Robo and Ror function in a common receptor complex to regulate Wnt-mediated neurite outgrowth in Caenorhabditis elegans

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Growing axons are exposed to various guidance cues en route to their targets, but the mechanisms that govern the response of growth cones to combinations of signals remain largely elusive. Here, we found that the sole Robo receptor, SAX-3, in \textit{Caenorhabditis elegans} functions as a coreceptor for Wnt/CWN-2 molecules. SAX-3 binds to Wnt/CWN-2 and facilitates the membrane recruitment of CWN-2. SAX-3 forms a complex with the Ror/CAM-1 receptor and its downstream effector Dsh/DSH-1, promoting signal transduction from Wnt to Dsh. sax-3 functions in Wnt-responsive cells and the SAX-3 receptor is restricted to the side of the cell from which the neurite is extended. DSH-1 has a similar asymmetric distribution, which is disrupted by sax-3 mutation. Taking these results together, we propose that Robo receptor can function as a Wnt coreceptor to regulate Wnt-mediated biological processes in vivo.

Wnts are secreted extracellular molecules that act through a number of distinct signaling pathways to regulate a variety of biological processes. Canonical Wnt signaling is activated by Wnt binding to the Frizzled receptor (Frz) and LDL-receptor–related protein families, which triggers nuclear translocation of β-catenin to regulate transcription of specific target genes (15). The kinase-like orphan receptor (Ror) proteins also serve as Wnt receptors signaling via novel noncanonical Wnt pathways and have been implicated in neuronal migration, neurite outgrowth, axon guidance, axonal pruning, axonal branching, and synapse formation (16–22).

Here, we found that instead of mediating Slit signaling, the Robo receptor binds and responds to Wnt to promote directional neurite outgrowth in \textit{C. elegans}. By forming a complex with the Ror receptor and Dsh effector, Robo promotes signal transduction from Wnt to Dsh. Taken together, our results reveal a mechanistic link between Robo receptor and Wnt-Ror-Dsh signaling.

Results

\textbf{Robo/SAX-3 Receptor Associates with Ror/CAM-1 Receptor}. The RME neurons are a set of four GABAergic motor neurons that innervate head muscles and regulate foraging movements in \textit{C. elegans} (23). Among them, RMEL and RMER only send out normal, suggesting that the Robo receptor may also respond to other cues.

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\textbf{Significance}

How a limited repertoire of secreted molecules pattern a large number of axon trajectories is an enduring mystery. Both Slit-Robo and Wnt-Ror pathways are involved in proper neurite extension, but whether and how these two signaling pathways are interacted is largely unknown. Here, we found that instead of mediating Slit signaling, Robo could bind and respond to Wnt ligand. By forming a complex with the Ror receptor and Dsh effector, the Robo receptor promotes signal transduction from Wnt to Dsh. The mechanistic link between the Robo receptor and Wnt-Ror-Dsh signaling revealed here will markedly advance our understanding of how guidance molecules interact in space and time to orchestrate the dynamic process of growth cone navigation through a complex cellular environment.

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The authors declare no conflict of interest.

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processes to the nerve ring region. In contrast, RMD and RMEV each send out an extra process, which runs down the dorsal and ventral cords, respectively, and then terminates around the middle of the body (Fig. 1A) (24). Using a GFP transgene driven by the GABAergic neuron-specific promoter Punc-25, we were able to visualize the morphology of RME neurons in unc-30 mutant animals (Fig. S1 A and B). unc-30 encodes a homeodomain protein that controls the specification of type D GABA neurons. In the absence of UNC-30, unc-25 expression in type D neurons is abolished; however, its expression remains unchanged in RME neurons. Therefore, using the Punc-25::GFP marker in the unc-30 mutant background, we were able to specifically follow the development of RME neurons in living animals at the single-cell level (Fig. 1B).

We previously reported that the Ror/CAM-1 receptor acts on RMD/V cells and transmits the CWN-2 signal, probably through a direct association, to Dsh/DSH-1 to regulate the posterior neurite outgrowth similar to RMED/V neurites. Arrow marks the vulva region. (Scale bar, 50 μm.)

**SAX-3 Functions with CAM-1 Receptor to Regulate Neurite Outgrowth.** Next, we tested whether sax-3 plays a role in RMD/V neurite outgrowth similar to cam-1. The sax-3(ky203) allele deletes the first exon of sax-3, which results in an ORF shift that covers the whole sax-3 gene and is likely a molecular null. sax-3(ky203) contains a nonsense mutation immediately after the transmembrane domain. In sax-3(ky123) and sax-3(ky203) mutant animals, we found that the neurite length of both RMD and RMEV is significantly reduced (Fig. 1 F–H). The defect in sax-3(ky203)...

![Image of sax-3 and cam-1 interactions](image_url)
mutants is almost as severe as in sax-3(ky123) mutants (Fig. 1 F and G), suggesting that sax-3(ky203) probably acts as a null or strong loss-of-function allele in neurite outgrowth. The temperature-sensitive allele sax-3(ky200) shows a weak neurite outgrowth defect at the restrictive temperature (25 °C), which is marginally alleviated when animals are cultivated at a more permissive temperature (22 °C) (Fig. 1 F and G). Thus, sax-3 is indeed involved in RME/D neurite outgrowth.

The association between SAX-3 and the cytoplasmic domain of CAM-1 hinted that Robo/SAX-3 may function with the CAM-1 receptor. If this is the case, we would expect that the loss of SAX-3 function would worsen the short neurite defect of cam-1 mutants, particularly those that lack the intracellular domain of CAM-1. Hence, we created double mutants between sax-3(ky203) and cam-1(ks52). In these double-mutant animals, the posterior neurite extension of RMD/V is completely blocked, suggesting a complete signaling transduction failure from CWN-2 to DSH-1 (Fig. 1 F and S2B). We further created cam-1(ks52);sax-3(ky200) doubles and found that the outgrowth of the posterior neurites of RMD/V neurites is also completely blocked (Fig. 1 I and S2B). The observation that double mutants carrying an intracellular null of cam-1 with a sax-3 null resemble single mutants with full loss-of-function of cam-1 raises the possibility that Robo/SAX-3 receptor cooperates with CAM-1 to transduce the Wnt signal to the cytosol. Our attempt to make double mutants of the cam-1(ks52) null with wild-type sax-3(ky203) was unsuccessful, suggesting a strong lethality. Indeed, when we constructed cam-1(ks52);sax-3(ky203) double animals with the wild-type sax-3 gene expressed extrachromosomally, we found that their progeny were arrested at the early larval stage. Given the fact that none of those dead larvae carried the wild-type sax-3 gene, we believe that they were cam-1(ks52);sax-3(ky203) doubles. The strong lethality of cam-1(null);sax-3(null) suggests that Ror/CAM-1 and Robo/SAX-3 may function together in other developmental processes as well (Fig. S2 C and D) (25).

The classic Frz receptors MIG-1 and CFZ-2 are also involved in RMD/V neurite outgrowth (17). Compared with cam-1, null mutants of cfz-2 or mig-1 only display mild neurite outgrowth defects (Fig. 1 I and S2B). In addition, mig-1;cfz-2 double mutants exhibit a more severe phenotype resembling cam-1 null (17), which suggests that CFZ-2 and MIG-1 function redundantly. When both SAS-3 and CFZ-2 receptors are removed, we found that the RMD/V neurite length is much shorter than in either sax-3– or cfz-2–null mutants (Fig. 1 I and S2B), suggesting a redundant interaction between these two genes. The neurite length in sax-3(ky203);mig-1(e1787) animals is similar to that in sax-3(ky203) single mutants (Fig. 1 I and S2B), suggesting that mig-1 may function in the same pathway as sax-3 (Fig. 1 I).

In addition to the shorter posterior neurite, ectopic anterior neurites are observed in sax-3 mutant animals (Fig. 1 H, white arrows). Additional phenotypic analysis showed that those ectopic anterior neurites are mainly derived from RME and RMER neurones and their growth is likely independent of Wnt-Ror-Dsh signaling (Fig. S2 E and F).

Sax-3 Acts Cell-Autonomously. A GFP construct driven by a 4-kb sax-3 promoter revealed a wide sax-3 expression pattern in the nervous system (Fig. 2 A and Fig. S3 A and B). The protein-coding region of sax-3 driven by this promoter fully restored the neurite length to wild-type level, suggesting that the expression pattern revealed by this promoter represents the endogenous sax-3 distribution (Fig. 2 B and C). To identify whether sax-3 is expressed in RMD/V neurones, we specifically highlighted the RME neurones with Punc-25:mCherry in the unc-30 mutant background and found that Psaax-3::GFP is present in both RMD and RMEV neurones (Fig. S3 B). Furthermore, we introduced nuclear localization sequence (NLS)-tagged GFP driven by sax-3 into worms. As shown in Fig. 2 A, double labeling with Punc-25:mCherry in unc-30 mutants revealed that the sax-3 gene is indeed expressed in both RMD and RMEV neurones. We then tested whether sax-3 functions in Wnt/CWN-2–responsive cells (RMD/V). When a wild-type copy of sax-3 was expressed in the whole nervous system using the pan-neuronal promoter Psnb-1, we found that the short posterior neurite outgrowth defects were efficiently rescued (Fig. 3 B and C). cam-1 is expressed in a subset of neurons including RMD/V (17). The significant rescuing activity of a sax-3 construct driven by the Pcam-1 promoter indicated that sax-3 can function in cam-1–expressing cells (Fig. 2 B and C). In unc-30 mutants, the Punc-25 promoter drives gene expression, specifically in RME neurones. With this approach, we examined whether expressing sax-3 only in RME cells was able to restore the RMD/V neurite length. Indeed, we found that the shortened RMD/V neurite phenotype was significantly rescued (Fig. 2 B and C). unc-47 encodes the GABA transporter in C. elegans. In the absence of the UNC-30 homeodomain protein, unc-47 gene expression is also restricted in RME neurones in the nerve ring region. When sax-3 was expressed in unc-30 mutant worms using the Punc-25 promoter, significant rescue activity was observed (Fig. 2 B and C). Thus, sax-3 can function cell-autonomously within RME cells to regulate neurite outgrowth. We additionally created a tissue-specific knockout of sax-3 in RME cells using the Punc-25 promoter to drive the CRISPR/Cas9 system (Fig. S3 C and D). We found that the RME neurite length is noticeably reduced in the RME-specific sax-3 knockout (Fig. S3 F). All together, the RMD/V neurite length is indistinguishable from wild-type, we did notice that some of the knockout animals display shorter RMD neurites (Fig. S3 F), RME cells are born earlier than the onset of unc-25 gene expression and the tissue-specific knockout efficiency with the current system is rather limited (26). The relatively weak (compared with sax-3 mutant animals) neurite extension defect in those RME-specific sax-3 knockout animals may due to the above limitations. In contrast, expression of the sax-3 gene driven by the Punc-86 promoter in neurons other than RME in the nerve ring did not rescue the short RMD/V neurite defect caused by sax-3 mutation (Fig. 2 B and C). The Pphil-17 promoter drives gene expression in glia-like sheath cells surrounding the nerve ring area. When we introduced sax-3 gene expression into the sheath cells using the Pphil-17 promoter, no rescue activity was observed (Fig. 2 B and C), suggesting that sax-3 probably does not function on close-by neurons or tissues to regulate RMD/V neurite growth. Together, these results provide evidence that sax-3 functions cell-autonomously within RME neurones to regulate neurite growth.

Slt1/SLT-1 Is Not Involved in RMD/V Neurite Outgrowth. The classic ligands for Robo receptors are Slit proteins. We wondered whether Robo/SAX-3 responds to Slit to regulate RMD/V neurite growth. C. elegans has a single Slit ligand, SLT-1. In null slt-1(eh15) animals, no obvious neurite outgrowth defect was detected (Fig. 2 D–F). The sltg-1 gene encodes the sole slit-GAP (Slit-Robo-GTPase activating protein), which is implicated in the Slit-Robo pathway (27–29). In animals carrying the loss-of-function allele sltg-1(ok300), the RMD/V neurite length is indistinguishable from wild-type (Fig. 2 D–F). Hence, the typical Slit-Robo pathway appears not play a significant role in RMD/V neurite outgrowth.

Slit may function redundantly with the Wnt-Ror-Dsh pathway, in which case slt-1 single mutants cannot reveal the functional requirement for Slit in RMD/V outgrowth. Elimination of the Wnt-Ror-Dsh pathway leads to complete blockade of RMD/V neurite growth. Thus, it is not feasible to reveal the redundant role of slt-1 by making double mutants between slt-1 and any of the cwn-2, cam-1, or dsh-1 nulls. Nevertheless, we created the slt-1(e15);cam-1(122) double mutant and found that its phenotype resembles the null cam-1(122) (Fig. 2D). In contrast, the cam-1(ks52) mutation, which affects the intracellular domain of CAM-1, causes a partial outgrowth defect. Hence, we further created the slt-1(e15);cam-1(ks52) double mutant and found that
the neurite outgrowth defect is comparable to the *cam-1(ks52)* single mutant (Fig. 2 D–F). This suggests that the Slit ligand is not acting redundantly with the Wnt-Ror-Dsh pathway to regulate neurite outgrowth.

**SAX-3 Binds to Wnt/CWN-2.** The noninvolvement of Slit suggests that the Robo/SAX-3 receptor may sense other ligands to mediate neurite outgrowth. Given that SAX-3 associates with CAM-1 and CAM-1 functions as a CWN-2 receptor during RMED/V neurite extension, we suspected that SAX-3 may respond to the Wnt/CWN-2 ligand instead. To test this possibility, we constructed C-terminal Myc-tagged CWN-2 (CWN-2–Myc) to examine whether SAX-3 can bind to CWN-2. When introduced into *cwn-2*(tsd1) mutant worms, this CWN-2–Myc-expressing construct significantly rescued the short neurite defect (Fig. S4 A and B), which suggests that the Myc tag does not affect CWN-2 function. We then coexpressed both CWN-2–Myc and SAX-3–Flag in HEK293T cells and performed co-IP tests. Considering that the SAX-3 receptor contains a transmembrane domain, we used Myri-GFP, a plasma membrane reporter, and the integral membrane protein connexin43 (CX43) as negative controls to eliminate the possibility that the membrane-anchoring feature of SAX-3 may be responsible for CWN-2 association. As shown in Fig. 3, CWN-2–Myc was specifically communoprecipitated with SAX-3–Flag but not with Myri-GFP or CX43. We further tested whether SAX-3 can directly bind to CWN-2 using GST pull-down assays. After affinity purification, the CWN-2–GST and SAX-3–Flag proteins were incubated together and the pull-down experiments were performed. We found that CWN-2–GST, but not GST alone, pulled down SAX-3–Flag (Fig. 3B).

We further divided SAX-3 into the ectodomain (ECD) and ICD and found that only full-length SAX-3 and the ECD bound with CAM-1 receptor? CAM-1 has been implicated in Wnt binding previously (30). Indeed, Flag-tagged CAM-1 receptor associated with functional CWN-2–Myc in a co-IP assay (Fig. 4A). When a similar amount of SAX-3 or CAM-1 protein was used to pull down CWN-2, we noticed that more CWN-2 protein was precipitated by CAM-1 (Fig. 4A and Fig. S4C), suggesting that CAM-1 may possess a higher binding affinity than SAX-3 for CWN-2 molecules. In the presence of both CAM-1 and SAX-3, we tested the CWN-2–Flag protein binding activity of individual fibronectin repeats and found that only full-length SAX-3 and the ECD bound with CAM-1 receptor? CAM-1 has been implicated in Wnt binding previously (30). Indeed, Flag-tagged CAM-1 receptor associated with functional CWN-2–Myc in a co-IP assay (Fig. 4A). When a similar amount of SAX-3 or CAM-1 protein was used to pull down CWN-2, we noticed that more CWN-2 protein was precipitated by CAM-1 (Fig. 4A and Fig. S4C), suggesting that CAM-1 may possess a higher binding affinity than SAX-3 for CWN-2 molecules. In the presence of both CAM-1 and SAX-3,
we found that the amount of precipitated CWN-2 protein was comparable to that observed with CAM-1 alone (Fig. 4A).

To test whether Wnt/CWN-2 molecules can be recruited by Robo/SAX-3 or Ror/CAM-1 receptors that are expressed on cell membranes, we first created a CWN-2::GFP-expressing construct. When introduced into worms, this CWN-2::GFP construct rescued the RMED/V neurite outgrowth defect in cwn-2 mutants (Fig. S4A and B), suggesting that the CWN-2::GFP protein is probably functional. When CWN-2::GFP protein was incubated with Drosophila S2 cells expressing freely diffusing mCherry, no GFP signal could be detected on the cell surface (Fig. 4B). In contrast, when either SAX-3::mCherry or CAM-1::mCherry was expressed in S2 cells, the CWN-2::GFP signal accumulated on the surface of the cells (Fig. 4B and C), suggesting that CWN-2 molecules could indeed be recruited to the cell surface by Robo/SAX-3 or Ror/CAM-1 receptor. When we transfected the S2 cells with an equal amount of sax-3::mCherry and cam-1::mCherry constructs, the cell surface CWN-2::GFP signal was increased compared with cam-1::mCherry transfection alone (Fig. 4B and C). Because SAX-3 binds to CAM-1, it is a reasonable assumption that by forming a receptor complex with CAM-1, SAX-3 may stabilize the association of CWN-2 with the cell membrane to facilitate CWN-2-mediated signal transduction.

**The Role of the Extracellular Domain of SAX-3 in Neurite Outgrowth.**

Next, we examined whether the association of CWN-2 with the extracellular domain of SAX-3 plays a role in neurite outgrowth in vivo. First, given the ability of SAX-3 to bind to CWN-2, we suspected that the extracellular domain of SAX-3, when over-expressed, may be able to sequester CWN-2 ligands away from CAM-1 and would therefore have a dominant-negative effect on CWN-2 signal transduction. In agreement with this prediction, when we expressed a SAX-3 protein composed only of the extracellular domain on RME cells, we found that the RMED/V neurites were shortened (Fig. S4D).

Second, the Ig1 and fibronectin domains of SAX-3 possess the CWN-2-binding activity, so we asked whether these domains are important for SAX-3-mediated neurite outgrowth. We created a series of constructs expressing various truncated SAX-3 proteins. In both sax-3(ky123) and sax-3(ky203) animals, we found that SAX-3 lacking either the Ig1 or fibronectin domain displayed rescue activity similar to full-length SAX-3 (Fig. 4D and E and Fig. S4E and F). In contrast, simultaneous deletion of both the Ig1 and fibronectin domains obviously decreased the rescue activity of SAX-3 (Fig. 4D and E and Fig. S4E and F). We noticed that a significant proportion of rescue activity remained even when both the Ig1 and fibronectin domains were removed (Fig. 4D and E and Fig. S4E and F), suggesting that the residual intracellular domain of SAX-3 also plays an important role in neurite outgrowth. To confirm the nonessential role of the Ig2,-3, and -4 domains, we used the CRISPR-Cas9 system to create the sax-3(ad392) allele, which has an in-frame deletion of the Ig2, Ig3, and Ig4 domains, while leaving the Ig1 and fibronectin domains intact. Compared with other sax-3 alleles, for example x1394, which contains a frame-shift mutation after Ig1, the sax-3(ad392) allele displays almost no outgrowth defects (Fig. S4G and H). Taken together, these results provide evidence that the
extracellular domain of SAX-3 receptor can sense CWN-2 during RMED/V neurite outgrowth.

**SAX-3 Binds to DSH-1.** Our previous study showed that the CAM-1 ICD binds to DSH-1 in yeast two-hybrid assays (17). Additional co-IP assays confirmed that CAM-1 ICD indeed binds to DSH-1 (Fig. S5A). Interestingly, when we incubated CAM-1 ICD and DSH-1 together with SAX-3 ICD, we found that the amount of DSH-1 protein associated with CAM-1 ICD was significantly increased (Fig. 5A and B). It has been proposed that the recruitment of Dsh protein to membrane receptors may trigger Dsh phosphorylation in mammals (20, 31). When DSH-1 was incubated with CAM-1 ICD and SAX-3 ICD, two DSH-1 protein bands with different sizes were observed (Fig. S5B). Treatment with calf intestinal alkaline phosphatase (CIAP) significantly decreased the level of the larger DSH-1 band (Fig. S5B). In addition, when we probed the DSH-1 protein with an antibody against phosphorylated Tyrosine (anti-pTyr), the anti-pTyr* band disappeared after CIAP treatment (Fig. S5B). Interestingly, when SAX-3 ICD was present, the level of phosphorylated DSH-1 associated with CAM-1 was significantly increased, implying that the DSH-1 signal can be enhanced by the SAX-3 receptor (Fig. S5 C and D).

How does SAX-3 help DSH-1 to associate with CAM-1? We expressed SAX-3 ICD and DSH-1 in HEK293T cells and found that SAX-3 ICD is associated with DSH-1 (Fig. 5C). The SAX-3 intracellular region contains three conserved cytosolic (CC) domains, which are CC1, CC3, and CC2 in sequence (4) (Fig. 5D). To identify the specific region involved in DSH-1 binding, we further performed structure-function analysis using various truncated forms of the SAX-3 protein. As shown in Fig. 5D, the membrane proximity region and the CC domains associated with DSH-1. Among the three CC domains, the CC2 domain appears to contain the strongest DSH-1 binding activity. Thus, SAX-3 may convey DSH-1 to CAM-1 through its association with DSH-1 via multiple binding sites.

**SAX-3 Forms a Complex with CAM-1 and DSH-1.** In addition to DSH-1, SAX-3 ICD also associates with CAM-1. Additional co-IP experiments with a series of truncated SAX-3 fragments suggested that both the membrane proximity region and the N-terminal portion containing the CC2 domain were able to bind to CAM-1 ICD (Fig. S5E). Conversely, in CAM-1, the kinase domain and the region close to the transmembrane motif were able to bind to SAX-3 (Fig. S5F).

Based on the above data, we wondered whether SAX-3, CAM-1, and DSH-1 function in the same complex. To test this theory, we performed a two-step co-IP. We first pulled down Flag-tagged CAM-1 ICD using anti-Flag antibody, then detected the presence of DSH-1 and SAX-3 ICD. Next, we immunoprecipitated the Flag-tagged sample with anti-His antibody to pull down the Myc-His double-tagged SAX-3 ICD fragment and analyzed the pull-down sample with anti-Myc and anti-Flag antibodies. As shown in Fig. 5E, both CAM-1 ICD and DSH-1 were present in the SAX-3 ICD-containing sample, suggesting that the CAM-1, SAX-3, and DSH-1 proteins form a complex. Consistent with this notion, we found that sax-3+/+; cam-1+/+; dsh-1+/+ triple heterozygote animals displayed evidently shortened neurites (Fig. 5F). In contrast, neither cam-1+/+; dsh-1+/+ double heterozygotes nor any of the sax-3+/+, cam-1+/+, dsh-1+/+ single heterozygotes showed any distinctive neurite outgrowth defect (Fig. 5F and Fig. S5G).

Furthermore, although SAX-3 without the membrane proximity region, the CC1–CC3 domains, or the CC2 domain possessed some rescue activity, SAX-3 lacking the intracellular domain almost completely lost its rescue capability (Fig. 5G and Fig. S5H), implying that the intracellular scaffolding activity is critical for SAX-3 function.

**SAX-3 Regulates the Asymmetric Distribution of DSH-1.** We further tested the function of SAX-3 in a cellular context in vivo. First, we examined the subcellular localization of the SAX-3 protein in developing RMED/V neurons. To do that, we created a functional
SAX-3::GFP construct driven by its endogenous promoter, which when introduced into sax-3(ky203) worms efficiently rescued the shortened neurite phenotype in sax-3(ky203) (Fig. 2 B and C). sax-3 expression begins around the midembryonic stage (350 min) and is restricted to the anterior region of an embryo (Fig. S6, Upper), where the neurons or neuroblast cells are clustered. As shown in Fig. S6, SAX-3::GFP is distributed on the membrane surfaces of those cells. When embryos progressed to the late-embryonic stage (500 min), we detected SAX-3::GFP on RME cells (Fig. S6, Lower, arrow). However, sax-3 is widely expressed in many neurons and the distinctive subcellular localization of SAX-3 on RMD/V neurons, if there is any, could easily be masked by the SAX-3::GFP signal from surrounding cells. Therefore, we generated SAX-3::GFP driven by the unc-25 promoter. By coexpressing Punc-25::mCherry in unc-30 to specifically visualize RME cells, we were able to detect the subcellular localization of SAX-3 protein on RME neurons. The posterior RMD/V neurites continuously extend during larval stages (17) and we found that SAX-3::GFP is distributed on the elongating neurites (Fig. 6 A and B, arrows). The SAX-3::GFP expression sometimes induces ectopic neurite growth and the SAX-3::GFP signal is localized on those ectopic neurites as well (Fig. 6B, arrowheads). Intriguingly, SAX-3::GFP is asymmetrically distributed on the posterior sides of RMD and RMEV cell bodies, from which the neurites are elongating (Fig. 6A and B).

Coincidentally, the functional DSH-1::GFP is also asymmetrically distributed on the growing neurites and the posterior side of RMD/V cell bodies (Fig. 6 C and E). When we removed sax-3 by sax-3(ky203) mutation, we found that the asymmetric distribution of the DSH-1::GFP on RMD/V neurites is disrupted (Fig. 6 D and E). In addition, the expression level of DSH-1::GFP on the extending neurites is somewhat decreased (Fig. S6 C–E). Taken together, these results provide evidence that sax-3 may promote directional neurite outgrowth by enhancing the asymmetric distribution of DSH-1 protein in vivo.

CWN-2 and CAM-1 Do Not Exert Their Regulatory Effect on Neurite Outgrowth by Patterning the Asymmetric Localization of DSH-1. We noticed that the overexpression of dsh-1 in RME cells induces ectopic neurites from both RMD and RMEV neurons (Fig. S6B, arrowheads), implying a correlation between dsh-1 expression level and neurite outgrowth capacity. When cam-1 or cwn-2 was removed, neurites were unable to extend, regardless of whether they were ectopic ones or regular posterior neurites from RMD/V cells. This suggests that the outgrowth activity executed by DSH-1 protein is dependent on upstream CWN-2 cues and the signaling receptor CAM-1. In cwn-2(ok895)-null animals, the asymmetric distribution of DSH-1 on RMD/V cells is not altered (Fig. 6F and Fig. S6F). In cam-1(ka52) animals, the intracellular domain of CAM-1 is largely removed, and the asymmetric distribution of DSH-1 is also unaffected (Fig. 6G and Fig. S6F). Furthermore, when we examined the SAX-3 localization in cwn-2(ok895) mutants, we found that the posteriorly enriched SAX-3::GFP pattern is indistinguishable from wild-type (Fig. S6G). Given the fact that RMD/V neurite outgrowth is greatly affected by the CWN-2 ligand and CAM-1 receptor, we would like to propose that the Wnt/CWN-2 ligand and Robo/CAM-1 receptor probably regulate neurite outgrowth by activating DSH-1. Meanwhile, Robo/SAX-3 receptor functions as a coreceptor with Robo/CAM-1 for Wnt/CWN-2 and patterns the specific subcellular localization of DSH-1 protein to facilitate directional neurite outgrowth in vivo.
Discussion

Genetics and biochemistry have identified the key molecules involved in axon guidance and provided important insights into what these molecules do. Here, we have identified a new mechanistic link between Robo receptor and the Wnt-Ror-Dsh pathway, which is certain to advance our understanding of how these molecules interact in space and time to orchestrate the dynamic process of growth cone navigation through a complex biological environment.

In agreement with our findings, evidence from multiple model systems has revealed that Robo receptors have Slit-independent functions. For example, Slits are not the only midline repellents in the mouse central nerve system, and many axons still cross normally in Slit triple-knockout embryos (32, 33). More recently, it was shown that mammalian Robo3 receptor does not bind to Slits. Robo3 also interacts with the netrin–DCC chemoattractive signaling pathway (10). In addition, NELL2, which is expressed by motoneurons in the mouse ventral spinal cord, acts as a repulsive ligand for Robo3 (7). Furthermore, while the Robo/SAX-3 receptor has a profound effect on nerve ring organization in C. elegans, the sole Slit plays a rather minor role in this process (14). sax-3 mutants have midline crossing defects in the ventral nerve cord; however, corresponding defects are not found in slit-1 mutants (34).

Previous studies hinted that Robo may function in Wnt-mediated processes. For example, Slit2-Robo1 cooperates with R-spondin1 to activate Wnt signaling and promote intestinal repair (35). Robo/SAX-3 is involved in the anterior–posterior cell migration of the C. elegans CAN neuron (34), which is strongly influenced by Wnt ligands and Ror receptor (16, 36). Both Slit/Slit-1 and Wnt/CWN-2 are expressed in the anterior region of the worm (17, 34), but only CWN-2 has a strong impact on the organization of the nerve ring bundle (14). Interestingly, both Robo/SAX-3 and Ror/CAM-1 function in SIA and SIB neurons to facilitate nerve ring placement (14), implying that Robo functions in the same cell as the Ror receptor. Here, we provide direct evidence that Robo/SAX-3 receptor binds to Wnt/CWN-2 ligand and may function in a receptor complex with Ror/CAM-1 receptor. Our findings offer a plausible explanation for how a Robo receptor participates in Wnt-mediated biological processes. The enhanced but not additive effect between CAM-1 and SAX-3 on the membrane association of CWN-2 hints that CAM-1 and Robo likely function in a common receptor complex and probably bind to the same CWN-2 molecule. The presence of SAX-3 receptor may therefore improve CWN-2 recognition, thus facilitating CWN-2 signal transduction. Taking all of the genetic and biochemical analyses together, a conceivable model is that Ror/CAM-1 acts as a major receptor to sense and respond to CWN-2 signal, while the SAX-3 receptor plays a more regulatory role in this process, possibly stabilizing the association of CWN-2 with membrane receptors.

The role of Frz receptor in RMED/V neurite outgrowth is intriguing and inspires us to think about how Wnt signaling can be regulated in a complex manner at the receptor level. The C. elegans genome encodes four Frz receptors. Among them, MIG-1 and CFZ-2 play redundant roles in Wnt signaling. Single mutations in either mig-1 or cfz-2 result in mild or variable neurite outgrowth defects (17). When both MIG-1 and CFZ-2 are removed, the neurite outgrowth defect is more severe, mimicking that observed in cam-1, cwn-2, or dsh-1 nulls (17). These data suggest that Frz receptors are also vital for Wnt signaling, but in a redundant manner. Interestingly, when both the SAX-3 receptor and CFZ-2 are absent, Wnt signaling is also reduced to a minimal level and the RMED/V neurites do not grow. Thus, SAX-3 can also function with Frz receptors to regulate Wnt signaling. The complex genetic interactions revealed by this and previous studies (17) suggest that different receptors, including Frz, Ror, and Robo, may form multiple receptor complexes by combining in various ways to respond to Wnt cues. In double mutants between a null allele of sax-3 and an allele of cam-1, in which the intracellular domain is disrupted, the phenotype
resembles the scenario in which CWN-2 signaling is completely blocked. Therefore, it is reasonable to propose that Robo receptor can assist both Rob and Frz receptors to transmit Wnt signals.

Robo receptors are single-pass transmembrane proteins with no autocatalytic or enzymatic activity in their intracellular region, with no intrinsic molecular features. They have an extracellular domain containing Robo domain. This domain is involved in binding to the Wnt ligands. The intracellular domain contains several conserved motifs including the PDZ binding motif. These motifs are involved in binding to various adapter proteins and scaffolding molecules to mediate their functions. Indeed, Robo receptors have been implicated in associations with Frizzled/ DCC/UNC-40 (3), Ephrin receptor (37), EVE-1 (38), and others (39, 40). What then is the biological significance of the Robo–Robo receptor complex in Wnt signaling? The directional outgrowth of RMED/V neurites require Robo/MIG-2 and Rac/CED-10, suggesting that the planar cell polarity (PCP) pathway may be activated (17). It is notable that recent work in vertebrates suggests that the role of Robo2 receptor, which serves as a Wnt5 receptor with Frizzled, is a newly identified coreceptor component of PCP (41).

Wnt5 induces Dsh phosphorylation through Robo1 and Robo2 (20). Although the function of Dsh phosphorylation in PCP remains unclear, the phosphorylation of Dsh is often used as a readout for Wnt activation (42) and multiple kinases have been found to associate with Dsh and phosphorylate it (43). The observations that the presence of Robo increases the amount of Dsh protein associated with Rob but does not alter the phosphorylation level of Dsh suggest that Robo can facilitate the recruitment of activated Dsh protein. The PCP pathway is critical for the generation of tissue polarity and directional information. These molecules are key to breaking symmetry and creating the complex organizational pattern of living organisms. In particular, the asymmetric subcellular localization of Dsh is tightly linked to the polarized action of a cell. For example, during the development of the Drosophila wing, Dsh protein specifically accumulates at the distal ends, but not at the proximal edges (44). In our study, the asymmetric distribution of Dsh fits well with the polarized neurite growth, and supports the notion that polarized Dsh distribution is crucial for polarized cell behavior. Like Dsh, Robo is also unevenly distributed. Without Robo, the asymmetric distribution of Dsh is disrupted, and the directional growth of RMED/V neurites is impaired. The Wnt ligand and Robo receptor regulate Dsh-mediated neurite outgrowth, but do not control the polarized distribution of Dsh protein. Therefore, we propose that Robo, as part of a membrane receptor complex with Rob, facilitates the establishment of a polarized signaling apparatus centered on Dsh, and thus converts the unidirectional Wnt signal to a polarized intracellular response. Intriguingly, the polarized subcellular localization of Robo/SAX-3 is not completely dependent on Wnt/CWN-2. Hence, the molecular identity of the signals that pattern the distribution of the Robo receptor remains to be determined. In C. elegans, the other four Wnts are able to bypass the loss of CWN-2 in mediating neurite outgrowth, suggesting that the functional requirement for CWN-2 in neurite outgrowth is not due to its intrinsic molecular features (17). Considering the pleiotropic functions of Wnts and the mechanistic conservation underlying Wnt signaling in many biological processes, the role of Robo receptor in facilitating Wnt signaling is likely to extend to different Wnts in a wide range of species.

Methods

Worm Strains and Genetics. Strain maintenance and genetic manipulation were performed as described previously (45); jsb76 is a transgenic line of Punc-25::GFP labeling GABAergic motor neurons. Mutants and transgenic fluorescence reporters used in this study are: LG I: eva-1(ok1133), mim-1(e1787); LGI: cam-1(ok52), cam-1 (gmr50), cam-1(gmr122), dsb-1(ok1445), jsb76, xdh121(Punc-25::mCherry); LGIV: unc-30(ju54), srg-1(ok300), cwn-2(ad1), cwn-2(ok895); LGV: czf-2(ok1120); LGV: sax-3(yh123), sax-3(yh200), sax-3(929), sax-3(934), st(1e16s). Additional transgenic lines are: xde12139(Pacr-2::GFP::rol-6), xde12326(Pacr-3::sax-2::GFP::rol-6), xde13236(Pacr-3::sax-2::GFP::rol-1::RFP), xde14362(Pacr-1::sax-2::GFP::rol-1::RFP), xde14362(Pacr-1::GFP::rol-1::RFP), xde14696(Pacr-2::sax-3::GFP::rol-1::RFP), xde14727(Pacr-2::GFP::rol-1::RFP), xde14727(Pacr-2::sax-3::GFP::rol-1::RFP).
mAb (1:5,000; Abmart), antiphosphotyrosine (4G10) mAb (1:1,000; Millipore), anti-GFP pAb (1:5,000; MBL), and anti-HA-tag mAb (1:5,000; MBL). For the CAM-1-Flag or SAX-3-Flag and CWN-2-Myc binding assays, the coinmunoprecipitated CWN-2–Myc protein was normalized with the input level.

GST Pull-Down Assay. GST and GST–CWN-2 were expressed in HEK293T cells, and the cells were lysed using 1% Nonidet P-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, pH 7.6) containing protease and phosphatase inhibitors. After 30 min on ice and centrifuged at 13,400 g for 15 min at 4 °C. The supernatants were incubated with glutathione Sepharose 4B (GE Healthcare) at 4 °C for 4 h. After washing with PBS three times, the Sepharose was incubated with purified Flag–SAX-3 at 4 °C overnight. The Sepharose was washed three times with PBS and boiled in SDS sample buffer.

S2 Immunostaining. S2 cells expressing SAX-3–mCherry, CAM-1–mCherry, or mCherry alone were harvested 48 h after transfection and incubated for 4 h in Sf-900 II serum-free medium (Gibco) containing CWN-2–GFP. The treated cells were then cultured on coverslips coated with poly-lysine (Sigma) for 2 h.

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