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UNC-6/netrin and its receptor UNC-5 locally exclude presynaptic components from dendrites

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Abstract

Polarity is an essential feature of many cell types, including neurons that receive information from local inputs within their dendrites and propagate nerve impulses to distant targets through a single axon. It is generally believed that intrinsic structural differences between axons and dendrites dictate the polarized localization of axonal and dendritic proteins[1]. However, whether extra-cellular cues also instruct this process *in vivo* has not been explored. Here we show that the axon guidance cue UNC-6/netrin and its receptor UNC-5 act throughout development to exclude synaptic vesicle and active zone proteins from the dendrite of the *Caenorhabditis elegans* motor neuron DA9, which is proximal to a source of UNC-6/netrin. In *unc-6/netrin* and *unc-5* loss-of-function mutants, presynaptic components mislocalize to the DA9 dendrite. In addition, ectopically expressed UNC-6/netrin, acting through UNC-5, is sufficient to exclude endogenous synapses from adjacent subcellular domains within the DA9 axon. Furthermore, this anti-synaptogenic activity is interchangeable with that of LIN-44/Wnt despite being transduced through different receptors, suggesting that extracellular cues such as netrin and Wnts not only guide axon navigation but also regulate the polarized accumulation of presynaptic components through local exclusion.

The *C. elegans* motor neuron DA9 elaborates a molecularly and functionally distinct axon and dendrite[2] (Fig. 1a, b). In wild-type animals, presynaptic components are excluded from the dendrite and accumulate in a stereotyped and discrete domain within the DA9 dorsal axon[3] (Fig. 1c, d). These presynaptic components include synaptic vesicle proteins such as RAB-3, SNB-1/synaptobrevin and SNG-1/synaptogyrin (Supplementary Fig. 1), the L-type voltage-gated calcium channel β-subunit CCB-1, and the active zone protein SYD-2/α-liprin (Supplementary Fig. 2). In exploring whether extra-cellular cues instruct this polarized localization, we found that these presynaptic proteins mislocalize to the DA9 dendrite in *unc-6/netrin* (*ev400*) and *unc-5*(*e53*) null mutants (Fig. 1e–h, and Supplementary Figs 1 and 2). This mislocalization defect is not enhanced in *unc-5;unc-6/netrin* double mutants, suggesting that UNC-5 and UNC-6/netrin function in the same pathway (Fig. 1h). A null mutation in the other principal UNC-6/netrin receptor, UNC-40, results in a minor mislocalization defect (Fig. 1h). We further observed that this mislocalization is partly

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suppressed by a mutation in the presynaptic assembly gene, *syd-2/liprin-α* (Supplementary Fig. 3), suggesting that SYD-2/α-liprin promotes the accumulation of GFP::RAB-3 in the DA9 dendrite. In addition to the mislocalization defect, the average number of GFP::RAB-3 puncta in the dorsal axon of DA9 is reduced in *unc-5* mutants compared with wild-type animals (Fig. 1e–g and Supplementary Fig. 4).

Netrins are evolutionarily conserved axon guidance molecules present in worms[4], flies[5] and mammals[6]. The activity of these secreted molecules is transmitted through two distinct cell-surface receptors: UNC-5 repels axons[7] whereas UNC-40/DCC/Frazzled[8–10] attracts axons to a source of UNC-6/netrin. Similar to mammals, UNC-6/netrin in *C. elegans* is expressed in many classes of ventral cells and its expression persists into adulthood[4]. The UNC-5 receptor is expressed in DA motor neurons, as indicated by antibody staining, transgene expression[11] and microarray analysis[12]. We confirmed that UNC-5 is expressed in DA9 with a transgenic line expressing dsRed driven by the *unc-5* promoter (Supplementary Fig. 5a–c).

To distinguish if UNC-5 is required for localization of presynaptic components in DA9 itself or in other cells like the postsynaptic partners of DA9 (VD/DD neurons and dorsal body-wall muscles), we performed cell-autonomous rescue experiments with various promoters. The *mig-13* promoter is expressed only in DA9 within the tail region at the early larval L1 stage[13] and a *mig-13::unc-5* transgene robustly rescues the mislocalization defect in *unc-5* mutant L1 and adult animals, suggesting that UNC-5 acts cell-autonomously in DA9 to exclude presynaptic components from the dendrite. Furthermore, we did not observe any rescue when UNC-5 was expressed in the postsynaptic partners of DA9 using the *unc*-25 and *unc*-129m promoters[14,15] (Fig. 2a and Supplementary Fig. 6). To substantiate these observations, we created *unc-5* mutant animals expressing a rescuing *unc-5::unc-5* transgene together with a cytoplasmic DA9 marker in a mosaic pattern. In two independent transgenic lines, we observed a strong correlation between the expression of *unc-5* in DA9 and rescue of the mislocalization defect, consistent with a cell-autonomous function for UNC-5 in DA9 (Fig. 2b). Using an UNC-5::YFP fusion construct expressed specifically in DA9, we observed a higher level of UNC-5::YFP in the dendrite and ventral axon than in the dorsal axon (Supplementary Fig. 7).

The disrupted distribution of presynaptic components in *unc-5* and *unc-6/netrin* mutants is observable at the early L1 larval stage, when the DA9 dendrite begins to form, and persists throughout the life of the animal (Supplementary Figs 1 and 2a–f). To differentiate whether the mislocalization of presynaptic components is a consequence of an early axodendritic polarity or guidance defect or a later developmental defect, we used a modified version of a silencing intron cassette (M. Chalfie, personal communication) to regulate *unc-5* temporally. We observed that culturing *unc-5* mutant animals expressing the *unc-5::intron::unc-5* transgene at 25 °C throughout development resulted in a significant rescue of the mislocalization defect in *unc-5* mutants, whereas culturing them at 16 °C led to no rescue, suggesting that the transgene produces functional UNC-5 at 25 °C but not at 16 °C (Fig. 2d and Supplementary Fig. 8a). A shift to the restrictive temperature at the L4 larval stage, after DA9 and surrounding neurons are fully developed, results in a mislocalization defect in the transgenic mutant animals that is comparable to *unc-5* mutants. Conversely, a shift to the permissive temperature is insufficient to rescue the mislocalization defect, suggesting that the defect is irreversible. Therefore, compromising the activity of *unc-5* in mature DA9 neurons leads to a mislocalization defect, suggesting a novel function for UNC-5 in maintaining the polarized localization of GFP::RAB-3 independent of early polarization and guidance.

To elucidate further whether this novel function of UNC-5 can be separated from its previously known role in axon guidance, we examined axon guidance in the DD and VD neurons using the same temperature-shift experimental paradigm. We found that a shift to the restrictive temperature at the L4 larval stage did not cause further errors in axon guidance, suggesting that UNC-5 is only required during the early outgrowth phase to guide axons, and is not required later to maintain axon trajectory (Supplementary Fig. 8b).

The two distinct roles of UNC-5 in axon guidance and GFP::RAB-3 localization is further supported by the following observations. First, approximately half of *unc-5* or *unc-6/netrin* mutant animals have no detectable DA9 guidance defects, yet the mislocalization defect is still observed (Supplementary Figs 9 and 10a). Second, *unc-5* and *unc-6/netrin* mutant animals with defective DA9 guidance do not display more penetrant mislocalization defects (Supplementary Fig. 10a). Third, UNC-129/TGF-β, like UNC-5 and UNC-6/netrin, is important for the dorsal guidance of DA neurons[15], and we observed that approximately half of *unc-129/Tgf-β* mutant animals have severe DA9 guidance defects but none exhibit dendritic GFP::RAB-3 (Supplementary Fig. 10b, c).

What cell biological processes do UNC-5 and UNC-6/netrin affect in localizing presynaptic components? It is possible that they are important for the establishment and maintenance of dendritic fate. Hence, we examined the localization of four dendritically localized proteins: CAM-1/ROR[16], a receptor tyrosine kinase that localizes somatodendritically in hippocampal cultures[17]; UNC-9/innexin, a structural component of invertebrate gap junctions[18]; F35D2.3/fibrillin[16]; and DYS-1/dystrophin[16]. In wild-type animals, CAM-1, UNC-9 and DYS-1 localize to the DA9 dendrite, cell body and ventral axon, whereas F35D2.3/fibrillin localizes exclusively to the dendrite. These localization patterns are unaffected in *unc-5* and *unc-6/netrin* mutants, suggesting that many aspects of axodendritic polarization are maintained in these mutants (Supplementary Fig. 11). These results are consistent with the late temporal requirement of UNC-5 for proper localization of presynaptic components.

An alternative possibility is that UNC-5 and UNC-6/netrin regulate accumulation of presynaptic components in the dorsal axon, and that reduced axonal accumulation may indirectly cause the mislocalization defect. However, we did not observe any significant difference when we compared the average number of axonal GFP::RAB-3 puncta between *unc-5* mutants with and without dendritic GFP::RAB-3 (Supplementary Fig. 3). We conclude that this model is unlikely to be true.

To test directly whether UNC-6/netrin provides instructive information for the localization of presynaptic components, we generated a posterior to anterior gradient of UNC-6/netrin near DA9 using the *egl-20* promoter[19] where the posterior segment of the DA9 dorsal axon is exposed to an abnormally high level of UNC-6/netrin. This ectopic expression of UNC-6/netrin causes DA9 guidance defects in a small fraction of animals (12.2 ± 3.2%). When we examined animals with normal DA9 guidance, we observed that the *egl-20::unc-6/netrin* transgene dramatically displaces GFP::RAB-3 anteriorly, creating an enlarged asynaptic zone in the posterior segment of the DA9 dorsal axon compared with wild-type animals (Fig. 3a–d, h). As the transgene does not affect the dorsal axon length of DA9 (Supplementary Fig. 12), it is unlikely that altered axonal outgrowth of DA9 leads to the enlarged asynaptic zone. We further observed that *unc-5* mutants expressing the *egl-20::unc-6/netrin* transgene do not have an enlarged asynaptic domain (Fig. 3e, f). In addition, the enlarged asynaptic domain is partly restored in *unc-5* mutants expressing the *mig-13::unc-5* transgene, demonstrating that UNC-5 acts cell-autonomously in DA9 to mediate ectopic UNC-6/netrin-induced exclusion of presynaptic components (Fig. 3g, h).

The striking similarity between the displacement of presynaptic components induced by the *egl-20::unc-6/netrin* and *egl-20::lin-44/wnt* transgenes[3] suggests that UNC-6/netrin and LIN-44/Wnt can both exclude synapses. We observed that the asynaptic domains in *lin-44/wnt*(*n1792*);Ex[P*egl-20::unc-6/netrin*] or *lin-17/fz*(*n671*);Ex[P*egl-20:: unc-6/netrin*] mutants are significantly larger than those in *lin-44/wnt* or *lin-17/fz* mutants alone, suggesting that ectopically expressed UNC-6/netrin is sufficient to rescue the mislocalization defect in *lin-44/wnt* and *lin-17/fz* mutants (Fig. 4a–e). We further observed a significant reduction in penetrance of the mislocalization defects in *unc-5* and *unc-6/netrin* mutants expressing *lin-44/wnt* under the control of the *unc-6* promoter (Fig. 4f). If UNC-6/netrin and LIN-44/Wnt have similar functions, one might expect that *unc-6/netrin;lin-44/wnt* double mutants would have more severe mislocalization defects. However, these double mutants exhibit a fully penetrant guidance defect such that the DA9 axon turns posteriorly (data not shown), precluding analysis of presynaptic localization. Collectively, these results suggest that UNC-6/netrin and LIN-44/Wnt play parallel roles in specifying the discrete presynaptic domain of DA9 by excluding presynaptic components from inappropriate compartments (Fig. 4g). Ventrally secreted UNC-6/netrin excludes presynaptic components from the DA9 dendrite, whereas LIN-44/Wnt secreted by the tail hypodermal cells performs a similar function in the posterior segment of the DA9 dorsal axon.

Here we demonstrate a novel role for UNC-6/netrin in providing spatial information for the exclusion of presynaptic components throughout development. Interestingly, UNC-6/netrin was recently shown to promote presynaptic formation in the amphid interneuron AIY in *C. elegans*[20]. These opposing effects of UNC-6/netrin on pre-synaptic formation might be explained by the different receptors used: UNC-5 in DA9 and UNC-40/DCC in AIY. The roles of these receptors in synaptic polarization parallel their contrasting functions in axon guidance, with UNC-5 functioning in repulsion and UNC-40/DCC in attraction[7–10].

In addition to its well-characterized function in axon guidance, UNC-6/netrin was recently implicated in the initial polarization of the *C. elegans* hermaphrodite-specific neuron (HSN) neuronal cell body[21]. However, it is unclear whether UNC-6/netrin is required for later stages of neuronal polarity. Our findings suggest that UNC-6/netrin and UNC-5 activity coordinate two temporally distinct functions in DA9: axons are first guided to the appropriate locations, and presynaptic components are later localized in a polarized manner. Consistent with this hypothesis, netrin is expressed in the adult mammalian nervous system long after axon guidance is complete[22].

The conventional view of synapse formation is that contact between synaptic partners triggers assembly of the pre- and post-synaptic apparatus through the interaction of adhesion molecules like neurexin/neuroligin, SynCAM and EphrinB/EphB receptor[23] across the synaptic cleft. However, extracellular cues such as members of the Wnt[24,25], fibroblast growth factor[26] and bone morphogen protein[27] families can also promote synapse formation. Our studies in DA9 suggest that negative regulators also pattern synaptogenesis by inhibiting the accumulation of presynaptic components in inappropriate subcellular domains. The UNC-6/netrin gradient is high ventrally and low dorsally[4], encompassing the dendrite and ventral axon of DA9. The LIN-44/Wnt gradient is high posteriorly and low anteriorly[28], effectively reaching the ventral axon, commissure and posterior region of the DA9 dorsal axon. Signalling through independent receptors, both UNC-6/netrin and LIN-44/Wnt, excludes presynaptic components, setting negative constraints on presynaptic formation in DA9 and forcing synapses to form in a discrete domain within the DA9 dorsal axon (Fig. 4g). Thus, inhibitory factors play essential roles in patterning the subcellular distribution of synapses.

METHODS

Temperature shift experiments

Animals were either cultured at 16 °C or 25 °C for multiple generations before being shifted to a different temperature. Experimental animals in the L3 and early L4 larval stages were placed at 16 °C for three days or 25 °C for two days before the phenotype was analysed. Scoring was performed in gravid adults with normal DA9 guidance for all experiments. *unc-5;mec-8* double mutants were analysed for experiments in Fig. 2d. It was later discovered that the temperature-dependent regulation of Ex[P*unc-5::intron:: unc-5*] was independent of the *mec-8* mutation (Supplementary Fig. 8a).

Constructs and transgenic worms

wyIs109: a *Xma*I–*Nhe*I PCR fragment containing *cfp* was subcloned into P*ttx-3::rab-3* pSM obtained from P*ttx-3::mcherry::rab-3* (ref. 20) to make P*ttx-3::cfp::rab-3*. A *Sph*I–*Asc*I fragment containing P*mig-13* (ref. 3) was subcloned into *cfp::rab-3* pSM derived from P*ttx-3::cfp::rab-3* described above to make P*mig-13::cfp::rab-3*. A *Kpn*I–*Apa*I fragment containing mCherry obtained from P*mig-13::lin-17::mcherry*[3] was subcloned into P*mig-13::gateway* pSM from P*mig-13::gateway::yfp*[3] to make P*mig-13::gateway::mcherry*. The *sng-1* entry clone was obtained from the ORFeome project (http://worfdb.dfci.harvard.edu/) and cloned into the destination vector P*mig-13::gateway::mcherry* using the gateway strategy with LR clonase (Invitrogen) to make P*mig-13::sng-1::mcherry*. P*mig-13::cfp::rab-3*, P*mig-13::sng-1::mcherry* and P*mig-13::snb-1::yfp*[3] were injected with P*odr-1::gfp* at 20 ng μl^{-1} and integrated into chromosome V using trimethylpsoralen/ultraviolet mutagenesis. *cfp* primers: 5′-TCCCCCGGGATGAGTAAAGGAGAAGAACTTTTCAC and 3′-CTAGCTAGCTTTGTATAGTTCATCCATGCCATG.

wyEx2055: a *Nhe*I–*Kpn*I fragment containing the *syd-2* genomic sequence from P*gcy-8::mCherry::syd-2* was subcloned into P*itr-1 pB::gfp* pSM to make P*itr-1 pB::gfp::syd-2*; a *Fse*I–*Asc*I PCR fragment containing P*itr-1 pB* was sub-cloned into *mcherry::rab-3* pSM from P*glr-3::mcherry::rab-3*. P*itr-1 pB::gfp::syd-2* was injected at 0.5 ng μl^{-1} with P*itr-1 pB::mcherry::rab-3* at 10 ng μl^{-1} with P*odr-1::gfp* at 20 ng μl^{-1} into N2 animals. P*itr-1 pB* primers: 5′-GAAAGGGGCCGCCATCTATTCCAGAGTTCGTTCCCGAGC and 3′-CTTTCCGGCGCGCCCAATTCGTGTGCTTCCACCACCAC.

wyEx1902: a *Sph*I–*Asc*I PCR fragment containing P*itr-1 pB* was subcloned into *mcherry::gateway* from P*mig-13::mcherry::gateway*. P*itr-1 pB::mcherry::gateway* was injected at 5 ng μl^{-1} with P*odr-1::gfp* at 20 ng μl^{-1} into N2 animals. P*itr-1 pB* primers: 5′- (ref. 3) and 3′-GAAAAGGGCGCGCCCAATTCGTGTGCTTCCACCAC.

wyEx1311, *wyEx1485*: a *Kpn*I–*Apa*I fragment containing the *unc-5* 3′untranslated region (UTR) was subcloned into P*mig-13::snb-1* pSM and a *Asc*I–*Kpn*I PCR fragment containing the *unc-5* genomic sequence was subcloned into P*mig-13::unc-5* 3′UTR pSM. The P*mig-13::unc-5* plasmid was injected at 2 ng μl^{-1} with P*odr-1::gfp* at 20 ng μl^{-1} into *unc-5;wyIs85* mutants. The two arrays are separate lines obtained in independent injections. Higher levels of the plasmid were toxic and there would be no germline transmission, whereas lower levels did not rescue the defect. *unc-5* primers: 5′-GAAAGGGGCGCCGCCATGGACGAAATCACAATCACAACACAAC and 3′-GAAGGGGTACCAGTGGGGACACAATTTGTGGAAAAGCTG; *unc-5* 3′UTR primers: 5′-GAAAGGGGTACCGCTCAATTTTTTGCACAAACACAACTAG and 3′-GAAAGGGGGCCCCGGTCTTTCTGCATAGAAAATCGC.

wyEx1498: an *Asc*I–*Kpn*I PCR fragment containing the *unc-5* genomic sequence and a *Sph*I–*Asc*I PCR fragment containing the P*unc-5* were subcloned into *sl2 dsred* pSM[20] to make P*unc-5::unc-5::sl2::dsred*. This plasmid was injected at 10 ng μl^{-1} with P*odr-1::gfp* at 20 ng μl^{-1} into *unc-5;lin-18;wyIs85* mutants.

wyEx1228, *wyEx2418*, *wyEx2419*: a *Sph*I–*Asc*I PCR fragment containing P*unc-5* was subcloned into *unc-5::unc-5* 3′UTR from P*mig-13::unc-5::unc-5* 3′UTR. P*unc-5::unc-5* was injected at 10 ng μl^{-1} with P*odr-1::gfp* at 20 ng μl^{-1} into *unc-5;wyIs85* mutants (*wyEx1228*). P*unc-5::unc-5* was injected at 8 ng μl^{-1} with P*mig-13::mcherry::gateway*[3] at 8 ng μl^{-1} with P*odr-1::gfp* at 20 ng μl^{-1} into *unc-5;wyIs85* mutants. *wyEx2418* and *wyEx2419* were two arrays obtained that had animals where UNC-5 was absent in DA9. Most of the other lines obtained had no animals where UNC-5 was absent in DA9. P*unc-5* primers: 5′-GAAAGGGCATGCTGAGCTTTTCCAAACTAGAGAGCTTC and 3′-GAAAGGGGCGCGCCTACTGGAATAGAAATTATGATTAGTGACAAACTTG.

wyEx1277: an *Eco*RI–*Apa*I PCR fragment containing the *unc-5* 3′UTR was subcloned into P*mig-13::snb-1::yfp* pSM[3] to make P*mig-13::snb-1::yfp::unc-5* 3′UTR. An *Asc*I–*Kpn*I PCR fragment containing the *unc-5* genomic sequence (similar to *wyEx1311*) and a *Sph*I–*Asc*I PCR fragment containing P*itr-1 pB* (similar to *wyEx2055*) were subcloned into *unc-5* 3′UTR pSM derived from make P*mig-13::snb-1::yfp::unc-5* 3′UTR. P*itr-1 pB::unc-5::yfp* was injected at 80 ng μl^{-1} with P*odr-1::gfp* at 20 ng μl^{-1} into N2 animals. *unc-5* 3′UTR primers: 5′-GAAAGGGAATTCGCTCAATTTTTTGCACAAACACAACTAG and 3′-GAAAGGGGGCCCCGGTCTTTCTGCATAGAAAATCGC.

wyEx1904, *wyEx1916*: a *Sph*I–*Asc*I PCR fragment containing P*egl-20* was sub-cloned into *unc-6* pSM derived from P*unc-6::unc*-6 (ref. 20). The P*egl-20::unc-6* plasmid was injected at 20 ng μl^{-1} with P*ttx-3::cfp* at 50 ng μl^{-1} into N2 animals. The two arrays were separate lines obtained from one injection. P*egl-20* primers: 5′-GAAAGGGCATGCAAGTTTCCCTTTTATTTTTGAAGTCATCC and 3′-GAAAGGGGCGCGCCTATTTCTGAAATTGAGATGTTTTAGAATTTC.

wyEx2093, *wyEx2094*: an *Asc*I–*Kpn*I PCR fragment containing the *lin-44* complementary DNA was subcloned into P*unc-6* pSM derived from P*unc-6::unc-6::mcherry*[20]. The P*unc-6::lin-44* plasmid was injected at 20 ng μl^{-1} with P*odr-1::gfp* at 20 ng μl^{-1} into *unc-5;wyIs85* mutants. The two arrays were separate lines obtained in the same injection. *lin-44* primers: 5′-GAAAGGGGCGCGCCATGCGAGCAGCTCCTTTTGATTTC and 3′-GAAAGGGGTACCTTAAAAAATTAGGCTTTTCGGCGGTG.

wyEx2306, *wyEx2308*: an *Asc*I PCR fragment obtained from pAC13, a gift from M. Chalfie, containing *mec-2 intron9* was subcloned into P*unc-5::unc-5* (similar to *wyEx1494*). This plasmid was injected at 10 ng μl^{-1} with P*odr-1::gfp* at 20 ng μl^{-1} into *unc-5;mec-8;wyIs85* mutants. The two arrays were separate lines obtained from one injection. *unc-5;wyIs85;wyEx2308* mutant animals were obtained by crossing *unc-5;mec-8;wyIs85;wyEx2308* animals with N2 animals. *mec-2 intron9* primers: 5′-GAAAGGGGCGCGCCCACCGCCTAAAGTGTAAGTTTTC and 3′-GAAAGGGGCGCGCCGACGGTGGCTCCTCACTGAAAAC.

wyEx1054, *wyEx2396*, *wyEx2430*: the *unc-9*, *F35D2.3* and *dys-1* entry clones were obtained from the ORFeome project (http://worfdb.dfci.harvard.edu/) and cloned into the destination vector P*itr-1 pB::gateway::yfp*[3] using the gateway strategy with LR clonase (Invitrogen) to make P*itr-1 pB::unc-9::yfp*, P*itr-1 pB::F35D2.3::yfp* and P*itr-1 pB::dys-1::yfp*. These plasmids were then injected at 10 ng μl^{-1} or 40 ng μl^{-1} (for *dys-1*) with P*odr-1::dsRED* or P*odr-1::GFP* at 20 ng μl^{-1} into N2 worms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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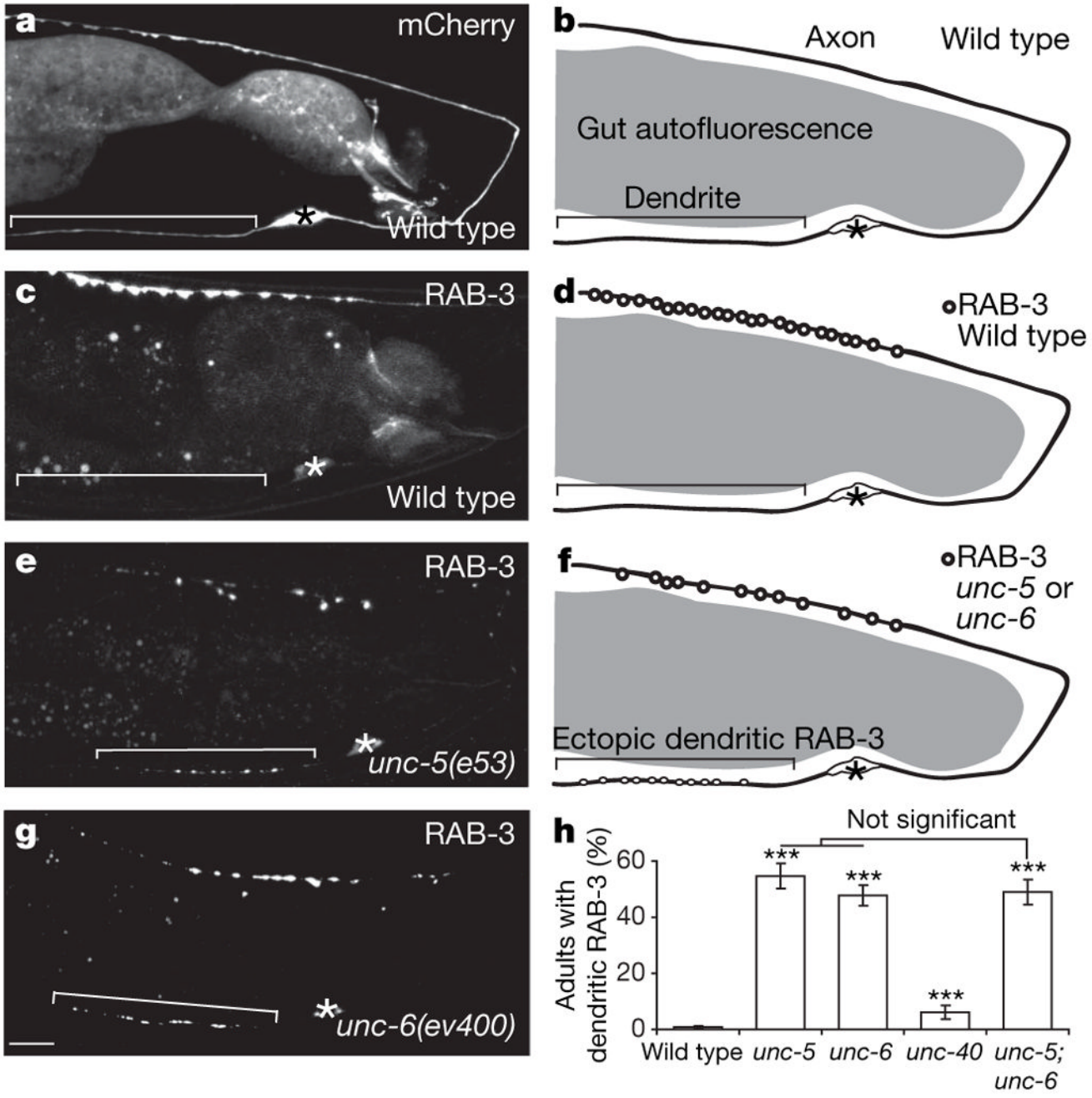

Figure 1. GFP::RAB-3 is mislocalized to the dendrite in *unc-5* and *unc-6/netrin* mutants

a–**d**, Micrographs and diagrams of representative wild-type adults expressing cytoplasmic mCherry (**a**, **b**) or GFP::RAB-3 (**c**, **d**). **e**–**g**, Micrographs and diagram of representative *unc-5* and *unc-6/netrin* mutant adults expressing GFP::RAB-3. Signal in the middle of the worm is gut autofluorescence. Anterior, left; dorsal, top; brackets, dendrites; asterisks, cell bodies. Scale bar, 10 μm. **h**, Penetrance of dendritic GFP::RAB-3 in adults with no DA9 guidance defects. Error bars, standard error of proportion; $n > 100$; ***$P < 0.0001$ (versus wild-type animals), χ^2 test.

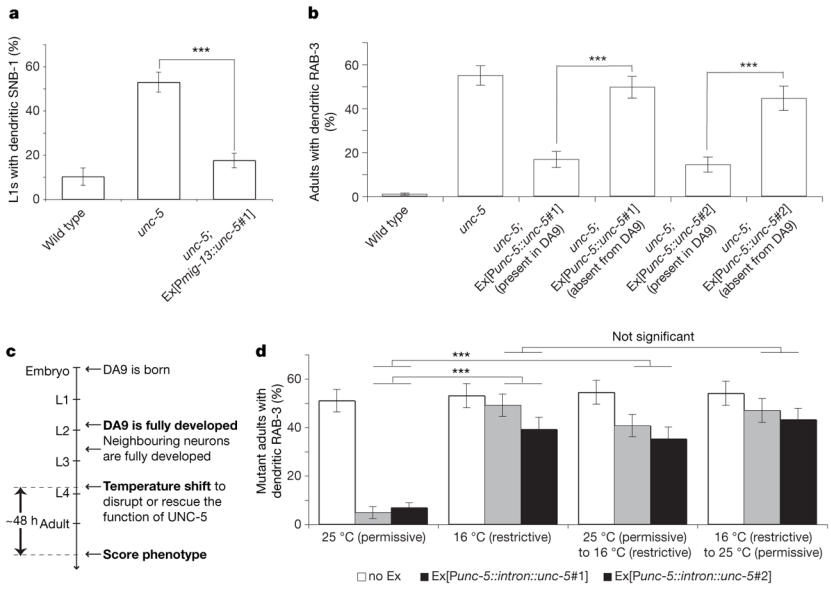

Figure 2. UNC-5 acts cell autonomously in DA9 and is required throughout development

a, Expression of UNC-5 in DA9 rescues the mislocalization defect in *unc-5* mutant L1 animals ($n > 100$). **b**, Expression of UNC-5 in other cells does not rescue the mislocalization defect ($n > 80$).

c, Experimental timeline. **d**, The *unc-5::intron::unc-5* transgene rescues the mislocalization defect of *unc-5;mec-8* mutant adults at 25 °C, not 16 °C. The mislocalization defect occurs when UNC-5 is inactivated early or late in development. Error bars, standard error of proportion; $n > 100$; ***$P < 0.0001$ (within each transgenic line), χ^2 test.

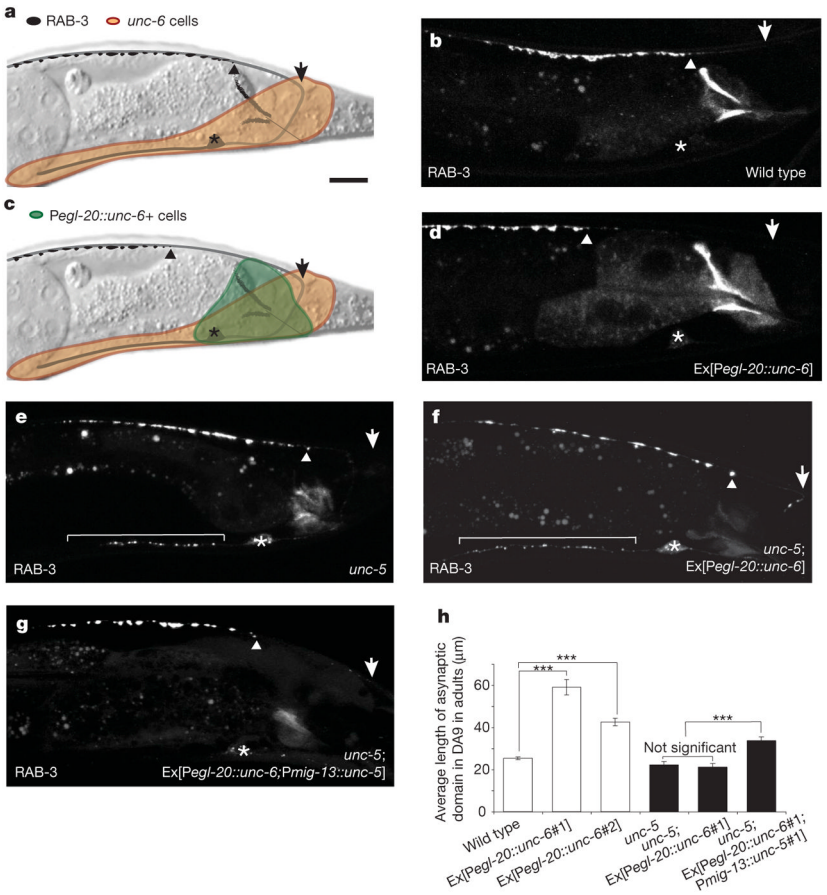

Figure 3. UNC-6/netrin is sufficient to exclude GFP::RAB-3 locally and acts through UNC-5

a, Endogenous UNC-6/netrin-expressing cells (beige). **c**, Ectopically expressed UNC-6/netrin (green). **b, d**, Representative wild-type L4 animal expressing GFP::RAB-3 in the absence (**b**) or presence (**d**) of ectopic UNC-6/netrin. **e**–**g**, Representative *unc-5* mutant adults expressing GFP::RAB-3 with (**f**) or without (**e**) ectopic UNC-6/netrin or, alternatively, ectopic UNC-6/netrin with the *mig-13::unc-5* transgene (**g**). Arrows, posterior end of dorsal axon; arrowheads, posterior border of presynaptic domain; brackets, dendrites; asterisks, cell bodies. Scale bar, 10 μm. **h**, Error bars, s.e.m. ($n > 50$); ***$P < 0.0001$, *t*-test.

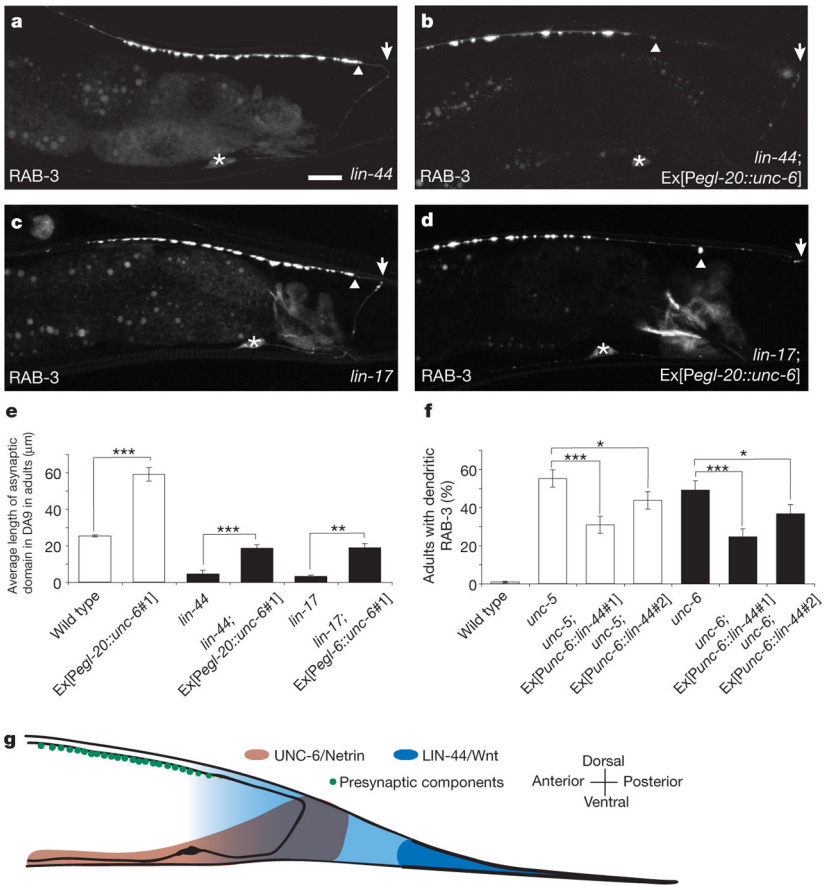

Figure 4. UNC-6/netrin and LIN-44/Wnt function interchangeably

a–**d**, Representative *lin-44/wnt* or *lin-17/fz* mutant adults expressing GFP::RAB-3 with (**b**, **d**) or without (**a**, **c**) ectopic UNC-6/netrin. Arrows, posterior end of dorsal axon; arrowheads, posterior border of presynaptic domain; brackets, dendrites; asterisks, cell bodies. Scale bar, 10 μm. **e**, Error bars, s.e.m. ($n > 50$). ***$P < 0.0001$, *t*-test. **f**, Error bars, standard error of proportion ($n > 100$); ***$P < 0.0001$; **$P < 0.005$; *$P < 0.05$; χ^2 test. **g**, Model for the roles of UNC-6/netrin and LIN-44/Wnt in subcellular patterning of presynaptic specializations in DA9.