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Zebrafish Prickle, a Modulator of Noncanonical Wnt/Fz Signaling, Regulates Gastrulation Movements

Michael T. Veeman,[1,2] Diane C. Slusarski,[3] Ajamete Kaykas,[1] Sarah Hallagan Louie,[1] and Randall T. Moon[1,*]
[1]Howard Hughes Medical Institute
Department of Pharmacology
Center for Developmental Biology
[2]Molecular and Cellular Biology Program
University of Washington School of Medicine
Seattle, Washington 98195
[3]Department of Biological Sciences
University of Iowa
Iowa City, Iowa 52242

Summary

In addition to the canonical Wnt/β-catenin signaling pathway, at least two noncanonical Wnt/Fz pathways have been described: the planar cell polarity (PCP) pathway in *Drosophila* [1] and the Wnt/calcium pathway in vertebrate embryos [2]. Recent work suggests that a vertebrate pathway homologous to the PCP pathway acts to regulate the convergent extension movements of gastrulation [3–7]. To further test this hypothesis, we have identified two zebrafish homologs of the *Drosophila* PCP gene *prickle* (*pk*) [8], both of which show discrete and dynamic expression patterns during gastrulation. Both gain and loss of *pk1* function cause defects in convergent extension. Pk1 localizes to both the cytoplasm and the cell membrane, and its normal localization is partially dependent on its C-terminal prenylation motif. At the cell membrane, Pk1 is frequently localized asymmetrically around the cell and can colocalize with the signaling molecule Dishevelled (Dsh). In overexpression assays, Pk1 is able to activate AP-1-mediated transcription and inhibit activation of Wnt/β-catenin signaling. Like noncanonical Wnts [9, 10], overexpression of Pk1 increases the frequency of calcium transients in zebrafish blastulae. Our results support the idea that a vertebrate PCP pathway regulates gastrulation movements and suggest that there is overlap between the PCP and Wnt/calcium pathways.

Results and Discussion

Zebrafish *pk* Sequence and Expression

In *Drosophila*, *pk* functions as part of a cassette of genes including *frizzled* (*fz*), *dishevelled* (*dsh*), and *strabismus* (*stbm*) to control the planar cell polarity of epithelial structures [1]. We have cloned two zebrafish *pk* genes, *prickle1* (*pk1*) and *prickle2* (*pk2*), which share the highly conserved PET and triple LIM domains of all *pk* homologs (Figure 1A) [8]. We note that the C termini of these and other Pk proteins contain a consensus signal for prenylation.

At the beginning of gastrulation, *pk1* RNA is expressed on the future dorsal side of the embryo (Figure 1B). By the 100% epiboly stage, *pk1* RNA is expressed strongly in axial tissue, but there is considerable lateral expression as well (Figure 1C). This expression is in both the ectoderm and the mesendoderm (Figure 1C, inset), but it is excluded from the most anterior lateral tissue. During somitogenesis, *pk1* is expressed most strongly in the posterior nonaxial mesoderm (Figures 1D–1H), but, by 24 hr, it is restricted to the lens of the eye (Figure 1I). Zebrafish *pk1* expression is reminiscent of that recently reported for a *Xenopus prickle* homolog [11]. It is also quite similar to zebrafish *wnt11* (*silberblick*) expression [4].

pk2 RNA is also localized to the dorsal side at the beginning of gastrulation, though less dramatically than *pk1* (Figure 1J). During gastrulation, *pk2* is expressed specifically in the axial mesoderm and around the blastopore (Figure 1K and inset). At the 6-somite stage, it is still expressed most strongly in the axial mesoderm and more diffusely in the neural plate (Figures 1L–1N). Quite broad and diffuse mesodermal and neural expression persists through the 18-somite (Figures 1O and 1P) and 24-hr (Figure 1Q) stages. The early notochord-specific expression of *pk2* is similar to that reported for two ascidian *pk* homologs cloned as targets of Brachyury [12]. Interestingly, there is partially complementary expression during early somitogenesis between *pk1*, which is strongest in the nonaxial mesoderm and the anterior tailbud, and *pk2*, which is strongest in the axial mesoderm and the posterior tailbud (compare Figure 1F with Figure 1N). While both *pk1* and *pk2* are expressed in tissues that undergo profound morphogenetic movements, *pk1* is much more widely expressed in early gastrulation (compare Figure 1C with Figure 1K).

pk1 Regulates Convergent Extension Movements

After showing that they reduce translation of their respective targets in vitro (Figure S1A), we next used morpholino antisense oligonucleotides [13] to reduce the function of *pk1* and *pk2*. Injection of the first *pk1* morpholino, pk1aMO, causes severe morphogenetic defects; the notochord and neural plate are short and wide at the 1-somite stage, and somitic and neural markers are compressed in the anteroposterior dimension and are widened mediolaterally at the 6-somite and 12-somite stages (Figures 2G, 2L, and 2Q). Early dorsoventral patterning is normal, as assayed by *goosecoid* expression at the shield stage (Figure 2B). There are no obvious effects on anteroposterior patterning in the neural tube, as assayed by *opl*, *pax2.1*, and *krox20* expression (Figures 2L and 2Q and data not shown). All neural and mesodermal markers tested show normal anteroposterior and dorsoventral patterning, except for the compression and widening of these markers, which are characteristic of a defect in convergent extension. Morphogenesis of the prechordal plate appears relatively normal (Figure 2G, inset), as seems reasonable given that *pk1* expression is excluded from the most anterior tissue. Overexpression of *pk1* RNA is also able to disturb morphogenetic

*Correspondence: rtmoon@u.washington.edu

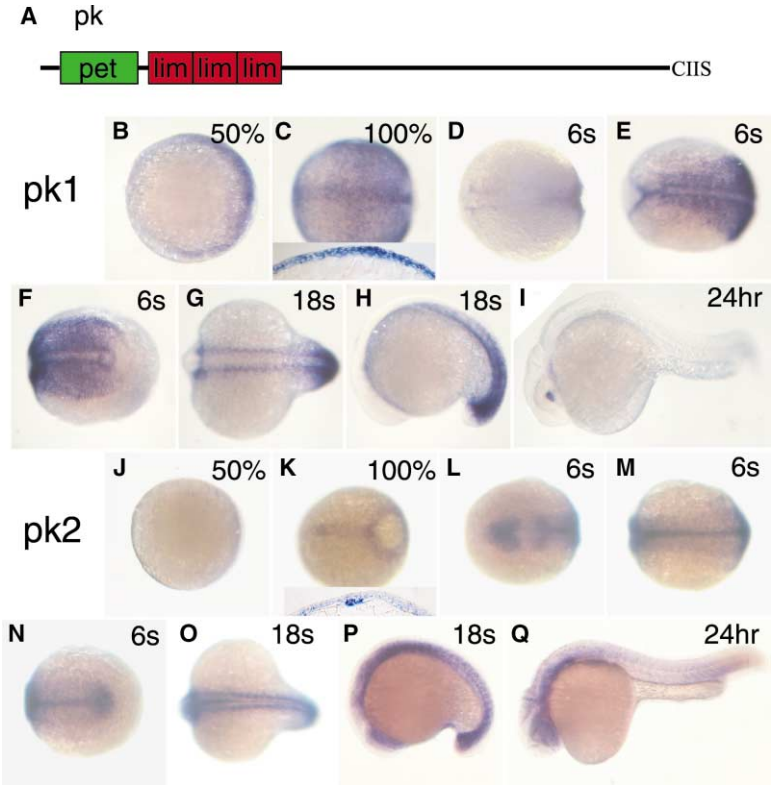

Figure 1. Domain Organization and Expression of Zebrafish *Prickle* Genes

(A) A cartoon of domains conserved in *pk* homologs. CIIS indicates the C-terminal tetrapeptide found in *Drosophila* and zebrafish *pk* genes that is a consensus motif for prenyl modification by farnesyl.

(B–Q) In situ hybridizations for (B–I) *pk1* and (J–Q) *pk2*. (B) and (J) are animal pole views at the shield stage; dorsal is oriented toward the right. (C) and (K) are dorsal views at 100% epiboly; anterior is oriented toward the left. The insets show a cross-section. (D) and (L) are anterior views at the 6-somite stage; dorsal is oriented toward the right. (E) and (M) are dorsal views at the 6-somite stage; anterior is oriented toward the left. (F) and (N) are posterior views at the 6-somite stage; dorsal is oriented toward the left. (G) and (O) are dorsal views at the 18-somite stage; anterior is oriented toward the left. (H) and (P) are lateral views at the 18-somite stage; anterior is oriented toward the left. (I) and (Q) are lateral views at 24 hr; anterior is oriented toward the left.

movements, and embryos with twisted notochords, malformed somites, and greatly widened posterior neural markers are frequently generated (Figures 2J, 2O, and 2T). The effect of *pk1* RNA overexpression is quite variable, and the prechordal plate is sometimes affected (Figure 2J, upper and lower insets).

The pk2aMO morphant phenotype is much less severe than pk1aMO (Figures 2C, 2H, 2M, and 2R). There is a very mild broadening of neural and mesodermal markers at the 6-somite stage, but it appears relatively normal at the 1-somite and 12-somite stages. The pk1aMO/pk2aMO combination is slightly more severe than pk1aMO alone, but it does not reveal any dramatic redundancy between *pk1* and *pk2* (Figures 2D, 2I, 2N, and 2S). A second *pk2* morpholino, pk2bMO, is similarly mild in phenotype (data not shown), so we focused on *pk1* for more detailed studies.

To confirm the validity of the pk1aMO phenotype, we designed a second nonoverlapping morpholino. pk1bMO causes a similar but milder phenotype, but only at higher doses and in a less penetrant fashion (data not shown). Most convincingly, moderate doses of pk1bMO show synergy with low doses of pk1aMO and give a more severe phenotype. Only 5% ($n = 63$) of embryos injected with 600 pg pk1aMO, and no ($n = 51$) embryos injected with 5 ng pk1bMO, showed a strong phenotype, whereas 44% ($n = 61$) of embryos coinjected with 600 pg pk1aMO and 5 ng pk1bMO showed a strong phenotype.

To confirm that a defect in cell movements underlies the *pk1* morphant phenotype, we performed gastrula transplant experiments to follow the movements of groups of labeled cells in wild-type and pk1aMO embryos. Wild-type cells transplanted dorsally into wild-type embryos converged and extended dramatically along the A-P axis of the embryo (Figure 2U). As predicted, comparable transplants of pk1aMO cells into pk1aMO hosts showed much less convergent extension (Figure 2X).

pk1aMO cells transplanted into wild-type hosts converged and extended relatively normally (data not shown), but it was unclear in this assay if the transplanted cells were moving actively or being passively moved by the surrounding wild-type cells. We thus examined cell shape during gastrulation as a more cell-intrinsic property. Dorsal ectodermal cells are largely mediolaterally elongated and aligned with one another in wild-type embryos (Figure 2V), but they are much less elongated and aligned in pk1aMO embryos (Figure 2Y), as has also been described for the convergent extension mutation *trilobite*/Strabismus [5]. pk1aMO cells transplanted into wild-type hosts are predominantly mediolaterally elongated (red cells in Figure 2W), whereas wild-type cells transplanted into pk1aMO hosts are more symmetrical and disarrayed (red cells in Figure 2Z). This suggests that the pk1aMO defect is not autonomous to single cells, although we cannot exclude that there could be cell autonomous effects in some other assay or region of the embryo.

Pk Subcellular Localization

It has been suggested that the main function of *Drosophila* Pk is to exclude Dsh from the proximal cell cortex [14]. That interpretation, however, relied on a heterologous assay in cultured osteosarcoma cells in which Wnt and Fz responses have not been characterized and in which the significance of Dsh localization is unclear.

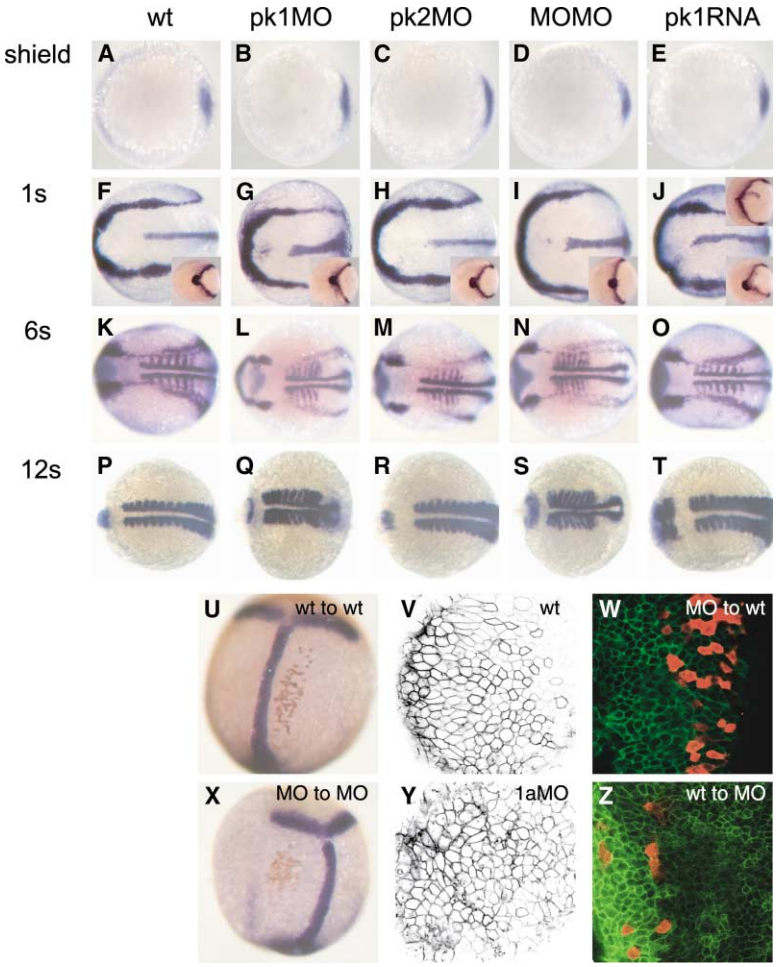

Figure 2. Pk1 Is Required for Convergent Extension

(A–T) In situ marker analysis of *pk* loss and gain of function. (A), (F), (K), and (P) are uninjected control embryos. (B), (G), (L), and (Q) are injected with the pk1aMO morpholino. (C), (H), (M), and (R) are injected with pk2aMO. (D), (I), (N), and (S) are injected with both pk1aMO and pk2aMO. (E), (J), (O), and (T) are injected with *pk1* RNA. (A–E) Expression of *gsc* in the dorsal organizer at the germ ring stage. Animal pole view; dorsal is oriented toward the right. (F–J) Expression of *dlx3* surrounding the neural plate and *ntl* in the presumptive notochord at the 1-somite stage. Dorsoanterior view; anterior is oriented toward the left. The inset is an anterior view of *CatL* expression in the anterior prechordal plate together with *dlx3* to mark the neural plate. (K–O) Expression of *dlx3*, *pax2.1*, and *myod* at the 6-somite stage. A dorsal view; anterior is oriented toward the left. *dlx3* surrounds the neural plate; *pax2.1* marks the midbrain/hindbrain boundary and is also expressed in lateral mesoderm. *myod* marks the somites and adaxial cells flanking the notochord. (P–T) Expression of *krox20* and *myod* in 12-somite stage embryos. A dorsal view; anterior is oriented toward the left. *myod* marks the somites. *krox20* marks rhombomeres 3 and 5, but only one stripe is visible in this perspective.

(U–Z) (U and X) Analysis of cell movements by transplantation of small groups of labeled cells from (U) wild-type donors to wild-type hosts and (X) morpholino donors to morpholino hosts. Blue staining indicates in situ hybridization for *pax2.1* and *ntl*, to orient the embryos. Brown staining indicates the transplanted cells. In three independent experiments, pk1aMO donor to pk1aMO host transplants consistently showed less extension along the A-P axis than comparably located transplants of wild-type cells in wild-type hosts. (V and Y) Membrane-targeted GFP localization (black) to visualize cell shape in (V) wild-type and (Y) pk1aMO embryos. (W) Red-labeled pk1aMO cells transplanted into membrane GFP-labeled wild-type hosts. (Z) Red-labeled wild-type cells transplanted into membrane GFP-labeled pk1aMO hosts. (V), (W), (Y), and (Z) are all oriented with anterior toward the top.

We have examined the subcellular localization of Pk1 and its potential effects on Dsh by expressing epitope-tagged constructs in *Xenopus* animal caps. In addition to having large and uniform cells, Dsh localization has been extensively characterized in these explants. GFP-tagged Pk1 is found diffusely in the cytoplasm, at the cell membrane, and in cytoplasmic and perinuclear punctae (Figure 3A). HA-tagged Pk1 shares the cortical and diffuse cytoplasmic staining of the GFP-tagged construct but lacks the punctate staining; this finding suggests that the punctate staining is an artifact of the GFP tag (data not shown). A mutant Pk1 with the prenylation motif deleted still localizes to the membrane, but it also shows increased cytoplasmic staining and bright nuclear localization (Figure 3D). We have not observed any change in Pk1 localization when coexpressed with a variety of Wnts and Frizzleds, nor have we seen any nonautonomous effects at the interfaces of cells expressing Pk1 juxtaposed with cells expressing Wnts or Frizzleds (data not shown).

In *Xenopus*, coexpression of Fz1 or Fz7 can relocalize DshGFP from cytoplasmic punctae to the cell membrane [15, 16]. Coexpression of Pk1 does not alter DshGFP localization in the presence or absence of Fz7 (compare Figures 3B with 3E and 3C with 3F). We used approximately stoichiometric levels of untagged *pk1* RNA in this experiment to ensure that the tag did not interfere with Pk1 function. This is at odds with the finding that *Drosophila* Pk can block the Fz-dependent translocation of Dsh to the membrane in an osteosarcoma cell line [14]. It is entirely consistent, however, with the observation that *Drosophila pk* mutation and overexpression only perturb the asymmetric localization of Dsh within the plane of the membrane, and not its initial recruitment to the membrane [14].

We observed that both Pk1GFP and membrane-associated Dsh are often irregularly localized around the cell periphery. Two-color localization experiments with Pk1GFP and DshHA in the presence of untagged Fz1 reveal substantial colocalization of Pk1 and Dsh (Figures 3G–3I). This supports the idea that Pk and Dsh are involved in a common process and is reminiscent of the early phase in *Drosophila* pupal wing development in which Pk and Dsh initially colocalize at the membrane

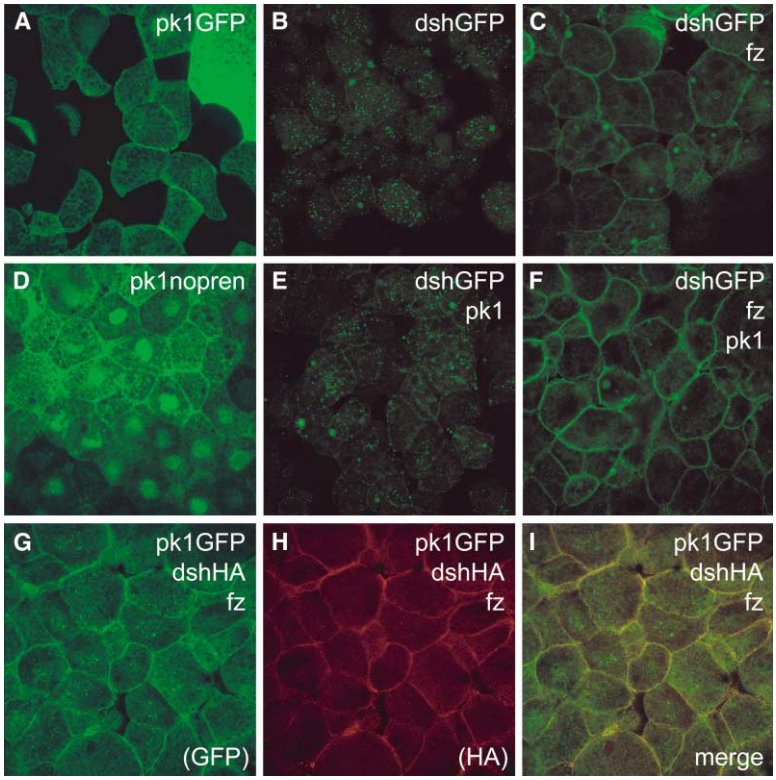

Figure 3. Pk1 Subcellular Localization in *Xenopus* Animal Caps

(A) Localization of Pk1GFP.
(B) Localization of DshGFP.
(C) Localization of DshGFP in an animal cap coexpressing DshGFP and untagged Fz7.
(D) Localization of Pk1noprenGFP (lacks prenylation motif).
(E) Localization of DshGFP in an animal cap coexpressing DshGFP and untagged Pk1.
(F) Localization of DshGFP in an animal cap coexpressing DshGFP, untagged Fz7, and untagged Pk1.
(G–I) An animal cap coexpressing DshHA, Pk1GFP, and Fz1. (G) Localization of Pk1GFP. (H) Localization of DshHA. (I) A merged image of Pk1GFP (green) and DshHA (red).

before later resolving into complementary domains [14]. It is also consistent with the proposed physical interaction of Pk and Dsh [16, 17].

Signaling Assays

We next tested zebrafish Pk1 in overexpression assays for the known Wnt/Frizzled-mediated signaling pathways. Activation of Jun N-terminal Kinase (JNK) has been implicated in PCP signaling [18–21]. We used an AP-1 reporter plasmid in 293T cells as a readout for JNK activation [22]. Pk1 can activate AP-1 activity approximately 4-fold, which is less than LMP-1, a strong viral activator of JNK, but similar to what we observe with Dsh (Figure 4A). Mutant Pk1 that lacks the prenylation motif is still able to activate AP-1 (Figure 4A). The actual role of JNK signaling is somewhat unclear in both *Drosophila* planar cell polarity and vertebrate convergent extension, but these results show a functional correlation between Pk1 and other components of PCP signaling.

In vertebrate embryos, noncanonical Wnt signaling has been proposed to involve intracellular calcium re-

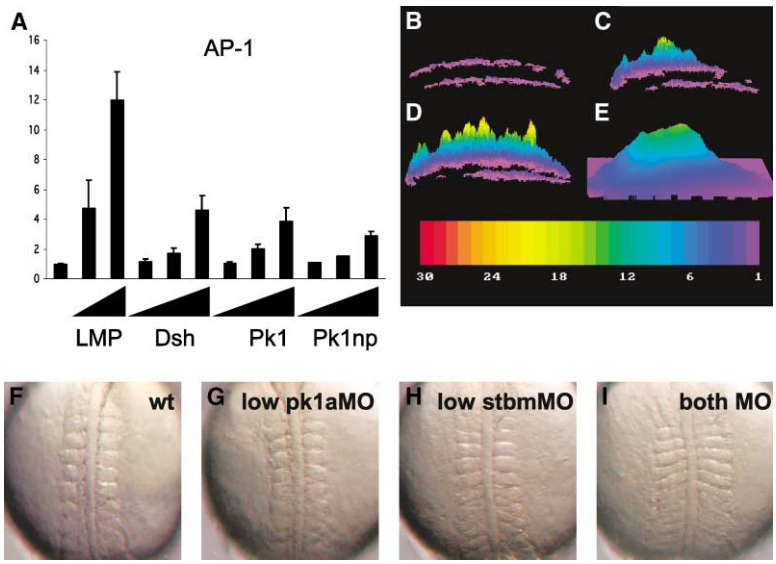

Figure 4. Signaling Assays

(A) AP-1 reporter assay in 293T cells. Low, mid, and high doses are 1, 10, and 100 ng, respectively, for zDsh2, Pk1, and Pk1nopren. Low and high doses are 1 and 10 ng, respectively, for LMP-1.
(B–D) Surface plots of calcium release activity generated from live zebrafish embryos. The height and color of the peaks indicates the number of calcium fluxes observed over the course of the experiment; the embryos are oriented in a lateral position. The color bar indicates the pseudocolor representation of the number of transients from low (purple, 1) to high (red, 30). (B) Endogenous calcium activity. (C) An embryo unilaterally overexpressing *pk1* RNA. (D) An rFz2-injected embryo.
(E) Localization of Texas red lineage tracer coinjected with Pk1 in (C).
(F–I) Dorsal views of live 10-somite stage embryos injected with the indicated morpholinos; anterior is oriented toward the top.

lease [9, 10, 23, 24]. Overexpression of Wnt5a or Fz2 increases the frequency of calcium transients in zebrafish blastulae [9, 10]. We have tested Pk1 in the same assay and find that overexpression of *pk1* RNA increases the frequency of calcium release by 2- to 3-fold over endogenous activity. Unilateral injection of *pk1* RNA (Figure 3C) stimulates calcium release in the region of the embryo also showing the coinjected lineage tracer (Figure 3E). Unlike rFz2 overexpression (Figure 3D), which stimulates an increase in calcium release shortly after injection, Pk1 overexpression does not stimulate calcium fluxes until after the approximate time of the midblastula transition (data not shown). This suggests that Pk1 action in this assay is less direct than Wnt5a or Fz2, which is consistent with the interpretation from *Drosophila* genetics that Pk is not a simple, linear signaling component [8, 14]. Given, however, that *Drosophila pk* is thought to be specifically involved in the PCP pathway, we interpret the ability of Pk1 to stimulate calcium transients to be an indication that the PCP pathway and the Wnt/calcium pathway may be overlapping.

We also tested whether Pk1 can influence canonical Wnt/β-catenin signaling. Pk1 alone has no effect on the Wnt/β-catenin reporter plasmid superTOPFLASH in 293T cells (Figure S1B). Pk1 can, however, modestly inhibit superTOPFLASH activation by Wnt but not constitutively active β-catenin (Figure S1B). A similar effect was seen in a *Xenopus* animal cap assay for induction of the direct Wnt/β-catenin target genes *siamois* and *xnr3* (Figure S1C). Comparable results have been observed with other noncanonical components such as *trilobite*/Strabismus [6] and Wnt5a [25], and this finding further correlates the gain-of-function activities of these proteins. We note, however, that there are no obvious Wnt/β-catenin gain-of-function phenotypes in *pk1* morphant or *trilobite* mutant [5] embryos, so it is possible that this effect, while useful for comparisons between signaling proteins, is not of physiological relevance.

Lastly, we have tested for genetic interactions between *pk1* and *trilobite*/Strabismus. At a range of doses, embryos injected with low doses of both pk1aMO and stbmMO showed a more severe convergent extension phenotype than embryos injected with either morpholino alone (Figures 4F–4I). Only 6% ($n = 52$) of embryos injected with 0.5–1.0 ng pk1aMO and 2% ($n = 44$) of embryos injected with 0.5–1.0 ng stbmMO showed a strong phenotype, whereas 91.5% ($n = 47$) of embryos injected with 0.5–1.0 ng each of pk1aMO and stbmMO showed a strong phenotype. While epistatic relationships between *pk* and other PCP genes remain unclear in this and other systems, this synergy is consistent with a close functional relationship between Pk and Stbm.

Conclusions

Our results show that a zebrafish homolog of the *Drosophila* PCP gene *prickle* is required for proper cell movements during gastrulation and further support the concept of a PCP-like pathway in vertebrate gastrulation. Our analysis of Pk signaling activities suggests that there is overlap between the PCP pathway and the Wnt/calcium pathway. The accompanying report by Takeuchi et al. [17] details similar results with a *Xenopus pk* homolog, especially with respect to a role for *pk* in convergent extension, Pk subcellular localization, and the absence of an obvious effect on Dsh localization. As is the case with *Drosophila*, however, much remains to be learned regarding vertebrate Prickle.

Supplemental Data

Supplemental Data including the Experimental Procedures and Figure S1 are available at http://images.cellpress.com/supmat/supmatin.htm.

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