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# Neural crest induction by paraxial mesoderm in *Xenopus* embryos requires FGF signals

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## SUMMARY

**At the border of the neural plate, the induction of the neural crest can be achieved by interactions with the epidermis, or with the underlying mesoderm. Wnt signals are required for the inducing activity of the epidermis in chick and amphibian embryos. Here, we analyze the molecular mechanisms of neural crest induction by the mesoderm in *Xenopus* embryos. Using a recombination assay, we show that prospective paraxial mesoderm induces a panel of neural crest markers (*Slug*, *FoxD3*, *Zic5* and *Sox9*), whereas the future axial mesoderm only induces a subset of these genes. This induction is blocked by a dominant negative (dn) form of FGFR1. However, neither dnFGFR4a nor inhibition of Wnt signaling prevents neural crest induction in this system. Among the FGFs, *FGF8* is strongly expressed by the paraxial mesoderm. FGF8 is sufficient to induce the neural crest markers *FoxD3*, *Sox9* and *Zic5* transiently in the animal cap assay. In vivo, FGF8 injections also expand the *Slug* expression domain. This suggests that FGF8 can initiate neural crest formation and cooperates with other DLMZ-derived factors to maintain and complete neural crest induction. In contrast to Wnts, eFGF or bFGF, FGF8 elicits neural crest induction in the absence of mesoderm induction and without a requirement for BMP antagonists. In vivo, it is difficult to dissociate the roles of FGF and WNT factors in mesoderm induction and neural patterning. We show that, in most cases, effects on neural crest formation were parallel to altered mesoderm or neural development. However, neural and neural crest patterning can be dissociated experimentally using different dominant-negative manipulations: while Nfz8 blocks both posterior neural plate formation and neural crest formation, dnFGFR4a blocks neural patterning without blocking neural crest formation. These results suggest that different signal transduction mechanisms may be used in neural crest induction, and anteroposterior neural patterning.**

Key words: FGF, WNT, FGF8, Paraxial mesoderm, *Xenopus* embryo, Neural crest, Neural patterning

## INTRODUCTION

The neural crest, a transitory population of cells that is characteristic of vertebrate embryos, forms at the border of the neural plate, posteriorly to the diencephalon. After induction, neural crest cells undergo an epithelial-to-mesenchymal transition and migrate into several locations to give rise to a large variety of derivatives (for a review, see Le Douarin and Kalcheim, 1999). Experimental manipulations in chick, fish and amphibian embryos have shown that both the ectoderm and the neural plate can give rise to neural crest cells when they are juxtaposed (Moury and Jacobson, 1989; Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; Woo and Fraser, 1998). However, in vivo, the neural crest forms adjacent to three different tissues, the non neural ectoderm, the neural plate and the underlying paraxial mesoderm, all of which thus constitute potential sources of neural crest inducers (Schroeder, 1970). Although many studies have focused on neural crest induction by the ectoderm in the chick embryo (Dickinson et al., 1995; Basch et al., 2000; Knecht and Bronner-Fraser, 2002), a pioneering study by Raven and Kloos (Raven and Kloos, 1945) showed that the paraxial mesoderm can induce neural crest formation in the ectoderm of amphibians. More recent studies also show that recombining the paraxial mesoderm with naive ectoderm in *Xenopus laevis* embryos results in potent neural crest induction in the ectodermal part of the explant and that excising the paraxial mesoderm results in lack of neural crest formation in vivo (Mancilla and Mayor, 1996; Bonstein et al., 1998; Marchant et al., 1998). In chick embryos, some data also indicate that the melanocytes, which are neural crest derivatives, are induced after neural plate-paraxial mesoderm recombination (Selleck and Bronner-Fraser, 1995). Although tested separately in these experimental assays, the possibility that the inducing activities from the ectoderm and the mesoderm might act in concert during normal development remains to be explored.

In the amphibian embryo, the current analysis of the molecular basis of ectoderm-neural tissue interactions results in a two-step model of neural crest induction detailed below (reviewed by Aybar and Mayor, 2002; Knecht and Bronner-Fraser, 2002). *Slug* was generally used in these studies as a

specific marker gene for neural crest development (Nieto et al., 1994; Mayor et al., 1995). In the first step of the model, in parallel to what happens during amphibian neural induction (Harland, 2000), BMP activity in the ectoderm must be attenuated by BMP antagonists. Neural crest forms after moderate BMP inhibition while neural tissue induction requires higher levels of inhibition (Marchant et al., 1998). However, the levels of *Slug* induction in these assays, using BMP antagonists alone, are very low compared with endogenous levels (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998). This suggests that in the embryo, additional factors are required for normal levels of *Slug* expression and neural crest induction/maintenance.

Co-injection of BMP antagonists with molecules such as Wnts (Wnt7b or Wnt8), fibroblast growth factors (eFGF or bFGF) or retinoic acid (RA) results in strong neural crest formation in ectodermal explants (animal caps) (Mayor et al., 1995; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Villanueva et al., 2002). Although these molecules do not induce neural crest by themselves in vitro, the in vivo overexpression of positive regulators of the Wnt, FGF or RA pathways expands neural crest-forming domains, whereas blocking these pathways prevents normal neural crest induction in both embryo and explant assays (Mayor et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Villanueva et al., 2002). Together, these data suggest a second phase of induction where partially neuralized ectoderm is specified to become neural crest either by Wnts, FGF, RA or a combination. However, this model does not specifically address the mechanism by which paraxial mesoderm might induce the neural crest. Furthermore, both FGF and Wnt proteins play important roles in mesoderm induction and paraxial mesoderm development (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994; Fisher et al., 2002; Vonica and Gumbiner, 2002) and reagents that affect neural crest induction might do so indirectly by their effects on the mesoderm (Mayor et al., 1995; Mayor et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998). Finally, all three classes of molecules implicated in neural crest induction are also important neural posteriorizing agents (Lamb and Harland, 1995; Bang et al., 1997; Bang et al., 1999; Kiecker and Niehrs, 2001; Kudoh et al., 2002). BMP antagonism results in the formation of anterior neural tissue that is not expected to form neural crest (Lamb et al., 1993; Knecht and Harland, 1997). This raises the possibility that posteriorization of this area into a neural crest-producing tissue would account for the *Slug* induction recorded after co-injecting Noggin/Chordin with Wnt/FGF/RA molecules. This correlation of neural crest induction with posterior identity has recently been demonstrated in embryos (Villanueva et al., 2002). Thus, whether induction of neural crest can occur independently from neural induction and patterning remains unclear.

In this study, we address two questions. First, what is the nature of the mesodermal signal(s) inducing neural crest in the ectoderm? Second, how is neural crest induction related to early anteroposterior (AP) patterning of the neural plate? To study the molecular mechanisms of neural crest induction by the paraxial mesoderm in the *Xenopus laevis* embryo, we focused on the neural crest-inducing properties of the dorsolateral marginal zone (DLMZ) on animal cap explants. Using various neural crest markers, we show that the DLMZ and the dorsal marginal zone (DMZ) exhibit qualitative differences in their inducing properties. In order to study the role of specific growth factor signaling in neural crest induction, we then took advantage of previously characterized molecular tools, consisting of broad range or more specific inhibitors of the Wnt and FGF pathways. These reagents include NFz8, GSK3, dnTCF3 and a truncated form of Dishevelled (Xdd1) for Wnt signaling, and SU5402, XFD and dnFGFR4a for FGF signaling (Amaya et al., 1993; Sokol, 1996; Mohammadi et al., 1997; Deardorff et al., 1998; Hongo et al., 1999; Deardorff et al., 2001), for reviews see (Galzie et al., 1997; Brantjes et al., 2002; Moon et al., 2002). We have also used these reagents in vivo to address whether neural crest formation can be uncoupled from repatterning of the mesoderm or changes in AP patterning of the neural plate.

## MATERIALS AND METHODS

### RNA injections

*Xenopus laevis* embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994) and analyzed according standard procedures described by Sive et al. (Sive et al., 2000). Nuclear targeted β-galactosidase (pCS2-*Nls-NlacZ*, 100-200 pg) mRNA was co-injected with the test mRNA for lineage tracing. Capped messenger RNAs were synthesized using the mMESSAGE mMachine kit (Ambion).

To block the response of the ectoderm to endogenous Wnt molecules, we injected mRNAs encoding either xNFz8, Glycogen Synthase Kinase 3 (GSK3), dnTCF3 or Xdd1. The pCS2-*xNFz8* encodes a wide spectrum dominant-negative Wnt receptor (Deardorff et al., 1998), Xdd1 is a truncated form of Dishevelled, which acts as a dominant-negative in both the canonical and the non canonical planar cell polarity (PCP) pathways (Sokol, 1996; Wallingford and Harland, 2002). The pCS2-*xGSK3*, pT7Ts-*dnTCF3*, p64T-*XWnt8* and pCS2-*dnXWnt8* plasmids have been described previously (Christian et al., 1991; Molenaar et al., 1996; Pierce and Kimelman, 1996; Hoppler and Moon, 1998). We blocked FGF signaling in the responding ectoderm using either a dominant-negative form of xFGFR1, constructed by S. Dougan (pCS2-*XFD-GFP*) similar to the XFD construct published by Amaya et al. (Amaya et al., 1991), or a truncated FGFR4a (p64T-*dnXFGFR-4a*) (Hongo et al., 1999), subcloned into pCS108. *XFGF8* (Christen and Slack, 1997) was subcloned into pCS107.

### Tissue recombination, SU5402 treatment of the recombinants

Stage 10-10.5 DLMZ or DMZ were recombined with stage 8-9 animal caps (Fig. 1A) (Bonstein et al., 1998). Dissections and culture were performed in 3/4 Normal Amphibian Medium (NAM) containing gentamycin (100 μg/ml). The recombinants were harvested when sibling embryos reached stage 18. For inhibition of FGF signaling by the SU5402 (Calbiochem) (Mohammadi et al., 1997), the recombinants were cultivated in 50 μM SU5402 diluted into 3/4 NAM (Shinya et al., 2001; Maroon et al., 2002). Controls were grown in DMSO diluted in 3/4 NAM.

### In situ hybridization

The in situ hybridization protocol was simplified by directly prehybridizing embryos younger than stage 20 after rehydration in PBT. The rest of the procedure remained unchanged. This shorter protocol allows a better staining of superficially located tissues (such as the neural crest).

The probes for *Slug*, *Twist*, *Snail*, *Krox20*, *Cpl-1* and *Otx2* have

**Table 1. Primers used in this study**

| Probe name | Sequence (upstream and downstream, respectively) | Number of cycles | Reference |
|---|---|---|---|
| Slug | 5′-TCCCGCCACTGAAAATGCCACGATC-3′<br>5′-CCGTCCTAAAGATGAAGGGTATTCCTG-3′ | 23-25 | Mizuseki et al. (1998) |
| Zic5 | 5′-AGAGAGGACTATACGCTAAC-3′<br>5′-GGTACATGAGAGCAGAGAAC-3′ | 23 | Nakata et al. (2000) |
| FoxD3 | 5′-CCAGAACAAGCCCAAGAACAGC-3′<br>5′-GAAGCAGTCATTCAGCGACAGG-3′ | 23 | This work |
| Snail | 5′-GGGGCTTACTACACACCTCTTGTC-3′<br>5′-AGATTTTACAGACGCAGGGCAG-3′ | 21 | This work |
| Sox9 | 5′-AACAGGAGTTCCATCAATCCCC-3′<br>5′-CTTTTGCTAAACCCCGTGTCAC-3′ | 25 | This work |
| FGF3 | 5′-GGCTGGAGAGAGAACCTAAGTATCC-3′<br>5′-TGTATGTTTCCGAGGCGTAAAGTC-3′ | 25 | This work |
| eFGF/FGF4 | 5′-CCGCTTTCTTTCCAGAGA-3′<br>5′-GGATATGAAACCCGATGC-3′ | 25 | Isaacs et al. (1992) |
| FGF8 | 5′-TGCGGAGACTGGTTACTACATCTG-3′<br>5′-TTCTGTGGTGTGGTGTCCCTTTGG-3′ | 25 | This work |
| FGFR1 | 5′-TCGCCCCTAAAACCAAAACG-3′<br>5′-TGTCATCATCATCATCGTCGTCC-3′ | 25 | This work |
| FGFR2 | 5′-ATGTCTTTCCCGTTCTGCCTGGTG-3′<br>5′-GTCGTTATCATCTTCATCATCCCC-3′ | 25 | This work |
| FGFR3 | 5′-ACAGAGCGAGCAGAAAAGCACC-3′<br>5′-CGTCACCAGATGATGGCAAGTC-3′ | 25 | This work |
| FGFR4a | 5′-TCACGAGTAACCTTCTAACGGCAC-3′<br>5′-CTTCCAGTTGGCTTCATCTTCG-3′ | 25 | This work |

been described elsewhere (Richter et al., 1988; Hopwood et al., 1989; Bradley et al., 1993; Lamb et al., 1993; Mayor et al., 1993; Grammer et al., 2000). The *Sox9* probe was a kind gift of R. Spokony and J-P. Saint-Jeannet (Spokony et al., 2002). *Zic5* and *FoxD3* in situ probes were derived from a *X. tropicalis* library made by A. Zorn (Khokha et al., 2003).

### RNA isolation and Reverse Transcriptase-PCR assay

Preparation of total RNA and RT-PCR assay were carried out as described previously (Condie et al., 1990). For each lane of one given experiment, 15-20 animal caps or six to eight recombinants were pooled and analyzed. One non-injected sibling embryo serves as a positive control in the first lane of each PCR gel. The absence of DNA contamination was verified by omitting the reverse transcriptase in an equivalent total embryo sample (lane 2 of the PCR gels). *EF1α* was used as a cDNA loading control. Primers for *EF1α*, *muscle actin*, *Krox20*, *Otx2*, *Xnot*, *MyoD* and *Twist* have been described elsewhere (Rupp and Weintraub, 1991; von Dassow et al., 1993; Ribisi et al., 2000) (*Xenopus* MMR database http://www.xenbase.org/XMMR/Welcome.html). Specific primers used in this study are described in Table 1. Each of them was designed using MacVector 6.5.3 from the sequences published in GenBank so that they do not crossreact with related genes.

## RESULTS

### The DLMZ and the DMZ induce distinct subsets of neural crest markers in the ectoderm

Mesoderm explants were dissected at stage 10 to 10.5 (Fig. 1A) and recombined with a stage 8-9 animal cap (AC) (Fig. 1B). Neural crest formation in the recombinants was first assessed by the induction of *Slug* expression in the recombinants, as reported by Marchant et al. (Marchant et al., 1998) and Bonstein et al. (Bonstein et al., 1998) (Fig. 1C). The DMZ was dissected above the pigment line, indicating the future dorsal lip at stage 10 or along an equivalent width at stage 10.5 (Fig. 1A). When analyzed using RT-PCR at stage 17-18, DMZ explants grown in isolation expressed the notochord marker *Xnot* but very little *muscle actin* (MA) (Fig. 1C, lane 6). After recombination with animal caps, the DMZ-AC did not show *Slug* expression (Fig. 1C, lane 7). By contrast, the paraxial mesoderm (DLMZ) (Fig. 1A) expressed both *muscle actin* and *Xnot* (Fig. 1C, lane 4), and DLMZ-AC recombinants showed a strong *Slug* signal (Fig. 1C, lane 5). Previous studies have shown that the inducing tissue in this system is the DLMZ and that neural crest forms from the animal cap (Bonstein et al., 1998; Marchant et al., 1998). *Xnot* expression in the DLMZ area corresponds to the lateral extension of the notochord domain at stage 10.5 (Yasuo and Lemaire, 2001).

If slightly larger DMZ explants were cut, extending beyond the stage 10.25 dorsal lip, they variably expressed *muscle actin* and *Slug* upon recombination (not shown). Thus, for consistency in the experiments illustrated in this study, we dissected the DMZ as a narrow band of tissue taken at stage 10-10.5, and cut DLMZs that contained robust *Slug* inducing activity.

To characterize the neural crest induced by the DLMZ in this explant assay in more detail, we analyzed several other genes in addition to *Slug*, all expressed mainly by the neural crest around stage 18 (Fig. 1C,D). *Snail* (Essex et al., 1993; Mayor et al., 1993), *Twist* (Hopwood et al., 1989), *Zic5* (Nakata et al., 2000), *Sox9* (Spokony et al., 2002) and *FoxD3* (Pohl and Knochel, 2001; Sasai et al., 2001) were all upregulated when the DLMZ was recombined with animal caps (Fig. 1C, lane 5). *FoxD3* responded in a very similar manner to *Slug*: in

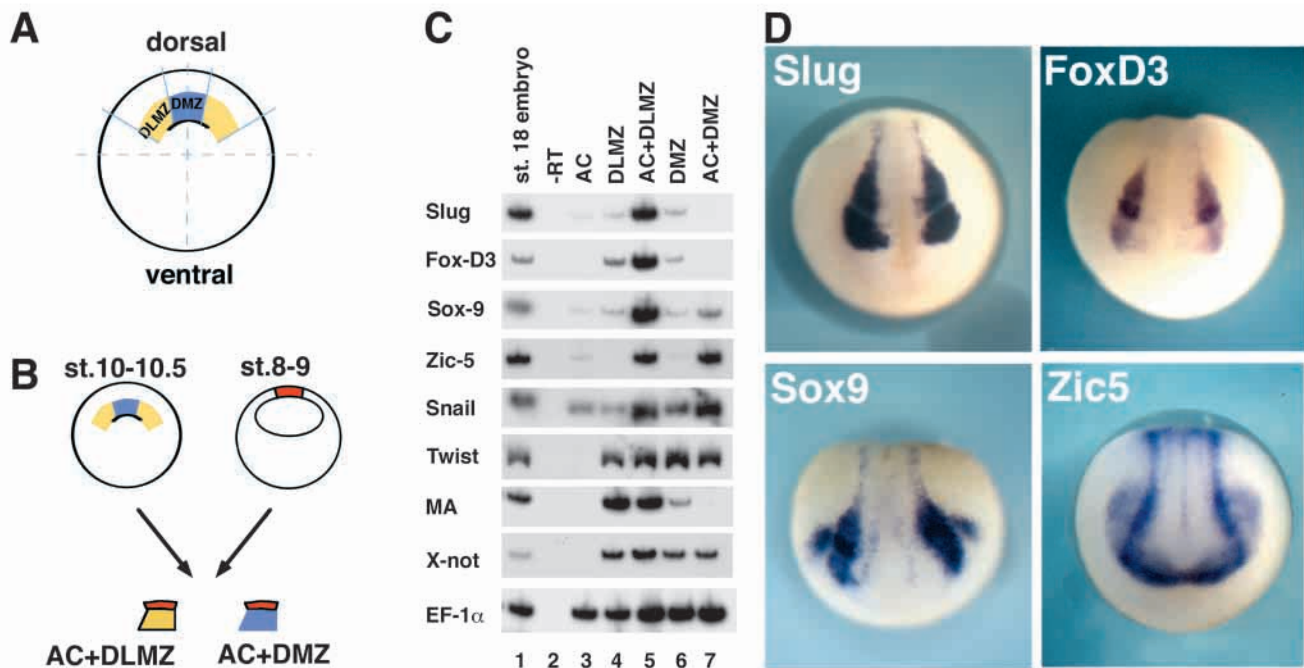


**Fig. 1.** Neural crest marker induction after recombining ectoderm to mesoderm. (A) The DMZ (blue) or the DLMZ (yellow) are dissected at stage 10-10.5 as depicted. (B) Each type of mesoderm explant is recombined to the animal cap ectoderm (AC, red) of stage 8-9 embryos, to form the DLMZ-AC and DMZ-AC recombinants, respectively. (C) RT-PCR analysis of gene expression in the stage 18 recombinants shows that the DLMZ-AC recombinants express a whole range of neural crest markers at stage 18 (*Slug, FoxD3, Sox9, Zic5, Snail* and *Twist*, lane 5), whereas the DMZ-AC recombinants express only a subset of them (lane 7). Lanes 1, 2: controls (see Materials and Methods). Lanes 3, 4, 6: isolated AC, DLMZ and DMZ, respectively. (D) In situ hybridization for the four most specific neural crest markers studied (see text) on normal embryos around stage 18 seen in dorsal view, anterior is towards the bottom. Note that *Slug* and *FoxD3* are restricted to the neural crest, whereas *Zic5* and *Sox9* are also expressed in other areas.

particular, neither was induced in the AC-DMZ recombinants (Fig. 1B, lane 7). Both showed weak expression in the mesoderm, corresponding to what was observed in vivo (Fig. 1C, lanes 4 and 6) (Linker et al., 2000; Sasai et al., 2001). By contrast, *Sox9*, *Zic5* and *Snail* expression were also upregulated in the AC-DMZ, although at a low level in the case of *Sox9*. Interestingly, *Snail* and *Zic5* induction was as strong with the DMZ as with the DLMZ, perhaps reflecting the normal expression of these genes in the midline of the anterior neural fold (Fig. 1D) (Linker et al., 2000; Nakata et al., 2000).

This analysis suggests that neural crest induction observed in this recombination assay reproduces the complexity of in vivo mechanisms. Because of their basal expression in the isolated animal caps and/or mesoderm explants, *Snail* and *Twist* were not analyzed further in this study. We focused on *Slug*, *FoxD3*, *Sox9* and *Zic5*, which were specifically upregulated in the recombinants.

## Blocking Wnt signaling does not prevent induction of neural crest by the DLMZ

The canonical Wnt pathway has been shown to be important in neural crest formation in other systems. In addition, the *Slug* promoter contains LEF-TCF binding sites suggesting a direct regulation by this pathway (Vallin et al., 2001). To test the hypothesis that the DLMZ requires Wnt signals to induce neural crest, we blocked the response of the ectoderm to Wnt signaling using the antagonists NFz8, GSK3 and dnTCF3. The xFz8 receptor has been shown to mediate the activity of Wnt1, Wnt2c, Wnt3a, Wnt5a, Wnt7b, Wnt8 and Wnt11 efficiently (Deardorff et al., 2001). NFz8, a truncated and diffusible form of xFz8, acts on gastrulation movements and neural plate patterning as expected for a Wnt antagonist, but does not prevent dorsal mesoderm specification (Deardorff et al., 1998). In contrast to NFz8, glycogen synthase kinase 3 (GSK3) and dnTCF3 prevent Wnt signaling in a cell autonomous manner (Brantjes et al., 2002; Moon et al., 2002).

In this series of experiments, positive controls of Wnt inhibiting activity showed that 400 pg of *NFz8* mRNA efficiently blocked XWnt8-induced secondary axis formation (100% reversal of double axis formation, after co-injecting 400 pg of *NFz8* and 50 pg p64T-*XWnt8* mRNAs, $n$=31, not shown). Moreover, the injected embryos displayed defects in dorsal neural tube closure, as shown when Wnt signaling is blocked (Wallingford and Harland, 2002). Thus, injections of 400 to 800 pg of NFz8 mRNA per embryo were generally used in the next experiments, although doses above 1 ng were also tested. Moreover, as Wnt antagonists, NFz8 and GSK3 overexpression is expected to anteriorize the neural plate and, later, increase cement gland formation (Deardorff et al., 1998; Kiecker and Niehrs, 2001). After injecting GSK3 or NFz8 (400 to 1600 pg) in the animal hemisphere of two- or four-cell stage embryos, the cement gland was enlarged in more than 96% ($n$>53) of the embryos (Fig. 2A). This phenotype was used as a routine control, when sibling embryos were analyzed for neural crest formation as described below.

*NFz8* or *GSK3* mRNAs were injected into in the animal hemisphere of two- to four-cell stage embryos, sometimes with *NlacZ* mRNA for lineage tracing. Animal caps were cut at

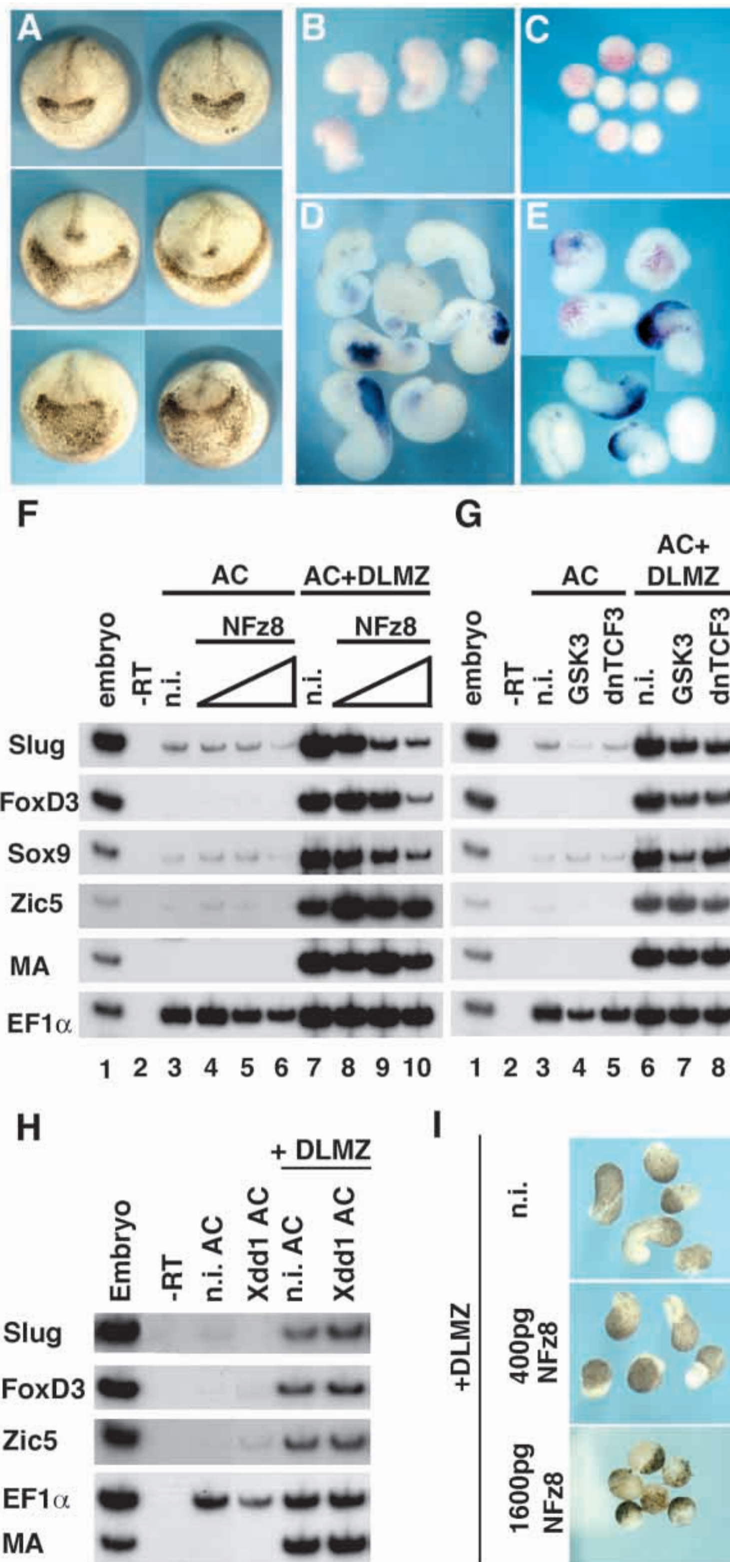


**Fig. 2.** Wnt signaling is not required for neural crest induction by the DLMZ. (A) The activity of different Wnt antagonists is monitored by analyzing cement gland formation in stage 20 injected embryos: Wnt antagonism results in a striking enlargement of the cement gland [top panels, control embryos; middle panels, *NFz8* mRNA treated (800 pg); bottom panel, *GSK3* mRNA treated (800 pg)]. (B-E) *Slug* expression in explants. (B) DLMZ, (C) AC, (D) AC-DLMZ control recombinants, (E) *NFz8* (800 pg) injected AC-DLMZ. The top four recombinants in E are also stained for β-galactosidase activity. Strong *Slug* expression is found in the ectoderm of about half of the recombinants in D and E. (F) RT-PCR analysis after injecting increasing amounts of *NFz8:* lanes 3-6 and 7-10, 0-400-800-1600 pg of *NFz8* mRNA injections in AC and AC-DLMZ, respectively. 400-800 pg injections do not block response to DLMZ signals. Lanes 1 and 2, controls (see Materials and Methods); n.i., non-injected. (G) *GSK3* (800 pg) or *dnTCF3* (1 ng) mRNA injections do not prevent the ectoderm to form neural crest in response to the DLMZ. (H) Xdd1 (1 ng) injections do not prevent neural crest marker induction either. (I) The AC+DLMZ recombinants elongate in the same way as controls even in presence of 400 pg of NFz8 (middle) but their elongation is abolished by injections of 1600 pg of NFz8 in the ectoderm (bottom).

stage 8-9 and recombined with uninjected DLMZ explants. The recombinants were grown up to stage 17-18, fixed and processed for in situ hybridization with a *Slug* antisense probe or for RT-PCR analysis. In some explants, β-galactosidase activity was revealed before the in situ procedure. After an equivalent treatment, isolated DLMZs or animal caps did not express *Slug* (Fig. 2B,C). In control recombinants, the animal cap-derived tissues exhibited *Slug* staining, either as a strong domain of expression or as individual dispersed cells (Fig. 2D). Weaker and more internal *Slug* expression was detected in the DLMZ-derived tissues (Fig. 2D), consistent with *Slug* being expressed in the mesoderm (Mayor et al., 2000).

We first blocked signaling by putative endogenous Wnt molecules using NFz8. After injections of 800 pg of NFz8 mRNA, a similar proportion of the recombinants exhibited *Slug* staining, being virtually identical to controls (Fig. 2E). This observation was confirmed by RT-PCR analysis. After recombination, control explants strongly expressed *Slug* and other neural crest markers ($n$=151, on average, 8-10 recombinants were used for each lane; Fig. 2F, lane 7). Moderate to high doses of NFz8, which are fully active in the biological tests described above, did not prevent the induction of any of the neural crest markers tested (400 pg/$n$=81 and 800 pg/$n$=76, Fig. 2F, lanes 8 and 9). This was also true when the explants were analyzed at stage 12, shortly after initial neural crest induction (not shown). In some cases, however, the induction of *Slug* and *Sox9* was reduced compared with controls (lane 9).

Massive doses of NFz8 resulted in inhibition of *Slug*, *FoxD3* and *Sox9*, but not of *Zic5* (1200-1600 pg/$n$=21, Fig. 2F, lanes 10). This was correlated with a striking lack of elongation of the recombinants, suggesting that these higher doses affect the development of the mesoderm itself rather than the response of the ectoderm (Fig. 2I). Although *Xnot* was expressed normally in the recombinants, *muscle actin* and *MyoD*, which were expressed at the same levels in the 0-800 pg NFz8 injected recombinants, were slightly diminished in the 1600 pg NFz8 injections (Fig. 2F-lane 10 and not shown). This suggests that other aspects of the specification of the DLMZ could also be perturbed by the highest doses of NFz8. Such perturbation could secondarily

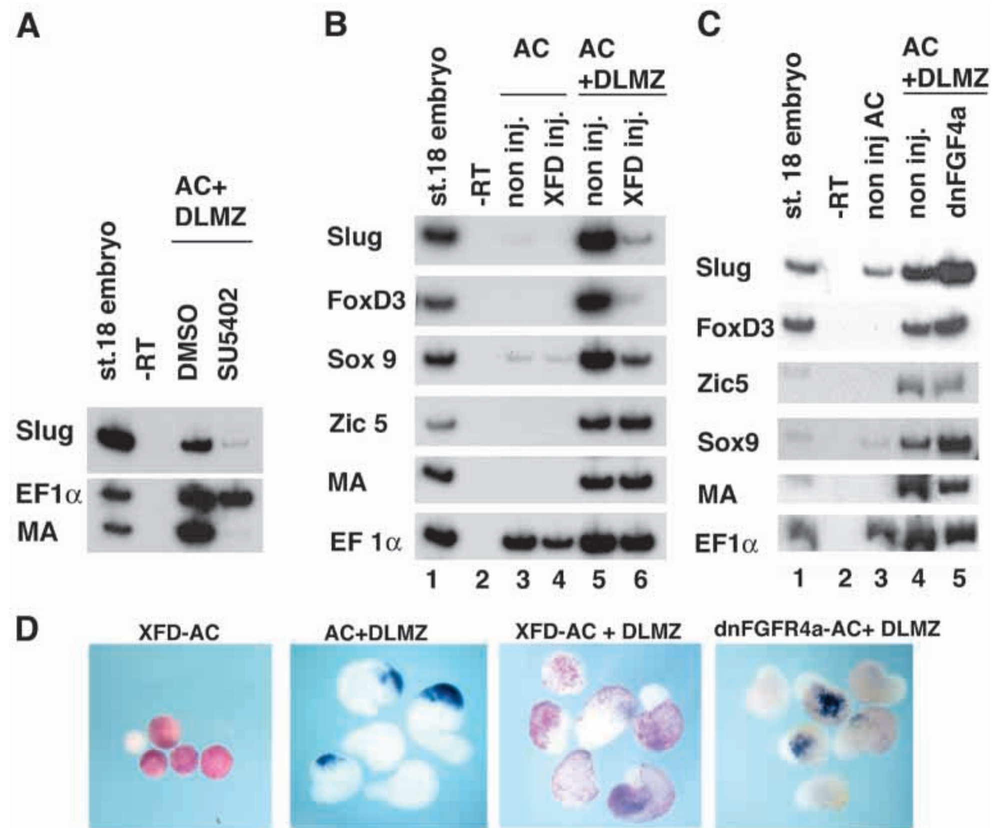


**Fig. 3.** FGF signaling is required for neural crest induction by the DLMZ. (A) RT-PCR analysis after SU5402 treatment of the recombinants shows the lack of *Slug* induction, as well as defective paraxial mesoderm development (*MA*). Lane 3, DMSO treatment; lane 4, SU5402 treatment. (B) *XFD* injections (500 pg) in the ectoderm prevent normal induction of the most specific neural crest markers *Slug* and *FoxD3* by the DLMZ. *Zic5* and *Sox9* are still induced. (C) Similar injections with *dnFGFR4a* (500 pg) do not prevent neural crest marker induction by the DLMZ. (D) Using situ hybridization, the recombinants show a strong downregulation of *Slug* expression after *XFD* injections (third panel) but not after *dnFGFR4a* injections (fourth panel). First panel, XFD-injected animal caps; second panel, control recombinants.

alter the DLMZ signaling activity and account for the reduction of neural crest induction seen in lane 10.

We thus focused on 400-800 pg NFz8 doses (lanes 8 and 9): the decrease in *Slug* and *Sox9* neural crest markers expression, in lanes 9, could either reflect the requirement for a Wnt signal acting directly on the ectodermal cells or a change in the DLMZ-inducing properties. To avoid Wnt-dependent changes in the signaling properties of the DLMZ, we blocked the response to the canonical and non canonical Wnt pathways intracellularly in the ectoderm, by injecting either GSK3 (300-400 pg/*n*=38 and 800-1000 pg/*n*=40), dnTCF3 (1 ng/*n*=10) or Xdd1 (1 ng/ *n*=10) (Fig. 2G-H and not shown). None of these blocked the induction of neural crest markers by the DLMZ (Fig. 2G, lanes 6-8 and Fig. 2H). However, the injection of NFz8 or GSK3 did modulate the expression of other genes, such as *Krox20* or *Otx2*, but not *Pax3* (not shown). We conclude that neither canonical nor PCP Wnt-dependent pathways are required directly for the ectoderm to respond to the DLMZ neural crest-inducing activity. Blocking Wnt signals by diffusible antagonists perturbs DLMZ development and most probably its signaling properties. However, if Wnt signaling is not perturbed in the mesoderm, the DLMZ can induce neural crest in the ectoderm, suggesting alternative or redundant pathways for neural crest induction.

## FGF signaling is required for neural crest induction by the DLMZ

FGFs bind to one of four tyrosine-kinase receptors, FGFR1-FGFR4, which lead to activation of MAP kinase or phosphatidyl inositol pathways, eventually modulating target gene expression (for a review, see Galzie et al., 1997). Blocking signaling by FGFRs, in vivo or in vitro, has employed either a truncated dominant-negative form of FGFR1, XFD (Amaya et al., 1993) or a synthetic inhibitor (SU5402) that binds to the kinase domain of FGFRs (Mohammadi et al., 1997).

In the first approach, we blocked FGF signaling in the explants by growing them in presence of 50 μM SU5402. Two DLMZs were dissected out of each stage 10 embryo and used to make two recombinants, one was cultivated in the SU5402 solution, the other in the control DMSO medium. RT-PCR analysis (Fig. 3A) showed that the SU5402 treatment completely suppressed *Slug* induction (Fig. 3A-lane 4, *n*=19). However, it also prevented normal development of the paraxial mesoderm from the DLMZ as shown by the lack of *muscle actin* expression. Under these conditions, the lack of *Slug* induction could be a secondary effect caused by abnormal DLMZ development.

To avoid perturbing FGF signaling in the DLMZ part of the recombinant, we injected XFD into the embryos used for animal cap explants (500 pg). The XFD-injected caps did not express *Slug* (Fig. 3D, XFD-AC) or the other neural crest markers (Fig. 3B, lane 3). When they were recombined with wild-type DLMZs, in situ analysis showed that most of *Slug* expression was lost (Fig. 3D, compare AC+DLMZ with XFD-AC+DLMZ). Both *Slug* and *FoxD3* induction were lost after XFD injections when analyzed by RT-PCR, whereas *Sox9* expression was only slightly diminished and *Zic5* expression was essentially unchanged (Fig. 3B, lanes 5 and 6, *n*=18). The loss of both *Slug* and *FoxD3*, the most specific neural crest markers, indicates that the DLMZ does not induce proper

neural crest if the ectodermal part of the explant is unable to respond to FGF signals. In addition, this experiment suggests that different mechanisms control *Sox9* and *Zic5* induction or maintenance. Another dominant-negative FGFR construct, *dnFGFR4a*, was tested to address potential specific roles of the distinct FGFRs. This mRNA perturbed several aspects of in vivo development (see below) but interestingly, *dnFGFR4a* injections resulted in normal expression of all the neural crest markers tested, both by in situ and RT-PCR analysis (Fig. 3C,D; *n*=36). Thus, different FGFRs are not equivalent and induction of crest by DLMZ may involve FGFR1 rather than FGFR4a activity.

### FGFs and FGFRs are expressed in the recombinants

We analyzed the expression of *FGF3*, *FGF4* (*eFGF*) and *FGF8* in explants during the period of neural crest induction, i.e. stages 10.25-14, using semi-quantitative RT-PCR (Aybar and Mayor, 2002). *FGF3*, *FGF4* and *FGF8* were detected in the isolated DLMZ but not in the isolated animal caps at all stages analyzed (Fig. 4A,C). In the DLMZ, the expression of FGF genes preceded that of myotome markers such as *MyoD* and *muscle actin*, which appeared around stage 12 (Fig. 4A, lanes 5 and 6), similar to *Slug* in the ectoderm (Linker et al., 2000). Thus, FGF genes and *FGF8* in particular are expressed in the DLMZ during gastrulation and early neurulation, and this expression is maintained without the need for external signals.

The expression of the different FGF receptors has been described in animal caps grown in isolation (Friesel and Dawid, 1991; Golub et al., 2000). Interestingly, this study showed that *FGFR1* and *FGFR4a* genes are expressed when animal caps are dissected but their expression is maintained only in the presence of ongoing FGF signaling (Friesel and Dawid, 1991). We therefore analyzed *FGFR1-FGFR4* expression in the different kinds of explants used in this study. *FGFR2* expression was maintained in the isolated animal caps, consistent with previous results (Fig. 4B, lane 3) (Friesel and Brown, 1992). This expression was also present in all the recombinants containing ectoderm or DMZ (Fig. 4B, lanes 5-7). FGFR3 was more specifically found in the DMZ-containing explants (Fig. 4B, lanes 6 and 7) but was expressed at much lower levels in DLMZ or AC-DLMZ recombinants (Fig. 4B, lanes 3-4). Most interestingly, *FGFR1* and *FGFR4a* expression was hardly detected in the isolated AC or DLMZ (Fig. 4B, lanes 3 and 4) but was present if both tissues were recombined (Fig. 4B, lane 5). This suggests that when the ectoderm and the DLMZ are in contact, interactions between the two parts of the recombinant sustain *FGFR1* and *FGFR4a* expression. To test whether this was due to an active FGF signaling in the recombinants, we cultivated them either in DMSO or in SU5402 as described above. Although this prevented *Slug* and *muscle actin* expression (Fig. 3), both FGFR1 and FGFR4a were normally expressed in these explants (Fig. 4D). We conclude that, non-FGF signals act in the AC-DLMZ recombinants to maintain FGFR expression when FGF signaling is blocked.

### FGF8 enhances neural crest formation in embryos and is sufficient to induce neural crest markers in explants

As FGF8 has recently been shown to be involved in neurogenesis without inducing mesoderm (Hardcastle et al., 2000), we decided to focus on this member of the family and analyze its potential activity in neural crest formation. We examined *FGF8* gene expression at gastrula and early neurula stages. *FGF8* appears initially as a ring around the blastopore and is reinforced dorsally by stage 11-11.5, when neural crest induction is thought to begin (Fig. 5E) (Christen and Slack, 1997). *FGF8* expression level is then enhanced in the dorsolateral mesoderm at stage 13 and onwards, whereas it is downregulated in the dorsal midline (Fig. 5E). FGF8 is thus a good candidate to mediate the FGF-dependent DLMZ activity on neural crest induction. To test this hypothesis in whole embryos, we analyzed *Slug* expression after *FGF8* mRNA injections. Compared with control sibling embryos (Fig. 5A), 50 pg of *FGF8* mRNA injections were followed by a strong increase in *Slug* expression (Fig. 5B, yellow arrows indicate the injected side). This upregulation was not correlated to an expansion of the *MyoD* domain (Fig. 5C,D, small red arrow). Interestingly, when the injected cells (*lacZ* staining) were located in the anterior part of the neural plate, this region expressed *Slug*, suggesting that these injections transformed the anterior neural fold into a more posterior structure (Fig. 5B, red arrow) (Christen and Slack, 1997). However, in the

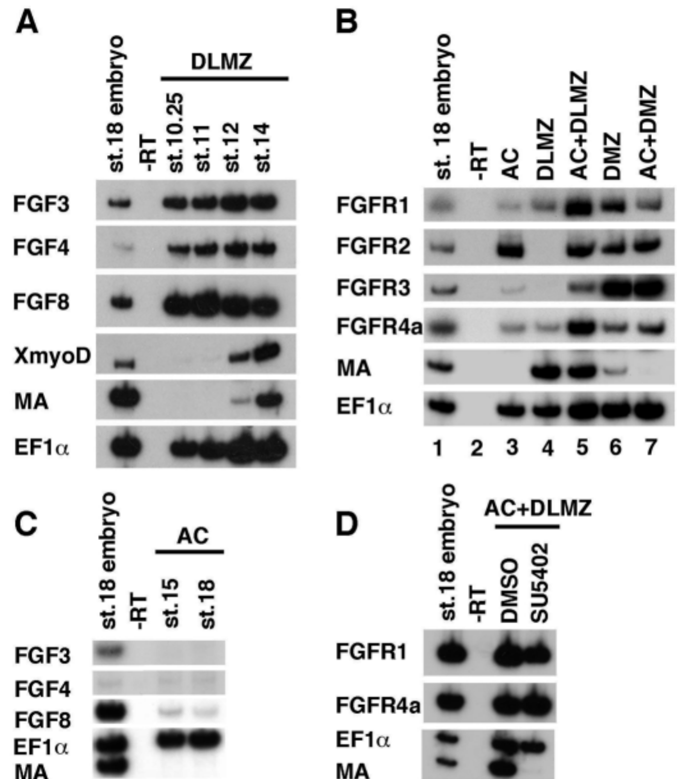


**Fig. 4.** FGF signaling in the recombinants. (A) *FGF3*, *FGF4* and *FGF8* are expressed in the DLMZ cultivated in isolation, from stage 10.25 to stage 14 (lanes 3-6; DLMZ dissected at stage 10.25 and cultivated up to the stage indicated). (B) FGFRs are differentially expressed in the DLMZ-AC (lane 5) and DMZ-AC (lane 7) recombinants. In particular, *FGFR1* and *FGFR4a* expression is maintained at stage 18 in AC-DMLZ recombinants (lane 5). (C) Isolated animal caps do not express FGF3, FGF4 or FGF8. (D) SU5402 treatment does not suppress FGFR1 and FGFR4a expression in the recombinants.

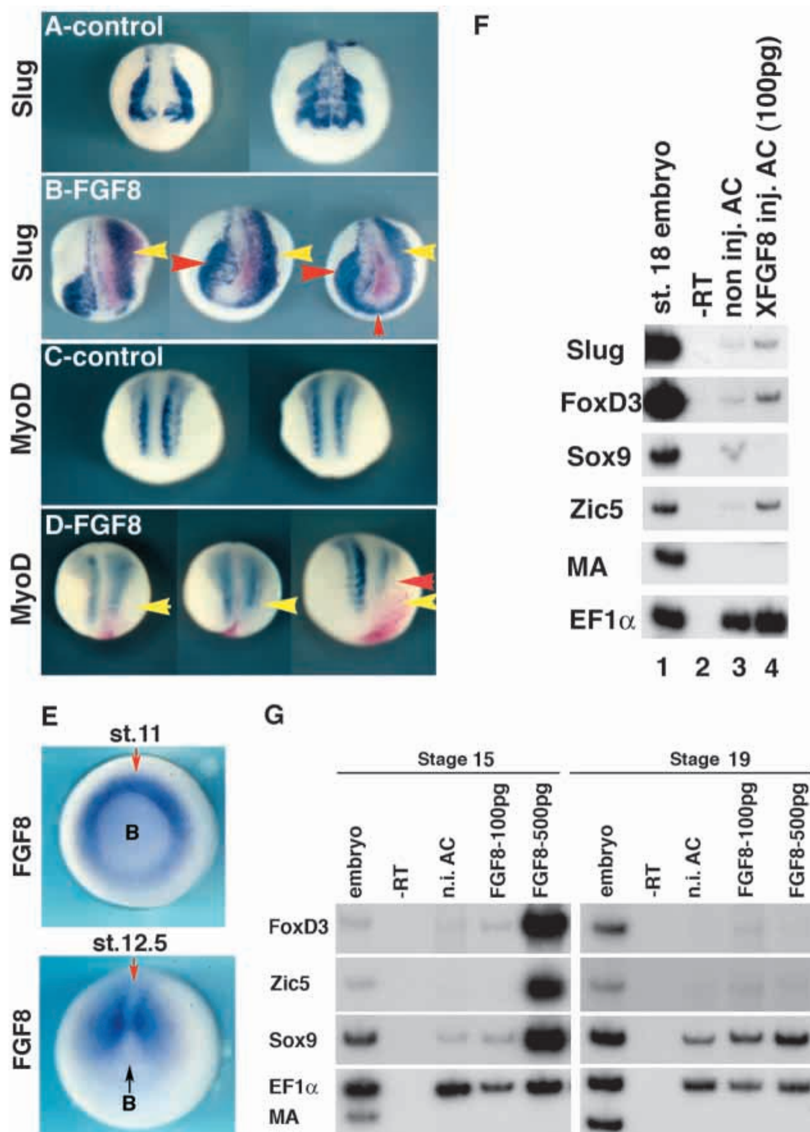


**Fig. 5.** FGF8 induces neural crest in vivo and in vitro. (A-D) In vivo injections of *FGF8* mRNA in one of two-cell stage embryos, analyzed by in situ hybridization for *Slug* (A,B) or *MyoD* (C,D) at stage 18-20. (A) Control embryos. (B) *FGF8* mRNA unilateral injections result in a strong overexpression of *Slug* on the injected side (yellow arrows) and sometimes in the contralateral side and the anterior neural fold (red arrowheads). (C) Control embryos. (D) *FGF8* mRNA injections (injected side indicated by yellow arrowheads) do not expand paraxial mesoderm, they even reduce it in some embryos (embryo on the right) (red arrowhead). (E) FGF8 mRNA is expressed as a ring around the blastopore at stage 11 (top), reinforced dorsally (red arrows). Later on, FGF8 is expressed in the DLMZ and downregulated in the midline (bottom, red arrow). (F) *FGF8* mRNA injections induce neural crest markers in animal caps. RT-PCR analysis shows the induction of *FoxD3* and *Zic5* by 100 pg of *FGF8* mRNA, but not of paraxial mesoderm formation. (G) When the caps are analyzed earlier (stage 15), increased doses of FGF8 induce strongly *FoxD3, Sox9* and *Zic5*. By stage 19, *FoxD3* and *Zic5* expression was not maintained.

embryo, co-factors from the surrounding tissues, such as the mesoderm or the ectoderm, could also be recruited for FGF8 activity on the neural crest.

To test FGF8 activity in a more defined assay, we injected animal caps with 100 pg of *FGF8* mRNA at the two- to four-cell stage into the animal pole, cut animal caps at stage 9 and grew them in isolation up to stage 17-18. This did not result in the induction of *muscle actin* expression, confirming that these injections do not induce paraxial mesoderm (Fig. 5D, lanes 3-4). Nonetheless, the neural crest markers *Zic5* and *FoxD3* were clearly induced by FGF8 injections. *Slug* expression was only slightly upregulated in the *FGF8*-injected animal caps, at much lower levels than in the recombination assay, and *Sox9* was not consistently present (Fig. 5F). This first result indicates that FGF8 alone is sufficient to induce the expression of some genes characteristic of the neural crest, although not as efficiently as the DLMZ. To understand FGF8 activity better, we then increased the injected doses and saw that 500 pg of FGF8 consistently induced *Sox9* expression when observed at stage 17-18 (data not shown). In addition, we found that, when the animal caps were analyzed at an earlier stage (stage 15), a very strong induction of *FoxD3*, *Sox9* and *Zic5* was obtained (Fig. 2G). However, when sibling animal caps from the same injection series were fixed a few hours later, at stage 19, the expression of all markers, except for *Sox9*, had vanished (Fig. 2G). This demonstrates that FGF8 is able to elicit a strong but transient induction of *FoxD3*, *Sox9* and *Zic5*. This is obtained in the absence of mesoderm induction and without need for additional downregulation of BMP signaling. By contrast, we did not obtain *Slug* induction above the background levels shown in Fig. 2F, and thus we conclude that FGF8 does not significantly induce this gene under our experimental conditions.

In conclusion, these data suggest that FGF8 alone is sufficient to mediate both the DLMZ-specific induction of *FoxD3* and the common DMZ/DLMZ induction of *Zic5* and *Sox9*. Second, because, in vivo, FGF8 injections show a potent *Slug* upregulation, we conclude that this aspect of FGF8 activity requires interactions with other DLMZ-specific factors. Moreover, in the AC-DLMZ or AC-DMZ recombinants, the expression of neural crest markers is induced and maintained, indicating that other molecules must reinforce and sustain FGF8 inductive activity.

### In vivo inhibition of Wnt or FGF signaling result in anteriorization of the neural plate prior to neural crest induction and affects paraxial mesoderm development

Previous studies have shown that both Wnt and FGF signals are required for normal expression of *Slug* in the *Xenopus* embryo (Mayor et al., 1997; LaBonne and Bronner-Fraser, 1998; Villanueva et al., 2002). However, these signaling molecules are also required for multiple steps of early development, such as mesoderm formation or neural plate AP patterning (Ribisi et al., 2000; Kiecker and Niehrs, 2001). We repeated the analysis of *Slug* expression under similar

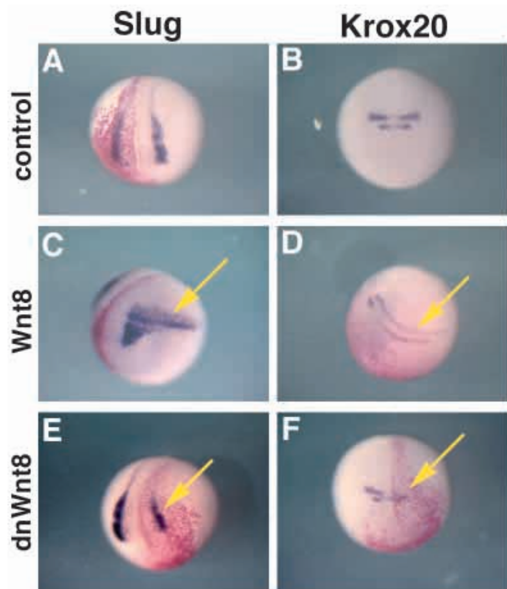


**Fig. 6.** In vivo analysis of neural crest and neural plate patterning after modification of Wnt signaling. (A,B) Control embryos stained for *Slug* and *Krox20* at stage 19. (C,D) *Wnt8* mRNA injections (50 pg) are followed by the extension of *Slug* and *Krox 20* domains together, in the posterior parts of the embryos (arrows). (E,F) *dnWnt8* mRNA injections (50 pg) result in reduction of both *Slug* and *Krox 20* expression (arrows).

experimental conditions (Fig. 6A) and also tested expression of the rhombencephalon marker *Krox 20* in parallel (Fig. 6B) (Bradley et al., 1993). Moreover, we analyzed mesoderm development in these assays, by staining the embryos simultaneously for *Slug* mRNA and with the monoclonal antibody 12-101, which stains differentiated muscle (Kintner and Brockes, 1984).

After overexpression of *Wnt8*, as previously shown (LaBonne and Bronner-Fraser, 1998), the *Slug* expressing domain was expanded posteriorly (Fig. 6A,C). However, *Krox20* was also expanded in a similar manner, showing that the whole rhombencephalon area was enlarged (Fig. 6B,D) and that the neural crest increase observed could be a consequence of modifications in neural patterning. Conversely, blocking Wnt signals with a dominant- negative form of *XWnt8* resulted in reduction of both *Slug* and *Krox20* (Fig. 6E,F), suggesting again that neural crest modifications observed previously could be interpreted in terms of general neural patterning (Villanueva et al., 2002).

To avoid the diffusible effects of NFz8, we also inhibited Wnt signaling cell-autonomously using GSK3 injected either in one half of the embryo or into the prospective neural fold at the 16-cell stage. Control injections did not alter *Slug* expression (Fig. 7A,D) or paraxial mesoderm formation (Fig. 7A). However, in both types of GSK3 injections, the decrease or a lack in *Slug* expression was correlated with altered paraxial mesoderm and neural patterning (Fig. 7B-F). Thus, in these in vivo assays, we have not been able to dissociate the effects of Wnt signaling on neural crest formation from those on neural plate and mesoderm patterning.

### Neural plate patterning and neural crest induction can be uncoupled in vivo

To understand better how Wnt or FGF signaling might affect neural crest formation by changing early neural patterning, we analyzed NFz8 and dnFGFR4a effects on AP neural pattern. We compared the AP neural pattern at stage 11.5 to *Slug* expression in sibling embryos fixed around stage 18. Blocking of Wnt signaling by *NFz8* mRNA injections, or of FGF signaling by *XFD* injections in vivo, resulted in a reduction of *Slug* expression together with perturbed gastrulation and neural plate formation when observed at stage 18 (Fig. 8A-C). However, by marked contrast, *dnFGFR4a* injections resulted in dramatic gastrulation defects without obvious downregulation of *Slug* expression levels (Fig. 8D). The *Slug* expressing domain was shifted around the blastopore in the most affected embryos, but we did not observe as strong a decrease in *Slug* staining as after NFz8 or XFD treatment. To test if this observation was correlated with distinct activities of these molecules on neural AP patterning, we fixed the injected embryos at stage 11.5, i.e. before neural crest induction is established, and when *Slug* expression is hardly detected (not shown). At this stage, neural AP pattern is already well established and the *Otx2* expression domain is restricted to the anterior part of the neural plate (Fig. 8E) (Kiecker and Niehrs, 2001). This domain corresponds to the presumptive forebrain and midbrain and does not give rise to neural crest. After NFz8 dorsal injections at the two-cell

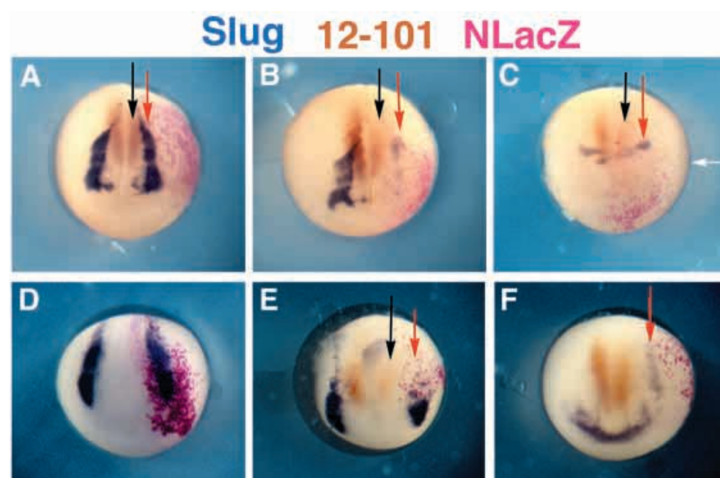


**Fig. 7.** In vivo GSK3 injections perturb neural crest, neural plate and mesoderm development. (A-C) Injections in one of two-cell stage embryos of *lacZ* (A) or *GSK3* (300 pg) plus *lacZ* mRNA (B,C). The *GSK3* injected side (white arrow, pink *lacZ* staining) displays greatly reduced *Slug* expression (B, red arrow, blue staining), abnormal *Krox20* expression (C, red arrow, blue staining) and reduction of the paraxial mesoderm marker 12-101 staining (B,C; black arrows, brown staining). Red and black arrows in A indicate normal staining for *Slug* (blue) and 12-101 (brown), respectively. (D-F) *GSK3* (150 pg) was injected into one dorsoanimal blastomere at the 16-cell stage to target one neural fold and reduce the effect on adjacent tissues. (D) Control β-galactosidase staining. (E) The injected area shows reduction in both *Slug* (red arrow) and 12-101 staining (black arrow). (F) Ectopic expression of the anterior neural plate marker *Cpl-1* is induced ectopically (red arrow), showing that neural patterning is also affected.

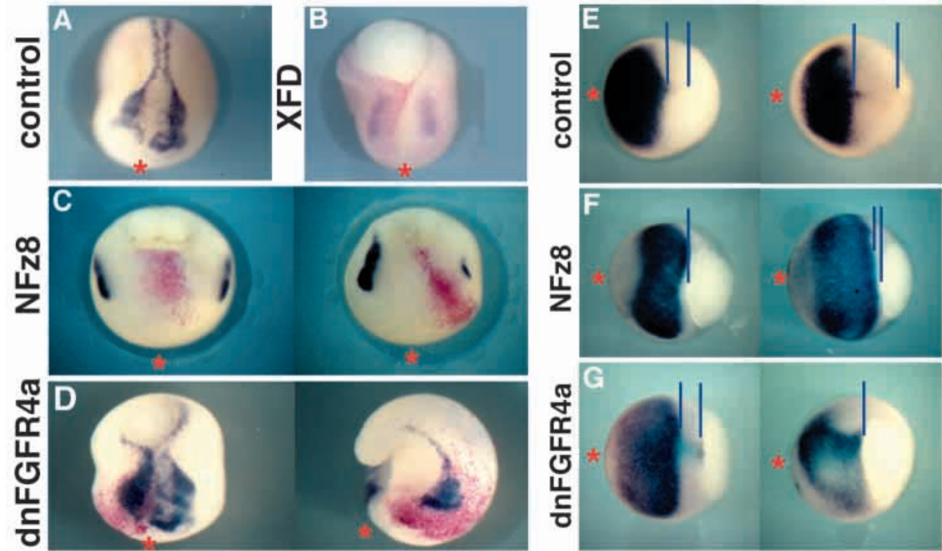


**Fig. 8.** Neural crest formation can be experimentally uncoupled from neural plate patterning. (A-D) Injected embryos were analyzed around stage 18 and stained for *Slug* expression. (A) Control embryo. (B) *XFD* injections result in gastrulation defects and loss of most *Slug* staining. (C) *NFz8* injections most often produce an abnormally shaped neural plate, gastrulation defects and reduced *Slug* expression. (D) *dnFGFR4a*-injected embryos show severe gastrulation defects but still present a strong *Slug* staining (the right hand embryo is shown in side view). (E,F) Similar injections were analyzed at stage 11.5-12 for *Otx2* expression. *Otx2* labels the area anterior to the neural crest-forming regions. It is found further from the blastopore as development proceeds (blue bars measure the distance between the posterior part of the *Otx2* domain and the blastopore; anterior is indicated by the red star). (E) Stage 11.5 (left) and stage 12 (right) control embryos. (F) *NFz8*-injected embryos show a strongly reduced posterior neural crest-forming domain. (G) *dnFGFR4a* injections result either in normal sized posterior domain (left) or strongly reduced ones (right). Both types of embryos will show a strong *Slug* expression at stage 18 (D). Red stars indicate anterior.

stage, we observed a clear anteriorization of the neural plate, assessed by the posterior border of the *Otx2* domain (Fig. 8F). The area between the blastopore and the posterior limit of the *Otx2*-positive area was reduced or absent (blue bars). This corresponds to a strong reduction of the neural crest-forming structures, the rhombencephalon and the spinal cord. Similarly, *dnFGFR4a* injections resulted either in a moderate reduction of the posterior structures (Fig. 8G, left embryo) or their complete absence (Fig. 8G, right embryo). When similar *dnFGFR4a*-injected embryos fixed at stage 18, the *Slug*-positive domain formed around the blastopore corresponded an area where *Krox20* was expressed. However, Krox20 was strongly reduced and abnormal (not shown). This result indicates that *dnFGFR4a* injections do perturb neural AP patterning deeply without resulting in a deficiency in *Slug* induction and maintenance. This implies that, in vivo, although we could not separate the roles of dnXWnt8, NFz8 or XFD injections on neural and neural crest patterning, these two phenomena can be uncoupled if we injected dnFGFR4a. We conclude that neural crest formation is not a strict consequence of proper neural plate patterning, although it is closely related to it, as signaling via FGFR4a is required for neural patterning but not for neural crest formation.

## DISCUSSION

In this study, we show that the paraxial mesoderm of *Xenopus* embryos induces neural crest by an FGF-dependent mechanism and that FGF8 is sufficient to induce neural crest markers in the naive ectoderm without requiring additional BMP antagonists. Moreover FGF8 can cooperate with additional factors to modulate *Slug* expression in vivo. Wnt signaling, via the canonical or the planar cell polarity pathways, is not necessary for the response of the ectoderm to the paraxial mesoderm. In examining the relationships between neural plate patterning and neural crest formation in vivo, we show that blocking Wnt signaling affects both AP neural patterning and neural crest formation; by contrast, a truncated FGFR4a acts on neural pattern but does not block neural crest induction and maintenance.

### The paraxial (but not the axial) mesoderm, induces a whole range of neural crest-specific genes in the ectoderm

Elegant experiments using albino *Xenopus* embryos have shown that the ectoderm can form neural crest in response to DLMZ signals and that the DMZ was a less efficient *Slug* inducer than the DLMZ (Bonstein et al., 1998; Marchant et al., 1998). We show here that the induction of *Slug* by mesoderm explants is closely correlated to the presence of *muscle actin* in the inductive tissue, i.e. to the presence of some paraxial tissue (Fig. 1). When DMZs are cut medially, they consistently fail to induce *Slug*. This suggests that the quantitatively lower activity of the DMZ reported previously might reflect some variability in the width of the explants. We also show that the DLMZ is able to induce a whole range of neural crest markers: *Slug*, *FoxD3*, *Sox9* and *Zic5* (Fig. 1C, lane 5). By contrast, the DMZ does not induce *Slug* or *FoxD3* but upregulates *Zic5* and *Sox9* expression at various levels (Fig. 1C, lane 7). The distinct inducing activities of the DLMZ and of the DMZ might be due either to a dorsal-to-lateral increasing gradient of neural crest inducing activity or to a different combination of inducing molecules produced by each kind of tissue. According to the first hypothesis, *Zic5* and *Sox9* genes would be upregulated by low levels of this inducer, whereas *Slug* and *FoxD3* activation would require a higher concentration. According to the second hypothesis, the DMZ would express a molecule able to induce *Zic5* and *Sox9* whereas the DLMZ would express an additional signal(s) required for inducing either the complete range of neural crest markers or *Slug* and *FoxD3* specifically. We therefore consider *Slug* and *FoxD3* to be most characteristic of

neural crest induction because they are specifically induced by the DLMZ and because their in vivo expression pattern is mostly restricted to the neural crest (Fig. 1D).

### Neural crest induction by the paraxial mesoderm requires functional FGF-FGFR1 signaling

Previous studies have shown that neural crest formation can be induced by a combination of BMP antagonists plus Wnt/FGF signals in *Xenopus* animal cap assays (Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998). Moreover, the same classes of molecules regulate the expression of *Slug* and *FoxD3*. *FoxD3* is induced by a combination of either chordin+bFGF or chordin+Wnt3a (Sasai et al., 2001). Similarly, *Slug* is upregulated in animal caps by combining chordin with either eFGF or XWnt8 (LaBonne and Bronner-Fraser, 1998). The regulation of *Zic5* and *Sox9* genes has not yet been studied, although these genes are required for neural crest development in vivo (Nakata et al., 2000; Spokony et al., 2002). Both Wnt and FGF signals are expressed in the paraxial mesoderm. They might play a role in mesoderm development itself, as well as mediating mesodermal signaling activities toward the ectoderm. These activities could be redundant and do not exclude the possibility that alternative mechanisms may also be active.

To analyze the mechanisms of action of the DLMZ, we blocked the response of the ectoderm to either endogenous Wnt or FGF signals, in the DLMZ-AC recombination assay (Fig. 2). Many previous studies have used secreted antagonists such as dnWnt8 to block Wnt signals in embryos or in explants: this results in downregulation of *Slug* in *Xenopus* (LaBonne and Bronner-Fraser, 1998) and blocks *Pax3* induction by the chick paraxial mesoderm (Bang et al., 1999). After NFz8 injections in the ectoderm of the recombinants, we only saw a moderate downregulation of *Slug*, *Sox9* and *Pax3* at high doses (Fig. 2; data not shown). By blocking the intracellular downstream canonical and PCP Wnt pathways, we show that none of the four neural crest markers analyzed depend directly on Wnt signaling to be induced by the DLMZ. Therefore, the effects of diffusible antagonists observed in these recombination assays might reflect a Wnt-dependent modulation or maintenance of the paraxial mesoderm-inducing activity, or indicate that the Wnt pathway may have an overlapping activity.

By contrast, blocking FGF-FGFR1 signaling, by injecting XFD in the ectoderm, strongly reduced the induction of *Slug*, *FoxD3* and to a lesser extent *Sox9* (Fig. 3). The induction of *Zic5* was unaffected by the XFD injections. The most affected genes corresponded to those specifically induced by the DLMZ but not by the DMZ. This suggests that the DLMZ-specific aspect of neural crest inducing activity requires functional FGF signaling, probably through FGFR1. FGF signaling is also required in vivo for normal neural crest formation as XFD injections strongly downregulate *Slug* expression (Mayor, 1997) (this work). Interestingly, we found that dnFGFR4a did not affect neural crest induction. In contrast to this observation, FGFR4a plays a prominent role in neurogenesis (Hardcastle et al., 2000). This raises the attractive possibility that different FGFRs might display different roles in neuronal versus neural crest development.

### FGF8 induces neural crest

We show that the DLMZ expresses *FGF3*, *FGF4* and *FGF8* at gastrula and early neurula stages (Fig. 4). We further show that FGF8 can account for the neural crest induction by the DLMZ, either alone or in cooperation with other DLMZ factors (Fig. 5). First, in vivo *FGF8* expression is detected at early gastrula stages as a ring around the blastopore, it is then reinforced in the DLMZ area. Second, *FGF8* mRNA injections in vivo are followed by a large increase in *Slug* expression without expansion of the paraxial mesoderm. Finally, *FGF8* injections in the animal cap assay induce the expression of neural crest markers without inducing mesoderm formation. This contrasts with previous studies using FGF4 (eFGF) or bFGF in similar assays, which showed (1) mesoderm induction, (2) absence of neural crest induction by FGF4 or bFGF alone and (3) requirement for co-expression with a BMP antagonist (LaBonne and Bronner-Fraser, 1998; Mizuseki et al., 1998). The unique properties of FGF8 on neural crest can be compared with its ability to induce neurogenesis without mesoderm induction, when it is expressed from blastula stages (Hardcastle et al., 2000). Other FGF molecules can also be direct neural inducers, but only if they are expressed after the period of competence to form mesoderm, and in tissue that has attenuated BMP signaling (Lamb and Harland, 1995). FGF8 is thus a good candidate for mediating FGF neural-specific roles during the period of early neural crest development defined by Aybar and Mayor (Aybar and Mayor, 2002).

### Is FGF8 a neural crest inducer?

To be considered a physiologically significant activity, a neural crest inducer must satisfy the following properties. First, it should be expressed by tissue(s) with a neural crest inducing potential, in early neurula stage embryos. *FGF8*, which is expressed in the paraxial mesoderm as early as stage 10, satisfies this first condition (Fig. 5E). By contrast, *Wnt1* expression is detected by stage 14, i.e. after *Slug* induction (Deardorff et al., 2001). Thus, Wnt1 and Wnt 3a, which act via Xfrizzled-3 and Kermit, are more likely to play later roles in neural crest development, such as maintenance of the induction or fate choice (Dorsky et al., 1998; Basch et al., 2000; Dorsky et al., 2000; Deardorff et al., 2001; Jin et al., 2001; Tan et al., 2001). Thus, in amphibians, the activity of an ectoderm-restricted Wnt, equivalent to the chick *Wnt6* gene, remains to be found (Garcia-Castro et al., 2002). The activity of β-catenin on early neural crest formation (LaBonne and Bronner-Fraser, 1998) could rather reflect a role of Wnt7b and Wnt 8, which are present in the early ectoderm/neurectoderm and paraxial mesoderm, respectively (Bang et al., 1999; Wu et al., 2003).

Second, the activity of the inducer should be necessary to obtain neural crest formation, although this can be missed if redundant pathways are activated in the same assay. We show here that FGF signaling is required to mediate paraxial mesoderm induction of *Slug* and *FoxD3* (Fig. 3). Active FGFR1 signaling is also necessary in vivo (Mayor et al., 1997). Further analysis by a selective knockdown of FGF8 will determine if FGF8 is specifically required in the DLMZ for neural crest induction or if other FGFs have overlapping activity.

In addition to these two properties, the neural crest-inducing activity could be mediated either by a single factor or a combination of molecules. Tested separately, these molecules might be able to evoke neural crest formation even if the robust induction of neural crest markers and further development of

neural crest cells might require additional inputs. In *Xenopus* animal cap assay, FGF8 induces *FoxD3*, *Sox9* and *Zic5* (but *Slug* is only very slightly upregulated) (Fig. 5F,G). Moreover, the induction by FGF8 in this assay is transient, showing the requirement for other factors to maintain and complete the induction of the full range of neural crest markers. It has been shown by similar experiments that, although they do not induce neural crest markers by themselves, Wnts, eFGF and bFGF synergize with noggin or chordin to induce neural crest (Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Mizuseki et al., 1998). The cooperation of FGF8 with other molecules such as BMP antagonists or Wnts in the maintenance of neural crest induction remains to be explored.

FGF8 activity must be modulated to become a potent crest-inducing activity, as FGF8 is expressed in both dorsal and dorsolateral marginal zones (Fig. 5), and these have qualitatively different neural crest-inducing activity (Fig. 1). FGF8 might account for DMZ-DLMZ common induction of *Zic5* and *Sox9*. However, in the recombinant assay, neither XFD nor dn FGFR4a injections prevented *Zic5* or *Sox9* induction, supporting the idea that redundant inducing mechanisms are provided by the DLMZ (Fig. 3). In addition, *Zic5* and *Sox9* are not restricted to the neural crest, but also expressed in the anterior neural fold and the prospective otic placode, respectively. They are thus expected to respond to neural crest specific inducers as well as to other signals (Fig. 1). Our study also revealed distinct regulation for *Slug* and *FoxD3*. Both genes were considered specifically induced by the DLMZ (Fig. 1) and this induction requires FGF signaling (Fig. 3). However, FGF8 is sufficient to induce expression of *FoxD3* but not of *Slug*. Cooperation of FGF8 with additional signals could account for the expansion of the *Slug* domain observed in the embryo (Fig. 5). Alternatively, our in vitro conditions might not induce the right relative levels of *FoxD3/Sox9/Zic5*: each of these factors is necessary for normal neural crest development and/or *Slug* expression. In particular, overexpression of FoxD3 can either increase or prevent *Slug* activation, suggesting that a fine balance is controlled in the embryo (Pohl and Knochel, 2001; Sasai et al., 2001). Finally, we cannot rule out the possibility that the neural crest induction we observed in the isolated ectoderm occurred secondarily to FGF8-induced neural tissue (Hardcastle et al., 2000), secondary to the formation of a border between the ectoderm and induced neural tissue. Further experiments will test if FGF8 is a direct neural crest inducer or if it switches on a developmental program eventually resulting into neural crest induction. However, by its neural crest inducing activity in the animal cap assay, FGF8 stands as an excellent candidate inducer when compared with previously proposed ones such as WNT8 or WNT7b, which do not act alone in this assay (Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998).

In the chick embryo, the ectoderm can induce neural crest from early neural tissue and WNT6 signaling seems necessary and sufficient to mediate this activity (Garcia-Castro et al., 2002). In *Xenopus*, blocking Wnt signaling strongly downregulates neural crest formation in vivo, whereas in animal cap assay, Wnt signals require additional downregulation of BMPs to act on *Slug* induction (LaBonne and Bronner-Fraser, 1998). Combined with our data, this suggests that, in vivo, both the ectoderm and the mesoderm participate in inducing the neural crest and that they have different requirements to achieve neural crest induction. The coordinate activity of both Wnt and FGF pathways may account for the robust neural crest formation observed in normal embryos.

### Neural crest induction and neural plate posteriorization

Neural crest induction is achieved experimentally by combining the same classes of molecules as those required for neural plate patterning: BMP antagonists, Wnts and FGFs. All three kinds of molecules have been shown to downregulate *Bmp4* expression or BMP4 activity, either in *Xenopus* or in chick embryos (Lamb et al., 1993; Lamb and Harland, 1995; Baker et al., 1999; Wilson et al., 2000). In addition, FGF and Wnts also posteriorize the neural plate (Lamb and Harland, 1995; Domingos et al., 2001; Kiecker and Niehrs, 2001). We show here: (1) that *Slug* expression in vivo strongly correlates to proper neural and mesoderm development (Figs 6, 7), but (2) that blocking FGFR4a signaling strongly affects the AP neural pattern without preventing robust *Slug* expression (Fig. 8). We conclude that although the AP position of the *Slug*-positive domain might vary under these conditions, *Slug* induction can occur independently of AP neural patterning. Thus, we postulate that the loss of *Slug* expression observed after blocking Wnt or FGFR1 signaling (Mayor et al., 1997; LaBonne and Bronner-Fraser, 1998) (this work) reflects a role of these pathways in neural crest formation, on top of their role on neural patterning (Villanueva et al., 2002).

In conclusion, our study shows that, in the *Xenopus* embryo, (1) normal early development of the neural crest can occur in a context of abnormal AP neural patterning in vivo, (2) the paraxial mesoderm induces neural crest by an FGF-dependent pathway and (3) FGF8 is likely to mediate this activity. Our data still agree with the two-signal model of neural crest induction, and even suggest a multiple-signal model: in this model, the neural crest would arise in a location where a ‘cocktail’ of positive regulators is expressed. We propose that simultaneous moderate downregulation of BMP4 signaling, upregulation of ectodermal-derived factors (Wnt) and mesoderm-produced FGFs provides this suitable environment.

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