Essential role of non-canonical Wnt signalling in neural crest migration

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Summary

Migration of neural crest cells is an elaborate process that requires the delamination of cells from an epithelium and cell movement into an extracellular matrix. In this work, it is shown for the first time that the non-canonical Wnt signalling [planar cell polarity (PCP) or Wnt-Ca^{2+}] pathway controls migration of neural crest cells. By using specific Dsh mutants, we show that the canonical Wnt signalling pathway is needed for neural crest induction, while the non-canonical Wnt pathway is required for neural crest migration. Grafts of neural crest tissue expressing non-canonical Dsh mutants, as well as neural crest cultured in vitro, indicate that the PCP pathway works in a cell-autonomous manner to control neural crest migration. Expression analysis of non-canonical Wnt ligands and their putative receptors show that *Wnt11* is expressed in tissue adjacent to neural crest cells expressing the Wnt receptor *Frizzled7* (*Fz7*). Furthermore, loss- and gain-of-function experiments reveal that Wnt11 plays an essential role in neural crest migration. Inhibition of neural crest migration by blocking Wnt11 activity can be rescued by intracellular activation of the non-canonical Wnt pathway. When Wnt11 is expressed opposite its normal site of expression, neural crest migration is blocked. Finally, time-lapse analysis of cell movement and cell protrusion in neural crest cultured in vitro shows that the PCP or Wnt-Ca^{2+} pathway directs the formation of lamellipodia and filopodia in the neural crest cells that are required for their delamination and/or migration.

Key words: Neural crest, Cell migration, Wnt, Wnt11, Fz7, Non-canonical, PCP

Introduction

Neural crest cells give rise to a variety of cell types, including neurons and glial cells in the peripheral nervous system, and connective tissues of the craniofacial structures. The neural crest is initially formed at the junction of the epidermal and neural ectoderm by mutual interaction between these tissues, and by signals from the mesoderm. Several molecules have been implicated in neural crest induction, including BMPs, Wnts, FGF, Notch and Retinoic Acid (for reviews, see Aybar and Mayor, 2002; Basch et al., 2004; Dorsky et al., 2000; Heeg-Truesdell and LaBonne, 2004; Huang and Saint-Jeannet, 2004; Knecht and Bronner-Fraser, 2002; Mayor and Aybar, 2001).

Once the neural crest is induced at the border of the neural plate, its cells delaminate and move along specific routes to their destination in the embryo. A number of molecules are known to participate in neural crest delamination and migration, such as cadherins, Rho GTPases, Noggin and several extracellular matrix molecules (Borchers et al., 2001; Bronner-Fraser et al., 1992; Henderson et al., 2000; Hoffmann and Balling, 1995; Kimura et al., 1995; Liu and Jessell, 1998; Nakagawa and Takeichi, 1995; Nakagawa and Takeichi, 1998; Perris and Perissinotto, 2000; Pla et al., 2001; Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld and Kalcheim, 2000; Takeichi et al., 2000; Vallin et al., 1998; Van de Putte et al., 2003; Yagi and Takeichi, 2000). However, the mechanisms by which extracellular signals are integrated with cell adhesion and cytoskeletal modification to orchestrate the cell movements underlying delamination and movement of the neural crest are still unclear.

Mesoderm is another tissue that undergoes extensive cell movement. In recent years, evidence has accumulated from studies in zebrafish and *Xenopus* embryos that supports the notion that the migration of mesodermal cells during gastrulation is dependent on factors similar to those involved in planar cell polarity (PCP) in *Drosophila*, which are activated by non-canonical Wnt signalling (for reviews, see Keller, 2002; Mlodzik, 2002; Myers et al., 2002; Ueno and Greene, 2003; Veeman et al., 2003b; Wallingford et al., 2002).

Non-canonical Wnt signalling (Planar Cell Polarity or Wnt-Ca^{2+}) affects convergent extension movements through a pathway similar to the *Drosophila* PCP pathway. One element in this pathway is the protein Dishevelled (Dsh); a domain of this protein is required for PCP and for convergent extension in vertebrates (Axelrod et al., 1998; Boutros et al., 1998; Heisenberg et al., 2000; Tada and Smith, 2000). Perturbation of non-canonical Wnt signalling disrupts the mediolateral elongation and alignment of dorsal mesodermal cells, and the mediolateral stabilization of cell protrusions (Wallingford et al., 2000). In addition, interference with the non-canonical Wnt

signalling pathway of zebrafish, *Xenopus* or mouse embryos, either genetically or by the use of morpholinos, produces defects in convergent extension of the mesoderm and failures in neural tube closure (Carreira-Barbosa et al., 2003; Curtin et al., 2003; Darken et al., 2002; Goto and Keller, 2002; Heisenberg et al., 2000; Jessen et al., 2002; Kibar et al., 2001; Kilian et al., 2003; Park and Moon, 2002; Rauch et al., 1997; Takeuchi et al., 2003; Veeman et al., 2003b).

Here, we present evidence that the non-canonical Wnt pathway regulates neural crest migration. We conclude from the effect of expressing different mutants of the Dsh proteins in *Xenopus* embryos that canonical (β-catenin-mediated) Wnt signalling participates in neural crest induction, whereas the non-canonical (PCP or Wnt-Ca^{2+}) pathway controls neural crest migration. Grafts of cells containing fluorescent markers and expressing specific Dsh mutants show that non-canonical Wnt signalling is essential for neural crest migration. We show that *Wnt11* is expressed in the ectoderm of *Xenopus* embryos in a region adjacent to the neural crest cells that expresses the Wnt receptor *Fz7*. Loss- and gain-of-function experiments of Wnt11 indicate that this ligand is required for neural crest migration in vivo. In addition, localized overexpression of Wnt11 in *Xenopus* embryos provokes an abnormal migration of the neural crest cells towards the region of high *Wnt* expression. Finally, by performing time-lapse analysis, we show that the non-canonical Wnt signal controls neural crest migration on a fibronectin substrate by stabilizing the protrusions of the migrating neural crest cells.

Materials and methods

Xenopus embryos, micromanipulation and whole-mount in situ hybridization

Xenopus embryos and dissections were obtained as described previously (Mancilla and Mayor, 1996), and embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For in situ hybridization antisense RNA probes, digoxigenin or fluorescein (Boehringer Mannheim) was used as a label. Specimens were stained using the method of (Harland, 1991). For neural crest dissection, we confirmed that the dissected tissue contained neural crest by performing an in situ hybridization for a neural crest marker in the donor embryo.

In vitro RNA synthesis and microinjection of mRNAs

All cDNA was linearized and transcribed, as described by Harland and Weintraub (Harland and Weintraub, 1985) (New England Biolabs). For injections and lineage tracing, mRNA was resuspended in DEPC-water, and co-injected into two- or eight-cell-stage embryos with fluorescein dextran or rhodamine dextran (FDX, RDX; Molecular Probes) using 8-12 nl needles as described by Aybar et al. (Aybar et al., 2003). The constructs used were *Slug* (Mayor et al., 1995); *Wnt11* (Ku and Melton, 1993); *Fz7* (Medina et al., 2000); dd1 and dd2 (Sokol, 1996); and Dsh-ΔN, Dsh-DEP+ and dnWnt11 (Tada and Smith, 2000).

In vitro culture, time lapse and immunostaining of neural crest cells

In vitro culture of neural crest cells was performed as described by Alfandari et al. (Alfandari et al., 2003). For time-lapse recordings of migrating neural crest, images were collected on a Nikon Eclipse E1000 Microscope using a Jenoptik/Jena cam. Images were collected every 2 minutes and time-lapse stacks were assembled and viewed in OpenLab software. Protrusive activity was quantified by counting new protrusions extending, existing protrusions withdrawn, or stable protrusions (present in both the first frame and the last frame of the movie). Phalloidin-rhodamine and microtubule staining was performed by incubating with phalloidin-rhodamine (Sigma-Aldrich), or with a monoclonal antibody against α-tubulin (Sigma-Aldrich) for 1 hour; the secondary antibody used was IgG-FITC (Sigma-Aldrich). Lamellipodia were counted as large when they occupied more than one-third of the cell border, and as normal when they were smaller than one-third of the cell border.

Scanning electron microscopy (SEM)

Embryos were microinjected and their neural crest cultured in vitro as described above. They were then fixed in 0.2 M cacodylate buffer and 1.5% glutaraldehyde, and rinsed in 0.1 M cacodylate buffer, as described previously (Sadaghiani and Thiebaud, 1987). Critical point drying was performed by using ethanol and liquid nitrogen.

Results

Neural crest induction requires canonical Wnt signalling, whereas non-canonical Wnt signalling is required for neural crest migration

Canonical and non-canonical Wnt pathways are dependent on the Dishevelled (Dsh) protein, but specific deletion mutants of Dsh allow the two kinds of signalling properties of the protein to be uncoupled. Fig. 1A shows the different Dsh mutants used in this study and their activities, as described for mesoderm development (Axelrod et al., 1998; Boutros and Mlodzik, 1999; Boutros et al., 1998; Smith et al., 2000; Sokol, 1996; Tada and Smith, 2000). *dd1* and *dd2* mutants disrupt both canonical Wnt (β-catenin) and non-canonical (PCP or Wnt-Ca^{2+}) signalling pathways in *Xenopus*, whereas Dsh-ΔN and Dsh-DEP+ do not affect canonical Wnt signalling but do interfere with PCP/Wnt-Ca^{2+} signalling (Rothbacher et al., 2000; Sokol, 1996; Wallingford et al., 2000). To examine the role of the PCP/Wnt-Ca^{2+} pathway on neural crest migration, we needed to be certain that the Dsh mutants used in this study specifically affect the PCP/Wnt-Ca^{2+} pathway without having an effect on the canonical Wnt pathway that might affect neural crest induction. The animal pole of one blastomere of a two-cell-stage embryo was injected with 1 ng of mRNA for *dd2* or *dd1*. The embryos were then fixed at a premigratory stage (stage 17) and the expression of the neural crest marker *Slug* analyzed by in situ hybridization. The injected cells were identified by the co-injection and immunostaining of FLDx. Inhibition of canonical Wnt signalling by *dd2* and *dd1* dramatically inhibited neural crest induction on the injected side (Fig. 1B-E). By contrast, injection of 1 ng mRNA coding for Dsh-ΔN or Dsh-DEP+ produced no effect in the expression of the neural crest marker *Slug* (Fig. 1F-I). Importantly Dsh-DEP+, a specific inhibitor of the PCP/Wnt-Ca^{2+} pathway, had no effect on neural crest induction as assessed by *Slug* expression (Fig. 1G,I), suggesting that inhibition of non-canonical Wnt signalling does not affect neural crest induction.

Based on these results, we decided to use Dsh-DEP+ and Dsh-ΔN to study neural crest migration. Embryos were injected as described, but fixed at stages when neural crest migration is taking place (stages 24-25). Injection of either construct produced a dramatic effect on neural crest migration as visualized by *Slug* expression (Fig. 2A,B). On the injected side, *Slug*-expressing cells were seen in a group on the surface of the embryo with no indication of cell migration (white arrowheads in Fig. 2A,B), whereas the uninjected side (the

control) showed the normal streams of cephalic neural crest cell migration (red arrowheads in Fig. 2A,B). To inhibit the PCP/Wnt-Ca^{2+} pathway specifically in neural crest cells, the following experiment was performed (Fig. 2C). FDX was injected at the one-cell stage, either alone or together with mRNA encoding Dsh-DEP+. Then, at the early neurula stage, the prospective neural crest was grafted into a normal embryo. Host embryos were then cultured to stage 26, when the distribution of the fluorescent neural crest cells was examined. Grafts of control neural crest cells show a normal distribution,

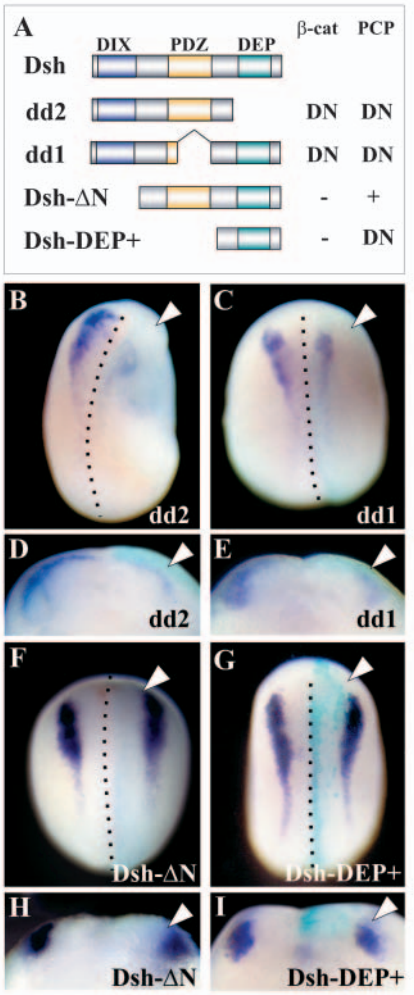

Fig. 1. Neural crest induction is dependent on canonical, but not non-canonical, Wnt signalling. (A) Several dishevelled (Dsh) mutants were used to distinguish between canonical (β-cat) and non-canonical (PCP) Wnt signalling. DN, dominant negative; –, no effect; +, activation. (B-I) mRNA coding for each of these mutants was injected at the two-cell stage into the animal region fated to become ectoderm, the embryos were cultured until the equivalent of stage 17 and the expression of the neural crest marker *Slug* was analyzed. B,C,F and G are dorsal views; D,E,H and I are sections; anterior is to the top. The injected side (arrowhead) was identified by the lineage marker FDX (pale green). (B,D) Embryo injected with 1 ng of *dd2* mRNA. Strong inhibition of the neural crest marker on the injected side is observed (35% of embryos showed inhibition of *Slug* expression, *n*=65; embryos with gastrulation defects were not included). (C,E) Embryo injected with 1 ng of *dd1* mRNA. Strong inhibition of the neural crest marker on the injected side is observed (37% of embryos showed inhibition of *Slug* expression, *n*=85; embryos with gastrulation defects were not included). (F,H) Embryo injected with 1 ng of *Dsh-ΔN* mRNA. Normal expression of the neural crest marker is observed on the injected side. Some embryos exhibited a weak inhibition of the expression of *Slug* (12% of embryos showed inhibited *Slug* expression, *n*=85). (G,I) Embryo injected with 1 ng of *Dsh-DEP+* mRNA. No effect on the expression of the neural crest marker is observed (*n*=55).

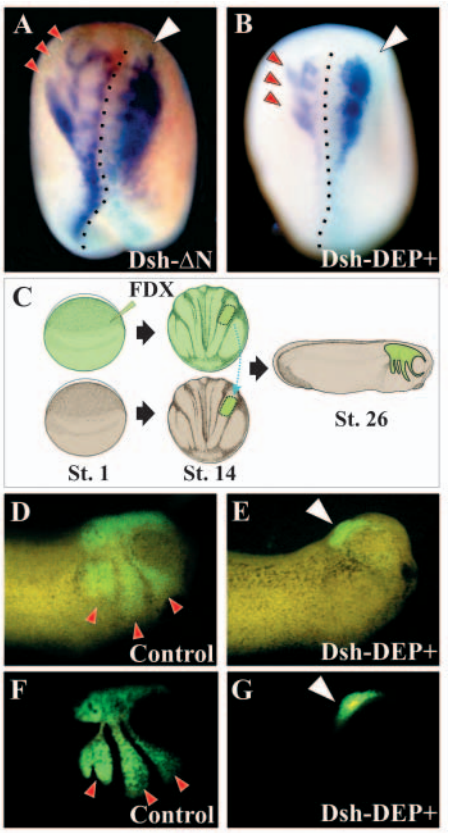

Fig. 2. Neural crest migration is dependent on normal non-canonical Wnt signalling. (A,B) Embryos were injected into the animal blastomeres at the 8-cell stage with 1 ng of mRNA coding for Dsh-ΔN (A) or Dsh-DEP+ (B). The embryos were cultured until stage 24, when the expression of the neural crest marker *Slug* was analyzed at postmigratory stages; the injected side (white arrowhead) was identified by FDX expression (pale green). The uninjected side shows the normal pattern of cephalic neural crest migration, which is indicated by the red arrowheads, each one pointing to the mandibular, hyoid and branchial neural crest, respectively. The injection of *Dsh-ΔN* and *Dsh-DEP+* led to a dramatic inhibition of neural crest migration (white arrowhead in A,B; 40%, *n*=60, and 45%, *n*=55, of embryos showed inhibition of neural crest migration, respectively). (C) One-cell-stage embryos were injected with mRNA coding for Dsh-DEP+, together with the fluorescent lineage tracer FDX (green). At the early neurula stage, the prospective cephalic neural crest were taken from the injected embryos and grafted into a normal uninjected neurula embryo. The migration of the neural crest was analyzed in vivo by following the fluorescence label until stage 26, when the cephalic neural crest has reached its final destination. (D,F) Control embryo showing the normal pattern of cephalic crest migration; 95% of grafted embryos exhibited normal migration, *n*=30. (E,G) Embryo grafted with neural crest taken from an embryo expressing Dsh-DEP+. No migration of the neural crest is observed on the operated side. Only 5% of grafted embryos showed normal migration, *n*=20.

with typical streams of migrating cephalic neural crest cells (red arrowheads in Fig. 2D,F). However, grafts of cells expressing Dsh-DEP+ showed complete inhibition of migration of the neural crest (white arrowhead in Fig. 2E,G), consistent with the phenotype shown in Fig. 2B.

Wnt11 is involved in controlling neural crest migration

Next, we analyzed the possible ligand of the non-canonical Wnt signalling involved in neural crest migration. Several members of the Wnt family (Wnt4, Wnt5a and Wnt11) have been proposed as activators of the non-canonical Wnt signalling pathway (reviewed by Kuhl, 2002). We have focused on Wnt11 as a possible candidate for a ligand that controls crest migration.

The expression of *Wnt11* was compared with that of the neural crest marker *Slug* at different times during development (Fig. 3). Our results show that just before migration of the neural crest (stage 17) *Wnt11* is expressed adjacent to the prospective migrating cells (Fig. 3A-C,E-G). The prospective neural crest, defined by expression of *Slug* (Fig. 3A,E), is adjacent to a continuous band of *Wnt11*-expressing cells flanking the prospective pathway of migration (Fig. 3B,F). Double in situ hybridization for *Slug* and *Wnt11* shows *Wnt11* expression at the most lateral side of *Slug* expression (Fig. 3C,G). The continuous band of *Wnt11* that borders the cephalic neural crest is not uniform; there are regions where *Wnt11* seems to be expressed more strongly or in a larger population of cells (compare black and white arrows in Fig. 3C). Once neural crest cells start to migrate (Fig. 3I), the *Wnt11*-expressing cells do not move, instead they remain on the dorsal aspect of the neural tube (Fig. 3J) while the neural crest cells move underneath them (Fig. 3K).

Although no specific Wnt11 receptor has been identified, there is some evidence that suggests that PCP Wnt signalling involves Fz7 (Carreira-Barbosa et al., 2003; Djiane et al., 2000; Medina et al., 2000; Sumanas and Ekker, 2001; Winklbauer et al., 2001). We examined the distribution of the Fz7 receptor in neural crest cells. Our results show expression of *Fz7* in different regions of the neural ectoderm, as has been described previously (Djiane et al., 2000; Wheeler and Hoppler, 1999), including in the pre-migratory neural crest (Fig. 3D,H) and the migrating crest cells (Fig. 3L). A comparison of *Fz7* and *Slug* expression indicates that *Fz7* is expressed in a subpopulation of neural crest cells located adjacent to the *Wnt11*-expressing cells in the ectoderm (Fig. 3E,G,H). Interestingly, these cells are probably the first cells to delaminate. In summary, early in neural crest migration, *Wnt11* is present in cells adjacent to the first migrating cells, which also express the receptor *Fz7* (Fig. 3M). Once the neural tube closes, the early migrating crest cells move away and beneath the *Wnt11*-expressing cells, so that later migrating cells come into contact with the source of Wnt11 signalling (Fig. 3N).

As the expression pattern of *Wnt11* occurs in the right place and at the right time to perform a key role in controlling neural crest migration, we investigated the effect of gain and loss of function. This was done by injecting the mRNA of Wnt11 and a dominant-negative form of it (Tada and Smith, 2000) (Fig. 4A). Embryos were injected into two dorsal blastomeres of an eight-cell stage embryo with 1 ng of *Wnt11* and 2 ng of dominant-negative *Wnt11* (*dnWnt11*) mRNA, and the neural crest marker *Slug* was analyzed before or after neural crest migration. Injected cells were identified by the pale blue colour which results from the immunostaining of the lineage marker FLDx. Injection of any of these constructs did not affect the expression of the neural crest marker *Slug* at the early neurula stage, even in those cases in which gastrulation was affected (stage 17, Fig. 4B,D), indicating that Wnt11 is not involved in neural crest induction. All further neural crest migration experiments were conducted only with embryos that showed normal blastopore closure. Inhibition of neural crest migration was observed after injection of *Wnt11* (Fig. 4C) or *dnWnt11* (Fig. 4E), with a similar phenotype

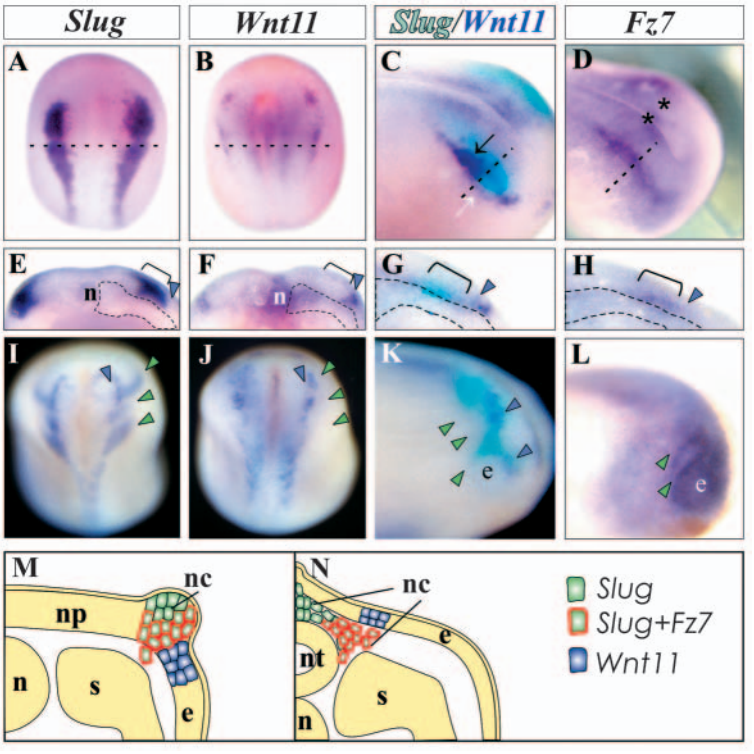

Fig. 3. *Wnt11* is expressed adjacent to the migrating neural crest. Simple and double in situ hybridization were performed for the neural crest marker gene *Slug*, *Wnt11* and *Fz7*, as indicated at the top of the figure. (A-D) Dorsal (A,B) and lateral (C,D) views of stage 16-17 embryos; dashed lines indicate the sections shown in E-H; white arrow, weaker *Wnt11* expression; black arrow, stronger *Wnt11* expression. (E-H) Sections of the embryos shown in A-D. Purple arrowhead indicates the region where *Wnt11* is detected. n, notochord; bracket, region of *Slug* and *Fz7* expression; dashed line marks the endomesoderm. (I-K) Dorsal (I,J) and lateral (K,L) views of stage 23 embryos. e, eye; purple arrowhead indicates the region where *Wnt11* is detected; green arrowhead shows the three streams of migrating neural crest. (M,N) Summary of *Slug, Fz7* and *Wnt11* expression. (M) Premigratory stages. *Wnt11* is expressed in the ectoderm adjacent to the neural crest just before migration starts. A subpopulation of the neural crest cells expresses *Fz7*. (N) Migratory stages. During neural crest migration, *Wnt11* is expressed next to the migrating neural crest. np, neural plate; nc, neural crest; s, somite; n, notochord; nt, neural tube; e, epidermis.

to that obtained when Dsh mutants were used to block the PCP/Wnt-Ca^{2+} pathway (Fig. 2A,B). As overexpression and inhibition of Wnt11 results in the same phenotype, we performed a rescue experiment by co-injecting these two molecules. Injection of *Wnt11* and of *dnWnt11* leads to an inhibition of neural crest migration in 30% and 35% of the embryos, respectively, whereas co-injection of both mRNAs reduces that inhibition to 10%, indicating a rescue in about one-third of the injected embryos (Fig. 4F,G). Taken together, these results support the conclusion that Wnt11 controls neural crest migration.

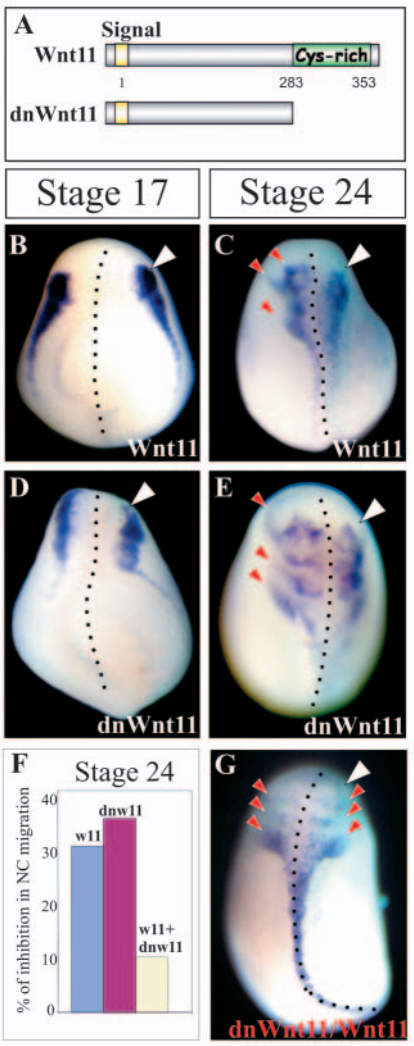

Fig. 4. Wnt11 activity is required for neural crest migration. (A) 1 ng of *Wnt11* or 2 ng of its dominant-negative mRNA were injected in one animal blastomere of an eight-cell embryo. (B-G) Embryos were cultured until the premigratory (B,D; indicated as stage 17) or migratory (C,E,G; indicated as stage 24) neural crest stages, when the expression of *Slug* was analyzed. All of the embryos are shown in dorsal view, anterior to the top. The injected side is indicated by a white arrowhead. Normal neural crest migration is indicated by red arrowheads. (B,C) Wnt11 overexpression. (B) 90% of embryos showed normal *Slug* expression, *n*=55. (C) 30% of embryos showed inhibited neural crest migration, *n*=55. (D,E) Expression of dominant-negative Wnt11. (D) 90% of embryos showed normal *Slug* expression, *n*=65. (E) 35% of embryos showed inhibited neural crest migration; *n*=65. (F) Summary of inhibition of neural crest migration after injecting *Wnt11* (w11), dominant-negative *Wnt11* (dnw11), or both (w11+dnw11). (G) Co-injection of *Wnt11* and its dominant-negative form. 90% of embryos showed normal neural crest migration, *n*=50.

Our observations that Wnt11 activity is required for neural crest migration and that *Wnt11* is expressed adjacent to the early migrating neural crest cells prompted us to test the hypothesis that Wnt11 influences neural crest migration by 'attracting' cells to regions with high Wnt11 levels. In order to analyze this proposition, the following experiment was performed in which Wnt11 was overexpressed in a localized manner opposite from its endogenous expression (Fig. 5A). Embryos were injected with 1 ng of *Wnt11* mRNA. To have a naive ectoderm expressing Wnt11, the animal cap was dissected at stage 9, but because at this stage the ectoderm is competent to respond to neural crest induction, the animal cap was cultured in vitro under a coverslip until the equivalent of stage 14, when the competence for neural crest induction has been lost (Bastidas et al., 2004; Mancilla and Mayor, 1996). With the purpose of generating a localized source of Wnt11 signalling, the cultured ectoderm was grafted into the neural plate region of a normal stage 14 embryo, but adjacent to the prospective neural crest cells, and neural crest migration was analyzed. Control uninjected grafts had no effect on migration of the neural crest (Fig. 5B,D); however, grafts that expressed Wnt11 block neural crest migration (dashed line in Fig. 5C,E; 87% of embryos showed an inhibited pattern of neural crest migration, *n*=22). When larger grafts were used, the most common outcome was the localization of the neural crest at the base of the graft, with a slight tendency to move into the graft (data not shown). The overexpression of Wnt11 in the normal neural crest migration pathway (Fig. 5F) leads to a local promotion of cell movement (Fig. 5G-N), but usually the cells are not able to continue their migration along the normal pathway; instead, they become stacked under the Wnt11-expressing graft or they invade the graft (Fig. 5K-N).

Finally, we analyzed whether the inhibition of neural crest migration by dnWnt11 could be rescued by intracellular activation of the PCP/Wnt-Ca^{2+} pathway in the neural crest cells (Fig. 6). The normal migration of the neural crest (Fig. 6B) was inhibited by the expression of dnWnt11 (Fig. 6C); however, when these embryos received a graft of neural crest taken from an embryo injected with Dsh-ΔN, an activator of the non-canonical pathway (Tada and Smith, 2000), complete rescue of neural crest migration was observed (Fig. 6D,E).

Non-canonical Wnt signalling controls cell protrusion in the migrating neural crest cells

The non-canonical Wnt pathway controls convergent extension of mesoderm during gastrulation movements by instigating a directional activity of the lamellipodia that favours movement in one direction (Carreira-Barbosa et al., 2003; Wallingford et al., 2000). Although no similar directionality of individual migratory neural crest cells has been described, we decided to investigate whether we could identify similar cell behaviour, controlled by non-canonical Wnt signalling. We cultured neural crest cells in vitro and analyzed their behaviour (Fig. 7A). In control explants, neural crest cells migrated normally (Fig. 7B), as described previously (Alfandari et al., 2003); however, in explants taken from embryos injected with 1 ng of *Dsh-DEP+* mRNA or 2 ng of *dnWnt11*, migration of cells was

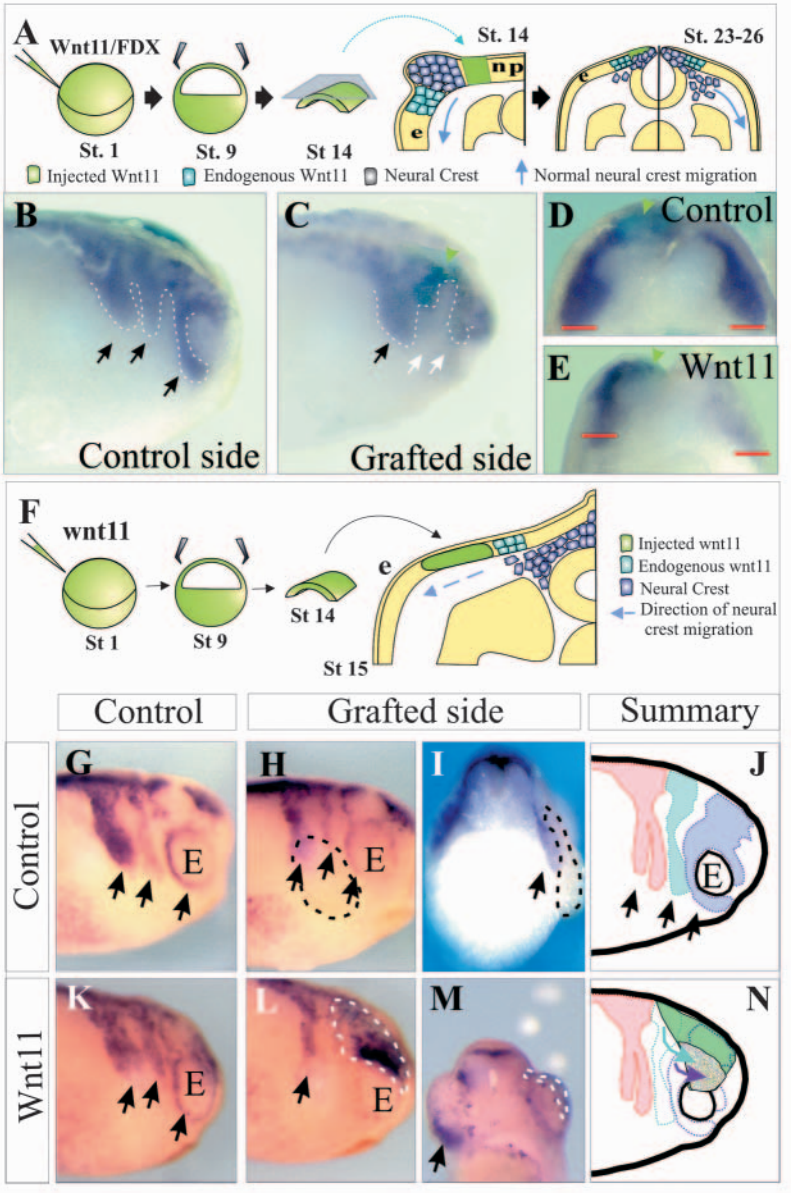

Fig. 5. Effect of localized overexpression of *Wnt11* mRNA on neural crest migration. (A) One-cell stage embryos were injected with *Wnt11* mRNA and FDX. At stage 9, the animal cap was dissected and cultured under a coverslip until the equivalent of stage 14. The ectoderm was grafted adjacent to the neural crest of a host embryo (stage 14), but into the neural plate side. Grafted embryos were cultured until stage 23-26, when neural crest migration was examined by *Slug* expression. Purple arrow indicates the normal migration of the neural crest cells. (B) Control side showing normal cephalic neural crest migration (dashed line), and the three streams of migration (black arrows). (C) Grafted side of the embryo shown in B. Note the inhibition in neural crest migration (dashed line and white arrows). The green arrowhead indicates the graft. (D,E) Sections from grafted embryos. Red bars indicate the ventral limit of neural crest migration; green arrowhead indicates the graft. (D) Graft of ectoderm injected with FDX; no effect on neural crest migration is observed. (E) Section of the embryo shown in B,C. Note the shorter distance of migration in the grafted side. The stronger signal in the grafted side is likely to be a consequence of all of the neural crest cells grouping in the dorsal region. Forty-five percent of embryos grafted with Wnt11-expressing cells showed inhibition of neural crest migration (n=26). (F) Similar experiment to the one described in A, but the Wnt11-expressing cells were grafted onto the normal pathway of neural crest migration. Grafted embryos were cultured until stage 26 when neural crest migration was examined by *Slug* expression. Purple arrow indicates the normal migration of the neural crest cells. (G-N) Anterior to the right. E, eye; dashed line indicates the limit of the graft; arrow indicates the migrating neural crest. (G,H,K,L) The operated (H,L) and control (G,K) sides of the same embryo are shown. (I,M) Sections of grafted embryos. (J,N) Summary of cephalic neural crest migration. Pink, branchial; blue, hyoid; purple, mandibular neural crest. Mixed colours indicate abnormal neural crest migration. The Wnt11-expressing graft is shown in green. Arrows indicate the proposed route of abnormal neural crest migration. (G-J) Control embryo that received an uninjected graft. (K-N) Embryos that received a Wnt11-expressing graft; 80% of the embryos with a Wnt11-expressing graft exhibited this phenotype, n=28.

strongly inhibited (Fig. 7C-E). To examine the effect at a cellular level, we performed a time-lapse analysis of the migrating neural crest cells. The number and shape of cell protrusions was counted in control and Dsh-DEP+ expressing cells in frames from time-lapse video movies (Fig. 7F-I). Our results indicate that in explants from Dsh-DEP+ expressing embryos, there were less cell protrusions than in control cells. The frequency of crest cells withdrawing rather than extending cell processes is greater in the Dsh-DEP+ cells than in the control cells (Fig. 7J). To extend these observations, we visualised actin microfilaments with phalloidin-rhodamine, and microtubules by immunostaining, and then analyzed the size and types of lamellipodia (Fig. 7K-P). In control cells, lamellipodia were larger and more polarized than in the Dsh-DEP+ expressing cells, whereas the Dsh-DEP+ expressing cells exhibited more filopodia than the control neural crest cells (Fig. 7Q). A typical control cell is shown in Fig. 7L,M (more than 50% of cells), while typical Dsh-DEP+ expressing cells are shown in Fig. 7O,P (more than 90% of cells, although most of the cells were found forming groups and very few were isolated). We also analyzed the morphology of the migrating neural crest by SEM (Fig. 7R-T). Control migrating cells exhibited large lamellipodia at the front of migration (yellow arrows in Fig. 7R,R′), while cells injected with *Dsh-DEP+* (Fig. 7S,S′) or *dnWnt11* (Fig. 7T,T′) exhibited long filopodia that frequently were connecting the more packed cells (red arrows).

Discussion

This study reveals that PCP/Wnt-Ca^{2+} signalling is involved in neural crest migration. We have not analyzed whether the PCP or Wnt-Ca^{2+} pathway is controlling neural crest migration because both pathways are inhibited by the same Dsh mutants (Sheldahl et al., 2003). Although there are several similarities with other instances in which PCP participates in cell

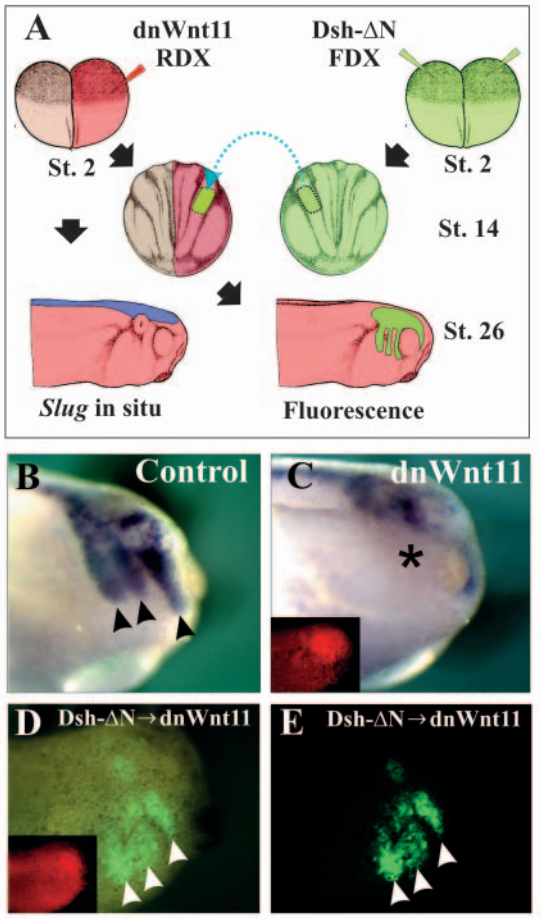

Fig. 6. Inhibition of neural crest migration by dominant-negative Wnt11 is rescued by activated Dsh. (A) Two-cell stage embryos were injected with *dnWnt11* mRNA and RDX (red), or with *Dsh-ΔN* and FDX (green). At stage 14, neural crest cells were taken from the *Dsh-ΔN*-injected embryo and grafted into the *dnWnt11*-injected embryo. Analysis of neural crest migration was performed by examining *Slug* expression and fluorescence. (B) Control side of embryo injected with *dnWnt11*. Arrowheads indicate normal neural crest migration. (C) Injected side of the embryo shown in B. Asterisk indicates the absence of neural crest migration. (Inset) RDX fluorescence. (D,E) Graft of Dsh-ΔN/FDX-expressing cells into an embryo injected with *dnWnt11*/RDX. (Inset) RDX fluorescence. Note the normal migration of the FDX-expressing cells. Seventy-five percent of embryos showed rescued neural crest migration; *n*=20.

migration, we also found some important differences. In vertebrates, there is compelling evidence that the non-canonical Wnt pathway controls aspects of gastrulation, cochlear hair cell morphology, heart induction, dorsoventral patterning, tissue separation, neuronal migration and cancer (for reviews, see Fanto and McNeill, 2004; Kuhl, 2002; Mlodzik, 2002; Strutt, 2003; Veeman et al., 2003a). However, the simplest and best-understood role of PCP is in the organization of hairs in the wing of *Drosophila.* Disruption of the PCP signal produces different phenotypes in which the orientation and the subcellular localization of the hair are affected (Eaton and Cohen, 1996; Gubb and Garcia-Bellido, 1982; Held et al., 1986; Strutt et al., 1997; Winter et al., 2001; Wong and Adler, 1993). An important feature of the PCP molecular mechanism is its effects on asymmetrically localized molecules. This has been particularly well studied in the wing, where *Flamingo*, *Diego*, *Frizzled*, *Dishevelled*, *Strabismus* and *Prickle* become localized along the proximodistal axis of the cells (Axelrod, 2001; Bastock et al., 2003; Feiguin et al., 2001; Shimada et al., 2001; Tree et al., 2002; Usui et al., 1999). A large body of evidence suggests that Fz has a key role in sensing positional information; although what is upstream of Fz remains unknown. It has been proposed that *ft* and *ds* control PCP upstream of Fz/Dsh pathways, and that a gradient of Ft activity sets up the initial asymmetrical localization (Ma et al., 2003; Rawls et al., 2002; Yang et al., 2001), but the origins of the gradient of Ft activity are unknown.

One of the most appealing models of positional information is based on the presence of a morphogen-like molecule that would activate Fz in a dose-dependent manner (Adler et al., 2000; Fanto et al., 2003). It has been suggested that such a molecule could belong to the Wnt family, as it binds to the Fz receptor and should be produced in a localized fashion. No such molecule has been found so far in the *Drosophila* PCP pathway, or in any vertebrate system. Our results show a clear localized expression of *Wnt11*, just adjacent to premigratory neural crest and just before the onset of crest delamination (Fig. 3). Other Wnt family members that participate in the non-canonical pathway (such as Wnt4 and Wnt5a) do not show a similar pattern of expression using in situ hybridization (J.D.C. and R.M., unpublished) (Torres et al., 1996; McGrew et al., 1992). Our functional studies using dnWnt11 also support the notion that this molecule is essential for neural crest migration, while the rescue of the effect of dnWnt11 by co-expression of *Wnt11* mRNA provides evidence for the specificity of this dominant-negative construct (Fig. 4F,G). Furthermore, the effect on neural crest migration of the dnWnt11 can be rescued by specific activation of non-canonical Dsh (Fig. 6).

Several observations suggest that the localized expression of *Wnt11* adjacent to the premigratory neural crest is essential for its function. First, overexpression of Wnt11 in one half of the embryo completely blocks normal neural crest migration (Fig. 4) and suggests a requirement for localized expression for this molecule. Second, when Wnt11 was expressed in a localized manner opposite to its normal expression, the neural crest cells migration was blocked (Fig. 5). Third, when Wnt11 was expressed in the normal route of crest migration the cells migrated actively under the Wnt11-expressing cells (Fig. 5). Taken together, these results show that localized expression of Wnt11 is required to activate the PCP/Wnt-Ca^{2+} pathway and to control neural crest migration. We do not know the molecular function of this localized Wnt expression in the neural crest cells, but the situation in *Drosophila* PCP suggests that a gradient of Wnt11 could determine the asymmetric expression of PCP molecules that direct crest migration. No asymmetrical localization of PCP proteins has been observed in any vertebrate system.

In addition to the evidence suggesting that Wnt11 is required for neural crest migration, our data based on Dsh mutants show that non-canonical Wnt signalling participates in neural crest migration. Dsh-DEP+, a dominant-negative form of Dsh that contains the DEP domain and lacks the DIX and PDZ domains, has been an incisive reagent for analysing the role of the non-canonical pathway in neural crest migration. This blocks the PCP/Wnt-Ca^{2+} pathway without affecting canonical signalling

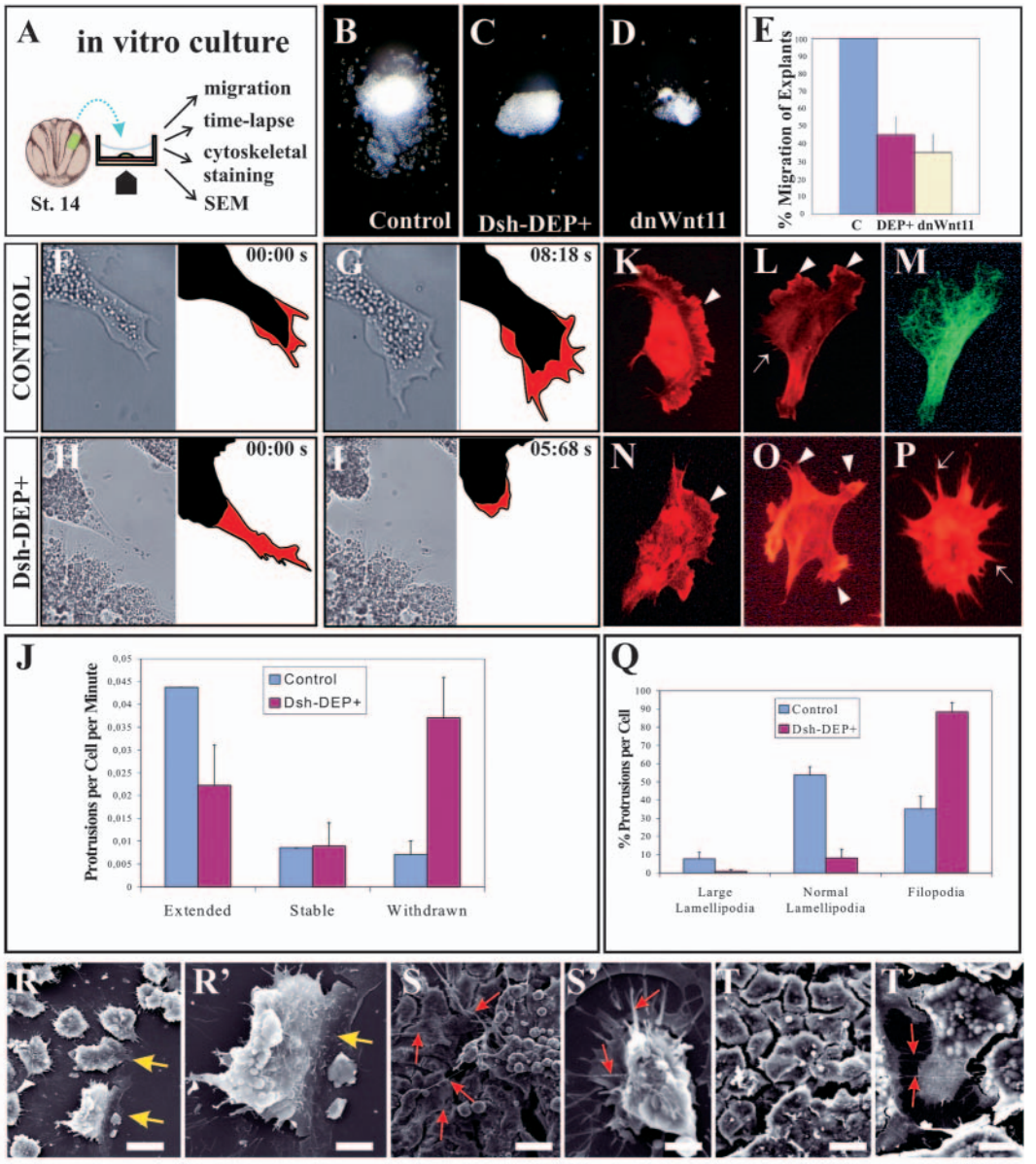

Fig. 7. Analysis of neural crest migration in vitro. (A) Neural crest was dissected from an early neurula and cultured in vitro on fibronectin for several hours. We analyzed migration, cell movement, cytoskeletal staining and cellular morphology. (B) Control neural crest explant, showing normal migration (n=60 explants). (C) Explant taken from an embryo injected with 1 ng of *Dsh-DEP+* mRNA. Note the absence of cell migration (n=30 explants). (D) Explant taken from an embryo injected with 2 ng of *dnWnt11* mRNA. Note the inhibition of cell migration (n=12 explants). (E) Summary of three independent experiments where the percentage migration was analyzed. One hundred pecent of the control explants showed a normal neural crest migration, whereas this process was inhibited in the *Dsh-DEP+* and *dnWnt11*-injected embryos. (F-I) Single time-lapse frames showing representative cell behaviour; right, diagrams of cells with time points. Cell bodies, black; lamellipodia, red. n=8 explants. (F,G) Control cells; (H,I) *Dsh-DEP+* injected cells. (J) Protrusive activity. Control cells extend more lamellipodia and *Dsh-DEP+* injected cells withdraw more protrusions. (K-T′) Analysis of lamellipodia was performed between 2 and 20 hours after culture, by staining actin with Phalloidin-rhodamine (K,L,N-P) or with an antibody against tubulin (M), or by SEM (R-T). Representative cells are shown for control (K-M) and *Dsh-DEP+* injected cells (N-P). (K,N) Representative large lamellipodia. (L,M,O) Representative normal lamellipodia. (M) Tubulin staining for the cell shown in L. (P) Representative cell extending filopodia as its main protrusion. (Q) Types of protrusion. Control cells show large lamellipodia and mainly normal lamellipodia, whereas *Dsh-DEP+* injected cells show mainly filopodia. (R,R′) Control neural crest; yellow arrow indicates a large lamellipodia at the front of migration. (S,S′) Neural crest injected with *Dsh-DEP+*; red arrow indicates filopodia. (T,T′) Neural crest injected with dominant-negative *Wnt11*; red arrow indicates filopodia. Scale bars in R-T′: 10 μm.

(Tada and Smith, 2000). It produces a strong inhibition of neural crest migration in vivo (Fig. 2) and in vitro (Fig. 7), either when injected into one side of the embryo or when specifically expressed in neural crest cells. Analysis of early neural crest markers shows no effect of Dsh-DEP+ on neural crest induction (Fig. 1), indicating that non-canonical signalling does not participate in neural crest induction. Another Dsh mutant, *dd1* is also able to block neural crest migration (data not shown), but it also interferes with the canonical Wnt pathway (Tada and Smith, 2000), and as a consequence reduces neural crest induction (Bastidas et al., 2004). Overexpression of Wnt11 dramatically affected neural crest migration, but also affected neural crest induction in some cases. This small effect could be explained either by an indirect effect on mesoderm or by inhibition of the canonical signal through the non-canonical Wnts (Torres et al., 1996; Prieve and Moon, 2003; Maye et al., 2004). Inhibition of canonical Wnt signalling by a dominant-negative form of Tcf3 does not inhibit neural crest migration (F. Romero and R.M., unpublished). There is convincing experimental evidence that shows that canonical Wnt signalling is involved in neural crest induction and cell differentiation (Dorsky et al., 1998; Garcia-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998; Lee et al., 2004; Lewis et al., 2004; de Melker et al., 2004; Tan et al., 2001;

Villanueva et al., 2002). Wnt signalling evidently plays a crucial role in neural crest development, in the canonical pathway in induction and in the non-canonical pathway in neural crest migration, as we have shown here.

If Wnt11 activates the non-canonical Wnt pathway in neural crest cells, these cells should express the appropriate Wnt receptor. Although the specific Wnt11 receptor is unknown, there is evidence for the involvement of Fz7 and Fz2 in non-canonical Wnt signalling, and of Fz7 in gastrulation (Carreira-Barbosa et al., 2003; Djiane et al., 2000; Kuhl et al., 2000; Medina et al., 2000; Sumanas and Ekker, 2001; Wang and Malbon, 2004; Winklbauer et al., 2001). We have shown that *Fz7* is expressed in the premigratory neural crest just before migration, as well as in the migrating neural crest. Comparison of the expression of *Fz7* with that of the neural crest marker *Slug* indicates that only a subpopulation of neural crest cells expresses the Wnt receptor. The *Fz7*-expressing cells correspond to those cells nearest to the *Wnt11*-expressing cells, which are likely to be the first cells to start migration. The expression pattern of *Fz7* correlates well with the proposal that this is the receptor of the Wnt11 signalling involved in neural crest migration; however, another receptor could also be involved. We have not investigated whether neural crest cells not adjacent to the *Wnt11*-expressing cells are able to receive the Wnt11 signal. Most reports suggest that Wnt works as short-range signalling molecule, but that depending on the cell context and the proteins involved, the range can be extended (reviewed by Christian, 2000; Arias, 2003).

The participation of PCP on cell movements during gastrulation has been very well characterized, although how Wnt controls cell movement remains unknown. Migration of the neural crest cells requires an epithelial-mesenchymal transition (EMT), an elaborate process that occurs in many steps. There is an initial delamination step that is essential for the second step of neural crest migration. Our results using Dsh and Wnt11 mutants, which show inhibition of cell movement both in vivo and in vitro, are compatible with an inhibition of delamination or posterior cell movement of the neural crest cells. Localized expression of Wnt11, by a graft of Wnt11-expressing ectoderm, shows an effect on crest migration that is dependent on the position of the graft. We propose that Wnt11 can trigger a cellular activity required for cell movement during delamination and/or cell migration, and that the crest cells require additional cues to translate this into an effective cell migration. The possibility that neural crest was induced in the graft is ruled out, as competence for neural crest induction is lost at the stage at which the tissue was transplanted (Mancilla and Mayor, 1996; Bastidas et al., 2004). It is still possible that Wnt11 promotes cell proliferation (Ouko et al., 2004), although this seems unlikely as cell numbers in our in vitro cultures did not increase after stimulating Wnt11 signalling. We, therefore, favour an effect of Wnt11 on neural crest migration, instead of cell proliferation. A recent report shows that PCP signalling controls the orientation of cell division during gastrulation (Gong et al., 2004). No analysis of cell division orientation during neural crest migration has been reported, but inhibition of the cell cycle blocks neural crest migration (Burstyn-Cohen and Kalcheim, 2002; Saka and Smith, 2001). Thus, it is possible that Wnt11 signalling controls cell migration by controlling cell divisions.

Neural crest migration in vitro and in vivo is blocked by Dsh and Wnt11 mutants, to a similar extent (Fig. 7). The ability to block cell migration in vitro suggests that the neural crest cells have already responded to Wnt11 signalling at the time of the dissection. This is possible, as the *Wnt11*-expressing cells are adjacent to the neural crest and it would be difficult to exclude them from an in vitro culture. Analysis of cell protrusions in migrating crest cells in vitro shows that non-canonical Wnt signalling is required to stabilize the lamellipodia. Inhibition of the PCP pathway increases the number of cells with filopodia with a less-polarized phenotype than the control neural crest cells. Similar functions for the PCP pathway and Wnt11 have been described during gastrulation in *Xenopus* and zebrafish embryos (Ulrich et al., 2003; Wallingford et al., 2000). We propose that Wnt11 controls cytoskeletal behaviour or cell adhesion properties in neural crest migration, and that it is required to generate the cell protrusions necessary for locomotion.

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