Interplay between Notch signaling and the homeoprotein *Xiro1* is required for neural crest induction in *Xenopus* embryos

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Summary

The neural crest is a population of cells that originates at the interface between the neural plate and non-neural ectoderm. Here, we have analyzed the role that Notch and the homeoprotein *Xiro1* play in the specification of the neural crest. We show that *Xiro1*, Notch and the Notch target gene *Hairy2A* are all expressed in the neural crest territory, whereas the Notch ligands *Delta1* and *Serrate* are expressed in the cells that surround the prospective crest cells. We have used inducible dominant-negative and activator constructs of both Notch signaling components and *Xiro1* to analyze the role of these factors in neural crest specification without interfering with mesodermal or neural plate development.

Activation of *Xiro1* or Notch signaling led to an enlargement of the neural crest territory, whereas blocking their activity inhibited the expression of neural crest markers. It is known that BMPs are involved in the induction of the neural crest and, thus, we assessed whether these two elements might influence the expression of *Bmp4*. Activation of *Xiro1* and of Notch signaling upregulated *Hairy2A* and inhibited *Bmp4* transcription during neural crest specification. These results, in conjunction with data from rescue experiments, allow us to propose a model wherein *Xiro1* lies upstream of the cascade regulating *Delta1* transcription. At the early gastrula stage, the coordinated action of *Xiro1*, as a positive regulator, and *Snail*, as a repressor, restricts the expression of *Delta1* at the border of the neural crest territory. At the late gastrula stage, *Delta1* interacts with Notch to activate *Hairy2A* in the region of the neural fold. Subsequently, *Hairy2A* acts as a repressor of *Bmp4* transcription, ensuring that levels of *Bmp4* optimal for the specification of the neural plate border are attained in this region. Finally, the activity of additional signals (WNTs, FGF and retinoic acid) in this newly defined domain induces the production of neural crest cells. These data also highlight the different roles played by BMP in neural crest specification in chick and *Xenopus* or zebrafish embryos.

Key words: *Xenopus*, *Iroquois*, Notch signaling, BMP, Neural crest, *Msx*, *Hairy2, Delta1, Snail*

Introduction

The neural crest is a unique and highly specialized population of cells found in all vertebrate embryos. The neural crest is generated at the border of the neural plate, and following closure of the neural tube these cells delaminate from the dorsal neural tube to migrate along different pathways. On reaching their destinations in the embryo, they differentiate into a wide variety of different cell types (reviewed by LaBonne and Bronner-Fraser, 1999; Mayor et al., 1999; Christiansen et al., 2000; Mayor and Aybar, 2001; Aybar and Mayor, 2002).

The generation of neural crest precursors is dependent on the interaction between the neural plate and the non-neural ectoderm (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; Mayor et al., 1997). From studies in chick, amphibian and zebrafish embryos, some of the signals involved in the induction of the neural crest have been identified, for example, BMPs, Wnts, FGF and retinoic acid (Liem et al., 1995; Selleck et al., 1998; Streit and Stern, 1999; Mayor et al., 1995; Mayor et al., 1997; LaBonne and Bronner-Fraser, 1998; Deardorff et al., 2001; García-Castro et al., 2002; Saint-Jeannet et al., 1997; Villanueva et al., 2002). However, the molecular interactions that are involved in these induction processes seem to be different in the chick to those in *Xenopus* and zebrafish embryos.

In the chick, blocking BMP activity inhibits neural crest development, and augmenting BMP activity, or its ectopic application, expands the neural crest population (Liem et al., 1995; Selleck et al., 1998). However, in *Xenopus* and zebrafish it appears that the early induction of neural crest cells depends on a gradient of BMP activity (reviewed by Chitnis, 1999; Aybar and Mayor, 2002). As such, neural crest cells are specified at the border between the neural plate and the epidermis, where intermediate concentrations of BMPs are established, i.e. where the BMP4 concentration is lower than that required to induce epidermis formation and above that which induces neural tissue (Morgan and Sargent, 1997; Marchant et al., 1998; Wilson et al., 1997; LaBonne and Bronner-Fraser, 1998; Villanueva et al., 2002; Nguyen et al., 1998).

The molecular mechanisms that underlie the differences in the way that BMP acts during neural crest induction in the

chick and in *Xenopus* or zebrafish are not understood. Thus, in order to study the role of BMP signaling on neural crest induction in *Xenopus*, and to compare it with what it is known in the chick, we have analyzed two different molecules implicated in the control of BMP4 transcription. The Notch/Delta signaling pathway is thought to influence neural crest development in zebrafish and chick by controlling BMP transcription (Endo et al., 2002; Cornell and Eisen, 2000; Cornell and Eisen, 2002). Indeed, Notch/Delta signaling has already been shown to be involved in a wide variety of other developmental processes, including neurogenesis, gliogenesis, somitogenesis, compartment boundary formation and eye development (reviewed by Artavanis-Tsakonas et al., 1999; Chitnis et al., 1995; Cho and Choi, 1998; Domínguez and de Celis, 1998; Kehl et al., 1998; Cavodeassi et al., 1999; Scheer et al., 2001). The Iro protein has been shown to control BMP transcription in the ectoderm and mesoderm of *Xenopus* embryos (Gómez-Skarmeta et al., 1998; Glavic et al., 2001; Glavic et al., 2002; Gómez-Skarmeta et al., 2001), and has been implicated in the development of the neural crest in zebrafish (Itho et al., 2002). The Iroquois genes participate in several developmental processes, including sensory organ development, compartment boundary formation in *Drosophila,* dorsal mesoderm formation, neural plate induction, dorsoventral patterning of the neural tube and midbrain-hindbrain development (Bürglin, 1997; Cavodeassi et al., 2001; Gomez-Skarmeta and Modolell, 2002; Leyns et al., 1996; Gomez-Skarmeta and Modolell, 1996; Papayannopoulos et al., 1998; Diez del Corral et al., 1999; Glavic et al., 2001; Kudoh and Dawid, 2001; Gomez-Skarmeta et al., 1998; Gomez-Skarmeta et al., 2001; Bellefroid et al., 1998; Bosse et al., 1997; Briscoe et al., 2000; Cohen et al., 2000; Glavic et al., 2002; Itoh et al., 2002).

Through conditional Notch/Delta and *iro1* gain- and loss-of-function strategies, we demonstrate that Notch/Delta signaling and the *iro1* protein in *Xenopus* play a direct role in neural crest induction by downregulating BMP4 transcription. Furthermore, a series of rescue experiments indicate that *iro1* acts upstream of Notch/Delta in the cascade of neural crest induction. We also show that *iro1* positively regulates *Delta1* transcription, in contrast to *Snail*, a gene that is specifically expressed in the neural crest and which negatively regulates *Delta1*. It should be mentioned that our experiments were performed using neural crest markers that are initially expressed only in the anterior neural crest. As a result, we discuss a model in which the interaction between *iro1*, Delta/Notch and *Snail* generates a pattern of gene expression in the anterior neural crest region that is required for the specification of these cells. Finally, our findings regarding the repression of BMP transcription through the activity of Notch/Delta signaling, and the ensuing induction of the neural crest, is in contrast to what has been observed in the chick, providing us with an explanation for the apparent differences between neural crest induction in chick and *Xenopus* embryos.

Materials and methods

Embryos, micromanipulation and dexamethasone treatment

Xenopus embryos were obtained as described previously (Gómez-Skarmeta et al., 1998) and staged according to Niewkoop and Faber (Niewkoop and Faber, 1967). Dissections were performed as described by Mancilla and Mayor (Mancilla and Mayor, 1996) and dexamethasone was employed as described by Kolm and Sive (Kolm and Sive, 1995). Dexamethasone was included in the culture medium at stage 2, 10 or 12 and maintained until the embryos were fixed.

Plasmid constructs and in vitro RNA synthesis

Inducible DNA constructs of *Xmsx1* were prepared by fusing the entire coding region of *Xmsx1* (amino acid residues 1-294) to the ligand-binding domain of the human glucocorticoid receptor (GR; amino acid residues 512-777). A dominant-negative DNA construct (*dnXmsx1*) was prepared by fusing the homeodomain region of *Xmsx1* (amino acid residues 156-294) to the GR domain. Coding sequences were amplified by PCR, using a high fidelity polymerase (Roche Molecular Biochemicals, Mannheim, Germany) and the following primers:

Xmsx1, 5′-ATGGGGGATTCGTTGTATGGATCGC-3′ and 5′-GAGCTCCGGACAGATGGTACATGCTGTATCC-3′; and

dnXmsx1, 5′-GAATTCATGAGCCCACCCGCCTG-3′ and 5′-GAGCTCCGGACAGATGGTACATGCTGTATCC-3′.

The PCR products were purified and cloned into pGEM-T Easy vector (Promega), digested with *Eco*RI/*Sac*I, and ligated with a *Sac*I/*Xho*I-digested GR fragment into a pCS2+ vector digested with *Eco*RI/*Xho*I. Both fusion constructs were automatically sequenced on both strands at the junctions (BRC, Cornell University, Ithaca, NY, USA).

The *Xiro1*, Notch, Delta, *Su(H),* SnailGR and *Snail* dominant-negative (SnailNGR) constructs have all been described previously (Gomez-Skarmeta et al., 2001; McLauglin et al., 2000; Aybar et al., 2003). All cDNAs were linearized and transcribed as described by Harland and Weintraub (Harland and Weintraub, 1985), using a GTP cap analog (New England Biolabs), and SP6, T3 or T7 RNA polymerases. After DNAse treatment, RNA was extracted with phenol-chloroform and precipitated with ethanol. *GFP* mRNA was used as a control for injections. For injection, mRNA was resuspended in DEPC-water and injected into two-cell stage embryos using 8-12 nl needles.

Microinjection of mRNAs and lineage tracing

Dejellied embryos were placed in 75% NAM containing 5% Ficoll. One blastomere of two-cell stage embryos was injected with different amounts of capped mRNA in a solution containing 1-3 μg/μl of lysine fixable fluorescein dextran, as previously described (Aybar et al., 2003)

RNA isolation and RT-PCR analysis

Total RNA was isolated from embryonic tissue by the guanidine-thiocyanate phenol-chloroform method (Chomczynski and Sacchi, 1987), and cDNA was synthesized using AMV reverse transcriptase (Roche Biochemicals) and an oligo(dT) primer. For PCR analysis, the primers for *H4* used were those described previously (Aybar et al., 2003). The primers used to analyze *Xenopus Delta1* expression amplify a 331 bp product corresponding to the 3′UTR region: 5′-GTCCTGGAGAGCAATATGCTCCAG-3′ and 5′-CCATTGTACTG-TGAACACAGCATGC-3′.

PCR amplification with these primers was performed over 30 cycles and the PCR products were analyzed on 1.5% agarose gels. PCR was performed simultaneously with RNA that had not undergone reverse transcription to control for genomic DNA contamination. Quantification of PCR bands was performed using ImageJ software (NIH, USA) on 8-bit grayscale JPG files. The values were normalized to the levels of *H4* from the same sample and expressed as relative intensities for comparison (sample/H4×10).

Whole-mount in situ hybridization, immunohistochemistry and Myc staining

Antisense RNA probes for *Xiro1* (Gómez-Skarmeta et al., 1998),

Xslug (Mayor et al., 1995), *Foxd3* (Sasai et al., 2001), *Hairy2A* (Wettstein et al., 1997), *Bmp4* (Hemmati-Brivanlou and Thomsen, 1995), *Xmsx1* (Suzuki et al., 1997), *Serrate* (Kiyota et al., 2001) and *Notch* (Coffman et al., 1990) were synthesized from cDNAs incorporating digoxigenin or fluorescein (Boehringer Mannheim) tags. Embryo specimens were prepared, hybridized and stained according to the method of Harland (Harland, 1991). The alkaline phosphatase substrates used were NBT/BCIP, or BCIP alone.

Antibody staining after in situ hybridization of the embryos was performed according to the method described by Turner and Weintraub (Turner and Weintraub, 1994), using a mouse anti-Myc monoclonal antibody from BabCo. The 12/101 polyclonal antiserum from the Developmental Studies Hybridoma Bank was used to label somites (Griffin et al., 1987).

Results

Elements of the Notch signaling pathway and the homeoprotein gene *Xiro1* are present in the neural crest territory

In order to examine the possible role of Notch signaling and of the homeoprotein gene *Xiro1* in the induction of the neural crest, we first analyzed the expression of *Xiro1*, *Delta1*, *Serrate*, *Hairy2A* and *Notch* in the presumptive crest territory, comparing their distribution with that of the neural crest marker *Xslug*. This analysis was performed using double whole-mount in situ hybridization and care was taken to follow individual embryos for the staining of both genes. At the late gastrula stage (stage 12-13), *Xiro1* expression was readily detected in the region of the neural plate, although weak expression could also be observed outside of the neural plate in the anterior region of the embryo (Fig. 1A,B; star). When the distribution of *Xslug*, characteristically expressed in the anterior neural crest cells, was visualized in the same embryos (Fig. 1C,E; arrowhead), it became evident that *Xiro1* is expressed in the neural plate, neural crest and tissue adjacent

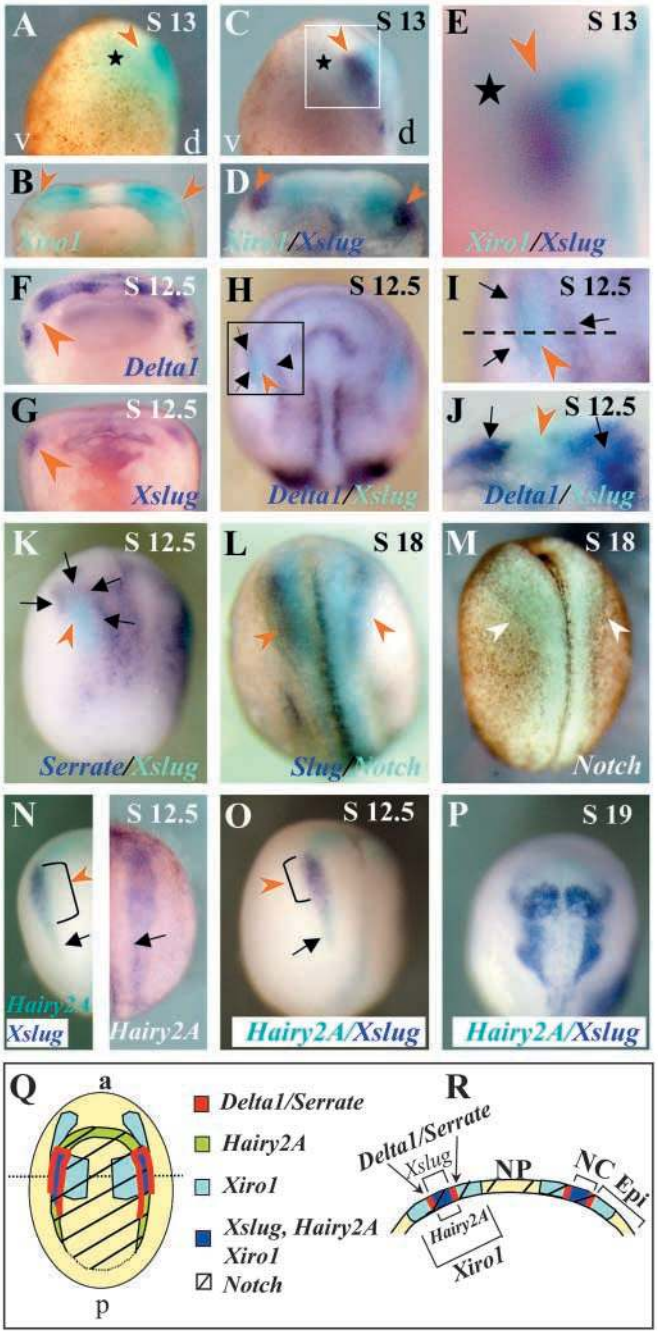

Fig. 1. Comparison of *Xiro1*, *Delta1*, *Serrate*, *Notch*, *Hairy2A* and *Xslug* expression. Embryos were fixed at late gastrula (stage 12.5-13) or mid-neurula stage (stage 18-19), and double or single in situ hybridization was performed for each gene. The stages and probes analyzed are indicated in each figure. Anterior is towards the top, and the sections are shown with the dorsal side towards the top. d, dorsal; v, ventral; orange arrowhead, neural crest. (A-E) Comparison of *Xiro1* and *Xslug* expression at the late gastrula stage. (A) Initial visualization of a double in situ hybridization of *Xiro1* (green). *Xiro1* has a dorsal or neural domain of expression, and transcripts are also found in the preplacode domain outside of the neural plate (star). (B) Section of an embryo stained as in A. (C) Visualization of *Xslug* in purple in the same embryo as shown in A, where *Xiro1* expression is in green. As the dorsal and placodal domain (star) are continuous, *Xiro1* overlaps with the neural crest territory (orange arrowhead). (D) Section of the embryo shown in C. (E) Higher magnification of the box highlighted in C. The continuity between the neural domain and the preplacode domain (star) is visible. (F-J) In situ hybridization for *Delta1* and *Slug*. (F,G) Late gastrula embryos were sectioned and divided in two groups. One group was stained for *Delta1* (F) and the other for *Xslug* (G). Note that *Slug* expression, in the neural crest (orange arrowhead), coincides with the gap in the expression of *Delta1* at the neural folds. (H,I) Double in situ hybridization for *Delta1* and *Xslug*; note that the cells expressing *Xslug* (arrowhead) are surrounded by cells expressing *Delta1* (arrows). (I) Higher magnification of the box highlighted in H. (J) Section of embryos along the plane indicated in I; note that the cells expressing *Xslug* are surrounded by *Delta1* expression in the deep layer of the ectoderm. (K) Double in situ hybridization of *Serrate* (purple) and *Xslug* (green), note that *Xslug* expressing cells (orange arrowhead) are surrounded by *Serrate* expressing cells (arrows) in the anterior neural crest region. (L,M) Expression of *Notch* can be seen in the neural plate and it overlaps with *Xslug* expression at the border of the neural plate (arrowheads). (N-P) Expression of *Hairy2A* (N,O: green in left panel and purple in the right panel of N, and green in O) can be detected in the entire neural fold, including the domain were *Xslug* is expressed (orange arrowhead). The bracket indicates the region in which *Slug* and *Hairy2A* expression overlap. The arrow (N,O) indicates the expression of *Hairy2A* in the prospective posterior neural crest. (P) The expression of both genes persists at the late neurula stage. A summary of the expression of all these genes is represented as a whole mount in Q, and in a section in R.

to the neural crest territory (Fig. 1C-E). The *Delta1* and *Serrate* genes have a very dynamic pattern of expression, although both are expressed in a similar manner. At the late gastrula stage (stage 12.5), *Delta1* is expressed along the neural anteroposterior axis, but there is a characteristic gap in its expression at the anterior neural plate border (Fig. 1F; arrowhead). In this tissue devoid of *Delta1*, *Xslug* is expressed (Fig. 1G; arrowhead). Double in situ hybridization for the *Delta1* and *Xslug* genes confirmed the complementary expression of these genes, the cells expressing *Xslug* are clearly surrounded by cells expressing *Delta1* (Fig. 1H,I). This expression pattern was more readily apparent in sections of the stained embryos (Fig. 1J). The same pattern was observed for *Serrate* expression, *Serrate*-positive cells surrounded those expressing *Xslug* (Fig. 1K). The expression of *Notch* is strong in the neural territory and, in contrast to *Delta1* and *Serrate*, it overlaps with the neural crest marker *Xslug* (Fig. 1L,M; arrowhead) (Coffman et al., 1993). Finally, from early in development *Hairy2A*, a downstream target of the Notch signaling pathway (Dawson et al., 1995; Wettstein et al., 1997), is expressed at the neural plate border, coinciding with the territory of *Xslug* expression (Fig. 1N,O; bracket and arrowhead). However, like *Delta1* and *Serrate*, *Hairy2A* expression extends into the posterior neural crest at stages when no *Slug* transcripts can be detected in these cells (Fig. 1N,O; arrow). At the late neurula stage, the expression of *Hairy2A* can be seen in the prospective forebrain region, whereas *Xslug* is expressed in the migrating neural crest (Fig. 1P).

In summary (Fig. 1Q,R), *Notch*, like *Xiro1*, is present in the neural plate and crest territory, where it could interact with *Delta1* and *Serrate*, which are present at the border of the prospective neural crest territory. The potential interaction of Notch with one of its ligands is compatible with the expression of the target gene *Hairy2A* in the crest cells.

The specific effect of Notch signaling on the neural crest

Based on the pattern of Notch expression and its ligands, we set out to determine whether Notch signaling might be involved in the induction of the neural crest. It has become clear that an interaction between the neural plate and the epidermis, and signals from the paraxial mesoderm, are involved in the induction of the neural crest (Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; Bonstein et al., 1998; Marchant et al., 1998; Monsoro-Burq, 2003). It has also been established that Notch signaling is involved in the development of the neural plate and mesoderm (Coffman et al., 1993). Thus, we took care not to interfere with the development of the mesoderm and the neural plate when studying the role of Notch signaling in the induction and development of the neural crest. It is known that the mesoderm is specified earlier than the neural tissues, and it has been reported that the neural plate is specified earlier than the neural crest (Smith and Slack, 1983; Servetnick and Grainger, 1991; Mancilla and Mayor, 1996; Woda et al., 2003). Therefore, in order to specifically study neural crest development, Notch signaling was interfered after the mesoderm and the neural plate had already been specified. For this reason, inducible constructs that activated or inhibited Notch signaling were used to control the timing of intervention.

We first analyzed the effect of activating Notch signaling at different developmental times on the formation of the mesoderm, neural plate and neural crest. Ligand activation of Notch results in the proteolytic cleavage of its transmembrane domain and the release of the cytoplasmic region (*NICD*) (Struhl and Adachi, 2000). *NICD* can then translocate to the nucleus, where it interacts with the transcriptional repressor Suppressor of Hairless (Su(H)), forming a transcriptional activator complex (Artavanis-Tsakonas et al., 1999). Here, we have used an inducible form of *NICD* (*NICDGR*) in order to control the time of its activation. We injected mRNA encoding *NICDGR* into one blastomere of a two-cell stage embryo, and induced its expression, by exposure to dexamethasone, immediately after the injection (stage 2), at the blastula stage (stage 6-8) or at the gastrula stage (stage 12). The development of the mesoderm was assessed by analyzing the expression of

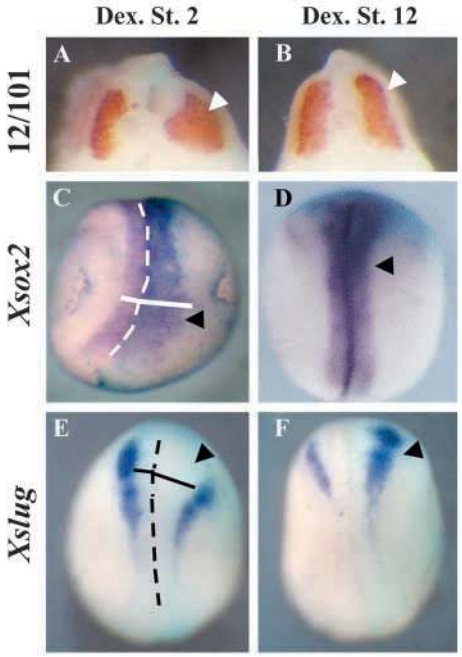

Fig. 2. Activation of Notch signaling on mesoderm, neural plate and neural crest development. Two-cell stage embryos were injected with *NICDGR* mRNA in one blastomere, treated with dexomethasone either directly after the injection (A,C,E) or at stage 12 (B,D,F), and cultured until stage 25 (A,B) or stage 18-19 (C-F). Subsequently, the expression of distinct markers was analyzed. The side of the injection is indicated with an arrowhead. (A,B) Immunostaining of somites with the 12/101 antiserum. Note the expansion of the somite in the injected side following early activation (A), and the normal morphology after late activation (B). Following activation at stage 2, 78% of embryos demonstrated somite expansion (n=70), whereas activation at stage 12 did not produce any expansion of the somites (0%; n=87). (C,D) In situ hybridization to visualize *Sox2* transcripts in the neural plate. Activation at stage 2 (C) leads to an expansion of the neural plate (65% of embryos with expanded neural plate; n=83), whereas activation at stage 12 (D) produces no effect on the neural plate (100% normal; n=92). (E,F) In situ hybridization to visualize *Xslug* transcripts in the cephalic neural crest. Activation at stage 2 (E) produces an inhibition in the expression of this neural crest marker (58% of inhibition; n=102), whereas activation at stage 12 (F) produces an expansion of *Xslug* expression (expanded in 75% of embryos; n=152).

the somite antigen 12/101; development of the neural plate and neural crest induction were assessed by analyzing *Sox2* and *Xslug* expression, respectively. As for non-inducible forms of activated Notch (Coffman et al., 1993), early activation of *NICDGR* provoked both the expansion of the somites and neural plate on the injected side (Fig. 2A,C), as well as the inhibition of the anterior neural crest (Fig. 2E). Similar results were obtained when *NICDGR* was activated prior to stage 8. By contrast, when induced at stage 12, *NICDGR* had no effect on somite or neural plate development (Fig. 2B,D), but rather a clear expansion of the neural crest markers was observed (Fig. 2F). These results indicated that to study the specific effects of Notch signaling on neural crest development, and to avoid any influence on the mesoderm or neural plate, all the Notch signaling constructs should be activated at stage 12. Indeed, using inducible constructs of Dlx proteins, an early effect was observed on neural plate and neural crest development, whereas a later induction produced alterations specific to the neural crest (Woda et al., 2003). Thus, in all the following experiments inducible constructs were activated at stage 12.

Notch signaling is required for neural crest specification in *Xenopus* embryos

Several molecular tools have been developed to modify the activity of the Notch signaling pathway at different levels (Coffman et al., 1993; Chitnis et al., 1995; McLaughlin et al., 2000). Thus we were able to analyze the effects of both gain- and loss-of-function on neural crest development. Activation, at stage 12, of a *NICD* (*NICDGR*), or of an inducible ankyrin activator fusion of *Su(H)* [*Su(H)ankGR*], provoked an expansion of the *Xslug* and *Foxd3* domains of expression (Fig. 3A,B,E,F). By contrast, the injection of mRNA encoding the dominant-negative $Delta^{Stu}$ (Dl^{Stu}) or *Su(H)DBMGR* into one blastomere of a two-cell embryo, and induction at the late gastrula stage (stage 12), inhibited the expression of the neural crest markers *Xslug* and *Foxd3* (Fig. 3C,D,G,H).

It has been shown that inhibition of BMP activity in *Xenopus* and zebrafish embryos leads to an expansion of the neural crest territory and an increase in *Xmsx1* expression (Marchant et al., 1998; Nguyen et al., 1998; Tríbulo et al., 2003). Thus, we analyzed the effect of activating or inhibiting Notch signaling on both *BMP4* and

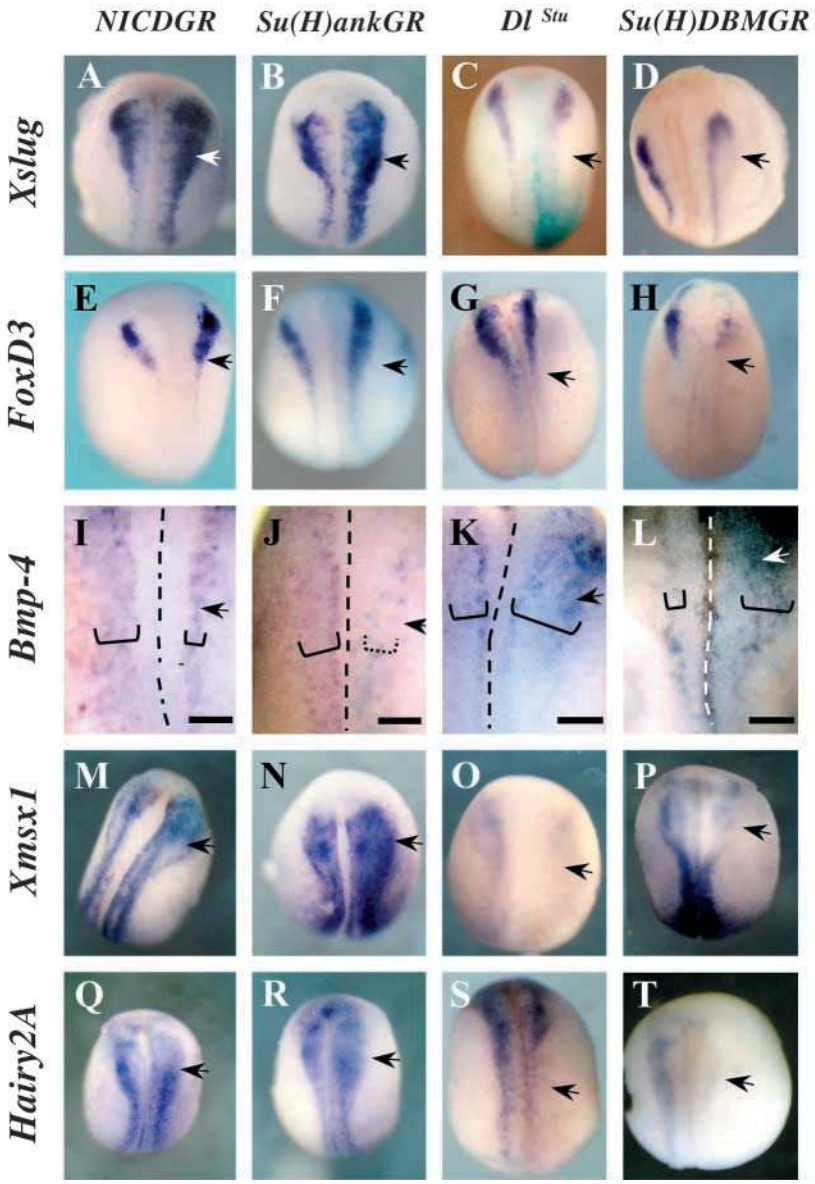

Fig. 3. Notch signaling is required for neural crest specification. Two-cell embryos were injected in one blastomere with 0.7 ng of *NICDGR* (A,E,I,M,Q), 0.7 ng of *Su(H)ankGR* (B,F,J,N,R), 1 ng of $Delta^{Stu}$ (C,G,K,O,S) or 0.25 ng of *Su(H)DBMGR* (D,H,L,P,T) mRNA, and the inducible constructs were activated at stage 12. *NICDGR* and *Su(H)ankGR* activate Notch signalling, and $Delta^{Stu}$ and *Su(H)DBMGR* inhibit Notch signalling. The expression of *Xslug*, *Foxd3*, *Bmp4*, *Xmsx1* and *Hairy2A* was analyzed at stage 17 or 18 by in situ hybridization, and the injected sides were visualized by alkaline phosphatase-mediated FITC immunodetection. The injected side is labeled with an arrow and all embryos are presented dorsally with the anterior to the top. (A,B,E,F) Note the expansion of *Xslug* (A,B) and *Foxd3* (E,F) expression on the injected side after activation of Notch signaling. (C,D,G,H) Note the inhibition in *Xslug* (C,D) and *Foxd3* (G,H) expression on the injected side, after inhibition of Notch signaling. (I-L) The domain of expression of *Bmp4* is highlighted in the neural folds by the brackets. Scale bar: 80 μm. Note the reduced expression domain after Notch activation (I,J), and the expansion and increase in the intensity of *Bmp4* expression on the injected side after Notch inhibition (K,L). (M-P) Expression of *Xmsx1*. Note the expansion in the *Xmsx1* expression domain after Notch activation (M,N) and the reduction of *Xmsx1* expression on the injected side after Notch inhibition (O,P). (Q-T) *Hairy2A* expression. Note the expansion in *Hairy2A* expression in the injected side after Notch activation (Q,R) and the decrease in *Hairy2A* expression on the injected side after Notch inhibition (S,T). Each experiment was performed at least twice with a minimum of 45 embryos. The effect seen in each experiment was observed in at least 70% of embryos.

Xmsx1 transcription. In contrast to the chick (Endo et al., 2002), activating Notch signaling, by inducing *NICDGR* and *Su(H)andkGR* expression, provoked the inhibition of *BMP4* expression (Fig. 3I,J) and the upregulation of *Xmsx1* transcription (Fig. 3M,N). In addition, inhibition of Notch signaling, by Dl^{stu} and *Su(H)DBMGR*, promoted the expansion of the *BMP4* expression domain (Fig. 3K,L), while inhibiting *Xmsx1* expression (Fig. 3O,P).

Finally, to confirm that these constructs were indeed acting on the Notch signaling pathway, we analyzed their effects on the expression of *Hairy2A,* a known target gene of Notch (Dawson et al., 1995). Each of the constructs that augmented Notch signaling provoked an expansion of the *Hairy2A* expression domain (Fig. 3Q,R). By contrast, those that inhibited Notch signaling diminished the expression of *Hairy2A* (Fig. 3S,T). Thus, we concluded that the activation of Notch signaling enlarges the neural crest territory and the domain of *Xmsx1* expression, while inhibiting *BMP4* transcription. Conversely, inhibition of Notch signaling produces exactly the opposite effect.

The Notch target gene *Hairy2A* is sufficient to induce neural crest cells in *Xenopus* embryos

Hairy2A is a vertebrate target of Notch signaling that belongs to the *Enhancer of Split* complex. This bHLH transcription factor can act as a transcriptional repressor and has been implicated in the repression of neuronal differentiation (Dawson et al., 1995; Wettstein et al., 1997). We analyzed whether overexpression of *Hairy2A* also influenced the expression of neural crest markers. Overexpression of *Hairy2A* repressed *N-tubulin* expression, a control for the activity of *Hairy2A* mRNA, at the sites where primary neurons form (Fig. 4A). As we had previously shown that an early activation of Notch signaling leads to an expansion of the somites and, in turn, to an indirect effect on neural crest induction, we took care of injecting the *Hairy2A* mRNA specifically into the blastomeres fated to become ectoderm. We performed the injection of *Hairy2A* mRNA into two animal blastomeres of an eight-cell stage embryo. In order to show that there was no effect on mesodermal development, the somite antigen 12/101 was analyzed. No effect on 12/101 was observed in the injected side (Fig. 4B). Interestingly, the same group of embryos that exhibited normal somite development showed an increase in *Xslug* expression (Fig. 4C). In addition, the expression of *Bmp4* was also decreased in these embryos, although the expression of *Xmsx1* augmented (Fig. 4D-F). These results suggest that the expansion of the neural crest population upon the activation of Notch signaling may be a consequence of the increase in *Hairy2A* expression provoked in these embryos.

The homeodomain protein gene *Xiro1* participates in neural crest development by controlling *Bmp4* and *Hairy2A* expression

We have shown that by influencing *Bmp4* transcription, Notch signaling is involved in specifying the neural crest. Another factor that is known to affect the early transcription of *BMP4* is *Xiro1* (Glavic et al., 2001; Gómez-Skarmeta et al., 2001). Given that *Xiro1* is co-expressed with the neural crest marker *Xslug*, and that the zebrafish Iroquois genes are involved in neural crest formation, we analyzed whether *Xiro1* might also influence *Xenopus* neural crest development. In order to overcome the early effects of *Xiro1* in mesoderm and neural plate development, inducible fusion constructs were used as described previously (Glavic et al., 2001; Gomez-Skarmeta et al., 2001; Glavic et al., 2002).

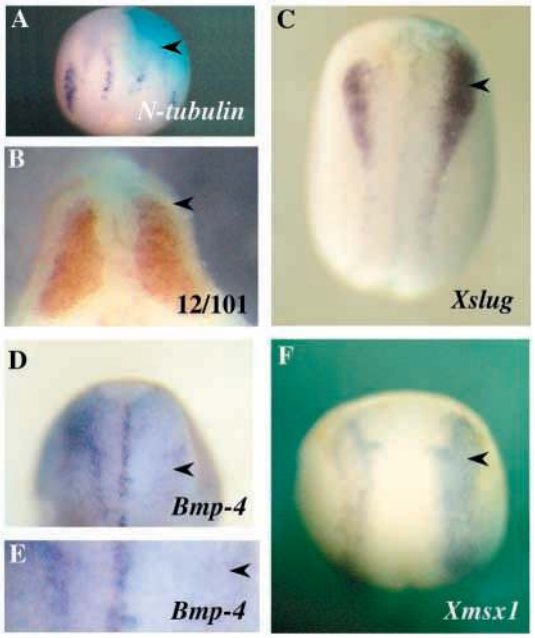

Fig. 4. The Notch target gene *Hairy2A* produces an expansion of the neural crest population. Eight-cell embryos were injected in two blastomeres with 1 ng *Hairy2A* mRNA, the arrowhead indicates the injected side, which was visualized by alkaline phosphatase-mediated FITC inmunodetection. (A) *N-tubulin* expression is clearly reduced. (B) Immunodetection of the somite antigen 12/101 analyzed at stage 25. Note that there is no difference in the staining between the injected and uninjected side. (C) The domain of *Xslug* expression is expanded on the injected side, whereas *Bmp4* is dramatically repressed (D,E). (E) Corresponds to a higher magnification of D. (F) *Xmsx1* expression is increased on the injected side. Each experiment was performed at least twice with a minimum of 35 embryos. The effect seen in each experiment was observed in at least 70% of embryos.

It has been shown that *Xiro1* acts as a transcriptional repressor (Glavic et al., 2001; Gomez-Skarmeta et al., 2001). However, when mRNA encoding both *Xiro1* (not shown) and its inducible repressor fusion (*HDGREnR*) was injected and then activated at stage 12, *Xslug* expression was augmented (Fig. 5A). Conversely, activation, at stage 12, of both the inducible dominant-negative fusion (*HDGR*) and the inducible activator fusion (*HDGRE1A*) inhibited *Xslug* expression (Fig. 5B,C). By contrast, transcription of *Bmp4* at the neural plate border was repressed in embryos injected with *HDGREnR* (Fig. 5D) but increased in embryos overexpressing *HDGRE1A* and *HDGR* (Fig. 5E,F). It should be noted that *Bmp4* has a complex and dynamic pattern of expression in the neural folds, and that the inhibition of *Xiro1* not only affects the levels of *Bmp4* expression but also its distribution. The expression of *Xmsx1* was augmented and expanded when *Xiro1* and *HDGREnR* was injected into embryos (Fig. 5G), whereas the levels of transcripts diminished and its expression pattern was disrupted in embryos injected with the mRNAs encoding for the activator and dominant-negative constructs (Fig. 5H,I). Finally, overexpression of *HDGREnR* de-repressed *Hairy2A*

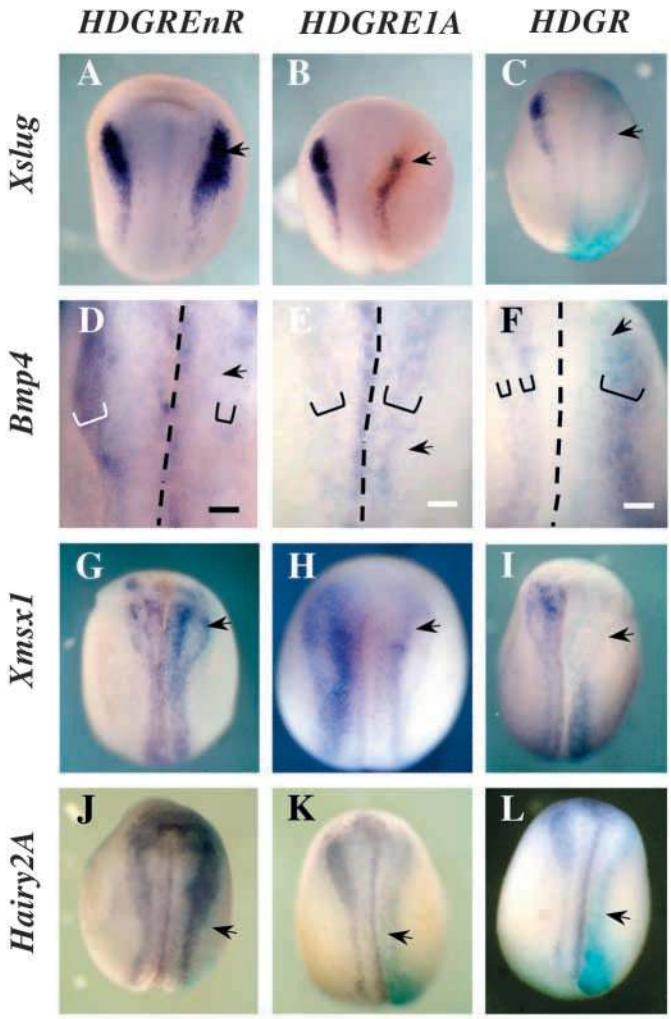

Fig. 5. *Xiro1* participates in the induction of neural crest cells. Two-cell embryos were injected in one blastomere with 1 ng of the inducible forms of a repressor of *Xiro1* (*HDGREnR*) (A,D,G,J), an activator form of *Xiro1* (*HDGRE1A*) (B,E,H,K), or with a dominant-negative form of *Xiro1* (*HDGR*) (C,F,I,L). The embryos were treated with dexomethasone at stage 12, and the expression of *Xslug*, *Bmp4*, *Xmsx1* and *Hairy2A* was analyzed by in situ hybridization. The injected side was visualized by Myc inmunostaining, or alkaline phosphatase-mediated FITC inmunostaining, and is indicated with an arrowhead. (A-C) *Xslug* expression. (A) An expansion of the *Xslug* expressing neural crest domain is observed. (B,C) *Xslug* expression is reduced on the injected side. (D-F) *Bmp4* expression. (D) A repression of *Bmp4* in the neural fold domain is indicated by the bracket. (E,F) The levels of *Bmp4* transcripts are augmented on the injected side and an expansion in the expression domain is also observed. Note that in F, the expression indicated by two small brackets on the uninjected side is transformed into a single big bracket on the injected side. Scale bar: 85 μm. (G-I) *Xmsx1* expression. (G) Note the expanded *Xmsx1* expression domain. (H,I) A reduction in the expression of *Xmsx1* can be seen in the neural fold region. (J-L) *Hairy2A* expression. (J) An expanded domain of *Hairy2A* expression is observed in the neural fold, whereas *Hairy2A* expression is inhibited by the injection of *HDGRE1A* and *HDGR* (K,L). Each experiment was performed at least twice with a minimum of 42 embryos. The effect seen in each experiment was observed in at least 65% of embryos.

expression in the neural fold (Fig. 5J), whereas injecting *HDGRE1A* and *HDGR* decreased *Hairy2A* expression (Fig. 5K,L). Thus, *Xiro1*, in addition to being involved in the expression of neural crest markers, also influences *Bmp4* and *Hairy2A* expression in the neural crest precursor domain.

Xiro1 is upstream of Notch signaling in the cascade that specifies neural crest cells

Having established that both *Xiro1* and Notch signaling are involved in the specification of the neural crest, we set out to investigate the relationship between these elements by performing rescue experiments. Activation of injected *Xiro1* dominant-negative mRNA (*HDGR*) at stage 12 clearly inhibited *Xslug* expression (Fig. 6A). By contrast, this effect was prevented, and in some cases *Xslug* expression was enhanced, if *HDGR* was co-injected with *Hairy2A* mRNA or with an activator fusion of Notch signaling (e.g. *Su(H)ankGR*; Fig. 6B,C). However, the inhibition of *Xslug* expression induced by blocking Notch signaling could not be rescued by activating the *Xiro1* gene (not shown). Taken together, these results suggest that Notch signaling and *Hairy2A* are likely to be downstream of *Xiro1* activity in specifying the neural crest. The inhibition of Notch signaling produced by *Su(H)DBMGR* repressed *Xslug* expression (Fig. 6D), an effect that was reversed by the co-injection of *Hairy2A* or *XmsxGR* mRNA (Fig. 6E,F). This suggests that the effect of suppressing Notch activity on neural crest specification depends mainly on *Hairy2A* and, in addition, that this Notch activity is likely to be upstream of *Xmsx1*. Finally, the enlargement of the *Xslug* expression domain produced by *NICDGR* (Fig. 6G) was reversed by blocking *Xmsx1* activity with an inducible dominant-negative construct of *Xsmx1*, *dnXmsxGR* (Fig. 6H). This observation provides further evidence that Notch signaling depends on *Xmsx1* activity to influence neural crest specification. In all rescue experiments, an unrelated mRNA such as GFP was co-injected, and no effects of GFP on rescue activity were observed (an example on the effect of *NICDGR* is shown; Fig. 6I).

Delta1 transcription is induced by *Xiro1* and repressed by *Snail* in the neural crest region

We have shown that *Xiro1* is likely to be upstream of Notch signaling and that the expression of *Xiro1* overlaps with that of *Delta1*. Therefore, we tested whether *Xiro1* could regulate the transcription of *Delta1*. When *HDGREnR* or *HDGR* mRNA was activated at stage 12, and cultured until stage 17, the activation of the *Xiro1* gene produced a moderate upregulation of *Delta1* expression in the neural crest region (Fig. 7A; arrowhead). By contrast, however, inhibition of *Xiro1* by *HDGR* expression produced a complete inhibition of *Delta1* expression, even at the border of the neural crest territory (Fig. 7B). Thus, we further examined the regulation of *Delta1* by Xiro by injecting one-cell embryos with *Xiro1* or *Xiro3* mRNA, dissecting out the animal caps from these embryos at stage 9, and then culturing these to the equivalent of stage 18, when the expression of *Delta1* was analyzed. Although no expression of *Delta1* was observed in control animal caps (Fig. 7C), *Delta1* transcripts were detected by in situ hybridization in animal caps injected with *Xiro1* or *Xiro3* mRNA (Fig. 7D,E). When analyzed by RT-PCR, low levels of *Delta1* mRNA could be detected in the control animal caps

(Fig. 7F,G), probably due to the expression of *Delta1* in the ciliary cells of the epidermis. However, after injection of *Xiro3* mRNA, a significant upregulation of *Delta1* mRNA expression was observed. Taken together, these results strongly suggest that *Xiro1* (and *Xiro3*) is able to activate *Delta1* transcription. However, it is likely that in the embryo other signals are present that repress *Delta1* transcription, which might explain why *Delta1* is only expressed in a sub-domain of *Xiro1* expressing cells.

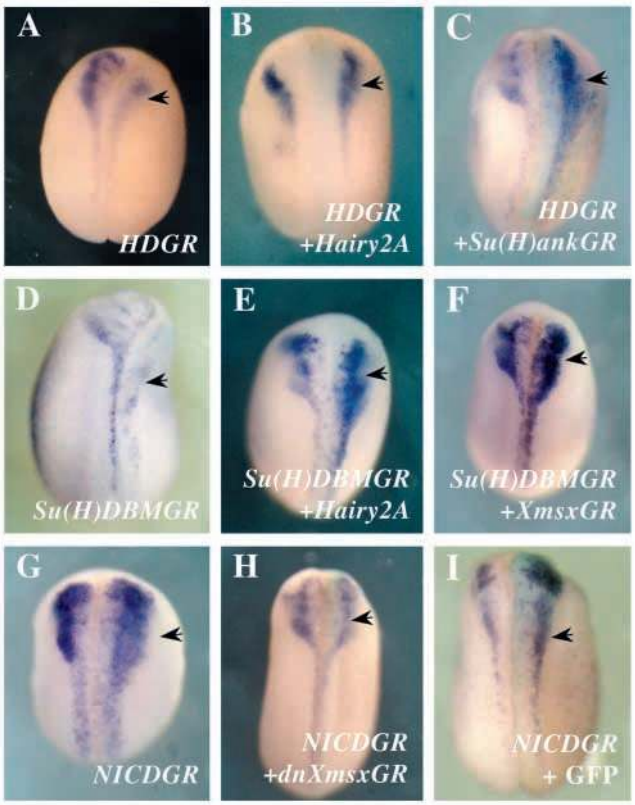

Fig. 6. *Xiro1* is upstream of Notch signaling in the specification of the neural crest. Embryos were injected with 1 ng *HDGR* (A) mRNA, and co-injected with 1 ng *Hairy2A* (B) or 1 ng *Su(H)ankGR* (C) mRNA. A second set of experiments was performed by injecting two-cell embryos in one blastomere with 0.25 ng *Su(H)DBMGR* (D) mRNA, and co-injecting 1 ng *Hairy2A* (E) or 0.7 ng *Xmsx1GR* (F) mRNA. Finally, a third set of experiments was performed by injecting one blastomere of a two-cell embryo with 1 ng of *NICDGR* (G) mRNA, and co-injecting 0.7 ng of *dnXmsxGR* (H). The embryos were treated with dexomethasone at stage 12, and the expression of *Xslug* was analyzed by in situ hybridization between stage 17 and 19. The injected side was visualized by alkaline phosphatase-mediated FITC inmunodetection and is indicated by an arrowhead. (A) *Xslug* expression was inhibited by *HDGR*. (B) The inhibition of *Xiro1* activity was rescued by co-injection of *Hairy2A*, reaching 89% recovery of *Xslug* expression (n=56). (C) A similar reversion of *Xiro1* inhibition was obtained by activating Notch signaling, 93% rescue of *Xslug* expression was observed (n=47). (D) *Xslug* expression was inhibited by *Su(H)DBMGR.* (E) The inhibition of the Notch signaling could be rescued by co-expression of *Hairy2A* (92% rescue; n=43). (F) The effect of inhibiting Notch signaling could be rescued by co-expression of *Xmsx1* (97% rescue; n=39). (G) Expansion of *Xslug* expression by injecting 1 ng *NICDGR*. (H) The effect of *NICDGR* was rescued by blocking *msx1* activity with *dnXmsxGR*, (92% rescue; n=45), whereas the effect of *NICDGR* was not rescued by the co-injection of GFP mRNA (I; 0% rescue; n=25).

The expression of *Delta1* and *Serrate* is restricted to the border of the neural crest region (Fig. 1). This observation suggests that a repressor of *Delta1* might be present in neural crest cells. Many transcription factors that act as transcriptional repressors have been identified (reviewed by Mayor and Aybar, 2001). One such factor is *Xsnail*, which also seems to be upstream of the genetic cascade of transcription factors that act in the neural crest territory (Aybar et al., 2003). Thus we tested whether *Xsnail* could repress *Delta1* transcription in the neural crest territory. Animal caps taken from embryos co-injected with *Xiro3* and *Xsnail* mRNA were cultured until the equivalent of stage 18, and their mRNA analyzed by RT-PCR. Strong inhibition of *Delta1* expression was observed in these animal caps when compared with controls or those injected with *Xiro3* mRNA alone (Fig. 7F,G). We have recently developed two specific dominant-negative constructs of *Snail*, one that contains the *Snail* zinc finger (*ZnfSnailGR*) and another that includes the N-terminal (*SnailNGR*) domain (Aybar et al., 2003). The mRNAs that encode these dominant-negative constructs were injected into one cell of a two-cell embryo, and the expression of *Delta1* was analyzed by in situ hybridization after their activation. The expression of *Delta1* was clearly upregulated in the injected side of the embryo injected with both *ZnfSnailGR* (Fig. 6H-J) or *SnailNGR* (not shown). We also examined the effect of inducing the expression of *SnailGR* at stage 12 and, in these embryos, a moderate but consistent inhibition of *Delta1* expression was observed in the ectodermal regions (not shown). Taken together, these results support the idea that *Snail* could repress *Delta1* transcription in the neural crest territory.

Discussion

We have analyzed the role that Notch signaling and *Xiro1* play in neural crest specification. The activation of these elements at the late gastrula stage using inducible constructs has enabled us to examine their specific effects on crest induction without producing any detectable effect on mesoderm or neural plate development. As a result, we have produced a schematic model of the molecular interactions involved in the generation of the neural crest in *Xenopus* embryos

Notch signaling in neural crest specification

In *Xenopus* embryos, the expression patterns of *Notch*, the Notch ligand *Delta1* and the Notch downstream gene *Hairy2A* suggest that these molecules might be implicated in the formation of the neural crest. Interestingly, in contrast to the homogenous expression described previously (Kiyota et al., 2001), we observed that another Notch ligand, *Serrate*, is expressed in a complex pattern very similar to that of *Delta1*. Thus, both ligands are expressed in cells that surround those expressing *Xslug* and hence they could activate Notch and, thus, *Hairy2A* in the neural folds. The restricted pattern of *Hairy2A* expression overlaps that of *Xslug*, suggesting that other elements either repress *Hairy2A* transcription in the adjacent epidermis and neural plate, or permit the expression of this gene in the neural fold region. One of these elements could be Notch itself.

In *Xenopus, Notch* is detected in neural tissue and is excluded from the non-neural ectoderm, thereby accounting for

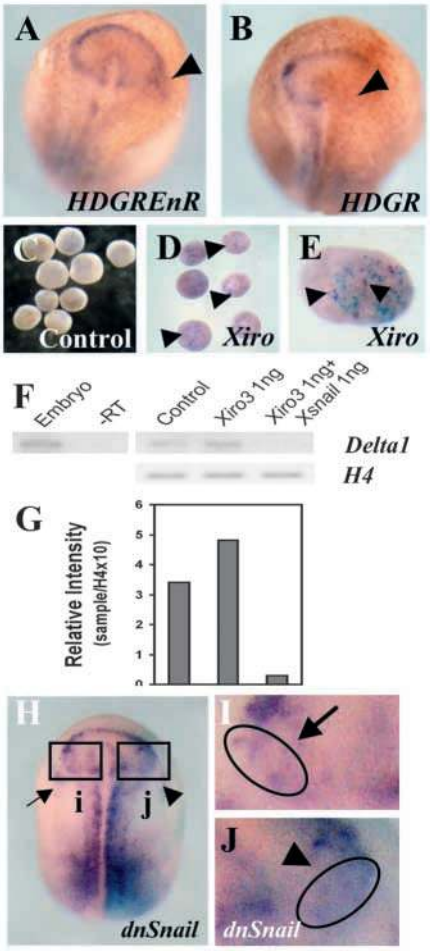

Fig. 7. *Delta1* expression is upregulated by *Xiro1* and down regulated by *Snail*. (A,B) Two-cell embryos were injected in one blastomere with 1 ng *HDGREnR* (A) or with 1 ng *HDGR* (B) mRNA. The embryos were treated with dexomethasone at stage 12 and the expression of *Delta1* was analyzed by in situ hybridization at stage 17. The injected side was visualized by alkaline phosphatase-mediated FITC immunodetection. (A) *HDGREnR* produces a moderate expansion of *Delta1* expression in the neural crest region (arrowhead), whereas *HDGR* leads to a complete inhibition of *Delta1* expression in the crest region (B; arrowhead). (C-E) Animal caps taken from stage 9 embryos were cultured until the equivalent of stage 18, and the expression of *Delta1* was analyzed by in situ hybridization. (C) In control animal caps, no expression of *Delta1* could be detected. (D,E) In animal caps taken from embryos injected with 1 ng *Xiro3* mRNA, *Delta1* expression was observed in 87% of the caps (*n*=57). (E) Higher magnification of the animal cap shown in D. (F) RT-PCR to analyze *Delta1* and *H4* mRNA. Arrowheads in D,E indicate *Delta1*-expressing cells. Left panel, control embryo and PCR in the absence of reverse transcriptase; right panel, mRNA taken from a control animal cap, a cap injected with 1 ng *Xiro3* mRNA, or a cap co-injected with 1 ng *Xiro3* mRNA and 0.7 ng *Xsnail* mRNA. (G) Quantification of data shown above in F. Note the increase in *Delta1* mRNA produced by *Xiro3*, and the complete inhibition produced by *Xsnail*. (H-J) Two-cell embryos were injected in one blastomere with 0.7 ng *dnSnail* mRNA. The embryos were treated with dexomethasone at stage 12, and the expression of *Delta1* was analyzed by in situ hybridization at stage 17. The injected side was visualized by alkaline phosphatase-mediated FITC inmunodetection. (H) *Delta1* expression is upregulated in the neural crest region. (I,J) Higher magnification of the neural crest region indicated by the box in H, where the staining was stronger on the injected (J) than on the uninjected side (I). Arrow, uninjected side; arrowhead, injected side. Each experiment was performed at least twice with a minimum of 52 embryos. The effect seen in each experiment was observed in at least 65% of embryos.

the absence of *Hairy2A* expression in the epidermis (Coffman et al., 1990) (this work). Our analysis of Notch signaling demonstrates that increasing Notch activity at the early gastrula stage produces an expansion of the neural crest territory. Interestingly, the increase in *Xslug* and *Foxd3* expression produced by Notch activation is in contrast to the repression of *Slug* upon changes in Notch activity previously described in the chick (Endo et al., 2002). In addition, inhibition of Notch signaling by $Delta^{Stu}$, or by a dominant-negative form of *Suppressor of Hairless*, produces a reduction in the number of *Xslug*- and *Foxd3*-positive cells. Furthermore, direct overexpression of the Notch target gene *Hairy2A* leads to the induction of neural crest cells. Thus, our results provide evidence of a role for Notch and its downstream elements in the specification of *Xenopus* neural crest.

The molecular mechanism by which Notch signaling controls the induction of the neural crest in the chick appears to involve the upregulation of *BMP4* expression, necessary for neural crest induction (Liem et al., 1995; Endo et al., 2002). However, in *Xenopus*, the activity of BMP is opposite to that of the chick, and a decrease in BMP activity relative to that seen in the non-neural ectoderm induces neural crest cells. Therefore, the observed increase of *Xslug* and *Foxd3* expression is most likely due to the repression of *Bmp4* transcription. Indeed, here we show that the activation of Notch represses *Bmp4* expression in *Xenopus* embryos. In addition, inhibition of Notch signaling by $Delta^{Stu}$, or by a dominant-negative form of *Suppressor of Hairless*, produces an increase in *Bmp4* transcription. Our analysis of the influence of Notch signaling on the BMP pathway further showed that the precise pattern of *Xmsx1* expression, a BMP target gene, is finely regulated in the neural crest precursor domain.

Contrary to our expectations, activation of Notch often produced an increase in *Xmsx1* expression, even though *Bmp4* transcription was inhibited. Accordingly, treatments that blocked Notch signaling, and that therefore activated *Bmp4* expression, produced embryos where *Xmsx1* expression was impaired. These results support the conclusion that *Xmsx1* expression is induced at a specific level of BMP activity (Tríbulo et al., 2003). We also observed that, when overexpressed in embryos, *Hairy2A* produced similar effects on *Xslug*, *Bmp4* and *Xmsx1* expression, and that it is able to rescue the effect of *Su(H)DBMGR* in blocking Notch signaling.

In conclusion, Notch signaling activates the expression of *Hairy2A* in the region of the neural folds, and thereby represses *Bmp4* transcription. This effect of Notch signaling is dependent on *Xmsx1* activity, as the inhibition of Notch by *Su(H)DBMGR* can be reversed by *Xmsx1*, and the effects produced by activating Notch can be blocked by a dominant-negative *Xmsx1* construct. Our results also provide a possible explanation for the apparent discrepancy in the role played by BMP in chick and *Xenopus* or zebrafish neural crest induction. At the time of neural crest induction, the levels of BMP at the neural plate border are high in both *Xenopus* and zebrafish, and low in the

chick. If we assume that an intermediate level is required to induce neural crest in all these vertebrates, then an increase in BMP levels in the chick would establish similar levels to those generated by a decrease in *Xenopus* and zebrafish. Thus, because of the initial differences in the levels of BMP in these two groups of organisms, the molecular machinery that induces neural crest formation (e.g. Notch/Delta, *Xiro1*) must adjust the specific levels of BMP by producing opposing effects on BMP expression. Thus, Notch/Delta signaling induces the neural crest by increasing BMP expression in the chick (Endo et al., 2002), and decreasing it in *Xenopus*.

The homeoprotein gene *Xiro1* in neural crest specification

Genes of the Iroquois family have been implicated in a variety of developmental processes, including dorsal mesoderm formation, neural induction, compartment specification in the eye imaginal disc of *Drosophila* and midbrain-hindbrain boundary formation (Glavic et al., 2001; Kudoh and Dawid, 2001; Papayannopoulos et al., 1998; Diez del Corral et al., 1999; Gomez-Skarmeta et al., 1998; Bellefroid et al., 1998; Bosse et al., 1997; Briscoe et al., 2000; Glavic et al., 2002; Itoh et al., 2002). Our results extend the role of *Xiro1* during development to that of neural crest specification. Indeed, it has already been demonstrated that *Xiro1* can bind to the *Bmp4* promoter, and, by acting as a repressor, it can inhibit *Bmp4* transcription in both the Spemanns' organizer and the neural plate (Gomez-Skarmeta et al., 2001; Glavic et al., 2001).

Our observations show that *Xiro1* is expressed in the neural crest territory and that its activation produces an enlargement of this territory. By contrast, inhibition of *Xiro1* leads to a reduction in the expression of neural crest markers. Like Notch signaling, *Xiro1* also represses *Bmp4* transcription and activates *Hairy2A* expression in the neural folds, as well as expanding the domain of *Xmsx1* expression. The effects of inhibiting *Xiro1* on neural crest specification can be reversed by activating Notch signaling, or by co-injecting the Notch target gene *Hairy2A*. Taken together, these results indicate that *Xiro1* activity is upstream of Notch signaling.

Although the regulation of Notch activity by *Xiro1* could operate at different levels, we have presented evidence that *Xiro1* can upregulate *Delta1* transcription. Activation of *Xiro1* in animal caps or whole embryos, led to an upregulation of *Delta1*, whereas impairing *Xiro1* produced an inhibition of *Delta1* expression in the neural crest territory. Thus, *Xiro1* seems to positively regulate *Delta1* expression. However, as the expression of *Delta1* and *Xiro1* do not completely overlap, additional factors must be required either to activate *Delta1* where *Xiro1* is not expressed, or to inhibit its expression in those cells expressing *Xiro1* but not *Delta1*.

Delta1 is excluded from the center of the prospective neural crest region, and its transcripts can only be seen at the border of the crest region. This pattern of *Delta1* expression suggests that a repressor is acting in the crest region. Many transcriptional repressors are expressed in the neural crest, including *Snail* (Aybar et al., 2003), *Slug* (LaBonne and Bronner-Fraser, 1999; Mayor et al., 2000), *Foxd3* (Sasai et al., 2001) and *Zic5* (Nakata et al., 2000). Moreover, *Snail* appears to be upstream in this genetic cascade (Aybar et al., 2003). We show here that *Snail* can repress *Delta1* expression in animal caps and in whole embryos, and that the inhibition of *Snail* activity provokes an upregulation of *Delta1* expression in the neural crest territory. Our results strongly suggest that the expression of *Delta1* in the neural crest could be patterned by the activity of *Snail*. It is worth mentioning that the effect of *Snail* on *Delta1* expression was not only seen in the ectoderm but also in the somites, where *Snail* is also expressed (Essex et al., 1993). Thus, it seems feasible that *Delta1* expression, which plays an important role in somite formation (Jen et al., 1997), could also be under the control of *Snail*. Indeed in *Drosophila*, *Snail* has been shown to represses *Delta* expression during the dorsoventral patterning of the embryo (Cowden and Levine, 2002; Ip and Gridley, 2002). It is also interesting to note that *Snail* is weakly expressed in the anterior neural fold at the early gastrula stage, but at the end of gastrulation, when *Delta1* is strongly expressed in the anterior neural fold, *Snail* expression is downregulated in that region (Aybar et al., 2003). This complementary pattern of expression between *Snail* and *Delta1* also supports the idea that *Snail* is indeed a repressor of *Delta1* transcription. Finally, *Snail* may not only serve to repress *Delta1* in the neural crest, overexpression of *Snail* induces the appearance of neural crest markers in animal caps and in whole embryos (Aybar et al., 2003). Indeed, it is likely that the influence of *Snail* on neural crest markers is independent of its repression of *Delta1*. It is important to mention that *Slug* or *Foxd3* are never expressed in the anterior neural fold, being also putative inhibitors of *Delta1* in the crest region.

The role of the Iroquois genes in establishing embryonic boundaries seems to be extended across this gene family. As mentioned before, Iroquois genes participate in the development of the imaginal disc compartment in *Drosophila* (Papayannopoulos et al., 1998; Diez del Corral et al., 1999; Cavodeassi et al., 1999), and, in *Xenopus*, *Xiro1* is involved in the formation of the midbrain-hindbrain boundary (Glavic et al., 2002). It is noteworthy that Notch signaling is also involved in both these processes (Papayannopoulos et al., 1998; Domínguez and de Celis, 1998). In *Drosophila*, the Iroquois genes influence Notch signaling through the expression of *Fringe*, thereby defining the dorsal and ventral compartments (Cavodeassi et al., 1999). In *Xenopus*, the Notch target genes *Hes1* and *Hes3* (Hirata et al., 2001), and the *Hes-related 1* gene (*Xhr1*) (Shinga et al., 2001), have been implicated in establishing the midbrain-hindbrain border, and in particular in midbrain development. Recently, *Xiro1* has been shown to be involved in the establishment of this region by controlling *Gbx2* and *Otx2* expression (Glavic et al., 2002). It is thus tempting to speculate that *Xiro1* might regulate *Hes1*, *Hes3* and/or *Xhr1* expression at the midbrain-hindbrain boundary. Here, we present evidence that *Xiro1* is also involved in the establishment of the boundary between the neural plate and the epidermis, i.e. the region in which the neural crest cells are generated.

A molecular model for neural crest induction

The data generated over the past years, together with our present observations, lead us to propose the following model for neural crest induction (Fig. 8). It should be noted that this model is predominantly based on data from the analysis of neural crest markers that are initially expressed only in the anterior neural crest. Therefore, additional studies using specific posterior neural crest markers should be carried out to determine whether our model is also valid for posterior neural crest cells.

At the early gastrula stage, the coordinate action of *Xiro1*,

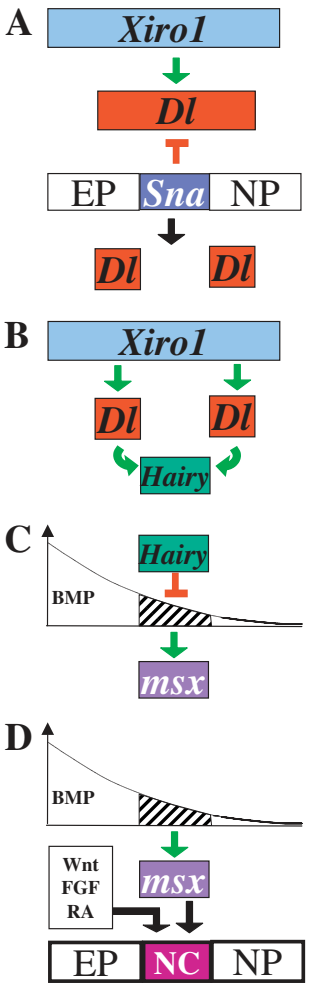

Fig. 8. A molecular model for neural crest induction. (A) At the early gastrula stage, *Xiro1* induces the expression of *Delta1* (and possibly *Serrate*) in a region that includes the neural crest territory. Later, *Snail* represses the expression of *Delta1* in the prospective neural crest region, leaving a ring of cells expressing *Delta1* at the border of the neural crest. (B) At the end of gastrulation, *Xiro1* continues upregulating *Delta1* at the border of the neural crest, and *Delta1* interacts with *Notch* to induce the expression of *Hairy2A* in the neural folds. (C) Later on, *Hairy2A* acts as a repressor of *Bmp4*, ensuring that the levels of *Bmp4* required to specify *Xmsx1* expression and the formation of the neural folds are established. (D) *Msx1*, induced in the neural folds by the threshold levels of BMP activity, in combination with other signals, such as WNTs, FGFs and retinoic acid (RA), induces the formation of the neural crest at the border of the neural plate.

as a positive regulator, and *Snail*, as a repressor, restricts the homogenous expression of *Delta1* to a ring of cells at the border of the neural crest territory (Fig. 8A). At the late gastrula stage, *Xiro1* continues to induce the expression of *Delta1* at the border of the neural crest territory, where *Delta1* interacts with Notch to activate *Hairy2A* in the neural fold region (Fig. 8B). Later in development, *Hairy2A* acts as a repressor of *Bmp4* transcription, ensuring that the optimal level of *Bmp4* to specify the neural plate border in this region is reached (Fig. 8C). This intermediate level of *Bmp4* in turn activates *msx1* expression, which is also required for the specification of the neural plate border (Tríbulo et al., 2003). Finally, the action of additional signals (WNTs, FGFs, retinoic acid) in this newly defined domain induces the production of neural crest cells (Mayor et al., 1995; Mayor et al., 1997; LaBonne and Bronner-Fraser, 1998; Deardorff et al., 2001; Villanueva et al., 2002; García-Castro et al., 2002).

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