An Extracellular Adhesion Molecule Complex Patterns Dendritic Branching and Morphogenesis

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SUMMARY

Robust dendrite morphogenesis is a critical step in the development of reproducible neural circuits. However, little is known about the extracellular cues that pattern complex dendrite morphologies. In the model nematode Caenorhabditis elegans, the sensory neuron PVD establishes stereotypical, highly branched dendrite morphology. Here, we report the identification of a tripartite ligand-receptor complex of membrane adhesion molecules that is both necessary and sufficient to instruct spatially restricted growth and branching of PVD dendrites. The ligand complex SAX-7/L1CAM and MNR-1 function at defined locations in the surrounding hypodermal tissue, whereas DMA-1 acts as the cognate receptor on PVD. Mutations in this complex lead to dramatic defects in the formation, stabilization, and organization of the dendritic arbor. Ectopic expression of SAX-7 and MNR-1 generates a predictable, unnaturally patterned dendritic tree in a DMA-1-dependent manner. Both in vivo and in vitro experiments indicate that all three molecules are needed for interaction.

INTRODUCTION

The establishment of a complex, type-specific dendrite morphology is crucial for many neurons to receive the appropriate inputs from their receptive fields and to function properly within a neural circuit. As with axon morphogenesis, the development of dendrites must be precisely regulated to achieve the appropriate structure. However, the immense complexity of dendrite morphology has made it far more challenging to study than the pathfinding of a single axon (Jan and Jan, 2010).

Dendrites receive extrinsic cues from the adjacent environment to guide their spatially regulated growth and branching. Several molecules that were originally identified as axon guidance signals also play important roles in directing dendritic morphogenesis. Cortical pyramidal neurons extend their apical dendrites toward the pial surface in response to the diffusible chemotactrant Semaphorin 3A (Polleux et al., 2000). In the mammalian retina, dendritic arbors of amacrine cells and retinal ganglion cells (RGCs) are strictly organized in defined laminae in the inner plexiform layer (IPL) (Sanes and Zipursky, 2010). A number of neuronal homotypic adhesion molecules, including Sdk1, Sdk2, Dscam, DcamL, and Cntn2, restrict arbors to specific sublaminae (Yamagata and Sanes, 2008, 2012). Repulsive mechanisms mediated by the transmembrane semaphorin Sema6A and its receptor, PlexA4, are also required for layer-specific arbor targeting (Matsuoka et al., 2012). Similarly, dendrites of projection neurons in the Drosophila olfactory system utilize graded expression of Semaphorin 1A and the differentially expressed leucine-rich repeat (LRR) protein Caps for precise glomeruli targeting (Hong et al., 2009; Komiyama et al., 2007). Dendrites can also avoid sister branches from the same neuron while coexisting with the arbors of their neighboring neurons. This self-avoidance phenomenon has been elegantly explained by the function of two classes of highly diversified, contact-dependent repulsive molecules: Down syndrome cell adhesion molecules (DSCAMs) in Drosophila and protocadherins in vertebrates (Lefebvre et al., 2012; Matthews et al., 2007; Schmucker et al., 2000; Soba et al., 2007; Wojtowicz et al., 2004, 2007).

However, our current understanding of spatial dendritic patterning is not yet complete. A key component still missing from the puzzle is the interaction between dendrites and their growth substrate. It has been shown that confining dendritic arbors to a precise two-dimensional surface by the growth substrate is necessary for further refinement of receptive fields through dendro-dendritic interactions (Han et al., 2012; Kim et al., 2012; Yasunaga et al., 2010). However, the molecular identities of the signals on the growth substrate and corresponding neuronal receptors that instruct the growth, directionality, and branching of dendrites remain largely elusive.

The multidendritic PVD neurons in the nematode Caenorhabditis elegans provide us with a unique opportunity to study these issues. PVs grow elaborate, highly branched, and well-organized dendritic arbors (Smith et al., 2010; Figure 1A). The cell bodies of the two PVD neurons, PVDL and PVDR, are derived postembryonically during the mid-L2 larval stage along the lateral midlines underneath the hypodermal cells on each side of the worm. During L2, each cell extends two 1st dendrites (antero- and posteriorly) and one ventrally oriented axon. By late L2/early L3, 2nd branches form perpendicular to the 1st branches. When the 2nd branches reach the lateral borders of the outer body wall muscles, most 2nd branches make 90° turns and then...
form collateral 3’ branches (Smith et al., 2010). All 3’ PVD branches extend along the same line that largely colocalizes with the sublateral nerve cords. Dendrite morphogenesis is completed in early L4 stage after 4’ branches have sprouted from the 3’ branches to form a network of candelabra-shaped processes. This structure follows general dendritic organization principles such as self-avoidance (Smith et al., 2010). Understanding how the PVD dendrites make decisions about growth and branching at stereotyped times and locations will provide insights into the genetic components involved in the regulation of dendrite development.

Several reports have been published on the intrinsic molecules that are involved in shaping and maintaining PVD morphology. These include transcription factors (Smith et al., 2010, 2013), cell trafficking components (Aguirre-Chen et al., 2011), a membrane fusion protein (Oren-Suissa et al., 2010), and factors involved in dendrite self-avoidance (Smith et al., 2012). Our lab previously identified a transmembrane LRR protein, DMA-1, that is required cell-autonomously in PVD for the formation of dendritic branches (Liu and Shen, 2012). DMA-1 is expressed most prominently in the PVDs and another pair of multidendritic sensory neurons, the FLP head neurons. Loss-of-function mutants of dma-1 show severely defective dendritic arbors, whereas overexpression causes overbranching. We reasoned that DMA-1 may be the neuronal receptor that signals the development of PVD dendritic branches, but the extrinsic ligands that instruct the precise patterning of the dendritic trees remained to be identified.

In this paper, we report the identification and characterization of a tripartite receptor-ligand complex of cell-surface proteins that plays an instructive role in directing the formation and growth of dendritic branches. We provide both genetic and biochemical evidence that SAX-7, a homolog of the vertebrate L1 cell adhesion molecule (L1CAM), and its coligand, MNR-1, form prepatterned signals in the skin hypodermal cells to direct dendrite morphogenesis through the neuronal receptor DMA-1.

**RESULTS**

**Mutations in sax-7, mnr-1, and dma-1 Cause Defects in PVD Dendritic Morphology**

We visualized the PVD neurons using a membrane-GFP marker expressed under the control of a cell-specific promoter.
SAX-7 and MNR-1 Function in the Hypodermal Cell as a Prepatterning Cue

To understand where SAX-7 and MNR-1 function to pattern PVD dendrites, we first examined the endogenous expression pattern of both genes by introducing C-terminal GFP tags followed by SL2-mCherry into fosmids containing the entire genomic loci (Tursun et al., 2009). SL2 sequences are trans-splicing sites for mRNAs, similar to the IRES sites in vertebrate systems (Zorio et al., 1994). These constructs revealed the cellular expression of the genes and pinpointed the subcellular localization of protein products. The mCherry signal revealed that SAX-7 was expressed in the hypodermal cells and in many neurons, but not in PVD (Figure 2D). Within the hypodermal cell, SAX-7::GFP showed specific localization to two sublateral stripes and the hypodermal-seam cell junctions (Figure 2C, arrows). In contrast, MNR-1 was exclusively expressed in the hypodermis (Figure 2H) and did not exhibit similar subcellular localization (Figure 2G).

In C. elegans, the PVD dendritic processes are positioned in a narrow space between the hypodermis and the internal organs similarly to mammalian somatosensory neurons (Albeg et al., 2011). Because neither SAX-7 nor MNR-1 was expressed in the PVD neurons, and both were instead found in the PVD growth substrate (the hypodermal cell), we hypothesized that they might function as extrinsic signals to pattern the PVD dendrites. To definitively test which cells require SAX-7 and MNR-1, we expressed SAX-7 and MNR-1 using tissue-specific promoters in their respective mutant backgrounds. Expressing SAX-7 in the hypodermal cells using the dpy-7 promoter completely rescued the sax-7 phenotype (Figure 2B), whereas expression of SAX-7 in the PVD neuron or muscles showed no rescue, suggesting that SAX-7 functions non-cell autonomously in the surrounding hypodermal cell (Figure 2I). Similarly, we found that expression of MNR-1 in the hypodermal cells alone also rescued the wy758 phenotype (Figure 2F), whereas expression in the PVD neuron resulted in no rescue (Figure 2J). These results are consistent with the expression analysis and suggest that both SAX-7 and MNR-1 function in the hypodermal cell to pattern the PVD dendrites.

Because sax-7 and mnr-1 mutants showed identical phenotypes and are both transmembrane proteins that function in the hypodermal cell, we considered the possibility that they function as coligands. Consistent with this hypothesis, sax-7; mnr-1 double mutants showed phenotypes identical to those of sax-7 and mnr-1 single mutants, without any apparent enhancement or suppression (Figure 2K). The two genes thus function in the same genetic pathway.

The requirement of SAX-7 in the hypodermal cell prompted us to examine its subcellular localization in more detail. We expressed a functional SAX-7::YFP fusion protein in the hypodermal cell under the dpy-7 promoter. We found that SAX-7::YFP was enriched at the hypodermal-seam cell junctions and also formed two longitudinal sublateral stripes on each side of the worm (Figure 3B), similar to what was observed for SAX-7 under the control of its own promoter (Figure 2C). These stripes are especially interesting because they are located along the edge of outer body wall muscles where the PVD 3′ branches are aligned (Albeg et al., 2011). Because the PVD dendrites were previously reported to associate with other neurons,
including those forming the sublateral nerve cord (Smith et al., 2010), we sought to tease apart the relationship among PVD, SAX-7, and other neurons in the sublateral cords by coexpressing mCherry in PVD (Figure 3A), SAX-7::YFP in the hypodermis (Figure 3B), and CFP in all neurons (Figure 3C). In the anterior half of the worm, it was apparent that the PVD 3 branches colocalized with the sublaterally enriched SAX-7 line, but not with other neuronal processes (Figure 3D). Expression and localization of SAX-7 was observed early in development during the L1 and early L2 stages, long before the formation of PVD branches (Figures S2A and S2B). The localization of SAX-7 was normal in mnr-1(wy758) mutants, where PVD dendritic organization was completely lost (Figures S2C–S2E). SAX-7 localization and PVD 3 branch structure were also unaffected in an axon guidance mutant, mig-10(ct41), in which a small portion of the sublateral nerve cord is missing (data not shown). Thus, hypodermal SAX-7 was expressed and localized independently of the presence of PVD branches or the sublateral neurons. Together, these observations support the hypothesis that the sublaterally enriched SAX-7, together with MNR-1,
The absence of 3 Morphogenesis with Time-Lapse Imaging

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Figure 3. SAX-7 Was Enriched in Sublateral Stripes in the Hypodermis

(A–D) Fluorescent images showing (A) PVD neuron, (B) SAX-7 localization, (C) all neurons, and
(D) overlay of the three colors. SAX-7 was localized to two sublateral longitudinal lines in the hypo-
dermal cells on each side of the worm. PVD 3 branches completely colocalized with the SAX-7
lines but did not fasciculate with any other neurons in the anterior part of the worm. Arrows, sublateral
stripes of enriched SAX-7 that colocalize with PVD 3 dendrites. The images on the right are zoomed-
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that reached the sublateral cord in sax-7, mnr-1, and dma-1 mutants went on to
initiate a T (22.9%, n = 48; 18.8%, n = 32; and 11.1%, n = 36, respectively; Figure
4A; Movies S2, S3, and S4), suggesting that these three molecules play an
important role in collateral branch formation along the 3 branch line.

Once a T had formed, the 3 branches exhibited dynamic growth and retraction.
In wild-type animals, the majority of the T-shaped branches were stabilized over
the course of filming; however, 43.3% (n = 30) of the Ts retracted to an L or I (Figure
4B; Movie S1). In sax-7, mnr-1, and dma-1 mutants, nearly all of the Ts re-
tracted to an L or I (81.8%, n = 11; 100%, n = 6; 100%, n = 4, respectively; Figure
4B; Movies S2, S3, and S4), suggestive of a failure to maintain the 3 branch.
However, due to the branch-initiation defects in these mutants, the number of Ts we could successfully image was low, and
therefore the difference in T retraction rate between wild-type and mutant worms did not reach statistical significance
(Figure 4B).

SAX-7, MNR-1, and DMA-1 do appear to contribute to overall branch stability, however. A majority of branches that reached the sublateral line in these three mutant worms retracted back toward the primary dendrite, often completely disappearing, even after L or T initiation (54.2%, n = 48; 68.8%, n = 32; and 83.3%, n = 36, respectively; Figure 4C). Such retractions of
branches that reached the sublateral line were significantly less frequent in wild-type worms (13.1%, n = 61; Figure 4C), sug-
gest that the mutants do exhibit a defect in branch stability. Additionally, although dma-1 mutant worms had an overall
reduced number of 2 branches (Liu and Shen, 2012), we observed frequent initiation and retraction of branches during
filming (Movie S4), suggesting that the lack of 2 branches in dma-1 mutants is due, in part, to reduced branch stabilization
rather than a loss of 2 branch initiation. Taken together, our
time-lapse images of the dynamic growth of dendritic branches showed that the sax-7, mnr-1, and dma-1 mutants exhibit
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Stripes in the Hypodermis

Figure 4

(A–D) Fluorescent images showing (A) PVD neuron, (B) SAX-7 localization, (C) all neurons, and
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Developmental Analysis of PVD Dendritic

Morphogenesis with Time-Lapse Imaging

The absence of 3 branches in sax-7, mnr-1, and dma-1 worms could arise from a failure in 3 branch initiation, stabilization, or
both. To further understand the phenotypes of sax-7, mnr-1, and
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to observe how the wild-type and mutant dendritic arbors are
established during development. We observed PVD branch formation
erelative to the sublateral nerve cord using worms expressing
ser2prom3::myr-mCherry and Prab-3::mCherry. Consistent with previous
reports, the majority of T-shaped 3 branches arose via
collateral formation from Ls in wild-type animals (Smith et al.,
2010). However, we also observed that a subset of Ts appeared
to form through direct bifurcation of the secondary branches
(10%, n = 30 branches). To understand the mutant defects, we
quantified transitions between the different growth stages of
the dendrites. In wild-type animals, nearly half of all of the
branches that reached the sublateral line went on to eventually
form a T (49.2%, n = 61 branches; Figure 4A; Movie S1). Of those
branches that formed an L at the sublateral line, the majority
(57.4%, n = 47) transitioned to Ts during filming (18% of
branches that reached the sublateral cord remained “I”s during
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markedly similar phenotypes, i.e., compromised branch stabili-
zation and a reduced ability to initiate 3 branches. These two
developmental phenotypes caused the 3° branches to be completely absent in all three mutants at later stages in development.

**Ectopic Expression of SAX-7 and MNR-1 Alters PVD Dendritic Morphology**

To further test whether SAX-7 and MNR-1 play instructive roles in directing the development of PVD dendrites, we expressed these proteins ectopically in various tissues to determine whether they could alter PVD morphology. We first expressed SAX-7 in seam cells using the nhr-81 promoter. Seam cells are egg-shaped, specialized epithelial cells that are arranged in longitudinal rows on the left and right sides of the worm body (Sulston and Horvitz, 1977). They penetrate the hypodermis and make contact with the PVD dendrites at their thickest parts, where the nucleus is located, but are buried in the hypodermal cell on their narrow ends. Ectopic seam cell expression of SAX-7 in sax-7(nj48) single mutants resulted in a striking, fully penetrant gain-of-function (gof) phenotype in which all PVD dendrites were restricted around the seam cells (Figure 5A). This gof phenotype was completely abolished in the mutant background of mnr-1(wy758). The branches displayed a phenotype identical to that of the mnr-1 single mutants, with no sign of following the seam cells (Figures 5B and 5D). We quantified the phenotype by counting the number of dendrites that grew outside the estimated positions of the seam cells in each worm. PVD morphology was indistinguishable between mnr-1 mutant worms that carried the SAX-7 gof transgene and those that did not (Figure 5D). However, when we put MNR-1 back into the hypodermal cells using the dpy-7 promoter, the gof phenotype could be fully rescued (Figure 5D).

These results show that MNR-1 is required for SAX-7 to exert its instructive function, consistent with our hypothesis that the two molecules function as coligands. But how can hypodermal MNR-1 assist the function of seam cell SAX-7? One possibility is that MNR-1 can function as a secreted molecule. We tested this hypothesis by expressing MNR-1 without its predicted C-terminal transmembrane domain, which should cause it to be secreted. Expressing this construct in the mnr-1(wy758) mutant resulted in a partial rescue. Many dendrites were able to recognize the sublateral SAX-7 cue and form T-shaped tertiary structures, but they failed to further form quaternary branches (Figure S3A). This result indicates that secreted MNR-1 is partially functional. To directly test whether MNR-1 can be secreted, we tagged full-length MNR-1 on its extracellular domain and expressed it in the hypodermal MNR-1 line that subsequently retracted away from the 3° line, as determined by the pan-neuronal mCherry signal.

In further experiments, we expressed SAX-7 and MNR-1 together in mechanosensory neurons, including PLMs and...
ALMs, using the mec-17 promoter in sax-7; mnr-1 double mutants. We chose PLMs and ALMs because they are part of the sublateral nerve cords that largely colocalize with the hypodermal SAX-7 and PVD 3' branches in the posterior half of the animal. If the prepatterned SAX-7 and MNR-1 are indeed instructing the formation of 3' branches in wild-type animals, expression of SAX-7 and MNR-1 in part of the sublateral nerve cords should partially rescue the 3' branching phenotype of the mutants. This is indeed what we observed. As shown in Figures 6A–6C, 2' PVD branches that reached the SAX-7- and MNR-1-expressing PLM and ALM neurons formed T-shaped collateral 3' branches (Figure 6B, arrowheads). We quantified the total number of 2' branches that reached the SAX-7- and MNR-1-expressing PLMs and ALMs, and observed significant rescue of the T-formation phenotype (Figure 6F). Instead of being nearly absent in the mutants, Ts were formed by about 50% of dendrites that made contact with the SAX-7- and MNR-1-expressing PLM or ALM neurons (Figure 6F). We also consistently observed abnormally long PVD dendrites that completely followed the PLM or ALM processes (Figure 6B, arrows), supporting the hypothesis that SAX-7 and MNR-1 together form a preferred substrate on which PVD branches can form and grow. When expressed independently, Pmec-17::sax-7 by itself produced a weaker yet significant gof phenotype in sax-7 single mutants, with some PVD dendrites forming Ts on the PLM and ALM neurons. This effect was fully abolished in the mnr-1 single and sax-7; mnr-1 double mutants, consistent with the notion that MNR-1 can be partially secreted from the hypodermal cell and is necessary for SAX-7 to exert its function in directing PVD dendrites. On the other hand, expressing MNR-1 in the sublateral neurons of mnr-1 single mutants produced an equally strong gof phenotype as expressing both SAX-7 and MNR-1 in the sax-7; mnr-1 double mutants, consistent with our observation of endogenous SAX-7 expression in these neurons (Figure 2C, arrowheads). This was fully abolished in sax-7 single or sax-7; mnr-1 double mutants (Figure 6F). These results strongly support the model that SAX-7 and MNR-1 must function together as coligands to instruct morphogenesis of the PVD dendrites, and that MNR-1 can be partially secreted.

We also expressed SAX-7 and MNR-1 in D-type motor neurons using the unc-47 promoter. The DDs and VDs form ventral-dorsal commissures with which PVD 2' branches sometimes fasciculate (Smith et al., 2010). The presence of SAX-7 and MNR-1 in the commissures significantly reduced the number of 2' branches growing in the wrong directions. Most 2' branches instead followed the commissures expressing SAX-7 and MNR-1 and grew symmetrically toward both the dorsal and ventral midlines.
ventral sides. Instead of stopping at the sublateral lines, where they normally turn and form 3' branches, the dendrites following the SAX-7- and MNR-1-containing commissures extended all the way to the ventral and dorsal nerve cords (Figure 6D, arrows). We quantified this phenotype by counting the percentage of 2' branches that crossed the sublateral nerve cords and found that 70% of branches did so in the worms with the transgene (Figure 6G). These results show that SAX-7 and MNR-1 form a preferred substrate that can stimulate the branching and growth of PVD dendrites. We expressed SAX-7 and MNR-1 in both PLM and ALM neurons, as well as in D-type motor neurons, and found that almost all PVD dendrites followed the neuronal processes that ectopically expressed these proteins (Figure 6E). Thus, we can reconstruct the PVD dendritic structure simply by ectopically expressing these instructive adhesion molecules as guidance cues, and we can conclude that the SAX-7/MNR-1 complex is sufficient to direct PVD dendrite growth.

**DMA-1 Is the Neuronal Receptor for SAX-7 and MNR-1**

We reported previously that mutation of a neuronal LRR gene, dma-1, causes severe defects in PVD 3' branches (Figure 1D; Liu and Shen, 2012), so we considered the possibility that DMA-1 is the neuronal receptor for hypodermal SAX-7 and MNR-1. DMA-1 functions autonomously in the PVD neuron (Liu and Shen, 2012). If DMA-1 is the receptor in the neuron and is responsible for responding to the SAX-7 and MNR-1 signal, then loss of DMA-1 should completely abolish all gof phenotypes produced by SAX-7 and MNR-1 ectopic expression. This was indeed the case (Figure 6F). Therefore, DMA-1 is the neuronal receptor for the SAX-7 and MNR-1 signal.
We crossed the integrated seam cell SAX-7 overexpression transgene with \textit{dma-1(wy686)} and found that the branches failed to follow the ectopic SAX-7 signal. Instead of wrapping around the seam cells, dendrites in the \textit{dma-1(wy686)} mutants extended toward the sublateral cords, as if the ectopic SAX-7 did not exist (Figures 5C and 5D). Similarly, dendrites in the \textit{dma-1} mutants did not respond to ectopically expressed SAX-7 and MNR-1 in PLM and ALM. Instead of being modified by the SAX-7 and MNR-1 ectopic expression transgene, the dendrite morphology in these worms was indistinguishable from that in all other \textit{dma-1(wy686)} mutant worms, and no rescue was observed for the 3/C14 branching phenotype (Figures 6F and S4).

To further explore these genetic interactions, we made \textit{dma-1(wy686); sax-7(nj48)} (data not shown), \textit{dma-1(wy686); mnr-1(wy758)} double mutants and \textit{dma-1(wy686); sax-7(nj48); mnr-1(wy758)} triple mutants (Figures S5A and S5B), and examined their PVD dendritic phenotypes. The PVD morphology in all of these mutants was indistinguishable from that in the \textit{dma-1} single mutants. This is consistent with the idea that SAX-7 and MNR-1 function as the upstream binding partners of DMA-1. Overexpression of DMA-1 in PVD causes a severe overbranching phenotype (Figure S5C; Liu and Shen, 2012). However, in \textit{sax-7} or \textit{mnr-1} mutants carrying the DMA-1 overexpression transgene, the PVD morphology was indistinguishable from that in \textit{sax-7} or \textit{mnr-1} single mutants (Figures S5D and S5E). Together, these results indicate that DMA-1 may be the receptor for SAX-7 and MNR-1.

**DMA-1 Physically Interacts with the SAX-7/MNR-1 Ligand Complex**

Because the genetic evidence strongly suggested that DMA-1 could be the neuronal receptor for SAX-7 and MNR-1, we tested whether DMA-1 physically interacts with SAX-7 and MNR-1 in vitro to form a receptor-ligand complex using a \textit{Drosophila} S2 cell aggregation assay (Zorio et al., 1994). We transfected S2 cells with C-terminal GFP-tagged SAX-7, MNR-1, or both, and mixed them with cells expressing RFP-tagged DMA-1. Strong aggregation was observed only when cells cotransfected with both SAX-7::GFP and MNR-1::GFP were mixed with cells expressing DMA-1::RFP (Figure 7C). Little aggregation was observed in all other groups, including ones in which cells expressing only SAX-7::GFP or MNR-1::GFP individually were mixed with DMA-1::RFP (Figures 7A–7D). This argues strongly that SAX-7 and MNR-1 form a coligand complex in \textit{cis} and interact with DMA-1 in an in \textit{trans} fashion.

We also performed coimmunoprecipitation (coIP) experiments to test for a direct physical interaction among these three molecules. Cells were transformed with hemagglutinin (HA)-tagged SAX-7, myc-tagged DMA-1 extracellular domain, and FLAG-tagged MNR-1. Again, DMA-1 and MNR-1 could only be
coprecipitated when lysates of SAX-7::HA, MNR-1::FLAG co-transfected cells were mixed with DMA-1::myc lysate and anti-HA beads (Figure 7E).

The Fibronectin III, but Not the Ig, Domains of SAX-7 Are Required for Tripartite Binding

All previously reported functions of SAX-7 require a homotypic interaction between its first two Ig domains (Pocock et al., 2008; Sasakura et al., 2005), whereas the fibronectin III (FnIII) domains are not necessary. We conducted a structure-function analysis of SAX-7 to test whether the same domains are required for binding in the SAX-7/MNR-1/DMA-1 tripartite complex. To our surprise, the FnIII domains were both necessary and sufficient to perform the functions of SAX-7 described in this study. Truncation constructs of SAX-7 without the first one, first two, or even all four Ig domains were all able to fully rescue the sax-7 mutant phenotype of PVD when expressed in the hypodermal cell using the dpy-7 promoter (Figures S6A and S6B). Conversely, deleting all five FnIII domains completely abolished its ability to rescue (Figures S6A and S6C). The same construct has been shown to be sufficient to rescue the axon-integration and cell-positioning phenotypes of sax-7 mutants (Pocock et al., 2008). The construct showed normal localization to the sublateral lines in the hypodermal cell (data not shown), but completely failed to rescue the PVD dendritic phenotype. We thus reason that it is the FnIII domains—not the Ig domains—that participate in the tripartite binding. In vitro binding experiments with S2 cells showed results consistent with this idea. Cells expressing MNR-1 and SAX-7 without Ig domains could still form large aggregates with DMA-1-expressing cells, whereas cells coexpressing MNR-1 and SAX-7 without FnIII domains completely failed to do so (Figures 7D, S6D, and S6E).

Together, these in vivo and in vitro experiments provide evidence that the three proteins form a tripartite complex, with SAX-7 and MNR-1 forming a coligand and DMA-1 interacting in trans. We thus propose a model in which SAX-7 and MNR-1 form prepatterned cues in the hypodermal cell and DMA-1 responds to this signal to direct the proper formation and growth of PVD dendrites (Figures 7F and S6F).

DISCUSSION

Environmental Signals Instruct Dendrite Morphogenesis

The precise spatial patterning of dendritic arbors requires delicate interactions between dendrites and their growing environment. Dendrite surface receptors must sense various types of extrinsic signals, such as global diffusible factors that determine their general orientation (Polleux et al., 2000), local CAMs on the growth substrate that defines their spatial territories (Han et al., 2012; Kim et al., 2012), and signals from other neurites, including axon terminals of their presynaptic partners (Sanes and Yamagata, 2009; Yamagata and Sanes, 2012) and dendrites from the same cell and neighboring arbor (Han et al., 2012; Lefebvre et al., 2012; Matthews et al., 2007; Soba et al., 2007).

To extend our molecular knowledge about the interaction between dendrites and their environment, we report the identification of three CAMs that form a tripartite receptor-ligand complex between skin hypodermal cells and developing neuronal dendrites. In the hypodermal cell, SAX-7/L1CAM, an Ig CAM, forms a precise subcellular pattern on which the dendrites later grow. To the best of our knowledge, such a precisely localized patterning signal has not been previously reported for dendrite development. However, there is evidence suggesting the existence of such molecular signals for precise spatial patterning of dendrites. For example, DA neurons in adult Drosophila establish a lattice-like morphology along the basement membrane between muscle fibers underneath the epidermis (Yasunaga et al., 2010). Multimodal somatosensory neurons in vertebrates also establish similar stereotyped rather than random morphologies (Hall and Treinin, 2011; Li et al., 2011). In the mammalian retina, dendritic arbors of amacrine cells and RGCs are strictly organized in defined laminae in the IPL (Sanes and Zipursky, 2010). Time-lapse imaging of the zebrafish retina revealed that at least some RGC dendrites elaborated branched arbors only when they reached their target layer, indicating the existence of branch-promoting factors arrayed within the IPL (Mumm et al., 2006; Yamagata and Sanes, 2012). Although specific instructive molecules have yet to be identified for these examples, the highly organized morphology and specific branching locations strongly suggest the existence of such extrinsic signals. In our system, the activity of SAX-7 requires another transmembrane protein, MNR-1, which also functions in the hypodermal cell (Salzberg et al., 2013). The cognate neuronal receptor for SAX-7 and MNR-1 is an LRR transmembrane protein, DMA-1, which promotes selective stabilization and further branching of the dendrites at specific locations predefined by the SAX-7 and MNR-1 signal. All three molecules have homologs in vertebrate genomes (Figure S1), supporting the notion that the molecular players as well as the organizing principles in dendrite morphogenesis might be conserved throughout evolution.

A Tripartite Complex of Adhesion Molecules

SAX-7/L1CAM has been intensively studied for its functions in maintaining neuronal integrity, axon outgrowth, and cell migration (Brümmendorf et al., 1998; Pocock et al., 2008; Sasakura et al., 2005; Zallen et al., 1999). Mutations in human L1 have been linked to a number of neurological abnormalities (Van Camp et al., 1996; Vits et al., 1994). Here, we report a function of SAX-7/L1CAM that is likely different from those that have been previously studied. First, rather than functioning as a recognition molecule in the neuron, we found that SAX-7 was expressed and subcellularly localized in the hypodermal cell, the substrate upon which the neuron grows. Second, the function of SAX-7 was exerted with the assistance of another novel transmembrane coligand, MNR-1. Third, although all previously reported functions of SAX-7 require homotypic interactions between the Ig domains (Pocock et al., 2008; Schürmann et al., 2001), the binding we report here required the FnIII domains of SAX-7.

Our genetic analyses suggest that the novel protein MNR-1 functions as the coligand of SAX-7 in patterned PVD dendrites. This is supported by the following observations: (1) sax-7 and mnr-1 mutants exhibited indistinguishable phenotypes, (2) both MNR-1 and SAX-7 were expressed and required in the hypodermal cells, (3) the gof activity caused by ectopic expression of
SAX-7 and MNR-1 required both molecules, and (4) SAX-7 only bound to DMA-1 in the presence of MNR-1. The genetic requirement of MNR-1 for SAX-7 gof phenotypes in tissues that did not express MNR-1 suggests that MNR-1 is partially secreted. This hypothesis is supported by the accumulation of fluorescently tagged MNR-1 in coelomocytes. More detailed genetic and protein structure-function analyses will be needed to fully elucidate MNR-1’s functions and the mechanisms of its secretion.

As we have previously reported, DMA-1 is a good candidate for the neuronal receptor that regulates the development of dendritic arbors (Liu and Shen, 2012). In this report, we present evidence that DMA-1 functions as the cognate neuronal receptor for SAX-7 and MNR-1. First, dma-1 mutants exhibited phenotypes in 3’ branch formation similar to those seen in sax-7 and mnr-1 mutants. Second, DMA-1 was absolutely required for the gof phenotypes caused by ectopic expression of SAX-7 and MNR-1, whereas overexpression of DMA-1 in sax-7 or mnr-1 mutants did not alter their PVD dendritic phenotype. Third, DMA-1 bound to the SAX-7/MNR-1 complex in vitro. We speculate that the difference in the number of branches formed in these mutants can be explained by the selective loss of adhesion. dma-1 mutants lack adhesion in the dendritic branches themselves, making branch growth and stabilization to any degree difficult to achieve (Movie S4). However, in the sax-7 and mnr-1 mutants, DMA-1 is still present in the dendritic branches. If DMA-1 can interact at least somewhat with other proteins (perhaps expressed on the surface of the gut or gonad), then in the absence of its preferred substrate (SAX-7/MNR-1), the branches may overgrow in an attempt to find the appropriate signal. In both wild-type animals and gof experiments, DMA-1-containing dendrites contacting a source of SAX-7 and MNR-1 led to a universal reduction of undirected branching (Figures 6B, 6D, and 6E), suggesting that formation of the tripartite receptor-ligand complex can both reinforce “appropriate” dendritic branch growth and suppress “inappropriate” growth in other locations.

A Model to Explain How the Tripartite Complex Patterns Dendritic Arbors in PVD

Collectively, our results are consistent with the following speculative model to explain the orderly branched PVD dendritic arbors. The PVD dendritic arbors are established in a sequential manner. First, 2’ branches are formed from the primary dendrite shaft. When the 2’ processes encounter the 3’ line, the high concentration of SAX-7 and MNR-1 activates DMA-1 on the neurite, possibly leading to both a tighter adhesion and signaling events that recruit cytoskeletal components necessary to form and maintain branches (Figures 7F and S6F). The exact mechanisms that lead to the decisions regarding dendrite growth, branching, or retraction in response to extrinsic signals remain to be characterized. However, the identification of surface receptor-ligand interactions opens the door for analysis of the elaborate underlying cellular processes, just as has been done in great detail for axon guidance.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

N2 Bristol was used as the wild-type strain. Worms were raised on OP50 Escherichia coli-seeded nematode growth medium plates at 20°C or room temperature according to a standard protocol (Brenner, 1974). All transgenes and plasmids are listed in Tables S1 and S2.

Isolation and Mapping of mnr-1(owy758) Mutant

The owy758 allele was isolated from an F2 semicolonial screen of 3,000 haploid genomes in the strain wtsy378. Worms were mutagenized with 50 mM ethyl methanesulfonate. SNIP-SNP mapping, rescue, and sequencing were performed using standard protocols (Matthews et al., 2007). This allele carries a nonsense G-to-A point mutation flanked by sequences TTTAAGGAAT and GCTCAGAAG.

Time-Lapse Imaging

Worms were raised at approximately 22°C and prepared for imaging as previously described (McCarter et al., 1999). Briefly, the worms were soaked in a solution of 0.1% tricaine and 0.01% levamisole (Sigma-Aldrich) in M9 for 20–30 min prior to imaging. The immobilized worms were then transferred with a glass hook to a drop of M9 containing 0.05 μg poly styrene microspheres (Polysciences) on a slab of 5% agarose in M9. The coverslip was then sealed with Vaseline. Images were acquired using a Zeiss Axio Observer Z1 microscope equipped with a Plan-Apochromat 63 x 1.4 objective (Carl Zeiss), Yokogawa spinning disk head, 488 and 561 diode lasers, and a Hamamatsu ImagEM EMCCD camera driven by MetaMorph (Molecular Devices). To follow dendrite branching, a stack of both GFP and mCherry images with 15 z planes at 0.5 μm intervals was acquired every 2 min. Maximum-intensity projections were created for analysis and presentation. Movies of similarly aged worms imaged just anterior to the cell body were scored for the time points at which branch initiation or retraction occurred to determine initiation and stabilization rates over the course of filming (~2–3 hr). Due to the infrequency of 3’ branch initiation in the mutants, a 3’ branch was counted as stabilized if it was present at the end of filming, rather than existing for a minimum lifespan.

S2 Cell Aggregation and CoIP Assays

Drosophila S2 cells were cultured in Schneider’s insect medium (Sigma) according to the manufacturer’s description and transfected using Effectene (QIAGEN). S2 cell aggregation assays were performed as previously described (Zorio et al., 1994). All plasmids used for transfection are listed in Table S3. The detailed experimental procedure is included in the Extended Experimental Procedures.

ACCESSION NUMBERS

The RefSeq accession number for the mnr-1 sequence reported in this paper is NC_003283.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, three tables, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.08.059.

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