

RESEARCH ARTICLE

A dynamic intracellular distribution of Vangl2 accompanies cell polarization during zebrafish gastrulation

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ABSTRACT

During vertebrate gastrulation, convergence and extension movements elongate embryonic tissues anteroposteriorly and narrow them mediolaterally. Planar cell polarity (PCP) signaling is essential for mediolateral cell elongation underlying these movements, but how this polarity arises is poorly understood. We analyzed the elongation, orientation and migration behaviors of lateral mesodermal cells undergoing convergence and extension movements in wild-type zebrafish embryos and mutants for the Wnt/PCP core component Vangl2 (Trilobite). We demonstrate that Vangl2 function is required at the time when cells transition to a highly elongated and mediolaterally aligned body. *vangl2* mutant cells fail to undergo this transition and to migrate along a straight path with high net speed towards the dorsal midline. Instead, *vangl2* mutant cells exhibit an anterior/animal pole bias in cell body alignment and movement direction, suggesting that PCP signaling promotes effective dorsal migration in part by suppressing anterior/animalward cell polarity and movement. Endogenous Vangl2 protein accumulates at the plasma membrane of mesenchymal converging cells at the time its function is required for mediolaterally polarized cell behavior. Heterochronic cell transplantations demonstrated that Vangl2 cell membrane accumulation is stage dependent and regulated by both intrinsic factors and an extracellular signal, which is distinct from PCP signaling or other gastrulation regulators, including BMP and Nodals. Moreover, mosaic expression of fusion proteins revealed enrichment of Vangl2 at the anterior cell edges of highly mediolaterally elongated cells. These results demonstrate that the dynamic Vangl2 intracellular distribution is coordinated with and necessary for the changes in convergence and extension cell behaviors during gastrulation.

KEY WORDS: Vangl2, Wnt/PCP, Cell movement, Cell polarity, Gastrulation, Zebrafish

INTRODUCTION

Gastrulation is a fundamental morphogenetic process that entails a variety of evolutionarily conserved cell movement behaviors to generate germ layers and shape them into the animal body plan. Studies in different model organisms have demonstrated that convergence and extension (C&E) gastrulation movements are crucial players that narrow the tissues in the mediolateral (ML) direction while extending them along the anteroposterior (AP) embryonic axis (Keller et al., 2000). Studies in chick, mouse, frog and zebrafish embryos revealed that C&E movements are driven by active cell migration and polarized cell intercalations (ML planar

and radial cell intercalations) of mediolaterally elongated cells (Keller et al., 2000; Myers et al., 2002b; Yin et al., 2008). Cell-cell and cell-extracellular matrix interactions also play essential roles in this process (Coyle et al., 2008; Dohn et al., 2013).

Although the cellular signals that guide C&E movements are not fully understood, the noncanonical Wnt/planar cell polarity (Wnt/PCP) signaling system is known to be a key actor. Originally discovered in *Drosophila melanogaster*, PCP signaling polarizes cells in the plane of epithelia of many tissues, including the eye, wing, abdomen and thorax (Adler, 2002; Das et al., 2002; Lawrence et al., 2004; Shimada et al., 2001; Strutt, 2001; Tomlinson and Struhl, 1999). In vertebrates, Wnt/PCP signaling is known to polarize both epithelial and mesenchymal tissues (Gray et al., 2011) and has been implicated in many cellular processes, such as C&E gastrulation movements, polarization of ciliary structures, neurulation, cartilage morphogenesis and stem cell expansion (Borovina et al., 2010; Bradley and Drissi, 2011; Kuss et al., 2014; Le Grand et al., 2009; Mahaffey et al., 2013; Wada and Okamoto, 2009; Wallingford et al., 2000; Zilber et al., 2013). Genetic studies in *Drosophila* led to the identification of several core PCP components, including the four-pass transmembrane protein Van Gogh (Vang; also known as Strabismus), the seven-pass transmembrane proteins Frizzled (Fz) and Flamingo (Fmi; also known as Starry night) and the cytoplasmic proteins Dishevelled (Dsh, or Dvl), Diego (Dgo) and Prickle (Pk) (Adler et al., 1997; Chae et al., 1999; Gubb et al., 1999; Taylor et al., 1998; Theisen et al., 1994; Usui et al., 1999; Vinson and Adler, 1987; Wolff and Rubin, 1998). PCP signaling in *Drosophila* produces, or is a consequence of, an asymmetric distribution of some core components at the apical cell membrane (Amonlirdviman et al., 2005; Axelrod, 2001; Lawrence et al., 2004; Ma et al., 2003). Vang and Pk are enriched on one side (proximal in the wing), whereas Fz, Dsh and Dgo are enriched on the opposite side (distal in the wing) of the cell along the planar polarity axis of the tissue, with Fmi present at both cell edges (Strutt and Strutt, 2008). As a result of intercellular interactions and intracellular feedback loops, the core protein complexes have been shown to concentrate into discrete membrane subdomains or ‘puncta’ (Strutt et al., 2011). A tight regulation of the composition of these complexes on opposite cell membranes, and consequently on adjacent membranes of two neighboring cells, is essential for the polarizing signal to be propagated across several cell diameters (Adler et al., 2000). Recent studies also implicate Wg and Wnt4 in providing long-range directional input in the *Drosophila* wing (Wu et al., 2013). Zebrafish embryos carrying mutations in several of the Wnt/PCP pathway core components and in Wnt11/Wnt5 ligands exhibit a characteristic morphogenetic phenotype, with shorter AP and broader ML body axes due to impaired C&E movements (Heisenberg et al., 2000; Kilian et al., 2003; Marlow et al., 1998).

In vertebrate embryos, C&E movements shape all the germ layers and are coordinated with mesendoderm internalization and epiboly movements. Mesodermal precursors, upon internalization via a

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blastopore, migrate away from it, and later turn their trajectories towards the nascent embryonic midline (Solnica-Krezel and Sepich, 2012). In the zebrafish gastrula, internalized mesodermal cells first move anteriorly, towards the animal pole, but at mid-gastrulation they alter their movement trajectories from anterior/animal to dorsal, marking the start of C&E (Sepich and Solnica-Krezel, 2005). At these early C&E stages, mesodermal cells undergo dorsal migration as individuals along irregular paths; however, as gastrulation progresses these cells become mediolaterally elongated and migrate dorsally as a group, faster and along straighter trajectories. This change fails to occur in Wnt/PCP signaling mutants, including *vangl2* (also known as *trilobite*) (Jessen et al., 2002), and polarized radial and planar ML intercalations are also impaired (Yin et al., 2008).

How Wnt/PCP signaling contributes to ML cell polarization, polarized cell migration and intercalation is incompletely understood. Increasing evidence suggests that this pathway acts as a cellular compass that coordinates polarity and individual cell behaviors with the AP and ML embryonic axes (Gray et al., 2011; Yin et al., 2008). Similar to the asymmetric distribution of the core PCP components in *Drosophila* epithelia, asymmetric localization of core PCP proteins expressed as fluorescent fusion proteins was observed in mediolaterally polarized cells during zebrafish gastrulation, with Pk-GFP and Dvl-GFP fusion proteins being enriched at the anterior and posterior cell membranes, respectively (Ciruna et al., 2006; Yin et al., 2008). By contrast, in *Xenopus* Dvl is enriched at the cell edges facing the notochord-somite boundary (Panousopoulou et al., 2013). Yet, C&E movements in *Xenopus* explants are controlled by PCP core proteins via the Septin cytoskeleton and its ability to regulate cortical actomyosin compartmentalization and the remodeling of cell-cell contacts (Shindo and Wallingford, 2014). Phosphorylated myosin II, a reporter of actomyosin-based contraction, was shown to be enriched at the anterior and posterior cell boundaries (Shindo and Wallingford, 2014), consistent with PCP core protein enrichment at these boundaries in zebrafish gastrulae. Asymmetric distribution of Vangl2 was observed in epithelia of the developing ear and skin in adult mouse (Devenport and Fuchs, 2008; Montcouquiol et al., 2006). However, the intracellular distribution of Vangl2 during gastrulation is not known.

In this study, we have addressed the dynamics of cell polarization by Vangl2 and its subcellular distribution during zebrafish gastrulation. First, we show that lateral mesodermal cells undergo a transition from a round to mediolaterally elongated morphology at a specific time during gastrulation and location relative to the dorsal midline. Because *vangl2* mutant cells fail to elongate and exhibit an abnormal anterior/animal pole bias in their cell body alignment and direction of movement, we propose that PCP signaling promotes effective dorsal migration in part by suppressing anterior/animalward cell polarity and movement. Second, our analyses of the endogenous Vangl2 distribution revealed its accumulation at the cell membrane preceding ML cell elongation and effective convergence to the dorsal midline. Third, based on heterochronic transplantation experiments, we propose that this membrane accumulation of Vangl2 at the onset of C&E is regulated by both a cell-intrinsic mechanism and a non-cell-autonomous signal, which is distinct from PCP signaling or other known gastrulation regulators. Finally, we show that Vangl2 accumulates more strongly at the anterior cell membranes in highly mediolaterally elongated mesodermal and neuroectodermal cells. These results demonstrate that the dynamic intracellular distribution of Vangl2 is coordinated with, and necessary for, the changes in C&E cell behaviors during gastrulation.

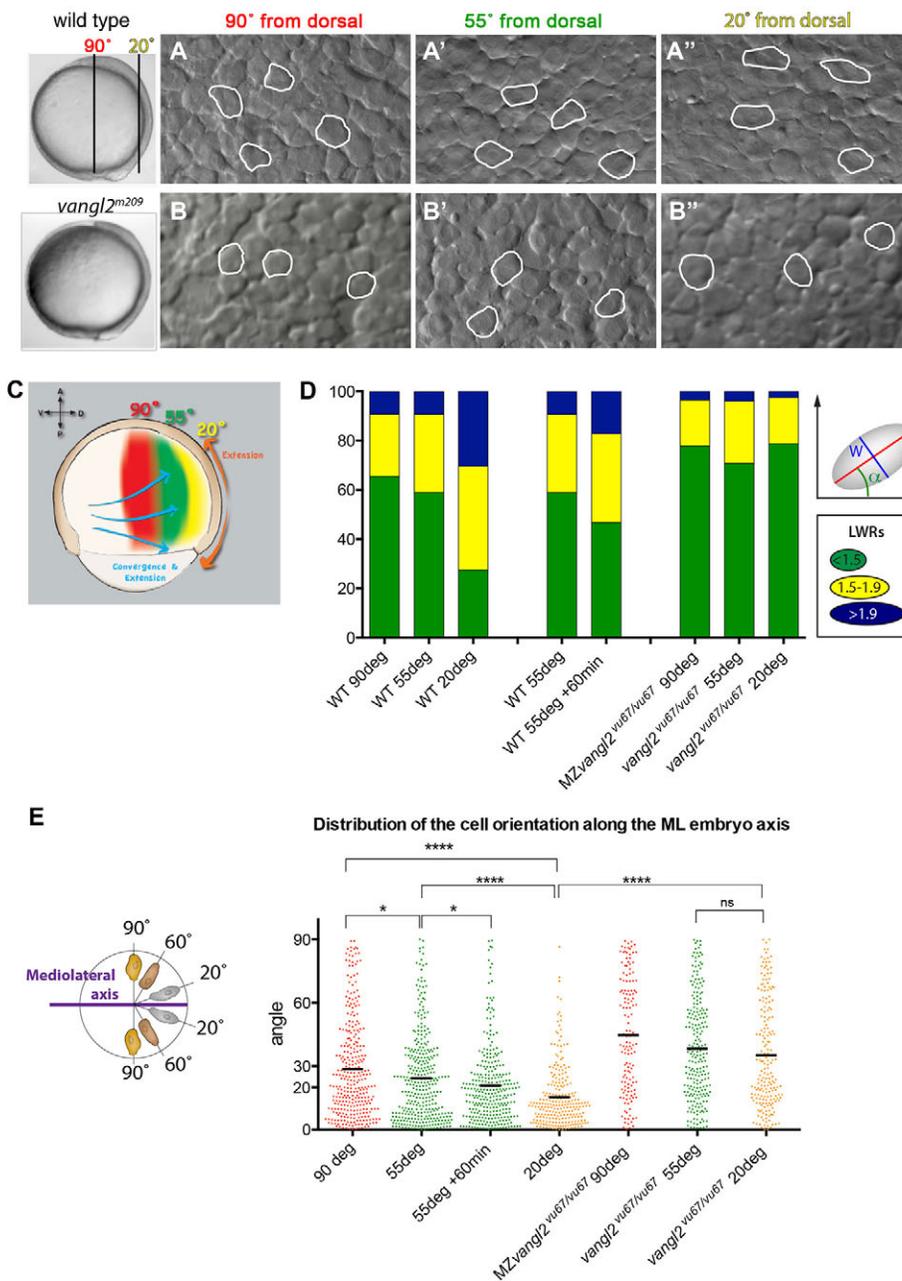
RESULTS

Spatiotemporal dynamics of ML elongation of mesodermal cells during C&E

During zebrafish gastrulation, lateral mesodermal cells converging towards the dorsal midline change their morphology and behavior in a location- and stage-dependent manner. This transformation depends on Wnt/PCP signaling and *vangl2* function (Jessen et al., 2002), although its timing and underlying cellular and molecular mechanisms remain to be determined. Towards this goal, we first analyzed changes in cell shape and alignment at different positions along the dorsoventral (DV)/ML axis of the wild-type (WT) gastrula by performing time-lapse analyses at late gastrulation stages (9-10 hpf). We determined cell elongation, as length to width ratio (LWR), in domains at different positions relative to the dorsal midline (expressed as angular degrees from the dorsal midline: 90°, 55° and 20°; Fig. 1C) (Topczewski et al., 2001). Our analyses at late gastrulation (9 hpf) showed a gradual cell elongation, or increase in the LWR, along the ML embryonic axis, with lower LWR values seen at 90° from the dorsal midline ($n=7$ embryos, 310 cells) and higher LWR values closer (20°) to the dorsal midline ($n=5$ embryos, 240 cells) (Fig. 1A-B",D). Additional analyses 55° from the dorsal midline demonstrated dynamic cell shape changes during the course of each time-lapse: LWR increased over the 60 min time-lapse ($n=7$ embryos, 341 cells, and $n=6$ embryos, 295 cells, respectively), suggesting that cell shape transformation occurs at this specific location and time during gastrulation (Fig. 1D). Analysis of cell shape changes in *vangl2* and maternal zygotic *vangl2* mutant gastrulae (MZ*vangl2*^{vu67/vu67} mutants were produced by genetically mosaic WT females harboring *vangl2*^{vu67/vu67} germ cells, generated by germline replacement and therefore lacking both maternal and zygotic *vangl2* function; see Materials and Methods) showed that mutant cells fail to undergo this change. At both 55° and 20° from the dorsal midline, mutant cells exhibited rounder shapes even when compared with those 90° from dorsal location in WT (Fig. 1D; 90°, MZ*vangl2*^{vu67/vu67} $n=2$ embryos, 140 cells; 55° *vangl2*, $n=3$ embryos, 226 cells; 20° *vangl2*, $n=3$ embryos, 193 cells).

Next, we examined when and where, relative to the changes in cell shape, the mesodermal cells align their long axes with the DV/ML gastrula axis. Analyzing the time-lapse data described above, we observed that in WT gastrulae the alignment of cell bodies along the ML embryonic axis occurred in a gradual manner. Cells located 90° from dorsal exhibited various degrees of ML alignment, whereas in the region 20° from dorsal almost all WT cells had their long axes aligned $\pm 20^\circ$ with the ML embryonic axis (Fig. 1E). By contrast, in *vangl2* and MZ*vangl2*^{vu67/vu67} gastrulae, cells failed to align mediolaterally, consistent with our previous studies (Jessen et al., 2002) (Fig. 1B-B",E). Notably, a significant fraction of *vangl2* mutant cells were aligned along the AP axis, and thus perpendicular to the ML cell alignment, the hallmark of C&E cell behavior (Jessen et al., 2002; Keller et al., 2000; Wallingford et al., 2000).

At the onset of gastrulation, internalized lateral mesodermal and endodermal cells initially move anteriorly, and at mid-gastrulation turn their trajectories towards the dorsal midline (Dumortier et al., 2012; Sepich and Solnica-Krezel, 2005). In order to better understand the relationship between the cell body alignment and this movement behavior, we analyzed the trajectories of WT and *vangl2* mutant cells. We analyzed cell trajectories in 9.5 hpf embryos for 60 min (1 min intervals) at 55° from the dorsal midline, where the transition in the cell shape occurs (Fig. 1D). Because cell behavior and trajectories vary along the AP axis (Sepich et al., 2005), we performed this analysis at different locations along the AP axis: animal, equatorial, and vegetal (anterior to, at the level of, and



posterior to the first somite, respectively) (Fig. 2B). We observed, as expected, a predominance of cell movements in the dorsal direction ($\pm 45^\circ$ from the ML axis) in both WT and *vangl2* mutant gastrulae (Fig. 2C). In WT, dorsal movement was most predominant in the equatorial domain as compared with the animal or vegetal domains. However, in the *vangl2* mutant gastrulae the proportion of anteriorly/animally directed movements was increased at all locations along the AP axis compared with WT embryos, with the greater increase in the vegetal domain. Moreover, the frequency of vegetally/posteriorly and ventrally oriented movements was decreased in *vangl2* mutant compared with WT gastrulae (Fig. 2C).

Next, we analyzed cell trajectories according to their 'next cell movement direction', defined as the direction (dorsal, animal, vegetal or ventral) that the cell adopts compared with its previous direction of movement (Fig. 2A) (Sepich et al., 2005). We thus pooled the cell movements according to their initial direction

(animal, dorsal, vegetal, ventral) and determined the direction of the next movement. In WT, the next cell movement was predominantly dorsal at all locations along the AP axis. Notably, in *vangl2* mutants, the fraction of cells moving anteriorly/animally was increased at all AP locations (Fig. 2D). Therefore, unlike WT, *vangl2* mutant cells did not elongate nor align in the ML embryonic axis, and they displayed a slightly anteriorly/animally biased body alignment and movement preference.

Vangl2 accumulates at the plasma membrane at a specific time during gastrulation

Given that the dynamic changes in cell shape and orientation during C&E depend on *vangl2* function (Jessen and Solnica-Krezel, 2004; Jessen et al., 2002) (Fig. 1), we examined the subcellular distribution of Vangl2 protein during this process. We generated a polyclonal antibody against the cytosolic C-terminal domain of Vangl2 (Li et al.,

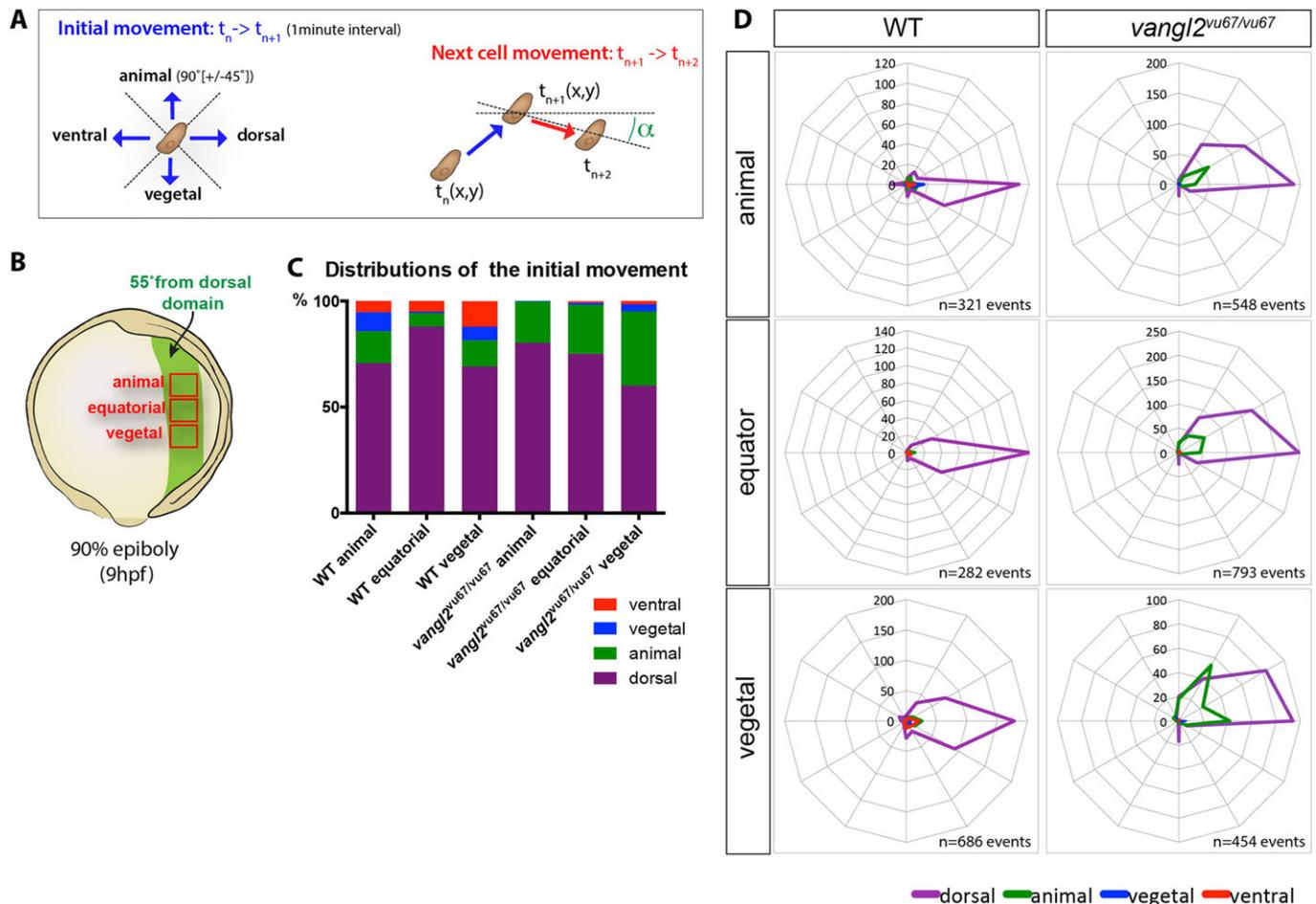


Fig. 2. Cell behaviors at different positions along the AP embryonic axis. (A) Definition of initial cell movement and next cell movement. (B) Drawing of a late gastrula embryo showing the animal, equatorial and vegetal locations examined. (C) Distribution of the directionality of all cell movements during the time-lapse (60 min, 1 min interval) in WT and *vangl2^{vu67/vu67}* embryos in anterior, equatorial and posterior locations. (D) The orientation of the next cell movement pooled in categories referring to previous/initial cell movement. On diagrams animal is to the top, dorsal to the right, vegetal to the bottom and ventral to the left. Scale indicates the number of movement events.

2013) (Fig. 3). Whole-mount immunofluorescence experiments revealed that Vangl2 was present at high levels at the membrane of mesodermal and ectodermal cells during late gastrulation beginning at 8 hpf (Fig. 3C,D,F). By contrast, at blastula (3 and 4 hpf) and early gastrula (6 hpf) stages the protein was preferentially detected in cytoplasmic puncta (Fig. 3A; supplementary material Figs S2 and S3). Low levels of Vangl2 protein were occasionally observed at cell membranes at and before shield stage (6 hpf) (supplementary material Fig. S3A-G). These data demonstrate that Vangl2 localization is dynamic during gastrulation and that it accumulates at the cell membrane at the stages when *vangl2* function is required for proper C&E cell behaviors.

Vangl2 membrane accumulation is independent of signaling pathways that pattern vertebrate gastrulae

We next investigated the mechanism underlying the membrane accumulation of Vangl2 at the onset of C&E movements. Gastrulation movements are both spatially and temporally coordinated with embryonic axis formation and patterning (Myers et al., 2002a). Morphogen gradients, including those of BMP and Nodal, are known to regulate the expression of some PCP genes during gastrulation (e.g. *wnt11*, *wnt5*) (Gritsman et al., 1999; Myers et al., 2002a). To test whether Vangl2 membrane accumulation is

dependent on Nodal signaling we performed whole-mount immunostaining on maternal zygotic *one-eyed pinhead^{tz257/tz257}* (*MZoep*) mutant gastrulae, in which the essential Nodal co-factor Cripto (Tdgf1 – ZFIN) is inactive (Gritsman et al., 1999). We found that Vangl2 was localized to cell membranes in *MZoep* mutants at early segmentation stages, indicating that Nodal signaling is not necessary for Vangl2 membrane localization (Fig. 4A,A'). Next, we asked whether Vangl2 distribution is regulated by the ventral to dorsal BMP activity gradient that limits C&E movements to the dorsolateral gastrula regions (Myers et al., 2002a). At early segmentation stages, Vangl2 was localized at cell membranes in *MZichabod* mutant embryos (Fig. 4B,B'), which have substantially reduced levels of β -catenin 2, fail to form the Spemann-Mangold gastrula organizer and consequently show strong upregulation of BMP signaling and severe ventralization (Kelly et al., 2000). Finally, downregulation of BMP signaling by injecting synthetic mRNA encoding the BMP antagonist Noggin, resulted in severe embryo dorsalization, but did not affect Vangl2 membrane localization (Fig. 4C,C') (Bauer et al., 1998; Fürthauer et al., 1999; Hammerschmidt et al., 1996). Together, these data indicate that the two key morphogens involved in embryo patterning, BMP and Nodal, are not essential for Vangl2 membrane accumulation during zebrafish gastrulation.

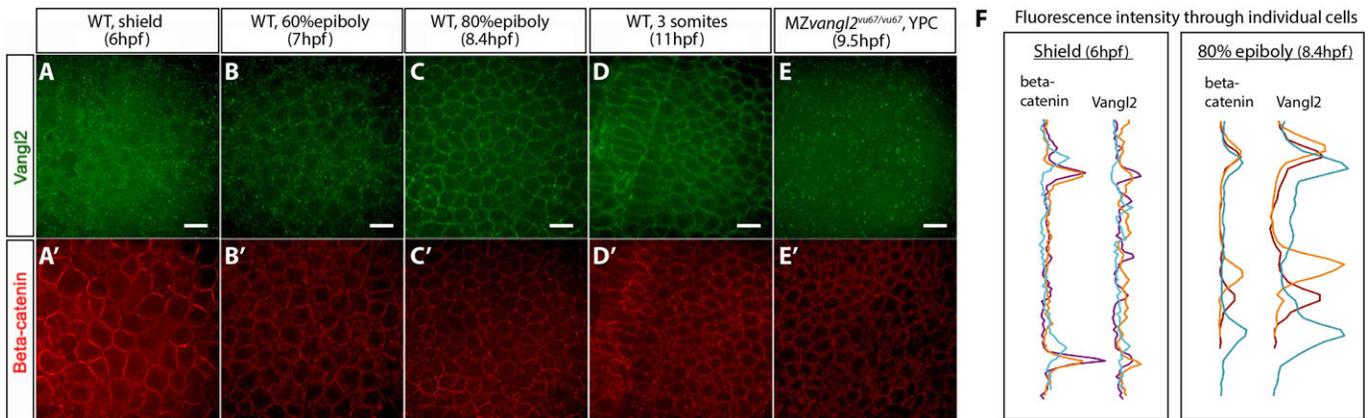


Fig. 3. Dynamic Vangl2 intracellular localization during gastrulation. (A-E') Confocal images of WT embryos showing the intracellular and membrane localization of Vangl2 (A-E) and β -catenin (A'-E'). (F) Quantification of Vangl2 and β -catenin fluorescence intensity in individual cells (three independent cells shown in lines of different color) at 6 hpf and 8.4 hpf, obtained using the Plot Profile tool (Fiji) through the length of the cell. Peaks correspond to cell membrane. Scale bars: 20 μ m.

To test further the possible relationship between ventral to dorsal gastrula patterning and Vangl2 we performed whole-mount immunostaining using anti-Vangl2 antibody at mid-gastrulation (8.4 hpf) and quantified membrane fluorescence intensity in ventral, lateral, and dorsal gastrula domains. We did not observe significant differences in the accumulation of Vangl2 at cell membrane between these domains (supplementary material Fig. S4). This provides further evidence against the ventrodorsal gradient of BMP, or another regional signal, regulating Vangl2 membrane accumulation during gastrulation.

We next tested whether Vangl2 membrane accumulation requires Wnt/PCP signaling. We observed that in 10.7 hpf WT embryos mosaically expressing *Xdd1*, a dominant-negative mutant form of *Xenopus* Dvl that impairs noncanonical Wnt signaling and C&E movements in frog and zebrafish (Jessen et al., 2002; Sokol, 1996; Wallingford et al., 2000), groups of cells expressing *Xdd1* did not present any perturbation in Vangl2 membrane localization compared with neighboring cells (Fig. 5A,A'). Likewise, we observed normal Vangl2 membrane localization at 10.7 hpf in *kny^{fr6/fr6} (gpc4)*, *MZscribble^{rw468/rw468} (landlocked)* and *MZfz7a^{e3/e3}, MZfz7b^{hu3465/hu3465} (fzd7a/b)* mutants (Fig. 5), which are all defective in PCP signaling (Quesada-Hernández et al., 2010; Topczewski et al., 2001; Wada et al., 2005). Vangl2

was also detected at cell membranes of WT cells transplanted into *MZvangl2^{vu67/vu67}* mutants, suggesting that Vangl2 membrane localization is independent of its presence at the cell membrane in the adjacent cell (Fig. 5E). These results indicate that key signaling pathways that pattern vertebrate gastrulae and/or regulate C&E movements, including BMP and Nodal and Wnt/PCP signaling, are not necessary for Vangl2 localization to the plasma membrane.

Vangl2 membrane accumulation at mid-gastrulation is regulated both cell-autonomously and by a stage-specific non-autonomous global signal

To understand whether Vangl2 membrane accumulation at the onset of C&E is a cell-autonomous process or is mediated by a non-cell-autonomous signal, we performed heterochronic transplantation experiments between WT embryos of different developmental stages (Fig. 6A). In the first set of experiments, cells were transplanted from sphere stage (4 hpf) donors into germ ring/shield stage (5.5-6 hpf) hosts. When donor embryos reached the shield stage and host embryos the 75-80% epiboly stage (8-8.4 hpf), Vangl2 protein localization was assessed by whole-mount immunostaining and its membrane localization was quantified in confocal microscopy images (Fig. 6B-C',G). The intensity of Vangl2 expression on cell

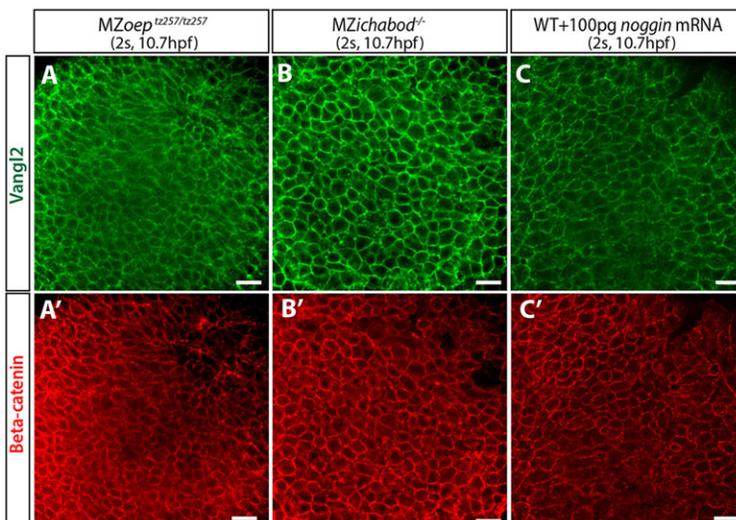


Fig. 4. Membrane localization of Vangl2 in embryos with patterning defects. Confocal images of whole-mount immunostaining with antibodies against zebrafish Vangl2 C-terminus (A-C) and β -catenin (A'-C') in *MZoeptz257/tz257* (A,A') and *MZichabod* (B,B') embryos, and WT embryos injected with 100 pg *noggin* synthetic mRNA (C,C'). Scale bars: 20 μ m.

membranes was normalized to background (the interior of the same cell). Homochronic transplantations with sphere stage donors and hosts were carried out as a control, and analyzed by Vangl2 staining at 8 hpf (Fig. 6F). These experiments indicated that, unlike in homochronic transplantations, the cells transplanted from 'younger' donors into 'older' host embryos showed significantly higher amounts of Vangl2 protein at the cell membrane than observed in the younger donor embryo from which they were removed (Fig. 6G). However, Vangl2 membrane localization levels in these younger donor cells did not reach the level observed in the surrounding older host cells. These results suggest that Vangl2 membrane localization is stimulated in the younger donor cells by a non-cell-autonomous signal when transplanted into an older WT host embryo, but that a cell-intrinsic mechanism is also involved.

To test this further, we performed a reverse heterochronic transplantation experiment by moving cells from an older (shield stage, 6 hpf) donor into a younger (sphere stage, 4 hpf) host embryo. Vangl2 membrane localization was assessed as described above, when the younger hosts reached shield stage and donor embryos the 75-80% epiboly stage (8-8.4 hpf) (Fig. 6D-E"). In these experiments, the level of Vangl2 membrane localization in the cells transplanted from older donors was lower than in the cells of the donor embryo, but higher than in cells of the younger host embryo (Fig. 6H). These results further support the involvement of a cell-autonomous mechanism of Vangl2 membrane accumulation at a specific stage of gastrulation. However, considering that Vangl2 protein levels at the membrane were lower in transplanted older cells than in the original donor embryos, non-autonomous global signals also regulate Vangl2 accumulation at the cell membrane at mid-gastrulation, when increasing C&E movements take place (Sepich et al., 2000).

Vangl2 localizes asymmetrically in highly polarized gastrula cells

Although a polarized intracellular distribution of Vangl2 has been observed in the developing ear and skin epithelia in the mouse (Devenport and Fuchs, 2008; Montcouquiol et al., 2006), whether this core Wnt/PCP component shows asymmetric localization during vertebrate gastrulation remains to be determined. Given the previously reported asymmetric localization of PCP components Pk and Dvl in vertebrate gastrulae at the anterior and posterior cell membranes, respectively, we hypothesized that Vangl2 is enriched at the anterior cell membrane, where Pk is enriched (Ciruna et al., 2006; Yin et al., 2008). To test this we used several approaches to

quantify the amount of Vangl2 protein at the anterior and posterior cell membranes.

First, we performed whole-mount immunofluorescence on WT embryos with anti-Vangl2 antibody, acquired confocal images and measured the fluorescence intensity at the cell membranes. This quantification based on the merged anterior/posterior and medial/lateral cell boundaries showed no obvious asymmetric pattern of Vangl2 protein distribution in the presomitic mesoderm (data not shown).

Second, we adopted the mosaic approach that allows the visualization of protein distribution asymmetries on single anterior or posterior membranes using a transgenic line expressing a zebrafish GFP-Vangl2 N-terminal fusion protein (Sittaramane et al., 2013). We transplanted a few cells from *Tg(vangl2:GFP-Vangl2)* embryos injected with *mCherry* synthetic mRNA (used as a control) into unlabeled WT host embryos. Confocal images were acquired at the dorsal location at 90% epiboly stage (9 hpf) when C&E movements are most dramatic, and at the 5-6 somite (s) stage (11.7-12 hpf) when cells are extremely polarized. We quantified the fluorescence intensity (FI) at the anterior and posterior cell membrane of isolated cells for both GFP-Vangl2 and mCherry proteins using the Plot Profile tool (Fiji) and calculated the ratio posterior/anterior membrane FI of GFP-Vangl2 and mCherry for each cell. At 9 hpf in the notochord GFP-Vangl2, like mCherry, did not show any significant difference in FI at the anterior and posterior cell membranes ($n=23$ cells, 4 embryos) (Fig. 7A,A',D). However, at segmentation stages (11.7-12 hpf), in the notochord ($n=23$ cells, 8 embryos) and neuroectoderm ($n=15$ cells, 4 embryos) we observed an anterior bias in GFP-Vangl2 localization, contrasting with the random anterior or posterior enrichment observed for mCherry protein (Fig. 7B-C',E).

As an alternative approach, we used mosaic expression of the human GFP-VANGL2 N-terminal fusion protein (construct provided by the T. Ogura lab, Tohoku University, Japan). Indicating that the GFP-VANGL2 protein has normal activity, after injection of synthetic *GFP-VANGL2* mRNA (25-75 pg/embryo) we observed partial but significant rescue of the axis elongation phenotype in *MZvangl2^{vu67/vu67}* (supplementary material Fig. S1), comparable with previously reported incomplete rescue of the *vangl2* mutant phenotype using synthetic WT zebrafish *vangl2* mRNA (Jessen et al., 2002). In addition we observed that, similar to the endogenous protein, GFP-VANGL2 became localized at the cell membranes at late gastrulation and segmentation stages (Fig. 7G). These results indicate that the

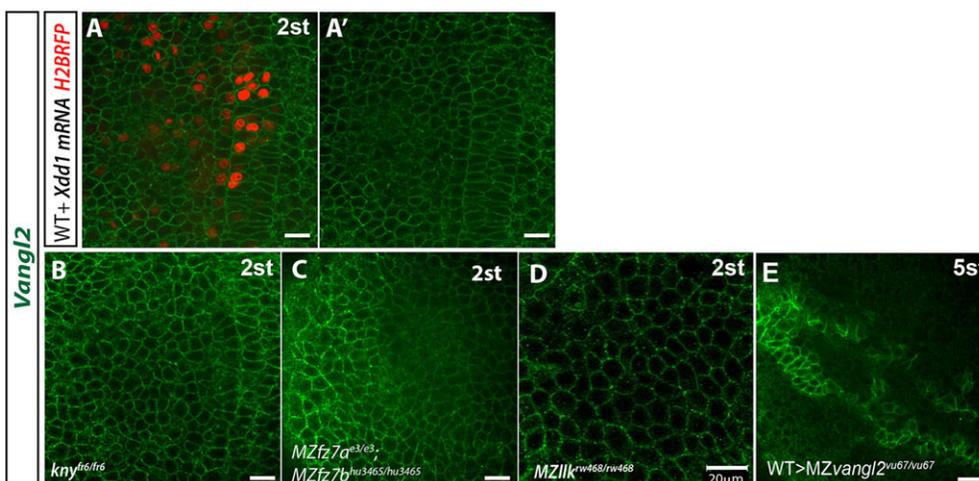


Fig. 5. Vangl2 membrane localization in PCP mutant backgrounds. Confocal images of whole-mount immunostaining with antibody against zebrafish Vangl2 C-terminus at 2 s stage (10.7 hpf) in embryos mosaically expressing Xdd1 and H2B-RFP (A,A') or in *kny^{tr6/tr6}* (B), *MZfz7^{ae3/ae3}*, *MZfz7b^{hu3465/hu3465}* (C), *MZllk^{rw468/rw468}* (D) or *MZvangl2^{vu67/vu67}* embryos (11.7 hpf, 5 s) containing transplanted WT cells (showing strong Vangl2 membrane expression) (E). Scale bars: 20 μ m.

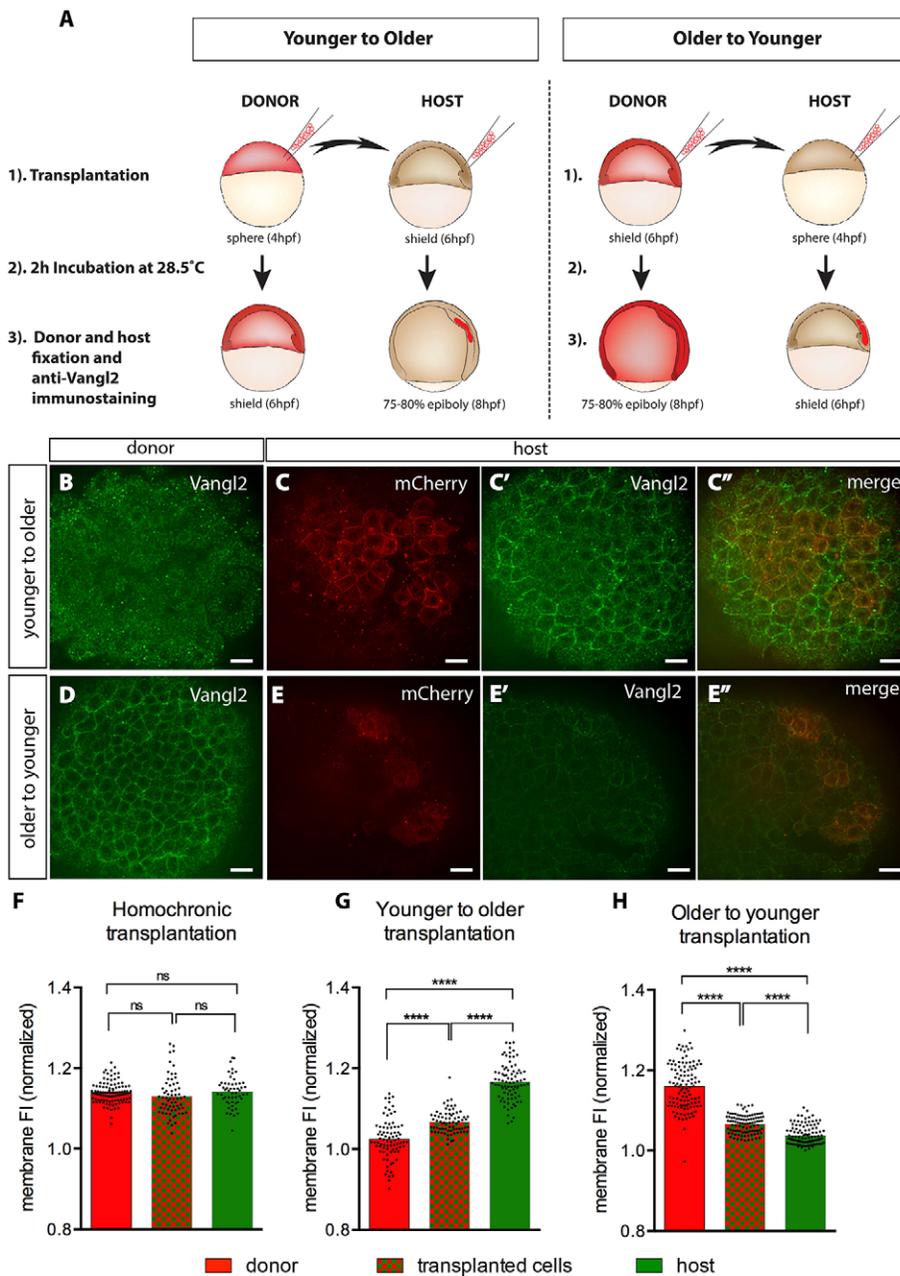


Fig. 6. Heterochronic cell transplantations.

(A) The experimental scheme. (B-E'') Confocal images of donor and host embryos after immunostaining for Vangl2 and mCherry. (B-C'') Results of transplantation from younger donor embryos into older hosts. (B) Donor embryo 2 h post transplantation, 6 hpf. (C-C'') Host embryo 2 h post transplantation, 8 hpf. (D-E'') Results of transplantation from older donor embryos into younger hosts. (D) Donor embryo 2 h post transplantation, 8 hpf. (E-E'') Host embryo 2 h post transplantation, 6 hpf. (C,E) mCherry staining shows the transplanted cells. (F-H) Quantitative data showing the membrane fluorescence intensity (FI) in transplanted cells, and endogenous cells in host and donor embryos. Quantitative data are from homochronic (control) and heterochronic cell transplantations. Bars represent the mean FI. **** $P < 0.0001$; ns, not significant; unpaired t -test with Welch's correction. Scale bars: 20 μ m.

activity of the GFP-VANGL2 fusion protein during gastrulation is comparable to that of the zebrafish Vangl2 protein.

To express GFP-VANGL2 in a mosaic fashion, we injected synthetic *GFP-VANGL2* mRNA into one blastomere of WT embryos at the 64-cell stage (Fig. 7F), after first titrating the mRNA to establish the concentration that would enable us to visualize fluorescence without causing gastrulation defects due to excess *VANGL2* expression (Jessen et al., 2002). *mCherry* mRNA was co-injected as a control. Confocal images were acquired during early segmentation and we focused on highly polarized notochord cells. The FI of GFP-VANGL2 and mCherry was quantified as above. Compared with mCherry, which exhibited random anterior/posterior enrichment, GFP-VANGL2 levels were consistently higher at anterior cell membranes (Fig. 7H,I; $n=12$ cells, 3 embryos).

Together, these lines of experimentation support the notion that Vangl2 is asymmetrically localized in highly polarized notochord

and neuroectoderm cells at early segmentation stages, but not in less polarized cells during gastrulation.

We next asked whether the anterior bias in Vangl2 membrane localization is dependent on functional Wnt/PCP signaling. We either injected a minimal dose of *GFP-VANGL2* into one cell of a 64-cell stage *MZfz7a^{e3/e3};MZfz7b^{hu3465/hu3465}* or *kny^{fr6/fr6}* mutant embryo, or we transplanted a few cells from *Tg(vangl2:GFP-Vangl2)* embryos co-injected with *Xddl* and *mCherry* mRNA into a WT host previously injected with the same dose of *Xddl* mRNA. We quantified the membrane FI exclusively in individual cells present in the notochord at the 5-6 s stage (11.7-12 hpf) as described above. Our data showed that perturbation of the PCP pathway in *MZfz7a^{e3/e3};MZfz7b^{hu3465/hu3465}* ($n=27$ cells, 7 embryos) and *Xddl*-injected embryos ($n=27$ cells, 8 embryos) did not result in a significant loss of the anterior bias in Vangl2 localization (Fig. 8A,A',C). By contrast, *kny^{fr6/fr6}* mutants showed significantly decreased Vangl2 polarization, although not a total loss of polarity ($n=43$ cells, 7 embryos) (Fig. 8B-C).

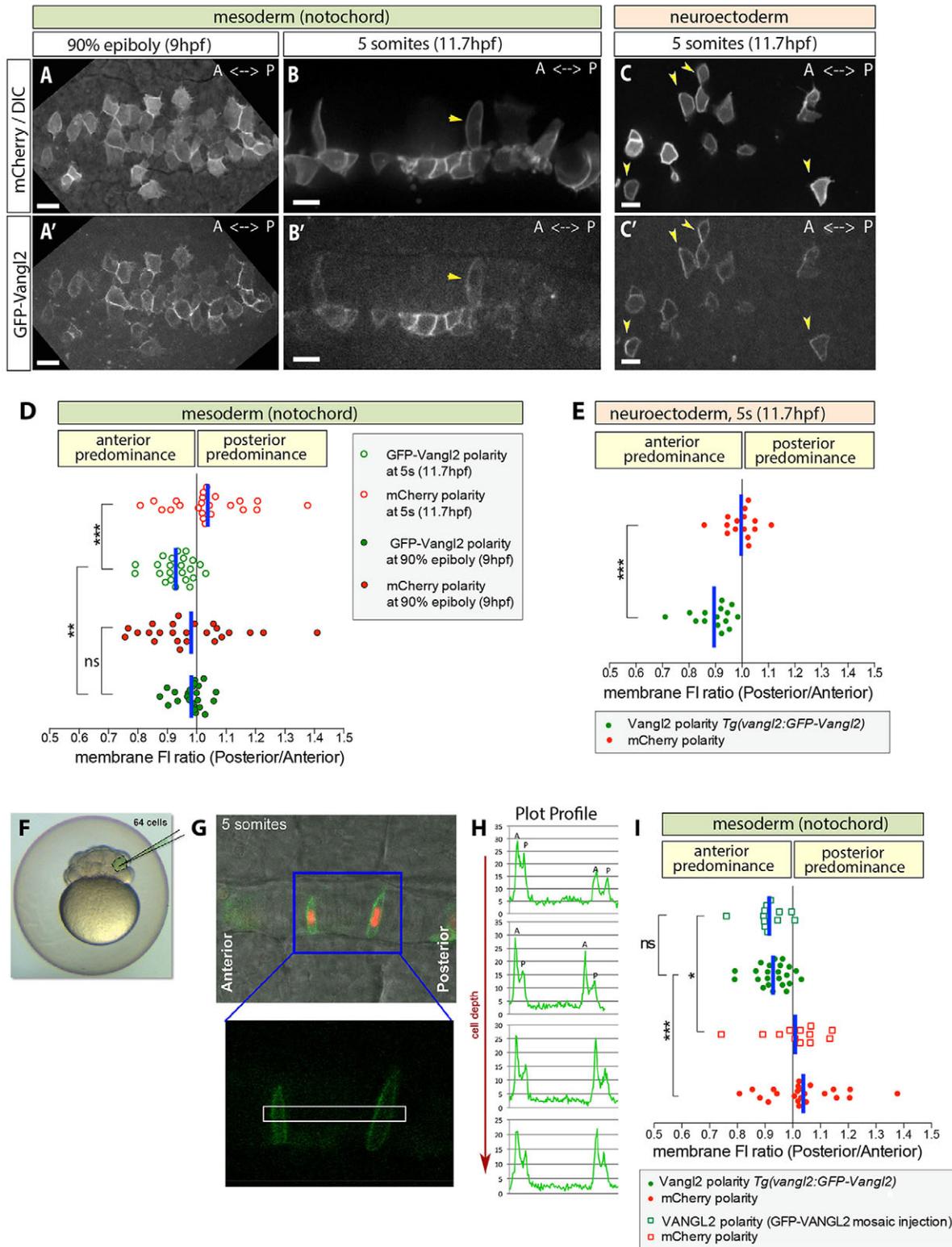


Fig. 7. Enrichment of Vangl2 at anterior membranes of mediolaterally elongated cells. (A-C') Live embryos at 90% epiboly stage (9 hpf; A,A') or 5 s stage (11.7 hpf; B-B'). mCherry- and GFP-Vangl2-expressing cells were transplanted from *Tg(vangl2:GFP-Vangl2)* embryos into the WT unlabeled host. Yellow arrowheads indicate the anterior cell membranes where Vangl2 is enriched. (D,E) GFP-Vangl2 and mCherry posterior/anterior membrane FI ratios of individual cells in the notochord at 90% epiboly stage (9 hpf) and at 5-6 s stage (11.7-12 hpf) (D) or in the neuroectoderm at 5-6 s stage (11.7-12 hpf) (E). The blue bars represent the average posterior/anterior FI ratios for each condition. (F-I) Vangl2 asymmetry analysis using GFP-VANGL2 mosaic expression. (F) Modified image of 64-cell stage embryo showing the mosaic synthetic mRNA injection performed. (G) Live embryos mosaically expressing GFP-VANGL2 (green)/H2B-RFP (red) in notochord cells. (H) Plot Profile (Fiji) quantification of FI of two cells (white rectangle in G) at four different z-planes (confocal optical slices). (I) Comparison of GFP-VANGL2, GFP-Vangl2 and corresponding mCherry membrane FI ratios of individual cells in the notochord at 5-6 s (11.7-12 hpf) stage. **P*<0.05, ***P*<0.01, ****P*<0.001; ns, not significant; unpaired *t*-test with Welch's correction. Scale bars: 20 μm in A,A'; 10 μm in B-C'.

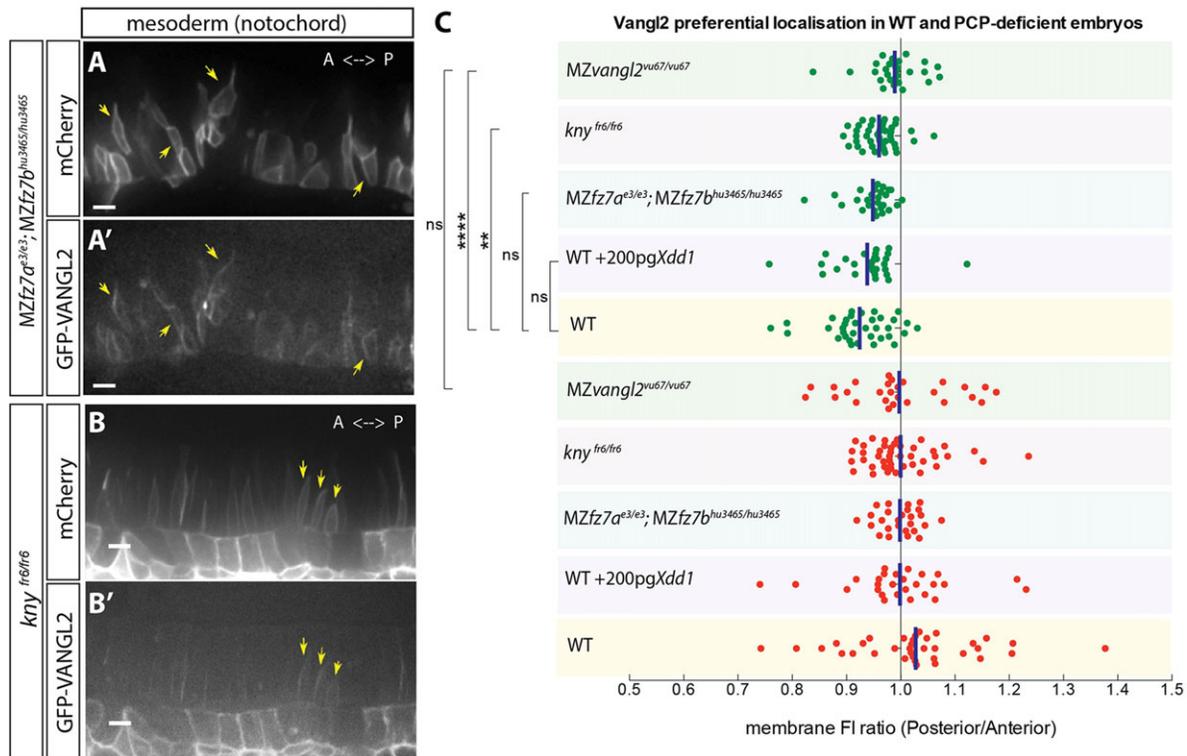


Fig. 8. Vangl2 membrane localization in embryos with defective PCP signaling. (A-B') Live embryos mosaically expressing GFP-VANGL2 and mCherry. Arrows indicate individual cells showing anteriorly biased Vangl2 membrane localization. (C) Quantification of mGFP (green) and mCherry (red) posterior/anterior membrane FI ratios obtained by analyzing individual cells in the notochord of 5-6 s stage (11.7-12 hpf) embryos in specified mutants or *Xdd1* RNA-injected embryos. The data plot shows the quantification of either GFP-Vangl2 from *Tg(vangl2:GFP-Vangl2)* cells in MZvangl2^{vu67/vu67} and *Xdd1*-injected embryos, or of GFP-VANGL2 in MZfz7a^{e3/e3}; MZfz7b^{hu3465/hu3465} and *kny^{fr6/fr6}* embryos. GFP-Vangl2 and GFP-VANGL2 quantification data are combined for the WT embryos. Blue bar indicates the average posterior/anterior FI ratio for each condition. ***P*<0.01, *****P*<0.0001; ns, not significant; unpaired *t*-test with Welch's correction. Scale bars: 10 μ m.

Given that Vangl2 showed reduced anterior bias at the membrane when PCP is perturbed, we next examined whether this bias is cell-autonomous. We transplanted cells expressing GFP-Vangl2 from *Tg(vangl2:GFP-Vangl2)* embryos injected with *mCherry* mRNA into unlabeled MZvangl2^{vu67/vu67} host embryos. We selected and imaged embryos that contained individual GFP-Vangl2/mCherry-expressing cells surrounded by MZvangl2^{vu67/vu67} host cells in the notochord. Quantification of the FI at the anterior and posterior membranes of these cells revealed that GFP-Vangl2 was not asymmetrically localized and presented a random membrane preference similar to that observed for mCherry (*n*=27 cells, 7 embryos) (Fig. 8C). This result revealed a non-cell-autonomous function in the preferential anterior membrane localization of Vangl2.

DISCUSSION

During C&E gastrulation movements in zebrafish, dorsally migrating mesodermal cells undergo a remarkable transformation from round cells migrating along complex trajectories to mediolaterally elongated cells that move efficiently along straighter paths in a Wnt/PCP-dependent manner (Sepich et al., 2005; Sepich and Solnica-Krezel, 2005). The Wnt/PCP pathway has been proposed to act as a cellular compass that links the embryonic AP or tissue proximodistal polarity to that of individual cells, with some pathway components accumulating at the anterior and others at the posterior (or proximal and distal) cell membranes (Adler, 2002; Adler et al., 2000; Amonlirdviman et al., 2005; Bastock et al., 2003; Devenport and Fuchs, 2008; Gao et al., 2011; Gray et al., 2011). However, the

sequence of these morphological transformations and how they are affected by Wnt/PCP signaling was not clear. In this study we delineate the sequence of the morphological transformations that the dorsally converging mesodermal cells undergo, identify the role of the core PCP component Vangl2 in these transformations, and the accompanying dynamic changes in the intracellular distribution of Vangl2 protein. We show that Vangl2 membrane accumulation preceding ML cell polarization relies on both cell-intrinsic and non-cell-autonomous mechanisms. Moreover, Vangl2 localizes preferentially at the anterior cell membrane in mediolaterally polarized notochord and neuroepithelial cells.

Using time-lapse analyses we visualized the entire morphological transformation from round to mediolaterally elongated mesodermal cells engaged in dorsal convergence. Our analysis of cell shape in different domains (90°, 55° and 20° from dorsal) along the ML axis is consistent with previous studies indicating that cells present a more elongated cell shape when closer to the dorsal midline (Myers et al., 2002a; Shih and Keller, 1992). Interestingly, we show that cells in a single location gradually increase their LWR over time. Concomitant with cell body elongation, ML cell alignment occurs gradually between the three ML domains and, also in time, in the same domain (Fig. 1E). The process of cell elongation and ML alignment could be regulated by a cell-intrinsic mechanism or by a stage-specific secreted signal(s), or both. We previously demonstrated that Vangl2 has both cell-autonomous and non-autonomous functions in ML cell elongation (Jessen et al., 2002). Rho kinase 2 (Rok2) and $\text{G}\alpha_{12/13}$, both regulators of the actin cytoskeleton, have been shown to regulate cell polarity and cell behavior during gastrulation, with

$G\alpha_{12/13}$ acting in a cell-autonomous manner and likely in parallel to Wnt/PCP (Lin et al., 2005), and Rok2 affecting cell shape cell-autonomously but ML cell alignment in a non-cell-autonomous manner acting downstream of Wnt/PCP (Lin et al., 2005; Marlow et al., 2002). Although a link between the cytoskeleton and Wnt/PCP has been established, stage/domain-specific signals instructing such cytoskeletal regulators remain to be clarified. Our study demonstrates that Vangl2 is essential for cells to undergo location- and time-specific cell elongation and alignment. As Rok2 has been proposed to act downstream of Wnt/PCP signaling, it will be important to understand the activity of this kinase in the morphological transformation of mesodermal cells during C&E described here.

Our analyses revealed that *vangl2* mutant mesodermal cells present an abnormal AP bias of cell alignment and trajectories (Figs 1 and 2). Previous cell behavioral analyses showed that at the onset of gastrulation the trajectories of lateral mesodermal cells are oriented anally/anteriorly, but these trajectories become dorsally biased at mid-gastrulation (Jessen et al., 2002; Sepich et al., 2005). It has been hypothesized that a repulsive signal from the blastopore/blastoderm margin or an attractive signal from the animal pole guides the early animal/anterior migration of mesodermal cells away from the blastopore. The signals underlying the 90° shift in movement direction are unknown, but Wnt/PCP signaling is not absolutely required for dorsalward movement (Sepich and Solnica-Krezel, 2005; Yin et al., 2008). Rather, a dorsal midline-derived chemokine has been hypothesized to explain the dorsal migration based on computational modeling (Sepich and Solnica-Krezel, 2005). Moreover, epiboly movements that occur simultaneously with C&E and are posteriorly/vegetally directed have a potential influence on cellular trajectories (Sepich and Solnica-Krezel, 2005; Warga and Kimmel, 1990). In our analysis at late gastrulation to the 1 s stage, WT cells located 55° from the dorsal domain exhibited a significant dorsal movement preference (Fig. 2). *vangl2* cells also migrated dorsally, although their trajectories were slightly anally/anteriorly biased in all analyzed domains along the animal-vegetal/AP axis (Fig. 2D). This anterior bias in *vangl2* mutant cell movement could be due to their persistent perception of the above discussed attractive or repulsive signal along the AP axis that guides the animal migration of lateral mesodermal cells at early gastrulation (Sepich and Solnica-Krezel, 2005). It is intriguing that Vangl2 and PCP signaling may promote effective dorsal migration in part by suppressing anterior/animalward cell polarity and movement. The nature of the signal guiding mesodermal migration away from the blastoderm margin is not known in zebrafish, but FGF8 was proposed to have this activity in the chick gastrula (Yang et al., 2002).

We detected Vangl2 protein, using an antibody specific to the zebrafish protein (Li et al., 2013), largely in cytoplasmic puncta at early gastrulation, but predominantly at the plasma membrane at mid-gastrulation, just preceding initiation of PCP-dependent ML cell elongation (Fig. 3; supplementary material Figs S2 and S3). Given that Vangl2 is a four-pass transmembrane protein, the punctate pattern observed at early gastrulation could be due to the accumulation of Vangl2 protein in vesicular cargoes and endoplasmic reticulum. In murine neural tissues, Vangl2 is selectively sorted into COPII vesicles by Sec24b for transport from the endoplasmic reticulum to the Golgi (Merte et al., 2010). Interestingly, Rack1 (Gnb211) protein interacts with Vangl2 and has been shown to regulate C&E during gastrulation, oriented cell division, and cellular polarization. Injection of *rack1*-MO prevented membrane localization of EGFP-Vangl2 fusion protein expressed in

zebrafish embryos (Li et al., 2011). However, it is still unclear which signal(s) triggers this interaction and it would be interesting to test whether *rack1* depletion affects the localization of endogenous Vangl2.

We addressed the mechanism of Vangl2 membrane accumulation by heterochronic transplantations. The amount of Vangl2 protein at the cell membranes in the cells transplanted from older to younger embryos was decreased compared with cells of the donor embryo, but it was higher in these cells than in the cells in the younger host embryo (Fig. 6). In the reverse experiment, in which groups of younger cells were transplanted into an older host, the amounts of Vangl2 protein at the membrane were higher in the transplanted cells than in the younger donor embryo, although they did not match the levels observed in the older hosts. Together, these results support the notion that the accumulation of Vangl2 protein at the plasma membrane during gastrulation depends both on cell-intrinsic as well as non-cell-autonomous mechanisms. Whereas, the extracellular cue(s) and cell-autonomous mechanism involved remain to be defined, we showed that neither of the global patterning regulators Nodal and BMP is required for Vangl2 localization to the cell membrane. Furthermore, Vangl2 accumulates at the membrane independently of location along the DV gastrula axis, implying that ventral-dorsal patterning does not regulate Vangl2 membrane accumulation. Finally, our experiments argue against the involvement of the PCP components Kny, Fz7a and Fz7b, Dvl and Scribble 1 in this process. However, other global signaling pathways could be involved. For instance, in this study we did not address the involvement of the fibroblast growth factor (FGF) pathway, which has been implicated in C&E movements in chick and frog embryos (Nutt et al., 2001; Yang et al., 2002). It is an intriguing possibility that post-translational modifications could regulate Vangl2 intracellular localization during gastrulation. Indeed, Vangl2 is subject to phosphorylation in its N-terminal domain under the control of Wnt5a in mouse limb development (Gao et al., 2011). Vangl2 phosphorylation is required for, and modulates, its activity. In zebrafish, phosphorylation of Vangl2 is a possibility that warrants further investigation.

Similar to *Drosophila*, in which some core PCP components assume an asymmetric distribution at the apical cell junctions (Amonlirdviman et al., 2005; Axelrod, 2001; Lawrence et al., 2004; Ma et al., 2003), studies in zebrafish using GFP fusion proteins have shown that, in the dorsal mesoderm and neuroectoderm, Pk-GFP and Dvl-GFP are asymmetrically localized at the anterior and posterior cell membranes, respectively (Ciruna et al., 2006; Yin et al., 2008). In the mouse limb mesenchyme, functional Wnt/PCP is essential for the asymmetric distribution of Vangl2 and proximodistal polarization of chondrocytes (Gao et al., 2011). In the present study, using different approaches to quantify mosaicly expressed GFP-Vangl2 protein at the cell membranes, we observed that Vangl2 protein is present at all cell membranes of mesenchymal cells during gastrulation, but is preferentially enriched at the anterior membrane of dorsal mesodermal and neuroectodermal cells. However, our methods did not allow us to detect Vangl2 protein asymmetry in less polarized cells undergoing C&E, including notochord (late gastrulation, 9 hpf). This result is consistent with previous data showing that Pk asymmetric membrane localization is detectable at 10 hpf and not at mid-gastrulation (8 hpf) (Yin et al., 2008).

In contrast to the *Drosophila* system, in which the asymmetry of core PCP components has been shown to be very pronounced in epithelial tissues, in zebrafish gastrulae these asymmetries are less clear, probably owing to the dynamic nature of mesenchymal cells during gastrulation. In *Drosophila*, the asymmetric accumulation of

the core PCP components at opposite cell membranes is a gradual process and is preceded by their accumulation at the membrane in uniform fashion (Strutt, 2002). This mechanism might be similar in vertebrates, in which asymmetric Wnt/PCP proteins distribution arise gradually and are detectable only in highly polarized cells. Furthermore, Vangl2 asymmetry at cell membranes in the axial mesoderm was not affected in *fz7a;fz7b* mutants or in embryos expressing Xdd1, and it was significantly reduced, but not abolished, in *kny* mutants. These observations are consistent with the Pk-GFP and Dvl-GFP asymmetries being reduced in *kny* and *vangl2* single mutants and almost completely absent in *kny;vangl2* compound mutants (Yin et al., 2008).

We interpret these results to mean that the asymmetric distribution of PCP components requires PCP signaling, but that even low levels of PCP signaling are sufficient. Interestingly, Vangl2 polarity was lost in WT cells transplanted into MZ*vangl2*^{vu67/vu67} gastrulae, suggesting that the asymmetric localization of Vangl2 is non-cell-autonomous (Fig. 8C). Since Vangl2 asymmetric localization was not lost in embryos with reduced Wnt/PCP signaling and cell polarity, but was lost in a *vangl2* null background, we hypothesize that it requires PCP signaling and/or the interaction of protein complexes containing Vangl2 and possibly Fmi/Celsr on opposite membranes of neighboring cells (Strutt and Strutt, 2008). Future studies will explore the molecular mechanisms underlying the emergence of core PCP protein intracellular asymmetries and how they effect the associated changes in cellular morphology and behavior during C&E movements.

MATERIALS AND METHODS

Zebrafish lines and husbandry

Wild-type zebrafish (AB*, Tübingen and hybrids) and fish carrying mutations in *vangl2* (*tri*^{vu67}), *fz7a*^{e3}, *fz7b*^{hu3495}, *kny*^{fr6} (Topczewski et al., 2001), *scribble 1b* [originally described as *landlocked*, *llk*^{rw468} (Wada et al., 2005)] were maintained according to standard procedures as described in Solnica-Krezel et al. (1994). Naturally spawned embryos were raised at 28–32°C and staged by morphology (Kimmel et al., 1995). *Tg(vangl2:GFP-Vangl2)* fish were a gift from Anand Chandrasekhar (University of Missouri, Columbia, MO, USA) (Sittaramane et al., 2013). MZ*vangl2*^{vu67/vu67} fish were obtained by germ cell transplantation as described by Ciruna et al. (2002). The experiments involving embryos and adult zebrafish conformed to the relevant regulatory standards.

Whole-mount immunostaining

Embryos at shield stage (6 hpf) to 5 s stage (11.7 hpf) were fixed in Prefer Fixative (Anatech) at room temperature for 30–50 min and rinsed in 1× PBS. Dechorionated embryos were incubated in permeabilization solution [0.5% (w/v) Triton X-100, 5% BSA, 5% serum, 2% DMSO, 1× PBS] for 20–30 min. Then, embryos were incubated for 1 h in blocking solution (0.1% Triton X-100, 5% BSA, 10% serum, 2% DMSO, 1× PBS). Primary and secondary antibodies were diluted in blocking solution. Embryos were incubated in primary antibody solution overnight and in secondary antibody solution for 1 h at room temperature. Embryos were rinsed and stored in 1× PBS at 4°C. Primary antibodies: rabbit polyclonal anti-zebrafish Vangl2 (made against a C-terminal peptide), 1:500; anti-β-Catenin (Sigma C7207), 1:250; anti-RFP (Allele Biotechnology, ACT-CM-MRRFP10), 1:500. Secondary antibodies: goat anti-rabbit Alexa-488 (Invitrogen-Molecular Probes, A11034), 1:500; goat anti-rabbit Alexa-647 (Invitrogen-Molecular Probes, A21245), 1:500; goat anti-mouse Alexa-568 (Invitrogen-Molecular Probes, A11031), 1:500; goat anti-rat Alexa-568 (Invitrogen-Molecular Probes, A11077), 1:500.

Microscopy image acquisition

For Nomarski time-lapse acquisition, live dechorionated embryos were mounted in 0.5% low-melting-point agarose (LMP, SeePlaque Agarose, Cambrex Bio Science, 50100) in 0.3× Danieau's buffer, in wells in a 1.5%

agarose block on glass-bottom Petri dishes (MatTek, P35G-1.0-14-C). For live imaging, embryos were kept at 28.5°C with a heating chamber. For time-lapse, z-stacks were collected with 30 s or 1 min intervals using a 40× objective (N.A 0.75). Images were acquired using a Zeiss LSM510 inverted confocal microscope or Olympus IX81 inverted microscope, Quorum spinning disc confocal and MetaMorph software (Molecular Devices).

Quantification of cell shape and alignment

Cell shape (LWR) and cell alignment (angle) analyses were performed using Fiji software (NIH). Cell membrane boundaries were drawn manually. Quantification of the major and minor axis of the cell (fitting ellipse function) and angle were estimated using Fiji. LWR is defined as major axis/minor axis.

Synthetic RNA synthesis and injections

RNA was synthesized using the Ambion mMESSAGE mMACHINE Kit for SP6 (AM1340) or T7 (AM1354) promoter. RNA concentrations were measured by NanoDrop and by gel electrophoresis. Injections were performed at the 1- or 64-cell stage. 40 pg or 200 pg *Xdd1* synthetic mRNA was injected into one cell at the 64-cell or 1-cell stage, respectively. *Xenopus noggin* synthetic mRNA (100 pg) achieved a strong dorsalization phenotype (Hammerschmidt et al., 1996).

Transplantation experiments

For homochronic/heterochronic transplantations donor embryos were injected with 100 pg *mCherry* synthetic mRNA at the 1-cell stage. For heterochronic transplantations, donors and host embryos were fertilized 2 h apart. 'Younger to older' transplantation was performed by moving cells from sphere stage (4 hpf) donor embryos into shield stage (6 hpf) host embryos. 'Older to younger' transplantation was performed in the reverse order. Embryos developed at 28.5°C until the host embryos reached 80% epiboly (8.4 hpf) and were fixed for 40 min in Prefer Fixative at room temperature. Homochronic control transplantations were performed at sphere stage and embryos were fixed at 80% epiboly. Whole-mount immunostaining was performed in individual tubes for each set of host-donor embryos. The image acquisition settings were identical for each embryo in the individual sets.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

All authors conceived and designed the experiments, analyzed and discussed the data, and discussed the manuscript. I.R. and J.R.J. performed the experiments. D.S.S., A.C. and L.S.-K. contributed reagents, materials and analysis tools. I.R. wrote the manuscript with the assistance of L.S.-K. and J.R.J.

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Supplementary material

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