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Epidermis-Derived Semaphorin Promotes Dendrite Self-Avoidance by Regulating Dendrite-Substrate Adhesion in *Drosophila* Sensory Neurons

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Summary

Precise patterning of dendritic arbors is critical for the wiring and function of neural circuits. Dendrite-extracellular matrix (ECM) adhesion ensures that the dendrites of *Drosophila* dendritic arborization (da) sensory neurons are properly restricted in a 2D space, and thereby facilitates contact-mediated dendritic self-avoidance and tiling. However, the mechanisms regulating dendrite-ECM adhesion *in vivo* are poorly understood. Here, we show that mutations in the semaphorin ligand *sema-2b* lead to a dramatic increase in self-crossing of dendrites due to defects in dendrite-ECM adhesion, resulting in a failure to confine dendrites to a 2D plane. Furthermore, we find that Sema-2b is secreted from the epidermis and signals through the Plexin B receptor in neighboring neurons. Importantly, we find that Sema-2b/PlexB genetically and physically interacts with TORC2 complex, Tricornered (Trc) kinase and integrins. These results reveal a novel role for semaphorins in dendrite patterning and illustrate how epidermal-derived cues regulate neural circuit assembly.

eTOC Blurb

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Author Contributions

S.M. designed and performed a genetic screen, confocal imaging, genetic and statistical analysis, co-immunoprecipitations, proximity ligation assays, and wrote the manuscript with help from other authors. S.Y. co-designed and performed co-immunoprecipitations. J.L. co-designed and performed immunohistochemistry with P-Trc antibody. S.H.Y. contributed to generating fly lines. P.S., P.J. and W.Z. contributed to generating several constructs. J.P., L.J. and Y.N.J. supervised the project development.

Meltzer et al. demonstrate that semaphorin ligand derived from epidermis is required to restrict dendrites into a 2D space, thereby facilitating self-avoidance. They further show that Trc/Fry and integrin signaling pathways are components of Sema-2b/PlexB signaling.

Introduction

Precise patterning of dendritic arbors is crucial for the development and function of the nervous system, but the mechanisms that instruct dendrite patterning are only beginning to be elucidated (Jan and Jan, 2010; Dong et al., 2014; Puram and Bonni, 2013). In humans, defects in dendritic morphology are linked to neurodevelopmental disorders including autism, Down syndrome, and Fragile X syndrome (Kaufmann and Moser, 2000; Kulkarni and Firestein, 2012). To refine the location and strength of synaptic or sensory inputs, neurons use various strategies to organize their dendritic arbors, including self-avoidance and tiling (Jan and Jan, 2010; Zipursky and Sanes, 2010; Zipursky and Grueber, 2013).

Self-avoidance, the phenomenon in which dendritic branches of the same neuron avoid overlapping with one another, ensures the non-redundant coverage of sensory or synaptic inputs. Tiling is the phenomenon in which the dendrites of neighboring neurons of the same type avoid overlapping with each other. In both vertebrates and invertebrates, contact-mediated self-repulsion is a well-studied mechanism underlying self-avoidance (Jan and Jan, 2010; Zipursky and Sanes, 2010; Zipursky and Grueber, 2013). In *Drosophila*, Down syndrome cell adhesion molecule (Dscam) mediates contact-dependent dendritic repulsion through homophilic interactions between identical isoforms of the protein on dendrites of the same neuron (Matthews et al., 2007; Soba et al., 2007; Hughes et al., 2007). In mammalian neurons, protocadherins (Pcdhs) mediate self-avoidance through isoform-specific homophilic interaction (Lefebvre et al., 2012; Chen and Maniatis, 2013).

Importantly, self-avoidance and tiling require not only contact-mediated interactions between dendrites, but also dendrite-ECM adhesion to restrict the dendrites onto a 2D X-Y plane so that dendrites can not stray along the Z-axis and escape contact-mediated repulsion (Han et al., 2012; Kim et al., 2012). Similar spatial restriction of dendrites has been found in *Drosophila* sensory neurons, fish somatosensory neurons, and within the mammalian retina (Jan and Jan, 2010; Sagasti et al., 2005; Perry and Linden, 1982; Zipursky and Sanes, 2010; Zipursky and Grueber, 2013). In the mammalian retina, laminar stratification of the neurites of retinal neurons is regulated by semaphorins (Sun et al., 2013; Matsuoka et al., 2011a; Matsuoka et al., 2011b). However, how dendrite-ECM adhesion is regulated *in vivo* remains largely unknown.

Drosophila dendrite arborization (da) sensory neurons are a powerful model system to study the molecular mechanisms underlying dendrite-ECM adhesion. *Drosophila* da neurons can be divided into four classes (classes I–IV), based on dendritic morphology and central axon projections (Grueber et al., 2002; Grueber et al., 2007). During development, dendrites of da neurons extend mainly in a 2D plane. Defects in dendrite-ECM adhesion lead to a detachment from the ECM and therefore to an increase in the number of non-contacting crossings (Kim et al., 2012; Han et al., 2012). Loss-of-function mutations in integrin subunits cause dendrites to detach from the ECM and become enclosed by the epidermal cell

membrane, resulting in excessive non-contacting self-crossings between dendrites (Kim et al., 2012; Han et al., 2012). Additionally, target of rapamycin complex 2 (TORC2) and the evolutionarily conserved protein kinase Tricornered (Trc) as well as its adaptor protein Furry (Fry) were found to be important for self-avoidance and tiling (Emoto et al., 2004), and were shown to function by regulating dendrite-ECM adhesion (Han et al., 2012). To gain insight into how dendrite-ECM adhesion is regulated, we conducted a genetic screen in *Drosophila* class IV da neurons to examine the contribution of cell surface proteins.

In this study, we show that mutations in the secreted semaphorin ligand *sema-2b* cause detachment of dendrites from the ECM, leading to an increase in non-contacting dendritic crossings in class IV da neurons. Sema-2b protein is expressed and secreted from epidermal cells, and signals locally through the PlexB receptor in neurons. Moreover, we show that the TORC2 complex and the Trc/Fry signaling pathway act downstream of the Sema-2b/PlexB signaling pathway, and that the PlexB receptor associates with Mys, a β subunit of integrins *in vitro* and *in vivo*. Our results reveal an important role for semaphorins in the precise arrangement of dendrites during development and show how epidermis-derived cues shape neural circuit assembly.

Results

Sema-2b Prevents Dendritic Crossings in Class IV da Neurons

In a screen designed to identify cell surface proteins important for the development of sensory neuron dendrites *in vivo*, we discovered that mutations in *sema-2b*, which encodes a secreted semaphorin ligand, led to a fully penetrant phenotype in which dendrites of class IV da neurons overlap with each other excessively (Figure 1B–D and 1H), whereas they normally avoid each other in wild-type larvae (Figure 1A and 1H). We examined the phenotypes of two mutant alleles of *sema-2b*: a P-element insertion called $sema\text{-}2b^{f02042}$ (Figure 1B; Thibault et al., 2004), and a FRT-derived genomic deletion called $sema\text{-}2b^{C4}$ (Figure 1C; Wu et al., 2011), which showed a stronger dendritic self-crossing phenotype than $sema\text{-}2b^{f02042}$ (Figure 1H). We also examined a heteroallelic combination of $sema\text{-}2b^{f02042/C4}$ mutants, which had an intermediate severity of the self-crossing phenotype in between that of $sema\text{-}2b^{f02042}$ and $sema\text{-}2b^{C4}$ mutants (Figure 1D and 1H). The *Drosophila* genome encodes two secreted semaphorin ligands, Sema-2a and Sema-2b, both of which often act in the same system (Wu et al., 2011; Joo et al, 2013). To determine if Sema-2a is required for preventing dendritic crossings, we examined *sema-2a* loss-of-function mutants and found that the loss of *sema-2a* did not lead to any increase in dendritic crossings (Figure 1E and 1H). Moreover, a double mutant of *sema-2a* and *sema-2b* did not show a more severe phenotype than *sema-2b* mutants alone (Figure 1F and 1H), suggesting that Sema-2b, but not Sema-2a, plays a major role in preventing dendritic self-crossings in class IV da neurons.

Dendrites of class IV da neurons are tightly confined between the basal surface of epidermal cells and the ECM, and branch out in a 2D plane, facilitating contact-mediated dendritic self-avoidance (Han et al, 2012; Kim et al., 2012). Loss of attachment to the ECM leads to enclosure of dendrites into epidermal cells and thus to an increase in the number of non-contacting dendritic crossings (Han et al, 2012; Kim et al., 2012). To understand the nature

of the dendritic crossings we observed in the *sema-2b* mutants, we tested whether these crossings involve direct contact between dendrites using *in vivo* high-resolution confocal imaging (Figure 1G; Figure S1A). In all mutants examined, we observed no significant increase in contacting crossings compared to wild-type controls (Figure 1H). However, the number of non-contacting crossings increased in *sema-2b* mutants and *sema-2a, sema-2b* double mutants (Figure 1H). In addition, we also found an increase in the number of non-contacting crossings in the class I da neurons in *sema-2b* mutants (Figures S1B–S1D). By contrast, we found no detectable defects in the overall axonal projections of class IV da neurons in the ventral nerve cord of the *sema-2b* mutants (Figure S2). These observations suggest that the loss of Sema-2b leads to non-contacting dendritic crossings, possibly by an impairment of dendrite-ECM adhesion.

To determine whether Sema-2b is required in class IV da neurons to prevent dendritic crossings, we generated single neuron clones homozygous for a *sema-2b* mutation in an otherwise heterozygous background using the mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo, 1999). Surprisingly, reducing Sema-2b function in class IV da neurons had no significant effect on dendritic self-crossings (Figures 1I–1K). Therefore, Sema-2b is not required in class IV da neurons for preventing self-crossing of dendrites (also see Figures 3J–3L).

Loss of Sema-2b Function Leads to a Progressive Increase in Detachment of Terminal Dendrites from the ECM

To investigate the spatial distribution of dendrites in relation to the ECM, we visualized class IV da neuron dendrites using a class IV-specific membrane marker *ppk-CD4-tdTom* (Han et al., 2011) and the ECM using *viking-GFP* (*vkg-GFP*) (Han et al., 2012) in live third instar larvae. Using a combination of high-resolution confocal imaging and 3D deconvolution, we reconstructed the positions of each dendrite relative to the ECM. At 120 hr after egg laying (AEL), wild-type dendrites of class IV da neurons at the dorsal midline were tightly attached to the ECM (Figure 2C and 2E). In *sema-2b* mutants, however, the percentage of enclosed dendritic length greatly increased (Figure 2D and 2E), suggesting that defects in dendrite-ECM adhesion contribute to increased non-contacting dendritic crossings.

Enclosure of growing dendrites by epidermal cells is a dynamic process that takes place during development (Han et al., 2012). To determine the stage during which Sema-2b is required for dendrite-ECM attachment, we characterized the dendrite enclosure in wild-type and *sema-2b* mutant larvae at different time points throughout development. In wild-type larvae, class IV da neuron dendrites establish their receptive fields by tiling the body wall completely and non-redundantly at 48 hr AEL (Parrish et al., 2007). We found that wild-type dendrites were tightly attached to the ECM from 48 hr AEL to 120 hr AEL (Figure 2A and 2E). Interestingly, in the *sema-2b* mutants, dendrites initially exhibit normal dendrite-ECM attachment at 48 hr AEL (Figure 2B and 2E). Defects in dendrite-ECM attachment of *sema-2b* mutants became evident at 72 hr AEL and became worse at 96 hr AEL, revealing that *sema-2b* mutants exhibit progressive loss of dendrite-ECM attachment after neurons have established their receptive fields (Figure 2E).

Since it has been shown that terminal dendrite branching increases between 72 hr and 96 hr AEL (Parrish et al., 2007), we reasoned that loss of terminal dendrite attachment to the ECM could be the main contributor to the observed increase in dendrite enclosure in the epidermis in *sema-2b* mutants. Indeed, we found that 72.2% of the enclosed dendrites (n=72) were terminal dendrites in *sema-2b* mutants, whereas only 25.0% of the enclosed dendrites (n=24) were terminal dendrites in wild-type animals (Figure 2F). In addition, we found that the overall morphology and total number of epidermal cells in the *sema-2b* mutants were the same as wild-type animals, suggesting that Sema-2b is not required for the development of epidermis (Figure S3).

Collectively, these data reveal that Sema-2b is required for the maintenance of dendrite attachment to the ECM in order to reduce non-contacting crossings (Figure 1G) and ensure non-overlapping coverage of dendritic fields.

Sema-2b is Secreted From Epidermal Cells to Regulate Dendrite-ECM Adhesion

Next, we examined the localization of Sema-2b proteins in the *Drosophila* larval body wall using a polyclonal antibody specific for Sema-2b (Sweeney et al. 2011). We found that Sema-2b was evenly distributed throughout the wild-type larval body wall (Figure 3A), and was completely absent in *sema-2b*^{C4} null mutants (Figure 3B).

To pinpoint the source of Sema-2b, we employed a Sema-2b reporter, *2b*^{L}*-τGFP*, with the *τGFP* expression driven by the *sema-2b* promoter (Wu et al. 2011). The *2b*^{L}*-τGFP* reporter resided in epidermal cells (Figure 3C) and colocalized with an epidermal-cell specific marker (Figure S4). We further confirmed the expression pattern using a more selective Sema-2b reporter, *2b-τMyc* (Wu et al. 2011). This reporter uses a shorter fragment of the *sema-2b* promoter to drive the expression of *τMyc* and labels a subset of cells in the developing *Drosophila* embryo that normally express a high level of Sema-2b (Wu et al. 2011). We found that *2b-τMyc* labeled a subset of epidermal cells in the midline of each abdominal hemisegment, suggesting that these cells express a high level of Sema-2b (Figures 3D and S4). These results suggest that that Sema-2b is expressed in epidermal cells, with a high level of expression in the midline of each segment.

We next tested if restoring Sema-2b in epidermal cells would be sufficient to rescue the dendrite crossing phenotype in the *sema-2b* mutants. To do this, we used the Gal4/UAS system in *Drosophila* to specifically express secreted Sema-2b in the epidermal cells using the epidermis-specific driver *Gal4*^{A58} in the *sema-2b* mutant background (Galko and Krasnow, 2004). Indeed, expression of Sema-2b specifically in the epidermis, but not in class IV da neurons, rescued the crossing defects observed in the *sema-2b* mutants (Figures 3E–3G). To test if the release of Sema-2b protein into the extracellular space is required for dendrite self-avoidance, we expressed a membrane-tethered Sema-2b^{TM} in all the epidermal cells. We found that Sema-2b^{TM} also rescued dendritic crossing defects in *sema-2b* mutants (Figure 3H–3I), suggesting that secretion of Sema-2b is not required for mediating dendrite self-avoidance. To further examine if locally produced Sema-2b is required to prevent dendrite crossing, we performed MARCM analysis to remove Sema-2b from epidermal cells and we found that dendrites innervating mutant *sema-2b* clones showed an increase in dendritic crossings (1.1 ± 0.2, N=26), compared to control clones (0.3 ± 0.1, N=26; Figures

3J–3L). Together, these results strongly suggest that Sema-2b is derived from epidermal cells and functions locally to regulate dendrite-ECM adhesion. Further, the fact that a clone of a single *sema-2b* mutant epidermal cell can produce the dendritic crossing phenotype in adjacent class IV da neuron dendrites strongly suggests that Sema-2b can not diffuse substantially for a distance longer than the diameter of an epidermal cell.

PlexB functions in the Sensory Neurons to Regulate Dendrite-ECM Adhesion

We next explored the mechanisms by which Sema-2b regulates dendrite-ECM adhesion in class IV da neurons. PlexB and Sema-1a have been shown to be receptors for Sema-2a and Sema-2b (Ayoob et al., 2006; Wu et al., 2011; Sweeney et al., 2011; Joo et al., 2013). *Sema-1a* loss-of-function mutants had normal dendrite morphology with no defects in dendritic crossing (Figure S5B). However, *plexB* loss-of-function mutants showed a large increase in the number of dendritic crossings (Figures 4A, 4B and 4E), suggesting that reducing PlexB function leads to a defect in dendrite-ECM adhesion.

PlexB is expressed in class IV da neurons during the embryonic stages of development (Zlatic et al., 2009). Therefore, we investigated if the PlexB receptor is required cell-autonomously in class IV da neurons to mediate dendrite-ECM adhesion. We used Gal4/UAS-based RNA interference (RNAi) to knock down PlexB in class IV da neurons using $Gal4^{21\text{-}7}$ (Song et al., 2007). Using two different RNAi lines, we observed a strong increase in the number of dendritic crossings (Figures 4C–4E, and Figures S5C–S5F), demonstrating that *plexB* acts cell-autonomously in class IV da neurons to regulate dendrite-ECM adhesion. Additionally, we found an increase in the number of non-contacting crossings when we knocked down *plexB* in class I da neurons (Figures S5G–S5I).

To determine if *sema-2b* and *plexB* function in the same genetic pathway, we examined *sema-2b/+*, *plexB/+*, and *sema-2b/plexB* animals and found an increase in non-contacting crossings but not in contacting crossings in the *sema-2b/plexB* animals compared to *sema-2b/+* and *plexB/+* animals (Figures 4F–4I). In addition, we did not find genetic interaction between *sema-2b* and a null allele of a transmembrane protein Off-track (Otk), which mediates downstream signaling of Sema-1a and PlexinA (Winberg et al., 2001), suggesting that *otk* is not involved in regulating dendrite-ECM adhesion (Figures S6E–S6G).

Sema-2b Genetically Interacts with TORC2 Complex and Trc/Fry

To uncover the components of Sema-2b/PlexB signaling involved in dendrite-ECM adhesion, we assayed for genetic interactions between *sema-2b* and other known regulators of dendrite-ECM adhesion, including TORC2 complex and the Trc/Fry signaling pathway (Han et al. 2012). Trc kinases are a subclass of the protein kinase A (PKA)/protein kinase G (PKG)/protein kinase C (PKC) (AGC) group of serine/threonine kinases (Hergovich et al., 2006). In *Drosophila*, components of the TORC2 complex function in the same pathway by phosphorylating and activating Trc (Koike-Kumagai et al., 2009). In class IV da neurons, loss of components in the TORC2 complex, or the loss of Trc or Fry leads to an increase in dendritic self-crossings and defects in dendrite-ECM adhesion (Emoto et al., 2004; Han et al. 2012; Koike-Kumagai et al., 2009). We tested if Target of Rapamycin (tor), SAPK-

interacting protein 1 (sin1), or rapamycin-insensitive companion of Tor (rictor), three components of TORC2 complex, genetically interact with *sema-2b*. We found strong genetic interactions between *sema-2b* and all three loss-of-function alleles (Figures 5A–5F and 5K). We further found that *sema-2b* also interacts genetically with *trc* and *fry* (Figures 5G–5K). These genetic interaction results suggest that the Sema-2b/PlexB signaling pathway likely functions together with TORC2 complex and Trc/Fry pathway to regulate dendrite-ECM adhesion.

Sema-2b/PlexB Signaling Regulates Dendrite-ECM Adhesion through Tricornered Kinase Activation

Next, we investigated whether Sema-2b/PlexB signaling functions upstream or downstream of the Trc/Fry signaling pathway. The function of the Trc/Fry signaling pathway is evolutionarily conserved, controlling dendrite growth and morphology in worms (Gallegos and Bargmann, 2004), flies (Emoto et al., 2004), and mammals (Ultanir et al., 2012; Rehberg et al., 2014). It has been previously shown that mammalian substrates of Trc/NDR1/2 kinase all contain the consensus sequence HXRXXS/T (Ultanir et al., 2012), which is absent in the *Drosophila* PlexB receptor. Therefore, we reasoned that PlexB is unlikely to be a direct substrate of Trc kinase.

To assess whether the Sema-2b/PlexB signaling pathway influences Trc kinase activity *in vivo*, we used a previously generated antibody that specifically detects phosphorylation on threonine residue 449 (T449) of Trc kinase, which is associated with maximal Trc kinase activation (Tamaskovic et al., 2003; Lee at al., 2014). In the larval body wall, Trc P-T449 immunoreactivity was present at high levels in the axons, dendrites and cell bodies of class IV da neurons (Figures 6A and 6D). In the *sema-2b* and *plexB* mutants, overall Trc P-T449 levels were greatly reduced (Figures 6B–6D), whereas we did not see any difference in the overall Trc expression levels in class IV da neurons of the *sema-2b* and *plexB* mutants (data not shown). Together, these results suggest that Sema-2b/PlexB signaling is required for Trc phosphorylation and activation in class IV da neurons.

If Sema-2b/PlexB signaling functions by promoting Trc kinase activation to regulate dendrite-ECM adhesion, we hypothesized that ectopic Trc activation in class IV da neurons of *sema-2b* mutants would mitigate the dendritic crossing phenotype. First, we tested if overexpressing wild-type Trc could rescue the dendritic crossing phenotype in *sema-2b* mutants. It has been shown that overexpressing wild-type Trc in a wild-type background is sufficient to activate Trc (Wu et al., 2013). Therefore, if overexpressing wild-type Trc could rescue the *sema-2b* mutant crossing phenotype, it would suggest that Trc functions downstream of Sema-2b/PlexB signaling, and that its activation is independent of Sema-2b/PlexB signaling. On the other hand, if overexpressing Trc does not rescue the *sema-2b* mutant crossing phenotype, it would suggest that Sema-2b/PlexB signaling is not only upstream of Trc, but is also required for its activation. Consistent with the second scenario, overexpressing wild-type Trc in *sema-2b* mutant background led to a more severe dendritic crossing defect (Figures 6E and 6H). Next, we overexpressed dominant negative Trc (S292AT449A), with alanine substitutions of Serine 292 (S292A) and Threonine 449 (T449A) to prevent phosphorylation and activation of Trc (Emoto et al., 2004). Consistent

with our hypothesis, overexpressing dominant negative Trc in *sema-2b* mutants also led to a more severe dendritic crossing defect (Figures 6F and 6H). Finally, overexpressing a constitutively active myristoylated Trc (Myr-Trc), which targets Trc to the membrane (Koike-Kumagai et al., 2009), significantly suppressed the dendritic crossing defects of the *sema-2b* mutants (Figures 6G and 6H).

Together, these results suggest that Sema-2b/PlexB signaling regulates dendrite-ECM adhesion through the Trc/Fry signaling pathway.

Mys Associates with PlexB and Likely Acts Downstream of the Sema-2b/PlexB Signaling Pathway

Loss of integrins in class IV da neurons causes defects in dendrite-ECM adhesion similar to those found in *sema-2b* and *plexB* mutants (Han et al., 2012; Kim et al., 2012). Because Trc promotes integrin-mediated adhesion in class IV da neurons (Han et al., 2012), we hypothesized that defects in integrin-mediated adhesion contribute to the increased dendrite-ECM detachment seen in *sema-2b* and *plexB* mutants. If so, we reasoned that increasing the level of integrin expression should rescue the *sema-2b* mutant dendritic crossing defects, since integrin overexpression is able to suppress dendritic crossing defects of Trc/Fry pathway mutants (Han et al., 2012). To test this prediction, we overexpressed an integrin α subunit encoded by *multiple edematous wings* (*mew*), and an integrin β subunit encoded by *myospheroid* (*mys*), both of which are required in class IV da neurons for dendrite-ECM adhesion (Han et al., 2012). In fact, overexpressing these two integrin subunits reduced the number of total dendritic crossings in *sema-2b* mutants to wild-type levels (Figures 7A–7C). It is worth noting that overexpressing integrins greatly reduced the number of non-contacting crossings, suggesting that dendrites become tightly attached to the ECM.

To test whether Mys and PlexB localize to dendrites, we expressed Mys-FLAG together with Myc-PlexB in class IV da neurons, and found that Mys-FLAG and Myc-PlexB strongly colocalize in the dendrites of class IV da neurons (Figure 7D; Pearson correlation coefficient 0.71 ± 0.02, n=5).

In mammals, activation of integrins signals through focal-adhesion kinase (FAK) family proteins to promote outgrowth of neurites (Ivankovic-Dikic et al., 2000), and semaphorin treatment has been shown to exert changes on Fak phosphorylation (Cho et al., 2012). Therefore, we tested if Fak is also required for dendrite-ECM adhesion in class IV da neurons. We examined the $fak56^{CG1}$ null allele and found no significant difference in the number of dendritic crossings (Figures S6A and S6D). Moreover, we did not find any significant genetic interaction of *fak56* and *sema-2b* mutations (Figures S6B–S6D). Our results suggest that Fak is not essential for regulating dendrite-ECM adhesion in class IV da neurons.

Plexin receptors have been shown to associate with various other receptors to mediate unique downstream signaling processes (Pasterkamp, 2012; Kolodkin and Pasterkamp, 2013). Whether the *Drosophila* PlexB receptor associates with other transmembrane receptors is unknown. Therefore, we asked if PlexB could associate with integrins by co-immunoprecipitations. We expressed FLAG-tagged Mys (Mys-FLAG) and Myc-tagged full-

length PlexB (Myc-PlexB) in the *Drosophila* S2 cell line (Schneider, 1972). As a control, we expressed Myc-tagged Dscam, a guidance receptor required for contact-mediated self-avoidance in class IV da neurons (Matthews et al., 2007; Soba et al., 2007; Hughes et al., 2007), to examine if Dscam interacts with Mys. All proteins were expressed robustly and colocalized with membrane tagged GFP (Figures S7A and S7B). We immunoprecipitated Myc-PlexB and Dscam-Myc and found that Mys-FLAG co-immunoprecipitated with Myc-PlexB, but not with Dscam-Myc (Figure 8A). Consistent with these results, immunoprecipitation of Mys-FLAG revealed robust co-immunoprecipitation of both the full-length Myc-PlexB (250kDa) and the cleaved extracellular region of Myc-PlexB (150kDa; Ayoob et al., 2006), but not Dscam-Myc (Figure 8B), demonstrating that the interaction between PlexB and Mys is specific.

To define the regions of Mys that interact with PlexB, we generated three FLAG-tagged Mys constructs encoding: the full-length Mys (FLAG-Mys), the Mys extracellular region with transmembrane domain (FLAG-MysEctoTM), and the Mys intracellular region with transmembrane domain (FLAG-MysEndoTM). Surface staining confirmed that these proteins were trafficked to the surface of S2 cells (Figure S7C). We found that immunoprecipitation of Myc-PlexB strongly co-immunoprecipitated FLAG-Mys and FLAG-MysEctoTM, but not FLAG-MysEndoTM (Figure 8C), suggesting that the formation of a complex between Mys and PlexB requires the extracellular region of Mys.

To test if Mys and PlexB interact on the surface of the cells, we performed surface staining and used an *in situ* proximity ligation assay (PLA), which can visualize specific protein-protein interactions (Söderberg et al., 2006). As a negative control, we generated FLAG-Dscam and confirmed its expression by western blotting and its membrane localization by surface staining (Figures S7D and S7E). We performed surface labeling of FLAG and Myc and found strong PLA signals only in the FLAG-Mys and Myc-PlexB co-expressed condition, and not in FLAG-Dscam and Myc-PlexB co-expressed condition (Figures 8E and 8F), demonstrating that Mys and PlexB interact on the surface of S2 cells.

Next, we examined if Mys and PlexB interact *in vivo* in dendrites of class IV da neurons. We expressed FLAG-Mys and Myc-PlexB (both tagged at the N-terminus) in class IV da neurons and performed *in situ* PLA (Figure 8G). As a negative control, coexpressing Mys-FLAG (tagged at the C-terminus) and Myc-PlexB should not give strong signals (Figure 8H). Indeed, even though FLAG-Mys is expressed at a lower level than Mys-FLAG (Figure S7F), we found a significantly higher level of PLA signals in dendrites expressing FLAG-Mys and Myc-PlexB, compared to those expressing Mys-FLAG and Myc-PlexB (Figure 8I). We found PLA signals in both the proximal regions and the distal regions of the dendritic fields, suggesting the interactions of Mys and PlexB occur in these areas.

Discussion

Self-avoidance and tiling are critical mechanisms governing the patterning of dendritic arbors. In *Drosophila* sensory neurons, these mechanisms depend on the dendrites being restricted onto a 2D plane through dendrite-ECM adhesion. We have uncovered a new role for Sema-2b/PlexB signaling in regulating dendrite-ECM adhesion to promote contact-

mediated self-avoidance. We demonstrate that Sema-2b secreted from epidermal cells acts on neuronal PlexB receptors. Furthermore, we identified the TORC2 complex, the Trc/Fry signaling pathway and integrins as components of a Sema-2b/PlexB signaling pathway (Figures 8J and 8K). Our study uncovers previously unknown functions of semaphorin in regulating dendrite-ECM adhesion in neurons to promote dendrite self-avoidance.

Epidermis Secreted Cues Shape Neuron Circuit Formation

Neurons interact with the complex environment that surrounds them at every step of neural development. For example, neuron-glia interaction has been studied in a variety of model organisms from worms to mammals (Corty and Freeman, 2013). Glia-secreted factors play an active role in neuronal migration, axon guidance, and synapse formation during development, and have been implicated in many neurodevelopmental disorders (Sloan and Barres, 2014). In vertebrates and *Drosophila*, the secretion of TGF-β ligands from glia regulates neuromuscular junction synapse formation and growth (Feng and Ko, 2008; Fuentes-Medel et al., 2012).

Compared to the extensive inquiries into neuron-glia interactions, the importance of neuron-epidermis interactions in neural circuit formation has only begun to be studied. Sensory neurons innervate the epidermis of both invertebrate and vertebrate organisms, and precise innervation must require diverse extrinsic signals. Two recent studies in *C.elegans* show that the epidermis generates pre-patterned cues to regulate sensory dendrite development (Dong et al., 2013; Salzberg et al., 2013). Our study provides evidence that semaphorin is an epidermis-derived ligand that instructs spatial patterning of sensory dendrites in *Drosophila*. In mammals, many members of the semaphorin family are expressed in sensory neurons, including those in dorsal root ganglia (Pasterkamp, 2012; O'Malley et al., 2014). It will be of great interest to determine whether epidermis-derived semaphorin also regulates morphogenesis of mammalian sensory neurons.

Role for Semaphorins in Regulating Dendrite-ECM Adhesion to Promote Self-Avoidance

For self-avoidance involving homotypic repulsion, spatial restriction is critical to ensure that contact-mediated interaction between neurites occurs (Zipursky and Grueber, 2013). Similar to class IV da neurons, spatially restricted systems for tiling and self-avoidance also exist in vertebrates, such as fish somatosensory neurons (Sagasti et al., 2005), mammalian cerebellar Purkinje cells (Kaneko et al., 2011), and mammalian retina (Wässle, 2004; Sanes and Zipursky, 2010). Indeed, semaphorin signaling is required for the stratification and symmetric arborization of starburst amacrine cell (SAC) dendrites in the developing mouse retina (Sun et al., 2013). Interestingly, SACs with defects in laminar stratification also show an increase in the number of self-crossings (Sun et al., 2013). In this study, we show that the spatial restriction mediated by dendrite-ECM adhesion is itself modulated by Sema-2b/PlexB signaling, thereby promoting contact-mediated self-avoidance in *Drosophila*. Our data suggests that Sema-2b/PlexB signaling does not directly promote contact-mediated dendritic repulsion, because the number of contacting crossings did not increase in the *plexB* mutants (Figure 4B and 4E). Conceptually, the problem of laminar stratification of neurites in vertebrate retina (Sanes and Zipursky, 2010; Kim et al., 2010) and the tethering of dendrites of *Drosophila* da neurons to the ECM are somewhat similar. In both cases, the

growth of the neurites has to be constrained to a 2D space to allow the contact-mediated self-avoidance mechanism to operate. It is therefore very intriguing that semaphorins are involved in both situations (Sun et al., 2013 and this study). In the future, it will be of interest to determine whether the semaphorin regulated dendrite-substrate adhesion observed in our study also occurs in the dendrites of mammalian neurons, and if so whether it also contributes to self-avoidance and tiling in that context.

New Insights into the Semaphorin Signaling Pathway

Semaphorin family proteins play essential roles in refinements of neural circuitry, including neuronal polarization, topographical mapping, dendrite arborization and axon sorting (Pasterkamp, 2012; Koropouli and Kolodkin, 2014). How a small number of proteins can generate such a diverse set of neuronal connections is an intriguing question. One mechanism by which semaphorins may achieve this feat is through activation of divergent downstream signaling pathways at different times during development. In the *Drosophila* embryonic central nervous system, PlexB receptors in mechanosensory axon terminals mediate both Sema-2b attraction and Sema-2a repulsion (Wu et al., 2011). At the embryonic stage, Sema-2a also acts through PlexB receptor to guide precise axon terminal projections of class IV da neurons (Zlatic et al., 2009). In our study, we found that Sema-2b, but not Sema-2a, acts on PlexB receptor to mediate dendrite adhesion to the ECM. How PlexB binding to Sema-2a and Sema-2b activates distinct downstream pathways is a question that awaits future studies.

Drosophila PlexB receptor has been shown to have opposite effects on cytoskeletal components by simultaneously inhibiting active Rac and enhancing RhoA signaling (Hu et al., 2001). Yet other components downstream of PlexB receptor are less well understood. Our study found that the activation of the Trc/Fry signaling pathway requires Sema-2b/PlexB signaling and provided genetic evidence that TORC2 and Trc activation is downstream of Sema-2b/PlexB signaling (Figure 8K). Our finding is consistent with a previous study that showed TORC2 complex associates with Trc and promotes its activation possibly by recruiting Trc to the membrane in the class IV da neurons (Koike-Kumagai et al., 2009).

The phenotype of the *plexB* mutants we observed was weaker than the phenotype of *sema-2b* mutants. It is possible that the *plexB* allele we used is not a complete null allele (Ayoob et al., 2006), or that there is an unidentified Sema-2b receptor that also mediates Sema-2b/PlexB signaling (Figure 8K). It was recently shown that Ret receptor mediates dendrite-ECM adhesion and that Ret also associates with Mys (Soba et al., 2015), and it would be interesting to determine if Ret mediates part of Sema-2b signaling.

In our model, we proposed that Trc/Fry signaling is upstream of integrins, based on three lines of evidence (Figure 8K). First, overexpressing integrins in *trc* and *fry* mutant background rescued their dendrite-ECM adhesion defect (Han et al., 2012). Second, the mammalian homologue of Trc, NDR2 kinase, can induce phosphorylation at the activity and trafficking-relevant site of β1-integrin to stimulate their trafficking to the cell membrane in neurons (Rehberg et al., 2014). Third, our previous study identified AAK1 (AP-2 associated kinase) and Rabin8, a GDP/GTP exchange factor (GEF) of Rab8 GTPase as two direct

substrates of NDR1 kinase in mammals, both of which function in intracellular vesicle trafficking (Ultanir et al., 2012). Thus, it is possible that Trc/Fry signaling is important for regulating membrane turnover of integrins in neurons. In the future, it will also be important to determine whether the mammalian NDR kinases mediate semaphorin signaling.

Previous studies show that crosstalk between semaphorin and integrin signaling leads to inhibition of integrin-mediated adhesion in both invertebrates and vertebrates (Tamagnone and Comoglio, 2004; Kruger et al., 2005; Cho et al., 2012). For example, semaphorin-mediated signaling decreases integrin-mediated attachment during vascular morphogenesis (Serini et al., 2003). It has also been observed that semaphorin 4D promotes the formation of focal adhesion complexes through RhoA and Rho kinases (Basile et al., 2007). Here, we found that in *Drosophila* sensory neurons Sema-2b/PlexB signaling acts in the same pathway as integrins, and showed that PlexB and Mys form a complex (Figure 8). The biological relevance of this association would be of interest for future studies. It is possible that the physical association of PlexB and Mys stabilizes the surface expression of integrins for binding to laminins in the ECM. It is also possible that the interaction induces conformational changes to facilitate signaling pathways downstream of PlexB and/or integrins.

Overall, our results demonstrate that epidermis-secreted semaphorins regulate contact-dependent self-avoidance by promoting dendrite-ECM association and provide insights into the downstream components of semaphorin signaling pathway.

Experimental Procedures

Live Imaging and Image Processing

Animals were reared at 25°C and 29°C for RNAi expe riments in density-controlled vials. Third instar larvae were mounted in glycerol and dendrites of class IV da neurons were imaged using a Leica SP5 laser scanning confocal microscope. For high-resolution imaging in the z axis, images were collected as described in Han et al., 2012. To quantify dendrite enclosure, images were deconvoluted using Autoquant (MediaCybernetics) and analyzed in Imaris (Bitplane) as described in Han et al., 2012.

Immunohistochemistry

Third instar larvae were dissected in PBS, fixed in 4% PFA for 20 min at room temperature, blocked with 5% normal goat serum, and stained with the primary antibodies in a 0.3% Triton X-100 solution overnight at 4°C, and subsequently with secondary antibodies in a 0.3% Triton X-100 solution for 3 hours at room temperature.

Statistical analysis

Two-tailed Student’s t-tests were used to compare two samples. A one-way ANOVA test was used to compare multiple samples.

See Supplemental Experimental Procedures for details on mutant alleles, MARCM analysis, image analysis, immunohistochemistry, immunoprecipitation, *in situ* proximity ligation assay and western blotting used in this paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Sema-2b/PlexB signaling confines dendrites in a 2D space.
- Sema-2b is secreted from the epidermis and functions through PlexB in neurons.
- Sema-2b/PlexB signaling regulates Tricornered kinase activity *in vivo*.
- An integrin β subunit associates with PlexB receptor.

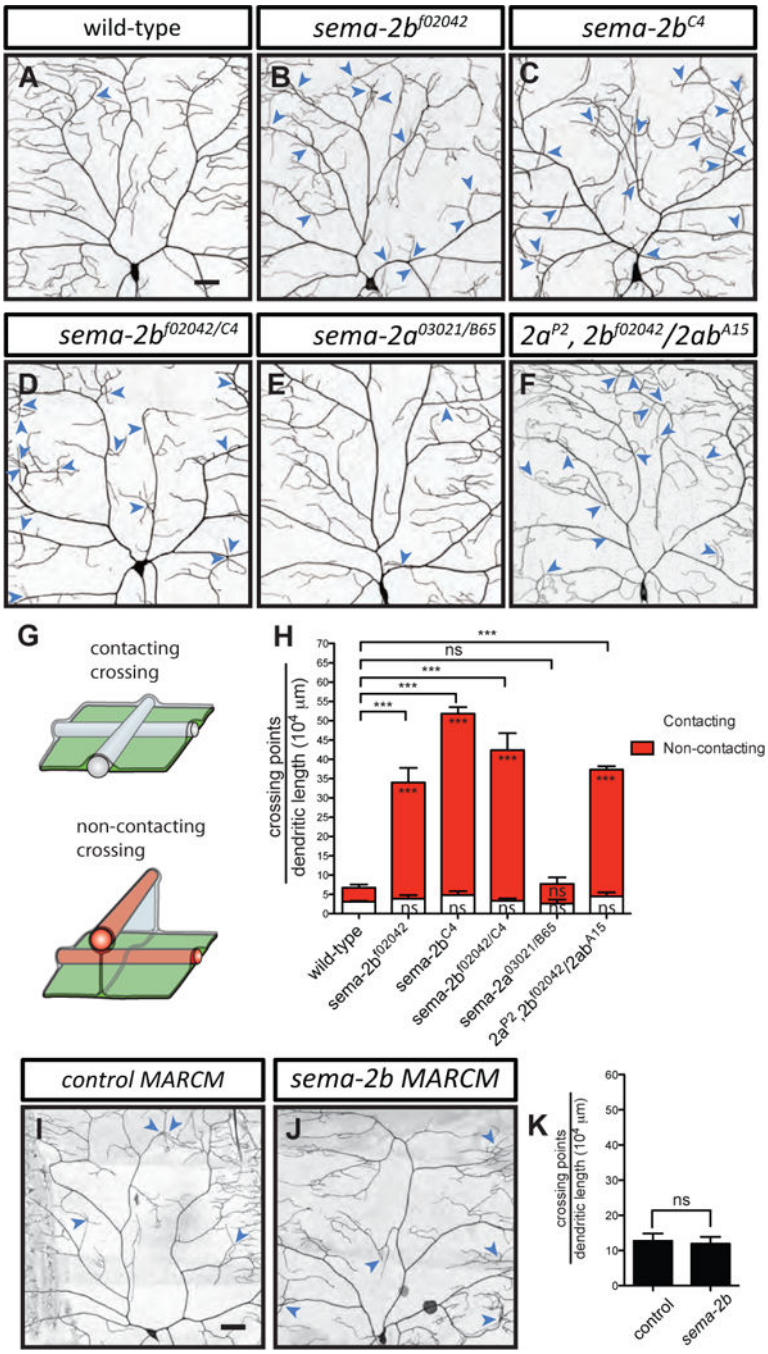

Figure 1. Sema-2b, but not Sema-2a loss of function leads to an increase in dendritic self-crossings

(A–F) Dendritic patterns of wild-type (A), *sema-2b*^{f02042} (B), *sema-2b*^{C4} (C), *sema-2b*$^{f02042/C4}$ (D), *sema-2a*$^{03021/B65}$ (E), and *sema-2a*^{P2}, *2b*^{f02042}*/2ab*^{A15} (F) class IV da neurons. Dendritic crossings are indicated by blue arrowheads. Scale bars represent 30 µm. Wild-type animals are *w*^{1118} carrying one copy of *ppk-CD4-tdTomato*.

(G) Schematic of a contacting crossing (top) and a non-contacting crossing (bottom). In the case of contacting crossings, both dendrites (white bars) are in the same X–Y plane. In the

case of non-contacting crossings, one or both dendrites (red bars) detach from ECM (green sheet) and become enclosed by the basal surface of epidermal cells (grey sheet).

(H) Quantification of crossing points normalized to total dendritic length in wild-type (n=6), *sema-2b*^{f02042} (n=4), *sema-2b*^{C4} (n=4), *sema-2b*$^{f02042/C4}$ (n=7), *sema-2a*$^{03021/B65}$ (n=4), and *sema-2a*^{P2}, *2b*^{f02042}/*2ab*^{A15} (n=4) mutant neurons. White bars represent the quantifications of contacting crossings and red bars represent the quantification of non-contacting crossings. Data are plotted as average ± SEM. ns, not significant, and ***p < 0.001 as assessed by one-way analysis of variance and Dunnett's test. The comparisons of the total number of crossings are labeled on top of the bars. The comparisons of the contacting crossings and non-contacting crossings are labeled in the white bars and red bars, respectively.

(I–J) Dendritic patterns of control (I) and *sema-2b*^{C4} (J) class IV neurons generated with MARCM. Scale bars represent 30 μm.

(K) Quantification of total crossing points normalized to total dendritic length in control (n=3) and *sema-2b*^{C4} (n=3) class IV da neurons. Data are plotted as average ± SEM. ns, not significant as assessed by a Student's t test.

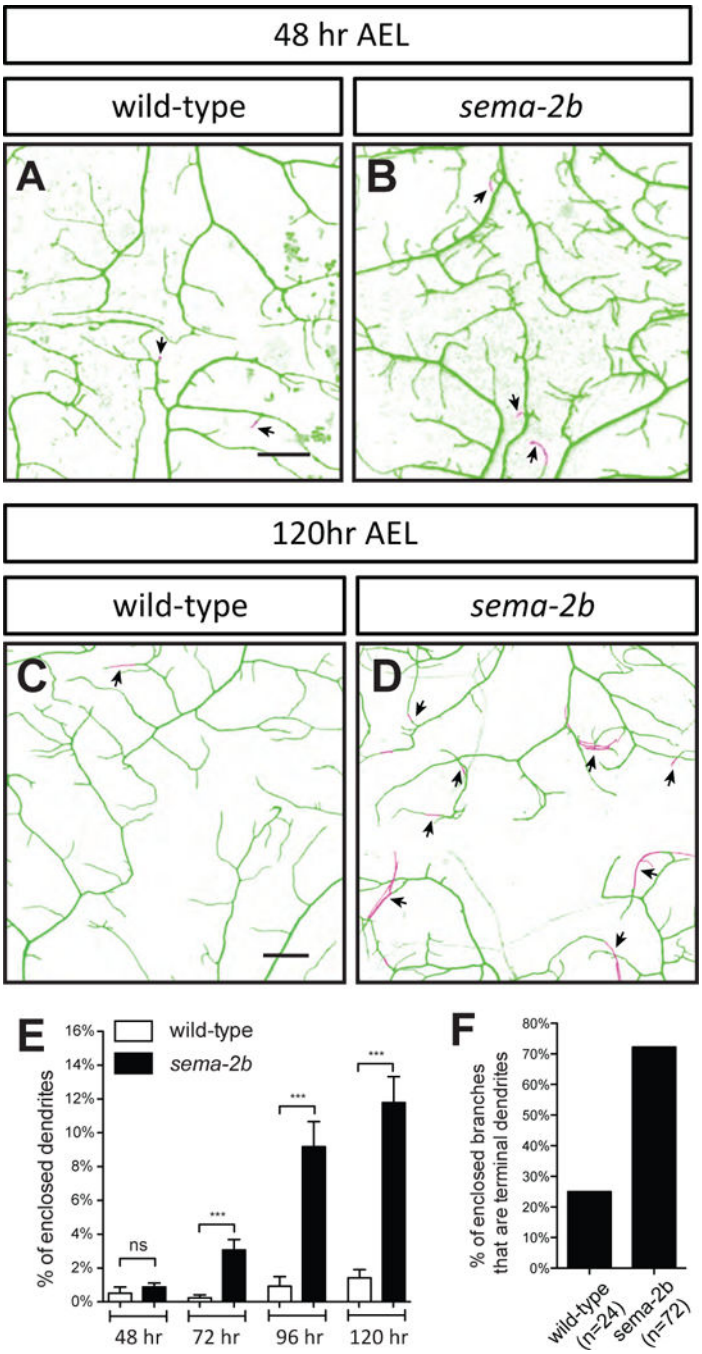

Figure 2. Enclosure of terminal dendrites by epidermal cells in *sema-2b* mutants

(A–B) Wild-type (A) and *sema-2b*$^{f02042/C4}$ (B) dendritic field imaged at 48 hr AEL. (C–D) Wild-type (C) and *sema-2b*$^{f02042/C4}$ (D) dendritic field imaged at 120 hr AEL. Wild-type animals are *w*^{1118} carrying one copy of *ppk-CD4-tdTomato*. Dendrites attached to the ECM are labeled in green and enclosed dendrites are labeled in magenta. The arrowheads point to terminal dendrites that are enclosed by epidermal cells. Scale bars represent 30 μm.

(E) Quantification of percentages of enclosed dendrites at 48 hr, 72 hr, 96 hr and 120 hr AEL in wildtype and *sema-2b*$^{f02042/C4}$ neurons. Data are plotted as average ± SEM. ***p <0.001 as assessed by a Student's t test.

(F) Percentages of detached dendrites that are terminal dendrites in class IV da neurons in wild-type and *sema-2b* mutants.

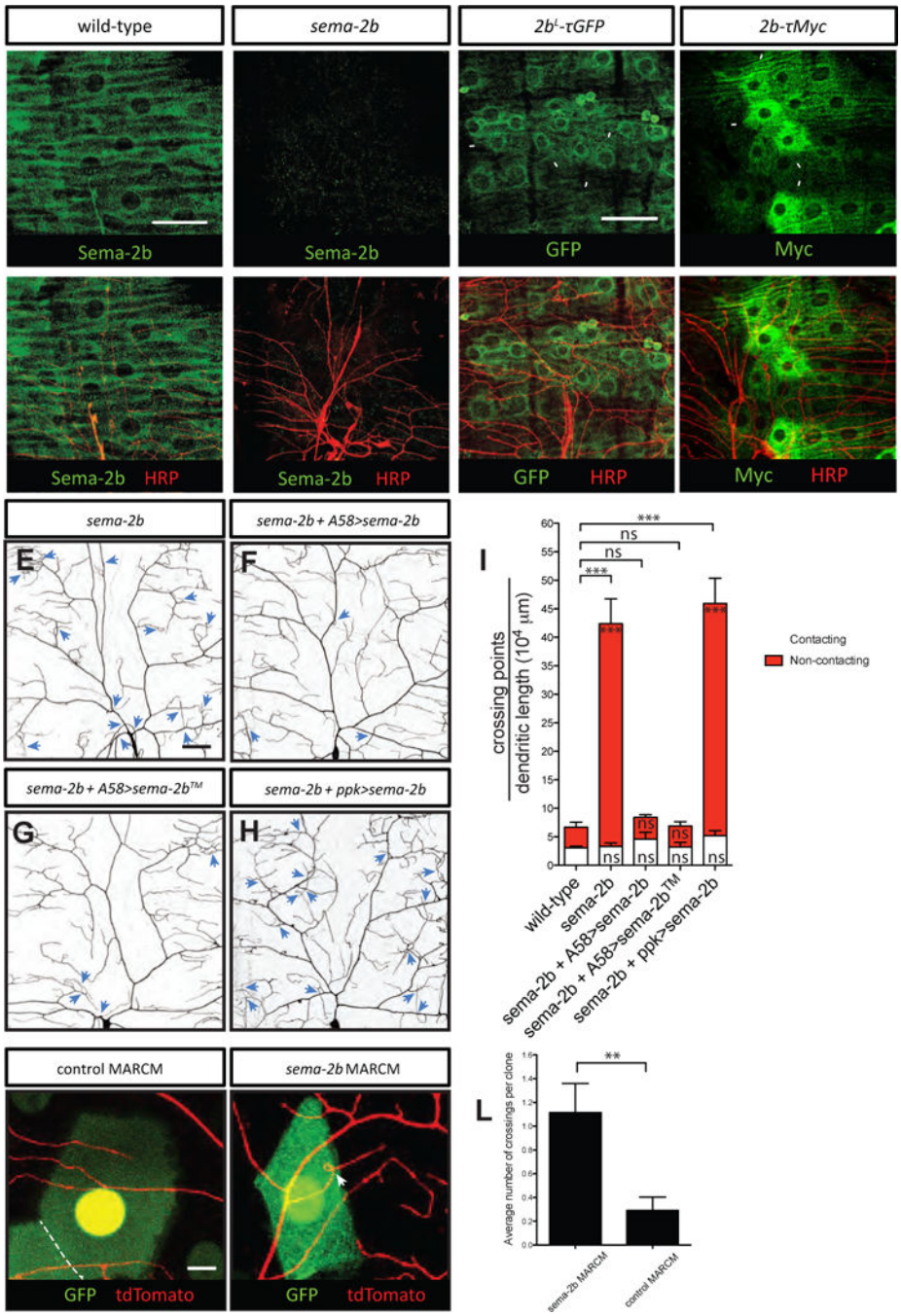

Figure 3. Sema-2b is derived from epidermal cells and acts at short range to regulate dendrite adhesion

(A–A′) Anti-Sema-2b immunostaining of a w^{1118} wild-type third instar fillet reveals strong expression in epidermal cells. Anti-HRP immunoreactivity labels neurons in the peripheral nervous system.

(B–B′) No detectable Sema-2b staining in *sema-2b*^{C4} mutants. Scale bar represents 50 µm.

(C–C′) The $2b^{L}$-*τGFP* reporter labels epidermal cells that express Sema-2b. $2b^{L}$-*τGFP* animals were immunostained for τGFP and anti-HRP, which labels neurons in the body wall. Some GFP positive epidermal cells are indicated by white arrowheads.

(D–D′) The *2b-τMyc* reporter labels a subset of Sema-2b-expressing epidermal cells. *2b-τMyc* animals were immunostained for τMyc and anti-HRP, which labels neurons in the body wall. Some τMyc positive epidermal cells are indicated by white arrowheads. All the staining images are positioned with the anterior side on the left and dorsal side on the top.
(E–I) Dendritic patterns and quantifications of class IV da neurons in *sema-2b*$^{f02042/C4}$ (E, n=7), *sema-2b* $^{f02042/C4}$ mutant with epidermis-expressing full length Sema-2b (F, n=5), epidermis-expressing membrane tethered Sema-2b (G, n=5), and neuronal-expressing full length Sema-2b (H, n=6). Dendritic crossings are indicated by blue arrowheads. Scale bars represent 30 μm. Wild-type animals are w^{1118} carrying one copy of *ppk-CD4-tdTomato*. White bars represent the quantifications of contacting crossings and red bars represent the quantification of non-contacting crossings. Data are plotted as average ± SEM. ns, not significant and ***p<0.001 as assessed by one-way analysis of variance and Bonferroni test. The comparisons of the total number of crossings are labeled on top of the bars. The comparisons of the contacting crossings and non-contacting crossings are labeled in the white bars and red bars, respectively.
(J–K) Dendritic patterns of control (J) and *sema-2b*^{C4} (K) epidermal clones (labeled by GFP) generated with MARCM. Class IV da neurons are genetically labeled by one copy of *ppk-CD4-td-Tomato*. Scale bars represent 10 μm. The boundary between two adjacent epidermal cells is marked by a white dashed line.
(L) Quantification of total crossing points among dendrites that are covered by control (n=24) and *sema-2b*^{C4} (n=26) class IV da neurons. Data are plotted as average ± SEM. ns, not significant as assessed by a Student’s t test.

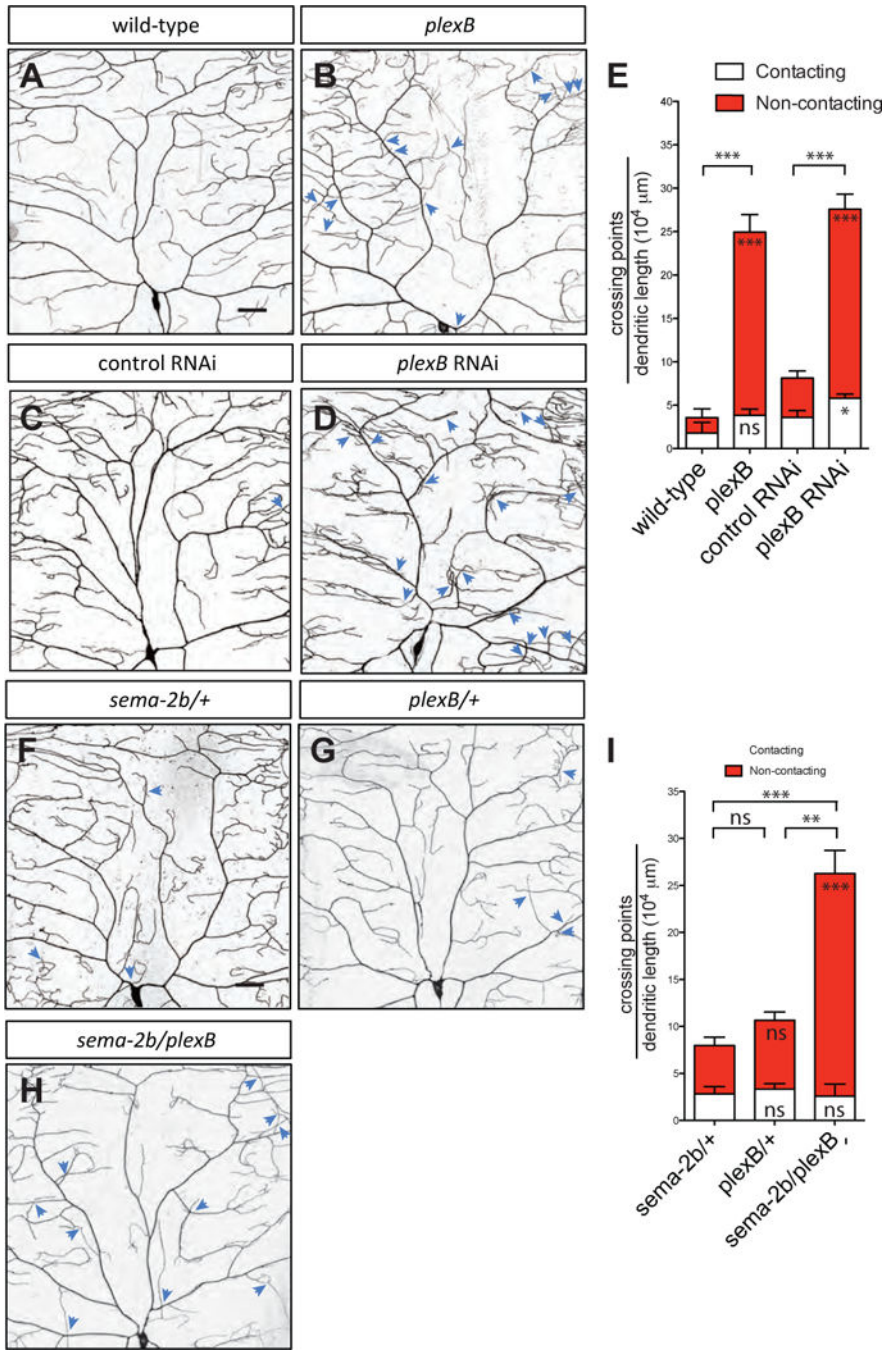

Figure 4. Plexin B is the Sema-2b Receptor that regulates dendrite-ECM adhesion

(A–D) Dendritic patterns of wild-type (A), *plexB*^{KG00878} (B), control RNAi (C) and *plexB RNAi* (D) class IV da neurons. Dendritic crossings are indicated by blue arrowheads. Scale bars represent 30 µm. Wild-type animals are *w*^{1118} carrying one copy of *ppk-CD4-tdTomato*. (E) Quantification of total crossing points normalized to total dendritic length in wild-type (n=4), *plexB*^{KG00878} (n=6), RNAi control (n=6), and *plexB RNAi* (n=6) neurons. Data are plotted as average ± SEM. ns, not significant, *p<0.05, ***p < 0.001 as assessed by a Student's t test. The comparisons of the total number of crossings are labeled on top of the

bars. The comparisons of the contacting crossings and non-contacting crossings are labeled in the white bars and red bars, respectively.

(F–H) Dendritic patterns of *sema-2b*^{C4}/+ (F), *plexB*^{KG00878}/+ (G), *and sema-2b*^{C4}/*plexB*^{KG00878} (H) class IV da neurons. Dendritic crossings are indicated by blue arrowheads. Scale bars represent 30 μm.

(I) Quantification of total crossing points normalized to total dendritic length in *sema-2b*^{C4}/+ (F), *plexB*^{KG00878}/+ (G), *and sema-2b*^{C4}/*plexB*^{KG00878} (H) class IV da neurons. White bars represent the quantifications of contacting crossings and red bars represent the quantification of non-contacting crossings. Data are plotted as average ± SEM. ns, not significant and **p < 0.01, ***p<0.001 assessed by one-way analysis of variance and Bonferroni test for all pairs of columns. The comparisons of the total number of crossings in each genotype to *sema-2b*^{C4}/+ are labeled on top of the bars. The comparisons of the contacting crossings and non-contacting crossings each genotype to *sema-2b*^{C4}/+ are labeled in the white bars and red bars, respectively.

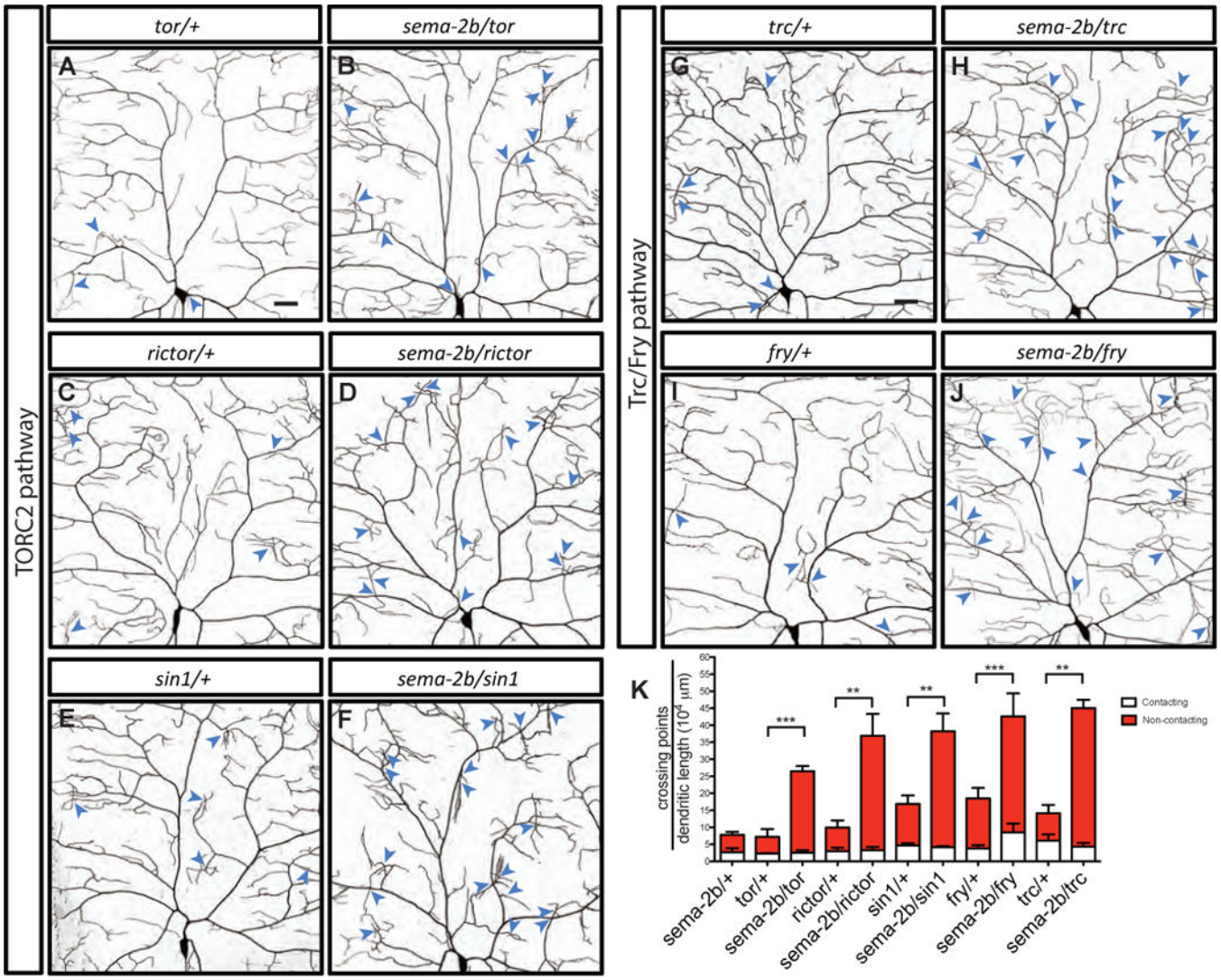

Figure 5. Sema-2b genetically interacts with TORC2 complex and Trc kinase

(A–J) Dendritic patterns of $tor^{\Delta P}$/+ (A), $sema\text{-}2b^{C4}/tor^{\Delta P}$ (B), $rictor^{\Delta 2}$/+ (C), $sema\text{-}2b^{C4}/rictor^{\Delta 2}$ (D), $sin1^{e03756}$/+ (E), $sema\text{-}2b^{C4}/sin1^{e03756}$ (F), trc^{1}/+ (G), $sema\text{-}2b^{C4}/trc^{1}$(H), fry^{1}/+ (I), and $sema\text{-}2b^{C4}/fry^{1}$ (J) class IV da neurons. Dendritic crossings are indicated by blue arrowheads. Scale bars represent 30 µm.

(K) Number of total crossing points normalized to total dendritic length in $sema\text{-}2b^{C4}$/+ (n=5), $tor^{\Delta P}$/+ (n=4), $sema\text{-}2b^{C4}/tor^{\Delta P}$ (n=5), $rictor^{\Delta 2}$/+ (n=5), $sema\text{-}2b^{C4}/rictor^{\Delta 2}$ (n=5), $sin1^{e03756}$/+ (n=5), $sema\text{-}2b^{C4}/sin1^{e03756}$ (n=7), trc^{1}/+ (n=4), $sema\text{-}2b^{C4}/trc^{1}$ (n=4), fry^{1}/+ (n=5), and $sema\text{-}2b^{C4}/fry^{1}$ (n=5) neurons. White bars represent the quantifications of contacting crossings and red bars represent the quantification of contacting crossings. Data are plotted as average ± SEM. **p < 0.01, and ***p < 0.001 as assessed by one-way analysis of variance and Bonferroni test.

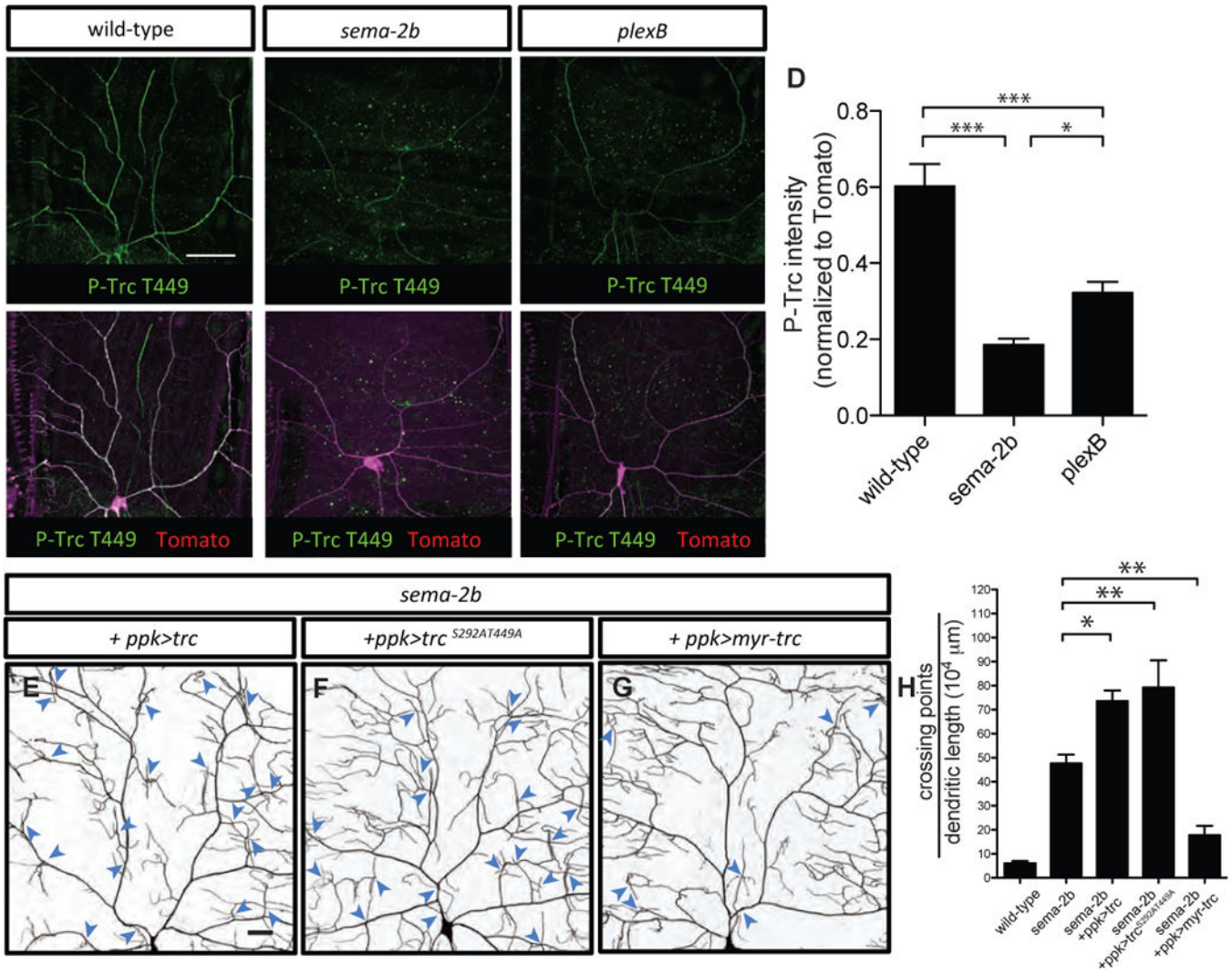

Figure 6. Trc kinase acts downstream of Sema-2b/PlexB signaling to promote dendrite adhesion

(A–C) Anti-phospho-Trc (P-T449) immunostaining of a wild-type w^{1118} third instar fillet reveals strong labeling in the wild-type class IV da neuron (A). Anti-phospho-TrcT449 shows weak labeling in *sema-2b*^{C4} (B) *and plexB*^{KG00878} (C) mutant class IV da neurons. Scale bar represents 50 μm. Class IV da neurons are genetically labeled by *ppk-CD4-td-Tomato*. All the staining images are positioned with the anterior side on the left and dorsal side on the top.

(D) Quantification of the level of P-T449 normalized to *ppk-CD4-td-Tomato* in the dendrites of the class IV da neurons in the wild-type, *sema-2b*^{C4}, *and plexB*^{KG00878} animals. ns, not significant, **p < 0.01, and ***p < 0.001 as assessed by one-way analysis of variance and Bonferroni test for all pairs of columns.

(E–G) Dendritic patterns of *sema-2b*$^{f02042/C4}$ mutants expressing wild-type Trc (E), phosphorylation-site mutated Trc (S292AT449A) (F), and myristoylated Trc (Myr-Trc) (G) in class IV da neurons. Dendritic crossings are indicated by blue arrowheads. Scale bars represent 30 μm.

(H) Quantification of total crossing points normalized to total dendritic length in *sema-2b*$^{f02042/C4}$ mutants expressing wild-type Trc (n=5), Trc^{S292AT449A} (n=5), and Myr-Trc (n=5) in class IV da neurons. Data are plotted as average ± SEM. *p < 0.05 and **p <0.01 as assessed by a one-way analysis of variance and Dunnett's test.

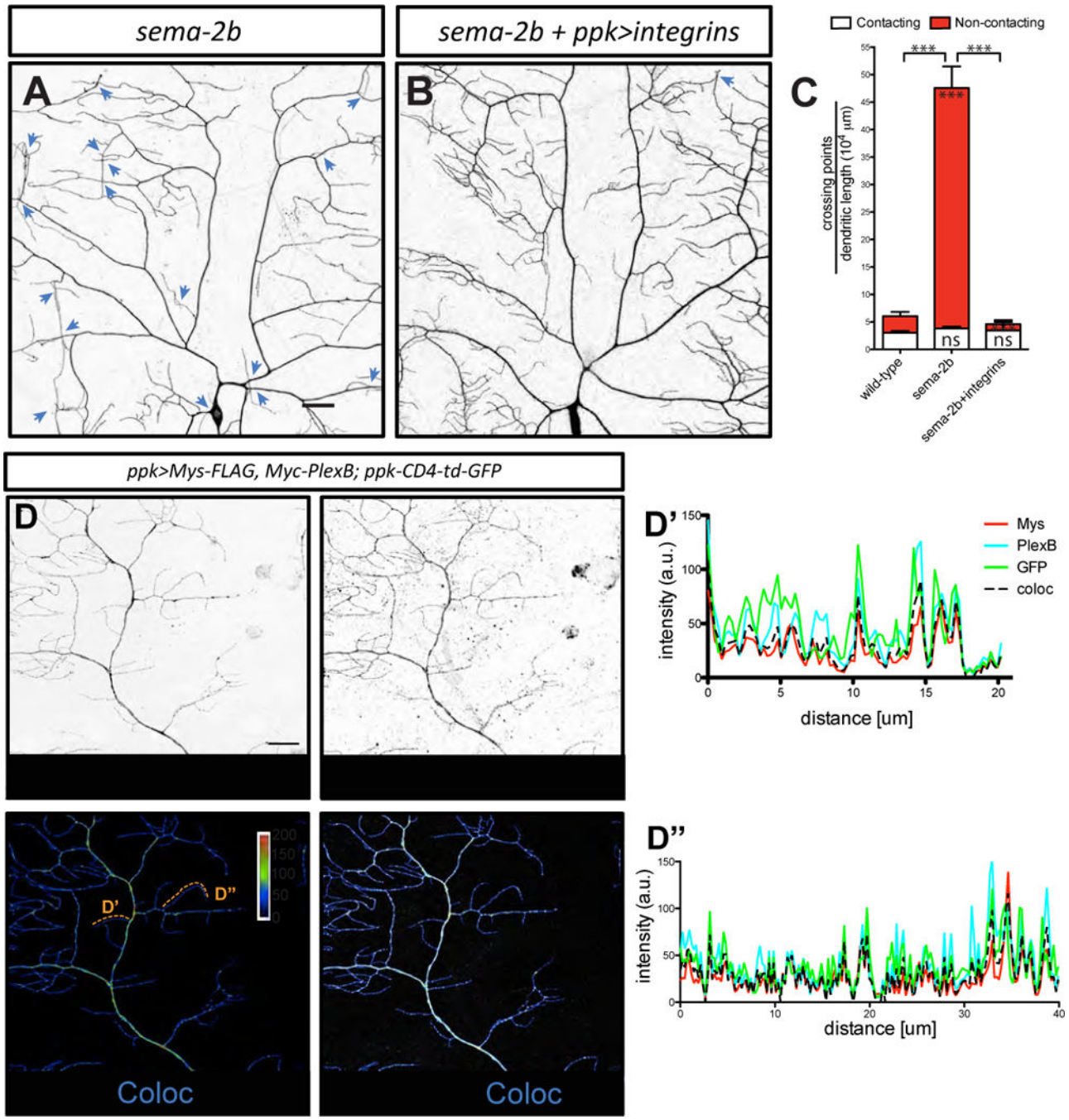

Figure 7. Integrins overexpression suppresses dendrite crossing defects in *sema-2b* mutant

(A–B) Dendritic patterns of class IV da neurons in *sema-2b*$^{f02042/C4}$ mutants (A) and *sema-2b*$^{f02042/C4}$ mutants with integrins (*UAS-mys* and *UAS-mew*) overexpressed in class IV da neurons (B). Dendritic crossings are indicated by blue arrowheads. Scale bars represent 30 µm.

(C) Quantifications of crossing points in wild-type (n=6), *sema-2b*$^{f02042/C4}$ (n=5) class IV da neurons and *sema-2b*$^{f02042/C4}$ with integrins overexpressed in class IV da neurons (n=4). Data are plotted as average ± SEM. ns, not significant and ***p<0.001 as assessed by one-

way analysis of variance and Bonferroni test for all pairs of columns. The comparisons of the total number of crossings in each genotype and wild-type animals are labeled on top of the bars. The comparisons of the contacting crossings and non-contacting crossings are labeled in the white bars and red bars, respectively.

(D) Colocalization of Mys-FLAG and Myc-PlexB in dendrites of class IV da neurons. Mys-FLAG and Myc-PlexB are co-expressed by *ppk-Gal4* driver. Class IV da neurons are labeled by *ppk-CD4-td-GFP*. Scale bar represents 20 m. The line plots of two terminal dendrites are show in D' and D".

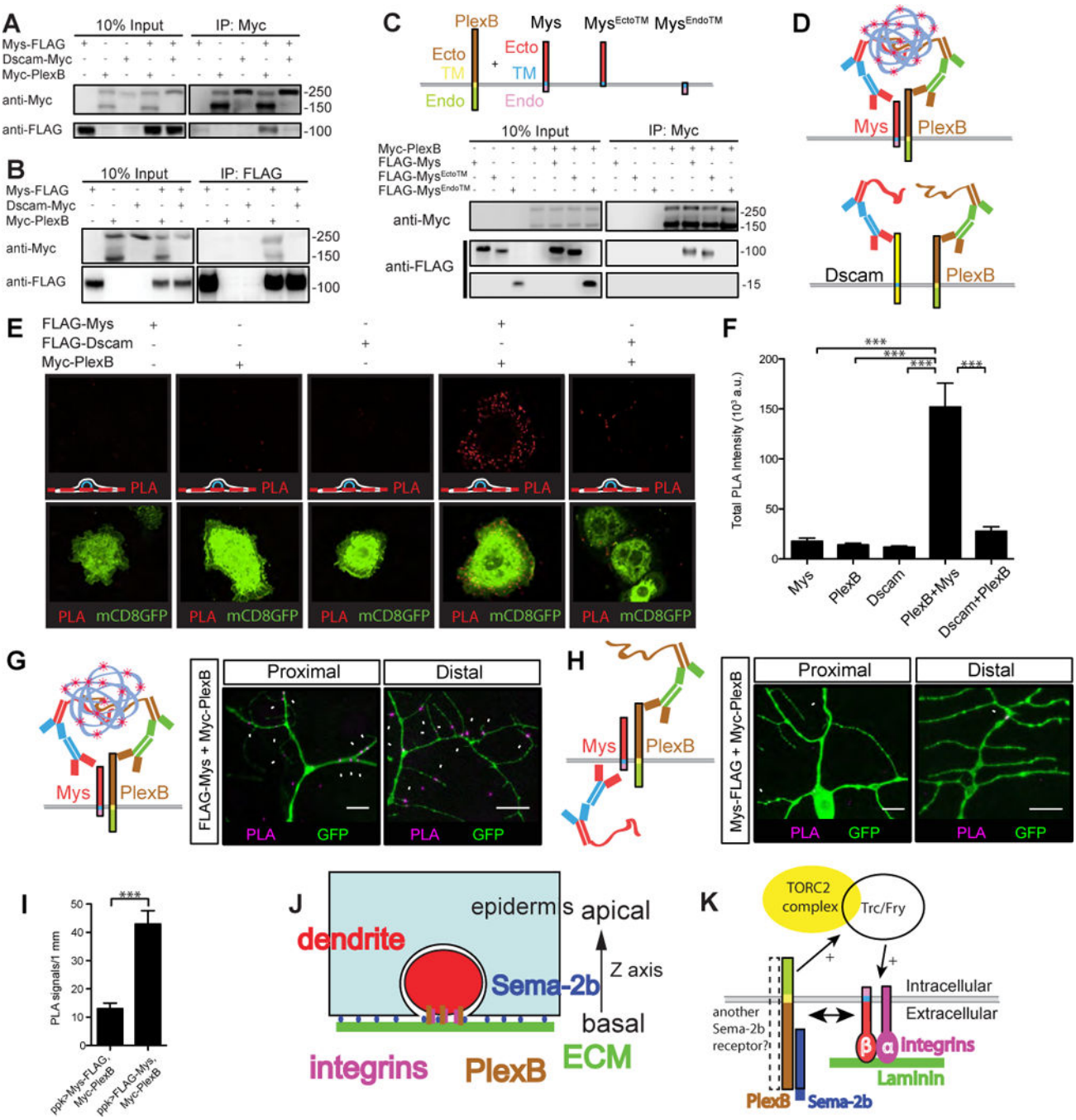

Figure 8. Mys, a β subunit of integrin, associates with PlexB

(A–B) Western blot showing co-immunoprecipitation of PlexB and Mys. Lysates from S2 cells expressing Mys-FLAG with Myc-PlexB or Dscam-Myc as depicted were incubated with anti-Myc antibody (A) or FLAG antibody bound beads (B). Immunoprecipitates were probed for the presence of Mys-FLAG (A), Myc-PlexB or Dscam-Myc (B).

(C) Western blot revealing the extracellular region of Mys is required for the association between Mys and PlexB. Lysates from S2 cells expressing either FLAG-Mys, FLAG-MysEctoTM, FLAG-MysEndoTM, and Myc-PlexB as depicted were incubated with Myc

antibody. Immunoprecipitates were probed for the presence of FLAG-Mys, FLAG-MysEctoTM and FLAG-MysEndoTM using anti-FLAG antibody.

(D–F) PlexB and Mys interaction detected by *in situ* PLA on the surface of S2 cells. (D) Diagram showing the principles of *in situ* PLA: if the two membrane proteins (Mys and PlexB) are within 40nm of each other, the secondary antibodies conjugated with oligonucleotides will be joined together and fluorescently labeled oligonucleotides will then be added to the site by a rolling circle amplification. Both Myc-PlexB and FLAG-Mys are tagged on the extracellular regions. (E) PLA signals in each condition. The interactions were visualized as red fluorescent dots and membrane GFP marked the transfected cells. A cartoon showing the position of the focal plane is shown on the lower left of the panel. (F) Quantification of total PLA intensity per GFP positive cell (n=40 in each condition). Data are plotted as average ± SEM. ***p<0.001 as assessed by one-way analysis of variance and Bonferroni test for all pairs of columns. Scale bars represent 10 μm. A.u. arbitrary unit.

(G–I) PlexB and Mys interaction detected by *in situ* PLA in dendrites of class IV da neurons. PLA signals in a neuron that co-expresses FLAG-Mys and Myc-PlexB are shown in (G). PLA signals in a neuron that co-expresses Mys-FLAG and Myc-PlexB are shown in (H). (I) Quantification of the number of PLA dots normalized to the dendritic length in each genotype. Data are plotted as average ± SEM. ***p<0.001 as assessed by a Student’s t-test. Staining images are positioned with the anterior side on the left and dorsal side on the top. Scale bars represent 10 μm.

(J) Diagram of a cross section of the ECM (green), epidermis (blue) and dendrites (red). Sema-2b ligands are produced and secreted by epidermal cells and diffuse throughout the whole dendritic field of class IV da neurons. Sema-2b signals through PlexB receptors in the neurons to regulate dendrite-ECM adhesion.

(K) A model of the downstream molecular mechanisms of how Sema-2b/PlexB signaling regulates dendrite-ECM adhesion through the activation of Trc kinase and the binding of integrins to laminin proteins in the ECM.