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-Ca2+] 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-Ca2+ 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-Ca2+) 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. Grants 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-AN, 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 cacadate 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-AN 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-AN 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-AN 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\textsuperscript{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-catenin) 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 un.injected 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,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. 3H) 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 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.
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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 (dnWnt11), or both (w11+dnWnt11). (G) Co-injection of Wnt11 and its dominant-negative form. 90% of embryos showed normal neural crest migration, n=50.

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 prevented (Fig. 7C-E). This result is consistent with the inhibition of neural crest migration observed in the animal cap assay (Fig. 4). Therefore, these results suggest that the non-canonical Wnt pathway is important for the directional migration of neural crest cells.

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. To test this hypothesis, 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.
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-Ca²⁺ signalling is involved in neural crest migration. We have not analyzed whether the PCP or Wnt-Ca²⁺ 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...
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., 2001; 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 molecular function of this localized Wnt expression in the neural crest has been found yet. Recent studies on the wing and how the gradient of Wnt11 could determine the asymmetric 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 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 in 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

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-∆N and FDX (green). At stage 14, neural crest cells were taken from the Dsh-∆N-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 dsWnt11. 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-∆N/DFX-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.

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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; 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. 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Development of the expression of Wnt11 and Wnt11 mutants, to a similar extent (Fig. 7). The ability to block cell migration in vivo 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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