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Left-right asymmetry in the chick embryo requires core planar cell polarity protein Vangl2

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Abstract

Consistent left-right patterning is a fascinating and biomedically important problem. In the chick embryo, it is not known how cells determine their position (left or right) relative to the primitive streak, which is required for subsequent asymmetric gene expression cascades. We show that the subcellular localization of Vangl2, a core planar cell polarity (PCP) protein, is consistently polarized, giving cells in the blastoderm a vector pointing toward the primitive streak. Moreover, morpholino-mediated loss-of-function of *Vangl2* by electroporation into chicks at very early stages randomizes the normally left-sided expression of *Sonic hedgehog*. Strikingly, Vangl2 morpholinos also induce a de-synchronization of asymmetric gene expression within the left and right domains of Hensen's node. These data reveal the existence of polarized planar cell polarity protein localization in gastrulating chick and demonstrate that the PCP pathway is functionally required for normal asymmetry in the chick upstream of *Sonic hedgehog*. These data suggest a new and widely-applicable class of models for the spread and coordination of left-right patterning information in the embryonic blastoderm.

Keywords

Left-right asymmetry; planar cell polarity; embryogenesis; Vangl2; chick

Introduction

Consistent left-right (LR) asymmetry of the heart and viscera is a crucial and fascinating aspect of embryonic development. Errors in this process result a clinically important class of birth defects (Burn, 1991; Peeters and Devriendt, 2006; Ramsdell, 2005). In recent years, a wealth of molecular information has been uncovered (Levin, 2006; Speder et al., 2007) illustrating how biophysical events in very early embryos result in asymmetric gene expression cascades, which ultimately direct the differential growth and morphogenesis of organs on the left (L) and right (R) side of the midline.

A key step in this process must orient the LR axis with respect to the dorso-ventral and anterior-posterior axes, so that the resulting asymmetry can be consistent among all normal members of a given species. The best conceptual analysis of this mechanism postulated the "F-molecule" model, whereby a structure that possesses intrinsic (biochemical) chirality is tethered with respect to the other two axes and thus nucleates consistent, asymmetric processes (Brown and Wolpert, 1990). While the molecular nature of this mechanism is

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currently controversial (Levin and Palmer, 2007; Tabin, 2005), it provides a satisfying picture of how cells on both sides of the midline can define the "Left" or "Right" direction.

By itself however, this is not sufficient. The genetic cascades establishing left-sided and right-sided domains of gene expression during gastrulation require that cells know not only what direction is L or R, but also on which side of the midline they are located. Unlike L vs. R direction, which is invariant throughout the blastoderm, position is different for cells on either side of the midline. Thus, mechanisms must exist to convert direction to position with respect to midline. In *Xenopus* embryos, a mechanism has been proposed in which the biased unidirectional localization of ion pump subunits in the early cleavage-stage embryo sets up an embryo-wide gradient of serotonin by an electrophoretic process (Esser et al., 2006; Fukumoto et al., 2005b; Levin et al., 2006). This demonstrates how intracellular directional information can be converted into an embryo-wide positional signal (serotonin concentration) by physiological mechanisms. However, this system relies on the holoblastic cleavage of the frog embryo where the first cleavage plane demarcates the prospective midline of the embryo, and intracellular localization events at this stage can redistribute components to the future L or R side.

How might this process occur in other types of embryos, such as amniotes, where intracellular events at early cleavages do not span the prospective midline? In the chick, where the first known asymmetries occur when there are tens of thousands of small cells in the blastoderm, some of the same molecular components, such as gap junctions, serotonin receptors R3 and R4, and H^+, K^+ pumps, are known to be required for LR patterning (Adams et al., 2006; Fukumoto et al., 2005a; Fukumoto et al., 2005b; Levin et al., 2002; Qiu et al., 2005). However, the serotonin model cannot apply without modification because the same kind of unidirectional redistribution of serotonin is not observed, and because in the small cells of the chick embryo, intracellular localization cannot directly result in L vs. R asymmetries across the embryonic midline. Thus, an additional mechanism must exist to derive LR position from subcellular direction in amniotes, fish, and similar types of embryos. The chick is also an especially interesting context for these experiments because it is a vertebrate model system in which cilia are very unlikely to play any role in establishment of asymmetry (Levin and Palmer, 2007; Manner, 2001).

In the chick embryo, the primary axis (and thus the LR midline) becomes apparent during the formation of the primitive streak in the blastoderm. The chick embryo's cells know their lateral position by early streak stages, since a coherent group of depolarized cells appears adjacent to the left side of the primitive streak on the left side during st. 2-2^+ (Levin et al., 2002); the maturation of the streak is followed by asymmetric gene expression during stages 3–5, at which point neurulation and the highly-conserved left-sided cascade of *Nodal-Lefty-Pitx* expression occurs (Logan *et al*., 1998). This suggests that cells in the blastoderm are able to perform the computation deriving L or R side location with respect to the midline. Although we have proposed that the orientation in the chick is known by the initiation of the streak (Levin and Mercola, 1998) or even much earlier (Levin and Palmer, 2007), LR-relevant intracellular polarization in chick cells has not yet been reported. Interestingly, there is also an as yet-unexplained coherence among the cells of the node, where even in LR-"randomized" embryos, the L and R sides of the node express (or do not express) asymmetric markers like *Sonic hedgehog* as a single domain – all published phenotypes to date include *Shh* on or off on either side of the node but never in a speckled manner – the L and R sides of the node behave as single domains.

Thus, we sought models of this process that would explain how cells convert intracellular directional information into position within the blastoderm, and also account for the observation that even when randomized, decisions to express right- or left-sided markers are

not made at the cell level but rather at the level of cell groups (half of the node). Interestingly, planar cell polarity (PCP) solves much the same problem in numerous other patterning contexts.

PCP is a mechanism for patterning an epithelium in a plane orthogonal to the apical basal polarity and is thought to occur in three steps (Tree et al., 2002). First, a directional cue initiates polarity that will orient the field with respect to the rest of the embryo. Next, this directional signal is interpreted by intracellular mechanisms to produce asymmetric subcellular localization of core PCP proteins. These asymmetries then spread across the entire cell field, perhaps by mutual inhibition and/or stabilization at cell-cell boundaries (reviewed in (Seifert and Mlodzik, 2007)), creating global parallel arrays of asymmetric intracellular protein localization. Finally, this subcellular asymmetry is interpreted by each tissue to carry out downstream differentiation and morphogenesis programs. The PCP pathway ensures coordinated cell behavior, whether to achieve directed movement in a plane, as in convergent extension during gastrulation in *Xenopus*, or to produce an oriented field of polarized structures, like the bristles of the *Drosophila* wing or the ciliary flow of respiratory epithelia (Wang and Nathans, 2007).

The parallels between PCP and LR patterning are striking (Aw *et al*., 2008; Aw and Levin, 2008a). In widespread taxa, including *Drosophila*, zebrafish, chick, frog, and mouse, LR patterning first occurs in an epithelium (Bisgrove et al., 2005; Coutelis et al., 2008; Levin, 2006; Speder et al., 2007) in which non-canonical Wnt-PCP mechanisms are also operating (Bodenstein and Stern, 2005; Gong et al., 2004; Muller and Hausen, 1995; Oteiza et al., 2008; Ross et al., 2005; Schwarz-Romond et al., 2002; Stern, 1982; Voiculescu et al., 2007; Wei and Mikawa, 2000). The geometry is clearest in the chick, where the blastoderm dorso-ventral axis corresponds to the epithelium's apical-basal axis, and LR asymmetry is imposed as a planar polarity within the embryonic field.

We tested the hypothesis that PCP signaling in the chick was required for normal LR asymmetry, focusing on the gene *Vangl2* - a vertebrate homolog of the *Drosophila* PCP gene *Strabismus*/*Van Gogh* (Carroll *et al*., 2003; Katoh, 2005, 2007; Torban *et al*., 2004). *Strabismus*/*Van Gogh* is one component of the non-canonic Wnt-PCP pathway, encoding a protein with a potential PDZ domain-binding motif at the C-terminus and two possible transmembrane domains at the N-terminus (Wolff and Rubin, 1998). Van Gogh is required to establish polarity in the wing (Bastock et al., 2003), eye (Rawls and Wolff, 2003), legs, and bristles of *Drosophila* (Wolff and Rubin, 1998). Strabismus participates in the regulation of two PCP processes at gastrulation stage of vertebrates, convergent extension and neural tube closure, in zebrafish (Jessen et al., 2002), *Xenopus* (Goto and Keller, 2002), and chick (Voiculescu et al., 2007).

Cell-level immunohistochemistry revealed localization of Vangl2 protein that was polarized along the medio-lateral axis; crucially, morpholino-induced knockdown of *Vangl2* randomized the expression of the normally left-sided marker *Sonic hedgehog*, and abolished the normal tight coherence among cells on each side of the node. These data implicate planar cell polarity pathways in the conversion and spreading of LR information within an early embryonic blastoderm.

Results

Vangl2 localization is consistently polarized within epithelial cells

To test the hypothesis that planar polarity proteins might provide spatial information in the blastoderm, chick embryos at early streak stages were examined for subcellular localization of Vangl2 protein by immunohistochemistry. Most cells exhibited an accumulation of

Vangl2 protein on the cell membrane with extension into the cytoplasm of individual cells, allowing us to determine the angle, relative to the primary axis of the embryo, of the vector connecting the center of cells to the center of the Vangl2 protein localization (Fig. 1). The polarization angle of Vangl2 vectors in cells on the left and right sides of the middle portion of the streak of four embryos (about 50–100 cells for each embryo) at stage 3 were calculated (Fig. 2). The Vangl2 vectors pointed toward the streak on both sides of embryos, with an average angle of 81° on the left side and an average angle of −110° on the right side (Table 1 and Table 2); the Vangl2 polarization was significantly different from a random distribution, as determined by the one-sample critical ratio test (Table 3, with Z = 9.2 for the left-side cells and 10.2 for the right-side cells). 57% of the Vangl2 vectors on the left side cells, and 62% of the Vangl2 vectors on the right side cells, fall into the sector (shaded in pink) between ±38° and ±142° (30°–150°, but discarding those vectors falling within ±8.4° of the edges to account for measurement error of ±8.4°, quantified in Table 2). Both are significantly different from the 28% expected to fall into that sector by chance (in the case of a random, non-polarized distribution of Vangl2 protein). The difference in average Vangl2 vector angles of L cells vs. R cells was also significantly different at $p<0.05$ (2-tailed, 2-sample T-test with unequal variance). We conclude that chick blastoderm cells on both sides of the streak possess a polarized accumulated Vangl2 protein on cell membrane, and that in a majority of cells, this points towards the midline of the embryo.

Morpholinos to Vangl2 randomize left sidedness of *Shh* expression

To test the hypothesis that planar polarization of Vangl2 protein is functionally required for normal patterning, we electroporated fluorescein-tagged morpholinos against *Vangl2* mRNA into whole chick embryos in culture at st. 1–3 (Fig. 3a). We processed the embryos at st. 5 (anatomical legend in Fig. 3b) for in situ hybridization with the normally left-sided marker *Sonic hedgehog* (*Shh*), a key readout of chick asymmetry that determines the sidedness of downstream asymmetric genes and ultimately, of the heart and visceral organ positioning (Levin *et al*., 1995a). Control embryos exhibited the normal left-sided expression of *Shh* in the node (Fig. 3c). Embryos electroporated with a basepair mismatch morpholino exhibited an incidence of 12% of aberrant *Shh* expression (absent or bilateral), indicating that the process of electroporation slightly disturbs LR patterning. In contrast, embryos electroporated at st. 3 with morpholinos targeting *Vangl2* exhibited a significantly greater number of aberrant *Shh* patterns including bilateral (Fig. 3d), or absent (Fig. 3e) patterns in the node (Table 4). Based on the randomization of *Shh* sidedness by loss-of-function treatment for *Vangl2*, and the known role of *Shh* as an upstream determinant of the sidedness of subsequent asymmetric cascades and organogenesis (Levin *et al*., 1995b;Levin *et al*., 1997;Pagan-Westphal and Tabin, 1998b;St Amand *et al*., 1998), we conclude that Vangl2 function is required for correct left-right patterning of the early chick.

When morpholinos were applied at st. 1, the effect was not significant (10% incorrect *Shh* expression, vs. 12% for control morpholino). The incidence rose to 25% when Vangl2 morpholinos were applied at st. 2, and to 41% when morpholinos were applied at st. 3. Based on these timing data, we conclude that the function of Vangl2 in directing asymmetric *Shh* expression likely takes place between st. 2 and st. 4.

Morpholinos to *Vangl2* disrupt coordination of *Shh* expression among node cells

The normal expression domains of *Shh*, whether left-sided (normal) or right-sided (specifically randomized by a variety of different treatments as in (Adams *et al*., 2006; Fukumoto *et al*., 2005a; Fukumoto *et al*., 2005b; Levin *et al*., 1995a; Levin *et al*., 2002; Meyers and Martin, 1999; Rodriguez Esteban *et al*., 1999; Wang *et al*., 2004)) exhibit solid expression throughout the region (e.g., Fig. 3f). Thus, in all perturbations of LR patterning described to date, as well as in the wild-type embryo, the right and left sides of Hensen's

node act as units – the decision to express *Shh* or not is made by the whole compartment (side of the node) as a single unit. In contrast, we observed that in embryos treated with Vangl2 morpholinos, node cells became desynchronized from their neighbors on each respective sides of the node: cells throughout the node made individual decisions as to whether or not to turn on *Shh*, resulting in a speckled pattern (Fig. 3g) instead of a coherent "yes" or "no" expression domain on each side. We conclude that Vangl2 function is required for the ability of the L and R sides of Hensen's node to synchronize their decisions to adopt left or right fates with respect to asymmetric gene expression.

Discussion

We examined the localization and function of the planar cell polarity gene *Vangl2* in chick embryos, with the goal of probing its role in left-right patterning. Immunohistochemistry of whole embryos at the single cell level revealed that accumulations of Vangl2 protein are polarized in individual cells. Relative to the cell's center, the accumulation generally points towards the streak in both left-side and right-side cells (Fig. 1–Fig. 2, Table 1 and Table 2); future studies will further characterize the molecular components of this accumulation and its relationship to other PCP components. Thus, a planar polarity protein serves as a readout of large-scale embryonic pattern within cells. Neither streak-ward nor "right" (or "left") directionality is sufficient for a cell to know it's position in the embryo. However, the existence of a cellular vector that reliably points towards the streak can be used by a cell, in conjunction with a chiral subcellular structure oriented with respect to the dorso-ventral and anterior-posterior axes, to determine where it is located relative to the midline. We propose a model (Fig. 4) of the process by which cells use the Vangl2 vector to derive their L or R position within the embryo. The relationship of this orientation to the complex PCP-related cell movements (Chuai *et al*., 2006;Voiculescu *et al*., 2007) in the chick blastoderm remains to be investigated.

A well-characterized morpholino reagent that strongly perturbs Vangl2-mediated PCP signaling in chick (Voiculescu et al., 2007) efficiently randomized the localization of the key left side determinant, *Sonic hedgehog* (Fig. 3, Table 4). We chose *Shh* as the optimal LR marker for these experiments because it is a very early asymmetric gene (and thus not affected by neural tube and other later embryonic outcomes of PCP disruption). Moreover, since it is upstream of other LR gene expression, randomization of *Shh* reveals an effect that is of consequence to the known downstream markers and organ *situs* (Levin, 1998; Levin *et al*., 1997; Logan *et al*., 1998; Pagan-Westphal and Tabin, 1998a). Upon a background of 12% induced by control electroporation (not entirely unexpected, given the known importance of membrane voltage in embryonic asymmetry (Levin et al., 2002)), the incidence of abnormal *Shh* sidedness from treatment with Vangl2 morpholino was more than 3-fold greater and thus easily detected (41%). There was no general toxicity induced by the morpholino at 3mM; moreover, no ectopic *Shh* outside the node was detected, nor was the LR-irrelevant *Shh* domain in the notochord perturbed, demonstrating a specific effect on the sidedness of the LR-relevant *Shh* domain and not simply on transcription of *Shh*. These loss-of-function data support a role for Vangl2 in left-right patterning. While previous studies (Chuai *et al*., 2006) have reported convergent-extension and other phenotypes during later embryogenesis, the randomization of *Shh* could not be a consequence of these events because asymmetric expression of this marker occurs prior to significant notochord extension and neurulation processes. Given the plane-polarized localization of Vangl2 protein and the known involvement of this protein in the PCP pathway, we propose that the role of Vangl2 in asymmetry is mediated via its function in PCP, although other roles cannot be ruled out.

Our data also reveal the temporal and functional position of Vangl2 in the LR signaling pathway. While introduction of morpholinos at st. 3 (just 4–6 hours prior to the appearance of asymmetric *Shh* expression) randomizes strongly (41%), exposure at st. 2 has a smaller effect (25%). Indeed, exposure at st. 1 has no significant effect. These data suggest that the function of morpholinos is quite limited, either due to their instability or to the compensatory up-regulation of mRNA levels by the embryo. The time-dependency of randomization by morpholinos suggests that Vangl2 functions most crucially for LR patterning around st. 3 (streak elongation), just prior to the determination of *Shh* sidedness in the node.

While a variety of mutations disrupt both PCP and LR patterning (reviewed in Table 1 in (Aw and Levin, 2009)), the Vangl2 mutant mouse (*looptail*) has not been reported to exhibit left-right asymmetry defects. It is possible that a different PCP protein fulfills this function in mice and future analysis of other targets may reveal a role for planar polarity in rodent asymmetry. Alternatively, mice – which have a very different architecture than even most mammals – may not use PCP for LR patterning. It is known that significant differences in asymmetric gene expression exist between mice and chick (Meyers and Martin, 1999;Schlueter and Brand, 2007), and asymmetric expression of *Shh* has not been reported in the mouse at all. Mouse embryos are thought to use ciliary motion as an important component of LR patterning (reviewed in (Basu and Brueckner, 2008)); in contrast, the chick not only acquires consistently-asymmetric gene expression (Levin *et al*., 1995b;Stern *et al*., 1995) and asymmetric transmembrane potential (Levin *et al*., 2002) prior to appearance of the node, but also does not have enough cilia in the node to perform chiral extracellular flow (Manner, 2001). Considerable controversy exists over the degree of conservation of different LR mechanisms among phyla (Aw and Levin, 2008b;Levin and Palmer, 2007;Tabin, 2005); the role of PCP in LR patterning remains to be tested in model species other than chick.

However, it is clear that a very wide variety of cells, including protozoa (Frankel, 1991), *Xenopus* blastomeres (Aw et al., 2008), and mammalian neutrophils in culture (Xu et al., 2007) possess intrinsic left-right directionality. What is necessary for asymmetric gene expression (such as that of *Shh*) in a multicellular organism is for cells to know whether they are on the Left or Right side of the embryonic field. Our model (Fig. 4) does not address the initiation (breaking) of asymmetry but illustrates how knowledge of direction within cells (which can be provided by any chiral molecule tethered with respect to the other 2 axes (Brown and Wolpert, 1990)) can be used to derive position with respect to the midline. This is an alternative (but not mutually exclusive) model to the serotonin-dependent system used in frog (Esser et al., 2006; Fukumoto et al., 2005b; Levin et al., 2006), and can apply to blastoderms of embryos like fish and mammals where lots of small cells (instead of big blastomeres) exist at the time of midline determination. However, it should be noted that amniotes (and other organisms) may in fact determine the midline long before the blastoderm stage, as is currently believed, since gynandromorphs derived from chromosome nondisjunction at the first cleavages after fertilization exhibit pigment and brain structure differences precisely across the animal's midline in chickens and numerous other creatures (see (Agate et al., 2003; Lillie, 1931) and discussion in (Levin and Palmer, 2007)).

Several key pieces of data remain to be obtained to flesh out this model. Very early left-right steps in frog embryos take place in an epithelium (Chalmers et al., 2003; Muller and Hausen, 1995), and indeed the early ion transport proteins are plane-polarized (Adams et al., 2006; Aw et al., 2008; Levin et al., 2002). Thus, future work must examine the subcellular localization of V-ATPase and H,K-ATPase proteins in chick blastoderm cells to determine how the PCP and bioelectrical signals interact. Additional components of such models, based on the identification of consistent subcellular asymmetry in individual neutrophil cells

(Xu et al., 2007), may include the basal body/centriole; these await high-resolution imaging in early chick. This would be likely to shed light on the intracellular chiral structure required by all models of consistent LR patterning. The nature of this component is still unknown, although previous work suggests that the cytoskeletal organizing center is a likely candidate (Aw *et al*., 2008; Aw and Levin, 2008a).

Our data highlight some still mysterious and little-discussed aspects of asymmetric gene expression in chick; this includes the mechanistic explanation of "randomization" (several possible outcomes on each side of the node) following loss-of-function of a specific upstream signal, and the non-equiprobable nature of asymmetric marker expression. As observed in numerous previous studies, right-sided expression of *Shh* was very rarely observed (absent and bilateral are much more common, as in Table 4); this statistical distribution is not specifically predicted (explained) by any available conceptual models.

A final aspect that must also be addressed is coordination among cells within a domain. In normal or randomized nodes, each side makes a coordinated (all or none) decision as to whether to express an asymmetric gene - the sides of Hensen's node act as synchronized compartments with respect to LR identity. Strikingly, Vangl2 morpholinos abolish this unity among cells on one side of the node, leading to a speckled *Shh* phenotype (Fig. 3g) that is not recapitulated by any perturbations of LR pathways reported to date. Thus, as in numerous other organisms (Lewis and Davies, 2002;Zallen, 2007), PCP appears to be involved in coordinating the behavior of cell groups into coherent domains as well as the linkage of subcellular cell polarization with that of macroscopic embryonic axes. The chick is an ideal system in which to explore the PCP-like mechanisms by which LR information is spread and coordinated in an embryonic blastoderm. While the details remain to be worked out, the recent identification of asymmetric cell movement around Hensen's node (Cui *et al*., 2009;Gros *et al*., 2009) suggests that effects on cell movement, as well as upon gene expression, of alterations of PCP need to be considered in formulating models of the spread of LR information throughout the blastoderm. The next generation of models in this field must begin to synthesize several neglected aspects of existing molecular data, and the insights of the PCP field are likely to facilitate deep insight into the fascinating process by which polarity and asymmetry is coordinated across size scales during embryogenesis.

Materials and Methods

Whole mount immunofluorescence at subcellular resolution

Chicken eggs (Charles River) were incubated at 38°C until the stage indicated in the text (Hamburger and Hamilton, 1992). Embryos were fixed in 4% paraformaldehyde for 6 hours, washed in PBS, and treated with 0.3% H_2O_2 in PBS for 30 min. Embryos were blocked in PBSTT (PBS with 0.1 % Triton-100x and 0.1 % Tween-20) with 2% BSA and 10% goat serum for 3 hours, and then incubated with primary antibody (Vangl2 at 1:500 dilution, (Montcouquiol et al., 2006)) overnight. After washing in PBS, embryos were incubated with HRP-conjugated secondary antibody (Jackson ImmunoResearch,) at 1:1000 dilution overnight, then with Alexa568-tagged Tyramide anti-HRP (Invitrogen) for 1 hour at 1:200 dilution. Embryos were further incubated with Alexa488 fluorescent conjugated phalloidin (Invitrogen) in PBS for one hour to label the cell boundary. Embryos were mounted in Vectashield (Vector Laboratories).

Confocal imaging

A Leica TCS SP2 confocal microscope was used for imaging. Regions about 150 μm × 150 μm in size, located at the equivalent anterio-posterior position on the left and right sides of the middle part of the streak (indicated in Fig. 1 and Fig. 2), were scanned at 100×

magnification with an image taken every 1 mm along the Z axis (proceeding from the dorsal to ventral direction). The exposure/gain/offset for the image with the brightest stain in one stack was determined automatically using Leica software, and applied to all the images in the stack. For each embryo, images for each fluorescent channel were taken sequentially.

Image analysis

Vectors, $\bar{v} = x\bar{\imath} + y\bar{\jmath}$, pointing from the center of the Vangl2 protein localization to the cell center, were selected for 50–100 cells in a single image selected from a Z stack. Cells were chosen randomly and only those in which the red stain unambiguously belonged to one cell were scored. In each scored cell, the angle θ ranging from −180° to 180°, formed between the vector and a line parallel to the x-axis of the image, was calculated and normalized to the streak direction. Thus θ is the angle between the vector and the streak from the anterior to posterior direction. The average angle was calculated from average values of sinθ and cosθ. The standard deviation of angle was also calculated from average values and standard deviation of sinθ and cosθ.

It proved impossible to achieve fully automated segmentation and processing of individual cells (in part because of the difficulty in getting contiguous cell surface stain, nuclear stain, and Vangl2 stain in the same plane of section in cells). Thus, the cell center and the center of the Vangl2 localization were chosen manually in order to draw vectors. In those sections in which nuclei could be observed, the center most often overlapped with the cell nucleus. In order to establish the maximum error inherent in this procedure, we independently measured 5 randomly chosen cells 15 times each. The data (Table 2) revealed that manual determination of Vangl2 vector angle is consistent (based on average spread) within ±8.4 degrees.

One sample critical ratio z test—The “One sample critical ratio z test” is designed to test whether a statistic proportion is significantly different from its corresponding population parameter (Fleiss, 1981). To test whether the vector, and thus the distribution of Vangl2 protein in individual cells, points towards or away from the streak, a one sample critical ratio z test for proportions was applied with expectation probability $P_E = 0.5$ for cells with random polarity; if Vangl2 protein accumulations were randomly distributed among cells, then the expected ratios for polarizing toward the streak ($\theta > 0$ for left cells and $\theta < 0$ for right cells) and polarizing away from the streak ($\theta < 0$ for left cells and $\theta > 0$ for right cells) would be 0.5 ($P_E = 0.50$). The experimental ratio is P_O, which equals the number of cells polarizing towards the streak divided by the total number of cells measured. For sample size of n, the Z value was calculated as:

$$Z = \frac{P_O - P_E}{\sqrt{\frac{P_E(1-P_E)}{n}}} \quad (1)$$

When $Z > 2$, $p < 0.05$, the result was considered significant.

Early chick culture and whole embryo-electroporation

The electroporation method was modified from (Voiculescu et al., 2008) and (Chapman et al., 2001). Embryos were detached from the vitelline membrane and moved to the electroporation chamber. The dorsal side was facing up, with a positive electrode (3 mm) at the bottom, and a negative electrode (3 mm) above. 0.5 ml of the morpholino electroporation mixture was deposited on top of the embryo. Square waves of 5 Volts, 5 pulses, 50 ms each pulse, with 500ms interval duration was applied to each embryo using an electroporation

apparatus (CUY21EDIT). The remaining electroporation mixture was washed off and the embryos were transferred to, and cultured on albumin-agar plates until fixation at st. 5.

Morpholinos

Fluorescein tagged splice morpholino for *Vangl2* had the sequence 5'-CAAGGGAATGGGAGCTCACCCTCGC-3', which reduces endogenous mRNA levels in the chick 50% by exon skipping and partial failure of an intron to be spliced out as checked by PCR (Voiculescu et al., 2007). As controls for toxicity and specificity, the morpholino and its 5 base pair mismatch control, 5'-CAAcGGAATcGGAcCTgACCCTgGC-3', were applied separately at 3.0 mM with 2/5 volume of 1.2 mg/ml RFP DNA plasmid and 1/10 volume of 60% sucrose.

Whole-mount in situ hybridization

In situ hybridization was performed as previously described (Nieto et al., 1996). *Sonic hedgehog* probe was used as in (Levin *et al*., 1995a). Chromogenic reaction times were optimized for signal : background ratio.

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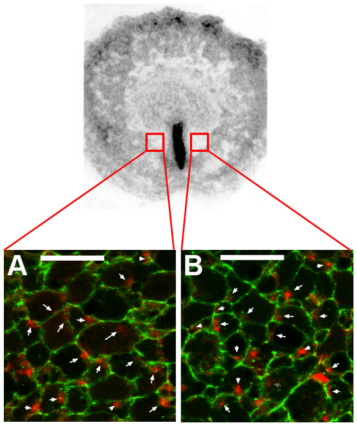

Figure 1. Vangl2 protein is localized to a discrete domain within chick cells

Chick embryos at st. 2–3 were processed for immunohistochemistry with an antibody to Vangl2 (red). Cell boundaries were revealed by phalloidin stain (green). Cells on the left (**a**) and right (**a**) of the primitive streak exhibited polarized accumulations of Vangl2 protein on cell membranes, with some extension into the cytoplasm of individual cells. We analyzed randomly chosen cells in which the accumulation of Vangl2 was unambiguously located within a single cell, by drawing a vector from the Vangl2 accumulation (white arrows) to the center of the cell. Relative to the cell center, the Vangl2 localization generally points towards the streak in cells on either side of the embryo. White arrows indicate Vangl2 vectors observed in typical cells (Table 1 and Table 2). Red lines schematize location of the field scored for panels A and B (not to scale with red square). Scale bar = 16 μm.

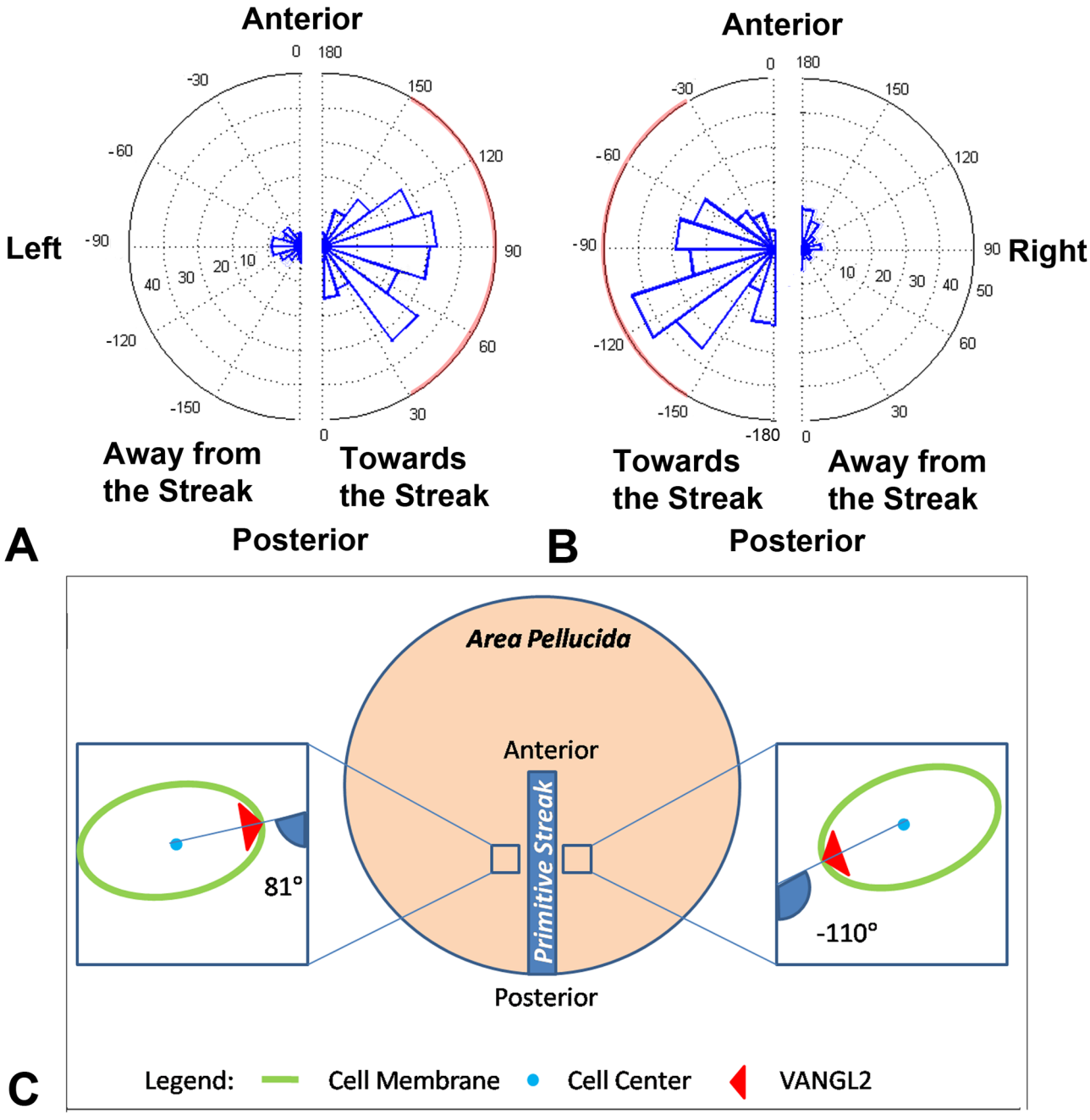

Figure 2. Vangl2 localization is polarized with respect to the streak

Vectors, $\bar{v} = x\bar{\imath} + y\bar{\jmath}$, pointing from the center of the Vangl2 protein localization signal to the cell center (−180°→180° axis along the streak), were computed for 50–100 cells in each embryo. Distribution of vectors is plotted in polar coordinates; length of each blue sector reflects (is proportional to) the number of cells with the corresponding angle of Vangl2. (**a**) Distribution of Vangl2 relative to streak for cells on the Left side of embryos. (**b**) Distribution of Vangl2 relative to streak for cells on the Right side of embryos. 57% of the Vangl2 vectors on the left side cells, and 62% of the Vangl2 vectors on the right side cells, fall into the sector (shaded in pink) between ±38° and ±142° (30°−150°, but discarding those vectors within ±8° of the edges to account for measurement error of ±8°, see Table 2).

This is significantly different from the 28% expected by chance (random, non-polarized distribution of Vangl2 protein). (**c**) Schematization of average alignment.

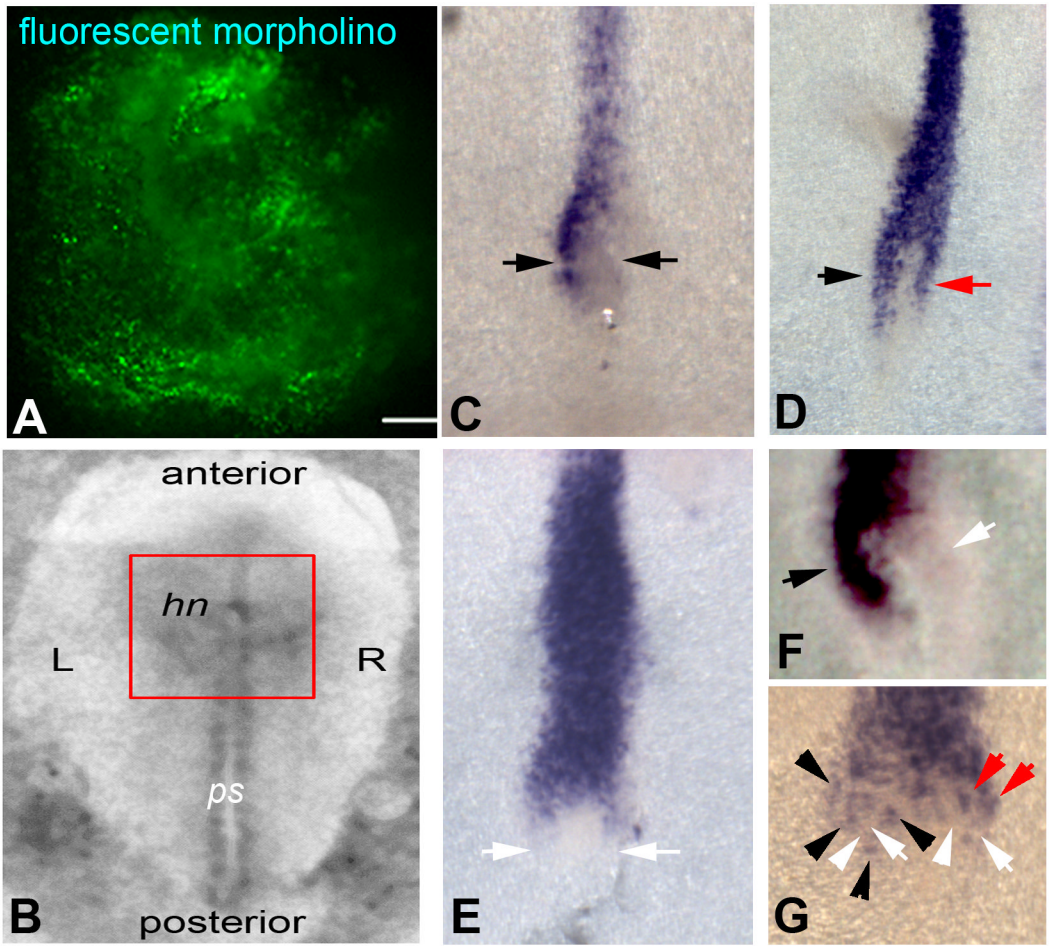

Figure 3. Vangl2 morpholino randomizes and desynchronizes *Shh* expression

Chicken embryos at st. 1–3 were transfected in culture with a fluorescein-tagged morpholino to *Vangl2* mRNA (**a**). They were then processed for in situ hybridization with a probe to the left determinant *Shh* (quantified in Table 4). Panels C–G show close-ups of the anterior streak, node, and emerging notochord as indicated in **b** (hn = Hensen's node; ps = primitive streak). Control embryos (only cultured, or cultured and electroporated with a control morpholino) exhibited the normal left-sided *Shh* pattern (**c**). In contrast, embryos treated with the Vangl2 morpholino often exhibited bilateral (**d**) or absent (**e**) expression of *Shh* at the node. In panel E, note the expression of *Shh* in the notochord anterior to the node, demonstrating that lack of *Shh* expression in Hensen's node is not due to a failure of in situ hybridization. While most published patterns of *Shh* expression, even when randomized by modulation of various pathways, exhibit expression that is entirely on or entirely off on each side of the Node (**f**), Vangl2 morpholinos often induced a speckled pattern in the node (**g**), indicating a failure of the cells on one side of the node to come to a synchronized (coherent) decision about their sidedness. White arrows indicate lack of *Shh* expression. Black arrows indicate normal expression. Red arrow indicates ectopic expression.

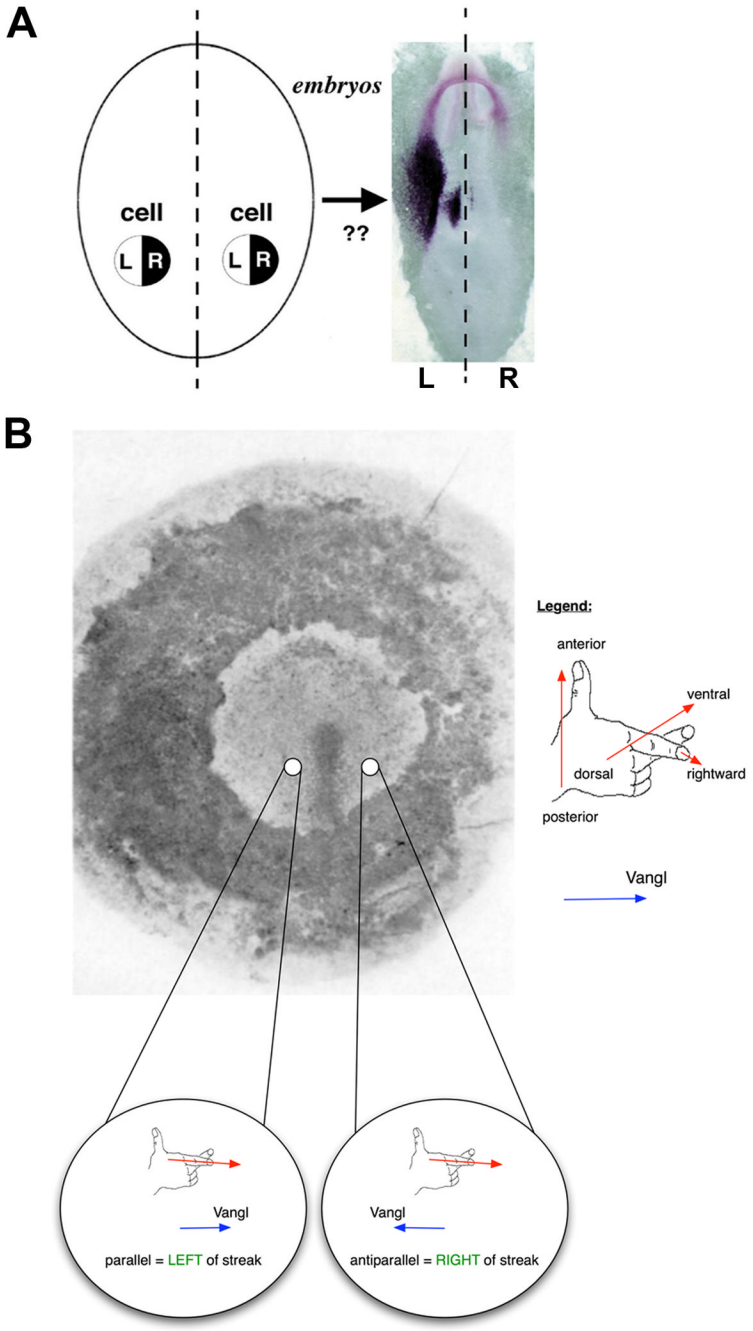

Figure 4. A model for using Vangl2 vector to derive position from direction

(**a**) Cells can derive direction (knowing which way is left-ward and which way is right-ward) by tethering a chiral intracellular component (schematized by a “right hand molecule”) with respect to the dorso-ventral and anterior-posterior axes, which are already known in the chick blastoderm (Brown and Wolpert, 1990). (**b**) To determine their position with respect to the midline (location on the left or right of the primitive streak), cells can compare the chiral cue with the Vangl2 vector shown in Fig. 1. If the vectors are parallel, the cell knows it’s located on the left side of the embryo and can execute the left-specific events (depolarization followed by *Shh* expression in the node). If the vectors are antiparallel, the cell knows that it must be located on the right side.

Table 1

Average and variability of Vangl2 polarization angles in cells along the left and right side of the streak

The orientation of angles is significantly different from a random distribution($p<0.01$); the difference in angle with respect to the primitive streak is significantly different between the L and R sides to $p<0.05$ (2-tailed, 2-sample T-test with unequal variance)..

	Average angle of Vangl2 vectors in cells on Left	Average standard deviation of angles of Vangl2 vectors in cells on Left	Average angle of Vangl2 vectors in cells on Right	Average standard deviation of angles of Vangl2 vectors in cells on Right
Embryo 1	63.83	68.93	−90.45	71.14
Embryo 2	82.14	117.11	−116.46	74.70
Embryo 3	83.03	119.41	−127.78	81.86
Embryo 4	94.07	94.29	−107.10	81.85
Mean	**80.77**	**99.93**	**−110.44**	**77.39**

Table 2

Quantification of error introduced by manual determination of cell center. Five different cells, comprising angles in the entire range, from −8.3° to 108.8°, were used for determination of Vangl2 angle, and each cell was independently measured 15 times. Such measurements exhibited an average spread of ±8.4.

Measured	Mean	Standard Deviation	Spread of Measurements
Cell 1	−94.1	2.8	9.3
Cell 2	−108.8	5.2	17.6
Cell 3	−74.9	5.9	19.8
Cell 4	−43.0	7.7	32.4
Cell 5	−8.3	10.4	4.8
Average	n/a	6.4	16.8 = ±8.4

Table 3

Intracellular localization of Vangl2 protein is polarized toward the primitive streak significantly

(Z = 9.2 for the Left cells and 10.2 for the Right cells). To test the significance of polarity, the one sample critical ratio test for proportions (Fleiss, 1981) was performed with expectation probability $P_E = 0.5$ for cells with random polarity. The experimental ratio is $P_O = N_{tw}/N_{total.}$ For sample size *n*, the Z value is calculated as: $Z = \frac{P_0 - P_E}{\sqrt{\frac{P_E(1-P_E)}{n}}}$. When Z > 2, p < 0.05, the result (the distribution of angle point towards or away from the streak) is significant. The polarities of Vangl2 in cells towards or away from the primitive streak of four embryos at st. 3 were evaluated individually and together.

On the Left Side of the Streak					
embryo:	**E1**	**E2**	**E3**	**E4**	**Total**
Number of cells measured	80	61	60	87	288
Vectors pointing away from the Streak	12	16	16	22	66
Vectors pointing towards the Streak	68	45	44	65	222
Z value	6.3	3.7	3.6	4.6	9.2

On the Right Side of the Streak					
embryo:	**E1**	**E2**	**E3**	**E4**	**Total**
Number of cells measured	41	96	55	84	276
Vectors pointing away from the Streak	11	10	15	17	53
Vectors pointing towards the Streak	30	86	40	67	223
Z value	3.0	7.8	3.4	5.5	10.2

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Table 4

***Shh* expression in embryos exposed to Vangl2 loss-of-function reagent**

Cultured embryos have a very low (2%) incidence of aberrant *Shh* expression. Electroporation with a control morpholino raised this to 12%. Electroporation with a morpholino to Vangl2 at st. 2 or st. 3 results in 25% or 41% respectively of incorrect *Shh* expression among embryos (both significant using χ^2 test to compare raw numbers). ns = not significant.

	Total N	Left-sided	Bilateral	Right-sided	Absent	% wrong *Shh*	Probability compared to ctrl morpholino
Control morpholino at st. 3	17	15	1	-	1	12%	ns
		(88%)	(6%)	(0%)	(6%)		
Culture only	41	40	1	-	-	2%	ns
		(98%)	(2%)	(0%)	(0%)		
Vangl2 morpholino at st. 1	20	18	2	-	-	10%	ns
		(90%)	(10%)	(0%)	(0%)		
Vangl2 morpholino at st. 2	24	18	3	-	3	25%	p = 0.013
		(74%)	(13%)	(0%)	(13%)		
Vangl2 morpholino at st. 3	32	19	8	-	5	41%	$p = 4.5 \cdot 10^{-15}$
		(59%)	(25%)	(0%)	(16%)		