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PTK7 recruits dsh to regulate neural crest migration

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PTK7 regulates planar cell polarity (PCP) signaling during vertebrate neural tube closure and establishment of inner ear hair cell polarity; however, its signaling mechanism is unknown. Here, we demonstrate a new function for PTK7 in *Xenopus* neural crest migration and use this system in combination with in vitro assays to define the intersection of PTK7 with the non-canonical Wnt signaling pathway that regulates PCP. In vitro, using *Xenopus* ectodermal explants, we show that PTK7 recruits dishevelled (dsh) to the plasma membrane, a function that is dependent on the PDZ domain of dsh, as well as on the conserved kinase domain of PTK7. Furthermore, endogenous PTK7 is required for frizzled7-mediated dsh localization. Immunoprecipitation experiments confirm that PTK7 can be found in a complex with dsh and frizzled7, suggesting that it cooperates with frizzled to localize dsh. To evaluate the in vivo relevance of the PTK7-mediated dsh localization, we analyzed *Xenopus* neural crest migration, as loss-of-function of PTK7 inhibits neural crest migration in whole embryos as well as in transplanted neural crest cells. Supporting the in vivo role of PTK7 in the localization of dsh, a PTK7 deletion construct deficient in dsh binding inhibits neural crest migration. Furthermore, the PTK7-mediated membrane localization of a dsh deletion mutant lacking PCP activity inhibits neural crest migration. Thus, PTK7 regulates neural crest migration by recruiting dsh, providing molecular evidence of how PTK7 intersects with the PCP signaling pathway to regulate vertebrate cell movements.

KEY WORDS: Planar cell polarity, Neural crest migration, *Xenopus,* dsh, fz7

INTRODUCTION

Wnt signaling controls diverse developmental processes such as axis formation, anteroposterior patterning and the development of the neural crest (Moon et al., 1997; Raible and Ragland, 2005; Wodarz and Nusse, 1998). Precise regulation is necessary for many developmental processes and misregulation of several components of the canonical Wnt signaling pathway has been implicated in cancer formation (Polakis, 2007). Conversely, reduction or loss of function of Wnt signaling leads to general developmental defects or loss of organs, demonstrating the need for a tight regulation of the levels of Wnt signaling (Logan and Nusse, 2004).

The Wnt signaling pathway is highly conserved among all metazoans. Wnt ligands bind to frizzled (fz) transmembrane receptors leading to accumulation and nuclear localization of β-catenin, which serves as a transcriptional co-activator for TCF/Lef transcription factors (Logan and Nusse, 2004; Wodarz and Nusse, 1998). In addition to this so called 'canonical' Wnt signaling pathway, fz receptors also activate alternative signaling pathways like the planar cell polarity (PCP) pathway, which defines the orientation of cells in the plane of an epithelium (Klein and Mlodzik, 2005; Seifert and Mlodzik, 2007). PCP signaling has been best characterized in *Drosophila*, where it determines for example the ommatidia organization in the eye and the bristle hair orientation in the wing (Axelrod and McNeill, 2002; Klein and Mlodzik, 2005). In vertebrates, PCP signaling is necessary for the orientation of the stereocilia bundles in the neurosensory epithelium of the inner ear, and dynamic convergent extension movements during gastrulation and neurulation (Wallingford et al., 2002; Wang and Nathans, 2007).

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Dishevelled (dsh) is a key regulator of both the canonical as well as the PCP signaling pathway and regulates cell fate specification as well as cell movements such as convergent extension. Dsh consists of three major conserved domains, the DIX, PDZ and DEP domain, that have been implicated in different downstream signaling events (Boutros and Mlodzik, 1999; Wallingford and Habas, 2005). The DIX domain is used for canonical Wnt signaling, whereas the DEP domain is involved in PCP signaling (Boutros et al., 1998; Habas et al., 2003; Itoh et al., 2000; Rothbacher et al., 2000). By contrast, the PDZ domain is shared by both pathways. As downstream effectors of dsh are distinct for canonical and PCP signaling, it remains unclear how dsh selectively activates one or the other pathway. In the vertebrate PCP pathway, the signaling mechanisms affecting the subcellular localization and choice of effectors proteins of dsh are not well defined.

PTK7 (protein tyrosine kinase 7) is a regulator of PCP signaling that could modulate the dsh localization as well as the interaction with pathway-specific effector proteins. PTK7 regulates PCP in the inner ear hair cells and during neural tube closure in mice (Lu et al., 2004). In *Xenopus*, PTK7 is required for neural convergent extension (Lu et al., 2004). PTK7 is a transmembrane protein containing seven extracellular immunoglobulin domains and a kinase homology domain. Although the kinase domain lacks the DFG triplet necessary for catalytic activity, its overall structure is evolutionary conserved from Hydra to humans (Kroiher et al., 2001; Miller and Steele, 2000). The signaling mechanism of PTK7 has not been characterized, but considering the structure and function of PTK7, it could affect the localization of dsh as well as its downstream signaling.

Neural crest migration is a new system to analyze the mechanistic role of PTK7 in vertebrate PCP signaling. Neural crest cells are induced at the border region of the neural plate and migrate on defined routes throughout the embryo, where they give rise to a variety of derivatives ranging from neurons and glia cells of the peripheral nervous system, to cartilage and pigment cells. Although canonical Wnt signaling plays a role in the induction, delamination and differentiation of neural crest cells (Schmidt and Patel, 2005;

Yanfeng et al., 2003), PCP signaling seems to be required for neural crest migration. De Calisto et al. have shown that a dsh mutant lacking PCP activity inhibits neural crest migration. Conversely, inhibition of neural crest migration by loss of Wnt11 function can be rescued by expressing a dsh mutant, which activates PCP signaling in neural crest cells (De Calisto et al., 2005). Furthermore, 'core-PCP signals' such as Van Gogh/strabismus, prickle and daam are expressed in cranial neural crest cells (Bekman and Henrique, 2002; Darken et al., 2002; Goto and Keller, 2002; Nakaya et al., 2004), indicating that the PCP signaling cascade is active during neural crest migration.

Here, we identify a new function for PTK7 in neural crest migration. By analyzing the signaling mechanism of *Xenopus* PTK7 in vitro in animal cap explants and in vivo in migrating neural crest cells, we provide evidence that PTK7 localizes dsh to the plasma membrane and that this function is required for neural crest migration.

MATERIALS AND METHODS

Construct design

Myc-tagged PTK7 (PTK7-myc) was amplified by PCR (forward primers 5′CG*GGATCC*ATGGGGCCGATTGTGCTC3′, reverse primers 5′CC*ATCGAT*ACCCTTGTGTCTTGCTGCC3′), cut with *Bam*HI and *Cla*I, and ligated into the respective restriction sites of pCS2-MT. HA-tagged PTK7 (PTK7-HA) was obtained from PTK7-myc by removing the myc-tag with *Cla*I and *Xba*I, and inserting an HA-tag generated using the following primers: forward primer, 5′CGATATCCCTACGATGTTCCAGAT-TATGCATGATAA3′; reverse primer 5′CTAGTTGCGTAATCCG-GTACATCGTAAGGGTAGT3′. The deletion of the predicted kinase domain of PTK7 (ΔkPTK7-myc) was introduced by PCR amplification of PTK7-myc using the following primers: forward, 5′CTTGTCGCCAGAGCTGTGTC3′; reverse, 5′TCTTCTGGCAGCA-AGACACAAG3′. RNA transcribed from PTK7 constructs cloned in pCS2 is significantly more potent than RNA generated from the constructs published by Lu et al. (Lu et al., 2004); therefore lower concentrations were injected.

For neural crest-specific expression, a minimal *slug* promoter, a700BA (Vallin et al., 2001), was excised with *Spe*I/*Bam*HI from a700BA-GFP (Vallin et al., 2001) and ligated into the *Spe*I/*Bam*HI sites of BS-I-Sce-II KS (Pan et al., 2006) generating *slug*-BS-I-Sce-II KS. To express PTK7 under the control of the slug promoter, full-length PTK7 was excised using *Sal*/*Bsp*E1, blunted and ligated into the *Bam*HI site of *slug*-BS-I-Sce-II KS generating *slug*-PTK7. For cloning of the kinase deletion mutant (*slug*-ΔkPTK7), full-length PTK7 was cut with *Sal*I/*Bsu*361, blunted and ligated into the *Bam*HI site of *slug*-BS-I-Sce-II KS. To express GFP in neural crest cells, the minimal *slug* promoter and the GFP-coding sequence were excised with *Spe*I/*Not*I from a700BA-GFP (Vallin et al., 2001) and inserted into the *Spe*I/*Not*I sites of BS-I-Sce-II KS. All constructs cloned in *slug*-BS-I-Sce-II KS were purified using QIAprep Spin Miniprep Kit (Qiagen) previous to injection in *Xenopus* embryos.

Xenopus injection and neural crest transplantation

Xenopus injection experiments were performed as previously described (Borchers et al., 2001). The PTK7 MO sequences were published elsewhere (Lu et al., 2004). Here, a 1:1 mixture of MO2 and MO3 was used at total concentrations indicated in the text. As a toxicity control, identical concentrations of control MO (Gene Tools) were used. To evaluate neural crest migration independently of earlier neural tube closure defects, PTK7 MO injected embryos with severe neural tube closure phenotypes were excluded.

For RNA injection experiments, capped mRNA was synthesized using the mMESSAGE mMACHINE Kit (Ambion) according to the manufacturer's instructions. The following published plasmids were used to synthesize RNA for dsh-GFP (Yang-Snyder et al., 1996), dshΔDIX-GFP, dshΔPDZ-GFP, dshΔDEP-GFP (Miller et al., 1999), dsh-myc (Sokol, 1996), fz7-myc (Winklbauer et al., 2001) and lacZ (Smith and Harland, 1991). For in situ hybridization, embryos were injected with RNA, plasmids or MO in one blastomere at the two-cell stage. *GFP* or *lacZ* RNA were used as lineage tracers as indicated in the text. To analyze protein localization in animal cap assays, embryos were injected in the animal pole at the one-cell stage.

Transplantation of neural crest cells was carried out as previously described (Borchers et al., 2000). Images were taken using a Leica MZFLIII microscope and a Leica DC500 camera (Leica Fire Cam 1.2.0 software for Macintosh).

Whole-mount in situ hybridization and immunostaining

β-Galactosidase staining and whole-mount in situ hybridization were performed as previously described (Borchers et al., 2002; Harland, 1991). Antisense probes were synthesized from the following published plasmids using a DIG-RNA labeling kit (Roche): *AP-2* (Winning et al., 1991), *engrailed* (Brivanlou and Harland, 1989), *twist* (Hopwood et al., 1989) and *Sox10* (Aoki et al., 2003). The PTK7 antisense probe was generated from full-length PTK7 (RZPD, IMAGp998I099552) linearized with SexA1.

For immunostaining on vibratome sections, embryos were fixed for 1 hour in MEMFA (3.7% formaldehyde, 0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$). Sectioning and immunostaining was performed as previously described (Borchers et al., 2000). Myc-tagged PTK7 was detected with anti-c-myc-Cy3 antibody (C6594, Sigma) and sections were imaged using an Axioplan 2 microscope (Zeiss) equipped with an AxioCam HRc digital camera.

Animal cap localization experiment

Ectodermal explants (animal caps) were prepared as described by Wallingford and Harland (Wallingford and Harland, 2001). At stage 10.5-11, animal caps were fixed in MEMFA and processed for immunostaining. Antibodies were used in the following dilutions: anti-c-myc-Cy3 (Sigma, C6594) 1:100, anti-HA (MMS-101P, Covance) 1:150, anti-GFP (AB290, Abcam) 1:1000, goat anti-mouse Alexa-fluor 488 nm (A11029, Invitrogen) 1:200, goat anti-mouse Cy5 (AB6563, Abcam) 1:200, and goat anti-rabbit IgG fluorescein isothiocyanate (FITC) (F7367, Sigma) 1:200. After immunostaining, protein localization was imaged by confocal microscopy using a LSM510 META confocal microscope with LSM510 META software (Carl Zeiss, MicroImaging).

Immunoprecipitation

For immunoprecipitation, 50 stage 11 embryos were homogenized in 500 μl NOP buffer (10 mM TrisHCl pH 7.8, 150 mM NaCl, 5% NP40) supplemented with protease and phosphatase inhibitors to a final concentration of 1 mM PMSF, 0.1 mM $NaVO_4$, 1 mM NaF and 1 mM β-glycerolphosphate. Inhibitor tablets (Roche) were added according to the manufacturer's instructions. Lysates were centrifuged at 16,000 ***g*** for 20 minutes at 4°C and the supernatant was pre-cleaned by a 30 minute incubation with Protein A sepharose (Amersham). After incubation with anti-myc antibodies (9E10, Sigma) for 1.5 hours at 4°C, protein A sepharose (15 μl of the bead volume) was added for 1 hour and protein complexes were precipitated by low-speed centrifugation (400 ***g***). Sepharose beads were washed three times with NOP buffer, boiled in Laemmli sample buffer and analyzed by western blotting using anti-HA antibodies (Covance).

RESULTS

PTK7 leads to membrane localization of dsh

PTK7 is necessary for disparate processes regulated by PCP signaling such as neural tube closure and hair cell polarity in the inner ear (Lu et al., 2004); however, its precise signaling mechanism is unknown. Although the genetic interaction of PTK7 with Vangl2 (Lu et al., 2004) suggests that PTK7 is an integral part of the PCP signaling cascade, so far its intersection with it has not been defined. One possibility is that PTK7 signals via dsh, which like PTK7 regulates neural convergent extension (Wallingford and Harland, 2001; Wang et al., 2006). A prerequisite for this would be that dsh co-localizes with PTK7 at the plasma membrane. To analyze whether this is the case, we employed the *Xenopus* animal cap assay, which has previously been used to demonstrate the interaction and co-localization of dsh with fz at the plasma membrane (Axelrod et al., 1998; Medina et al., 2000; Medina and Steinbeisser, 2000;

Sheldahl et al., 1999; Yanagawa et al., 1995). Animal caps are ectodermal explants of blastula stage *Xenopus* embryos that allow the analysis of the cellular localization of overexpressed tagged proteins. For colocalization assays, *Xenopus* embryos were injected with RNA coding for the respective tagged constructs in the animal pole of a one-cell stage embryo. At blastula stage, the animal cap was dissected and cultured until gastrula stages, when cellular protein localization was determined using a laser scanning microscope. GFP-tagged dsh is predominantly localized in the cytoplasm of animal caps (Fig. 1A,G), but is translocated to the plasma membrane if myc-tagged fz7 is co-expressed (Fig. 1C,G). To examine whether PTK7 translocates dsh to the plasma membrane, myc-tagged PTK7 was co-expressed with GFP-tagged dsh. Like fz7, PTK7 can recruit dsh-GFP from the cytoplasm to the membrane (Fig. 1E,G). To further confirm the co-localization of PTK7 with dsh in a biochemical assay, we used glycerol gradient density centrifugation (see Fig. S1 in the supplementary material). This method allows the separation of proteins according to their molecular weight, and protein complex formation can be detected as a shift to higher molecular weight fractions. Indeed, the dsh protein tailed into higher density fractions in the presence of PTK7 (see Fig. S1 in the supplementary material), indicating complex formation of dsh with PTK7. Thus, the animal cap localization assay as well as the glycerol gradient density centrifugation indicate that PTK7 connects with the Wnt signaling pathway at the level of dsh.

The kinase domain of PTK7 and the PDZ domain of dsh are required for co-localization

To identify the molecular domains necessary for the interaction of PTK7 with dsh, deletion mutants were tested for co-localization in animal caps. As the intracellular domain of PTK7 contains a conserved tyrosine kinase motif, we analyzed whether its deletion abolishes translocation of dsh. Indeed, in contrast to overexpression of wild-type PTK7, this deletion mutant (ΔkPTK7) was not able to translocate dsh to the plasma membrane (Fig. 1F,G). Furthermore, this mutant also failed to shift dsh to higher molecular weight fractions in *Xenopus* lysates separated by glycerol gradient centrifugation (see Fig. S1C,D in the supplementary material). Taken together, this indicates that the kinase motif of PTK7 is required for dsh recruitment to the plasma membrane.

Dsh contains different functional domains that are involved in canonical and non-canonical Wnt signaling, respectively (Wallingford and Habas, 2005). To identify which of these domains are necessary for the PTK7-dependent dsh-translocation, we expressed GFP-tagged deletion mutants of the DIX, the PDZ and the DEP domain of dsh (Fig. 2A) in animal caps. In the absence of PTK7 expression, all mutant dsh proteins were mainly localized in the cytoplasm (Fig. 2B,D,F,H). However, co-expression of PTK7 transferred ΔDIX- as well as ΔDEP-dsh to the membrane (Fig. 2C,G,H). In the case of the ΔDIX-dsh-injected caps, a residual cytoplasmic staining remained that was not apparent in ΔDEP-dsh injected caps. By contrast, ΔPDZ-dsh was not translocated to the plasma membrane in the presence of PTK7 (Fig. 2E,H), indicating that the PDZ domain is required for function. In summary, these data show that the tyrosine kinase domain of PTK7 as well as the PDZ domain of dsh are necessary for the translocation of dsh to the plasma membrane.

PTK7 is part of a fz7/dsh complex and is required for fz7-mediated dsh localization

The ability of PTK7 to control dsh localization suggests that the two proteins might interact. We tested for binding by co-expressing HA-tagged PTK7 with myc-tagged dsh and immunoprecipitating with

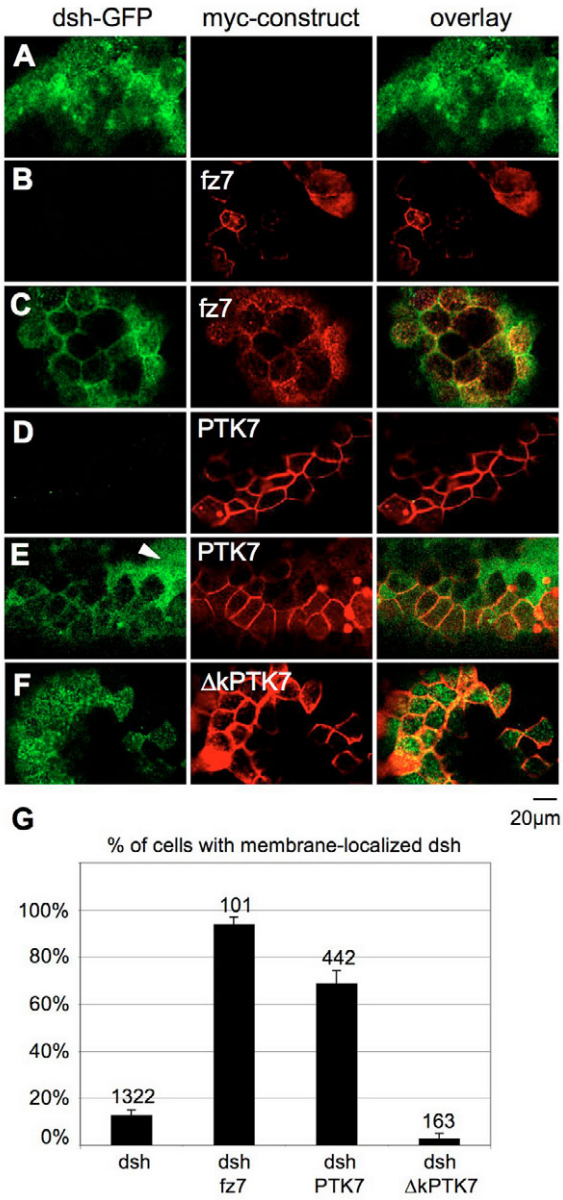

Fig. 1. PTK7 recruits dsh to the plasma membrane. Animal caps were injected with different tagged RNAs, and protein localization was analyzed by confocal microscopy. The GFP-tagged dsh (green, left), the co-expressed myc-tagged protein (red, middle) and the merged pictures (right) are shown. (**A**) GFP-tagged dsh is localized to the cytoplasm of animal caps injected with 100 pg *dsh-GFP* RNA. (**B**) Myc-tagged fz7 is predominantly membrane localized in animal caps injected with 100 pg *fz7-myc* RNA. (**C**) Co-injection of 100 pg *dsh-GFP* and 100 pg *fz7-myc* RNA leads to membrane recruitment of dsh. (**D**) PTK7 is membrane localized in animal caps injected with 500 pg myc-tagged *PTK7* RNA. (**E**) Animal caps co-injected with 100 pg *dsh-GFP* RNA and 500 pg *PTK7-myc* RNA show membrane-recruitment of dsh. Cells that do not express PTK7 in the membrane do not show membrane-localization of dsh (white arrowhead). (**F**) Animal caps injected with 250 pg RNA coding for a PTK7 mutant lacking the conserved kinase domain (ΔkPTK7) and 100 pg *dsh-GFP* RNA do not show membrane localization of dsh. (**G**) Graph summarizing the percentage of cells with membrane-localized dsh. For colocalization assays using PTK7 or fz7, only cells in which these proteins were membrane localized were analyzed. To determine the number of cells with cytoplasmic dsh localization DAPI co-staining was used. The total number of cells is indicated above each column.

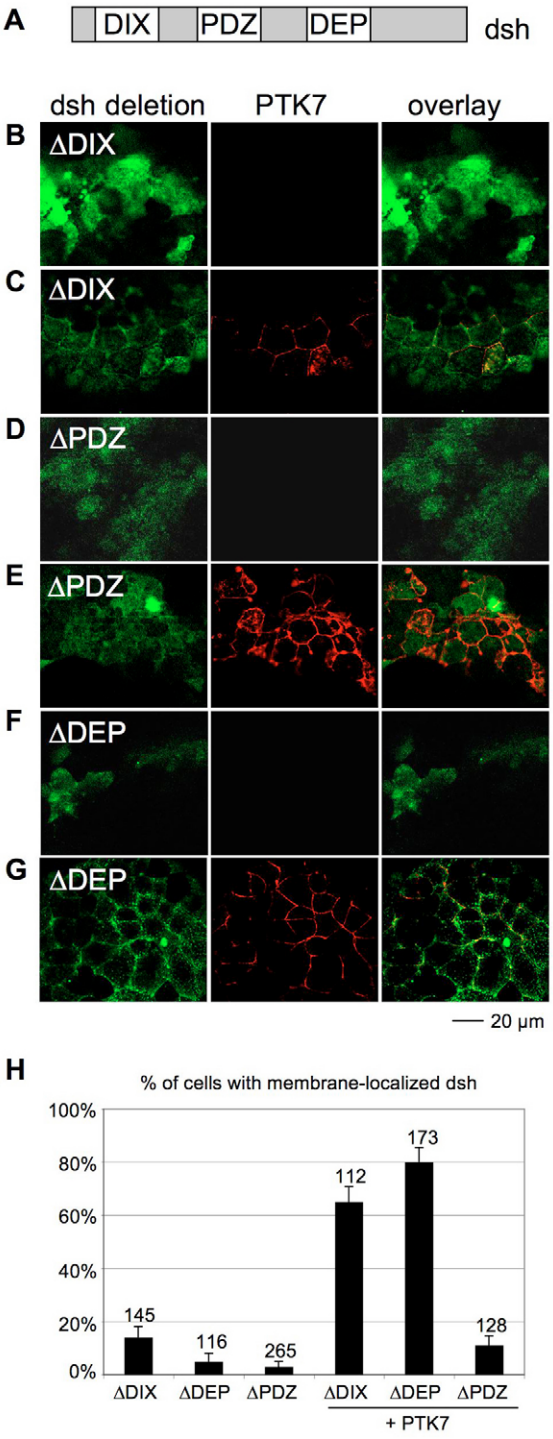

Fig. 2. The PDZ domain is necessary for PTK7-dependent membrane translocation of dsh. (**A**) Dsh protein structure indicating the DIX, PDZ and DEP domain. RNA of GFP-tagged deletion mutants of these domains were expressed alone or in combination with wild-type *PTK7* RNA in animal caps. Protein localization was determined by confocal microscopy. (**B**) Animal cap injected with 100 pg *ΔDIX-GFP* RNA showing cytoplasmic protein localization. (**C**) Co-injection of 500 pg *PTK7-myc* RNA partially translocates the cytoplasmic ΔDIX-GFP protein to the membrane. (**D**) Animal caps injected with 100 pg *ΔPDZ-GFP* RNA show only cytoplasmic localization of the protein. (**E**) The same is seen after co-expression with *PTK7-myc* RNA. (**F**) Animal caps injected with 100 pg *ΔDEP-GFP* RNA express the protein in the cytoplasm, whereas (**G**) co-injection of 100 pg *PTK7-myc* RNA leads to membrane recruitment. (**H**) Graph summarizing the percentage of cells with membrane-localized dsh. Only cells with membrane expression of PTK7 were analyzed for dsh localization. The total number of cells is indicated on each column.

either myc- or HA-antibodies. Independent of the antibodies used, we could not detect co-immunoprecipitation of PTK7 and dsh (data not shown), indicating that additional molecules are required for PTK7-mediated dsh localization. A likely candidate is fz7, which, like PTK7, is also able to recruit dsh to the plasma membrane. To test whether PTK7 forms a complex with dsh and fz7, we expressed HA-tagged PTK7 with either myc-tagged dsh or myc-tagged fz7, or a combination of the two (see Fig. S2 in the supplementary material). Although HA-tagged PTK7 was co-precipitated with myc-tagged fz7 in one out of three experiments, we detected only robust co-precipitation in combination with myc-tagged dsh and myc-tagged fz7 (see Fig. S2 in the supplementary material), indicating that PTK7 is part of a protein complex that includes fz7 and dsh.

If PTK7 forms a complex with fz7 and dsh, PTK7 could affect the fz7-mediated dsh recruitment. To test this, we analyzed animal caps, which endogenously express PTK7 (see Fig. S3 in the supplementary material), how loss of PTK7 function interferes with the fz7-mediated dsh recruitment. In the presence of control morpholino oligonucleotides (MOs), fz7 recruits GFP-tagged dsh to the plasma membrane (Fig. 3A,D). However, the PTK7 MO abolished the fz7-mediated dsh translocation (Fig. 3B,D). This could be rescued by co-injection of *PTK7* RNA lacking the MO binding site (Fig. 3C,D).

As fz-mediated dsh membrane localization correlates with hyperphosphorylation of dsh (Rothbacher et al., 2000), we further examined whether PTK7 loss of function also affects the phosphorylation status of dsh. Animal caps expressing myc-tagged dsh alone or in combination with PTK7 show only a single band in western blots using anti-myc antibodies. However, an additional high molecular weight band representing hyperphosphorylated dsh is detected in lysates expressing dsh with fz7 (Fig. 3E). Interestingly, this fz7-mediated hyperphosphorylation of dsh is inhibited by the PTK7 MO, indicating that PTK7 is required for the fz7-mediated dsh hyperphosphorylation. Thus, these data support that PTK7 is part of a dsh-fz7 complex required for dsh localization and phosphorylation.

PTK7 functions in cranial neural crest migration

To further evaluate the in vivo relevance of the PTK7-mediated dsh membrane localization, we focused on *Xenopus* neural crest migration. Recently, PCP signaling has been implicated in the regulation of neural crest migration (De Calisto et al., 2005). As PTK7 is expressed in premigratory (Fig. 4A,B) as well as migratory cranial neural crest cells (Fig. 4C), we analyzed whether PTK7 also functions in neural crest development. *Xenopus laevis* embryos were injected with PTK7 MO and *GFP* RNA as a lineage tracer in one blastomere at the two-cell stage and neural crest migration was analyzed at neurula and tadpole stages using in situ hybridization for different neural crest markers. Starting at the neurula stage, injection of the PTK7 MO inhibited neural crest migration. Although *twist*-positive cells were induced, they failed to migrate in PTK7 MO-

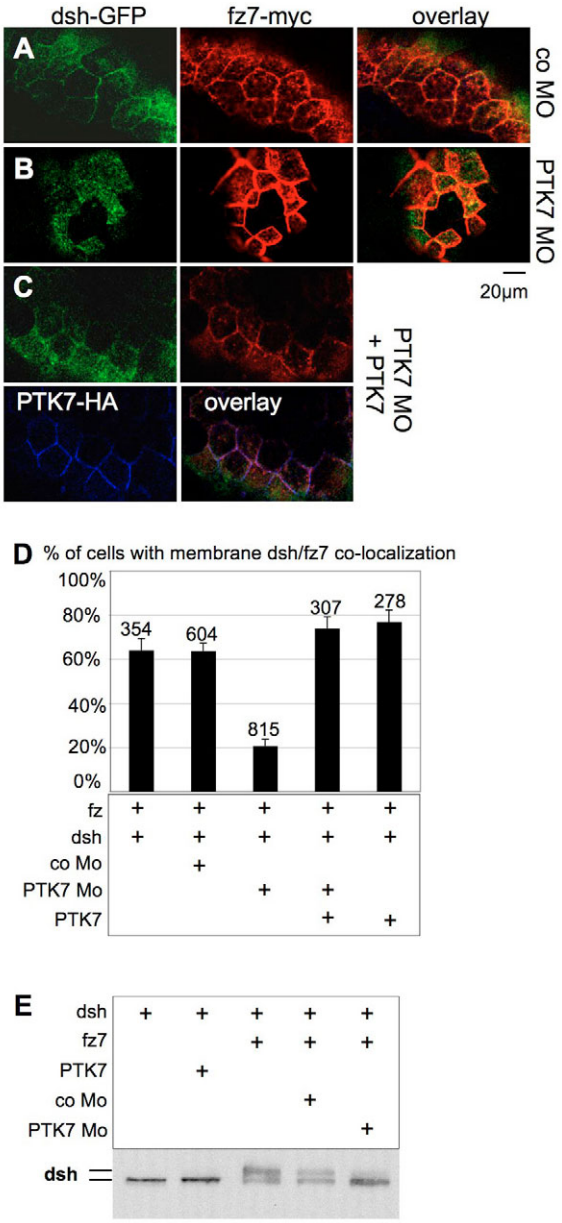

Fig. 3. PTK7 is required for fz7-mediated dsh recruitment and phosphorylation. (A-D) PTK7 is required for fz7-mediated dsh recruitment to the plasma membrane. GFP-tagged dsh is shown in green, myc-tagged fz7 in red, HA-tagged PTK7 in blue. (**A**) GFP-tagged dsh is localized to the plasma membrane in animal caps injected with 100 pg *dsh-GFP* RNA, 100 pg *fz7-myc* RNA and 20 ng control MO. (**B**) GFP-tagged dsh is not recruited to the plasma membrane in animal caps injected with 100 pg *dsh-GFP* and 100 pg *fz7-myc* RNA and 20 ng PTK7 MO. (**C**) Co-injection of 100 pg wild-type *PTK7* RNA lacking the MO binding site rescues dsh-localization of animal caps injected with 100 pg *dsh-GFP* RNA, 100 pg *fz7-myc* RNA and 20 ng PTK7 MO. (**D**) Graph summarizing the percentage of cells with simultaneously membrane-localized dsh and fz7. The total number of cells is indicated on each column. (**E**) PTK7 is required for fz7-dependent hyperphosphorylation of dsh. Embryos were injected with 100 pg *dsh-myc* RNA, 100 pg *fz7* RNA, 500 pg *PTK7-myc* RNA, 20 ng control MO or 20 ng PTK7 MO in the combinations indicated. Animal caps were cut at stage 9 and their lysates were analyzed by western blotting using anti-myc antibodies. Hyperphosphorylated dsh is detected as a second high molecular weight band. One representative experiment of three independent experiments is shown.

injected embryos (Fig. 4H), whereas induction and migration of *twist*-positive cells was normal in embryos injected with the control MO (Fig. 4F). At tadpole stages, a few migrating neural crest cells are found in the PTK7 MO-injected embryos; however, their number as well as their migration distance is dramatically reduced compared with the control (Fig. 4J,K). Similar migration defects are also seen with other neural crest markers such as AP-2 (Fig. 4L,M) and Sox10 (data not shown), whereas the expression of the midbrain-hindbrain marker engrailed is not affected at these MO concentrations (Fig. 4I, right embryo). Thus, the PTK7 MO does not lead to morphological changes, but rather seems to specifically affect neural crest development.

To verify that the neural crest migration defect is caused by loss of PTK7 function, we performed rescue experiments. Embryos were injected with PTK7 MO in combination with increasing concentrations of wild-type *PTK7* RNA lacking the MO-binding sites. Embryos were injected in one blastomere at the two-cell stage using *lacZ* RNA as a lineage tracer and neural crest migration was analyzed by *twist* in situ hybridization at tadpole stages. Fig. 3O summarizes a total of three injection experiments showing a concentration-dependent rescue effect of the PTK7 MO phenotype by wild-type *PTK7* RNA. This demonstrates that the MO phenotype is specific to loss of PTK7 function.

In contrast to loss of PTK7 function, overexpression of PTK7 did not affect neural crest migration (Fig. 5). Different concentrations of myc-tagged and untagged *PTK7* RNA were injected in one blastomere at the two-cell stage and neural crest migration was analyzed at neurula and tadpole stages. Neither neural crest induction nor neural crest migration were affected by overexpression of PTK7 (Fig. 5A-D), although the tagged PTK7 protein was detected by western blotting and immunostaining (Fig. 5E,F). Thus, PTK7 is required for neural crest migration; however, an excess of the protein seems not to disturb neural crest development. In summary, these data show that PTK7 is expressed and probably directly required in neural crest cells to enable their migration.

Transplanted neural crest cells require PTK7 for migration

To address the function of PTK7 specifically in migrating neural crest cells, we used neural crest transplantation assays (Borchers et al., 2000). Embryos were co-injected with PTK7 MO and *GFP* RNA in one blastomere at the two-cell stage. At early neurula stages (14-16), the fluorescent neural crest was removed and transplanted into control embryos from which the local neural crest had been removed (Fig. 6A). To distinguish 'non-migrating' grafts (Fig. 6D) from 'migrating' grafts (Fig. 6B) the GFP fluorescence of the transplanted cells was monitored at different time points. Five hours after transplantation, grafts injected with the control MO showed streams of migrating neural crest cells (Fig. 6B,F). However, most grafts injected with the PTK7 MO did not migrate (Fig. 6D,F). Twelve hours after grafting, the number of migrating PTK7 MO grafts (Fig. 6E) increased, but was still significantly lower compared with control MO grafts (Fig. 6F). Furthermore, even if PTK7 MO grafts migrated, they showed fewer migrating cells and did not migrate as far to the ventral side as did control grafts (Fig. 6C,E). The PTK7 MO phenotype is rescued by co-injection of wild-type *PTK7* RNA lacking the MO binding sites (Fig. 6F), although the rescue effect is not as pronounced as in whole embryo injection experiments, which is probably due to the more challenging experimental procedure. In summary, the transplantation assay shows that transplanted neural crest cells require PTK7 for migration.

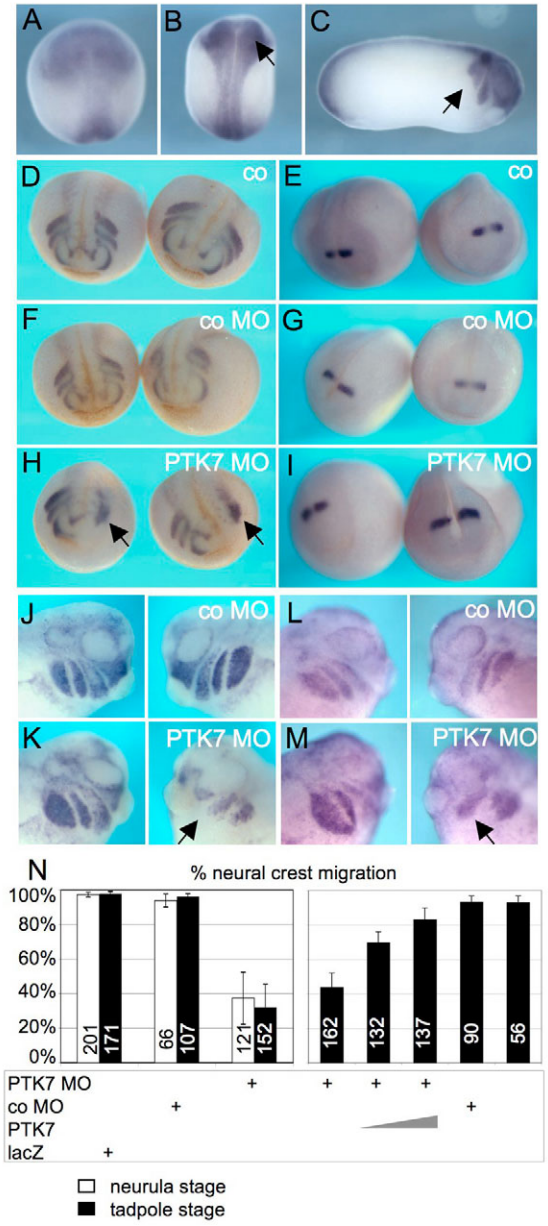

Fig. 4. PTK7 is expressed in cranial neural crest cells and required for their migration. (A-C) PTK7 expression pattern in *Xenopus laevis* detected by whole-mount in situ hybridization. (**A**,**B**) PTK7 is broadly expressed in premigratory neural crest cells at early neurula stages (black arrow). (**C**) At stage 26, PTK7 expression is detected in migrating cranial neural crest cells (black arrow). (D-M) Embryos injected in one blastomere at the two-cell stage with different constructs in combination with 100 pg *GFP* RNA as a lineage tracer. Neural crest migration was analyzed at neurula stages using the neural crest marker *twist* (D,F,H) or the midbrain-hindbrain marker *engrailed* (E,G,I). The injected side is shown on the right. (**D**,**E**) GFP-injected embryos. (**F**,**G**) Embryos co-injected with 10 ng control MO and *GFP* RNA. (**H**,**I**) Embryos co-injected with 10 ng PTK7 MO and *GFP* RNA. (**J-M**) Tadpole embryos analyzed by in situ hybridization with the neural crest markers *twist* (J,K) or *AP-2* (L,M). The injected side is shown on the right. (J,L) Tadpoles injected with 10 ng control MO und 100 pg *GFP* RNA. (K,M) Tadpoles injected with 10 ng PTK7 MO and 100 pg *GFP* RNA. Inhibition of cranial neural crest migration is marked by arrows in H,K,M. (**N**) Graph summarizing the MO injection experiments. Left graph summarizes five independent experiments (analyzing neurula stages) and six independent experiments (analyzing tadpole stages), respectively. The right graph summarizes three independent rescue experiment. The percentage of migrating neural crest cells was determined by *twist* in situ hybridization. Columns are labeled with the number of injected embryos.

Neural-crest-specific expression of ΔkPTK7 inhibits neural crest migration

To study the function of PTK7 in neural crest migration isolated from earlier developmental defects, we targeted the expression of PTK7 to neural crest cells. PTK7 constructs were expressed under the control of the neural crest-specific *slug* promoter, so that only the cells that are already specified to become neural crest cells are affected. Regions of the *slug* promoter that are sufficient to drive neural-crest-specific expression (Vallin et al., 2001) were used to express GFP (Fig. 7A,B), full-length PTK7 (Fig. 7C,D) and a deletion mutant of the kinase motif, ΔkPTK7 (Fig. 7E,F). ΔkPTK7 was used as a PTK7 antimorph, as it failed to recruit dsh to the plasma membrane in animal cap assays (Fig. 1F) and caused similar neural crest migration defects as the PTK7 MO in whole embryos (injection of 50 pg *ΔkPTK7* RNA caused 44% neural crest migration defect, *n*=122). Plasmids were injected together with *lacZ* RNA as a lineage tracer in one blastomere at the two-cell stage and embryos were analyzed for twist expression at neurula and tadpole stages. As plasmid injections result in mosaic expression, the effects are generally not as strong as MO or RNA injections. Nevertheless, neural crest-specific expression of ΔkPTK7 significantly inhibited the migration of twist-expressing neural crest cells (Fig. 7E,F), whereas the expression of GFP or PTK7 rarely affected neural crest migration (Fig. 7A-D,G). Although we cannot completely rule out the possibility that PTK7 does also affect neural crest induction, these data demonstrate that PTK7 has an independent function in neural crest migration.

Recruitment of the DEP domain by PTK7 is required for neural crest migration

Having established that PTK7 is required for neural crest migration, we can use this system to analyze the in vivo relevance of the PTK7-mediated dsh membrane localization. By targeting the kinase deletion mutant of PTK7, ΔkPTK7, to neural crest cells, we have already demonstrated that failure of dsh localization (Fig. 1F,G) inhibits neural crest migration (Fig. 7F,P). Next, to characterize the genetic interaction of PTK7 with dsh in migrating neural crest cells, we targeted the expression of PTK7, as well as of different dsh mutants, to neural crest cells using the *slug* promoter system. First, we analyzed how dsh, ΔPDZ dsh and ΔDEP dsh affect neural crest migration. We used ΔPDZ dsh, because this mutant abolishes the PTK7-mediated translocation, and ΔDEP dsh, because the DEP domain is required for PCP activity and affects neural crest migration (De Calisto et al., 2005; Wallingford and Habas, 2005). Single injections of all dsh constructs and a single injection of *slug*-PTK7 produced only very mild defects (Fig. 7H,I,P). The same holds true for co-injection of *slug*-PTK7 with *slug*-dsh or *slug*-ΔPDZ (Fig. 7L-P). However, the combination of *slug*-PTK7 with *slug*-ΔDEP inhibited neural crest migration to an extent that is comparable with expression of *slug*-ΔkPTK7 (Fig. 7J,K,P), which cannot recruit dsh to the plasma membrane. This suggests that, although ΔDEP dsh can be

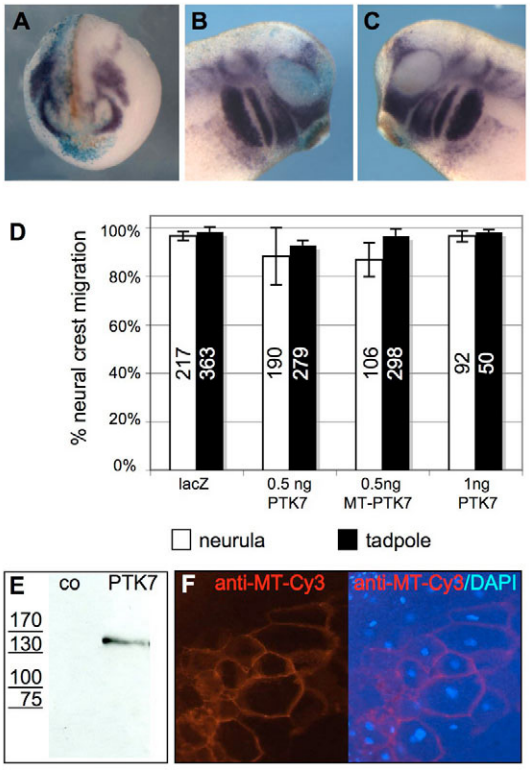

Fig. 5. PTK7 overexpression does not affect neural crest migration. (**A-C**) Embryos were injected with 1 ng *PTK7* RNA and 50 pg *lacZ* RNA in one blastomere at the two-cell stage and analyzed for *twist* expression by in situ hybridization at neurula (A) and tadpole stages (B,C). Light blue lacZ staining marks the injected side. *Twist*-positive neural crest cells show normal migration at neurula (A) and tadpole stages (B,C). (B) Injected side of a tadpole stage embryo. (C) Uninjected side. (**D**) Graph summarizing the percentage of *twist*-expressing embryos injected with 50 pg *lacZ* RNA, 0.5 ng *PTK7* RNA, 0.5 ng myc-tagged *PTK7* RNA or 1 ng *PTK7* RNA at neurula and tadpole stages. Numbers on the columns indicate the number of injected embryos. (**E**) Confirmation of PTK7 expression using western blotting (co, uninjected controls) or (**F**) immunostaining on transverse sections of neurula stage embryos injected with 0.5 ng myc-tagged *PTK7* RNA. In F, membrane localization can be detected with a Cy3-coupled myc antibody (left panel). Right panel shows an overlay with the DAPI staining.

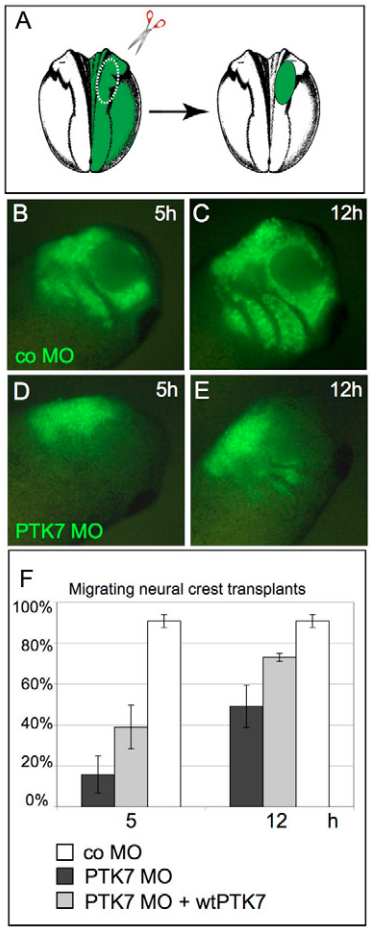

Fig. 6. PTK7 is necessary for the migration of transplanted neural crest cells. (**A**) Transplantation assay. Two-cell stage embryos are injected with MO and 100 pg *GFP* RNA. At early neurula stage, premigratory neural crest cells are transplanted into uninjected control embryos from which the endogenous neural crest had been removed. (**B**,**C**) Transplant injected with 10 ng control MO showing normal neural crest migration 5 hours (B) and 12 hours (C) after transplantation. (**D**) A transplant injected with 10 ng PTK7 MO does not migrate 5 hours after transplantation, but (**E**) shows some migrating cells 12 hours after transplantation. (**F**) Graph summarizing a total of four independent experiments (n=73) using grafts injected with 10 ng control MO (n=29), 10 ng PTK7 MO (n=29) or 10 ng PTK7 MO co-injected with 1 ng *PTK7* RNA, lacking the MO-binding sites (n=15).

recruited by PTK7, its lack of PCP activity inhibits neural crest migration. Co-expression of wild-type dsh, which can be translocated but does not lack the DEP domain, does not affect neural crest migration. The same holds true for ΔPDZ, which is not translocated by PTK7. Thus, these data indicate that PTK7 is required to localize a functional DEP domain to the plasma membrane to enable neural crest migration.

DISCUSSION

PCP signaling in vertebrates regulates complex cell movements, but the molecular mechanisms that guide these processes are not well understood. Here, we show that PTK7, a regulator of vertebrate PCP signaling, is required for neural crest migration, and we provide in vitro as well as in vivo evidence that PTK7 regulates PCP signaling by recruiting dsh to the plasma membrane.

Neural crest cells migrate extensively on defined routes throughout the embryo to give rise to a range of derivatives. However, the signaling mechanisms guiding these organized cell movements are not well characterized. As overexpression of dsh mutants, which are defective in PCP signaling, inhibits neural crest migration in *Xenopus*, this indicates that PCP signaling plays a role (De Calisto et al., 2005). Although, most PCP effectors are expressed at the right time and location for a function in neural crest migration (Bekman and Henrique, 2002; Darken et al., 2002; Goto and Keller, 2002; Nakaya et al., 2004), their function in this process has so far not been analyzed. Here, we identify PTK7 as the first regulator of PCP with a function in neural crest migration. Loss of function of PTK7 inhibits neural crest migration, and transplantation assays show that neural crest cells directly require PTK7 for

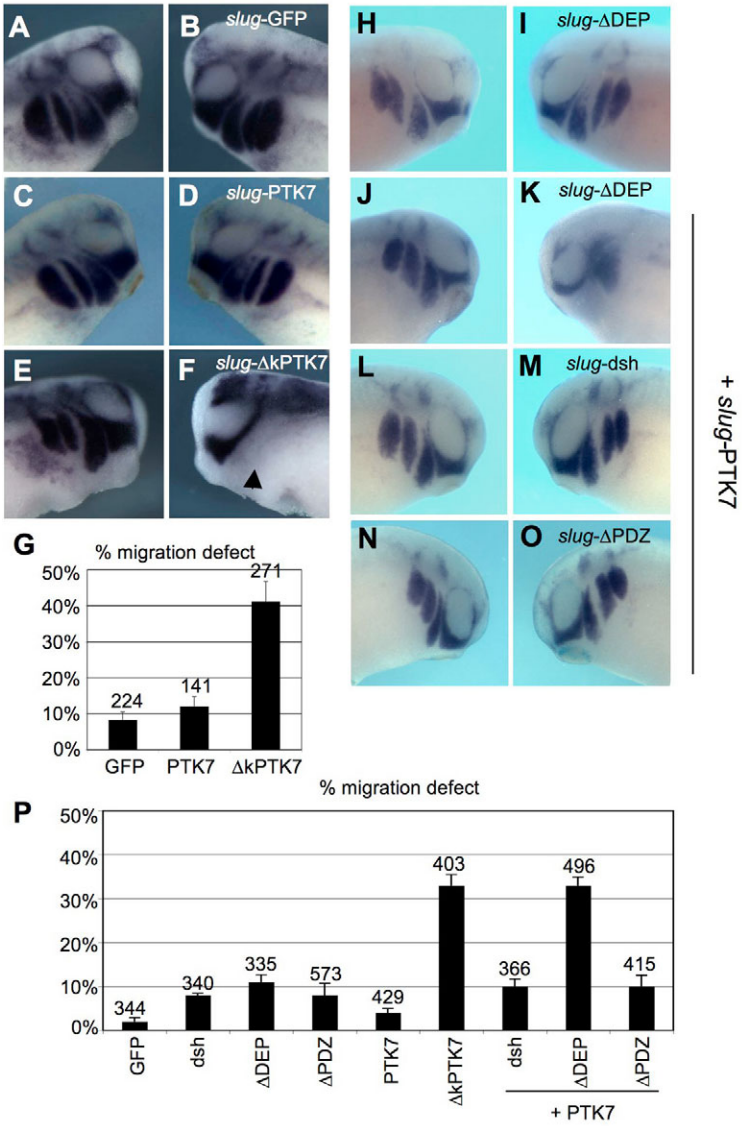

Fig. 7. Neural-crest-specific expression of PTK7 and dsh constructs. Two-cell stage embryos were injected in one blastomere with plasmids containing a minimal *slug* promoter driving either the expression of GFP (A,B), wild-type PTK7 (C,D) or ΔkPTK7 (E,F). (A-F) Tadpole stage embryos analyzed by *twist* in situ hybridization. The injected side is presented in the right panel (B,D,F). (**A**,**B**) Embryo injected with 100 pg *slug*-GFP plasmid. (**C**,**D**) Embryo injected with 100 pg *slug*-PTK7 plasmid. (**E**,**F**) Embryo injected with 100 pg *slug*-ΔkPTK7. Arrowhead in F indicates the inhibition of neural crest migration. (**G**) Graph summarizing the percentage of migrating neural crest cells in five independent injection experiments. The number of injected embryos is indicated on each column. (**H**,**I**) Embryo injected with 50 pg *slug*-ΔDEP. (**J-O**) Embryo injected with 50 pg *slug*-PTK7 together with 50 pg *slug*-ΔDEP (J,K) or 50 pg *slug*-dsh (L,M) or 50 pg *slug*-ΔPDZ (N,O). (**P**) Graph summarizing three dsh and PTK7 co-injection experiments. The number of injected embryos is indicated on each column.

migration. Furthermore, this function is independent of the role of PTK7 in neural tube closure, as demonstrated by neural crest-specific expression.

The data presented here, suggests that PTK7 regulates neural crest migration by recruiting dsh to the plasma membrane. Dsh occupies a key position at the crossroad of all branches of the Wnt signaling cascade and several lines of evidence indicate that PTK7 channels dsh signaling towards PCP signaling. First, PTK7 affects processes regulated by PCP signaling such as convergent extension/neural tube closure and inner ear hair cell polarity in vertebrates (Lu et al., 2004). Second, PTK7 displays heteroallelic interaction with another regulator of PCP signaling, Vangl2 (Looptail mice) (Lu et al., 2004). Third, PTK7 does not activate β-catenin signaling, but does activate JNK signaling. Neither in TOPFLASH reporter assays (data not shown) nor in *Xenopus* laevis double axis assays (0% in n=168) does PTK7 activate canonical Wnt signaling. However, PTK7 overexpression in *Xenopus* animal caps activates the phosphorylation of JNK (see Fig. S4 in the supplementary material), indicating PCP activity (Boutros et al., 1998; Habas et al., 2003; Li et al., 1999; Yamanaka et al., 2002). Thus, these data suggest that PTK7-mediated dsh recruitment activates PCP signaling.

In vivo evidence supports a model in which PTK7 recruits dsh to the plasma membrane, thereby regulating neural crest migration (Fig. 8A). This is experimentally supported by two observations. First, the PTK7 MO as well as ΔkPTK7, which both abolish PTK7-dependent dsh-recruitment, inhibit neural crest migration (Fig. 8B). Second, overexpression of wild-type *slug*-PTK7 together with *slug*-ΔDEP dsh, which is deficient in PCP activity, but can be recruited to the membrane, inhibits neural crest migration to the same extent as *slug*-ΔkPTK7 (Fig. 8C). Hence, if the DEP domain is missing or dsh cannot be localized to the plasma membrane, neural crest migration is inhibited (Fig. 8B,C). In contrast to RNA injection experiments, slug-driven expression of single dsh mutants did not affect neural crest migration. This could be caused by the late onset as well as the mosaic nature of the expression. Thus, overexpressed dsh proteins cannot effectively compete with endogenous dsh, unless ectopic PTK7 is simultaneously provided. Conversely, excess PTK7 does not affect neural crest migration unless it translocates ΔDEP dsh to the plasma membrane. Thus, PTK7 may function by anchoring dsh to the plasma membrane, which then regulates neural crest migration via its DEP domain probably by activating JNK signaling.

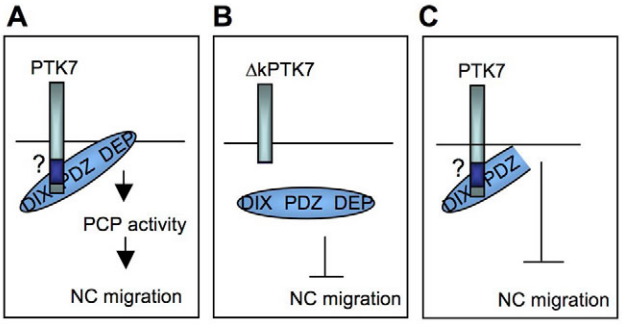

Fig. 8. Model of the role of PTK7 in neural crest migration. (**A**) Overexpression of PTK7 recruits dsh to the plasma membrane, leading to activation of PCP and enabling neural crest migration. The interaction of PTK7 and dsh may involve fz7 (indicated as ?). (**B**) Deletion of the conserved kinase domain of PTK7 inhibits membrane localization of dsh and inhibits neural crest migration. (**C**) ΔDEP dsh can be recruited to the membrane by PTK7, but lacks PCP activity and therefore blocks neural crest migration.

The mechanism by which PTK7 mediates dsh recruitment is unclear. Our data suggest that PTK7 is part of the fz-dsh complex that localizes dsh to the plasma membrane. First, PTK7 is co-precipitated with fz7/dsh in *Xenopus* lysates and HEK293 cells (data not shown). Second, PTK7 loss of function inhibits the fz7-mediated dsh recruitment and hyperphosphorylation. Alternatively, as the binding activity of PTK7/fz7/dsh is weak, PTK7 may contribute to the fz/dsh interaction at the signaling level possibly by promoting dsh phosphorylation. As the kinase domain of PTK7 lacks amino acid residues crucial for kinase activity (Kroiher et al., 2001; Miller and Steele, 2000), this scenario could involve additional PTK7-binding partners that mediate dsh phosphorylation. Independent of the molecular nature of the PTK7 interaction with fz/dsh – be it binding, signaling or both – it also remains to be seen whether PTK7 exclusively interacts with fz7 or whether other fz family members can replace it. The latter is supported by the finding that animal caps express other fz family members, including fz8 (see Fig. S3 in the supplementary material), which, like fz7, recruits dsh to the plasma membrane (Rothbacher et al., 2000). Furthermore, loss of function of fz7 does not affect the PTK7-mediated dsh localization in animal caps (data not shown), suggesting that either PTK7 can recruit dsh independently of fz7 or indeed that other fz family members can also interact with PTK7. As fz7 is expressed at the right time and place for a function in neural crest migration (De Calisto et al., 2005), it probably takes part in the in vivo PTK7-fz-dsh interaction. Future experiments will be needed to address the molecular composition, as well as the function, of the PTK7-fz-dsh interaction in neural crest migration.

Our findings supports the notion that PCP signaling is not limited to epithelia, but is also found in moving mesenchymal tissues such as migrating neural crest cells (Kuriyama and Mayor, 2008). Neural crest cells migrate over long distances, but also stay in contact with each other (Teddy and Kulesa, 2004). Thus, PCP signaling may pattern migrating cells or orient cell protrusions. Indeed, PCP factors determine the polarization of neural crest cells by lamellipodia formation (De Calisto et al., 2005; Kuriyama and Mayor, 2008) and PTK7 could mediate this by activating dsh-JNK signaling at precise cellular locations. Additionally, as PTK7 contains extracellular immunoglobulin domains, which have been implicated in homophilic binding (Lu et al., 2004; Pulido et al., 1992), PTK7 may affect cell clustering or cell contact persistence. The latter was shown for fz7, which modulates local cell contact persistence to coordinate cell movements during zebrafish gastrulation (Witzel et al., 2006). Furthermore, PTK7 could also function as a receptor for signals that guide migrating neural crest cells. Indeed, functional analyses of the *Drosophila* PTK7 ortholog (otk, off-track) indicate that PTK7 is a co-receptor for semaphorin/plexins (Winberg et al., 2001), which are guidance cues for migrating neural crest cells in vertebrates (Gammill et al., 2007; Gammill et al., 2006; Yu and Moens, 2005). The next challenge will be to further dissect the signaling mechanism of vertebrate PTK7 and analyze whether it can also respond to extracellular signals to guide neural crest migration.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/24/4015/DC1

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