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Dishevelled genes mediate a conserved mammalian PCP pathway to regulate convergent extension during neurulation

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The planar cell polarity (PCP) pathway is conserved throughout evolution, but it mediates distinct developmental processes. In *Drosophila*, members of the PCP pathway localize in a polarized fashion to specify the cellular polarity within the plane of the epithelium, perpendicular to the apicobasal axis of the cell. In *Xenopus* and zebrafish, several homologs of the components of the fly PCP pathway control convergent extension. We have shown previously that mammalian PCP homologs regulate both cell polarity and polarized extension in the cochlea in the mouse. Here we show, using mice with null mutations in two mammalian *Dishevelled* homologs, *Dvl1* and *Dvl2*, that during neurulation a homologous mammalian PCP pathway regulates concomitant lengthening and narrowing of the neural plate, a morphogenetic process defined as convergent extension. *Dvl2* genetically interacts with *Loop-tail*, a point mutation in the mammalian PCP gene *Vangl2*, during neurulation. By generating *Dvl2* BAC (bacterial artificial chromosome) transgenes and introducing different domain deletions and a point mutation identical to the *dsh1* allele in fly, we further demonstrated a high degree of conservation between Dvl function in mammalian convergent extension and the PCP pathway in fly. In the neuroepithelium of neurulating embryos, Dvl2 shows DEP domain-dependent membrane localization, a pre-requisite for its involvement in convergent extension. Intriguing, the *Loop-tail* mutation that disrupts both convergent extension in the neuroepithelium and PCP in the cochlea does not disrupt Dvl2 membrane distribution in the neuroepithelium, in contrast to its drastic effect on Dvl2 localization in the cochlea. These results are discussed in light of recent models on PCP and convergent extension.

KEY WORDS: Mouse, Planar cell polarity, Convergent extension, Neurulation

INTRODUCTION

Many of the conserved genetic pathways and cell biological mechanisms required during eukaryotic development have been uncovered over the past few decades, leading to a basic understanding of fundamental processes required during embryogenesis. During evolution, these conserved pathways and mechanisms are adapted and modified according to the demands of the evolved body plan. Two examples of conserved pathways are the Wnt and planar cell polarity (PCP) pathways, which share some common components (Cadigan and Nusse, 1997; Keller, 2002; Mlodzik, 2002; Myers et al., 2002; Tree et al., 2002a; Veeman et al., 2003a; Wallingford et al., 2002). The Wnt pathway was first defined in *Drosophila*, where it is required for the anteroposterior (AP) polarity of the body segments and cell fate determination (Cadigan and Nusse, 1997). In *Xenopus*, the Wnt pathway participates in axis determination and dorsoventral (DV) patterning (Cadigan and Nusse, 1997). In mammals, an expanded pathway has diverse effects on cell proliferation and patterning during gastrulation and organogenesis (Huelsken and Birchmeier, 2001; Wang and Wynshaw-Boris, 2004; Yamaguchi, 2001). Similarly, the conserved PCP pathway was first uncovered in *Drosophila*, where it regulates the polarity of a cell within the plane of the epithelium, perpendicular to the apicobasal axis of the cell. Manifestations of this polarity includes uniformly oriented parallel arrays of cellular extensions called trichomes on the epithelial cells of the wing and precisely coordinated orientation of ommatidial units of the compound eye (Strutt, 2002; Tree et al., 2002a; Veeman et al., 2003a). In *Xenopus* and zebrafish, an homologous pathway regulates convergent extension, a coordinated extension of the AP axis with concomitant narrowing of the mediolateral (ML) axis (Carreira-Barbosa et al., 2003; Darken et al., 2002; Goto and Keller, 2002; Keller, 2002; Kinoshita et al., 2003; Park and Moon, 2002; Takeuchi et al., 2003; Veeman et al., 2003b; Wallingford et al., 2002; Wallingford et al., 2000). During convergent extension in these animals, several PCP proteins have been shown to control the polarity of lamellipodial protrusions that drive cell intercalation (Jessen et al., 2002; Wallingford et al., 2000). In zebrafish, the PCP pathway also appears to regulate convergent extension, at least in part, through determining the orientation of cell division (Gong et al., 2004).

The multifunctional protein Dishevelled (Dsh in fly, XDsh in *Xenopus*, and Dvl1, Dvl2 and Dvl3 in mammals) is involved in both the canonical Wnt signaling pathway as well as the PCP pathway (Mlodzik, 2002; Strutt, 2002; Tree et al., 2002a; Veeman et al.,

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2003a; Wallingford et al., 2002; Wallingford and Habas, 2005). In the canonical Wnt pathway, Dsh is important in transmitting a signal initiated from Wnt binding to its receptor Frizzled and co-receptor Arrow/Lrp5/6 on the cell surface. As a consequence, the cytoplasmic protein Armadillo/β-catenin becomes stabilized and is transported into the nucleus to activate transcription (He et al., 2004; Wang and Wynshaw-Boris, 2004). In the PCP pathway in fly, Dsh participates with a different set of proteins such as Strabismus (Stbm), Prickle (Pk), Diego (Dgo) and Flamingo (Fmi) (Das et al., 2004; Jenny et al., 2005; Strutt, 2002; Tree et al., 2002a; Veeman et al., 2003a). Although the precise mechanisms through which Dsh determine PCP is not well defined, an asymmetric plasma membrane localization of Dsh and the other PCP members appears to be both required for and dependent upon proper establishment of the PCP pathway in the fly (Axelrod, 2001; Bastock et al., 2003; Das et al., 2004; Jenny et al., 2003; Jenny et al., 2005; Rawls and Wolff, 2003; Strutt et al., 2002; Strutt, 2002; Tree et al., 2002a; Tree et al., 2002b; Veeman et al., 2003a).

Recent genetic studies have also provided evidence that a putative PCP pathway exists in mammals (Curtin et al., 2003; Hamblet et al., 2002; Kibar et al., 2001; Montcouquiol et al., 2003; Murdoch et al., 2001; Wang et al., 2005; Wang et al., 2006). *Loop tail* (*Lp*) is a point mutation of *Ltap* or *Vangl2*, a homolog of the fly PCP component *Stbm* (Kibar et al., 2001; Montcouquiol et al., 2003; Murdoch et al., 2001), while *Crash* and *Spin cycle* (*Crsh, Scy*) are distinct mutations of *Celsr1*, a homolog of the PCP pathway member *fmi* (Curtin et al., 2003). Frizzled 3 (*Fzd3*) and frizzled 6 (*Fzd6*) encode two of the Frizzled receptors in mammals (Wang et al., 2006). *Lp*, *Crsh*, *Scy*, $Fzd3^{-/-}$; $Fzd6^{-/-}$ and $Dvl1^{-/-}$; $Dvl2^{-/-}$ mutants result in misorientation of stereocilia of sensory hair cells in the cochlea. The uniform orientation of the stereocilia on hair cells in the cochlea has been proposed to be a manifestation of PCP (Lewis and Davies, 2002). Indeed, we found that a transgenic Dvl2-EGFP protein capable of rescuing the stereocilia orientation defects in $Dvl1^{-/-}$; $Dvl2^{-/-}$ mutants displayed asymmetric plasma membrane localization that is disrupted in *Lp/Lp* embryos (Wang et al., 2005). Others reported a similar *Vangl2*-dependent asymmetric localization of Fzd3 and Fzd6 in the sensory hair cells, utricles and cristae (Wang et al., 2006), suggesting a conserved PCP pathway as the underlying mechanism in coordinating stereocilia orientation. In addition, *Lp*, *Crsh, Scy*, $Fzd3^{-/-}$; $Fzd6^{-/-}$ and $Dvl1^{-/-}$; $Dvl2^{-/-}$ mutants all result in a unique severe neural tube closure defect in which the entire neural tube from mid-brain to tail fails to close, a congenital defect termed craniorachischisis in humans (Curtin et al., 2003; Hamblet et al., 2002; Kibar et al., 2001; Murdoch et al., 2001; Wang et al., 2006). The cause of craniorachischisis in these mutants has been inferred to be failure of convergent extension, because, in *Xenopus*, overexpression of wild-type or mutant forms of XDsh and Stbm (which blocks convergent extension) usually results in similar neural tube closure defects (Copp et al., 2003; Darken et al., 2002; Goto and Keller, 2002; Ueno and Greene, 2003; Wallingford and Harland, 2002). However, the developmental mechanisms disrupted by the loss of conserved mammalian PCP pathway members during neurulation have not been investigated. There is no experimental evidence that the neural tube defect in these PCP mutant mice results from convergent extension defects, and if so, it is not clear how PCP regulates convergent extension in mammals.

In this study, we provide evidence that during neurulation, *Dvl1* and *Dvl2* are required for concomitant lengthening and narrowing of neural plate, a morphogenetic process that resembles convergent extension. Using a *Dvl2* BAC transgene expressing a functional Dvl2-EGFP protein, we found that subcellular Dvl2 localization in neuroepithelium is consistent with its function in convergent extension. Intriguingly, although *Lp* genetically interacts with Dvl genes during neurulation and in cochlea development, *Lp* appears to have no effect on Dvl2 localization in the neuroepithelium, in contrast to its drastic effect on Dvl2 distribution in the cochlea (Wang et al., 2005). We found that part of the N-terminal DIX domain, which is essential for Dvl2 function in the Wnt pathway (Capelluto et al., 2002), was largely dispensable for its function during neurulation or recruitment to the plasma membrane. By contrast, the C-terminal DEP domain, which is solely required for Dsh/Dvl function in PCP/convergent extension in flies and *Xenopus* (Axelrod et al., 1998; Boutros et al., 1998; Rothbacher et al., 2000; Wallingford and Harland, 2001; Wallingford et al., 2000), was essential for Dvl2 function in neurulation and for plasma membrane localization. To further test whether we can genetically distinguish PCP and convergent extension, we produced a *Dvl2* BAC transgene carrying a point mutation identical to the *dsh1* allele in fly, which specifically abolishes the PCP pathway. Our analysis of the mutant transgene suggests stringent conservation of Dvl function in convergent extension and PCP.

MATERIALS AND METHODS

Length-to-width ratio (LWR) measurement in neurulating embryos

Embryos derived from appropriate crosses were dissected at E8.5 and yolk sacs were removed for PCR genotyping. Embryos were fixed in 4% paraformaldehyde at 4°C overnight and stored in PBS. After counting somite numbers, embryos were transferred into an empty dish and allowed to extend naturally. Embryos were photographed with a Spot CCD camera mounted on a Leica MZFL *III* dissection microscope. Images of both dorsal and ventral view of each embryo were acquired and imported into ImageProPlus, and length-to-width ratio measurements of the trunk were made as follows. On each embryo, a line was drawn along the body edge from the base of the allantoic bud to the base of the headfold. The average of the traces on the left and right sides was used for the length measurement, the average distance between these two traces was determined by ImageProPlus and used for the width measurement. Averages of the LWR derived from ventral and dorsal view image were taken as the final LWR of the embryo. At least three embryos of each genotype at each somite stage were measured. Statistical analysis was performed by using the Mann-Whitney U-test through GraphPad InStat software.

Generating *Dvl2-EGFP* and mutant *ΔDIX-EGFP*, *ΔDEP-EGFP* and *dsh1-EGFP* BAC transgenes

A BAC clone containing the *Dvl2* genomic sequence, derived from a PCR screen of a BAC library from Genome Systems, was characterized for genomic integrity and potential rearrangement by using 11 overlapping PCR reactions that covered the entire *Dvl2* genomic region. The existence of at least two flanking genes on the 3′ and 5′ ends were confirmed by designing specific PCR primers based on the sequence information in the mouse genome database. Modifications of the BAC, including insertion of two LoxP sites and EGFP-coding sequence in-frame to the last codon of *Dvl2* were performed as described (Lee et al., 2001). *Dvl2-EGFP1* had an EGFP cassette fused in-frame to the last codon of *Dvl2* and a LoxP site inserted into intron 2 for genotyping purpose (see Mouse strains and genotyping), whereas *Dvl2-EGFP2* contained an additional LoxP site inserted in the 3′ flanking gene *Acadvl* (acyl-Coenzyme A dehydrogenase, very long chain). Using BAC recombineering techniques, *ΔDIX-EGFP* and *ΔDEP-EGFP* deletion mutant transgenes were generated by removing sequence encoding amino acids 67-159 and amino acids 442 to 736, respectively. *dsh1-EGFP* point mutation was generated by replacing AAG with ATG to introduce a K to M substitution at amino acid 446. Modified BACs were purified and pronuclear injection was performed as described to create transgenic mice (Lee et al., 2001).

Mouse strains and genotyping

Mice carrying *Lp* mutation was obtained from the Jackson Laboratory (Bar Harbor, ME) and genotyped as described (Murdoch et al., 2001). Genotyping of the targeted *Dvl1* and *Dvl2* alleles was performed as described (Hamblet et al., 2002). To differentiate the *Dvl2-EGFP* BAC transgene from the endogenous wild-type *Dvl2*, a pair of primers flanking intron 2 were used (E2F, TGCTTCAATGGAAGGGTTGTC; E3R, TTCTGTCCGAGACTCATGGG) for PCR (94°C for 20 seconds, 56°C for 20 seconds, 72°C for 30 seconds; 35 cycles). Endogenous wild-type *Dvl2* allele gave rise to a PCR product of 370 bp, while the *Dvl2-EGFP* or *dsh1-EGFP* transgenes, owing to the insertion of the 34 bp LoxP site in intero 2, gave rise to a PCR product of 404 bp. The targeted *Dvl2*-null allele would not be amplified in this PCR reaction. The same PCR was used to genotype for *ΔDEP-EGFP* and *dsh1-EGFP* transgenes. To genotype and differentiate *ΔDIX-EGFP* transgenes from the endogenous *Dvl2* and *Dvl2* null alleles, a three primer PCR genotyping strategy was used (I1F, TTGCTCACTGAGTGCCTCTTGC; E3R, TTCTGTCCGAGACTCATGGG; R3, GCCATGTTCACTGCTGTCTCTC).

Confocal microscopy

To visualize native GFP signal from *Dvl2-EGFP* or *dsh1-EGFP* mice, embryos or cochleas of appropriate stages were dissected and briefly fixed in 4% paraformaldehyde at 4°C for 1 hour. Embryos were then counterstained with Alexa Fluor 568 phalloidin (A-12380, Molecular Probe) according to the manufacturer's protocol. For antibody staining, embryos were fixed in 4% paraformaldehyde at 4°C overnight. After blocking in PBS + 5% normal goat serum, embryos were stained with an anti-GFP antibody (A-6455, Molecular Probe, 1:1000) and a donkey anti-rabbit IgG antibody (A-21206, Molecular Probe, 1:1000). Embryos were counterstained with Alexa Fluor 568 phalloidin. For analyzing planar cell polarity of the organ of Corti, whole-mount organs of Corti were stained with rhodamine or fluoroscein-conjugated phalloidin. Image was acquired using an Olympus FV 1000 confocal scanning microscope.

RESULTS

Dvl1$^{-/-}$; *Dvl2*$^{-/-}$ mutants disrupt concomitant lengthening and narrowing of neural plate

As previously reported, 2-3% of *Dvl2*$^{-/-}$ single mutants display spina bifida, while *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants display craniorachischisis (Hamblet et al., 2002). *Dvl2*$^{-/-}$; *Dvl3*$^{-/-}$ double mutants also display craniorachischisis (J.W., N.S.H. and A.W.-B., unpublished). However, *Dvl1*$^{-/-}$ (Lijam et al., 1997) and *Dvl3*$^{-/-}$ single mutants or *Dvl1*$^{-/-}$; *Dvl3*$^{-/-}$ double mutants (J.W. and A.W.-B., unpublished) do not display neural tube defects. These findings indicate that *Dvl2* is the most important mammalian Dvl gene for neural tube closure and is sufficient by itself for normal neural tube closure. By contrast, *Dvl1* and *Dvl3* are not sufficient by themselves for normal neural tube closure, but contribute significantly when *Dvl2* is completely missing.

In an effort to determine the cause of the craniorachischisis defect in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants, we performed in situ hybridization to determine dorsoventral (DV) patterning as defective DV patterning in the spinal cord is known to cause severe neural tube closure defects (Copp et al., 2003; Ybot-Gonzalez et al., 2002). We found normal dorsal expression of *Wnt1*, *Pax3* and *Engrailed2* and ventral expression of *sonic hedgehog* (*shh*) in the spinal cord of *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants (data not shown). These results suggest that the failure of neural tube closure in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants is not due to abnormal DV patterning. Furthermore, the normal expression of *En2*, a known Wnt target gene, suggests that the canonical Wnt pathway was not globally disrupted in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants.

Recent studies in *Xenopus* have indicated an essential role of XDsh-mediated convergent extension morphogenetic movement in neural tube closure (Wallingford and Harland, 2002). In *Xenopus*, convergent extension results in coordinated elongation of the AP axis and narrowing of the ML axis, and is reflected as a continuous increase of the length-to-width ratio (LWR) during neurulation (Wallingford et al., 2002; Wallingford and Harland, 2002). To determine whether *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants display defects consistent with a disruption of convergent extension, we adopted the methods used in *Xenopus* (Wallingford and Harland, 2002) and measured the LWR of neurulating mouse embryos (Fig. 1; Materials and methods). In control embryos, we found that the LWR continuously increased during neurulation (Fig. 1E). However, when we systematically measured *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutant embryos, we found consistent, significant reduction of LWR from the earliest time point where we could perform this analysis (Fig. 1E). More strikingly, from the five- to seven-somite stages, immediately before the first apposition of neural folds at closure 1 at the 7-somite stage in control embryos (Fig. 1C) (Ybot-Gonzalez et al., 2002), there was virtually no increase of the LWR in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutant embryos.

This result reveals that, like *Xenopus*, convergent extension, which is defined as concomitant lengthening and narrowing of a tissue (Wallingford et al., 2002), occurs during mouse neurulation and is affected in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants prior to the time at which neural tube closure defects are observed. As suggested in *Xenopus* (Wallingford and Harland, 2002), the resulting wider neural plate in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants may also be incompatible with normal neural tube closure in the mouse.

Null (Pinson et al., 2000) and hypomorphic (Kokubu et al., 2004) alleles of the Wnt co-receptor *Lrp6* result in the less severe neural tube defects exencephaly or spina bifida, implicating the canonical Wnt pathway in neural tube closure. In addition, the canonical Wnt pathway is crucial for the elongation of the AP axis through, at least in part, activating brachyury (*T*) expression (Yamaguchi et al., 1999). Therefore, we analyzed the expression of *T* to confirm that the reduced LWR was not due to the loss of function of the canonical Wnt pathway. In the early headfold stage, *T* is expressed in the notochord, node and primitive streak. In *Wnt3a*-null mice, *T* expression was reported to be lost in the anterior primitive streak. The loss of *T* expression was interpreted as the cause of axis shortening in *Wnt3a*$^{-/-}$ mutants (Yamaguchi et al., 1999). However, in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants, *T* expression in the anterior primitive streak appeared to be normal, suggesting that the reduced LWR was not due to a failure of the canonical Wnt pathway (Fig. 2A,B) and that reduced dose of Dvl in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants does not cause global loss of function of Wnt signaling.

In contrast to the primitive streak, we observed a different pattern of *T* staining in the notochord. In the wild-type embryos, the notochord was slender and there were no *T*-positive cells outside of the notochord (Fig. 2A). In the *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants, however, the notochord was wider, and there were also a few *T*-positive cells outside of the notochord (Fig. 2B). As lengthening of the notochord in the mouse was argued to be driven by convergent extension-like cell movements in the absence of cell division (Beddington, 1994), the different pattern of *T* staining in the *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants suggests a reduction of PCP-dependent convergence of the notochord cells towards midline.

Lp/Lp embryos also display disruption of convergent extension

To test the validity of this hypothesis, we examined *Lp* mutant that harbors a loss-of-function mutation in the mouse PCP gene *Vangl2* (Kibar et al., 2003; Kibar et al., 2001; Murdoch et al., 2001). When we

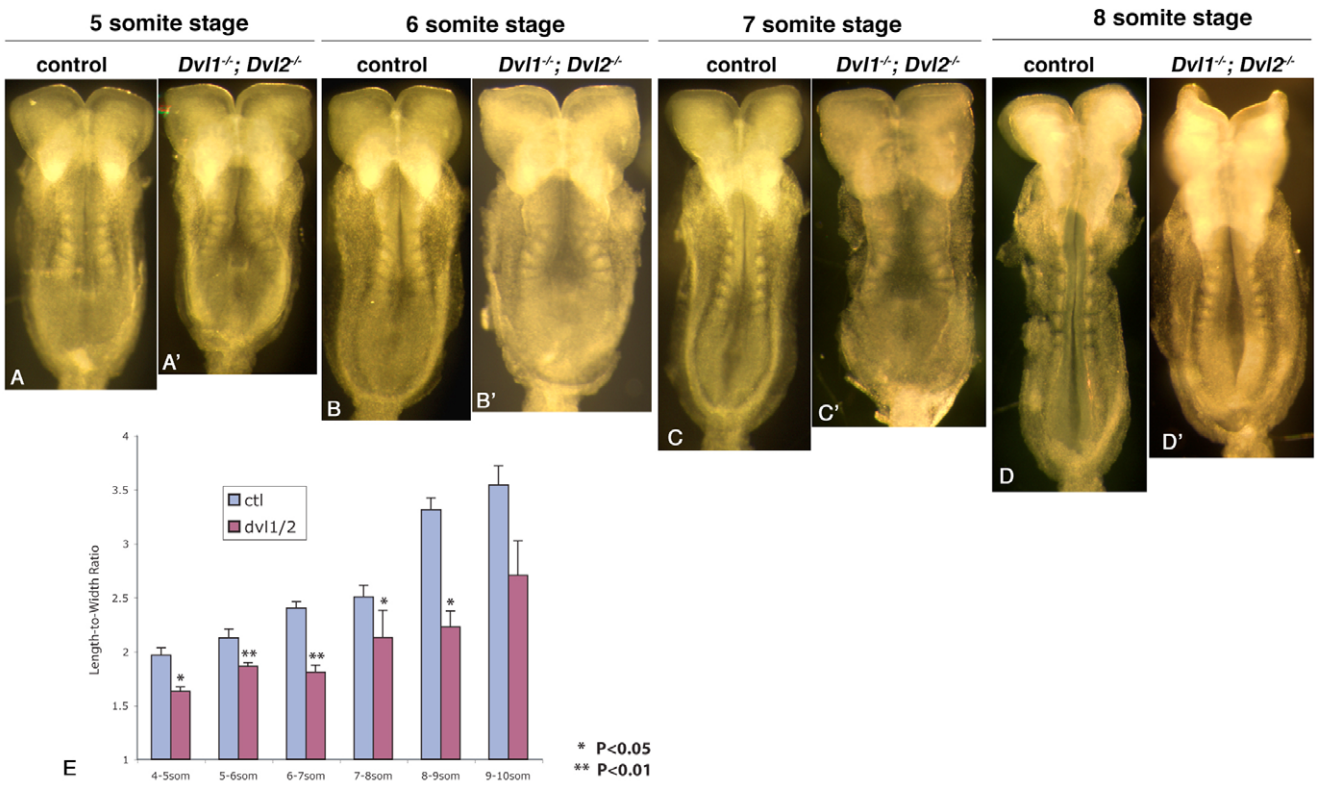

Fig. 1. *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants displayed reduced length-to-width ratio during neurulation. (**A-D**) Five- to eight-somite stage *Dvl1*$^{-/-}$; *Dvl2*$^{+/-}$ or *Dvl1*$^{-/-}$; *Dvl2*$^{+/+}$ control embryos. (**A'-D'**) *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants of equivalent somite stages. Floor plates are expanded in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants. (**E**) Length-to-width ratio (LWR) changes of control and *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutant embryos at different somite stage during neurulation. Each point represents the average of at least three separate embryos. The error bars represent standard deviation. **P*<0.05; ***P*<0.01.

analyzed *T* expression in the *Lp/Lp* mouse embryos, we observed a similar broadening of the notochord, as well as stranding of *T*-positive cells outside of the notochord, further evidence that these defects could be due to a disruption of convergent extension (Fig. 2C,D). Furthermore, when we determined the LWR in neurulating *Lp/Lp* mutant embryos, we found that, similar to *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants, *Lp/Lp* embryos displayed a significant reduction of the LWR prior to closure 1 (Fig. 3A). *Lp/+* mutants display kinky or looped tails but nonetheless manage to close their neural tubes in most cases, and displayed a moderate reduction of LWR. Accompanying the reduced LWR, *Lp/+* embryos displayed delayed closure of neural tube: their neural tubes often remained unfused at the 8- to 9-somite stage, while in the same stage wild-type embryos, neural tube has fused extensively along the AP axis (Fig. 3B,C). These results provide further evidence that the mammalian PCP pathway regulates convergent extension to effect neural tube closure in the mouse.

Dvl2 and *Lp* genetically interact during neurulation

As *Dsh* and *Stbm* interact with each other and both are involved in the PCP in fly and convergent extension in *Xenopus* and zebrafish (Darken et al., 2002; Goto and Keller, 2002; Park and Moon, 2002; Wallingford and Harland, 2002; Wallingford et al., 2000), we determined whether *Dvl* and *Lp* genetically interact during mouse neurulation. We crossed *Dvl1*$^{+/-}$; *Dvl2*$^{+/-}$; *Lp/+* triple heterozygotes with *Dvl2*$^{-/-}$ homozygotes, and genotyped the surviving offspring (Table 1). Out of 48 pups that survived, 50% of the expected *Dvl2*$^{-/-}$ homozygotes were recovered (expected six, recovered three), consistent with our previous observation that 50% of *Dvl2*$^{-/-}$ mutants die at birth because of heart defects (Hamblet et al., 2002). Reducing the dose of *Dvl1* by half did not affect the viability of *Dvl2*$^{-/-}$ mutants as we were still able to recover 50% of the expected *Dvl1*$^{+/-}$; *Dvl2*$^{-/-}$ mutants (Table 1). However, reducing the dose of *Lp* by half had much more deleterious effect on the viability of *Dvl2*$^{-/-}$ mutants, as we recovered no neonates that were *Dvl2*$^{-/-}$; *Lp/+*, either with or without additional *Dvl1* mutation.

When we dissected the embryos from this mating at midgestation stages, we found that *Dvl1*$^{+/-}$; *Dvl2*$^{-/-}$ and *Dvl2*$^{-/-}$ mutants had closed neural tubes. By contrast, all *Dvl1*$^{+/-}$; *Dvl2*$^{-/-}$; *Lp/+* and *Dvl2*$^{-/-}$; *Lp/+* showed craniorachischisis, a phenocopy of the *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ and *Lp/Lp* mutants (Fig. 3F,G). This result indicates that *Dvl2* and *Lp* genetically interact and suggests they function together in the mouse to promote convergent extension during neurulation. Consistent with this hypothesis, *Dvl2*$^{-/-}$; *Lp/+* embryos displayed a severe reduction of the LWR prior to closure 1, resembling *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ and *Lp/Lp* mutants (Fig. 3E).

Association of neural tube closure defect with PCP defect in the cochlea

In addition to the neural tube closure defect, we recently found that *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants and *Dvl2*$^{-/-}$; *Lp/+* also display disruption of the uniform stereociliary bundle orientation in the cochlea (Wang et al., 2005), a manifestation of mammalian PCP (Klein and Mlodzik, 2005; Lewis and Davies, 2002). The association of these two phenotypes has also been reported in two other mammalian PCP mutants identified so far, *Lp* and *Celsr*, strongly implying that a conserved PCP pathway regulates both hair cell polarity and neural tube closure.

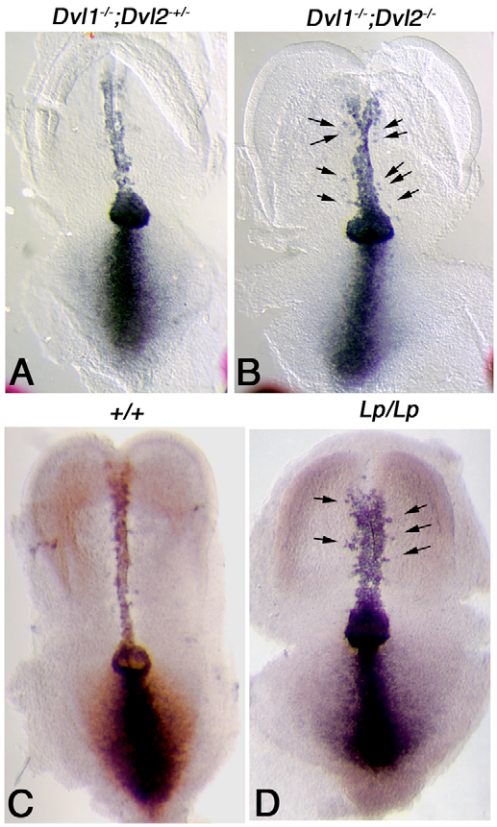

Fig. 2. In situ hybridization analysis of *Brachyury* (*T*) in early headfold stage *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ and *Lp/Lp* mutant embryos. (**A**) *T*, a known downstream target gene of the canonical Wnt pathway, was expressed in the notochord, node and primitive streak in control embryos (*Dvl1*$^{-/-}$; *Dvl2*$^{+/-}$). (**B**) In *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants, *T* expression in the node and primitive streak was maintained, suggesting normal activity of the Wnt pathway. However, *T* expression in the notochord was abnormally widened. *T*-positive cells were also observed outside of the notochord (arrows), consistent with a disruption of cell movement towards the central midline. Compared with wild-type littermates (**C**), *T* expression was also widened in the notochord of *Lp/Lp* embryos (**D**). *T*-positive cells were also observed outside of the notochord (D, arrows).

We further found that both defects can be modified by genetic background variation. Although both defects were close to 100% penetrant in an inbred 129 SvEv background, *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants showed incompletely penetrant neural tube closure defects in a mixed 129 SvEv/C57B6 background. When F1 *Dvl1*$^{+/-}$; *Dvl2*$^{+/-}$ mice, derived from an intercross between inbred 129 *Dvl1*$^{-/-}$; *Dvl2*$^{+/-}$ mice and wild-type C57B6 mice, were back-crossed to 129 *Dvl1*$^{-/-}$; *Dvl2*$^{+/-}$ mice, 13% of the expected F2 *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants survived to adults (out of 557 weaning age progeny genotyped, 86 *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ expected, 11 recovered), indicating successful neural tube closure during embryogenesis. This result suggests that multiple dominant modifiers from C57B6 background act together to rescue this phenotype. Interestingly, in all *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants, either recovered at E18.5 or as adults, where the neural tube defects were rescued, the stereociliary bundles in cochlea also displayed normal polarity (data not shown), while all E18.5 *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants that showed craniorachischisis also displayed stereociliary polarity defects (n=10) (see Wang et al., 2005). The strict association between neural tube closure and stereociliary polarity in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants indicates that both phenotypes can be modified by genetic background variation to the same extent, further arguing a highly conserved genetic circuitry underlies both mammalian convergent extension and PCP.

Plasma membrane distribution of Dvl2 and its dependence on the DEP domain during neurulation

During fly wing development, it has been reported that Dsh proteins are localized asymmetrically on the plasma membrane (Axelrod, 2001; Bastock et al., 2003). This asymmetric membrane distribution is both dependent upon and important for the proper establishment of planar cell polarity (Axelrod, 2001; Bastock et al., 2003). We recently generated a *Dvl2* bacterial artificial chromosome (BAC) transgene that contains an EGFP-coding sequence inserted in-frame to the last codon of *Dvl2* (Wang et al., 2005) and found that transgenic Dvl2-EGFP protein displayed a Vangl2-dependent polarized membrane distribution in the organ of Corti. Furthermore, the transgene was also able to rescue the cochlea elongation and stereocilia orientation defects in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants (Wang et al., 2005), indicating that the *Dvl2* BAC transgene can fully substitute for the endogenous *Dvl2* function during inner ear development.

We further found that this *Dvl2* BAC transgene, as well as an additional *Dvl2* BAC transgene (*Dvl2-EGFP2*, Fig. 4A) that contains two flanking LoxP sites, was also able to rescue *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ and *Dvl2*$^{-/-}$; *Lp/+* mutants to fertile adults, suggesting that the encoded Dvl2-EGFP fusion protein could also substitute for the endogenous Dvl2 during neurulation (Fig. 4B). Consistent results were acquired from all six independent lines derived from the two transgenes, suggesting BAC transgenic approach is a reliable method to produce transgenic protein capable of replacing endogenous Dvl2, presumably owing to its ability to recapitulate more consistently the endogenous gene expression pattern and level (Lee et al., 2001).

We investigated whether the Dvl2 protein localized in a polarized fashion during mouse neurulation using these *Dvl2* BAC transgenes. In neurulating embryos of different somite stages, we observed the Dvl2-EGFP transgenic protein primarily localized to the plasma membrane in the neuroepithelium (Fig. 5A,E,I). The more diffuse apparent distribution of Dvl2-EGFP and F-actin in some cells in Fig. 5A-D was the result of capture of a single focal plane immediately underneath the apical plasma membrane, presumably reflecting a close association of Dvl2-EGFP to the apical plasma member. When the entire series of z stacks were examined (data not shown), it was clear that both Dvl and actin were localized to the lateral plasma membrane as individual optical sections are scanned more basally through the cell. Nevertheless, we could not find any evidence for an asymmetric pattern of Dvl2-EGFP distribution on the plane of the neuroepithelium (Fig. 5A,E,I; data not shown). Furthermore, the *Lp* mutation did not affect the membrane localization of Dvl2-EGFP as we observed the same membrane distribution pattern in *Lp/Lp* mutants (Fig. 5B,F,J; data not shown). Consistent results were observed in multiple transgenic lines using confocal microscopy of Dvl2-EGFP from live (Fig. 5I,J) and briefly fixed embryos (Fig. 5E,F), or from permanently fixed embryos stained with an anti-GFP antibody (Fig. 5A,B). In addition, although manipulating the activity of a number of PCP homologs in *Xenopus* and zebrafish could alter the cell orientation and elongation along the ML axis (Jessen et al., 2002; Veeman et al., 2003b; Wallingford et al., 2000), our analysis did not reveal obvious differences of cell polarity in the

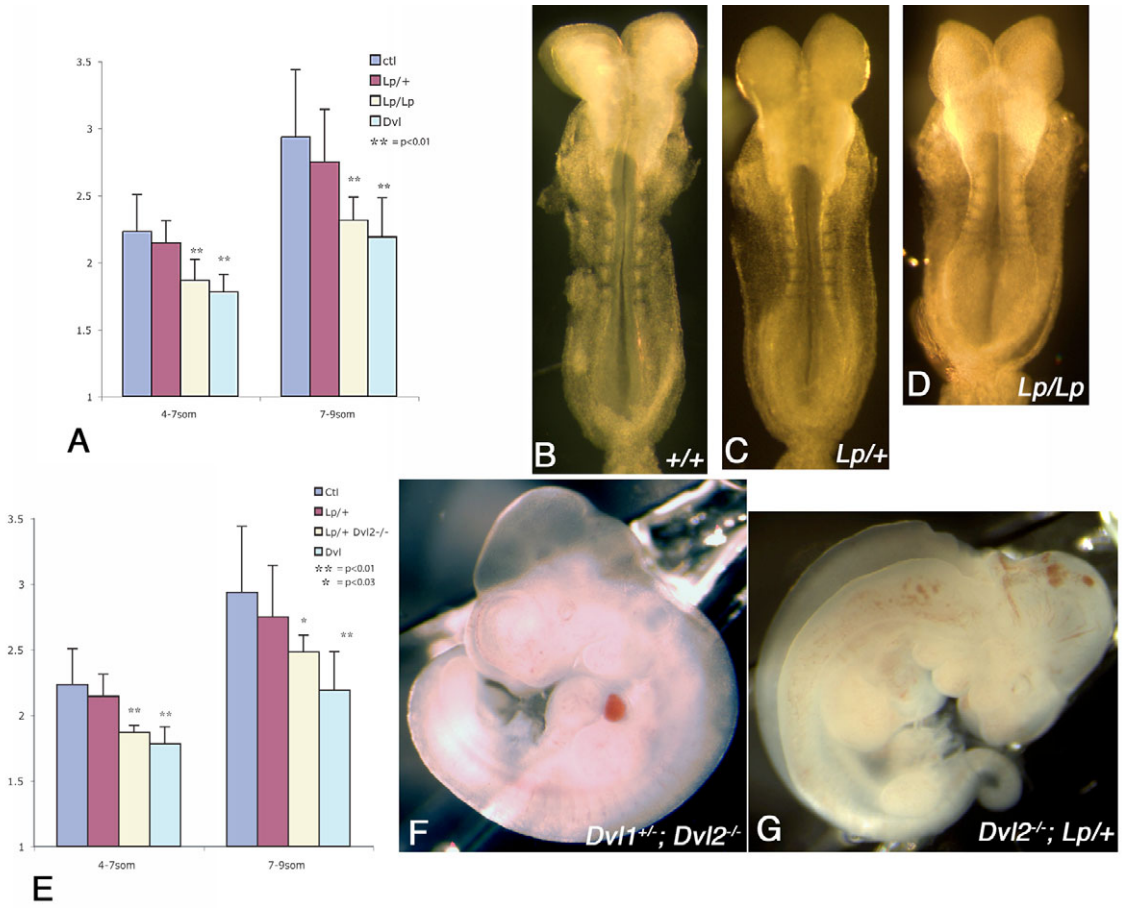

Fig. 3. Genetic interaction between *Dvl2* and *Lp* and reduced length-to-width ratio (LWR) in *Lp/Lp* and *Dvl2*$^{-/-}$; *Lp/+* embryos during neurulation. (**A**) Similar to *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ embryos, *Lp/Lp* mutants displayed significant reduction of LWR during neurulation, while *Lp/+* embryos displayed intermediate reduction. At the eight-somite stage, some of the neural folds were already apposed and started to fuse at the dorsal midline in wild-type embryos (**B**). Parallel to the reduction of LWR, however, the neural folds were still open in *Lp/+* embryos (**C**) and widely apart in *Lp/Lp* embryos (**D**) of the same somite stage. Similar to both *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ and *Lp/Lp* mutants, all *Dvl2*$^{-/-}$; *Lp/+* embryos also displayed significant reduction of LWR during neurulation (**E**). From a cross between *Dvl1*$^{+/-}$; *Dvl2*$^{+/-}$; *Lp/+* and *Dvl2*$^{-/-}$ mice, all progeny that were *Dvl2*$^{-/-}$ or *Dvl1*$^{+/-}$; *Dvl2*$^{-/-}$ (**F**) displayed normal neural tube closure at E10.5. By contrast, all *Dvl1*$^{+/-}$; *Dvl2*$^{-/-}$; *Lp/+* and *Dvl2*$^{-/-}$; *Lp/+* embryos displayed craniorachischisis (**G**). The error bars represent s.d.

neuroepithelium of wild-type and *Lp/Lp* embryos (compare Fig. 5E,F with 5G,H). We note that in *Xenopus*, ML cell polarity in the mesoderm undergoing convergent extension is easy to observe because as the cells intercalate, they align and elongate mediolaterally (Shih and Keller, 1992). By contrast, neural cells elongate only episodically during convergent extension in *Xenopus*, so at any given time only a few cells will be elongated and appear mediolaterally polarized (Elul et al., 1997). Thus, it may be more difficult to assess mediolateral polarity in neural cells with static confocal imaging.

Two of the conserved domains in Dsh/Dvl, DIX and DEP, play different roles in the Wnt pathway and the PCP/convergent extension (Axelrod et al., 1998; Boutros et al., 1998; Rothbacher et al., 2000; Wallingford and Habas, 2005; Wallingford et al., 2000). In *Xenopus*, mediolateral intercalation in the mesoderm requires the C-terminal DEP domain but not the N-terminal DIX domain of Xdsh (Wallingford and Harland, 2001; Wallingford et al., 2000). To confirm that mammalian neurulation requires the same domain of Dvl2, we generated mutant *Dvl2* BAC transgenes identical to the ΔDEP mutant in *Xenopus* (*ΔDEP-EGFP*, Fig. 4A) (Rothbacher et al., 2000; Wallingford and Harland, 2001; Wallingford and Harland, 2002; Wallingford et al., 2000). When crossed into *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ background, *ΔDEP-EGFP* completely failed to rescue the neurulation defects (Fig. 4C; eight *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$;*ΔDEP-EGFP* expected, six recovered, all of which displayed craniorachischisis). By contrast, a *ΔDIX-EGFP*

Table 1. Neonates derived from genetic cross between *Dvl1*$^{+/-}$; *Dvl2*$^{+/-}$; *Lp/+* female and *Dvl2*$^{-/-}$ male mice

	Dvl1$^{+/-}$; *Dvl2*$^{+/-}$; *Lp/+* × *Dvl2*$^{-/-}$							
Genotype	*Dvl1*$^{+/+}$; *Dvl2*$^{+/-}$; *Lp/+*	*Dvl1*$^{+/-}$; *Dvl2*$^{+/-}$; *Lp/+*	*Dvl1*$^{+/+}$; *Dvl2*$^{+/-}$; *+/+*	*Dvl1*$^{+/-}$; *Dvl2*$^{+/-}$; *+/+*	*Dvl1*$^{+/+}$; *Dvl2*$^{-/-}$; *Lp/+*	*Dvl1*$^{+/-}$; *Dvl2*$^{-/-}$; *Lp/+*	*Dvl1*$^{+/+}$; *Dvl2*$^{-/-}$; *+/+*	*Dvl1*$^{+/-}$; *Dvl2*$^{-/-}$; *+/+*
Observed (48 total)	14	10	6	12	0	0	3	3
Expected (48 total)	6	6	6	6	6	6	6	6

transgene, in which part of the DIX domain was deleted, could still fully rescue the neurulation defect in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants (Fig. 4F; 12 *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$*;ΔDIX-EGFP* expected, 15 recovered, all of which displayed normal neural tube closure). As the *ΔDIX-EGFP* transgene removed a VKEEIS motif in the N-terminal region that is essential for Dvl2 function in the canonical Wnt pathway (Capelluto et al., 2002), this result also confirmed that the neural tube closure defect in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants was not due to the loss of function of Wnt signaling.

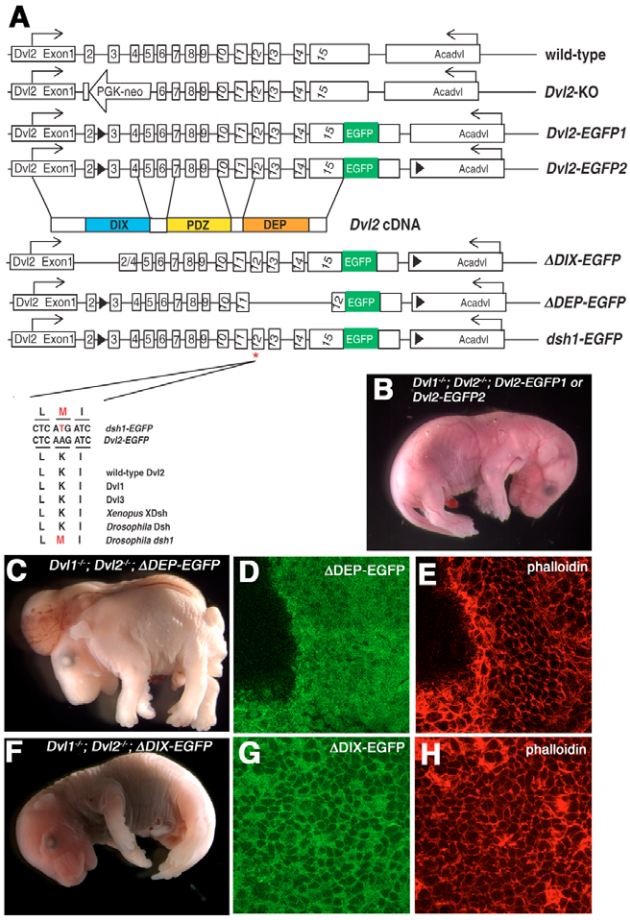

Fig. 4. Domain requirement of Dvl2 for its involvement in convergent extension and membrane distribution during neurulation. (**A**) Schematic diagram of the genomic structure of the endogenous wild-type *Dvl2* allele, the targeted *Dvl2* null allele (*Dvl2 KO*), the two wild-type *Dvl2* BAC transgenes, *Dvl2-EGFP1* and *Dvl2-EGFP2*, and three mutant *Dvl2* BAC transgenes: *ΔDIX-EGFP*, *ΔDEP-EGFP* and *dsh1-EGFP*. Although *Dvl2-EGFP1* had an EGFP cassette fused in-frame to the last codon of *Dvl2* and a LoxP site inserted into intron 2 for genotyping purpose (see Materials and methods), *Dvl2-EGFP2* contained an additional LoxP site inserted in the 3′ flanking gene *Acadvl* (acyl-coenzyme A dehydrogenase, very long chain). *ΔDIX-EGFP* and *ΔDEP-EGFP* deletion mutant transgenes were generated by removing sequence encoding amino acids 67-159 and 442-736, respectively. *dsh1-EGFP* point mutation was generated by replacing AAG with ATG to introduce a K→M substitution at amino acid 446. Lower panel showed sequence alignment of the wild-type *Dvl2-EGFP* and the modified *dsh1-EGFP* BAC transgenes, as well as endogenous mouse Dvl1, Dvl2, Dvl3, *Xenopus* XDsh, *Drosophila* Dsh and the *dsh1* mutation identified in fly. Although *Dvl2-EGFP1*, *Dvl2-EGFP2* (**B**) and *ΔDIX-EGFP* (**F**) can fully rescue the neural tube closure defects in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants, *ΔDEP-EGFP* transgenes completely failed to rescue the neural tube defects in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants (**C**). Confocal analysis indicated that although ΔDIX-EGFP was primarily localized to the membrane (**G**,**H**), ΔDEP-EGFP was evenly distributed in the cytoplasm (**D**,**E**).

When we investigated the distribution of the mutant Dvl2 protein during neurulation, we found that although ΔDIX-EGFP was still primarily membrane localized (Fig. 4G), indistinguishable from wild-type Dvl2-EGFP, ΔDEP-EGFP was evenly distributed in the cytoplasm (Fig. 4D). This result is consistent with existing literature and confirmed the requirement of the DEP domain in targeting Dvl2 to the plasma membrane during mammalian neurulation.

A point mutation identical to the fly dsh1 allele disrupted the ability of *Dvl2-BAC* to rescue the neurulation defect in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$

The result above suggested that DEP-dependent plasma membrane localization of Dvl2-EGFP could be a pre-requirement for its involvement in neural convergent extension and neural tube closure. However, there were differences in Dvl2-EGFP localization in the neuroepithelium and that in the organ of Corti, where Dvl2-EGFP showed an asymmetric membrane distribution that was disrupted in *Lp/Lp* mutants, reminiscent of fly PCP establishment (Fig. 6E) (see Wang et al., 2005). We wondered whether this could be due to a difference in how Dvl proteins function during convergent extension and PCP establishment. To further address this issue, we tested whether the point mutation *dsh1* identified in fly, which specifically abolished the PCP (Axelrod et al., 1998; Boutros et al., 1998), might affect convergent extension in mammals. The *dsh1* mutation results in a K to M missense mutation in the C-terminal DEP domain (Axelrod et al., 1998; Boutros et al., 1998). Using BAC recombineering, we introduced an identical mutation into *Dvl2-EGFP* BAC (Fig. 4A) and produced transgenic mice. All *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$*; dsh1-EGFP* embryos recovered at E9.5 or later displayed craniorachischisis (Fig. 7A,D, eight expected, six recovered, all of which displayed craniorachischisis), suggesting that the *dsh1* mutation completely disrupted the ability of Dvl2 to function during convergent extension. In addition, consistent with our proposal that convergent extension underlies cochlea elongation (Wang et al., 2005), we observed a shorter and wider cochlea in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$*; dsh1-EGFP* embryos (Fig. 6E,F). However, in either wild-type or *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ background, this mutation did not appear to appreciably affect the membrane localization of Dvl2-EGFP (Fig. 7B,C) in neuroepithelium during neurulation.

To determine whether *dsh1* also affects the PCP establishment in mammals, we analyzed the stereociliary bundle orientation in the cochlea. In P0 (postnatal day 0) control pups, the stereociliary bundles of sensory hair cells in the cochlea showed uniform orientation (Fig. 7G,I). By contrast, in a P0 *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$*; dsh1-EGFP* cochlea, we observed a disruption of the uniform stereocilia orientation and a less recognizable form of stereocilia (Fig. 7H,J), resembling the *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants. Occasional mis-alignment of inner hair cells, which has also been found in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ and *Dvl2*$^{-/-}$*; Lp/+* mutants (Wang et al., 2005), was also observed (Fig. 7J and Fig. 5D′, arrows).

In fly wing development, the *dsh1* mutation was thought to disrupt PCP establishment through abolishing DSH-EGFP localization to the plasma membrane (Axelrod, 2001). To investigate how the *dsh1* mutation might disrupt PCP in mammals, we examined its distribution during stereociliary bundle formation. In E16.5 *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$*; Dvl2-EGFP* embryos, before stereociliary polarity could be detected, wild-type Dvl2-EGFP was already localized to the distal membrane (Fig. 6C,C′), similar to that in the more mature hair cells

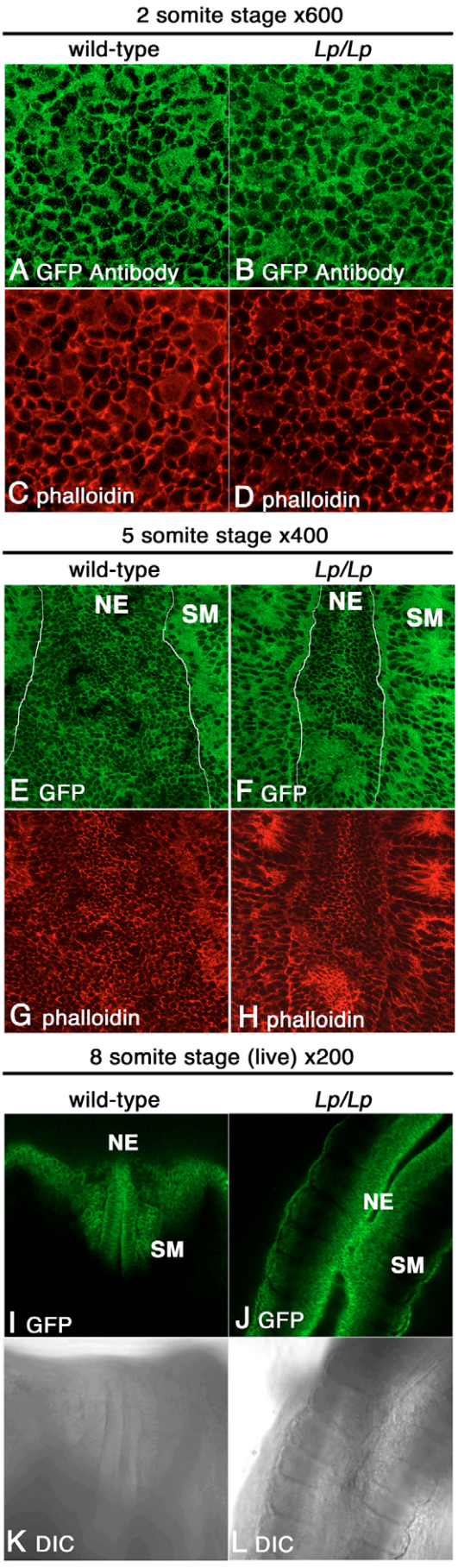

Fig. 5. Dvl2-EGFP was primarily localized to the membrane in the neuroepithelium during neurulation. Single optical sections from laser-scanning confocal microscopy of neuroepithelium from neurulating embryos showed that, in the wild-type background, Dvl2-EGFP was primarily localized to the plasma membrane in the neuroepithelium (A,E,I). Consistent results were observed in wild-type and Lp/Lp mutant two-somite stage embryos stained with an anti-EGFP antibody (**A**,**B**) or through direct visualization of Dvl2-GFP signal from briefly fixed five-somite stage embryo (**E**,**F**) or live eight-somite stage embryos in culture (**I**,**J**). The apparently diffuse cytoplasmic distribution of Dvl2-EGFP and actin in some cells in A,B was due to scanning of the focal plane immediately underneath the apical plasma membrane during confocal laser microscopy. Wild-type and mutant embryos from the two- and five-somite stage were also stained with Phalloidin (**C**,**D**,**G**,**H**) to label F-actin. When the entire series of *z* stacks were examined (data not shown), it was clear that both Dvl and actin are localized to the lateral plasma membrane as individual optical sections are scanned more basally through the cell. Curiously, Dvl2-EGFP and actin distribution also appeared to be more diffuse in the somatic mesoderm at the five-somite stage (E-H). Overall Dvl2-EGFP distribution in the neuroepithelium was not altered in the *Lp/Lp* mutants (B,D,F,H,J,L; data not shown). (**K,L**) DIC images of live wild-type (K) or Lp/Lp mutant (L) embryos in culture. NE, neuroepithelium; SM, somatic mesoderm.

at E18.5 (Fig. 6A,A′). However, in E16.5 *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$*; dsh1-EGFP* embryos, most dsh1-EGFP lost its membrane localization (Fig. 6D,D′, arrowheads) and formed aggregates in the apical cytoplasm, suggesting that the *dsh1* mutation affected Dvl function in mammalian PCP establishment through interfering with its localization to the membrane. Intriguingly, the dsh1-EGFP aggregates were not evenly distributed throughout the apical cytoplasm but rather restricted primarily to the distal side of hair cells. Furthermore, the cytoplasmic dsh1-EGFP distribution was largely rescued by increasing the dose of wild-type Dvl: in phenotypically wild-type E18.5 *Dvl1*$^{-/-}$; *Dvl2*$^{+/-}$*; dsh1-EGFP* cochlea, dsh1-EGFP protein was primarily localized to membrane at the distal side of hair cells (Fig. 6B,B′), although its localization appeared to be somewhat punctate when compared with wild-type Dvl2-EGFP (Fig. 6A,A′). Similar differences were also noticed at E16.5 (data not shown). Presumably, the presence of wild-type Dvl3 and Dvl2 protein may account for the partial rescue of dsh1-EGFP localization defects.

It is interesting to note that the *Lp* mutation also affected Dvl2 membrane localization but in a different fashion: in E16.5 *Lp/Lp* mutants, Dvl2-EGFP displayed randomized and reduced apical membrane localization but rarely formed aggregates in the apical cytoplasm (Fig. 6E). Nevertheless, both the randomized membrane distribution of Dvl2-EGFP in *Lp/Lp* mutants and the loss of membrane distribution of dsh1-EGFP in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants correlated with disruption of the uniform stereociliary polarity during later development (Montcouquiol et al., 2003; Wang et al., 2005). Collectively, these results demonstrated the importance of polarized Dvl2 membrane localization in mammalian PCP establishment in hair cells, but not in neuroepithelium during neurulation.

DISCUSSION

Recent studies have revealed that convergent extension in *Xenopus* and zebrafish requires several proteins whose homologs are involved in establishing PCP in fly, suggesting that a mechanism highly similar to the PCP pathway regulates convergent extension

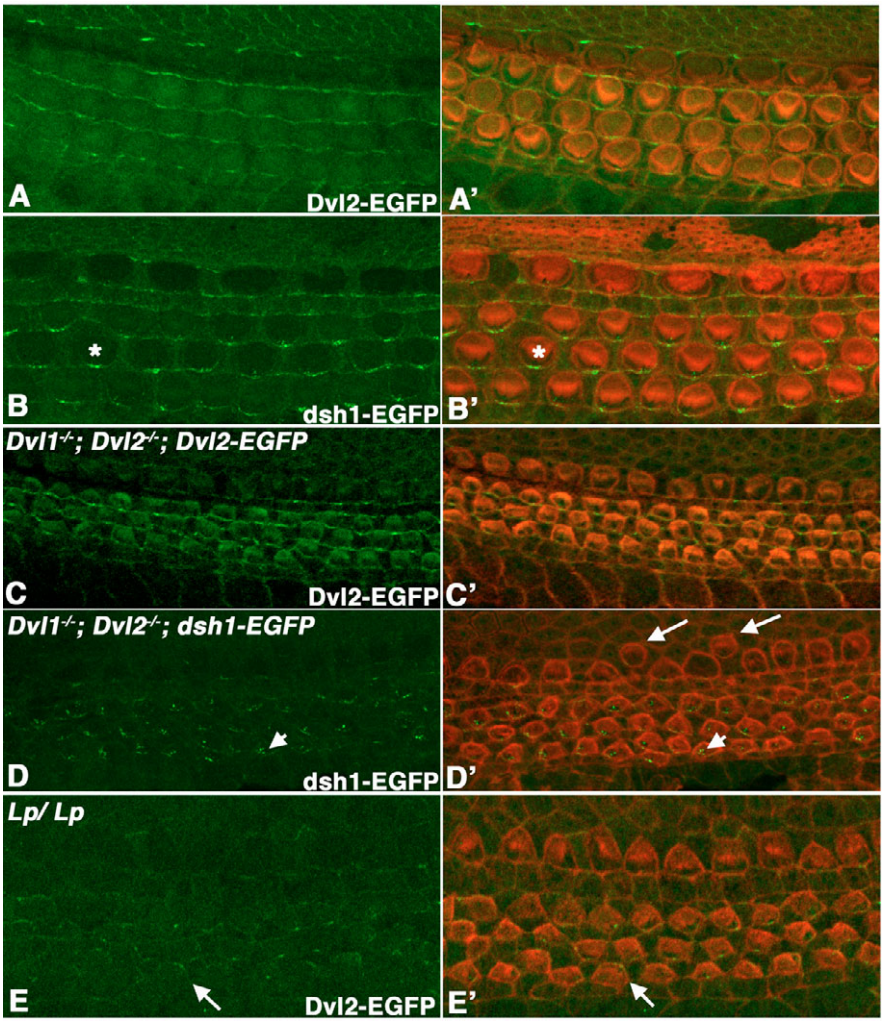

Fig. 6. Localization of Dvl2-EGFP and dsh1-EGFP in the organ of Corti. (**A-E**) Confocal scanning at the apical surface of hair cells in whole-mount organ of Corti at E18.5, showing the native Dvl2-EGFP (A,C,E) or dsh1-EGFP (B,D) signal. (**A'-E'**) are the overlay of Dvl2-GFP (green, A',C',E') or dsh1-EGFP (green, B',D') and hair cell membrane and stereociliary bundles (red) outlined with phalloidin staining. At E18.5 in an otherwise wild-type background, dsh1-EGFP localization along the distal membrane appeared to be more punctate (asterisk in B and B') when compared with wild-type Dvl2-EGFP (A,A'). By contrast, in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ background at E16.5, dsh1-EGFP localization on the membrane was mostly lost and accumulation in the cytoplasm was observed (arrowheads in D,D'). *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$; *dsh1-EGFP* mutants also display mis-alignment of inner hair cells (arrows in D'). Although wild-type Dvl2-EGFP could maintain an even distribution along the distal membrane in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ background at E16.5 (C,C'), its membrane localization was reduced and no longer restricted to the distal side (arrows in E,E') in *Lp/Lp* mutants at this stage.

(Mlodzik, 2002; Myers et al., 2002; Tada et al., 2002; Veeman et al., 2003a). However, significant differences have also been observed that may represent evolutionary adaptation in each pathway for the distinct downstream cellular and developmental process (Mlodzik, 2002; Tada et al., 2002; Veeman et al., 2003a). Our results further demonstrate that convergent extension in the neuroepithelium and PCP co-exist in the mouse and may have co-evolved by using the same set of core PCP proteins.

Using LWR measurements and *T* in situ of neurulating *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ embryos, we demonstrate an essential function of mammalian *Dvl* genes in a coordinated lengthening of the AP axis and narrowing of the ML axis, a morphogenetic process closely resembling convergent extension in *Xenopus*. By contrast, although Dsh/Dvl are also essential in the canonical Wnt pathway, our in situ analysis on *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ embryos did not reveal global loss of function of Wnt signaling during neurulation, presumably because the remaining Dvl3 activity is sufficient to compensate for the loss of Dvl1 and 2 in Wnt signaling. These results are also consistent with our interpretation that the neural tube phenotype of the *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutant is not secondary to defects in the canonical Wnt pathway, but to defects in the PCP pathway. Further support for this interpretation derives from our findings that similar defects in LWR and *T* localization were also observed in *Lp/Lp* and *Lp/+; Dvl2*$^{-/-}$ mutants. *Lp* is a mutation of the *Stbm* homolog *Vangl2*, which is a core PCP pathway component but not part of the canonical Wnt pathway (Darken et al., 2002; Goto and Keller, 2002; Kibar et al., 2001; Murdoch et al., 2001). Finally, the fact that *ΔDIX-EGFP* BAC transgenes with a deletion of the VKEEIS motif essential for Dvl2 involvement in the canonical Wnt pathway (Capelluto et al., 2002) was still able to fully rescue the lethality of *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants, while *ΔDEP-EGFP* and *dsh1-EGFP* transgenes completely failed to do so, indicated that the neural tube closure defect in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ was not due to the loss of function of Wnt signaling, but solely to the disruption of the PCP pathway.

Our analyses of LWR suggest that neural plate undergoes coordinated lengthening and narrowing throughout neurulation, resembling convergent extension that occurs during *Xenopus* neural tube closure. Furthermore, three mouse mutants, *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$, *Lp/Lp* and *Lp/+; Dvl2*$^{-/-}$, all displayed similar severe neural tube closure defects. In each case, significant reductions in LWR were found before the seven-somite stage, a crucial time of neurulation where elevating neural folds appose at dorsal midline. These findings suggest that the mammalian PCP pathway is the driving force behind this convergent extension-like morphogenesis process and that, as in the *Xenopus*, a disruption of this process is the cause rather than the result of neural tube closure defects. However, it is important to note that our data do not imply a similar type of cell movement is involved in mammalian neurulation. Although convergent extension in *Xenopus* is driven by cell intercalation (Elul and Keller, 2000; Elul et al., 1997; Shih and Keller, 1992;

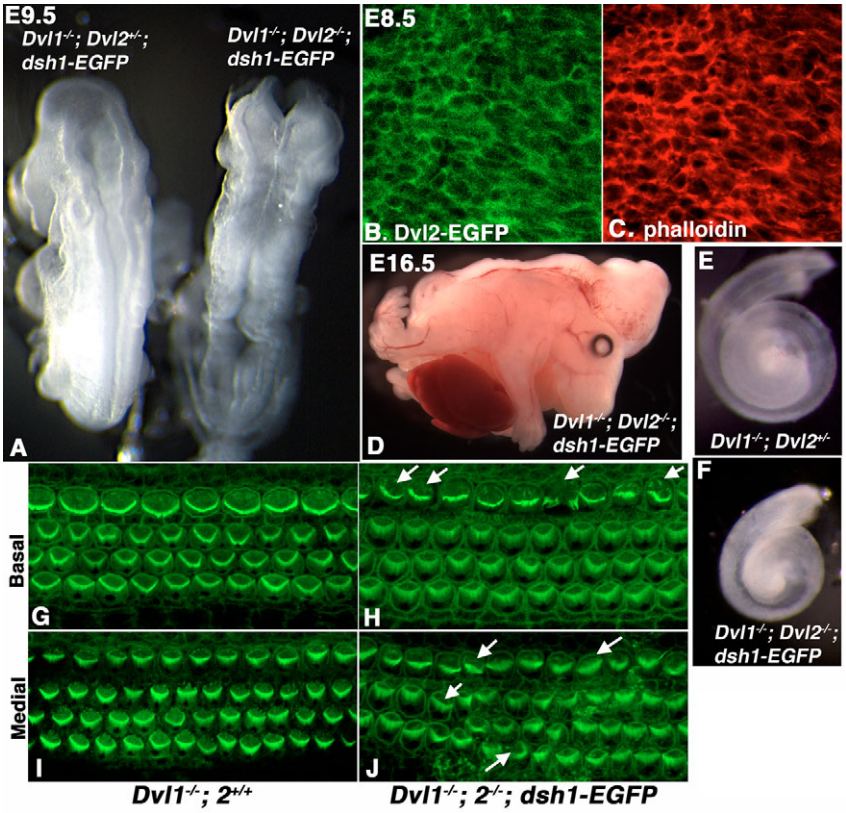

Fig. 7. *dsh1-EGFP* failed to rescue the craniorachischisis, cochlear elongation and stereociliary polarity phenotypes in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ embryos. (**A**,**D**) *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$; *dsh1-EGFP* embryos (right in A) displayed craniorachischisis at E9.5 and E16.5, suggesting that dsh1-EGFP was not able to compensate for the convergent extension defect in neurulation *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants. (**B**) Confocal analysis indicated that dsh1-EGFP was primarily localized to the plasma membrane in the neuroepithelium. (**C**) Confocal analysis of phalloidin staining. (**E**,**F**) Cochlea recovered at p0 from *Dvl1*$^{-/-}$; *Dvl2*$^{+/-}$ and *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$; *dsh1-EGFP* mice, indicating that *dsh1-EGFP* also failed to rescue the cochlea elongation defect in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ mutants that was also thought to be due to disruption of convergent extension. (**G**,**I**) Confocal scan at either the base (G) or medial region (I) showing uniform stereociliary orientation in the organ of Corti of a p0 *Dvl1*$^{-/-}$; *Dvl2*$^{+/+}$ mouse. (**H**,**J**) However, in the organ of Corti of a *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$; *dsh1-EGFP* mouse, the stereociliary bundles were mis-oriented (arrows) in some hair cells. Mis-alignment in the outer hair cells could also be observed.

Wallingford and Harland, 2001), it can also be accomplished through directed migration or oriented cell division in zebrafish (Gong et al., 2004; Solnica-Krezel et al., 1995; Wallingford et al., 2002). The exact cell behavior behind mammalian neural convergent extension remains to be determined.

It has been argued that a highly conserved pathway analogous to the PCP pathway in fly regulates convergent extension in *Xenopus* and zebrafish. Our results extend and strengthen this notion in mammals by demonstrating that disruption of neural convergent extension and PCP establishment co-exist in three mouse mutants: *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$, *Lp/Lp* and *Lp/+; Dvl2*$^{-/-}$ (see also Wang et al., 2005). Based on the similar neural tube closure defect, we infer that mutations of the mammalian PCP homologs *Celsr1* and *Fzd3/6*, which cause PCP defects in the ear, also lead to convergent extension defect during neurulation (Curtin et al., 2003; Wang et al., 2006). Our finding that the missense point mutation *dsh1* identified in fly that specifically disrupts the PCP pathway (Axelrod et al., 1998; Boutros et al., 1998) also abolishes the function of Dvl2 to regulate convergent extension and stereociliary polarity suggested that Dsh/Dvl functions through very conserved mechanisms in convergent extension and establishment of PCP. This notion is also supported by that fact that genetic background variation modifies neural tube closure and stereociliary polarity defects to the same extent.

Using BAC transgenes that express Dvl2-EGFP fusion proteins capable of replacing endogenous Dvl2 function, we found that Dvl2-EGFP is primarily localized to the plasma membrane in neuroepithelium, a prerequisite for its involvement in convergent extension (Park et al., 2005). Similar to the findings in fly and *Xenopus*, we found that the C-terminal DEP domain was required for Dvl2-EGFP localization to the plasma membrane and its ability to mediate neural tube closure and presumably, convergent extension during neurulation. By contrast, although Vangl2 is known to bind to Dvl and the *Lp* mutation weakens the binding (Torban et al., 2004), we did not find any change of Dvl2-EGFP distribution in the neuroepithelium of neurulating *Lp/Lp* mutants, indicating that the membrane localization of Dvl2 in neuroepithelium is not crucially dependent upon Vangl2 function. This observation is in direct contrast to our previous findings in cochlea where Dvl2-EGFP displayed an *Lp*-dependent asymmetric membrane distribution that correlates with the localization and orientation of stereocilia, resembling the establishment of PCP in fly wing cells (Wang et al., 2005). Similarly, our current results with the *dsh1-EGFP* transgene also revealed a disparity between neuroepithelium undergoing convergent extension and sensory hair cells during PCP establishment. In fly, the *dsh1* point mutation disrupts membrane localization of Dishevelled (Axelrod, 2001). Here, we found that the same point mutation abolished Dvl2-EGFP membrane localization in the cochlea sensory hair cells in *Dvl1*$^{-/-}$; *Dvl2*$^{-/-}$ embryos and concomitantly, its ability to establish PCP. By contrast, although the *dsh1* mutation also disrupted Dvl2-EGFP function in convergent extension during neurulation, we could not detect any significant change of dsh1-EGFP distribution in the neuroepithelium. Collectively, these data suggested that plasma membrane distribution of Dvl2-EGFP might be necessary for its involvement in convergent extension, but not sufficient. Like Dvl2, it has recently been shown that Fzd3 and Fzd6 display *Lp*-dependent asymmetric localization in the in the inner ear, but their distribution in the neuroepithelium has not been reported (Wang et al., 2006).

If Dvl proteins function in convergent extension and PCP through highly conserved mechanisms, then why would the *Lp* and *dsh1* mutation have different effects on Dvl2-EGFP localization in neuroepithelium undergoing convergent extension and sensory hair cells undergoing PCP establishment? One possibility is that another *Vangl* homolog, *Vangl1*, codes for a protein that allows for the membrane localization of Dvl2 in

neuroepithelium but not the inner ear. In zebrafish, Vangl1 and Vangl2 have largely non-overlapping patterns of expression but similar biochemical function (Jessen and Solnica-Krezel, 2004). *Vangl1* expression has not been carefully examined in the developing mammalian neural tube or inner ear.

The second possibility is that in neuroepithelium undergoing convergent extension, Dvl2-EGFP is distributed at specific locations that depend upon Vangl2 and a functional PCP pathway, but our imaging techniques are not sensitive enough to recognize them. To resolve this, we are using different *Cre* transgenes and the floxed *Dvl2-EGFP2* transgenes to generate chimeric embryos that consist of individual Dvl2-EGFP cells surrounded by non-GFP cells. Our preliminary results showed no polarized distribution of Dvl2-EGFP that could be correlated with either the AP or ML axis of the embryo. Similar studies in *Xenopus* also did not reveal consistent polarized membrane localization of Dsh in mesodermal cells undergoing convergent extension (Park et al., 2005).

A third possibility is that although Dvl functions similarly in PCP establishment and convergent extension, different cellular processes demand distinct modes of action. For example, during polarity establishment in the well-aligned sensory hair cells, polarized distribution of Dvl and other PCP components dictates the precise location of stereociliary assembly within the hair cells. By contrast, in cells undergoing dynamic cell rearrangement and intercalation, the localization of Dvl and other PCP components is relatively mobile to mediate temporary cytoskeleton assembly that underlies cell-cell adhesion and generates traction (Ulrich et al., 2005; Wallingford and Harland, 2002). Supporting evidence comes from the observation that in *Xenopus*, *XDsh* mutant that blocks convergent extension also leads to less stable membrane protrusions (Wallingford et al., 2000). This view is also consistent with the proposal that PCP signaling serves as a permissive factor in convergent extension, while the alignment and orientation of cell intercalation may be determined by the AP polarity (Ninomiya et al., 2004). In this scenario, membrane localization of Dvl in the neuroepithelium may represent only a steady state distribution that does not require the function of Vangl2, nor confer polarity.

To understand more thoroughly the function of the PCP pathway in mammals, it will be important to find out not only the similarities, but also the differences between PCP and convergent extension at the cellular and molecular level. In this regard, it is interesting to note that convergent extension in *Xenopus* and zebrafish also involves Wnt/Ca^{2+} signaling, a pathway that has not been implicated in PCP determination in fly but nonetheless requires the function of PCP proteins such as Dsh and Pk (Veeman et al., 2003a). Analyzing the function of the Wnt/Ca^{2+} pathway in the mouse may lead to a better understanding of how the core PCP proteins co-evolved to regulate both convergent extension and PCP determination.

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