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Live imaging and conditional disruption of native vertebrate planar cell polarity

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Abstract

Tissue-wide coordination of polarized cytoskeletal organization and cell behaviour, critical for organ morphogenesis and tumour progression, is controlled by asymmetric membrane localization of non-canonical Wnt/planar cell polarity (PCP) signalling components. Understanding the dynamic regulation of PCP thus requires visualization of these core proteins. In vertebrates, immunohistochemical studies have provided snapshots of PCP within tissues while exogenous, fluorescently-tagged reporters have been introduced for live imaging of cell polarity. However, the functionality and spatiotemporal relevance of static and exogenous PCP readouts remain uncertain. Here we fluorescently tag an endogenous core PCP component, Vangl2, in zebrafish. We report on the authentic regulation of vertebrate PCP, through live imaging of this native sfGFP-Vangl2 protein during embryogenesis. We couple sfGFP-Vangl2 with conditional zGrad GFP-nanobody degradation methodologies for tissue-specific interrogation of PCP function. Together, our studies provide crucial insights into the establishment and maintenance of vertebrate PCP and create a powerful experimental paradigm for future investigations.

Introduction

The non-canonical Wnt/planar cell polarity (PCP) signalling pathway controls polarized, uniform orientation of cells within a plane of a tissue. PCP pathway has been shown to regulate a number of developmental and homeostatic processes by directing collective asymmetric modification of the cell cytoskeleton, leading groups of cells to either move or divide in a shared direction, or position their organelles such as the basal body asymmetrically¹⁻⁵. Molecular genetic studies in *Drosophila* have identified essential roles for asymmetric membrane localization of opposing PCP signalling proteins in both the establishment and intercellular propagation of polarity⁶.

Despite identification of a multitude of PCP-regulated morphogenetic processes, characterization of vertebrate PCP at the cellular and molecular level is still lacking. This is challenging, because detailed observation requires live imaging capabilities and the ability to differentiate asymmetric protein localization across membranes of adjacent cells. In vertebrates, immunohistochemical studies have provided snapshots of PCP within tissues^{7,8} while exogenous, fluorescently-tagged reporter proteins have been introduced for mosaic imaging of PCP. Although great complexities in the dynamic regulation of PCP have been reported across tissues and polarized cell behaviours,⁹⁻¹² the functionality and spatiotemporal accuracy of these static and exogenous readouts remain uncertain. The biological relevance of studies utilizing exogenous, fluorescently-tagged PCP molecules remains to be determined, as overexpression of PCP proteins can disrupt cell polarity^{11,13} and functionality of published PCP fusion proteins has not been validated via rescue of orthologous mutant phenotypes.^{12,14-16} Furthermore, non-physiological expression of exogenous proteins can mask subtle localization patterns or signalling dynamics. This is highlighted by observations that in many occasions, co-expression of another PCP component is required for asymmetric membrane localization of the fluorescent fusion protein of interest.¹⁷⁻²⁰

To understand the dynamic regulation of native PCP, we have aimed to overcome limitations imposed by exogenous cell polarity reporters by imaging and manipulating the endogenous zebrafish Vangl2 protein, a core PCP component and essential regulator of vertebrate planar polarity.^{21,22} Using CRISPR/Cas9 gene editing, we introduced a superfolder GFP (sfGFP) tag onto the N-terminus of endogenous Vangl2 to generate a functional and native PCP reporter protein. By performing detailed embryonic cell transplantation and single-cell level analyses, we documented the dynamic sub-cellular localization of Vangl2 over the course of neural tube

formation, revealing novel associations of sfGFP-Vangl2 positive cell membranes with polarized basal bodies in floorplate cells. Furthermore, we exploited zGrad²³ GFP-specific protein degradation methodologies to conditionally interrogate sfGFP-Vangl2 function in floor plate cells, demonstrating an essential role for Vangl2 in both the establishment and maintenance of polarized basal body positioning as well as an inherent and surprising directionality to the intercellular propagation of PCP. Our work highlights the importance and utility of studying endogenous proteins in the context of PCP, and opens the door to conditional analysis of PCP function beyond embryonic development.

Results and Discussion

To visualize the dynamic regulation of endogenous PCP signalling in zebrafish, we used CRISPR/Cas9 genome editing to target a superfolder green fluorescent protein (sfGFP) onto the N-terminus of Vangl2, an essential and membrane-localized PCP signalling protein (Fig. 1a; targeting details in Methods section). Embryos carrying the knock-in allele showed prominent sfGFP signal in the brain and spinal cord at 22 hours post fertilization (hpf) (Fig. 1b). In striking contrast to the *vangl2*^{m209/209} loss-of-function mutant phenotype, which is embryonic lethal²¹, fish homozygous for the *vangl2*^{sfGFP} allele or trans-heterozygote for *vangl2*^{sfGFP} and *vangl2*^{m209} alleles are viable, fertile and appear morphologically normal (Fig. 1c). Closer inspection revealed that *vangl2*^{sfGFP/sfGFP} embryos are slightly but significantly shorter than wildtype siblings at 24 hpf (Fig. 1d). Interestingly, heterozygote *vangl2*^{m209/+} embryos are similarly shorter than wildtype, which has not been previously reported. *Vangl2*^{sfGFP/m209} transheterozygote embryos demonstrate a further shortening of the body axis but are significantly less affected than the *vangl2*^{m209/m209} mutants (Fig. 1d). Altogether these data indicate that, although mildly hypomorphic, the sfGFP-

Vangl2 fusion protein is functional and can therefore be used to interrogate endogenous PCP signalling dynamics.

To determine the onset of Vangl2 expression, we imaged *vangl2^{sfGFP/sfGFP}* embryos (hereafter referred to as *vangl2^{sfGFP}*) through blastula and gastrula stages. Weak Vangl2 membrane localization could be observed in a subset of cells as early as high stage (3.3 hpf), which became more ubiquitous through early epiboly stages (4.6 hpf; Fig. 1e). Interestingly, weak Vangl2 membrane-localization was also observed within enveloping layer (EVL) cells at these stages (Supplementary Fig. 1a). These observations are consistent with a role for Vangl2 prior to gastrulation, perhaps in promoting tissue cohesion in margin cells during early blastoderm morphogenesis.²⁴ Vangl2 expression levels increased, and membrane localization became more prominent with the onset of gastrulation at shield stage, persisting through 75% epiboly and bud stages (Fig. 1e, Supplementary Fig. 1b-e). This corresponds to the known role for Vangl2 in the regulation of convergence and extension movements.²¹ Although immunohistochemical analyses of Vangl2 expression have yielded similar observations¹⁴ the superior consistency, sensitivity and live-imaging capabilities of sfGFP-Vangl2 expression highlight its utility for experimental studies.

In addition to membrane-localized Vangl2, we also observed punctate intracellular accumulations, which became prominent at the onset of gastrulation when they concentrated into large cytosolic aggregates (Fig. 1e). To investigate the identity of these compartments, we analysed Vangl2 localization in relation to known markers of intracellular sorting organelles (Fig. 1f-i and Supplementary Fig. 1b-e). In published studies, early and late endosome markers Rab5 and Rab7 colocalize with PCP components Flamingo/Celsr and Frizzled-3a in *Drosophila* wing, mouse keratinocytes and zebrafish neural tube.^{10,15,25-27} The recycling endosome marker

Rab11 also colocalizes with Flamingo/Celsr in mouse skin and in *Drosophila* wing, and with Vangl2 in cell culture.^{15,28,29} However, vertebrate studies have used exogenous PCP reporters, which might not recapitulate the endogenous subcellular protein localization and may induce trafficking artifacts. While we observed that Rab5c colocalized with some of the smaller Vangl2 puncta at both shield and 90% epiboly stages (Fig. 1f and Supplementary Fig. 1b), and that Rab11a colocalized with Vangl2 at the cell membrane but not within the cytoplasm (Fig. 1h and Supplementary Fig. 1d), the majority of Vangl2 puncta colocalized with mCherry-Rab7 (Fig. 1g and Supplementary Fig. 1c) suggesting late endosomal transport to lysosomes or the trans-Golgi network (TGN). Prior to gastrulation the zebrafish Golgi is fragmented,³⁰ and whereas we could see occasional sfGFP-Vangl2 signal in the proximity of the TGN reporter dsRed-GalT, Vangl2 was predominantly localized elsewhere in the cell (Fig. 1i and Supplementary Fig. 1e). Taken together these results suggest that, at early gastrulation stages, Vangl2 is targeted to late endosomes for degradation.

Following gastrulation, strong Vangl2 expression was observed in the central nervous system, whereas only weak sfGFP-Vangl2 signal could be detected in surrounding axial and paraxial mesodermal lineages (Figs. 1b, 2a). Previous work has demonstrated that exogenous GFP-tagged Vangl2 or its cytoplasmic binding partner Prickle polarize anteriorly in neuroepithelial cells in zebrafish and *Xenopus*.^{9,12,14,16,26,31} To characterize the asymmetric distribution of endogenous Vangl2, we performed cell transplantation at blastoderm stages to generate clones of *vangl2*^{sfGFP} cells within wildtype host embryos and imaged Vangl2 localization in individual neuroepithelial cells at multiple time points during neural tube formation (Fig. 2b). At all stages, weak sfGFP-Vangl2 signal could be observed across the entire neuroepithelial cell membrane, with bright membrane sfGFP-Vangl2 domains enriched in puncta at anterior cell surfaces (Fig.

2c, Supplementary Fig.2a, Supplementary Movie 1). Vangl2 localization varied considerably between cells, including apical and basal distribution patterns, as well as localization into cell protrusions (Fig. 2c and Supplementary Fig.2a). Previously, exogenous Vangl2 reporter localization has been quantified along single cellular planes. To gain a global view of Vangl2 distribution, we performed 3-dimensional quantification of sfGFP-Vangl2 membrane-enrichment in comparison to an RFP membrane reporter (mRFP) using the spot function in Imaris (detailed description in Methods). We plotted the subset of membrane spots containing the highest sfGFP-Vangl2 and mRFP fluorescent intensities across anterior-posterior and apico-basal cell axes (Supplementary Fig.3A) and found that, despite the variation between cells, Vangl2 was significantly enriched at the anterior membrane at all stages analysed (Fig. 2d). Vangl2 localization was less uniformly polarized along the apico-basal axis, with Vangl2 showing strongest apical enrichment at the 13 somite-stage concomitant to neural tube midline formation¹⁶ (Fig. 2e).

To examine the dynamics of Vangl2 protein localization, we performed time-lapse imaging of *vangl2^{sfGFP}* chimeric embryos between 10-13 somite stages. Persistent sfGFP-Vangl2 enrichment was observed along anterior membrane domains (Supplementary Movie 1), supporting evidence from multiple *Drosophila*, *Xenopus* and mouse studies (predominantly using exogenous reporters) that PCP components concentrate into potential “signalosomes” on the cell membrane.^{9,16,20,28,32–35} In *Xenopus*, exogenous Vangl2 and Prickle reporters accumulate with actomyosin at shrinking neuroepithelial cell junctions⁹ and, in mesodermal cells undergoing convergence and extension movements, Prickle2 colocalizes with the LifeAct filamentous actin reporter.^{35,36} These observations suggest that Vangl2/PCP directly influences cytoskeletal reorganization. To investigate the relationship between anterior Vangl2 enrichment and actin

organization, we co-expressed a mCherry-LifeAct reporter in *vangl2^{sfGFP}* cells. Live imaging revealed that the majority of neuroepithelial cell protrusive activity and LifeAct localization was restricted to apical and basal surfaces, and not to anterior membranes where sfGFP-Vangl2 was enriched (Supplementary Movies 2-3). However, sfGFP-Vangl2 could be observed accumulating within active cellular protrusions at the anterior membrane (Supplementary Fig.2a, Supplementary Movie 4), consistent with known roles for PCP and Vangl2 in regulating filopodia and cell protrusive activity during gastrulation and facial branchiomotor neuron migration.^{11,12,37} Together, although the presence of bright sfGFP-Vangl2 puncta supports the notion that PCP signalling functions in specific subdomains on the anterior membrane, the presence of Vangl2 membrane enrichment was not predictive of a particular membrane behaviour.

To further understand how neighbouring cells affect asymmetric Vangl2 localization into different membrane-domains, we performed cell transplantation experiments to generate clones of *vangl2^{sfGFP}* cells within a *vangl2^{tk50f}* loss-of-function host embryo (Fig. 2f). In this PCP-deficient environment, *vangl2^{sfGFP}* cells clustered together, suggesting that Vangl2 expressing cells are able to recognize and preferentially interact with one other. While Vangl2 remained localized to the cell membrane, planar polarized membrane enrichment was lost and Vangl2 appeared to concentrate at cell protrusions (Fig. 2i and Supplementary Fig.2b). We picked a subset of the most mediolaterally aligned cells to quantify asymmetric Vangl2 localization and found no difference in the distribution of the brightest Vangl2 and mRFP spots along the anterior-posterior or apico-basal axes (Fig. 2g-h; Supplementary Fig. 3d). Loss of polarity in *vangl2^{sfGFP}* positive clones supports a cell non-autonomous regulation of vertebrate PCP. The

180 persistence of Vangl2 in cell protrusions suggests that it might function in mediating local
181 signalling that is independent of its role in global anterior-posterior neural tube planar polarity.

182 The re-organization and re-establishment of cell polarity over the course of cell division remains
183 a poorly understood feature of vertebrate PCP. In zebrafish, asymmetric localization of
184 exogenous Prickle-GFP reporter molecules is lost upon neuroepithelial cell division, but is re-
185 established as daughter cells reintegrate into the neuroepithelium.¹⁶ In mouse epidermis, different
186 studies have shown PCP components to be internalized during mitosis or to persist on cell
187 membranes in a polarized manner.^{15,38} Exogenous Vangl2, specifically, has been shown to
188 persist on cell membranes and to get trans-internalized from neighbouring cell membranes into
189 the dividing cell.¹⁰ To determine the fate of endogenous Vangl2 during mitosis, we imaged
190 *vangl2^{sfGFP}* neuroepithelial cells over the course of cell division. Notably, asymmetric sfGFP-
191 Vangl2 distribution was lost upon cell rounding, but Vangl2 remained at the cell membrane
192 (Supplementary Movie 3-4). We observed sfGFP-Vangl2 enriched at the poles of dividing cells,
193 as well as to basolateral membrane projections that are maintained during division
194 (Supplementary Movies 3-4). Although some intracellular sfGFP-Vangl2 signal was observed in
195 dividing cells, we did not observe significant endocytosis of membrane Vangl2 or trans-
196 endocytosis between neighbouring cells (Supplementary Movies 3-4). Reacquisition of Vangl2
197 polarization in daughter cells was not obvious immediately upon division (Supplementary Movie
198 2). Of interest, sfGFP-Vangl2 positive membrane protrusions from distant neighbours could be
199 observed interacting with dividing cells (Supplementary Movie 4). Out of 24 cells that had an
200 anterior Vangl2 positive protrusion, 11 were touching a dividing cell, and the same was observed
201 for 15/32 apical protrusions (total n=112 cells). Our results show that Vangl2 remains on the

202 membrane but loses its asymmetry during mitosis, and that re-establishment of cell polarity may
203 be regulated by contacts from neighbouring cells.

204 In addition to cytoskeletal organization, polarized basal body (BB) positioning has been
205 identified as a conserved readout of PCP from insects to vertebrates.³⁹ In zebrafish floorplate
206 cells, BBs are localized to the posterior apical membrane by 24 hpf, and the establishment of BB
207 polarity requires Vangl2.^{13,26,40} To examine the relationship between Vangl2 localization and BB
208 positioning, we imaged the floorplate of *vangl2^{sfGFP}* embryos at 22 and 32hpf. In the sagittal
209 plane, sfGFP-Vangl2 was enriched on the anterior apical cell membrane (Fig. 3a-b), in
210 accordance with observations using an exogenous GFP-Vangl2 transgenic reporter.^{12,26}

211 However, in some floorplate cells apically enriched Vangl2 appeared to extend anteriorly
212 towards neighbouring cells (Fig. 2a, Fig. 3a-b). This bright apical Vangl2 signal was closely
213 associated with BBs labelled with Centrin-mCherry, and with Arl13b-positive cilia (Fig. 3a-b).

214 We imaged individual transplanted *vangl2^{sfGFP}* floorplate cells and found that Vangl2 was indeed
215 localized asymmetrically on anterior cell membranes, and that in some cells it further localized
216 to an anterior apical projection (Fig. 3c). Labelling BBs in transplanted *vangl2^{sfGFP}* cells with
217 Centrin-mCherry confirmed the proximity of anterior Vangl2 with posteriorly polarized BBs
218 (Fig. 3d). Furthermore, labelling of host cell BBs confirmed that Vangl2-positive apical
219 membrane closely associated with the posterior BB of neighbouring cells (Fig. 3e, 11/17 cells).

220 Of interest exogenous Frizzled3a, a putative intracellular binding partner of Vangl2, has been
221 shown to be enriched posteriorly into puncta at the base of cilia in zebrafish.²⁶ This localization
222 pattern suggests that PCP activity could act to physically deform the floorplate cell membranes at
223 the site of BB docking, perhaps influencing the posterior tilting of the floorplate motile cilia.¹³

224 To determine whether Vangl2 also controls the maintenance of BB polarity, we took advantage
225 of zGrad GFP-nanobody protein degradation methodologies to conditionally manipulate
226 endogenous sfGFP-Vangl2 protein levels.²³ We first confirmed that zGrad is non-toxic to
227 embryos, and that *zGrad*-injected *vangl2^{sfGFP/sfGFP}* embryos phenocopy *vangl2* loss-of-function
228 mutants (Supplementary Fig. 4a-b). Furthermore, because *vangl2* mutants are subject to maternal
229 effects¹⁶, we investigated the consequences of zGrad expression across a full genotypic spectrum
230 of maternal and zygotic *vangl2^{sfGFP}* alleles. Strikingly, sequential zGrad-mediated degradation of
231 maternal and zygotic *vangl2^{sfGFP}* gene products yielded progressively more severe phenotypes as
232 measured by embryo length and axial length-width ratios (Fig. 4a, Supplementary Fig. 4c),
233 recapitulating maternal and zygotic *vangl2* mutant phenotypes. This highlights the sensitivity and
234 functionality of sfGFP-Vangl2 zGrad protein degradation methods.

235 To conditionally manipulate sfGFP-Vangl2 protein levels within the floorplate, we generated a
236 ubiquitously expressed *Tg(β-actin2::loxP-mCherry-STOP-loxP-zGrad)* transgene and crossed it
237 to the *Tg(foxj1a::iCre)* line⁴¹, which expresses Cre recombinase (activating zGrad) specifically
238 within motile-ciliated cell lineages. At 32 hpf, when BB polarity has been established, sfGFP-
239 Vangl2 was still present at the membrane of experimental *zGrad; foxj1a::iCre; vangl2^{sfGFP}*
240 floorplate cells (data not shown). However, mosaic loss of sfGFP-Vangl2 could be observed by
241 48 hpf (Fig. 4b), allowing us to analyse both cell-autonomous and non-autonomous roles for
242 Vangl2 in the maintenance of BB polarity. Images were scored for the loss of Vangl2 in
243 individual cells and their immediate anterior and posterior neighbours. Irrespective of their
244 Vangl2 expression status, cells surrounded by sfGFP-negative neighbours demonstrated a loss of
245 BB polarity when compared to control populations. Strikingly, the presence of sfGFP-Vangl2 in
246 a posterior neighbouring cell was associated with restored BB polarity in Vangl2-negative cells,

even if the posterior neighbour demonstrated abnormal BB positioning (Fig. 4c-d). In contrast, the presence of sfGFP-Vangl2 in anterior neighbours did not restore polarity of Vangl2 negative cells. These results demonstrate that Vangl2 is required for the maintenance of BB polarity, and that Vangl2 functions non-cell autonomously to propagate PCP in a posterior to anterior direction.

Taken together, our work provides essential new insights into the establishment and maintenance of vertebrate PCP. Live imaging of endogenous Vangl2 has authenticated broad principles predicted using exogenously introduced reporter molecules, including organization of PCP across the embryonic anterior-posterior axis and concentration of core PCP proteins into ‘signalosomes’ at the cell membrane. It has also yielded novel findings that 1) demonstrate dynamic redistribution of Vangl2 within discrete subcellular membrane domains over the course of gastrulation and neurulation, 2) suggest tissue-specific contexts for published tenets regarding membrane recycling of PCP proteins during mitosis or their association with the actin cytoskeleton, and 3) indicate an inherent and surprising directionality to the intercellular propagation of PCP. Outside of *C. elegans*,⁴² functional fluorescently-tagged PCP knock-in alleles have not been widely reported. Our *vangl2^{sfGFP}* allele thus represents an invaluable resource that, when combined with embryonic cell-transplantation, live-imaging and conditional zGrad degradation methodologies, establishes a powerful experimental paradigm for future investigations into vertebrate cell polarity.

Methods

Animal husbandry

Zebrafish husbandry and experimental protocols were approved by the Hospital for Sick Children's Animal Care Committee, and all protocols were performed in accordance with Canadian Council on Animal Care guidelines. Wild-type zebrafish from TU strains were used. The *vangl2* mutant allele *tri*^{tk50f} was used for cell transplantation experiments²¹, and the *tri*^{m209} allele was used in to validate the *vangl2*^{sfGFP} allele functionality because of the ability to use a published PCR mediated genotyping strategy^{21,43}. Tg(*foxj1a::iCre*) line has been previously described⁴¹. Embryos from natural matings were grown at 28°C unless otherwise indicated.

sfGFP-Vangl2 targeting strategy

Target Selection

A previously validated guide RNA (gRNA) was used to target the sequence surrounding the start codon of *vangl2* (targeting sequence GGATAACGAGTCGCAGTACTCGG)⁴⁴. As a proof of principle, a solution containing 300 mM KCl, 100 ng *vangl2* gRNA, 200 ng recombinant Cas9 (PNA Bio cat. CP01) and 10% phenol red was incubated at 37°C for 5 minutes to form ribonucleoprotein complexes (RNPs), and injected into wild-type embryos at the 1-cell stage. Embryos were (i) scored for *vangl2/trilobite* phenotypes at 28 hpf and (ii) assessed for loss of a ScaI restriction site by PCR to gauge mutagenesis efficiency.

293 *Targeting Vector Construction*

294 Prior to vector construction, male and female Tu wild-type animals were screened by PCR for
295 polymorphisms surrounding *vangl2* exon 2. Left and right arms of homology of ~1 - 1.2 kb in
296 length were amplified from fin clips and subjected for Sanger sequencing. Fish containing highly
297 similar amplicons were selected for use in targeting experiments (4 males and 4 females). A ~2.2
298 kb amplicon surrounding *vangl2* exon 2 was amplified from these wildtype fish and cloned into
299 pDONR221. A superfolder GFP (sfGFP) was amplified from sfGFP-N1 (a kind gift from
300 Michael Davidson and Geoffrey Waldo, Addgene #54737)⁴⁵ and cloned into the *vangl2* targeting
301 intermediate with a 3x(GGGGS) flexible linker by megaprimer PCR⁴⁶ and verified for in-frame
302 inclusion. Site-directed mutagenesis was performed on this intermediate to abolish the *vangl2*
303 gRNA targeting site to prevent vector from being cleaved *in vivo*. This final targeting
304 intermediate was amplified and cloned into XhoI and EcoRI sites of pKHR5 (a kind gift from D.
305 Grunwald, Addgene #74593). All targeting intermediates and final clones were fully verified by
306 Sanger sequencing.

307

308 *Knock-In Injections*

309 A 2.5 µl mix containing 20 ng of *vangl2* targeting vector, 1.25 U of I-SceI (NEB cat. R0694S),
310 0.5x Cutsmart buffer and 10% phenol red was prepared. A second 2.5 µl mix containing 300 mM
311 KCl, 100 ng *vangl2* gRNA and 200 ng recombinant Cas9 (PNA Bio cat. CP01) was incubated at
312 37°C for 5 minutes. The two mixes were combined together and one nanolitre was injected into
313 incrosses between pre-designated Tu wild-type animals. Mosaic GFP fluorescence was observed
314 in several F0 embryos which were separated and grown to adulthood. Adult F0 animals were
315 outcrossed to wildtype Tu fish and screened for GFP+ fluorescence. A founder transmitting 36%

of GFP⁺ embryos was identified, and F1 embryos were screened by PCR using the following primers, F: CCGCGCTCTCCAGTCCGTCA, R: CGAGAGCTGCGTGAGTGTGAA, to ensure precise editing of the *vangl2* locus.

Line Establishment and Maintenance

The F1 fish were incrossed to produce homozygous F2 fish. Embryos were screened either visually by GFP fluorescence or genotyped using the primers listed above.

Transgenesis

Transgenesis was performed using standard Gateway-compatible procedures⁴⁷. The zGrad open reading frame was amplified from pCS2(+)-zGrad (a kind gift from H. Knaut; Addgene #119716) and cloned into pDONR-P2rP3 to generate p3E-zGrad. To generate Tg(*βactin2::loxP mCherry STOP loxP zGrad*) zebrafish, p5E-*βactin2*⁴⁷, pME-loxP mCherry STOP loxP (a gift from K. Kwan), and p3E-zGrad were recombined into pDEST Tol2 pA2 transgenesis vector. Embryos were injected at the one-cell stage with 25 pg of assembled transgene and 25 pg of Tol2 mRNA. Embryos were sorted at 48 hpf for mCherry reporter expression and were subsequently grown to adulthood. Individuals were bred to TU wild-type zebrafish to generate stable F1 lines. Clones were fully verified by Sanger sequencing.

Plasmids and embryo microinjections

Rab5c, 7 and 11a plasmids were a kind gift from B. Link, mCherry-LifeAct-7 was a kind gift from M. Davidson (Addgene #54491), pCS2(+)-zGrad was a gift from H. Knaut (Addgene #119716)²³, pME-mTagBFP-CAAX was a gift from N. Cole (Addgene # 75149)⁴⁸, zebrafish

codon-optimized mScarlet was a kind gift from T. Thiele, centrin-mCherry was a kind gift from A. Salic and GalT-RFP was a kind gift from J. Wallingford. N-terminal mCherry-linker was cloned into the Rab plasmids and mCherry-LifeAct and mTagBFP-CAAX were cloned into pCS2+ using Gateway technology (Thermo Fisher Scientific). The plasmids were linearized and sense-strand-capped mRNA was synthesized with the mMESSAGE mMACHINE system (Ambion). 15 pg membrane-localized monomeric RFP (mRFP), 20 pg mTagBFP-CAAX, 30 pg mCherry-LifeAct, 5-25 pg mCherry-Centrin, 2.5 pg Arl13b-mScarlet, 10 pg mCherry-Rab5c, 10 pg mCherry-Rab7, 15 pg mCherry-Rab11a, 10 pg GalT-RFP and 50-100 pg zGrad were injected at one-cell stage.

Cell transplantations

Cell transplantations were performed at mid-blastula stages, as described previously⁴⁹. For the early somite stage imaging, embryos were grown at 25°C after transplantations.

Imaging

Brightfield embryonic imaging was performed on Leica M80 microscope with a MC170 HD camera (Leica). Wide-field fluorescence imaging was performed on a Zeiss AXIO Zoom V16 microscope (EMS3/SyCoP3, Zeiss) and ORCA-Flash 4.0 C1140-22C camera (Hamamatsu). Confocal imaging was performed on Leica TCS SP8 Lightning confocal with Leica DMI8 microscope, 40x/1.3 water immersion objective and HyD detectors using the 405, 488 and 552 laser lines (Leica). The images were deconvolved using the Lightning module. All time-lapse imaging was performed using a resonant scanner with 16 times averaging. Maximum intensity projections and time lapse images were recorded using Imaris (Bitplane).

Measurements of embryonic lengths and L/W ratio

Embryo lengths were measured by manually drawing a line from the front of the head to the tip of the tail in ImageJ. Embryonic length/width ratios were calculated by measuring the widest and longest points of *krox20* and *myoD* expression. Statistical testing was performed using GraphPad Prism (GraphPad Software).

Quantification of asymmetric Vangl2 membrane-localization

Comparison of Vangl2 and mRFP membrane enrichment was performed using the spot function in Imaris. In short, membrane-signals for mRFP or sfGFP were decorated with spots, and the spots for the two channels were merged. A reference point was placed in the centre of each cell to determine the anterior-posterior (X) and apical-basal (Y) axis. The position of each spot was determined as their distance to the reference point. Mean fluorescence intensity for mRFP and sfGFP within each spot was calculated, and the 10% of brightest spots for each fluorophore were plotted against their normalized X and Y positions using GraphPad Prism (GraphPad Software). Statistical testing was performed using GraphPad Prism (GraphPad Software).

BB positioning quantification

Basal body position was defined as a ratio between the distance of the basal body from the posterior membrane and the anterior-posterior floor plate cell length normalized to 1. Measurements were done in Imaris and statistical testing was performed using GraphPad Prism (GraphPad Software). The values were divided into three categories: posterior (0.66-1), medial (0.33-0.66) and anterior (0-0.33).

Whole-mount in situ hybridization

Antisense RNA probes were prepared using DIG RNA labelling kit (Roche) from linearized DNA. Whole mount RNA *in situ* hybridizations were performed according to Rose et al.⁵⁰. Samples were genotyped after imaging.

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Author Contributions

M.J. and C.W.B. performed Vangl2 targeting, M.J. performed all Vangl2 knock-in line validation and Vangl2 imaging experiments and analysis, C.W.B. generated the zGrad transgene, B.C. supervised research studies, M.J. and B.C. wrote the manuscript with input from C.W.B.

Declaration of Interests

The authors declare no competing interests.

Supplementary Movie Titles

Supplementary Movie 1. Vangl2 enrichment on anterior membranes is dynamic over time

Supplementary Movie 2. Vangl2 enrichment on the anterior membrane does not colocalize with filamentous actin

Supplementary Movie 3. Vangl2 localizes to anterior apical membranes and to basal membrane extensions in dividing cells

Supplementary Movie 4. Vangl2 anterior polarization is lost during cell division

Figure 1. Jussila et al.

Figure 1. sfGFP-Vangl2 is functional and membrane localized during early zebrafish development

(a) A schematic illustration of the sfGFP-Vangl2 knock-in targeting strategy.

(b) Wide-field fluorescence images demonstrating sfGFP-Vangl2 expression in *vangl2*^{sfGFP/+} and *vangl2*^{sfGFP/sfGFP} embryos at 28 hours post fertilization (hpf).

(c) Lateral view of wild-type, *vangl2*^{sfGFP/sfGFP}, *vangl2*^{sfGFP/m209} and *vangl2*^{m209/m209} embryos at 24 hpf.

(d) Embryo length quantification at 24 hpf. Data shows representative experiments combined from independent crosses that were repeated 2-4 times. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test (**** P <0.0001). Wild-type $n=14$, *vangl2*^{sfGFP/+} $n=22$, *vangl2*^{sfGFP/sfGFP} $n=20$, *vangl2*^{m209/+} $n=82$, *vangl2*^{sfGFP/m209} $n=72$, *vangl2*^{m209} $n=63$.

(e) Live confocal images of *vangl2*^{sfGFP/sfGFP} (hereafter referred as *vangl2*^{sfGFP}) embryos at blastula through early gastrula stages, as indicated. Ectodermal cells were imaged in gastrulating embryos. Embryos were injected with mRNA coding for a membrane-localized monomeric RFP (mRFP) reporter. All sfGFP images were acquired using identical settings.

(f-i) Live confocal images of shield staged *vangl2*^{sfGFP} embryos injected with mRNA coding for mCherry-Rab5c (f), mCherry-Rab7 (g), mCherry-Rab11a (h) or GalT-RFP (i) reporter constructs. Arrows point at cytoplasmic Vangl2 puncta in close proximity to respective reporters, and arrowheads point at isolated Vangl2 puncta.

Figure 2. Jussila et al.

Figure 2. Planar polarized enrichment of Vangl2 on anterior neuroepithelial membranes is controlled by cell non-autonomous PCP signalling

- (a) Confocal images comparing sfGFP-Vangl2 and mRFP localization at 22 hours post fertilization (hpf) in dorsal (top) and lateral (bottom) orientations. Anterior is to the left in all images. Strong Vangl2 expression is observed on cell membranes in the neural tube (NT) including the floor plate (FP), while only weak expression is observed in somites (S) and notochord (NC).
- (b) A schematic illustrating transplantation of mRFP-labelled *vangl2^{sfGFP}* donor cells into WT (wild-type) host embryos, at sphere stage. Chimeric embryos were imaged dorsally, at positions between the 2nd and 8th somite.
- (c) Confocal images of mRFP and sfGFP-Vangl2 localization in the developing spinal cord of chimeric embryos at 4-somite, 10-somite and 13-somite stages of development. Maximum intensity projections are shown. Arrows point to Vangl2 enrichment on anterior neuroepithelial cell membranes, and arrowheads to anterior apical membranes at the neural midline.
- (d-e) Distribution of the brightest sfGFP-Vangl2 and membrane-RFP spots along anterior-posterior (d) and apical-basal (e) axes of *vangl2^{sfGFP}* neuroepithelial cells, quantified at four consecutive stages of neural tube morphogenesis. Data from multiple transplanted *vangl2^{sfGFP}* cells was pooled (3-5 somites, n = 7; 7 somites, n = 7; 8-10 somites, n = 6; 13 somites, n = 5) and axial lengths normalized to 1. Details of the quantification can be found in the Methods section. Statistical analysis was performed using a two-tailed Mann-Whitney test.
- (f) A schematic illustrating transplantation of mRFP-labelled *vangl2^{sfGFP}* donor cells into *vangl2^{tk50f}* loss-of-function host embryos, at sphere stage.
- (g-h) Distribution of the brightest sfGFP-Vangl2 and membrane-RFP spots along anterior-posterior (g) and apical-basal (h) axes of *vangl2^{sfGFP}* neuroepithelial cells transplanted into a *vangl2^{tk50f}* mutant host, quantified at two consecutive stages of neural tube morphogenesis. Data from multiple transplanted *vangl2^{sfGFP}* cells was pooled (10 somites, n = 7; 12-13 somites, n = 9).
- (i) Confocal images of mRFP and sfGFP-Vangl2 localization in *vangl2^{sfGFP}* neuroepithelial cells within *vangl2^{tk50f}* mutant hosts, at 10-somite and 13-somite stages of development. Maximum intensity projections are shown. Arrows point at Vangl2 enrichment on cell protrusions.

Figure 3. Jussila et al.

Figure 3. Vangl2-positive anterior membrane closely associates with planar polarized basal bodies of floor plate cells

(a) Confocal images of sfGFP-Vangl2 localization in relation to basal body (Centrin-mCherry) and membrane (mTagBFP) reporters within floorplate cells of a *vangl2^{sfGFP}* embryo at 22hpf. Lateral view, anterior to the left.

(b) Confocal images of sfGFP-Vangl2 localization in relation to cilia axoneme (Arl13b-mScarlet) and membrane (mTagBFP) reporters within floorplate cells of a *vangl2^{sfGFP}* embryo at 32hpf. Lateral view, anterior to the left.

(c) Confocal image comparing sfGFP-Vangl2 and mRFP localization in floorplate cells of a 23-somite staged chimeric embryo (*mRFP* mRNA-injected *vangl2^{sfGFP}* cells transplanted into WT hosts). Maximum intensity projections, dorsal (top) and lateral (bottom) views are shown, anterior to the left. Arrows point at Vangl2 enrichment on anterior cell membranes. Arrowhead points at Vangl2 enriched membrane protruding from the anterior apical surface.

(d) Confocal image comparing sfGFP-Vangl2 and Centrin-mCherry localization in floorplate cells of a 15-somite staged chimeric embryo (*Centrin-mCherry* mRNA-injected *vangl2^{sfGFP}* cells transplanted into WT hosts). Maximum intensity projections, dorsal (top) and lateral (bottom) views are shown, anterior to the left. Arrows point at Vangl2 on the anterior membrane that is closely associated with a basal body docked on the posterior membrane. Asterisk indicates anterior unlabelled host cell that is not Centrin-mCherry positive.

(e) Confocal image comparing sfGFP-Vangl2 localization in relation to basal body (Centrin-mCherry) and membrane (mTagBFP) reporters in neighbouring floorplate cells of a 15-somite staged chimeric embryo (*vangl2^{sfGFP}* cells transplanted into WT hosts injected with *Centrin-mCherry* and *mTagBFP* mRNA). Maximum intensity projections, dorsal (top) and lateral (bottom) views are shown, anterior to the left. Arrow points at Vangl2 on the anterior membrane of a donor cell that is closely associated with a basal body docked on the posterior membrane of a host cell.

Figure 4. *Jussila et al.*

Figure 4. Conditional degradation of sfGFP-Vangl2 reveals a cell non-autonomous requirement for Vangl2 in maintaining basal body polarity

(a) Quantification of embryo lengths for *zGrad* mRNA-injected and control embryos at 24 hpf. Maternal and zygotic genotypes are as indicated. Data is pooled from three independent experiments. Control n from left to right: n=53, n=62, n=61, n=36. *zGrad* n from left to right: n=55, n=51, n=53, n=54. Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparisons test.

(b) Confocal images of sfGFP-Vangl2 expression in relation to basal body (Centrin-mCherry) and membrane (mTagBFP) reporters within floorplate cells of control *vangl2^{sfGFP};Tg(loxP-mCherry-STOP-loxP-zGrad)* embryos (left) and experimental *vangl2^{sfGFP};Tg(loxP-mCherry-STOP-loxP-zGrad);Tg(foxj1a-iCre)* embryos (right) at 48 hpf. Dorsal view, anterior to the left. Arrows indicate residual sfGFP-Vangl2 expression in a subset of floorplate cells following *zGrad* expression. Asterisks indicate mispolarized basal bodies following *zGrad* expression.

(c) Quantification of basal body localization within the posterior, medial or anterior thirds of floor plate cells for control and *vangl2^{sfGFP};Tg(loxP-mCherry-STOP-loxP-zGrad);Tg(foxj1a-cre)* embryos described in (b). Cells were categorized as A-D based on the presence or absence of sfGFP-Vangl2 signal within both the cell and its immediate neighbours, as illustrated in (d). Control n=164, A n=256, B n=20, C n=29, D n=19.

(d) A schematic illustrating cell categories quantified in (c). Grey colour indicates cells that have lost sfGFP-Vangl2 signal; green colour indicates residual sfGFP-Vangl2 expression.

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