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RESEARCH REPORT

Four specific immunoglobulin domains in UNC-52/Perlecan function with NID-1/Nidogen during dendrite morphogenesis in *Caenorhabditis elegans*

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ABSTRACT

The extracellular matrix is essential for various aspects of nervous system patterning. For example, sensory dendrites in flies, worms and fish have been shown to rely on coordinated interactions of tissues with extracellular matrix proteins. Here we show that the conserved basement membrane protein UNC-52/Perlecan is required for establishing the correct number of the highly ordered dendritic trees in the somatosensory neuron PVD in *Caenorhabditis elegans*. This function is dependent on four specific immunoglobulin domains, but independent of the known functions of UNC-52 in mediating muscle-skin attachment. Intriguingly, the four conserved immunoglobulin domains in UNC-52 are necessary to correctly localize the basement membrane protein NID-1/Nidogen. Genetic experiments further show that *unc-52*, *nid-1* and genes of the netrin axon guidance signaling cassette share a common pathway to establish the correct number of somatosensory dendrites. Our studies suggest that, in addition to its role in mediating muscle-skin attachment, UNC-52 functions through immunoglobulin domains to establish an ordered lattice of basement membrane proteins, which may control the function of morphogens during dendrite patterning.

KEY WORDS: Dendrite, Nervous system development, *C. elegans*, Netrin, Nidogen, Perlecan

INTRODUCTION

Neurons receive signals through often elaborately sculpted dendritic arbors, first documented by Santiago Ramón y Cajal in the late 19th and early 20th century (Ramón y Cajal, 1894). To understand dendrite arbor morphogenesis we study the stereotypically patterned somatosensory PVD neuron in *Caenorhabditis elegans* (Fig. 1A) (Smith et al., 2010; Albeg et al., 2011). Many mechanisms required for dendritic morphogenesis are conserved during PVD dendrite patterning, including the combinatorial use of different levels of transcription factors (Smith et al., 2013) and a role for molecular transport (Aguirre-Chen et al., 2011; Albeg et al., 2011; Taylor et al., 2015; Zou et al., 2015). Other factors or processes involved in dendritic morphogenesis include the proprotein convertase KPC-1/Furin (Schroeder et al., 2013; Salzberg et al., 2014; Dong et al., 2016), the EFF-1 fusogen (Oren-Suissa et al., 2010; Zhu et al., 2017), the claudin-like membrane protein HPO-30 (Smith et al., 2013) and the unfolded protein response (UPR) (Wei et al., 2015; Salzberg et al., 2017). The netrin axon guidance signaling cascade, which comprises the secreted UNC-6/Netrin cue and the UNC-40/DCC and UNC-5/hUNC5 receptors, plays an important role in self-avoidance of PVD dendrites (Smith et al., 2012).

In addition to the above, largely cell-autonomous cues, the epidermal MNR-1/Menorin and SAX-7/L1CAM cell adhesion molecules and the muscle-derived diffusible LECT-2/Chondromodulin II act in a complex through a leucine-rich repeat transmembrane receptor DMA-1/LRR-TM on PVD dendrites (Liu and Shen, 2011; Dong et al., 2013; Salzberg et al., 2013; Díaz-Balzac et al., 2016; Zou et al., 2016). More recently, it has been suggested that muscle sarcomeres pattern PVD quaternary branches by localizing the basement membrane protein UNC-52/Perlecan to these muscle structures, which in turn pattern the hemidesmosome-like fibrous organelles in the epidermis to localize the SAX-7 cell adhesion molecule (Liang et al., 2015). Whether UNC-52 serves functions in PVD patterning independently of shaping quaternary dendrites or of its structural functions to localize fibrous organelles remained to be determined.

RESULTS AND DISCUSSION

Four immunoglobulin domains in UNC-52 function to pattern PVD dendrites

Since PVD dendrites are shaped by extracellular factors originating from muscle and epidermis, we systematically tested genes encoding components of the fibrous organelles, which physically link muscles and epidermis (Hresko et al., 1994), for their involvement in PVD patterning (Fig. S1). We included genes that function in muscle [e.g. *pat-2/α-integrin* (Williams and Waterston, 1994)], the extracellular matrix [e.g. the heparan sulfate proteoglycan *unc-52/perlecan* (Rogalski et al., 1993, 1995)] as well as the epidermis [e.g. the intermediate filament protein *mua-6/IFA-2* (Hapiak et al., 2003)] (Fig. S1). Compromising the function of genes encoding components of the fibrous organelles significantly reduced the number of secondary (2°), tertiary (3°) and quaternary (4°) dendritic branches (Fig. S1C-E). However, the proportions of the menorah-like PVD dendritic arbors remained unchanged as determined by the number of 4° branches per 2° branch (Fig. S1F). This conclusion is further supported by limited effects on the average length of branches but a reduction in the aggregate length of 2°, 3° and 4° dendritic branches under most conditions (Fig. S1G-L). Thus, components of fibrous organelles are important for controlling the number of menorah-like dendritic arbors per animal, without significantly disrupting their overall structure.

A mutation in *unc-52*, suggested to be important for patterning of 4° PVD dendrites (Liang et al., 2015), showed a previously unappreciated reduction in the number of 2° branches (Fig. 1A,B).

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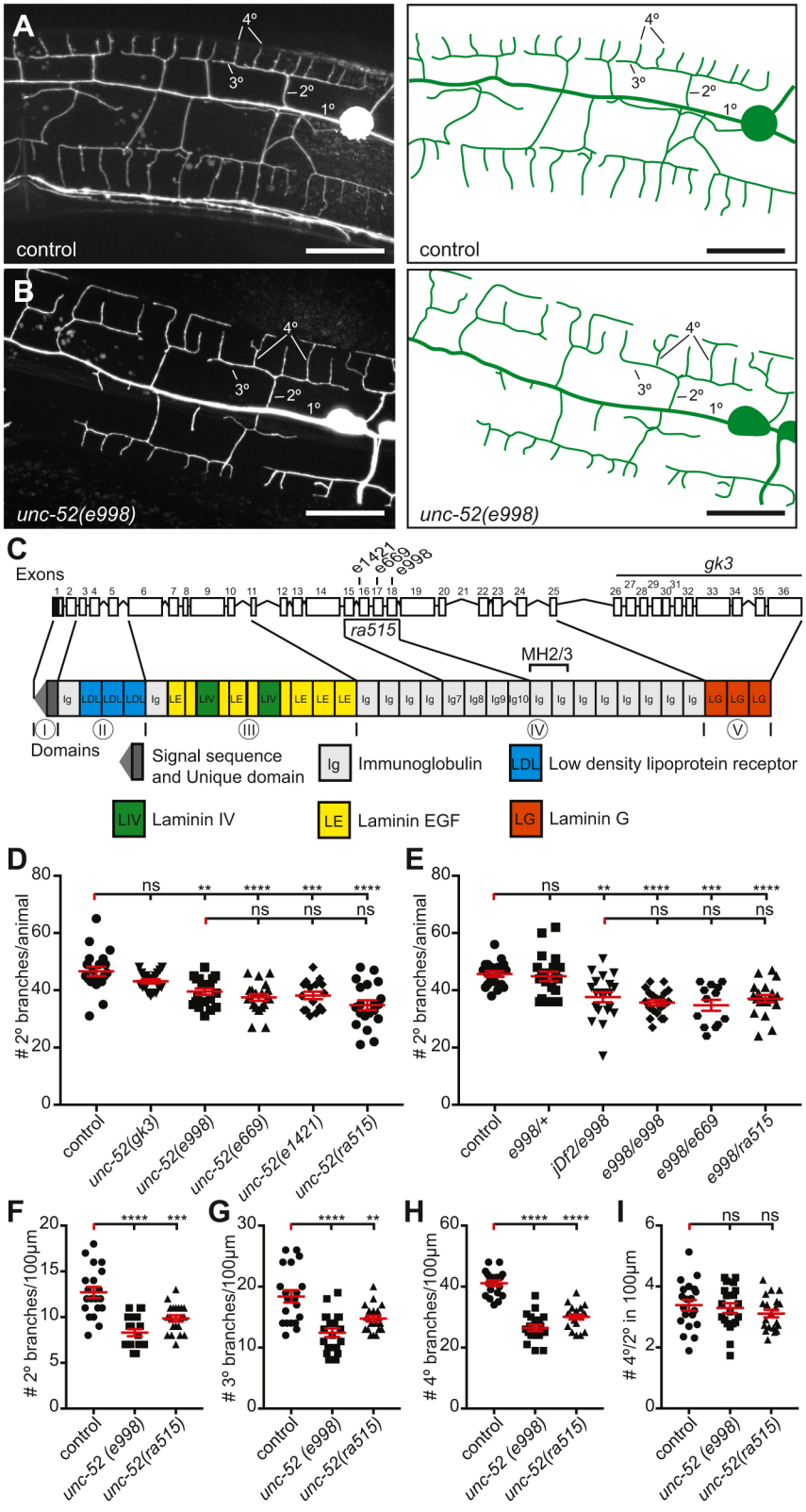

Fig. 1. Four immunoglobulin domains in UNC-52 function to pattern PVD dendrites. (A,B) Fluorescence micrographs of wild-type and *unc-52(e998)* mutant animals with schematics. Note the stereotypic patterning of PVD with 1°, 2°, 3° and 4° dendrites in wild-type animals. Anterior is to the left and ventral down. Scale bars: 20 µm. (C) *unc-52* genomic organization and UNC-52 protein structure. Exons are numbered, horizontal lines denote the extent of deletions, and alleles are indicated. Note that some Ig domains of domain IV, including #7-10, are encoded by individual exons. The location of the antigen recognized by the MH2 and MH3 monoclonal antibodies is shown. (D-I) Quantification of morphometric characteristics of PVD dendrites in the genotypes indicated, including four alleles affecting domain IV: *e1421*, a splice acceptor site mutation in exon 16; *e669* and *e998*, nonsense mutations in exon 17 and 18, respectively; *ra515* and *gk3*, in-frame deletions that delete Ig domains #7-10 or domain V (and thus all long forms of UNC-52), respectively. In D,E, the total number of 2° branches per animal was determined, whereas in F-H the number of 2°, 3° and 4° branches in a 100 µm segment anterior to the PVD cell body was determined. The ratio of 4° per 2° branch serves as a measure for menorah proportion (I). Error bars denote mean±s.e.m.; ns, not statistically significant (P>0.05); **P<0.005, ***P<0.0005, ****P<0.0001. N=20 animals.

UNC-52/Perlecan is a conserved multidomain protein of the extracellular matrix (Rogalski et al., 1993, 1995) (Fig. 1C). Domain I is Perlecan specific, whereas domains II, III, and V comprise LDL (low-density lipoprotein receptor) modules, laminin EGF (epidermal growth factor) repeats, and laminin G domains, respectively (Fig. 1C). Domain IV is conserved from cnidarians to humans (Warren et al., 2015) and defined by a string of immunoglobulin (Ig) domains. The *unc-52* locus in *C. elegans* is alternatively spliced to produce short (domains I-III), medium (domains I-IV), and long (domains I-V) UNC-52 forms (Rogalski et al., 2001). The medium forms show additional alternative splicing of four Ig domains within domain IV (Fig. 1C), where the Ig

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domains are assembled in different combinations to produce distinct medium isoforms (Rogalski et al., 1995) (Fig.S2A).

Using the total number of 2° branches as a proxy for the number of menorahs, we found a reduction of 2° branches in several additional *unc-52* alleles, except the *gk3* deletion allele that deletes domain V (Fig. 1C,D, Fig. S2A). There are more PVD 2° branches located on the right and dorsal sides of the animals than on the left and ventral sides, respectively (Smith et al., 2010). These asymmetries persist in *unc-52* mutants (Fig. S2B,C), suggesting that *unc*-52 is equally required for dorsal/ventral and left/right pattering of PVD 2° branches. The changes in the number of 2° branches in *unc-52* mutants are developmental rather than maintenance defects because, in contrast to wild-type animals, the number of 2° branches did not increase in *unc-52* mutants during development from the L3 to the L4 larval stage (Fig. S2D). We conclude that (1) *unc-52* functions to establish the appropriate number of 2° branches during development; (2) four Ig domains in domain IV are important for this process; and (3) domain V and hence the long forms of UNC-52 are dispensable for this function. The latter conclusion is reminiscent of findings reported for the correct formation of 4° branches in PVD (Liang et al., 2015).

Null alleles of *unc-52* display an embryonic lethal phenotype due to the failure of muscles to attach to the body wall at the twofold embryonic stage (Rogalski et al., 1993). We found that the *unc-52(e998)* allele behaved as a genetic null for the 2° branching phenotype of PVD dendrites because animals in which this allele was placed over a deficiency of the *unc-52* region [*unc-52(e998)/jDf2*] did not display a more severe phenotype than *unc-52(e998)* homozygous animals (Fig. 1E). Moreover, transheterozygous animals between *unc-52(e998)* and *unc-52(e669)* or *unc-52(ra515)* also failed to display enhancement (Fig. 1E), suggesting that they are likely to act as genetic null alleles for the 2° PVD branching defect as well. Finally, morphometric analyses of *unc-52(e998)* and *unc-52(ra515)* revealed no statistical differences (Fig. 1F-I). We conclude that the PVD patterning defect is the null phenotype due to loss of specific medium UNC-52 forms.

UNC-52 function in PVD patterning is separate from its function in muscle patterning

Most *unc-52* alleles show disorganized body wall muscle structure, resulting in progressive paralysis as the animals enter adulthood owing to compromised muscle attachment (Rogalski et al., 1995). Thus, the PVD dendrite defects in *unc-52* mutant animals could be a secondary consequence of structural body wall muscle defects, as has been suggested for the defects in 4° PVD dendrites (Liang et al., 2015). Alternatively, the PVD defects in *unc-52* mutant animals could be due to loss of specific UNC-52 splice variants. Intriguingly, *unc-52(ra515)* animals do not display locomotion or morphogenetic defects at any stage (Mullen et al., 1999; Merz et al., 2003), but do show defects in PVD dendritic branch morphology similar to those seen in other *unc-52* alleles (Fig. 1F-I).

To determine whether PVD patterning defects correlate with structural defects in body wall muscle, we analyzed body wall muscle morphology in different *unc-52* alleles. Using polarizing light microscopy, which identifies the A and I bands in muscle (Waterston et al., 1980), and phalloidin staining, which labels the F-actin-containing thin filaments, we detected the well-documented defects in sarcomere organization in *unc-52(e998)* mutants. By contrast, we never observed similar defects in *unc-52(ra515)* animals or wild-type controls (Fig. 2A-C). We also investigated patterning of fibrous organelles with antibodies against PAT-3/β-integrin (a muscle component of fibrous organelles), UNC-52/Perlecan (an ECM component of fibrous organelles), myotactin (an epidermal component of fibrous organelles) and a MUA-6::RFP reporter that labels the intermediate filaments in the epidermis (Hapiak et al., 2003) (Fig. 2A). As previously reported (Mullen et al., 1999), we observed severe disorganization of the body wall muscle in the uncoordinated *unc-52(e998)* allele when we stained with antibodies against β-integrin or Perlecan (Fig. 2D). However, we did not observe structural irregularities on the epidermal side of the fibrous organelles in *unc-52(e998)* animals, as myotactin staining and the MUA-6::RFP reporter appeared indistinguishable from wild type (Fig. 2D). This suggests that the epidermal part of the fibrous organelles remains unperturbed in these *unc-52* mutant alleles. No comparable patterning defects of any type were observed in *unc-52(ra515)* or wild-type animals (Fig. 2D). Therefore, the reduction in the number of 2° branches in PVD dendrites in *unc-52* mutants is unlikely to be secondary to structural muscle defects but instead the result of specific molecular interactions that involve the four Ig domains (#7-10) of UNC-52.

UNC-52 is expressed in muscle (Rogalski et al., 1993) but also serves essential functions in the epidermis during early development (Spike et al., 2002). Using cell-specific rescue experiments, we found that expression of an *unc-52* cDNA from muscle but not epidermis consistently rescued the *unc-52* mutant phenotype (Fig. S2E-I). Thus, UNC-52 may function from muscle to pattern PVD dendrites or, alternatively, UNC-52 could undergo post-translational modifications specific to body wall muscle, such as the well-known modifications of UNC-52/Perlecan by heparan sulfates, a type of extracellular glycosaminoglycan. In support of this idea, we found that mutating a crucial enzyme in heparan sulfate biosynthesis, the *N*-deacetylase-*N*-sulfotransferase *hst-1/NDST*, resulted in a PVD mutant phenotype indistinguishable from that of *unc-52(ra515)* (Fig. S2J).

UNC-52 functions with NID-1 to pattern PVD dendrites

Nidogen is a conserved multidomain ECM protein comprising three globular domains separated by a rod-like domain (Fig. 3A). Mouse nidogen and perlecan display biochemical interactions (Hopf et al., 1999, 2001). Thus, we examined whether the *C. elegans* nidogen ortholog NID-1, which serves important roles in neuronal (Kim and Wadsworth, 2000) and synaptic patterning (Ackley et al., 2003) in *C. elegans*, is also important for patterning of PVD dendrites. We found that the PVD phenotype of a *nid-1* null mutant was (1) not statistically different from the *unc-52* mutant phenotype and (2) not enhanced in a double mutant (Fig. 3B-D), suggesting that both genes act in the same genetic pathway.

To determine whether UNC-52 is required for the localization of NID-1, we stained *unc-52(ra515)* mutant animals, which lack Ig domains #7-10, with antisera against NID-1. We found that the localization of NID-1 was severely affected and failed to consistently colocalize with the M lines and dense bodies of the body wall muscle as visualized with an antibody against β-integrin (Fig. 3E). Conversely, the localization of UNC-52 or PAT-3/β-integrin was unaffected in the *nid-1* null mutant (Fig. S3A,B). NID-1 localization also appeared completely normal in *unc-52(gk3)* animals (which lack domain V) (Fig. S3C), consistent with the absence of defects in PVD patterning in this allele (Fig. 1D). These results suggest that Ig domains #7-10 in domain IV, but not domain V, of UNC-52 are required for NID-1 localization. Surprisingly, NID-1 localization appeared normal in the *nid-1(cg118)* allele, which is predicted to encode a NID-1 protein with an in-frame deletion of the G2 globular domain (Fig. S3C) (Kang and Kramer, 2000). Thus, the G2 domain, which can bind mouse perlecan

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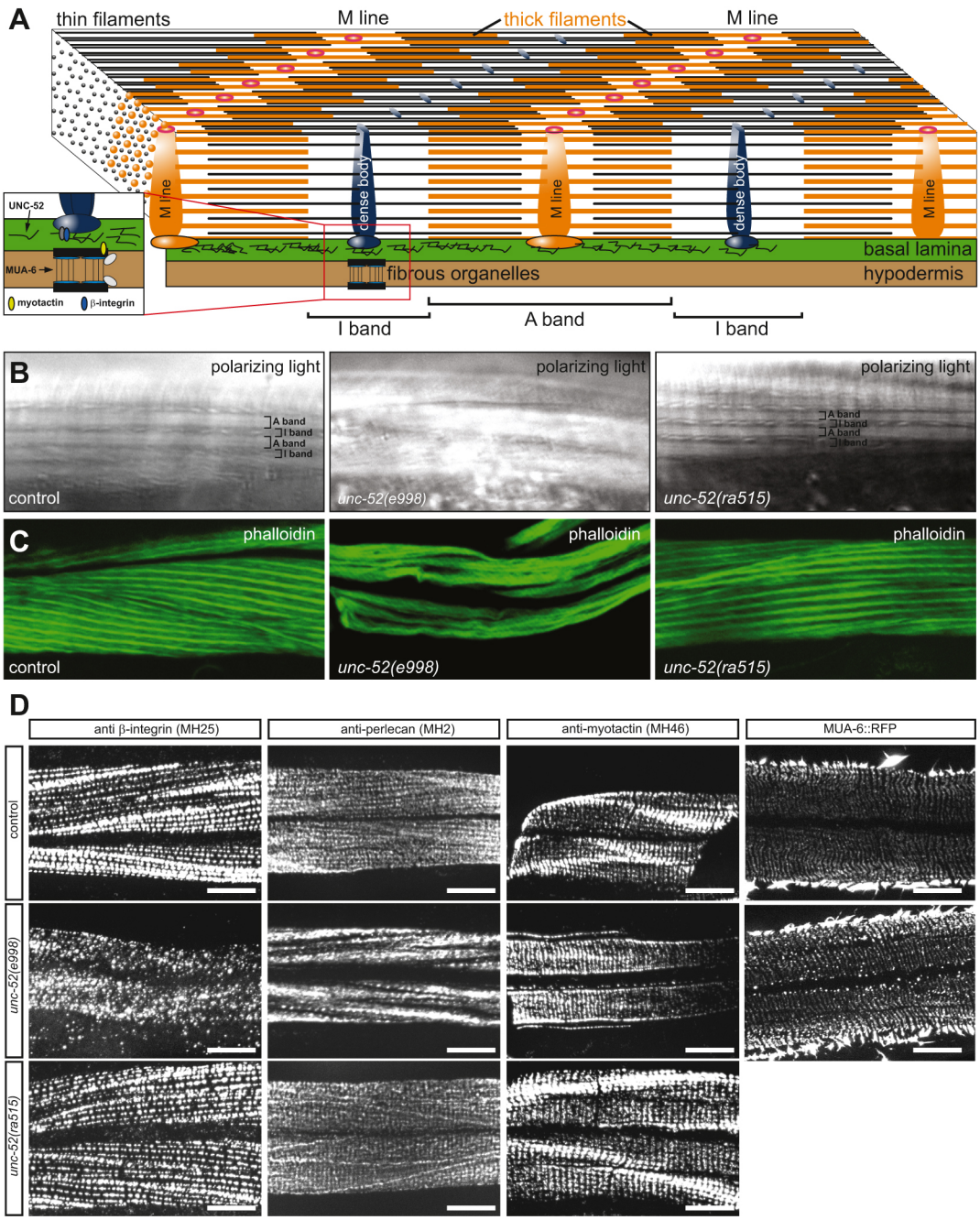

Fig. 2. UNC-52 function in PVD patterning is separate from its function in muscle patterning. (A) Schematic of the *C. elegans* body wall muscle [modified after Altun and Hall (2009)]. The fibrous organelles are not exclusive to dense bodies, but also localize to M lines and elsewhere. (B) Micrographs of body wall muscle using polarizing light in animals of the indicated genotypes. (C) Fluorescence micrographs of the indicated genotypes stained with Alexa Fluor 488-conjugated phalloidin. (D) Fluorescence micrographs of the indicated genotypes stained with monoclonal antibodies (Waterston et al., 1980) against PAT-3/β-integrin (MH25), UNC-52/Perlecan [MH2 (similar results were obtained with the equivalent MH3 antibody, not shown)], epidermal myotactin (MH46) or containing a MUA-6::RFP reporter. Scale bars: 20 µm.

in vitro (Hopf et al., 1999, 2001), is individually not required for NID-1 and UNC-52 to interact *in vivo*.

UNC-52 and NID-1 function with UNC-6 signaling to shape PVD dendrites

The secreted UNC-6/Netrin ligand functions through the two receptors UNC-40/DCC and UNC-5/hUNC5 alone or in combination to control axonal guidance in both invertebrates and vertebrates (Bradford et al., 2009). Mutations in *unc-6*, *unc-40* and *unc-5* result in fewer 2° branches in PVD dendrites (Smith et al., 2012). We confirmed these results (Fig. 4A) and also found that the PVD 2° branching defects could be rescued by transgenic UNC-40 expression in PVD but not in motor neurons, where *unc-40* functions to pattern commissures (Chan et al., 1996) (Fig. 4B). Thus, UNC-40 is likely to function cell-autonomously to regulate the number of 2° PVD branches, and the observed defects are not an indirect consequence of motor neuron commissure defects of *unc-40* mutants. The phenotypes of *unc-52; unc-6* and *unc-40; unc-52* double mutants were not more severe than the single mutants, suggesting that *unc-52* acts genetically in the netrin pathway

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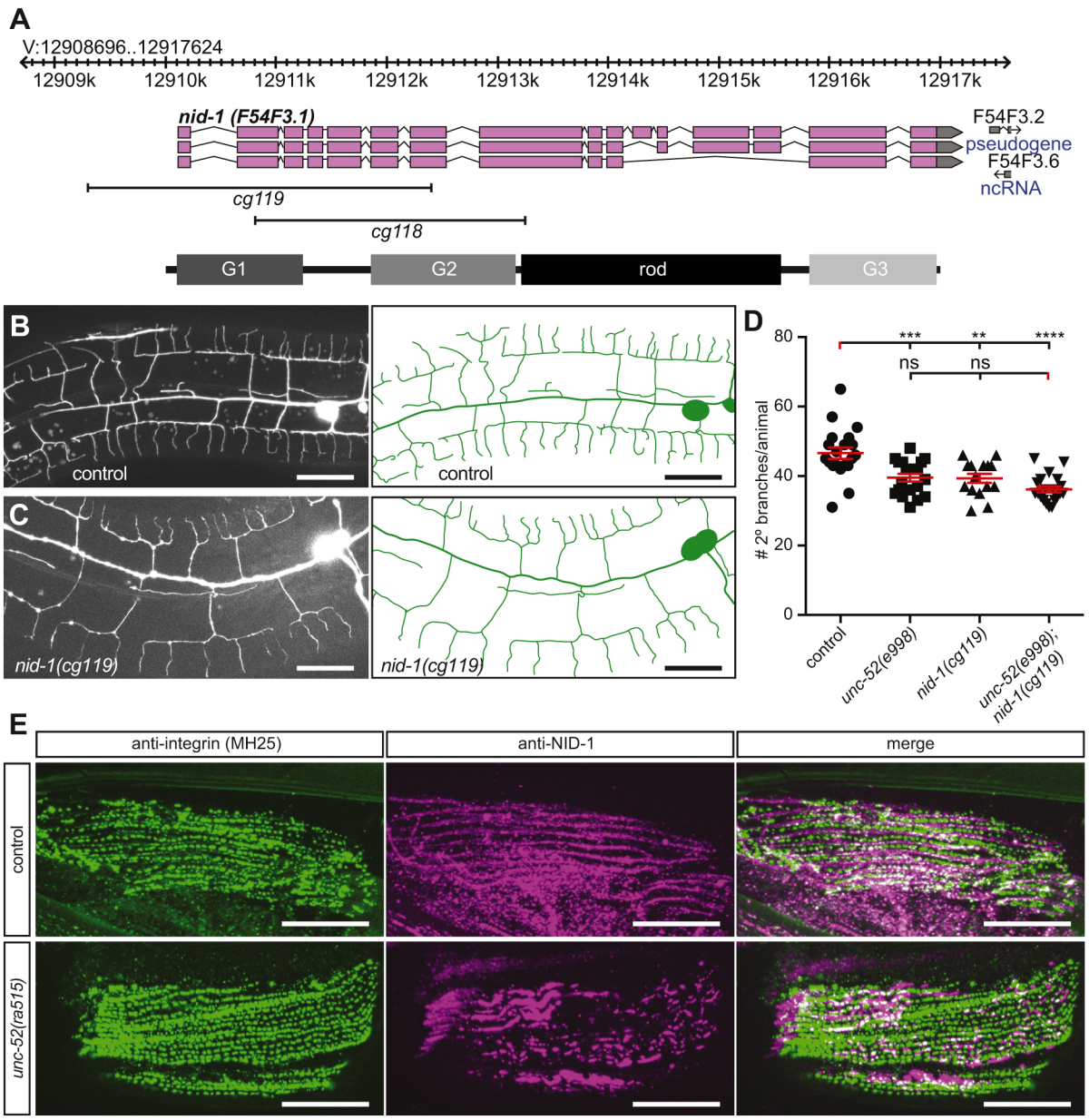

Fig. 3. UNC-52 functions with NID-1 to pattern PVD dendrites. (A) Schematic of the *nid-1* locus showing the exon-intron structure, the globular (G1-G3) and connecting rod protein domains. NID-1 antisera were raised against the G3 globular domain. (B,C) Fluorescence micrographs of wild-type control and *nid-1(cg119)* mutant animals with schematics. Anterior is to the left. (D) Quantification of morphometric characteristics of PVD dendrites in the genotypes indicated. Error bars denote mean±s.e.m.; ns, not statistically significant ($P>0.05$); **$P<0.005$, ***$P<0.0005$, ****$P<0.0001$. $N=20$ animals. (E) Fluorescence micrographs of the indicated genotypes stained with a monoclonal antibody against PAT-3/β-integrin (MH25) (Waterston et al., 1980) or an antiserum against NID-1. Scale bars: 20 µm.

(Fig. 4A). Moreover, *nid-1* behaved genetically like an *unc-52* mutant because it also failed to enhance a mutation in *unc-40* (Fig. 4A). We conclude that *unc-52* and *nid-1* act in a common pathway with netrin signaling. These findings are consistent with studies showing that the netrin receptor *unc-40* functions together with *nid-1* to guide axons (Kim and Wadsworth, 2000), and a report that *unc-52* is required for the *unc-6*-dependent subcellular localization of an UNC-40::GFP reporter in HSN neurons (Tang and Wadsworth, 2014).

Conclusions

We show that four specific Ig domains of the conserved basement membrane protein UNC-52/Perlecan function to localize the basement membrane protein NID-1/Nidogen and correctly pattern PVD 2° dendrites. The four Ig domains are part of domain IV, a domain common to all known perlecan proteins, which is composed of a string of Ig domains that are alternatively spliced in *C. elegans* and mice (Iozzo et al., 1994). Both *unc-52* and *nid-1* act genetically in the *unc-6* pathway. Surprisingly, UNC-52 acts from muscle to pattern PVD dendrites. NID-1 has been shown to be concentrated at the edges of the muscle quadrants (Kang and Kramer, 2000), and we observe enrichment for some fibrous organelle components (e.g. MUA-6::RFP) and, possibly, UNC-52 along the edges of the muscle (Fig. 2D). Thus, molecular interactions between specific UNC-52 splice variants and NID-1 could shape the basement membrane lattice as part of specialized fibrous organelles along the edges of the muscle quadrants. Alternatively, muscle-derived UNC-52 could be

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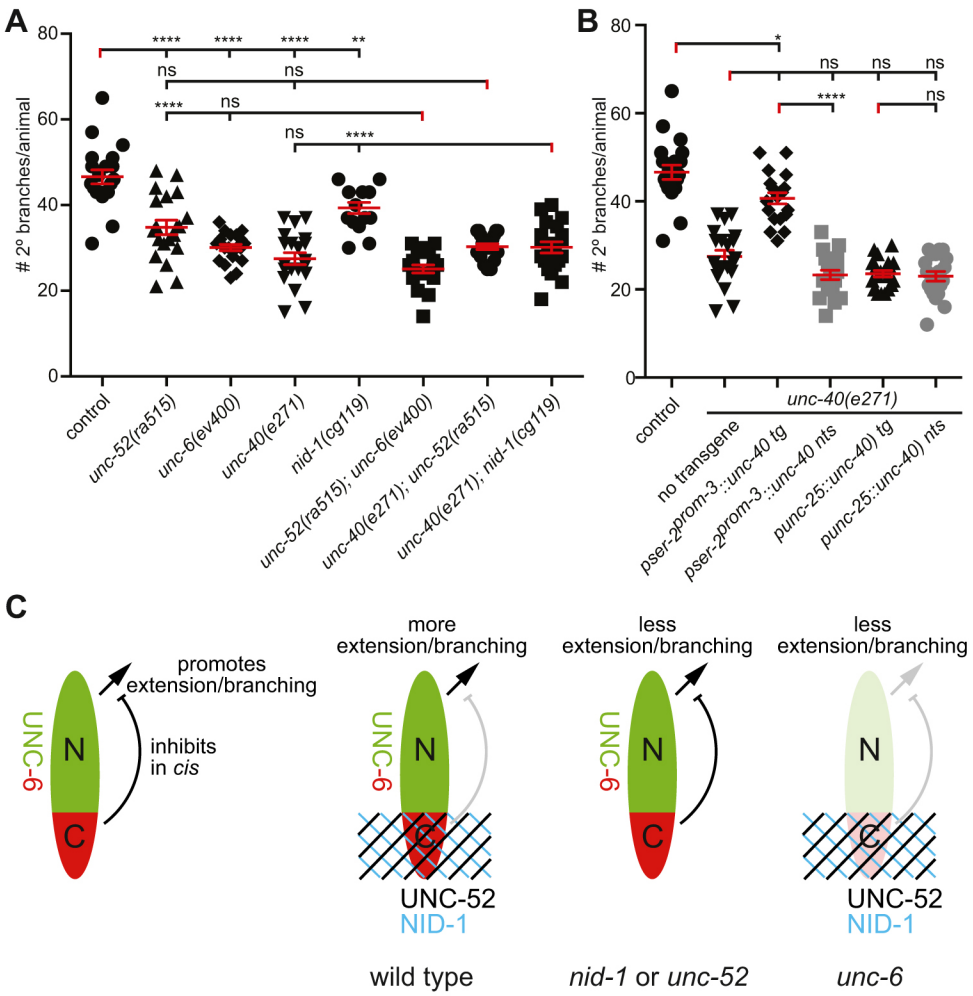

Fig. 4. UNC-52 and NID-1 function with UNC-6 signaling to shape PVD dendrites. (A,B) Quantification of the total number of 2° branches per animal of the genotypes indicated. In B, *unc-40(e271)* rescuing transgenes include constructs expressing *unc-40* cDNA under control of the PVD-specific *pser-2 prom-3* promoter (Tsalik et al., 2003) or the *punc-25* promoter specific for GABAergic neurons (i.e. D-type motor neurons) (Jin et al., 1999). Error bars denote mean±s.e.m.; ns, not statistically significant ($P>0.05$); *$P<0.05$, **$P<0.005$, ***$P<0.0005$, ****$P<0.0001$. $N=20$ animals. (C) Model of UNC-6/Netrin, UNC-52/Perlecan and NID-1/Nidogen function, all of which promote the extension/formation of 2° branches. In wild-type animals, the UNC-52–NID-1 basement membrane lattice prevents the UNC-6 C-terminal domain from inhibiting the extension/branch-promoting activities of UNC-6. Removal of UNC-52 or NID-1 results in more effective inhibition and thus less extension/branching. The same is true for complete removal of UNC-6.

incorporated into the epidermal basement membrane at a distance from its place of biosynthesis and modification in the muscle, similar to what has been shown for laminin α subunits (Huang et al., 2003).

How could two basement membrane proteins in conjunction with netrin signaling control the number of 2° branches? The Perlecan-Nidogen complex and netrin signaling normally promote the formation of 2° branches. Nidogen links collagen and laminin complexes (Mak and Mei, 2017) and thus may affect the distribution, availability or presentation of the secreted laminin-related UNC-6/Netrin morphogen. For example, UNC-6 could normally be incorporated in the basement membrane lattice in a manner that inhibits/shields its C-terminal domain, which is known to inhibit branching, likely in *cis* (Lim and Wadsworth, 2002; Wang and Wadsworth, 2002) (Fig. 4C). Compromising the specific UNC-52–NID-1 complex could release or change the presentation of UNC-6 and allow the C-terminal domain to exert its inhibitory function, resulting in fewer branches (Fig. 4C). Further work will be required to resolve this question. Altogether, our work provides insight into the function of the alternatively spliced Ig domains of UNC-52/Perlecan in localizing NID-1/Nidogen and, possibly, specific basement membrane signaling complexes during dendrite patterning.

MATERIALS AND METHODS

Strains, RNAi and transgenic rescue

Worms were cultured as previously described (Brenner, 1974). N2 (Bristol) was used as the control strain. Alleles used include *e998*, *e1421*, *e699*, *ra515* and *gk3* for *unc-52*, *nid-1(cg119)*, *nid-1(cg118)*, *unc-6(ev400)* and *unc-40(e271)*. RNAi-mediated gene knockdown experiments were performed as described (Timmons and Fire, 1998). Transgenic animals were created using standard protocols (Mello et al., 1991). For additional information, including the primers used to construct the full-length *unc-52A* cDNA, see the supplementary Materials and Methods and Table S1.

Immunohistochemistry

Whole-mount freeze-fracture antibody staining was performed using the protocol described by Duerr (2006). Briefly, animals were fixed using methanol and acetone and immunodetection was accomplished using primary antibodies MH2, MH3 (both UNC-52/Perlecan), MH25 (PAT-3/β-integrin) and MH46 (myotactin) (Francis and Waterston, 1991) at 1:100 dilution. Secondary antibody Alexa Fluor 555 donkey anti-mouse (Thermo Fisher Scientific, A-31570) was used at 1:500.

Microscopy and morphometric analyses

L4/young adult animals were placed in 1 mM Levamisole and mounted on a 5% agarose pad. Animals were imaged using a Zeiss Axio imager Z.1 compound microscope under control of Zeiss Axiovision software. Maximum intensity projections were generated of each animal at 40× magnification with an exposure time of 120 ms and a slice separation of 0.6 µm. Images were exported in a 16-bit tiff format, converted into 8-bit images in ImageJ (NIH), and subjected to morphometric analysis using the NeuronJ plugin (ImageJ) as described (Salzberg et al., 2013). Statistical analysis was performed using one-way ANOVA with Tukey correction for multiple comparisons (GraphPad, Prism v7).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.C., L.T.H.T., H.E.B.; Formal analysis: K.C., H.E.B.; Investigation: K.C., C.A.D.-B.; Resources: C.A.D.-B., B.D.A.; Data curation: K.C., H.E.B.; Writing - original draft: K.C., H.E.B.; Writing - review & editing: K.C., C.A.D.-B., L.T.H.T., B.D.A., H.E.B.; Visualization: K.C., H.E.B.; Supervision: H.E.B.; Project administration: H.E.B.; Funding acquisition: H.E.B.

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Supplementary information
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