UNC-6 and UNC-40 promote dendritic growth through PAR-4 in *Caenorhabditis elegans* neurons

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Abstract

Axons navigating through the developing nervous system are instructed by external attractive and repulsive cues. Emerging evidence suggests the same cues control dendrite development, but it is not understood how they differentially instruct axons and dendrites. We studied a *C. elegans* motor neuron whose axon and dendrite adopt different trajectories and lengths. We found that the guidance cue UNC-6 (Netrin) is required for both axon and dendrite development. Its repulsive receptor UNC-5 repelled the axon from the ventral cell body, whereas the attractive receptor UNC-40 (DCC) was dendritically enriched and promotes antero-posterior dendritic growth. Although the endogenous ventrally secreted UNC-6 instructs axon guidance, dorsal or even membrane-tethered UNC-6 could support dendrite development. Unexpectedly, the serine-threonine kinase PAR-4 (LKB1) was selectively required for the activity of the UNC-40 pathway in dendrite outgrowth. These data suggest that axon and dendrite of one neuron interpret common environmental cues with different receptors and downstream signaling pathways.

The precise assembly of neural circuits during development is a prerequisite for intact behavior in the adult organism. Assembly begins with symmetry breaking of each neuron when the neuron decides when and where to form an axon and dendrites, resulting in two molecularly, morphologically and functionally distinct compartments that are each fundamental to its function. Whereas many attractive and repulsive cues determining axonal trajectories have been identified, less is known about cues promoting or limiting dendrite growth.

Recently, several *in vivo* studies indicated that axon guidance molecules are also involved in dendrite development. Semaphorin (Sema)-3A secreted at the cortical marginal zone attracts the apical dendrites of pyramidal neurons toward superficial layers[1]. In *Drosophila melanogaster* olfactory glomeruli, discrete groups of olfactory receptor neuron axons and projection neuron dendrites must connect. A Sema-1a gradient in the antennal lobes cell-autonomously targets projection neuron dendrites to the correct location in the glomeruli[2]. Netrin and Slit, initially described as axon guidance molecules, have since been shown to regulate dendrite guidance[3,4]. In zebrafish, midline netrin1 guides octavolateralis efferent neuron dendrites across it without affecting neuronal fate or axon guidance[5]. Fly motor neuron axons and dendrites respond to Slit and Netrin in both outgrowth and midline

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guidance events[6,7]. However, as these dendrites extend from axon segments bilaterally of the midline, it is impossible to distinguish the molecules' effects on axons and dendrites. As dendrites respond distinctly to these cues, there must be mechanistic differences in the cell's interpretation of its environment when forming an axon versus a dendrite. Hence the challenge ahead of us appears not only to be identifying new genes, but also teasing apart how a neuron interprets identical molecular cues in different ways.

One mechanism by which cues could be interpreted distinctly is by implementing task-specific downstream molecules. Multiple signaling pathways downstream of UNC-6 (Netrin) in axon guidance have been explored, starting with different receptors. The UNC-5 receptor binds UNC-6 to repel axons. In *C. elegans*, this response allows ventral motor neurons to dorsally guide their axons away from the ventral UNC-6 source toward their muscle targets[3]. In mouse, Unc5h3 is required for the formation of the corticospinal tract and its guidance at the pyramidal decussation[8]. UNC-6's attractive function is mediated by the receptor UNC-40 (DCC), which guides axons toward ventral UNC-6 in *C. elegans*, chick and mouse[3,9,10]. However, a neuron's response specificity to UNC-6 exceeds receptor choice. In *C. elegans*, a bifurcation of pathways downstream of UNC-40 has been identified. The UNC-40 cytoplasmic portion consists of three P domains (P1, P2 and P3), which associate with specific downstream molecules that may deliver distinct signals to the actin cytoskeleton. The functions of the actin-binding protein UNC-34 (Enabled) in axon guidance and filopodium outgrowth have been shown *in vitro* and *vivo* in *C. elegans* and *Drosophila* neurons[11,12]. In UNC-6 signaling, UNC-34 acts downstream of the P1 domain. MIG-10 (Lamellipodin) and UNC-34 cooperate to promote filopodium outgrowth in the *C. elegans* neuron HSN[13]. In parallel, the UNC-40 P2 domain acts through the Rac GTPase CED-10 and the actin-binding protein UNC-115 (abLIM). Both P1 and P2 domain–dependent pathways contribute to filopodium formation, but their activity is not entirely redundant, as within these pathways each protein preferentially contributes to filopodium formation, number or orientation. Hence the molecular diversity orchestrates distinct events in UNC-6 signaling downstream of UNC-40 (refs. 14,15).

Several molecules have been identified as acting downstream of vertebrate DCC. These include kinases such as focal adhesion kinase (FAK), the Src family kinase Fyn and phospholipase C (PLC)-γ and phosphatidylinositol-3-OH kinase (PI3K)pathways[16,17]. DCC has also been shown to interact with the Rac1-activating guanine nucleotide exchange factor DOCK180 and with unconventional myosin X, which stimulates filopodium formation and modulates neuritic DCC localization[18,19]. p130[CAS] has been proposed to link the Src family kinases downstream of DCC with the small GTPases Rac1 and Cdc42 (ref. 20). These findings highlight the commonalities between pathways used for axon guidance and other cell motility events.

Here we show how specificity can be achieved while generating a dendrite using the same extracellular cues used for axonal development. We use the *C. elegans* motor neuron DA9 to describe how UNC-6 regulates dendrite outgrowth. Its function in dendrite development is executed by short-range signaling through UNC-40, whereas the repulsive receptor UNC-5 is critical for axon guidance. We also show that PAR-4 is required for dendrite, but not axon, outgrowth in DA9 and acts downstream of UNC-6 andUNC-40. Therefore, the axon and dendrite of one neuron interpret common environmental cues with different receptors and downstream signaling pathways.

RESULTS

UNC-6 and UNC-40 are required for DA9 dendrite development

DA9 is an embryonically born, cholinergic, DA-class motor neuron. The somata of DA motor neurons located in the ventral nerve cord extend a single axon posteriorly and form a commissure to reach the dorsal nerve cord. In the dorsal cord, the DA axons grow anteriorly and form *en passant* synapses with a subset of dorsal body wall muscles and ventral D reciprocal inhibitory neurons[21]. DA motor neuron axon outgrowth and neuromuscular junction formation occur embryonically. DA9 is the most posterior of DA neurons. It has a single, unbranched dendrite that extends anteriorly in the ventral nerve cord and receives inputs from command interneurons and sensory neurons[21]. To characterize DA9 dendrite growth, we used a fluorescently labeled transmembrane protein driven under a promoter[22] (*Pmig-13::mig-13::gfp*) that, in the tail, is predominantly expressed in DA9 during larval stages 1 through 4 (L1–L4), and cytoplasmic mCherry expression under a DA9-specific promoter[23] that is expressed predominantly after L4 (*Pitr-1::mCherry*). Using these markers, we measured dendrite length from its anterior tip to the soma at different developmental time points. Unlike axon development, we found that most dendrite outgrowth in DA9 takes place postembryonically (Fig. 1a–g). In L1 larvae, when the axon has already completed its dorsal and anterior growth, the dendrite is very short (Fig. 1b). The dendrite continues to grow throughout larval development (Fig. 1c–f), occupying an increasing proportion of the tail (Supplementary Fig. 1 and Supplementary Table 1). Hence, dendrite growth is not a secondary effect of body size, as it is not directly coupled to the worm's growth. The rate of dendrite extension falls off in older adults. Dendrite development in DA9 is temporally and spatially distinct from axon outgrowth and guidance, and so it is an appropriate system in which to study mechanisms differentially specifying the developing dendrite.

In asking what processes underlie DA9 dendrite outgrowth, we considered UNC-6 as a candidate. UNC-6 is an evolutionarily conserved axon guidance cue in invertebrates and vertebrates[24]. In *C. elegans*, ventral epidermoblasts and then ventral cord pioneer neurons provide UNC-6 as a diffusible cue[25]. Its expression by these cells presumably creates a ventral to dorsal gradient providing directional information for axons. Neurons with dorsal somata use UNC-6's attractive receptor UNC-40 to guide their axons up the gradient toward the ventral cord, while ventral neurons send their axons down the gradient through the repulsive activity of the UNC-5 signaling pathway[3,26].

The DA9 axon migrates dorsally and its dendrite grows along the ventral cord, suggesting that its axon uses the UNC-5 repulsive pathway. Consistent with this, we have previously found that DA9 dorsal guidance is partially affected in *unc-6* or *unc-5* mutants[27]. To understand how UNC-6 affects dendrite growth, we measured DA9 dendrite length at various developmental stages. We found that the DA9 dendrite in *unc-6* mutants was shorter compared with that in wild-type worms (Fig. 2). This defect could be detected in all postembryonic developmental stages (Fig. 2e, f). Similar phenotypes were found in *unc-40* mutants and in worms lacking the previously characterized signaling molecules UNC-34 and UNC-115 (Fig. 2e, f). Of note, *unc-5* mutants, which exhibit defects in axon dorsal guidance[27], showed a weak outgrowth phenotype in L4 stage, but an otherwise normal dendrite length (Fig. 2d, e). The dendrite orientation was normal in all the mutants mentioned above. The average dendrite length in DA9 neurons with misguided versus properly routed axons in *unc-6* mutants was not significantly different, suggesting that axon guidance and dendrite growth are independent events (Supplementary Fig. 2). To test whether *unc-6* mutant dendrites have lost their molecular identity, we examined the subcellular distribution of two somato-dendritically localized proteins, fibrillin and dystrophin. Their polarized distribution was unaffected in *unc-6* mutants, suggesting that the

remaining neurite maintains dendritic characteristics[27]. These data suggest that the high concentration of UNC-6 promotes DA9 dendrite growth along the ventral nerve cord.

UNC-40 acts cell-autonomously and localizes dendritically

To establish whether UNC-40 acts cell-autonomously in DA9, we created mosaic *unc-40* worms by expressing wild-type *unc-40* cDNA under the *mig-13* promoter in *unc-40* mutants. The *mig-13* promoter is expressed in only DA9 and VA12 in the tail of the worm, with expression in VA12 becoming evident after the L3 stage[23]. Expression of *unc-40* in DA9 fully rescued dendrite outgrowth (Fig. 3), suggesting that UNC-6 signaling is acting in DA9 itself to promote dendrite outgrowth.

To further test whether UNC-6–UNC-40 signaling is the rate-limiting molecular event for DA9 dendrite growth, we examined worms wild-type for *unc-40* expressing *mig-13::unc-40*. We reasoned that the dendrite of these worms might be longer than wild type if the signaling strength of the UNC-6–UNC-40 pathway instructs growth. Indeed, we observed that transgenic worms showed significantly longer DA9 dendrites compared with wild-type worms. If this UNC-40 'gain-of-function' effect represents overactivation of endogenous UNC-6 signaling, the dendrite overextension should be UNC-6 dependent. Indeed, the overextension phenotype was significantly suppressed in *unc-6* worms ($P = 0.0001$; Fig. 3). Hence, UNC-6–UNC-40 signaling is necessary and sufficient for dendrite outgrowth in DA9, and signaling through UNC-40 represents the 'rate-limiting' step.

As UNC-40 acts cell-autonomously, we investigated whether it acts in a specific subcellular compartment. It has previously been observed in some signaling events during neuronal development that the receptor localizes specifically to its site of activity[28,29]. We tested this in DA9 by expressing a functional *unc-40::gfp* fusion. We found that UNC-40 was markedly enriched in the dendrite and soma compared to the axon (Supplementary Fig. 3a). To address the mechanism responsible for spatially restricting UNC-40, we asked whether the receptor localization may be subject to its own ligand, as has been shown to be the case for UNC-40 localization during the polarization of the soma of neuron HSN and in neuron AIY[13,28]. We examined UNC-40::GFP localization in *unc-6* mutants and observed that the dendritic restriction of UNC-40 was no longer fully maintained. Instead, we saw UNC-40 localizing in the dendrite and axon in many individuals (Supplementary Fig. 3b). This suggests that downstream UNC-40 signaling might be restricted to the dendrite through UNC-40 subcellular localization *in vivo*. However, dorsal muscle expression of *unc-6* revealed that *unc-6* was not sufficient to relocalize dendritic UNC-40 to the axon and that even low levels of dendritic UNC-40 were sufficient to support dendrite outgrowth (Fig. 4 and Supplementary Fig. 3c).

UNC-6 action is local and gradient-independent

UNC-6 produced by ventral neurons is secreted and diffuses to produce a putative ventro-dorsal gradient. This gradient is required for ventral axon guidance in, for example, AVM, PDE and HSN neurons[3]. However, it has also been reported that UNC-6 (Netrin) can act locally on the cell surface, without diffusing. This has been shown in the crossing of commissural axons at the *Drosophila* midline, and immobilized Netrin-1 has recently been shown to produce traction with axonal growth cones that is sufficient to reorient them[30,31]. We attempted to test whether the UNC-6 gradient is required for anterior dendrite growth. We disrupted the endogenous gradient by expressing *unc-6* in dorsal muscles of *unc-6* mutants using a fragment of the *unc-129* (*tgf-β*) promoter (Fig. 4a–d). This manipulation should result in a reverse gradient (dorsal high, ventral low). Unexpectedly, the presence of UNC-6 in a changed distribution was sufficient to completely rescue the dendrite truncation, suggesting that UNC-6 distribution is not important for its function in promoting DA9

dendrite growth (Fig. 4a). Notably, this artificial dorsal-high UNC-6 gradient worsened the axon phenotype. In *unc-6* worms, 24% of axons failed to reach the dorsal cord, indicating that UNC-6 is important, but not essential, for DA9 guidance. In *unc-6* mutants carrying the *unc-129::unc-6* transgene, 49% of axons failed to reach the dorsal cord, suggesting that the artificial gradient further prevents the axons' dorsal migration, possibly through UNC-5 (Supplementary Fig. 4).

Given that a ventro-dorsal UNC-6 gradient is not required, we asked whether UNC-6 from neurons adjacent to DA9 might be sufficient. To test this, we expressed the membrane-tethered UNC-6 extracellular domain under its own promoter. This manipulation fully rescued the *unc-6* mutant dendrite defect, and expression of membrane-tethered UNC-6 resulted in dendrite overshooting in wild-type worms, suggesting that UNC-6 is acting as a short-range signal (Fig. 4a, e, f). Consistent with this, UNC-6 is expressed by neurons in the ventral nerve cord that fasciculate with the DA9 dendrite. Among these are the interneuron AVA and the motor neuron VA12 (refs. 21,25). Notably, *unc-6* mutants expressing membrane-tethered UNC-6 showed a penetrance of axon guidance phenotypes similar to that of the *unc-6* mutants alone, suggesting that axon guidance requires secreted UNC-6 (Supplementary Fig. 4). Hence, UNC-6 from neighboring neurons is probably promoting dendrite outgrowth.

These results highlight how differently UNC-6 affects DA9's axon and dendrite development. Axon guidance requires a ventro-dorsal UNC-6 gradient and is mediated by the UNC-5 receptor, whereas short-range UNC-6 is sufficient to promote adjacent

PAR-4 acts downstream of UNC-6 and UNC-40

Genes identified acting downstream of UNC-6 and UNC-40 in *C. elegans* include the actin-binding proteins UNC-34, UNC-115 and MIG-10 (ref. 14). We tested whether dendrite outgrowth was mediated by any of these genes. We found that mutants for *unc-34* and *unc-115*, but not *mig-10*, showed a shortened dendrite (Fig. 1 and data not shown). Similar to the *unc-40* phenotype, the *unc-34* phenotype is slightly more severe than the *unc-6* phenotype.

These data suggest that molecules originally described in axon guidance also function during dendrite outgrowth[1,6]. Given that dendrite and axon growth seem to be mediated by common pathways, we tested a gene originally identified in early embryonic polarity and more recently in axon outgrowth, the serine-threonine kinase gene *par-4* (*lkb1*)[32,33]. Unexpectedly, *par-4* mutants exhibited normal axon outgrowth and guidance (Fig. 5a, b). However, they showed shortened dendrites, a phenotype similar to but less severe than that of *unc-6*, *unc-40* and *unc-34* mutants (Fig. 5a, b). DA9-specific *par-4* cDNA expression in *par-4* mutants was able to rescue the defect (Fig. 5c). Furthermore, *par-4* overexpression in wild-type worms was sufficient to cause marked dendritic overshooting (Fig. 5c). Hence, like *unc-40*, *par-4* is acting cell-autonomously in DA9 and is necessary and sufficient for dendrite outgrowth.

As the *par-4* phenotype resembles that of *unc-6* signaling mutants, we asked whether these genes were acting in parallel or in a hierarchical pathway. With few exceptions, double mutants for genes within one genetic pathway show a phenotype not significantly different from that of the more severe of the two mutants. If *par-4* and *unc-6* are acting independently, *par-4; unc-6* or *unc-40; par-4* double mutants should show a significantly shorter dendrite than single mutants. The *unc-40; par-4* mutants showed a dendrite length not significantly different from that of *unc-40* mutants alone, and the *par-4; unc-6* mutant dendrite length was not significantly different from that of *unc-6* mutants (Fig. 6a). We

found this also to be the case at L4 and adult stages (Supplementary Fig. 5) and conclude that these genes are likely acting in the same pathway.

One could imagine several ways in which UNC-6, UNC-40 and PAR-4 could be hierarchically organized, given their functional and molecular characteristics. PAR-4 is a serine-threonine kinase that was cloned in a screen for genes mediating the asymmetric localization of P-granules in early embryonic worm development[34]. Furthermore, PAR-4 has been shown to modulate the localization of the zinc finger protein MEX-5 (ref. 35). Hence, PAR-4 could act upstream of UNC-6 and UNC-40 and be responsible for the asymmetric distribution of UNC-40 or other proteins required for dendrite outgrowth downstream of UNC-6 and UNC-40. The second possibility would be that PAR-4 acts downstream of UNC-6 and UNC-40 to regulate cytoskeletal modulators. This second scenario would resemble PAR-4's function in mouse axon formation, in which PAR-4 is activated by BDNF and phosphorylates the SAD-A and SAD-B kinases[33,36].

To establish a hierarchy between PAR-4 and UNC-6, we carried out several experiments. If PAR-4 were acting upstream of UNC-40 in regulating its localization, we would expect UNC-40 localization to change in *par-4* mutants, as in *unc-6* mutants. However, UNC-40 localization was unaltered in *par-4* mutants (data not shown). If PAR-4 were acting upstream of UNC-40 without affecting its localization, we would expect *unc-40* expression in *par-4* mutants to rescue dendrite outgrowth. However, the same *unc-40* cDNA that rescued in *unc-40* mutants did not in *par-4* mutants (Fig. 6b). If PAR-4 acts downstream of UNC-6 and UNC-40, its overexpression should rescue. Overexpression of *par-4* in both *unc-6* and *unc-40* mutants rescued dendrite outgrowth and resulted in marked overshooting (Fig. 6b). These data taken together indicate that PAR-4 is a new type of effector of UNC-6.

Two pathways downstream of UNC-40 in attractive axon guidance have already been described, one of which is UNC-34 dependent, and one of which is UNC-115 dependent[14]. To determine whether PAR-4 acts in either of these or represents a third pathway, we constructed double mutants of *par-4* with either *unc-34* or *unc-115*. The phenotype of *unc-34; par-4* double mutants was strongly enhanced (Fig. 6a). The severe dendrite truncation indicates that in mutating these genes we have tapped into the two main pathways responsible for dendrite outgrowth in DA9. In contrast, the *par-4; unc-115* double mutants show a dendrite length similar to that of *par-4* and *unc-115* single mutants (Fig. 6a), suggesting they function in one genetic pathway. Furthermore, the dendrite overshooting produced when expressing *par-4* in DA9 is blocked by a mutation in *unc-115*, suggesting that *par-4* acts upstream of *unc-115* (Fig. 6c).

We wondered whether *par-4* acts in the same pathway in other neurons that have been characterized as having *unc-40-* and *unc-34*-dependent growth. One such neuron is the touch receptor AVM, whose process makes a ventral turn in part owing to the attractive guidance of *unc-6* (ref. 12) (Fig. 7a). In *unc-34* mutants, 3% of worms had guidance defects and failed to turn ventrally (Fig. 7b, c). Whereas we observed no ventral guidance defects in *par-4* mutants, the incidence of ventral guidance defects increased to 10% in *unc-34; par-4* double mutants. Furthermore, the double mutants showed a 10% incidence of processes that terminate before the pharynx terminal bulb, which was not observed in single mutants or wild-type worms (Fig. 7d). These data suggest that *par-4* acts downstream of *unc-40* and in parallel with *unc-34*.

Next we sought to identify how *par-4* is regulated by *unc-40*. While no enzymatic activity has been associated with *unc-40*, three evolutionarily conserved cytoplasmic motifs, P1, P2 and P3, have been identified[37,38]. P1 and P2 have previously been identified as mediating netrin attraction through *unc-34*, *unc-115*, *ced-10* and *mig-10* (refs. 14,15). We asked

whether *par-4* also promotes outgrowth through one of the P motifs. To do this, we characterized the gain-of-function phenotypes in AVM when expressing myristoylated *unc-40* (*myr::unc-40*) in touch cells. Overexpression of *myr::unc-40* caused defects in soma morphology, axon guidance and branching in 90% of worms (Supplementary Fig. 6). A partial suppression and trend toward suppression of these gain-of-function phenotypes was achieved by deleting the P1 and P2 motifs, respectively (*myr::unc-40ΔP1* and *myr::unc-40ΔP2*), but less so upon deleting the P3 motif. Losing *unc-34* did not further suppress excess outgrowth in the *myr::unc-40ΔP1* background, confirming that *unc-34* acts through the P1 motif. Similarly, we confirmed that loss of *unc-115* did not further suppress excess outgrowth in *myr::unc-40ΔP2* worms, suggesting that *unc-115* acts through the P2 motif. When we inspected these strains in *par-4* mutants, we found that loss of *par-4* suppressed *myr::unc-40ΔP1* gain-of-function phenotypes but, like *unc-115*, did not suppress excess outgrowth in *myr::unc-40ΔP2* worms (Supplementary Fig. 6). This suggests that *unc-40* regulates *par-4* through its intracellular P2 motif and is consistent with *par-4* acting upstream of *unc-115*.

We conclude that short-range UNC-6 signaling regulates dendrite outgrowth in DA9 by means of a previously undescribed pathway involving UNC-40 acting upstream of PAR-4. This extends the role of UNC-6, originally described as an axon outgrowth and guidance cue, and more recently as a regulator of synaptic trafficking, to another domain of neural development. This trifunctional capacity within one cell is presumably achieved by means of specificity in downstream signaling molecules, such as PAR-4.

DISCUSSION

Dendrite growth and guidance is a key event in neural circuit formation. We have identified UNC-6 as regulating dendrite outgrowth by a pathway in which local UNC-6 signals through UNC-40 and PAR-4.

UNC-6 functions in multiple events during DA9 development

The contribution of UNC-6 to axon guidance of DA class motor neurons has previously been described[3,39]. In *C. elegans*, lateral and dorsal neurons in the nerve ring use UNC-40 to grow toward UNC-6. Ventral motor neurons, including the DA class, use ventral UNC-6 as a repulsive cue for dorsal guidance through UNC-5. Hence UNC-6 governs the dorsal guidance in DA motor neurons, which is the first event in their morphogenesis.

The trafficking of axon-specific molecules, such as presynaptic vesicles and active zone proteins, appears to take place simultaneously with axon growth and is also mediated by UNC-6 in an UNC-5-dependent fashion. In *unc-6* and *unc-5* mutants, vesicle-associated and active zone proteins are detected in the dendrite, whereas in wild-type worms, their localization is restricted to a zone within the dorsal axon[27]. The discovery that a diffusible molecule might instruct the molecular polarity of a neuron was unexpected. In particular, the fact that UNC-6 acts as a local inhibitor is noteworthy, as the contribution of other diffusible molecules to synapse formation has been primarily positive. It is also of note that the exclusion or 'repulsion' of synaptic components is UNC-5 dependent, as is the axonal growth-cone repulsion away from ventral UNC-6 (ref. 27).

We have found that dendrite outgrowth is another event orchestrated by UNC-6 during DA9 development. Postembryonically, UNC-6 acts as a growth-promoting signal on the dendrite, much like the outgrowth-promoting function by which Netrin was originally identified as the UNC-6 homolog in the chick floor plate[9]. UNC-6 mediates these three functions in DA9 by using distinct downstream signaling pathways.

UNC-6 differentially regulates axon and dendrite growth

The fact that axons and dendrites use identical cues to instruct distinct growth trajectories creates the conundrum of how a neuron can interpret the same external cues to generate different subcellular responses. Conceptually, the answer must lie in the spatial confinement and temporal regulation of signaling components. Although the temporal aspect remains poorly understood, we have uncovered several mechanisms by which a motor neuron differentiates its response to UNC-6 during dendrite and axon outgrowth.

First, *unc-40* mutants, but not *unc-5* mutants, show dendrite truncations. Hence the primary receptor mediating dendrite outgrowth is UNC-40, whereas both axon guidance and dendritic exclusion of presynaptic components are regulated by UNC-5. Therefore, axon and dendrite growth are regulated by distinct UNC-6 receptors (Supplementary Fig. 7a). The next question is how the distinct receptors are confined to subcellular compartments. It turns out that UNC-40 is targeted to the dendrite in part by UNC-6 during dendrite outgrowth; that is, UNC-6 compartmentalizes DA9 to allow a receptor-specific response. It is possible that UNC-6 recruits or retains dendritic UNC-40 by direct binding. Such ligand-dependent receptor localization has been observed in *C. elegans* for UNC-6 and LIN-44 (Wnt)[23,28]. As *unc-6* cannot relocalize UNC-40 away from the dendrite, it is likely that other molecules, such as integrins or the clathrin adaptor protein AP1, contribute to UNC-40's dendritic localization[40,41].

Second, we found that membrane-tethered UNC-6 is sufficient for dendrite outgrowth, suggesting that UNC-6 is acting at short range. This is in contrast to the long-range, UNC-6 gradient–dependent growth and guidance events during DA9 axon development. Rather than acting as an orienting cue, UNC-6 acts at the surface of UNC-6-positive neighboring neurons, allowing the DA9 dendrite to grow along them. This is reminiscent of *Drosophila* Netrin at the ventral midline, where Netrin is not required for attracting axons toward the midline, but rather for promoting their growth across it[30]. Several pieces of our data suggest that a second pathway also promotes dendrite outgrowth. Among them, the dendrite was significantly shortened, but not lost, in all single mutants. Furthermore, both *unc-34* and *unc-40* phenotypes were more severe than that of *unc-6*, suggesting they respond to another ligand as well. The *unc-40* cDNA expression was able to partially rescue the *unc-6* phenotype, also speaking to its having an *unc-6*-independent function. Ventral UNC-6 seems to have a permissive function in dendrite outgrowth, but does not convey directional (antero-posterior) information. This would suggest that there is an unidentified directional signal that is acting in conjunction with UNC-6. The ability of the *Pmig-13::unc-40* overexpression to have effects in wild-type but not *unc-6* worms suggests that *unc-40* also has a nonautonomous role in DA9 dendritic extension.

PAR-4 acts downstream of UNC-40

We found that PAR-4 is a new type of downstream component of UNC-6–UNC-40 signaling in promoting dendrite outgrowth in DA9. PAR-4 is among a group of genes having asymmetrical effects on intra-cellular events, such as cell-division, apical-basal epithelial cell polarization and neurite extension[42]. These genes were first described in a *C. elegans* screen for 'partitioning-defective' worms showing defects in early cell divisions, P granule distribution and intestinal development[34,43]. Its fly and mouse homologs *lkb1* and *LKB1* have since been implicated in cleavage spindle placement, centrosome localization and downstream events such as asymmetric cell division, neuronal migration and axon outgrowth[44,45]. *LKB1* conditional mutants were used to demonstrate that LKB1 is required cell-autonomously for axon outgrowth and phosphorylates SAD-A and SAD-B kinases in cortical neurons *in vivo*[33]. As mutants of *sad-1*, the worm homolog of mammalian SAD kinases, do not show a truncated dendrite, PAR-4 is likely acting through other genes (data

not shown). Both the fact that UNC-115 and PAR-4 are regulated by means of the UNC-40 P2 motif and the absence of enhancement observed in the genetic interaction of the *par-4; unc-115* mutant suggest that *par-4* is acting in one genetic pathway with the actin-binding protein *unc-115*. As *unc-115* loss of function suppresses the excess outgrowth resulting from *par-4* overexpression, this may be one avenue by which UNC-6 signals to the cytoskeleton by way of PAR-4 (Supplementary Fig. 7b).

The use of distinct signaling mechanisms within one neuron in response to one extracellular signal explains how a cell can generate local responses independently of each other. In DA9, a dendrite growth response is promoted in the neurite in which UNC-40 is localized preferentially and is present at the tip of the growing neurite. UNC-40 responds to local UNC-6 and acts through genes that do not contribute to axon growth or guidance in DA9. To achieve this response specificity, a simultaneous suppression of the dorsal guidance response is probably achieved by excluding the UNC-5 receptor or its downstream players from the leading edge of the dendrite[27]. Whereas UNC-5 mediates axon guidance and outgrowth in DA9, other neurons, such as VD inhibitory motor neurons, extend axons in the ventral cord and send dendrites to the dorsal cord using UNC-6–UNC-5 signaling, suggesting that different cells can use similar mechanisms in different ways to specify axon and dendrite outgrowth.

Another example of one guidance cue differentially regulating dendrite and axon development within one neuron was reported in cortical pyramidal neurons. The growth of apical dendrites toward the pial surface is mediated by the diffusible chemoattractant Sema-3A, which also acts as a chemorepellent for cortical axons[1,46]. In this case, whereas it seems that one receptor is mediating both axon and dendrite responses, a soluble guanylate cyclase is asymmetrically localized to the developing dendrite and is required for the chemo-attractive effect of Sema-3A. Thus, asymmetric localization of intra-cellular signaling components and receptors are mechanisms by which axons and dendrites can be patterned in response to one extracellular cue. As the study of axon and dendrite growth and guidance has not continued to uncover more extrinsic cues in recent years, it is likely that neurons use a plethora downstream signaling mechanisms in a neurite-specific fashion, such as the one described here, which we have yet to discover.

ONLINE METHODS

Strains and genetics

Worms were raised on OP50 *Escherichia coli*–seeded NGM plates at 22 °C, excepting *par-4(it47ts)* and *par-4(it47ts)*–derived strains, which were raised at the permissive temperature 16 °C and analyzed at 25 °C. The following mutant strains were obtained through the Caenorhabditis Genetics Center: CF896 *dpy-20(e1282) IV; muIs42 (Pmig-13::mig-13::GFP; dpy-20 +)*, CB271 *unc-40(e271) I*, MT464 *unc-5(e53) IV*, NW434 *unc-6(ev400) X*, TV2918 *unc-34(gm104) V*, KK184 *par-4(it47ts) V* and SK4005 *zdIs5 I*. CX3079 *unc-115(ky275) X* was obtained from E. Lundquist's laboratory. CX4834 *zdIs4 IV; kyEx456 (Pmec-7:: myr::unc-40)*, CX5999 *zdIs4 IV; kyEx639 (Pmec-7::myr::unc-40ΔP1)* and CX5997 *bad-1(ky592) V; zdIs4 IV; kyEx637 (Pmec-7::myr::unc-40ΔP2)* were obtained from the Bargmann laboratory, and CX5997 was outcrossed to eliminate *bad-1(ky592)*. N2 Bristol was used as the wild-type reference strain.

par-4 husbandry and temperature shift experiments

par-4(it47ts) worms were raised at the permissive temperature of 16 °C. The PAR-4 requirement is surpassed at the four-cell stage[43], and *par-4(it47)* worms show delayed growth at the permissive temperature. L4 worms were picked onto a plate and let grow to

gravid adults and lay eggs, which were shifted to 25 °C at 36 h after picking the L4 stage. These eggs hatched and were raised at 25 °C until the L3 stage, at which point they were imaged for dendrite length.

Cloning and constructs

Expression clones were made in a derivative of pPD49.26 (ref. 47) with additional cloning sites from C.I. Bargmann (Rockefeller University). The following plasmids and strains were generated using standard techniques: *wyEx1902 (Pitr-1::mCherry*[27], *wyEx2501* (*Pmig-13::unc-40*), *wyEx2462 (Pmig-13::unc-40::gfp)*, *wyEx2885*(*Punc-6::unc-6::nlg-1TM::mCherry*), *wyEx3249 (Punc-129::unc-6)* and *wyEx3250 (Pitr-1::par-4::gfp)*. We used the co-injection markers *Podr-1::gfp* or *Podr-1::dsRED* at 20 ng μl^{−1}. Transgenes were introduced into *C. elegans* as previously described[48]. *Pmec-7::myr::unc-40ΔP3* plasmid was obtained from the Bargmann lab and injected at 50 ng μl^{−1} into *zdIs4 IV* worms *(wyEx4206)*. For *Pmig-13::unc-40* (*wyEx2501*), a SphI–AscI fragmentcontaining *Pmig-13* (ref. 23) was subcloned into *unc-40* pSM from *Pttx-3::unc-40. Pmig-13::unc-40* was injected at 20 ng μl^{−1} into *unc-40(e271)* worms. This array was subsequently outcrossed from *unc-40(e271)* worms into the N2 background. For *Pmig-13::unc-40::gfp (wyEx3208)*, an SphI–AscI fragment containing *Pmig-13* was subcloned into *unc-40::gfp* pSM from *Pttx-3::unc-40::gfp. Pmig-13::unc-40:: gfp* was injected at 5 ng μl^{−1} into N2 worms. *Punc-6unc-6::nlg-1TM::mCherry (wyEx2885)* was injected at 15 ng μl^{−1} with the *Punc-122::rfp* co-injection marker at 20 ng μl^{−1}. For *Punc-129::unc-6 (wyEx3249)*, an SphI–AscI PCR fragment containing a *Punc-129* promoter fragment driving expression in dorsal muscle[23] cloned into *Punc-6::unc-6* was injected at 10 ng μl^{−1} into *unc-6(ev400)* worms. For *Pitr-1::par-4::gfp* (*wyEx3250*), an SphI–AscI PCR fragment containing *Pitr-1pB*[23] was subcloned into *par-4::gfp* pSM and was injected at 20 ng μl^{−1} into N2 worms. *par-4* cDNA (K. Kemphues, Cornell University) was amplified using the following primers: 5′-ACATGGCGCGCCATGGATGCTCCGTCGACATCCTCGGGAGC ACA-3′; 5′-CCGGTACCGGAGCACTATCGGTACGAGAACG-3′.

Microscopy methods, equipment and settings

For all imaging of DA9, worms were immobilized using 10 mM levamisole (Sigma), and for imaging and measuring body length 0.3 M 2,3-butanedione monoxime (BDM) was used. All images were taken at 25 °C. Images of fluorescently tagged fusion proteins were captured in live *C. elegans* using a Plan-Apochromat ×63, numerical aperture 1.4, oil immersion, differential interference contrast objective on a Zeiss LSM510 confocal microscope. Images were acquired using LSM510 software release 4.0 SP1 in Expert mode using the following settings: pixel depth, 8 bits; stack size (pixels), $1024 \times 512 \times$ number of slices (1.8 μm slice depth or scan interval); objective, Plan-Apochromat ×63, 1.4 numerical aperture, oil immersion, differential interference contrast; pixel time, 3.2 μs; main beam splitter 405/488/543/633; dichroic beam splitter 1 635 visible; dichroic beam splitter 2 545; Wavelength, 488 nm; 100% transmission; filter, channel 2, band pass 505–530 nm; pinhole, channel 2, 248 μm. Detector gain, 658; amplifier offset, 0; amplifier gain, 1. Images were processed in Adobe Photoshop CS2 version 9.0 for brightness and contrast. Quantification of dendrite length and body length was carried out by imaging with a Zeiss Axiophot with AxioCam/Axiovision digital imaging. Dendrite length was measured on these images from the anterior point of process extension from the cell body to the process tip. Body length was measured from tip of head to tail.

Statistical analysis

Statistical analysis was performed using two-tailed Student's *t*-tests, Pearson's χ^2-test or Mann-Whitney *U*-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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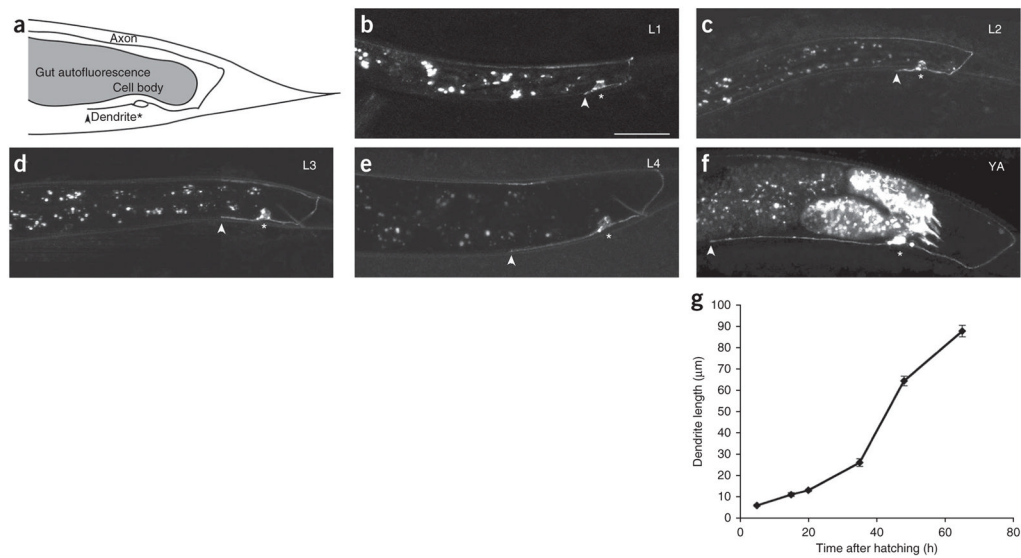48. Mello C, Fire A. DNA transformation. Methods Cell Biol. 1995; 48:451–482. [PubMed: 8531738]

Figure 1.
Development of the DA9 motor neuron in wild-type *C. elegans*. (**a**) Schematic diagram of DA9. The DA9 cell body is located in the tail. Its axon extends posteriorly in the ventral cord, dorsally through a commissure and anteriorly in the dorsal cord. The ventrally located soma elaborates a single, unbranched dendrite anteriorly. Gut autofluorescence, within gray patch, should be disregarded in all images. Asterisk, cell body; arrowhead, tip of dendrite. (**b–f**) Lateral view of wild-type worms expressing *Pmig-13::mig-13::gfp* in DA9, at developmental stages L1–L4 and young adult (YA). (**b**) An L1 worm. Worms hatch with a fully formed axon, while the dendrite is a stump. The dendrite orientation is already determined by L1. (**c–f**) The dendrite continues to grow anteriorly throughout the life of the worm (images L2–YA). (**g**) Growth curve of dendrite over time. Early larval dendritic growth takes place at a steady pace (L1–L3), followed by a growth spurt between L4 and YA and slowing in older adults. All pictures are lateral view with ventral down and anterior to the left; all quantification from anterior edge of soma to anterior tip of dendrite. Scale bar, 20μm. *n* 20. Error bars, s.e.m.
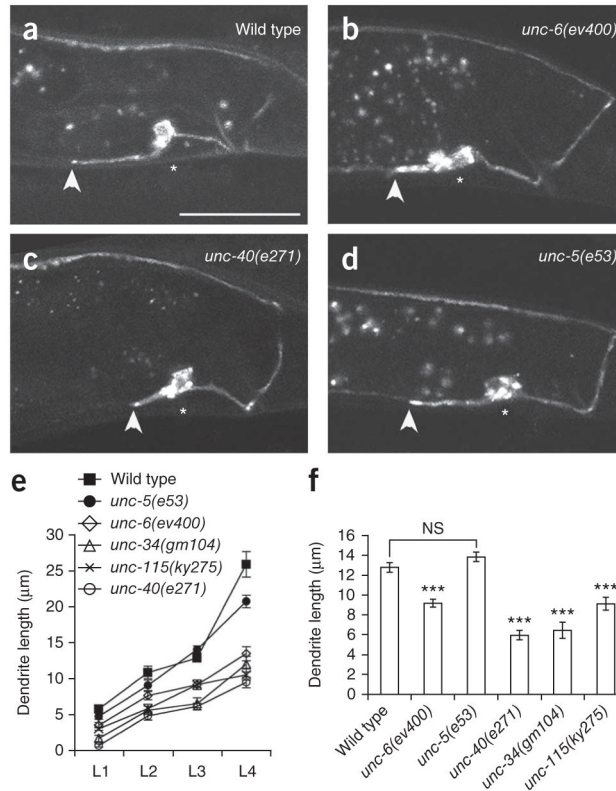
Figure 2.
UNC-6 (Netrin) regulates dendrite outgrowth through UNC-40 (DCC). (**a–d**) Micrographs of wild-type and *unc-6*, *unc-40* and *unc-5* mutants expressing *Pmig-13::mig-13::gfp*, revealing truncated dendrites in *unc-6* and *unc-40* mutants. (**e**) Growth curves showing dendrite length in L1–L4 wild-type and *unc-6* signaling mutants. A minimal defect in dendrite growth in L1 worms increases over larval growth, with a trend toward *unc-40* signaling mutant phenotypes being more severe than *unc-5*. (**f**) Dendrite lengths in L3 worms. All pictures are lateral views with ventral down and anterior to the left, all quantification from anterior edge of soma to anterior tip of dendrite. Scale bar, 20 μm. *n* 20. Error bars, s.e.m. ***$P < 0.001$; NS, not significant; Student's *t*-test.
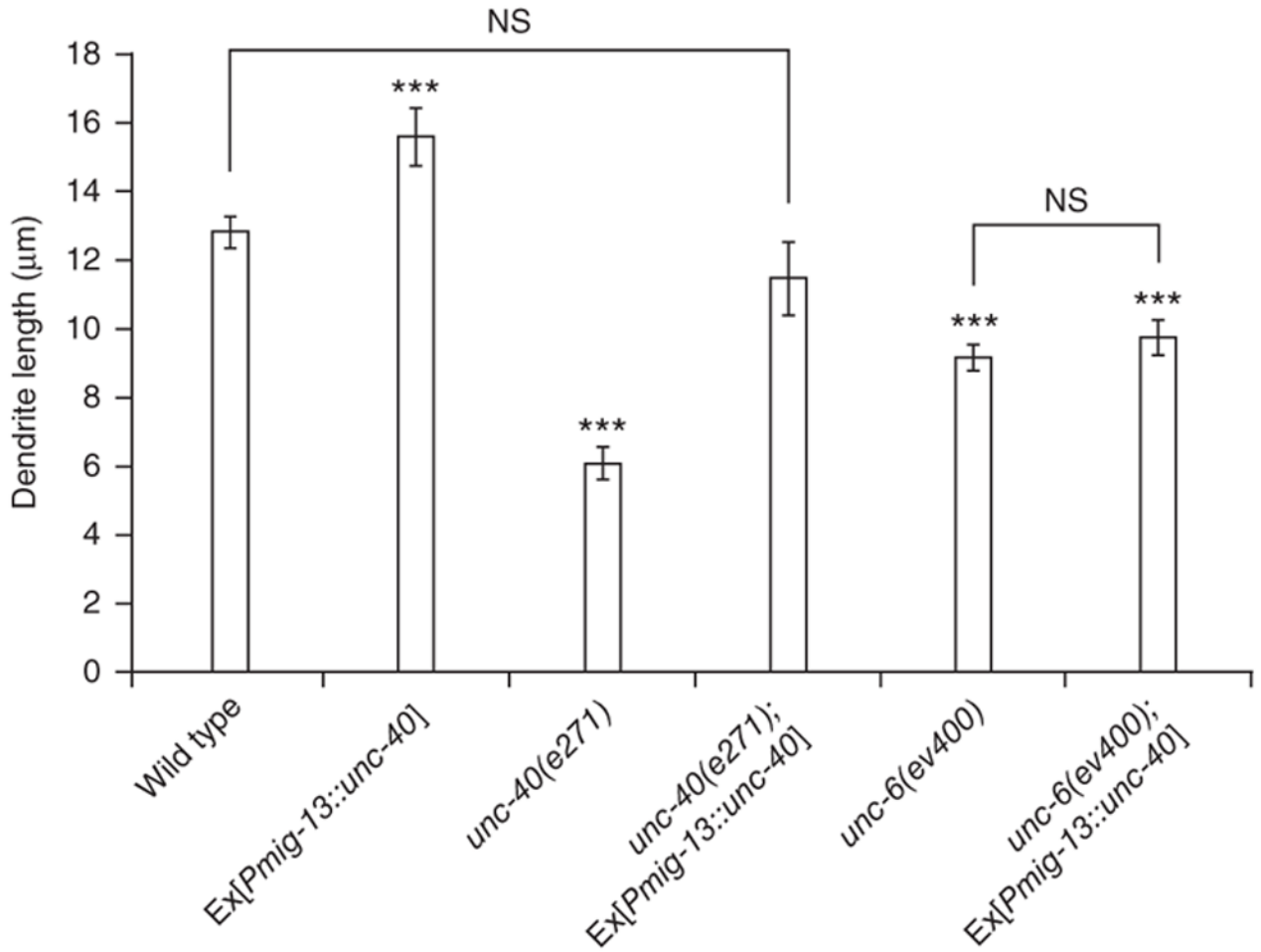
Figure 3.
UNC-40 is necessary and sufficient for dendrite outgrowth. Graph of dendrite lengths in wild-type and with UNC-40 transgenes in various mutant background. All quantification is from anterior edge of soma to anterior tip of dendrite. n 20. Error bars, s.e.m. ***$P <$ 0.001; NS, not significant; Student's t-test.
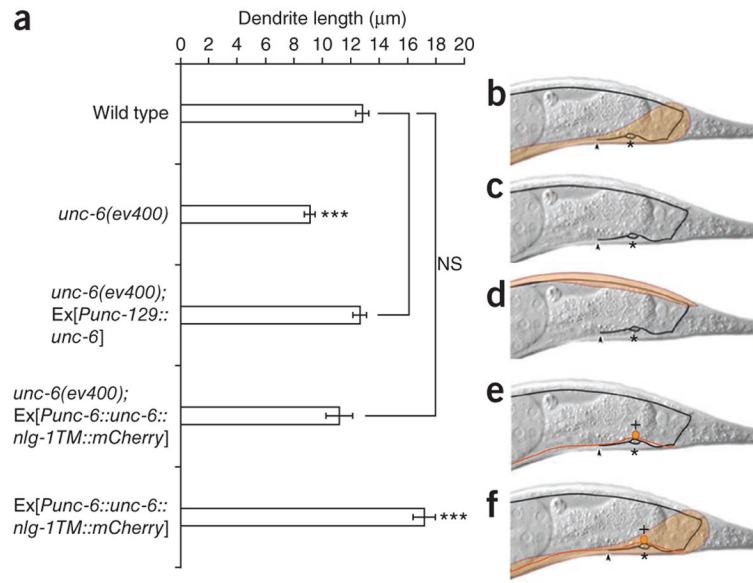
Figure 4.
Local, non-graded UNC-6 signaling is sufficient for dendrite outgrowth. (**a**) Graph of dendrite length in different genetic backgrounds. (**b**–**f**) Schematics of the corresponding UNC-6 expression pattern (orange shading) in each background. Dendrite lengths in wild-type (**b**) and *unc-6* worms (**c**), as well as rescue of outgrowth by dorsal muscle *unc-6* expression (*unc-6(ev400)*; Ex[*Punc-129::unc-6*]) (**d**) and membrane-tethered *unc-6* expression (*unc-6(ev400)*; Ex[*Punc-6::unc-6::nlg-1TM:: mCherry*]). The soma of the neighboring VA12 motor neuron (red) is marked by + (**e**). Overexpression of membrane-tethered *unc-6* is sufficient for dendrite overshooting (**f**). All quantification from anterior edge of soma to anterior tip of dendrite. *n* 25. Error bars, s.e.m. ***$P < 0.001$; NS, not significant; Student's *t*-test.
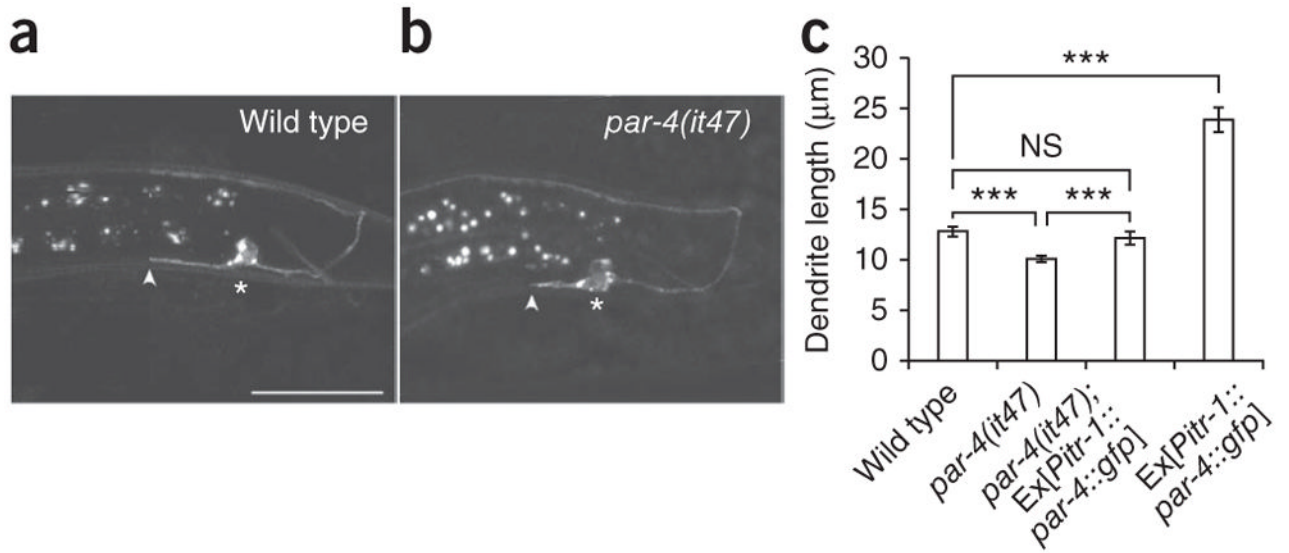
Figure 5.
PAR-4 (LKB1) is necessary and sufficient for dendrite outgrowth. (**a**, **b**) Lateral view of wild-type and *par-4* mutants expressing *Pmig-13:: mig-13::gfp* at L3 stage. (**c**) Graph of dendrite length in L3 wild-type, *par-4*, *par-4* rescue (*par-4(it47)*; Ex[*Pitr-1::par-4::gfp*]) and *par-4* overexpression (Ex[*Pitr-1::par-4::gfp*]). All worms are L3 stage, all pictures are lateral view with ventral down and anterior to the left, all quantification from anterior edge of soma to anterior tip of dendrite. Scale bar, 20 μm. *n* ≥ 30. Error bars, s.e.m. ***P < 0.001; NS, not significant; Student's *t*-test.
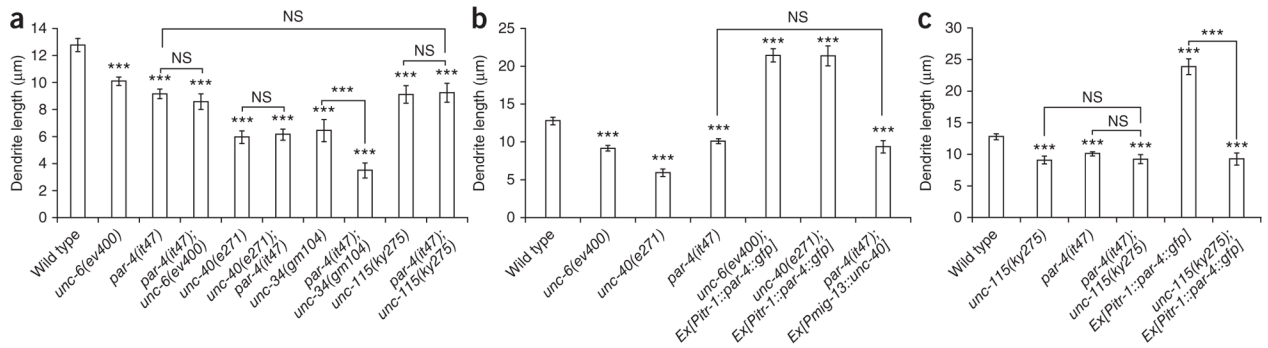
Figure 6.
PAR-4 acts downstream of UNC-6 and UNC-40. (**a**) Graph of dendrite length in L3 worms
in *par-4*, *unc-6*, *unc-40*, *unc-34* and *unc-115* mutants and their double mutants with *par-4*.
The *par-4; unc-6*, the *unc-40; par-4* and the *par-4; unc-115* mutants do not enhance,
whereas *unc-34; par-4* mutants show a marked enhancement in dendrite truncation. (**b**)
Dendrite length in L3 worms expressing a *par-4* cDNA construct in *unc-6* and *unc-40*
mutants (*unc-6(ev400)*; Ex[*Pitr-1::par-4::gfp*] and *unc-40(e271)*; Ex[*Pitr-1::par-4::gfp*]
and worms expressing *unc-40* cDNA in a *par-4* mutant (*par-4(it47)*; Ex[*Pmig-13::unc-40*]).
Overexpression of *par-4* can compensate for *unc-6* or *unc-40* loss, whereas *unc-40*
overexpression does not rescue *par-4*. (**c**) Graph of dendrite length in L3 worms. *par-4;
unc-115* double mutants do not enhance compared to single mutants, and the overextension
caused by Ex[*Pitr-1::par-4::gfp*] expression is suppressed in *unc-115* mutants, suggesting
that *unc-115* acts downstream of *par-4*. All quantification is from anterior edge of soma to
anterior tip of dendrite. $n \geq 20$. Error bars, s.e.m. ***$P < 0.001$; NS, not significant;
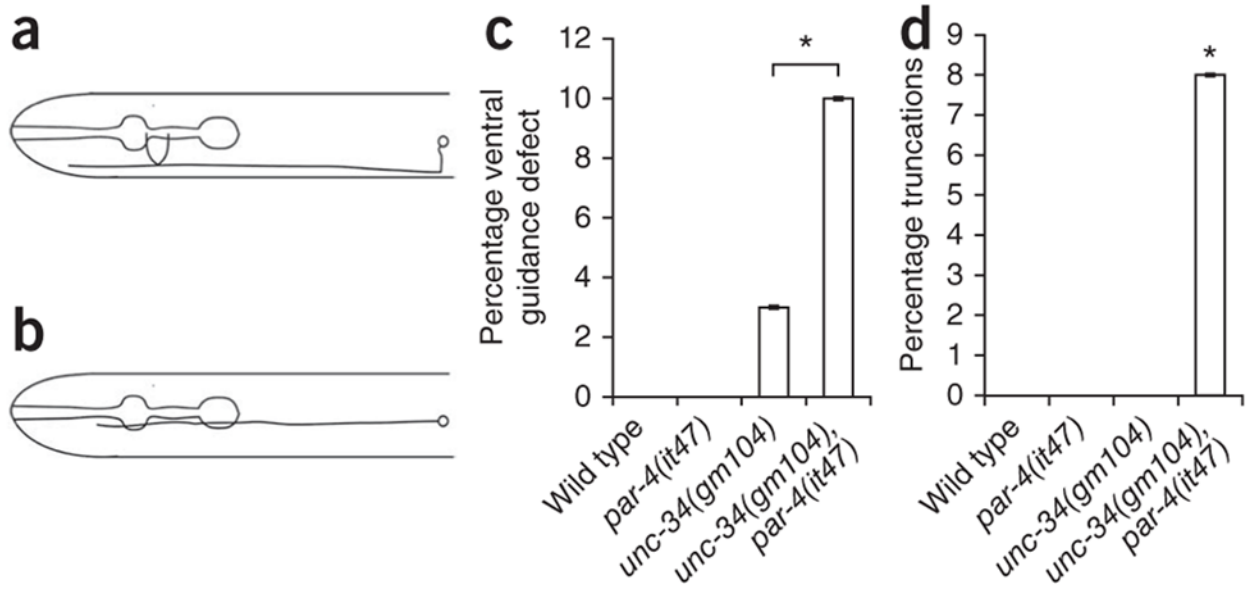Student's *t*-test.

Figure 7.
unc-34 and *par-4* act in parallel in AVM development. (**a**) The AVM soma is located
laterally in the worm and extends a process which first migrates ventrally, then anteriorly
into the head (ventral down, anterior to the left). (**b**) *unc-34* and *unc-40* mutants show a
guidance defect in which the AVM process fails to make a ventral turn before extending[12].
(**c**) Graph of percentage of guidance defects in AVM. Wild-type and *par-4* single mutants do
not show ventral guidance defects, whereas *unc-34* mutants show a low-penetrance defect.
The frequency is enhanced in *unc-34; par-4* double mutants. (**d**) Graph of percentage of
outgrowth defects in the AVM process. Wild-type, *par-4* and *unc-34* single mutants
consistently have AVM processes that grow beyond the pharynx terminal bulb, whereas
par-4, unc-34 double mutants show a low-penetrance outgrowth defect. *n*   100. Error bars,
standard error of proportion; **$P < 0.01$, *$P < 0.05$, χ^2 test.