

ARTICLE

Polarity Proteins Are Required for Left–Right Axis Orientation and Twin–Twin Instruction

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Summary: Two main classes of models address the earliest steps of left–right patterning: those postulating that asymmetry is initiated via cilia-driven fluid flow in a multicellular tissue at gastrulation, and those postulating that asymmetry is amplified from intrinsic chirality of individual cells at very early embryonic stages. A recent study revealed that cultured human cells have consistent left–right (LR) biases that are dependent on apical-basal polarity machinery. The ability of single cells to set up asymmetry suggests that cellular chirality could be converted to embryonic laterality by cilia-independent polarity mechanisms in cell fields. To examine the link between cellular polarity and LR patterning in a vertebrate model organism, we probed the roles of apical-basal and planar polarity proteins in the orientation of the LR axis in *Xenopus*. Molecular loss-of-function targeting these polarity pathways specifically randomizes organ situs independently of contribution to the ciliated organ. Alterations in cell polarity also disrupt tight junction integrity, localization of the LR signaling molecule serotonin, the normally left-sided expression of *Xnr-1*, and the LR instruction occurring between native and ectopic organizers. We propose that well-conserved polarity complexes are required for LR asymmetry and that cell polarity signals establish the flow of laterality information across the early blastoderm independently of later ciliary functions. *genesis* 50:219–234, 2012. © 2011 Wiley Periodicals, Inc.

Key words: left–right asymmetry; Vangl2; Par6; conjoined twins; heterotaxia; ion flux

INTRODUCTION

During development, embryos acquire specific patterns in three orthogonal directions, which ultimately align

with the dorsal–ventral, anterior–posterior, and left–right (LR) axes. Although considerable insight has been generated into the molecular mechanisms of patterning along the anterior–posterior and dorsal–ventral axes, much remains to be understood about how the LR axis is consistently oriented with respect to the other two (Aw and Levin, 2008, 2009; Levin, 2005, 2006; Speder *et al.*, 2007; Tabin, 2005; Vandenberg and Levin, 2010b). Externally, the vertebrate body-plan possesses bilateral symmetry, but this is coupled with a strikingly conserved LR asymmetry of the internal organs including the morphogenesis and placement of the heart, liver, gall bladder, stomach, and lungs. Abnormalities in laterality form a class of human birth defects that can affect the health of individuals (Casey, 1998; Casey and Hackett, 2000; Kosaki and Casey, 1998; Peeters and Devriendt, 2006). Individuals with heterotaxia, a condition in which the placement of each organ is determined independently, often have serious medical problems because of the failed connections of major blood vessels. In contrast, individuals with situs inversus, the complete mirror inversion of all body organs, are typically healthy.

Two major classes of models have been proposed to explain how consistent asymmetries of the LR axis originate. In the first type of model (Basu and Brueckner,

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2008; Tabin and Vogan, 2003), the LR axis is not oriented until late gastrula/early neurula stages when cells isolated in a small pocket of the embryo [termed the node (mouse), gastrocoel roof plate (GRP; *Xenopus*), or Kupffer's Vesicle (zebrafish)] develop primary cilia that beat in a clockwise manner [reviewed in (Basu and Brueckner, 2008)]. The tilting of the cilia, together with this stereotypical motion, is proposed to set up asymmetric fluid flows that are transduced into a cascade of asymmetric gene expression by direct movement of a morphogen (Tanaka *et al.*, 2005) or activation of Ca^{++} signaling in sensory cilia (McGrath *et al.*, 2003). This is proposed to ultimately lead to asymmetric gene expression and biased organ placement [reviewed in (Tabin and Vogan, 2003)]. In the second class of models, a chiral cytoskeletal organizing element (an intracellular structure such as the centriole) becomes oriented with respect to the other two orthogonal axes within the first few cell cleavages, generating a cytoskeleton able to consistently guide the asymmetric intracellular localization of key maternal components by motor proteins (Aw *et al.*, 2008; Danilchik *et al.*, 2006; Qiu *et al.*, 2005). Such asymmetric distributions of ion channels and pumps give rise to a voltage differential consistently biased along the early LR midline (Adams *et al.*, 2006; Levin *et al.*, 2002), which subsequently drives the establishment of a physiological gradient of prenervous serotonin (5HT) (Fukumoto *et al.*, 2005a; Fukumoto *et al.*, 2005b), ultimately controlling the expression of asymmetric genes by an HDAC-dependent intracellular receptor (Carneiro *et al.*, 2011).

The ciliary model predicts that primary organizers induced later in development should have normal asymmetry since cilia generated at nodes possess their own fixed chirality determined by their molecular structure. In contrast, the cytoplasmic model suggests that the early cleavage events (allowing alignment of intracellular transport events with major embryonic axes) are indispensable for normal asymmetry. We recently showed that organizers whose induction is delayed by as little as four cell divisions give rise to embryos with randomized asymmetry, except when they are induced in a blastoderm containing a primary organizer that was present at the first cleavage (Vandenberg and Levin, 2010a). The inability of organizers to orient asymmetry without instruction from an organizer that had participated in the early-cleavage events led us to propose a "big brother effect"—LR instruction between two organizers embedded in the same blastoderm. We had speculated that planar cell polarity (PCP) is a plausible mechanism for the coordination of organizers' orientation within a cell sheet.

PCP has been more broadly proposed as a mechanism that could be used to orient the LR axis across a wide variety of embryonic architectures (Aw and Levin, 2009; Vandenberg and Levin, 2009). Several recent studies demonstrated a role for PCP in LR asymmetry (Antic

et al., 2010; Borovina *et al.*, 2010; Ferrante *et al.*, 2009; Maisonneuve *et al.*, 2009; May-Simera *et al.*, 2010; Oteiza *et al.*, 2010; Ross *et al.*, 2005; Song *et al.*, 2010; Zhang and Levin, 2009). This has often been interpreted as being due to the actions of PCP in positioning cilia at the node or GRP (Hashimoto *et al.*, 2010; Maisonneuve *et al.*, 2009; Okada *et al.*, 2005). However, inhibition of the PCP protein Vangl2 randomizes asymmetry in chick embryos (Zhang and Levin, 2009), which do not use cilia to establish asymmetry (Gros *et al.*, 2009; Manner, 2001). Likewise, in *Xenopus*, aspects of PCP are already consistently asymmetric at a stage well before ciliary flow appears (Ohkawara and Niehrs, 2011).

PCP has been shown to affect tissue organization and polarity (Chung *et al.*, 2009; Reynolds *et al.*, 2010). Importantly, overexpression of PCP proteins can lead to inappropriate accumulation of other members of the pathway, producing comparable phenotypes to those induced when PCP proteins are downregulated (Amonlirdviman *et al.*, 2005). Most previous studies of PCP and LR asymmetry focused on the protein Vangl2. However, other members of the PCP pathway, including disheveled, diversin, and RSG1, a PCP effector gene, have yet to be examined for a role in LR asymmetry (Gray *et al.*, 2009; Schwarz-Romond *et al.*, 2002).

The first step in establishing PCP is to position planar polarity proteins at the apical surface of epithelial cells (Tree *et al.*, 2002). The Par6/aPKC complex is one apical-basal polarity (ABP) complex that has been shown to play this role, and both Par6 and aPKC have been implicated in asymmetric cell divisions in multiple animals and in embryo-wide asymmetries in *C. elegans* (Burgess, 2008; Guo and Kemphues, 1996; Petronczki and Knoblich, 2001). Previous studies indicate that ABP proteins such as the FERM domain protein Lulu, which is apically localized and required for actin reorganization, are required for orientation of the LR axis in the mouse (Lee *et al.*, 2010). Further, a recent study discovered that single neutrophils in culture display a consistent LR asymmetry (Xu *et al.*, 2007). HL60 cells preferentially extend their pseudopodia to the left of a line connecting the nucleus and centrosome, demonstrating that consistent asymmetries do not require fluid flow in a multicellular node or ciliary motion. The HL60 cells' biases were shown to be dependent on Par6 and aPKC, which is consistent with the requirement of orienting the LR axis relative to the other two axes. Similar data on intrinsic chirality were recently shown for a wide range of other cell types (Wan *et al.*, 2011).

Thus, it becomes important to determine what roles PCP may have in LR-relevant events that do not involve cilia. Interestingly, no studies linking PCP to LR asymmetry have investigated what happens to the LR axis when PCP is altered in cells that do not contribute to the ciliated node or GRP (Vandenberg and Levin, 2010b). Hence, we probed the role of PCP in early, cilia-inde-

Table 1
Antibodies and Concentrations Used for Immunohistochemistry

Antibody	Primary concentration	Secondary	Secondary concentration	Detection	Type of section
Dsh2 (BioMol)	1:50 in 5% goat serum + 1.5% milk	Goat anti-rabbit, conjugated to Alexa 647 (Jackson ImmunoResearch)	1:200 in PBS + 1.5% BSA	Direct via fluorescent secondary antibodies	Paraffin, 10 μ m
5HT (Millipore)	1:500 in 10% goat serum	Goat anti-rabbit, HRP conjugated (Jackson ImmunoResearch)	1:500 in 10% goat serum	HRP substrate (Moss Biologicals)	Gelatin-albumin, 100 μ m
ZO-1 (Invitrogen)	1:50 in 10% goat serum	Goat anti-rabbit, conjugated to Alexa 647 (Jackson ImmunoResearch)	1:100 in 10% goat serum	Direct via fluorescent antibodies	Whole GRP from dorsal explants
Acetylated tubulin (Sigma)	1:50 in 10% goat serum	Goat anti-mouse, conjugated to Alexa 555 (Jackson ImmunoResearch)	1:100 in 10% goat serum	Direct via fluorescent antibodies	Whole GRP from dorsal explants

pendent events in normal singleton embryos and during twin-twin LR instruction. Here, we report a role for ABP and PCP proteins including Par6, Vangl2, aPKC, and others in the orientation of the LR axis in *Xenopus*. Disruption of ABP and PCP by overexpression or inhibition of proteins in these pathways resulted in heterotaxia. Importantly, these effects were observed even when altered expression was limited to cells that do not contribute to the GRP. Disrupted ABP or PCP results in early alterations of the normal localization of 5HT and tight junctional integrity, both known to be crucial for LR asymmetry. Moreover, we observed that both ABP and PCP were required for the LR instruction of a late organizer by an early one in a conjoined twin model. Taken together, the data suggest multiple roles for PCP in laterality; the spatial and temporal aspects of these mechanisms highlight novel, cilia-independent functions during the crucial early events underlying LR patterning.

RESULTS

Alterations in Apical-Basal and Planar Cell Polarities Induce Heterotaxia

Previous studies of single cells suggest that ABP proteins could be involved in fundamental, well-conserved mechanisms of establishing LR asymmetry. To determine whether these same proteins are involved in the orientation of the LR axis in vertebrates, we expressed dominant negative (DN) forms of Par6 and aPKC, two well-characterized proteins necessary for the establishment of ABP, in *Xenopus* embryos. All mRNAs were titrated to levels low enough to enable normal development and dissociate subtle patterning roles from house-keeping functions. Injection of either DNPar6 or DNAPKC mRNAs at one cell specifically induced heterotaxia—an independent randomization of individual organ situs observed at stage 45 (Fig. 1, Table 2). This occurred in the absence of generalized malformations

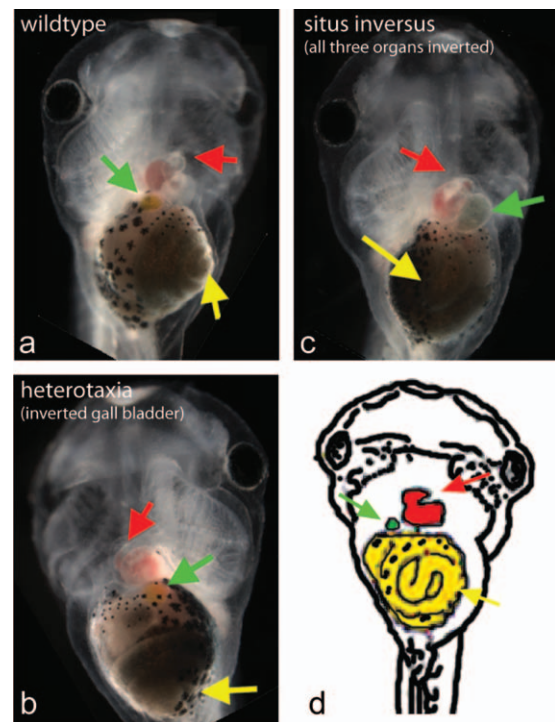


FIG. 1. Alterations in apical-basal polarity affect organ situs, including the loss of concordance of the gall bladder with other organs. **a–c:** Organ position in *Xenopus* embryos at stage 45. **a:** Wildtype organ situs in an untreated embryo, with the stomach (yellow arrow) coiling to the embryo's left, the gall bladder (green arrow) on the embryo's right, and the heart apex (red arrow) located on the embryo's left. **b:** Heterotaxic organ situs in an embryo injected with DNAPKC. In a small percentage of embryos, only the position of the gall bladder is affected. **c:** Situs inversus observed in an embryo injected with DNPar6, marked by the inverted position of all three organs. **d:** Schematic showing the wildtype position of three asymmetric organs, the heart (red), stomach (yellow), and gall bladder (green).

or alterations in the dorso-anterior index. To verify that these constructs were affecting ABP, we examined the expression of an apical marker, cytokeratin, and the

basolateral marker β 1-integrin. In embryos injected with DNPar6, we observed an expansion of basolateral surface markers and a loss of apical surface markers (Supporting Information Fig. 1), as well as defects in the

Table 2
Heterotaxia Is Induced When ABP or PCP Is Disrupted

Construct/ Treatment	(%) Heterotaxia	χ^2 , P -value	(%) toxicity
Untreated	2–4 ($n = 173$ – 248)		8–15
DNPar6	25 ($n = 207$)	66.5, $P < 0.001$	28
DNaPKC	27 ($n = 269$)	48.7, $P < 0.001$	21
Const active CDC42	12 ($n = 52$)	5.4, $P < 0.05$	80
DN CDC42	33 ($n = 82$)	59.1, $P < 0.001$	84
Vangl2 MO	15 ($n = 298$)	25.7, $P < 0.001$	26
RSG1	19 ($n = 183$)	15.6, $P < 0.001$	22
Diversin	12 ($n = 194$)	17.7, $P < 0.001$	47
Disheveled	21 ($n = 48$)	26.5, $P < 0.001$	80

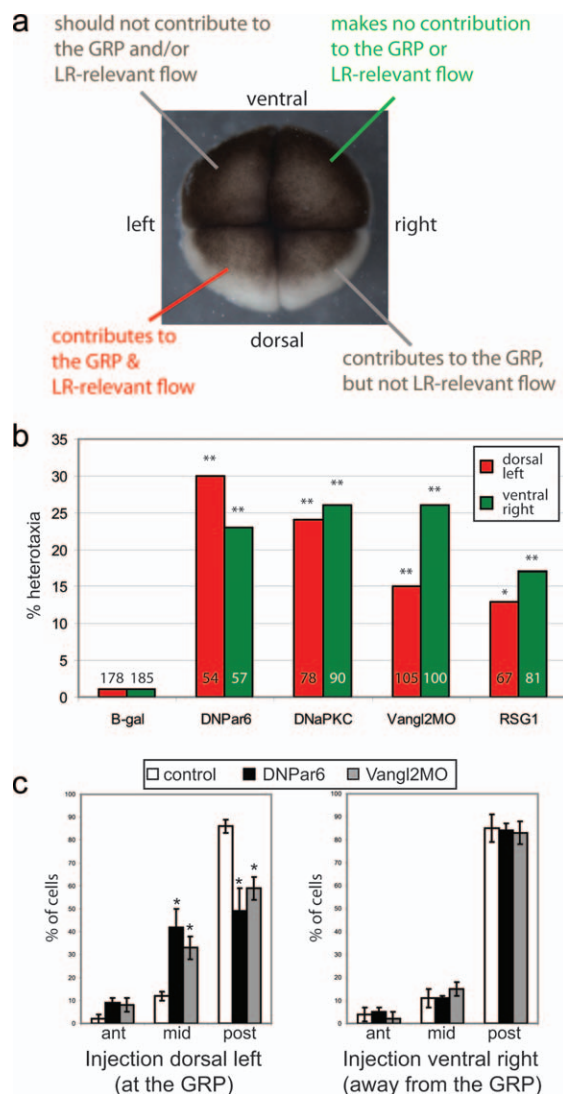


FIG. 2.

pigmented apical surface (data not shown), similar to what was seen previously with expression of DNAPKC (Chalmers *et al.*, 2005). We conclude that normal LR patterning requires Par6 and aPKC, and disruption of these ABP proteins alters not just apical-basal domains but also LR asymmetry.

To determine whether the effects we observed were due to changes in the expression of Par6 and aPKC specifically, or were fundamentally due to alterations in ABP, we examined the effect of several additional mRNA constructs on the orientation of the LR axis. Expression of other ABP-disrupting reagents, including a constitutively active and DN CDC42, also induced significant levels of heterotaxia (Table 2). Unsurprisingly, some of these reagents induced measurable toxicity; only embryos with completely normal dorso–anterior indices were included for analysis. We conclude that establishment of LR asymmetry is dependent on intact ABP.

Previous studies using Vangl2 morpholinos (MO) have shown that downregulation of the PCP protein Vangl2 can disrupt orientation of the LR axis (Antic *et al.*, 2010; Song *et al.*, 2010; Zhang and Levin, 2009). We confirmed this observation, finding that injection of a Vangl2 MO at the one cell stage induced heterotaxia in 15% of embryos (Table 2). To determine whether these effects were due to specific actions of Vangl2, or if they were indicative of a more general role of PCP in the orientation of the LR axis, we overexpressed diversin, disheveled, and

FIG. 2. Altering apical-basal or planar cell polarity in cells that do not contribute to the GRP randomizes asymmetry. **a:** Fate mapping experiments of the four-cell embryo have shown that right ventral cells do not contribute to flow at the GRP, and left dorsal cells contribute to the GRP and are required for LR-relevant flow (Blum *et al.*, 2009; Schweickert *et al.*, 2007; Vick *et al.*, 2009). **b:** Five constructs were injected into single blastomeres of the four-cell embryo and then scored at stage 45 for laterality defects. Red bars indicate heterotaxia rates when injected into the dorsal left cells, which are required for cilia-induced flow at the GRP. Green bars indicate heterotaxia rates when injected into the ventral right cells, which do not contribute to flow at the GRP. For all constructs and each blastomere position, at least 50 embryos were examined for organ situs. * $P < 0.01$, ** $P < 0.001$ relative to controls. Numbers on graph indicate total sample sizes that were scored for LR phenotypes. See Supporting Information Figure 2 for incidence of heterotaxia associated with injections targeted to each one of the four blastomeres. **c:** Cilia positioning assays were performed as described in (Antic *et al.*, 2010). Similar to the results reported there, we observed that disruption of PCP signaling via injection of Vangl2MO in dorsal blastomeres significantly alters cilia position, with fewer cells demonstrating posterior localization of cilia. Disruption of ABP signaling via expression of DNPar6 in dorsal cells has similar effects. However, alteration of ABP or PCP signaling in cells that do not contribute to the GRP had no effect on cilia position in cells at the GRP. On both graphs, the X-axis indicates position of the cilia (ant = anterior third of the cell, mid = middle, post = posterior third of the cell.) * $P < 0.01$ relative to controls, ANOVA with Bonferroni post-hoc analysis. Each group (controls, DNPar6, Vangl2MO) at each position (at the GRP, away from the GRP) included at least 100 individual cells from at least four different embryos.

Rem/Rab-Similar GTPase 1 (RSG1). We observed that altered expression of diversin, disheveled, or RSG1 induced heterotaxia at similar frequencies as those observed with expression of Vangl2 MO (Table 2).

Alterations of ABP or PCP in Cells That Do Not Contribute to the Ciliated Node (GRP) Induce Heterotaxia

Elegant reverse fate-mapping experiments indicate that only the dorsal blastomeres' descendants contribute to the GRP (Blum *et al.*, 2009) and only those cells on the left side of the GRP are required to generate asymmetry-relevant flows (Vick *et al.*, 2009). Therefore, the four-cell embryo contains one blastomere giving rise to daughter cells that contribute to flow at the GRP (left dorsal), one blastomere with daughter cells that do not contribute to the GRP and are not required for LR relevant flow (right ventral), and two other blastomeres with daughter cells that may make minor contributions to flow at the GRP, depending on the accuracy of the first two cleavage furrows (left ventral and right dorsal; see Fig. 2a and Supporting Information Fig. 2).

To definitively test whether ABP and PCP pathways are required for LR patterning because of their roles in orienting ciliary motion with respect to two axes, we examined the ability of ABP- and PCP-disrupting reagents to randomize when targeted to cells that either do or do not contribute to nodal flow. Single blastomeres of four-cell stage embryos (oriented both by blastomere size and pigmentation patterns) were injected with the mRNA or morpholino of interest, together with a β -gal lineage tracer to allow subsequent verification of the targeting of injections (Supporting Information Fig. 2). Expression of DNPar6, DNaPKC, RSG1, or Vangl2MO in right ventral cells induced heterotaxia at rates similar to those obtained when GRP-relevant blastomeres were targeted (Fig. 2b).

In the GRP, motile cilia are typically localized to the posterior portion of the cell; the positioning of the cilia is required to establish leftward flow, and cilia remaining at the center of the cell cannot perform this task (Schweickert *et al.*, 2007). Previous studies using a cilia positioning assay have shown that inhibition of Vangl2 in the GRP prevents the localization of cilia to the posterior of the cell (Antic *et al.*, 2010). We used the same assay to determine whether disruption of ABP or PCP signaling in cells that do not contribute to the GRP alters the position of cilia at the GRP. We observed a decrease in the number of cilia positioned at the posterior third of cells when DNPar6 or Vangl2MO was targeted to the GRP (Fig. 2c). Crucially, however, injection of either of these reagents into ventral blastomeres, which do not contribute to the GRP, had no effect on cilia position at the GRP. From these data, we conclude that the orientation of the LR axis requires broadly

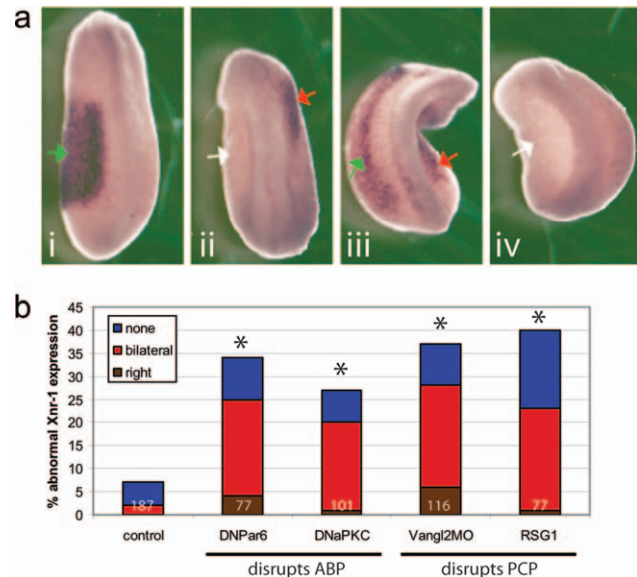


FIG. 3. Asymmetric gene expression is affected when apical-basal or planar cell polarity is disrupted. **a:** Xnr-1 localization in embryos injected with DNPar6. In all images, green arrows indicate correct left-sided expression, red arrows indicate ectopic expression, and white arrows indicate absent expression. a.i: Normal left-sided expression of Xnr-1, as observed in the majority of untreated embryos. a.ii: Right-sided expression. a.iii: Bilateral expression. a.iv: Absent Xnr-1 expression. Similar localization patterns were observed for other reagents that disrupt ABP and PCP signaling (not shown). **b:** Graph of inappropriate Xnr-1 expression observed in untreated and injected embryos. All groups are significantly different from controls ($P < 0.001$). Numbers on graph indicate sample sizes.

intact ABP and PCP with no special status for these processes in cilia-dependent events at the GRP.

ABP and PCP Proteins Function Upstream of Xnr-1

To determine where in the known LR pathway ABP and PCP proteins are playing a role, we examined whether disruption of either of these pathways alters asymmetric gene expression. Xnr-1 is normally expressed only on the left side of the embryo at approximately stage 20 (Sampath *et al.*, 1997). Expression of DNPar6, DNaPKC, RSG1, and Vangl2MO all disrupted the typical asymmetric expression of Xnr-1 (Fig. 3). These results indicate that ABP and PCP pathways feed into the well-conserved transcriptional cascades regulating organ situs (Levin and Mercola, 1998a; Yost, 2001) and are functioning upstream of known asymmetric gene expression in the LR pathway.

Expression of DNPar6 or Vangl2MO Disrupts Asymmetric Protein Localization in Early Cleavage Stages

We next asked how early the polarity pathways play a role in asymmetry, as it has been suggested that PCP is a

mechanism used very early to amplify cellular chirality information onto multicellular fields in the blastoderm (Aw and Levin, 2009). To determine whether alterations in ABP or PCP has an effect during the first few cleavage stages, we examined the early localization of disheveled-2 (*dsh2*) in embryos injected with DNPar6 or Vangl2MO. Dsh2 is a key component of the Wnt signaling pathway and is involved in both canonical and non-canonical (PCP) signaling (Wharton, 2003). In untreated embryos, we found that localization of *dsh2* protein had two apparent asymmetries: first, in embryos cut along the animal-vegetal axis, there was a bias observed with significant enrichment in the animal pole (Fig. 4a.i). Second, when examining cross-sections collected from regions in the animal pole, we observed that the majority of embryos displayed an asymmetric expression whereby the right ventral blastomere had a stronger signal than the other three (Fig. 4a.ii). In embryos expressing DNPar6, sections cut along the animal-vegetal axis still revealed a bias toward the animal pole, although in approximately one-third of embryos, there was a slight shift downward, so that the strongest signal was no longer observed at the apical-most surface of the embryo (Fig. 4a.iii). Additionally, in cross-sections collected from the animal pole, we found that expression of Vangl2MO altered the asymmetric distribution among blastomeres such that two, three, or all four blastomeres were now found to have strong *dsh2* expression (Fig. 4a.iv). We conclude that the effects of our polarity-disrupting reagents are already manifest within the embryo by the second cleavage, and that the normal localization of at least some proteins (e.g., Dsh2) at this stage is downstream of polarity establishment.

Intact ABP and PCP Are Required for Correct Localization of Prenervous Serotonin

How might ABP and PCP contribute to early events controlling LR patterning? To link these aspects of cell polarity to known steps in LR patterning, we examined the effect of DNPar6 and Vangl2MO on two mechanisms known to be important at early stages. The first is the localization of serotonin (5HT) at stage 5/6. It has previously been shown that the movement of maternal 5HT from an initially homogenous distribution to a gradient enriched in a small group of right-ventral blastomeres at the ~32 cell stage is required for normal LR patterning (Adams *et al.*, 2006; Carneiro *et al.*, 2011; Esser *et al.*, 2006; Fukumoto *et al.*, 2005b). In control embryos, 5HT signal becomes localized to a single blastomere (Fig. 4b.i). In contrast, in embryos where ABP or PCP was targeted, the 5HT signal was disrupted, maintaining distribution throughout the embryo and failing to localize (Fig. 4b.ii,iii). These results indicate that both ABP and PCP are required to establish the normal early redistribution of the crucial LR patterning molecule serotonin.

Disruption of ABP and PCP Affects Tight Junction Integrity

A second mechanism functioning early in LR patterning is tight-junctional integrity. The Par6 complex and other polarity proteins are required to establish and maintain tight junctions (TJs) in epithelial sheets (Broman *et al.*, 2006; Hurd *et al.*, 2003; Joberty *et al.*, 2000) and their integrity is required for orientation of the LR axis in several species including *Xenopus* and chick (Brizuela *et al.*, 2001; Simard *et al.*, 2006; Vanhove *et al.*, 2006). To determine whether alterations in ABP and PCP affect TJ integrity in the early cleavage stage *Xenopus* embryo, we performed a biotin-labeling assay on embryos at stage 6. In control embryos, the biotin signal typically penetrated ~1 cell depth or less (Fig. 5a). In contrast, about 50% of the embryos injected with either DNPar6 or Vangl2MO showed penetrance of the TJ probe further into the embryo, often several cell layers deep (Fig. 5b,c). We conclude that the ABP and PCP pathways are required to establish or preserve TJ integrity, an important downstream step of LR patterning.

Par6 and Vangl2 Are Required for the “Big Brother” Effect in Conjoined Twins

Having observed that PCP and ABP are required for LR patterning in normal embryos, we next examined the role of polarity proteins in the fascinating process of LR instruction of one primary organizer by another in the context of conjoined twins. We hypothesized that all cells of the developing embryo are oriented in a kind of planar polarity, and that an induced (secondary) organizer uses this PCP information to align its LR axis with respect to the blastoderm. To test this hypothesis, we injected one-cell embryos with either DNPar6 or Vangl2MO to disrupt ABP or PCP, and then induced secondary organizers at midblastula transition by injecting the transcription factor *XSiamois* at the 16-cell stage. Although embryos with intact ABP and PCP gave rise to conjoined twins with normal LR asymmetry, we found that preinjection of either polarity-disrupting construct resulted in randomized heart situs in the induced twin (Fig. 6). We conclude that the instruction of the secondary twin by the primary organizer requires ABP and PCP.

To gain further insight into the mechanistic role of polarity in this process, we next asked whether the “big brother effect” would occur if polarity were altered in only a small number of cells: is planar polarity necessary for the primary organizer to be able to send the orienting signal, for the secondary organizer to receive the signal, or both? To address this question, we coinjected DNPar6 or Vangl2MO with *XSiamois* at the 16-cell stage, thus targeting the polarity disruption exclusively to the cells belonging to the ectopic organizer (Supporting Information Fig. 3). We found that expression of either construct solely at the location of the induced

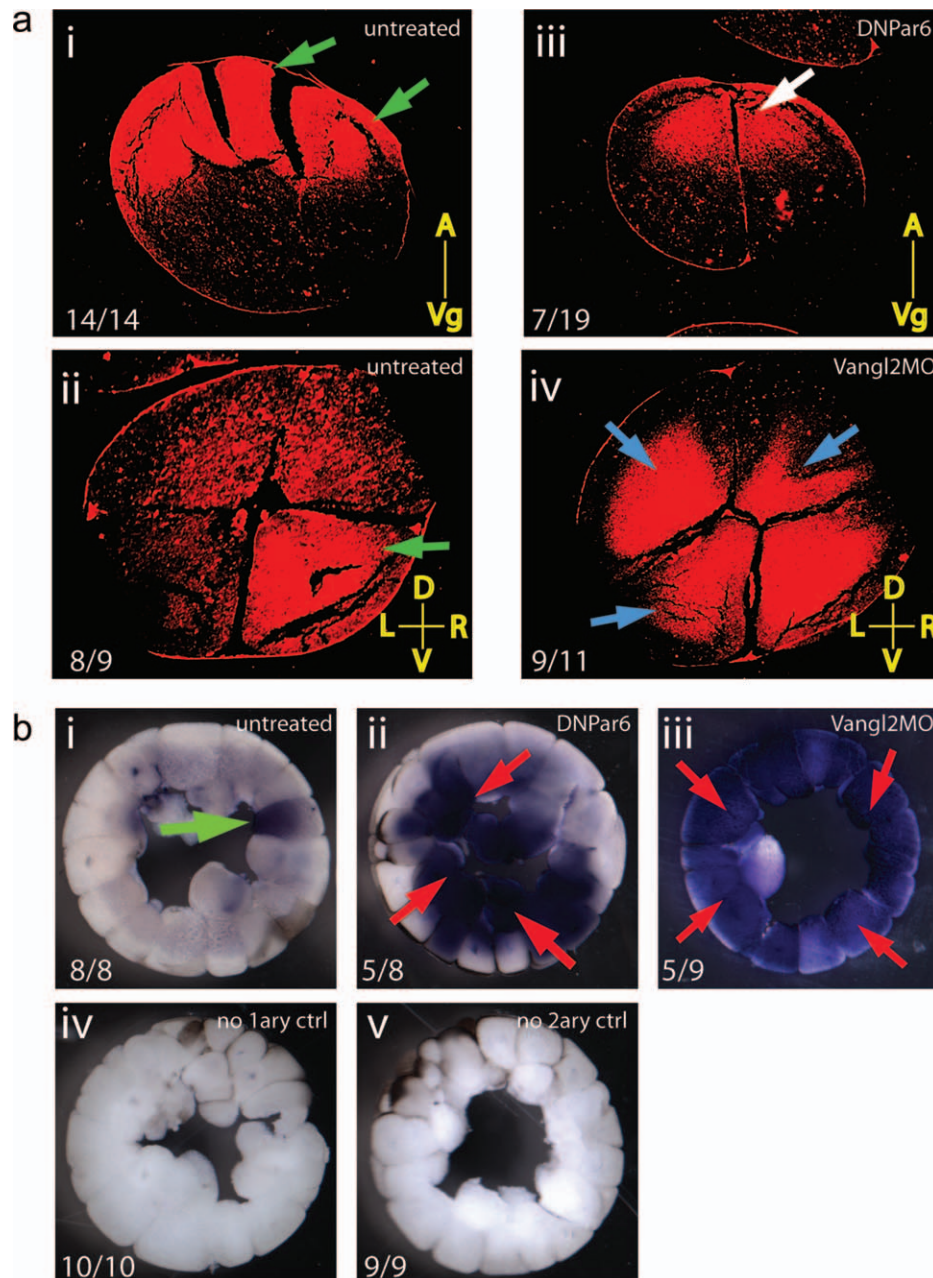


FIG. 4. Asymmetric protein localization in cleavage stage embryos is affected by DNPar6 and Vangl2MO. **a:** Localization of dsh2 protein in four-cell embryos. **a.i:** Animal-vegetal sections through uninjected embryos show strong localization of disheveled-2 at the animal-most position (green arrows) in 100% of embryos. **a.ii:** In 89% of controls, dsh2 was localized strongly to the right ventral blastomere (green arrow). **a.iii:** In embryos injected with DNPar6, 37% show a shifted localization of dsh2 protein away from the extremes of the animal pole (white arrow). **a.iv:** Injection of Vangl2MO disrupted the asymmetric localization of dsh2 in 89% of embryos, with abnormal localization in two, three, or all four blastomeres (blue arrows). Numbers on panels indicate the number of embryos observed with the phenotype over the total number of embryos examined. Yellow lines indicate orientation of the sections (A, animal; Vg, vegetal; D, dorsal; V, ventral; L, left; R, right). **b:** Expression of 5HT in stage 5/6 embryos. Green arrows indicate appropriate expression; red arrows signal inappropriate 5HT localization. **b.i:** 5HT was localized to a single blastomere in uninjected embryos. **b.ii:** Injection of DNPar6 caused a mislocalization of 5HT throughout the embryo. **b.iii:** Similarly, embryos expressing Vangl2MO showed 5HT localized to most blastomeres. **b.iv:** Embryos treated for immunohistochemistry without primary antibody or **b.v:** without secondary antibody show no unspecific staining. Numbers on panels indicate the number of embryos observed with the phenotype over the total number of embryos examined.

organizer randomized heart situs in the induced twin (Fig. 6). These results suggest that intact PCP in the primary organizer is not sufficient for the instructive orien-

tation—the secondary axis must also have normal PCP if it is to receive correct LR patterning information and orient itself within the blastoderm.

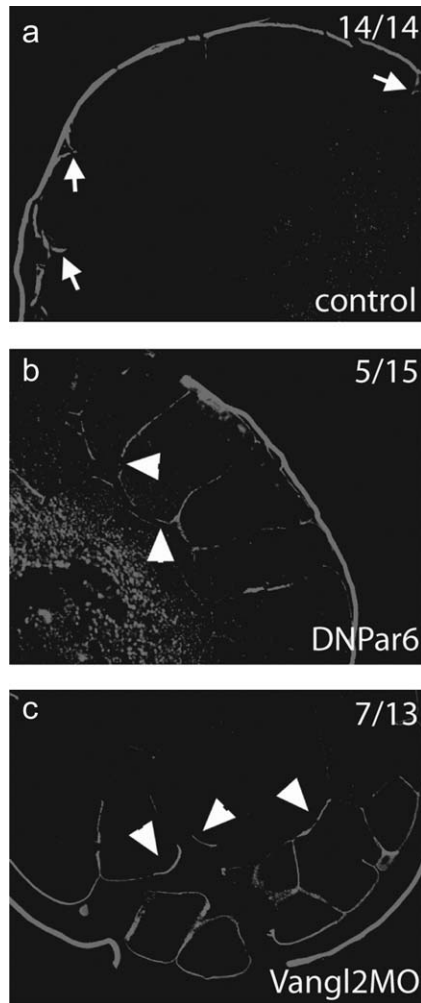


FIG. 5. Tight junction integrity is disrupted by expression of either DNP6 or Vangl2MO. Using a biotin permeability assay, embryos were examined for tight junction integrity. **a:** Tight junctions were maintained in control embryos, determined because very little biotin was able to penetrate into the embryos. Arrows indicate areas where biotin signal penetrates the embryo; this signal was never observed more than 1 cell depth. **b:** In embryos injected with DNP6 or **(c)** Vangl2MO, the biotin signal was observed several cell layers deep in the embryo (arrowheads), indicating that tight junction integrity was affected. Similar results were obtained with embryos cultured in Mg^{2+}/Ca^{2+} -free medium, which is known to break tight junctions (data not shown). This could be interpreted as showing that tight junctions never formed properly in these embryos, or alternatively that tight junctions were disrupted. Numbers on panels indicate the number of embryos observed with the phenotype over the total number of embryos examined.

DISCUSSION

Here, we have shown that ABP and PCP signaling pathways are required for the proper orientation of the LR axis in *Xenopus*. Furthermore, our results indicate that these pathways act independent of cilia; a result that is consistent with studies of PCP in chick and other data that indicate the LR axis can be oriented in the absence

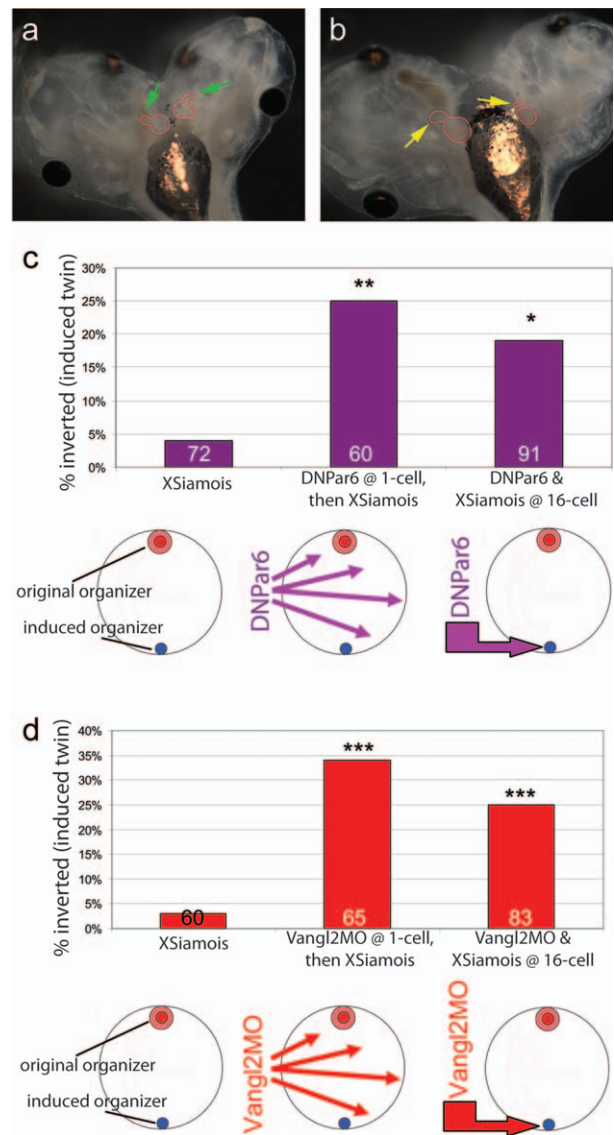


FIG. 6. Alterations in ABP or PCP randomize heart situs in conjoined twins, regardless of whether polarity is disrupted in one or both twins. **a:** Normal heart looping in both twins (green arrows) after injection with XSiamois only. **b:** Inverted heart looping in both twins (yellow arrows) when XSiamois was paired with DNP6. **c:** XSiamois was injected into a single left ventral vegetal blastomere at the 16-cell stage, inducing a secondary organizer (blue circle) directly across from the primary organizer (red circles). A subset of these embryos was injected with DNP6 at the one-cell stage, allowing this construct to be expressed in most cells, and then injected with XSiamois at 16-cells. A second subset of embryos was coinjected with both DNP6 and XSiamois into a single left ventral vegetal blastomere at the 16-cell stage. Inverted heart loops were observed in the induced twins when DNP6 was injected, regardless of where this construct was introduced. Numbers indicate sample sizes examined, $*P = 0.01$, $**P < 0.01$. **d:** The same groups were examined as in (c), but this time examining the effects of Vangl2MO. Again, inverted heart loops were observed in the induced twins expressing Vangl2MO, regardless of whether it was injected at one-cell or coinjected at 16 cell with XSiamois. Numbers indicate sample sizes examined, $***P < 0.001$.

of cilia [reviewed in (Oviedo and Levin, 2007; Speder *et al.*, 2007; Vandenberg and Levin, 2010b)]. Finally, we have demonstrated that ABP and PCP signaling is required for patterning of the LR axis in induced twins, likely because these mechanisms are needed for communication of orientation cues between the original and the secondary axis.

ABP Proteins: A Link Between Asymmetries in Single Cells and Vertebrate Embryos

Previous studies show that the Par6/aPKC complex plays important roles in oocyte maturation, maintenance of cell morphology, the asymmetric distribution of proteins to the apical and basolateral surfaces, and the regulation of asymmetric cell divisions in *Drosophila*, *C. elegans*, *Xenopus*, and mammals (Burgess, 2008; Chalmers *et al.*, 2005; Etienne-Manneville and Hall, 2003; Huttenrue *et al.*, 2004). Additionally, single cells in culture utilize the Par6/aPKC complex to establish and/or maintain LR-like asymmetries, as LR biases were randomized when ABP was disrupted (Xu *et al.*, 2007). Here, we show that ABP machinery is required for orientation of the LR axis in a vertebrate species. Our results closely match the effects of mutant Par6 and aPKC in single cells in culture (Xu *et al.*, 2007), supporting the hypothesis that LR asymmetry is an ancient phenomenon and that the mechanisms utilized to orient the LR axis are highly conserved (Levin and Palmer, 2007; Vandenberg and Levin, 2010a). Importantly, while we have focused on the effects of Par6 and aPKC, other ABP proteins, including CDC42, can also randomize LR asymmetry (Table 2). Collectively, these results suggest that the effects we observed on LR asymmetry are not specific to the Par6/aPKC complex, but a more general effect of altered ABP signaling.

Functional Data Implicate PCP in the Orientation of the LR Axis

Downstream of ABP are a group of signaling proteins involved in PCP, which establish polarity perpendicular to the apical-basal axis (McCaffrey and Macara, 2009; Tree *et al.*, 2002). In the *Xenopus* oocyte, disruption of ABP via depletion of aPKC mRNA alters the localization of Vangl2, a PCP protein (Cha *et al.*, 2011), indicating that an intimate relationship between these signaling proteins exists in the early embryo. A large number of PCP proteins have been well characterized in biological processes including hair and bristle patterning, *Drosophila* wing shape, ommatidial positioning and rotation in the fly eye, and vertebrate gastrulation [reviewed in (Simons and Mlodzik, 2008)]. The noncanonical Wnt signaling pathway, including molecules such as frizzled, disheveled, nonclassical cadherins, and downstream factors such as strabismus (also called van Gogh or

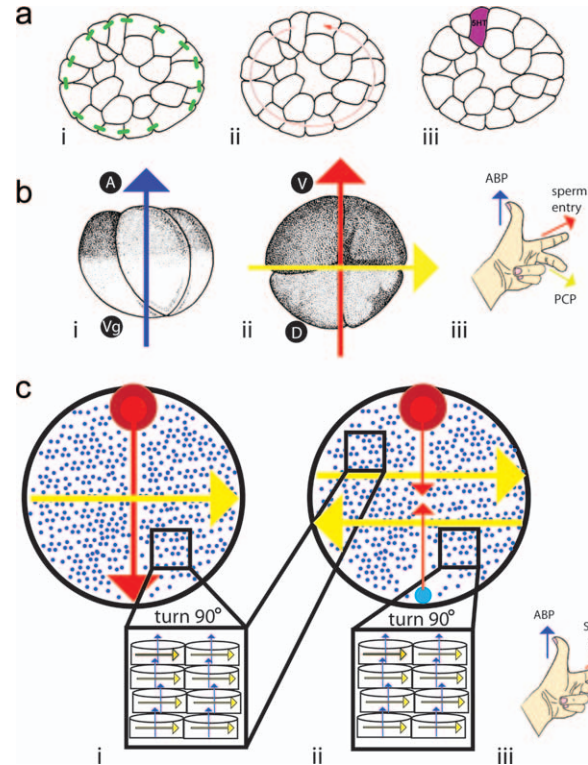


FIG. 7. Schematic model for the role of polarity proteins in the orientation of the LR axis. **a:** ABP proteins are needed to maintain epithelial integrity via tight junctions (green bars, panel i). This allows physiological gradients, including biophysical ones, to be established (red arrow, panel ii). Ultimately, this, plus a chiral cytoskeleton described in (b), allows for the asymmetric localization of 5HT (purple, panel iii). Thus, ABP is at the center of a well-studied mechanism required for the generation of asymmetry in the early cleavage stage embryo. **b:** Consistent, biased orientation of 4-cell embryos can be achieved with ABP proteins, which generate an apical-basal axis along the animal (a) and vegetal (Vg) axis (blue arrow, panel i), the sperm entry point, which generates the dorsal (D) ventral (V) axis (red arrow, panel ii), and PCP proteins, which establish LR differences (yellow arrow, panel ii). Together (iii), these three signals work to distinguish all three axes. **c:** In embryos that establish their axes when thousands of cells are present, one axis is set by ABP proteins (blue arrows and small blue dots marking the tips of arrows coming out of the plane of the page). The organizer (red circle) marks the dorsal-ventral axis (red arrow), and PCP proteins orient the LR axis perpendicular to the other two (yellow arrow, panel i). When a secondary organizer (large blue circle, panel ii) is introduced across from the original one, the dorsal-ventral axis is shifted 180°, which also causes a shift in PCP proteins (yellow arrows, panel ii). Together (iii), ABP proteins, signals from Spemann's organizer (like *XSiamois*), and PCP proteins work to distinguish all three axes.

Vangl) and prickle, are responsible for establishing PCP in epithelial sheets (Axelrod and McNeill, 2002).

Several studies have linked PCP signaling to the orientation of the LR axis (Antic *et al.*, 2010; Borovina *et al.*, 2010; Ferrante *et al.*, 2009; Maisonneuve *et al.*, 2009; May-Simera *et al.*, 2010; Oteiza *et al.*, 2010; Ross *et al.*, 2005; Song *et al.*, 2010; Zhang and Levin, 2009). Most of these examined the role of Vangl in LR patterning of

chick, fish, mouse, and frog embryos using a variety of molecular tools. Our experiments extended these findings by examining the effects of manipulating several PCP proteins on the LR axis, particularly probing a role for PCP in regions of the embryo outside of the ciliated GRP. Most previous studies examining a link between PCP and the LR axis have focused on the effects of Vangl on ciliary placement [reviewed in (Santos and Reiter, 2010)]. Studies in other organ systems indicate that PCP may play a role in the docking of basal bodies to the apical membrane, a process that is important for cilia growth and motility (Lee *et al.*, 2011). However, the requirement for Vangl2 in normal LR patterning in the chick (Zhang and Levin, 2009), which orients the LR axis in a cilia-independent manner (Gros *et al.*, 2009; Manner, 2001), and our data showing that PCP in regions well outside of the cilia-bearing cells of the GRP is still required for normal LR asymmetry, both suggest that PCP has additional roles in laterality that do not involve cilia.

Antic and coworkers disrupted Vangl2 specifically in the cells that contribute to the GRP (Antic *et al.*, 2010), and the genetic mutants utilized in previous studies lack Vangl in all cells of the embryo (Borovina *et al.*, 2010; Song *et al.*, 2010). These experiments did not test cilia/node-independent functions of PCP by asking whether alterations in PCP outside of the node/GRP could affect orientation of the LR axis. This is important, because many of the “ciliary” proteins implicated in LR patterning have other nonciliary roles including intracellular ones [reviewed in (Armakolas *et al.*, 2010; Levin, 2003; Levin and Palmer, 2007)]. To address this issue, we specifically examined the effect of Vangl2 morpholinos, and other constructs that inhibit PCP and ABP signaling, in cells that do not contribute to the GRP. We found that altered PCP or ABP signaling was effective in randomizing the LR axis, whether it occurred in cells that contributed to the GRP or not (Fig. 2b). This result suggests that, similar to the results in chick (Zhang and Levin, 2009), PCP has a cilia-independent role in LR asymmetry. We observed normal GRP cilia when PCP or ABP signaling was disrupted in cells that do not contribute to this structure (Fig. 2c)—a result not compatible with hypotheses about impairment of GRP ciliary orientation by PCP defects in distant cells. This is consistent with data reported by Antic *et al.* (2010), where cilia were only disrupted in GRP cells within clones expressing the Vangl2MO; cilia localization in uninjected cells—near the injected cells, and still within the GRP—was unaffected, confirming a local model of cilia polarization by PCP.

Our results indicate that even species that do appear to use cilia at some point in the LR pathway, such as *Xenopus* (Vick *et al.*, 2009), still use PCP for other aspects of LR orientation that are distinct from the roles cilia and fluid flow play in establishing LR asymmetry.

The ability of PCP-disrupting reagents to specifically randomize the LR axis when not contributing to the cells needed for GRP flow strongly suggests that ciliated cells have no privileged requirement for PCP in LR patterning; these results are instead more compatible with the need for consistent PCP throughout the blastoderm, disruption of which in any region is sufficient to impact global coordination of LR polarity within the embryo.

Polarity Proteins Maintain TJ Integrity, a Requirement for LR Axis Orientation

As early as the 64-cell stage, the *Xenopus* embryo has characteristics of an epithelium, including the expression of TJs, desmosomes, and apical-basal domains (Muller and Hausen, 1995). Maintaining the integrity of the epithelium is required for the proper orientation of the LR axis, likely because when TJs are disrupted, the ability of the epithelium to establish electrophysiological gradients is diminished (Aw *et al.*, 2010). Polarity proteins are required to establish and maintain TJs in epithelial sheets (Hurd *et al.*, 2003; Joberty *et al.*, 2000). Our results indicate that alterations in either ABP or PCP affect TJs and epithelial integrity in cleavage stage embryos (Fig. 5), suggesting that these pathways could disrupt LR asymmetry also via this second mechanism (Fig. 7a).

The ultimate consequence for disrupting ABP or PCP pathways is the mislocalization of the LR signaling molecule, 5HT (Fig. 4b). In normal embryos, 5HT becomes strongly concentrated in a single right ventral blastomere at stage 5/6 (Adams *et al.*, 2006; Fukumoto *et al.*, 2005b), and in this position is thought to repress the expression of laterality-related genes, leading to their appearance solely on the left side (Carneiro *et al.*, 2011; Levin, 2006; Levin *et al.*, 2006). The establishment of a chiral cytoskeleton and ion flux pathways are both upstream of asymmetric 5HT localization (Adams *et al.*, 2006); therefore, ABP and PCP pathways could be disrupting LR asymmetry via either or both mechanisms.

ABP and PCP Are Necessary for Intracellular Protein Localization and LR Events at the Multicellular Level

The early cleavage stage embryo has a chiral cytoskeleton, resulting in biased protein distribution among the blastomeres (Aw *et al.*, 2008), which is a plausible means of generating asymmetry intracellularly (Levin and Palmer, 2007). PCP originates intracellularly and PCP proteins are known to interact with—and cause biases in—the cytoskeleton (Aw and Levin, 2009; Djiane *et al.*, 2005; Li *et al.*, 2008; Mirkovic and Mlodzik, 2006). In fact, a recent study of *Xenopus* oocytes demonstrates that alterations in either ABP or PCP signaling disrupt the microtubule cytoskeleton; only when ABP and PCP proteins are normally expressed can the

stable architecture of the oocyte be maintained (Cha *et al.*, 2011). For this reason, we propose that the polarity machinery used in ABP and PCP could be a widely conserved intracellular mechanism used by vertebrates to orient the LR axis.

The cytoskeleton has been shown to be integral to the process of establishing the body axes and asymmetries. In *C. elegans* embryos, the sperm entry point provides polarity cues specifying the anterior-posterior axis, which is maintained by several Par molecules (Goldstein and Hird, 1996; Guo and Kemphues, 1996), and the microtubule-dependent localization of ion channels is crucial for generating consistent neuronal asymmetry (Chang *et al.*, 2011). The sperm centrosome has, therefore, been proposed as a cytoskeletal mechanism to set up asymmetric cell divisions in the worm (Goldstein and Hird, 1996). In *Xenopus*, the sperm entry point marks the ventral side of the embryo (Scharf and Gerhart, 1980); after fertilization, cortical rotation localizes elements to the dorsal side of the embryo that are needed for dorsal structures to form (Chang *et al.*, 1996; Nagano *et al.*, 2000). Only when this process occurs is the dorsal-ventral axis established, and the LR axis is then oriented with respect to the other two.

Considering our data and the findings from studies of single cells (Xu *et al.*, 2007), we propose that ABP proteins could be involved in the earliest steps to define the LR axis (Fig. 7b). ABP and PCP proteins are, therefore, plausible candidates to act as the theoretical “F-molecule” that has been proposed to tether the LR axis with the dorsal-ventral and anterior-posterior axes (Brown and Wolpert, 1990). Previous studies implicate the cytoskeleton as the origin of LR asymmetry (Aw *et al.*, 2008; Danilchik *et al.*, 2006; Qiu *et al.*, 2005), and the localization of the Par6 complex during early development of *Xenopus* embryos suggests that ABP proteins play a role in generating asymmetry during the first cell divisions (Choi *et al.*, 2000) via regulation of the cytoskeleton (Brazil and Hemmings, 2000). Loss-of-function experiments depleting ABP proteins in *Xenopus* oocytes support this conclusion (Cha *et al.*, 2011). Additionally, CDC42, which is activated by the Par6/aPKC complex, is required for orientation of the microtubule organizing center (Tzima *et al.*, 2003), providing an additional link between ABP proteins and the cytoskeleton.

Our previous results showed that a late-induced organizer can only orient its LR axis properly when an original organizer was present during the first cell cleavages, indicating that the early organizer transmits positional information to the secondary organizer (Vandenberg and Levin, 2010a). Here, our results implicate ABP and PCP signaling in the transfer of information from one twin to the other; expression of either DNPar6 or Vangl2MO disrupted direction of heart looping in twins resulting from late-induced organizers (Fig. 6).

Importantly, even disruption of PCP or ABP signaling in a small number of cells in the induced twin was sufficient to alter LR patterning.

Early *Xenopus* embryos have the benefit of large blastomeres that are all in contact, allowing for the direct flow of morphogens and proteins between cells, whereas many species appear to establish the LR axis when thousands of cells are present. In these animals, the architecture of the embryo precludes the ability to establish the LR axis by intracellular transport of determinants across the midline (Levin, 2004). The conjoined twin model (where a secondary organizer is induced *de novo* in a multicellular field) is a good analog of the environment for patterning of the LR axis in such species: with large numbers of cells present when the second organizer is induced, each cell does not intrinsically know whether it is on the left or right side of the embryo, but must orient itself within a field of cells. Our results position ABP and PCP signaling as integral in this process (Fig. 7c); together with other studies showing a role for Vangl in LR patterning in many animals, our findings support a role for these proteins in a widely conserved intracellular mechanism used by vertebrates with different embryonic architectures to orient the LR axis.

CONCLUSIONS

We show a requirement for ABP and PCP signaling to properly orient the LR axis. Disruption of either pathway, via over- or misexpression of a large panel of mRNAs or a Vangl2 morpholino, randomized organ placement in *Xenopus* tadpoles. Importantly, expression of several of these constructs outside of the GRP was sufficient to disrupt LR patterning, suggesting that the roles of PCP and ABP in LR patterning are cilia independent. Altered ABP and PCP signaling also affected asymmetric expression of *Xnr-1*, altered TJ integrity, and prevented the specific localization of 5HT in cleavage stage embryos. Finally, both ABP and PCP are required for the instructive “big brother effect” observed when late-induced organizers are properly oriented within their own LR axis by an early organizer. Polarity proteins are, therefore, involved in several aspects of asymmetry generation and may be useful in orienting the LR axis in a variety of species with different embryonic architectures at the time of axes specification.

MATERIALS AND METHODS

Animal Rearing

Xenopus laevis embryos were collected and fertilized in vitro according to standard protocols (Sive *et al.*, 2000) in 0.1× Modified Marc's Ringers (MMR) pH 7.8 + 0.1% Gentamycin. *Xenopus* embryos were housed at 14–18°C and staged according to (Nieuwkoop and

Faber, 1967). All experiments were approved by the Animal Care and Use Committee at Tufts University and were conducted according to the Guidelines for the Care and Use for Laboratory Animals.

Microinjection of Molecular Constructs (mRNAs and Morpholinos)

Constructs included: DNPar6, a human Par6 construct missing the N-terminus, i.e., the region that interacts with aPKC (Etienne-Manneville and Hall, 2001; Tzima *et al.*, 2003); DNaPKC, a rat PKC ζ construct lacking the kinase domain (Xu *et al.*, 2007); RSG1-GFP, a human RSG1 construct fused to a GFP sequence (Gray *et al.*, 2009); DNCD42, a *Xenopus* DN Cdc42 mutant T17N (Broman *et al.*, 2006); constitutively active human CDC42 mutant Cdc42-L61 (Lamarche *et al.*, 1996); Disheveled-GFP, a wildtype *Xenopus* Disheveled fused to GFP (Rothbacher *et al.*, 2000; Wallingford *et al.*, 2000); and wildtype mouse Diversin (Moeller *et al.*, 2006). All constructs except RSG1-GFP were subcloned into pCS2 vectors; RSG1-GFP was maintained in CS107, a related vector. Linearized template was transcribed using a mMessage mMachine SP6 transcription kit (Ambion).

For all microinjections of mRNA constructs, capped synthetic mRNAs (Sive *et al.*, 2000) were dissolved in water containing a lineage tracer and injected into embryos in 3% Ficoll using standard methods (50–100 msec pulses with borosilicate glass needles calibrated for a bubble pressure of 50–70 kPa in water) at the specified stage. For induction of conjoined twins, *XSiamois* was injected into a left ventral vegetal blastomere at the 16-cell stage (Nascone and Mercola, 1997). At stage 22, twin embryos were examined under fluorescence and sorted for left- and right-side induced twins; only left-sided twins were used for analysis (Vandenberg and Levin, 2010a). At stage 45, conjoined twin embryos were further sorted into “complete” twins, i.e., twins with two fully developed hearts and heads, and “incomplete” twins, i.e., twins with only one heart and an underdeveloped second head.

Vangl2 morpholinos (MO) were obtained from GeneTools using the sequences previously found to achieve effective knockdown (Antic *et al.*, 2010; Mitchell *et al.*, 2009). The morpholino was diluted in distilled water and ~40 ng was injected. The Vangl2MO was constructed with a FITC tag, allowing lineage tracing after injection.

Laterality Assay

At stage 45, *Xenopus* embryos were analyzed for position (situs) of the heart, stomach, and gall bladder according to (Levin and Mercola, 1998b). Heterotaxia was defined as the reversal in position of one or more organs. Only embryos with a normal dorso-anterior

index (DAI = 5) were scored to prevent confounding of randomization caused by midline defects (Danos and Yost, 1995). Conjoined twins were analyzed at stage 45 for situs of the heart for both twins.

Percent heterotaxia was calculated as number of heterotaxic embryos divided by the total number of scorable embryos. A χ^2 test with Pearson correction for increased stringency was used to compare absolute counts of heterotaxic embryos. Percent toxicity was calculated as the number of unscorable (dead or DAI \neq 5) divided by the total number of treated embryos.

In situ Hybridization

Whole mount in situ hybridization was performed using standard protocols (Harland, 1991). In situ hybridization probes against Xnr-1 (the *Xenopus* nodal) mRNAs (Sampath *et al.*, 1997) were generated in vitro from linearized template using DIG labeling mix (Roche, Branford, CT).

Immunohistochemistry

Four-cell embryos were fixed in MEMFA (Sive *et al.*, 2000), embedded, and sectioned on a Leica M2255 microtome (paraffin) or Leica VT1000S vibratome (gelatin-albumin). For fluorescence microscopy, either cross-sections or sections along the plane of the animal-vegetal axis were collected to allow comparisons of protein expression between the four blastomeres as well as along the animal-vegetal axis. The samples were blocked, incubated with primary antibodies, washed, and incubated with secondary antibodies (see Table 1 for details). Controls included primary-only and secondary-only conditions. All sections were incubated for the same durations in all experiments.

Cilia positioning assays were performed on st. 16–17 embryos according to previous methods (Antic *et al.*, 2010). Briefly, immunohistochemistry was performed for cilia and the location of cilia in GRP cells was determined to be in the anterior, middle, or posterior third of the cell. A total of 110–225 cells were examined in at least four embryos for each treatment.

TJ Assay

An assay using cell membrane impermeable biotin (Aw *et al.*, 2010) was used to determine whether TJ integrity was maintained. Briefly, at st. 6, control and injected embryos were cooled to 10°C and incubated in a fresh solution of 1 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in 0.1× MMR with 10 mM HEPES (pH 7.8) for 12 min. Embryos were then washed, fixed, embedded in paraffin, and sectioned at 5 μ m on a Leica M2255 microtome. Biotin was visualized using anti-streptavidin-Alexa 555 antibodies (Molecular Probes).

Cell Lineage Tracer

For some experiments, mRNA encoding β -galactosidase (β -gal) was coinjected with other mRNAs or injected alone into specific blastomeres at the stages indicated. At stage 45, tadpoles were scored for heterotaxia, overdosed with tricaine, fixed for 30 min in MEMFA, washed, and stained with X-gal (Roche Applied Sciences, Indianapolis, IN) at 37°C.

Microscopy and Image Collection

For immunohistochemistry and the visualization of FITC signals in Vangl2MO-injected twins, an Olympus BX-61 with a Hamamatsu ORCA AG CCD camera, controlled by MetaMorph software, was used for imaging. For all other experiments, images were collected with a Nikon SMZ1500 dissection microscope with a Retiga 2000R camera and ImageQ software. Photoshop[®] was used to orient, scale, and improve clarity of images. Data were neither added nor subtracted; original images are available on request.

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