using the analyze tool. Resultant fluorescent intensity values were generated with prism graphical software.

**isIM super-resolution imaging and analysis.** Animals were immobilized with 0.4mM levamisole and mounted on 10% agarose pads in M9 buffer. Z stack images were captured using a Hamamatsu sCMOS camera on a Leica iSIM and a 100x, 1.45 NA objective. Images were deconvolved and processed using Metamorph and Fiji/ImageJ.

**Spinning disk confocal microscopy:** Animals were immobilized with 0.4mM levamisole and mounted on 10% agarose pads in M9 buffer. Volumes over time were captured using an automated TiE inverted fluorescence microscope platform with an X1 spinning disk head (Yokogawa) and DU-897 EM-CCD (Andor Technology), piezo-electric Z stage (Mad City Labs) and Apo TIRF 100x 1.49 NA oil immersion objective (Nikon Instruments, Inc.). Analysis of fluorescence intensity was accomplished using NIS-Elements and Fiji/Image J software.
Quantification of fluorescence intensity in growing vs. non-growing dendrites: L2-L3 larvae were immobilized with 0.4mM levamisole and mounted on 10% agarose pads in M9 buffer for imaging with the spinning disk confocal microscope. Z-stacks of PVD dendrites were imaged at either 1 min or 1.30 minute intervals with the 100X 1.49 NA oil immersion objective. PVD dendrites (2°, 3° and 4°) undergoing outgrowth with a succession of rapid extensions and retractions during the length of the time lapse videos were analyzed. NIS elements was used for measurements of LifeAct::GFP from a 1µm ROI at the tips of growing dendrites. In the same videos, fluorescence intensity was measured for an identical 1µm ROI of PVD branches (1°, 2° and 3°) that were not undergoing outgrowth. Fluorescence intensity measurements for the outgrowth and no-outgrowth datasets were normalized against the maximum overall LifeAct::GFP value (Figure 5).

Quantification of fluorescence intensity in 3° dendrites undergoing self-avoidance: Late L3 larvae were immobilized as above for imaging in the spinning disk confocal microscope. Z-stacks of PVD dendrites were imaged at either 1 or 1.30 minute intervals with the a 100X objective. Contact is defined by the point at which the maximum ferret (i.e., gap) between adjacent PVD 3° dendrites in the mCherry channel approached zero. Nikon NIS Elements was used to obtain LifeAct::GFP and mCherry fluorescence measurements from a 1µm ROI at the tips of the left and right 3° dendrites undergoing contact. Fluorescence intensity measurements of LifeAct::GFP and mCherry for the opposing dendrites were normalized against the maximum value for each fluorophore and plotted vs time (Figure 6A-E).

For comparisons of LifeAct::GFP signals at the tips of 3° dendrites before and during contact, fluorescence intensities were determined from a 5 µm ROI centered at the point of contact and
from a contiguous control (non-contact) 5 µm ROI positioned either to the left or right of the contact region (Figure 6F). The change in fluorescence intensity (ΔF) for each contact and control region was calculated as the difference between the signal at contact (F_t) and the fluorescence value at two time points prior to contact (F_{t-2}) normalized to F_{t-2} or \[ ΔF = \frac{F_t - F_{t-2}}{F_{t-2}} \] [34]. For all measurements, the cytoplasmic mCherry marker was monitored to measure the Max Feret (in this case, maximum distance between two points) between the tips of adjacent 3° dendrites and the point of contact determined when the Max Feret approaches zero. Statistical comparisons (N = 8) were performed with a 2-way ANOVA with Bonferroni correction.

Statistics: Student’s t test was used for comparisons between 2 groups and ANOVA for comparisons between three or more groups with either Tukey posthoc or Bonferroni correction for multiple comparisons. Kolmogorov-Smirnov test was performed for comparisons between frequency distributions of 3° dendrite length and gaps between 3° dendrites (Figures 2C and 8F, G).

Table 1.

| Primer name       | Primer sequences                                      |
|-------------------|-------------------------------------------------------|
| myrunc-5 Vec. For.| CGGCCCGCGGATAACAAATTTTCA                               |
| myrunc-5 Vec. Rev.| GGATCCCCATCCCGGGAT                                   |
| myrunc-5 Frag. For.| CCCGGGATGGGATCCATGgatatcttactgactgactgactgctagcAAAC |
| myrunc-5 Frag. Rev.| TGTATCCCGCGGCGCTATTTGTATAGTTCCATCCATGCCCCATG         |
| nmy-1 linker F1   | GGATGCCCCCTCCgtacagtctccatg                           |
| mlc-4 Frag. For. | TCCGCAGGGGGAGGTATGGCCTCCCGCAAAACC |
|-----------------|----------------------------------|
| mlc-4 Frag. Rev.  | agaaattgttatccTTAAGCCTCATCCTTGTTGGTTCC  |
| mlc-4DD F1    | CATGTTCGATCAGGCTCAAATTCAAG |
| mlc-4DD R1    | GCGAACACATTGTCATCGGCTCTTTTG |
| nmy-1 linker R1 | GCAGGGGGAGGTGATGGGTGACCTGCAGTAC |

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helped with spinning disk imaging and edited videos, L.S. and D.M.M. wrote the paper with input from coauthors.
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FIGURE LEGENDS

Figure 1: UNC-6/Netrin signaling mediates sister dendrite self-avoidance.

(A-B) Representative images and tracings of PVD sensory neurons in wild-type (A) and unc-6 (B) L4-stage larvae. PVD morphology was visualized with PVD::GFP or PVD::mCherry markers. PVD cell body marked with an asterisk. (A) 1°, 2°, 3° and 4° dendritic branches and single axon (blue arrowhead) are denoted. (B) Arrowheads point to overlaps between adjacent 3° dendrites that failed to self-avoid in unc-6 mutants. Scale bars are 5 µm. C) Summary of self-avoidance in 3° PVD dendrites denoting growth, contact and retraction. (D) Model of self-avoidance mechanism involving reciprocal contact-dependent repulsion mediated by UNC-6/Netrin and its receptors, UNC-40/DCC and UNC-5[9].

Figure 2: Constitutively active UNC-5 drives hyper-retraction of 3° PVD dendrites.

(A-D) Representative images and tracings of 3° dendrites anterior to PVD cell body in (A) wild type (red) and (B) PVD::MYR::UNC-5 (blue) (Late L3-early L4 animals) (C) Gaussian fit to distribution of lengths of PVD::mCherry-labeled 3° dendrites showing shorter average length for MYR::UNC-5 (n = 156, 7 animals) versus wild type (n = 159, 8 animals), p = 0.03, Kolmogorov-Smirnov statistical test. (D) Schematics of intact, wild-type UNC-5 receptor vs membrane-associated, constitutively-activated myristylated-UNC-4 (MYR::MYR-5). (E-F) Dynamicity of 3° dendrites in wild-type vs MYR::UNC-5-expressing PVD neurons labeled with PVD::GFP. (E) Schematic (top) depicts displacement of the tip of a 3° dendrite relative to a fiduciary mark (vertical dashed red line). Scale bar is 10 µm. Representative plots of displacement for wild-type (red) and MYR::UNC-5 (blue) 3° dendrites over time. (F) The dynamic saltatory growth and retraction of 3° dendrites in the wild type vs the relative quiescence of MYR::UNC-5-expressing PVD neurons.
is correlated with elevated standard deviation of tip movement from a fiduciary point in wild-type vs MYR::UNC-5 expressing PVD neurons (p = 0.04 from Student’s t-test, n = 4). (G) Self-avoidance defects (percentage of overlapping 3° branches) are rare (< 5%) in wild type and MYR::UNC-5. The unc-6 self-avoidance defect is rescued by PVD expression of MYR::UNC-5. Error bars denote mean and M. ***p<0.0001. n ≥ 15. from ANOVA with Tukey’s posthoc test. Scored in a PVD::mCherry background.

**Figure 3. Mutations in genes that promote actin polymerization disrupt PVD dendrite self-avoidance.**

(A) Representative images (top) and schematics (bottom) of the actin cytoskeleton (Green) labeled with PVD::ACT-1::GFP (A) and microtubules (Magenta) marked with pdes-2::TBA-1::mCherry. PVD cell bodies (asterisks) and axons (arrowheads) are noted. (B) Representative images of PVD::GFP for wild type, unc-34, mig-10, unc-73, wsp-1 and arx-5RNAi. Asterisks denote PVD cell bodies and arrowheads point to overlaps between adjacent PVD 3° dendrites. (C-E) Self-avoidance defects are plotted in a PVD::GFP background (mean and SEM) as the percentage of overlapping 3° dendrites for each genotype. ***p<0.001, *p<0.05, ANOVA with Tukey’s posthoc test, n ≥ 15 animals for all backgrounds. (C) Self-avoidance defects in unc-73, mig-10 and unc-34/Ena/VASP exhibit significant self-avoidance defects. unc-34/Ena/VASP mutants are rescued by PVD expression of UNC-34 (PVD::mCherry::UNC-34). Myristoylated UNC-5 (PVD::MYR::UNC-5) fails to rescue the self-avoidance defects of an unc-34 mutant. (D) PVD self-avoidance defects for arx-5RNAi, unc-6, wsp-1 and unc-6; wsp-1 mutants and compared to wild-type and E.V. *(Empty Vector) RNAi controls. ***p<0.001, NS = Not Significant, ANOVA with Tukey’s posthoc test, n ≥ 15 animals. Scale bar is 10 µm.
Figure 4. Localization and trafficking of PVD::UNC-34::mCherry puncta in PVD.

(A-B) Leica iSIM images of PVD neurons (late L3 larvae) labeled with PVD::LifeAct::GFP (green) and PVD::mCherry::UNC-34 (Magenta). Merge shown at top right. Insets depict punctate mCherry::UNC-34 in 1°, 2° and 3° PVD dendrites. (B) Note punctate mCherry::UNC-34 near tips (white arrowheads) of 3° dendrites. Scale bars are 5 µm. (C-D) Kymographs of PVD::mCherry::UNC-34 in PVD 3° dendrite captured in the Leica iSIM microscope. Moving (green arrowheads) vs stationary (red arrowheads) mCherry::UNC-34 puncta are denoted. The distal end of the 3° dendrite is indicated by an asterisk and a red dashed arrow line points toward the PVD cell soma. Scale bar is 10 µm.

Figure 5. The actin cytoskeleton is highly dynamic in PVD dendrites.

(A) Representative images of PVD::LifeAct::GFP and PVD::mCherry labeled 2° dendrite undergoing outgrowth through time (late L3 larvae). (B) Fluorescent intensity was measured from regions of PVD dendrites undergoing outgrowth vs no-outgrowth (see Materials and Methods). ***p<0.001, N = 118 measurements from 6 dendrites. (C) Representative image showing enriched LifeACT::GFP signal in distal, growing PVD dendrites labeled with PVD::mCherry. (D) Rapid changes in the normalized fluorescence intensity of PVD::LifeAct::GFP versus the cytoplasmic marker, PVD::mCherry, were quantified as the standard deviation of the normalized fluorescent intensity measured at the tips of growing dendrites in a wild-type background. See Movie S3 and Materials and Methods) N=22. (E) Normalized fluorescence intensity measurements of PVD::LifeAct::GFP (green) vs cytoplasmic PVD::mCherry (red) at the tip of a growing late L3 stage PVD 3° dendrite from a representative time lapse movie.
**Figure 6**: (A-D) Fluorescent intensity traces in wild-type PVD dendrites during 3° dendrite contact based self-avoidance. Fluorescent intensity measurements of PVD::LifeAct::GFP and PVD::mCherry were acquired from a 1µm region at the tip of the growing and contacting left and right 3° dendrites (Late L3-L4 animals). Measurements were normalized against the maximum intensity during a ~10 minute interval that includes at least one contact event. The cytoplasmic mCherry signal was used to measure the gap between the left and right 3° dendrites undergoing contact and plotted against time. The period of contact (vertical shading) corresponds to a minimum value for the gap (0-0.14µm) between contacting left and right 3° dendrites. (E) Representation of 3° dendrites with color schemes to indicate left and right 3° dendrites and regions quantified. (F) Measurements from contact and non-contact (control) regions of 3° dendrites undergoing self-avoidance did not detect a significant increase in LifeAct::GFP at contact between the tips of adjacent 3° dendrites. Normalized LifeAct::GFP fluorescence intensity values from these regions (see Materials and Methods) were plotted vs time, N = 8, NS = Not Significant, 2-way ANOVA with Bonferroni correction.

**Figure 7. NMY-1/non-muscle myosin II mediates dendrite self-avoidance.**

(A) Representative images (left) and tracings (right) of wild-type and nmy-1(sb113) labeled with PVD::GFP. PVD cell soma (asterisks) and overlapping 3° dendrites (arrowheads) are noted. The PDE neuron is marked with an asterisk and is adjacent to the PVD cell-body. (B) NMY-1 fused to GFP via a linker peptide (PVD::GFP::NMY-1) (top) expressed in PVD with PVD::mCherry (bottom) rescues self-avoidance defects (C) of nmy-1(sb113). Scale bars are 5µm. (D) Quantification of self-avoidance defects in wild type and nmy-1. Self-avoidance defects for nmy-
mutants and *nmy-1* cell-specific RNAi (csRNAi) increase the percentage of overlapping 3° branches. PVD expression of GFP-labeled NMY-1 (PVD::GFP::NMY-1) rescues the *nmy-1*(*sb113*) self-avoidance defect. Scatter plots with mean and SEM, ***p<0.01, One way ANOVA with Tukey’s posthoc test. N = 15-20 animals per genotype. All defects were quantified in a PVD::GFP background. Scale bar is 5 µm.

**Figure 8. Phosphomimetic activation of myosin regulatory light chain, MLC-4, shortens 3° PVD dendrites and rescues the unc-6 dendritic self-avoidance defect.**

(A) Phosphorylation of Regulatory Light Chain (RLC) induces a conformational change that activates the myosin protein complex composed of myosin heavy chain (MHC), essential light chain (ELC) and RLC. (B) Threonine and serine phosphorylation sites (red) in conserved regions of *C. elegans*, *Drosophila* and human myosin RLCs. (C) Phosphomimetic construct, PVD::GFP::MLC-4-DD, fused to GFP with a linker peptide for expression with PVD promoter. (D-E) Representative images (top) and schematics (bottom) of PVD morphology anterior to cell body (asterisk) denoting 3° dendrites for (D) wild type (red) and (E) MLC-4DD (blue) (L3 stage larvae). Wild-type image also used in Figure 2A. Scale bars are 10 µm. 3° dendrite length (F) is shorter and gaps between 3° dendrites (G) is wider for MLC-4DD versus wild type. Gaussian fit was plotted with bin center values (µm) on the X-axis and the number of values that fall under each bin center on the Y axis, ***p<0.001, Kolmogorov-Smirnov statistical test for cumulative distributions of PVD 3° dendrite lengths and length of gaps in MLC-4DD (n = 141, 6 animals) versus wild type (n = 156, 8 animals). (J) PVD expression of MLC-4DD rescues the *unc-6* mutant self-avoidance defect, ***p<0.0001, One way ANOVA with Tukey’s posthoc test. Mean and SEM are shown. n ≥9 animals per genotype. Quantified with PVD::mCherry. Scale bar is 10 µm.
Figure 9. Opposing pathways regulate dendrite outgrowth and retraction.

Representative images and schematics of PVD in (A) kpc-1/kpc-1, (B) kpc-1/+; unc-6/+ and (C) kpc-1 +/+ dma-1; unc-6/+; PVD soma (asterisks) and overlapping 3° dendrites (arrowheads) are noted. (D) Quantification of self-avoidance defects for each genotype. Note that all mutant alleles are recessive but that kpc-1/+; unc-6/+ double heterozygotes show a strong self-avoidance defect that is suppressed in kpc-1 +/+ dma-1; unc-6/+ triples. ***p<0.0001, one way ANOVA with Tukey’s posthoc test. Mean and SEM are indicated. N >7 animals per genotype. PVD is labeled with ser2prom3::GFP for all images. Scale bar is 10 μm. Mutant alleles were: kpc-1(xr58), unc-6(ev400) and dma-1(wy686). (E) Model of opposing pathways that promote actin polymerization to define dendrite length. KPC-1 antagonizes surface expression of DMA-1 that interacts with an epidermal receptor composed of SAX-7/CAM, MNR-1 and LECT-2 and functions with HPO-30 to promote actin polymerization and dendrite outgrowth. UNC-6/Netrin interacts with UNC-5 to activate UNC-34/Ena/VASP which functions with WSP-1/WASP and the Arp2/3 complex to drive F-actin assembly for NMY-1/myosin-dependent dendrite retraction in the self-avoidance response.
SUPPORTING INFORMATION.

Imaging and quantification of self-avoiding 3° dendrites: L3 animals of strain CLP928 twnEx382[Pser2.3::LifeAct::EGFP, Pser2.3::mCherry, Pgcy-8::gfp] [34] were immobilized for time-lapse imaging with 1µl of agarose beads (Polybead carboxylate microspheres, Polysciences, Catalog # 15913-10, 0.05 µm) and mounted on a 10% agarose pad. Z-stacks of PVD dendrites (0.5 um depth, 5 slices) were imaged at 35 second intervals with a 40X objective on a Nikon spinning disk confocal microscope. Contact between the 3° dendrites was established as a function of Max Feret (maximum distance between 2 points). Max Feret (in pixels) was measured in the mCherry channel (cysotolic marker) with NIS elements and contact defined as by the time-point at which the Max-Feret approaches zero. LifeAct::GFP and mCherry signals were measured for a ROI of 2 µm at the tips of the left and right dendrites in a single Z plane. Left and right fluorescence intensities were summed for comparison to a control ROI (2 µm). The change in fluorescence intensity (ΔF) for each contact and control region was calculated as the difference between the signal at contact (F_t) and the fluorescence value at two time points prior to contact (F_{t-2}) normalized to F_{t-2} or ΔF = (F_t - F_{t-2})/F_{t-2} [34]. These values were computed for contact and control ROIs for LifeAct::GFP and mCherry. Statistical tests used for comparison between the datasets (LifeAct::GFP contact vs control, mCherry contact vs control, LifeAct::GFP contact vs. mCherry contact) were 2-way ANOVA with Bonferroni correction and multiple t-tests with a False Discovery Rate of 1%. Both these tests yielded similar results.

Supplemental Figure 1. Mutations in unc-34/Ena/VASP and unc-73/Trio disrupt PVD dendrite morphology.
(A) Quantification of 1° dendrite outgrowth and (B) number of 2° dendrites in wild-type, *unc-34*, *unc-73*, *wsp-1* and PVD::mCherry::UNC-34;*unc-34*. Defective 1° dendrites show altered alignment and/or extension defects. Mutations in *unc-34* and *unc-73* but not *wsp-1* exhibit defects in 1° dendrite outgrowth and fewer 2° dendrites. Note that cell-autonomous expression of UNC-34::mCherry does not rescue 1° and 2° dendritic defects but does restore self-avoidance (Figure 3C). (C) Representative images of PVD morphological defects in an *unc-34* mutant. Note premature termination of 1° dendrites (red arrowheads). Scale bars are 10 µm. Mutant alleles are *unc-34*(gm104), *unc-73*(rh40), *wsp-1* (gm324).

**Supplemental figure 2.** (A) Schematic (left) of adjacent 3° dendrites and representative time lapse series (35 sec intervals) of PVD::mCherry and PVD::LifeAct::GFP in 3° dendrites showing self-avoidance response. Point of contact is indicated by an asterisk and period of contact denoted with dashed blue outline. Scale bar is 10 µm. (B) Schematic depicting ROIs for fluorescence intensity measurements at the tips of adjacent (left vs right) 3° dendrites that undergo contact and at an adjacent non-contact (control) region. (C-E) Graphical representation of the change in fluorescence intensity at each time point vs that of the t = -2 time interval before contact at t = 0; measurements were normalized to th[34] (See Materials and Methods). Normalized fluorescence intensity values were plotted against time (min) for (C) LifeAct::GFP contact vs control, (D) LifeAct::GFP contact vs mCherry contact, (E) mCherry contact vs mCherry control. The values at each time-point were compared by 2-way Anova with Bonferonni correction, NS (Not Significant) N=14.
Movie S1: Time-lapse video representing the dynamic movement of 3° dendrites in a wild-type background. Images of PVD::GFP were recorded (late L3-early L4) at 15 sec intervals with a 100X objective in a Nikon spinning disk microscope (see Materials and Methods). The entire length of the video is 240 seconds. The movie was edited using NIS elements software with Bayes advanced denoising. Green arrowheads denote the tips of 3° dendrites. Scale bar is 25 µm.

Movie S2: Time-lapse video of 3° PVD dendrites in a MYR::UNC-5 background. Images of PVD::GFP in the MYR::UNC-5 strain were recorded (late L3-early L4) at 15 sec intervals with a 100X objective in a Nikon spinning disk microscope (see Materials and Methods). The entire length of the video is 12 minutes. Boxes denote tips of 3° dendrites showing limited outgrowth. The movie was edited using NIS elements software with Bayes advanced denoising. Scale bar is 10µm.

Movie S3: Time-lapse video showing the dynamicity of LifeAct::GFP during in 3° PVD dendrites. Images of PVD::mCherry (left) and PVD::LifeAct::GFP (right) and were collected at 1.3 min intervals with a 100 X objective in a Nikon spinning disk microscope (see Materials and Methods). The entire length of the video is 60 minutes. The movie was edited using NIS elements with automatic deconvolution. Scale bar is 5 µm. Spherical objects are autofluorescent granules.

Movie S4: Time-lapse video demonstrating the rapid accumulation of LifeAct::GFP after contact between 3° sister dendrites. Images of PVD::mCherry (left) and PVD::LifeAct::GFP (right) and were collected at 1.3 min intervals with a 100 X objective in a Nikon spinning disk microscope (see Materials and Methods). The entire length of the video is 60 minutes. The movie
was edited using NIS elements software with automatic deconvolution. Arrows track the moving 3° dendrite undergoing contact and retraction. Scale bar is 5 µm.

Movie S5: Time-lapse video demonstrating the rapid accumulation of LifeAct::GFP after contact between 3° sister dendrites. Images of PVD::LifeAct::GFP were captured at 15 second intervals using a 100X objective in a Nikon TIRF microscope for a total of 25 minutes at the L3-L4 stage (see Materials and Methods). The movie was edited using NIS elements with Bayes advanced denoising. The bottom panel depicts a fluorescence intensity profile through time (bottom) for the ROI (green). Scale bar is 5 µm.

Movie S6: Time-Lapse video demonstrating the trafficking of UNC-34::mCherry puncta in PVD 3 dendrites. Images of PVD::mCherry::UNC-34 were captured continuously on a single frame using a 100X objective in a iSIM microscope for 5 minutes (See Materials and Methods). The movie was edited using a Metamorph software and deconvolved using Fiji software. Scale bar is 5 µm.
Figure 1: PVD $3^\circ$ dendrites undergo self-avoidance in an UNC-6/Netrin dependent manner

A. wild-type

B. unc-6

C. outgrowth $\rightarrow$ contact $\rightarrow$ retraction

UNC-6 expressing cells
Figure 2: Constitutively active UNC-5 drives hyper-retraction of 3° PVD dendrites.

A. Wild-type

B. MYR::UNC-5

C. Length of 3° dendrites (2μm bins)

D. 

E. 

F. 

G.
Figure 3: Mutations in genes that promote actin polymerization disrupt PVD dendrite self-avoidance.

A. PVD::ACT-1::GFP

B. wild-type

unc-34(gm104)
mig-10(ct41)
unc-73(rh40)
wsp-1(gm324)
arx-5(RNAi)

C. 

| Treatment                  | % Overlap |
|----------------------------|-----------|
| Wild-type                  | 0         |
| unc-73                     |           |
| mig-10                     |           |
| unc-34                     |           |
| PVD::UNC-34MYR::UNC-5MYR::UNC-5 | ***      |
| MYR::UNC-5                 | ***       |
| unc-34                     | ***       |

D. 

| Treatment                  | % Overlap |
|----------------------------|-----------|
| Wild-type                  | 0         |
| Empty vector               |           |
| arx-5 RNAi                 | ***       |
| unc-6                      | NS        |
| wsp-1                      |           |
| unc-6; wsp-1               |           |
Figure 4: Localization and trafficking of PVD::UNC-34::mCherry puncta in PVD.

A.

PVD::LifeAct::GFP

PVD::mCherry::UNC-34

Merge

B.

PVD::LifeAct::GFP

PVD::mCherry::UNC-34

Merge

C.

Time

anterograde

D.

3° dendrite
Figure 5: The actin cytoskeleton is highly dynamic in PVD dendrites

A. PVD::Lifeact::GFP
   PVD::mCherry

B. Graph showing normalized fluorescent intensity (A.U.) for GFP and mCherry.

C. Merge of PVD::Lifeact::GFP and PVD::mCherry images.

D. Graph showing S.D. of normalized fluorescent intensity (A.U.) for GFP and mCherry.

E. Graph showing normalized fluorescent intensity (A.U.) over time (min) for GFP and mCherry.
Figure 6: Dynamic changes in actin polymerization during contact based retraction

A. B. C. D.

E. F.

-2 -1 0 1 2 3 4 5 6
-40 -20 0 20 40 60 80

gap(μm)

left GFP right GFP left mCherry right mCherry

ΔF/F %

control contact
Figure 7: NMY-1/non-muscle myosin II mediates dendrite self-avoidance.

A. wild-type

nmy-1(sb113)

B. PVD::GFP::NMY-1; nmy-1(sb113)

C. PVD::GFP::NMY-1; nmy-1(sb113)

D. % overlap

*** ***

NS

** **

NS

5 μm 5 μm

merge

merge
Figure 8: Phosphomimetic activation of myosin regulatory light chain, MLC-4, shortens 3° PVD dendrites and rescues the unc-6 dendritic self-avoidance defect.

A. Diagram showing the activation of MLC-4 and MLC-4DD.

B. Table showing the sequence of MLC-4 in different species:

- C. elegans: MLC-4
- D. melanogaster: MRLC-C
- H. sapiens: MRLC2

C. Diagram showing the GFP-linked linker and MLC-4DD.

D. Images of wild-type and MLC-4DD dendrites.

E. Images showing the lengths of dendrites.

F. Graph showing the length of 3° dendrites (2μm bins) for wild-type and MLC-4 DD.

G. Graph showing the length of gaps between 3° dendrites (2μm bins) for wild-type and MLC-4 DD.

H. Graph showing the percentage overlap between wild-type, unc-6, MLC-4DD, and MLC-4DD; unc-6.

*** P < 0.001

NS = Not Significant
Figure 9: Opposing pathways regulate dendrite outgrowth and retraction.

A. kpc-1

B. kpc-1 +/+ dma-1; unc-6/+ 

C. kpc-1/+; unc-6/+ 

D. % overlap

E. 3 Dendrites 

OUTGROWTH

RETRACTION

KPC-1

F-Actin

WSP-1

Arp2/3

NMY-1/Myosin

UNC-5

UNC-34

UNC-40

HPO-30

LECT-2

MNR-1

SAX-7/L1CAM

NS

wild-type

kpc-1

kpc-1/+ 

dma-1/+ 

unc-6/+ 

kpc-1/+; unc-6/+ 

kpc-1 +/+ dma-1; unc-6/+ 

kpc-1 +/+ dma-1; unc-6/+ 

** ** **** ** ** ** **

epidermis