Vangl2 promotes the formation of long cytonemes to enable distant Wnt/β-catenin signaling

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Wnt signaling regulates cell proliferation and cell differentiation as well as migration and polarity during development. However, it is still unclear how the Wnt ligand distribution is precisely controlled to fulfill these functions. Here, we show that the planar cell polarity protein Vangl2 regulates the distribution of Wnt by cytonemes. In zebrafish epiblast cells, mouse intestinal telocytes and human gastric cancer cells, Vangl2 activation generates extremely long cytonemes, which branch and deliver Wnt protein to multiple cells. The Vangl2-activated cytonemes increase Wnt/β-catenin signaling in the surrounding cells. Concordantly, Vangl2 inhibition causes fewer and shorter cytonemes to be formed and reduces paracrine Wnt/β-catenin signaling. A mathematical model simulating these Vangl2 functions on cytonemes in zebrafish gastrulation predicts a shift of the signaling gradient, altered tissue patterning, and a loss of tissue domain sharpness. We confirmed these predictions during anteroposterior patterning in the zebrafish neural plate. In summary, we demonstrate that Vangl2 is fundamental to paracrine Wnt/β-catenin signaling by controlling cytoneme behaviour.
Long-distance cell-cell communication is essential for development and function of multicellular organisms. During embryogenesis, shape-forming signals, called morphogens, are produced at a localized source and act both locally and at a distance to control morphogenesis. In a concentration-dependent manner, morphogens orchestrate the cellular fates in their signaling range by controlling the gene expression of key transcription factors. A tightly regulated distribution of morphogens is a prerequisite to allowing their precise spatial and temporal function during embryogenesis in tissue patterning and organ development.

Morphogens of the Wnt signaling family are a class of secreted ligands, that can transduce their signals through several distinct pathways to regulate a diverse array of developmental processes. The best-characterized Wnt pathway is the Wnt/β-catenin dependent signaling pathway. Wnt ligands together with Frizzled receptors and the co-receptors Lrp5/6 stabilize the key downstream target β-catenin. The co-transcription factor β-catenin, together with TCF/LEF transcription factors, mediates many cellular processes such as cell differentiation and proliferation and determines tissue patterning along the anteroposterior (AP) body axis. The planar cell polarity (PCP) signaling pathway is a β-catenin-independent pathway in the Wnt signaling network. The Wnt/PCP pathway regulates cytoskeleton remodeling by activation of c-Jun N-terminal kinases (JNK) and members of the Rho-family GTPases, such as Cdc42 and RhoA to direct cellular morphogenesis, tissue polarity, and cell migration. Within a cell, β-catenin-dependent and PCP-dependent Wnt signaling are well known to act in a mutually repressive manner and inhibiting one will typically upregulate the other.

In vertebrates, the transduction of Wnt signaling pathways begins when a Wnt ligand binds to its receptors at the cell membrane. However, the question of how a Wnt moves from a producing cell to fulfill its paracrine function in a tissue remains highly debated. The transport of signal components such as ligands and receptors can be facilitated by signaling filopodia known as cytonemes. Indeed, recent high-resolution imaging experiments in zebrafish demonstrated that specialized cytonemes are fundamental in Wnt trafficking in vertebrates. The regulated generation and function of cytonemes is critical as it impacts directly on the signaling range and signaling gradient. Specifically, the number and length of cytonemes generated by a Wnt source cell influence events, such as zebrafish neural plate patterning during embryogenesis. However, the molecular mechanism regulating these attributes during zebrafish gastrulation to allow neural plate patterning is still unclear.

Unlike in flies, Wnt-dependent activation of the receptor-tyrosine kinase-like orphan receptor 2 (Ror2) is thought to act as a crucial receptor of the Wnt/PCP pathway, and in turn drives de novo biogenesis of Wnt8a-positive cytonemes. The subsequent formation of cytonemes is influenced by activation of cytoskeletal regulators, such as the small Rho GTPase Cdc42, which controls actin polymerization. Ror2 is thought to function by forming a protein complex with other PCP regulatory proteins at the plasma membrane, at a sub-membrane region, or at cell–cell junctions. This PCP core complex can include the key component, the four-pass transmembrane protein, Van-Gogh-like (Vangl) 1/2 in vertebrates. As Vangl2 lacks any known receptor or enzymatic activity, protein–protein interaction domains of Vangl2 are likely to modulate downstream signaling. In mouse, Wnt ligands bind to both Ror2, which recruit CK1δ/ε through Dvl. Subsequently, CK1δ/ε activates Vangl2 by phosphorylation, similarly CK1 phosphorylates Vangl2/strabismus in Drosophila. Supporting a functional role for Vangl2, its knockdown inhibits axon outgrowth by inhibition of filopodia formation. It has been suggested that this downstream signaling pathway is triggered in a Wnt concentration-dependent manner in mouse limb development. During zebrafish gastrulation, Vangl2 is asymetrically localized at the plasma membrane and localizes to forming protrusive membranes. It is currently unclear if the Vangl2-positive filopodia perform signaling functions in tissue organization.

Here we show that Vangl2—together with Wnt8a and Ror2—is loaded on cytoneme tips. Vangl2 positive cytoneme tip complex activates JNK signaling to increase cytoneme length and the number of cytoneme contacts. We find that Vangl2 function during cytoneme emergence is vital for paracrine Wnt/β-catenin signaling in both human tissue culture and zebrafish embryo. Concordantly, blockage of Vangl2 function or JNK signaling leads to quickly collapsing signaling filopodia. Consequently, impairment leads to a reduction of both Wnt dissemination and paracrine Wnt signaling in human cancer tissue culture, the zebrafish embryo and the mouse intestinal crypt. Based on our findings, we developed a mathematical model of how changes in cytoneme length and contacts would affect embryogenesis. This mathematical model of morphogen distribution in the zebrafish gastrula predicts that increased Wnt cytoneme length and number of contacts leads to extended signaling range, and to altered patterning with fuzzy compartment boundaries. We confirm these predictions in vivo during zebrafish neural plate patterning. These findings suggest that the activity of the Vangl2-positive PCP complex determines the emergence of Wnt-positive cytonemes during development and tissue homeostasis in vertebrates.

### Results

**Vangl2 together with Wnt8a and Ror2 form the cytoneme tip complex.** The PCP signaling component Ror2 is an essential regulator of Wnt8a-positive cytoneme emergence. We investigated the interconnected role of additional PCP family members on cytoneme regulation. The four-transmembrane PCP protein Vangl2 is a PCP core member that is localized at the tips of filopodia extending from both neurons, as well as in forming membrane protrusions, such as gastrula cells during zebrafish gastrulation. Upon Wnt activation, Ror2 recruits CK1δ/ε, which phosphorylates Vangl2 to activate downstream signaling, such as c-Jun N-terminal kinase (JNK) signaling.

To analyse the localization and function of PCP components together with Wnt8a, we first sought to establish an in vitro test system to monitor cytoneme behavior using zebrafish PAC2 fibroblast cells in culture. First, we asked if PAC2 cells transported endogenous Wnt8a by filopodial protrusions. We found that these fibroblasts have numerous, dynamic filopodial protrusions and that endogenous Wnt8a can be detected on these filopodia, similar to over-expressed fluorescently-tagged Wnt8a (Fig. 1A, B and Supplementary Fig. 1A, B). Therefore, we define filopodial protrusions as Wnt8a-bearing cytonemes when they co-localize with fluorescently-tagged Wnt8a along the filopodium or at the filopodium tip (Fig. 1A, B). Next, we asked if filopodia can also be decorated with Van-Gogh protein. As there is no suitable antibody for immunohistochemistry against zebrafish Vangl2 available, we stained the Wnt signaling active gastric cancer cells AGS with a human anti-Vangl2 antibody and, indeed, could find that endogenous hVangl2 is also localized to the tip of filopodia of AGS cells (Supplementary data Fig. 1C). We measured fluorescent intensity along the PAC2 cytonemes (Fig. 1G) and found that glycosphatidylinositol (GPI)-anchored, membrane-bound mCherry (mem-mCherry) was evenly distributed along the PAC2 cytonemes (Fig.1G) and found that endogenous Wnt8a can be detected on these protrusions as Wnt8a-bearing cytonemes when they co-localized for immunohistochemistry against zebrafish Vangl2 available, we stained the Wnt signaling active gastric cancer cells AGS with a human anti-Vangl2 antibody and, indeed, could find that endogenous hVangl2 is also localized to the tip of filopodia of AGS cells (Supplementary data Fig. 1C). We measured fluorescent intensity along the PAC2 cytonemes (Fig. 1G) and found that glycosphatidylinositol (GPI)-anchored, membrane-bound mCherry (mem-mCherry) was evenly distributed along the length of cytonemes from tip to base, whilst Wnt8a-GFP was
cytonemes (Fig. 1D, J), whereas GFP-Vangl2 can be also seen at the tip (Fig. 1K). We used an N-terminal deletion mutant lacking the phosphorylation sites, ΔN-Vangl2, in which 10 Ser/Thr are replaced by Ala (Vangl210A) to block Vangl2-mediated signaling21. Ectopic expression of the Van-gl2ΔN and Vangl210A to a reduction in the number of cytonemes and an increase in the number of short cytonemes (Fig. 2B). While in control cells only 23% of cytonemes were >10 µm, in the Vangl2-overexpressing cells 68.9% were >10 µm, and 43.8% were >20 µm (Fig. 2M). Ror2/Vangl2 double-transfected cells had a similar and significant increase in Wnt8a positive cytonemes length (Fig. 2D, Supplementary Figs. 3 and 4). We found that 23% of the observed cytonemes were 10 µm or longer (Fig. 2M). Activation of Vangl2 caused the average cytoneme length to increase significantly by 187.3% (Fig. 2C, L, M and Supplementary Fig. 2B). While in control cells only 23% of cytonemes were >10 µm, in the Vangl2-overexpressing cells 68.9% were >10 µm, and 43.8% were >20 µm (Fig. 2M). Ror2/Vangl2 double-transfected cells had a similar and significant increase in Wnt8a positive cytonemes length (Fig. 2D, L and Supplementary Fig. 2B), and in addition displayed more branching (Fig. 2D). Vangl2 lacking the phosphorylation sites exhibit a dominant negative effect on signaling21. So we used an N-terminal deletion mutant of Vangl2 (ΔN-Vangl2) lacking the two Ser/Thr phosphorylation clusters and the all-phospho Vangl2 mutant S5Δ76 (Vangl2) to block Vangl2-mediated signaling21. Ectopic expression of the Vangl210A led to a significant reduction in the number of cytonemes and ΔN-Vangl2 and Vangl210A to a reduction in the number of long cytonemes, and an increase in the number of short cytonemes (Fig. 2E, G, K and Supplementary Fig. 2A, B). Vangl210A also showed significant reduction in all filopodia numbers predominantly localized to the tip of the cytoneme (Fig. 1B, H). Next, we transfected plasmids encoding Ror2-mCherry and GFP-Vangl2 to investigate the localization of these PCP proteins in the PAC2 cells. Ectopically expressed Ror2-mCherry was membrane localized (Fig. 1C) with a slight increase in fluorescence of Wnt8a intensity at the tip (Fig. 1L). GFP-Vangl2 and Ror2-mCherry expression was membrane localized and present all along the cytonemes (Fig. 1D, J), whereas GFP-Vangl2 can also be seen at the filopodia tip of PAC2 cells (Supplementary Fig. 1D). GFP-Vangl2 accumulated dynamically together with Wnt8a at cytoneme tips (Fig. 1E) with an increase in fluorescence intensity at the tip (Fig. 1K). We find that in the presence of over-expressed untagged Vangl2, Ror2-mCherry accumulated more strongly at the cytoneme tip (Fig. 1F, L). To further characterize the interactions between Wnt8a, Vangl2, and Ror2, we used fluorescence cross-correlation spectroscopy (FCCS) to determine the formation of a receptor complex17. We performed an FCCS analysis at the plasma membrane of PAC2 cells expressing GFP-Vangl2 and Ror2-mCherry (Fig. 1M). We found the cross-correlation of Vangl2 and Ror2-mCherry if treated with Wnt5a compared to the untreated control in accordance with previous published biochemical data in mouse20,23 (Fig. 1N). Similarly, we observed cross-correlation of Vangl2 and Ror2, when PAC2 cells were treated with Wnt8a protein (Fig. 1O). We conclude that Wnt8a can induce the formation of a Ror2/Vangl2 complex. This shows that Wnt8a induces a Ror2/Vangl2 complex at the tip of the cytoneme, suggesting a potential role in emergence and function of Wnt cytoneme.
**Fig. 2 Vangl2 controls the emergence of Wnt8a cytonemes in fibroblasts.** A–J Wnt8a-positive PAC2 zebrafish fibroblasts transfected with indicated constructs, imaged and analysed live 24 h post-transfection. Yellow arrows indicate examples of Wnt8a positive cytonemes. Scale bar = 10 µm.

K Number of Wnt8a positive cytonemes per cell (n = number). (n per condition = 25, 9, 14, 6, 25, 14, 31, 36, 13 cells).

L Length of Wnt8a positive cytonemes in PAC2 cells (µm). (n per condition = 139, 52, 32, 21, 131, 65, 51, 51, 11 cytonemes).

M Breakdown of the percentage of Wnt8a positive cytoneme lengths into 0–5, 5–10, 10–15, 20+ µm categories. Graphs represent mean and standard error of the mean. K, L Two-sided Kruskal–Wallis tests with Bonferroni correction for multiple tests. Statistical significance: p ≤ 0.05. SEM = 1. Corresponding dot plots are shown in Supplementary Fig. 2, and analysis of PAC2 filopodia is shown in Supplementary Figs. 3 and 4. Source data are provided as a Source Data file.
and ΔN-Vangl2 and Vangl210A showed reduction in all filopodia length compared to control (Supplementary Figs. 3 and 4). Co-expression of Ror2 did not reverse the phenotype (Fig. 2F, H, K, L, M), suggesting that Vangl2 functions downstream of Ror2.

The I-BAR protein insulin receptor tyrosine kinase substrate p53 (IRSp53) plays an essential role in filopodia formation, and connects filopodia initiation and maintenance by assembling the actin scaffold. IRSp53 is localized to the tips of filopodia and overexpression leads to the de novo formation of filopodia. IRSp53 has four lysine residues in the actin-binding sites mutated to glutamic acid, which strongly reduce filopodia formation. Overexpression of IRSp53 reduced the number and decreased the length of cytonemes (Fig. 2I), and Vangl2 activation could not compensate for the loss of IRSp53 function (Fig. 2J–M and Supplementary Figs. 2 and 3J, K), suggesting that Vangl2-mediated induction of cytonemes operates upstream of the filopodia nucleation machinery.

Vangl2 controls the length of Wnt8a cytonemes during zebrafish gastrulation. We next investigated the role of Vangl2 in cytoneme regulation in vivo in zebrafish embryos. First, we asked if Vangl2 can be localized to filopodia of embryonic cells during zebrafish gastrulation. To do this, we analysed the localization of Vangl2 using a transgenic line expressing a zebrafish GFP-Vangl2 N-terminal fusion protein under the control of the Vangl2 promoter. In Tg(vangl2:GFP-Vangl2) embryos injected with mRNA of mem-mCherry, we found that epiblast cells have dynamic filopodial protrusions and that GFP-Vangl2 can be detected on these filopodia with an accumulation at the tips (Fig. 3A, A’ and Supplementary movie 1A, 1B). Overexpression of Ror2 leads to the formation of more cytonemes in vivo and Vangl2 is similarly localized to the cytonemes of Ror2-positive cells (Fig. 3B, B’ and Supplementary movie 2A, 2B). Next, we generated clones of cells in intact zebrafish embryos by micro-injecting mRNA of mem-mCherry and wnt8a-GFP, together with either vangl2, ΔN-vangl2, the all-phospho mutant vangl210A, and IRSp53K mRNA at the 8–16-cell stage. At 5hpf (50% epiboly), we imaged individual clones of cells in the zebrafish gastrula (Fig. 3C–G). In particular, we focused on visualization of Wnt8a-positive cytonemes within the embryo. We found that, like what occurred in cultured PAC2 cells, expression of vangl2 mRNA led to significantly longer cytonemes per cell compared to control (Fig. 3C, D, H, I and Supplementary Fig. 5A, B). The average length of cytonemes significantly increased by 39.6% (Fig. 3I, J). The cytonemes in Vangl2-expressing embryonic cells were likewise found to branch abnormally, form multiple contact points and extend over larger areas between cells compared to control (Fig. 3D, Supplementary Fig. 5C, and Supplementary movie 3, 4). Expression of ΔN-vangl2 or vangl210A did not significantly change the length of cytonemes compared to control however, it significantly reduced the number (Fig. 3E, F, H, I and Supplementary movie 5), suggesting that the observed lengthening and cytoneme induction requires Vangl2 function. Next, we used the IRSp53K mutant to block filopodia formation. Co-expression of IRSp53K and vangl2 led to a significant reduction of filopodia including Wnt8a-positive cytonemes, and at the same time cells start to form more tubular structures (Fig. 3G)—similar to our observation in PAC2 fibroblasts (Fig. 2I, J and Supplementary movie 6).

Next, we asked if the increased average length of cytonemes upon Vangl2 activation leads also to an increase of contact sites. Therefore, we quantified the number of contact sites of one cytoneme on the receiving cells, and found that a cytoneme on average contacts the neighboring cell only once (Fig. 3K–M and Supplementary movie 3). However, we found that this number doubles in Vangl2 expressing cells, and in some cases, we counted over five contact sites on various cells by a single cytoneme (Fig. 3K–M and Supplementary Movie 4). This effect could not be observed after overexpression of ΔN-vangl2 or the vangl210A mutant (Fig. 3M, Supplementary movie 5). Furthermore, the effect of Vangl2 overexpression can be reversed by blockade filopodia by reducing IRSp53 function (Fig. 3M). Therefore, we conclude that Vangl2 is necessary but not sufficient for cytoneme induction, and it regulates both Wnt8a cytoneme length and cell contact sites—both in vitro and in vivo.

Vangl2-mediated JNK activation is required to stabilize cytonemes. We next wanted to investigate the mechanisms by which Vangl2 regulates cytoneme stability. Wnt/PCP can transduce signals through the receptors Frizzled and Ror2 to Rac-JNK and RhoA-ROCK signaling cascades in a context-dependent manner. Apart from its nuclear functions, JNK also directly regulates the cytoskeleton by phosphorylation of diverse cytoplasmic targets including proteins directly interacting with the actin cytoskeleton, as well as microtubule-associated proteins. Similarly, the RhoA-ROCK signaling cascade can induce actin cytoskeletal reorganization and cell movement. In addition, there is crosstalk between these pathways; RhoA can activate JNK during convergent extension movement in Xenopus, and loss of RhoA can be rescued by over-expression of JNK.

We recently reported results from a cell-culture-based screen to identify kinases that regulate cytoneme formation. In this screen, in addition to Ror2, we identified several key family members of the JNK signaling pathway including MKK4 and JNK3 as positive regulators of Wnt8a cytoneme length. After receiving external signals, MAP kinase kinases (MKK) phosphorylate and activate c-Jun N-terminal kinases (JNK). In turn, the JNKs phosphorylate a number of transcription factors, primarily components of AP-1. In our screen, forced expression of MKK4 led to an increase in the average length of cytonemes by 36.4%, whereas JNK3 expression resulted in an increase of 19.7%. To further probe the involvement of JNK signaling, we carried out a reporter assay in HEK293T cells, which express a very low level of endogenous Wnt ligands and a defined set of Fzd receptors. In cells with low JNK activity, the KTR-mCherry reporter is localized to the nucleus (Supplementary Fig. 6A). However, upon activation of JNK signaling, phosphorylation of the JNK-KTR-mCherry reporter causes it to translocate to the cytoplasm. Control HEK293T cells have low JNK activity, shown by nuclear localization of KTR-mCherry (Fig. 4A, B). Transfection of Ror2, Vangl2 or a combination of both did not significantly change the ratio of cytoplasmic to nuclear signal (Fig. 4A, B). Remarkably, addition of either Wnt5a or Wnt8a protein with Ror2 and Vangl2 led to a significant increase in JNK activity in Ror2/Vangl2 expressing cells, as seen by movement of KTR-mCherry from nucleus to cytoplasm (Fig. 4A, B). This suggests that Wnt protein with Ror2 and Vangl2 initiates JNK signaling in HEK293T cells. Wnt8a did not activate JNK signaling in cells transfected with ΔN-Vangl2 (Fig. 4B). This indicates that Vangl2 is a key downstream element in Ror2/Wnt activated JNK signaling in HEK293T cells.

We observed that Wnt8a cytonemes form and retract within some tens of minutes (Supplementary Movie 7). To assess the role of JNK signaling in this process, we treated Wnt8a-GFP/mem-mCherry transfected PAC2 cells with a small molecule inhibitor of JNK kinase activity. In vertebrates SP600125 specifically inhibits all three JNKs (JNK1–3) within minutes without inhibition of ERK1 or ERK2, phospho-p38, or ATF2. We then recorded and analysed the effect of JNK.
blockage on protrusion length and number (Fig. 4C–E). We found that Wnt cytonemes collapse and retracted following the addition of SP600125 (Fig. 4C), whereas cytonemes in DMSO-treated PAC2 cells are unchanged. A time course revealed that over the course of 120 min, control cells showed no significant change in the number or length of signaling filopodia (Fig. 4D, E and Supplementary Fig. 6B). However, JNK inhibition caused a significant reduction in average relative protrusion length and...
number after 1 h. Vangl2-expressing cells, which had longer protrusions (as in Fig. 2L) prior to the addition of SP600125, also had a significant change in the number and length of cytonemes compared to untreated Vangl2-expressing fibroblasts. A detailed analysis of cytoneme lengths showed a specific loss of extremely long signaling filopodia as a result of JNK inhibition (Supplementary Fig. 6B). We also tested the involvement of RhoA/ROCK signaling as it can be similarly activated downstream of PCP signaling to regulate cytoskeletal re-arrangement. To do so, we treated cytoneme-bearing cells with Y-27632, an antagonist of Rho-associated kinase (RhoA/ROCK)\(^{31}\). While JNK inhibition had a significant effect at 60 min, Y-27632 treatment produced only a modest and non-significant reduction of protrusion length after 5 h (Supplementary Fig. 6C–G). We conclude that JNK signaling is the primary signaling cascade required for fast cytoneme formation downstream of Wnt/Vangl2/Ror2 signaling and it contributes to the formation of long Vangl2-positive cytonemes.
Vangl2-controlled cytonemes regulate Wnt/β-catenin signaling in neighboring cells. Next, we investigated the effect of Vangl2 on cytoneme-mediated Wnt protein delivery by investigating Wnt/β-catenin signal activation in the receiving cells (Fig. 5). To measure paracrine signal activation, we used a sensitive reporter system of AGS gastric cancer cells—which are primed for Wnt/β-catenin signaling—transiently transfected with a Wnt/β-catenin reporter with seven TCF-responsive elements driving expression of nuclear mCherry (7xTCF-nls-mCherry)42. AGS cells have also been shown to exhibit Ror2-dependent Wnt8a cytonemes17. We co-cultivated AGS cells transiently expressing combinations of Wnt8a, Vangl2, Ror2, and ΔN-Vangl2 together with the STF-mCherry reporter cells (Fig. 5A, B). The expression of nuclear mCherry significantly increased in the receiving cells when Wnt8a was expressed in the source cells (Fig. 5Bii, C). This suggests that Wnt8a can activate the Wnt/β-catenin pathway in a paracrine way. Expression of Vangl2 alone in the source cells did not induce a Wnt/β-catenin response in the receiving cells (Fig. 5Biii, C). However, Wnt8a/Vangl2 co-transfection, which we showed in zebrafish cells increased cytone- nate contact points and length, could significantly increase STF reporter activation in receiving cells in comparison to control and Wnt8a only (Fig. 5Biv, C). The significant increase of Wnt signal transmission to the responding cells is dependent on Vangl2, because co-transfection of Wnt8a/ΔN-Vangl2 showed no detectable effect on reporter activation compared to Wnt8a expressing source cells (Fig. 5Bv, C). Next, we tested if the Vangl2-dependent increase of paracrine Wnt/β-catenin signal activation depends on Ror2 function. We found that expression of Wnt8a/Ror2/Vangl2 in the producing cells further increased reporter activation in the receiving cells (Fig. 5Bvi, C), and this was abrogated by co-expression with ΔN-Vangl2 or kinase-dead Ror2 (Ror2K) (Fig. 5Bvii, Bviii, C). These data indicate that the activity of Wnt8a in source cells is markedly enhanced by two factors known to increase cytone number and length, consistent with the transmission of the Wnt8a signal by cytonemes. At this point, we could not exclude that formation of longer cytonemes and increased activation of the Wnt8a signal in neighboring cells by activation of Vangl2 in the source cells are two independent events, acting in parallel. Therefore, we blocked cytone formation in cells expressing Wnt8a and Vangl2 by co-expression of the mutant form of IRRSP53K (Fig. 5D, E). We find that blockage of filopodia formation in these cells reduces significantly the paracrine signaling activity by Vangl2/Wnt8a expressing cells. The signaling capacity of Wnt8a/Vangl2/IRSP53K is similar to the control cells (Fig. 5D, E), suggesting that the Vangl2-regulated Wnt8a transport requires mainly cytonemes.

Stem cells are highly dependent on extrinsic cues derived from their microenvironment; however, it is still unclear how signaling is controlled within such a niche environment. Wnt/β-catenin signals are an essential component of a wide range of stem-cell niches, including that of the gastrointestinal epithelium43. The intestinal stem-cell niche is regulated by both Wnts and RSPO3, which are supplied predominantly by stromal myofibroblasts44,45.

To test for the requirement for cytone-mediated Wnt transport in stem cell regulation, we investigated the influence of VANGL on WNT cytonemes in the mouse intestinal crypt. In particular, the stem cells at the bottom of intestinal crypt require constant Wnt/β-catenin signaling for tissue homeostasis16–19. In vivo, subepithelial myofibroblasts, described as PDGFRA+ telocytes, are the major source of physiologically relevant WNTs to maintain these intestinal crypts44,50,51. We have shown the ability of these myofibroblasts to provide essential WNTs is compromised when they lack cytonemes as a result of siRNA mediated ROR2 inhibition17. We used crypt organoids as an optimal system to test our hypothesis that telocytes also require VANGL to generate cytonemes to distribute WNT proteins in the mouse intestinal crypt. VANGL1 and VANGL2 expression in telocytes was reduced by siRNA-mediated knockdown (Supplementary Fig. 7A). The number of filopodia was significantly reduced after Vangl1 knockdown and even more markedly reduced after double knockdown of Vangl1/Vangl2 (Fig. 6A, B). In parallel, we found a reduction in filopodia length in all three knockdown experiments with the most significant reduction after double knockdown of Vangl1/Vangl2 (Fig. 6C). Next, we used an organoid formation assay to analyse the requirement for VANGL-dependent WNT cytonemes (Fig. 6D). Organoids of WNT-deficient Porcn−/− crypt cells need to be co-cultivated with WT Wnt-producing telocytes for maintenance. Telocytes interact with crypt organoids and supply them with signaling factors such as WNT proteins. Notably, we were not able to observe long filopodia in telocytes upon knockdown of Vangl1/Vangl2 or IRRSP53 (Fig. 6A–D). After simultaneous knockdown of Vangl1 and Vangl2 in the WNT-producing telocytes, we further observed a strong decrease in the number of organoids (Fig. 6E). In addition, knockdown of IRRSP53 led to a similar reduction in organoid counts (Fig. 6F). This suggests that the WNT cytonemes from the telocytes are required for the induction and maintenance of the intestinal crypt organoids and that VANGL1/ VANGL2 are crucial for their formation.

Simulation predicts an important role for Vangl2-controlled cytonemes in the zebrafish gastrula. On the basis of these findings, we hypothesized that Vangl2 function in the Wnt source cells is crucial for Wnt dissemination via cytonemes which we examined in silico. To quantitatively test the consequences of altered Vangl2 function on gradient formation and tissue patterning in the zebrafish neural plate, we created an agent-based simulation of morphogen distribution via cytonemes using the Chaste modeling software52,53. First, we generated a 2D model of the zebrafish gastrula based on the positional information of every cell during the first 10h of zebrafish gastrulation54,55, representing a portion of the overall gastrula. We defined the population of marginal cells as Wnt8a source cells and the overlying epiblast cells as Wnt-receiving neural plate cells (Fig. 7A, B). The simulation takes into account...
ligand transport by cytonemes, ligand decay and the migration and proliferation of epiblast cells using the agent-based simulation approach (Fig. 7C–F). We employed cytonemes as the exclusive transport mechanism from the producing marginal source cell group to the target cell group. We made the assumption that all cells are initially at the animal pole then migrate and intercalate to produce a thin tissue, which covers the yolk during the epiboly movement and Wnt transport process. We found that cytonemes can distribute Wnt8a in a graded manner in the dynamically evolving target tissue of the zebrafish embryo during gastrulation. Cells receiving a high concentration of Wnt8a acquire hindbrain fate, according to a pre-defined Wnt
threshold, whereas, cells receiving a lower concentration acquire forebrain/midbrain fate. We further find the formation of a stable boundary between midbrain and hindbrain (MHB) due to a sharp drop of the morphogen concentration across the boundary. Next, we tested two scenarios with varying ligand concentration, cytoneme length, and contact sites (to match experimentally observed properties), based on our in vivo measurements after alteration of Vangl2 function (Fig. 3). We found that increasing ligand concentration (in these simulations by a factor of 10) within the morphogenetic field leads to an anterior shift of MHB in comparison to the control situation (Fig. 7G, H). We found that increasing lengths of cytonemes per cell after expression of Vangl2 in the source cells (by the experimentally determined factor of 33%) leads to a slight anterior shift of the MHB (Fig. 7I). Remarkably, lengthening of cytonemes and increasing the number of contact sites and the ligand concentration—comparable to the co-expression of Vangl2 and Wnt8a (Fig. 3D)—led to a significant broadening of the hindbrain territory (Fig. 7J). Notably, we also found the MHB becomes less distinct, (Fig. 7B), whereby there are considerably more cells exhibiting a fate incongruous with that defined by their position relative to the computed boundary. Finally, we asked the question if the observed phenotype observed after increasing the length of Wnt8a positive cytonemes (Fig. 7J) can be rescued by reducing the capability of cells to form cytonemes. Therefore, we addressed the question of how reduction of the mean cytoneme number would affect the positioning of the MHB in a morphogenetic field with increased Wnt ligand concentration and Vangl2 controlled cytonemes. We find that the MHB shifts back towards a posterior position, however, a full rescue of the phenotype could not be observed in our simulations (Fig. 7K). Our analysis of the simulations, we predicted a strong increase in the range of Wnt signal activation as well as a loss of robust boundaries in the neighboring tissue, when Vangl2 function is accelerated in the Wnt8a source cells.

Vangl2 activity influences neural plate patterning in zebrafish neurogenesis. To test our prediction from the simulation, we analysed the consequences of Vangl2 function on the Wnt signaling range in zebrafish embryogenesis. In detail, we wanted to understand if the changes to cytoneme length and contact sites, as a result of Vangl2 alterations, impacted on neural plate patterning as suggested by agent-based simulation. First, we analysed the formation of the Wnt source tissue. The zebrafish embryonic margin functions as a major signaling source for Wnt and Fgf in the early gastrula. The transcription factor Notail (Ntl; ortholog in mouse TBXT, formerly known as T or brachyury) is essential for the induction of signaling factors such as Wnt8a and Fgf8a at the embryonic margin. Ntl expression is detected early in development in Wnt8a positive mesodermal progenitor cells and is required for body axis formation. Wnt8a and Ntl act in a positive autoregulatory loop to reinforce their expression. Therefore, we analysed the expression pattern of Ntl at the embryonic margin after altering of expression of Wnt8a and Vangl2. At 60% epiboly, the overexpression of vangl2, and more drastically, wnt8a with vangl2, caused an abnormal broadening and ectopic expression pattern of ntl (Fig. 8A). To link the phenotype observed after co-expression of vangl2/wnt8a to cytoneme appearance, we performed an experiment in which we reduce cytoneme formation by IRSp534K overexpression in embryos expressing vangl2 and wnt8a. We found that reduction of cytoneme number in Vangl2/Wnt8a expressing embryos rescued partially the increase of the ntl expression domain (Fig. 8A).

Next, we mapped the expression pattern of neural plate markers. gbx1 is a further direct Wnt signaling target in the gastrulating embryo and a marker for hindbrain identity. gbx1 mRNA expression can be detected at the marginal region at 7hpf (60–80% epiboly) (Fig. 8A). By measuring the intensity of the gbx1 expression from vegetal to animal pole, we were able to measure the width of the expression pattern, as well as the sharpness of the MHB (Fig. 8B). Wnt8a expression leads to a broadening of the expression pattern of gbx1 compared to control and a reduction in the sharpness of the MHB (Fig. 8A, B). Vangl2 alone has little effect on gbx1 expression, with similar expression distance and sharpness of boundaries to control. However, wnt8a and vangl2 overexpression together leads to a more exaggerated broadening to the gbx1 expression domain pattern compared to control and wnt8a mRNA alone. This phenotype requires phosphorylation of Vangl2 as the Vangl210A mutant shows a similar phenotype like in the control embryos. Our data suggests that the Wnt/β-catenin signaling range is broadened, most likely due to the increase of cytoneme length and number of contacts of cytonemes by Vangl2 as observed earlier (Fig. 3). In addition, there is a reduced sharpness of the midbrain-hindbrain boundary (MHB), as a result of more ectopic gbx1 expression further from the original expression domain. As predicted from our simulation, in synergy, Wnt8a and Vangl2 leads to a broadening and less sharp gbx1 expression domain, suggesting that the increase in cytoneme length and contact points may affect the signaling range and capability to signal to further cells. This has an impact on the specification of the brain primordia, for example, the gbx1 positive hindbrain primordium. Our simulation predicts that a reduction of cytonemes leads to a partial rescue (Fig. 7K). Indeed, blockage of cytoneme formation by IRSp534K overexpression rescues partially the phenotype observed in wnt8a and vangl2 expressing embryos as previously observed (Fig. 8A, B).

By 24hpf, primordial brain boundaries have formed due to activation of specific markers for brain regions. pax6a is expressed at the forebrain primordium and at the hindbrain primordium and rhombomeres. Addition of Wnt8a posteriorizes the brain, leading to reduction of forebrain and midbrain primordia structures (Fig. 8C, D). The observed posteriorization is an indication of increased Wnt/β-catenin signaling. Vangl2 overexpression leads to similar anteroposterior patterning as control. However, overexpression of wnt8a plus vangl2 leads to significantly reduced forebrain and midbrain structures, with complete abolishment of forebrain primordia structures in some cases. This is more severe than wnt8a alone. Emergence of
convergent extension (CE) phenotypes with addition of PCP vangl2 is only evident after the addition of wnt8a (Fig. 8C, E). Therefore, as well as a CE phenotype, Wnt8a and Vangl2 causes a neural plate patterning phenotype, with reduction of forebrain and midbrain primordia structures. Finally, we asked if the observed alteration of AP patterning can be linked to cytoneme emergence. Therefore, we reduced the formation of cytonemes in the Wnt8a/Vangl2 expressing embryos by co-expression of IRSp534K. We find that the observed posterization phenotype is rescued (Fig. 8C, D). However, the CE phenotype shown by an open neural tube phenotype could not be rescued. We speculate that due to the reduction of filopodia formation per se, cell migration could be affected. Overall, Vangl2 alterations leads to changes in early neural plate patterning, as outlined by changes to ntl and gbx1 expression. At later stages, CE and AP patterning phenotypes of the neural tube are present. We suggest that the
observed effect on AP patterning of the zebrafish brain anlage is a result of increased Wnt cytoneme length and cytoneme contacts, impacting on an increased Wnt/β-catenin signaling activation range.

Discussion
The PCP signaling pathway was initially characterized in Drosophila and orchestrates cell polarity across an epithelium. For example, in Drosophila, PCP is responsible for the coordinated and consistent orientation of hairs and bristles. In vertebrates, PCP homologues regulate the orientation of inner ear sensory hair cells, hair follicles of the skin, and epithelial cells bearing multiple motile cilia. PCP is further involved in regulating cell migration as seen during convergent extension during gastrulation, differential adhesion across cells, orientation of cytoskeletal elements, and positioning of cell extensions, such as filopodia. Core members of the Wnt/PCP signaling in vertebrates are, for example, Vangl and Ror2. The transmembrane factor Ror2 serves as a Wnt co-receptor helping to relay the signal to Vangl. After activation by the Wnt5a ligand, Ror2 forms a ligand-receptor complex to which Dvl and CK1δ is recruited. CK1δ phosphorylates Dvl and the two Ser/Thr phosphorylation clusters in the cytoplasmic N-terminal region of the Vangl2 protein. In mouse, mutants for the core PCP component Vangl2 exhibit open neural tubes (craniorachischisis). Similarly, human mutations in both VANGL1/2 are associated with spina bifida. In zebrafish, the vangl2 mutant trilobite exhibits a broadened body axis, owing to similar defects in convergent extension (CE) movements during development. Therefore, Vangl2 together with Ror2 take center stage in the PCP signaling pathway in vertebrates.

Vangl2 and cytoneme formation. Here, we demonstrate that Vangl2 has an essential function during formation of Wnt cytonemes and thus in the distribution of Wnt ligands across vertebrate tissues. The mechanism of cytoneme-based ligand transport has been observed in many tissues. Initially, cytonemes have been described in various tissues of Drosophila transporting Fgf, Dpp, and HH signaling components. Recent data suggest that cytonemes are also used to mobilize signaling components in vertebrates. Shh is transported by cytonemes in the chick limb bud and uses a Cdc42-independent mechanism. Cytonemes are also fundamental for transporting Wnt signals. We have shown that cytonemal transport of Wnt8a is essential during neural plate patterning during zebrafish gastrulation. Wnt is loaded on cytonemes and can be found at the cytonemal tip. In chick, there is evidence that also the Wnt receptor Frizzled7 (Fzd7) is required for somite formation and Fzd7 puncta could be detected on cytonemes emitting from the ectodermal layer. Activation of Wnt signaling in stem cells can be similarly mediated via Wnt receptor positive cytonemes. These findings are similar to observations in Drosophila, in which cytonemes containing Fzd receptors extend from myofibrilasts to pick up Wg signal from the wing disc. However, the molecular mechanism underlying cytoneme formation is still unclear.

Here, we show that Vangl2 can be seen together with Wnt8a and Ror2 on cytoneme tips of PAC2 fibroblasts as well as zebrafish epiblast cells in vivo. These data are supported by observations in zebrafish epiblast cells and zebrafish hindbrain motor neurons, in which Vangl2 localizes to filopodia tips. Similarly, in the mouse neural tube, Vangl2 and Frizzled3 are enriched on the tips of growth cone filopodia. However, the function of Vangl2 in cytonemes is yet to be elucidated.

We provide evidence that Vangl2 regulates the appearance of Wnt-bearing cytonemes: Vangl2 activation induces specifically long, branched cytonemes, which carry Wnt protein at their tips. To reduce with Vangl2 function, we expressed the N-terminal truncated form of Vangl2 and the Vangl210A mutant. Both tools have been shown to inactivate Vangl2 signaling. We decided against the usage of the zebrafish vangl2 mutant trilobite or a Morpholino-based knock-down approach, because our experimental strategy demanded the analysis of the cytoneme-generating cells without interfering with the cytoneme-receiving cells. Therefore, a mosaic expression strategy was preferable. To complement our analysis in zebrafish embryos, we knocked-down Vangl1/2 in mouse telocytes by an siRNA approach (Fig. 6). We find that reduction of Vangl2 signaling causes significantly fewer cytonemes, demonstrating a function for Vangl2 in cytoneme mediated Wnt transport. Besides its function in cell polarity and coordinated cell migration, Vangl2 has been suggested to play an important role in cellular protrusion formation. In Drosophila, knock-down of vangl and prickle lead to the formation of very few and short Fgf cytonemes. In Vangl2−/−/Loop-tail mouse mutants, filopodia were unable to extend. In hippocampal neurons, Vangl2 was found to regulate dendritic branching, with Vangl2 knockdown leading to reduced spine density and dendritic branching. Vangl2 is enriched at membrane domains that are developing these large protrusions compared with non-protrusive domains. Interestingly, Vangl2 has been suggested to de-stabilize protrusions, whereas Fzd3a is required to stabilize the same extensions. However, the nature of these protrusions is unclear. Here, we investigated the function of Vangl2 on cytonemes—small, slender protrusions, which form and retract within minutes and are loaded with signaling proteins. We show that Vangl2 function is essential for the formation of long Wnt cytonemes.

PCP/Vangl2 signaling regulates JNK signaling. The mechanism of how Vangl2 regulates cytoneme appearance is unknown. Our data suggests that cytoneme emergence can be modulated by an
intrinsic signaling cascade and Rac/Jun N-terminal kinase (JNK) and Rho-associated kinase (ROK) participate in the Wnt-mediated PCP pathway. The Rac/JNK signaling cascade is an intracellular relay pathway and is essential in regulating both the cytoskeleton and cell adhesiveness. At the core of this cascade are the stress-activated MAP kinase kinases MKK4 and MKK7 that activate JNK to modulate cytoskeletal and nuclear events. During dorsal closure in Drosophila, JNK signaling regulates the formation of actin and myosin dependent protrusions. We have previously identified several members of the Rac/JNK signaling family as regulators of Wnt8a positive cytonemes. We showed that overexpression of MKK4 and JNK3
led to the formation of longer Wnt8a cytonemes in PAC2 cells. Furthermore, we have shown that blockage of Rac1 (and Cdc42) mediated signaling by ML141 leads to a collapse of Wnt cytonemes within 2h. Here, we show that Vangl2-positive cytonemes are retracted within minutes after JNK signal inhibition (Fig. 4).

Indeed, there is evidence of Vangl2 mediated regulation of JNK activity. Vangl2/PCP signaling leads to activation of JNK and c-Jun by phosphorylation. Furthermore, Vangl2 regulates cell adhesion by regulating Rac1/JNK activity at adherens junctions. Similarly, it has been suggested that Vangl2 promotes phosphorylation of c-Jun and AP-1 in zebrafish. It has been further...
Fig. 8 Vangl2 function is crucial for anteroposterior patterning of the zebrafish neural plate. A In situ hybridization analysis of indicated markers in 60% epiboly zebrafish embryos (6.5 hpf) injected with Wnt8a, Vangl2 and Wnt8a/Vangl2, Wnt8a/Vangl2/IRSp534K, Wnt8a/Vangl2/IRSp534K. Scale bar indicates 200μm. (nt: n = 35, 16, 6, 12, 13, 19 embryos) (gbx: n = 6, 19, 3, 11, 22, 19 embryos). a = animal pole, v = vegetal pole. Black arrowheads indicate width of gbx1 expression. Asterisks indicate ectopic expression. B Box and whisker plot of sexual dimorphism from in situ hybridization (gray values in %) across the hindbrain primordium from vegetal (v) to animal (a) pole in embryos injected with indicated constructs at 60% epiboly (6.5 hpf). Control- light blue, wnt8a- orange, vangl2 - gray, wnt8a/vangl2- yellow, wnt8a/vangl2/IRSp534K - dark blue. C In situ hybridization to mark pax6 expression in the primordia of the forebrain and the hindbrain in embryos injected with mRNAs for the indicated constructs at 24 hpf: control, wnt8a, vangl2, and wnt8a/vangl2/IRSp534K. Scale bar indicates 100 μm. Horizontal black line indicates length of forebrain and midbrain primordium. Yellow arrowheads show the width of the hindbrain primordium, indicating the extent of convergent and extension of these cells. (n = 13, 19, 8, 10, 7 embryos). D Box and whisker plot of maximum width of hindbrain primordium in control (blue), Wnt8a (orange), Vangl2 (gray), Wnt8a/Vangl2 (yellow) and Wnt8a/Vangl2/IRSp534K (green). Measured from anterior forebrain pax6 expression to the position of the midbrain-hindbrain boundary (MHB) shown by horizontal black line (C). (n = 6, 12, 4, 10, 7 embryos). E Box and whisker plot of maximum width of hindbrain primordium in control (blue), Wnt8a (orange), Vangl2 (gray), Wnt8a/Vangl2 (yellow) and Wnt8a/Vangl2/IRSp534K (green). Measured from maximum width of hindbrain pax6 expression shown by yellow arrowheads (C). (n = 5, 11, 5, 8, 7 embryos). D, E Data represented as box and whisker plots. Whiskers define the minimum and maximum values. Bounds of box indicate the 25th and 75th percentile. Center line indicates the median. Cross indicates mean. Outliers and inner points shown. Statistical significance: p ≤ 0.05. D, E One-way ANOVA tests plus Tukey’s post-hoc test. SEM = 1. Source data are provided as a Source Data file.

suggested that p62 is required to recruit and activate JNK through an evolutionarily conserved VANGL2–p62–JNK signaling cascade in Xenopus and human breast cancer cells.87 We conclude that Vangl2/PCP activates Rac1/JNK signaling to regulate cytoneme generation in the Wnt source cell.

In addition to JNK signaling, Wnt/Vangl stimulation can also induce activation of RhoA-dependent signaling. RhoA/Rock regulate cell migration88—specifically convergence and extension (CE) movements—by mediating Wnt/PCP signaling in zebrafish and Xenopus gastrula67,89–91. Although there are reports of cross-regulation and synergism, RhoA/Rock signaling has been rather implicated in changes in cell shape, orientation, and polarity whereas Rac/JNK signaling is involved in filopodia formation92. This notion is supported by our observation, that JNK signaling is essential for controlling the formation of highly dynamic cytonemes, whereas RhoA/ROCK signaling seems to be more important for adjusting the number of filopodia93 or cytonemes34 presumably as an adaption to an altered cell morphology. Indeed, we found that Cdc42 can regulate the number of cytonemes34 similar to RhoA, which regulates cell shape and protrusion lifespan in CE movements92. Therefore, we hypothesize that Vangl2 activates Rac/JNK signaling to regulate the elongation and collapse of Wnt cytonemes within minutes and, therefore, influences the dynamic distribution of Wnt ligands on cytonemes.

In addition to the intrinsic function of Vangl2 on Rac/JNK, it has been suggested that Vangl2/PCP signaling induces protrusion formation by influencing the composition of the extracellular matrix in Drosophila and zebrafish35,76,78. In the zebrafish vangl2 mutant trilobite, longer and thicker protrusions were observed. However, it is unclear if these extensions are filopodia, which are used for signaling or nanotubes forming intracellular bridges94. In Drosophila, it has been suggested that the PCP components prickle and vangl are essential for Fgf cytonemes formation. After blockade of prickle/vangl function, cytonemes were reduced in number and length when pknRNAi or VangRNAi were expressed95. In these flies, the composition of the extracellular matrix (ECM) was altered, suggesting that prickle and vangl are involved in maintaining normal levels of glycans such as Dally, Dlp, and laminin. However, a molecular mechanism explaining the relation between PCP signaling and ECM composition is unclear.

Vangl2 cytonemes and paracrine signaling and tissue patterning. Recently, we compared modes of signaling transport, specifically we compared diffusion-based transport to cytoneme-regulated transport95. We found that cytoneme-based transport is required for patterning in quickly developing tissues such as during zebrafish gastrulation. Diffusion-based distribution requires significantly more time to establish a robust pattern in the receiving tissue. In particular, the time taken for the distribution to form is inconsistent with the development time checkpoints during gastrulation. Thus, we here exclude diffusion as a transport mechanism for Wnt8a signaling in this context.

We further could show that Wnt distribution by cytonemes is essential during a very narrow time window of 2 h to achieve neural plate patterning during early zebrafish gastrulation95. This raised the question of how to reach a sufficiently high flux of Wnt protein into the field by fast cytonemes, given the fact that only a few cytonemes form per Wnt producing cell. Here we show that Vangl2 activation is a crucial regulator of cytone behavior regarding length, and growth properties, and the ability to branch out with the potential to contact multiple target cells. This observation became relevant for the generation of the morphogenetic field in our simulation. When the simulation assumed that all cytonemes deliver the ligand to a single neighboring cell and we take into account the actual number of cytonemes, we observed only minimal differences in the concentration of the ligand within the morphogenetic field. However, as soon as we allow multiple contact sites per cytoneme over a longer time, we increase the level of activation in the target cells combined with a wider patterning activation (Fig. 7). We concluded that for generating a signaling gradient employing the mechanism we describe it is essential to regulate all parameters of cytonemes. In particular, the net change in the flux of Wnt8a from producing to receiving tissue controls the range of the gradient and is directly proportional to the filopodia number and length but more importantly, also to the number of contact events per cytoneme. We observed that activation of Wnt cytoneme transport by Vangl2 and Ror2 led to significant upregulation of Wnt/β-catenin signaling in the neighboring cells, whereas blockage of Ror2 or Vangl2 function led to a strong reduction of paracrine Wnt signaling. Consequently, activation of Vangl2 and Wnt8a led to a synergistic upregulation of paracrine Wnt/β-catenin signaling and in posteriorization of the neural tube during zebrafish gastrulation. In future work, the predictions of our model could be validated by investigating paracrine signal activation and tissue patterning. We argue Vangl2 function is essential to control cytoneme length and contact events given the short time window in which Wnt signaling is required for neural plate patterning.

Cytonemes are taking center stage in cell-cell communication in invertebrates87, and there is an increasing number of examples similarly describing the essential function of these signaling filopodia in vertebrates15. However, our understanding of how
were maintained at 28 °C in Leibovitz L-15 media (Gibco). PAC2 cells14 were
Methods
We propose that PCP signaling is a crucial mechanism for controlling Wnt8a cytonemes. In summary, we show that traf
neural plate patterning in the developing zebra
their transport. Finally, we show that Vangl2 activity controls that also these Wnts utilize Vangl1/2-dependent cytonemes for
gradient operating in the intestinal crypt and provide evidence
Animal Care and Use Committee. Cat#13778
Vangl1 and Vangl2 siRNAs (Dharmacon Cat# J-057276-09-0002 and Cat# J-
ARTICLE NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-021-22393-9
(AGS) cells were maintained at 37 °C with 5% CO2 in RPMI-1640 media (Gibco).
Animal care regulations and all embryo experiments were performed before 120 h
contrast with organoids, cell cultures were photographed 24 h following co-culture. Antibody staining. The IF staining were performed according to the published protocol88. In particular, PAC2 and AGS cells were plated on coverslips and depending on the experiment transfected with the zfWnt8a-mCherry plasmid fused with Fugene. Twenty-four hours later, cells were washed once in prewarmed PBS, then fixed using 0.25% glutaraldehyde and 4% PFA (for anti-Wnt8a antibody) or 0.2% glutaraldehyde (for anti-Vangl2 antibody) at 4 °C. Cells were washed in PBS, and blocked and permeabilised using 0.1% Triton X-100, 5% rabbit or goat serum, and 0.2 M glycine in 1 h at room temperature. Cells were washed again, then incubated with anti-Wnt8a antibody (1:50 dilution, catalog number: MBS9216179, MyBio-Sem). Coverslips were then inverted and incubated with an anti-rabbit secondary polyclonal antibody conjugated to Alexafluor 488 or a donkey anti-goat AlexaFluor 647 (both 1:1000 dilution, Abcam, catalog number: ab150077 and ab150313, respectively) and Phalloidin TRITC/FITC (Merck) for 1 h at room temperature. Coverslips were washed and mounted using ProLong Mountant (Invitrogen).

Fluorescent intensity along cytoneme. Fiji software was used to plot the pixel intensity along a selected line image within an image. A line was assigned from the base to the tip of cytoneme as defined by a membrane marker and the pixel intensity was plotted. The selection line was copied to the ROI manager and the measurement repeated for the second channel.

We describe Vangl2/PCP-induced cytonemes as transport carriers for Wnt8a in zebrafish. In cell culture experiments, we use PAC2 fibroblasts and HEK299Tc cells to provide further evidence for the importance of Vangl2-dependent regulation of cytonemes in Wnt trafficking. In addition, we show that human gastric cancer cells AGS process paracrine Wnt signaling via cytonemes, which are influenced by Vangl2 activity. Then, we use murine intestinal stroma cells, which express multiple Wnts to maintain the Wnt gradient operating in the intestinal crypt and provide evidence that also these Wnts utilize Vangl1/2-dependent cytonemes for their transport. Finally, we show that Vangl2 activity controls neural plate patterning in the developing zebra
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differently, approx. 50 cells in 5 embryo or 30 cells of three independent transfection experiment were randomly chosen for analysis. In tissue culture as well as in the zebrafish embryos, the numbers and lengths of filopodia and cytonemes were measured manually from base to tip of protrusions in Fijii. Continuous nanotube structures from cell to cell or thicker membrane extensions were discounted.

**Microinjection of mRNA and DNA constructs.** Capped sense mRNA was generated from linearized plasmid using the mMessage mMachine SP6 & T7 Transcription Kits (Invitrogen). mRNA was microinjected at the 16-cell stage at 200 ng/µl, to generate clonal expression, then imaged live intact embryos from 50% epiboly. GFP-Vangl2 DNA, and mRNA was microinjected at 2–4 cell for clonal expression for in situ experiments.

**KTR-mCherry based NKn reporter assay and analysis.** HEK293T cells were transfected with JNK reporter KTR-mCherry and either or, GFP-Vangl2, xRO2 and Shh RNAi plasmids. mRNA and Wnt8a and Wnt5a mouse recombinant protein was added and incubated at 37 ºC for 24 h before imaging. For analysis, mean gray values were recorded for 3× ROI in the nucleus and 3× ROI in the cytoplasm per cell and an average was taken. The ratio of cytoplasmic/nuclear signal was then recorded and normalized to 1 for the control.

**Inhibitor treatment.** Cells were transfected as described above. Twenty micro-molar of JNK inhibitor SP600125 (Sigma), dissolved in DMSO, was added to the media. Cells were imaged immediately and 1 and 2 h after treatment. Y27632 (ROCK inhibitor) was dissolved in water and added to the media at 10 µM for 1, 5, or 24 h.

**STF reporter co-culture assay and fluorescent intensity analysis.** AGS cells were transfected with either STF reporter 7xTRE Super TOPFlash-NLS-mCherry/Lrpf-GFP or plasmids of interest. After 24–48 h, cells were then trypsinized and seeded together for another 24 h. STF reporter expression was imaged on a Leica widefield microscope. Nuclei STF reporter expression was thresholded in FIJI and relative fluorescence of nuclei was measured. A nuclear DAPI staining was used to ensure that equal number of cells were present in each frame analysed.

**Generation of in situ probes and in situ hybridization.** notal, gbx1, and pax6a digoxygenin and FITC antisense probes were generated from linearized plasmids using an RNA labeling and detection kit (Roche)99. Probes were purified on ProbeQuant G50 Micro Columns (GE Healthcare).

**Data availability**

The ICGS data that support the findings of this study are available in Dryad, Dataset https://doi.org/10.5061/dryad.cpxvswp. Additional data that support the findings of this study are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions
L.B. and S.S. designed, performed and analyzed all experiments except where noted and wrote the manuscript. S.R. performed the IF staining and the STF assay. G.G. and D.M.V. performed the intestinal organoid studies, B.D.E. and K.C.A.W. designed and performed the simulations.

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