Reiterated Wnt signaling during zebrafish neural crest development

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Summary

While Wnt/β-catenin signaling is known to be involved in the development of neural crest cells in zebrafish, it is unclear which Wnts are involved, and when they are required. To address these issues we employed a zebrafish line that was transgenic for an inducible inhibitor of Wnt/β-catenin signaling, and inhibited endogenous Wnt/β-catenin signaling at discrete times in development. Using this approach, we defined a critical period for Wnt signaling in the initial induction of neural crest, which is distinct from the later period of development when pigment cells are specified from neural crest. Blocking Wnt signaling during this early period interfered with neural crest formation without blocking development of dorsal spinal neurons. Transplantation experiments suggest that neural crest precursors must directly transduce a Wnt signal. With regard to identifying which endogenous Wnt is responsible for this initial critical period, we established that *wnt8* is expressed in the appropriate time and place to participate in this process. Supporting a role for Wnt8, blocking its function with antisense morpholino oligonucleotides eliminates initial expression of neural crest markers. Taken together, these results demonstrate that Wnt signals are critical for the initial induction of zebrafish neural crest and suggest that this signaling pathway plays reiterated roles in its development.

Key words: Zebrafish, Neural crest, Wnt

Introduction

The neural crest is a population of ectodermally derived cells that migrate throughout the vertebrate embryo to contribute to a variety of cell types, including neurons and glia of the peripheral nervous system and pigment cells. The characterization of the signaling mechanisms that trigger the initial formation of neural crest remains a major issue in the field. A classic model of neural crest induction theorizes that inductive signaling events from underlying mesoderm localize crest cells to the epidermal–neural–ectoderm boundary (Raven and Kloos, 1945). Neural crest cells can also be induced through interactions between adjacent neural and non-neural ectoderm (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995). Data from several studies suggest that multiple, sustained signaling events are required for induction of neural crest cells (reviewed by Le Douarin and Kalcheim, 1999). Lineage tracing studies have shown that neural crest cells are continuously produced at the lateral edges of the neural plate, suggesting neural crest promoting signals are present after the initial induction events (Selleck and Bronner-Fraser, 1995). Studies of mouse, frog, chick and zebrafish neural crest development have implicated several families of secreted signaling molecules in this process, including Bone Morphogenetic Proteins (BMPs), Fibroblast Growth Factors (FGFs), retinoic acid and Notch (Liem et al., 1995; Marchant et al., 1998; Mayor et al., 1995; Mayor et al., 1997; Bang et al., 1997; Nguyen et al., 1998; Cornell and Eisen, 2000; Endo et al., 2002; Villanueva et al., 2002; Monsoro-Burq et al., 2003).

The Wnt family of secreted signaling molecules, which has been shown to modulate cell proliferation, fate, and behavior in both vertebrates and invertebrates (reviewed in Cadigan and Nusse, 1997), has multiple roles in neural crest development (reviewed by Dorsky et al., 2000a; Yanfeng et al., 2003). Several studies have implicated Wnts in neural crest formation. Overexpression of various Wnts induces ectopic expression of neural crest markers in *Xenopus* neuralized animal caps and embryos, while expression of dominant-negative Wnts represses neural crest markers (Saint-Jeannet et al., 1997; Chang et al., 1998; Labonne and Bronner-Fraser, 1998, Bang et al., 1999; Tan et al., 2001; Villanueva et al., 2002). In addition, it was recently shown that inhibition of Wnt signaling in vivo blocks expression of avian neural crest markers, and that addition of soluble Wnt is sufficient to induce neural crest from neural tube explants (García-Castro et al., 2002). It should be noted that none of these studies identified which specific Wnt molecules are required at particular stages of development for neural crest induction or maintenance. Mice null for Wnt1

and Wnt3A show a loss of neural crest derived cell types, including peripheral sensory neurons and pigment cells (Ikeya et al., 1997). However, these ligands are expressed after the initial appearance of neural crest, and expression of markers for pre-migratory neural crest are not changed in mutant mice. These results suggest that if a Wnt signal is needed for initial neural crest induction, another Wnt ligand must be involved. Wnt signals are involved in early patterning of the embryo, imparting posterior character on neuroectoderm (McGrew et al., 1995; McGrew et al., 1997; Bang et al., 1997; Lekven et al., 2001; Erter et al., 2001; Kiecker and Niehrs, 2001) and ventral character on mesoderm (Christian et al., 1993; Hoppler et al., 1996; Marom et al., 1999). It is thus not clear whether Wnt-mediated effects on neural crest induction are direct or indirect.

There are several lines of evidence suggesting that Wnt/β-catenin signaling is also involved later in neural crest cell fate specification after crest cells have been formed. Activation of Wnt/β-catenin signaling in pre-migratory zebrafish neural crest cells promotes pigment cell determination at the expense of neurons and glia, while inhibition of the Wnt/β-catenin signaling pathway promotes neuronal and glial cell fates (Dorsky et al., 1998). These data support a model in which Wnt signaling is required for the pigment cell lineage. However, they do not identify which Wnts are involved in this fate decision. As noted, Wnt1/Wnt3A knockout mice are deficient in several neural crest derivatives, including pigment cells (Ikeya et al., 1997), but this is probably due to lack of early expansion rather than effects on later cell fate decisions. In addition, it has recently been shown that Wnts influence the development of crest-derived pigment cells in chicks (Jin et al., 2001), and that Wnt/β-catenin signaling is necessary for mouse pigment cell differentiation (Hari et al., 2002). Wnts may also promote the proliferative expansion of melanocyte precursors as well as promote their differentiation (Dunn et al., 2000; Yasumoto et al., 2002). Together these studies suggest that Wnts are used at sequential stages of neural crest development, both initially in crest induction and subsequently in cell fate determination.

In this study we address how reiterated Wnt signaling influences neural crest development in zebrafish. By inducing expression of a Wnt/β-catenin signaling pathway inhibitor, we identify specific stages of development during which Wnt/β-catenin mediated signaling is required cell-autonomously for neural crest induction. We also identify a specific Wnt, Wnt8, and demonstrate that it is crucial for initial neural crest induction. What emerges from our data is the concept of a reiterated Wnt/β-catenin signaling mechanism. The Wnt/β-catenin signaling pathway, probably in conjunction with other secreted factors such as BMPs and FGFs, functions early to induce the pre-migratory neural crest cells. Later, secreted Wnt signals provide environmental cues during crest cell migration to specify which cells will adopt the pigment cell fate.

Materials and methods

Fish maintenance and transgenic fish

Fish were maintained as described (Westerfield, 1994). Wild-type fish used were of the AB strain. The stable transgenic line carrying the Wnt pathway inhibitor was generated as follows. The dominant-negative T-cell Factor 3 reporter construct (hsΔTcf-GFP) was generated by replacing the N-terminus of zebrafish Tcf3a (Pelegri and Maischein, 1998; Dorsky et al., 1999) with GFP and placing the fusion construct under the control of the zebrafish hsp70 promoter (Halloran et al., 2000). N-terminally truncated Tcf3 is a dominant repressor of Wnt-mediated transcription (Molenaar et al., 1996). The linearized plasmid for this construct was injected into 1-2 cell zebrafish embryos. These fish were reared to adulthood. Adult siblings were inter-crossed to identify founders with stable genomic integration of the transgene (Tg (hsp70:ΔTCF-GFP)w26). Transgenic TOPdGFP fish (Tg (TOP:dGFP)w25) express d2EGFP under control of four Lef binding sites and have been described previously (Dorsky et al., 2002).

Morpholino injections

Anti-sense Morpholino oligonucleotides (GeneTools) were dissolved in 1X Danieau's buffer (Nasevicius and Ekker, 2000) for a stock concentration of 20 ng/nl. Morpholino oligonucleotides (MOs) used in this study have the same sequences as wnt8 MO1 and MO2 (Lekven et al., 2001). Each MO was injected into embryos at the 1-2 cell stage using an ASI pressure injector (ASI Systems).

In-situ hybridization

Whole-mount in-situ hybridization was performed as described (Thisse et al., 1993). Digoxigenin-labeled RNA antisense probes were prepared from templates encoding *gsc* (Stachel et al., 1993), *wnt8.1* and *wnt8.2* (Lekven et al., 2001), *pax3* (Seo et al., 1998), *foxd3* (Odenthal and Nüsslein-Volhard, 1998; Kelsh et al., 2000), *sox10* (Dutton et al., 2001), *mitfa* (Lister et al., 1999), *crestin* (Rubenstein et al., 2000; Luo et al., 2001), *dlx2* (Akimenko et al., 1994), and *huC* (Kim et al., 1996). Following in-situ hybridization, embryos were cleared in 70% glycerol. Embryos were photographed using an Olympus SZX12 dissecting microscope and DP12 camera.

Antibody staining

To detect Foxd3 expression, fixed embryos were stained with anti-Foxd3 rabbit antisera (1:1000) with 20% goat serum, and then incubated with anti-rabbit Alexa568-conjugated secondary antibodies. To detect Hu expression, fixed embryos were stained with mouse monoclonal anti-Hu antibody (Molecular Probes; 1:1000) with 10% goat serum, followed by incubation with anti-mouse Alexa568-conjugated secondary antibody. All incubations were performed either at room temperature for 4 hours or at 4°C for 12-16 hours. Following antibody staining, serial washes in PBS+0.1% TritonX-100 were performed to reduce background. Staining was visualized with a Zeiss LSM 510 Pascal confocal microscope.

Transplantation assays

Donor embryos were labeled at the 1-4 cell stage with rhodamine-dextran. Cells from blastula stage donor embryos were transplanted to mediolateral regions of shield-stage, unlabeled host embryos (Moens and Fritz, 1999). After transplantation, embryos were placed in embryos medium (Westerfield, 1994) containing 50 units penicillin and 5 μg streptomycin at 28.5°C. To activate expression of the hsΔTcf-GFP, embryo incubation temperature was shifted to 37°C for 1 hour. Transplanted cells were visualized using a Zeiss compound microscope.

Results

Expression of ΔTcf inhibits expression of Wnt targets

To identify where and when Wnt/β-catenin signals might be involved in zebrafish neural crest induction, we generated a transgenic line that allowed conditional expression of a truncated form of the zebrafish *tcf3* gene *headless* (Pelegri and Maischein, 1998; Dorsky et al., 1999; Kim et al., 2000). We replaced the N-terminal domain of TCF with GFP, eliminating

the β-catenin binding domain and producing a dominant inhibitor of Wnt signaling (Molenaar et al., 1996). The fusion gene was then placed under control of the zebrafish *hsp70* promoter (Halloran et al., 2000). To confirm that this hs-ΔTcf construct can affect Wnt signaling in vivo, we tested its ability to block a well-characterized action of Wnt8 in zebrafish: ventrolateral mesoderm patterning (Lekven et al., 2001; Erter et al., 2001). The expression of the homeobox gene *gsc* is restricted to the dorsal region of the marginal zone by post-MBT Wnt signaling prior to gastrulation (Laurent et al., 1997). Activating the hs-ΔTcf transgene at 4hpf resulted in radial *gsc* expression 2 hours later at shield stage, as expected if it inhibited Wnt8 (Fig. 1B, compared with control, A). This indicates that global heat shock-induced expression of ΔTcf functions is expected to antagonize Wnt signaling during mesodermal patterning, as shown previously (Pelegri and Maischein, 1998).

To determine whether hs-ΔTcf represses transcription of Wnt targets in vivo, ΔTcf was induced by heat shock in fish that were transgenic for TOPdGFP, a Wnt-responsive reporter construct that contains a destabilized variant GFP under the control of four Lef binding sites (Dorsky et al., 2002). Since the destabilized GFP reporter has different codon usage than the GFP tag used in the ΔTcf construct, its expression can be specifically detected by in-situ hybridization. At 24hpf, embryos were exposed to heat shock for 1 hour at 37°C, and then sorted into two pools, those expressing ΔTcf and control siblings not expressing ΔTcf, based on GFP expression. The GFP associated with hs-ΔTcf is ubiquitously expressed at very high levels and localized in cell nuclei, which is readily distinguishable from TOPdGFP, which is fainter, cytoplasmic, and has a restricted expression domain. At 2 or 6 hours after heat shock, TOPdGFP is expressed in the brain and spinal cord of control embryos (Fig. 1C,E). In contrast, TOPdGFP is visibly reduced by 2 hours after heat shock in ΔTcf-expressing embryos, and is further attenuated at 6 hours after heat shock (Fig. 1D,F), suggesting that ΔTcf remains active over this time. Consistent with these observations, nuclear GFP expression in hs-ΔTcf embryos remains visible at 6 hours after heat shock (data not shown). These results indicate that upon rapid induction of ΔTcf expression, the transgene quickly represses target gene transcription in a manner detectable by in-situ hybridization, and this repression is maintained for at least 6 hours following heat shock treatment.

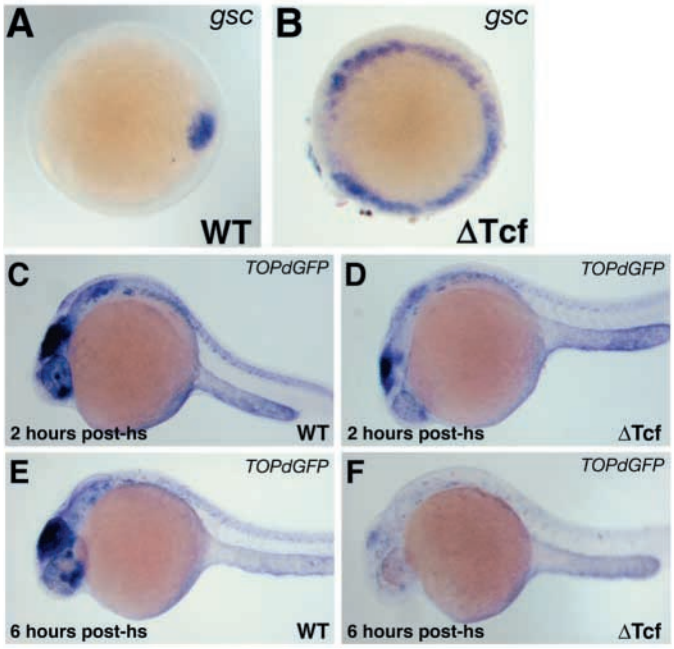

Fig. 1. Induction of hs-ΔTcf expression blocks zygotic Wnt signaling in vivo. (A) In control (non-transgenic) embryos at shield stage following 2 hours of heat shock at 37°C, *gsc* expression is normally limited to the axial mesoderm (animal pole view, dorsal to the right). (B) In transgenic ΔTcf-expressing sibling embryos the mesoderm is dorsalized, as indicated by radial *gsc* expression 2 hours after heat shock at 37°C. (C-F) TOPdGFP in-situ hybridization of heat shocked embryos expressing the ΔTcf transgene (D,F) compared with non-transgenic siblings (C,E). Embryos were fixed following 2 hour (C,D) and 6 hour (E,F) recovery periods. TOPdGFP is down regulated throughout the embryo, notably in the brain and spinal cord.

Canonical Wnt signaling is required for neural crest induction

If Wnt signals are indeed necessary for zebrafish neural crest induction, then inhibition of Wnt/β-catenin mediated signaling should result in loss of neural crest. We globally blocked Wnt signaling at various stages of early neural development by activating expression of ΔTcf in transgenic fish (Fig. 2). Embryos were incubated at 37°C for 45 minutes, and then returned to 28.5°C before fixing and staining with an antibody that recognizes zebrafish Foxd3 (fkd6) (Odenthal and Nusslein-Volhard, 1998), revealing neural crest cells flanking the neural plate. At this stage, *foxd3* appears to be expressed in virtually all pre-migratory neural crest cells, completely overlapping with *snail2/slug* (Kelsh et al., 2000). Wnt/β-catenin signaling is crucial for neural crest induction from the end of gastrulation (bud stage) through the 3-somite stage since ΔTcf inhibits expression of Foxd3 at these stages (Fig. 2D,E, compared with control Fig. 2A,B). In contrast, Foxd3 expression is comparable to that of wild-type siblings upon expression of the inhibitor 1 hour later at the 6-somite stage (Fig. 2C, compared with control, Fig. 2F). Expression of the neural crest marker *sox10* as assayed by in-situ hybridization is similarly affected (data not shown). We conclude that there is a temporal limit to the requirement for Wnt/β-catenin mediated signaling in neural crest induction, and that cells lose sensitivity to transgene activation between the 3- and 6-somite stages.

To assess the effect of ΔTcf expression on the dorsal/ventral patterning of the neural tube, we assayed expression of a pan-neuronal marker, Hu (Marusich et al., 1994). Hu protein is expressed in the trigeminal ganglia, primary motor neurons and dorsal Rohon–Beard sensory neurons (Kim et al., 1996). While the global loss of Wnt/β-catenin signaling in response to ΔTcf results in loss of Foxd3 expression (Fig. 3B, compared with control Fig. 3A), Hu expression is unaffected in transgenic embryos (Fig. 3D) or their wild-type siblings (Fig. 3C) after heat-activation at the 3-somite stage. Notably, differentiation of dorsal Rohon–Beard sensory neurons (white arrow and arrowhead) and ventral motor neurons (asterisk) of the spinal cord is unaffected. Hu expression is found in the same cells that express the transgene (Fig. 3E, yellow cells). These results suggest that the requirement for Wnt/β-catenin mediated

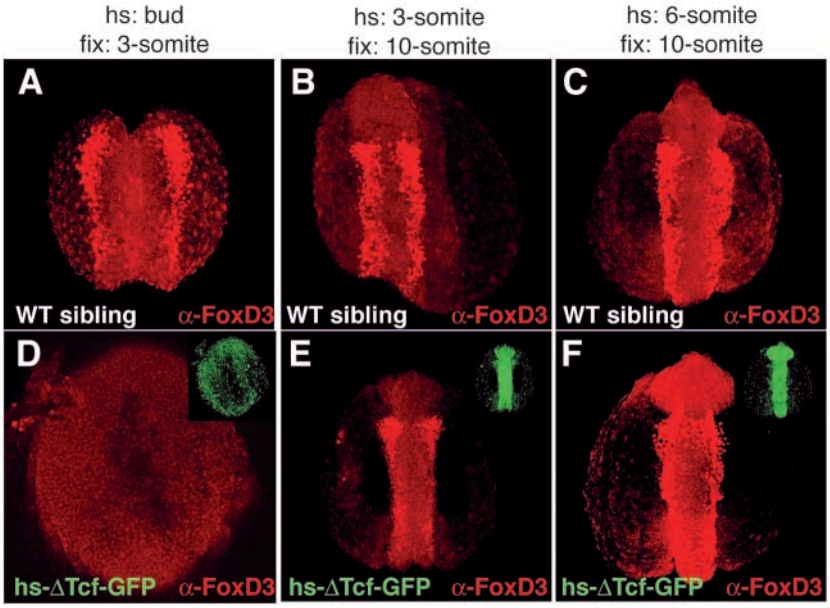

Fig. 2. Temporal limit for the Wnt/β-catenin signaling requirement. Foxd3-positive neural crest cells, identified by anti-Foxd3 primary antibody (red), flank the neural plate after heat activation at bud (A), 3-somite (B), and 6-somite stages (C). Transgenic embryos show significant loss of Foxd3 expression upon heat activation of hs-ΔTcf (green) (D,E), while Foxd3 expression is comparable to that of wild-type siblings after induction at 6-somites (F). All images are dorsal views, anterior to top, of stacked 10X confocal series after fixation at 3-somites (A,D) or 10-somites (B,C,E,F). Expression of the transgene is shown in the insets (D,E,F).

signaling at this stage is specific for neural crest and not indirectly through its role in overall patterning of neuroectoderm.

Wnt signaling is required cell-autonomously in neural crest precursors for crest induction

The requirement for canonical Wnt signaling in the induction of neural crest may be cell-autonomous, in which neural crest precursors must directly receive a Wnt signal and activate expression of Wnt target genes to establish their identity. Alternatively, the requirement may be non-autonomous; for example, neural crest precursors might be induced by other signals derived from the neural plate after this tissue has received posteriorizing Wnt signals. To distinguish between these two possible scenarios, we tested the ability of transplanted neural crest precursors to rescue the loss of neural crest in ΔTcf transgenics. Small numbers of cells were transplanted into the neural crest fate map position at shield stage. As a control, cells were transplanted from wild-type donors into wild-type host embryos. The transplantation process does not affect expression of Foxd3 in transplanted neural crest precursors (Fig. 4A and inset). If the requirement for Wnt response were cell-autonomous, transgenic neural crest precursors will be unable to express Foxd3 in wild-type embryos after activation of the ΔTcf transgene at bud stage. Consistent with this hypothesis, ΔTcf transgenic cells were unable to form neural crest cells in wild-type hosts, although cells were intercalated between wild-type cells expressing Foxd3 (Fig. 4B,C). In reciprocal transplants, we observed Foxd3-positive nuclei derived from wild-type transplanted cells in the ΔTcf transgenic host background (Fig. 4D,E,F). As shown, Foxd3-positive nuclei within the clone of cells derived from wild-type donors do not co-localize with host ΔTcf-positive nuclei. Taken together, these

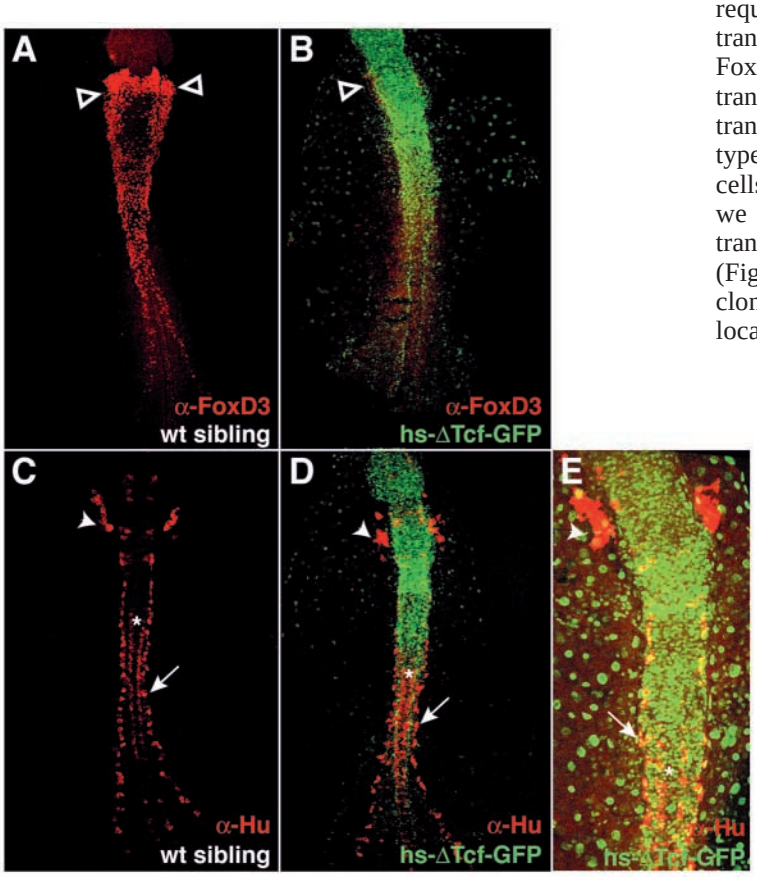

Fig. 3. Loss of canonical Wnt signaling does not alter Hu expression. (A,B) Foxd3-positive neural crest cells (outline empty arrowhead), identified by anti-Foxd3 primary antibody (red), flank the neural plate after heat activation at the 3-somite stage. As shown in (B), transgenic embryos show significant loss of Foxd3 expression upon heat activation of hs-ΔTcf (green) as compared to with wild-type siblings (A). (C,D,E) Anti-Hu antibody, which recognizes the pan-neuronal marker Hu, was were used to identify Hu-positive cells in heat-activation treated embryos (red). Normal expression is detected in the trigeminal ganglia (white arrowhead), Rohon–Beard sensory neurons (white arrow), and primary motorneurons in the spinal column (asterisk) in ΔTcf embryos (D) as compared to with their wild-type siblings (C). Overlap between Hu and transgene expression is seen at higher power (E). All images are dorsal views of flat-mounted embryos fixed at the 10-somite stage, anterior to top, of stacked confocal series obtained at 10X (A-D) or 20X (E).

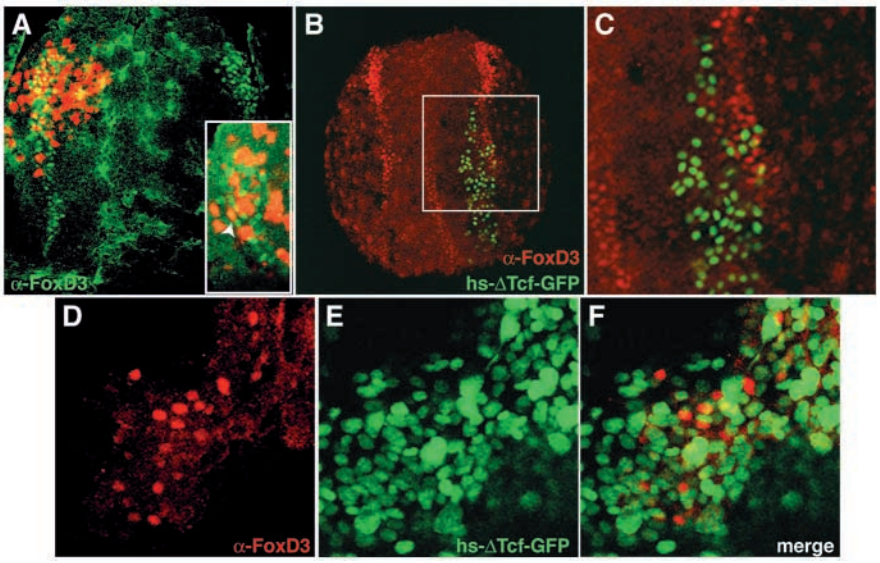

Fig. 4. Canonical Wnt/β-catenin signaling is required in neural crest precursors cell-autonomously. (A) As a control, rhodamine dextran-labeled wild-type neural crest precursors (red) were transplanted into a wild-type host embryo. The transplanted cells express Foxd3, detected with anti-Foxd3 antibody (green) when transplanted into a wild-type host embryo. Inset is a single confocal slice to indicate co-localization in single transplanted cell (white arrowhead). (B,C) Transgenic crest precursors fail to express Foxd3 when transplanted into a wild-type environment. Embryo shown is a dorsal view with anterior to the top. Inset in (B) is magnified in (C). (D-F) Wild-type cells express Foxd3 when transplanted into a transgenic environment. (D) Foxd3-positive nuclei, detected with anti-Foxd3 primary antibody (red), are observed in transplanted cells from wild-type donor embryos. (E) GFP-positive nuclei (green) are observed in the ΔTcf background after heat-activation of the transgene. (F) Stacked confocal images have been merged to show of failure of Foxd3-positive nuclei to co-localize with background GFP expression.

results suggest that cells require reception of a Wnt signal to be induced as neural crest.

Wnt8 is involved in neural crest induction

The temporal requirement for canonical Wnt signaling during neural crest development determined by the preceding data corresponds with the expression of zebrafish *wnt8*. Zebrafish *wnt8* is a bicistronic gene, yielding two transcripts (*wnt8.1* and *wnt8.2*) with overlapping but distinct expression domains early in gastrulation (Kelly et al., 1995; Lekven et al., 2001). We performed in-situ hybridization to determine the correspondence of *wnt8* to the earliest identified marker of the neural crest domain, *pax3*. Both *wnt8.1* and *wnt8.2* transcripts are expressed in proximity to, and partially overlapping with, *pax3*, demonstrating that they may play a role in neural crest induction (Fig. 5).

To test the importance of Wnt8 in the initial induction of neural crest, we used antisense MOs to interfere with the translation of Wnt8.1 and 8.2. Embryos were carefully staged by counting somites to discount any possible delays in development after MO injection. Injection of MOs against *wnt8.1* at the 1-2 cell stage resulted in significant loss of expression of *pax3*, *foxd3* and *sox10* (Fig. 6B,E,H, compared with controls, Fig. 6A,D,G). Expression of *pax3* completely overlaps with *foxd3* at this stage, and in addition is expressed in some cells of the dorsal neural plate (J.W.R., unpublished). *sox10* expression is found in a subset of cells that express *foxd3* (Dutton et al., 2001). This effect appears to be accompanied by lateral expansion of the neural plate and disruption of the notochord, as indicated by *foxd3* expression in the notochord, consistent with previously described roles for *wnt8* in zebrafish development (Erter et al., 2001; Lekven et al., 2001). The loss of early neural crest marker expression by *wnt8.1* MO was dose-dependent (data not shown), nearing 100% at 20 ng injected. In contrast, blocking *wnt8.2* function showed no loss of any of these markers at all concentrations tested (Fig. 6C,F,I). Embryos co-injected with *wnt8.1* MO and *wnt8.2* MO showed complete loss of neural crest marker expression and similar A/P axis patterning defects, as previously described (Lekven et al., 2001). These data support a critical role for Wnt8 at early stages of neural crest induction.

To assess the requirement for Wnt8 in later specification of neural crest derivatives, we again blocked Wnt8.1 function by injection of antisense MO and assayed for the expression of various neural crest markers. Injection of 20 ng *wnt8.1* MO blocked neither expression of *sox10* in pre-migratory neural crest at the 10-somite stage nor *sox10* expression in migratory crest at 24hpf (Fig. 7B,D, compared with uninjected controls, Fig. 7A,C). Similarly, injection of *wnt8.1*MO did not affect either expression of *mitfa*, a melanophore-specific neural crest marker (Fig. 7F, compared with control, Fig. 7E) or *dlx2*, a marker of cranial neural crest (Fig. 7H, compared with control, Fig. 7G) (Akimenko et al., 1994). These data suggest the loss of neural crest observed at the 3-somite stage upon elimination

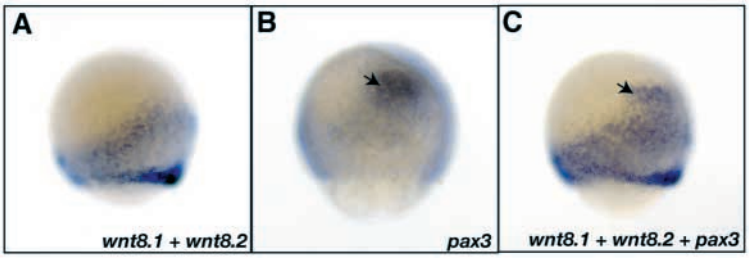

Fig. 5. *Wnt8* and *pax3* show overlapping expression in the presumptive neural crest domain. (A) Whole-mount in-situ hybridization at the 80% epiboly stage reveals expression of *wnt8* transcripts at the margin and along the dorsal side of the embryo. (B) Expression of *pax3* in the presumptive neural crest (arrow). (C) The *wnt8* expression domain is in proximity to, and partially overlapping with, expression of *pax3* in the presumptive neural crest region (black arrow). All images are lateral views, with dorsal to the right, of 80% epiboly stage embryos after hybridization.

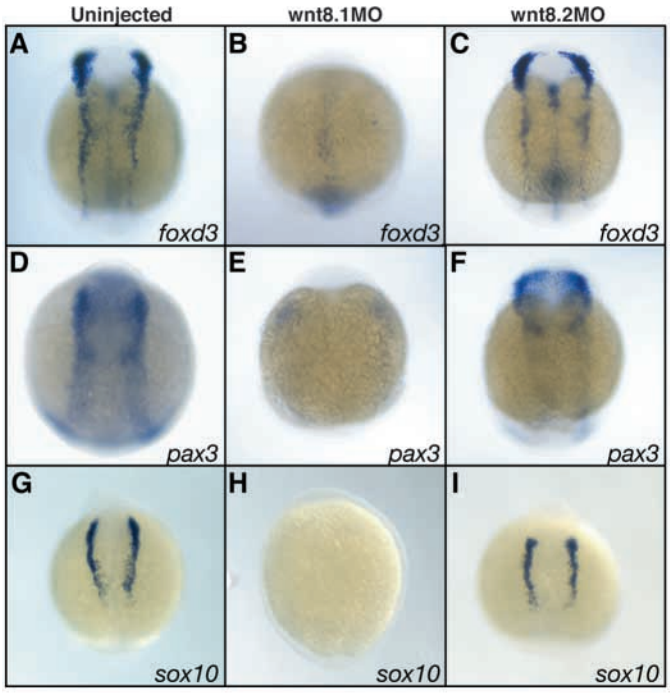

Fig. 6. Wnt8 is required for neural crest induction in vivo. Whole-mount in-situ hybridization at the 6-somite stage reveals loss of early neural crest markers *foxd3* (A-C), *pax3* (D-F), and *sox10* (G-I) upon injection of 20 mg/ml wnt8.1 Morpholino oligonucleotides (B,E,H). Such loss was not detected upon injection of 20 mg/ml wnt8.2 Morpholino (C,F,I), suggesting it does not have a role in neural crest induction. Uninjected controls (A,D,G) show normal expression of markers in the neural crest; *foxd3* is also expressed in the notochord. All images are dorsal views, anterior to top, of 3-somite embryos.

of functional Wnt8 recovers by the 10-somite stage. This result is consistent with the temporal limits for Wnt/β-catenin mediated signaling established by the ΔTcf transgenic experiments. It is also consistent with the restriction of *wnt8* mRNA expression to the tailbud during somitogenesis (Kelly et al., 1995) (data not shown).

Differential response of neural crest gene expression to loss of canonical Wnt signaling

To test whether Wnt/β-catenin signaling is required for proper specification of neural crest-derived cell types later in development, we used the stable ΔTcf transgenics to block canonical Wnt/β-catenin signaling at the onset of neural crest migration. Activation of the transgene for 1 hour at 18hpf resulted in dramatic loss at 24hpf of *mitfa*, a specific marker of crest-derived melanophores (Lister et al., 1999), compared with wild-type siblings (Fig. 8B, compared with control, Fig. 8A), consistent with previous results that *mitfa* is a direct target of Wnt/β-catenin signaling (Dorsky et al., 2000b). In addition, expression of *dlx2* in cranial neural crest cells migrating into the developing branchial arches is substantially reduced upon transgene activation (Fig. 8F,H, compared with controls, Fig. 8E,G). In contrast, transgene activation at this stage did not eliminate expression of more broadly expressed markers of migrating crest, such as *sox10* (Fig. 8D, compared with control, Fig. 8C) and *crestin* (not shown), although some reduction of *sox10* expression was consistently observed. These data support a model where Wnt/β-catenin mediated signaling is involved in specification of certain neural crest lineages, including the pigment cell and possibly craniofacial lineages, although Wnt signals no longer appear to be required for general neural crest identity as cells migrate from the dorsal neural tube. This Wnt/β-catenin requirement for melanophore and cranial crest specification appears to be independent of the requirement for Wnt8 in early crest induction.

Discussion

We present here evidence that Wnt signaling plays stage-specific roles during neural crest induction in zebrafish. *wnt8* is expressed at the right time and place to be involved in the initial induction of neural crest, and antisense MOs block induction. Using the hs-ΔTcf transgenic line, we have identified a critical period during which neural crest formation is disrupted that corresponds to the time when *wnt8* perturbation affects this process. Transplantation studies with this transgenic line demonstrate that neural crest cells require the reception of the Wnt signal directly. These studies confirm and extend previous findings demonstrating a specific requirement for Wnt signaling in neural crest induction. Bang et al. (Bang et al., 1999) previously demonstrated that overexpression of Wnt8 induces expanded expression of *pax3* in *Xenopus* embryos. Additional studies have demonstrated that misexpression of other Wnts, including *wnt1*, *wnt3a* and *wnt7b*, induces expanded expression of *Xenopus* neural crest markers (Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Villanueva et al., 2002), and treatment of chick explants with soluble *Drosophila* Wingless protein promotes neural crest formation (García-Castro et al., 2002). Together these studies have implicated a Wnt signal in neural crest formation but did not specifically identify which ligand was necessary.

Our studies are the first to identify a specific Wnt necessary for neural crest induction in zebrafish. However, *wnt8* is apparently needed only during the initial phase of neural crest formation, and perhaps other Wnts are needed after this period. In mouse embryos, loss of *Wnt1* and *Wnt3a* does not block the initial induction of neural crest but affects subsequent neural crest expansion (Ikeya et al., 1997). Although Wnts are thought to be involved in regulating proliferation of neural tissue (McMahon and Bradley, 1990; Dickinson et al., 1994; Megason and McMahon, 2002), proliferation of neural crest precursors is reported to be unaffected in *Wnt1*/*Wnt3a* knockout mice (Ikeya et al., 1997). These results suggest the possibility that there are several stages of neural crest induction regulated by different Wnts: an early phase involving *wnt8* and a later phase utilizing *wnt1* and *wnt3a*. Other Wnts, such as *wnt3*, *wnt6*, *wnt7* and *wnt10b*, may also be involved.

Several studies have implicated Wnts in promoting ventral/posterior patterning of mesoderm and neuroectoderm. Blocking *wnt8* in zebrafish results in loss of posterior brain and spinal cord (Erter et al., 2001; Lekven et al., 2001). In addition, there is an anterior expansion of posterior markers when zebrafish *tcf3* genes are inactivated (Kim et al., 2000; Dorsky et al., 2003), including an anterior shift of neural crest (Itoh et al., 2002). Since relief of Tcf3 repressor activity is a result of Wnt signal transduction, loss of *tcf3* function is thought to behave similarly to Wnt activation (Brannon et al., 1997; Kim

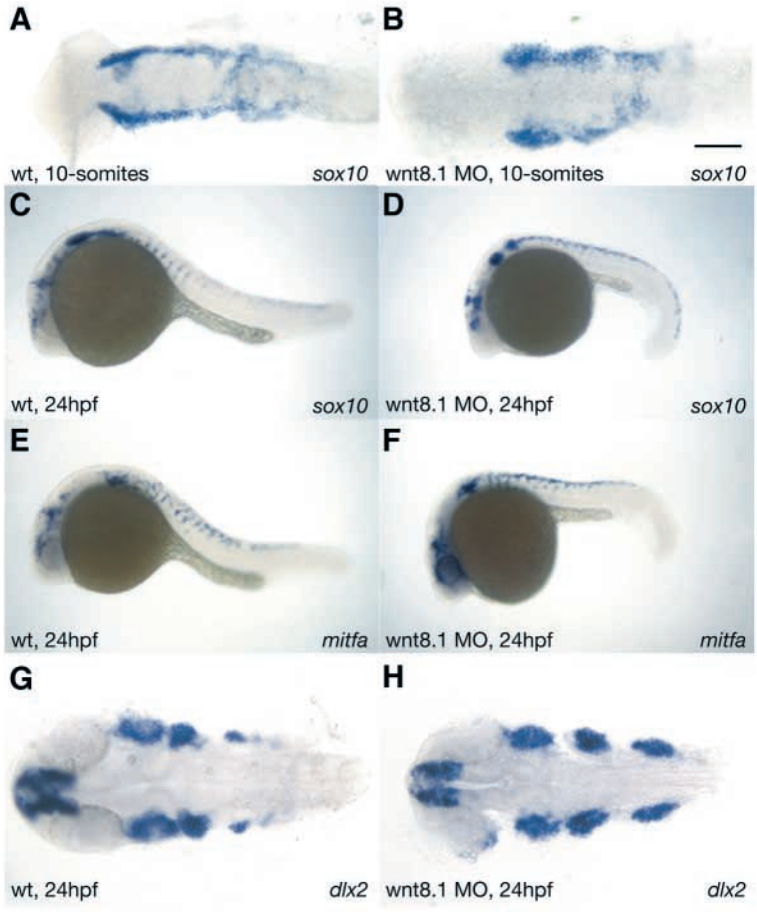

Fig. 7. Functional Wnt8 is not required for late expression of neural crest markers. Whole-mount in-situ hybridization for neural crest marker, *sox10*, on 10-somite (A,B) and 24hpf (C,D) stage embryos. (E,F) Whole-mount in-situ hybridization for melanophore-specific marker, *mitfa*. (G,H) Whole-mount in-situ hybridization for cranial neural crest marker, *dlx2*. Injection of 20 mg/ml wnt8.1 antisense Morpholino oligonucleotides does not significantly reduce or eliminate the expression of any of these neural crest markers. All images of uninjected control siblings (A,C,E,G) and wnt8.1MO-injected embryos (B,D,F,H) are lateral views (B-F) or dorsal views (A-B,G-H) with anterior to the left. Scale bar: 100 μm in (A,B); 200 μm in (C-F); 50 μm in (G,H).

et al., 2000). Together these observations suggest the possibility that the effects we see on neural crest are indirect: interfering with Wnt signals might simply convert posterior neural tissue to anterior, from which no neural crest would normally be produced. However, our experiments with the ΔTcf transgenic line suggest that this is not the case. We have found that neural crest development can be blocked at a time when the development of dorsal spinal Rohon–Beard sensory neurons is unaffected. This result implies that we have not simply eliminated the domain from which neural crest cells are derived, since neural crest cell precursors arise intermingled among Rohon–Beard neurons (Cornell and Eisen, 2000). In addition, cell transplantation experiments suggest that neural crest precursors specifically require activation of the Wnt/β-catenin signaling pathway, and that small groups of wild-type cells can form neural crest in the correct position even when Wnt signaling is blocked in all other cells of the host embryo. These results suggest that Wnt-mediated induction of zebrafish neural crest is probably independent of its roles in anterior/posterior patterning, as has been recently suggested for FGF-mediated induction of neural crest in *Xenopus* (Monsoro-Burq et al., 2003).

At first glance, the phenotype of the *headless/tcf3* mutant, in which neural crest markers are expanded anteriorly, appears to contradict our results with the ΔTcf transgenic line, in which neural crest markers are

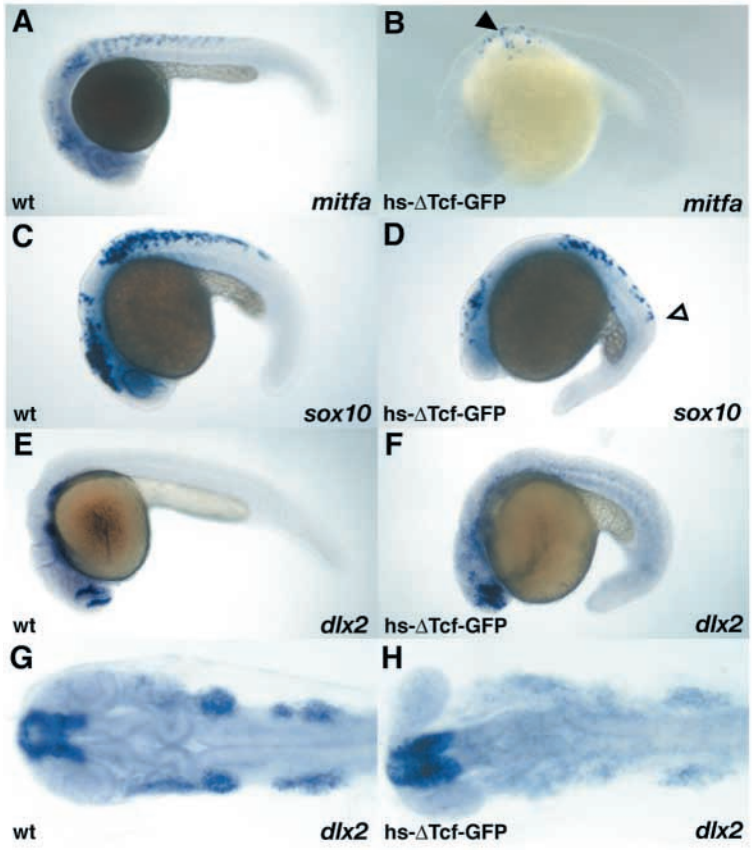

Fig. 8. Wnt/β-catenin signaling is required for *mitfa* expression and branchial arch expression of *dlx2* upon crest migration. (A,B) Whole-mount in-situ hybridization for melanophore-specific marker, *mitfa*. (C,D) Whole-mount in-situ hybridization for neural crest marker, *sox10*. (E-H) Whole-mount in-situ hybridization for cranial neural crest marker, *dlx2*. Black arrowhead indicates few remaining *mitfa*-expressing cells. Open arrowhead indicates normal pattern of *sox10*-expressing neural crest. While telencephalon and diencephalon expression is unchanged in ΔTCF transgenic embryos, *dlx2* expression is observed in the developing branchial arches. All images are lateral views, anterior to the left, of wild-type siblings (A,C,E,G) and hsΔTcf-GFP transgenic embryos (D,E,F,H) after heat shock at the 18-somite stage and fixation at the 26-somite stage, except (G,H) which are dorsal views of flat-mounted embryos, anterior to the left.

lost. However, the *headless* mutation results in a loss-of-function of a repressor while the ΔTcf transgenic results in a gain-of-function of a repressor, which is consistent with the different phenotypes. Furthermore, describing neural crest cells as 'expanded' in *headless* mutants is perhaps inaccurate, as the neural crest domain instead is shifted anteriorly. Finally, the ΔTcf transgene is likely to act as a dominant repressor in the place of all members of the TCF/Lef family, not just Tcf3.

There are several caveats to the interpretations of the data we present here. Although *wnt8* is expressed at the right place and time to be involved in the induction of neural crest cells from ectoderm and *wnt8* MO injection blocks neural crest formation, we cannot eliminate the possibility that activation of the ΔTcf transgene is instead blocking signaling from some other Wnt. The period we identified as critical using the transgenic line roughly corresponds to the period of *wnt8* expression; however, exactly when Wnts are needed cannot be easily determined by transgene activation. Although it takes several hours to inactivate the TOPdGFP reporter, suggesting that the Wnt requirement for neural crest formation might be later than the period in which we performed the heat shock, this multicopy reporter transgene may not respond with the same kinetics as endogenous genes. Currently identified zebrafish Wnts known to be expressed in the dorsal neural tube, such as *wnt1*, *wnt3a* and *wnt10b*, are expressed too late to be involved in this initial phase of neural crest induction, and a deletion eliminating zebrafish *wnt1* and *wnt10b* has little effect on neural crest (Lekven at al., 2003). Future studies identifying other zebrafish Wnt genes and their functions will be needed to fully address this point. Another caveat is that the ΔTcf transgene may additionally act to repress genes not regulated by Wnt signals. Future studies using other tools to block Wnt signals, such as identifying and eliminating Frizzled receptors expressed at the right place and time, or expressing other reagents that interfere with Wnt signals such as *axin* or kinase-dead *gsk3*, are needed.

Both BMP and Notch signaling have been implicated in neural crest induction in zebrafish, but these signaling pathways may be required at different times from Wnt signaling. Mutations in zebrafish *bmp2b* result in loss of both neural crest and Rohon–Beard cells (Barth et al., 1999; Nguyen et al., 2000). Disruption of Notch signaling also results in loss of neural crest but instead concomitantly increases the number of Rohon–Beard cells (Cornell and Eisen, 2000). In contrast, blocking Wnt signals can interfere with neural crest without affecting Rohon–Beard cells, suggesting that Wnts maintain a role independent of Notch regulation.

Previous studies have implicated Wnt signaling in the specification and differentiation of pigment cells from neural crest (Dorsky et al., 1998; Dunn et al., 2000; Jin et al., 2001; Hari et al., 2002), and that *mitfa*, a gene encoding a bHLH transcription factor necessary and sufficient for pigment cell formation, is a direct target of the Wnt pathway (Dorsky et al., 2000b; Takeda et al., 2000; Widlund et al., 2002). We demonstrate here that the Wnt requirement for *mitfa* expression is to some degree temporally separable from Wnt regulation of neural crest induction, suggesting that reiterated Wnt signaling plays sequential roles in neural crest development. When the Δ-Tcf transgene is activated at the 18-somite stage, *mitfa* expression is almost completely eliminated. An alternative explanation is that transgene activation at this stage specifically blocked the formation of a subpopulation of melanogenic neural crest cells, consistent with the observed reduction of *sox10*-positive cells. However, our previous results demonstrated that zebrafish melanogenic neural crest cells have already segregated from the neural tube by the 18-somite stage (Raible and Eisen, 1994; Raible and Eisen, 1996), suggesting this possibility is less likely.

Our results also suggest that Wnt signals promote *dlx2* expression in branchial arches. Unfortunately, the poor long-term survival of heat-shocked transgenic animals has not let us assess the final effects of transgene activation on cartilage differentiation. Although ectomesenchyme that gives rise to craniofacial cartilages is traditionally thought of as derived from neural crest, some studies suggest that these cells are a distinct population (e.g. Dutton et al., 2001). Although our results might suggest that Wnt signals are needed for ectomesenchyme as well as other neural crest derivatives, the inference that these cells thus have common origins should be reached with caution since Wnt signals have widespread use during embryogenesis.

One way the same signaling pathway could play different roles at different times is if different genes are induced in the context of other signaling pathways. Indeed, neural crest induction has been proposed to occur after a combination of signals, including both Wnts and BMPs (LaBonne and Bronner-Fraser, 1998). Moreover, Wnts work with FGF during anterior/posterior neural patterning (McGrew et al., 1997). Another possibility is that genes induced by initial Wnt exposure modify the response to subsequent Wnt signals. A good candidate for such a role would be *sox10*, which we show here requires Wnt signals for its initial expression. Sox10 is necessary for the development of a subset of neural crest derivatives, including pigment cells (Southard-Smith et al., 1998; Pingault et al., 1998; Dutton et al., 2001; Honore et al., 2003; Aoki et al., 2003), and directly regulates the *mitf* promoter in vitro and in vivo (Verastegui et al., 2000; Bondurand et al., 2000; Lee et al., 2000; Potterf et al., 2000; Elworthy et al., 2003).

The use of the same signaling pathways in different places and at different times is common throughout development, and understanding how specificity is generated in particular contexts remains a major challenge. It is interesting to consider that BMPs or Notch may also have sequential roles within the neural crest, first needed for induction and subsequently in generation of neuronal or glial sublineages (Shah et al., 1996; Reissmann et al., 1996; Varley and Maxwell, 1996; Morrison et al., 2000; Wakamatsu et al., 2000). Wnts, BMPs and Notch also play roles in the survival of neural crest cells (Graham et al., 1996; Maynard et al., 2000; Ellies et al., 2000; Brault et al., 2001; Hasegawa et al., 2002). Understanding the specifics of how Wnt signals regulate distinct steps in neural crest induction and pigment cell specification might reveal general mechanisms for reiterated signaling during development.

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