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Indian hedgehog signaling is required for proper formation, maintenance and migration of *Xenopus* neural crest

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ABSTRACT

Neural crest induction is the result of the combined action at the neural plate border of FGF, BMP, and Wnt signals from the neural plate, mesoderm and nonneural ectoderm. In this work we show that the expression of *Indian hedgehog* (*Ihh*, formerly named *Banded hedgehog*) and members of the Hedgehog pathway occurs at the prospective neural fold, in the premigratory and migratory neural crest. We performed a functional analysis that revealed the requirement of *Ihh* signaling in neural crest development. During the early steps of neural crest induction loss of function experiments with antisense morpholino or locally grafted cyclopamine-loaded beads suppressed the expression of early neural crest markers concomitant with the increase in neural and epidermal markers. We showed that changes in *Ihh* activity produced no alterations in either cell proliferation or apoptosis, suggesting that this signal involves cell fate decisions. A temporal analysis showed that Hedgehog is continuously required not only in the early and late specification but also during the migration of the neural crest. We also established that the mesodermal source of *Ihh* is important to maintain specification and also to support the migratory process. By a combination of embryological and molecular approaches our results demonstrated that *Ihh* signaling drives in the migration of neural crest cells by autocrine or paracrine mechanisms. Finally, the abrogation of *Ihh* signaling strongly affected only the formation of cartilages derived from the neural crest, while no effects were observed on melanocytes. Taken together, our results provide insights into the role of the *Ihh* cell signaling pathway during the early steps of neural crest development.

Introduction

The neural crest (NC), a vertebrate transient and multipotent embryonic cell population, is generated at the lateral borders of the neural plate. The NC cells delaminate from the dorsal neural tube and migrate extensively to several parts of the embryo, giving rise to a wide variety of cell types, including melanocytes, cartilage, connective tissue, neurons and glia of the peripheral nervous system, and neuroendocrine cells (Le Douarin and Kalcheim, 1999). The combined action of FGF, BMP and Wnt signals that originated from the neural plate, mesoderm and nonneural ectoderm are integrated at the neural plate border to induce and specify the neural crest cells (reviewed in Aybar and Mayor, 2002; Basch et al., 2004; Sauka-Spengler and Bronner-Fraser, 2006; Steventon et al., 2005). It has been demonstrated that these secreted molecules mediate tissue interactions during the early steps of neural crest development. These signals first activate neural border specifiers, then the neural crest specifiers are triggered (e.g. *Snail1*, *Snail2*, *FoxD3*, *Sox9-10*, etc.) during the NC premigratory phase (Meulemans and Bronner-Fraser, 2004), and later these genes are essential for further NC development involving NC specification maintenance or cell migration. Our understanding of the intricate regulatory network remains preliminary. Additional signals are also required to support the initial development of the neural crest, such as the juxtacrine signal Notch/Delta, retinoic acid, or noelin-1 (Barembaum et al., 2000; Endo et al., 2002; Glavic et al., 2004).

The roles and the relative importance of each cell signaling involved in the process of neural crest induction are still controversial. The participation of BMP as a neural crest inducer appears to be insufficient (LaBonne and Bronner-Fraser, 1998; Liem et al., 1995; Marchant et al., 1998; Nguyen et al., 1998) while the role of Wnt as an inducer has been demonstrated for different animal models (Deardorff et al., 2001; Garcia-Castro et al., 2002; Heeg-Truesdell and Labonne, 2006; Saint-Jeannet et al., 1997; Wu et al., 2003). The plurality of signaling molecules required for neural crest induction is not completely understood, but has introduced the idea that some signals could control particular processes or induce specific cell precursors among the multiple cell populations derived from this tissue.

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Genetic and pharmacological research has demonstrated that Hedgehog (Hh) signaling is essential for the correct development of facial and neural crest-derived structures in the avian embryo (Benouaiche et al., 2008). The experimental reduction of Sonic hedgehog signaling affected the proliferation and survival of cells in the chicken neural tube and cranial neural crest (Ahlgren and Bronner-Fraser, 1999; Ahlgren et al., 2002). Studies conducted in mammalian embryos have shown that the severity of craniofacial defects related to holoprosencefaly correlates with the stage in which interruption in Shh signaling occurs (Chiang et al., 1996; Cordero et al., 2004). In the zebrafish embryo Hh signaling is also required for cranial neural crest morphogenesis and chondrogenesis (Wada et al., 2005). In the absence of Hh signaling through inactivation of the Hh receptor Smoothened (Smo) a complete loss of cranial skeletal elements in zebrafish embryos was observed (Chen et al., 2001). Cartilage elements were lost or mislocalized after experimental treatments with cyclopamine, an alkaloid antagonist of Hh signaling, administered to whole embryos during the gastrulation period (Eberhart et al., 2006). Recently, through the characterization of *con/disp1* mutants, it was demonstrated that multiple and temporally separated Hh-signaling events are required for the development of neurocranial skeleton in zebrafish. In these mutant embryos neural crest cell condensations failed to maintain the expression of key transcription factors for chondrogenesis (Schwend and Ahlgren, 2009).

The three members of the Hh family of secreted factors, *Sonic hedgehog (Shh), Indian hedgehog* (*Ihh*, orthologous to the mammalian *Indian hedgehog* and formerly called *Banded hedgehog*), and *Desert hedgehog* (*Dhh*, orthologous to the mammalian *Desert hedgehog* and formerly called *Cephalic hedgehog*) were found in *Xenopus* embryos (Ekker et al., 1995). *Shh* the most studied member in all species, plays crucial roles in animal developmental processes and in the homeostasis of adult tissues (Chari and McDonnell, 2007; Ruiz i Altaba et al., 2002; Wang et al., 2007). In *Xenopus*, *Shh* is required for neural tube patterning (for a review see Ruiz i Altaba et al., 2003), during limb development and regeneration (Endo et al., 1997; Endo et al., 2000; Yakushiji et al., 2009), in hypaxial muscle development (Martin et al., 2007), axonal guiding (Gordon et al., 2010), and during amphibian intestinal remodeling (Ishizuya-Oka et al., 2006). On the other hand, *Ihh* and *Dhh* displayed novel and distinct expression patterns. Interestingly, *Ihh* expression was observed in peripheral regions of the neural plate and, at that time, it was implicated in the direct induction of the cement gland (Ekker et al., 1995) and in endochondral ossification but the precise role of *Ihh* signaling in *Xenopus* neural crest development has yet to be elucidated.

Here, we investigated the role of the *Ihh* signaling pathway in the early development of the neural crest in *Xenopus* embryos. Single and double in situ hybridization revealed that *Ihh* and different components of the Hh pathway are expressed in the prospective neural crest. Gain- and loss-of-function experiments showed that *Ihh* signaling is specifically involved in the early induction of the neural crest precursors while the specification of Shh-dependent structures remains unaffected. A detailed analysis was carried out to establish the temporal requirement of *Ihh* signaling. A combined embryological and molecular approach enabled us to show the participation of *Ihh* during neural crest migration. Collectively, we describe findings that offer an insight into the *Ihh* signaling role during neural crest induction, specification and migration in the amphibian embryo.

Material and methods

Embryonic manipulation, RNA microinjection and lineage tracing

Embryos were obtained by standard procedures (Aybar et al., 2003) and staged according to Nieuwkoop and Faber (1967). Tissue dissections were performed as previously described (Aybar et al., 2003; Bonano et al., 2008); mRNA synthesis, microinjection, and lineage tracing were performed as described (Aybar et al., 2003; Bonano et al., 2008). In vitro transcribed mRNA was synthesized from pT7TSXbhh (Ekker et al., 1995), pT7TSXbhhN (Lai et al., 1995), and pT7TSXbhhΔN-C (Lai et al., 1995) by standard procedures.

Cyclopamine treatments

In order to assess Hh activity, resin (Bio-Rex RG 501-8X resin, Bio-Rad, USA) or Heparin-Sepharose (Amersham, USA) beads were soaked with 20–50 μM of cyclopamine (N-aminoethyl aminocaproyl dihydrocinamoyl cyclopamine, LC Laboratories, USA) and grafted into embryos as previously described (Bonano et al., 2008; Honore et al., 2003). Cyclopamine-soaked beads were implanted in the right neural fold region (stages 11.5, 12, 14, and 18), or in the right branchial arch region (stage 23) and the final position of beads was carefully examined under a stereoscopic microscope after the in situ hybridization procedure. BSA- or vehicle-soaked beads were grafted as control in sibling embryos at the same position.

Morpholino antisense oligonucleotide and DNA constructs

A morpholino antisense oligonucleotide (*IhhMO*) was designed and synthesized against *Xenopus laevis Ihh* including the initiation start site (from +1 to +24 bp) with the sequence 5′-CAGCACAA CCTTGGGCAACTGCAT-3′ (Gene Tools, LLC). Doses of 10–25 ng/embryo were microinjected with a lineage tracer into one blastomere at the 8- or 16-cell stage embryos. A control antisense morpholino oligonucleotide (*CoMO*) composed of a random sequence (5′-CCTCTTACCT CAGTTACAATTTATA-3′, Gene Tools, LLC) was injected as a control. The *Ihh* ORF from pT7TS*Ihh* was used as a template for DNA constructs and in vitro mRNA synthesis. For the rescue of morpholino knock-down experiments a DNA construct was prepared by changing only the codon sequence targeted by the *IhhMO* antisense oligonucleotide. Special care was taken to mutate the DNA sequence without changing the wild type *Ihh* amino acid sequence. This construct, named *CRIhh* (CR, Codon Replacement), introduced 6 mismatches in the nucleotide sequence recognized by the morpholino and was made by high fidelity PCR using the following primers: 5′-GGATCC ATGCAACTACCCAAAGTCGTTCTGCTTCTC-3′ and 5′-CTCGAGTCAGCT TTCCAACTGGACAATTC-3′ (underlined, BamHI and XhoI restriction sites, respectively). The PCR products were purified, A-tailed and cloned into pTOPO-TA (pCR II-TOPO) (Invitrogen) to produce pTOPOIICR*Ihh* which was BamHI/XhoI-digested and ligated into a pCS2+ vector digested with EcoRI/XhoI. Both fusion constructs were sequenced on both strands at junction sites by automated DNA sequencing.

In order to test the efficacy of *IhhMO* in vivo, a *IhhGFP* fusion was generated by high fidelity PCR using pT7TS*Ihh* as the template and the following primers: 5′-GGATCCATGCAGTTGCCCAAGGTTGTGC-3′ (underlined, BamHI restriction site) and 5′-CCATGGTGCCTCCAG TCTTGGCAGCGG-3′ (underlined, NcoI restriction site). A fragment containing the N-region of *Ihh* encoding 196 amino acid residues of *Ihh* was cloned into the pTOPO-TA vector, amplified and then cloned directionally into BamHI and NcoI pCS2+EGFP vector to produce the *IhhGFP* construct. In vitro transcribed mRNA of this construct was co-injected with different amounts of *IhhMO* and the GFP fluorescence was observed under a Leica fluorescence stereomicroscope. An additional construct, named *CRIhhGFP*, was synthesized to complement the testing of *IhhMO* efficacy. This construct carries 6 mismatches and conserves the amino acid coding of wt*Ihh*. The *IhhGFP* construct was used as a template to amplify by high fidelity PCR a 5′-region that was fused to GFP. The primers used were: 5′-GGATCCATGCA**ac** T**a**CCCAA**a**GT**c**GT**t**CTGCTTCTC-3′ (underlined, BamHI restriction site; lowercase and bold letters indicate mismatched bases) and 5′-CCATGGTGCCTCCAGTCTTGGCAGCGG-3′ (underlined NcoI restriction site).

In order to specifically direct the overexpression of *Ihh* to the neural crest cells, the α*3000 Snail2* promoter (Vallin et al., 2001) was fused upstream to the full length *CRIhh* sequence. The α*3000CRIhh* DNA construct was prepared from BamHI/XhoI digested pTOPO-*CRIhh* and ligated into SB*XlpSlα3000-GFP plasmid (kindly donated by Dr. Florence Broders). The α*3000CRIhh* plasmid was purified, diluted in DEPC distilled water and 200–400 pg/embryo were microinjected into one blastomere of 8- to16-cell embryos. Fluorescence was observed since stage 13.

Cartilage staining

For cartilage staining, injected and control embryos were fixed in formaldehyde at stages 45–47, washed with PBS and stained overnight in 0.2% Alcian blue/20% acetic acid in ethanol. Embryos were washed extensively with ethanol and bleached with a 1% KOH solution. Finally, the embryos were washed with 20% glycerol/2% KOH for 20 min and dehydrated through a glycerol series into 80% glycerol. Skin and muscles were manually dissected out and cranial cartilages were isolated and photographed.

RNA isolation from embryos and RT-PCR analysis

Total RNA was isolated from whole embryos or embryonic tissues after microsurgery and cDNA were synthesized as previously described (Aybar et al., 2003). The primers designed for this study were: *Ihh*: for: 5′-TACGGAATGCTGGCTAGG-3′, rev: 5′-GCTGAGTTAGAGGAG CATAAGCC-3′; *Smo*: for: 5′-GGTGCCTTGGATCTGTTGGA-3′, rev: 5′-TGGGTTCTGACACTGGATGC-3′; *Ptc1*: for: 5′-AGCACATGTTTGCACC TGTC-3′, rev: 5′-GCTTCTTTGTTGGGTGGTGT-3′; *Gli3*: for: 5′-CTTCC TGAACCCTCAGTTCC-3′, rev: 5′-CACTAGCAGATGAGCTGCTG-3′; *MyoD*: for: 5′-GGACATGGAAGTCACTGAGG-3′, rev: 5′-ATACCTGCACTCAAC TGTCC-3′; *EF1*α, for: 5′-CAGATTGGTGCTGGATATGC-3′, rev: 5′-ACTG CCTTGATGACTCCTAG-3′; *Sox10*: for: 5′-CTTCCCCCTCATGCTCACTC-3′, rev: 5′-TGCTTCACGAATGCACACAG-3′; *Pax3*: for: 5′-AGGAGACCGGATC CATCAGA-3′, rev: 5′-TGGAGCTTACCGGGTTGTCT-3′; *Ptc1* (qPCR): for: 5′-CCCTTTGGAGTTGCTGGAAG-3′, rev: 5′-CCGATGGGTTCAAACAAGGT-3′; *H4* (qPCR) for: 5′-GATAACATCCAGGGCATCAC-3′, rev: 5′-TAACCTCC GAATCCGTACAG-3′. PCR amplification with these primers was performed over 28–30 cycles and the PCR products were analyzed on 1.0% agarose gels. The gels were photographed and the semi-quantitative analysis was carefully performed using the software ImageJ (NIH, USA) as previously described (Tribulo et al., 2003). As a control, PCR was performed with RNA that had not been reverse-transcribed to check for DNA contamination. Real-time RT-PCR analysis of *Ptc1* expression was performed using *Ptc1* (qPCR) primers as indicated and the Mezcla Real real-time PCR Mix (Biodynamics SRL, Argentina) on a Bio-Rad CFX 96 (Bio-Rad, CA, USA). Retrotranscription reaction for each sample was performed using random primers, 1 μg of total RNA, 400 U MMLV-RT (Promega, USA) in a total volume of 30 μl. The real time PCR reaction mixture consisted of 10 μl of 2× Mezcla Real mix, 100 nM forward and reverse *Ptc1* (qPCR) primers, and 2 μl of a 5× dilution of the RT reaction. The cycling conditions were as follows: denaturation at 95 °C (10 s), annealing at 60 °C (15 s), and extension at 72 °C (15 s). Reactions were optimized considering RNA and primers concentrations, reaction conditions, and the fact that a single specific product was amplified as confirmed by melting curve analysis. All samples were run in triplicates and included a control without template. The histograms in each figure are representative cases of at least three independent experiments. In each case, *ornithine decarboxylase* (*ODC*) was used as an internal reference (data not shown). Each *Ptc1* expression level has been normalized to the level of *ODC*, and presented as a ratio to the neural crest *Ptc1* expression level.

In situ hybridization, TUNEL and immunohistochemistry

Antisense probes containing Digoxigenin-11-UTP or Fluorescein-12-UTP were prepared for *Ihh* (Ekker et al., 1995), *Gli3* (Marine et al., 1997), *Ptc1* (Koebernick et al., 2001; Perron et al., 2003), *Smo* (Koebernick et al., 2001; Perron et al., 2003), *Snail2* (Mayor et al., 1995), *Snail1* (Aybar et al., 2003), *FoxD3* (Sasai et al., 2001), *Msx1* (Tribulo et al., 2003), *Sox2* (Dr. R.M. Grainger, personal communication), *Trp2* (Aoki et al., 2003), *Pintallavis* (also known as *FoxA4a*), (Ruiz i Altaba et al., 1993), *XK81a* (Jonas et al., 1985), *Paraxis* (Carpio et al., 2004) and *Nkx6.2* (EST clone Xl461d15, kindly donated by Dr. Naoto Ueno) by in vitro transcription. Specimens were prepared, hybridized, and stained as previously described (Aybar et al., 2003). As control, sense probes were synthesized and hybridized and did not produce any staining. Apoptosis was detected by TUNEL staining according to the procedure previously described (Hensey and Gautier, 1998; Tribulo et al., 2004). For cartilage staining, embryos were fixed at stages 45–47 and processed as previously described (Bonano et al., 2008). Rabbit Polyclonal anti-phosphohistone-3 (Upstate Biotechnology) was used to analyze mitotic cells according the method previously described (Bonano et al., 2008; Turner and Weintraub, 1994).

Results

Expression of Ihh and Hh signaling pathway components in the neural crest

Although the expression pattern of *Ihh* has already been reported for *Xenopus* embryos (Ekker et al., 1995), we decided to analyze and compare it with the expression of different components of the Hh signaling pathway and with specific neural crest gene markers. Single and double in situ hybridizations were performed in embryos at different stages in order to complement previously published expression patterns (Fig. 1). At the end of gastrulation (stage 12.5) the expression of *Ihh* was observed at low levels in the dorsal anterior region of the embryo. This expression occurred mostly in the anterior neural plate and at the lateral border (Fig. 1A, arrowheads). At the midneurula stage (stages 14–19) the expression increased in the neural plate and neural plate borders (Fig. 1B) but was not observed along the midline (arrow). Sibling embryos stained with sense *Ihh* probes showed no labeling (Fig. 1B, inset). Transversal sections of stage 16 embryos revealed that *Ihh* expression occurred mainly in the internal ectodermal layers corresponding to the neural plate and prospective neural crest and in the somitic mesoderm (Fig. 1I). At stage 14, a double in situ hybridization of *Ihh* and the neural crest marker *FoxD3* showed a clear overlapping expression of both genes in the neural crest region (Fig. 1M) in whole mount and in sectioned embryos (Fig. 1M'). At stages 21–23, neural crest cells migrate and the expression of *Ihh* was detected in the three migrating cranial neural crest streams (Fig. 1C, arrowheads) and in the otic and optic vesicles. From stage 22 onwards, *Ihh* expression appeared localized in a conspicuous chevron-shaped band within each somite (Fig. 1C). We also analyzed the expression of other members of the Hedgehog signaling pathway. The whole-mount in situ hybridization spatial analysis of *Patched-1* (*Ptc1*) showed that this receptor is expressed in early neurula stage embryos in the anterior region of the neural plate and neural plate border (Fig. 1D). Transverse sections revealed *Ptc1* expression in the internal ectodermal layer reminiscent of *Ihh* expression pattern (Fig. 1J). *Smoothened* (*Smo*) transcripts were detectable at low levels from early neurula stage (stage 12.5) onwards in a pattern similar to *Ihh* (Figs. 1E and F). At mid-neurula stages (stages 15–19) the transverse sections showed *Smo* expression in the internal ectodermal layer and in the underlying mesoderm (Fig. 1K). By stage 22, expression was localized in the head region, in the optic and otic vesicles and in the cephalic migratory neural crest streams (Fig. 1G). The expression of Hedgehog effector genes *Gli2* and *Gli3* was also analyzed by single and double in situ

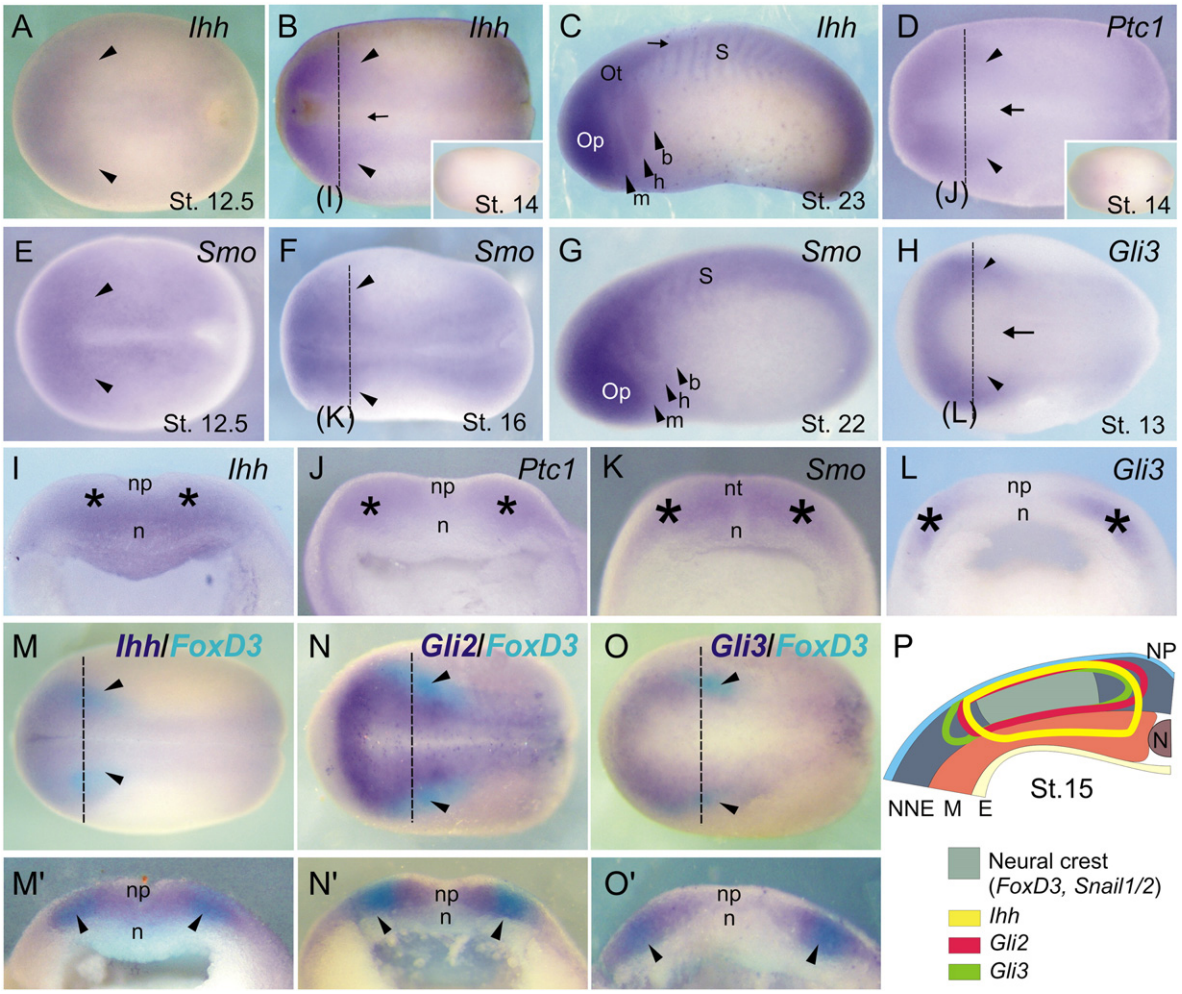


Fig. 1. *Xenopus laevis Ihh* and Hh pathway components are expressed in the neural crest. (A, B, D–F and H) Dorsal views of *Xenopus laevis* embryos, anterior side is on the left. (C and G) Lateral views. (A) *Ihh* transcripts are first detected since the late gastrula stage (stage 12.5) in the lateral domains of the neural plate (arrowheads). (B) During neurulation *Ihh* is expressed in the neural plate and in the neural plate border (arrowheads). No expression is observed in the neural plate midline (small arrow). No staining was observed in the sense probe control (inset). The line (I) indicates the site of transverse section shown in I. (C) Stage 23 embryos show *Ihh* expression in the cranial neural crest migratory streams (arrowheads), mandibular (m), hyoid (h) and branchial (b), in the optic (Op) and otic (Ot) vesicles, and in the somites (s, arrow). (D) *Ptc1* in situ hybridization in a stage14 embryo. *Ptc1* expression is observed in the neural folds (arrowheads) and in the lateral neural plate, while expression is absent in the midline (arrow). No staining was observed using the *Ptc1* sense probe (inset). The line (J) indicates the site of transverse section shown in J. (E) *Smo* transcripts are observed in the neural plate and neural plate border in stage 12.5 embryos. (F) Stage 14 embryos show *Smo* expression in the neural plate and neural folds (arrowheads). The line (K) indicates the site of transverse section shown in K. (G) In stage 22 embryos *Smo* is expressed in the neural crest migratory streams (arrowheads), optic vesicle and somites. (H) *Gli3* transcription factor is expressed in stage 13 embryos at the lateral border of the neural plate and neural folds (arrowheads). The line (L) indicates the site of transverse section shown in L. (I–L) Transverse section of embryos displayed in B, D, F, and H showing *Ihh* (I), *Ptc1* (J), *Smo* (K), and *Gli3* (L) expression. Asterisks indicate neural crest tissue; np, neural plate; n, notochord. (M–O) Double in situ hybridizations for *Ihh* (purple) and *FoxD3* (turquoise), *Gli2* (purple) and *FoxD3* (turquoise), and *Gli3* (purple) and *FoxD3* (turquoise). (M'–O') Transverse sections of embryos displayed in M–O showing that *Ihh*, *Gli2*, and *Gli3* expressions overlap with *FoxD3* in the neural crest territory. (P) Schematic diagram summarizing the expression of *Ihh*, *Gli2*, *Gli3* and neural crest markers *FoxD3*, *Snail1/2* in the left half of a midneurula stage transversally sectioned embryo. Yellow (*Ihh*), red (*Gli2*) and green (*Gli3*) lines encircle the areas of expression for each gene. NP, neural plate; N notochord; NNE, non-neural ectoderm; M, mesoderm; E, ectoderm.

hybridization. *Gli3* expression appeared in the neural fold region (Fig. 1H) and in the internal ectodermal layer at midneurula stages (Figs. 1L and O). *Gli2* transcripts were observed in the neural plate and neural plate border overlapping with the expression of the *FoxD3* marker in the most lateral domain of *Gli2* expression (Figs. 1N, N'). *Gli3* expression was found more lateral than *Gli2* and overlapping with *FoxD3* expression in its central domain of expression (Figs. 1O and O').

We also analyzed the expression of *Ihh* and members of the Hedgehog pathway by RT-PCR in neural crest, intermediate mesoderm or non-neural ectoderm explants isolated from embryos at mid-neurula stage (stage 16). As the intermediate mesoderm interacts with the neural crest region during neurulation (Bonano et al., 2008; Steventon et al., 2009), we considered it interesting to establish the expression of Hedgehog components in this tissue. The results confirmed that *Ihh* and members of the Hedgehog cell signaling pathway *Ptc1*, *Smo*, and *Gli3* genes are expressed in the neural crest tissue (Fig. 2A). *Ihh*, *Ptc1*, *Smo* and *MyoD* expression was detected in the intermediate mesoderm. Moreover, we independently analyzed *Ptc1* expression levels by qPCR as readout of Hedgehog signaling in different regions of stage 13 embryos. We were able to detect *Ptc1* expression that is indicative of an active Hedgehog signaling pathway in the neural crest tissue. In comparison with the neural crest *Ptc1* levels, the notochord, intermediate mesodermal tissue, and the non-neural ectoderm showed almost similar expression levels. The neural plate showed the highest expression level, reaching almost 3-fold the neural crest level. We conclude that *Ihh* and the Hedgehog *Smo* and *Gli* genes are expressed in the neural crest, that the *Ihh* signaling is active in the neural crest and that the *Ihh* signal is also available from the subjacent mesoderm.

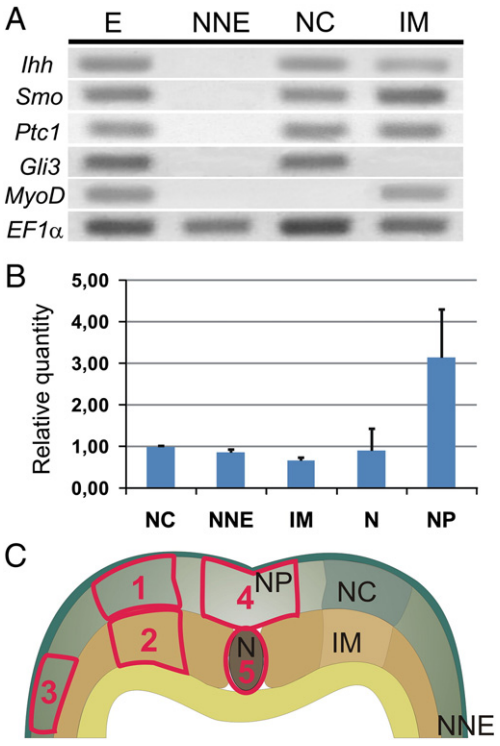


Fig. 2. Analysis of *Ihh* and Hh members' expression. (A) RT-PCR analysis of the expression of *Ihh*, *Smo*, *Ptc1*, *Gli3* and *MyoD* in neural crest, intermediate mesoderm and non-neural ectoderm explants. Explants were dissected out from stage16 embryos (see Material and methods). *EF1α*, loading control. (B) Ptc1 expression levels were analyzed as a read-out of Hedgehog signaling activity in different embryonic regions, as indicated in C. Each *Ptc1* expression level has been normalized to the level of *ODC*, and presented as a ratio to the neural crest *Ptc1* expression level. (C) Schematic drawing depicting a transverse section of a typical midneurula embryo indicating the dissected explants: 1, NC, neural crest explants; 2, IM, intermediate mesodermal explants; 3, NNE, non-neural ectoderm; 4, NP, neural plate; 5, N, notochord.

Ihh signaling is required for neural crest initial development

The expression patterns of *Ihh* and Hedgehog pathway components strongly suggest that *Ihh* signaling could have a role during the initial steps of neural crest development. We investigated the activity of *Ihh* by examining the effects of knocking down its expression using an antisense morpholino oligonucleotide directed against the initial codons of *Ihh* (*IhhMO*). We first analyzed the expression of GFP fusion constructs. *IhhGFP*-injected embryos (Figs. 3A and A') and control morpholino- and *Ihh*GFP-coinjected embryos (Figs. 3B and B') showed GFP fluorescence. Then we analyzed *IhhMO* oligonucleotide efficiency and confirmed that *IhhMO* was able to inhibit the translation of the GFP-tagged form of *Ihh* in a dose-dependent manner (*IhhGFP*, Figs. 3C and D). We also prepared an *IhhGPF* fusion construct carrying a 6-bp mutation in the morpholino recognition site (*CRIhhGFP*). The expression of this construct was not affected when it was coinjected with *IhhMO* at the same doses (Figs. 3E and F).

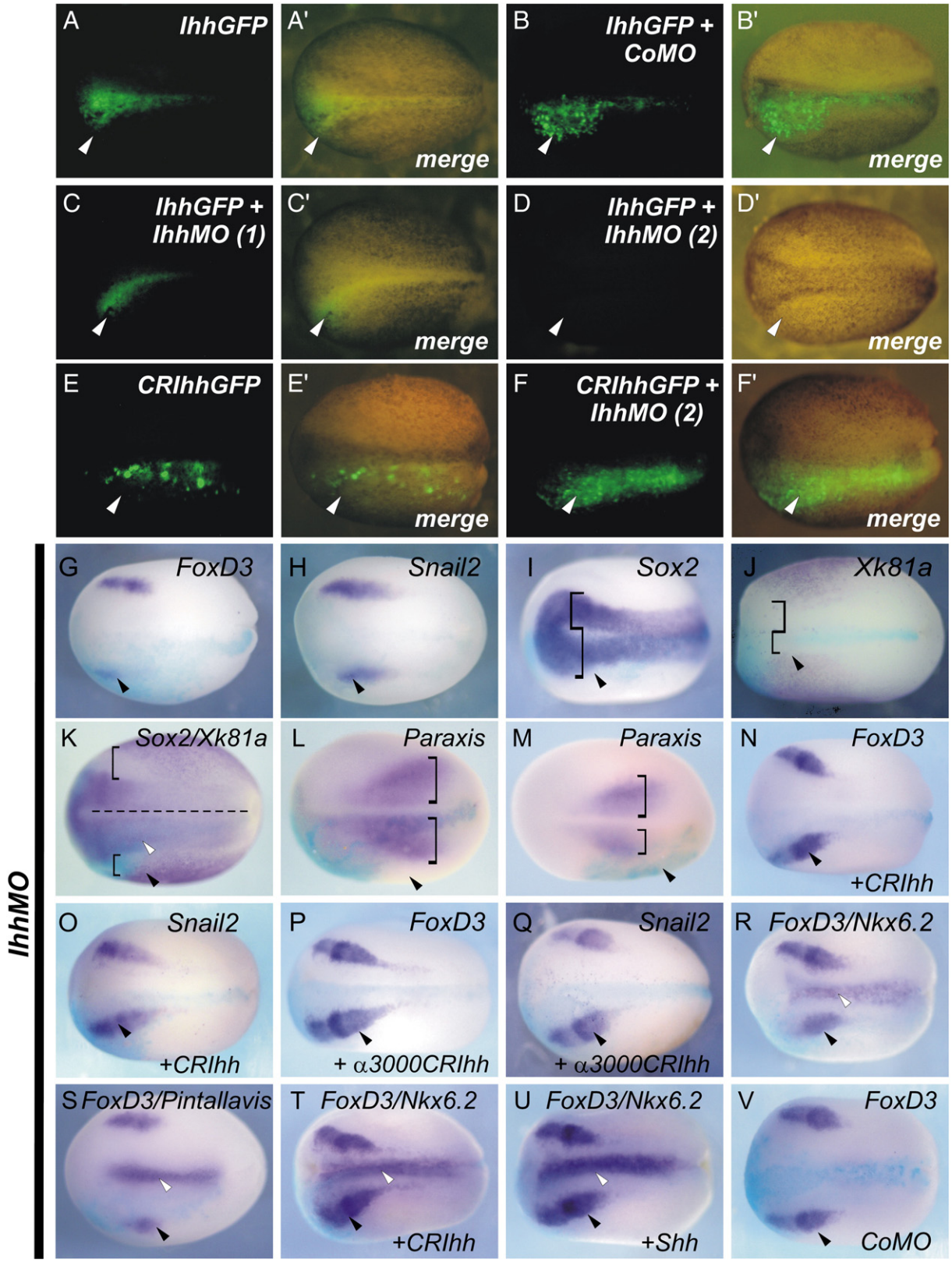
To assess the participation of *Ihh* signaling during neural crest induction, embryos were injected at the 8-cell stage in one dorsal blastomere with *IhhMO* (25 ng/embryo) (Figs. 3G–U) or an equivalent amount of a control morpholino (*CoMO*, Fig. 3V). The embryos injected with *CoMO* showed normal morphology and expression of the neural crest marker *FoxD3* (Fig. 3V, 100%, n = 29), while embryos injected with equivalent amounts of *IhhMO* failed to form the neural fold and showed an inhibition of *FoxD3* and *Snail2* expression at the injected side (Fig. 3G, 72% n = 98 and 3H, 75% n = 120; *Snail1* not shown, 71% of inhibition n = 32). In the *IhhMO* injected embryos the neural plate markers *Sox2* (Fig. 3I, 66%, n = 45) and the epidermal marker *XK81a* were expanded (Fig. 3J, 69% of expansion, n = 57), probably as a consequence of the reduction in the neural crest, and no overlapping between the neural plate and the epidermis was observed by double in situ hybridization of *Sox2* and *XK81a* markers (Fig. 3K, reduced NC domain in 78% of embryos, n = 27). In order to rule out the possibility of effects on mesodermal tissue we evaluated the expression of the mesodermal marker *Paraxis* (Carpio et al., 2004). We observed no effect on *Paraxis* expression in the injected side when embryos were microinjected in one dorsal blastomere of 8–16 cell embryos (Fig. 3L, 93%, n = 27), which indicates that the effects produced by *IhhMO* were directed only to the ectodermal tissue. When embryos were injected targeting the microinjection to the mesoderm (vegetative region of blastomere D1.1, Hirose and Jacobson, 1979) in one ventral blastomere the expression of *Paraxis* was found to be slightly reduced (Fig. 3M, 91%, n = 35). These results show that *Ihh* depletion in the ectoderm promotes a specific loss of neural crest progenitors, thus suggesting that this cell signal is involved in the early specification of the neural crest cells.

To evaluate the specificity of the morpholino effects, we decided to determine whether the phenotype of *Ihh*-depleted embryos could be rescued by restoring the *Ihh* function. We co-injected an *Ihh* construct (*CRIhh*) carrying a 6-bp mutation within the recognition motif of *IhhMO* in order to avoid hybridization between the morpholino and the coinjected mRNA. When *IhhMO* was co-injected with *CRIhh* mRNA a strong rescue in *FoxD3* and *Snail2* expression (Fig. 3N, 72% n = 98 and 3O, 70% n = 80) was observed. This effect was specifically produced by the coinjection of *Ihh* mRNA but not by the coinjection of unrelated mRNAs like GFP mRNA (not shown). Thus, we concluded that *IhhMO* was able to specifically knock down *Ihh* expression and therefore abolish its activity. Besides, the co-injection of *IhhMO* with *wtIhh*, *NIhh* (not shown) and a *Snail2*-promoter driven *CRIhh* construct restored to normal the expression levels of the neural crest markers *FoxD3* and *Snail2* (Fig. 3P 86%, n = 77and 3Q, 81%, n = 76). In order to rule out the possibility that *Ihh* knock-out could affect the specification of the embryonic midline, we simultaneously evaluated by double in situ hybridization the expression of the *FoxD3* marker gene along midline markers *Nkx6.2* and *Pintallavis* (*FoxA4a*). We observed that while neural crest expression of *FoxD3* was strongly reduced the expression of midline markers remained unaffected (Fig. 3R, 84% n = 32 and 3S, 85% n = 35, respectively). The effect on neural crest induction was rescued by the co-injection of *CRIhh* and by the microinjection of *Shh* mRNA targeted to the neural plate border. In both cases, the increase in *Ihh* activity or the ectopically targeted activity of *Shh* at the neural plate border rescued neural crest induction without affecting embryonic midline specification (Fig. 3T, 93% n = 30 and Fig. 3U, 89% n = 28).

We also analyzed the effects on neural crest specification of the *Ihh* dominant negative construct *IhhΔN-C*. This construct was found to block in vivo the activity of *Ihh* and N*Ihh* (Lai et al., 1995). We observed that the overexpression of *IhhΔN-C* mRNA (1–3 ng/embryo) diminished the expression of *FoxD3* (Fig. 4A, 59% n = 49) and *Snail2* neural crest markers (Fig. 4B, 55%, n = 56) and increased the neural plate and epidermis territories expressing *Sox2* (Fig. 4C, 48% n = 34) and *XK81a* (Fig. 4D, 51% n = 39), respectively. The effect of *IhhΔN-C* was rescued when *wtIhh* and *NIhh* were coinjected (Fig. 4E, 81% n = 53 and Fig. 4F, 62% n = 55). Taken together, the effect of *IhhMO* and *IhhΔN-C* overexpression indicates that *Ihh* signaling is required during the initial steps of neural crest formation.

In order to establish whether *Ihh* signaling might participate in the control of neural crest specification, we microinjected *Ihh* and *NIhh* mRNAs into the animal pole of a dorsal blastomere of 4-cell stage embryos. *NIhh* is an active N-terminal fragment of *Ihh* that mimics its function (Lai et al., 1995). *Ihh* injection led to an expansion of the neural crest territory analyzed by the expression of *FoxD3* (Fig. 5A, 71% n = 62), *Snail2* (Fig. 5B, 69% n = 65) and *Snail1* (not shown, 77% n = 43). This effect also produced a concomitant reduction in the *Sox2* (Fig. 5C, 57% n = 67) neural plate marker and the *XK81a* epidermal

marker (Fig. 5D, 55% n = 64). Similar effects on neural crest expression were observed when we overexpressed *CRIhh* (not shown, 84% n = 67) and *NIhh* mRNAs (Figs. 5E–H, *FoxD3* 61% n = 80; *Snail2* 58% n = 72; *Sox2* 60% n = 53, and *Xk81a* 55% n = 54). With the overexpression of *wtIhh*, double in situ hybridization for *Sox2* and *XK81a* showed that the expansion of neural crest territory was accompanied by a concomitant reduction in the neural plate and epidermal domains (Fig. 5I, 68% n = 28). The *Snail2* promoter-driven overexpression of *Ihh* (Fig. 5J) also led to a marked increase in neural crest territory evidenced by the expression of the *FoxD3* marker (Fig. 5K, 100% n = 64 and Fig. 5L 97%, n = 36). No changes were observed in the expression of the midline marker *Nkx6.2* in these α*3000CRIhh*-injected embryos (Fig. 5L).



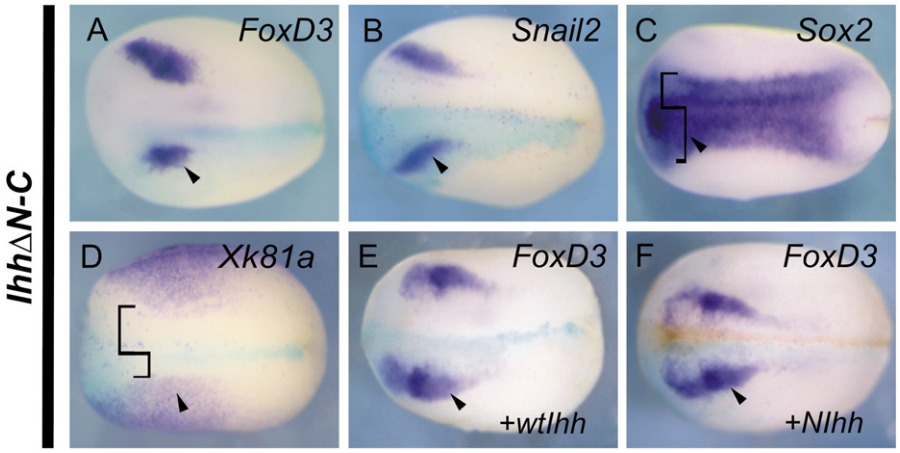


Fig. 4. *Ihh* is required for neural crest specification (continuation). (A–F) Analysis of the overexpression of dominant negative *Ihh*ΔN-C on neural crest specification. (A and B) *Ihh*ΔN-C-injected embryo shows a diminished expression of *FoxD3* and *Snail2* markers. (C and D) Expression of the neural plate marker *Sox2* and the epidermal marker *XK81a* is expanded on the *Ihh*ΔN-C-injected side. (E and F) *FoxD3* expression was rescued in embryos co-injected with wt*Ihh* (E) or N*Ihh* construct (F). Arrowheads indicate the injected side. Brackets indicate the width of the neural plate (C), and the width of the neural plate plus the neural crest domain (D).

These observations suggest that the expansion of the neural crest as a result of the ectopic expression of *Ihh* is produced by the transformation of the epidermis and of part of the neural plate region into prospective neural crest cells.

Ihh signaling is not involved in the control of cell proliferation or apoptosis in the neural crest

It has been previously shown that the apoptotic process is involved in the development of the neural crest (Honore et al., 2003; Tribulo et al., 2004). Moreover, the Hedgehog cell signaling pathway has been implicated in the control of cell proliferation in many cell systems also including neural crest cells (Ahlgren et al., 2002; Dyer and Kirby, 2009; Fu et al., 2004; Marcelle et al., 1999), etc.). We observed significant changes in the area expressing neural crest markers when we activated or blocked the expression or function of *Ihh*. There is a possibility that these changes were produced by changes in the cell proliferative status or by changes in the apoptotic process. To rule out such possibility we therefore decided to investigate cell proliferation and apoptosis during *Ihh* gain or loss of function.

The microinjection of *Ihh* or *Ihh*MO produced no detectable changes in the cell proliferation status assessed by the detection of a phosphorylated form of H3 histone (Fig. 6A n = 24; Fig. 6C n = 26). We were also unable to find significant changes in apoptotic cell death evaluated by TUNEL staining between the control side and the treated side of *Ihh*- or *IhhMO*-injected embryos (Fig. 6B, 94% n = 18; Fig. 6D 0%, n = 21), or in embryos injected with *CoMO* (0% increase in TUNEL staining, n = 20, not shown). Taken together, these results led us to conclude that *Ihh* signaling does not play a role in the control of cell proliferation or apoptosis, and that the alterations observed in the neural crest territories after changes in the activity of the *Ihh* pathway could represent ectodermal cell fate decisions.

Temporal analysis of Ihh requirement for neural crest induction

Antisense morpholino technology provides a very specific way of inhibiting the expression of a single gene. However, this approach is limited since it is difficult to control the timing of morpholino activity. In order to precisely control the moment of *Ihh* signaling blocking we used the Hedgehog-specific antagonist cyclopamine. Cyclopamine is a steroidal alkaloid found in the plant *Veratrum californicum* which interrupts the hedgehog pathway activation by binding directly to the Smoothened (Smo) transmembrane receptor (Chen et al., 2002).

We assessed the inhibition of *Ihh* signaling by using cyclopamine specifically delivered at the neural plate border at three different moments of development. To achieve this goal acrylic beads soaked with different amounts of cyclopamine were grafted into the right prospective neural plate border of stage 11.5 (Figs. 7A and B), stage 12 (late gastrula, Figs. 7C–G), and midneurula stage embryos (stage 14, Figs. 7I and J). Embryos were cultured until different stages and the expression of gene markers was analyzed by in situ hybridization. Treatment of stage 11.5 embryos with 20 μM cyclopamine-soaked beads markedly downregulated *Snail1* (Fig. 7A, 64% n = 22), *Snail2* (Fig. 7B, 56% n = 18) and *Msx1* (Fig. 7C, 66% n = 18) expression in the neural crest territory. A similar effect on the expression of the same marker was observed when cyclopamine-soaked beads were grafted at stage 12 embryos (Fig. 7D, *FoxD3*, 67% n = 41, and Fig. 7E, *Snail2*, 56% n = 43, respectively). The pharmacological inhibition by

Fig. 3. *Ihh* is required for neural crest specification. (A–F) In vivo efficiency of *Ihh* antisense morpholino oligonucleotide (*Ihh*MO). Dorsal views of *Xenopus laevis* embryos under a fluorescence stereoscopic microscope, anterior side is on the left. White arrows indicate the injected side. (A'–F') Fluorescence and clear field images of each embryo are superposed and shown in merged images. (A and A') Embryo injected with mRNA encoding *Ihh*GFP (1 ng/embryo) showing GFP fluorescence. (B and B') Embryo injected with *Ihh*GFP mRNA (1 ng/embryo) and CoMO (30 ng/embryo). (C, C', D and D') Embryos injected with *Ihh*GFP mRNA (1 ng/embryo) and *Ihh*MO (C and C', low dose (1), 10 ng/embryo; D and D', high dose (2), 20 ng/embryo). No embryo shows GFP fluorescence at a high dose of *Ihh*MO. (E and E') Embryo injected with CR*Ihh*GFP mRNA showing GFP fluorescence. (F and F') Embryo injected with CR*Ihh*GFP mRNA and a high dose (2) of *Ihh*MO (20 ng/embryo). The expression of CR*Ihh*GFP was not affected by the presence of the morpholino oligonucleotide. (G–V) Analysis of *Ihh*MO effects on neural crest specification. Dorsal views of *Xenopus laevis* embryos, anterior side is on the left. Arrows indicate the injected side. (G and H) *Ihh*MO-injected embryos show inhibition of *FoxD3* and *Snail2* neural crest markers. (I and J) Expression of the neural plate marker *Sox2* and the epidermal marker *XK81a* is expanded on the *Ihh*MO-injected side. The brackets indicate the width of neural plate (I), and the width of neural plate plus neural crest domain (J). (K) Double in situ hybridization for *Sox2* and *XK81a* genes evidenced that *Ihh*MO led to the reduction in the prospective neural crest domain in the injected side. The brackets indicate the width of the neural crest domain. (L and M) Expression of the mesodermal marker *Paraxis*. No effect was observed in a dorsal blastomere of 8–16 cell embryos (L). The targeting of *IhhMO* to the mesoderm by microinjection of the vegetative region of blastomere D1.1 produced a reduced *Paraxis* expression in the injected side (M). (N and O) Co-injection of *Ihh*MO and CR*Ihh* mRNA rescues *FoxD3* and *Snail2* expression, respectively. (P and Q) The co-injection of CR*Ihh* specifically driven by the *Snail2* promoter (α*3000CRIhh*) rescues the expression of *FoxD3* and *Snail2* neural crest markers. (R and S) The microinjection of *Ihh*MO into a dorsal blastomere of stage 16 embryos decreases the expression of *FoxD3* in the neural crest but produces no effects on the midline markers (double in situ hybridization, white arrowheads, *Nkx6.2* and *Pintallavis-FoxA4a*). (T and U) The effects of *Ihh*MO on the expression of the neural crest marker *FoxD3* is rescued by the directed co-injection of CR*Ihh* (Q) or *Shh* to the neural fold region (U). (V) CoMO-injected embryos show normal expression of *FoxD3*.

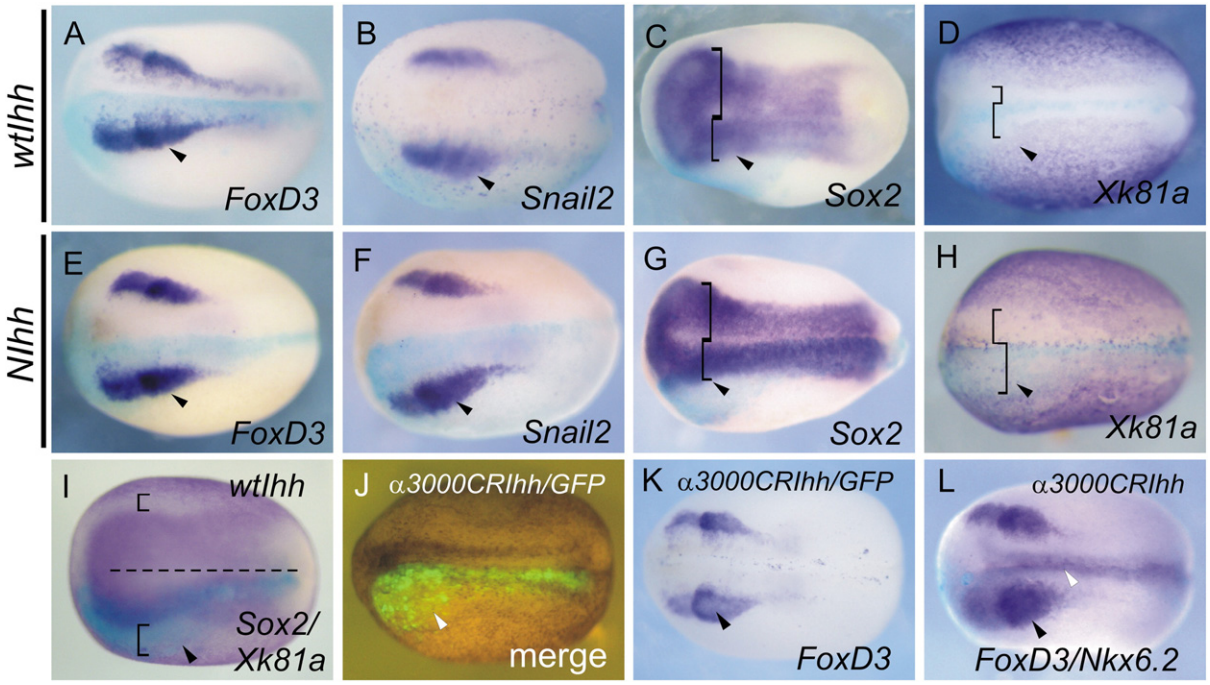


Fig. 5. *Ihh* participates in the early neural crest specification. Dorsal views of *Xenopus laevis* embryos, anterior side is on the left. Injected side is indicated by an arrowhead. wt*Ihh*-injected embryos show increased expression of *FoxD3* (A) and *Snail2* (B). The expression of the neural plate marker *Sox2* (C) and the epidermal marker *XK81a* (D) appear reduced on the injected side. (E–H) The injection of *NIhh* mRNA increases the expression of *FoxD3* (E) and *Snail2* (F) while the markers *Sox2* (G) and *XK81a* are reduced (H). (I) Double in situ hybridization for *Sox2* and *XK81a* genes evidenced the expansion of the prospective neural crest domain in the injected side. The brackets indicate the width of the neural crest domain. (J–L) The *Snail2* promoter-driven (*α3000CRIhh*) overexpression of the *CRIhh* construct increases the expression of *FoxD3* in the neural crest. The same embryo shown in J was hybridized for *FoxD3* and is depicted in K. (L) *α3000CRIhh*-injected embryos show increased *FoxD3* expression and normal expression of *NKx6.2* in the embryo midline (double in situ hybridization). Brackets indicate the width of the neural plate (C and G), and the width of the neural plate plus the neural crest domain (D and H).

cyclopamine produced an increase in the expression of the neural plate marker *Sox2* (Fig. 7F, 63% n = 44) and *XK81a* (Fig. 7G, 61% n = 39) expressed in tissues adjacent to the neural crest. Although the cyclopamine-soaked beads were implanted into the right neural plate border and this location is far from the embryo midline, we decided to test for possible effects on the midline gene marker *Nkx6.2* (Fig. 7H). We observed no effects on *Nkx6.2* expression while cyclopamine local treatment only reduced the neural crest expression of *FoxD3* (Fig. 7H, 96% n = 26). No effect on the expression of the neural crest marker *FoxD3* was observed when BSA-soaked beads were grafted at stage 12 (Fig. 7I, 95% n = 22).

When cyclopamine-soaked beads were grafted on the neural crest region at the midneurula stage (stage 14), a weaker effect was observed on the treated side of the embryos and the expression of neural crest markers *FoxD3* and *Snail2* showed only a mild but consistent downregulation (Fig. 7J, 59% n = 39, and Fig. 7K, 70% n = 30). No effects on the expression of *FoxD3* was found when a BSA-soaked bead was grafted on stage 14 embryos (Fig. 7L, 86% n = 21).

The analysis of the expression of the components of the Hedgehog pathway suggested that this cell signaling could be active at the neural plate border during the earliest stages of neural crest induction

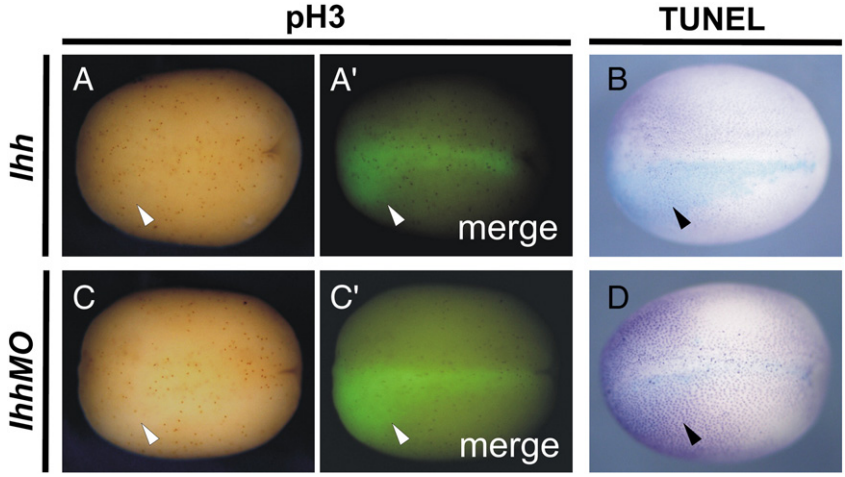


Fig. 6. *Ihh* gain- and loss-of-function produce no changes in cell proliferation and apoptosis. Dorsal views of *Xenopus laevis* embryos, anterior side is on the left. (A and C) The mitotic nuclei were visualized by whole-mount immunostaining using anti-phospho-Histone H3 antibody. White arrows indicate the injected side. (B and D) Apoptotic nuclei were labeled by TUNEL. *Ihh*- and *IhhMO*-injected sides show no changes in cell proliferation of apoptosis compared with the respective control sides. Black arrowhead indicates the injected side.

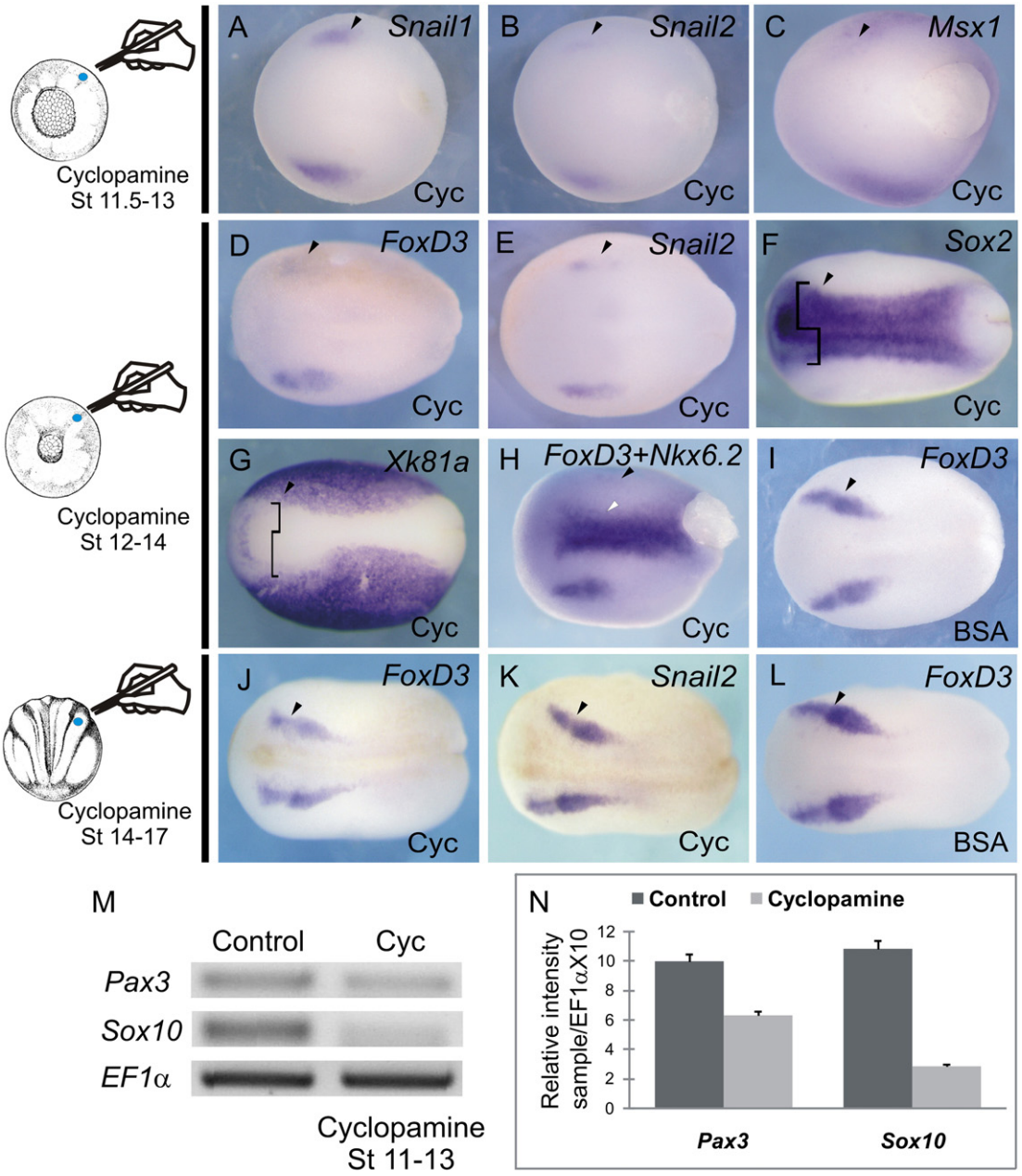


Fig. 7. Temporal requirement of *Ihh* signaling during early neural crest development. Dorsal views of *Xenopus laevis* embryos, anterior side is on the left. Stage 11.5 (A–C), 12 (D–H) or 14 (J, K) embryos were grafted on the right neural fold with a cyclopamine-soaked bead. Embryos were cultured until stage 13 (A–C), 14 (D–H) or 17 (J and K), when the expression pattern of marker genes was analyzed. Arrowheads indicate the grafted side. (A–C) Early treatment of neural folds with cyclopamine leads to a reduction in the expression of neural crest markers *Snail1* (A), *Snail2* (B) and *Msx1* (C). (D–H) Cyclopmine-soaked beads grafted in stage 12 also produced a decrease in the expression of neural crest markers *FoxD3*, *Snail2* (D and E) and an expansion of the neural plate (F) and prospective epidermis (G) on the treated side. Cyclopamine-soaked beads grafted on the right side of embryos produced no change in the expression of the midline marker *Nkx6.2* (double in situ hybridization). (J and K) Cyclopamine-loaded beads grafted at stage 14 produced a less intense decrease in the expression of *FoxD3* and *Snail2* markers on the treated side. (I and L) No changes in the expression of *FoxD3* were observed when BSA-soaked beads were grafted on stage12 or stage14 embryos. (M) Neural plate border explants were dissected out at stage 11 and incubated until stage 13 in 3/8 NAM solution or 3/8 NAM solution containing 20 μM cyclopamine, and the expression of *Pax3* and *Sox10* was analyzed by RT-PCR. (N) Quantification of the gel is shown in M, where the results are expressed as Relative Intensity (sample/EF1α × 10).

(stages 11 and 12). We decided to assess the effects of cyclopamine in neural plate border explants by analyzing the expression of early specification neural crest gene markers (Fig. 7M). We assessed *Pax3* and *Sox10* gene markers as they are considered a neural plate border specifier and neural crest specifier, respectively (Meulemans and Bronner-Fraser, 2004). The neural plate border explants were dissected out from stage 11 embryos and incubated in 20 μM cyclopamine until stage 13 when they were processed for RT-PCR. Cyclopamine-treated explants showed a reduced expression of both *Pax3* and *Sox10* markers. Notably, the effect of cyclopamine on *Sox10* was more intense as the expression of this marker showed approximately a 3.5-fold reduction compared to control explants (Fig. 7M).

Thus, the pharmacological blockade by cyclopamine was able to reproduce the phenotype obtained by the directed microinjection of morpholino oligonucleotides, showing that *Ihh* activity is required for the early steps of neural crest induction. Furthermore, these results suggest that at midneurula stages the specification of neural crest is still in progress.

Ihh signaling is necessary for the maintenance of neural crest specification

It has been shown that the mesoderm plays a role in neural crest specification and maintenance (Bonano et al., 2008; Bonstein et al., 1998; Mancilla and Mayor, 1996; Marchant et al., 1998; Monsoro-Burq et al., 2003; Steventon et al., 2009). Our results suggest that there is a later requirement for *Ihh* signaling during specification in the midneurula stages (Fig. 7). We decided to analyze the participation of mesoderm since the early steps of neural crest induction and the participation of *Ihh* signaling from the late gastrula to the late neurula stages.

NC explants dissected at late gastrula stage (stage 12) and immediately fixed the expressed *FoxD3* marker (Fig. 8B). *FoxD3* expression was observed both in explants that were dissected out at stage 12 and in explants incubated until stage 16 containing (NC + M) or not (NC) the underlying mesodermal tissue (Figs. 8C and D). When NC + M explants were incubated in the presence of 50 μM cyclopamine the *FoxD3* expression was downregulated (Fig. 8E).

A strong expression of *FoxD3* was observed in neural crest explants dissected at stage 14 or 17 and fixed immediately (Figs. 8F and J). When equivalent stage14 neural crest explants with no mesoderm were cultured until the equivalent of stage 18, the expression of *FoxD3* was notoriously reduced (Fig. 8G). Conversely, in the neural crest explants containing the subjacent mesoderm (NC + M) the expression of *FoxD3* was strong and similar to control explants (Fig. 8H). When these NC + M explants were incubated with 50 μM cyclopamine the expression of *FoxD3* almost completely disappeared (Fig. 8I). At later stages, in NC explants that were dissected containing no mesoderm at stage 17 and cultured to equivalent stage 23, the expression of *FoxD3* was lost (Fig. 8K), while the explants containing mesoderm conserved the expression of this gene (Fig. 8L). In contrast, in the NC + M explants cultured until stage 23 in 50 μM cyclopamine the expression of *FoxD3* was abolished (Fig. 8M).

Taken together, these findings strongly suggest that mesodermal requirement increases from gastrulation to late neurula stages and that *Ihh* signaling is required during the same period.

Ihh signaling is required for neural crest migration

Ihh transcripts were found to be expressed in the premigratory and migratory neural crest (Figs. 1 and 2). Moreover, our NC explants culture approach revealed that the mesoderm–neural crest relationship is progressively required from early to late neurulation stages to sustain the specification status of neural crest cells (Fig. 8). In these NC explants a Hedgehog signal is also required. All these findings suggest that *Ihh* signaling might also participate in the migratory process. We blocked the Hedgehog pathway using cyclopamine-loaded beads implanted in the neural crest region of stage 18 embryos, when neural crest cell

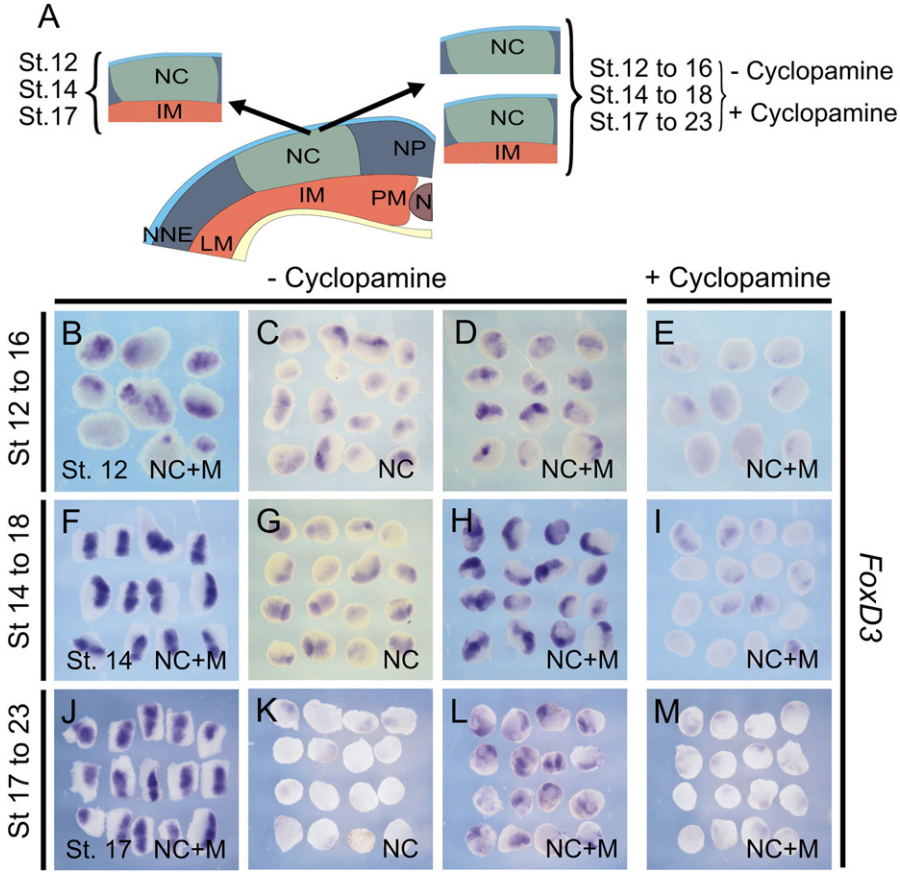


Fig. 8. *Ihh* signaling is necessary for neural crest maintenance. (A) Neural crest explants (NC) were prepared by dissection of the neural crest only, or by including the underlying mesoderm (NC + M) in the explants. Groups of explants were fixed immediately after excision (left), at stages 12, 14 or 17. Groups of explants (NC only or NC + M) were cultured until stages 16, 18 or 23 in the presence or absence of cyclopamine. (B, F and J) NC explants removed from embryos at different stages express *FoxD3* when fixed at the moment of dissection. (C and D) NC explants dissected from stage 12 embryos and cultured until stage 16 express *FoxD3* at similar levels regardless of the presence of the mesoderm. (D) The NC explants isolated at stage 12 and cultured until stage 16 in the presence of 20 μM cyclopamine lose *FoxD3* expression. (G) NC explants dissected from stage 14 embryos show a mild inhibition of *FoxD3* expression when they were cultured in the absence of mesodermal tissue. (H) A strong expression of *FoxD3*, similar to control explants, was observed in NC + M explants. (I) Culture of NC + M explants dissected from stage 14 embryos in 20 μM cyclopamine abolishes the expression of *FoxD3* neural crest marker. (K) NC explants dissected at stage 17 without mesoderm and cultured until stage 23 lose *FoxD3* expression compared with NC + M explants (L), revealing that signals from the mesoderm are required for the maintenance of specification. (M) Cyclopamine treatment of NC + M explants isolated from stage 17 embryos produced inhibition of *FoxD3* expression.

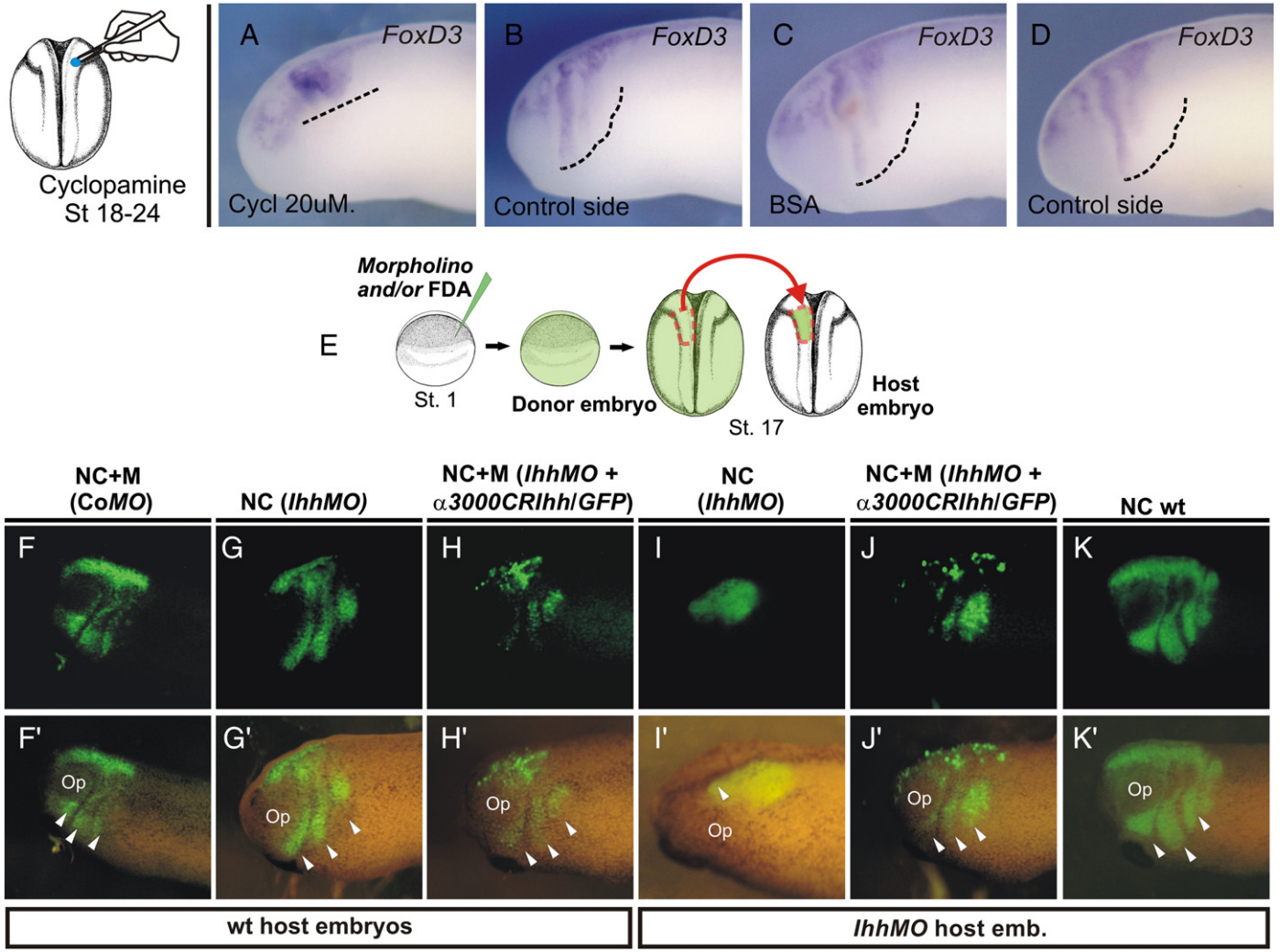


Fig. 9. *Ihh* signaling is required for neural crest migration. The anterior side of the embryos is on the left. (A) Stage 18 embryos were grafted on the right neural crest region with a 20 μM cyclopamine-soaked bead. Embryos were cultured until stages 21–23 and the expression pattern of marker genes was analyzed. *FoxD3* expressing neural crest cells show arrested migration and accumulated laterally to the hindbrain. The leading edge of migration is indicated by dashed lines. (C) Embryos grafted with control BSA-soaked beads show normal neural crest migration compared with the control untreated side (D). (E) Schematic drawing indicating the experimental procedure for the analysis of *Ihh* participation during neural crest migration. One-cell stage embryos were microinjected with *CoMO*, *Ihh*MO, α*3000CRIhh* construct and lineage tracer. When embryos reached stage 17, neural crest explants containing the underlying mesodermal tissue (NC + M) or neural crest tissue alone (NC) were grafted into wild type or *IhhMO* injected host embryos. (F and F') *CoMO*-injected NC + M explants show normal migration when transplanted into wild type host embryos. (G and G') *Ihh*MO-injected NC explants grafted into normal wild type host embryos show normal neural crest migration. (H and H') *Ihh*MO- and α*3000CRIhh*-coinjected NC + M explants grafted into wild type embryos show normal neural crest migration. The *CRIhh* construct rescued neural crest and produced a normal migratory cell population. The mesodermal source of *Ihh* is able to support the migration. (I and I') *IhhMO*-injected NC explants grafted onto *IhhMO*-injected host embryos show no migration. (J and J') *IhhMO*- and α*3000CRIhh*-coinjected NC + M explants grafted onto wild type embryos show normal neural crest migration. (K and K') Wild type NC explants grafted into *IhhMO*-injected host embryos show normal migration. The neural crest production of *Ih*h is able to sustain normal cell migration.

migration is about to begin. We observed that cyclopamine produced a clear effect on neural crest cell migration as in the treated side the front of migration, revealed by *FoxD3* expression, migrated less than in the control side (Fig. 9B, 72% n = 42). Control BSA- or saline solution-soaked beads produced no effect on neural crest migration (Fig. 9C, 81% n = 33).

As *Ihh* was also expressed in the mesoderm underlying the neural crest (Figs. 1 and 2), we then wanted to determine the role of this mesodermal source in the migratory process. In order to assess its participation, we performed a transplantation approach transferring neural crest tissue (NC) or neural crest tissue containing the underlying mesodermal tissue (NC + M) to stage 17 wild type- or *IhhMO*-injected embryos. Donor embryos were microinjected with *IhhMO*, α*3000CRIhh* or *CoMO* and FDA as a lineage tracer and neural crest explants were grafted orthotopically onto host embryos (Fig. 9E). When *CoMO*-injected neural crest grafts were transplanted to normal wild type embryos a normal cranial neural crest migration pattern was observed (Fig. 9F, 86%, n = 18). Strikingly, when *IhhMO*-injected NC neural crest explants containing no underlying mesoderm were grafted onto normal host embryos the migration pattern was completely normal, suggesting that the *Ihh* signal emanating from the subjacent mesoderm rescued neural crest migration (Fig. 9G, 75%, n = 20). The migration of neural crest cells from a *Ihh*MO-injected NC + M graft was rescued by the co-injection of the α*3000CRIhh*/GFP constructs (Fig. 9H, 78%, n = 18). When lineage tracer-injected NC normal explants were transplanted to *IhhMO*-treated embryos a normal neural crest migration could be seen (Fig. 9K, 77%, n = 22). In contrast, *IhhMO*-injected NC grafts transplanted into *IhhMO*-injected embryos showed no cranial neural crest migration (Fig. 9I, 100%, n = 19). Additionally, the migratory behavior of NC + M explants taken from *IhhMO*-injected embryos was rescued by the coinjection of α*3000CRIhh* and a normal migratory pattern in the cephalic neural crest was observed (Fig. 9J, 90%, n = 18).

Taken together, these observations indicate that *Ihh* signaling is required for the normal migration of cranial neural crest cells. Furthermore, our findings suggest that *Ihh* could act in both an autocrine and a paracrine manner to support neural crest migration.

Ihh is required for craniofacial derivatives development

We decided to investigate the effect of *Ihh* knockout on neural crest derivatives after the injection of *Ihh* morpholino antisense oligonucleotides (Fig. 10A). The analysis of cartilage morphology by Alcian Blue staining of *IhhMO*-injected embryos showed a severe reduction in craniofacial cartilages in the *BhMO*-injected side (Fig. 10A, 66% n = 80). The gross morphology analysis of craniofacial cartilages revealed that neural crest-derived cartilages (Meckel's, ceratobranchial and ceratohyal cartilages) were markedly reduced. The injection of *CoMO* showed no effect on craniofacial cartilage size or morphology (Fig. 10F). The effect of *IhhMO* was rescued by the co-injection of *Snail2* promoter-directed *CRIhh* construct (α*3000CRIhh*, Fig. 10B, 79% n = 56). We also assessed the late participation of Hh signaling in craniofacial cartilage development. We grafted cyclopamine beads in the right branchial arch region of stage 23 embryos. We observed that in the cyclopamine-treated side the development of craniofacial cartilages was severely affected (Fig. 10D, 95%, n = 44). The control grafting of BSA-loaded beads in the branchial region of sibling embryos produced no effects (Fig. 10E, 100%, n = 40). We also observed that the injection of *IhhMO* produced no effects either on *Trp2* expression (Fig. 10I, 100% n = 40) or on melanocyte pattern (Fig. 10G, H, 100% n = 31). In the *IhhMO* treated embryos no significant differences in melanocyte number or pattern were observed between injected and control sides. These results show that *Ihh* signaling is required for the normal formation of specific neural crest derivatives such as cartilage but not for the formation of other derivatives like melanocytes.

Discussion

Neural crest development is a highly orchestrated process that requires the contribution of various cell signaling pathways and involves the participation of different embryonic tissues. In this study, we showed that early induction, maintenance of specification and migration require *Ihh* cell signaling. Significant progress has been achieved in understanding the role of *Shh* in different developmental processes and animal models but the roles of other Hedgehog molecules (i.e. *Ihh* and *Dhh*, formerly named *Banded hedgehog* and *Cephalic hedgehog*, respectively) during embryogenesis are less known.

We report here the analysis of the *Ihh* expression pattern and the expression of Hh pathway members *Ptc-1*, *Smo* and the effectors *Gli2* and *Gli3*. The comparative analysis of the expression with neural crest and neural plate markers confirmed the expression of these genes in the neural crest during the late gastrula and neurula stages. These results extend and complement the previously reported expression pattern (Ekker et al., 1995) and suggest that new roles could be assumed by *Ihh* at the neural plate border that are distinct from those attributed to *Shh* (Brewster et al., 1998; Franco et al., 1999; Lopez et al., 2003; Ruiz i Altaba, 1998).

The expression patterns of *Ihh* and Hh components in the neural fold suggest that this signaling pathway plays a role in neural crest specification in *Xenopus* embryos. *Ihh* knock-down using a specific morpholino oligonucleotide or the pharmacological inactivation by cyclopamine-soaked beads grafted on the neural fold caused depletion of neural crest precursors. In contrast, the overexpression of *Ihh* produced an increase in the size of the neural crest territory. These results are in agreement with the findings of Franco et al. (1999), who showed an increased expression of neural fold prepattern gene *Zic2* after the injection of *Ihh* or *Shh* in 2-cell stage embryos. They also reported that *Ihh* or *Shh* injection increased the size of the neural plate and reduced the expression of the *Snail2* neural crest marker (Franco et al., 1999). This represents an unexpected result as *Zic2* gene is anti-neurogenic and a promoter of neural crest induction (Brewster et al., 1998; Nakata et al., 1998). In this work, extreme care was taken to direct *Ihh* overexpression to the lateral aspect of the neural plate and the neural plate border region by the injection of one dorsal blastomere of 8- to 16-cell stage embryos, or using the *Snail2* promoter (α*3000*, Vallin et al., 2001) to drive the expression of an *Ihh* construct. Moreover, the cyclopamine-soaked beads were implanted specifically into the neural fold region. These experimental approaches allowed us to assess the participation of *Ihh* signaling exclusively in the neural crest while the specification of dorsal midline remained unaffected. However, we also showed that the ectopic action of *Shh* at the neural plate border could rescue the loss of *Ihh* at the neural fold to support neural crest initial specification. Our results suggest that *Ihh* possibly has a role in the neural plate that could be different from the one in the neural crest. This suggests the possibility that *Ihh* could activate molecular mechanisms at the neural fold that support the counterbalance that apparently exists during the specification of ectodermal cells that control the neural versus neural crest fate decision, manifested when one territory increases its size at the expense of another. The lack of change in the cell proliferation or apoptosis status in *Ihh*- or *Ihh*MO-injected embryos reinforces the participation of the *Ihh* signaling cascade as a switch in cell fate decisions that take place in the ectoderm during the induction of the neural crest cell population. Conversely, an increased apoptosis rate has been described in chick embryo when *Shh* was inhibited (Ahlgren and Bronner-Fraser, 1999).

The early requirement of Hedgehog signaling for a correct development of the neural crest appears to be a feature of amphibian embryos. In the chick embryo the increased activity of Shh abrogates NC induction (Liem et al., 1995; Selleck et al., 1998). In addition, the disruption of dorsal–ventral neural tube patterning by cyclopamine-blocking of Hedgehog signaling after NC ablation induced migratory cardiac neural crest-like cells (Hutson et al., 2009). It has been shown for zebrafish

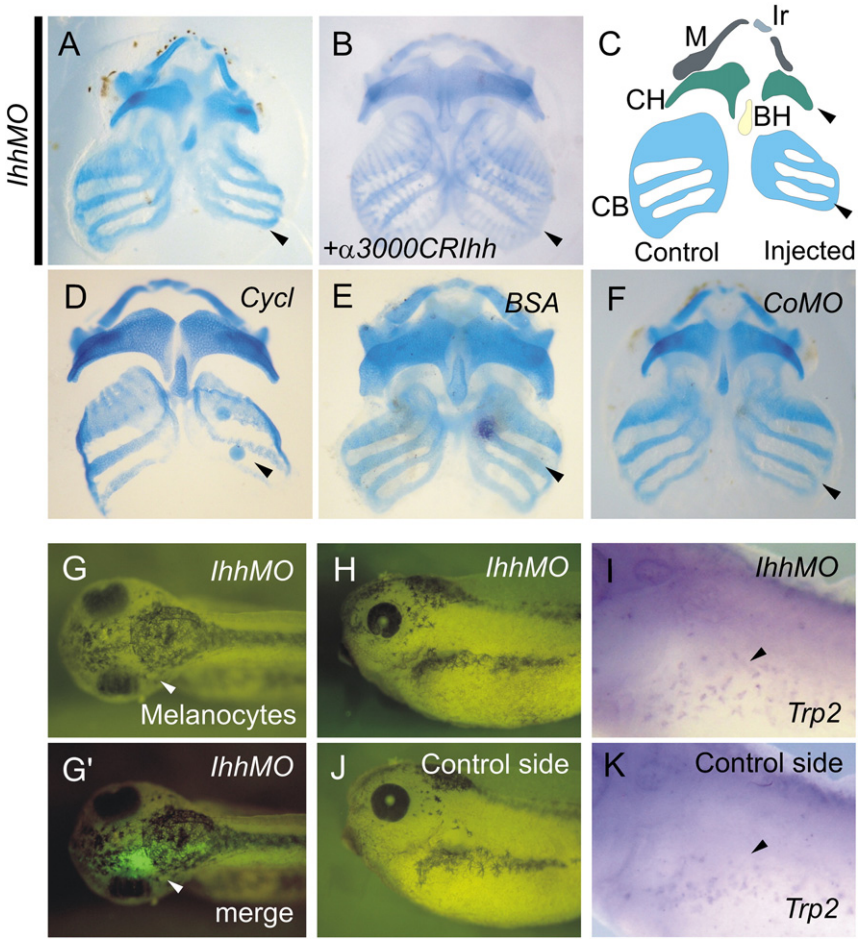


Fig. 10. *Ihh* participates in the formation of neural crest derivatives. (A–F) Craniofacial cartilages, Alcian blue staining. (A) *Ihh*MO-injected side of stage 45 embryos (arrowhead) present a marked reduction of Meckel's, ceratohyal and ceratobranchial cartilages. (B) The coinjection of *IhhMO* and specific *Snail2* promoter-driven (α*3000*) expression of *CRIhh* rescues normal cartilages morphology. (C) Schematic representation of *IhhMO* effects on *Xenopus* head cartilages. (D) Stage 23 embryos were grafted on the right branchial arch region with 20 μM cyclopamine-soaked beads, cultured until stage 46 and stained with Alcian blue. The ceratobranchial cartilage was the most affected by this treatment. (E) Control BSA-soaked beads grafted in the branchial region of stage 23 embryos produced no effect on craniofacial cartilages. (F) Control morpholino (*CoMO*)-injected embryos show no changes in cartilage morphology. (G–I) *IhhMO* produced no effects on pigment cell development (G–H) or *Trp2* expression (I) compared with their respective non-injected sides (G', J and K). (A–F) Anterior side is at the top. M, Meckel's cartilage; Ir, infrarrostral cartilage; CH, ceratohyal cartilage; BH, basihyal cartilage; CB, ceratobranchial cartilage.

embryos that most neural crest-derived craniofacial cartilages are lost or mislocalized by treatment with cyclopamine during the gastrulation period, but this requirement of Hh signaling is indirect and acts by specifying the stomodeal ectoderm before the neural crest cells arrive at its vicinity (Eberhart et al., 2006; Wada et al., 2005). Cyclopamine treatments of zebrafish embryos during early neurulation and neural crest specification also resulted in reduced mandibular cartilage (Schwend and Ahlgren, 2009). It has been suggested that although genetic studies in zebrafish have shown that *Shh* is involved in the formation of craniofacial cartilages, this role could be shared with *Shhb* (formerly named *tiggy-winkle hedgehog*, *twhh*), which is expressed in a similar pattern (Wada et al., 2005). In agreement with the published experimental data in zebrafish studies, we also found that late treatment with cyclopamine disrupts craniofacial cartilage formation. However, more studies are needed to discriminate between different Hedgehog signaling molecules since *Shh* and *Ihh* are both expressed in the branchial arch region (Ekker et al., 1995, this work) and also to determine their relative contribution to chondrogenesis.

To additionally understand the participation of *Ihh* signaling during neural crest development, we evaluated the temporal requirement of this signaling at different times of neural crest formation. Previous studies have demonstrated that the competence window for *Xenopus* neural crest induction is established from stages 10+ to 13 (Mancilla and Mayor, 1996). Our analysis using cyclopamine-treated stage 11 explants and cyclopamine-soaked beads grafted at stages 11.5, 12 and 14 and neural crest explants taken from stages 12, 14 and 17 embryos showed that *Ihh* signaling requirement goes beyond the ectodermal competence period and lasts until the midneurula stage. This agrees with previous observations that demonstrated the requirement of BMP, Wnt and Edn1 as maintenance signals from the subjacent mesoderm that sustain the neural crest specification status (Bonano et al., 2008; Steventon et al., 2009). When we analyzed the effects of cyclopamine on neural fold explants at the onset of neural crest induction we were able to establish that both *Pax3*, a gene considered a neural fold specifier, and *Sox10*, a neural crest specifier (Meulemans and Bronner-Fraser, 2004), showed a decreased expression. We found that *Sox10* expression was more strongly inhibited than *Pax3* expression, which suggests that the *Ihh* signaling pathway is probably able to selectively regulate certain components of the genetic cascade that controls neural crest induction, although this requires further investigation.

Ihh is expressed in the premigratory and migratory neural crest cells in *Xenopus* embryos. Here we showed that *Ihh* signaling is continuously required for neural crest migration and that this signal functions in both autocrine and paracrine modes. In contrast, in zebrafish embryos it has been shown that cyclopamine treatment during cranial neural crest migration caused little effects on the development of pharyngeal arch elements, revealing a differential requirement for Hh signaling (Schwend

and Ahlgren, 2009; Wada et al., 2005). Shh blocked neural crest cell adhesion and migration in chick neural tube explants in vitro by a mechanism independent from the Patched–Smoothened–Gli signaling pathway, and independently of its mitogenic or inductive activities (Testaz et al., 2001). In contrast, our results showing the blocking of neural crest migration by cyclopamine-soaked beads suggest that the canonical hedgehog signaling is required, as it is also indicated by zebrafish *Smo* and *Gli* mutations for the development of neural crest-derived DRG neurons (Ungos et al., 2003).

In our transplantation assays the *Ihh* source located in the somitic mesoderm was able to promote and maintain neural crest migration. It has been demonstrated that the mesoderm is involved in the neural crest initial induction (Bonstein et al., 1998; Marchant et al., 1998; Steventon et al., 2009), and in recent years the relationship between neural crest and surrounding tissues has increased in importance since those tissues provide essential signaling molecules required for migration and later development (Ahlgren and Bronner-Fraser, 1999; Bonano et al., 2008; Brito et al., 2006; Kimmel et al., 2001; Steventon et al., 2009)

In this work we showed that early *Ihh* abolition by the morpholino oligonucleotide and a late cyclopamine treatment in the branchial arch region at the end of neural crest migration (stage 23) impaired the formation of cranial neural crest-derived cartilages, although no changes were found in the generation of other trunk neural crest derivatives such as pigment cells. This observation agrees with a previous report that shows that *Xenopus* cranial neural crest cells are sensitive to cyclopamine treatment while trunk neural crests are resistant to treatment (Dunn et al., 1995). Species-specific requirements for Hh signals appear to be operating since in zebrafish embryos the genetic and pharmacological inhibition of Hh signaling leads directly to loss of dorsal root ganglia neurons derived from trunk neural crest, although there were no obvious effects on the level of expression of the *crestin* marker, on the pattern of neural crest migration or in the development of pigment cells and fin ectomesenchyme (Ungos et al., 2003).

Our results support an early role for *Ihh* in the neural crest development signaling based on the use of a specific morpholino to abolish its expression and temporal analysis of cyclopamine local effects. This leads us to propose that, among the multiplicity of signals operating in the neural crest early specification, *Ihh* signaling might participate in the establishment of different cell subtypes in the premigratory and migratory neural crest. Thus, the initial loss of the neural crest affected the size of cranial neural crest-derived structures, and the late effect of cyclopamine suggest that *Ihh* is required for the molecular events of craniofacial development, although this possibility requires further investigation. In zebrafish it has been shown that Hh-signaling is also required for the expression of transcription factors involved in chondrogenesis and osteogenesis differentiation (Schwend and Ahlgren, 2009). In addition, in mouse embryos, *Ihh* (the mammalian homologous of *Xenopus Ihh*) is expressed in the proximal region of Meckel's cartilage, which is entirely of neural crest origin, undergoes endochondral ossification and contributes to sclerotic ossicles (Iwasaki et al., 1997). Moreover, *Ihh* signaling also participates in the endochondral ossification during *Xenopus* limb formation (Moriishi et al., 2005).

In summary, our findings demonstrate the embryonic requirement of *Ihh* signaling for neural crest development. However, further research is necessary to understand the molecular mechanisms underlying its participation in each neural crest event. The transcription factors *Gli2* or *Gli3*, in their activator or repressor forms, appear as interesting candidates acting in the transduction of *Ihh* signaling in neural crest cells, but such possibility still remains to be explored.

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