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Vangl2 Promotes Wnt/Planar Cell Polarity-like Signaling by Antagonizing Dvl1-Mediated Feedback Inhibition in Growth Cone Guidance

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SUMMARY

Although a growing body of evidence supports that Wnt-Frizzled signaling controls axon guidance from vertebrates to worms, whether and how this is mediated by planar cell polarity (PCP) signaling remain elusive. We show here that the core PCP components are required for Wnt5a-stimulated outgrowth and anterior-posterior guidance of commissural axons. Dishevelled1 can inhibit PCP signaling by increasing hyperphosphorylation of Frizzled3 and preventing its internalization. Vangl2 antagonizes that by reducing Frizzled3 phosphorylation and promotes its internalization. In commissural axon growth cones, Vangl2 is predominantly localized on the plasma membrane and is highly enriched on the tips of the filopodia as well as in patches of membrane where new filopodia emerge. Taken together, we propose that the antagonistic functions of Vangl2 and Dvl1 (over Frizzled3 hyperphosphorylation and endocytosis) allow sharpening of PCP signaling locally on the tips of the filopodia to sense directional cues, Wnts, eventually causing turning of growth cones.

INTRODUCTION

Nervous system function depends on precise organization of axonal connections established during embryogenesis. Much of this axonal organization is established along major anatomical axes, such as anterior-posterior (A-P), dorsal-ventral (D-V) (equivalent to medial-lateral), and inferior-superior. Molecular guidance cues provide directional information for navigating axons during both pathfinding and target selection (Tessier-Lavigne and Goodman, 1996; Dickson, 2002; Zou and Lyuksyutova, 2007). Although many axon guidance molecules and their receptors have been identified, the signal transduction mechanisms leading to directed growth cone turning remain unsolved. In particular, where exactly signaling components are localized in the growth cone and how they respond to guidance cues and interact with each other to create asymmetric signaling leading to turning are unknown in virtually all axon guidance systems.

Multiple subpopulations of commissural axons first project along the dorsoventral axis toward the ventral midline, then cross the floor plate to the contralateral side of the spinal cord and turn either anteriorly or posteriorly along the longitudinal axis. Wnt-Frizzled signaling is required for anterior turning of the dorsal-most populations of commissural axons after they have crossed the midline (Lyuksyutova et al., 2003; Wolf et al., 2008; Zou and Lyuksyutova, 2007). Wnt proteins are secreted glycoproteins, a subset of which are expressed by the ventral midline cells in the floor plate of the spinal cord, where they are expressed in an anterior-posterior gradient. Wnts attract postcrossing commissural axons and the loss-of-function mutation of a Wnt receptor, Frizzled3, results in the randomized turning of commissural axons along the A-P axis after midline crossing (Lyuksyutova et al., 2003; Wolf et al., 2008).

Wnt-Frizzled signaling activates several pathways and plays multiple roles in development and function (Logan and Nusse, 2004; Zou, 2004). Among the known signaling pathways that mediate Wnt functions, the planar cell polarity (PCP) pathway is an appealing candidate for Wnt-mediated axon guidance because of its ability to introduce cellular asymmetry in response to environmental cues (Zou, 2004). PCP refers to cell and tissue polarity along the planar axis of epithelia or mesenchymal cell sheets, perpendicular to the apical-basal axis (Wang and Nathans, 2007; Zallen, 2007; Goodrich, 2008; Simons and Mlodzik, 2008). PCP signaling pathway is highly conserved and regulates the polarized cellular and tissue morphology exhibited in a number of processes, including orientation of epithelial prehair in the *Drosophila* wing, directed cell movement during vertebrate gastrulation and the polarized organization of mammalian stereocilia of cochlear hair cells. Furthermore, in *C. elegans* Wnts are instructive signals for PCP and control spindle orientation during neuroblast division (Goldstein et al., 2006).

The PCP signaling pathway involves two sets of regulators, the Frizzled/Flamingo core group and the Fat/Dachsous PCP system (Simons and Mlodzik, 2008). The Frizzled/Flamingo group of conserved core components include the seven transmembrane domain protein Frizzled (Fzd), the atypical cadherin with seven-pass transmembrane domains Flamingo/starry night (Fmi/Stan or Celsrs in vertebrates), the four-pass transmembrane protein Van Gogh or Strabismus (Vang/Stbm or Vangl), the Fzd-binding intracellular protein Dishevelled (Dsh, Dvl), the ankyrin repeat protein Diego (Dgo), and the Lim domain protein Prickle (Prkl, Pk). Furthermore, it is known that PCP signaling leads to activation of c-Jun N-terminal kinase (JNK) and c-Jun by phosphorylation (Boutros et al., 1998; Yamanaka et al., 2002). Until now, very little is known about the biochemical functions of the PCP signaling components and their cell biological mechanisms of action with the exception that some components directly bind

to each other and in some cases their proper subcellular locations are correlated to proper PCP signaling. For example, Fzd and Dvl colocalize to the distal membrane of the epithelial cells of the *Drosophila* wing and Vang and Pk are localized to the proximal membrane. How their subcellular localization is regulated biochemically and what signaling effects these cellular localizations trigger or reflect are completely unknown.

We report here that the core PCP components are present in commissural axon growth cones at the time they are making anterior turns and in addition to *Frizzled3*, both *Celsr3* and *Vangl2* are required for proper A-P guidance of commissural axons in vivo. PCP components, Frizzled3 and Vangl2, mediate Wnt5a-stimulated commissural axon growth in culture. Wnt5a activates JNK signaling in commissural neurons, and JNK activity is required for A-P guidance of commissural axons. We uncovered a Dvl1-mediated negative feedback loop upon Wnt-Frizzled activation. This feedback loop involves Dvl1-induced Frizzled3 hyperphosphorylation, which causes accumulation of Frizzled3 on the plasma membrane. Point mutations of Frizzled3 result in prolonged and enhanced PCP signaling. Vangl2 antagonizes Dvl1 inhibition by reducing Frizzled3 phosphorylation and cell surface accumulation. It has been shown that Dvl binds to AP-2 and clathrin-mediated endocytosis of Fzd is required for PCP signaling (Sato et al., 2010; Yu et al., 2007). When expressed in commissural axon growth cones, Fzd3 and Dvl1 are localized primarily in intracellular vesicles or puncta by themselves. When coexpressed, they target each other to the plasma membrane. Vangl2, which is primarily localized on plasma membrane, again antagonizes the Dvl1-mediated Frizzled3 accumulation on the growth cone plasma membrane. Finally, Vangl2 protein is found predominantly on the growth cone plasma membrane and highly enriched on the tips of stable or growing filopodia or in patches of plasma membrane where new filopodia emerge in live growth cones. We propose that the antagonistic interaction between Vangl2 and Dvl1 on Frizzled3 phosphorylation and internalization (and thus PCP signaling) maybe a general biochemical mechanism used to create asymmetric signaling in setting up planar cell polarity. And, in neuronal growth cones, this opposing interaction makes the tip of the filopodia more sensitive to guidance cues by allowing Wnt/PCP signaling to enter the growth cones via these tips.

RESULTS

PCP Components Are Expressed in Commissural Axon Growth Cones

During embryogenesis, commissural axons make a series of changes in trajectory en route to the brain. They first project along the dorsoventral (D-V) axis of spinal cord then turn anteriorly toward the brain after midline crossing. Commissural axons are not responsive to Wnts before crossing but become attracted to Wnts after exiting midline and turn anteriorly following a Wnt protein gradient secreted along the floor plate of the spinal cord (see Figure S2A available online) (Lyuksyutova et al., 2003; Wolf et al., 2008).

To test whether the Wnt-PCP pathway is involved in anterior-posterior guidance of postcrossing commissural axons, we first analyzed the expression patterns of the core PCP genes in the developing spinal cord using in situ hybridization (the PCP pathway and its components are listed in the table in Figure S1B). We found that the transcripts of all core PCP components (Figures S1C–S1H) are expressed in commissural neurons at mouse E11.5, a time when many axons are turning anteriorly. *Celsr1* and *Celsr2* were expressed in the ventricular zone (data not shown) whereas *Celsr3* transcripts were found selectively expressed in the mantle zone of the spinal cord (Figure S1C) and were particularly abundant in the areas encompassing commissural neuron cell bodies and regions expressing the Netrin-1 receptor *DCC* (Figure S1I), a marker for commissural neurons (Keino-Masu et al., 1996). As previously reported, *Fzd3* mRNA was broadly expressed in the spinal cord, including the mantle zone where commissural neuron cell bodies reside (Figure S1D) (Lyuksyutova et al., 2003). Both *Vangl1* and *Vangl2* mRNAs were also expressed broadly in the spinal cord (Figures S1E and S1F). *Prkl2* expression was observed in the dorsal commissural neurons as well as in the ventral spinal cord (Figure S1H), whereas *Prkl1* was expressed primarily in the ventrolateral regions of the spinal cord (Figure S1G). The *Dsh* genes were widely expressed in the central nervous system, as previously reported (Tissir and Goffinet, 2006).

We then performed immunohistochemistry on mouse E11.5 spinal sections to characterize the expression of PCP proteins. Commissural axons have a precrossing and a postcrossing segment (green and red segments in Figure S1A, respectively) and a short crossing segment through the floor-plate (FP). Because the spinal cord is a bilaterally symmetric (Figure S6A), there are pre- and postcrossing segments of commissural axons on both sides of the spinal cord. TAG-1 is expressed on the precrossing and crossing segments in the spinal cord (Figure S1M) and L1 delineates postcrossing axons or growth cones (Figure S1N) (Dodd et al., 1988; Zou et al., 2000). We found that Celsr3 mRNAs are broadly expressed in the spinal cord but the proteins are clearly expressed in the postcrossing segment (Figure S1K), along with Fzd3 protein (Figure S1L). We tested the specificity of these signals by staining E11.5 spinal sections of wild-type or knockout embryos. The postcrossing staining of the antibodies is diminished in *Celsr3* and *Fzd3* homozygous mutants (see Figures S6B and S6C, respectively). Vangl2 protein has previously been shown to also be present in the post crossing axons of the spinal cord (Torban et al., 2007).

JNK (Figure S1B) is a downstream kinase of PCP signaling and PCP signaling is commonly measured by increased phosphorylation of JNK and/or Jun (Boutros et al., 1998). We found that phosphorylated-JNK is present on commissural axons, as shown by coimmunoreactivity with TAG-1 (Figures S1P–S1S, short arrowheads) and is enriched in the postcrossing segment of the E11.5 spinal cord (Figures S1O and S1S, long arrows). In addition, to test whether PCP signaling components are present in axonal growth cones (the motile sensing tips of axons), we analyzed their distribution in dissociated commissural neuron cultures (Augsburger et al., 1999). We found that Celsr3, Fzd3, Vangl2, and Dvl are all present in dorsal commissural neurons and their growth cones (Figures S1T–S1W, respectively). Taken together, PCP components are expressed in the developing spinal cord in the right spatiotemporal pattern to be potential regulators of A-P guidance of commissural axons.

Anterior-Posterior Guidance Defects of Commissural Axons in the *Looptail* Embryos

Previous work showed that Fzd3 is required for proper A-P guidance of postcrossing commissural axons (Lyuksyutova et al., 2003). Fzd3 is a known component in multiple Wnt signaling pathways, including canonical/β-catenin, Wnt-Ca^{2+}, Wnt-PKC, Wnt-PI3Kinase, and PCP signaling. To address whether PCP signaling is required for A-P guidance of commissural axons in vivo, we analyzed other mouse mutants with deficiency in PCP signaling. A well-known PCP mutant mouse, the *loop-tail* mouse, has a point mutation in the *Vangl2* gene (S464N). This point mutation abolishes the function of the Vangl2 protein and makes the mutant protein unstable. We analyzed the Vangl2 protein level in spinal lysates from wild-type, heterozygous and *Lp/Lp* embryos and found that the mutant Vangl2 protein was nearly absent in the homozygotes (see Figure S6E). Because the *loop-tail* mouse has an open neural tube (caused by convergent extension defects), and because the roof plate is an essential source of morphogens, we examined the patterning and cell-fate markers in *Lp* mutants. A schematic of the dorsal progenitor domains and postmitotic neurons in the developing spinal cord is shown in Figure 1A. Staining of Pax7, which defines the dorsal progenitor domains dp3, dp4, dp5, dp6, and part of the ventral progenitor domain p0, appeared indistinguishable in the +/+, *Lp*/+, and *Lp/Lp* embryos (Figures 1B–1D). Nkx2.2 immunostaining for pMN and p3 domains showed no defects in heterozygous and homozygous mutant embryos (Figure S4A). The postmitotic dorsal interneuron (dI) markers Lhx1/5 for dI2, dI3, and dI4 were not affected, either (Figures 1E–1G). Finally, the staining pattern of Islet1 marking the dI3 and some motor neuron populations also appeared normal (Figure S4A).

We next examined the trajectory of commissural axons in transverse sections using TAG-1 and L1 staining (Figure 1H). TAG-1 staining showed that the dorsal-ventral projection of precrossing commissural axons are normal in *Lp*/+ and *Lp/Lp* embryos despite the open neural tube (Figures 1I–1K). After crossing the axons grew within the ventral and lateral funiculis in all three genotypes, as shown by L1 staining (Figures 1L–1N).

To analyze the postcrossing trajectory of commissural axons in the *Lp* mouse, we injected DiI in "open-book" preparations at E11.5, the time when many commissural axons are making their anterior turn (Figure 1O; Figures S2B and S2C). We found that commissural axons lost A-P directionality after midline crossing in *Lp/Lp* embryos (Figure 1R). This phenotype is fully penetrant in homozygous mutants. Of the 70 DiI injection sites in 11 *Lp/Lp* embryos, 94.6% (±SEM 2.92%) of the labeled axons showed aberrant A-P trajectory (Figure 1S). In these injections, about half of these axons projected anteriorly (up) and the other half posteriorly (down), suggesting that growth along the longitudinal axis was intact, but up or down directionality was lost in homozygous mutants. The heterozygous mutants showed partially penetrant but strong phenotypes. In the 15 heterozygous littermates analyzed, 68.2% (±SEM 5.14%) of the 96 injection sites showed aberrance, and only one-third of the injection sites showed normal anterior turned (Figure 1S).

To further test whether the PCP signaling pathway is responsible for proper A-P guidance of commissural axons, we analyzed the *Celsr3* knockout mice. There were no observable differences in the Pax7, Nkx2.2, Lhx1/5, and Islet1 staining patterns in all three genotypes (Figures S3A–S3F and S4B). TAG-1 and L1 staining in transverse sections appear identical. (Figures S3G–S3L). However, when examined with DiI injections in "open-book" preparations, *Celsr3* null embryos showed severe defects in anterior-posterior guidance, while the wild-type and heterozygous littermates are normal. 90.0% (±SEM 10.0%) and 94.6% (±SEM 2.34%) of the injection sites in *Celsr3*+/+ and *Celsr3*+/− embryos, respectively, showed normal anterior turning (Figure S3M top and middle panels respectively, Figure S3N). However, in *Celsr3*−/− embryos, only 6.00% (±SEM 3.88%) of DiI injection sites were normal. 94% of injection sites (50 injection sites in 7 homozygous embryos) showed "perfect" randomization of growth along the A-P axis (Figure S3M, lower panel, and Figure S3N).

Therefore, *Vangl2* and *Celsr3* are both required for A-P guidance of commissural axons, phenocopying the *Fzd3* null mice.

PCP Components Mediate Wnt5a-Stimulated Outgrowth of Commissural Axons

To directly test the function of PCP components in Wnt-stimulated outgrowth of commissural axons, we delivered DNA constructs expressing PCP components in commissural neurons and tested the response of commissural axons to Wnt5a in dissociated culture. We used Wnt5a here because Wnt5a attracts commissural axons after midline crossing (Lyuksyutova et al., 2003; Domanitskaya et al., 2010). After DNA constructs were injected into the central canal and electroporated to the dorsal margins of the spinal cord (Figure S2D), the spinal cord was dissected into an "open-book" configuration and then the dorsal spinal cord margin including the progenitor domains was dissociated as previously described (Augsburger et al., 1999) (Figure S2D). More than 90% of the electroporated and dissociated neurons were TAG-1 immunoreactive, confirming that they are commissural neurons (Wolf et al., 2008).

Control neurons expressing EGFP only exhibited a smaller, but still significant increase of ~15% in axon growth in response to Wnt5a (Figures 2B and 2C). This may be due to the presence of low endogenous levels of PCP components in these axons following 24 hr of culture. However, when we cultured the commissural neurons expressing Fzd3-EGFP, Wnt5a enhanced axon length by ~36% within 24 hr (Figures 2B and 2C). Neurons coexpressing Fzd3 and Vangl2 also showed a similar increase in average axon length in the presence of Wnt5a. It is possible that the level of endogenous Vangl2 protein is at a saturating level and therefore overexpression Vangl2 does not increase the Wnt5a response. In the absence of Wnt5a, Fzd3, and Vangl2 expression in precrossing neurons did not affect the growth of commissural axons compared with controls (Figures 2A and 2C). Similarly, co-expression of both Fzd3 and Vangl2 showed no effect on axon length in the absence of Wnt5a, either (Figure 2A, last panel). EGFP-Vangl2 expression alone, in the absence of Fzd3, did not exhibit a statistically significant increase in axon length following Wnt5a stimulation.

To test whether Vangl2 is required for Wnt5a-stimulated outgrowth of commissural axons, we electroporated a Vangl2 shRNA construct to downregulate the endogenous Vangl2 protein. We found that when Frizzled3-mCherry and the scrambled shRNA were co-expressed in commissural neurons, commissural axon outgrowth was stimulated by Wnt5a

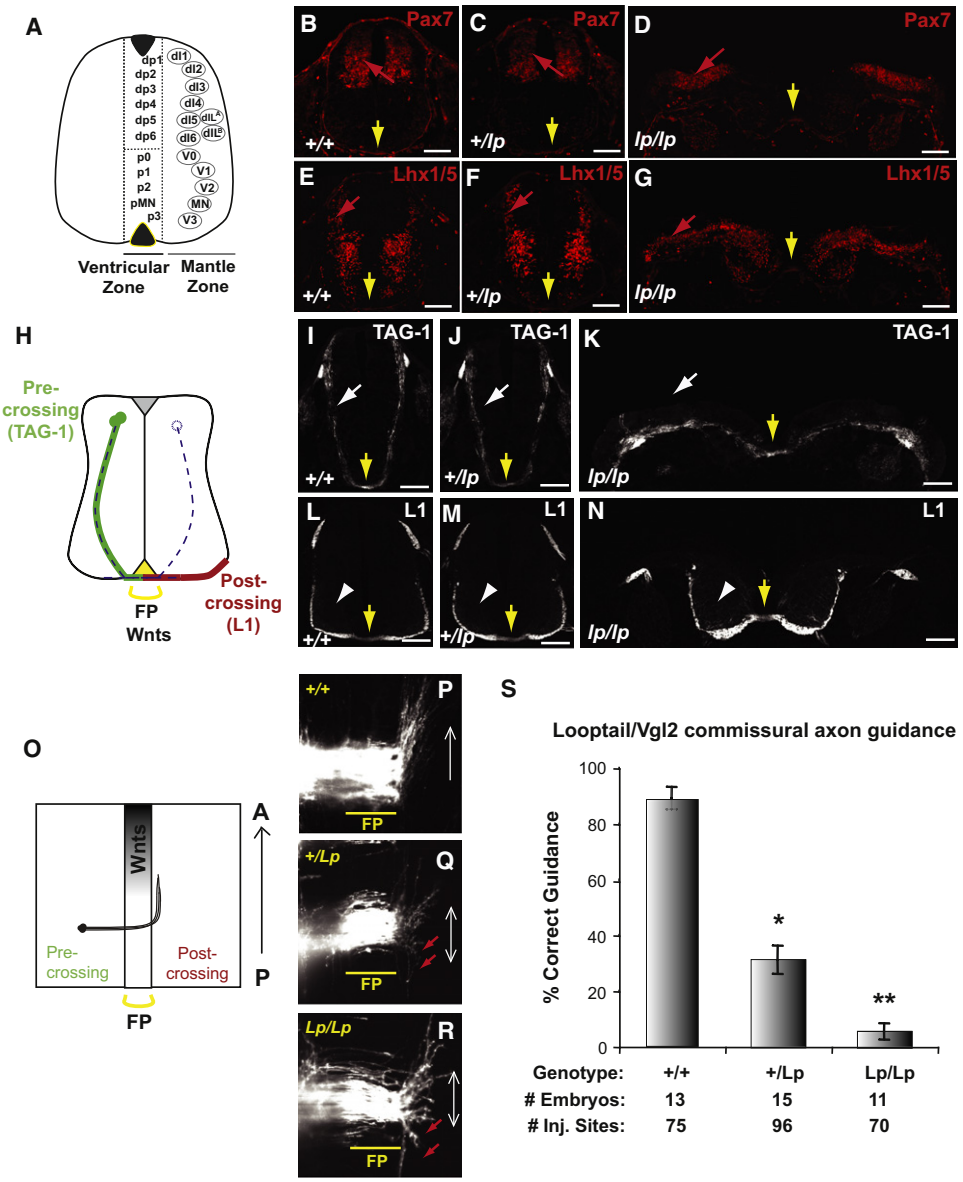

Figure 1. A-P Guidance Defects of Commissural Axons In *Looptail* Embryos

(A) Schematics of E11.5 mouse transverse spinal cord section showing cell fate markers. dI: dorsal interneurons; MN: motor neurons; dp: dorsal progenitor; p: progenitor.

(B–G) Pax7 and Lhx1/5 expression. Red arrow: cell bodies expressing indicated transcription factors; Yellow arrow: floor plate.

(H) Schematics of mouse E11.5 transverse section showing commissural axon trajectory.

(I–N) Dorsoventral trajectory of commissural axons in Lp spinal cords shown by TAG-1 immunostaining of E11.5 in *Vangl2*+/+, *Vanlg2*+/*Lp* and *Lp*/*Lp* embryonic sections (I–K) and L1 immunostaining (L–N). White arrow: precrossing commissural axons; Yellow arrow: location of the floor plate; White arrowhead: postcrossing commissural axons.

(O) Schematics of commissural axon trajectory in the open-book prep as revealed by DiI tracing.

(P–R) A-P guidance defects in *Vangl2*+/*Lp* and *Lp*/*Lp* embryos. Red arrows indicate aberrant posteriorly projecting axons.

(S) Quantification of DiI injection sites *Vangl2*+/+, *Vanlg2*+/*Lp* and *Lp*/*Lp* embryos. Scale bars, 100 μm.

See also Figures S1–S4.

(Figures 2D and 2E). However, when Frizzled3-mCherry was co-expressed with the Vangl2 shRNA, commissural axon outgrowth can no longer be stimulated by Wnt5a (Figures 2D and 2E).

To further assess whether PCP components respond to Wnt protein in growth cones, we examined the distribution of the endogenous Frizzled3 and Vangl2 in dissociated commissural neurons using immunocytochemistry (Figure S5). We found that Frizzled3 and Vangl2 are distributed evenly in the growth cone and along the shaft of the commissural neurons (Figures S5A–S5C). However, upon addition of Wnt5a into the culture, both Frizzled3 and Vangl2 become concentrated in the growth cone (Figures S5D–S5F, 30 min after Wnt5a addition) and

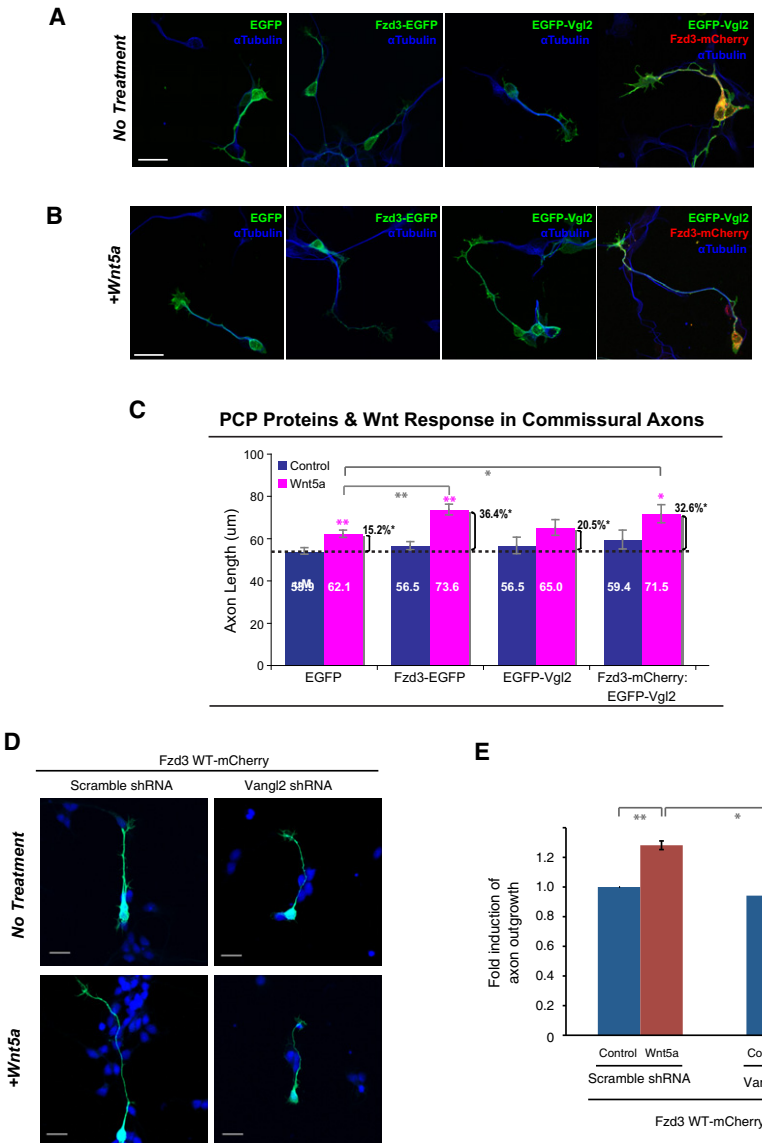

Figure 2. Frizzled3 and Vangl2 Mediate Wnt-Stimulated Commissural Axon Outgrowth

(A) Commissural axon expressing Frizzled3 and Vangl2 or both after 24 hr of culture in the absence of Wnt5a.

(B) Commissural axon expressing Frizzled3 and Vangl2 or both after 24 hr of culture in the presence of Wnt5a. Scale bars: 20 μm.

(C) Quantification of axon lengths. Data are the mean ± SEM. *p value of <0.005, **p value of <0.00005.

(D) Commissural axon expressing Frizzled3-mCherry and scramble shRNA or Vangl2 shRNA. Scale bars: 20 μm. Green indicates electroporated cells that express EGFP. Blue is DAPI staining.

(E) Quantification of axon lengths. Data are the mean ± SD of values from three independent experiments. *p value of <0.05, **p value of <0.005.

See also Figure S5.

60 min later, both Frizzled3 and Vangl2 protein are highly concentrated in the growth cone (Figures S5G–S5I). These results suggest that in the presence of Wnt proteins, endogenous PCP components are present in the commissural neuron growth cone and these components may mediate Wnt response.

JNK Is Activated by Wnt5a in Commissural Axons and Required for Their A-P Guidance

Phosphorylation of Jun and JNK is a classic and commonly used readout of JNK activation (Boutros et al., 1998; Jaeschke et al., 2006). Phospho-JNK appeared highly enriched in postcrossing regions of the commissural axons in vivo (Figure S1O). We tested whether JNK is activated in commissural neurons via Wnt5a stimulation by measuring the levels of phospho-Jun and whether JNK is required for A-P guidance of commissural axons. Commissural neurons were cultured for 36 hr and subject to bath application of Wnt5a (Figure 3A). After 30 min of treatment, we found that endogenous phospho-Jun level was increased by 4-fold (Figure 3B). The levels of the endogenous Fzd3 and Vangl2 proteins remained unchanged following the addition of Wnt5a (anti-Fzd3 and anti-Vangl2 IB, Figure 3A). The specificity of Fzd3 and Vangl2 antibodies for western blot analysis was validated using knock and mutant mouse line (Figures S6D and S6E). We also further analyzed the distribution of activated JNK in E13 rat spinal cord using western blot (Figures 3C and 3D). Levels of both activated JNK1 and JNK2 were indeed much higher in the ventral spinal cord (V.sc) as compared with the dorsal spinal cord (D.sc), (phospho-JNK IB, Figure 3D), though no difference in total JNK was observed, consistent with immunohistochemistry results (T.sc) (total JNK IB) (Figure 3D; Figure S1O).

Due to their high redundancy and importance in many earlier developmental processes, analyzing whether JNK activity is required for anterior-posterior (A-P) guidance of commissural axons using knockout mice of JNK gene families was not feasible. Therefore, we applied JNK inhibitors, JNK inhibitor-I, and SP600125 in the spinal "open-book" explant assay at a time when rat commissural axons are making anterior turning decisions (E13) (Figure 3E). These inhibitors specifically block all three JNKs (JNK1-3) in vertebrates. We analyzed the post-crossing trajectory of rat E13 commissural axons in "open-book" explants treated with JNK inhibitors by DiI injection after culturing for one day. We found that inhibiting JNK activity lead to anterior-posterior (A-P) randomization of commissural axons. In control explants, approximately 91% (±SEM 5.57%) of injection sites showed correct guidance as compared with 39% (±SEM 6.39%) when JNK is inhibited (Figure 3E). About two-thirds of the injection sites showed randomized A-P

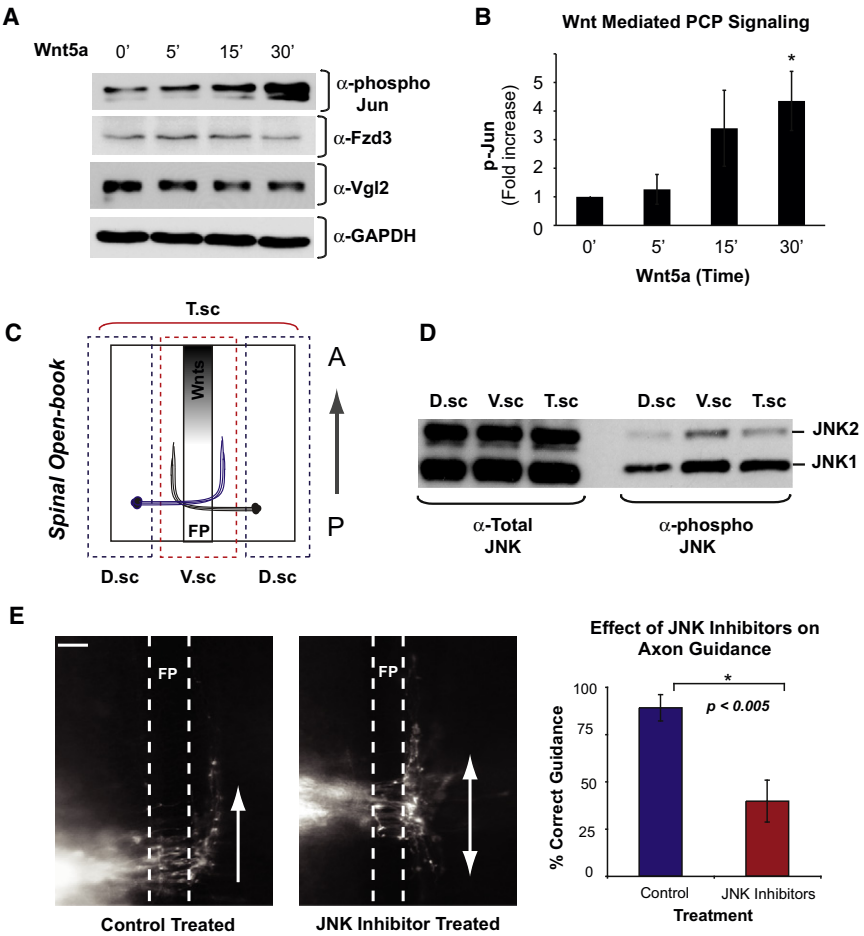

Figure 3. Wnt5a Activates JNK in Commissural Neurons and JNK Activity Is Required for A-P Guidance of Commissural Axons

(A) Wnt5a increases JNK activity in dissociated commissural neurons.

(B) Quantification of p-Jun levels after addition of Wnt5a. Data are the mean ± SD of values from three time independent experiments. *p value of < 0.05.

(C) Schematic of E13 rat spinal cord indicating regions used for western blot analysis (sc, spinal cord).

(D) Western blot analysis of dorsal spinal cord (D.sc) enriched with precrossing axons, ventral spinal cord (V. sc) enriched with crossing and post-crossing axons and total spinal cord (T.sc) using anti-JNK (left) and anti-phosphorylated-JNK (right) antibodies.

(E) Inhibition of JNK activity (with JNK inhibitor-I and SP600125) caused A-P randomization of commissural axons.

See also Figure S6.

projections after midline crossing. Therefore, a downstream effector of PCP signaling, JNK, is required for the A-P guidance of commissural axons.

Vangl2 Antagonizes Dvl1's Feedback Inhibition of Fzd3-Mediated Wnt/PCP Signaling

To elucidate how core PCP components function cell autonomously, we first explored how Dvl1, Vangl2, and Fzd3 interact with each other. It is known that Dvl is a central cytoplasmic molecule in the activation of Wnt-PCP signaling. In noncanonical signaling, Fzd is thought to recruit Dvl to the plasma membrane and Dvl then subsequently activates the downstream signaling, such as JNK, Rac1, and RhoA (Gao and Chen, 2010) (table in Figure S1B). When Wnt5a was added to HEK293T cells expressing Fzd3-mCherry, we found that p-Jun was indeed increased (Yao et al., 2004; Yu et al., 2007) (Figure 4A, lanes 1–3). However, upon coexpression of Fzd3 and Dvl1, we found unexpectedly a Wnt5a-dependent downregulation of p-Jun level (Figure 4A, lanes 4–6), revealing a feedback inhibition mechanism in PCP signaling. With 30 min of Wnt5a addition p-Jun signal is reduced in Fzd3 and Dvl1 coexpressing cells (Figure 4A, lane 6).

Van Gogh is thought to have opposite function of Dvl in PCP signaling. In addition, Van Gogh and Dvl are found on the membranes on opposite sides of many epithelial cells, where PCP signaling is essential for establishing tissue polarity (Simons and Mlodzik, 2008). We asked if Vangl2 could affect this Dvl1-mediated feedback on PCP signaling. We found when Vangl2 is present with Fzd3 and Dvl1, the p-Jun signaling was prolonged and was not diminished after 30 min of Wnt stimulation (Figure 4C, lanes 1–3).

A Vangl2 mutant, the *looptail*, has an S464N amino acid substitution, which affects its ability to bind Dvl (Torban et al., 2004b) (Figure S6F). Triple transfection of Lp, Fzd3 and Dvl1 followed by Wnt5a stimulation resulted in a rapid decrease of p-Jun signal similar to Dvl1 and Fzd3 co-transfection, rather than prolonged signaling (Figure 4C, lanes 4–6). Therefore, Vangl2 is able to antagonize Dvl1, whereas mutant Vangl2, Lp, fails to. Furthermore, S464 in Vangl2 is a likely phosphorylation site (Figure 4C, anti-Flag IB; Figure S7C).

Dvl and Vgl Have Antagonistic Functions on Fzd3 Hyperphosphorylation

Because Frizzled endocytosis has been shown to be required for PCP signaling, to understand how Dvl1 and Vangl2 may antagonize each other (Sato et al., 2010; Yu et al., 2007), we set out to analyze the cell surface levels of Frizzled3. We transfected Fzd3-mCherry in HEK293T cells then surface biotinylated and immunoprecipitated (IP) the surface molecules with strepavidin-conjugated beads to obtain membrane-localized proteins. These surface fractions were then analyzed by immunoblotting (IB) with anti-mCherry IB. There were two different bands of Fzd3-mCherry in cell-surface membrane fraction (Figure 4A, Avidin IP, lanes 1–3). These two bands represent phosphorylation states of the Frizzled3 protein because when the membrane fractions were subjected to protein phosphatase I treatment the upper Fzd3-mCherry band disappeared (Figure 4B). Phosphorylation of Fzds has been shown previously in *Xenopus* oocytes and the

Drosophila eye (Djiane et al., 2005; Yanfeng et al., 2006); furthermore, in *Xenopus* embryos phosphorylation of XFzd3 is XDsh dependent. We found when Fzd3-mCherry was co-transfected with Dvl1-EGFP, the total Fzd3 phosphorylation was indeed increased as previously reported (data not shown), and interestingly only the p-Fzd3 band was present at the membrane when Dvl1 was overexpressed (Figure 4A, Avidin IP, lanes 4–6). To verify that the precipitation was specific to only biotinylated proteins, we performed a no-biotin membrane label control and found that Fzd3-mCherry was absent (not shown). To verify that only the cell surface proteins were biotinylated, we performed western blot using the precipitates pulled down by the strepavidin-conjugated beads with antibody against the cytosolic protein GAPDH. GADPH was absent in the membrane IP fraction though the proteins were present in the total cell lysate input (not shown).

We next asked whether Vangl2 has any effect on the Dvl1-induced hyperphosphorylation of Fzd3 at the cell membrane. We triple transfected Fzd3-mCherry, Dvl1-EGFP, and 3xFlag-Vangl2 and found that coexpression with Vangl2 resulted in a reappearance of the unphosphorylated Fzd3 band at the membrane (Figure 4C, Avidin IP and anti-mCherry IB, lanes 1–3). Therefore, Vangl2 either prevents Dvl1-dependent phosphorylation of Fzd3 at the membrane or promoted the dephosphorylation of membrane bound Fzd3 that initially resulted from Dvl1 activation. In either case, the presence of Vangl2 reduces p-Fzd3 at the membrane. When Vangl2 was transfected with Fzd3 in the absence of Dvl1, only the non-phospho-Fzd3 was present at the membrane (Figure S7D, lanes 1–3). When we triple transfected the mutant Vangl2, 3xFlag-Lp, with Fzd3-mCherry and Dvl1-EGFP, we did not observe the non-phosphorylated band of Fzd3-mCherry, suggesting that the mutant Vangl2, Lp, cannot antagonize the phosphorylation of Fzd3 induced by Dvl overexpression (Figure 4C, lanes 4–6). 3x-Flag-Lp and Fzd3-mCherry transfection in the absence of Dvl1 resulted in the same Fzd3 banding patterns as Fzd3-mCherry alone (Figure S7D, lanes 4–6).

To confirm that Vangl2 is required for promoting PCP signaling, we developed a human shRNA construct to Vangl2 and expressed in the HEK293T cells and measured Wnt5a stimulated PCP signaling (Figure 4E). We found that when Vangl2 is knockdown, Wnt5a can no longer effectively induce c-jun phosphorylation (Figure 4E, lanes 4–6). To assess whether HEK293T cells are Wnt5a responsive without the expression of PCP components, we transfected EGFP alone then added Wnt5a. We found 30 min after Wnt5a stimulation a mild baseline increase in phospho-Jun signal was induced in our control EGFP transfected cells (Figure S7E, lanes 1 and 2, and Figure S7F). However, the expression of the PCP component Fzd3-EGFP or 3xFlag-Vangl2, consistently enhanced the Wnt5a induced phospho-Jun signal (Figure S7E, lanes 3 and 4, and Figure S7F). Frizzled3 hyperphosphorylation can only be induced by Dvl1 but not Dvl2 (Figure 4F). The ratio of the upper to lower bands of Frizzled3 increases in the presence of increasing levels of Dvl1 (Figure 4F, lanes 1–3) but not of Dvl2 (Figure 4F, lanes 1, 4, and 5).

Lack of Frizzled3 Hyperphosphorylation Correlates with Higher PCP Signaling Activity and Prolonged Wnt5a Response

To verify that Dvl1 induces Frizzled3 hyperphosphorylation and test the effects of Frizzled3 hyperphosphorylation, we made a series of combinations of point mutations (Figure 5A) with increasing number of the putative phosphorylation sites mutated (up to seven sites) as previously observed in *Xenopus* oocytes (Yanfeng et al., 2006). We found that the band shift caused by Dvl1 becomes more and more reduced with increasing number of mutation sites, suggesting that these amino acids are true phosphorylation sites (Figure 5B). Consistent with our hypothesis, increasing the number of phosphorylation sites did increase the level of c-jun phosphorylation (Figure 5B). We also found that the baseline of c-jun phosphorylation is higher when Frizzled3 phosphorylation mutation is expressed (with seven sites mutated) and that Wnt5a induced stronger and longer-lasting PCP signaling when Frizzled3 hyperphosphorylation is blocked (Figure 5C). Therefore, Dvl1-induced Frizzled3 hyperphosphorylation is indeed inhibitory to PCP signaling and probably causes the rapid decay of PCP signaling. Vangl2, which reduced Frizzled3 phosphorylation, thus promotes Frizzled3 signaling, likely via promoting endocytosis.

Dvl and Vgl Have Antagonistic Functions on Fzd3 Membrane Localization

It has been proposed that Frizzled internalization is necessary for PCP signaling and phenotypes (Gagliardi et al., 2008; Yu et al., 2007). However, it has not been directly tested whether the cell surface level of Fzd changes upon Wnt addition. Our results showed that Dvl1 and Vangl2 affect the total levels of Fzd3 at the plasma membrane in opposite ways. In the presence of dvl1, the surface IP showed approximately 3-fold more Fzd3 at the membrane than Fzd3-mCherry transfected alone (Figure 5D, lanes 1 and 2). However, when Vangl2 was present, Fzd3 surface levels were not increased, but rather reduced, suggesting that the Vangl2 promotes Fzd3 internalization (Figure 5D, lanes 2 and 3).

Both Fzd3 and Vangl2 Can Localize Dvl1 to Plasma Membrane of Commissural Axon Growth Cones

It is known that both Fzd3 and Vangl2 can bind to Dvls (Wong et al., 2003; Torban et al., 2004a). To further characterize the interactions of these PCP components during axon guidance, we analyzed the subcellular localization of PCP components in commissural axon growth cones. We coexpressed fluorescent fusion proteins of Fzd3, Dvl, and Vangl2 individually and in combination in dissociated commissural neurons by electroporation (Figure S2D). Frizzled3-mCherry showed both punctated distribution (intracellular vesicles) and smooth distribution (plasma membrane), consistent with its being a 7-pass transmembrane protein (red arrowhead in Figure 6A). Dvl1-EGFP alone in commissural neurons was found present only in intracellular punctates in both peripheral and central domains of growth cones (green arrowhead in Figure 6B). mCherry-Vangl2 was largely on plasma membrane with very small portion in intracellular vesicles, although it appeared to be aggregated in patches of plasma membrane on the filopodia and certain "hot spots" in the lamellipodia (large red arrow in Figure 6C). However, upon coexpression of Fzd3-mCherry and Dvl1-EGFP, the punctate staining of both decreased and the smooth plasma membrane staining increased (large red, green, or yellow arrows in Figures 6D–6F). Only the central domain or the core of the axon shaft shows some residual Dvl1-EGFP puncta, which may represent nascent Dvl-EGFP protein being transported along the

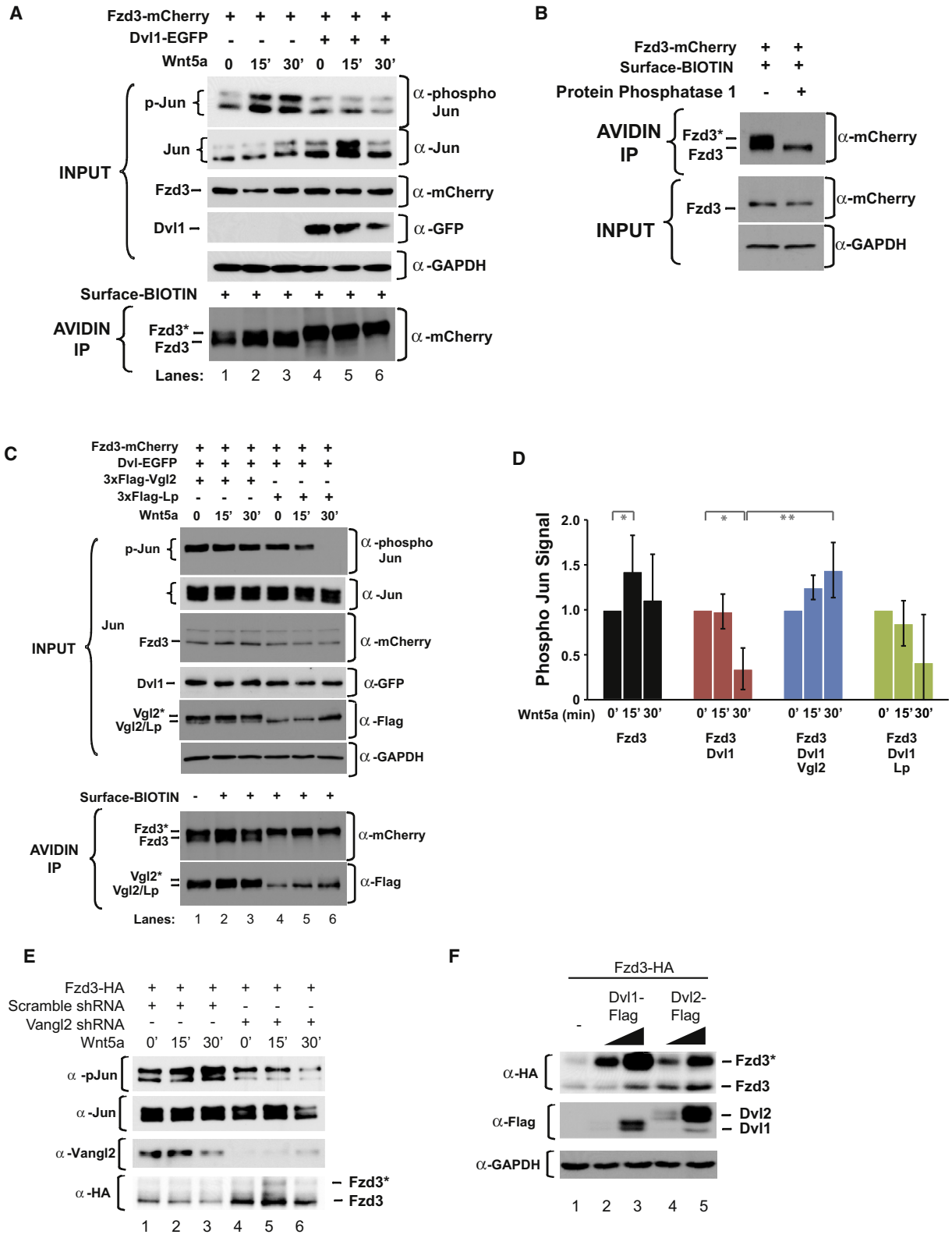

Figure 4. Vangl2 Antagonizes Dvl1's Feedback Inhibition

(A) JNK activity upon Wnt5a addition when Fzd3-mCherry was transfected alone or together with Dvl1-EGFP into HEK293T cells.

(B) Protein phosphatase 1 treatment of cell surface Fzd3-mCherry precipitated after surface biotinylation.

(C) Vangl2 prolongs the PCP signaling, but not Lp mutant.

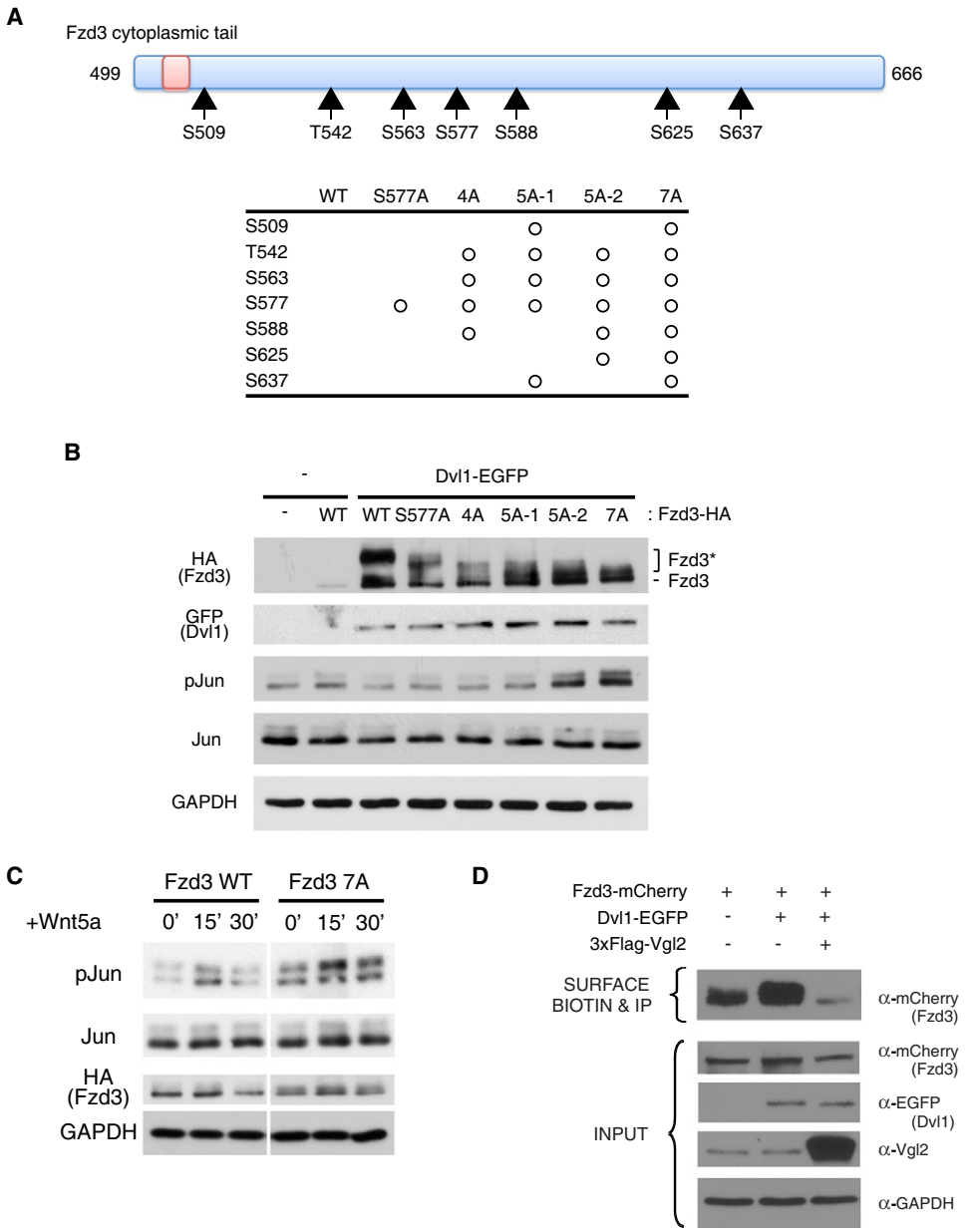

Figure 5. Frizzled3 Hyperphosphorylation Enhances and Prolongs PCP Signaling

(A) Putative phosphorylation sites in Frizzled3 cytoplasmic domain and the combinations of Frizzled3 point mutations created by site-directed mutagenesis.

(B) Increasing the number of phosphorylation site mutations correlated with reduction of the level of Frizzled3 hyperphosphorylation and increase of PCP activity.

(C) Frizzled3 phosphorylation mutation Frizzled3 7A resulted in higher PCP signaling level and prolong PCP activity.

(D) Fzd3 cell surface levels in the presence of Dvl1 or Vangl2.

microtubules, where it is unlikely to participate in signaling (green arrowheads in Figures 6E and 6F). Most of the Dvl1-EGFP becomes targeted to the plasma membrane (large green arrow in Figure 6E), compared with mostly puncta staining in Dvl1-EGFP only (green arrowhead in Figure 6B). This finding is in part similar to previous studies in other cellular contexts where Dvl was found recruited to the membrane by Fzds in the *Drosophila* epithelia (Axelrod et al., 1998). More importantly,

(D) Quantification of phospho-Jun level after normalizing time zero to the value of 1. The data were mean ± SD of six independent experiments of Fzd3, four experiments of Dvl1, three experiments of Vangl2 and Lp. *P value of < 0.05, **P value of < 0.005; t test.

(E) Knocking down Vangl2 with shRNA abolished Wnt5 stimulated PCP signaling.

(F) Dvl1, not Dvl2, induced mobility shift of Fzd3.

See also Figure S7.

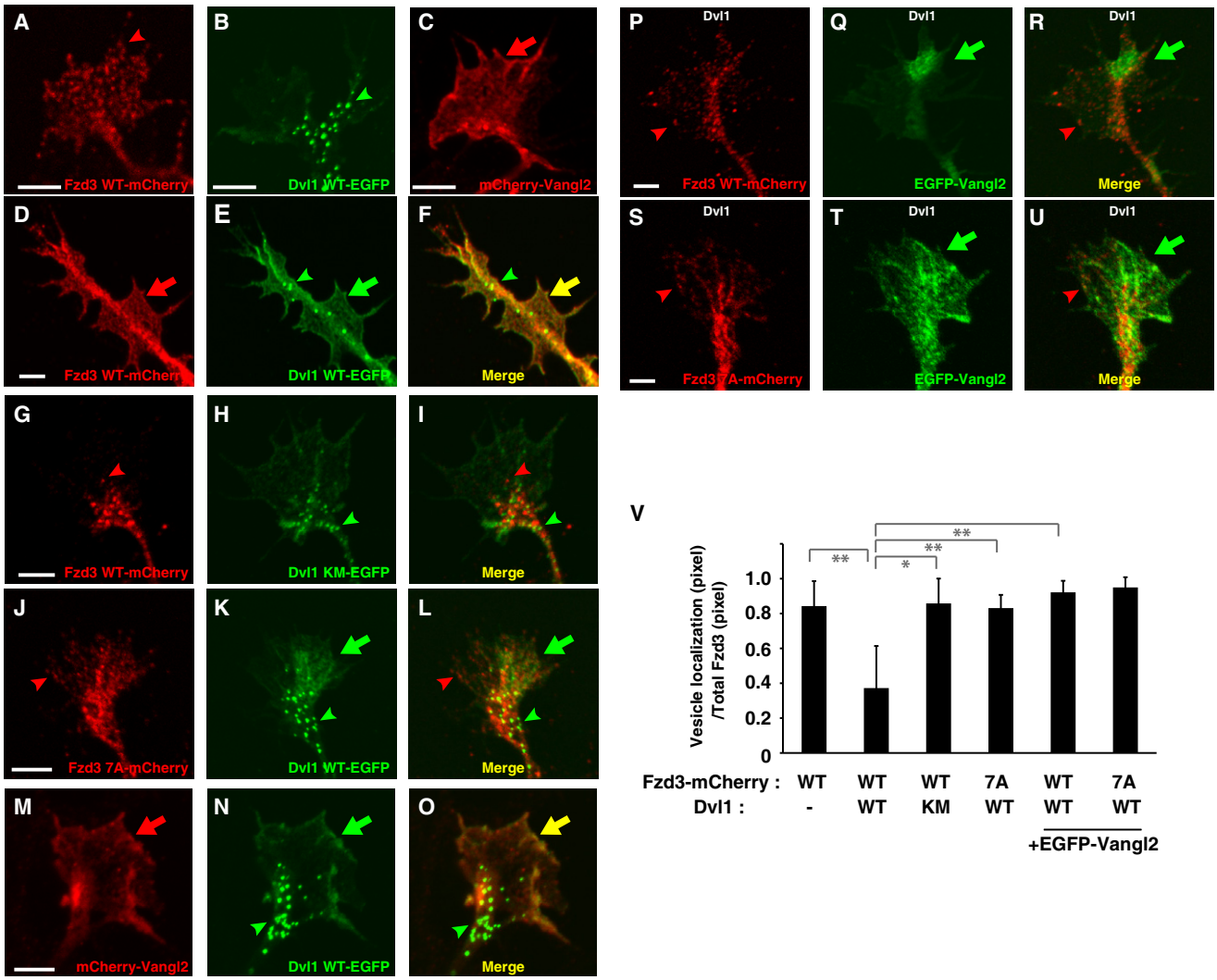

Figure 6. Both Fzd3 and Vangl2 Can Localize Dvl1 to Plasma Membrane of Commissural Axon Growth Cones

(A–C) Localization of Frizzled3-mCherry (A), Dvl1-EGFP (B), and mCherry-Vangl2 (C) in commissural axon growth cones. Arrowheads: intracellular vesicles; large arrows: plasma membrane localization.

(D–F) Localization of coexpressed Frizzled3-mCherry and Dvl1-EGFP.

(G–I) Localization of coexpressed Fzd3-mCherry and Dvl1 KM mutant-EGFP.

(J–L) Localization of coexpressed Frizzled3 (7A)-mCherry and Dvl1-EGFP.

(M–O) Localization of coexpressed mCherry-Vangl2 and Dvl1-EGFP.

(P–R) Localization of coexpressed Frizzled3-mCherry, EGFP-Vangl2 and Dvl1-HA.

(S–U) Localization of coexpressed Frizzled3 (7A)-mCherry, EGFP-Vangl2 and Dvl-HA. Scale bars: 5 μm.

(V) Quantification of Fzd3 vesicle localization. The pixel number of vesicle-like Fzd3-mCherry signal is divided by total pixel number of Fzd3-mCherry signal. Data are mean ± SD of ten growth cones. *p value of <0.01, **p value of <0.0005.

membrane localization of Dvl has been shown to be critical and specific to activation of PCP signaling (Axelrod et al., 1998; Park et al., 2005), and our data shows that Fzd3 coexpression with Dvl1 results in the membrane localization of Dvl in our commissural neuron growth cone, which suggest a protein localization consistent with the activation of PCP signaling.

We show here that coexpression with Dvl1 also resulted in Fzd3-mCherry stabilization at the plasma membrane (large red and yellow arrows in Figures 6D and 6F, 6V). A mutant Dvl1 (with DEP domain mutation) is unable to promote Fzd3 phosphorylation at the membrane (Figure S7B, lane 6). This lysine 438 to a methionine substitution in Dvl1 (Figure S7A) has been shown to be essential for PCP signaling and Dvl membrane association (Boutros et al., 1998; Moriguchi et al., 1999; Park et al., 2005; Simons et al., 2009). We expressed the Dvl1 (KM)-EGFP with Fzd3-mCherry and asked whether Fzd3 and Dvl1 mutant could be cotargeted to plasma membrane. Our results showed that Fzd3-mCherry localization in commissural neurons does not change when coexpressed with Dvl1 (KM)-EGFP (red arrowhead in Figures 6G and 6I, 6V) nor can Fzd3 recruit the DEP domain mutant Dvl1 to the plasma membrane (green arrowhead in Figures 6H and 6I). Because our results suggest that

Dvl1-induced Frizzled3 phosphorylation promotes plasma membrane localization (Figure 5D), we tested the localization of the mutant Frizzled3, Frizzled3 (7A), with most of the phosphorylation sites mutated (Figure 5) by coexpressing Frizzled3 (7A) with Dvl1 (Figures 6J–6L). We found that although Dvl1 was still able to target to plasma membrane (large green arrow in Figure 6K), Frizzled3 (7A) is mostly vesicular (red arrowheads in Figures 6J and 6L, 6V). No Dvl1-EGFP puncta were found in the peripheral domain of growth cone. Dvl1-EGFP puncta were only observed in the central domain of growth cone or the core of the axon shaft (green arrowheads in Figures 6K and 6L). This is consistent with the hypothesis that Frizzled3 hyperphosphorylation regulates its membrane localization and suggests that Dvl1 is likely still activated in the absence of Frizzled3 hyperphosphorylation, further supporting the model that Frizzled3 hyperphosphorylation correlates with Frizzzled3 inactivation and is not required for the PCP activity. In fact, the Frizzled3 hyperphosphorylation mutation caused higher PCP activity (Figure 5C).

Vangl1 and Vangl2 have been shown to bind Dvls (Park and Moon, 2002; Suriben et al., 2009; Torban et al., 2004b). We therefore asked whether Vangl2 can target Dvl1 to the plasma membrane. We found that Vangl2 can also target Dvl1 to the plasma membrane in the commissural growth cone (large red, green, and yellow arrows in Figures 6M–6O). mCherry-Vangl2 itself is localized primarily to the plasma membrane in commissural neurons (Figure 6C). When coexpressed with mCherry-Vangl2, Dvl1-EGFP becomes translocated to the membrane (large green arrow in Figure 6N and large yellow arrow in Figure 6O). Note that Dvl1-EGFP puncta, which can be observed in the peripheral domain of growth cones when expressed alone (green arrowhead in Figure 6B), can no longer be see in the peripheral domain when mCherry-Vangl2 is coexpressed and can only be seen in the central domain or the core of axon shaft (green arrowhead in Figure 6N). The mCherry-Vangl2 in the central domain or the core of axon shaft may be newly synthesized Vangl2 protein and may not participate in signaling. Therefore, Vangl2 may compete with Frizzled3 for Dvl1 on the plasma membrane, because Vangl2 is mainly on the plasma membrane. When Vangl2 removes Dvl1 from Frizzled3, Frizzled3 may become less hyperphosphorylated and may localize to the intracellular vesicles (red arrowhead in Figure 6A) rather than stay on the plasma membrane (large red arrow in Figure 6D). To test this, we did triple expression of Frizzled3-mCherry, EGPF-Vangl2 and Dvl1-HA. When Vangl2 and Fzd3 are coexpressed, Fzd3 is present more in intracellular vesicles and less on the plasma membrane in the commissural growth cone even in the presence of Dvl1-HA (red arrowhead in Figures 6P and 6R, 6V), compared with mostly plasma membrane localization when only Frizzled3-mCherry and Dvl1-EGFP are coexpressed. Therefore, we hypothesize that Vangl2 may compete with Frizzled3 for binding to Dvl1 and promote Frizzled3 internalization to allow PCP signaling to occur. Interestingly, we noticed that EGFP-Vangl2 was often found asymmetrically distributed in half or a smaller region of the growth cone in Vangl2, Frizzled3, and Dvl1 triple-transfected growth cones (large green arrow in Figures 6Q and 6R) and this asymmetric distribution was not observed in the Vangl2, Frizzled3 mutant (7A), and Dvl1 triple-transfected growth cones (Figures 6S–6U).

Vangl2 Protein Is Highly Enriched on Tips of Live Growth Cone Filopodia

Because Vangl2 may promote Frizzled3 endocytosis and thus PCP signaling by antagonizing Dvl1-induced Frizzled3 hyperphosphorylation and plasma membrane localization, we sought to pinpoint the precise localization of Vangl2 protein in live growth cones. We coexpressed EGFP-Vangl2, Frizzled3-mCherry, and Dvl1-HA in commissural neurons and used spinning disk confocal imaging to observe the localization of Vangl2 and Frizzled3. We found that Vangl2 protein is highly enriched in the plasma membrane in live growth cones, just as observed with mCherry-Vangl2 in fixed cells, and that it is also highly enriched in the tips of the growth cone filopodia (Figure 7A; see also Movie S1). Frizzled-mCherry is mostly localized in the intracellular vesicles (Figure 7B), similar to what we have observed in fixed cells when all three components are expressed (Figures 6M–6O). However, Frizzled3-mCherry can also be observed enriched on the tips of filopodia where EGFP-Vangl2 is enriched (Figures 7B and 7C), and where Vangl2 may promote Frizzled3 endocytosis. By looking at many individual filopodia at different time points in the confocal movies, we noticed that the filopodia that are elongating or stable tend to have high levels of Vangl2 expression and the shortening filopodia do not have high levels of Vangl2 protein (Figures 7D, 7E, and 7H; see also Movie S1). We quantified 149 filopodia from 8 growth cones and found that 36.9% of the filopodia tips with enriched EGFP-Vangl2 are moving forward (filopodia elongating). 46.8% of the filopodia tips with enriched EGFP-Vangl2 are stable (not elongating not shrinking). 16.3% of the filopodia tips with enriched EGFP-Vangl2 are moving forward (filopodia shrinking). On the other hand, 8.8% of the filopodia tips with no/low EGFP-Vangl2 are moving backward (mostly due to filopodia shrinking and occasionally due to shrinking of lamellipodia). 43.3% of the filopodia tips with no/low EGFP-Vangl2 are stable (not elongating not shrinking). 47.8% the filopodia tips with no/low EGFP-Vangl2 are moving backward (mostly due to filopodia shrinking and occasionally due to shrinking of lamellipodia). Interestingly, we observed similar behavior with our without Wnt5a protein addition, suggesting that the EGFP-Vangl2 localization to the tips may be an intrinsic property of these planar cell polarity proteins (data not shown).

Furthermore, new filopodia emerge from the hot spots of lamellipodia membrane where Vangl2 protein is present at higher concentration, suggesting that Vangl2 may also be able to initiate filopodia (Figures 7F–7H). All of the newly formed filopodia (58 newly formed filopodia) have highly enriched EGFP-Vangl2 (Movie S1). Taken together, Vangl2 may locally promote Frizzled3 internalization and PCP signaling on the tips of the growth cone filopodia by antagonizing Dvl1-induced Frizzled3 hyperphosphorylation and plasma membrane localization on the tips.

DISCUSSION

Our study shows that PCP components mediate Wnt attraction in commissural axons and reveals a cell-autonomous mechanism of how Van Gogh and Dvl may exert antagonistic biochemical and cell biological functions during cell polarity signaling. Our model suggests that when Wnt binds to Frizzled3 on the surface

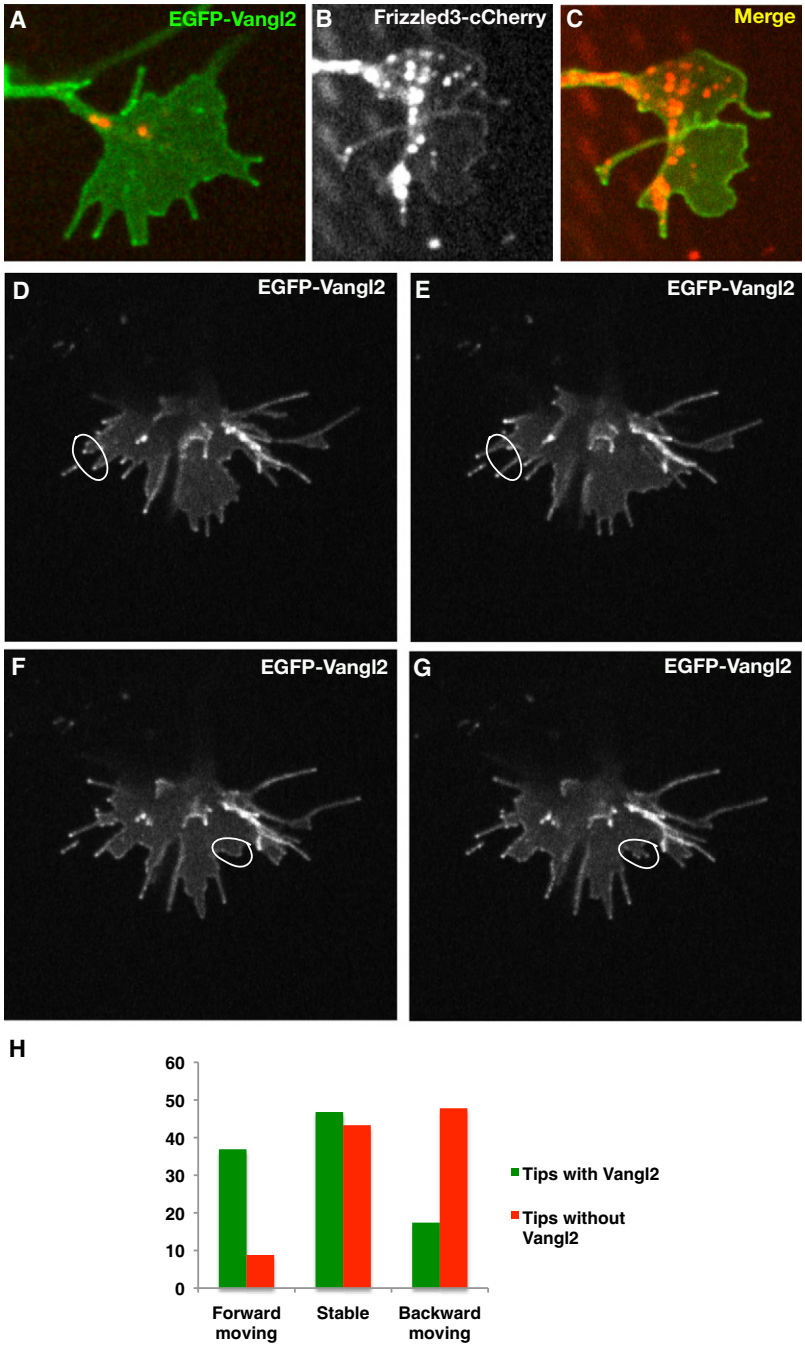

Figure 7. Vangl2 Protein Is Enriched on the Tips of Growth Cone Filopodia

(A–C) Localization of EGFP-Vangl2 and Fzd3-mCherry on the tips of filopodia in the presence of Wnt5a. Dvl1-HA is also expressed in this commissural neuron.

(D–G) Time-lapse imaging of EGFP-Vangl2 in the growth cone of commissural neuron. Frizzled3-mCherry and Dvl1-HA are also coexpressed. The circle indicates the area that new filopodia are about to form.

(H) Quantification of the filopodia with enriched EGFP-Vangl2 and their behavior.

See also Movie S1.

Evidence that Guidance Molecules May Enter the Growth Cone via the Tips of Filopodia

The growth cone is an elaborate apparatus that senses chemical cues in the environment and transmits signals to steer the growth of neuronal axons. Although many axon guidance cues have been discovered and their receptors identified, very little is known about how the cues are sensed and where the signals enter the growth cone. It has been proposed that the filopodia of growth cones explore the environment and the long filopodia are thought to be used to growth cones to maximize the span to sample greater concentration drop on gradient. Many studies also suggest that intracellular second messengers, calcium and cAMP, can be locally elevated in the filopodia, although it is hard to detect where does the calcium enter the filopodia or where does cAMP level first rises. Studies of actin dynamics and actin binding proteins showed that anticapping protein, Ena/VASP, is localized on the tip of the filamentous actin in the filopodia. The intracellular protein, Ena/VASP, is under the control of a number of axon guidance systems, such as Netrin and Slit, suggesting that the tips of the filopodia would be a place where signals enter the growth cone. However, until now no axon guidance receptor or receptor signaling component has been found enriched on the tips of the growth cone filopodia. We show here that an axon guidance receptor or a component of a receptor complex, Vangl2 plus Frizzled3, is enriched on the tips of growth cone filopodia. We propose that Vangl2 is a regulator of Frizzled3, which is a Wnt-binding receptor itself and the presence of the Vangl2 protein on the filopodia tip suggest that PCP signaling enters growth cone through these tips.

of commissural axon growth cones, it activate PCP signaling via a Disheveled (either Dvl1 or another Dvl). The activated Dvl1, however, subsequently negatively feeds back by promoting Fzd3 phosphorylation and accumulation of Fzd3 on plasma membrane, inhibiting PCP signaling. Vangl2, likely activated by Wnt via Frizzled or a different receptor, reduces Fzd3 phosphorylation and promotes Fzd3 internalization and PCP signaling. Vangl2 is highly enriched in the tips of the growth cone filopodia and may trigger Frizzled3 internalization and thus PCP signaling locally on the filopodia tips.

Wnts and PCP Signaling

Whether PCP signaling requires Wnt proteins is still under debate at least in some of the PCP signaling events, such as the *Drosophila* pupa wing epithelium. While testing whether the PCP components mediate Wnt functions in axon guidance, we show here that PCP components respond to Wnts in axonal

growth cones and are required for Wnt-mediated attraction and directional growth. In addition, we also found that PCP components, Dvl1 and Vangl2, have opposing effects on Wnt stimulated PCP signaling, measured by c-jun phosphorylation, with Dvl1 feedback inhibiting PCP and Vangl2 antagonizing the Dvl1-induced inhibition. Therefore, at least in growth cone guidance, Wnts appear to signal through the PCP pathway or a PCP-like pathway.

Cell Autonomous Interactions of Vangl2 and Dvl1 in PCP Signaling

PCP is a potent cell signaling system, which regulates polarized tissue morphogenesis and directed cell movement, now including growth cone guidance. In recent years, progress has been made on non-cell autonomous mechanisms, which involve cell-cell interactions. For example, Flamingo has been shown to bind to Van Gogh in a neighboring cell via the extracellular domain as well as Fzds, helping to establish an asymmetry (Chen et al., 2008; Devenport and Fuchs, 2008). On the other hand, the cell-autonomous mechanisms remain elusive, such as how Vgl and Dvl perform antagonistic functions to create asymmetric signaling. Cell autonomous mechanisms are clearly important regardless of whether they are upstream or downstream of non-cell autonomous events because asymmetric signaling activities within the cell are an essential part of the cell polarity signaling.

The antagonistic interaction between Vangl2 and Dvl1 may be a general mechanism in PCP signaling. This biochemical and cell biological mechanism provides a potential answer to why Van Gogh is usually not colocalized with Fzd/Dvl and they are often found on opposite sides of cells. In the *Drosophila* wing epithelial cells, Fzd/Dvl is absent on the proximal side of the cell where Van Gogh is enriched. It is possible that Van Gogh actively removes Fzd from the plasma membrane and Dvl actively keeps Fzd on the plasma membrane. This also implies that the proximal and the distal sides may have different types of PCP signal output. Furthermore, if Flm recruits Van Gogh to the proximal membrane from the neighboring cell and activates Van Gogh on the proximal membrane, Fzd will be excluded from the proximal membrane because of Van Gogh's function of promoting endocytosis. This may explain how cell-cell interaction is translated into intrinsic asymmetry.

Fzd3 Phosphorylation and Endocytosis

Although Fzd3 has been shown to be phosphorylated, how Fzd3 phosphorylation affects PCP signaling has not been elucidated (Djiane et al., 2005; Yanfeng et al., 2006). We show here that hyperphosphorylated Fzd3 is enriched on the plasma membrane and hypo-or nonphosphorylated Fzd3 is internalized. In addition, we identified several amino acids, which are necessary for hyperphosphorylation. Fzd3 can be phosphorylated on at least six to seven amino acids and our results also demonstrated that Fzd3 is indeed phosphorylated on multiple sites and unphosphorylatable Frizzled3 mutant enhanced and prolonged PCP signaling, consistent with our model. For example, our results suggest that at least the plasma membrane bound form of Fzd3 tends to be hyperphosphorylated and dephosphorylation of Fzd3 correlates with endocytosis and may represent an active form of Fzd3. If our model is correct, in the *Drosophila* wing epithelial cells, the distal membrane, where Fzd is present on the cell surface may be the area where PCP signaling output, or at least JNK, is inactivated (Simons and Mlodzik, 2008). By this prediction, Fzd protein on the distal membrane maybe phosphorylated or hyperphosphorylated. One such inactivating kinase could be atypical PKC, which phosphorylates Ser 500 of Fzd1 (Djiane et al., 2005). Furthermore, Fzd phosphorylation may affect how it interacts with other components. For example, the aPKC site of the Fzd intracellular domain in the Dvl interacting domain. Therefore, the phosphorylation state may affect Fzd's ability to bind to Dvl and thus block PCP signaling. For example, binding to Dvl could be a requirement for signaling and/or endocytosis. These questions will be addressed in future studies.

Vangl2 May Mediate Another Input of Wnt Signaling in PCP

Our study introduces the question of what regulates Vangl2 localization and activity. Several studies suggest that Flamingo and Frizzled can regulate Vangl2 in a non-cell autonomous manner via the extracellular domains. In this study, we also observed that a Flamingo homolog, Ceslr3, is required for normal A-P guidance. Future studies will address whether Flamingo plays similar roles in growth cone guidance, which may likely involve axon-axon interactions. Another Wnt receptor, Ror2, may be a plausible candidate that may regulate the localization (Minami et al., 2010) and activity of Vangl2. Vangl2 appears to be phosphorylated on serines and threonines (Figure 5C, anti-Flag IB; Figure S7C). Ror2 is a receptor tyrosine kinase and if Ror2 regulates Vangl2 via phosphorylation, it would need to involve other kinases. Because both Van Gogh and Fzd bind to Dvl, it is appealing to propose that Vangl2, once activated, may simply compete with Frizzled3 for Dvl1 binding and once Dvl1 is taken away from Frizzled3, Frizzled3 can no longer stay on the plasma membrane and will be endocytosis to allow PCP signaling to enter the cell. Future studies will test this hypothesis.

Cell Polarity Signaling Pathways May Convey Asymmetric Signaling in Growth Cone Guidance

Although many families of axon guidance molecules have been identified, how they signal to provide directional control of axon growth is still unknown. This study shows that the planar cell polarity signaling mediates Wnt function in A-P axon guidance. In navigating growth cones, the dynamic nature of these structures precludes the formation of stable adhesion junctions, therefore, the type of stable and prolonged cell-cell interaction observed in a stationary sheet of epithelial tissue is likely minor or nonexistent among growth cones. Therefore, navigating growth cones may rely on more sensitive cell autonomous mechanisms to detect and, more importantly, amplify the difference between the two sides of the growth cone. It should be noted that another important cell polarity signaling pathway, the apical-basal polarity pathway, which controls polarity perpendicular to the planar axis in epithelial cells may also provide additional guidance function. Atypical PKC (aPKC), a key component of the apical-basal polarity signing, was shown required for A-P guidance of commissural axons (Wolf et al., 2008). aPKC regulates of PCP signaling in the *Drosophila* eye, suggesting that these two pathways may be intimately coordinated to mediate growth cone guidance to potentially amplify signaling

asymmetry in the growth cone (Djiane et al., 2005). Future studies will address the interactions between PCP and apical-basal polarity signaling systems.

EXPERIMENTAL PROCEDURES

Open-Book Preparation and DiI Axon Labeling

Rat E13 spinal-open books were prepared as previously described (Wolf et al., 2008) and cultured for 5–6 hr ex vivo then for an additional 18 hr with either control or JNK inhibitors (25 μM JNK inhibitor-I and 50 μM SP600125) to obtain axons that are making their anterior-posterior turning decision before fixation with 4% PFA. Mouse E11.5 spinal cord open books were prepared and fixed immediately. Next, to visualize anterior posterior projection of commissural axons DiI labeling was used in the spinal open book preparation. Mouse open-book assay and DiI injections and data quantification were completed as previously described (Zou et al., 2000; Lyuksyutova et al., 2003). The number of injections sites and embryos used are indicated in Figure 1S and Figure S3N.

In Situ Hybridization

Mouse E11.5 embryos were fixed overnight at 4°C in 4% DEPC-treated PFA, frozen and sectioned. The in situ hybridization of the spinal sections were performed as previously described (Lyuksyutova et al., 2003), using digoxigenin-labeled riboprobes (Roche). All specific probes were obtained by RT-PCR from E11.5 mouse mRNA and subcloned into TOPO II vector (Invitrogen).

Immunohistochemistry

E11.5 mouse embryos of all wild-type, heterozygous, knock out and mutant embryos were fixed in 4% PFA for 2 hr on ice, frozen in OCT and sectioned at 14 μm slices for immunostaining. Immunostaining of spinal cord sections were performed as described previously (Lyuksyutova et al., 2003).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one movie, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2011.01.002.

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