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# Planar cell polarity proteins differentially regulate extracellular matrix organization and assembly during zebrafish gastrulation

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ABSTRACT

Zebrafish gastrulation cell movements occur in the context of dynamic changes in extracellular matrix (ECM) organization and require the concerted action of planar cell polarity (PCP) proteins that regulate cell elongation and mediolateral alignment. Data obtained using *Xenopus laevis* gastrulae have shown that integrin–fibronectin interactions underlie the formation of polarized cell protrusions necessary for PCP and have implicated PCP proteins themselves as regulators of ECM. By contrast, the relationship between establishment of PCP and ECM assembly/remodeling during zebrafish gastrulation is unclear. We previously showed that zebrafish embryos carrying a null mutation in the four-pass transmembrane PCP protein *vang-like 2* (*vangl2*) exhibit increased matrix metalloproteinase activity and decreased immunolabeling of fibronectin. These data implicated for the first time a core PCP protein in the regulation of pericellular proteolysis of ECM substrates and raised the question of whether other zebrafish PCP proteins also impact ECM organization. In *Drosophila melanogaster*, the cytoplasmic PCP protein Prickle binds Van Gogh and regulates its function. Here we report that similar to *vangl2*, loss of zebrafish *prickle1a* decreases fibronectin protein levels in gastrula embryos. We further show that Prickle1a physically binds Vangl2 and regulates both the subcellular distribution and total protein level of Vangl2. These data suggest that the ability of Prickle1a to impact fibronectin organization is at least partly due to effects on Vangl2. In contrast to loss of either Vangl2 or Prickle1a function, we find that *glypican4* (a Wnt co-receptor) and *frizzled7* mutant gastrula embryos with disrupted non-canonical Wnt signaling exhibit the opposite phenotype, namely increased fibronectin assembly. Our data show that *glypican4* mutants do not have decreased proteolysis of ECM substrates, but instead have increased cell surface cadherin protein expression and increased intercellular adhesion. These data indicate that Wnt/Glypican4/Frizzled signaling regulates ECM assembly through effects on cadherin-mediated cell cohesion. Together, our results demonstrate that zebrafish Vangl2/Prickle1a and non-canonical Wnt/Frizzled signaling have opposing effects on ECM organization underlying PCP and gastrulation cell movements.


## Introduction

Several morphogenetic cell movements operate during gastrulation to shape tissues into an embryo with recognizable anterior–posterior and dorsal–ventral body axes (Keller, 2005; Solnica-Krezel, 2005). Here, establishment of planar cell polarity (PCP) is required for polarized cell behaviors including directed migration and mediolateral intercalation (Keller, 2002; Jessen and Solnica-Krezel, 2005). Genes regulating PCP were first described in *Drosophila melanogaster* where they influence cell polarity in epithelial tissues such as the wing (Goodrich and Strutt, 2011). Vertebrate homologs of fly PCP genes regulate changes in gastrula cell morphology and orientation in relation to the dorsal embryonic midline (Jessen et al., 2002). Loss of function of the zebrafish (*Danio rerio*) PCP proteins Vang-like 2 (Vangl2) and Prickle1a disrupts cell polarity resulting in misshapen embryos that are shorter and broader than wild type (Solnica-Krezel et al., 1996; Sepich et al., 2000; Jessen et al., 2002; Carreira-Barbosa et al., 2003; Veeman et al., 2003). Other proteins required for PCP during zebrafish gastrulation include non-canonical Wnts and their co-receptor Glypican4, Frizzled receptors, and downstream components Disheveled and Rho family small GTPases (Jessen and Solnica-Krezel, 2005). Similar to the fly wing epithelium, it is thought that zebrafish Vangl2 and Prickle1a function to oppose or antagonize Frizzled/Disheveled signaling and thereby polarize cell behaviors (Roszko et al., 2009). However, molecular mechanisms underlying

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the establishment of PCP during gastrulation are unclear as are the functional relationships between Vangl2, Prickle1a, and components of the Wnt signaling pathway.

Gastrulation movements occur in the context of a dynamic extracellular matrix (ECM) network that is also capable of affecting PCP and morphogenesis and data suggest that PCP proteins themselves regulate ECM organization (Marsden and DeSimone, 2001; Goto et al., 2005; Dzamba et al., 2009; Latimer and Jessen, 2010). Regulation of ECM organization during gastrulation could influence a variety of polarized cell behaviors requiring cell–matrix interactions such as membrane protrusive activity (Davidson et al., 2006; Coyle et al., 2008). Knockdown of fibronectin expression causes PCP-like gastrulation phenotypes characterized by shortened and broadened embryos (Davidson et al., 2006; Latimer and Jessen, 2010). Work from *Xenopus laevis* has demonstrated that interactions between fibronectin and the α5β1 integrin receptor are essential regulators of polarized cell behaviors during gastrulation (Marsden and DeSimone, 2003). Notably, integrin activation suppresses inappropriate membrane protrusive activity at anterior–posterior cell domains thus contributing to cell polarization (Davidson et al., 2006). This indicates that proper cell–matrix interactions are essential for PCP. The concept that PCP proteins themselves could influence matrix organization was established when it was shown that ectopic expression of frog Van Gogh, Prickle, or Frizzled disrupts fibronectin fibril assembly on mesodermal tissue surfaces (Goto et al., 2005). The ability of these three disparate PCP proteins to cause apparently similar ECM phenotypes suggests that in frog, PCP proteins cooperate to affect polarized matrix assembly. Further work demonstrated that frog animal caps injected with dominant-negative Wnt11 fail to assemble fibronectin fibrils (Dzamba et al., 2009). This phenotype was rescued by co-injection with a Disheveled construct (minus the DIX domain) indicating that downstream non-canonical Wnt signaling is required for fibrillogenesis (Dzamba et al., 2009). Here, it was proposed that Wnt11 signaling affects fibronectin assembly by increasing cadherin adhesive activity and subsequently mechanical tension between integrins and fibronectin (Dzamba et al., 2009).

In contrast to frog, it is unclear how ECM assembly and remodeling during zebrafish gastrulation influence PCP. It is known that a fibronectin-containing ECM network is first detected near mid-gastrulation and separates epiblast tissue from the underlying hypoblast (Latimer and Jessen, 2010). As gastrulation proceeds, fibrillar fibronectin is assembled adjacent to mesodermal cells at both ectodermal and endodermal tissue interfaces (Latimer and Jessen, 2010). Notably, the time frame of increased ECM assembly during zebrafish gastrulation correlates with the onset of ectodermal and mesodermal PCP and increased directed cell migration (Jessen et al., 2002; Sepich et al., 2005). How might zebrafish PCP proteins regulate ECM assembly and remodeling during gastrulation? Our previous in vivo data implicated membrane type-1 matrix metalloproteinase (Mmp14) in the regulation of PCP and demonstrated a strong genetic interaction between zebrafish *mmp14* and *glypican4* (Coyle et al., 2008). Mmp14 is a member of the metzincin superfamily of zinc endopeptidases and is capable of cleaving a variety of ECM and non-ECM protein substrates (Sato et al., 1994; Zucker et al., 2003). ECM proteolysis by human MMP14 results in α5β1 integrin-mediated endocytosis and turnover of extracellular fibronectin (Shi and Sottile, 2011). MMP14 is activated intracellularly and endocytic and recycling pathways tightly regulate its expression at the cell surface (Remacle et al., 2003; Steffen et al., 2008). Therefore, regulation of cell surface Mmp14 proteolytic activity and ECM remodeling might provide a mechanism whereby migrating zebrafish gastrula cells influence the formation of polarized membrane protrusions. Indeed, utilizing migratory human cancer cells as a model, we determined that loss of VANGL2 function disrupts endocytosis of cell surface MMP14 causing increased pericellular proteolysis and invasion of ECM substrates (Cantrell and Jessen, 2010; Williams et al., 2012a, 2012b). We subsequently showed that zebrafish *vangl2*-null mutant embryos exhibit increased Mmp14 proteolytic activity and decreased immunolabeling for fibronectin in gastrula-stage embryos (Williams et al., 2012a). Together, our in vitro and in vivo findings support the hypothesis that Vangl2-dependent regulation of proteolysis and ECM organization is required for PCP.

The goal of the present study was to determine how loss of Prickle1a function and disruption of non-canonical Wnt signaling impact fibronectin assembly and to identify underlying mechanisms. Similar to Vangl2, we demonstrate that Prickle1a is required for fibronectin assembly during zebrafish gastrulation. We report that Prickle1a directly binds and positively regulates the expression of Vangl2. In contrast to Vangl2 and Prickle1a, we show that the non-canonical Wnt/Glypican4/Frizzled7 signaling branch of the PCP pathway inhibits fibronectin assembly through negative regulation of cadherin-mediated cell adhesion. Our results provide the first evidence that Vangl2/Prickle1a and non-canonical Wnt signaling function by distinct mechanisms to regulate ECM organization in the zebrafish embryo.

## Materials and methods

### *Zebrafish husbandry and embryo manipulation*

Adult zebrafish were maintained under standard conditions, and embryos were collected after natural spawnings. Embryos were raised in egg water (ultra pure water, 60 mg/L Instant Ocean) and staged according to morphological criteria (Kimmel et al., 1995). Strains utilized in this study: wild type (AB* and TL), *vangl2*/*trilobite*$^{m209}$ (Solnica-Krezel et al., 1996; Jessen et al., 2002), *glypican4*/*knypek*$^{m119}$ (Solnica-Krezel et al., 1996; Topczewski et al., 2001), and maternal-zygotic (MZ) *frizzled7a/7b* (Quesada-Hernandez et al., 2010).

### *Morpholinos, mRNA, and embryo microinjection*

The *vangl2* (Williams et al., 2012a), *prickle1a* (Carreira-Barbosa et al., 2003), *mmp14a/b* (Coyle et al., 2008), and *N-cadherin*/*cdh2* (Lele et al., 2002) morpholinos (MOs) were obtained from Gene Tools, LLC and have been described previously. The *vangl2* and *mmp14a/b* MOs were validated by our lab and shown to cause both PCP and convergence and extension phenotypes in gastrula stage embryos (Coyle et al., 2008; Williams et al., 2012a). The *cdh2* and *prickle1a* MOs were validated by other groups and re-validated by our lab. In our hands, both of these MOs consistently produced loss of function phenotypes identical to the published phenotypes (Lele et al., 2002; Carreira-Barbosa et al., 2003; Veeman et al., 2003; Harrington et al., 2007; Warga and Kane, 2007). Typical MO doses were 5 ng (*vangl2*), 8 ng (*prickle1a*), 5 ng each (*mmp14a* and *mmp14b*), and 6 ng (*cdh2*) per embryo. Synthetic mRNA encoding full-length zebrafish Cdh2 and Prickle1a were generated from pCS2+ vector clones using Ambion's Sp6 mMessage mMachine kit. Single cell stage embryos were injected with mRNA and MO using standard procedures (Gilmour et al., 2002) and grown in egg water until they reached the appropriate stage.

### *Transmission electron and confocal microscopy*

Tailbud stage wild type, *vangl2*, and *glypican4* mutant embryos were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, dechorionated, and processed for TEM following standard procedures. Thick cut cross-sections (500 nm) were used to verify

embryo orientation before generating thin cut cross-sections (~80 nm). Images were taken using a Philips/FEI T-12 microscope. For confocal imaging, zebrafish fibronectin and laminin immunolabeling was performed as described (Latimer and Jessen, 2010; Williams et al., 2012a). Labeling using anti-pan-cadherin (1:100; Abcam) was performed as follows: embryos were fixed overnight at 4 °C using 4% paraformaldehyde, blocked using 2% normal donkey serum in PBS containing 0.1% Triton X-100, and labeled with secondary antibody. Embryos were further washed in 0.1% Triton X-100 in PBS and 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Life Technologies) was used to detect nuclei. Embryos were positioned on glass bridge slides with two or three coverslips stacked on slide ends and mounted with Aqua Poly-Mount (PolySciences). In each confocal imaging experiment, fields lateral to the dorsal notochord near the embryo equator were captured using an Olympus FV1000 laser scanning confocal microscope using a 40× oil objective and a 3× digital zoom. Images correspond to ~0.01 $mm^2$ per field ($x$, $y$) and optical thicknesses ($z$) ranging from 0.7 to 0.9 μm, with the thickness consistent between each experimental condition (i.e. mutants, morphants, mRNA injected embryos, and negative controls). Images for each embryo are shown as flattened $z$-dimension image stacks. For all experiments, fibronectin, laminin, and pan-cadherin images were obtained from at least ten control and ten experimental embryos. Immunolabeling using an anti-Vangl2 antibody (1:100; gift from L. Solnica-Krezel) was performed similar to above except that embryos were fixed for 2 h at room temperature in Prefer fixative (Anatech Ltd.). Membrane/membrane-associated Vangl2 and cytoplasmic Vangl2 fluorescence intensity were quantified using ImageJ software (rsb.info.nih.gov/ij). Here, the intensity of cell surface Vangl2 immunolabeling in tailbud stage ectodermal and mesodermal cells was quantified at numerous cell junctions and compared to the intensity of Vangl2 expression within the cytoplasm of the same cell populations. High magnification 40× images were used to demarcate and quantify Vangl2 expression in these different domains. Statistical analysis was done using Microsoft Excel to perform a two-tailed Student's $t$-test.

*Cell culture, transfection, and co-immunoprecipitation*

Chinese Hamster Ovary (CHO) cells were obtained from ATCC and grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Zebrafish *vangl2* and *prickle1a* cDNAs were expressed from pCS2+ vectors in CHO cells transiently transfected via polyethylenimine (Polyscience, Inc.) as previously described (Ehrhardt et al., 2006). Cells were lysed in 1% CHAPS lysis buffer (Amresco, Inc.) supplemented with protease inhibitor cocktail (Sigma), clarified by centrifugation, and quantified by BCA (Thermo Fisher Scientific). Whole-cell lysates were boiled 5 min following addition of Laemmli sample buffer. For co-immunoprecipitation, equal volumes of lysate were incubated with anti-Vangl2 antibody for 1.5 h at 4 °C with end-over-end rotation, followed by incubation with Protein A/G Magnetic Beads (Thermo Fisher Scientific) for an additional 45 min. After magnetic separation and extensive washes, beads were suspended in Laemmli sample buffer and boiled 5 min.

*Embryo lysis, cell fractionation, and embryonic protease assay*

For zebrafish embryo whole-cell lysates, pools of approximately fifty phenotypically similar embryos were chemically dechorionated with Pronase (Merck), deyolked, lysed in RIPA (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, protease inhibitor cocktail (Sigma)), and clarified by centrifugation. For cell fractionation, pools of approximately twenty phenotypically similar embryos were harvested as previously described (Van Raay et al., 2011). Briefly, embryos at the 10-somite stage were dechorionated with Pronase, deyolked, and lysed by trituration in TKM buffer (50 mM Tris, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA) supplemented with protease inhibitor cocktail. Lysates were clarified by centrifugation at 900 g, and the resulting supernatant was further fractionated into cytoplasmic and membrane fractions by centrifugation at 100,000 g for 1 h at 4 °C. Whole-cell lysates and fractions were boiled for 5 min following addition of Laemmli sample buffer. Wild type and *glypican4* mutant embryo protein extracts were prepared and analyzed for proteolytic activity as previously described (Williams et al., 2012a).

*Western blotting*

Immunoprecipitations, whole-cell lysates, and cell fractions were separated by SDS-PAGE and transferred to nitrocellulose or PVDF membranes (Perkin Elmer). Non-specific binding to membranes was blocked with 5% non-fat milk in TBS–Tween (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20), and membranes were incubated overnight with primary antibody in milk at 4 °C. Membranes were then incubated with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.), developed using ECL substrate (Thermo Fisher Scientific), and exposed to X-ray film (Denville Scientific). Antibodies used were anti-Fibronectin (1:1000; Sigma), anti-myc (1 μg/ml; 9E10; Vanderbilt Antibody & Protein Resource), anti-Vangl2 (1:1000), anti-pan-cadherin (1:1000; Abcam), and anti-GAPDH (1:30,000; Ambion). Densitometry was performed using the ImageJ software, and for statistical analysis, two-tailed Student's $t$-tests were performed using Microsoft Excel. All western blot data was repeated three times or as indicated in the figure legends.

*Aggregation assay*

Cells were tested for their ability to aggregate in hanging drop suspension cultures. Tailbud stage wild type and *glypican4* mutant embryos were mechanically dechorionated and dissociated with 0.25% trypsin/EDTA (Sigma). Cells were washed and suspended in L15 medium/15% FCS, and 30 μl volumes were suspended as hanging drops from the lid of a 24-well culture dish and allowed to aggregate overnight at 25 °C. To eliminate evaporation within the drops, wells were filled with PBS. For analysis, cells were dissociated by pipetting ten times through a 200 μl pipette tip, and bright-field images were taken at 10× magnification. Cell clusters greater than 0.25 $mm^2$ were counted from at least five fields of view per experiment.

*Semi-quantitative PCR*

Total RNA was isolated from late somitogenesis stage wild type, *vangl2*, and *glypican4* mutant embryos with the RNAqueous Kit (Ambion) and reverse transcribed with SuperScript III Reverse Transcriptase (Life Technologies). The resulting cDNA was used as a template for PCR with primers specific for zebrafish *cdh1*, *cdh2*, *cdh5*, and *ef1α*.

## Results

*Knockdown of vangl2 or prickle1a decreases total fibronectin protein*

Our previous data showed that *vangl2*-null mutant embryos exhibit increased Mmp14 proteolytic activity and reduced immunolabeling of fibronectin at the end of gastrulation (Williams et al., 2012a). To further understand this phenotype we examined

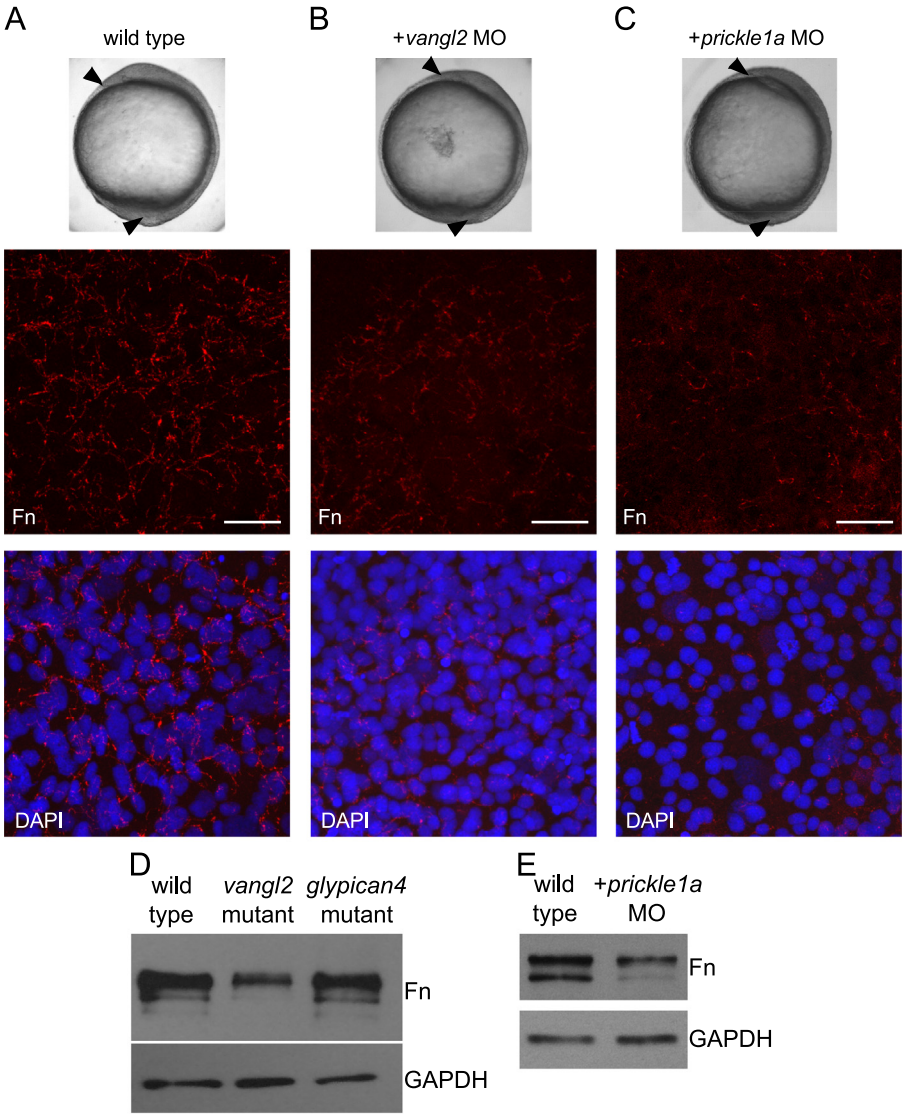


**Fig. 1.** Knockdown of *vangl2* and *prickle1a* decreases fibronectin. (A–C) Top panels show morphological convergence and extension phenotypes at tailbud stage (see arrowheads) of wild type, *vangl2* morphant, and *prickle1a* morphant embryos. Middle/bottom panels show confocal images of fibronectin (Fn) immunolabeling without and with nuclear DAPI staining. Scale bars=20 μm. (D and E) Fn and GAPDH western blots of 10 μg total protein extract obtained from tailbud stage wild type, *vangl2* mutant, *glypican4* mutant, and *prickle1a* morphant embryos.

embryos at mid-gastrulation when fibronectin fibrils are apparent (Latimer and Jessen, 2010) but *vangl2* function is not required (Sepich et al., 2000, 2005; Jessen et al., 2002). Here, wild-type embryos were injected with a previously validated *vangl2* antisense MO oligonucleotide (Williams et al., 2012a) followed by fibronectin immunolabeling at 80% epiboly (Fig. S1). This *vangl2* MO produces a convergence and extension phenotype in 100% of injected embryos (Williams et al., 2012a). Our data show that while *vangl2* MO-injected wild-type embryos consistently had reduced fibronectin at the end of gastrulation (Fig. 1A and B), at mid-gastrulation *vangl2* knockdown did not affect ECM organization (Fig. S1). Western blot analysis confirmed that indeed *vangl2* mutant embryos have a marked loss of total fibronectin protein at the end of gastrulation (Fig. 1D). Together, these results indicate that the requirement for Vangl2-dependent regulation of both fibronectin assembly and PCP occurs near late gastrulation.

Given that Van Gogh and Prickle are thought to function in a protein complex during establishment of PCP in the fly wing (Tree et al., 2002; Goodrich and Strutt, 2011), we next determined whether zebrafish Prickle1a also affects fibronectin organization at the end of gastrulation. Again, wild-type embryos were injected with a validated *prickle1a* MO (Carreira-Barbosa et al., 2003) and fibronectin immunolabeling was performed using tailbud stage embryos. Similar to Vangl2, loss of Prickle1a function causes a significant reduction in fibronectin protein as indicated by both immunofluorescence and western blot (Fig. 1C and E). These data suggest that a functional interaction between Vangl2 and Prickle1a might regulate proteolytic ECM remodeling during gastrulation.

### *Prickle1a binds Vangl2 and regulates its localization*

It is known that fly Van Gogh and Prickle physically interact and are capable of regulating each other's subcellular distribution (Bastock et al., 2003), and we reasoned that a similar relationship exists between the zebrafish proteins. First, we demonstrated that indeed zebrafish Vangl2 and Prickle1a form a protein complex that can be co-immunoprecipitated from CHO cells (Fig. 2A). Second, we showed that the cell surface/cytoplasmic ratio of Vangl2 protein is reduced in tailbud stage wild-type embryos injected with *prickle1a* MO (Fig. 2B and C). Lastly, we found that MO-mediated knockdown of *prickle1a* causes a reduction in total Vangl2 protein levels (Fig. 2D). Together, these results suggest

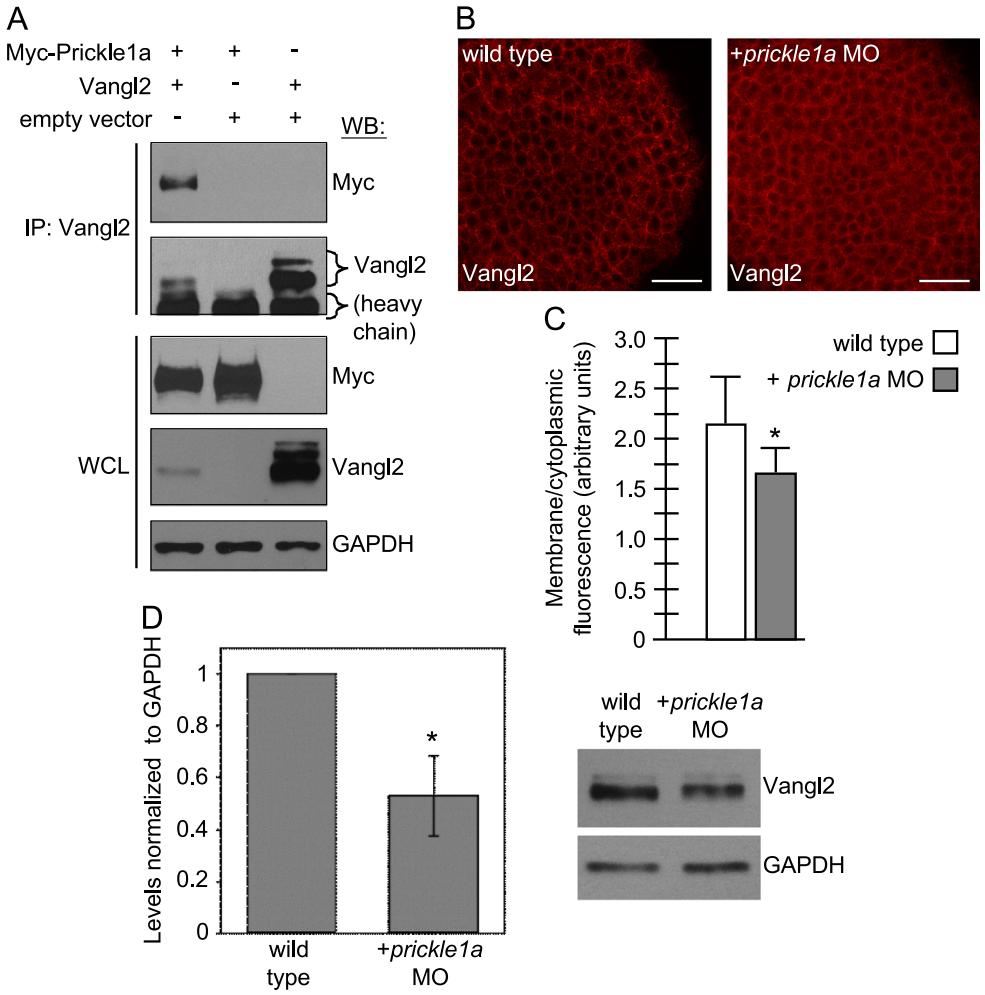


**Fig. 2.** Prickle1a binds Vangl2 and regulates its expression. (A) Western blot (WB) analysis after Vangl2 antibody immunoprecipitation of transfected CHO cell protein extracts. Incubation of protein extracts with Vangl2 antibody co-immunoprecipitates both Vangl2 and Myc-Prickle1a. Whole cell lysates (WCL) demonstrate successful transfection and expression of specific proteins. (B) Confocal images of Vangl2 expression in tailbud stage wild type and *prickle1a* morphant embryos. Scale bars=20 μm. (C) Quantification of membrane versus cytoplasmic Vangl2 expression; 465 wild type control measurements from three embryos; 742 *prickle1a* morphant measurements from four embryos ($^*p < 0.0001$). (D) Western blot analysis of Vangl2 protein expression normalized to GAPDH in wild type and *prickle1a* morphant embryos. Bar graph represents data from three independent experiments and the blot depicts results from one of those experiments ($^*p < 0.007$).

that Prickle1a-dependent changes in fibronectin organization are due at least in part to negative effects on Vangl2 function. Previous studies in zebrafish have shown that ectopic Prickle1a activity decreases the level of another interacting protein, Disheveled (Carreira-Barbosa et al., 2003), suggesting that Prickle1a can regulate the stabilization and/or degradation of its binding partners. We found that overexpression of synthetic zebrafish *prickle1a* mRNA in vivo also caused a reduction in total Vangl2 protein levels (Fig. S2).

*Loss of glypican4 or frizzled7 function increases fibronectin assembly*

Since loss of either Vangl2 or Prickle1a disrupts ECM assembly, we next determined how non-canonical Wnt/PCP signaling impacts fibronectin assembly. To do this we utilized both *glypican4* and MZ*frizzled7a/7b* homozygous mutant embryos because of their severe convergence and extension phenotypes (Topczewski et al., 2001; Quesada-Hernandez et al., 2010). Glypican4 is a glycosylphosphatidylinositol-anchored membrane protein that is required for PCP and functions with frizzled as a Wnt co-receptor during zebrafish and frog gastrulation (Topczewski et al., 2001; Ohkawara et al., 2003). The *glypican4*-null mutant phenotype is almost identical to that of *wnt5b/wnt11* double mutant embryos (Kilian et al., 2003) supporting the notion that loss of Glypican4 severely disrupts non-canonical Wnt/PCP signaling. Our data show that both *glypican4* and MZ*frizzled7a/7b* mutant embryos have increased fibronectin assembly (Fig. 3A–C). In each case, fibronectin labeling intensity was increased. These data demonstrate a striking difference in fibronectin between *glypican4* and *vangl2* loss of function embryos, a finding that was repeated for laminin (Fig. S3). We corroborated these results at the ultra structural level by using transmission electron microscopy to show that, compared to wild-type controls, *glypican4* mutant embryos have increased ECM while *vangl2* mutant embryos have decreased ECM (Fig. 4A–C). Here, it is clear that despite having similar morphological convergence and extension phenotypes (Solnica-Krezel et al., 1996; Marlow et al., 1998), tailbud stage *glypican4* and *vangl2* mutant embryos have clear differences in ECM organization.

*glypican4 mutant embryos do not have increased proteolytic activity*

In the fly wing epithelium, antagonistic interactions between Van Gogh and Frizzled/Disheveled signaling regulate formation of actin rich hairs on the distal side of each apical membrane (Strutt and Strutt, 2009). We hypothesized that opposite of *vangl2* mutant embryos that have increased Mmp14 activity, disrupted Wnt signaling in *glypican4* mutant embryos might decrease matrix

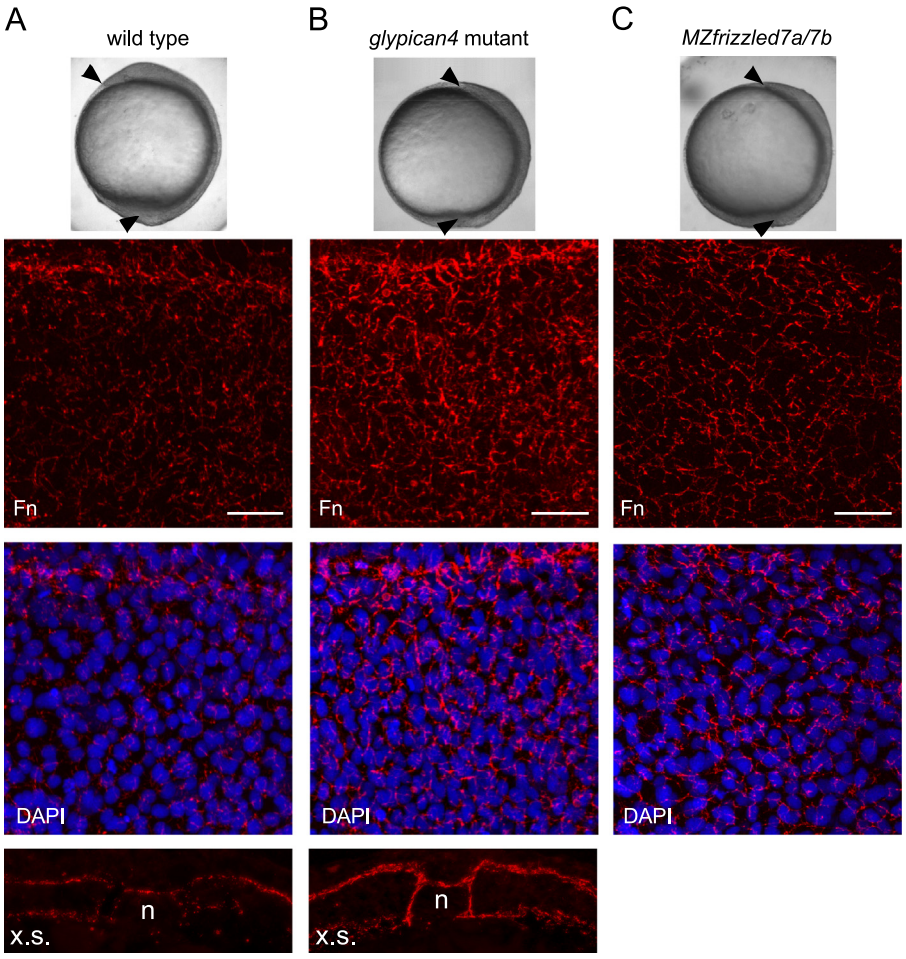


**Fig. 3.** *glypican4* and *frizzled7* mutant embryos have increased fibronectin assembly. (A–C) Top panels show morphological convergence and extension phenotypes at tailbud stage (see arrowheads) of wild type, *glypican4* mutant, and MZ*frizzled7a/7b* mutant embryos. Middle two panels show confocal images of fibronectin (Fn) immunolabeling without and with nuclear DAPI staining. Scale bars = 20 μm. The bottom images in (A) and (B) show Fn expression in cross-sections (x.s.) of tailbud stage embryos (n, notochord).

metalloproteinase activity and therefore increase fibronectin assembly. This concept was supported by our data showing that knockdown of *mmp14* increases total fibronectin protein levels as indicated by immunolabeling and western blot (Fig. 5A and B). However, in contrast to loss of Mmp14 function, *glypican4* mutant embryos have fibronectin protein levels that are comparable to wild type (Fig. 1D). In addition, as quantified using our established in vitro ECM substrate cleavage assay (Williams et al., 2012a), protein extracts isolated from *glypican4* mutant embryos have normal levels of protease activity towards gelatin and collagen IV substrates (Fig. 5C). These results indicate that increased fibronectin immunolabeling in *glypican4* mutant embryos is likely due to changes in fibrillogenesis and not fibronectin protein levels. Work on convergent extension movements during frog gastrulation has shown that changes in cadherin function and tissue tension regulate the formation and assembly of fibronectin fibrils (Dzamba et al., 2009). In zebrafish, both E-cadherin (Cdh1) and N-cadherin (Cdh2) are expressed during gastrulation and are capable of influencing convergence and extension (Babb et al., 2001; Babb and Marrs, 2004; Warga and Kane, 2007). To determine whether manipulation of cadherin function alters fibronectin assembly in zebrafish, we injected wild-type embryos with either *cdh2* synthetic mRNA or a validated *cdh2* MO (Lele et al., 2002). We chose *cdh2* because it is more broadly expressed throughout the late gastrula stage embryo than *cdh1* (Babb et al., 2001; Warga and Kane, 2007). Consistent with data from frog (Dzamba et al., 2009), our results demonstrate that ectopic Cdh2 expression leads to precocious fibril assembly while loss of Cdh2 function decreases fibronectin matrix assembly (Fig. 6A–C). This suggests that altered cadherin expression and intercellular adhesion might contribute to the fibronectin phenotype observed in *glypican4* and *frizzled7* mutant embryos.

### *glypican4 regulates fibronectin fibrillogenesis through cadherin-mediated cell adhesion*

To identify a mechanism whereby Glypican4 could impact cadherin function, we first examined *cadherin* gene expression. We performed semi-quantitative RT-PCR on wild type, *vangl2* mutant, and *glypican4* mutant embryos and found that for each mutant, the transcription of *cdh1*, *cdh2*, and *cdh5* is comparable to wild type (Fig. S4). We next examined cadherin protein expression by immunolabeling tailbud stage embryos using a pan-cadherin antibody predicted to recognize the C-terminus of Cdh1, Cdh2, Cdh4, and Cdh6. Of these, only *cdh1* and *cdh2* are expressed during early zebrafish embryonic development (Thisse et al., 2001). Plasma membrane cadherin labeling appeared increased in *glypican4* mutant embryos compared to wild-type controls (Fig. 7A–C). In contrast, *vangl2* mutant embryos had modestly decreased membrane cadherin expression as compared to wild type (Fig. 7C). Cadherin western blots on membrane fractionated protein samples corroborated these findings (Fig. 7D and E). Here, we detected a significant increase in cadherin protein localized to the membrane fraction of total protein isolated from *glypican4*

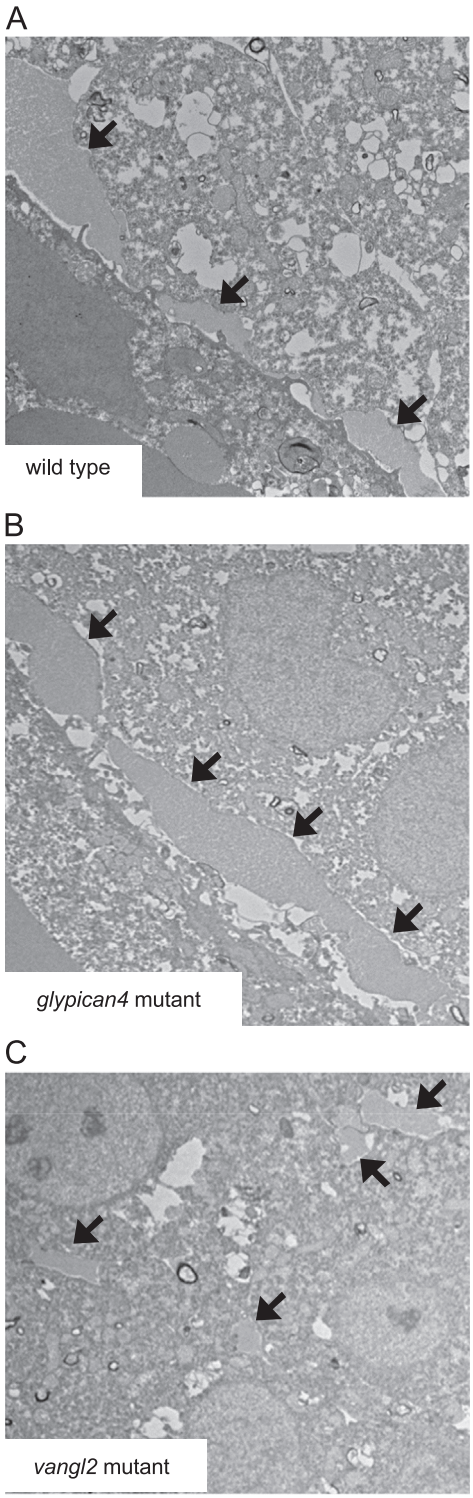


**Fig. 4.** *glypican4* and *vangl2* mutant embryos have distinct patterns of fibronectin organization. (A–C) Transmission electron microscopic images (4400× magnification) of tailbud stage wild type, *glypican4* mutant, and *vangl2* mutant embryos. Images are of thin cut cross-sections taken near the middle or equator of each embryo. Arrows denote the location of ECM (outlined with dashed circles).

mutant embryos and decreased membrane cadherin expression in *vangl2* mutant versus control embryos. To confirm whether Cdh2 contributes to the *glypican4* fibronectin phenotype, we injected *glypican4* mutant embryos with *cdh2* MO and assessed both pan-cadherin and fibronectin immunolabeling. Knockdown of *cdh2* expression in *glypican4* mutants consistently decreased plasma membrane cadherin labeling (Fig. 8A–C) and was sufficient to rescue the increased fibronectin fibrillogenesis phenotype (Fig. 8D–F). These results strongly suggest that deregulated plasma membrane cadherin expression is responsible for abnormal matrix assembly in zebrafish embryos with defective non-canonical Wnt/PCP signaling.

There is increasing evidence that cell–cell adhesion is a key regulator of tension and mechanical force during zebrafish gastrulation (Puech et al., 2005; Barone and Heisenberg, 2012; Maitre et al., 2012). In regards to matrix assembly, cadherin-mediated cell adhesion could create tension that is transmitted through integrins to bound fibronectin dimers thereby exposing cryptic sites and promoting fibril assembly across cell surfaces (Dzamba et al., 2009; Weber et al., 2011). Therefore, if our model is to be correct, cells isolated from tailbud stage *glypican4* mutant embryos should exhibit increased intercellular adhesion. We performed a hanging drop assay whereby cells from wild type or *glypican4* mutants were isolated by trypsin digestion and allowed to coalesce overnight. Repeated pipetting was used to gently dissociate the cells and the remaining clusters were counted. Our data show that *glypican4* mutant cells exhibit significantly more clustering than controls (Fig. 9A and B). Taken together, these data demonstrate that increased membrane cadherin expression and cell–cell adhesion are responsible for precocious fibronectin matrix assembly in *glypican4* mutant embryos.

## Discussion

### *Vangl2, Prickle1a, and regulation of fibronectin remodeling*

The identification of zebrafish with loss of function genetic mutations in *vangl2* has led to a detailed understanding of how this core PCP protein regulates cell polarity and directed migration in the gastrula embryo (Solnica-Krezel et al., 1996; Sepich et al., 2000, 2005; Jessen et al., 2002). However, it is unclear how Vangl2 interacts with other PCP proteins to promote PCP and gastrulation cell movements. Prickle is a LIM and PET domain-containing cytoplasmic PCP protein that is also required for polarity in fly epithelia (Gubb et al., 1999; Tree et al., 2002; Strutt and Strutt, 2007). Here, a physical interaction between Van Gogh and Prickle is considered an important event in regulating asymmetric PCP protein expression and establishing cell polarity (Bastock et al., 2003; Jenny et al., 2003). In zebrafish, knockdown of *prickle1a* using MOs causes a convergence and extension phenotype reminiscent of *vangl2* mutant embryos (Carreira-Barbosa et al., 2003; Veeman et al., 2003). Notably, *prickle1a* knockdown in a *vangl2*-null mutant background causes a stronger convergence and extension defect (Carreira-Barbosa et al., 2003) indicating that while Vangl2 and Prickle1a are both required for gastrulation cell movements, their functions are not entirely redundant. We have previously reported that loss of Vangl2 function increases Mmp14-mediated proteolysis of ECM substrates including fibronectin (Williams et al., 2012a). Our data now show that similar to *vangl2* mutant embryos, *prickle1a* morphants exhibit reduced fibronectin protein levels and we hypothesize that this is due, at least in part, to Prickle1a-dependent effects on Vangl2. We propose that Prickle1a is required to stabilize cell surface Vangl2 expression, and without Prickle1a, Vangl2 is internalized and degraded by an as yet unknown mechanism. Therefore, similar to the situation in the fly wing, loss of Prickle1a during zebrafish gastrulation would result in reduced Vangl2 expression, membrane localization, and function.

Our data show that similar to loss of zebrafish *prickle1a*, overexpression of *prickle1a* mRNA also decreases Vangl2 total protein levels as indicated by western blot. While this contrasts

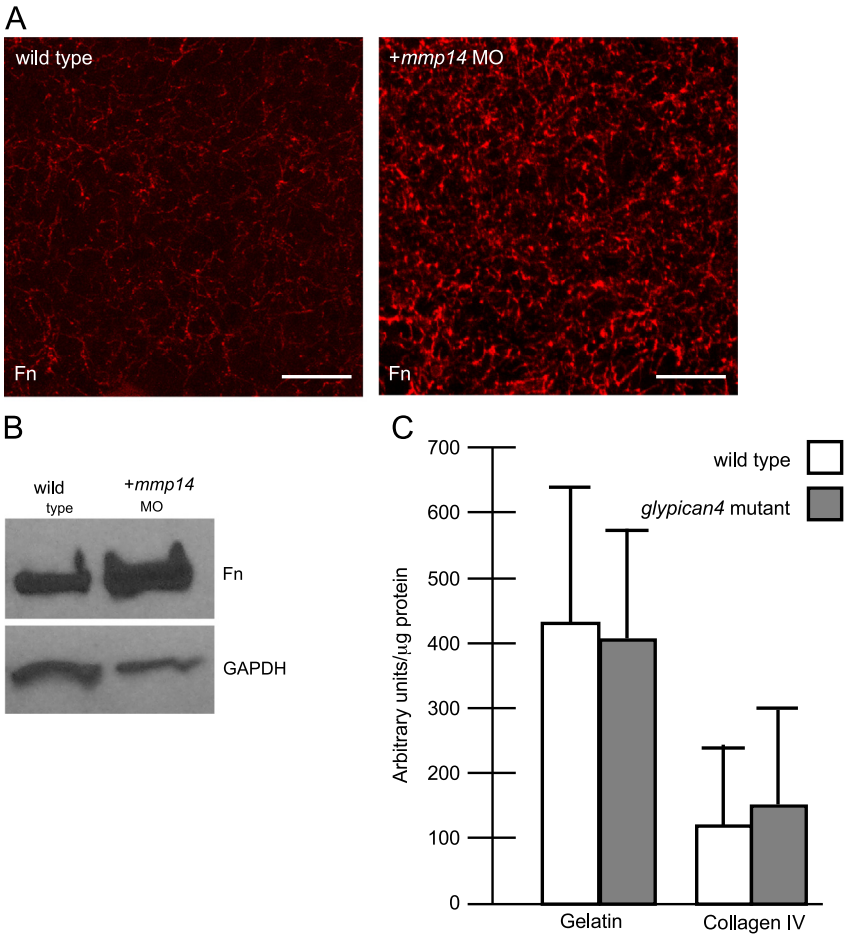


**Fig. 5.** *glypican4* mutant embryos have normal proteolytic activity. (A) Confocal images of fibronectin (Fn) expression in tailbud stage wild type and *mmp14* morphant embryos. Scale bars = 20 μm. (B) Fn and GAPDH western blot of 10 μg total protein extract obtained from wild type and *mmp14* morphant embryos. (C) Quantification of proteolytic activity in wild type and *glypican4* mutant protein extracts incubated with either gelatin or collagen IV fluorogenic substrates. Here, triplicate experiments were performed using three pools (50 embryos per pool) of total protein prepared from tailbud stage wild type or *glypican4* mutant embryos.

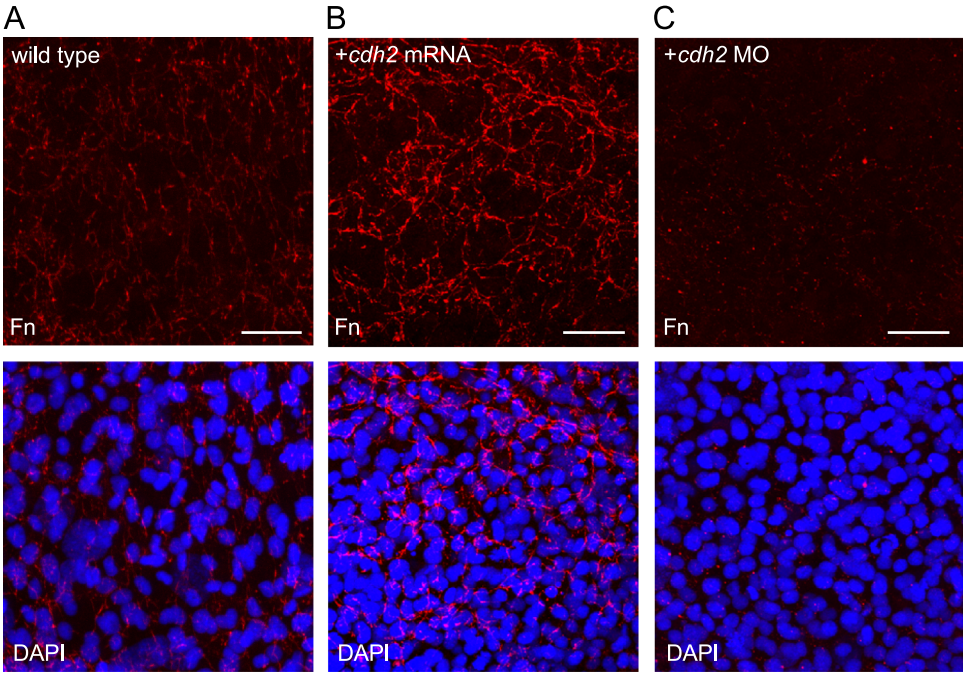


**Fig. 6.** Manipulating Cdh2 expression alters fibronectin fibrillogenesis during gastrulation. (A–C) Confocal images of fibronectin (Fn) expression (without or with nuclear DAPI staining) in wild type, synthetic *cdh2* mRNA (200 pg) injected, and *cdh2* morphant embryos. Scale bars = 20 μm.

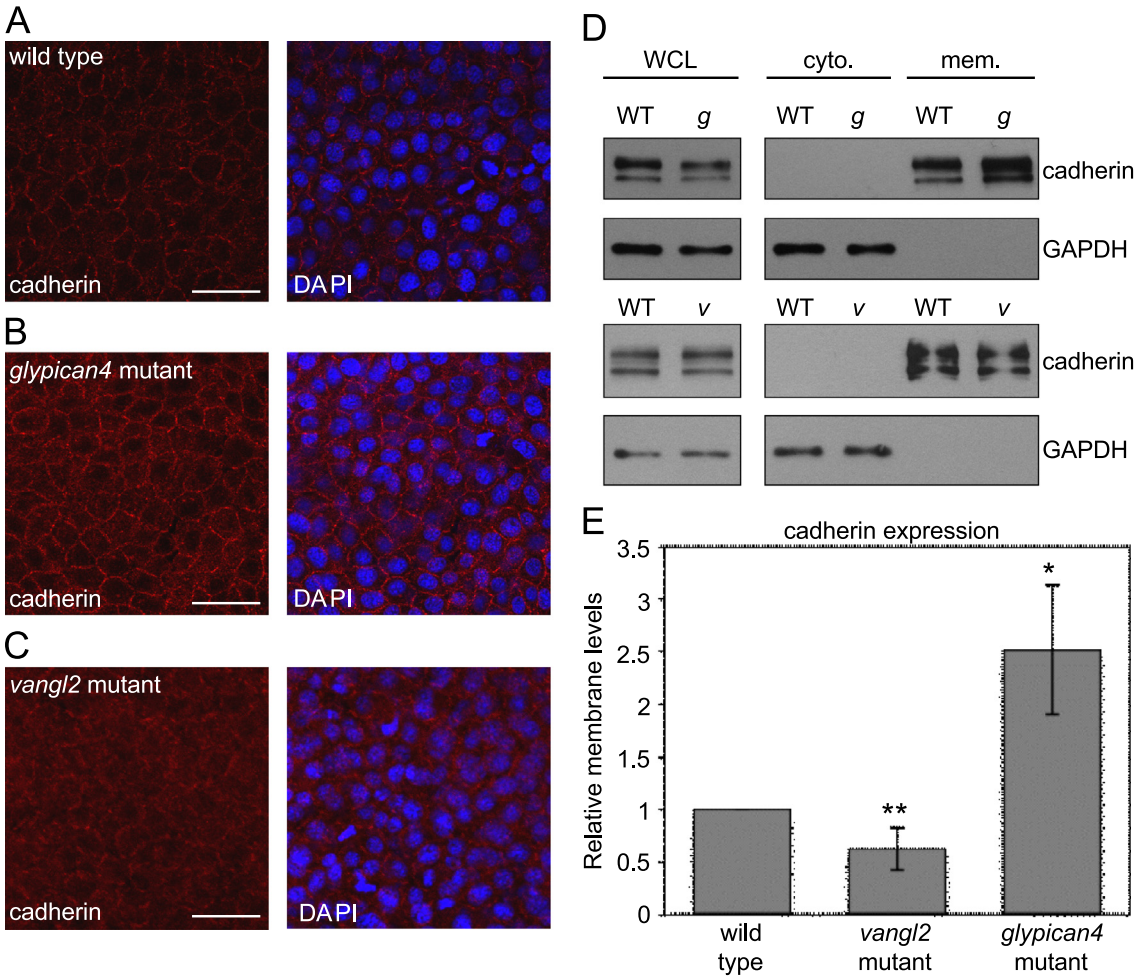


**Fig. 7.** *Glypican4* mutant embryos have increased levels of membrane cadherin protein. (A–C) Confocal images of cadherin expression (without or with nuclear DAPI staining) in tailbud stage wild type, *glypican4* mutant, and *vangl2* mutant embryos. Scale bars=20 μm. (D) Western blot analysis of cadherin expression in embryonic cytoplasmic (cyto.) and membrane (mem.) protein fractions. Cadherin and GAPDH expression in whole cell lysates (WCL) prior to fractionation are also shown. WT, *g*, and *v* denote protein extracts obtained from wild type, *glypican4* mutant, and *vangl2* mutant embryos, respectively. (E) Quantification of western blot fractionation data from triplicate experiments displayed as relative membrane cadherin expression normalized to GAPDH levels in WCL ($^{**}p < 0.02$, $^{*}p < 0.009$).

data from fly demonstrating that overexpression of *prickle* increases apicolateral accumulation of a Van Gogh (Strabismus)–YFP fusion protein (Bastock et al., 2003), these results are consistent with data showing that injection of *prickle1a* mRNA does not rescue the *vangl2* mutant phenotype (unpublished observations). Current data suggest that the amount or activity of core PCP proteins is regulated by feedback loops whereby the formation of protein complexes at the cell surface protects these proteins from degradation (Tree et al., 2002; Strutt et al., 2013). We propose that in zebrafish, both gain and loss of Prickle1a disrupts such a feedback mechanism with the end results being disruption of Vangl2 function. However, further work is needed to understand the specific molecular and cellular consequences of excess Prickle1a and to determine whether Prickle1a overexpression impacts the subcellular and perhaps asymmetric localization of zebrafish Vangl2.

### *Non-canonical Wnt signaling and regulation of cadherin-mediated cell adhesion*

Inhibition of Wnt11 in the frog embryo disrupts matrix assembly causing fibronectin to localize at cell boundaries rather than across cell surfaces (Dzamba et al., 2009). In this previous study, it was shown that non-canonical Wnt signaling alters fibrillogenesis through effects on the downstream proteins Disheveled and Rho family small GTPases. In contrast, our data show that inhibition of Wnt signaling in *glypican4* and MZ *frizzled7a/7b* mutant zebrafish embryos results in precocious fibronectin assembly during gastrulation. Accordingly, while the study by Dzamba et al. demonstrates that interference with Wnt11 signaling causes decreased cadherin mediated adhesion (Dzamba et al., 2009), our data show that in zebrafish embryos loss of Wnt signaling results in increased cell surface cadherin expression and cell adhesion. Therefore, it is now clear that during gastrulation, changes in cadherin function (positive or negative) directly impact matrix assembly required for convergence and extension cell movements. Though we have shown that *cdh2* mRNA overexpression is sufficient to induce fibrillogenesis and *cdh2* knockdown can suppress fibronectin assembly during gastrulation, our study does not identify the cadherin whose membrane expression is increased in *glypican4* mutant embryos. While Cdh2-dependent cell adhesion has been specifically linked to the directed migration of lateral mesodermal cell populations (von der Hardt et al., 2007), both Cdh1 and Cdh2 regulate zebrafish convergence and extension movements (Babb et al., 2001; Babb and Marrs, 2004; Warga and Kane, 2007) and are thus capable of influencing ECM assembly.

Because we did not detect changes in total cadherin protein expression, our data suggest that trafficking of plasma membrane cadherin might be disrupted in *glypican4* mutant embryos. The ability of PCP proteins to regulate Cdh1 expression through effects on membrane trafficking has been reported in a variety of

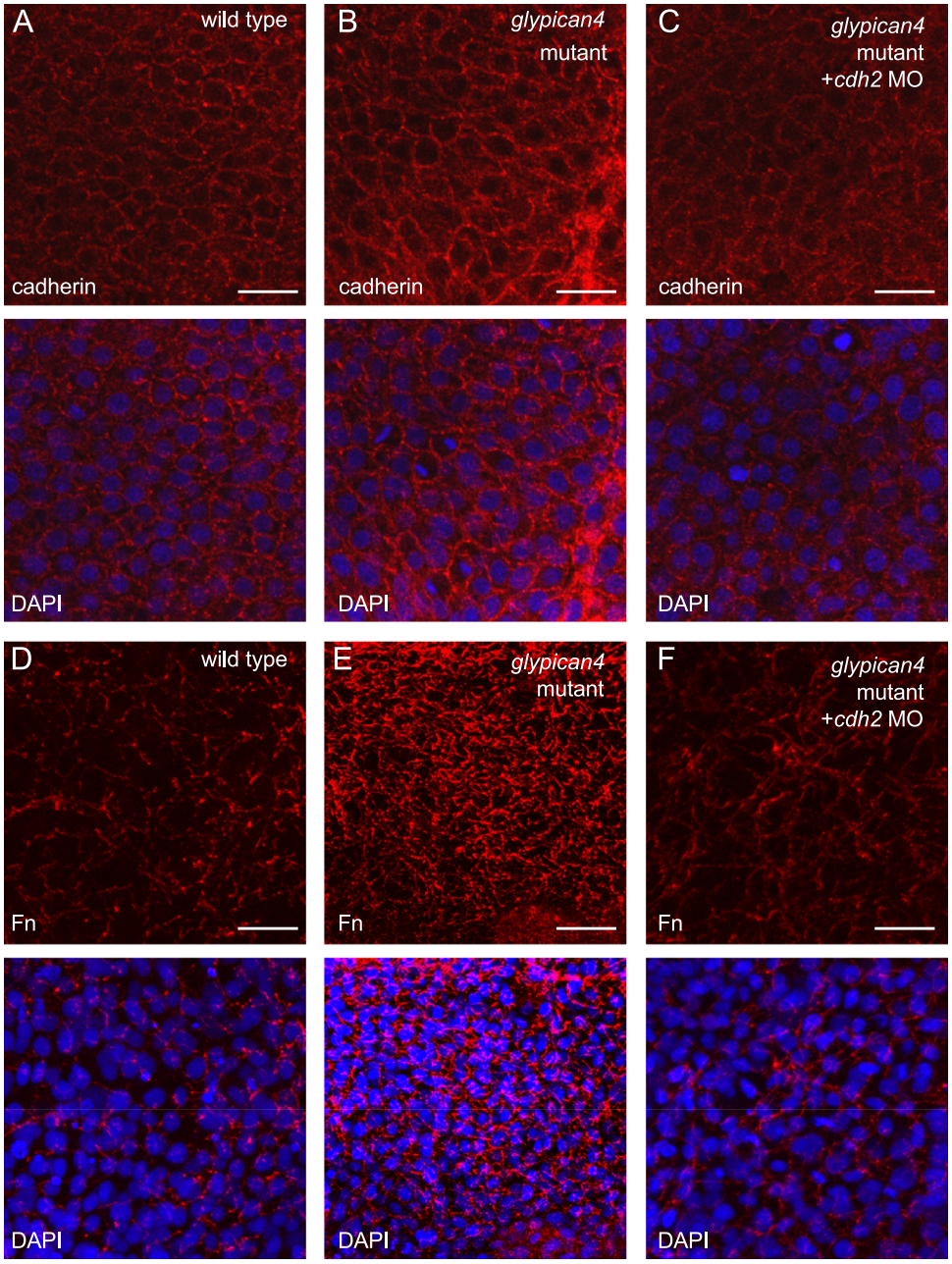


**Fig. 8.** Knockdown of *cdh2* suppresses the fibronectin phenotype in *glypican4* mutant embryos. Confocal images of cadherin (A–C) or fibronectin (Fn) (D–F) expression (with or without nuclear DAPI staining) in tailbud stage wild type, *glypican4* mutant embryos, and *glypican4* mutants injected with *cdh2* MO. Scale bars = 20 μm.

contexts (Classen et al., 2005; Ulrich et al., 2005; Dzamba et al., 2009; Chacon-Heszele et al., 2012). For example, Wnt11 was shown to influence Cdh1 endocytosis through a mechanism requiring Rab5c (Ulrich et al., 2005). However, it is unclear how non-canonical Wnt signaling might regulate cell surface levels of Cdh2. Recent data show that p120-catenin regulates Cdh2 by inhibiting its endocytosis (Kowalczyk and Nanes, 2012) or affecting its adhesive binding function (Gumbiner, 2005). Notably, p120-catenin binds cadherins (Kowalczyk and Nanes, 2012) and the Disheveled-interacting protein Frodo/Dapper (Cheyette et al., 2002; Gloy et al., 2002), and it was shown that Wnt signaling through Disheveled regulates p120-catenin stability (Park et al., 2006). Moreover, p120-catenin and Dapper2 function are linked to the regulation of gastrulation movements in frog and fish, respectively (Paulson et al., 1999; Waxman et al., 2004). These data suggest that Wnt signaling could affect p120-catenin function to control Cdh2 and ECM assembly.

### *PCP in migrating gastrula cells–Roles for proteolysis and cell adhesion*

It has become clear that the collective movement of polarized cell populations (during embryogenesis and cancer) requires coordinated regulation of cell–matrix interactions, proteolytic ECM remodeling, and cell–cell adhesion (Friedl and Gilmour, 2009). During collective migration, integrins function as transmembrane receptors for ECM proteins such as fibronectin while proteases cleave ECM. Rapid remodeling of cadherin-mediated cell adhesion allows dynamic changes in cell coupling while maintaining coherent movement of cell populations (Friedl and Gilmour, 2009). However, despite advances in the characterization of integrins, Mmp14, and cadherins during collective cell movement, their molecular relationship with PCP proteins is unclear. During zebrafish gastrulation, convergence and extension cell movements of both ectodermal and mesodermal cells require

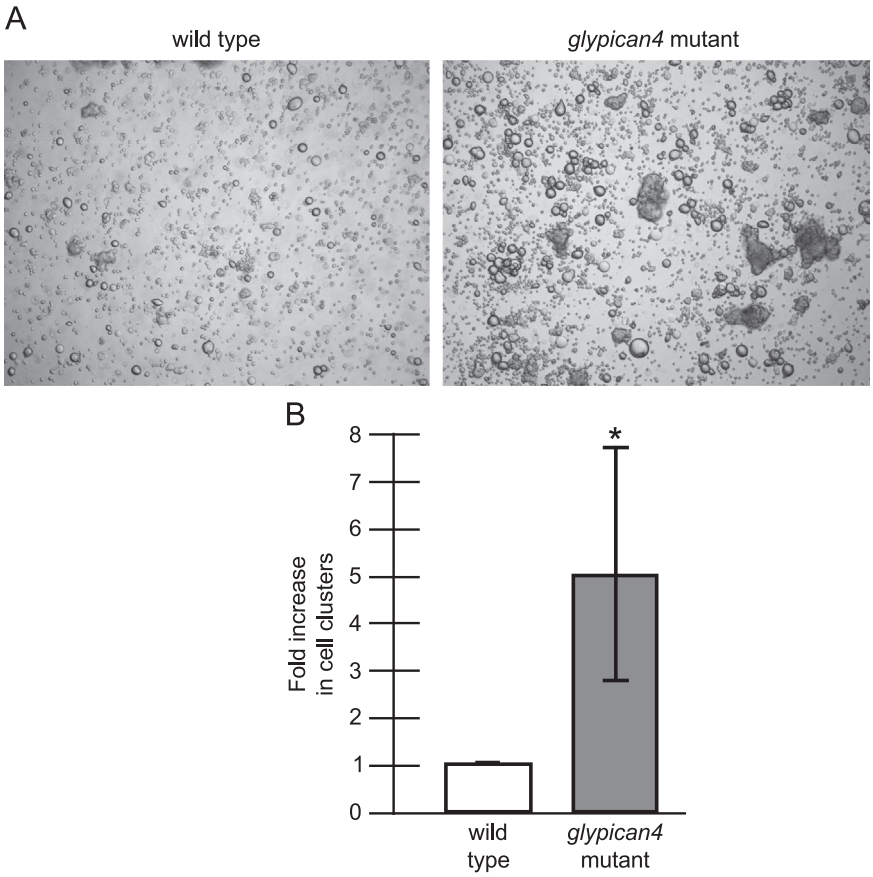


**Fig. 9.** *glypican4* mutant embryos have increased intercellular adhesion. (A) Representative 10× images of hanging drop cellular clusters from wild type and *glypican4* mutant embryos after repeated pipetting and dissociation. (B) Quantification of the fold increase in cell clusters in *glypican4* mutants (*$p < 0.05$). This assay was performed in triplicate, and five 10× images were analyzed per experiment.

PCP, and each of these germ layers is associated with a layer of fibronectin matrix (Latimer and Jessen, 2010). Unlike cancer progression, where migrating tumor cells must utilize membrane-tethered matrix metalloproteinases to degrade ECM and facilitate invasion of host tissues (Hotary et al., 2000, 2006; Sabeh et al., 2004), fibronectin does not represent a physical barrier to cell migration in the zebrafish gastrula. Here, we suggest that PCP-dependent regulation of cell surface Mmp14 activity provides a mechanism to influence cell–matrix interactions underlying the formation of membrane protrusions. Increased Mmp14 proteolysis of fibronectin in *vangl2* mutant embryos (Williams et al., 2012a) is predicted to reduce cell–matrix adhesion and increase membrane protrusive activity. Indeed, we know that Mmp14 regulates PCP and that *vangl2*-null mesodermal cells form inappropriate membrane protrusions that are not oriented along the path of directed migration (Jessen et al., 2002; Coyle et al., 2008). Vangl2-dependent Mmp14 endocytosis (Williams et al., 2012a) is therefore predicted to decrease fibronectin cleavage and increase or stabilize cell–matrix interactions. In the frog gastrula, increased cell–matrix interactions are necessary to suppress inappropriate membrane protrusive activity (Davidson et al., 2006). It is also important to note that Mmp14 has non-ECM protein cleavage substrates capable of impacting cell migration including cadherins (Covington et al., 2006). Our data indicate that *vangl2* mutant embryos have decreased membrane cadherin protein expression and future work will determine whether this is due to increased Mmp14 proteolytic activity.

Current models predict that opposing or antagonistic interactions between Vangl2/Prickle and non-canonical Wnt signaling regulate gastrulation convergence and extension cell movements (Roszko et al., 2009). However, mechanisms underlying the complex relationship between these PCP proteins remain undefined. Embryos double mutant for *vangl2* and either *wnt11* or *glypican4* exhibit almost synergistic PCP phenotypes (Heisenberg and Nusslein-Volhard, 1997; Marlow et al., 1998) suggesting that these proteins use distinct signaling mechanisms and downstream effector proteins. Unlike the loss of fibronectin observed in *vangl2* and *prickle1a* morphant embryos, we have shown that *glypican4* and *frizzled7* mutants have increased ECM assembly. As demonstrated, this latter phenotype is unlikely due to effects on Mmp14 activity but rather due to increased cadherin-mediated cell adhesion and tissue tension. We propose that Wnt signaling regulates the formation of polarized membrane protrusions in part by inhibiting cell adhesion at specific cellular domains. This idea is supported by data showing that protrusive activity is suppressed near sites of increased cadherin adhesion (Desai et al., 2009; Borghi et al., 2010; Theveneau et al., 2012). Furthermore, data obtained from frog explants demonstrates that differential cell adhesion can drive polarized protrusive activity and affect tissue tension required for ECM assembly (Dzamba et al., 2009; Weber et al., 2012).

## Conclusions

Our data demonstrate that Vangl2 and its binding partner Prickle1a are required for fibronectin assembly while Glypican4/Frizzled7-mediated non-canonical Wnt signaling antagonizes fibrillogenesis. Vangl2 and Glypican4 are key regulators of PCP and despite their similar loss of function morphological phenotypes the relationship between these proteins and the molecular

mechanisms whereby they control cell migration are unclear (Marlow et al., 1998; Topczewski et al., 2001; Jessen et al., 2002). This study shows for the first time that Vangl2 and Glypican4 regulate distinct downstream signaling events that, despite their differential effects on fibronectin organization, are both essential for PCP and convergence and extension cell movements. We propose that during zebrafish gastrulation, PCP-dependent integration of cell–matrix and cell–cell interactions might function to coordinate the directed migration of polarized cell populations.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.08.027.

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