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Duplication of a single neuron in *C. elegans*
reveals a pathway for dendrite tiling
by mutual repulsion

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Running title: Dendrite tiling in *C. elegans*

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Summary:

Simple cell-cell interactions can give rise to complex cellular patterns. For example, neurons of the same type can interact to create a complex patchwork of non-overlapping dendrite arbors, a pattern known as dendrite tiling. Dendrite tiling often involves mutual repulsion between neighboring neurons. While dendrite tiling is found across nervous systems, the nematode *C. elegans* has a relatively simple nervous system with few opportunities for tiling. Here, we show that genetic duplication of a single neuron, PVD, is sufficient to create dendrite tiling among the resulting ectopic neurons. We use laser ablation to show that this tiling is mediated by mutual repulsion between neighbors. Furthermore, we find that tiling requires a repulsion signal (UNC-6/Netrin and its receptors UNC-40/DCC and UNC-5) that normally patterns the PVD dendrite arbor. These results demonstrate that an apparently complex cellular pattern can emerge in a simple nervous system merely by increasing neuron number.

Introduction:

Neurons are complex machines that rely on their diverse geometries for proper function. Their precisely sculpted shapes include intricate patterns of dendrite arborization. Two key mechanisms of dendrite patterning are self-avoidance and tiling. Dendrite self-avoidance is mediated by repulsion between sister dendrites of a single neuron, ensuring that they splay apart to cover a large area (Cameron and Rao, 2010; Grueber and Sagasti, 2010). Self-avoidance requires adhesion molecules from large diverse families—thousands of spliced isoforms of Dscam1 in *Drosophila*, and dozens of combinations of protocadherin proteins in the mammalian retina and cerebellum—such that each neuron recognizes only itself and not its neighbors (Grueber and Sagasti, 2010; Hughes *et al.*, 2007; Lefebvre *et al.*, 2012; Matthews *et al.*, 2007; Soba *et al.*, 2007; Zipursky and Grueber, 2013). In contrast, dendrite tiling refers to the constrained growth of dendrite arbors from neighboring neurons such that they occupy distinct territories with minimal overlap. Tiling arises from combinations of mechanisms that regulate cell body spacing and dendrite arbor size. Here, we will focus on the role of mutual repulsion in tiling.

Notably, the molecules that have been implicated in tiling by mutual repulsion do not exhibit the diversity of isoforms characteristic of dendrite self-avoidance molecules—these include cadherin in *Drosophila* mechanosensory dendrites (Gao *et al.*, 2000; Grueber *et al.*, 2002), as well as Dscam2 and Plexin-Semaphorin in tiling-like interactions between axonal or synaptic regions respectively (Cameron and Rao, 2010; Millard and Zipursky, 2008; Mizumoto and Shen, 2013). Yet, despite their molecular differences, both self-avoidance and tiling can involve mutual repulsion between dendrites, raising the question whether a single pathway could, in

principle, mediate both. Indeed, pioneering studies showed that self-avoidance molecules can be experimentally engineered to mediate tiling by mutual repulsion. Forced expression of a single version of Dscam1 in *Drosophila* da neurons or protocadherin in mouse retinal starburst amacrine cells caused the neurons to aberrantly begin to tile, presumably by rendering them unable to distinguish self from nonself (Hughes *et al.*, 2007; Lefebvre *et al.*, 2012; Matthews *et al.*, 2007; Soba *et al.*, 2007). These results suggest a model in which tiling can emerge merely as a secondary byproduct of self-avoidance: each neuron “tries” to use self-recognition and repulsion to prevent its own dendrites from crossing but, because its neighbors also use the same molecules, it cannot distinguish self from nonself, with the result that neighbors end up tiling. It is important to note, however, that additional tiling mechanisms are already present in *Drosophila* and mouse, where these experiments were performed.

A strong prediction of the emergent tiling model is that it should be possible to create tiling by mutual repulsion in an organism where it does not normally exist, by using the endogenous mutual repulsion pathways that normally mediate dendrite self-avoidance. We reasoned that we could test this prediction by taking advantage of the highly simplified nervous system of *C. elegans*. Notably, several phenomena related to tiling have been described in this system, but are not known to involve mutual repulsion. The mechanosensory neurons ALM and PLM extend sensory processes into non-overlapping domains in the anterior and posterior portions of the animal, respectively, reminiscent of dendrite tiling (Gallegos and Bargmann, 2004). However, this differs in key respects from tiling of dendrite arbors by mutual repulsion: (1) the neurites are not branched and do not form arbors; (2) the neurites do not approach one another, as ALM lies in the dorsal portion of the body and PLM lies ventrally; and (3) it does not involve mutual

repulsion, as laser ablation or genetic removal of ALM does not lead to a change in the PLM neurite territory (Gallegos and Bargmann, 2004). Another related phenomenon is synaptic tiling, in which the neurites themselves do not change shape or size but the positioning of their synaptic fields shows mutual antagonism (Mizumoto and Shen, 2013). In fact, most *C. elegans* neurons extend non-branched dendrites, and only a few (PVD, FLP, IL2) can form branched arbors that exhibit self-avoidance. Intriguingly, PVD and FLP have been shown to form non-overlapping arbors, but it was not known whether these arise by mutual repulsion (Albeg *et al.*, 2011; Smith *et al.*, 2010). In some cell fate mutants, another neuron is converted to a PVD-like identity, and rare mosaic animals were used to show that this PVD-like neuron can form a dendrite arbor that does not overlap with that of FLP in the head or the endogenous PVD in the body (Smith *et al.*, 2013). These results suggested that PVD neurons, in particular, might be capable of undergoing dendrite tiling by mutual repulsion.

We therefore focused on the PVD neurons, a pair of bilaterally symmetric touch-sensitive cells that each extends a dendrite arbor covering the entire body of the animal on its left or right side, positioned just under the skin (Figure 1A). Primary dendrites originating from each PVD cell body extend along the anterior-posterior axis, running along the lateral nerve cord and terminating posterior to the head at a structure called the nerve ring (Smith *et al.*, 2010). Secondary, tertiary, and quaternary dendrites branch in orthogonal arrays from the primary dendrite to form repeating units of menorah-like structures that cover the body wall (Oren-Suissa *et al.*, 2010). The tertiary dendrites exhibit self-avoidance, which is mediated by the guidance molecule UNC-6/Netrin and its cell-surface receptors UNC-40/DCC and UNC-5 (Smith *et al.*, 2010; Smith *et al.*, 2012). Because there is only one PVD neuron on each side of the animal,

PVD neurons do not normally have other PVD neighbors with which to tile. Thus, we asked what would happen to the PVD dendrite arbors if we increased the number of PVD neurons on each side of the body.

Results:

Increasing the number of PVD neurons leads to spatially restricted dendrite arbors

We generated four additional PVD neurons using the cell lineage mutant *lin-22*. *lin-22* encodes a transcription factor that directs the lateral epidermal seam cells V1-V4 to generate hypodermal cells (hyp), while the V5 seam cell produces PVD as well as other cells (Horvitz *et al.*, 1983; Wrischnik and Kenyon, 1997). Loss of LIN-22 causes V1-V4 to adopt the lineage of the V5 seam cell; *lin-22* mutants recently were shown to generate four pairs of PVDs (PVD1-PVD4) in addition to the normal PVD pair (PVD5) (Liang *et al.*, 2015). Consistent with these observations, in the course of a genetic screen we serendipitously isolated three new *lin-22* alleles (*hmn23*, *hmn44*, and *hmn59*) as displaying extra PVDs; here, we use the reference allele *n372* throughout this work.

Because PVD1-5 all express the same markers, we could not resolve individual dendrite arbors using GFP. Therefore, we developed an optical cell marking strategy in which we expressed the photoconvertible fluorescent protein kaede (PVD::*kaede*) in all PVDs and then photoconverted a single PVD (Ando *et al.*, 2002). Because PVD dendrites are very thin, we were concerned that the photoconverted material might not efficiently fill the entire dendrite arbor. Therefore, as a control, we photoconverted PVD in wild-type animals, determined the

time necessary to label the entire arbor, and conducted all experiments under these conditions (Figure S1).

Using this method in *lin-22* animals, we found that PVD5 forms a spatially restricted arbor that terminates near the neighboring PVD cell body (Figure 1B). The dendrites often appeared to be packed more densely (Figure S2; average number of quaternary dendrites in a sampled region $\pm$ SEM = 11.2 ± 0.4 , wild type; 18.8 ± 1.7 , *lin-22*). We also observed quaternary dendrites occasionally interdigitating with dendrites from neighboring PVDs (Figures S2B and S2C, orange brackets), reminiscent of the way dendrites interdigitate while maintaining uniform interdendrite spacing in regions of overlap between retinal ganglion cells (Dacey, 1989; Wässle *et al.*, 1983). We observed some examples of fasciculation between neighboring dendrites, typically involving primary dendrite branches, although instances involving quaternary branches were also identified (Figure S2C, arrowheads). All of these observations are consistent with the idea that each PVD neuron is attempting to elaborate a normally-sized arbor in a restricted area. Due to these complexities, we decided to quantify the linear anterior-posterior extent of each arbor as a simple, robust proxy for tiling. We measured the distance from a PVD cell body to its most distant dendrite along the anterior-posterior axis, and normalized these measurements to the distance from the PVD cell body to the nerve ring (NR) to account for differences in body size. We found that PVD5 dendrites terminate far short of their normal end point at the nerve ring ($P < 0.0001$) (Figure 1C). PVD1-4 dendrite arbors behave similarly, with each arbor terminating near the neighboring PVD cell body and only partially overlapping with adjacent arbors (Figure 1D), thus dividing up the body into a series of distinct territories.

PVD dendrite territories form by mutual repulsion

We hypothesized that the reduced arbor size reflects mutual repulsion between PVDs. To test this hypothesis, we laser ablated PVD1-4 soon after their birth and measured the extent of the mature PVD5 arbor (Figures 2A and 2C). Under these conditions, PVD5 arbors extended farther than in non-ablated controls ($P = 0.0004$), showing that arbor size is indeed determined by mutual repulsion between neighbors. 14/14 PVD5 arbors terminated beyond the normal position of the PVD4 cell body, and 2/14 arbors extended to the nerve ring (Figures 2A and 2C). This extent of growth is probably an underestimate – as shown in Figure 2A, higher-order PVD5 dendrite branches between the ablated cell bodies were sometimes absent, possibly reflecting either a decrease in dendrite growth rate due to effects of the *lin-22* mutation or tissue damage caused by ablation that delayed or inhibited dendrite growth. Indeed, we found that the dendrite extent of PVD5 continued to increase beyond the time point used for our measurements (Figure S3A). In a second experiment designed to test for the role of repulsion, we laser ablated two or three PVDs in *lin-22* animals and distinguished between the remaining PVDs by photoconverting PVD5 (Figures 2B and 2D). In all five cases, the non-ablated PVDs extended their dendrites to completely fill the body wall, yet did not overlap with one another (Figures 2B and 2D). Consistent with the notion that PVD dendrite arbors tile by mutual repulsion, we conducted time-lapse imaging experiments of growing PVD dendrites in *lin-22* and observed contact-and-repulsion events between dendrites that appear to originate from neighboring PVDs (Figure S3B). Together, these experiments show that the spatially restricted PVD territories observed in *lin-22* reflect tiling by repulsion.

Dendrite tiling in PVD uses the same molecules as self-avoidance

How might PVD tiling arise? We considered two hypotheses. First, it is possible that a mechanism for tiling by mutual repulsion between neighbors is used in the development of the wild-type PVD and can be co-opted for PVD-PVD tiling. FLP is a touch-sensitive neuron in the head that elaborates a dendrite arbor similar to that of PVD in the body (Figure 3A) (Smith *et al.*, 2010). FLP and PVD arbors occupy distinct territories and do not overlap (Albeg *et al.*, 2011; Smith *et al.*, 2010). The mechanism underlying this pattern has not been identified; in particular, it is not known whether it requires nonself recognition or mutual repulsion. Similarly, the bilaterally symmetric PVDs (PVDL and PVDR) occupy distinct territories on the left and right sides of the body and do not overlap. We reasoned that if FLP-PVD or PVDL-PVDR tile by mutual repulsion, then the molecules they use could also mediate tiling of ectopic PVDs.

To test this hypothesis, we performed laser ablation of FLP and asked whether PVD dendrites extended into the FLP dendrite territory. FLP ablation was performed in wild-type first larval stage (L1) animals before PVD neurons are born, and the resulting PVD dendrite arbors were examined three days later when the animals reached adulthood (1-day adults) and again two days later (3-day adults). Conversely, we also ablated the PVD neuron in wild-type second larval stage (L2) animals, shortly after PVD neurons are born, and examined the FLP dendrite arbor in 1-day and 3-day adults. In all cases, we did not observe PVD primary dendrites extending into the head when FLP was ablated ($n = 7$) (Figures 3B and S4) or any change in FLP dendrite arbor size when PVD was ablated ($n = 7$) (Figures 3C and S4). These results strongly suggest that the non-overlapping FLP and PVD territories reflect the presence of an anatomical or molecular barrier, such as a ridge of tissue or the localized presence of non-permissive growth

signals. An alternative possibility is that dendrite extension was prevented by tissue damage caused by the laser ablation; however, at least in the case of the PVD ablations this is unlikely, as the ablated cell body is $\sim 500\ \mu\text{m}$ from the FLP dendrites. Importantly, when we ablated PVD on one side of wild-type animals (Figure S4), or PVD1-4 in *lin-22* mutants (Figure 2), we did not observe invasion of PVD dendrites from the contralateral side, suggesting that PVDL and PVDR respect a barrier at the midline (data not shown). Thus, we observed no evidence for tiling via nonself recognition and repulsion in wild-type animals.

Second, we considered the hypothesis that the PVD self-avoidance molecules (UNC-6/Netrin and its receptors DCC/UNC-40 and UNC-5 (Smith *et al.*, 2012)) could result in the emergence of tiling when extra neurons are introduced. To test this idea, we generated *lin-22; unc-6* mutants using either of two alleles of *unc-6* (*e78* and *ev400*), with the prediction that loss of UNC-6 would abolish dendrite tiling and result in overlapping PVD dendrite territories. Indeed, loss of UNC-6 disrupted PVD tiling ($P = 0.0008$ and 0.004 for *e78* and *ev400* respectively) (Figures 4 and S5A), as did the simultaneous loss of both Netrin receptors UNC-40 and UNC-5 ($P = 0.002$). In contrast, while loss of either UNC-40 or UNC-5 alone has been shown to disrupt self-avoidance (Smith *et al.*, 2012), these single mutants showed negligible or mild tiling defects ($P = 0.3$ and 0.06 respectively), suggesting that these receptors act redundantly in the case of tiling (Figures 4B and S5). Taken together, our results strongly suggest that endogenous self-avoidance molecules can create dendrite tiling by mutual repulsion in an organism where it does not normally exist.

Discussion:**Dendrite tiling can emerge as a byproduct of self-avoidance**

Here, we show that repulsive signaling pathways that normally pattern the dendrite arbor of a single neuron can give rise to dendrite tiling when the number of neurons is increased. These results are consistent with previous observations in which another neuron was converted to the PVD fate and, in rare genetic mosaics, was shown to form a dendrite arbor that did not overlap with that of the endogenous PVD (Smith *et al.*, 2013). Our results are surprising because the development of an apparently complex neuronal pattern like tiling might have been expected to require the evolution of new, dedicated molecular pathways. That is, natural selection would have favored animals bearing gene variants that promoted dendrite tiling. In contrast, our results support a possible alternative model in which dendrite tiling was not directly subject to natural selection, but rather arose as a byproduct of mutual repulsion pathways that evolved to mediate dendrite self-avoidance.

This model resembles Gould and Lewontin's classical concept of 'exaptation', in which a structural feature emerges as a secondary byproduct of another — their architectural metaphor involved the spandrels that form when an arch is built beneath a domed ceiling (Gould and Lewontin, 1979). Importantly, the idea that tiling is a byproduct of self-avoidance does not mean it lacks functional importance in the nervous system. Indeed, a central tenet of exaptation is that these new byproduct structures are exceptionally well poised to impart new functionality. Classic examples include feathers, which are hypothesized to have evolved as insulators and only later contributed to flight, and bones, which are hypothesized to have evolved as phosphate storage organs and only later contributed to mechanical support (Gould and Vrba, 1982).

Similarly, tiling could have arisen as an evolutionary "spandrel" but, once present, provided an important functional contribution to neuronal circuits by allowing better spatial resolution of incoming signals. Interestingly, in systems where tiling was detrimental, there would have been selective pressure to reduce tiling without affecting self-avoidance — strategies for this might have included the extensive splicing of *Dscam1*, which occurs only in a sublineage of arthropods, and the use of the expanded protocadherin gene cluster in mammals (Armitage *et al.*, 2012; Chen and Maniatis, 2013).

Netrin receptors act in dendrite repulsion across species

The use of Netrin signaling in *C. elegans* seems, on its face, to be different from the more well-established dendrite repulsion signaling mediated by *Dscam1*. However, it is worth noting that *Dscam* itself is a Netrin receptor — both the *Drosophila* and mammalian forms of *Dscam* bind Netrin with affinities comparable to that of its canonical receptor UNC-40/DCC (Andrews *et al.*, 2008; Ly *et al.*, 2008) — and DSCAM can physically interact with UNC5C/UNC-5 to transduce Netrin-mediated repulsion signals (Purohit *et al.*, 2012). Intriguingly, Slit/Robo signaling — which often acts in conjunction with Netrin signaling during axon guidance — was recently shown to act as a dendrite repulsion signal in the mammalian cerebellum (Gibson *et al.*, 2014). It is therefore interesting to speculate whether dendrite repulsion pathways in *C. elegans*, *Drosophila*, and mammals all share a common origin with pathways used in axon guidance.

Non-overlapping dendrite arbors can be established by mutual repulsion or by barriers

Finally, our results suggest the existence of at least two distinct mechanisms that prevent overlap between dendrite arbors. First, tiling can be established by mutual repulsion, as occurs

among the ectopic PVDs. Interestingly, although our study and others show that mutual repulsion alone can explain tiling (Hughes *et al.*, 2007; Lefebvre *et al.*, 2012; Matthews *et al.*, 2007; Soba *et al.*, 2007), observations in the retina suggest that tiling is not always so simple. The dendrite arbors of some retinal ganglion cells exhibit mutual repulsion at their edges — first, they overlap less than if they were oriented randomly (Wässle *et al.*, 1981); second, in regions where they do overlap, dendrite branches interdigitate rather than crossing (Dacey, 1989); and third, when neurons are killed, the remaining arbors reorient towards the vacated territory (Eysel *et al.*, 1985) — yet genetically ablating most of these cells does not lead to expanded growth of the remaining dendrite arbors, as a simple mutual repulsion model would predict (Eysel *et al.*, 1985). Rather, some other mechanism seems to constrain the overall growth of their dendrite arbors. Conversely, the dendrite arbors of retinal horizontal and bipolar cells exhibit extensive overlap between neighbors — suggesting an absence of mutual repulsion — yet these arbors nevertheless expand or reduce in size when cell number is decreased or increased, respectively, implying some other mechanism that coordinates the dendritic territory of a neuron with that of its neighbors (Lee *et al.*, 2011; Poché *et al.*, 2008). These experiments indicate that, in the retina, mutual repulsion alone is neither necessary nor sufficient to determine the extent of overlap between neighboring dendrite arbors, but may have been combined with additional mechanisms that contribute to more complex developmental interactions among dendrites.

Second, tiling can be established by a barrier, as seems to occur between PVD and FLP. Importantly, recent studies show that local anatomical cues from epithelia or muscles can play an important role in shaping dendrite arbors, including imposing restrictions on the size of a dendrite arbor (Dong *et al.*, 2013; Liang *et al.*, 2015; Parrish *et al.*, 2009; Salzberg *et al.*, 2013).

The presence of anatomical cues that do not require nonself recognition and repulsion may explain the seemingly paradoxical result that some classes of Dscam1 mutant neurons continue to tile normally despite failing to self-avoid: what has been grouped together as “tiling” may in fact be a mixture of repulsion-mediated effects, which are a secondary byproduct of self-avoidance, and restricted growth due to local anatomical cues, which provide an entirely independent way to ensure non-overlapping arbors.

Experimental Procedures:

Strains were constructed in the N2 background and cultured under standard conditions (Brenner, 1974; Stiernagle, 2006). In addition to the wild-type strain N2, the mutations, transgenes, and strains used in this study are described in Tables S1-S3. For CHB1374, only animals lacking the hT2 balancer were analyzed.

Microscopy and Image Processing

Paneled image stacks were collected on a DeltaVision Core imaging system (Applied Precision) with a UApo 40×/1.35 NA oil-immersion objective and a Photometrics CoolSnap HQ2 camera (Roper Scientific). 1-day adults were mounted on an agarose pad with 10-50 mM sodium azide or 10 mM levamisole. For optical cell marking, photoconversion was performed by targeting 1-2 50 ms pulses of a 406 nm laser at 10% power at a PVD cell body. The animals were immediately recovered from the slides to standard Nematode Growth Medium (NGM) plates containing OP50 and incubated for ≥ 2 h to permit diffusion of photoconverted material. Animals were again mounted on slides and imaged in yellow (excitation (EX) 513 nm/emission (EM) 559 nm) and red channels (EX 575 nm/EM 632 nm).

Deconvolution and analysis of images were performed with Softworx (Applied Precision) and ImageJ (National Institutes of Health, Bethesda, MD). Maximum brightness projections were obtained using contiguous optical sections. Individual panels of maximum brightness projections were stitched together using the Stitch tool in Softworx. Projections were adjusted for brightness, contrast, and were pseudo-colored in Photoshop (Adobe). Because the quaternary PVD dendrites are much thinner than the PVD cell body, and thus harder to visualize, nonlinear

gamma settings were applied to the entire image using the Curves adjustment tool in Photoshop. Merged color images were assembled using the Screen layer mode in Photoshop.

PVD dendrite arbors were manually reconstructed using the Pencil Tool in Illustrator using a stitched maximum-brightness image projection of the entire animal. Figures were assembled using Photoshop CS5.1 and Illustrator CS5.1.

Laser ablations

PVD ablations in *lin-22* animals (Figures 2 and S3A): *lin-22* animals in the late second larval stage (L2) or third larval stage (L3) expressing the PVD-specific marker *ser-2prom3::kaede* (PVD::*kaede*, CHB422) were anesthetized with 10 mM sodium azide and mounted on agarose pads between a slide and a coverslip. Ablations were timed to target newly born PVD neurons in which dendrite extension was not yet observed. PVD neurons were identified by PVD::*kaede* under a 100× oil objective using a Zeiss Axioplan microscope. PVDs were killed with a pulsed nitrogen laser (Laser Science, Inc. VSL-337) focused through a dye cell containing coumarin (5mM in methanol), which shifted the wavelength of the laser from 337nm to 435nm. The PVD-ablated animals were recovered and then imaged two or more days later, as 1-, 3-, and 5-day adults, using photoconversion as described where necessary (see Figure S3A).

FLP and PVD ablations in wild-type animals (Figures 3 and S4): FLP neurons were ablated in animals expressing PVD::*mCherry* and *mec-3::GFP* (CHB1226). Since FLP neurons are born during embryogenesis – much earlier than PVD neurons, which are born in late L2 – we ablated FLP in first larval stage (L1) or early L2 animals, prior to PVD neuron birth (assayed by the lack

of expression of PVD::mCherry). For PVD neuron ablations, we laser ablated newly born PVD neurons in CHB1226, about 30 h after plating starvation-synchronized L1 animals. FLP- and PVD-ablated animals were imaged as 1-day and 3-day adults in the green channel (EX 475 nm/EM 525 nm) for FLP and red channel (EX 575 nm/EM 632 nm) for PVD.

Quantification of PVD and FLP dendrite arbors

Dendrite arbor extent was quantified using the segmented line tool in ImageJ using stitched maximum brightness projections. Dendrite extent was measured as the distance from the labeled PVD cell body to its most anterior or posterior dendrite branch, normalized to the distance between the *lin-22* PVD5 cell body or wild-type PVD and the nerve ring. As a reference, the mean distance between the *lin-22* PVD5 (or wild-type PVD) cell body and the nerve ring in Day 1 adults across all genotypes in this paper ($n = 202$) was $475.4 \pm 7.0 \mu\text{m}$ (mean $\pm$ SEM). Thus, x-axis values are arbitrary normalized distance units. PVD1-4 cell body positions labeled on the schematized animal immediately below the x-axes represent the average PVD positions of *lin-22* ($n372$) animals. We used Student's t-test to determine *P*-values.

For FLP dendrite arbor measurements (Figure 3B), dendrite extent was measured as the distance from the FLP cell body to its most posterior dendrite branch, normalized to the distance between the FLP cell body and the nerve ring. The PVD and FLP cell body positions in Figure 3B labeled on the schematized animal immediately below the x-axes represent the average distance between PVD and nerve ring in the unablated animal, normalized to the distance between the FLP cell body and the nerve ring.

Quantification of photoconverted fluorescence intensity along the primary dendrite

Wild-type 1-day adult animals expressing PVD::*kaede* were imaged in yellow (EX 513 nm/EM 559 nm) and red channels (EX 575 nm/EM 632 nm) before, 1 min and 120 min after *kaede* photoconversion in the PVD cell body (Figure S1). To quantify the fold difference between red and yellow (R/Y) fluorescence intensities, summed projections through the optical stack were used to measure the fluorescence intensities along the length of the primary dendrite. After subtracting the background noise, which was determined for individual image panels by measuring the mean intensity value in a $20 \times 20 \mu\text{m}$ area, the R/Y fluorescence intensity ratio was calculated along the primary dendrite. The R/Y data was smoothed using a central moving average of data points contained within a $15 \mu\text{m}$ interval.

Quantification of quaternary dendrite branch density

To quantify dendrite branch density in Figure S2D, we counted the number of quaternary dendrites located in an $80 \mu\text{m}$ region immediately posterior to the PVD5 cell body in wild-type and *lin-22* 1-day adult animals. Only dendrite branches on one side of each animal (either dorsolateral or ventrolateral) were included. For clarity of presentation, data points with identical values in Figure S2D were nudged to prevent them falling exactly on top of each other.

Time-lapse imaging of PVD dendrite growth

Time-lapse imaging of PVD dendrite growth was conducted using a modified version of the protocol described in (Smith *et al.*, 2010). The L3-stage individual (CHB2163) was first immobilized using 13 mM levamisole + 0.05% tricaine in M9 buffer, and then mounted onto an agarose pad containing the same concentration of levamisole and tricaine. Image stacks (0.75

μm/step) were collected every 2.5 min on the DeltaVision Core imaging system using a PlanApo 60×/1.42 NA oil-immersion objective. Figure S3B panels were created using maximum-brightness projections of the image stack at the indicated timepoints.

Statistical analysis

We used Student's t-test to compare statistical significance of two independent groups. $P < 0.05$ was considered significant. All data are shown as mean $\pm$ SEM.

Author contributions:

Z.C.Y. and M.G.H conceived the ideas and designed the experiments. Z.C.Y. performed the experiments and analyzed the data. Z.C.Y. and M.G.H. wrote the manuscript.

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Figure Legends:**Figure 1. *lin-22* mutants generate multiple PVD neurons.**

(A) Wild-type animal (head is to the left) visualized by PVD::*kaede*. Arrowheads indicate the anterior and posterior extents of the PVD arbor. Bottom: tracing of PVD dendrites.

(B) *lin-22* (*n372*) individual expressing PVD::*kaede* following photoconversion of PVD5. Red arrowheads indicate extent of the PVD5 arbor. * : cell body of a head neuron, OLL, which also expresses the PVD marker.

(C) Quantification of the anterior dendrite extent of PVD, in wild-type (blue lines; $n = 12$) and PVD5 in *lin-22* (red lines; $n = 19$) animals. $P = 5E-13$ by Student's t-test. Gray lines, population average.

(D) Anterior and posterior dendrite extents of PVD1 ($n = 3$), PVD2 ($n = 6$), PVD3 ($n = 6$) and PVD4 ($n = 9$) dendrite arbors in *lin-22*. Circles represent PVD cell body positions.

x-axis values in (C) and (D) represent normalized distance units; dendrite extent measurements are normalized to the distance from the PVD5 cell body to the nerve ring (see Methods). Scale bars, 50 μm . See also Figures S1 and S2.

Figure 2. Restricted PVD dendrite arbors reflect mutual repulsion.

(A) *lin-22* animals expressing PVD::*kaede* following ablation of PVD1-4. Orange ovals indicate approximate locations of ablated cell bodies. Scale bar, 50 μm .

(B) *lin-22* animals expressing PVD::*kaede* following ablation of PVD2-4 and photoconversion of PVD5. Orange ovals indicate approximate locations of ablated cell bodies. Scale bar, 50 μm .

(C) Quantification of the anterior dendrite extent of PVD (wild type; blue lines) or PVD5 (*lin-22*; red lines), following PVD1-4 ablation (red lines, bottom row) or controls (red lines, middle row). Gray lines, population average.

(D) Quantification of the dendrite extent of PVD5 (red) and unablated PVDs (green) ($n = 5$, individuals are shown in Figures 2B). Circles represent cell body positions.

x-axis values in (C) and (D) as in Figure 1. See also Figure S3.

Figure 3. FLP and PVD establish dendrite arbors using a shared boundary.

(A) Schematic representation of FLP (red) and PVD (blue) neurons and their dendrite territories (shaded areas).

(B) Quantification of PVD primary dendrite length in wild-type and FLP-ablated animals bearing FLP::GFP (*mec-3pro:GFP*) and PVD::mCherry (CHB1226). $P = 0.5$ by Student's t-test.

(C) Quantification of FLP posterior dendrite length in wild-type and PVD-ablated animals bearing FLP::GFP (*mec-3pro:GFP*) and PVD::mCherry (CHB1226). $P = 0.09$ by Student's t-test.

x-axis values in (B) and (C) as in Figure 1. See also Figure S4.

Figure 4. UNC-6/Netrin signaling is required for dendrite tiling.

(A) *lin-22* (*n372*); *unc-6* (*e78*) individual expressing PVD::kaede following photoconversion of PVD5. Labels and scale bar as in Figure 1.

(B) Quantification of the anterior dendrite extent of PVD5 in genotypes indicated, normalized to average dendrite extent in corresponding *lin-22* (+) controls (red lines). Gray lines, population average. x-axis values as in Figure 1. P -values by Student's t-test.

See also Figure S5.

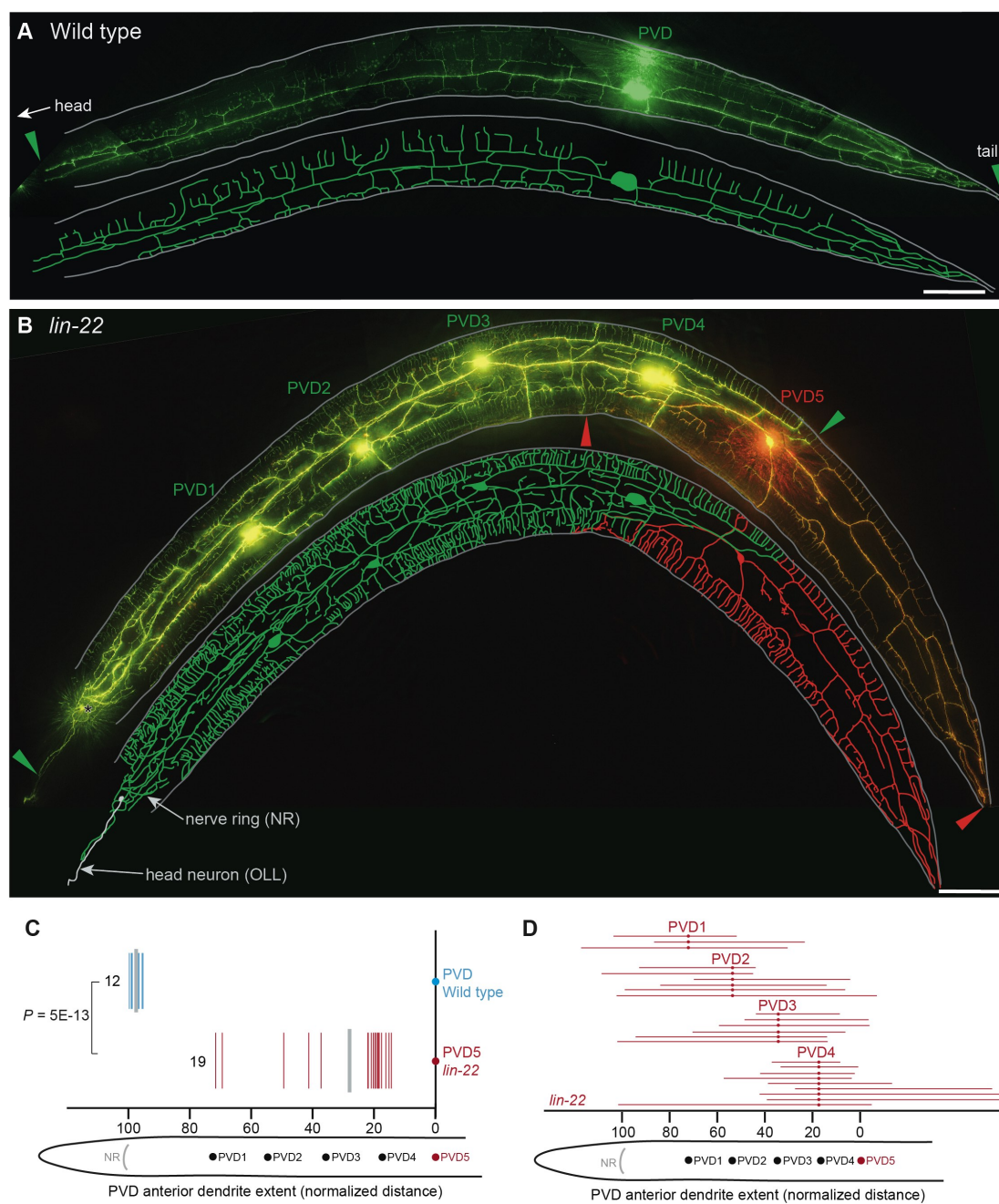
Figure 1

Figure 2

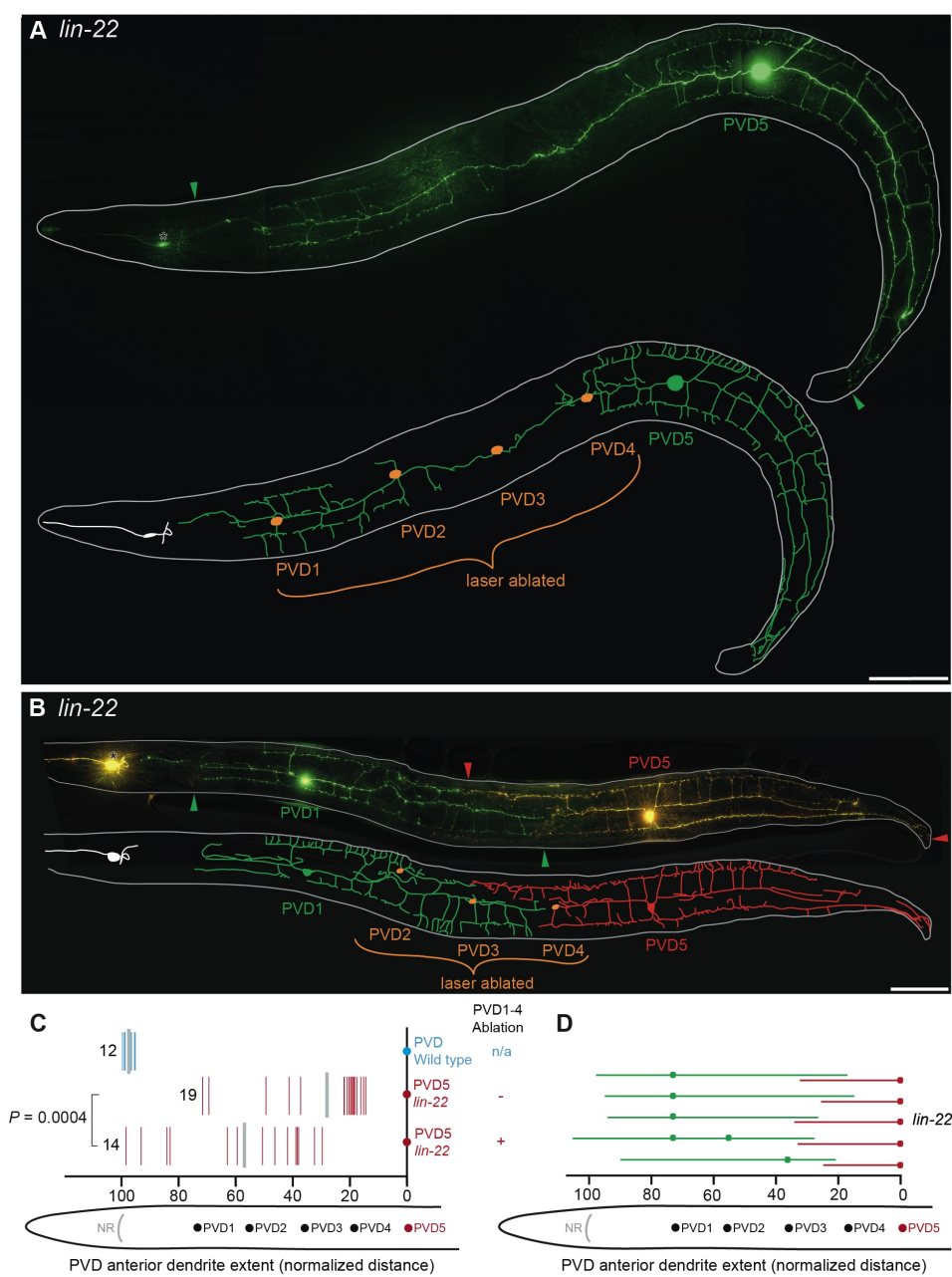


Figure 3

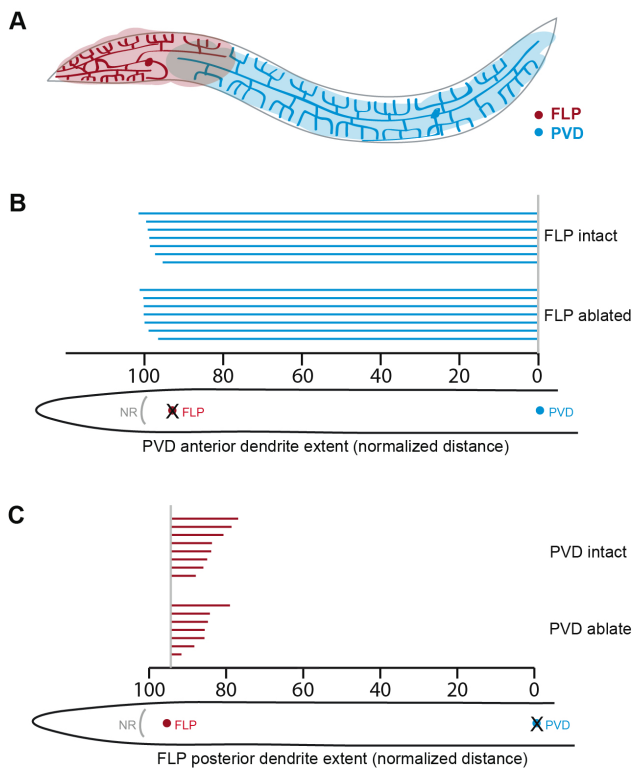
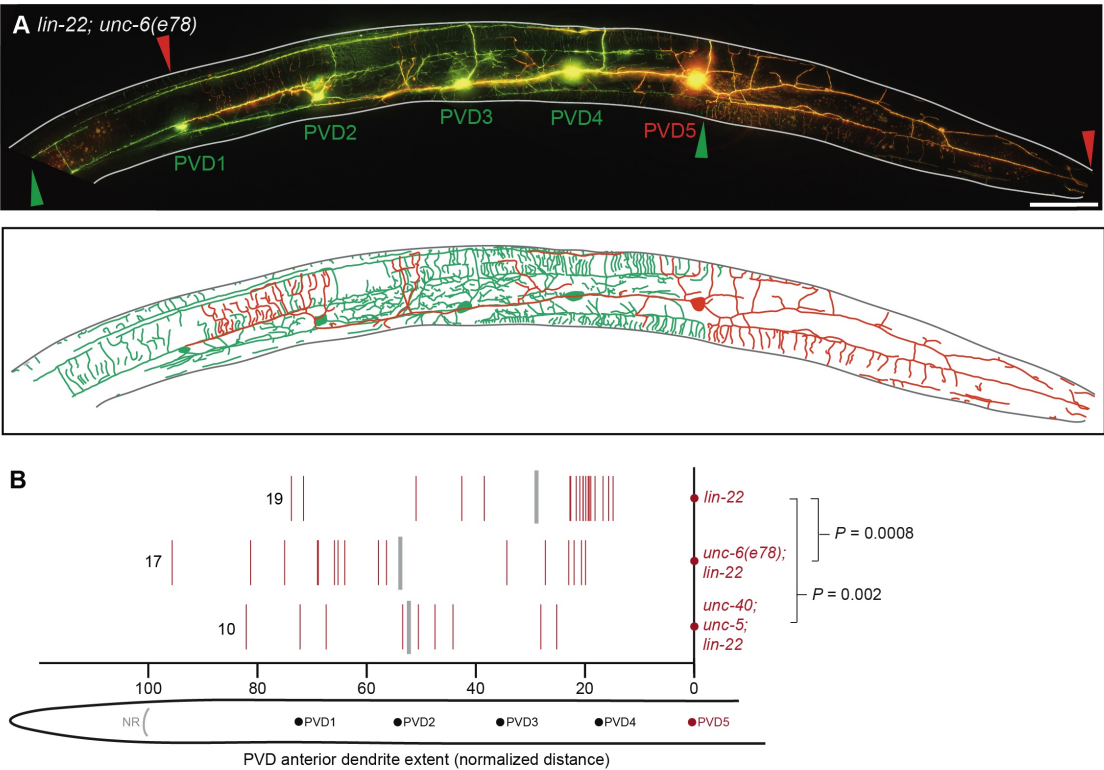


Figure 4



SUPPLEMENTAL INFORMATION

“Duplication of a single neuron in *C. elegans* reveals a pathway for dendrite tiling by mutual repulsion”

Zhiqi Candice Yip and Maxwell G. Heiman

Figures S1-S5

Tables S1-S3

Figure S1

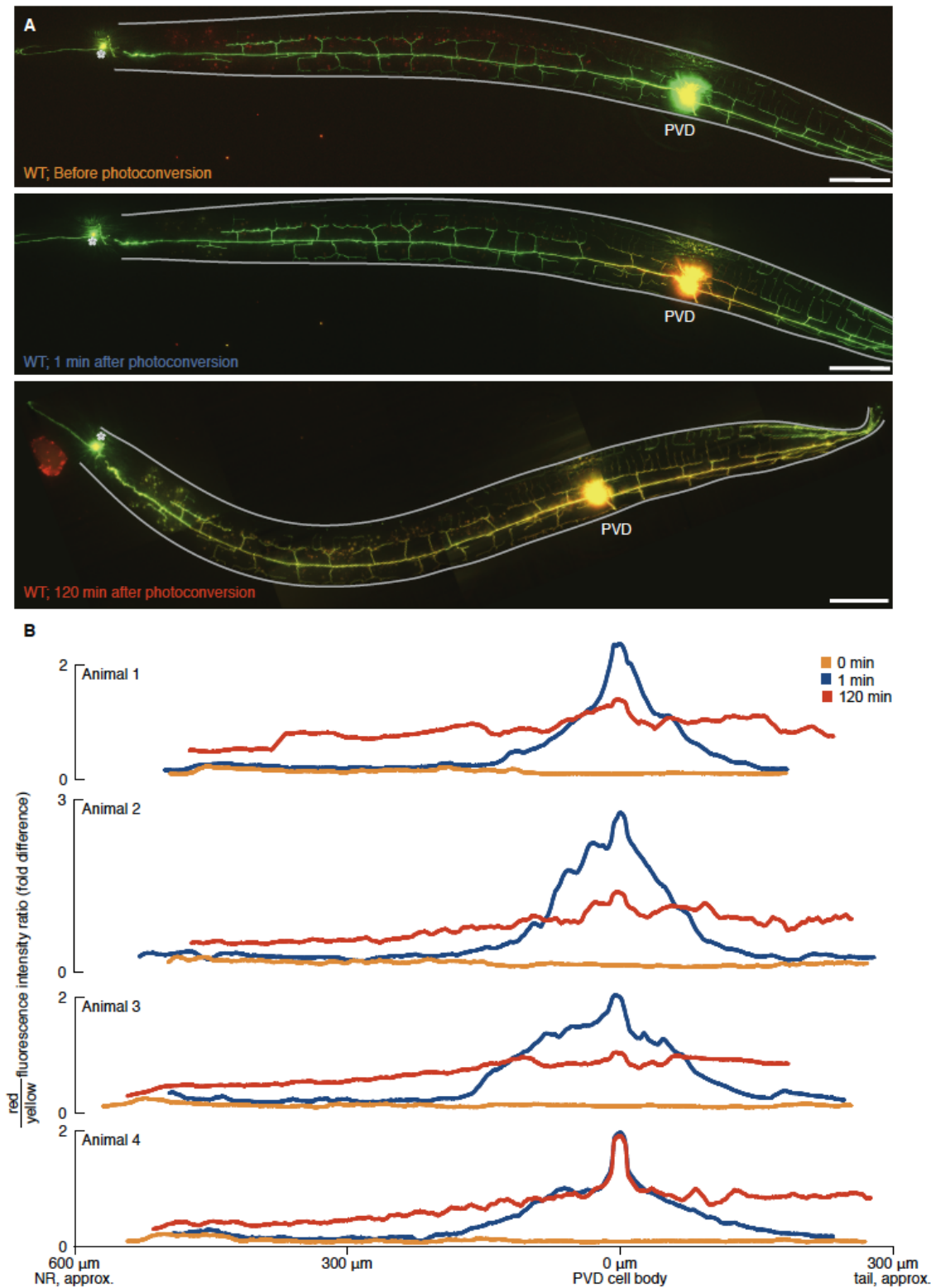


Figure S1. Photoconverted kaede labels the wild-type PVD primary dendrite within 2 h.**Related to Figure 1.**

(A) Wild-type animal expressing PVD::kaede imaged before photoconversion (top), or 1 min (middle) or 120 min (bottom) after photoconversion of the PVD cell body. The red-to-yellow (R/Y) fluorescence intensity ratio is shown in Figure S1B, Animal 1. Scale bars, 50 μ m.

(B) Quantification of fold difference of R/Y fluorescence intensity along the PVD primary dendrite before photoconversion (orange), or 1 min (blue) or 120 min (red) after photoconversion in four representative animals.

Figure S2

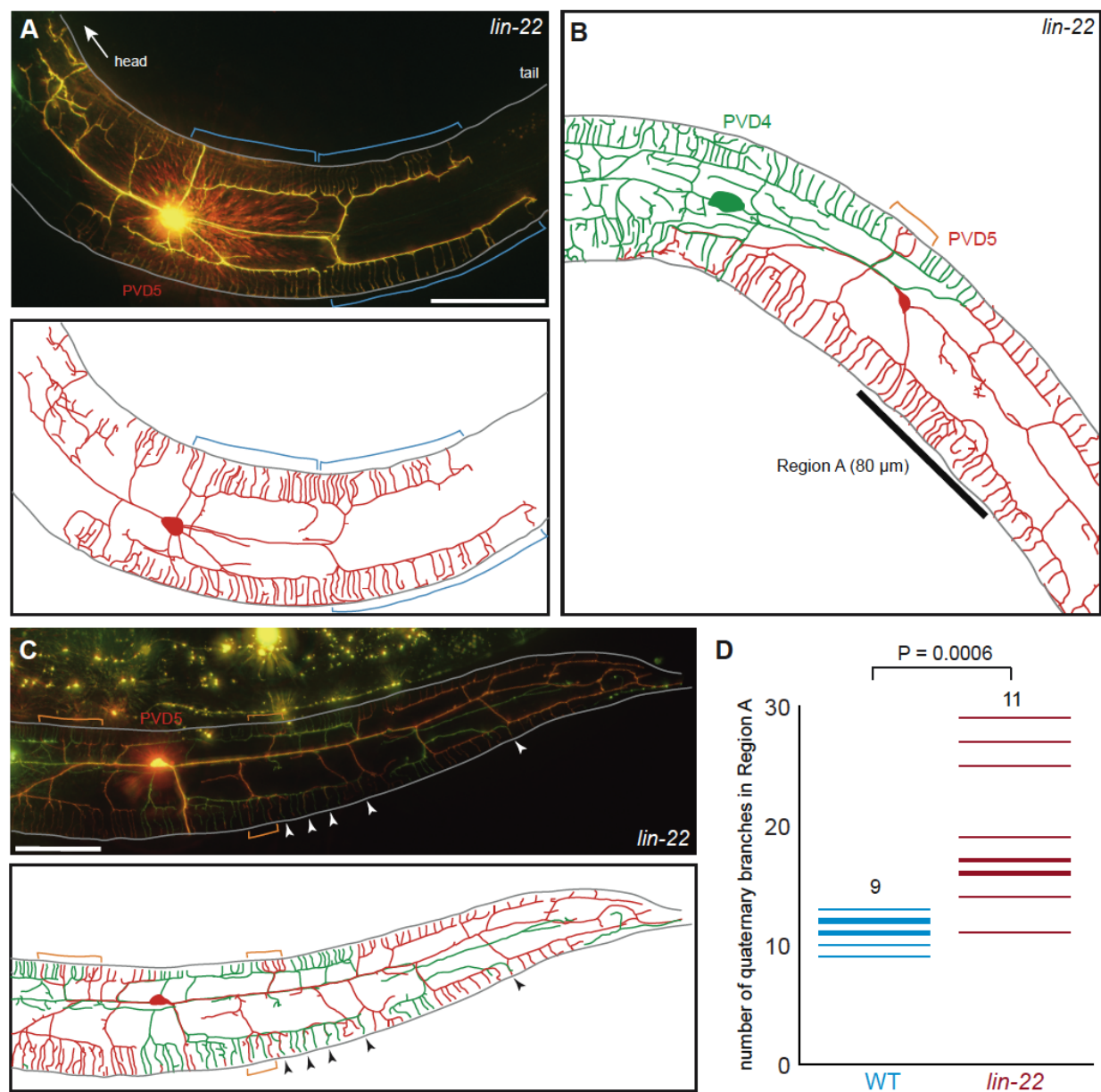


Figure S2. *lin-22* PVDs extend dense dendrite branches that interdigitate and fasciculate with dendrites emanating from neighboring PVDs. Related to Figure 1.

lin-22 PVDs extend long tertiary branches containing a high density of quaternary dendrites (**A**, blue brackets) compared to wild-type PVD (Figure 1A). *lin-22* PVD dendrites also interdigitate with dendrites from neighboring PVDs (**B and C**, orange brackets), and, in rare instances, quaternary branches fasciculate with neighboring PVD dendrites (**C**, arrowheads). (**B**) Magnification of dendrite arbors in Figure 1B (from the main text) showing a high density of quaternary dendrites. (**D**) Quantification of the number of PVD quaternary dendrites in an 80 μm region (“Region A”, as indicated in Figure S2B) located immediately posterior to the PVD5 cell body in wildtype and *lin-22* animals expressing PVD::*kaede*. Scale bars, 50 μm .

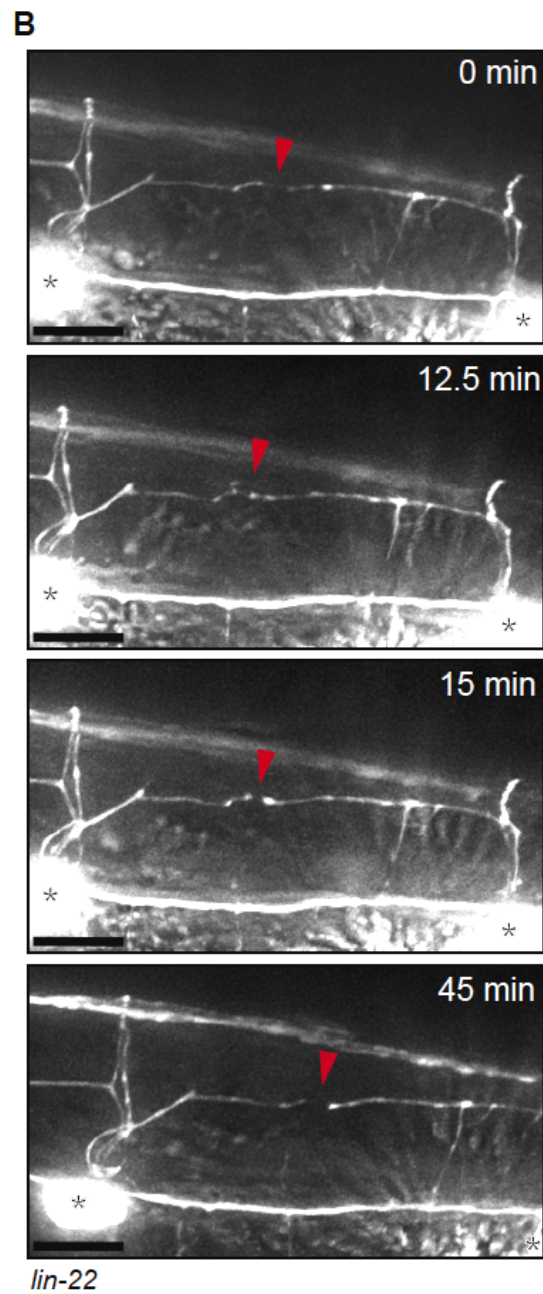
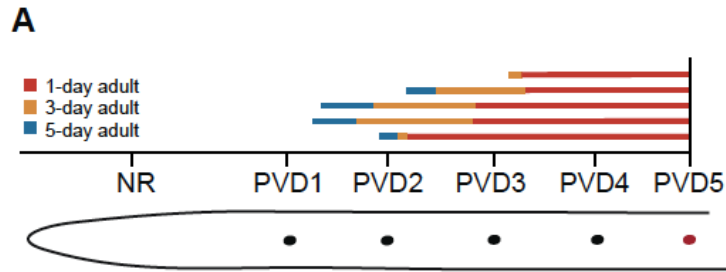
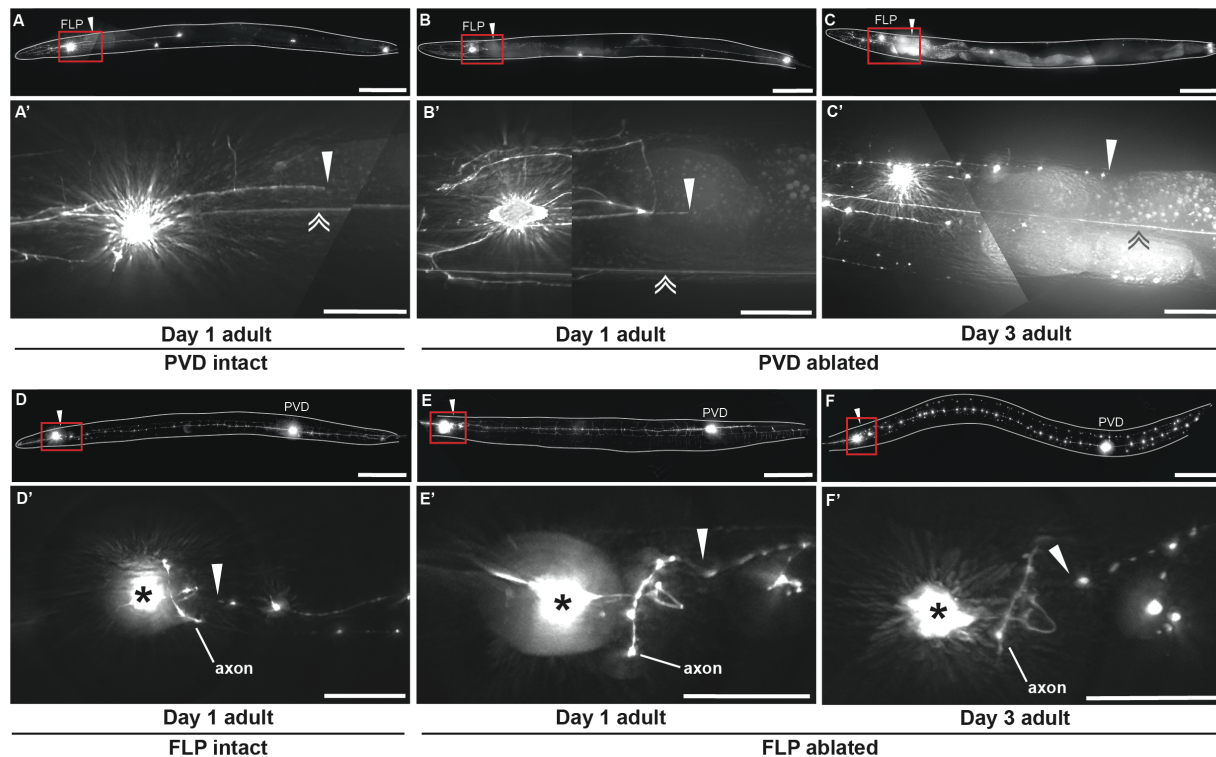
Figure S3

Figure S3. PVD dendrites undergo contact-mediated repulsion in *lin-22* animals. Related to Figure 2.

(A) PVD5 dendrites continue growing during adulthood after ablation of PVD1-4. PVD5 dendrite extent was measured in PVD1-4-ablated *lin-22* animals ($n = 5$) as 1-day adults (red, corresponding to the time point used in Figure 2C), 3-day adults (orange), and 5-day adults (blue). x-axis values as in Figure 1. (B) Time-lapse imaging of a contact-and-repulsion event between PVD dendrites in a single L3 *lin-22* animal (CHB2163) at timepoints indicated, visualized with PVD::mCherry. Asterisks indicate PVD cell bodies. Red arrows indicate the site of contact and repulsion between dendrites. Scale bars, 10 μm .

Figure S4**Figure S4. FLP and PVD do not tile by repulsion. Related to Figure 3.**

(A - C) Representative images of FLP::GFP in wildtype (CHB1226) of the following: Day 1 adult with PVD intact (**A and A'**); Day 1 adult with PVD ablated (**B and B'**), and the same animal 2 days later (**C and C'**). Arrowheads indicate posterior dendrite extent of FLP, and double arrowheads indicate sensory processes belonging to other neurons.

(D - F) Representative images of PVD::mCherry in wildtype (CHB1226) of the following: Day 1 adult with FLP intact (**D and D'**); Day 1 adult with FLP ablated (**E and E'**), and the same animal 2 days later (**F and F'**). Asterisks indicate OLL neuron cell bodies. Labeled axon belongs to OLL.

(A – F) Scale bars, 100 μm . (A' – F') Scale bars, 20 μm .

Figure S5

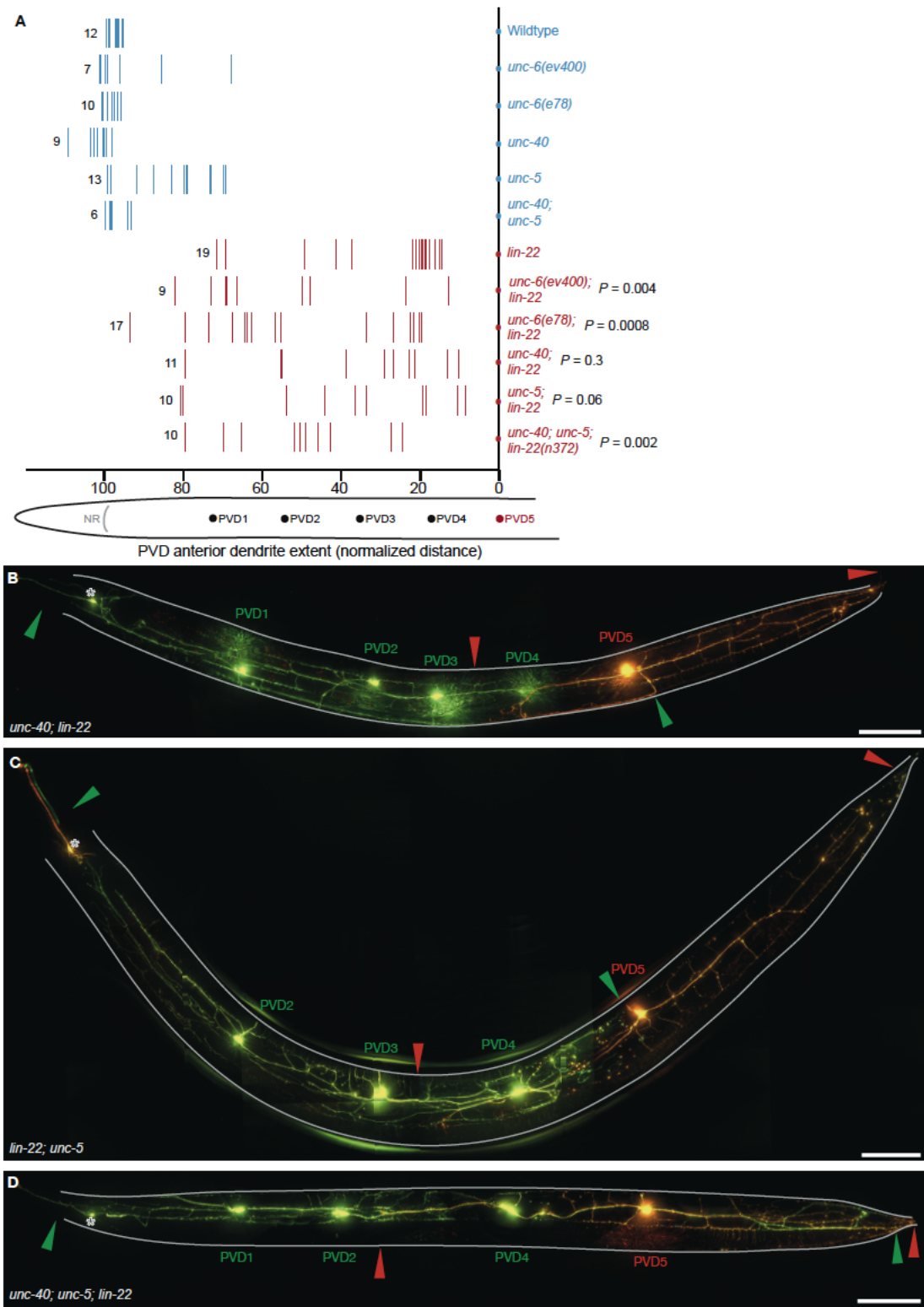


Figure S5. UNC-6/Netrin signaling is required for dendrite tiling. Related to Figure 4.

(A) Quantification of PVD5 anterior dendrite extents in two *unc-6* alleles (*ev400* and *e78*), *unc-40* (*e271*), and *unc-5* (*e152*), and *unc-40* (*e271*); *unc-5* (*e152*) double mutants (red and blue lines). x-axis values as in Figure 1. *P*-values (by Student's t-test) normalized to *lin-22* (+) animals (blue lines). (B) Dendrite tiling is normal in *unc-40*; *lin-22* mutants ($P = 0.3$ by Student's t-test, normalized to *unc-40* animals). (C) Dendrite tiling is partially normal in *unc-5*; *lin-22* mutants ($P = 0.06$ by Student's t-test, normalized to *unc-5* animals). (D) Dendrite tiling is disrupted in *unc-40*; *unc-5*; *lin-22* mutants ($P = 0.002$ by Student's t-test, normalized to *unc-40*; *unc-5* double mutant animals). Green and red arrowheads indicate the anterior and posterior extents of the PVD1-4 and PVD5 arbors, respectively. Scale bars, 50 μm .

Table S1. Strains used in this study. See Experimental Procedures.

All strains are available upon request.

strain	genotype
CHB446	<i>lin-22 (hmn23)</i> IV
CHB449	<i>lin-22 (hmn44)</i> IV
CHB375	<i>hulIs34</i> II; <i>lin-22 (hmn59)</i> IV; <i>oyIs44</i> V
CHB392	<i>hmnEx113</i>
CHB422	<i>lin-22 (n372)</i> IV; <i>hmnEx133</i>
CHB947	<i>unc-40 (e271)</i> I; <i>hmnEx113</i>
CHB856	<i>unc-40 (e271)</i> I; <i>lin-22 (n372)</i> IV; <i>hmnEx133</i>
CHB946	<i>unc-5 (e152)</i> IV; <i>hmnEx113</i>
CHB855	<i>lin-22 (n372)</i> <i>unc-5 (e152)</i> IV; <i>hmnEx133</i>
CHB613	<i>unc-6 (e78)</i> X; <i>hmnEx133</i>
CHB581	<i>lin-22 (n372)</i> IV; <i>unc-6 (e78)</i> X; <i>hmnEx113</i>
CHB945	<i>unc-6 (ev400)</i> X; <i>hmnEx133</i>
CHB1376	<i>lin-22 (n372)</i> IV; <i>unc-6 (ev400)</i> X; <i>hmnEx133</i>
CHB1377	<i>unc-40 (e271)</i> I; <i>unc-5 (e152)</i> IV; <i>hmnEx133</i>
CHB1374	<i>unc-40 (e271)/hT2</i> I; <i>lin-22 (n372)</i> <i>unc-5 (e152)</i> IV; <i>hmnEx133</i>
CHB1226	<i>uIs22</i> V; <i>hmnEx605</i>
CHB2163	<i>lin-22 (n372)</i> IV; <i>otIs138</i> X; <i>hmnEx605</i>

Table S2. Transgenes used in this study. See Experimental Procedures.

allele (s)	constructs	references and notes
<i>hmnEx113, hmnEx133</i>	pCY2	This study; injected at 25 ng/μl
<i>hmnEx605</i>	pCY76	This study; injected at 25 ng/μl
<i>huIs34</i>	<i>dat-1::GFP</i>	(H. Korswagen, unpublished)
<i>otIs138</i>	<i>ser-2prom3::GFP, rol-6</i>	(Tsalik et al., 2003)
<i>oyIs44</i>	<i>odr-1::RFP, lin-15 (+)</i>	(P. Sengupta, unpublished; Heiman and Shaham, 2009)
<i>uIs22</i>	<i>mec-3::GFP + dpy-20 (+)</i>	(Zhang et al., 2002). Gift from Irini Topalidou.

Table S3. Plasmids used in this study. See Experimental Procedures.

Plasmids and sequences are available upon request.

plasmid	description	notes
pCY2	<i>ser-2</i> prom3:kaede	<i>ser-2</i> prom3 after (Tsalik et al., 2003); kaede from pMH22 (Heiman and Shaham, 2009)
pCY76	<i>ser-2</i> prom3:mCherry	

Supplemental references:

Heiman, M.G. & Shaham, S. (2009) DEX-1 and DYF-7 establish sensory dendrite length by anchoring dendritic tips during cell migration. *Cell*, **137**, 344-355.

Tsalik, E.L. et al. (2003) LIM homeobox gene-dependent expression of biogenic amine receptors in restricted regions of the *C. elegans* nervous system. *Dev Biol*, **263**, 81-102.

Zhang, Y. et al. (2002) Identification of genes expressed in *C. elegans* touch receptor neurons. *Nature*, **418**, 331-335.