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Prickle 1 regulates cell movements during gastrulation and neuronal migration in zebrafish

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SUMMARY

During vertebrate gastrulation, mesodermal and ectodermal cells undergo convergent extension, a process characterised by prominent cellular rearrangements in which polarised cells intercalate along the medio-lateral axis leading to elongation of the antero-posterior axis. Recently, it has become evident that a noncanonical Wnt/Frizzled (Fz)/Dishevelled (Dsh) signalling pathway, which is related to the planar-cell-polarity (PCP) pathway in flies, regulates convergent extension during vertebrate gastrulation. Here we isolate and functionally characterise a zebrafish homologue of *Drosophila prickle (pk)*, a gene that is implicated in the regulation of PCP. Zebrafish *pk1* is expressed maternally and in moving mesodermal precursors. Abrogation of Pk1 function by morpholino oligonucleotides leads to defective convergent extension movements, enhances the *silberblick (slb)/wnt11* and *pipetail (Ppt)/wnt5* phenotypes and suppresses the ability of Wnt11 to rescue the *slb* phenotype. Gain-of-function of Pk1 also inhibits convergent extension movements and enhances the *slb* phenotype, most likely caused by the ability of Pk1 to block the Fz7-dependent membrane localisation of Dsh by downregulating levels of Dsh protein. Furthermore, we show that *pk1* interacts genetically with *trilobite* (*tri*)*/strabismus* to mediate the caudally directed migration of cranial motor neurons and convergent extension. These results indicate that, during zebrafish gastrulation Pk1 acts, in part, through interaction with the noncanonical Wnt11/Wnt5 pathway to regulate convergent extension cell movements, but is unlikely to simply be a linear component of this pathway. In addition, Pk1 interacts with Tri to mediate posterior migration of branchiomotor neurons, probably independent of the noncanonical Wnt pathway.

Key words: Wnt signalling, Planar cell polarity, Convergent extension, Gastrulation, Neuronal migration, Zebrafish

INTRODUCTION

Morphogenetic movements driven by co-ordinated cell-shape changes coupled with directed cell migration, lead to the establishment of the body axis during early development. During vertebrate gastrulation, the large rearrangements of cell groups that generate the three germ layers overtly shape the embryonic axis. One such movement is convergence and extension (CE) in which mesendoderm and ectoderm undergo cell intercalations along the medio-lateral axis that narrow the tissues dorsalwards (convergence) and consequently extend them along the anterior-posterior axis (extension) (Concha and Adams, 1998; Keller et al., 2000; Warga and Kimmel, 1990). Cells undergoing medio-lateral intercalations are elongated along their medio-lateral axis and this is closely associated with polarised protrusive activity and re-organisation of the actin cytoskeleton (Shih and Keller, 1992).

Recent functional studies in *Xenopus* and genetic analyses of gastrulation mutants in zebrafish have revealed that a noncanonical Wnt pathway is involved in the regulation of CE. This pathway is related to the planar cell polarity (PCP) pathway that mediates the establishment of cell polarity in the plane of epithelia in *Drosophila* (reviewed in Adler, 2002; Mlodzik, 2002; Tada et al., 2002; Wallingford et al., 2002a). In vertebrates, the secreted glycoproteins Wnt11/Silberblick (Slb) and Wnt5/Pipetail (Ppt) act as ligands (Heisenberg et al., 2000; Rauch et al., 1997; Tada and Smith, 2000), although a Wnt ligand mediating PCP has yet to be found in *Drosophila*. Shared components of these pathways include: Fz receptors; an intracellular signal transducer, Dsh; a 4-pass transmembrane protein, Van gogh/Strabismus/Trilobite (Vang/Stbm/Tri); small GTPases RhoA and Cdc42; and a RhoA effector, Rho kinase 2 (Darken et al., 2002; Djiane et al., 2000; Goto and Keller, 2002; Habas et al., 2001; Heisenberg et al., 2000; Jessen et al., 2002; Marlow et al., 2002; Park and Moon, 2002; Tada and Smith, 2000; Wallingford et al., 2000). In vertebrates, a formin-like protein, Daam1, functions between Dsh and RhoA to regulate the actin cytoskeleton (Habas et al., 2001). Moreover,

downstream of the small GTPases, the activation of Jun-N-terminal kinase (JNK) appears to be required for proper CE movements in vertebrates and for establishing PCP in *Droshophila* ommatidia (Park and Moon, 2002; Yamanaka et al., 2002) (reviewed in Mlodzik, 1999).

prickle (*pk*) is one of a core group of PCP genes that controls planar polarity in the eye, leg and wing of *Drosophila* (Gubb et al., 1999). *pk* encodes an intracellular protein containing three LIM domains and a conserved 'PET' domain (for Prickle, Espinas and Testin). Epistasis analyses have demonstrated that Pk is required for some aspects of Fz/Dsh-mediated PCP signalling, but is not placed in a linear cascade with Fz and Dsh. Recent studies indicate that Pk regulates the subcellular distribution of Fz through binding to Dsh, thereby localising the Fz/Dsh complex to one side of the epithelial cells (Tree et al., 2002).

Recently, it has become evident that genes involved in CE may also be involved in mediating cell migration in the CNS. For instance, the PCP gene *stbm/tri* is required not only during CE, but also for proper migration of branchiomotor neurons (Bingham et al., 2002; Jessen et al., 2002). This raises the intriguing possibility that vertebrate homologues of other PCP genes might participate in the regulation of CE movements as well as during neuronal migration.

In this study, we analyse the function of a zebrafish homologue of the *Drosophila* PCP gene *pk* during gastrulation and neuronal migration. Zebrafish *pk1* is expressed in moving mesodermal cells and overlying neuroectodermal cells during gastrulation, and functions together with Slb/Wnt11 and Ppt/Wnt5 to regulate CE movements in both mesendoderm and ectoderm. Possibly analogous to its role in flies, we find that Pk1 can destabilise Dsh and thereby block the ability of Fz to target Dsh to the cell membrane. In addition to its role in CE, *pk1* also mediates the migration of cranial motor neurons and shows a strong genetic interaction with Tri in this process. These results show that Pk1 modulates different types of cell behaviour during CE and neuronal migration.

MATERIALS AND METHODS

Zebrafish lines

Alleles used were ppt^{ta98}, kindly provided by Henry Roehl (MPI Tübingen), tri^{m209}, slb^{tx226} and an *isl1*-GFP transgene, kindly provided by Hitoshi Okamoto (Higashijima et al., 2000). For injection studies, mutant embryos were obtained by crossing homozygous *slb*, heterozygous *ppt* and heterozygous *tri* carriers.

Cloning of zebrafish *pk*

Est sequence highly homologous to *Xenopus pk* (M. T. and N.U., unpublished) was used to design primers for RT-PCR from zebrafish gastrula embryos. The isolated fragment was used to screen a zebrafish-shield library (kindly provided by Michael Rebagliati) to isolate a full-length cDNA for zebrafish *pk1*. The accession number of *pk1* is AY286492. For injection studies, the coding region of *pk1* was cloned into pCS2+.

In situ hybridisation

Antisense RNA probes were produced with a digoxygenin RNA-labelling kit (Boehringer Mannheim) according to the manufacturer's instructions using plasmids containing cDNA for *pk1* (this study), *ntl* (Schulte-Merker et al., 1994), *hgg1* (Thisse et al., 1994), *dlx3* (Akimenko et al., 1994), *papc* (Yamamoto et al., 1998), *myoD* (Weinberg et al., 1996), *emx1* (Morita et al., 1995), *rx3* (Chuang et al., 1999), *pax2.1* (Krauss et al., 1991), *chordino (chd)* (Schulte-Merker et al., 1997), *bmp2b* (Nikaido et al., 1997), *sna2* (Thisse et al., 1995) and *krox20* (Oxtoby and Jowett, 1993). Whole mount, in situ hybridisation was performed essentially as previously described (Barth and Wilson, 1995). Expression of GFP was detected using an anti-GFP polyclonal antibody (AMS Biotechnology), essentially as described previously (Shanmugalingam et al., 2000).

RNA and morpholino antisense oligonucleotide (Mo) injection

RNAs encoding zebrafish Wnt11 (Heisenberg et al., 2000), zebrafish Fz7 (El-Messaoudi and Renucci, 2001), *Xenopus* Dsh-GFP (Rothbacher et al., 2000) and Pk1 were synthesised essentially as described (Smith, 1993). All injections were performed on one-cell-stage embryos.

A Mo against *pk1* (*pk1*-Mo) was designed over the initiation methionine of Pk1: 5′-GCCCACCGTGATTCTCCAGCTCCAT-3′. A control Mo including four mismatched nucleotides [underlined, pk1-Mo (Mis)] is as follows: 5′-GCCCGCCATGATTCTCCAACTTCAT-3′. Injection of the control Mo in wild-type embryos causes no defective phenotype (data not shown). The specificity of *pk1*-Mo was determined by co-injection of RNA encoding GFP-tagged with amino acid sequences that include sequence corresponding to the *pk1*-Mo but not *pk1*-Mo (Mis). *pk1*-Mo but not *pk1*-Mo (Mis) prevented production of GFP (data not shown).

Cell transplantation

For transplantations, donor embryos were injected with either rhodamine-dextran (MW 10,000, Molecular probe) plus 5 pg *pk1* RNA or fluorescein-dextran (MW 10,000, Molecular probe) at the one-cell stage. Cells were taken from late-blastula host embryos and transplanted into deep regions of the germ ring of host wild-type embryos as described previously (Heisenberg et al., 2000). Tailbud-staged embryos were mounted in 1.5% methylcellulose and image analysis was performed using Openlab software.

Analyses for sub-cellular protein localisation

To monitor Dsh localisation, embryos at the one-cell stage were injected with 200 pg RNA encoding Dsh-GFP either with or without 50 pg *fz7* RNA and either with or without 5 pg *pk1* RNA and were mounted in 1% agarose at 40% epiboly. Image analysis of living embryos was carried out using a Leica DMLFS confocal microscope with a 63× water-immersion lens. We were unable to monitor Pk1 localisation in living embryos because expression of a GFP-tagged version of Pk1, even at moderate doses caused embryos to develop abnormally at early stages. Therefore, to analyse Pk1 localisation embryos were injected with 25 pg of RNA encoding Venus-Pk1 (Venus is an EYFP-derivative that was kindly provided by Atsushi Miyawaki) and fixed at 40% epiboly for anti-GFP antibody staining.

Western-blot analysis

To monitor the levels of Dsh protein, embryos were injected with 200 pg RNA encoding myc-Dsh either with or without 50 pg *fz7* RNA and either with or without 5 pg *pk1* RNA at the one-cell stage. Blastoderms from 20 embryos at 40% epiboly were collected for western-blot analysis after removal of the yolk according to a protocol kindly provided by Carl-Philipp Heisenberg (personal communication). Protein from the equivalent of five blastoderms was subject to SDS-PAGE (8% acrylamide gel) and then blotted to a PVDF membrane (Amersham). The membrane was reacted with 9E10 anti-myc monoclonal antibody (Santa Cruz Biotechnology) and subsequently with anti-mouse IgG conjugated with HRP followed by detection with ECL (Amersham). For loading control, the membrane was counter-stained with anti-β-tubulin monoclonal antibody (Sigma) and visualised with NBT and BCIP.

RESULTS

A planar polarity gene *pk1* is expressed maternally and in moving mesodermal precursors

To investigate whether homologues of the *Drosophila* PCP gene *pk* function during vertebrate gastrulation, we isolated a full-length clone of a zebrafish *pk* gene from a shield library. The predicted protein encoded by zebrafish *pk1* shares 85% and 82% amino acid identity to *Xenopus* XPk-A (Wallingford et al., 2002b) in a highly conserved PET domain of unknown function and three LIM domains, respectively (Fig. 1A). Phylogenetic analysis on the basis of the conserved PET and LIM domains shows zebrafish *pk1* is more closely related to *Drosophila pk* and *espinas* than to another closely related gene *testin* (data not shown).

pk1 is expressed maternally (Fig. 1B), and zygotic expression is initiated on the dorsal side of the embryo at pregastrula stages (Fig. 1C) and spreads throughout the germ ring by the shield stage (Fig. 1D). As gastrulation proceeds, expression becomes restricted to dorsal involuted cells and to overlying ectodermal cells, predominantly in axial tissues (Fig. 1E,F). By the tail-bud stage, expression is downregulated in the anterior ectoderm and becomes restricted to presomitic mesoderm, the posterior neuroectoderm and the lateral edge of the neural plate (Fig. 1G). The expression in the mesoderm and ectoderm is conserved in *Xenopus* (Wallingford et al., 2002b) and this prompted us to investigate whether Pk1 is involved in the regulation of gastrulation movements.

Interfering with Pk1 function disrupts CE during gastrulation

To analyse the function of Pk1, we first employed an antisense approach using morpholino oligonucleotides against *pk1* (*pk1*-Mo) to reduce the level of endogenous Pk1 protein (Nasevicius and Ekker, 2000). Injection of 3 ng *pk1*-Mo led to a shorter body axis with a curled down tail at pharyngula stage (99%, *n*>500) (Fig. 2A,B). At higher doses, injected embryos exhibited a more severe phenotype with shorter trunk and tail, but occasionally this was associated with cell death in the brain at later stages (data not shown). Thereafter, we used a moderate dose (3 ng) for further analyses of the *pk1* morphant phenotype.

Although the curly tail phenotype of *pk1* morphants is not obviously indicative of CE defects, analyses with various markers revealed that gastrulation cell movements were affected. At tail-bud to 2-somite stages, *pk1* morphants showed a slightly posteriorly located prechordal plate (hgg1), wider neural plate (*dlx3* and *pax2.1*), a shorter, wider notochord (ntl) and laterally expanded presomitic and head mesoderm (*papc* and *sna2*) (Fig. 2C-J). This phenotype is reminiscent of the CE mutants *slb*, *ppt*, *knypek* (*kny*) and *tri* in which gastrulation cell movements are disrupted but dorso-ventral and antero-posterior patterning remains unaffected (Hammerschmidt et al., 1996; Heisenberg and Nusslein-Volhard, 1997; Heisenberg et al., 2000; Jessen et al., 2002; Kilian et al., 2003; Rauch et al., 1997; Solnica-Krezel et al., 1996; Topczewski et al., 2001). Consistent with this, in *pk1* morphant embryos, the dorsal marker *chd* and ventral marker *bmp2b* are unchanged (Fig. 2K-N) and the telencephalic marker *emx1* and midbrain marker *pax2.1* are expressed in the correct positions, although their expression domains are laterally expanded (Fig. 2O-R), reflecting reduced convergence of the neural plate (Fig. 2G,H). These results indicate that *pk1* is required for proper gastrulation CE movements of both mesendoderm and ectoderm but that it has no obvious role in the specifying cell fates.

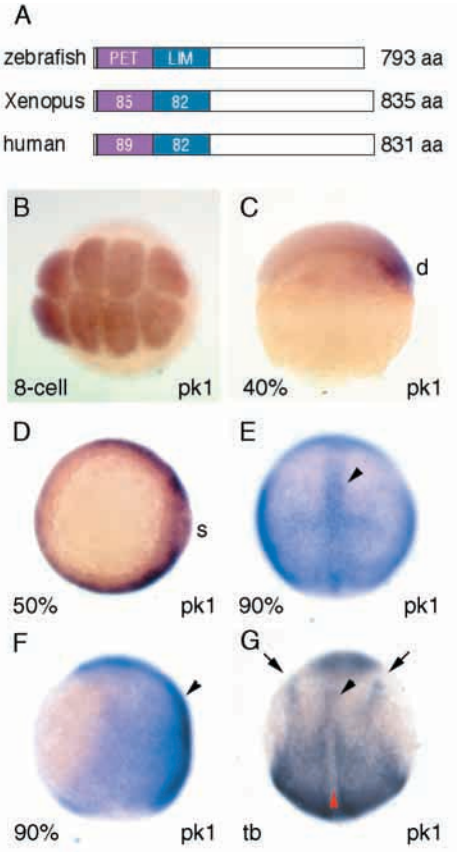

Fig. 1. *pk1* is expressed maternally and in migrating mesodermal precursors in zebrafish. (A) Homology of Prickle proteins between zebrafish (Pk1), *Xenopus* (XPk-A) (Wallingford et al., 2002b) and human (BAB71198). The numbers refer to the percentage amino-acid identities between the different orthologues. (B-E) Expression of *pk1* at early stages. *pk1* is expressed maternally at the eight-cell stage (B, animal view), on the dorsal side at ~40% epiboly (C, lateral view with dorsal to the right), and around the germ ring at 50% epiboly (D, animal view with dorsal to the right). At 90% epiboly (E, dorsal view, F, lateral view, anterior is up), expression is in dorsal involuted cells and in overlying ectodermal cells and highlighted in the axial cells (arrowheads). At tailbud stage (G, dorsal view, anterior is up), expression is restricted to the presomitic mesoderm, posterior neuroectoderm, the lateral edge of neural plate (arrows) and anterior axial mesodermal cells (black arrowhead), but slightly downregulated in the posterior axial mesodermal cells (red arrowhead). d, dorsal; s, shield.

Pk1 regulates CE movements by modulating the Wnt/PCP pathway

The CE phenotype caused by interfering with Pk1 function is similar to those in *slb/wnt11* and *ppt/wnt5* loss-of-function mutants (Heisenberg et al., 2000; Kilian et al., 2003; Rauch et al., 1997). In *Drosophila*, *pk* genetically interacts with the Fz/PCP pathway to establish epithelial polarity (Gubb et al., 1999). This led us to test the possibility that vertebrate Pk regulates CE through interaction with the Wnt/PCP pathway. First, we tested if there was any genetic interaction between

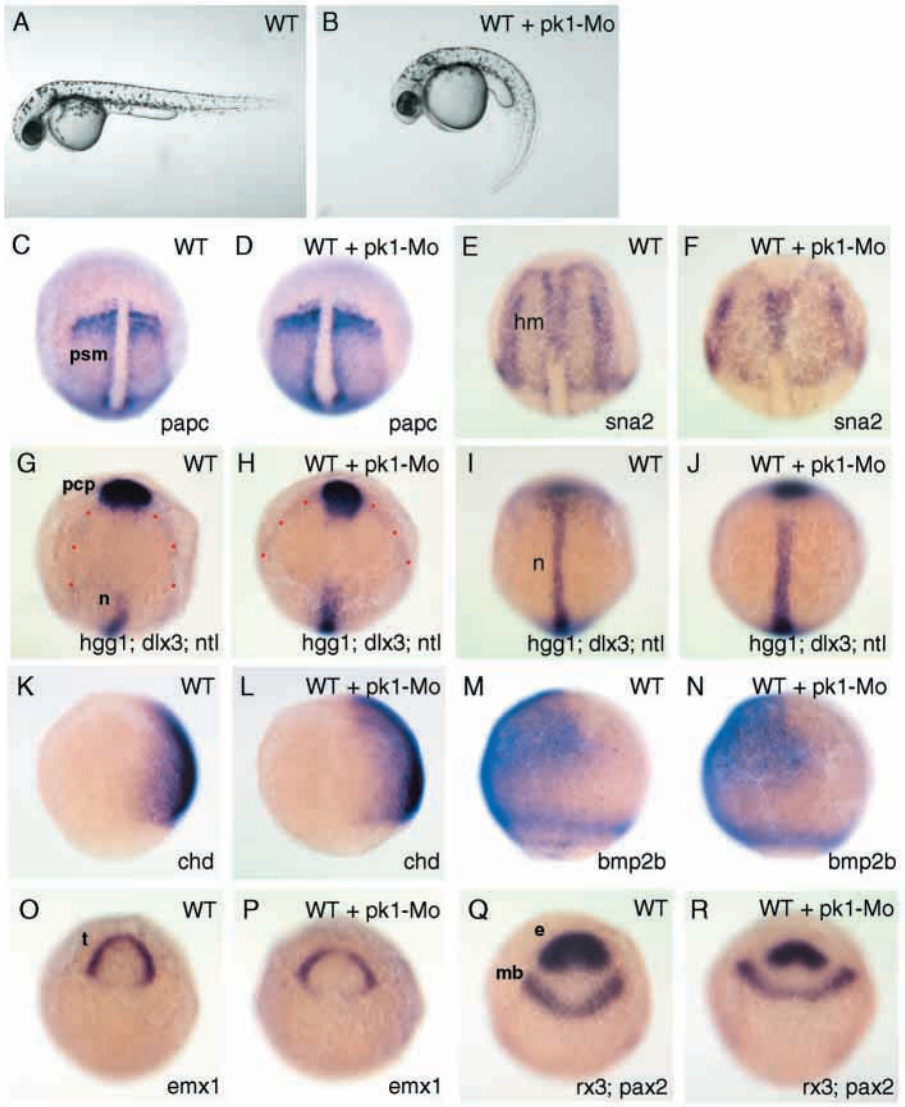

Fig. 2. *pk1* morphants exhibit defects in convergent extension movements. (A,B) Lateral views of pharyngula stage living wild-type (A) and *pk1*-morphant (B) embryos. The *pk1*-morphant embryo (B) shows a slightly compressed trunk with a curled down tail. (C-J,O-R) Dorsal views of tailbud-stage wild-type (WT) and *pk1*-morphant (WT + *pk1*-Mo) embryos. (K-N) Lateral views of 80% epiboly wild-type (WT) and *pk1*-morphant (WT + *pk1*-Mo) embryos. Anterior is up and genes analysed are indicated bottom right. Dots outline the prospective neural plate. e, eye field; mb, prospective midbrain; t, prospective telencephalon; psm, presomitic mesoderm; hm, head mesoderm; n, prospective notochord, pcp, prechordal plate.

slb and *pk1*. Injection of *pk1*-Mo in *slb* embryos enhanced the CE defect compared to either mutant/morphant alone, with the consequence that the prechordal plate remained posteriorly located beneath the neural plate (50%, *n*=92) (Fig. 3A,B). This result indicated that Pk1 may function in the Wnt/PCP pathway or in parallel to this pathway.

Given that Pk is an intracellular protein, it might act downstream of Wnt signals. To test this possibility, we assessed whether elevated Wnt11 activity can be suppressed by interfering with Pk1 function. Injection of *slb*$^{-/-}$ embryos with a low dose of *wnt11* RNA (10 pg) either fully rescued the phenotype (Heisenberg et al., 2000; and data not shown) or occasionally leads to lateral mispositioning of prechordal plate (Fig. 3C) (91%, *n*=45), possibly resulting from hyperactivation of the pathway. These phenotypes were suppressed in embryos with reduced Pk1 function (88%, *n*=58) (Fig. 3D), indicating that Pk1 function promotes Slb activity.

Because *ppt/wnt5* is expressed in similar domains to *pk1*, is required for CE movements in the posterior region of the gastrula, and genetically interacts with *slb/wnt11* (Kilian et al., 2003), we examined whether Pk1 also modulates the activity of Ppt. Both *ppt*$^{-/-}$ embryos and *pk1* morphants have a shorter body axis (Fig. 4A,C,E), in which somites are wider and thinner when compared to wild-type (Fig. 4B,D,F). *ppt/wnt5* mutant embryos with reduced Pk1 function showed a phenotype much more severe than either single mutant/morphant with greatly compressed wider somites (42%, *n*=118) (Fig. 4G,H), indicating that Ppt and Pk1 function redundantly in regulating CE in the posterior region.

Together, these results support the notion that Pk1 modulates the activity of the Wnt/PCP pathway, thereby influencing regulation of CE movements during gastrulation.

Gain-of-function of *pk1* causes defective CE movements by modulating the Wnt/PCP pathway

To complement our analysis of cell movements in embryos with reduced Pk1 activity, we investigated the consequences of increased levels of Pk1. Ubiquitous over-expression of *pk1* RNA, even at low dose (5 pg), caused abnormal cell aggregation at blastula stages precluding analysis of cell migration during gastrulation (data not shown). To overcome these early defects, we assayed the behaviour of small groups of cells over-expressing Pk1 in a wild-type environment. To achieve this, differentially labelled wild-type and Pk1-overexpressing cells were transplanted into the germ rings of wild-type host embryos (Fig. 5A). Following transplantation, convergence and extension movements redistribute the wild-type cells along the antero-posterior axis by tailbud stage (Fig. 5B) (Heisenberg et al., 2000). In comparison to wild-type, cells overexpressing Pk1 show less dorsal convergence and less spread along the antero-posterior axis (70%, *n*=40) (Fig. 5B). This indicates that elevated Pk1 activity inhibits cells from undergoing proper gastrulation movements.

The fact that Pk1 may act downstream of the Wnt/PCP pathway to regulate CE movements during gastrulation led us to test whether the *slb* phenotype could be rescued by overexpression of *pk1* RNA. Given the problems associated with *pk1* overexpression, we injected *pk1* RNA at a dose (0.5

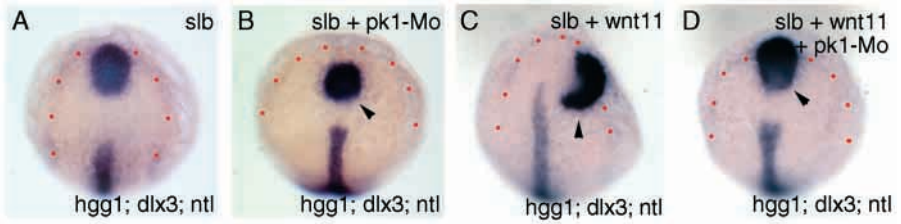

Fig. 3. Abrogation of Pk1 function enhances the *slb* phenotype and suppresses elevated Wnt11 activity. (A-D) Dorsal views showing prechordal plate position (arrowheads) in a $slb^{-/-}$ embryo (A), a $slb^{-/-}$ embryo injected with 3 ng of *pk1*-Mo (B), a $slb^{-/-}$ embryo injected with 10 pg *wnt11* RNA (C) and a $slb^{-/-}$ embryo injected with 10 pg *wnt11* RNA plus 3 ng of *pk1*-Mo (D). Dots outline the prospective neural plate. The positions of the prechordal plate are indicated by arrowheads.

pg) that has no effect in wild-type embryos (*n*=30) (Fig. 5C). Surprisingly, rather than rescue, this enhanced the severity of the *slb* phenotype (27%, *n*=107) (Fig. 5D,E), implying that excess Pk1 compromises Wnt/PCP-pathway-dependent cell movements. Taken together, the loss-of-function and gain-of-function studies with Pk1 indicate that Pk1 regulates CE movements by modulating the Wnt/PCP pathway, but that it is not simply a positive or negative linear component of this pathway.

Next, we attempted to assay if Pk1 modulates the Wnt/PCP pathway by regulating the subcellular localisation of components of this pathway. In the *Drosophila* wing, asymmetric localisation of the Fz-Dsh complex at the distal edge of each cell determines cell polarity within the plane of the epithelia (Axelrod, 2001; Strutt, 2001). During this process, Pk regulates localisation of Fz/Dsh by inhibiting the complex from forming at the proximal edges of the cells (Tree et al., 2002). In vertebrates, membrane localisation of Dsh in cells undergoing CE (Wallingford et al., 2000) is dependent on Fz (Axelrod et al., 1998; Rothbacher et al., 2000; Umbhauer et al., 2000). Therefore, we examined whether Pk1 affects the localisation of the Fz/Dsh complex in zebrafish embryos. When Dsh-GFP is expressed in animal pole blastomeres, it predominantly localises to the cytoplasm, sometimes associated with vesicles-like structures (100%, *n*=10) (Fig. 5F). This presumably reflects the requirement of Dsh to localise to vesicles for canonical Wnt signalling (Capelluto et al., 2002). In response to Fz7, Dsh is targeted to the membrane (90%, *n*=30) (Fig. 5G), but this is inhibited by increasing Pk1 activity (100%, *n*=21) (Fig. 5H). The predominantly cytoplasmic localisation of Pk1 (Fig. 5I) remains unchanged in the presence of Fz7 (data not shown). These observations, together with the fact that cytoplasmic Dsh-GFP becomes faint and hazy when Pk activity is increased (Fig. 5H), raised the possibility that Pk1 activity may destabilise Dsh, thereby blocking Fz7-mediated membrane localisation of Dsh.

To test if Pk1 affects the levels of Dsh protein, we quantified myc-tagged Dsh in the presence of Fz7 with or without Pk1. Western blot analysis revealed that the levels of Dsh are significantly lower in the presence of Pk1 (Fig. 5J). These data indicate that the disruption/degradation of the Fz/Dsh complex by Pk1 may contribute to the ability of exogenous Pk1 to negatively regulate the Wnt/PCP pathway.

pk1 genetically interacts with *tri/stbm*

The ability of Pk1 to regulate CE by modulating the Wnt/PCP pathway is similar to that of Tri/Stbm (Jessen et al., 2002). We therefore investigated whether there is any genetic interaction between *pk1* and *tri/stbm* in regulating CE movements. In the

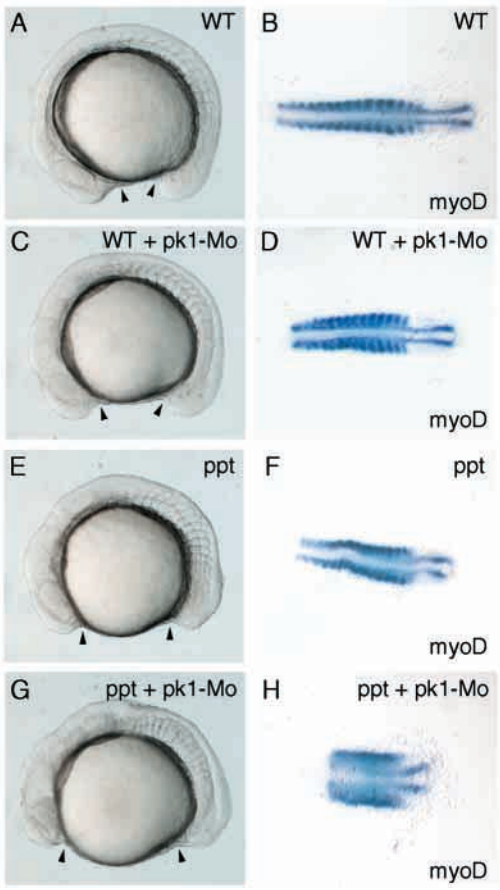

Fig. 4. Abrogation of Pk1 function enhances the *ppt* phenotype. Lateral (A,C,E,G) and dorsal (B,D,F,H) views of 14-somite embryos with anterior to the left. *myoD* expression shows the shape of the somites in (B,D,F,H). Arrowheads indicate the most anterior and posterior extent of the axis. (A,B) Wild-type embryos. (C,D) Wild-type embryos injected with 3 ng of *pk1*-Mo. (E,F) $ppt^{-/-}$ embryos. (G,H) $ppt^{-/-}$ embryos injected with 3 ng of *pk1*-Mo.

progeny of crosses between heterozygous *tri* carriers, approximately one quarter of embryos injected with 3 ng *pk1*-Mo showed a more severely compressed body axis as compared to *tri* homozygotes (Fig. 6A,B,G-J; Table 1). In addition, a further half of the injected population exhibited a phenotype indistinguishable from homozygous *tri* embryos. To confirm that the *tri*-like phenotype arose from abrogation of Pk1 activity in heterozygous $tri^{+/-}$ embryos, we injected *pk1*-Mo in embryos from crosses between heterozygous *tri* female and wild-type

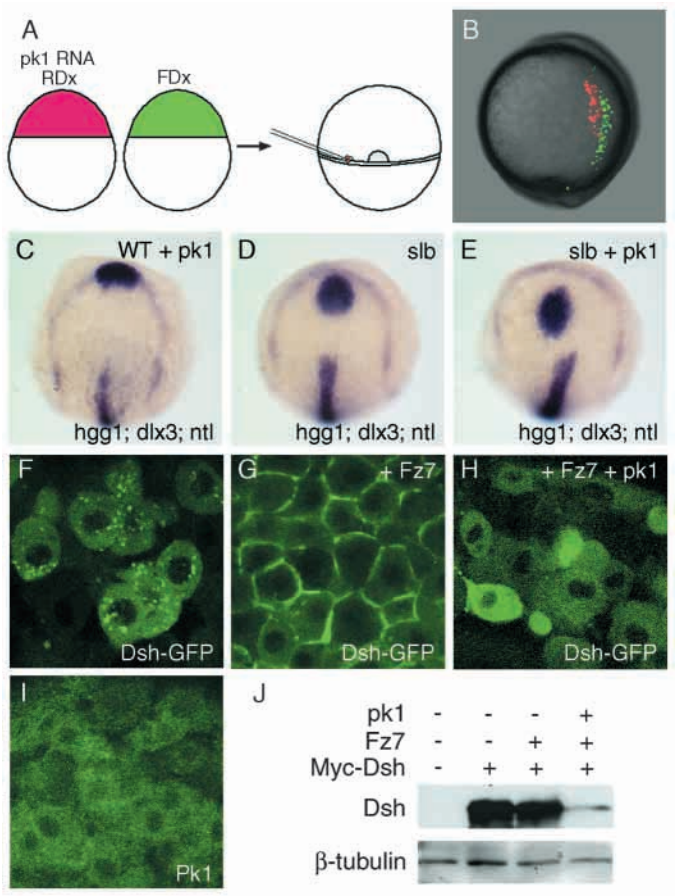

Fig. 5. Overexpression of *pk1* causes defective morphogenetic movements and enhances the *slb* phenotype. (A) Schematic of the experiment. Cells from embryos that received 5 pg *pk1* RNA and rhodamin-dextran (RDx) and cells from embryos that received fluorescein-dextran (FDx) were transplanted simultaneously into the germ rings of wild-type, shield-stage hosts. (B) Lateral view of a living, tailbud-stage host embryo with dorsal to the right. Pk1-expressing cells (red) are positioned more laterally compared to wild-type cells (green). (C-E) Gain-of-function of *pk1* enhances the *slb* phenotype. Dorsal views of a tailbud-stage embryo injected with 0.5 pg of *pk1* RNA (C), a $slb^{-/-}$ embryo (D) and a $slb^{-/-}$ embryo injected with 0.5 pg *pk1* RNA (E). The position and shape of prechordal plate (*hgg1*) in relation to the anterior edge of neural plate (*dlx3*) in the *pk1* morphant (C) are similar to wild-type (see Fig. 2I). The prechordal plate is displaced caudally in the $slb^{-/-}$ embryo (D) and even further caudally in the $slb^{-/-}$ embryo injected with the *pk1*-Mo (E). (F-H) Confocal images of animal pole cells of embryos at 40% epiboly injected with 200 pg RNA encoding Dsh-GFP (F), 200 pg RNA encoding Dsh-GFP and 50 pg *fz7* RNA (G) and 200 pg RNA encoding Dsh-GFP, 50 pg *fz7*RNA and 5 pg *pk1* RNA (H). Dsh preferentially localises to vesicles (F) but relocates to the membrane in the presence of Fz7 (G). The Fz7-induced membrane localisation of Dsh is inhibited by Pk1 (H). (I) Subcellular localisation of Pk1 in animal-pole cells of 40% epiboly embryos injected with 25 pg RNA encoding Venus-Pk1 (Pk1 tagged with a modified version of EGFP). Pk1 localises in the cytoplasm. (J) Western-blot analysis of the levels of tagged-Dsh in embryos injected with 200 pg RNA encoding myc-Dsh along with 50 pg *fz7* RNA in the presence or absence of 5 pg *pk1* RNA. The blot was detected with either an anti-myc antibody for Dsh or an anti-β-tubulin antibody for loading control. The level of Dsh is reduced by *pk1*.

male fish. About 40% of injected embryos showed a *tri*-like phenotype, more severe than wild-type embryos injected with *pk1*-Mo (Fig. 6C-F, Table 1). These results suggest that Pk1 and Tri function in the same genetic pathway.

Pk1 functions with Tri to mediate tangential migration of motor neurons

In addition to the regulation of CE, Tri function is required for tangential migration of facial (nVII) branchiomotor neurons (Bingham et al., 2002). This activity of Tri appears to be independent of the Wnt/PCP pathway (Jessen et al., 2002). Our demonstration of a strong genetic interaction between *pk1* and *tri* during CE prompted us to test the involvement of *pk1* in the regulation of neuronal migration. Initially we examined the temporal and spatial expression of *pk1* in relation to the position of branchiomotor neurons. *pk1* is expressed strongly in ventro-lateral regions of the hindbrain except at the level of rhombomere 4 (r4)-r6, where weaker expression is detected more medially, in the vicinity of migration pathway of the newborn facial motor neurons (Fig. 7A,B and data not shown). Thus *pk1* is expressed in the right place and at the right time to locally influence branchiomotor-neuron migration.

In embryos injected with *pk1*-Mo, nVII motor neurons accumulated in r4 and failed to migrate to r6-r7 (Fig. 7C,D, Table 2), confirming a requirement for Pk1 function in branchiomotor-neuron migration. Next, we tested for genetic interaction between *pk1* and *tri* during branchiomotor-neuron migration. To do this, embryos from crosses between heterozygous $tri^{+/-}$ females and males carrying the *Isl1*-GFP transgene were injected with a dose of *pk1*-Mo (1 ng), which does not affect neuronal migration in wild-type embryos (data not shown and Table 2). About 35% of embryos (the majority of presumed $tri^{+/-}$ heterozygotes) injected with the low dose of *pk1*-Mo (Fig. 7D,F, Table 2) showed defects in neuronal migration. Consistent with the fact that *pk1* is not required for correct regional pattering of the neuroectoderm (Fig. 2O-R), *krox20* and *hoxb1a* were still expressed appropriately in rhombomeres 3, 4 and 5 in *pk1* morphants (Fig. 7G-J). These results show that Pk1 and Tri function together to mediate the tangential migration of facial motor neurons.

Table 1. Genetic interaction between *pk1* and *tri* in the regulation of CE

		Phenotype* (%)				
Genotype	*pk1*-Mo (ng)	Wild type	*pk1*-Mo	*tri*-like	*tri* (severe)	$n^{\dagger}$
$tri^{+/-}\times tri^{+/-}$	–	76	–	24	–	226
$tri^{+/-}\times tri^{+/-}$	3	–	24	51	25	263
$tri^{+/-}\times$TL	–	100	–	–	–	76
$tri^{+/-}\times$TL	3	–	51	49	–	226

*Phenotypes were scored at 30 hpf according to the definition in Fig. 5 [wild type (A); *pk1*-Mo (C); *tri*-like (E,G); *tri* (severe) (I)].
†Number of embryos.

DISCUSSION

In this study, we have shown that Pk1 function is required, together with Slb/Wnt11 and Ppt/Wnt5, to regulate mesendodermal and ectodermal CE movements. Furthermore,

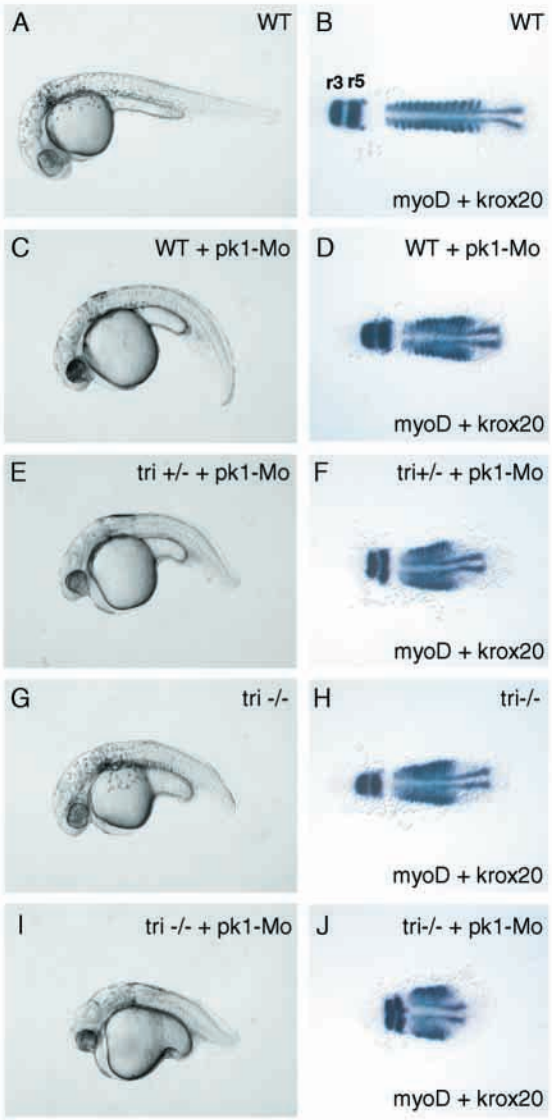

Fig. 6. *pk1* and *tri* show a strong genetic interaction in regulating convergent extension. Lateral views of pharyngula-stage embryos (A,C,E,G,I) and dorsal views of 14-somite embryos (B,D,F,H,J) with anterior to the left. *myoD* expression shows the shape of the somites and *krox20* the position of rhombomeres (r3 and r5) in (B,D,F,H,J). (A,B) Wild-type embryos. (C,D) Wild-type embryos injected with 3 ng of *pk1*-Mo. (E,F) *tri* heterozygous embryos injected with 3 ng of *pk1*-Mo. (G,H) *tri* homozygous embryos. (I,J) *tri* homozygous embryos injected with 3 ng of *pk1*-Mo.

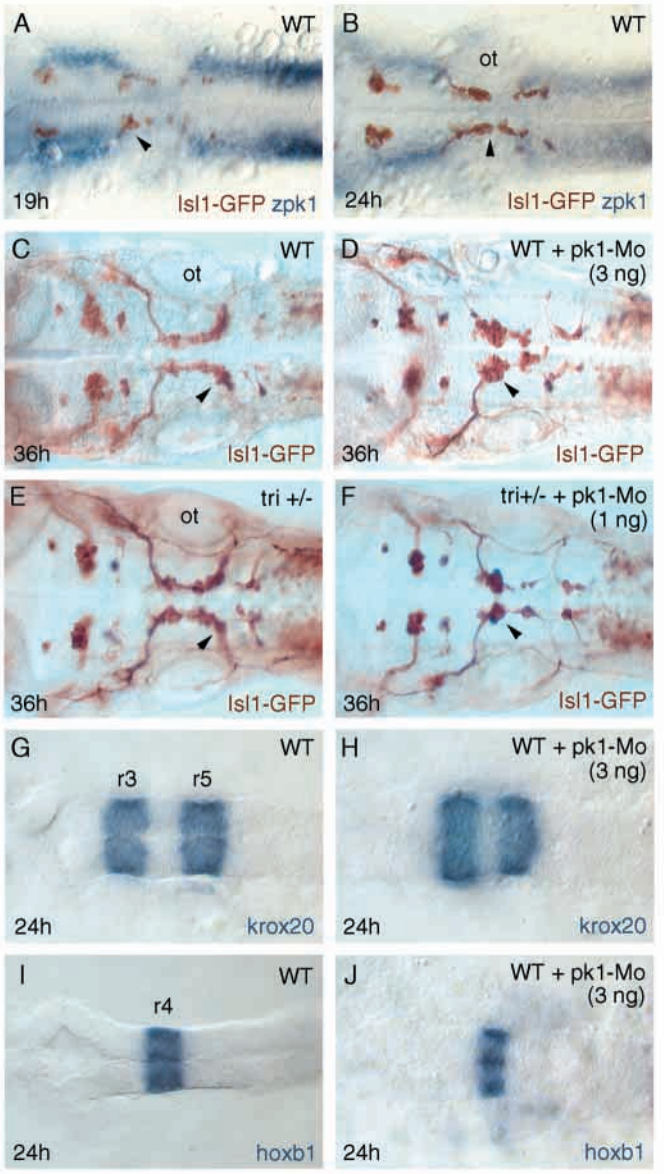

Fig. 7. Pk1 functions with Tri to mediate tangential neuronal migration. Dorsal views of hindbrains of embryos in which an *isl1*-GFP transgene labels cranial motor neurons (brown). Anterior to the left. Stage is indicated bottom left. (A,B) Expression of *pk1* (blue) in relation to motor neurons (brown). The arrowheads indicate newly born (A) and migrating (B) facial motor neurons. *pk1* is expressed at high levels anterior and posterior to the domain of facial motor neuron migration. In this domain, *pk1* is expressed weakly in the vicinity of the migrating cells. (C-F) Wild-type embryo (C), embryo injected with 3 ng of *pk1*-Mo (D), *tri* heterozygous embryo (E) and *tri* heterozygous embryo injected with 1 ng of *pk1*-Mo (F). Migration of facial motor neurons (arrowheads) is disrupted in the embryo with severe abrogation of Pk1 function (D) and in the embryo with partial loss of Tri and Pk1 function (F). (G-J) Dorsal views of hindbrains of wild-type embryos (G,I) and of embryos injected with 3 ng of *pk1*-Mo (H,J). ot, otic vesicle; r3-5, rhombomeres 3-5.

gain-of-function of Pk1 also impairs CE movements and enhances the *slb* loss-of-function phenotype, probably because Pk1 can block the Fz7-induced membrane localisation of Dsh. Finally, we showed that *pk1* genetically interacts with *tri/stbm* to mediate both tangential neuronal migration and CE. These results reveal Pk1 to be a key player in mediating cell movements in the vertebrate embryo.

pk1 regulates CE movements by modulating the noncanonical Wnt/PCP pathway

Our analysis of Pk1 function adds to a growing body of evidence that a molecular pathway involving noncanonical Wnts and homologues of fly PCP genes (Adler, 2002; Mlodzik, 2002) regulates cell movements underlying CE. Although Pk1 acts together with Wnt11 and Wnt5, it is not simply a linear

Table 2. Genetic interaction between *pk1* and *tri* in the regulation of neuronal migration

Genotype	pk1-Mo (ng)	Position of nVII neurons (%) r4	r5	r6-r7	$n^{\dagger}$
TL×*isl1*-GFP	–	–	–	100	36
TL×*isl1*-GFP	3	55	27	18	78
TL×*isl1*-GFP	1	2	6	92	51
$tri^{+/-}$×*isl1*-GFP*	–	–	10	90	99
$tri^{+/-}$×*isl1*-GFP*	1	23	19	58	231

Embryos either uninjected or injected with indicated amounts of *pk1*-Mo were fixed for anti-GFP staining at 36 hpf and were scored based on the final position of nVII branchiomotor neurons.

*Assuming that 50% of these embryos are heterozygous *tri* carriers.

†Number of embryos.

component of the Wnt/PCP pathway because both loss- and gain-of-functions of Pk1 enhance the *slb* mutant phenotype. Consistent with this conclusion, in *Drosophila* PCP, Pk is a context-dependent positive or negative modulator of Fz/PCP signalling, rather than just a downstream component of the pathway (Adler et al., 2000; Gubb et al., 1999; Tree et al., 2002).

In the *Drosophila* wing, Pk inhibits the Fz/Dsh complex from forming on the proximal edges of the epithelial cells, thereby functioning in a feedback loop that amplifies differences between Fz/Dsh levels on adjacent cells (Tree et al., 2002). Similarly, we have shown that vertebrate Pk1 can disrupt the Fz7-dependent membrane localisation of Dsh, despite the fact that Pk1 is neither localised to the membrane nor recruited to the membrane by Fz-7. In addition, increasing Pk1 activity alters the stability of exogenous Dsh. Considering that Pk binds directly to Dsh in vitro (Tree et al., 2002), Pk might dissociate Dsh from the membrane to the cytoplasm by direct binding and, subsequently, mediate the degradation of Dsh by unknown mechanisms. Alternatively, Pk1 might destabilise Dsh at the membrane through an indirect mechanism. In this scenario, Pk1 might co-operate with a factor at the membrane that, in turn, binds to Dsh and leads to dissociation from Fz.

One such candidate is the four-pass transmembrane protein Tri/Stbm/Van Gogh (Jessen et al., 2002; Taylor et al., 1998; Wolff and Rubin, 1998). In *Drosophila stbm* mutants, like *pk* mutants, Fz is symmetrically localised in the membrane (Strutt, 2001), and *stbm* interacts genetically with *pk* (Taylor et al., 1998). These observations indicate that, in flies, Stbm functions with Pk to establish PCP. Supporting a similar interaction in vertebrates, we show that heterozygous $tri^{+/-}$ embryos injected with *pk1*-Mo exhibit a *tri*-like phenotype. This reveals a strong genetic interaction between *pk1* and *tri* in the regulation of CE. Taken together with evidence that both Stbm and Pk1 can bind to Dsh and activate JNK in cultured cells (Park and Moon, 2002; Tree et al., 2002), it seems likely that Pk and Stbm function by similar mechanisms in the regulation of vertebrate CE and in the establishment of PCP in *Drosophila*.

pk1 and *tri/stbm* regulate neuronal migration independently of the Wnt/PCP pathway

In addition to disrupting CE, abrogation of Pk1 activity disrupts the tangential migration of hindbrain branchiomotor neurons. As during CE, there is a strong genetic interaction between Pk1 and Tri in the regulation of neuronal migration. However, unlike in *tri* and *pk1* mutants/morphants, branchiomotor neuron migration is unaffected by either *slb, ppt* and *kny* mutations or by overexpression of a dominant-negative form of Dsh which efficiently suppresses the Wnt/PCP-mediated CE movements (Bingham et al., 2002; Jessen et al., 2002). These observations raise the intriguing possibility that, regardless of the presence or absence of Wnt/PCP pathway signalling, Pk might act in the same molecular pathway as Tri to regulate cell behaviours that underlie CE and tangential neuronal migration.

pk is expressed in the local environment through which the nVII branchiomotor neurons migrate while *tri* is expressed more broadly in the hindbrain (Park and Moon, 2002). It is intriguing that *pk1* expression is relatively low in r4 and r5, where the nVII neurons undergo tangentially oriented caudal migrations, but higher in lateral regions of r6 (and more caudal rhombomeres), where the neurons change from tangential to laterally-directed radial migration (Chandrasekhar et al., 1997; Higashijima et al., 2000). The fact that even a subtle increase or decrease in Pk1 activity affects cell movements in pregastrula and gastrula embryos raises the intriguing possibility that changes in Pk1 activity could influence different aspects of neuronal movement. For instance, a low level of Pk1 between r4 and r6 might function together with cues (e.g. Studer, 2001) that facilitate tangential cell migration whereas high levels of Pk1 activity in r6 might modulate cues that inhibit further tangential migration and/or promote radial migration. We suggest that Pk1 might act as an intracellular sensor that mediates attractive/repulsive cues in a manner dependent on ubiquitously expressed Tri.

Possible interactions between genes involved in PCP/CE and neuronal migration

How might Stbm and Pk regulate tangential migration of facial motor neurons independent of the Wnt/PCP pathway? The Robo/Slit pathway is a candidate for exhibiting functional interaction with Stbm/Pk. Slit guides the migration of axons and neurons through its receptor Robo in both *Drosophila* and vertebrates (Brose et al., 1999; Hutson and Chien, 2002; Kidd et al., 1999; Wu et al., 1999; Zhu et al., 1999). Indeed, in the zebrafish hindbrain, *slit2* is expressed in the midline floor plate, *slit3* is expressed in branchiomotor neurons (Yeo et al., 2001) and three *robo* genes are expressed, overlapping with *pk1*, in the environment through which the nVII neurons migrate (Lee et al., 2001). Moreover, overexpression studies (Yeo et al., 2001) indicate that Slit/Robo signalling might influence CE movements through acting as a repulsive cue that modulates cell behaviour at the midline of the gastrula. Indeed, as cells approach the midline they lose bipolar protrusive activity and adopt monopolar cell morphology (Elul and Keller, 2000). Given that Stbm/Pk and Slit/Robo could both be involved in the same discrete cell migrations, it will be interesting to test the possibility that these genes interact to influence cell behaviour.

Finally, *flamingo* (*fmi*), a core PCP gene that encodes a seven-pass transmembrane protein with extracellular cadherin repeats, can also function independent of Fz/Dsh signalling. Although the role of *fmi* in the establishment of PCP is

dependent on Fz and Dsh (Shimada et al., 2001; Usui et al., 1999), *fmi* also regulates dendrite outgrowth independent of the Fz/Dsh pathway (Gao et al., 2000). The Fz/Dsh-dependent and independent activities of Fmi, Stbm and Pk lead us to speculate that PCP genes might co-ordinate the behaviour of large populations of cells in a Wnt/PCP-dependent fashion, whereas they might confer directionality to either migration or process outgrowth of small groups of cells independent of the Wnt/PCP pathway. As yet, there is little data on the roles of vertebrate *fmi* genes and it will be interesting to determine if they do function in the same pathways as Stbm and Pk during CE and neuronal migration.

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Note added in proof

Moon's and Ueno's groups have also recently reported that *prickle* is required for CE cell movements during gastrulation in zebrafish and *Xenopus* (Veeman et al., 2003; Takeuchi et al., 2003).

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