Regulation of Axon Guidance by the Wnt Receptor Ror/CAM-1 in the PVT Guidepost Cell in *Caenorhabditis elegans*

Jason Chien,* Ranjan Devkota,* Nebeyu Yosef,† and Catarina Mörck*,1
*Department of Chemistry and Molecular Biology, University of Gothenburg, 405 30, Sweden and †Faculty of Medicine Research Program Unit Translational Cancer Biology, University of Helsinki, FI-00014, Finland
ORCID ID: 0000-0001-9207-6376 (C.M.)

ABSTRACT The *Caenorhabditis elegans* ventral nerve cord (VNC) consists of two asymmetric bundles of neurons and axons that are separated by the midline. How the axons are guided to stay on the correct sides of the midline remains poorly understood. Here we provide evidence that the conserved Wnt signaling pathway along with the Netrin and Robo pathways constitute a combinatorial code for midline guidance of PVP and PVQ axons that extend into the VNC. Combined loss of the Wnts CWN-1, CWN-2, and EGL-20 or loss of the Wnt receptor CAM-1 caused >70% of PVP and PVQ axons to inappropriately cross over from the left side to the right side. Loss of the Frizzled receptor LIN-17 or the planar cell polarity (PCP) protein VANG-1 also caused cross over defects that did not enhance those in the *cam-1* mutant, indicating that the proteins function together in midline guidance. Strong *cam-1* expression can be detected in the PVQs and the guidepost cell PVT that is located on the midline. However, only when *cam-1* is expressed in PVT are the crossover defects of PVP and PVQ rescued, showing that CAM-1 functions nonautonomously in PVT to prevent axons from crossing the midline.

KEYWORDS *C. elegans*; ventral nerve cord; midline guidance; guidepost cell; Wnt; Netrin; Robo pathways

IN humans or other bilaterally symmetric animals, the nervous system is divided into two mirror image halves that are separated by the midline. During the development of the nervous system, some axons do not cross the midline at all, while other axons cross the midline exactly once. The choice for axons to cross or not to cross the midline is dependent on the expression of specific receptors in the axons that recognize and bind to attractive or repulsive guidance cues provided by other cells (Tessier-Lavigne and Goodman 1996). In vertebrates, UNC-6/Netrin and Sonic Hedgehog can attract axons to grow toward the midline (Charron *et al.* 2003), while Slit and Semaphorins repel growth cones away from the midline (Zou *et al.* 2000). Slit repels cells and axons that express Robo receptors (Dickson 2002). In *Drosophila*, all neurons express *robo*, but axons that cross the midline do not have Robo at the cell surface (Kidd *et al.* 1998). The levels of Robo are regulated by Commissureless (Comm). When a neuron expresses Comm, it will form a complex with Robo that will be degraded in lysosomes. The axons of these neurons are then allowed to cross the midline (Keleman *et al.* 2002, 2005). When the axon has crossed the midline, the expression of *comm* is lost and Robo will reappear at the surface and prevent the axon from recrossing. Vertebrates lack a Comm ortholog; instead a divergent member of the Robo family, Robo3/Rig-1, prevents axons from responding to Slit without downregulating or degrading Robo receptors (Sabatier *et al.* 2004).

The *Caenorhabditis elegans* Robo receptor SAX-3 is involved in midline guidance and worms also lack a Comm ortholog. The kinesin-related protein VAB-8 regulates SAX-3 levels in neurons that migrate along the anterior/posterior axis (Levy-Strumpf and Culotti 2007; Watari-Goshima *et al.* 2007) but it is not known whether it also does so in axons that cross the midline.

The Wnt family of secreted glycoproteins regulate anterior/posterior and dorsal/ventral guidance of cells and axons

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[1]Corresponding author: Department of Chemistry and Molecular Biology, Medicinaregatan 9E, University of Gothenburg, 405 30 Gothenburg, Sweden. E-mail: catarina.morck@cmb.gu.se

together with the Netrin pathway (Herman *et al.* 1995; Zecca *et al.* 1996; Rocheleau *et al.* 1997; Thorpe *et al.* 1997; Whangbo *et al.* 2000; Goldstein *et al.* 2006; Levy-Strumpf and Culotti 2014). There are 19 Wnts in humans, seven in *Drosophila*, and five in *C. elegans* that can signal through a canonical β-catenin pathway, or via the planar cell polarity (PCP) or the Wnt/calcium pathways (Wodarz and Nusse 1998). Wnt signaling is also known to regulate axon crossing at the midline. In *Drosophila*, midline axons that express the Wnt receptor Derailed are repelled away from the posterior commissure due to the presence of Wnt5 (Yoshikawa *et al.* 2003). In addition, loss of the zebrafish homolog of the Wnt receptor *Frizzled-3a* (*zfzd3a*) or *Wnt8b* causes the expansion of *slit-2* expression in the telencephalon and as a consequence, axons fail to cross the anterior midline of the brain (Hofmeister *et al.* 2012; Hofmeister and Key 2013).

In this study we show that Wnt signaling is involved in midline guidance of ventral nerve cord (VNC) axons in the nematode *C. elegans*. The *C. elegans* VNC consists of two asymmetric bundles of neurons and axons that are separated by the midline. The left side contains only four neurons (PVPR, PVQL, AVKR, and HSNL) and the right side contains >50 neurons (White *et al.* 1976). The first neuron that extends an axon on the right side is the pioneering AVG neuron. The remaining neurons on the right side follow in a specific order. The left side is pioneered by the PVPR interneuron, followed by the PVQL interneuron (Durbin 1987). The HSN motor neurons that control egg laying are the last neurons to send out their axons into the VNC during the L2–L4 larval stages (Adler *et al.* 2006). Most axons in the VNC do not cross the midline and those that do, cross the midline only once, for example PVPL and PVPR that cross over posterior to the PVT neuron (Wadsworth and Hedgecock 1996). The nematode midline is a dynamic structure that during embryogenesis and early larval stages is composed of motor neuron cell bodies and in later larval stages and adults of a ridge of hypodermal cells (Hao *et al.* 2001; Boulin *et al.* 2006). The PVT neuron is positioned on the midline and acts as a guidepost cell for PVP and PVQ. It secretes Netrin, and ablation of PVT causes abnormal crossovers of PVP and PVQ (Ren *et al.* 1999; Aurelio *et al.* 2002).

Here we report that loss of three *C. elegans* Wnts (CWN-1, CWN-2, and EGL-20), the two Wnt receptors (the Frizzled receptor LIN-17 and the receptor tyrosine kinase of the Ror family CAM-1) and PCP protein VANG-1 caused crossover defects of PVP and PVQ axons. Genetic analysis revealed that the Wnt pathway functions together with the Netrin and Robo pathways to guide cells away from the midline. Strong *cam-1* expression can be detected in PVQ and PVT. Surprisingly, only when *cam-1* is expressed in PVT are the crossover defects of PVP and PVQ axons rescued and this rescuing activity requires the intracellular domain of CAM-1. In addition, we show that LIN-17 and VANG-1 also function in PVT. These data suggest that CAM-1, LIN-17, and VANG-1 function nonautonomously in PVT to repel axons away from the midline.

Materials and Methods

C. elegans genetics

Worms were cultured as previously described (Brenner 1974). Hermaphrodites were used for all experiments. All strains were maintained at 20°, unless otherwise noted. The following strains were used: LGI: *lin-17*(*n677*), *lin-44*(*n1792*), *mig-1*(*e1787*), *mom-5*(*gm409*), and *unc-40*(*e271*); LGII: *cam-1*(*ak37*), *cam-1*(*cw82*), *cam-1*(*gm122*), *cam-1*(*ks52*), *cam-1*(*sa692*), *cwn-1*(*ok546*), *dsh-1*(*ok1445*), *dsh-2*(*ez25*), *mig-5*(*rh147*), and *mig-14*(*ga62*); LGIV: *ced-10*(*n1993*), *cwn-2*(*ok895*), and *egl-20*(*n585*); LGV: *cfz-2*(*ok1201*) and *mom-2*(*ok591*); and LGX: *lin-18*(*e620*), *sax-3*(*ky123*), *slt-1(ok255)*, *unc-6*(*ev400*), *vang-1*(*tm1422*), and *bar-1*(*ga80*).

The double and triple mutants created in the genetic interaction studies were sequenced to confirm that all mutations were present.

Transgenic animals

The following already published transgenic strains were used in this study:

hdIs26[*Podr-2*::*CFP*; *Psra-6*::*dsRed2*] (Hutter 2003), *gmIs65* [*Psra-6*::*mCherry*; *Ptph-1*::*GFP*] (Teuliere *et al.* 2014), *oyIs14* [*Psra-6*:: *GFP*]; *otIs37*[*Punc-47*(*del*)::*GFP*] (Aurelio *et al.* 2002), and *cwIs6*[*Pcam-1*::*cam-1*::*GFP*] (a kind gift from Wayne Forrester)

DNA plasmid constructs and transgenic lines

See Supplemental Material, File S1 for more information. A table containing reagents used in this manuscript can also be found in the Supplemental Files as Reagent Table.

Scoring of crossover defects

The PVP and PVQ neurons were visualized within the same young adult worm using the *hdIs26*[*Podr-2*::*CFP*; *Psra-6*::*dsRed2*] reporter, whereas PVQ and HSN neurons were visualized with the *gmIs65*[*Psra-6*::*mCherry*; *Ptph-1*::*GFP*] reporter. The midline guidance defects of HSNs, PVPs, and PVQs were classified according to where the crossovers occurred along the VNC. A crossover near the head was defined as one that occurred at or anterior to the AVG and RIF neurons. A crossover in the middle was defined as one that occurred between the AVG and the PVT neurons. A crossover in the tail was defined as one that occurred at or posterior to the PVT neuron.

Fluorescence microscopy

Worms were anesthetized in 10 mM levamisole. The images from A–C in Figure 5 were acquired using a Zeiss LSM700inv laser scanning confocal microscope with a Plan-Apochromat 20× objective lens. A Zeiss Axioskop 2 microscope was used otherwise to examine the worms. Images were collected using an ORCA-ER charge-coupled device (CCD) camera (Hamamatsu) and Openlab imaging software (Improvision).

Data availability

All data are within the paper and the supporting information files. See File S1 for information about DNA plasmids and

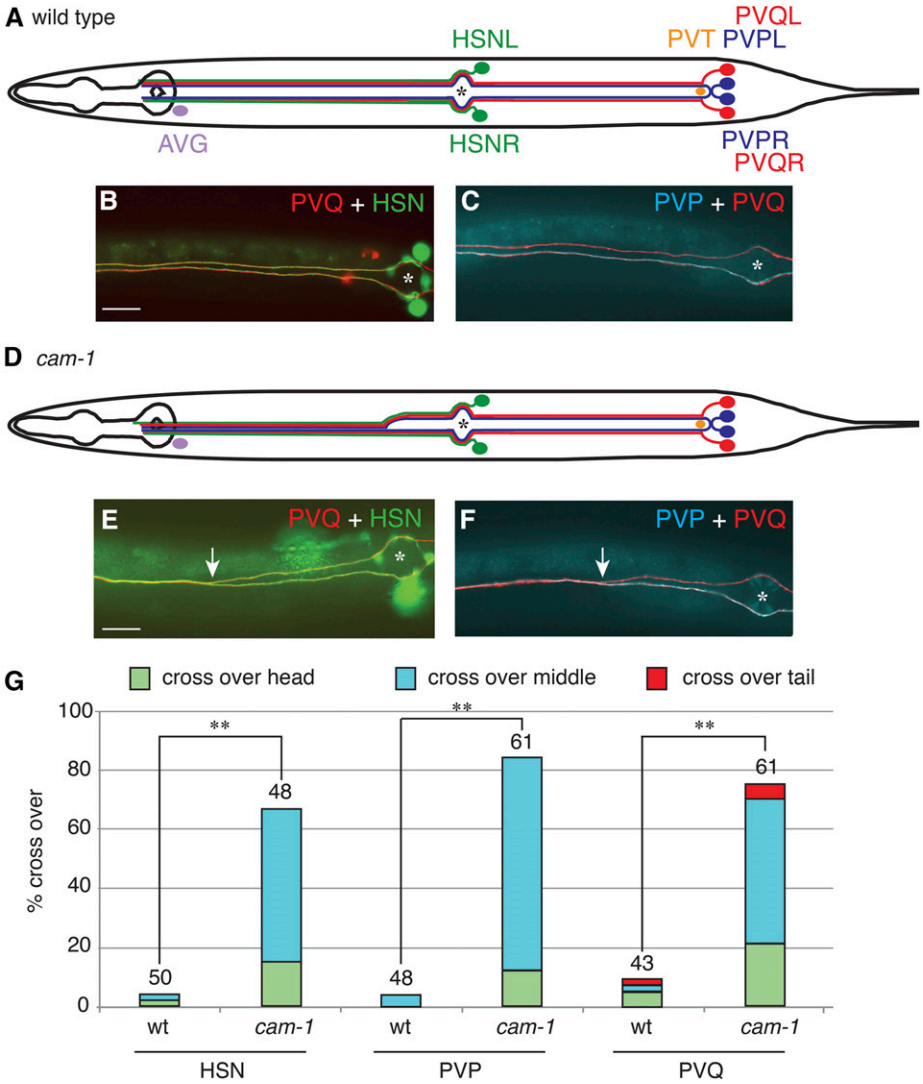

Figure 1 CAM-1 prevents axons in the VNC from inappropriately crossing the midline. (A) Diagram of a wild-type worm showing the anatomy of PVPL/R, PVQL/R, and HSNL/R neurons and axons. The PVP and PVQ neurons have their cell bodies in the tail and extend axons anteriorly to the nerve ring in the head. The HSN neurons are located slightly posterior to the vulva in the midbody and project axons ventrally to the midline where they join the VNC. In wild-type worms, the PVP axons cross the midline posterior to the PVT neuron shortly after they have projected their axons, while PVQ and HSN axons do not cross. In the young adults when the scoring was performed, the left and right axons of PVPs and PVQs are actually very close to each other and only begin to separate a quarter of the length between the vulva and the tail (see Figure 7A). This diagram aims to highlight the fact that PVP axons cross over in the tail. AVG and PVT neurons are guidepost cells in the VNC. (B) The anatomy of the HSN and PVQ neurons in a wild-type worm. The HSNs express GFP and the PVQs express mCherry. (C) The anatomy of the PVPs (blue) and PVQs (red) in a wild-type animal. (D) Diagram of representative midline guidance phenotypes in a *cam-1* (*gm122*) null mutant, with the PVP, PVQ, and the HSN axons crossing over at locations between the head and the vulva. (E and F) PVP, PVQ, and HSN axons in the left tract cross over to the right side (the site of crossover is indicated by the white arrows). (G) Quantification of crossover defects of PVP, PVQ, and HSN axons in *cam-1*(*gm122*) mutants. Green bars show crossover anterior to the AVG neuron (head), blue bars between the AVG and PVT (middle), and red bars posterior to PVT. The number of animals scored for each genotype is indicated above each bar. Ventral view, anterior is to the left. The vulva is marked with an asterisk. * $P = 0.01–0.05$, ** $P < 0.01$ (Fisher's exact test). [*Psra-6*::*mCherry*; *Ptph-1*::*GFP*] was used for the visualization of PVQs (mCherry) and HSNs (GFP) in B and E and *hdIs26*[*Podr-2*::*CFP*; *Psra-6*::*dsRed2*] was used to visualize PVPs (CFP) and PVQs (dsRed) in C and F.

transgenic lines. See Reagent Table for information about reagents used in this paper. Reagents are available upon request.

Results

The Wnt receptor CAM-1 prevents axons from crossing the midline

Proper axon guidance requires the synergy of different signaling pathways. The Netrin and Robo pathways have previously been implicated in the midline guidance of *C. elegans* VNC axons (Ishii *et al.* 1992; Keleman *et al.* 2002), but it is unclear whether other pathways are involved. We decided to examine whether the Wnt pathway is implicated in midline guidance in *C. elegans* and first studied whether loss of the Wnt receptor CAM-1 caused crossover defects of VNC axons. *cam-1* encodes a receptor tyrosine kinase belonging to the Ror family that is thought to bind Wnt glycoproteins via its cysteine rich domain (CRD) (Forrester *et al.* 2004). Almost 80% of the PVQ axons and 70% of the HSN axons cross over from the left to the right side in *cam-1*(*gm122*) null mutants. The PVP neurons normally cross the midline only once (Figure 1A), but in *cam-1* mutants, the left axon crossed over at one additional site in >80% of the worms. Most often the axons crossed the midline at various locations in the middle region of the worm (Figure 1B). Interestingly, theses PVP and PVQ defects were already present in newly hatched L1 larvas (data not shown), suggesting that the defects are developmental and not maintenance in nature. Previous studies suggest that the PVP and PVQ axons serve as substrates for the HSN axons in the VNC (Garriga *et al.* 1993), and HSN guidance defects likely depend on earlier guidance errors of PVP and PVQ. We decided therefore to focus the remaining studies on the PVP and PVQ axons.

A detailed analysis of different *cam-1* alleles revealed that the severity of crossover phenotypes correlated with the strength of the alleles. The null allele *gm122* is a nonsense mutation that introduces a premature stop codon in the

middle of the Wnt binding CRD domain (Figure 2A). The *sa692* allele is a missense mutation that changes a conserved cysteine within the CRD domain to tyrosine, whereas *ak37* has a large deletion, removing sequences from the kringle domain to the C terminus (Figure 2A). These three alleles exhibited highly penetrant midline guidance defects (Figure 2, B and C). The *cw82* allele is a missense mutation in a conserved residue in the signaling kinase domain, and *ks52* is a deletion allele that removes the kinase domain (Figure 2A). These two alleles had a milder phenotype compared to the other alleles (Figure 2, B and C).

The CWN-1, CWN-2, and EGL-20 Wnts, the LIN-17 Wnt receptor, and the PCP protein VANG-1 also regulate midline guidance

Next, we asked whether other Wnt signaling genes are involved in midline guidance. There are five Wnt genes in *C. elegans* (*cwn-1*, *cwn-2*, *egl-20*, *lin-44*, and *mom-2*) (Shackleford *et al.* 1993; Herman *et al.* 1995; Maloof *et al.* 1999) and four Frizzled receptor genes (*cfz-2*, *lin-17*, *mig-1*, and *mom-5*) (Sawa *et al.* 1996; Rocheleau *et al.* 1997; Ruvkun and Hobert 1998). In addition, there is a single ortholog of Ryk, a receptor tyrosine kinase-like molecule with an extracellular Wnt-binding Wnt-inhibitory factor (WIF) domain (Inoue *et al.* 2004). We first asked whether PVP and PVQ crossed the midline inappropriately in four of the single Wnt mutants (*cwn-1*, *cwn-2*, *egl-20*, and *lin-44*). MOM-2 specifies endodermal cell fates and the null mutant is lethal (Rocheleau *et al.* 1997). Very few *mom-2* homozygous progeny produced from *mom-2* heterozygous animals survived to the scoring stage of young adult ($n = 10$ with PVP crossover defect of 10% and PVQ crossover defect of 10%), precluding us from further analyzing the role of *mom-2* in midline guidance. Only loss of *cwn-2* caused crossover defects that were significantly different from wild-type worms (Figure 3). Next, we created double and triple mutant combinations with *cwn-1*, *cwn-2*, and *egl-20* and found that there was a significant increase of crossovers in the *cwn-1*; *cwn-2* double mutant and in the *cwn-1*; *cwn-2*; *egl-20* triple mutant, where >80% of PVP and PVQ exhibited crossover defects (Figure 3). This result indicates that CWN-1, CWN-2, and EGL-20 act together to prevent VNC axons from crossing the midline. We also studied the ortholog of *Drosophila* and human Wntless, *mig-14*, which is required for secretion of Wnts (Pan *et al.* 2008). We only observed mild PVP and PVQ defects (~30%) in *mig-14*(*ga62*) mutants, indicating that there is still enough Wnts secreted in this hypomorphic allele to prevent erroneous crossovers (Figure 3).

Next, we studied the Wnt receptors and found that only mutations in one of the four Frizzled genes, *lin-17*, caused significant defects, where 40% of the PVP and PVQ axons crossed the midline. To test whether *cam-1* and *lin-17* function in the same or in parallel pathways, we created a *lin-17*; *cam-1* double mutant. This mutant exhibited the same penetrance of defects as the *cam-1* single mutant (Figure 3), indicating that they function in the same pathway. Furthermore, a mutation in *cwn-2* failed to enhance the midline phenotype in either a *lin-17* or *lin-44* mutant, suggesting that these genes work in the same pathway (Figure 3).

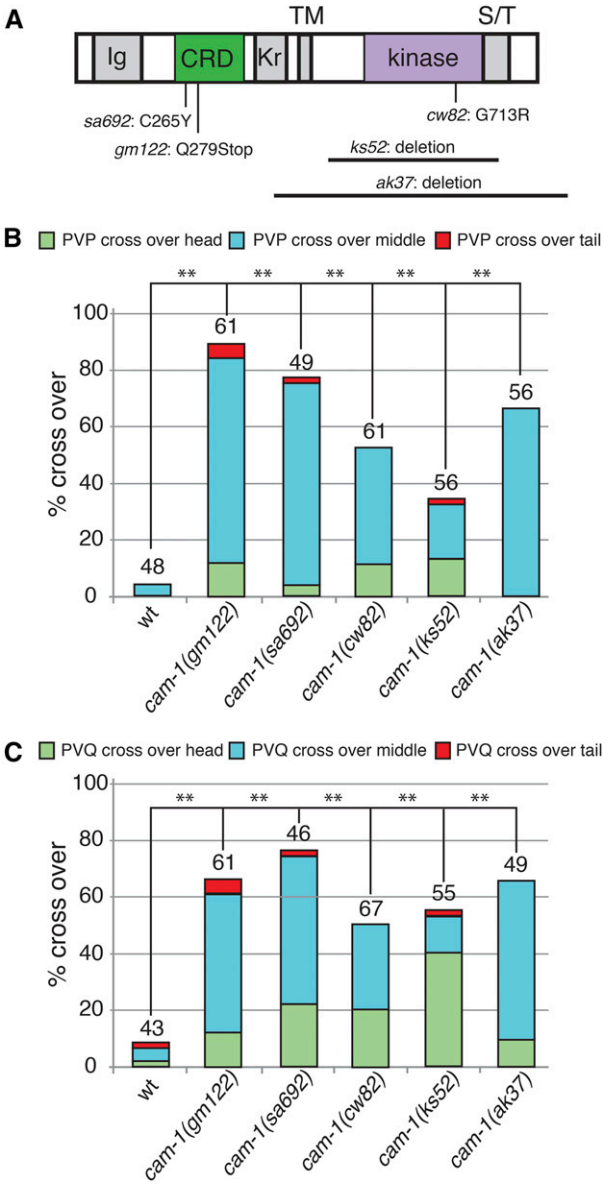

Figure 2 All five *cam-1* alleles exhibit PVP and PVQ crossover defects. (A) *cam-1* coding sequence with five *cam-1* alleles annotated below. CAM-1 is a transmembrane protein with three extracellular domains [*i.e.*, immunoglobulin (Ig) domain, cysteine-rich domain (CRD), and Kringle domain (Kr)] and two intracellular domains [*i.e.*, kinase domain and serine/threonine (S/T)-rich domain]. The null allele *gm122* is a nonsense mutation that introduces a premature stop codon in the middle of the Wnt binding CRD domain. The *sa692* allele is a missense mutation that changes a conserved cysteine within the CRD domain to tyrosine, whereas *ak37* has a large deletion, removing sequences from the kringle domain to the C terminus. The *cw82* allele is a missense mutation in a conserved residue in the kinase domain and *ks52* is a deletion allele that removes the kinase domain. (B and C) Percentage of PVP and PVQ crossovers in different *cam-1* alleles. Green bars show crossover anterior to the AVG neuron (head), blue bars between the AVG and PVT (middle), and red bars posterior to PVT. The number of animals scored for each genotype is indicated above each bar. ** $P < 0.01$ (Fisher's exact test).

Axon pathfinding is primarily mediated by the PCP pathway that regulates cytoskeletal dynamics by activating the

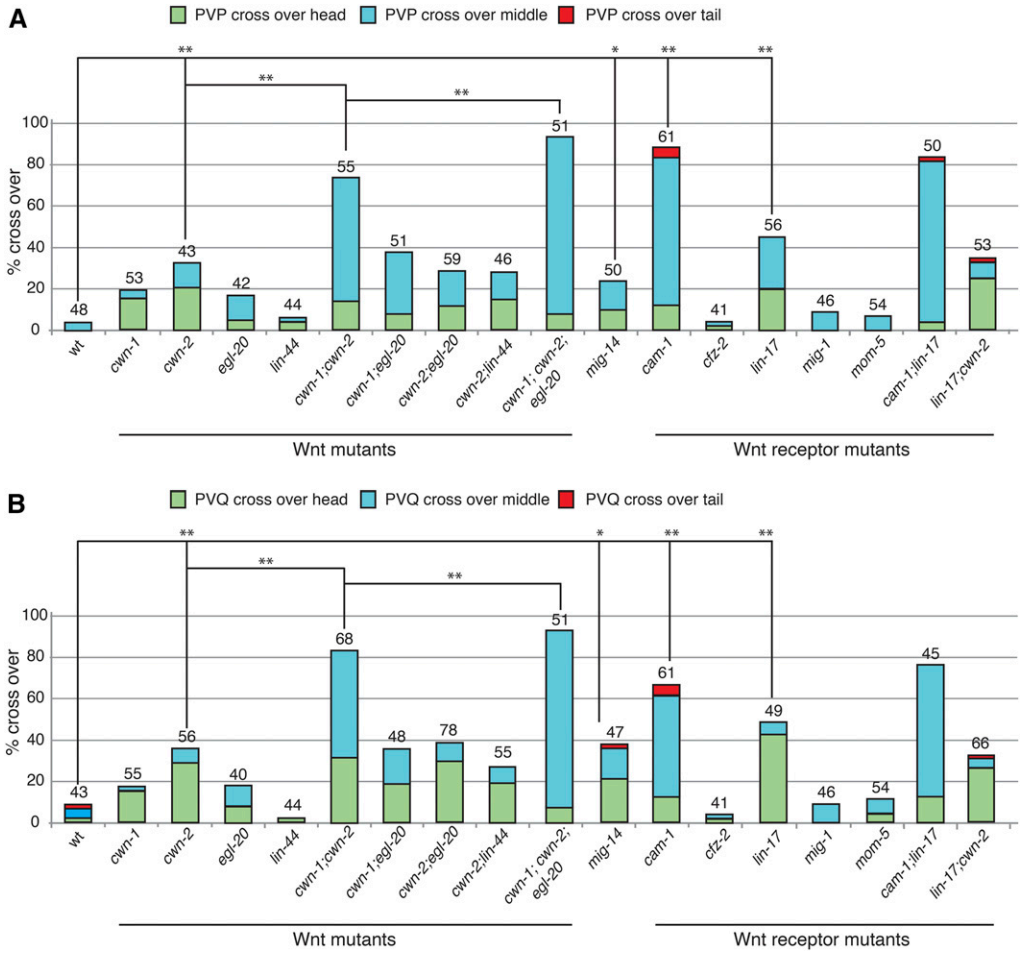

Figure 3 The Wnts CWN-1, CWN-2, EGL-20, and the Wnt receptor LIN-17 regulate midline guidance. Quantification of crossover defects of PVP (A) and PVQ (B) in single, double, and triple mutant combinations of the Wnt mutants *cwn-1*, *cwn-2*, *egl-20*, and *lin-44*, the Wntless homolog mutant *mig-14*, and the Wnt receptor mutants *cfz-2*, *lin-17*, *mig-1*, and *mom-5*. Green bars show crossover anterior to the AVG neuron (head), blue bars between the AVG and PVT (middle), and red bars posterior to PVT. The number of animals scored for each genotype is indicated above each bar. Single mutants were compared with wild type, double, and triple mutants with the single mutants combined. * P = 0.01–0.05, ** $P < 0.01$ (Fisher's exact test).

small GTPases Rho, Rac, and Cdc42 (Schlessinger *et al.* 2009; Zou 2012). In the PCP pathway, Frizzled receptors associate with Dishevelled phospho proteins, the transmembrane protein Van Gogh/Strabismus, and its adaptor protein Prickle and the atypical cadherin Flamingo/Starry night (Rosso *et al.* 2005; Zou 2012). The *C. elegans* Flamingo ortholog FMI-1 was recently shown to have severe VNC guidance defects, where 81% of PVPR crossed over to the right tract and the PVQ axons crossed the midline multiple times (Steimel *et al.* 2010). We asked whether any of the three *C. elegans* dishevelled genes (*dsh-1*, *dsh-2*, and *mig-5*), the Van Gogh ortholog *vang-1*, and the Rac ortholog *ced-10* also exhibit midline guidance defects. The single dishevelled mutants displayed only mild defects, indicating that they either function redundantly or are not involved in midline guidance. A more detailed analysis of the overlapping functions of dishevelleds proved difficult, as worm strains containing mutations in two or three dishevelled needed to be kept in a heterozygous state and no homozygous progeny reached the scoring stage of young adult. On the other hand, both *vang-1* and *ced-10* mutants exhibited significant defects, suggesting that the PCP pathway plays an important role in *C. elegans* midline guidance (Figure 4). Furthermore, a mutation in *vang-1* failed to enhance the crossover phenotype in either a *cam-1* or *lin-17* mutant (Figure 4), consistent with the idea that VANG-1 works together with CAM-1 and LIN-17 in midline guidance.

CAM-1, LIN-17, and VANG-1 function nonautonomously in PVT to regulate midline guidance of PVP and PVQ

To examine where CAM-1 functions to regulate midline guidance, we first asked whether *cam-1* is expressed in PVP and PVQ or in nearby cells where CAM-1 might function nonautonomously. For the *cam-1* expression studies, we used a strain that has an integrated full-length *Pcam-1*::*cam-1*::*GFP* construct (a kind gift from Wayne Forrester) (Figure 5B). *cam-1* is broadly expressed in many cell types, including muscle, hypodermis, and neurons (Forrester *et al.* 1999), making direct identification of relevant cells difficult. To determine whether *cam-1* is expressed in PVP and PVQ neurons, we crossed the *Pcam-1*::*cam-1*::*GFP* strain with *hdIs26*[*Podr-2*::*CFP*; *Psra-6*::*dsRed2*], which expresses CFP in PVP and dsRed in PVQ, and asked whether the different fluorophores colocalized. We detected strong CAM-1 expression in PVQ but only weak expression in PVP (Figure 5, A–C). The *Pcam-1*::*cam-1*::*GFP* transgene rescues the migration defects of ALM, BDU, CAN, and HSN cell bodies in the *cam-1*(*gm122*) null mutant (Kim and Forrester 2003), and we found that it also rescues *cam-1* midline guidance defects (Figure 5, K and L).

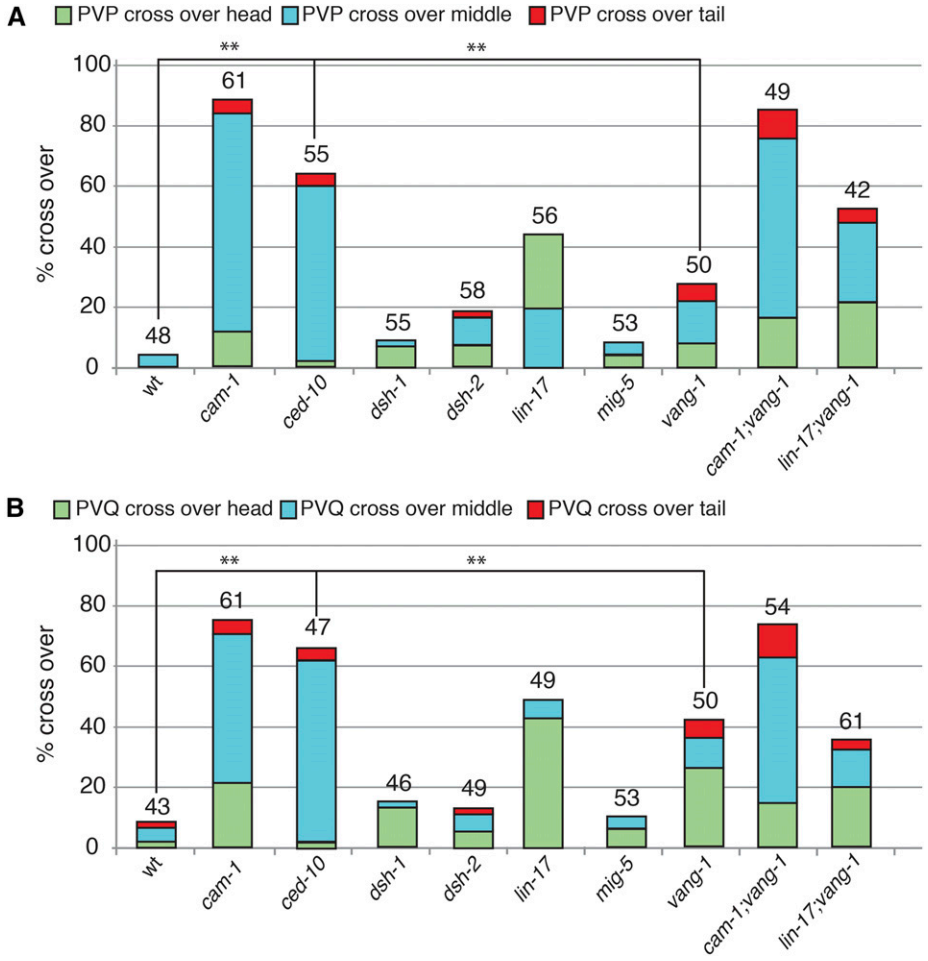

Figure 4 PVP and PVQ crossover defects in mutants in the Wnt PCP pathway. Quantification of crossover defects of PVP (A) and PVQ (B) in the Wnt PCP pathway mutants *ced-10*, *dsh-1*, *dsh-2*, *mig-5*, and *vang-1*. Green bars show crossover anterior to the AVG neuron (head), blue bars between the AVG and PVT (middle), and red bars posterior to PVT. The number of animals scored for each genotype is indicated above each bar. ** $P < 0.01$ (Fisher's exact test).

Next, we examined whether CAM-1 functions autonomously in PVP and PVQ and expressed *cam-1* complementary DNA (cDNA) with the same promoters used in the expression study. The *odr-2* (Chou *et al.* 2001) and the *sra-6* (Troemel *et al.* 1995) promoters were used to express *cam-1* in PVP and PVQ, respectively. The same promoters were previously used to express *flamingo/fmi-1* to rescue the PVP and PVQ crossover defects in *fmi-1* mutants (Steimel *et al.* 2010). To our surprise, neither of the *cam-1* constructs rescued the midline phenotype of *cam-1* mutants significantly (Figure 5, K and L).

We then decided to express *cam-1* in cells located in close proximity to the PVPs and PVQs, such as hypodermal cells and the guidepost cell PVT, which is located on the midline between the two VNC tracts. We used the *elt-3* promoter to express *cam-1* specifically in the hypodermis (Gilleard *et al.* 1999), but this construct failed to rescue PVP or PVQ crossovers in *cam-1* mutants (Figure 5, K and L). Finally, we examined whether CAM-1 is expressed and functions in PVT. For colocalization studies, we created a *mCherry*-tagged *cam-1* transgene (*Pcam-1*::*cam-1*::*mCherry*) and crossed it with *oyIs14* [*Psra-6*::*GFP*]; *otIs37*[*Punc-47*(*del*)::*GFP*], which expresses GFP in PVT, PVQ, and other neurons (Figure 5D). Our results show that CAM-1 is expressed in PVT (Figure 5, D–F).

To study whether CAM-1 functions in PVT, we first had to identify a promoter element that could drive expression of *cam-1* cDNA specifically in PVT. Hobert *et al.* (1999) previously studied the regulatory regions of the *lim-6* gene and found that the third intron in the gene (*lim-6int3*::*GFP*) can drive expression in only four neurons: PVT, DVB, RIS, and AVL (Figure 5G). We fused different parts of the 1.2-kb intron to the GFP gene and identified a 300-bp region, *lim-6int3_short*, that drove expression only in PVT (Figure 5I). We detected strong GFP expression in PVT in embryos as early as in the comma stage, well before the PVP neurons extend their axons (Figure 5, G–J). Interestingly, a *cam-1* cDNA expressed with this PVT-specific promoter element significantly rescued PVP and PVQ crossover defects in *cam-1* mutants (Figure 5, K and L), suggesting that CAM-1 functions nonautonomously in the PVT to prevent other axons from crossing the midline.

The PVT neuron runs along the entire length of the animal and we found that the trajectory of the PVT axon appeared normal in *cam-1* mutants (data not shown), suggesting that the guidance errors of PVP and PVQ axons do not result from a guidance defect of PVT axon *per se*. We considered two scenarios of how CAM-1 may function in PVT. One possibility is that CAM-1 expressed from PVT acts as a sink for the Wnts. Alternatively, CAM-1 acts as a signaling receptor in

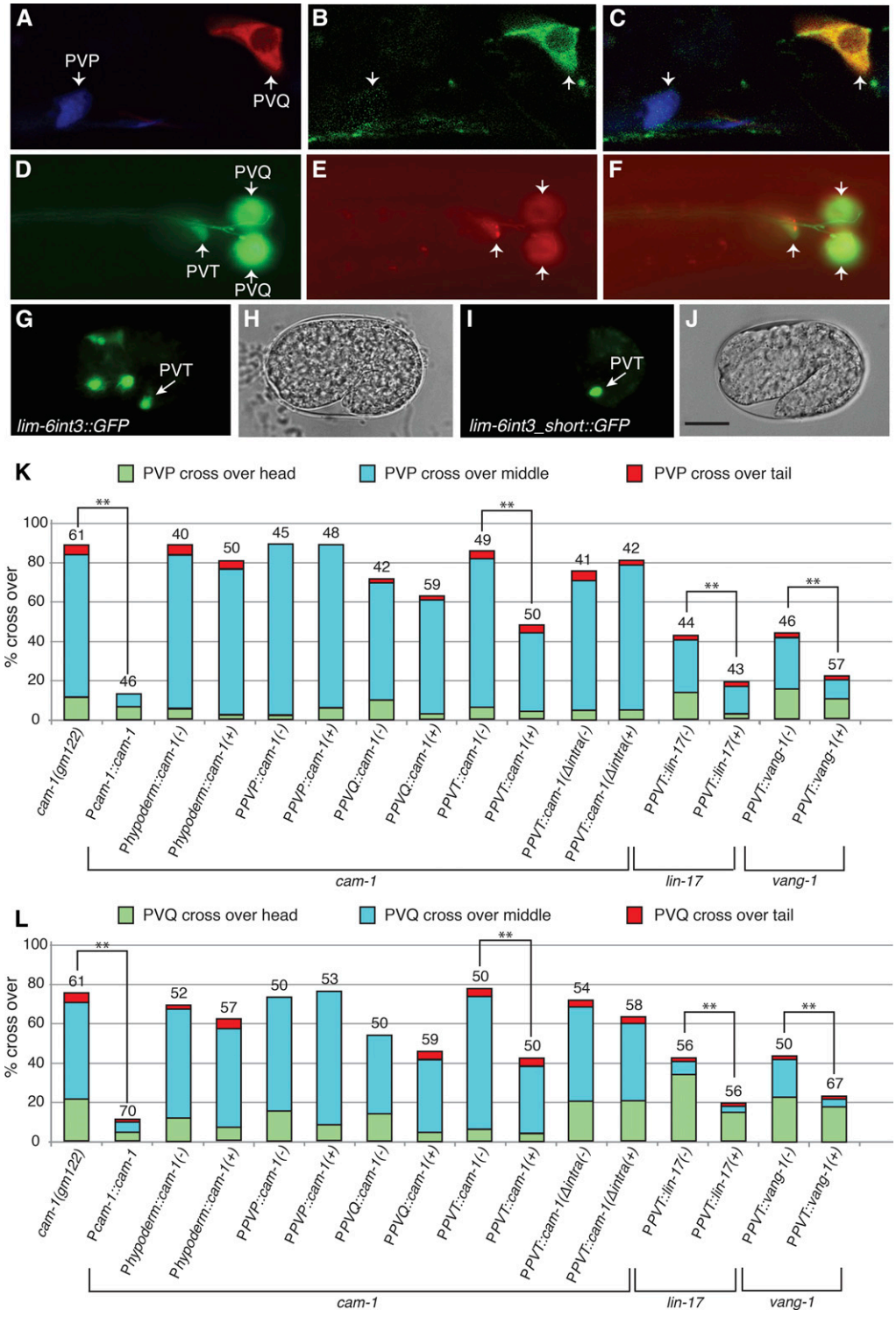

Figure 5 *cam-1* is expressed and functions in the guidepost cell PVT. (A) PVP and PVQ neurons in a L4 larva were visualized with *hdIs26*[*Podr-2*::*CFP*; *Psra-6*::*dsRed2*] that expresses CFP in PVPs and dsRed in PVQs. (B) Expression of *Pcam-1*::*cam-1*::*GFP* in the same worm. (C) Overlay of A and B shows that *cam-1* is strongly expressed in PVQs and barely detectable in PVPs. (D) *oyIs14*[*Psra-6*::*GFP*]; *otIs37*[*Punc-47*(*del*)::*GFP*] in a L2 larva shows expression of GFP in PVT and PVQs. (E) Expression of *Pcam-1*::*cam-1*::*mCherry* in the same worm. (F) Overlay of D and E shows that *cam-1* is expressed in PVT and PVQs. The images in A–C were acquired with a Zeiss LSM700inv laser scanning confocal microscope with a 20× objective. For images in D–F, we used an Axioscope 2, 40× objective. (G) Expression of *lim-6int3*::*GFP* in an embryo at the comma stage. Expression is seen in AVL, DVB, RIS, and PVT (white arrow). (H) Differential interference contrast (DIC) image of the same embryo as in G. (I) *lim-6int3_short*::*GFP* is only expressed in PVT (white arrow). (J) DIC image of the same embryo as in I. Bar, 100 μm. Quantification of crossover defects of PVP (K) and PVQ (L) in nontransgenic *cam-1*(*gm122*) mutants compared to *cam-1* mutants carrying transgenes containing *cam-1* cDNA expressed from either its own promoter *Pcam-1* (multiple tissue), a hypodermis-specific promoter (*Pelt-3*), a promoter expressed in PVP (*Podr-2*), a promoter expressed in PVQ (*Psra-6*), or from a promoter containing elements that drive expression specifically in PVT (*lim-6int_short*). Green bars show crossover anterior to the AVG neuron (head), blue bars between the AVG and PVT (middle), and red bars posterior to PVT. The number of animals scored for each genotype is indicated above each bar. At least two independent, nonintegrated transgenic lines were generated for each construct. ** $P < 0.01$ (Fisher's exact test).

PVT. To distinguish between these two possibilities, we generated a *cam-1* transgene that lacks the signaling intracellular domain and expressed it specifically in PVT [P*PVT*::*cam-1*(*Δintra*)]. This truncated CAM-1 transgene failed to rescue *cam-1* midline defects, suggesting that CAM-1 signaling activity in PVT is essential for midline guidance (Figure 5, K and L).

Given that CAM-1, LIN-17 and VANG-1 function in the same pathway, we examined whether LIN-17 and VANG-1 also function in PVT. Consistent with this idea, PVT-specific expression of LIN-17 or VANG-1 significantly rescued the midline defects in a *lin-17* mutant or a *vang-1* mutant, respectively (Figure 5, K and L). However, transgenic expression of LIN-17 in PVT failed to rescue midline defects in a

cam-1(gm122) mutant (data not shown), suggesting that LIN-17 and CAM-1 are not functionally interchangeable in PVT.

The Wnt pathway interacts with the Netrin and Robo pathways to regulate midline guidance

We next asked whether the Wnt pathway interacts with the Robo or Netrin pathways that are known to regulate midline guidance. In *C. elegans*, loss of the Slit homolog *slt-1* causes 20% of PVQ axons to cross the midline (Hao *et al.* 2001), while in mutants that lack the Slit receptor SAX-3, 55% of PVQ and HSN axons cross over (Zallen *et al.* 1998). The Netrin homolog UNC-6 is expressed and functions during three different phases of development of the VNC. In the first stage, the ventral hypodermis forms an UNC-6 gradient that is crucial for correct positioning of the VNC neuron cell bodies. In the next stage, the midline AVG and PVT neurons express UNC-6 to direct the pioneer axons from the right and left sides of the VNC. In the last stage, neurons in the lateral lumbar ganglion express UNC-6 to organize the later developing VNC axons (Wadsworth and Hedgecock 1996; Ren *et al.* 1999).

For our genetic interaction studies, we examined null mutants of *slt-1*, *sax-3*, *unc-6*, and its receptor *unc-40* and created the following double mutants: *cam-1*; *slt-1*, *lin-17*; *slt-1*, *cam-1*; *unc-6*, *unc-40*; *cam-1*, *lin-17*; *unc-6*, and *lin-17*; *sax-3*. We also tried to make a *cam-1*; *sax-3* double mutant, but failed since it was synthetic lethal. In *cam-1*, *lin-17*, and *sax-3* single mutants, the PVP and PVQ axons most often crossed over in the midbody, somewhere between the AVG and the PVT neurons, while in *unc-6* and *unc-40* mutants, the crossovers occurred most frequently in the tail, posterior to the PVT cell body (Figure 6). The *cam-1*; *unc-6* double mutant exhibited significantly higher penetrance of overall crossover defects, and *cam-1*; *unc-40* had a significant increase of crossovers in the tail, suggesting that *cam-1* functions in parallel to the Netrin pathway. *lin-17*; *sax-3* and *lin-17*; *unc-6* also had significantly more crossovers and the majority occurred in the tail, which indicate that *sax-3* and *unc-6* work in parallel to *lin-17* in midline guidance (Figure 6). On the other hand, a mutation in *slt-1* failed to enhance the crossover phenotype of either a *cam-1* or a *lin-17* mutant (Figure 6), suggesting that SAX-3 has SLT-1 independent function in midline guidance.

Killing the guidepost cell PVT causes crossover defects

Our data and previous studies (Ren *et al.* 1999) suggest that the PVT neuron signals to axons in the VNC to prevent them from crossing over to the opposite side during their outgrowth. Not only is PVT secreting UNC-6 that guides the PVP and PVQ axons, but our results also show that CAM-1 is expressed and functions as a signaling receptor in PVT.

PVT functions to both establish and maintain proper guidance of VNC axons. Laser ablation of PVT in embryos causes disorganization of the VNC (Ren *et al.* 1999) and ablation of PVT during the L1 larval stage causes axons to "flip over" to the other tract (Aurelio *et al.* 2002). The flip-over phenotype is distinct from the crossover phenotype because it occurs when a segment of the axon crosses over to the opposite side and is typically observed postembryonically, suggesting a maintenance defect of the PVT. Since the study performed by Ren *et al.* (1999) was done on only five embryos, we decided to ablate a larger number of PVT neurons (N = 130) during embryogenesis to study in more detail how PVT prevents erroneous crossovers during the development of the VNC. To kill PVT, we used a genetic ablation technique developed by Chelur and Chalfie (2007). In this system, the human Caspase-3 is expressed as two subunits that are only active when they are associated via the interaction of the antiparallel leucine-zipper domains. We expressed both subunits under the control of the same PVT-specific element that we used for our expression and rescue experiments. The two constructs were injected into the strain *otIs37*[*Punc-47*(*del*)::*GFP*]; *gmIs65*[*Psra-6*::*mCherry*; *Ptph-1*::*GFP*] that expresses *mCherry* in PVQ and GFP in PVT, HSN, and other neurons. We found that the caspase constructs fail to abolish GFP expression in PVT in all worms: 17% of the worms had no detectable GFP expression, 52% had weak expression, and 31% had normal expression. Since it is unclear whether PVTs in the worms with weak expression are functional or not, we decided to exclude this group of worms from our analysis. We scored the crossovers of the PVQ axons in animals with or without GFP expression in the PVT (Figure 7D). When PVT was present and expressed GFP at high levels, 12% of the PVQ axons crossed over in the tail. When PVT was absent, the penetrance was even higher: 4% crossed over in the head, 38% crossed over in the middle, and 25% crossed over in the tail. We also noticed that in 8% of worms where the PVT was absent, the PVQ axons flipped over instead of crossed over, which indicates that the PVT might have been alive and functioning during embryogenesis, but died later in the L1 stage (Figure 7D, see Figure 7, A–C for wild type, crossover, and flipover phenotypes). Consistent with our previous results that CAM-1 is acting in the PVT, caspase ablation of PVT in *cam-1*(*gm122*) mutants failed to further enhance the PVQ defects (Figure 7D). By contrast, *cam-1*; *unc-6* double mutants exhibited significantly higher crossover defects compared to worms in which the PVTs were killed (Figure 7D).

Given that CAM-1 and UNC-6 are both expressed in the PVT, we wondered whether there is a crosstalk between the Wnt and Netrin pathways as was observed in the *C. elegans* distal tip cell (Levy-Strumpf and Culotti 2014). We tested whether UNC-6 expression is regulated by CAM-1 by generating a *PVT*::*unc-6*::*gfp* transgene that was then crossed into *cam-1* mutants. We found that there was no obvious difference of UNC-6 expression in PVT between wild type and *cam-1* mutants (data not shown).

Discussion

The Wnt receptor CAM-1 functions nonautonomously in PVT to regulate midline guidance

The Wnt family of secreted glycoproteins regulates a wide range of developmental processes in metazoans, including body axis patterning, cell fate specification, cell proliferation,

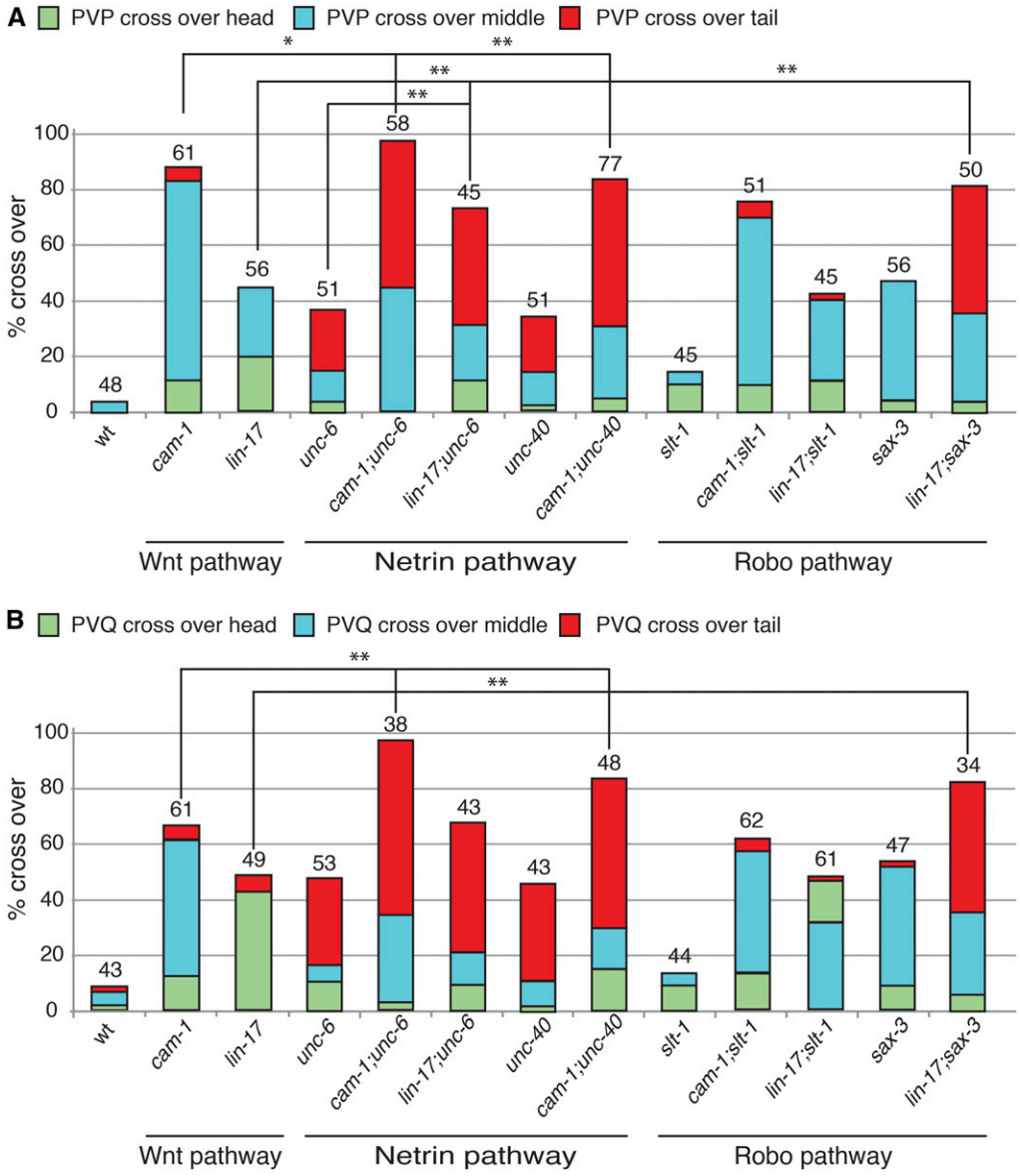

Figure 6 The Wnt pathway acts in parallel to the Robo and Netrin pathways in midline guidance. Quantification of crossover defects of PVP (A) and PVQ (B) in single and double mutant combinations of the Wnt pathway mutants *cam-1* and *lin-17*, Netrin pathway mutants *unc-6* and *unc-40*, and the Slit pathway mutants *slt-1* and *sax-3*. Green bars show crossover anterior to the AVG neuron (head), blue bars between the AVG and PVT (middle), and red bars posterior to PVT. The number of animals scored for each genotype is indicated above each bar. Single mutants were compared with wild type and double mutants with the single mutants combined. The *P*-values shown on the graph are pairwise comparisons of the total percentage of crossover defects between the single and double mutants. Comparisons of crossover defects in the tail between these genotypes are also highly significant: $P < 0.01$ between *cam-1* and *cam-1*; *unc-6*; $P < 0.01$ between *cam-1* and *unc-40*; *cam-1*, and $P < 0.01$ between *sax-3* and *lin-17*; *sax-3*. * $P = 0.01$–0.05, ** $P < 0.01$ (Fisher's exact test).

cell migration, and axon guidance (van Amerongen and Nusse 2009). Here we report that the *C. elegans* Wnts CWN-1, CWN-2, and EGL-20 and the Wnt receptors LIN-17 and CAM-1 function to prevent axons in the VNC from inappropriately crossing the midline. CAM-1 belongs to the Ror family of receptor tyrosine kinases (RTKs) that have an extracellular CRD Wnt-binding domain and an intracellular signaling kinase domain (Forrester *et al.* 2004; Green *et al.* 2007). Our observation that PVP and PVQ axons in *cam-1* kinase-defective alleles *cw82* and *ks52* exhibited crossover defects suggests that CAM-1 kinase activity is important for midline guidance and that CAM-1 acts as a signaling receptor in PVPs and PVQs. However, our tissue-specific expression of CAM-1 using the *odr-2* and *sra-6* promoters failed to rescue *cam-1* defects in PVPs and PVQs, respectively.

The reason for the inability of these promoters to rescue the midline defects in *cam-1* could depend on the fact that they are not restricted to PVPs and PVQs. Recent studies have reported that CAM-1 can have both autonomous and nonautonomous activities that are antagonistic to each other, for example in ALM polarity, where CAM-1 acts autonomously in the ALM neuron as a Wnt signaling receptor and nonautonomously to antagonize Wnts, presumably by sequestering them (Chien *et al.* 2015). CAM-1 could play similar roles in midline guidance where expression of CAM-1 in PVPs and PVQs may promote Wnt signaling, whereas CAM-1 expressed from surrounding cells may inhibit Wnt signaling. However, CAM-1 expressed from its own promoter, which is broadly expressed, completely restored the midline phenotype of *cam-1* mutants to wild-type levels. These data argue against the hypothesis above and prompted us to examine whether CAM-1 acts in cells located close to the PVPs and PVQs. Interestingly, we found that expression of CAM-1 exclusively in the guidepost neuron PVT rescued PVP and PVQ crossover defects in *cam-1*

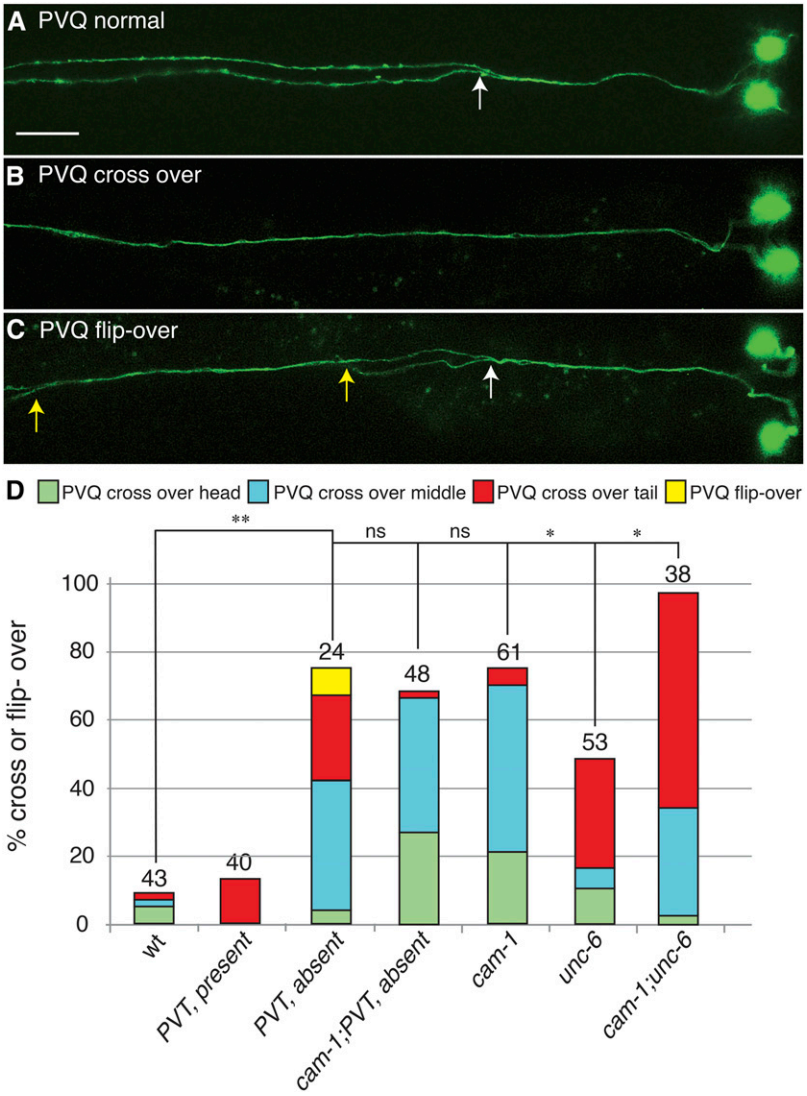

Figure 7 Killing the PVT neuron with human Caspase-3 causes the PVQ axons to exhibit both crossover and flip-over defects. (A) Wild-type PVQ axons in young adults are very close to each other and only begin to separate a quarter of the length between the vulva and the tail as indicated by the white arrow. (B) Crossover of PVQ axons in the tail region. Note the lack of the separation between the right and left axons, resulting in fused axons on one side of the VNC. (C) Flipover defect of PVQ axons. A segment of the axon usually flips over to the opposite side posterior to the vulva (as indicated by the yellow arrows). The axons still manage to separate a quarter of the length between the vulva and the tail as indicated by the white arrow. The PVQ neurons in A–C are visualized with *oyIs14* [*Psra-6*:: *GFP*]). (D) Quantification of crossover and flipover defects of PVQ. Human Caspase-3 was expressed specifically in PVT in wild-type worms containing fluorescent markers for PVT and PVQs. The transgenic worms were divided into two groups based on the presence or absence of GFP in PVT and then scored for PVQ crossover or flip-over defects. The penetrance of crossovers and flipovers in worms with PVT ablation were compared to wild type, *cam-1*, *unc-6*, and *cam-1*; *unc-6* double mutants. Green bars show crossover anterior to the AVG neuron (head), blue bars between the AVG and PVT (middle), red bars posterior to PVT, and yellow bars show flipovers along the VNC. The number of animals scored for each genotype is indicated above each bar. * P = 0.01–0.05, ** $P < 0.01$ (Fisher's exact test).

mutants, suggesting that CAM-1 acts cell nonautonomously in PVT to regulate midline guidance. Nevertheless, the trajectory of the PVT axon appeared normal in *cam-1* mutants (data not shown), suggesting that the guidance errors of PVP and PVQ axons do not result from a guidance defect of PVT axon *per se*.

Consistent with cam-1, lin-17 and vang-1 acting genetically in the same pathway, our transgene rescue analysis also shows that LIN-17 and VANG-1 function in PVT. Previous studies have suggested that CAM-1 can function as a coreceptor for LIN-17 to control the translocation of acetylcholine receptors to the synapses (Jensen *et al.* 2012). CAM-1 has also been shown to interact with the four-pass transmembrane protein VANG-1. VANG-1 lacks an obvious Wnt binding domain (Park *et al.* 2004), but genetic evidence suggests that it can act as a CAM-1 coreceptor for EGL-20 in ground polarity signaling and during developmental pruning (Green *et al.* 2008; Hayashi *et al.* 2009). The lack of an enhanced phenotype of the *lin-17*; *vang-1* double mutant is surprising and argues against the simple model that LIN-17 and VANG-1 are the sole coreceptors for CAM-1. It is possible that another unidentified CAM-1 coreceptor acts along with LIN-17 and VANG-1 in PVT to regulate midline guidance of PVP and PVQ axons. Since the rescue from the PVT-specific promoter is not complete, CAM-1, LIN-17, and VANG-1 may have functions outside of PVT.

PVT is born at ~290 min of development, on the ventral midline just anterior to the rectum. Then it sends out an anterior process toward the head before the bilaterally symmetric PVPs and PVQs extend their axons into the VNC at ~480 min of development (Wadsworth and Hedgecock 1996). We hypothesized that loss of CAM-1 in PVT may alter Wnt signaling at the midline in a way that attracts the PVP and PVQ axons on the left side to cross over to the right side. We considered two scenarios of how CAM-1 may function in PVT. One possibility is that CAM-1 expressed in PVT sequesters excess Wnts from the midline to prevent inappropriate attraction for PVPs and PVQs. Another, nonmutually exclusive possibility is that CAM-1 signaling from PVT generates a repulsive response for the PVPs and PVQs. To test the first possibility, we asked

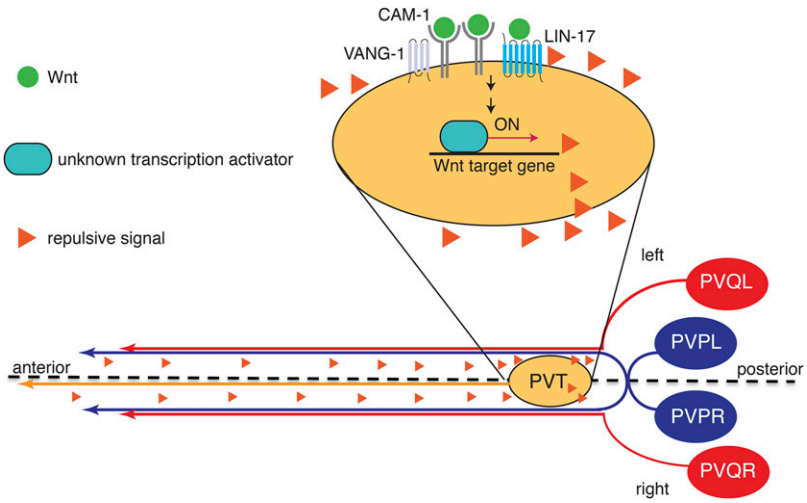

Figure 8 Model. This model proposes that, in the presence of the Wnts, CAM-1 and its putative coreceptors LIN-17 and VANG-1 in the PVT neuron induce transcription of a repulsive signaling molecule that in turn repels PVP and PVQ axons away from the midline anterior to the PVT (dashed line).

whether removing Wnts from the midline by expressing excess levels of the Wnt receptor LIN-17 from PVT could rescue *cam-1* defects. This transgene, despite displaying rescuing activity in a *lin-17* mutant, failed to rescue a *cam-1* mutant. Furthermore, a *cam-1* transgene lacking the intracellular domain failed to rescue *cam-1* defects, suggesting that CAM-1 signaling activity in PVT is essential for midline guidance (Figure 8).

Why is CAM-1 expressed at high levels in PVQ but only weakly in PVP and what is the function of CAM-1 in these cells? The PVP axons cross over to the opposite sides just posterior to the PVT cell body before turning anteriorly, while the PVQ axons project anteriorly without crossing over. It is possible that the differential expression of CAM-1 results in different responsiveness to the repulsive Wnt signal at PVT, leading to axons of PVPs, but not those of PVQs, to cross over posterior to PVT. However, another mechanism must exist to allow PVP axons to exit the midline anterior to PVT.

The signaling downstream of CAM-1 in PVT remains to be elucidated. CAM-1 has been proposed to signal to downstream disheveled DSH-1 to regulate the level of acetylcholine receptors at the neuromuscular junction (Jensen *et al.* 2012) and to promote the outgrowth of motor neurons (Song *et al.* 2010) in a noncanonical β-catenin-independent pathway. Loss of either one of the three *dishevelled* genes did not generate a midline phenotype, but it is possible that the Dishevelleds are required redundantly for midline guidance. A mutation in β-catenin *bar-1* yielded few crossover defects (data not shown). However, mutations in genes in the PCP pathway such as *vang-1* and *ced-10* did result in a significant midline phenotype, consistent with CAM-1 signaling through a noncanonical pathway in PVT to regulate midline guidance.

The combinatorial code of Wnt, Netrin, and Robo pathways mediates axon guidance at the C. elegans midline

Precise axon targeting usually requires inputs from multiple guidance pathways. For instance, the Wnt pathway regulates anterior/posterior and dorsal/ventral guidance of cells and axons together with the Netrin pathway in *C. elegans* (Levy-Strumpf and Culotti 2014). Here we provide evidence that the Wnt pathway also functions together with the Netrin and Robo pathways during *C. elegans* midline guidance.

Our genetic interaction studies revealed that the Wnt and Robo pathways function in parallel since loss of *sax-3*, which encodes the Robo receptor of the Slit pathway, increased the penetrance of crossover phenotypes in *lin-17* mutants. The majority of PVP and PVQ crossovers in *cam-1*, *lin-17*, or *sax-3* single mutant occurred in the anterior or middle regions of the worm. By contrast, *lin-17*; *sax-3* double mutants displayed more frequent crossover phenotype in the tail compared to the single mutants. Therefore, we considered crossovers in the tail to be more severe than those that took place more anteriorly along the VNC.

Interestingly, eliminating the function of Netrin/UNC-6 or its receptor UNC-40 also resulted in a penetrant crossover phenotype in the tail that is further enhanced by loss of CAM-1, indicating that the Netrin and Wnt pathways function together for midline guidance. Taken together, our genetic interaction studies point to a combinatorial code of Wnt, Netrin, and Robo pathways that mediate axon guidance at the *C. elegans* midline.

The enhanced phenotype in *cam-1*; *unc-6* double mutant suggests that CAM-1 and UNC-6 have separate functions despite being both expressed in the PVT. However, expression of a constitutively active caspase in PVT did not cause as penetrant a PVQ crossover defect as was observed in the *cam-1*; *unc-6* double mutant. This discrepancy may be due to the inefficiency of our PVT ablation; only 17% of the worms showed no detectable GFP expression in PVT. Furthermore, the PVTs in these worms may retain residual signaling activity despite lacking GFP expression. We observed an expected inverse correlation between the intensity of GFP expression in PVT and the severity of the midline phenotype; the lower PVT expression is correlated with higher penetrance of midline phenotype and vice versa. Chelur and Chalfie (2007) showed that chromosomal integration of the caspase constructs increased the ablation of touch sensory neurons from 60 to nearly 100%. It is possible that killing of

PVT would be more efficient if we also integrate our constructs. An alternative explanation for the milder phenotypes using caspase ablation of PVT could be that CAM-1 and UNC-6 have additional functions outside of PVT. For example, it is known that the pioneer AVG neuron and RIFL also express *unc-6* during embryogenesis and it has been suggested that these two neurons generate an UNC-6-labeled pathway on the right side of the ventral midline (Wadsworth *et al.* 1996). CAM-1 is essential for correct cell migration and specifies the final position of many migrating cells. In *cam-1* mutants, cells that migrate posteriorly often terminate their migration prematurely and cells that migrate anteriorly, for example the HSN neurons, often migrate too far (Forrester and Garriga 1997). It is possible that mispositioned cells in *cam-1* mutants can disrupt the development of the VNC.

PVT is important for development and maintenance of the VNC

PVT has both embryonic and postembryonic roles in regulating midline guidance. CAM-1 and UNC-6 function in PVT during embryogenesis to establish the VNC, whereas the ZIG proteins function postembryonically to prevent flipovers (Aurelio *et al.* 2002). The early and late roles of PVT are distinct because loss of *cam-1*, *unc-6*, and *zig* genes yield qualitatively different phenotypes. Loss of *cam-1* or *unc-6* results in mostly single crossovers and these defects can be detected in newly hatched L1s. On the other hand, loss of *zig* genes results in flipover, where a section of the axon crosses over to the other side. ZIG proteins are transmembrane proteins with two extracellular immunoglobulin (Ig) domains and they have been proposed to maintain VNC architecture by antagonizing the homophilic attraction between contralaterally analogous axons (Aurelio *et al.* 2002). This same attractive force could allow an inappropriately crossing axon to adhere to its analogous axon when the embryonic PVT signaling is compromised during the establishment of the VNC. This could explain why we observed mostly single (as opposed to multiple) crossovers in our mutant analysis. In conclusion, our study highlights PVT as a signaling hub for both Wnt and Netrin signaling pathways and nicely illustrates how a guidepost neuron employs different mechanisms at different developmental time points to ensure proper guidance of other neurons.

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