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Neural crest specification by noncanonical Wnt signaling and PAR-1

Olga Ossipova and Sergei Y. Sokol*

SUMMARY

Neural crest (NC) cells are multipotent progenitors that form at the neural plate border, undergo epithelial-mesenchymal transition and migrate to diverse locations in vertebrate embryos to give rise to many cell types. Multiple signaling factors, including Wnt proteins, operate during early embryonic development to induce the NC cell fate. Whereas the requirement for the Wnt/β-catenin pathway in NC specification has been well established, a similar role for Wnt proteins that do not stabilize β-catenin has remained unclear. Our gain- and loss-of-function experiments implicate Wnt11-like proteins in NC specification in *Xenopus* embryos. In support of this conclusion, modulation of β-catenin-independent signaling through Dishevelled and Ror2 causes predictable changes in premigratory NC. Morpholino-mediated depletion experiments suggest that Wnt11R, a Wnt protein that is expressed in neuroectoderm adjacent to the NC territory, is required for NC formation. Wnt11-like signals might specify NC by altering the localization and activity of the serine/threonine polarity kinase PAR-1 (also known as microtubule-associated regulatory kinase or MARK), which itself plays an essential role in NC formation. Consistent with this model, *PAR-1* RNA rescues NC markers in embryos in which noncanonical Wnt signaling has been blocked. These experiments identify novel roles for Wnt11R and PAR-1 in NC specification and reveal an unexpected connection between morphogenesis and cell fate.

KEY WORDS: Noncanonical Wnt signaling, Neural crest, Dishevelled, *Xenopus*, PAR-1, Microtubule-associated regulatory kinase, Cell polarity

INTRODUCTION

The neural crest (NC) comprises stem-cell-like cells that form in vertebrate embryos at the neural plate border, migrate to diverse locations in the body and differentiate into multiple cell types (Anderson, 1997; Crane and Trainor, 2006; Knight and Schilling, 2006; Le Douarin and Dupin, 2003; Sauka-Spengler and Bronner-Fraser, 2008). NC is specified by the combined action of several embryonic signaling pathways, including the Wnt, FGF, BMP and Notch pathways, and NC fates are maintained by a network of specific transcription factors. Once formed, NC cells undergo epithelial-mesenchymal transition (EMT) and migrate to many destinations in the body to contribute to diverse cell types, including face cartilage, melanocytes and the peripheral nervous system (Acloque et al., 2009; Heeg-Truesdell and LaBonne, 2004; Kuriyama and Mayor, 2008; Thiery et al., 2009; Yang and Weinberg, 2008). The large number of human diseases that are associated with NC abnormalities, including craniosynostosis, Waardenburg and Hirschsprung's syndromes and cancers, draw considerable attention to studies of the mechanisms of NC development (Crane and Trainor, 2006; Heeg-Truesdell and LaBonne, 2004).

One pathway that is essential for NC specification in all vertebrate models examined is the Wnt pathway. Canonical Wnt signaling triggers β-catenin/TCF-dependent gene transcription and regulates cell proliferation and cell fate (Cadigan and Peifer, 2009; Clevers, 2006). The involvement of this pathway in NC formation was first established by genetic studies of *Wnt1*/*Wnt3a* double-knockout mice and in gain-of-function experiments in *Xenopus* (Ikeya et al., 1997; Saint-Jeannet et al., 1997), and was subsequently extended to other models (Dorsky et al., 1998; Garcia-Castro et al., 2002; Hari et al., 2002; Lewis et al., 2004; Wu et al., 2003). The transcription of many NC-specific genes, including *Snail2*, *Snail* and *Twist*, has been shown to depend on β-catenin/TCF (Garcia-Castro et al., 2002; Howe et al., 2003; LaBonne, 2002; Sauka-Spengler and Bronner-Fraser, 2008; Vallin et al., 2001; Wu et al., 2003), further supporting the model that NC formation involves the Wnt/β-catenin pathway.

Noncanonical Wnt ligands, such as Wnt5a and Wnt11 (Angers and Moon, 2009; van Amerongen and Nusse, 2009), do not stabilize β-catenin or activate TCF-dependent transcription, but regulate morphogenetic processes that involve changes in cell shape and motility, which are sometimes referred to as planar cell polarity (PCP) (Ciani and Salinas, 2005; Komiya and Habas, 2008; Saneyoshi et al., 2002; van Amerongen et al., 2008; Winklbauer et al., 2001). The signaling from Wnt5 or Wnt11 is thought to involve Ror and Ryk receptors (Grumolato et al., 2010; Hikasa et al., 2002a; Lin et al., 2010; Lu et al., 2004; Mikels et al., 2009; Minami et al., 2010), small Rho GTPases (Habas et al., 2003; Habas et al., 2001), Rho-associated kinase (Marlow et al., 2002; Winter et al., 2001), c-Jun N-terminal kinases (Boutros et al., 1998; Lisovsky et al., 2002; Pandur et al., 2002) and intracellular calcium (Sheldahl et al., 2003; Slusarski et al., 1997; Witze et al., 2008). Although noncanonical Wnt pathways have been shown to function in NC cell migration (Carmona-Fontaine et al., 2008; De Calisto et al., 2005; Matthews et al., 2008b), their importance for NC specification has remained unclear.

Craniofacial defects in *Wnt5a* knockout mice (Yamaguchi et al., 1999), and in *wnt11* (*silberblick*) (Heisenberg et al., 2000; Heisenberg et al., 1996) and *wnt5* (*pipetail*) (Piotrowski et al., 1996) zebrafish mutant embryos suggest possible roles for

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noncanonical Wnt signaling in NC development. The results of our study support the view that noncanonical signaling from Wnt11R is essential for NC specification in *Xenopus* embryos and that it might act by changing the localization and activity of the polarity kinase PAR-1.

PAR proteins are conserved regulators of cell polarity that interact with several embryonic signaling pathways, including the Wnt pathway (Doe and Bowerman, 2001; Goldstein and Macara, 2007; Knoblich, 2008; Ohno, 2001). PAR-1 associates with Dishevelled (Dvl, or Dsh) and participates in Frizzled-dependent Dvl recruitment (Ossipova et al., 2005; Sun et al., 2001). We show that PAR-1 is itself required for NC specification and can rescue NC defects in embryos with inhibited Wnt5 and Wnt11 signaling. These findings identify PAR-1 as a molecular target for noncanonical Wnt signaling and reveal an unexpected causal connection between cell polarization and the NC cell fate.

MATERIALS AND METHODS

DNA constructs and RNA synthesis

pCS2-Myc-PAR-1A, pCS2-Myc-PAR-1KD and GFP-PAR-1A in pXT7 have been described (Ossipova et al., 2005; Ossipova et al., 2007). Flag-PAR-1A and Flag-PAR-1A-KD have been generated by subcloning the PAR-1 coding region into the *Xho*I and *Not*I sites of pCS2-Flag (Hikasa and Sokol, 2011). Capped synthetic RNA for microinjection was generated using the mMessage mMachine Kit (Ambion) from the following DNA templates: pCS2-Myc-PAR-1A, pCS2-nucβGal (Ossipova et al., 2005), pCS2-Ror2 and pCS2-Ror2ΔC (Hikasa et al., 2002b), pSP64-XWnt11, pSP64-dnWnt11, pCS2-ΔN-Dsh, pCS2-DshDEP+ (Tada and Smith, 2000), pXT7-dnWnt5/11 (Choi and Sokol, 2009), pSP64T-XWnt5a (Moon et al., 1993), pSP64T-Xfz3 (Shi et al., 1998), pSP64T-Xwnt3a (Wolda et al., 1993) and Noggin (Lamb et al., 1993).

Embryo culture and microinjections, morpholino oligonucleotides and RT-PCR analysis

Fertilization and embryo culture were performed as described (Itoh et al., 2005). Embryos were microinjected in 1/3 × MMR containing 2% Ficoll 400 (Pharmacia) in the animal pole with 5 nl solution per blastomere at the four- to eight-cell stage, and cultured in 0.1 × MMR until the desired stages. PAR-1 morpholino (MO) (5′-TCGGCAGCGGTGTCCTGGTG-GTCAT-3′), referred to as PAR-1BY MO (Ossipova et al., 2005), or control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′) was injected at 5-10 ng per blastomere. Further MOs were: PAR-1 MO2, 5′-TCAT-CCCGATACTGAAATTACCAAC-3′; Wnt11R MO1, 5′-CTTCATCT-TCAAAACCCAATAACAA-3′; Wnt11R MO2, 5′-ACTGTATCCAAA-GAGAGTTCCGAGG-3′; and Ror2 MO, 5′-ATTCTGGCTCCTGGTC-CTGGACATC-3′. Wnt11R MOs were injected at 5-30 ng, whereas Ror2 MO was used at 20 ng per injection. The *wnt11r*:GFP construct containing the Wnt11R MO1 target sequence upstream of the GFP coding sequence was created by PCR-based mutagenesis in the pXT7 vector as described (Itoh et al., 2005).

For RT-PCR, total RNA was extracted from whole embryos and animal cap explants using RNeasy columns (Qiagen) and treated with RNase-free DNase I to remove genomic DNA. cDNAs were prepared using the Superscript First-Strand Synthesis System (Invitrogen). PCR primers for *Twist*, *Snail2* (Mayor et al., 1995), *MyoD*, *EF1α* (Kibardin et al., 2006), *Sox3* (Chalmers et al., 2002), *Pax3* and *Sox8* (de Croze et al., 2011) have been described. *FoxD3* primers were: forward, 5′-TCCATCAT-CAAGTCTGAGCC-3′; reverse, 5′-ATAGTTGACGTGTTACCTGC-3′. The number of PCR cycles for each primer pair was determined empirically to maintain amplification in the linear range.

In situ hybridization, lineage tracing and immunocytochemistry

In situ hybridization and X-Gal staining were carried out using standard techniques (Harland, 1991) with the following antisense probes: *FoxD3* (Sasai et al., 2001), *Sox2* (Mizuseki et al., 1998), *Slug/Snail2* (Mayor et al., 1995), *Sox8* (O'Donnell et al., 2006), *Sox9* (Cheung and Briscoe, 2003), *MyoD* (Hopwood et al., 1989), *AP2* (Luo et al., 2002) and epidermal type I keratin *XK70* (Winkles et al., 1985). *Pax3* and *Hairy2a* antisense probes were generated with T7 RNA polymerase from *pXT7-Xpax3* (GenBank accession number BC108573) and pBluescript-*Xhairy2a* (Shibata et al., 2005). Results have been quantified as a percentage of embryos with the described phenotypic change.

For cryosections, RNAs encoding Myc- or GFP-tagged proteins were injected into the animal region of four-cell albino embryos. Embryos were manually devitellinized at stage 10.5 and fixed in Dent's solution for 2 hours. Indirect immunofluorescence on cryosections was performed essentially as described (Fagotto and Gumbiner, 1994). Embryos were cryosectioned using a Leica CM3050 cryostat. Images were digitally acquired on a Zeiss Axiophot microscope. The following antibodies were used: anti-GFP (B2, Santa-Cruz, 1:200), rabbit anti-occludin [a gift of S. Citi (Cordennonsi et al., 1997)] and anti-Myc (9E10) monoclonal antibodies as hybridoma supernatants (Roche, 1:50). Secondary antibodies were conjugated with Alexa Fluor 488 (Molecular Probes, 1:200). Imaging was performed on a Zeiss Axiophot microscope with the Apotome attachment at 400× magnification. A representative section of an experimental group containing 10-15 embryos is shown.

Immunoprecipitation, western analysis and protein purification

Immunoprecipitation (IP) and western blotting were carried out with embryo lysates essentially as described (Gloy et al., 2002; Itoh et al., 1998). The following antibodies and reagents were used: anti-Flag (M2) agarose beads (Sigma), anti-Myc monoclonal antibodies as hybridoma supernatants of 9E10 cells (Roche), anti-β-catenin 8E7 (Millipore), anti-Flag M2 (Sigma), anti-XTCF3N (Zhang et al., 2003), anti-hDvl2 (Itoh et al., 2005), anti-Tau-pS262/S356 (Seubert et al., 1995), anti-GFP (B2, Santa Cruz) and anti-tubulin (B512, Sigma).

Immune complex kinase assays of M2 agarose-precipitated Flag-PAR-1 from stage 10-11 embryo lysates are described in (Ossipova et al., 2005). Each reaction contained 1-3 μg of recombinant human Tau protein as a substrate. Human Tau in pET29b vector [a gift of P. Klein, (Hong et al., 1997)] was produced in *E. coli* BL21* and purified using benzonase endonuclease (Sigma) as described (Lindwall and Cole, 1984).

RESULTS

Modulation of neural crest markers by noncanonical Wnt signaling

To evaluate the effects of noncanonical Wnt signaling on premigratory NC, two animal blastomeres of the four- to eight-cell embryos were injected with different doses of *Wnt11* RNA. These injections were targeted to presumptive ectoderm and did not have major effects on gastrulation. The analysis of NC-specific markers at premigratory stages (stage 15/17) was carried out by in situ hybridization. *Wnt11* expanded *FoxD3* (61%, *n*=101) (Sasai et al., 2001), *Snail2* (formerly *Slug*) (50%, *n*=57) (Mayor et al., 1995) and *Sox8* (76%, *n*=13) (O'Donnell et al., 2006) in the NC territory, adjacent to the neural plate border (Fig. 1A-C). No significant decrease of the pan-neural marker *Sox2* was observed (*n*=53), although some embryos showed slightly expanded neural plate at the injected side (Fig. 1D and supplementary material Fig. S1A), as evidenced by the shifted expression of *Hairy2* (Glavic et al., 2004). *Wnt11* also expanded *Pax3* (57%, *n*=96) and *AP2* (30%, *n*=33), which are early regulators of NC development that define the neural plate border (Bang et al., 1999; Luo et al., 2002; Monsoro-Burq et al., 2005; Sato et al., 2005) (Fig. 1E and supplementary material Fig. S1B), and reduced epidermal keratin *XK70* in non-neural ectoderm (supplementary material Fig. S1C; 30%, *n*=12). These effects were largely restricted to the neural plate border, i.e. the prospective NC territory, as ectopic marker expression has not been detected in the neural plate or the epidermis.

We next examined NC markers in embryos in which Wnt11 function has been blocked by dominant-negative (dn) constructs. C-terminally truncated dnWnt5/11 and dnWnt11 proteins, which

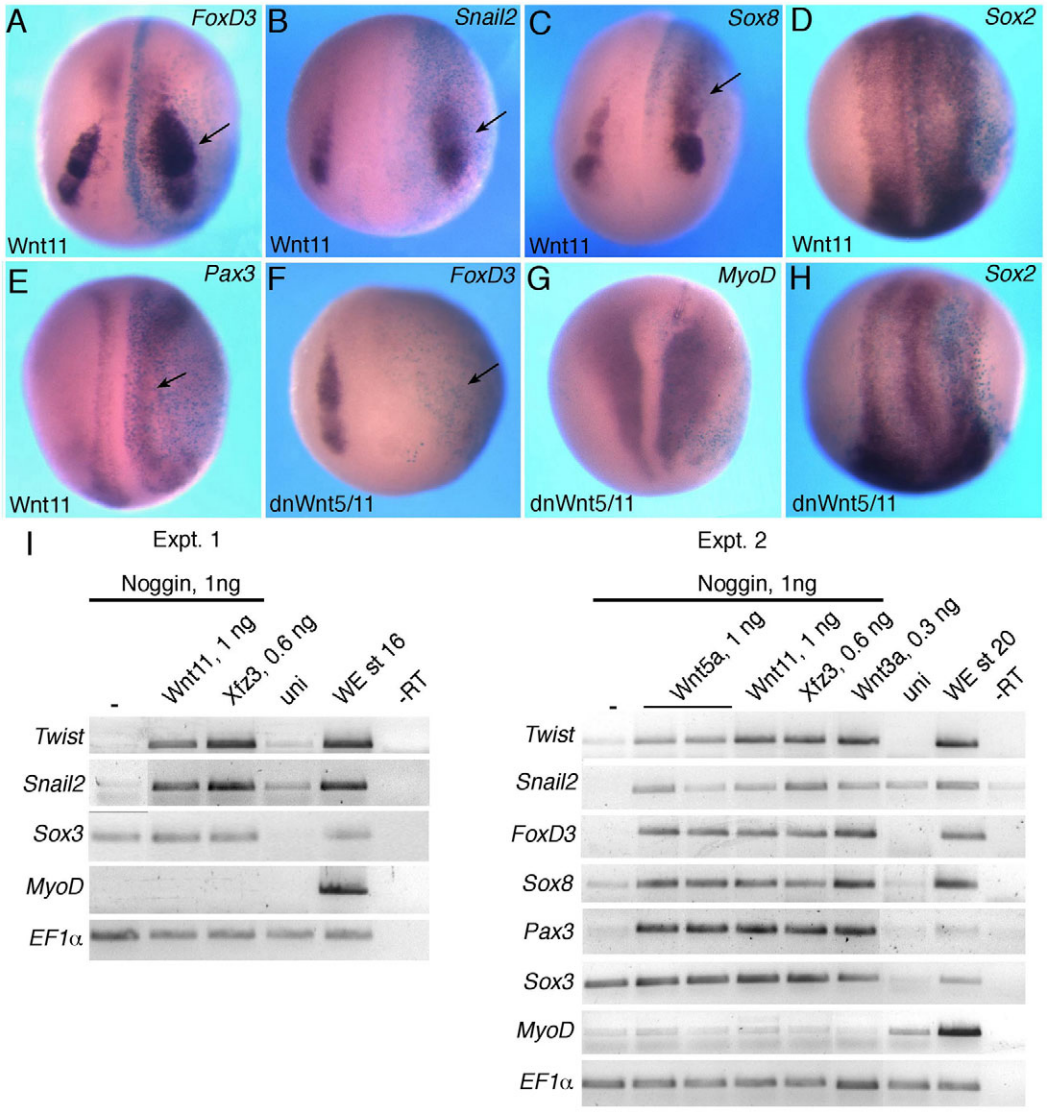

Fig. 1. Modulation of neural crest markers by noncanonical Wnt signaling. (**A-H**) Albino *Xenopus* embryos were injected animally with 0.5-1 ng *Wnt11* (A-E) or *dnWnt5/11* (F-H) mRNA at the four-cell stage, cultured until stage 14/16 and the expression of *FoxD3* (A,F), *Snail2* (B), *Sox8* (C), *Sox2* (D,H), *Pax3* (E) and *MyoD* (G) analyzed by in situ hybridization. β-galactosidase is a lineage tracer (light blue) marking the injected side. Arrows point to altered marker expression. Dorso-anterior view is shown. (**I**) RT-PCR analysis of gene expression in neuralized animal caps. Animal caps were isolated from embryos at stage 9, injected with RNA as indicated, and cultured until stage 16/20 for RT-PCR analysis. *Snail2*, *Twist*, *FoxD3*, *Sox8* and *Pax3* are induced by Wnt signaling in the absence of significant changes in the pan-neural marker *Sox3*. –, no Wnt or Frizzled stimulation; uni, uninjected animal caps; WE, whole embryos; –RT, no reverse transcriptase.

behave as dominant-negative mutants blocking Wnt5 and Wnt11 signaling (Choi and Sokol, 2009; Tada and Smith, 2000), strongly reduced the NC-specific marker *FoxD3* (94%, *n*=53 for dnWnt5/11; 45%, *n*=33 for dnWnt11) in ectoderm (Fig. 1F and supplementary material Fig. S1D). Importantly, the pan-neural marker *Sox2* (*n*=53) and somite-specific *MyoD* (*n*=37) did not decrease, indicating that neural induction and mesoderm formation remain unaffected (Fig. 1G,H and supplementary material Fig. S1E).

We next studied NC markers in ectoderm explants that have been neuralized by the presence of Noggin, a BMP inhibitor, essentially as described (LaBonne and Bronner-Fraser, 1998). RT-PCR analysis revealed that both Wnt11 and Wnt5a upregulated *Twist* and *Snail2* expression at the relevant developmental stages (Fig. 1I), similar to the effect of Wnt3a and Fz3 (Deardorff et al., 2001; Saint-Jeannet et al., 1997). Wnt11 and Wnt5a injections also activated other NC markers, including *FoxD3*, *Sox8* and *Pax3*. Neither Wnt protein significantly altered *MyoD* or the pan-neural marker *Sox3*, confirming our conclusion that noncanonical Wnt proteins do not affect mesoderm and neural tissue formation in these experiments (Fig. 1I). We did not observe any effects of Wnt11 on ectodermal cell proliferation at stage 12-14 using antibodies against phospho-histone H3 (data not shown). These observations reveal an essential role for Wnt5/11-like proteins in NC specification.

Lack of effect of Wnt5a and Wnt11 on canonical Wnt signaling

Although Wnt5 and Wnt11 proteins are usually considered β-catenin independent (Du et al., 1995), they have been reported to stabilize β-catenin under certain experimental conditions (Cha et

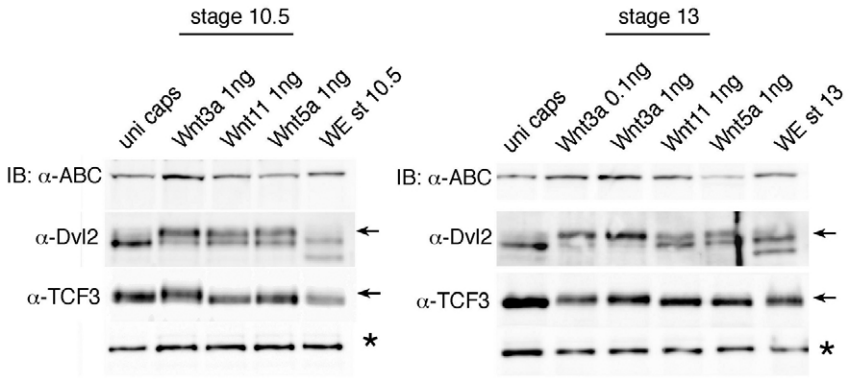

Fig. 2. Wnt5a and Wnt11 do not stabilize β-catenin or trigger TCF3 phosphorylation in ectodermal cells. *Xenopus* embryos were injected with the indicated RNAs into animal pole blastomeres at the two-cell stage. Animal caps were isolated at stage 9 and cultured until stage 10/10.5 or stage 13/14 for western analysis (IB) with the indicated antibodies. Asterisk indicates a non-specific band that serves as a loading control. Arrows point to the position of phosphorylated TCF3 and phosphorylated Dvl. Uni, uninjected; WE, whole embryo; ABC, unphosphorylated ('activated') β-catenin.

al., 2009). We therefore assessed whether Wnt5a and Wnt11 stimulate β-catenin-dependent processes in gastrula ectoderm (Fig. 2). Consistent with published data, Wnt3a increased β-catenin levels and triggered TCF3 phosphorylation (Hikasa et al., 2010), but neither Wnt5a nor Wnt11 had this activity in ectodermal cells at stage 10/10.5 or 13/14, even at high doses of injected RNA (Fig. 2; data not shown). By contrast, all Wnt ligands were able to promote Dvl phosphorylation (Yanagawa et al., 1995), as measured by the appearance of a double band corresponding to *Xenopus* Dvl2. Thus, Wnt5a and Wnt11 do not stimulate canonical β-catenin-dependent signaling in gastrula ectoderm.

Neural crest specification in embryos with manipulated Ror2 and Dvl function

Different domains of Dvl are responsible for signaling specificity of the two main branches of the Wnt pathway (Axelrod et al., 1998; Boutros et al., 1998; Sokol, 2000; Tada and Smith, 2000). ΔN-Dsh rescues the loss of Wnt11 function in *Xenopus* and zebrafish embryos without affecting β-catenin signaling, whereas Dsh-DEP+ specifically inhibits the PCP-like pathway (Heisenberg et al., 2000; Tada and Smith, 2000). To examine Dvl involvement in premigratory stages of NC development, ΔN-Dsh and Dsh-DEP+ RNAs were targeted by microinjection into prospective ectoderm

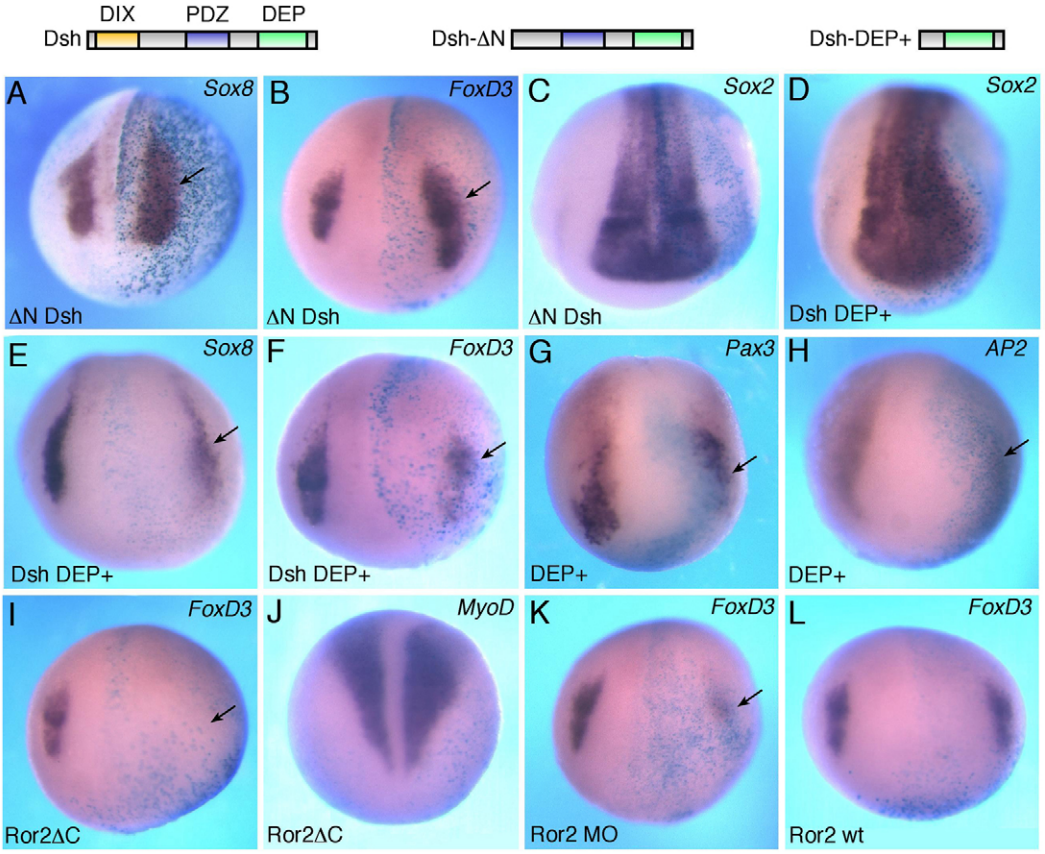

Fig. 3. Neural crest markers in embryos with modulated Dvl and Ror2 function. (**A-H**) *Xenopus* embryos were injected with the indicated Dvl (Dsh construct) RNAs (1 ng each) and processed for in situ hybridization as described in Fig. 1. Schematic representations of the Dvl mutant constructs are shown at the top. Dsh-ΔN activates *Sox8* (A) and *FoxD3* (B) but not *Sox2* (C). Dsh-DEP+ slightly expands *Sox2* (D) but inhibits *Sox8* (E), *FoxD3* (F), *Pax3* (G) and *AP2* (H). (**I-L**) The effects of Ror2 interference on NC marker expression. Ror2ΔC and Ror2MO, but not wild-type Ror2, inhibit *FoxD3* (I,K,L). *MyoD* is not affected by Ror2ΔC (J). Arrows point to altered marker expression. Dorso-anterior view is shown.

and their effects on NC induction assessed by in situ hybridization. ΔN-Dsh expanded the expression of *FoxD3* (60%, *n*=48) and *Sox8* (40%, *n*=37) at the injected side (Fig. 3A,B). By contrast, Dsh-DEP+ decreased the expression of *FoxD3* (42%, *n*=64), *Sox8* (67%, *n*=37) and *Snail2* (58%, *n*=24) (Fig. 3E,F; data not shown). Dsh-DEP+ also reduced the early markers *Pax3* (44%, *n*=45) and *AP2* (66%, *n*=18) (Fig. 3G,H). The size of the neural plate (marked by *Sox2*) was not significantly affected, indicating that neural induction was normal (Fig. 3C,D). Thus, these Dvl constructs alter NC markers in a manner that is consistent with their ability to regulate noncanonical signaling.

We next aimed to elucidate which Wnt receptor mediates the observed effects and tested the involvement of Ror2, which has been implicated in Wnt5a signaling and is expressed in the NC (Grumolato et al., 2010; Hikasa et al., 2002a; Matsuda et al., 2001; Mikels and Nusse, 2006; Nishita et al., 2006; Schambony and Wedlich, 2007). We found that Ror2ΔC, a dominant-negative form of Ror2 that lacks the intracellular domain (Hikasa et al., 2002a; Mikels and Nusse, 2006), strongly inhibits *FoxD3* (69%, *n*=106; Fig. 3I) and *Sox8* (41%, *n*=12; supplementary material Fig. S2A). Consistent with a previous report (Hikasa et al., 2002a), Ror2ΔC did not affect *MyoD* or *Sox2* gene expression (Fig. 3J and supplementary material Fig. S2C). Similarly to the dominant-negative construct, a morpholino oligonucleotide (MO) complementary to the 5′ untranslated region of *Xenopus* Ror2, but not a control MO, blocked *FoxD3* expression (70%, *n*=50; Fig. 3K; data not shown). By contrast, wild-type Ror2 did not significantly affect the NC markers (Fig. 3L and supplementary material Fig. S2B). The lack of NC expansion in response to Ror2 possibly indicates limiting levels of endogenous Wnt ligands. Together, these observations suggest that Wnt5 and Wnt11 might act through Ror2 during NC specification in the early embryo.

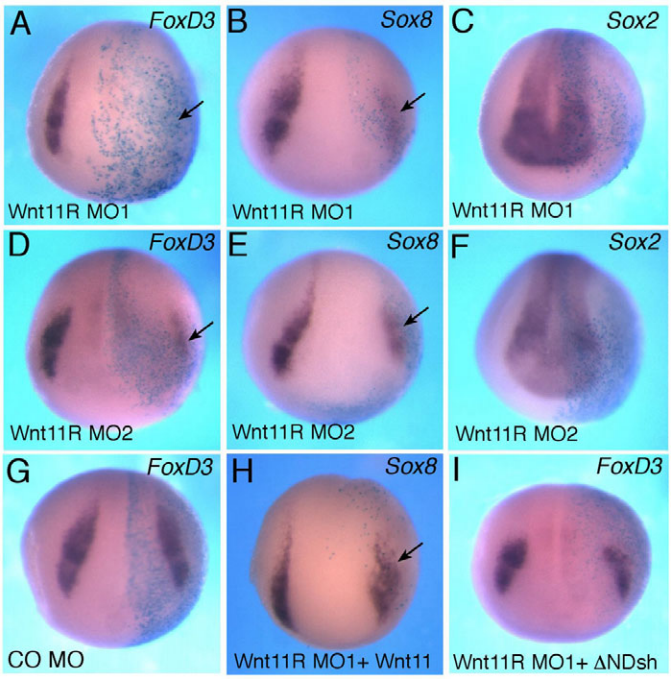

Fig. 4. Wnt11R is essential for neural crest specification. (**A-F**) *Xenopus* embryos were injected with the indicated MOs or RNAs and processed for in situ hybridization as described in Fig. 1. Wnt11R MO1 (A-C) and MO2 (D-F) inhibit *FoxD3* (A,D) and *Sox8* (B,E) but do not significantly affect *Sox2* (C,F). (**G**) Control MO (CO MO) does not alter *FoxD3*. (**H**,**I**) *Wnt11* and ΔN-Dsh RNAs partially suppress the inhibitory effect of Wnt11R MO1 on *Sox8* (H) and *FoxD3* (I).

Wnt11R is required for neural crest specification

To identify a potential noncanonical Wnt ligand responsible for NC specification, we searched for Wnt proteins that are expressed in close proximity to the NC territory. One of the Wnt genes that is expressed in neuroectoderm, adjacent to the NC, is *Wnt11R*, which is closely related to *Wnt11* (Garriock et al., 2005; Matthews et al., 2008a). Previous studies implicated Wnt11R in the formation of tail fin, a NC derivative, and in NC migration (Garriock et al., 2005; Garriock and Krieg, 2007; Matthews et al., 2008a). Since the previously used Wnt11R MO (Garriock et al., 2005) has three mismatches with the *Wnt11R* cDNA sequence from public databases (GenBank accession number AY695415) and would be predicted to confer only a partial phenotype, we designed two new MOs that are expected to block the translation of *Wnt11R* RNA and assessed their effects on the premigratory NC population (Fig. 4 and supplementary material Fig. S1F, Figs S3, S4). Wnt11R MO1 efficiently inhibited the in vivo translation of an mRNA containing the 5′UTR sequence of *Wnt11R* fused to the *GFP* coding sequence (supplementary material Fig. S3). Wnt11R MO1, but not the control MO, interfered with *FoxD3* and *Sox8* expression at the injected side in a dose-dependent manner with high penetrance (>75%; *n*=90 for *FoxD3*, *n*=30 for *Sox8*; Fig. 4A,B). Wnt11R MO2, with non-overlapping sequence, had similar effects on *FoxD3* (61%, *n*=39) and *Sox8* (92%, *n*=25) (Fig. 4D,E). The Wnt11R MOs did not inhibit *Sox2*, but we have observed some expansion of *Sox2* on the injected side (Fig. 4C,F; data not shown). We also noticed that Wnt11R depletion resulted in the partial inhibition of *Pax3* and *AP2* (supplementary material Figs S1, S4), whereas a control MO injected at the same dose did not affect these markers. The partial effect suggests that *Pax3* and *AP2* might be under the control of multiple Wnt ligands or other signaling pathways.

As an additional control for specificity, Wnt11R MO1 was co-injected with *Wnt11* or ΔN-Dsh RNAs. We observed that both RNAs were able to partially rescue the NC defect. About 50% of embryos co-injected with 10 ng of Wnt11R MO1 and 1 ng of *Wnt11* RNA expressed *Sox8* (*n*=98), whereas approximately one-third of embryos co-injected with Wnt11R MO1 and ΔN-Dsh expressed *FoxD3* (*n*=122) (Fig. 4H,I). These experiments suggest that Wnt11R functions at the neural plate border to induce the NC fate.

Wnt11 signaling influences PAR-1 localization and activity

Noncanonical Wnt signaling has been proposed to involve PAR-1/MARK, a serine/threonine protein kinase that binds Dvl (Ossipova et al., 2005; Sun et al., 2001). Consistent with this role, PAR-1 is required for convergent extension movements in *Xenopus* embryos (Kusakabe and Nishida, 2004; Ossipova et al., 2005). To further assess the involvement of PAR-1 in Wnt signaling, we studied whether Wnt ligands can alter PAR-1 enzymatic activity or subcellular localization. Both Wnt5a and Wnt11 caused the redistribution of PAR-1 from the basolateral cortex to the cytoplasm without significant changes in protein levels (Fig. 5A,B and supplementary material Fig. S5A). This effect was accompanied by the alteration of epithelial cell morphology. Similar to the noncanonical Wnts, Wnt3a also affected PAR-1 distribution (data not shown). By contrast, dnWnt5/11, dnWnt11 and overexpressed β-catenin did not significantly alter the localization of PAR-1 at the basolateral cortex

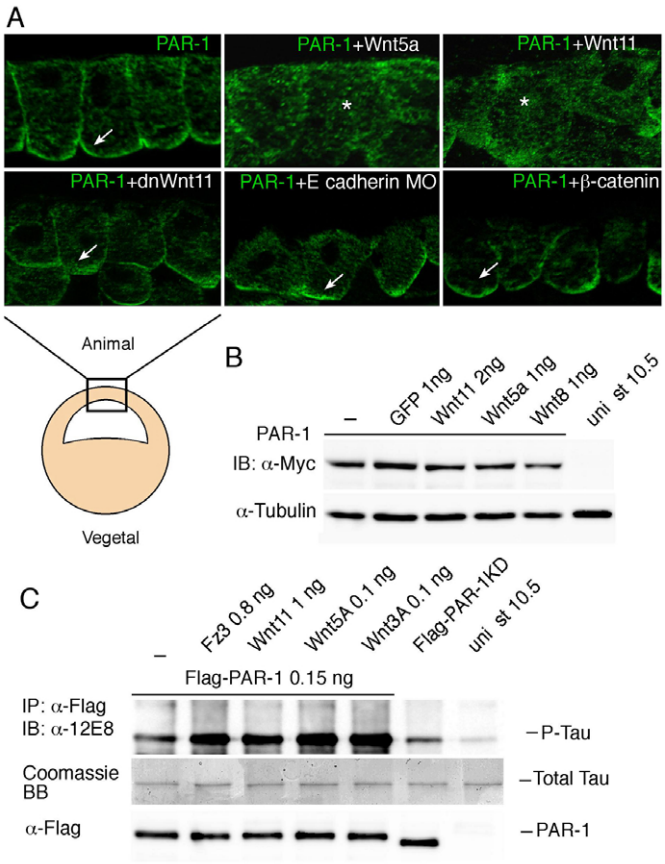

Fig. 5. Wnt5 and Wnt 11 influence PAR-1 localization and activity. (**A**) *Wnt5a* or *Wnt11* RNAs (1 ng each) trigger the relocalization of Myc-PAR-1 (co-injected as 0.3 ng of RNA) from the basolateral cortex to the cytoplasm in embryonic ectoderm (arrow, anti-Myc staining). The asterisk indicates cytoplasmic staining. By contrast, PAR-1 distribution is not significantly affected by dnWnt11, E-cadherin depletion or β-catenin overexpression (lower panels). The location of the superficial ectoderm cells used for this analysis is illustrated beneath. (**B**) Western analysis reveals comparable Myc-PAR-1 levels in the presence of Wnt or control *GFP* RNAs in stage 10.5-11 embryonic lysates. α-tubulin is a loading control. (**C**) Wnt signaling increases Flag-PAR-1 protein kinase activity as assessed by hTau phosphorylation at Ser262/356 detected by a phospho-specific antibody. Recombinant hTau protein was added at 1 μg per sample. Amounts of precipitated PAR-1 were assessed by anti-Flag antibody.

(Fig. 5A; data not shown). A previously characterized E-cadherin MO (Nandadasa et al., 2009) used at phenotypically active doses did not alter PAR-1 localization either, despite a pronounced effect on cell adhesion and epithelial morphology (Fig. 5A). Moreover, despite a pronounced effect on PAR-1, Wnt11 did not change the basolateral localization of occludin in double-immunostaining experiments (supplementary material Fig. S5B). These observations suggest that Wnt proteins have a specific effect on PAR-1 localization.

We next assessed how Wnt ligands modulate PAR-1 enzymatic activity in vivo. PAR-1 activation by Wnt proteins and Fz3 was assessed in Flag-PAR-1-expressing embryos in an immune complex kinase assay with human (h) Tau as a phosphorylation substrate (Nishimura et al., 2004). Co-expression of Wnt5a, Wnt11 or Wnt3a increased the ability of PAR-1 to phosphorylate hTau when measured using a phosphopeptide-specific antibody (Fig. 5C). We conclude that Wnt signaling dissociates PAR-1 from the cell cortex and upregulates its enzymatic activity.

PAR-1 is involved in neural crest specification

Since PAR-1 binds Dvl and PAR-1 activity and localization are regulated by noncanonical Wnt ligands, we hypothesized that PAR-1 is a mediator of noncanonical signaling during NC specification. This hypothesis was tested in gain- and loss-of-function experiments. *PAR-1* RNA enhanced *FoxD3* (54%, *n*=95), *Snail2* (65%, *n*=26) and *Sox8* (54%, *n*=61) (Fig. 6A-C), whereas *Sox2* was largely unaltered (Fig. 6D). We next used a PAR-1 MO that was previously characterized and shown not to affect β-catenin signaling (Kusakabe and Nishida, 2004; Ossipova et al., 2005), suggesting that the canonical pathway is not involved. This PAR-1 MO, but not the control MO, suppressed *Sox8* (90%, *n*=44), *FoxD3* (82%, *n*=86) and *Snail2* (83%, *n*=36) with high efficiency (Fig. 6E-H), without an effect on *Sox2* (Fig. 6I). A second, non-overlapping, PAR-1 MO2 also inhibited *FoxD3* (73%, *n*=15) but did not change *Sox2* (Fig. 6J; data not shown). *PAR-1* RNA and MOs also triggered the expected changes in *Pax3* (Fig. 7A,B) and *AP2* (supplementary material Fig. S6), supporting an essential role of PAR-1 in the early stages of premigratory NC development. No changes in ectodermal cell proliferation have been detected in cells with manipulated PAR-1 function (Ossipova et al., 2009), suggesting that PAR-1 has a primary effect on cell fate.

To evaluate whether PAR-1 is a downstream mediator of noncanonical Wnt signaling, we tested whether PAR-1 is able to rescue NC marker defects in embryos injected with *dnWnt5/11* RNA (Fig. 7C,D). Importantly, PAR-1 overexpression was sufficient to partially restore *FoxD3* expression in *dnWnt5/11* RNA-injected embryos (45%, *n*=18), consistent with the view that PAR-1 functions downstream of Wnt5/11 signaling (Fig. 7E). Together, these experiments indicate that PAR-1 might mediate noncanonical Wnt signaling during NC specification.

DISCUSSION

In this study we show that noncanonical Wnt signaling, and specifically Wnt11R, is essential for NC-specific gene expression at premigratory stages of NC development. We find that Wnt proteins regulate the localization and the activity of the Dvl-associated protein kinase PAR-1, which itself plays a key role in regulating NC specification (Fig. 7E). This involvement of PAR-1, a cell polarity determinant, in NC formation reveals an unexpected connection between morphogenetic events and transcriptional control.

Our observations contrast with previous studies that have implicated noncanonical Wnt signaling in NC cell migration but not specification (Berndt et al., 2008; Carmona-Fontaine et al., 2008; De Calisto et al., 2005; Kuriyama and Mayor, 2008; Matthews et al., 2008a). Although we do not know why our results are different, the effect of Wnt11 on NC gene expression might have previously been overlooked because it likely requires higher levels of the interfering Wnt and Dvl (Dsh) constructs than are needed for highly sensitive cell migration assays. In a search for a specific Wnt ligand responsible for this signaling, we have shown that Wnt11R is required for NC specification. Of interest, *Wnt11R* is a true ortholog of other vertebrate *Wnt11* genes (Garriock et al., 2007). Genetic analyses have demonstrated a role for Wnt11 in the ureteric branching of the mouse kidney (Majumdar et al., 2003) and in heart morphogenesis (Nagy et al., 2010), but its role in NC development has not been examined. Consistent with the role in NC specification, *Xenopus* Wnt11R is expressed in the neural plate, adjacent to the NC territory (Matthews et al., 2008a). Additionally, vertebrate Ror2 and PAR-1/MARK homologs are also expressed in early neuroectoderm, at the neural plate border, and later in

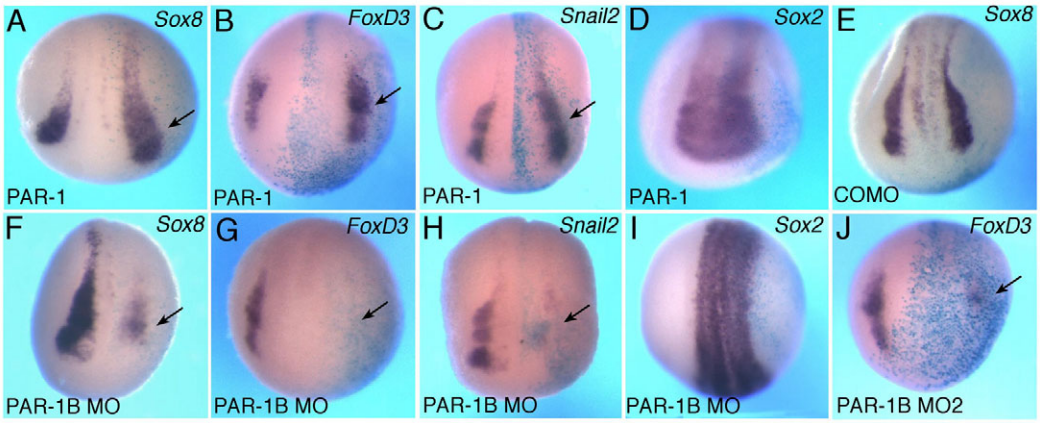

Fig. 6. PAR-1 plays an essential role in neural crest specification. *Xenopus* embryos were injected with RNAs or MO and in situ hybridization analysis was carried out as described in Fig. 1. (**A-D**) *PAR-1* RNA (0.3 ng) upregulates *Sox8* (A), *FoxD3* (B) and *Snail2* (C) but does not affect *Sox2* (D). (**E-I**) Effects of PAR-1 depletion. (E) Control MO (COMO) does not change *Sox8*. (**J**) PAR-1B MO2, which has a different sequence, has a similar effect to PAR-1B MO (G). Arrows indicate a change in marker gene expression. Dorso-anterior view is shown.

migrating NC populations, as revealed in previous studies of *Xenopus* and mouse embryos (Hikasa et al., 2002a; Matsuda et al., 2001; Ossipova et al., 2002).

The Wnt11R pathway leading to NC specification appears to involve Dvl and could be similar to the PCP pathway identified in *Drosophila* studies (Simons and Mlodzik, 2008), although the involvement of a Wnt ligand, a Ror receptor or a PAR-1 homolog in fly PCP has not been conclusively demonstrated. When the vertebrate homologs of the 'core' PCP components, such as Prickle, Dishevelled, Frizzled and Strabismus/Van Gogh-like, are modulated, gain- and loss-of-function effects are often indistinguishable. For example, the embryonic body axis is shortened due to defective convergent extension when Strabismus or Prickle proteins are either overexpressed or depleted (Darken et al., 2002; Park and Moon, 2002; Takeuchi et al., 2003; Veeman et al., 2003), interfering with the subsequent analysis of the pathway. Our observations establish a new molecular assay in which the gain- and loss-of-function effects of noncanonical signaling are easily distinguished, thus eliminating the complication of morphology-based assays. This assay provides a straightforward means to identify additional pathway components by assessing a role in NC formation for other candidate proteins that affect PCP in *Drosophila* or convergent extension in vertebrates.

Our findings reveal an essential role for the epithelial polarity protein PAR-1 in NC development. Although the involvement of polarity proteins in cell migration is highly predictable (Imai et al., 2006; McCaffrey and Macara, 2009), their function at the premigratory stage was unexpected. Based on the effect of noncanonical Wnt proteins on PAR-1 localization and activity, and the ability of PAR-1 to rescue the NC defect in embryos in which noncanonical Wnt signaling has been inhibited, we propose that PAR-1 mediates Wnt11R signaling during NC specification (Fig. 7E). The direct interaction of PAR-1 and Dvl implies a direct mechanism, which remains to be investigated. The hypothesis that PAR-1 functions downstream of Wnt11R is also supported by the ability of PAR-1 MO to block *FoxD3* induction by Fz3, which affects PAR-1 localization (data not shown) and has been implicated in noncanonical signaling (Witze et al., 2008) (supplementary material Fig. S6D-F). It is currently unknown how PAR-1 is regulated by Wnts and which proteins mediate its effects on NC marker expression, but possible mechanisms might involve the segregation of specific fate determinants after asymmetric mitosis or the polarization of the cytoskeleton, leading to mechanotransduction. These mechanisms are likely to involve small Rho GTPases, which have been reported to function in NC specification (Broders-Bondon et al., 2007; Guemar et al., 2007) and can be regulated by both Wnt and PAR signaling (Habas et al., 2001; Schlessinger et al., 2009). Recently, RhoGAP has been shown to regulate

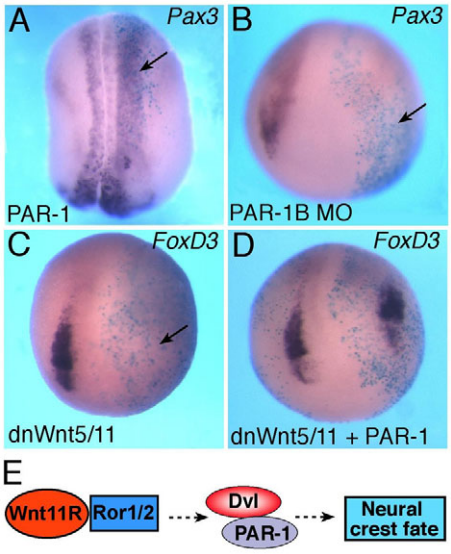

Fig. 7. PAR-1 functions in the Wnt5/11 pathway upstream of *Pax3*. (**A**) *PAR-1* RNA promotes *Pax3* expression (stage 17). (**B**) PAR-1 is required for *Pax3* expression (stage 14/15). (**C**,**D**) PAR-1 rescues the *FoxD3* defect in embryos that were injected with *dnWnt5/11* RNA. Dorso-anterior view, except A (dorsal view). Arrows indicate altered gene expression. (**E**) Model for Wnt11R signaling during NC specification. Dvl and the associated PAR-1 protein kinase are essential regulators of NC-specific transcription in response to Wnt11R/Ror2 signaling. Dashed lines represent indirect effects via unknown intermediates.

transcriptional events by binding to specific transcription factors and controlling tissue-specific transcription (Mammoto et al., 2009; Mammoto and Ingber, 2010).

Combined with previous studies (Ikeya et al., 1997; Saint-Jeannet et al., 1997), our observations support the view that vertebrate NC forms in response to signaling by both canonical and noncanonical Wnt ligands, although the details of these processes remain to be determined. Of interest, both noncanonical Wnt ligands and polarity proteins can regulate EMT and cell migration (Imai et al., 2006; Minichiello et al., 1999; Nishita et al., 2006; Witze et al., 2008), two processes that are characteristic for NC cell behavior. Since NC cells form at the boundary of neural and non-neural ectoderm, it is possible that the regulation of cadherin-mediated cell adhesion contributes to the process of NC fate specification, in addition to its known function in NC migration (Kashef et al., 2009; Park and Gumbiner, 2010; Taneyhill, 2008). Of note, both Wnt11 signaling and the RhoV/Chp GTPase, which is required for NC formation, have been implicated in the maintenance of adherens junctions in zebrafish embryos (Tay et al., 2010; Ulrich et al., 2005). Further studies are needed to examine the functions of cadherins and other cell junction components in NC specification.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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