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RESEARCH ARTICLE

Functional Analysis of *Hairy* Genes in *Xenopus* Neural Crest Initial Specification and Cell Migration

Guillermo A. Vega-López,[1] Marcela Bonano,[1] Celeste Tríbulo,[1,2] Juan P. Fernández,[1] Tristán H. Agüero,[1] and Manuel J. Aybar[1,2]*

[1]Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT
[2]Instituto de Biología "Dr. Francisco D. Barbieri", Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Chacabuco, San Miguel de Tucumán, Argentina

Background: **Neural crest formation is one of the fundamental processes in the early stages of embryonic development in vertebrates. This transient and multipotent embryonic cell population is able to generate a variety of tissues and cell types in the adult body. *hairy* genes are transcription factors that contain a basic helix–loop–helix domain which binds to DNA. In *Xenopus* three *hairy* genes are known: *hairy1*, *hairy2a*, and *hairy2b*. The requirement of *hairy* genes was explored in early neural crest development although the late requirements of these genes during neural crest maintenance, migration and derivatives formation are still unknown.** **Results:** **In this work, we extended the analysis of *Xenopus hairy* genes expression patterns and described new domains of expression. Functional analysis showed that *hairy* genes are required for the induction and migration of the neural crest and for the control of apoptosis. Moreover, we showed that *hairy* genes function as transcriptional repressors and that they are down-regulated by bone morphogenetic protein-Smad signaling and positively regulated by the Notch/Delta-Su(h) pathway.** **Conclusions:** **Our results indicate that *hairy* genes have a functional equivalence between them and that they are required for multiple processes during neural crest development. *Developmental Dynamics 244:988–1013, 2015*.** © **2015 Wiley Periodicals, Inc.**

Key words: neural crest; cell specification; morpholino; DAPT; transcriptional repressor

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Introduction

The neural crest is a vertebrate transient and multipotent embryonic cell population that arises from the lateral borders of the neural plate during late gastrulation and neurulation. The formation of the neural crest is important due to the variety of tissues and cell types that it generates in the adult body. Neural crest cells undergo an epithelial to mesenchymal transition and after extensive migration throughout the embryo they differentiate into a high number of derivative cell types and tissues including melanocytes, neurons and glia of the peripheral nervous system, face cartilage, smooth muscle, and neuroendocrine cells (Le Douarin and Kalcheim, 1999; Gammill and Bronner-Fraser, 2003; Rogers et al., 2012). The molecular mechanisms responsible for early inductive events such as cell fate decision in the ectoderm, initial cell precursor specification and specification maintenance are not yet completely understood. In *Xenopus* embryos, neural crest induction requires cell signals including bone morphogenetic protein (BMP) signaling and Wnt/FGF signaling (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Rogers et al., 2012). Along with these primary inducers other cell signaling pathways also play a relevant role in early neural crest development (Barembaum et al., 2000; Endo et al., 2002; Glavic et al., 2004a; Bonano et al., 2008; Agüero et al., 2012). At the time of induction, the prospective neural crest is flanked by the neural plate and the nonneural ectoderm and is located above the intermediate mesoderm. The spatial position of each of these tissues in relation to the prospective neural crest and the signaling molecules they secrete imposes an additional level of complexity to the understanding of the neural crest induction phenomenon. Neural crest induction by these secreted signals is first mediated by the combined action of *msx1*, *pax3*, and *zic1* transcription factors at the neural border (Tribulo et al., 2003; Monsoro-Burq et al., 2005; Sato et al., 2005). Then, these neural border specifier genes could activate several transcription factors implicated in the specification process such as *foxd3* (Sasai et al., 2001) and *snail* factors (Mayor et al., 1995; Aybar et al., 2003; Barrallo-Gimeno and Nieto, 2005), among others. Despite recent advances, still little is known about the way in which the complex network of cell signaling molecules and transcription effectors are able to regulate neural crest developmental process.

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*Correspondence to: Manuel J. Aybar, INSIBIO (CONICET–Universidad Nacional de Tucumán), Chacabuco 461, T4000ILI–San Miguel de Tucumán, Tucumán, Argentina. E-mail: mjaybar@fbqf.unt.edu.ar

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hairy genes belong to the HES (*hairy* and *enhancer-of-split*) transcription factors family that encodes helix–loop–helix (HLH) proteins with a basic DNA binding domain. In *Xenopus*, three hairy genes are known (Dawson et al., 1995; Iso et al., 2003; Tsuji et al., 2003). *hairy1* is expressed in the neural plate and pronephric mesoderm (Andreazzoli et al., 2003; Taelman et al., 2006). *hairy2*, the ortholog of mouse *hes1*, is expressed in the deep layer of the Spemann-Mangold organizer and at the neural plate border from late gastrulation onward (Tsuji et al., 2003), and has been suggested to act as a transcription effector of Notch/Delta signaling during neural crest induction (Glavic et al., 2004b). It was also shown that *hairy2* downregulates the HLH-containing *id3* gene in the neural crest through attenuation of BMP signaling (Nichane et al., 2008a). The requirement of *hairy2* in a morpholino-based analysis has shown that its activity is required at an early period to maintain the proliferative and multipotential status of the neural crest progenitors (Nagatomo and Hashimoto, 2007). Late requirements of *hairy* genes during specification, maintenance, or in neural crest cell migration are still unknown.

As neural crest cells migrate through areas with differential gene expression and signaling patterns, they acquire postmigratory identities and fates. A combination of factors is required to promote and maintain neural crest migration; the precise mixture varies from region to region and over time in a given region. The key point is that proper migration of the neural crest is critical for morphogenesis and some important components of the migration regulatory machinery have been revealed recently (Theveneau and Mayor, 2012a; Pegoraro and Monsoro-Burq, 2013). However, the participation of *hairy* genes in neural crest migration remains unknown.

In the present work, the comparative expression pattern of the three *Xenopus hairy* genes has been determined and new expression domains are described. To analyze the role of *hairy* genes, conditional gain and loss of function studies were performed by inducible chimeric proteins. The in vivo microinjection of mRNA of chimeric inducible proteins, and the use of antisense oligonucleotides demonstrated that *hairy* genes are required for the induction and migration of the neural crest in the embryos and for the control of apoptosis. Analyses revealed that the *hairy2* gene functions as a transcriptional repressor in neural crest induction and that it is partially able to induce neural crest cells from naïve ectodermal cells in vitro. The results obtained in this study showed that *hairy* genes have unique characteristics that distinguish them from the previously described functions in other species, have a functional equivalence between them and are required for the various early processes during neural crest development: initial induction, maintenance, specification, migration, and differentiation of certain derivatives.

Results

Comparative Expression Pattern of *hairy* Genes

The *Xenopus hairy1* gene was initially found to be expressed in the ectoderm (Dawson et al., 1995), and *hairy2a* (Turner and Weintraub, 1994; Davis et al., 2001) and *hairy2b* were found to be expressed in the organizer mesoderm and in the ectoderm. However, a vis-à-vis comparison of the expression of three *Xenopus hairy* genes is lacking and their expression during mid- to late neurula stages is unknown. The spatial and temporal expression patterns of *Xenopus hairy* genes (*hairy1*, *hairy2a*, *hairy2b*) were analyzed by single and double whole-mount in situ hybridization of embryos. Zygotic transcription became apparent during gastrulation and was initially found in the ectoderm. The *hairy1* gene first expression appeared restricted to thin lines located in the lateral boundaries of the neural folds (Fig. 1A, black and white arrowheads). *hairy2a* and *hairy2b* genes became visible at early neurula stages restricted to the area of the lateral and anterior neural fold (Fig. 1E,I, white and yellow arrowheads) and were slightly expressed at the midline of the neural plate (Fig. 1E,I, black arrowheads). When embryonic development proceeded, the expression of *hairy1* continued to be localized surrounding the neural fold and lines of expression extended toward the posterior part of neural fold. A strong expression appeared in the anterior neural plate (Fig. 1C, asterisk, this expression was maintained throughout all midneurula stages). At neurula stages, *hairy2a* and *hairy2b* genes were expressed in the neural plate midline and in the anterior and lateral neural folds (Fig. 1F,J, white, yellow and black arrowheads) this expression increased and extended to the posterior region of embryos (Fig. 1G,K, black and yellow arrowheads). It is interesting to note that *hairy2a* was expressed in wider domains and more strongly than *hairy2b* in the anterior neural plate (Fig. 1F,J). We observed the apparition of transverse stripes of expression in the neural plate at the midneurula stage (stage 16, Fig. 1G,K, green arrowheads) labeling the prospective limit forebrain–midbrain, midbrain–hindbrain, and hindbrain–spinal cord, and the presomitic segmentative mesoderm. After the closure of the neural tube, *hairy1* expression was observed in the optic, olfactory, profundal, and epibranchial placodes (Fig. 1D, red, green, yellow and white arrowheads, respectively). In contrast, the expression of the *hairy2a* and *hairy2b* genes is located in the optical vesicle and in the neural crest cells migration pathways (Fig. 1H,L, red and black arrowheads). This latter expression has never been shown for *hairy2* genes.

To better define *hairy* gene expression patterns, we compared them with other ectodermal gene markers and performed cross sections to achieve greater precision of the domains of the gene expression within the embryo. A complementary expression was found between *hairy1* and the neural crest marker *foxd3* (Fig. 1M,M′). *hairy1* was observed as a fine line in the most dorsal region of the neural plate (Fig. 1M′, black arrowhead). A fine line of expression was also observed in the lateral and external border of the prospective neural crest that expresses the *foxd3* marker (white arrowhead). On other hand, we noticed an overlap in the expression pattern of *hairy1* and the neural plate marker gene *sox2* in the most anterior region of the neural plate at the prospective forebrain (Fig. 1N,N′). *hairy2a* expression overlaps with the neural crest marker *foxd3* (Fig. 1O,O′, asterisk) only in the territories that will originate the neural crest. The expressions of *hairy2a* and *sox2* were complementary because they were not superimposed in the lateral region of the neural plate (Fig. 1P,P′). Only a line of *hairy2a* expression overlaps with *sox2* in the anterior edge of the neural plate (Fig. 1P). Meanwhile, the expression pattern of *hairy2b* showed great similarity to the *hairy2a* gene with respect to the domains they occupy in the dorsal ectoderm of the embryo at the neural crest and in the anterior neural plate (Fig. 1Q–1R′).

hairy Genes Are Regulated by Notch and BMP Pathways

The expression patterns we found suggest that members of the *Xenopus hairy* family are participating in the specification of the

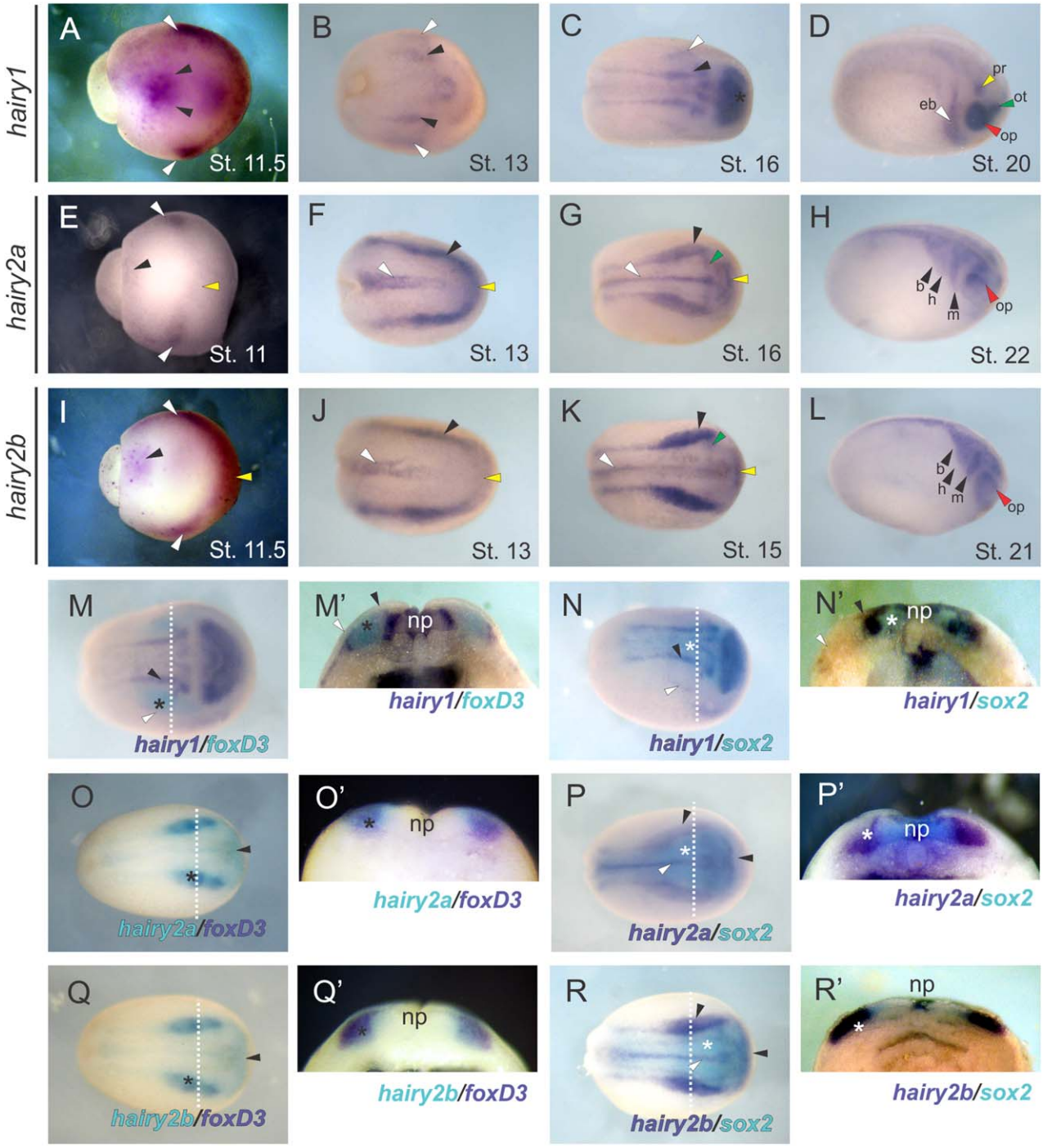

Fig. 1. Comparative expression pattern of *hairy* genes. Whole-mount in situ hybridization analysis of spatio-temporal hairy genes expression. A–C, E–G, I–K, M, and O–S: Dorsal view of *Xenopus laevis* embryos; anterior side is on the right. N: Dorso–lateral view. D, H, L: Lateral view. M′, N′, O′,Q′, R′: Transversal sections. Dashed lines in M, N, O, P, Q and R indicate the sites of transverse section shown in M′, N′, O′, P′ Q′ and R′, respectively. **A:** *hairy1* transcripts are first detected since the early neurula stage (stage 11.5) in the dorsal region of embryos at the neural plate (black arrowheads) and neural plate borders (white arrowheads). **B,C:** During neurulation (stages 13–16), *hairy1* is expressed in the neural plate borders (arrowheads) and surrounding the anterior neural plate (asterisk). At stage 16 the increased expression in the anterior neural plate is remarkable. **D:** Stage 20 embryo. Arrowheads show the expression from the dorsal to the anterior–ventral region. The red arrowhead shows the anterior expression in the optic vesicle. Arrowheads show the expression in different placodes (see text). **E:** *hairy2a* transcripts are first detected since the early neurula stage (stage 11) in the dorsal region of embryos at the neural plate border (yellow and black arrowheads) and in the prechordal mesoderm (white arrowheads). **F,G:** During neurulation (stages 13–16), *hairy2a* expression is predominant in the anterior neural fold (yellow arrows), in the neural crest region (black arrowheads) and in the prospective floor plate (white arrowheads). **H:** Stage 22 embryo. The expression of *hairy2a* can be seen in the cephalic streams of migrating neural crest cells (black arrowheads) and in the optic placode (red arrowhead). **I:** Early expression of *hairy2b* can be observed in the anterior neural fold (yellow arrow), in the lateral neural fold (black arrowhead) and in the posterior part or the neural plate (white arrowhead). **J,K:** During neurulation (stages 13–16), the expression of *hairy2b* can be observed in the prospective neural crest region (black arrowheads), in the anterior neural fold (yellow arrowhead), in the prospective floor plate (white arrowheads), and a transversal expression in the middle of the neural plate (green arrowhead). **L:** Stage 21 embryo. *hairy2b* expression is shown in migratory cephalic neural crest (black arrowheads) and the optic placode (red arrowhead). **M–R:** Double in situ hybridizations for hairy and *foxd3*, or *sox2*. M′–N′: Transverse sections of St. 15 neurula embryos displayed in M–N showing that *hairy1* (M′, purple, arrowheads) expression is surrounded by *foxd3* (neural crest expression, turquoise, asterisk), and *hairy1* expression (N, purple) is located at the outer limit (arrowheads) of the neural plate domain revealed by *sox2* gene expression (turquoise, white asterisk). O′–R′: Transverse sections of St. 15 neurula embryos displayed in O–S showing that *hairy2a* and *hairy2b* (turquoise) expression overlaps with *foxd3* (purple) in the neural crest territory (O′ and R′, black asterisk: *foxd3* neural crest expression) and is complementary with *sox2* (turquoise) expressions (P′,R′). np, neural plate.

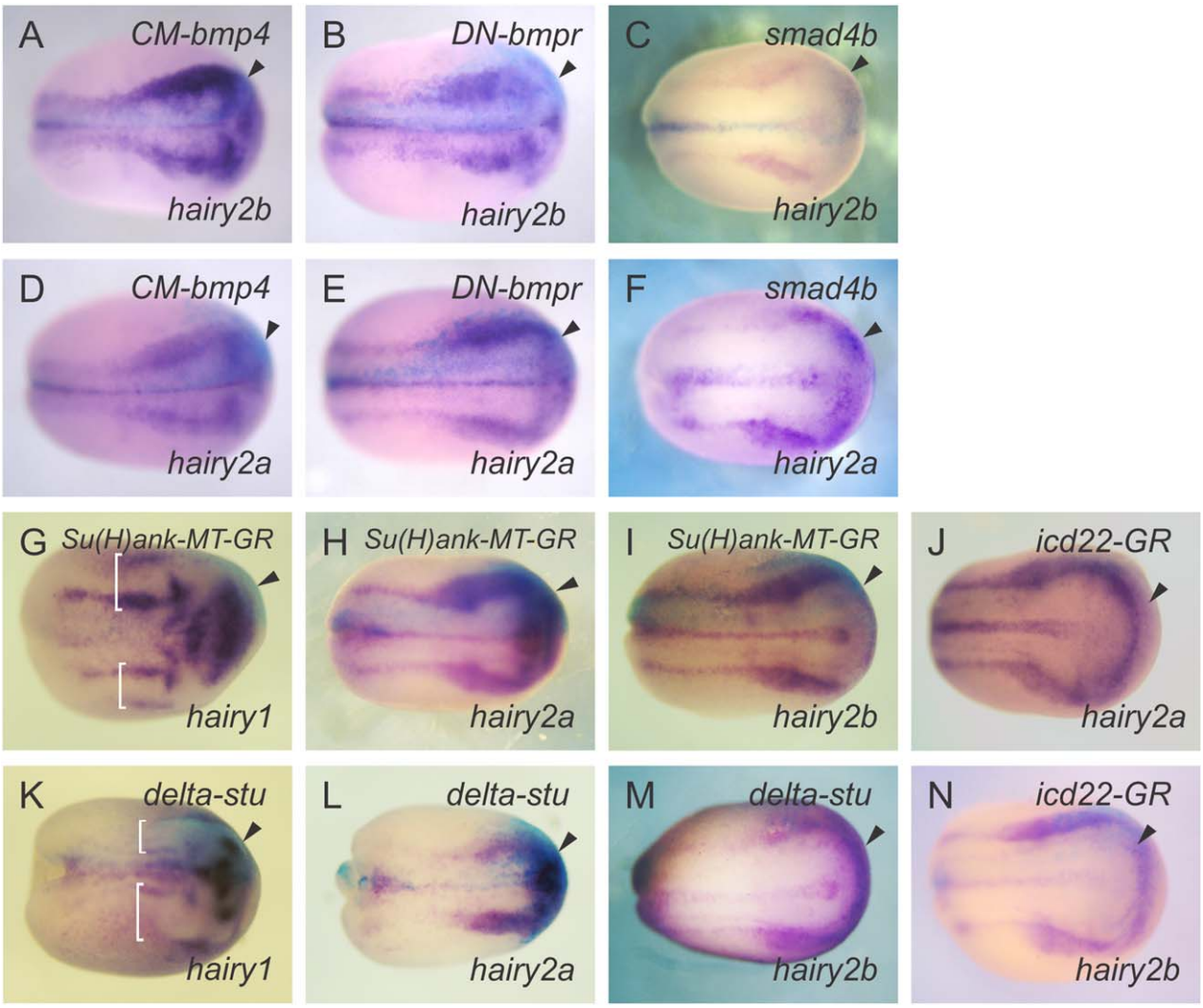

Fig. 2. *hairy* genes are regulated by Notch and BMP pathways. **A–F:** One blastomere of a 8- to 16-cell stage embryo was injected with mRNA of *CM-bmp4* (250 pg; A, D) or *DN-bmpr* (500 pg; B, E). Note the expansion of *hairy2b* or *hairy2a* markers (respectively, arrowheads). In *smad4* (1 ng) a decreased expression of *hairy2a* (C) or *hairy2b* (F) can be observed with respect to the control. **G–I:** Microinjection of *Su(H)Ank-MT-GR* mRNA (700 ng). Note the expansion of the expression of *hairy1* (G), *hairy2a* (H), and *hairy2b* (I) on the injected side with respect the control. **K–M:** Microinjection of delta-Stu mRNA (1 ng). Note the decrease in *hairy1* (K), *hairy2a* (L) and *hairy2b* (M). **J,N:** Microinjection of *icd22-GR* (700 ng). Note the expansion in *hairy2a* (J) and *hairy2b* (N) expressions.

early neural crest. Then, we decided to investigate the relationships between *hairy* genes and other factors involved in the genetic cascade of neural crest induction. It has been proposed that *BMP* signaling could be one of the most important events in the early induction of neural crest. We analyzed the existence of a hierarchical regulatory relationship between *hairy2* genes and the *BMP* pathway, which was analyzed for both genes after interfering with the activity of the *BMP* signaling pathway at different levels (ligand, receptor, and effector transcription factor). BMP activity was altered by using the dominant negative of *BMP* receptor type I (*dnBMPR*, Graff et al., 1994; Suzuki et al., 1994) with a dominant negative ligand *BMP4* (*CM-BMP4*, Hawley et al., 1995; Tribulo et al., 2003) or *Smad4b* (Chang et al., 2006). The two first tools blocked *BMP* signaling and the transcription factor *Smad4b* increased the activity of the signaling pathway. Blocking *BMP* signaling expanded the expression territory of *hairy2b* and *Hairy2a*, and also increased their expression level (Fig. 2A,B,D,E). In contrast, increasing *BMP* pathway activity by *Smad4b* led to a decreased expression of *hairy2* genes (Fig. 2C, *hairy2a*, 84% n = 19, and Fig. 2F, *hairy2b*, 70% n = 13) during the process of induction in *Xenopus* neural crest.

It has been shown that the *hairy1* and *hairy2a* genes are targets of the *Notch/Delta* signaling pathway (Davis and Turner, 2001; Umbhauer et al., 2001; Stancheva et al., 2003; Lopez et al., 2005) and that *hairy2a* changes its expression by activation or blocking of this pathway (Glavic et al., 2004b). In this work, we extended the analysis to *hairy1* and *hairy2b* genes. The *Notch/Delta* pathway was activated with the constitutive activator construct *Su(H)-Ank-MT-GR* (Wettstein et al., 1997) and was inhibited by the dominant negative version of the *Delta* ligand, named *Delta-Stu* (Chitnis et al., 1995). First, we assessed the *hairy2a* response to an increase or decrease in *Notch/Delta* activity. It was found that the gene increased its expression when the pathway was activated (*Su(H)Ank-MT-GR*, Fig. 2H, 58% n = 26) and decreases it with a

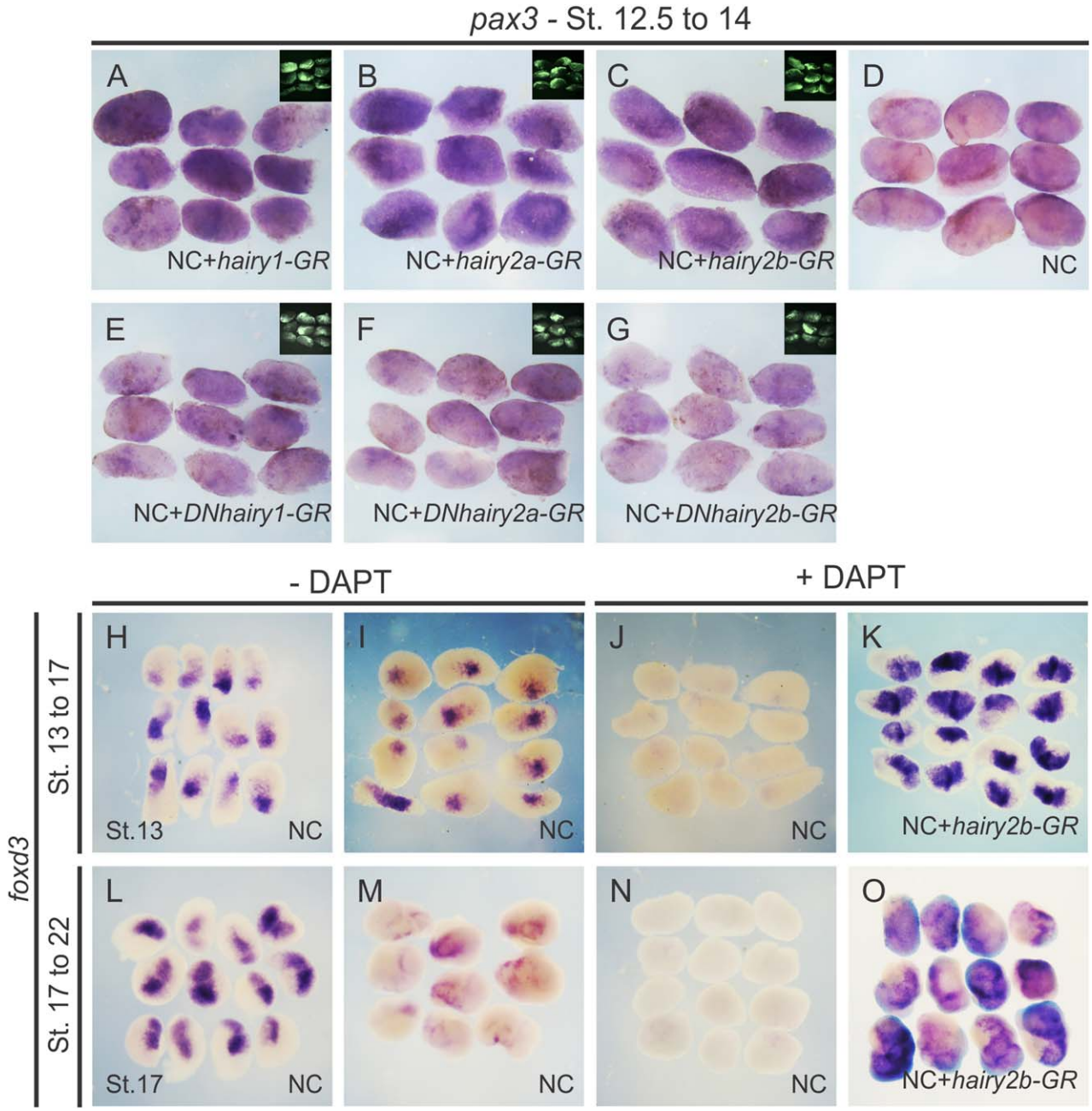

Fig. 3. Temporal requirement of *Notch/Delta* signaling during early neural crest development. A–G: Groups of NC explants were isolated from *hairy* constructs mRNA injected embryos and cultured from stage 12.5 to 14. Inset in A–C and E–G: the fluorescent picture verifies the injection. **A–C:** *hairy-GR* activator constructs inject explants strongly express *pax3*. **D–G:** *DNhairy-GR* constructs inject explants showed no changes with respect to control NC (D). H–O: Neural crest explants (NC) were prepared by dissection of the neural crest. Groups of explants were fixed immediately after excision (left), at stages 13 or 17. Groups of explants were cultured until stages 17 or 22 in the presence or absence of DAPT (see text for details). **H,L:** NC explants removed from embryos at different stages express *foxd3* when fixed at the moment of dissection. **I:** NC explants dissected from stage 13 embryos and cultured until stage 17 express *foxd3*. **J:** The NC explants isolated at stage 13 and cultured until stage 17 in the presence of 100 μM DAPT lose *foxd3* expression. **K:** The *hairy2b-GR* mRNA strongly rescued *foxd3* expression of DAPT treated NC explants. **M:** NC explants dissected at stage 17 cultured until stage 23 shown *foxd3* expression. **N:** DAPT treatment of NC explants isolated from stage 17 embryos produced inhibition of *foxd3* expression. **O:** The *hairy2b-GR* mRNA strongly rescued *foxd3* expression of DAPT treated NC explants.

blocking of the signaling pathway (*Delta-Stu*, Fig. 2L, 53% n = 36). Moreover, an expansion of the expression of *hairy1* and *hairy2b* genes was produced by the activation of *Notch/Delta* signaling by *Su(H)Ank-MT-GR* (*hairy1*, Fig. 2G, 48% n = 36, and *hairy2b*, Fig. 2I, 50% n = 16) or *ICD22-GR* (*hairy2a*, Fig. 2J, 43% n = 23 and *hairy2b*, Fig. 2N, 81% n = 21). Conversely, inhibition of *Notch* signaling with *Delta-Stu* decreased the expression of *hairy1* and *hairy2* genes (*hairy1*, Fig. 2K, 47% n = 32, and *hairy2b*, Fig. 2M, 30% n = 21). These results show that *hairy* genes are able to respond to changes in the activity of the *Notch/Delta* signaling pathway.

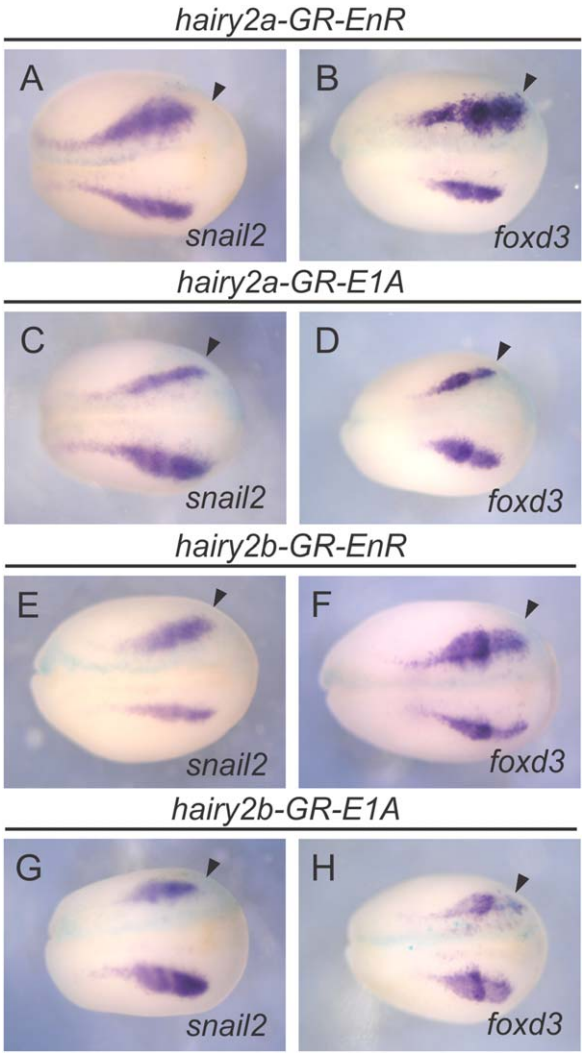

Fig. 4. *hairy* genes work as transcriptional repressor. **A–H:** One blastomere of a four- to eight-cell stage embryo was injected with 700 pg of mRNA of *hairy2a* and *hairy2b* repressor construct (A,B, E,F) or the *hairy2a* and *hairy2b* activator construct (C,D, G,H), treated with dexamethasone at stage 12.5 and fixed at stage 16, and the expression of neural crest markers *snail2* (A,C,E,G) and *foxd3* (B,D,F,H) were analyzed. Arrowhead shows injected side. Note that the *snail2* repressor construct (*hairy2-GR-EnR*) produced an expansion in neural crest markers on the injected side (A,B, E,F) while the *hairy2a* and *hairy2b* activator lead to inhibition in the expression of the markers (C,D, G,H).

Notch/Delta-hairy Signaling is Necessary for the Maintenance of Neural Crest Specification

Our results suggest that there is an earlier requirement for Notch/Delta signaling during specification in the neurula stages (Fig. 2) at the neural plate border during the earliest stages of neural crest induction. First, we analyzed the participation of Notch/Delta signaling in neural fold explants by evaluating the expression of early specification neural crest gene marker *pax3* (Hong and Saint-Jeannet, 2007). To avoid effects of the overexpression at early stages, we used a *hairy* gene construct fused to the ligand binding domain of the human glucocorticoid receptor (GR).

The neural fold explants were dissected out from stage 12.5 embryos and incubated with or without dexamethasone until stage 14 when they were processed for in situ hybridization (Fig. 3A,E, respectively). The overexpression by directed microinjection of *hairy* genes in their full length version caused in the isolated neural crest explants an increase in the expression of *pax3* (Fig. 3J–L). Conversely, other sets of neural crest explants were injected with the mRNA of *DNhairy1-GR*, *DNhairy2a-GR*, and *DNhairy2b-GR* constructs. Each construct is composed of the N-terminal region of the protein (amino acid residues 1-106, 1-106, 1-108, respectively), comprising only the corresponding bHLH domain (see Experimental Procedures for details). The overexpression of *hairy* genes produced only a slight decrease in the expression of *pax3* in neural crest explants (*hairy1*, Fig. 3A, 67% n = 27, *hairy2a*, Fig. 3B, 75% n = 36,and *hairy2b*, Fig. 3C, 83% n = 30). When dominant negatives constructs were overexpressed, the expression of the neural plate border specifier marker (*pax3*) showed only a slight decrease in its expression (*DNhairy1*, Fig. 3E, 80%, n = 45; *DNhairy2a*, Fig. 3F, 79%, n = 29; *DNhairy2b*, Fig. 3G, 77%, n = 30) with respect to the control explants (Fig. 3D). The explants approach provided an opportunity to study the response of the neural crest in the absence of additional signals in the embryonic context. Thus, the gain and loss of function of the *hairy* genes shows that their activity is required for the early steps of neural crest induction.

Our results suggest that there is a later requirement for *hairy2b* gene during specification in the midneurula stages (Figs. (5 and 7)). We decided to analyze the requirement of *notch* signaling using the pharmacological inhibitor DAPT (Geling et al., 2002; Lassiter et al., 2010) and its relationship with *hairy2b* from the late gastrula to the late neurula stages. Neural crest explants dissected at the late gastrula stage (stage 13) and immediately fixed showed normal expression of the *foxd3* marker (Fig. 3H). *foxd3* expression was observed in explants that were dissected out at stage 13 and incubated until stage 17 containing neural crest and the underlying mesodermal tissue (NC, Fig. 3I).When neural crest explants containing the underlying mesoderm were incubated in the presence of 100 μM DAPT, *foxd3* expression was downregulated (Fig. 3J). In contrast, in the neural crest explants isolated from *hairy2b-GR* injected embryos cultured until stage 17 in 100 μM DAPT and dexamethasone, the expression of *foxd3* was rescued (Fig. 3K). A strong expression of *foxd3* was observed in neural crest explants dissected at stage 17 and fixed immediately (Fig. 3L). When stage 17 neural crest explants were cultured until the equivalent of stage 22, the expression of *foxd3* was notoriously reduced (Fig. 3M). When these neural crest explants were incubated with 100 μM DAPT, the expression of *foxd3* disappeared (Fig. 3N). In contrast, in the neural crest explants isolated from *hairy2b-GR* injected embryos cultured until stage 23 in 100 μM DAPT and dexamethasone, the expression of *foxd3* was conserved (Fig. 3O). Taken together, these findings strongly suggest that *hairy2b* is able to rescue the inhibition of *notch* signaling and that it is required from the gastrulation to late neurula stages to maintain neural crest specification.

hairy Genes Work as Transcriptional Repressor

To better understand the molecular mechanisms that underlie the activity of *hairy* genes during neural crest development, the N-terminal region including the bHLH domain of *hairy2* genes was fused to the activation domain of the human type 5 adenovirus E1a protein (Lillie and Green, 1989) or to the repressor domain from *Drosophila* Engrailed protein (Jaynes and O'Farrell, 1991). One blastomere of a four-cell stage embryo was injected with

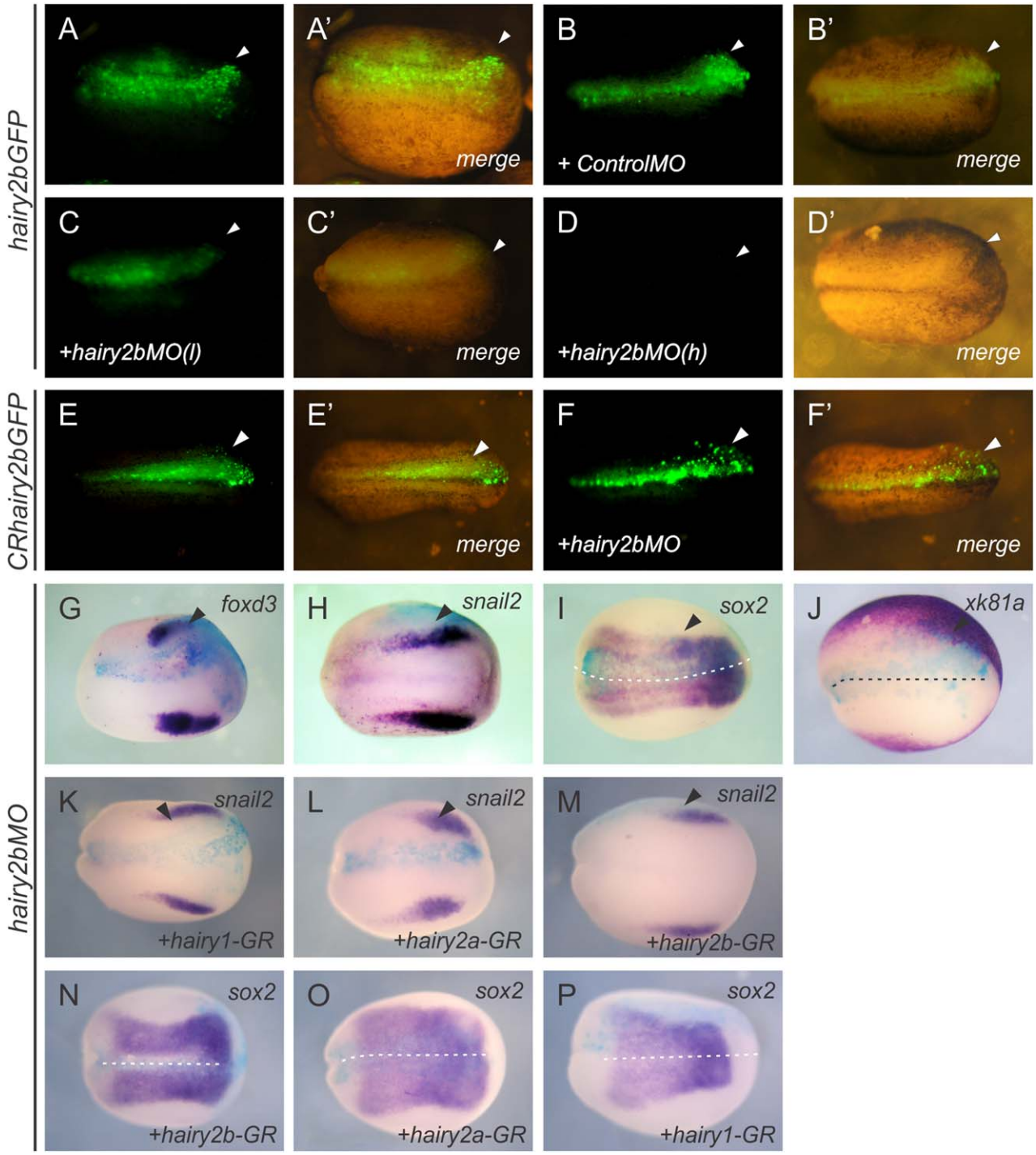

Fig. 5. *hairy2b* is required for early neural crest specification in *Xenopus* embryos. A–P: Dorsal views. **A–F:** In vitro and in vivo efficiency of *hairy2b* antisense morpholino oligonucleotide (*hairy2bMO*). Embryos under a fluorescence stereo microscope; anterior side is on the right. White arrowheads indicate the injected side. A′,B′,D′,E′,F′: Fluorescence and clear field images of each embryo are shown in merged images. A–A′: Embryo injected with mRNA encoding *hairy2bGFP* (1 ng/embryo) showing GFP fluorescence on the treated side. B–B′: Embryo injected with *hairy2bGFP* mRNA (1 ng/embryo) and control antisense morpholino oligonucleotide (ControlMO, 20 ng/embryo). C–C′,D–D′: Embryos injected with *hairy2bGFP* mRNA (1 ng/embryo) and *hairy2bMO* (C–C′, low dose (l), 5 ng/embryo; D and D′, high dose (h), 10 ng/embryo). No embryo shows GFP fluorescence at a high dose of *hairy2bMO*. E,F: Embryo injected with mRNA encoding *CRhairy2bGFP* (E–E′, 1 ng/embryo) alone or co-injected with *hairy2bMO* (F–F′, high dose (h), 10 ng/embryo) showing GFP fluorescence on the treated side. **G–P:** Analysis of *hairy2bMO* effects on neural crest early specification. Dorsal views, anterior side is on the right. Black arrowheads indicate the injected side. G,H: *hairy2bMO*-injected embryos show inhibition of *foxd3* and *snail2* neural crest markers, respectively. I,J: The expression of the neural plate marker *sox2* and the epidermal marker *xk81a* are expanded on the *hairy2bMO*-treated side. K,P: Co-injection of *hairy2bMO* and *hairy1-GR* or *hairy2a-GR* or *hairy2b-GR* mRNA rescues, *snail2* (K–M) and *sox2* (N–P) expression. The hairy genes rescue the expression of *foxd3* in the neural crest (K–M) and the expression of *sox2* in the neural plate (N–P).

mRNA of these constructs and at stage 12.5 the embryos were incubated with dexamethasone to activate the inducible chimeric protein. Then, the expression of several neural crest markers were analyzed at the midneurula stage. The injection of the transcription repressor constructs *hairy2a-GR-EnR* and *hairy2b-GR-EnR* resulted in an enlargement of the territory of expression of the *snail2* marker (Fig. 4A, 52% n = 19, and Fig. 4C, 53% n = 23) and *foxd3* (Fig. 4B, 61% n = 23 and Fig. 4D, 60% n = 20). Conversely, injecting the transcription activator constructs *hairy2a-GR-E1A* and *hairy2b-GR-E1A* led to an inhibition of the expression of the *snail2* marker (Fig. 4G, 57% n = 23, and Fig. 4E, 46% n = 19) and *Foxd3* (Fig. 4H, 52% n = 21, and Fig. 4F, 60% n = 20). Thus, as the repressor construct produced the same phenotype as the wild-type *hairy* gene when overexpressed while the opposite effect was produced by the activator construct, we concluded that *hairy* genes probably function as transcriptional repressors in the neural crest induction process.

hairy2b is Required for the Development of the Neural Crest

The expression patterns of *hairy* genes show that they are expressed in the neural crest or in neighboring tissues. We decided to use antisense morpholino oligonucleotides to knock down the expression of the *hairy2b* gene because it is the one that is expressed at a higher level on the neural crest. We designed an antisense morpholino (*hairy2bMO*) which specifically binds to a 25nt sequence between the AUG site and nt +25. The efficiency and specificity of *hairy2bMO* were assessed by evaluating its action against the expression of a green fluorescent protein (GFP) fusion construct (*hairy2bGFP*). Embryos that were injected with *hairy2bGFP* mRNA (Figs. 5A, 4A′, 100% n = 15) or coinjected with *hairy2bGFP* mRNA and control morpholino (20 ng/embryo) showed high levels of GFP fluorescence (Figs. 5B, 4B′, 100% n = 12). Then we analyzed the efficiency of *hairy2bMO* oligonucleotide and confirmed that it was able to inhibit the translation of the GFP-tagged form of *hairy2b* in a dose-dependent manner (Fig. 5C,C′ 1 ng/embryo of *hairy2bGFP* and 5 ng/embryo of *hairy2bMO*, 67% n = 15; and Fig. 5D,D′ 1 ng/embryo of *hairy2bGFP* and 10 ng/embryo of *hairy2bMO*, 94% n = 15). We also prepared a fusion construct carrying mismatches in the morpholino recognition site without changing *hairy2b* protein sequence (*CRhairy2bGFP*). The expression of this construct (*CRhairy2bGFP* mRNA, 1 ng/embryo) was not affected when it was co-injected with *hairy2bMO* at the higher dose (Fig. 5E,E′ 100% n = 15; and Fig. 5F,F′ 10 ng/embryo of *hairy2bMO*, 100% n = 15).

To evaluate the participation of *hairy2b* gene during neural crest induction, embryos were injected at the eight-cell stage in one dorsal blastomere with *hairy2bMO* (10 ng/embryo; Fig. 4G–P). The injected embryos showed a reduced neural fold as well as inhibition of *foxd3* and *snail2* expression at the injected side (Fig. 5G, 68% n = 22 and Fig. 5H, 55% n = 33). In these embryos, the neural plate marker *sox2* (Fig. 5I, 57%, n = 42) and the epidermal marker *xk81a* were expanded in the injected side (Fig. 5J, 75% of expansion, n = 36), probably as a consequence of the reduction in neural crest territory. These results show that the *hairy2b* factor is required in the development of neural crest cells.

Next, in the absence of *hairy2b* function caused by the morpholino, we explored the capacity of the other *hairy* genes to rescue the phenotypes. In all the cases, the coinjection of *hairy2bMO* with *hairy1*, *hairy2a*, or *hairy2b* rescued neural crest gene marker expression (*hairy1-GR*, Fig. 5K, 66% n = 15; *hairy2a-GR*, Fig. 5L, 60% n = 25, and *hairy2b-GR*, Fig. 5M, 67% n = 18) and neural plate markers (*hairy1-GR*, Fig. 5N, 70% n = 20; *hairy2a-GR*, Fig. 5O, 56% n = 25, *and hairy2b-GR*, Fig. 5P, 68% n = 19). This result suggests that a functional equivalence between the three members of the *hairy* gene family exists as each member was able to compensate the effects of *hairy2b* depletion.

hairy Genes Participate in the Early Formation of Neural Crest Cells

Morpholino oligonucleotides can efficiently block the expression of maternal and zygotic transcripts, but this approach is limited to the control of the timing of morpholino activity. Moreover, *hairy* genes are expressed and have functions in the mesoderm. We chose a strategy for the functional analysis of *hairy* genes that is directed spatially and temporally by using constructs fused to the GR. The use of such an inducible construct avoids effects on the mesoderm at the early stages, as any effects on this tissue could indirectly impact the neural crest.

The overexpression by directed microinjection of *hairy* genes in their full length version caused an increase in the specific neural crest gene markers *snail2* and produced a larger prospective neural crest territory (*hairy1*, Fig. 6A, 79% n = 41; *hairy2b*, Fig. 6E, 73% n = 15 and *hairy2b*, Fig. 6I, 35% n = 51). Concomitantly, the overexpression of *hairy* genes caused a decrease in the expression of neural plate markers (*sox2*, Fig. 6B, 54% n = 52; Fig. 6F, 61% n = 51, and Fig. 6J, 56% n = 16) and in the prospective epidermis (*xk81a*, Fig. 6C, 76% n = 35; Fig. 6G, 81% n = 22, and Fig. 6K, 61% n = 28). Double in situ hybridization analysis using *sox2* and *xk81a* markers, confirmed that both territories were reduced compared with the control side of embryos (*hairy1*, Fig. 6D, 54%, n = 11; *hairy2a*, Fig. 6H, 73%, n = 11; *hairy2b*, Fig. 6L, 64%, n = 11).

In addition, the in vivo overexpression of dominant negative inducible proteins was carried out using the *DNhairy1-GR*, *DNhairy2a-GR*, and *DNhairy2b-GR* constructs. The overexpression of the inducible constructs that were activated at stage 12 with dexamethasone caused a reduction in the neural crest territory, shown by the specific gene marker *snail2* (Fig. 6M, 67% n = 68; Fig. 6Q, 66% n = 15, and Fig. 6U, 61% n = 86). The overexpression of dominant negative forms of *hairy* genes led to increased neural plate and prospective epidermal territories revealed by the specific markers *sox2* (Fig. 6N, 63%, n = 47; Fig. 6R, 70%, n = 34, and Fig. 6V, 56%, n = 72) and *xk81a* (Fig. 6O, 53% n = 34; Fig. 6S, 77% n = 23 and Fig. 6W, 57% n = 23). Double in situ hybridization analysis using *sox2* and *xk81a* markers confirmed that both territories were increased compared with the control side of embryos (*DNhairy1*, Fig. 6P, 54%, n = 11; *DNhairy2a*, Fig. 6T, 64%, n = 11; *DNhairy2b*, Fig. 6X, 73%, n = 11). The effects observed in the loss of function experiments by the use of the dominant negative constructs were rescued by co-injection of each full length *hairy* molecule, respectively (Fig. 6P′, 87% n = 32, Fig. 6T′, 93% n = 31 and Fig. 6X′, 88% n = 33). In each instance, the rescue of *foxd3* expression in the neural crest was observed. This result suggests that the complete molecule could biochemically displace the dominant negative form under the conditions used and that dominant negative effects were specific to the neural crest. These results are in contrast to what was observed when we overexpressed the full length version of each *hairy* gene and

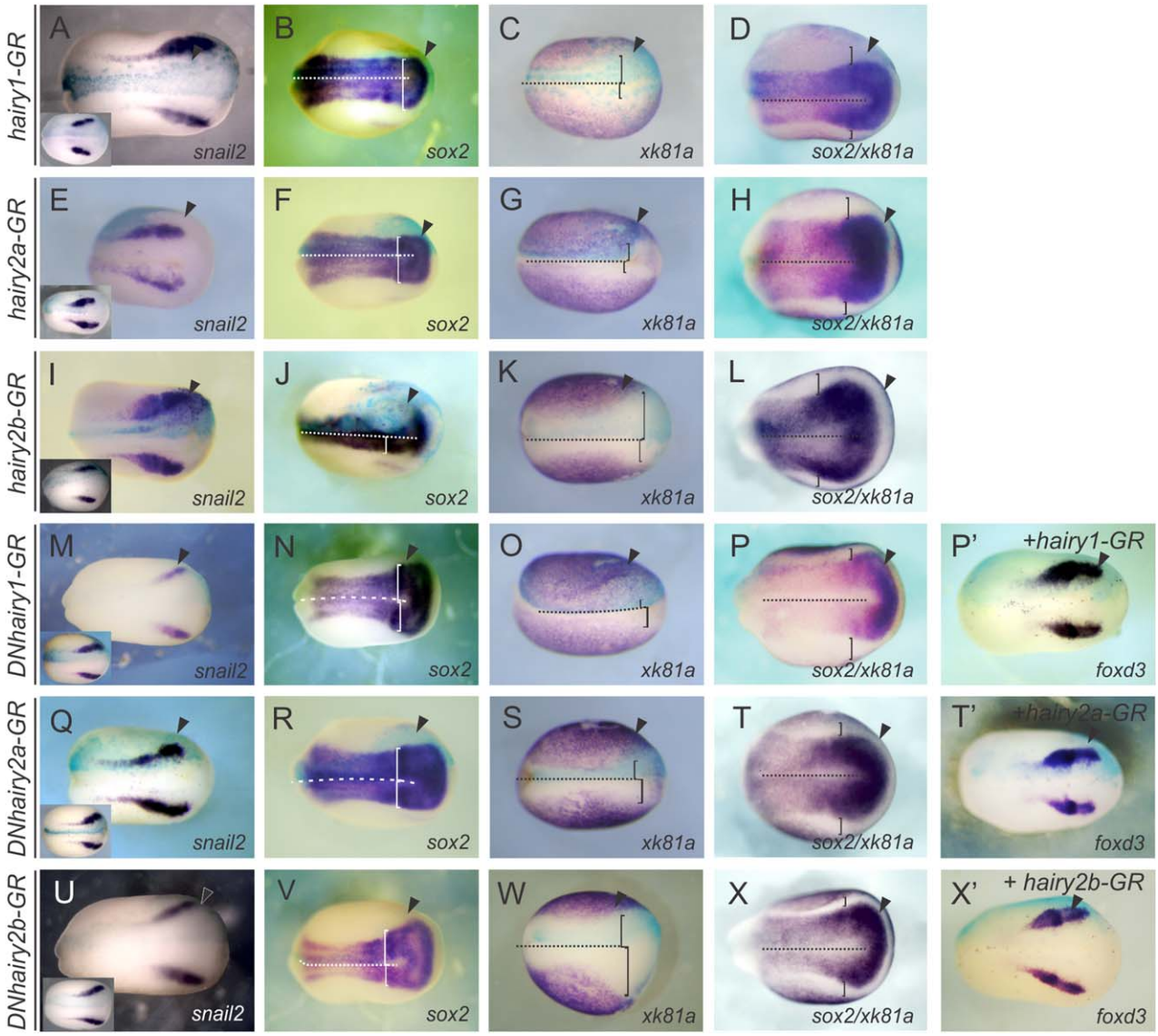

Fig. 6. *hairy* genes participate in the early formation of neural crest cells. **A–X:** Dorsal views of *Xenopus laevis* embryos; anterior side is on the right. The injected side is indicated by an arrowhead. Embryos were injected into one blastomere at the 8- to 16-cell stage with 700 ng of mRNA, treated with dexamethasone stage 11 until neurula stages and fixed, and the expression of several markers was analyzed by in situ hybridization. No change was observed in the control injected embryos nonincubated with dexamethasone (insets in A, E, I, M, Q, and U). A–L: Gain of function. *hairy* activator constructs mRNA-injected embryos show increased expression of *snail2* (A,E,I) while neural plate marker *sox2* (B,F,J) and epidermis marker *xk81a* (C,G,K) are reduced. D,H,L: Embryos labeled by double in situ hybridization for *sox2* and *xk81a* genes showing the increase in prospective neural crest region (black brackets) in the injected side (arrowheads). M–X: Loss of function. Conversely, *DNhairy* constructs mRNA-injected embryos show that neural crest markers *snail2* are reduced in the injected side (M,Q,U) while neural plate marker *sox2* (N,R,V) and the epidermis marker *xk81a* (O,S,W) are slightly increased. P′, T′, X′: Rescue of *DNhairy* by coinjecting mRNA of *hairy* activator constructs. The phenotype of the neural crest marker *foxd3* was rescued by the co-injection of *hairy1-GR* (P′), *hairy2a-GR* (T′) and *hairy2b-GR* (X′). P,T,X: Embryos labeled by double in situ hybridization for *sox2* and *xk81a* genes showing the decrease in prospective neural crest territory (black brackets indicate width) in the injected side.

similar to those observed using the *hairy2bMO* (Fig. 5). The loss of function of *hairy* genes showed that they are required for the normal development of the neural crest.

Cell Fate in the Absence of Cell Proliferation

The results of *hairy* overexpression could be due to either a change in the ectodermal cell fate or changes in the cell proliferation status of the neural crest cells. To differentiate these two possibilities, we carried out the overexpression in the presence of aphidicolin and hydroxyurea (HUA), two strong inhibitors of cell proliferation (Harris and Hartenstein, 1991; Sharpe and Goldstone, 1997; Aybar et al., 2003; Fernandez et al., 2014). Embryos injected with inducible constructs of *hairy* genes were treated with dexamethasone and HUA at stage 11 until neurula stages (the same stage for overexpression results in Fig. 6), they showed an expanded neural crest territory (*foxd3*, Fig. 7A, *hairy1-GR* 64%, n = 14; Fig. 7B, *hairy2a-GR* 54%, n = 11; Fig. 7C, *hairy2b-GR* 64%, n = 11), a reduction in neural plate (*sox2*, Fig. 7G, *hairy1-GR* 55% n = 20; Fig. 7H, *hairy2a-GR* 50%, n = 10; Fig.

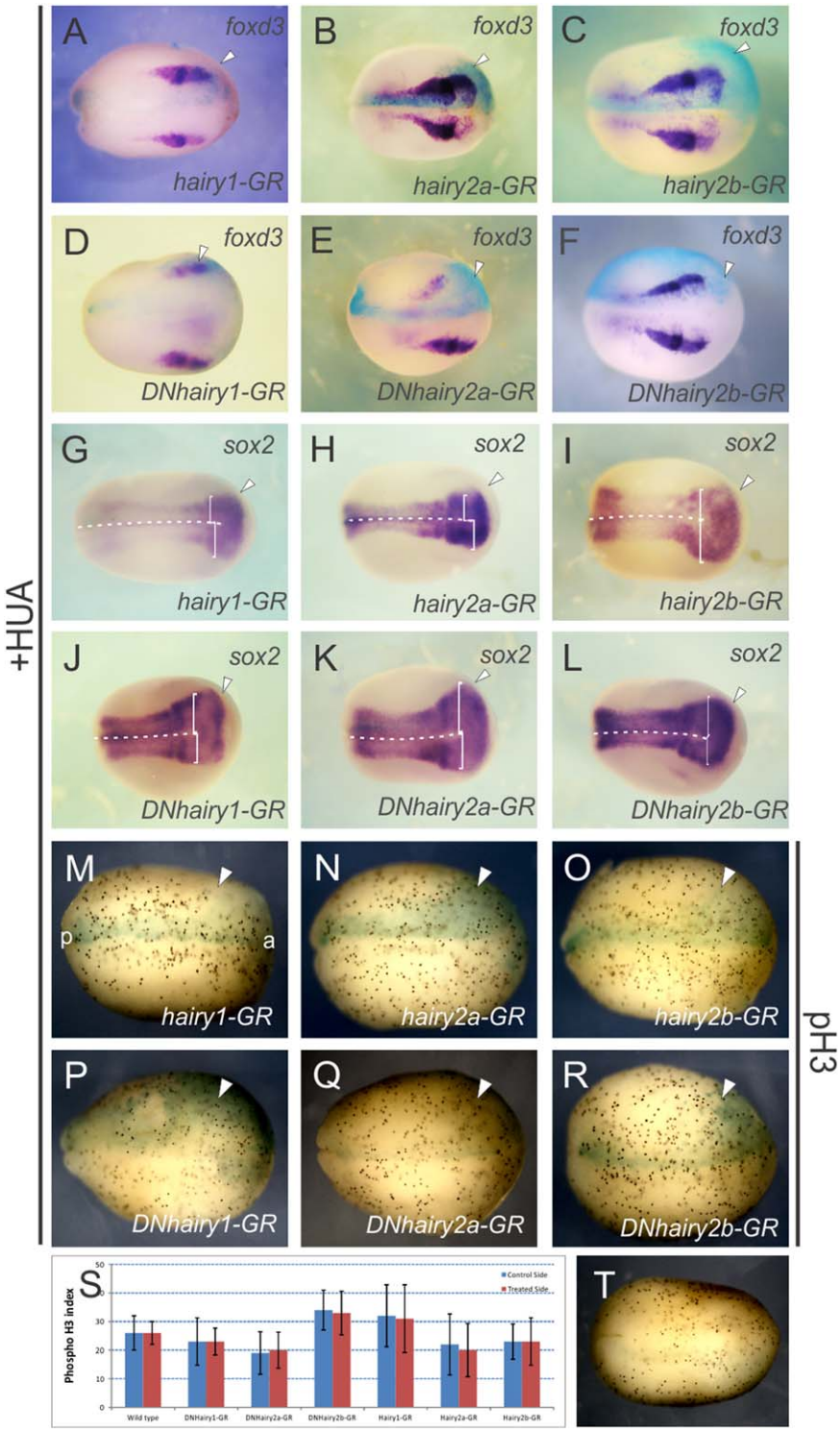

Fig. 7. *hairy* genes do not control cell proliferation during neural crest specification. **A–R:** Dorsal views of *Xenopus laevis* embryos; anterior side is on the right. The injected side is indicated by an arrowhead. Embryos injected into one blastomere at the 8- to 16-cell stage with 700 ng of *hairy* constructs mRNA were treated with dexamethasone and HUA (hydroxiurea 20 mM, aphidicolin 150 μM) at stage 11 until neurula stages and fixed. A–L: *hairy* inducible constructs mRNA or *DNhairy* constructs mRNA-injected embryos were incubated in the presence of HUA inhibitors mix until the neurula stage. The expression of *foxd3* was analyzed by in situ hybridization. *hairy* inducible constructs produced an expanded *foxd3* labeling (A–C) and *DNhairy* constructs caused a decrease in *foxd3* labeling (D–F). *hairy* constructs reduced the expression of *sox2* marker in the neural plate (G–I) and *DNhairy* constructs produced an expansion in *sox2* labeling (J–L). The presence of HUA caused no changes in the *foxd3* and *sox2* phenotype after hairy gain or loss of function. M–R: Whole-mount anti-phospho H3 immunohistochemistry labeling was performed as indicated in the Experimental Procedures section. The injected side can be recognized by the fluorescence of the lineage tracer fluorescein dextran and is indicated by a white arrowhead. **S:** Quantification of phospho-H3 labeling for *hairy* constructs-microinjected embryos. No significant changes were observed in cell proliferation caused by the microinjection of *hairy* or *DNhairy* inducible constructs. **T:** No significant anti-histone H3 staining was observed in embryos treated only with HUA.

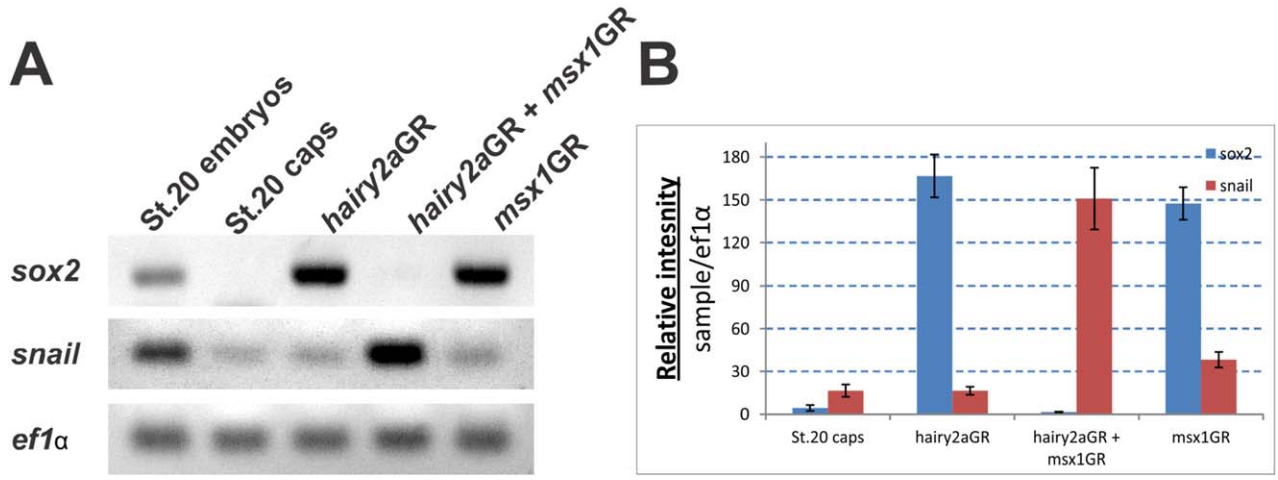

Fig. 8. *hairy2a* activity on naive ectodermal tissue. Each blastomere of a 2-cell stage embryo was injected with 1 ng of inducible construction mRNA of *hairy2a*GR, *msx1GR* or a mixture of both genes. The embryos developed until stage 8, and then animal caps were isolated and incubated with dexamethasone from equivalent stage 11 until stage 20. Total mRNA was isolated and analyzed by RT-PCR. **A:** The expression of ectodermal tissue markers *sox2* (neural plate) and *snail1* (neural crest) was assessed. The expression of *ef1α* was analyzed as a loading control for each sample. **B:** Quantitation of gene expression results shown in panel A. The results are expressed as relative intensity values (calculated as the ratio between each sample band intensity and its corresponding *ef1α* band intensity × 100).

7I, *hairy2b-GR* 66%, n = 42), and reduced prospective epidermis (*xk81a*, Fig. 7M, *hairy1-GR* 70% n = 20; Fig. 7N, *hairy2a-GR* 60% n = 20; Fig. 7O, *hairy2b-GR* 80%, n = 20), indicating that these effects occur independently of changes in cell proliferation. In contrast, embryos injected with the dominant negative forms of *hairy* genes and treated with dexamethasone and HUA showed a neural crest territory smaller than the control area (*foxd3*, Fig. 7D, *DNhairy1-GR* 58%, n = 19; Fig. 7E, *DNhairy2a-GR* 78%, n = 19; Fig. 7F, *DNhairy2b-GR* 60%, n = 10) and expansion of the neural plate (*sox2*, Fig. 7J, *DNhairy1-GR* 75%, n = 20; Fig. 7K, *DNHairy2a-GR* 55%, n = 20, Fig. 7L, *DNHairy2b-GR* 57%, n = 21), indicating that the effects observed are not due to changes in cell proliferation.

The state of cell proliferation in ectodermal cell populations was assessed by the in situ detection of a phosphorylated form of histone H3 (Fig. 7M–T). Under the conditions of gain (mRNA microinjection of full length *hairy* inducible constructs) and loss of function of *hairy* genes (mRNA microinjection of dominant negative inducible constructs), we found no significant changes in the state of proliferation in the ectodermal populations (Fig. 7M–R). These results were quantified by measuring areas in the control side and in the treated side of injected embryos (Fig. 7S) and showed no significant differences between both sides. Thus, we conclude that the expansion in the neural crest territory or in neighboring tissues due to overexpression of *hairy* genes or their dominant negative forms, respectively, is not due to an increase in cell proliferation but instead is the consequence of changes in cell fate. Embryos treated with HUA and stained with histone H3 showed no significant labeling (Fig. 7T).

hairy2a Activity on Naive Ectodermal Tissue

The animal cap assay was conducted to establish whether ectopic overexpression of the *hairy2a* gene, without the influence of other signals and factors present in the whole embryo, is sufficient to induce neural crest markers in naïve ectodermal tissue. The overexpression was performed by microinjection of *hairy2aGR* mRNA into the animal pole of two-cell stage embryos, and then the animal cap ectoderm was dissected out at the early blastula stage. The inducible protein in explants was activated at stage 11, and animal caps were processed for reverse transcriptase-polymerase chain reaction (RT-PCR) at stage 20. In control (noninjected) animal cap explants the expression of neural tissue specific marker *sox2* could not be detected, unlike a basal expression of *snail1* and the *ef1α* factor, which was used as loading control for mRNA (Fig. 8A). Also as a control, PCR was performed with RNA that had not been reverse-transcribed (-RT) to check for DNA contamination (data not shown). When *hairy2a* gene was overexpressed the *sox2*, a pan neural tissue marker, expression was observed but only a slight expression of *snail1* a neural crest marker. The *msx1* gene is involved in the induction of neural crest in vivo (Tribulo et al., 2003) but very poorly in vitro (Monsoro-Burq et al., 2005). In this work, we also evaluated the effect of *msx1* overexpression in isolated animal cap ectoderm and found that it has a similar ability to *hairy2a* to induce neural tissue. Because the expression of each gene separately did not seem to be sufficient to exclusively induce in vitro neural crest tissue, we decided to assess whether both could act synergistically. The *hairy2a* plus *msx1* overexpression in animal cap ectoderm led to a high expression of the *snail1* neural crest marker (Fig. 8A) in the absence of *sox2* neural marker. These results showed that both inducible constructs together are able to induce neural crest tissue and that *msx1* activity is also required for this induction.

hairy Genes Are Involved in the Survival of Neural Crest Cells

Previous studies indicated that apoptosis is important for the correct formation of the neural crest during the midneurula stages of *Xenopus* embryos. We explored whether *hairy* genes are involved in controlling apoptosis of neural crest cells by TUNEL (TdT-mediated dUTP Nick-End labelling) in whole embryos. We assessed *hairy* genes by overexpression of microinjected mRNA of inducible constructs upon their activation with dexamethasone at stage 15 (midneurula) once the initial induction of neural crest cells had been completed to not interfere with this process. It was

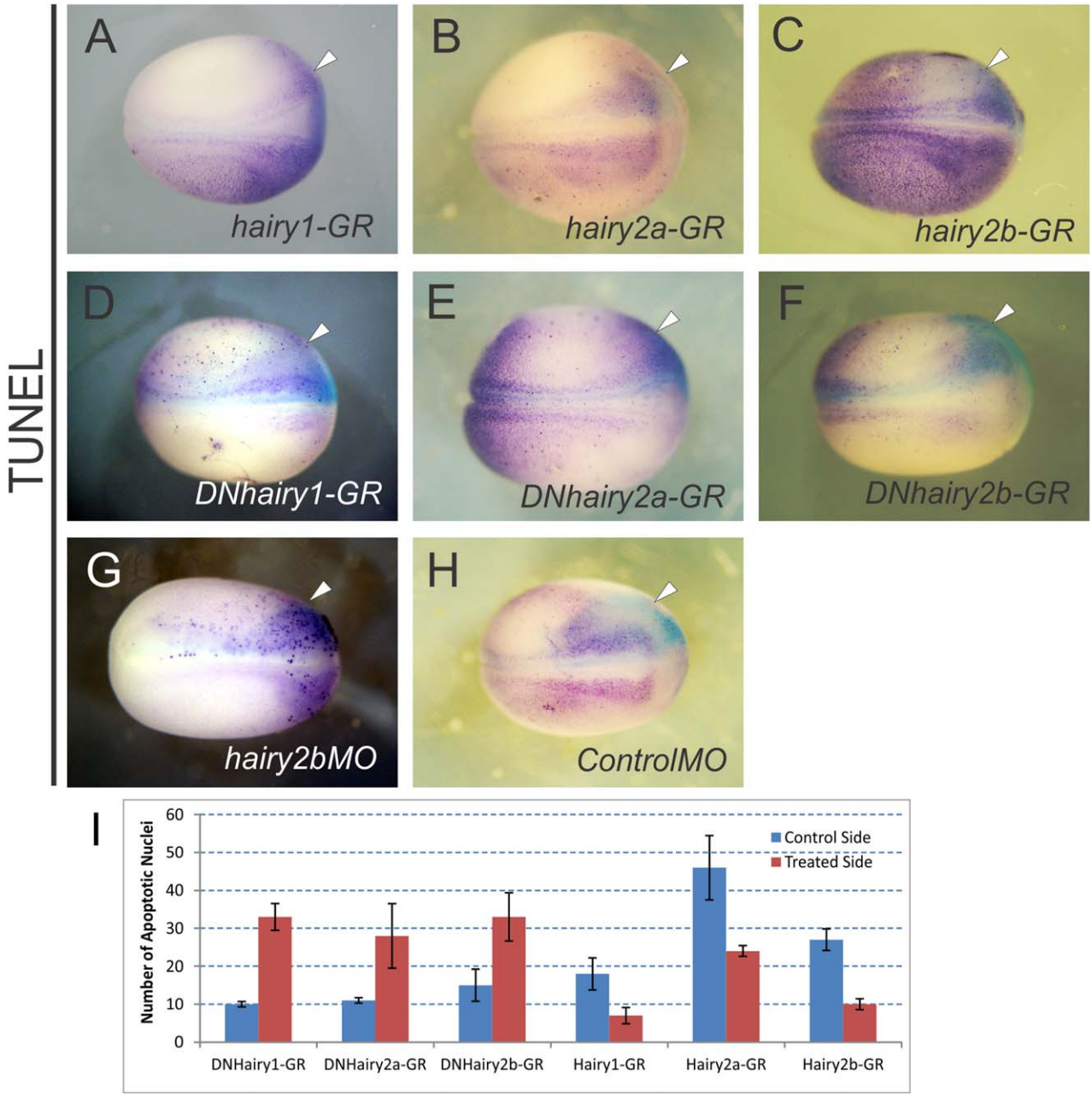

Fig. 9. *hairy* genes participate in the control of apoptosis during neural crest initial development. **A–H:** Dorsal views of *Xenopus laevis* embryos; anterior side is on the right. The injected side, indicated by an arrowhead, is also recognizable by the presence of the lineage tracer FDx (turquoise). Wholemount TUNEL labeling was performed as indicated in the Experimental Procedures section. Embryos were injected into one blastomere at the 8- to 16-cell stage with 700 ng of *hairy* inducible constructs mRNA (A–C), *DNhairy* constructs mRNA (D–F), *hairyMO* (G), or *ControlMO* (H), incubated until stage 17 and fixed. For inducible constructs, dexamethasone was added at stage 14, after the initial specification of neural crest. I: Quantification of TUNEL labeling for hairy construction-microinjected embryos (see Experimental Procedures section). The changes in apoptosis status caused by the microinjection of hairy constructions mRNA were statistically significant (Student *t*-test: $^{**}P \leq 0.05$; $^{*}P \leq 0.1$).

observed that overexpression of the three *hairy* genes caused inhibition in the normal apoptotic pattern in the injected side of the embryo (Fig. 9A, *hairy1-GR* 55%, n = 11; Fig. 9B, *hairy2a-GR* 75%, n = 8; Fig. 9C, *hairy2b-GR* 85%, n = 7), whereas injection of dominant negative forms of *hairy* genes produced an increase in the number of apoptotic nuclei in the injected side (Fig. 9D, *DNhairy1-GR* 31% n = 13; Fig. 9E, *DNhairy2a-GR* 86% n = 7; Fig. 9F, *DNhairy2b-GR* 60% n = 15). Additional evaluation of apoptosis in *hairy2bMO*-injected embryos showed an increase in apoptotic nuclei (Fig. 9G, *hairy2bMO*, 67%, n = 12) compared with control morpholino (Fig. 9H, *CoMO*, 15%, n = 13). These results indicate that *hairy* genes may act as antiapoptotic factors in the neural crest of *Xenopus* embryos.

hairy Genes in the Control of Neural Crest Migration

The expression pattern analysis revealed that *hairy* genes are expressed during the migration phase of neural crest cells and

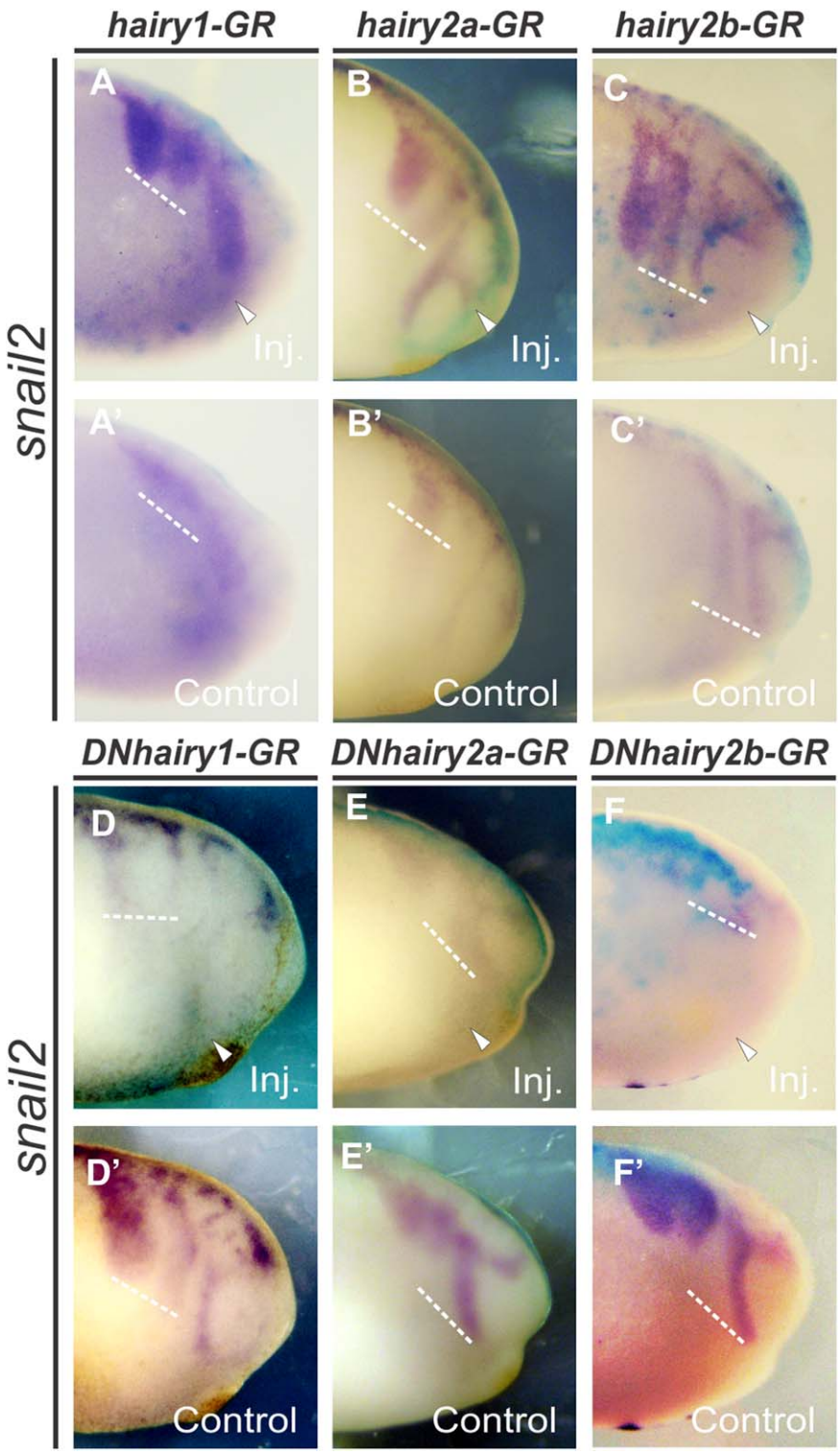

Fig. 10. *hairy* genes are involved in the control of neural crest migration. **A–F:** One blastomere of a 8- to 16-cell stage embryo was injected with 700 ng of inducible constructs mRNA and FDA as a linage tracer (turquoise, arrowhead). Embryos were fixed at stage 21–22. The overexpression of the hairy inducible constructs increased the migration of cephalic neural crest cells (*snail2*, A–C) compared with the control side (A′–C′). The overexpression of the *hairy* inducible dominant negative constructs reduced the migration of the cephalic neural crest (*snail2*, D,E) in comparison with the control side (D′–E′). The leading edge of cephalic stream migration is indicated by a dashed line.

suggests that they may have relevant functions in controlling this process. We took advantage of inducible constructs to assess the participation of *hairy* genes as they were activated after prospective neural crest cells had completed the process of induction, initial specification and apoptosis and before the onset of cell migration (St. 18). The overexpression of *hairy* genes at the

beginning of cell migration caused a greater advance of the migration front on the injected side, revealed by the expression of neural crest markers *snail2* (*hairy1-GR*, Fig. 10A, 60% n = 30; *hairy2a-GR*, Fig. 10B, 64% n = 36; *Hairy2b-GR*, Fig. 10C, 53% n = 60). In contrast, microinjection of dominant negative forms of *hairy* genes showed a decrease in the migration of the neural crest that was observed as a delay in the migration front of the neural crest cranial streams (*snail2*, *DNhairy1-GR*, Fig. 10D, 57% n = 37; *DNhairy2a-GR*, Fig. 10E, 61% n = 23; *DNhairy2b-GR*, Fig. 10F, 62% n = 23). Our results show that *hairy* genes are involved in the control of neural crest cells migration.

hairy2 Genes Are Differentially Required for the Neural Crest Migration Process

Because it remains yet to figure out the way in which *hairy* genes are involved in the migration of neural crest cells, if they act in the early process of epithelial mesenchyme transition, or in the later processes of cell adhesion, or in the migration itself, we took advantage of inducible constructs to evaluate two different moments of the migratory process. To assess the involvement of *hairy2* genes during the earliest step of neural crest migration, embryos were injected at the 8- to 16-cell stage in one dorsal blastomere with mRNA of dominant negative inducible constructs (Fig. 11A–D). The embryos whose constructs were activated at stage 17, close to the onset of migration, and fixed at stage 21 showed a strong inhibition of migration at the injected side, as revealed by the marker gene *snail2* (*DNhairy2a*, Fig. 11A, 69% n = 23, and *DNhairy2b*, Fig. 11C, 70% n = 24). On the other hand, in the injected embryos activated with dexamethasone at stage 20, when migration is effectively occurring, at stage 23 showed only a weak inhibition of migration at the injected side shown by the marker *snail2* (*DNhairy2a*, Fig. 11B, 35% n = 23, and *DNhairy2b*, Fig. 11D, 26% n = 23). We also carried out an approximation of co-injection of a dominant negative form of *hairy2* genes and a different *hairy* gene to rescue migration phenotypes. The dominant negative effects on migration assessed by the ectodermal marker *snail2* were rescued by the co-injection of a *hairy* gene and the activation of chimeric proteins at the onset of migration (stage 17, *DNhairy2a* + *hairy2b*, Fig. 11E, reduced migration 32% n = 22, and *DNhairy2b* + *hairy2a*, Fig. 11G, reduced migration 36% n = 25) and once the migration has already started (stage 20, *DNhairy2a* + *hairy2b*, Fig. 11F, reduced migration 18% n = 27, and *DNhairy2b* + *hairy2a*, Fig. 11H, reduced migration 22% n = 26). These results suggest that *hairy* genes are required more actively during the early stage of migration of the neural crest, possibly to prepare the cells for the epithelial to mesenchymal transition or to begin the migration itself, rather than for the period in which migration is taking place.

hairy2b Gene is Required for Neural Crest Migration

To assess whether the *hairy2b* gene acts through a cell-autonomous mechanism in the migration of the cephalic neural crest, we injected embryos with *DNhairy2b-GR* plus a lineage tracer and transplanted the neural crest region orthotopically into stage 17 wild-type embryos. The chimeric inducible protein was activated by dexamethasone at stage 18. Only the transplanted tissue was treated, which eliminated possible effects of the overexpression on neighboring tissues that could have also been affected. When FDA-injected neural crest grafts were transplanted into normal wild-type embryos a normal cranial neural crest migration pattern was observed (Fig. 12A, 87%, n = 15). *DNhairy2b*-injected neural crest explants were grafted onto normal host embryos and the migration pattern was normal when the inducible proteins were not activated (Fig. 12B–B′, 73%, n = 15). In contrast, neural crest cells from a *DNhairy2b-GR*-injected neural crest grafted and incubated in dexamethasone showed lack of migration (Fig. 12C, 67%, n = 15). Taken together, these observations indicate that the *hairy2b* gene is required for the normal migration of cranial neural crest cells and suggest that it could act in a cell-autonomous manner to support neural crest migration.

Analysis of the *hairy2b* Role in the Formation of Neural Crest Derivatives

We analyzed the effect of the loss of function of the *hairy2b* gene on the formation of the neural crest derivatives like craniofacial cartilage and pigmented cells. We found that when *hairy2b* expression is blocked there are alterations in the morphology and size of Meckel's (M), ceratohyal (CH), and ceratobranchial (CB) cartilages (Fig. 13B, 47%, n = 30). The *hairy1* gene is not a target sequence of *hairy2bMO* and we have shown its functional equivalence during neural crest development (Figs. (5 and 7)). We conducted a rescue experiment through the co-injection of *hairy2bMO* and *hairy1-GR* inducible construct. The co-expression was able to restore the craniofacial cartilage phenotype so that it was indistinguishable from cartilage of normal larvae (Fig. 13D, 93%, n = 30). We also observed that the overexpression of *hairy2b-GR* produced no effects on melanocyte pattern (stage 38, Fig. 13E, 100% n = 25, and stage 45, Fig. 13G, 100% n = 20). In the *DNhairy2b-GR* treated embryos no significant differences in melanocyte number or pattern were observed between injected and control sides (stage 38, Fig. 13F, 100% n = 25, and stage 45, Fig. 13H, 100% n = 20). These results show that the *hairy2b* gene 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 cells constitute a fascinating population of multipotent migratory cells that contribute to the formation of a wide range of derivative cell types of vertebrate embryos. As a consequence of the large contribution of neural crest to many specific cell types, organs, and body systems, this cell population is involved in several human disorders. Therefore, the study of the basic developmental biology of neural crest cells is a critical step to improve our understanding of these conditions and generate new diagnostic and therapeutic strategies. The interest of the scientific community in neural crest cells is increasing and the study of this cell population is attracting more attention from many research groups. During the last 15 years, great progress has been achieved regarding the molecular events that regulate the processes leading to the emergence of the neural crest cell population, known as neural crest induction. At present, it is accepted that the induction events involve at least 5 signaling pathways: BMP, FGF, Wnt, retinoic acid, and Notch. The growing diversity of studies in this area has established the idea that the normal

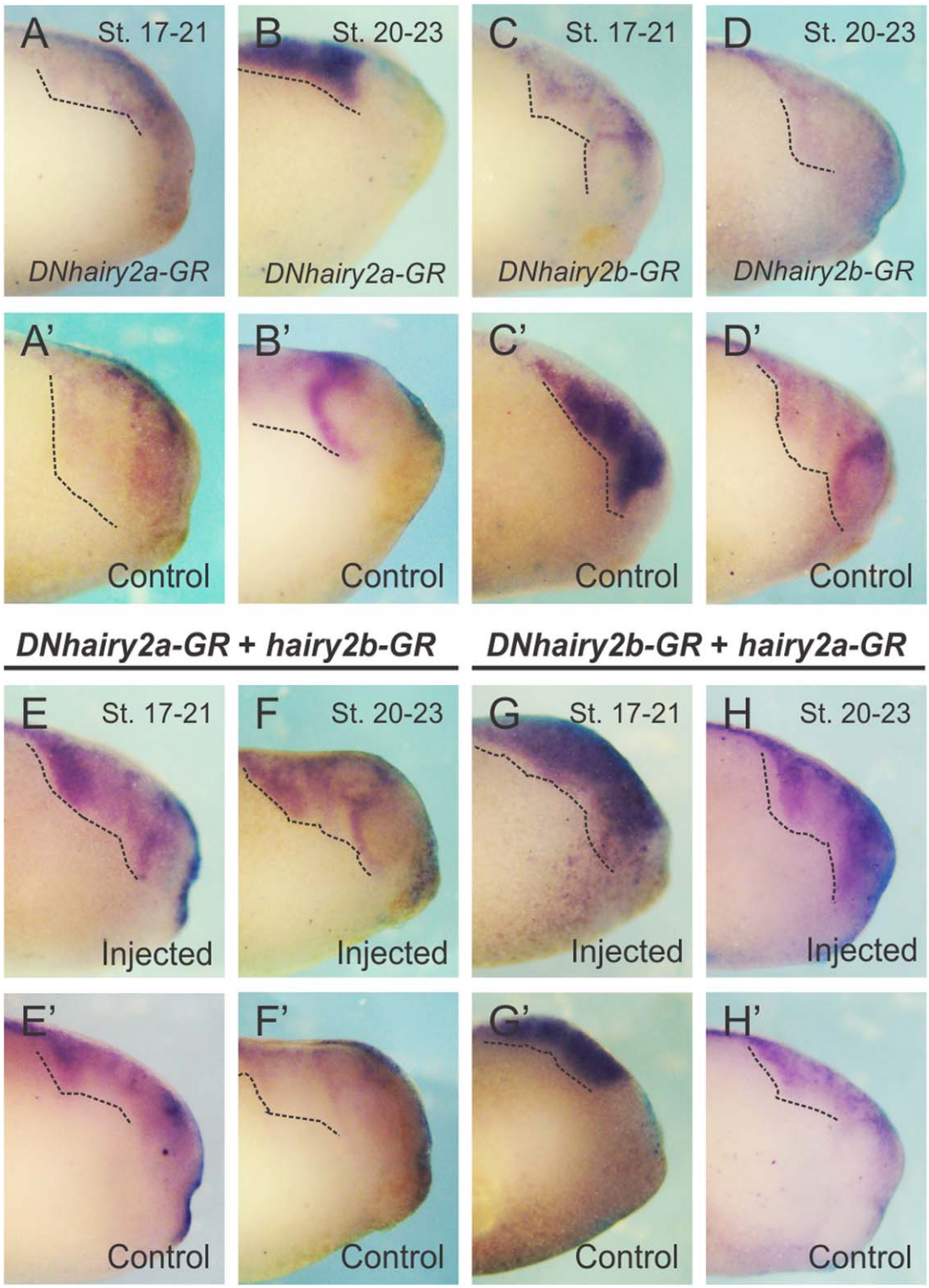

Fig. 11. *hairy* genes are differentially required for the neural crest migration process. The anterior side of the embryos is on the right. One blastomere of a 8- to 16-cell stage embryo was injected with mRNA encoding inducible constructions of *hairy2* genes and were cultured until stage 17 or 20 when the construction was activated with dexamethasone. After activation, embryos were cultured until stages 21 or 23, respectively, and the expression pattern of the *snail2* marker was analyzed. Black arrowheads indicate the injected side, which can be also recognized by the turquoise FDA staining. **A,C:** The overexpression of the dominant negative inducible constructs markedly decreased the migration of cephalic neural crest cells when dexamethasone was added at the beginning of neural crest cell migration (stage 17), in comparison with the control side (A′–D′). **B,D:** Neural crest cephalic migration was slightly inhibited when *DNhairy2* inducible constructs were activated at stage 20 when the migration was occurring. The leading edge of cephalic streams migration is indicated by a dashed line. **E–H:** Co-injection of *DNhairy2* mRNA and mRNA encoding a different *hairy2* gene inducible construct. E,G: The effects of reduced migration of *DNhairy2a* on the expression of the ectodermal marker *snail2* were rescued by the co-injection of *hairy2b*. F,H: The effects of reduced migration of *DNHairy2b* on the expression of the ectodermal marker *snail2* were partially rescued by directed co-injection of *hairy2a*.

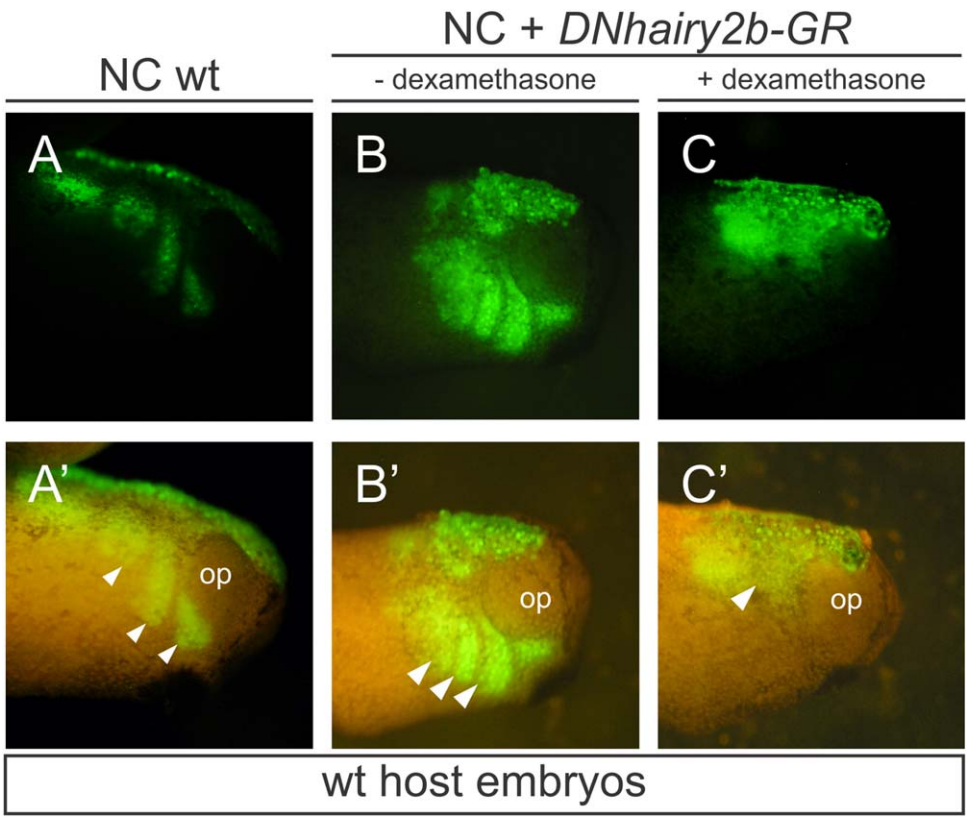

Fig. 12. *hairy2b* participates during neural crest migration. **A–C:** Transplantation of neural crest made in stage 16–17 embryos cultured until stage 23–24. Lateral views. Anterior side of the embryos is on the right. The three streams of cranial neural crest migration heads are indicated by white arrowheads. Transplanted tissues came from embryos injected with FDA (3.5 μg / embryo) (A–A′); 700 pg mRNA of *DNHairy2b-GR* without dexamethasone (B–B′) and with dexamethasone (C–C′). A–A′: FDA-neural crest transplant in normal recipient embryos. Note cell migration and migratory streams (arrowhead in A′). B–B′: Neural crest transplantation containing the inducible protein *DNHairy2b-GR* with FDA in normal recipient embryos ithout the addition of dexamethasone. Note the normal migration, similar to the one performed only with FDA (arrowheads in B′). C–C′: Transplant identical to (B) with the addition of dexamethasone. Note that neural crest migration is inhibited. Op, optic vesicle. All photographs taken through a fluorescence microscope were captured under the same conditions.

function of the Notch pathway is central to most events of cell fate decisions in the development of animals (Artavanis-Tsakonas et al., 1995; Cornell and Eisen, 2005; Bray, 2006; Louvi and Artavanis-Tsakonas, 2006) and that alterations in this signaling pathway are involved in many diseases including Alagille syndrome (defect in the neural crest cells development of heart), T-acute lymphoblastic leukemia cells (T-ALL), cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and multiple sclerosis, among other diseases (Roca and Adams, 2007; Sharma et al., 2007; Brosnan and John, 2009; Ayata, 2010).

The *hairy* genes, which are part of the Hairy and Enhancer of Split (HES) family, have been described in many vertebrate animals. They encode transcriptional regulators of the bHLH-O class (basic Helix-Loop-Helix-Orange), but the molecular details of how they control transcription are still poorly understood. *hairy* genes are nuclear effectors that have been shown to act in some developmental events (Davis and Turner, 2001; Iso et al., 2003; Fischer and Gessler, 2007), but there are new processes in which they could be essential. Here, we discuss the results of the biological roles of *hairy* genes in *Xenopus laevis* neural crest development and mechanisms of action as well as some still unanswered questions about the function and regulation of these proteins during neural crest development.

hairy Genes Expression in *Xenopus* Embryos

The *hairy1* gene is expressed in the neural plate and defines dorsally and ventrally the neural crest boundaries during the neurulation stages. This work describes in detail and for the first time the early *hairy1* expression in the prospective neural crest, highlighting that this gene is also expressed in areas limiting the prospective placodes that surround the prospective neural crest domain (Fig. 1A–D, Streit, 2004; Brugmann and Moody, 2005; Schlosser, 2006). The posterior and dorsal placodes are those that later develop the lateral line, and the otic and epibranchial placodes (Pieper et al., 2011). The anterior placodes comprise the optic vesicle and the olfactory and profoundal placodes. The profoundal placodes develop neuronal precursors, which migrate and are assembled with the neural crest cells to form ophthalmic sensory neurons (Pieper et al., 2011). Different studies in *Xenopus* have suggested the involvement of the Notch/Delta pathway in developing embryonic placodes (Glavic et al., 2004b; Zaghloul and Moody, 2007; Ogino et al., 2008), which could be directly related to the expression of *hairy1* and could play a role in the formation of these structures. Furthermore, studies have involved the mouse homolog gene related to *hairy1*, *HES1 (Drosophila)*, with embryonic development of otic, epibranchial, optic, and olfactory placodes (Cau et al., 2000; Hatakeyama et al., 2006; Ikeda et al., 2007; Jayasena et al., 2008). Therefore, *hairy1* could play a role

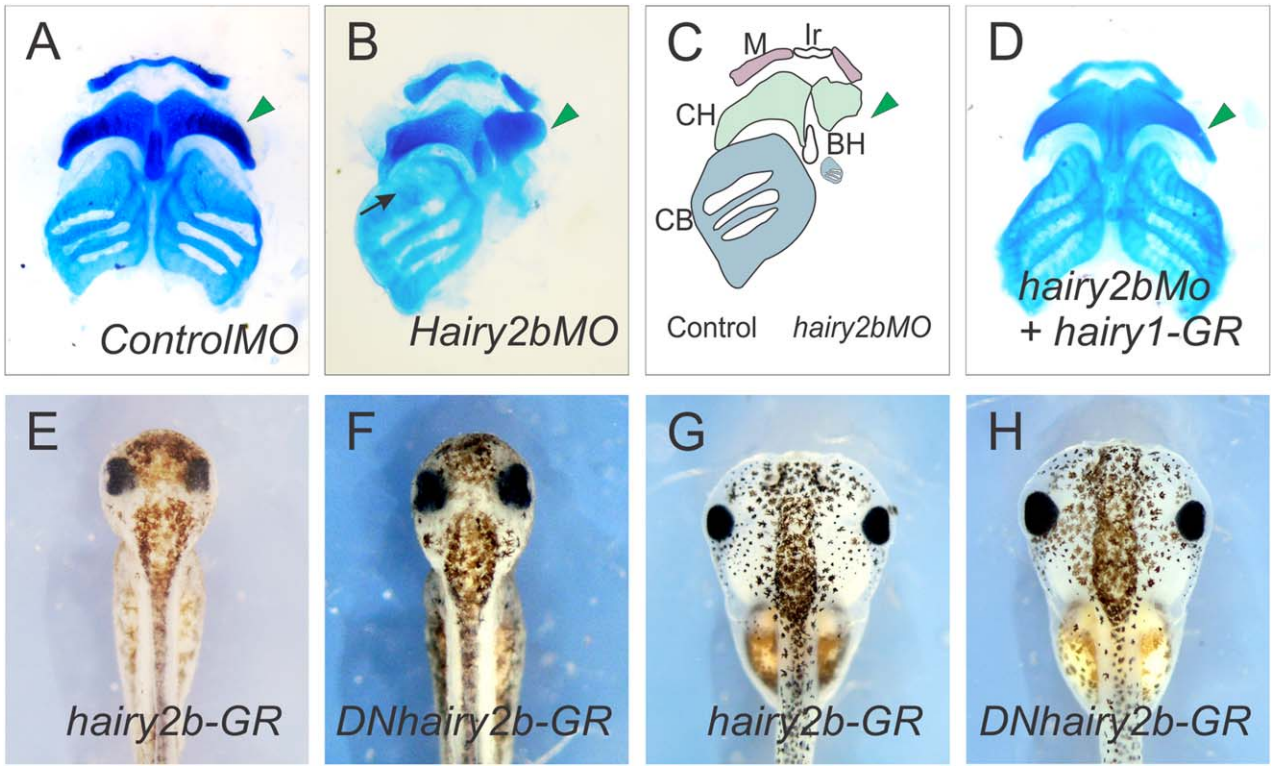

Fig. 13. *hairy* genes are involved in the formation of craniofacial cartilage derived from neural crest. Embryos were injected in one blastomere at the 16-cell stage; cartilage was visualized after Alcyan-blue staining and analyzed at stage 45. **A,B,D:** Anterior part is toward the top, ventral view. Arrowheads indicate the injected side. At stage 45, *hairy2bMO*-injected (B) embryos show a reduction in cranial cartilage while *ControlMO*-injected (A) embryos show no changes in the same cartilage. **C:** Diagram of the effects of *hairy2bMO* on head cartilage of a *Xenopus* tadpole. CH, ceratohyal cartilage; CB, ceratobranchial cartilage; BH, basihyal cartilage; Ir, infrarrostral; M, Meckel cartilage. **E–H:** Normal cartilage formation was completely rescued by co-injection of *hairy2bMO* and *hairy1-GR* mRNA. E,F: Analysis of melanocyte (Stage 38) formation in embryos injected with 700 ng of *hairy2b-GR* (E,G) and *DNhairy2b-GR* (F,H) mRNA. Injected embryos do not show inhibition in melanocyte development.

in the formation of the outer boundary of the tissue corresponding to the neural crest or in the formation of placodes themselves, a role that could add to the already established one in primary neurogenesis. Our results also showed that *hairy1* expression closely resembles *delta1* or *delta2* expression during neurogenesis in *Xenopus* dorsal ectoderm (Chitnis et al., 1995; Glavic et al., 2004b).

Detailed analyses performed in this study on the expression pattern of *hairy2a* and *hairy2b* genes showed that they are expressed at the neural fold and outer layer of the floor plate within the neural plate. This study provides a detailed description of the *hairy2a* expression pattern (Fig. 1), which until this work had only been described until mid neurula stages (Turner and Weintraub, 1994). A previous work of (Murato et al., 2007) showed by RT-PCR analysis that *hairy2b* is expressed more abundantly than *hairy2a* during early development in *Xenopus laevis*, particularly in the neural crest domain, while both genes are expressed similarly in the prospective floorplate. Our expression study indicated that *hairy2* genes could also be involved in neural crest cell migration. Previous studies also suggested that some *hairy*-related genes are also expressed in the tissue edges (i.e., in *Drosophila* embryos) and control their development (Davis and Turner, 2001). In vertebrates, it was found that *hairy* genes control processes in which demarcation of the territory is essential, a characteristic that could resemble in many ways the case of mesodermal segmentation in the formation of somites (Davis and Turner, 2001; Umbhauer et al., 2001; Iso et al., 2003; Fischer and Gessler, 2007). These findings and our results suggest that the expression of *hairy*-related genes could play essential roles at an initial stage in the formation of tissue boundaries that could serve to establish the different cell domains (neural, neural crest and epidermis) in the embryonic ectoderm.

hairy Genes Control Cell Fate in the Absence of Cell Proliferation

The changes observed in the neural crest territory in the experiments of loss and gain of function for the three *hairy* genes were accompanied by an expansion or a compensatory decrease in the adjacent ectodermal territories, neural plate, and prospective epidermis (Fig. 7). Moreover, the results showed that the activity of hairy genes could lead to a change in cell fate in territories contiguous to the neural crest as well. This behavior could be explained by considering the model that proposes the existence of an embryonic region which is called the competency area that defines the region where the neural crest can be induced even when transiently is specifying another cell fate (Bastidas et al., 2004; Steventon et al., 2009). According to our results in amphibian embryos, *hairy* genes do not participate in the control of cell proliferation but in controlling cell fate, which was observed in the study of hairy gene function in the presence of a cell proliferation inhibitors mixture (HUA, Fig. 7). Thus, an increase or decrease in *hairy* gene activity was followed by a proportional change in the size of neural crest territory and an opposite change in adjacent territories. In

the inhibited proliferation situation, the changes observed in the size of each cell population may be interpreted as being caused by the adoption of a different cell fate. These results were confirmed by cell proliferation assays using the detection of mitosis marker, the phosphorylated histone H3 protein, which is the predominant form in the chromatin of cells that are replicating their DNA (Turner and Weintraub, 1994; Saka and Smith, 2001) (Fig. 7). This work confirmed that the growth of the neural crest territory was not due to increased cell proliferation, so that it may be inferred that *hairy* genes have the ability to transform prospective epidermal cells and neural ectodermic cells into neural crest cells. In our results, there was no completely ectopic formation of neural crest in an area away from it. This suggests that *hairy* genes alone are not capable of directing the generation of neural crest as would do a "master gene" or "field selector gene" (Carrol et al., 2004). According to the temporal and spatial pattern of cell division during *Xenopus* embryogenesis it is known that the concentration of mitotic cells is localized in the neural plate and prospective neural crest rather than in the nonneural ectoderm (Harris and Hartenstein, 1991; Saka and Smith, 2001). However, our observations suggest that *hairy* genes, despite having expression in the neural crest, are not involved in cell cycle regulation, which maintains a balance in the number of cells in the ectodermal population of ectoderm during development. This behavior, which is consistent with the expression of the gene in this region, is similar to that observed for genes such as *snail1* (Sargent and Bennett, 1990), *snail2* (Mayor et al., 1995), *foxd3* (Sasai et al., 2001), among others. Moreover, the ability of *hairy2a* to induce *snail2* expression suggests that *hairy2a* could be one of the genes located at the top of the genetic cascade of neural crest induction (Mayor and Aybar, 2001; Steventon and Mayor, 2012; Theveneau and Mayor, 2012b).

Our results showed for the first time that *hairy1*, *hairy2a*, and *hairy2b* genes alone do not affect the process of cell proliferation during the early stages of neural crest development. Moreover, in the control of cellular proliferation, it has been observed that when *hairy2a* and *hairy2b* function are eliminated with a 1:1 mixture of morpholino oligonucleotides against both genes the cell proliferation rate decreases (Nagatomo and Hashimoto, 2007). This different result could be explained as the microinjection of morpholino does not allow specific temporal control of gene action during the study and thus other cellular processes could have been indirectly affected. Other researchers demonstrated that overexpression of steroid-inducible *hairy2b* constructs or a DNA-binding-mutant increased cell proliferation in both cases (Nichane et al., 2008b). The differences in the protein sequence/activity of the injected constructs (wild-type and bHLH-containing constructs) in this work may be the main reason for the differences previously observed regarding cell proliferation. Apparently, the solely mutation of the basic domain could not be sufficient to produce changes in the cell proliferation rate compared with wild-type construct (Nichane et al., 2008b). Also, the differences in the activation stage of inducible proteins could explain the differences, which could in turn suggest that there is a very precise control of cell proliferation with respect to the timing of the activity of the *hairy2b* gene.

hairy Genes in the Control of Apoptosis in Neural Crest

Apoptosis is required for normal development to occur (Baehrecke, 2002). Studies of cell death in *Xenopus* have identified an apoptotic program that starts at the beginning of gastrulation and remains active in many tissues (Sible et al., 1997; Stack and Newport, 1997; Malikova et al., 2007), particularly in the neural crest (Hensey and Gautier, 1997). It has been proposed that the neural crest is a population of cells that presents a high degree of apoptosis because it is in a highly dynamic state of specification and undergoes constant evaluation and remodeling to give rise to derivatives (Tribulo et al., 2004). In this work, the TUNEL technique demonstrated that *hairy* genes have anti-apoptotic activity in the ectodermal tissue (Fig. 9). Based on the normal expression of *hairy* genes during neural crest induction and on experiments using inducible constructs, we could suggest that one activity of these genes in neural crest cells is to act as antiapoptotic factors to promote cell survival, thus probably contributing to correct cellular specification. *hairy* genes could be part of the apoptotic control gene network. It has been described that different factors are part of the *Xenopus* programmed cell death gene network which includes the anti-apoptotic factor *bcl2* (Cosulich et al., 1996; Johnston et al., 2005; De Marco et al., 2010), the pro-apoptotic factor *bax* (Sachs et al., 1997, 2004; Kluck et al., 1999), *cytochrome C* (Kluck et al., 1997), *apaf-1* (Newmeyer et al., 2000), *caspases* (Cosulich et al., 1999; Rowe et al., 2005), among others. Because they are transcription factors, *hairy* genes could exert transcriptional control of these genes directly involved in the execution of the apoptotic program. Recently other factors were described that promote cell survival in the early development of *Xenopus* neural crest such as *snail2* (Tribulo et al., 2004), *sox10* (Honore et al., 2003), and cell signaling pathway Edn1/Ednra (Poelmann and Gittenberger-de Groot, 2005; Bonano et al., 2008), among others, which act in opposition to the proapoptotic factor *msx1* (Tribulo et al., 2004) to maintain the balance that allows the existence of an adequate number of neural crest cell precursors in the correct state of specification. It would be interesting to determine the relationship between these regulatory genes (*snail2*, *sox10*, *ednra*, etc.) and those that directly control the apoptotic process (*bcl2*, *bax*, *caspases*, etc.).

Requirement of *hairy* Genes in the Formation of Neural Crest Derivatives

Total or partial loss of the Notch/Delta pathway in mouse embryos leads to a considerable decrease in the size or even the disappearance of neural crest craniofacial cartilage derivatives (Akimoto et al., 2010; Levi et al., 2010; Siar et al., 2010). It was earlier shown that the *hairy2b* gene reduces neural crest derivatives such as chondrogenic precursors and a *Xenopus laevis* melanocyte marker (Nichane et al., 2008a). Our experiments in *Xenopus* embryos, carried out by assessing the loss of function of the antisense oligonucleotide injection *hairy2bMO*, showed a requirement of this gene similar to those previously described (Fig. 13). In addition, our results showed that the loss of function of the *hairy2b* gene can be rescued by co-injection with the inducible *hairy1* gene construction. Our results indicate that only the craniofacial cartilage precursor cell population is affected but not melanocyte precursors (Fig. 13), although this should be strengthened by further molecular studies.

Members of the *hairy* Gene Family Exhibit Functional Equivalence

The *hairy* gene expression patterns in the ectoderm and the high similarity in their sequences (this work, Turner and Weintraub, 1994;

Dawson et al., 1995; Tsuji et al., 2003) suggest that *hairy* genes could present a functional equivalence. In this study, the function of *hairy2b* gene was blocked with an antisense oligonucleotide (*hairy2bMO*, Fig. 5, Heasman et al., 2000; Ekker and Larson, 2001). This loss of *hairy2b* gene function was rescued by the co-injection of *hairy1-GR*, *hairy2a-GR*, or *hairy2b-GR* when inducible chimeras were activated at stage 12 (Fig. 5). These results suggest that, in *Xenopus* embryos, at least the main functions attributed to the *hairy2b* gene can also be performed by the *hairy2a* gene and even by the *hairy1* gene, for example, in the early specification of neural crest cells. The functional equivalent behavior observed in *hairy* family genes in *Xenopus* as well as the pattern of expression characteristic of each of these genes would suggest that changes in this gene family may be associated with the model known as Duplication-Degeneration-Complementation (DDC; Force et al., 1999; Minguillon et al., 2003). In the classical model, it is proposed that a common ancestral *hairy* gene suffers duplication, and this duplication must first be fixed before it is observed for gene evolution. Once set, two possible destinations are generally conceived. In the "nonfunctional" one, a mutation may result in the creation of a pseudogene which very likely is to be degraded. In the second, "neofunctionalization," after a time of selection, the new allele of the gene can gain new functions and new sites of expression. In the DDC model, there could probably be a third destination in which both functional genes remain relatively related to their inherent potential and then suffer a "sub-functionalization." Their roles or functions would become partitioned between duplicate copies and thus increase the fitness of the organism by removing conflicts between two or more functions. Other examples of gene families that are consistent with the DDC model are *engrailed* (Force et al., 1999), *beta-globin* (Aguileta et al., 2004), *sox* (Navratilova et al., 2010), *hoxb1* (McClintock et al., 2002), and the *alpha subunit of Na + / K + ATPase* genes (Serluca et al., 2001), among others.

hairy Genes Are Involved in Cell Migration Processes

This study describes that *hairy2a* and *hairy2b* genes are expressed in the migratory neural crest (Fig. 1) and this fact suggests that both genes are functioning in this stage, so it was in our interest to assess the requirement of *hairy* genes during migration. To evaluate their role, we took advantage of the special characteristics of inducible constructs, which were activated only after the neural crest precursors were formed so as not to alter their number and initial specification state. Overexpression and activation of *hairy* chimeras in stage 18 when neural crest migration is just beginning produced an increase in the migration of neural crest cells which was observed as a faster advance of the front of migration on the treated side (Fig. 10). Blocking the activity of *hairy* genes with the dominant negative constructs in similar conditions led to a reduction in neural crest migration. These early effects were stronger than the observed changes produced by the overexpression and activation of inducible proteins when migration was occurring. *hairy* genes could be involved in the migration process by two different mechanisms. In a cell-autonomous mechanism, neural crest cells themselves require *hairy* genes to carry out the migration process. In a non–cell-autonomous mechanism, neural crest neighboring tissues directly or indirectly require *hairy* genes that are expressed in the neural crest. The results obtained in the experiments of treated neural crest cells grafting into a normal recipient embryo to evaluate *hairy* genes functions in the migratory phase indicate that the *hairy2b* gene is required for cranial neural crest cells to migrate properly, and that this requirement is taking place through a cell-autonomous mechanism (Fig. 12).

Moreover, it is also likely that *hairy* genes could also be required to control genes involved in cytoskeletal remodeling and cell shape change needed for starting the migration itself (Wunnenberg-Stapleton et al., 1999; Lucas et al., 2002) and for the expression of genes involved in the migration process such as extracellular matrix molecules and their receptors, cell adhesion molecules, and guide molecules. Examples of these guide molecules are Neuropilin/semaphorin 3 (Gammill et al., 2007), EPHR/ephrin (Wehrle-Haller and Weston, 1997; Nakamoto, 2000; Mellott and Burke, 2008), cadherins (Perris and Perissinotto, 2000; Snider et al., 2007; Acloque et al., 2009), among others. In this study, using different tools and experiments, we demonstrated for the first time that *hairy* genes are also required in the process of neural crest migration in *Xenopus laevis* embryos. However, further experiments are still needed to determine the mechanisms by which they act to promote epithelial to mesenchymal transition, the acquisition of a migratory control, the need to address a target tissue over long distances, or even the entrance and colonization of target tissues (Carmona-Fontaine et al., 2008; Kulesa et al., 2010).

Transcriptional Activity of *hairy2* Genes During Neural Crest Induction: Structure–Function Relationship in *hairy* Genes

The effects observed on molecular phenotypes indicate that overexpression of full length and dominant negative Hairy proteins (Fig. 6) were able to retain their structural and conformational properties despite being fused to a heterologous protein domain. In turn, GR domain functionality was demonstrated by the ability of the chimeric proteins to differentially affect developmental stages that occur in a temporal succession. The dominant negative *hairy* genes constructed in this work showed that the choice of protein domains to achieve the loss of function was adequate. As in other examples (LaBonne and Bronner-Fraser, 2000; Sasai et al., 2001; Glavic et al., 2002; Aybar et al., 2003; Tribulo et al., 2003), and many references therein), these dominant negatives allowed us to achieve specific effects which were the opposite to those obtained by overexpression of the whole protein. An interpretation of the observed effect is that the overexpressed bHLH domain is structurally and functionally enough to bind to its target sequences in DNA by competition and block the activities of the native gene in the process of neural crest induction. The Hairy dominant negative constructs in this work were different from those previously reported by Nichane et al. (2008a) as they kept the Orange domain in the DNA construct. This biochemical competition was particularly revealed given that overexpression of a whole molecule as well as its dominant negative showed notorious effects on neural crest marker genes expression (Fig. 7), that were able to completely be rescued by the co-injection of wild-type *hairy* genes. These results also indicate that Orange, WRPW and other potential structural domains or motifs located in the C-terminal region of the Hairy molecules are fundamental for their biological functions during neural crest development (Nakatani et al., 2004; Tsuji and Hashimoto, 2005; Sun et al., 2007). bHLH genes encode for proteins that are classified into three groups according to their structural and biochemical characteristics: class A are transcriptional activators, class B are bHLH proteins with a leucine zipper, and

class C are transcriptional repressors (Murre et al., 1994; Fisher and Caudy, 1998; Massari and Murre, 2000). Although *hairy* genes are classified as transcriptional repressors judging by the presence of bHLH in their primary structure (Tsuji et al., 2003), recent studies on these transcription factors suggest that the molecular mechanisms are not always limited to negative transcriptional regulation but may even become transcriptional activators (Bae et al., 2000; Sun et al., 2001; Giagtzoglou et al., 2003; Ju et al., 2004; Fischer and Gessler, 2007). Another example of a dual functional transcription factor gene is *foxd3*, which shows both repressor and activator activity depending on the cellular context (Sutton et al., 1996; Guo et al., 2002; Lee et al., 2006; Steiner et al., 2006; Yaklichkin et al., 2007). Based on these considerations, we decided to evaluate the transcriptional activity of the *hairy2* gene in the neural crest induction process, which was still unknown. To analyze precisely the way in which *hairy2* genes regulate neural crest induction, inducible chimeric proteins were generated in which Orange and WRPW domains were replaced by an activator domain of a viral protein (E1A) and the repressor domain of engrailed protein (EnR). This experimental approach has been used and validated to analyze the transcriptional activity of several transcription factors (Jaynes and O'Farrell, 1991; Glavic et al., 2001; Tamashiro et al., 2008; Tran et al., 2010). Our results showed that *hairy2* repressor constructs produce the same phenotypes that were observed when the wild-type *hairy2* genes were overexpressed (whole molecule, Figs. (3 and 5)). Thus, we can say that *hairy2* genes are repressors of their own target genes in the process of neural crest induction. Thus, it can also be proposed that it is possible that the transcriptional repression of other repressor genes could be favorable for the induction of neural crest observed when overexpressing the Hairy genes. Our findings in this regard are coincident with the overexpressions of other genes, also proposed as transcriptional repressors that are expressed in the neural crest during the same stage, for example, *snail1*, *snail2*, *foxd3*, *pax3*, among others. (LaBonne and Bronner-Fraser, 1998; Sasai et al., 2001; Aybar et al., 2003; Monsoro-Burq et al., 2005; Sato et al., 2005). Based on the above, it could be speculated that the phenomenon of transcriptional repression is a key feature for the proper development of the neural crest. It has been postulated that the transcriptional repression conducted by *hairy* genes can be performed by two different mechanisms. One mechanism is the "active repression" mediated by the co-repressor Groucho. In turn, the Groucho repressor interacts with Hairy domain WRPW. Thus, in association with Groucho, Hairy protein binds directly to the N-box and actively represses gene expression (Ohsako et al., 1994). The other mechanism could be a regulation of "dominant-negative" type. Most bHLH factors such as Mash1 bind to the E-Box (CANNTG) and activate gene transcription. Hairy proteins form heterodimers with such nonfunctional bHLH activators and inhibit activity by a mechanism called competitive inhibition or blockade of binding target sequences in DNA (Fischer and Gessler, 2007). Considering our results, perhaps the mechanism of "active suppression" could be the mechanism by which Hairy genes exert their function in the process of neural crest induction.

Relationships of *hairy* Genes in the Genetic Cascade of Neural Crest Induction

It has been reported that *hairy2a* gene expression in the prospective neural crest is altered by an appropriate activity of BMP4 for the induction of the neural crest (Glavic et al., 2004a). In this study we decided to expand knowledge of the relationship of *hairy* genes with the BMP pathway and thus evaluate how experimental interference on BMP pathway activity affects *hairy2a* and *hairy2b* gene expression in the neural crest. Our results showed that ectopic expression of a dominant negative form of BMP4 called CM-BMP4 (Hawley et al., 1995) or the mutated type I BMP receptor called dnBMPR (Graff et al., 1994; Suzuki et al., 1997) block the BMP signaling pathway that results in the increased expression of *hairy* genes. Moreover, the overexpression of the intracytoplasmic component of the BMP signaling pathway *smad4b* (Chang et al., 2006), which increases the activity of the pathway, decreased *hairy2* gene expression (Fig. 2). It is apparent that changes in BMP pathway activity can affect *hairy2* gene expression. One possible explanation for our results is that BMP pathway activity changes affect the activity gradient of this molecule in the ectodermal tissue that would be required for the induction of neural crest (Mayor and Aybar, 2001; Tribulo et al., 2003). Thus, for example, a decrease in the activity of BMP flattens the gradient laterally extending the intermediate values area, which has been shown to be required for the specification of the neural crest and the neural fold and thus explains the increased *hairy* gene expression in this territory (Tribulo et al., 2003; Linker et al., 2009). Induction models of neural crest have been proposed in which at the onset of neurulation an inhibited BMP activity is needed (Tribulo et al., 2003; Steventon et al., 2009). An alternative explanation might consider direct regulatory effects of BMP/Smad pathway on *hairy* genes expression. At present, despite the great progress made, there is still no accurate and detailed picture of how control occurs downstream of these cellular signaling pathways.

Although many vertebrate HES family genes are regulated by the Notch signaling pathway, a few such as zebrafish *her5* (Geling et al., 2003) or mouse *Hes6* and *Hes3* (Bae et al., 2000; Hirata et al., 2000; Koyano-Nakagawa et al., 2000) seem to work independently of the Notch pathway, unlike *hairy* (*h*) genes in fly (Fisher and Caudy, 1998; Davis and Turner, 2001). This evidence suggests that target genes of the Notch pathway act without activating the canonical connection in this way and would be activated by other functional pathways simultaneously. The key point to determine what factors govern target gene activation of the Notch pathway in different development contexts can be found in regions of the promoters of responsive genes in this pathway. For example, *Drosophila HES* genes (Bailey and Posakony, 1995) contain a Su(H) paired binding site (SPS) responsive to this transcriptional activator gene of the Notch signaling pathway (Hsieh et al., 1996; Nellesen et al., 1999; Krejci and Bray, 2007). This consists of two high-affinity binding sites in opposite orientations, typically separated by 15–17 base pairs. SPS is a site whose response element modulates the activity of target genes of the Notch pathway. This SPS site, which was first found in *Drosophila* and then in different species, has been evolutionarily conserved. It has been reported that this site is present in *Xenopus laevis hairy2* (Davis et al., 2001) as well as in *her6* and *her9*, zebrafish orthologs of *Xenopus hairy1* and *hairy2*, and also in zebrafish, blowfish, and spotted puffer *HER1* gene (Gajewski and Voolstra, 2002; Brend and Holley, 2009). In mouse, *Hes1* is activated by the binding of the homolog of Su(H) and KBF2/RBP-J kappa to its promoter (Jarriault et al., 1995; Nam et al., 2007; Friedmann and Kovall, 2010).

The presence of the SPS site was shown in human *HES4* and *HES1*, which are orthologs of *Xenopus hairy1* and *hairy2*

(Gajewski and Voolstra, 2002; Katoh, 2004; Nam et al., 2007), and was also recently shown in arthropods (Rebeiz et al., 2012). Our results revealed that *Xenopus hairy1*, *hairy2a*, and *hairy2b* genes respond to changes in the regulation of the Notch/Su(H) pathway (Figs. (2 and 3)). This coincides with previous observations (Glavic et al., 2004b) showing this effect on *hairy2a* exclusively during the induction process of the neural crest in *Xenopus* laevis. Surprisingly, Nichane et al. (2008a) suggested that there would be no regulatory link between the Notch pathway and the *hairy2b* gene, while an SPS site was found in the *Xenopus* promoter of the *hairy2a* gene (Davis and Turner, 2001) and in other *HES* family members (ESR1 and Esr10) (Lamar and Kintner, 2005). However, the existence of this SPS site genes in *Xenopus laevis hairy1* and *hairy2b* has not been confirmed yet.

Final Considerations

The involvement of the *hairy2* gene in the induction of the early neural crest process was proposed previously (Nagatomo and Hashimoto, 2007; Nichane et al., 2008a, 2010) as well as the specification of the lens (Murato and Hashimoto, 2009). However, in the work of (Nagatomo and Hashimoto, 2007) no comparative or differential studies of activities of each of the *Xenopus hairy2* genes were conducted. Furthermore, the study by Nichane et al. (2008b) determined that the specification of neural crest progenitors are executed by the Notch/Su(H) signaling pathway, which contradicts previous evidence (Glavic et al., 2004b) and the results described in this study. The main aspects highlighted in this work are the following. In the *Xenopus laevis* embryo, the normal early development of the neural crest requires the activity of the Notch/Su(H)/Hairy pathway, which would be integrated to other cell signals running autonomous action through its *hairy2* genes in neural crest cells. The genes studied would be perfectly integrated into the proposed two-stage model for neural crest induction (Huang and Saint-Jeannet, 2004). A model of multiple signals is also suggested in which the neural crest originates in a precise position where there is a coordinated and integrated drive of upregulators. This action would take place in circumstances in which a moderate activity of the BMP signal and the coupling with the timing of a Notch/Su(H)/Hairy signal input from the ectoderm provides a suitable environment for the normal development of the neural crest cell population (Fig. 14).

Experimental Procedures

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; mRNA synthesis, microinjection, and lineage tracing were performed as previously described (Aybar et al., 2003; Bonano et al., 2008). In vitro transcribed mRNA was synthesized from pCS2+ by standard procedures. Microinjections were performed in specific blastomeres to direct the in vivo overexpression and activity of each *hairy* chimeric protein to a precise stage and embryo territory, thus allowing the experimental analysis of ectodermal cell populations' specification without detectable indirect effects on development processes occurring in the mesodermal tissue.

DAPT Treatments

To assess Notch activity, explants of neural crest embryos were cultured with 100 μM of DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester, Sigma, St Louis, MO). DAPT explants of neural crest were cultured until different stage (stages 13 to 17, and 17 to 22), finally the DAPT explants of neural crest were process using and situ hybridization procedure.

DNA Constructs and Morpholino Antisense Oligonucleotide

The development of hormone-inducible proteins allows an accurate control when they perform their actions (Hollenberg et al., 1993; Kolm and Sive, 1995) and this has a widespread use (Glavic et al., 2004b; Tribulo et al., 2004; Gestri et al., 2005; Sato et al., 2005; Wawersik et al., 2005; Chang and Harland, 2007; Zaghloul and Moody, 2007; Li et al., 2009; de Croze et al., 2011; Kawasaki-Nishihara et al., 2011, and many references therein). In our case, because the *hairy* genes are expressed and function during early gastrulation (Lopez et al., 2005), it was necessary to use this type of molecular tools to prevent early effects and thus specifically analyze the role and requirement of these genes only during the neural crest development process, which occurs at later stages. This precise temporal control was combined with a specific blastomere-directed microinjection approach. Structural bioinformatic predictions allowed us to identify various functional domains of *hairy* genes, which in turn facilitated the rational design and construction of inducible chimeric proteins. Recombinant proteins were assembled into a vector pCS2+, allowing further exploit of its characteristics for in vitro transcription to generate mRNAs translated with high efficiency by having sequences which mimic globin mRNA in *Xenopus* (Rupp et al., 1994; Turner and Weintraub, 1994).

To temporally control the overexpression of *hairy2b*, *hairy2a*, and *hairy1*, the ligand binding domain of the human glucocorticoid receptor was fused downstream to the full length of *hairy* sequences (*hairy1*: BC070988, *hairy2a*: AF383159, *hairy2b*: AF383160). Also, a fragment containing the N-region of *hairy* genes (106aa of *hairy1*, 106aa of *hairy2a*, and 108aa of *hairy2b*) were cloned into the pTOPO-TA vector, amplified and then cloned directionally into EcoRI and SacI, upstream to the GR. The plasmids containing the inducible constructs were used to in vitro transcribe capped mRNA for microinjection. Each dorsal blastomere of 8- to16-cell embryos was microinjected with 700 pg of mRNA.

A morpholino antisense oligonucleotide was designed and synthesized against *Xenopus laevis hairy2b* (*hairy2bMO*) including the initiation start site (from + 1 to + 25 bp) with the sequence 5′-GCTTCTCCATACTATCTGCAGGCAT-3′ (Gene Tools, LLC). Doses of 10 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′-CCTCTTACCTCAGTTACAATTTATA-3′, Gene Tools, LLC) was injected as a control.

To test the efficacy of *hairy2bMO* in vivo, a *hairy2bGFP* fusion was generated by high fidelity PCR using *pBSSK+hairy2b* as the template and the following primers: 5′-GAATTCATGCCTGCAGA TAGTATGGAGAAGC-3′(underlined, *Eco*RI restriction site)

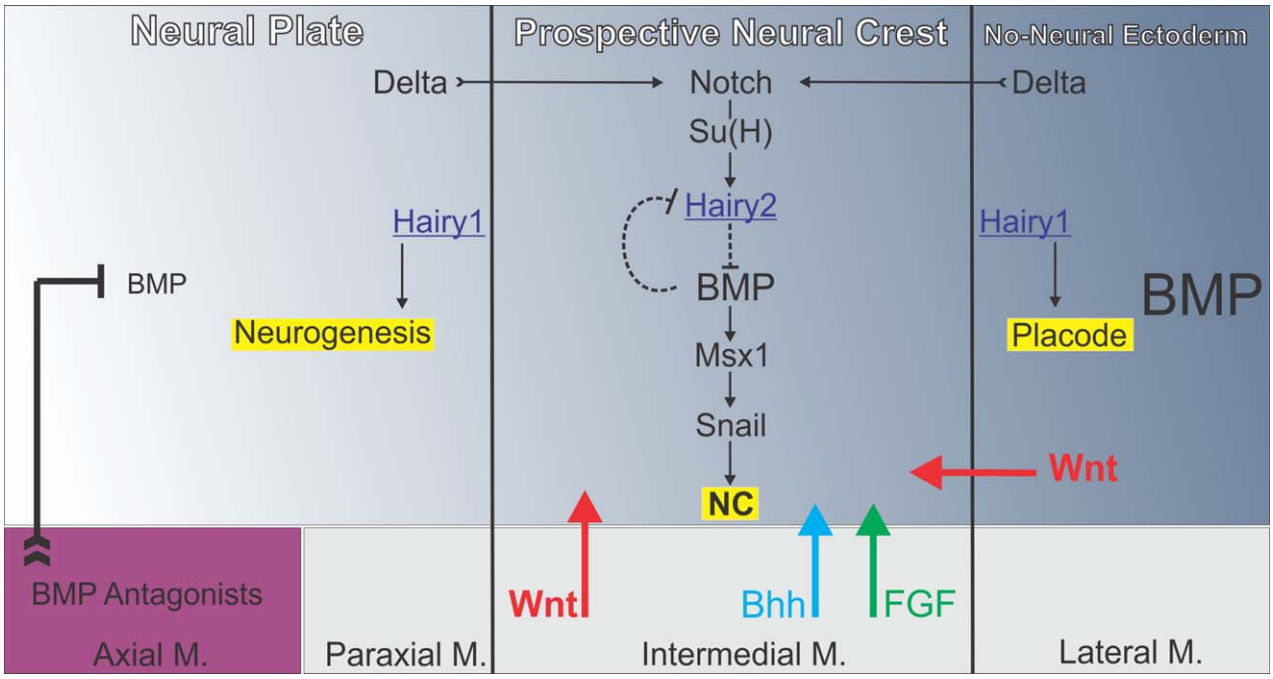

Fig. 14. Diagram representing a unified model of the signals involved in neural crest development. Three ectodermal domains are shown (neural plate, neural crest and nonneural ectoderm) and four underlying mesodermal domains (axial, paraxial, intermediate, and lateral). The blue gradient represents BMP activity in the ectoderm. In the lateral domain to the neural crest Notch/Delta signal activates the *hairy1* gene that is also involved in neurogenesis in the neural plate and in the specification of the placodes in the nonneural domain. Some regulatory relationships described in this work involving *hairy* genes are shown in the neural crest region. References: Arrows indicate activation or positive relationships; truncated arrows indicate inhibitory or negative relationships. M, mesoderm. Modified from Huang and Saint-Jeannet (2004).

and 5′-GAGCTCGCACTGAGGGGTCGGC-3′ (underlined, *Sac*I restriction site). A fragment containing the N-terminal region encoding 110 amino acid residues of *hairy2b* was cloned into the pTOPO-TA vector, amplified and then cloned directionally into EcoRI and SacI of *pCS2+EGFP* vector to produce the *hairy2bGFP* construct. In vitro transcribed mRNA of this construct was co-injected with different amounts of *hairy2bMO* and GFP fluorescence was observed under a Leica fluorescence stereomicroscope. An additional construct, named *CRhairy2bGFP*, was synthesized to complement the testing of *hairy2bMO* efficacy. This construct carries 6 mismatches and conserves the amino acid coding of the wild-type *hairy2b* gene.

For the rescue of morpholino knock-down experiments a DNA construct was prepared by changing only the codon sequence targeted by the *hairy2bMO* antisense oligonucleotide. The *hairy2b* ORF from *pBSSK+hairy2b* was used as a template for additional DNA constructs. Special care was taken to mutate this *hairy2b* DNA sequence without changing the wild-type amino acid sequence. This construct, named *CRhairy2b* (CR, codon replacement), introduced six mismatches in the nucleotide sequence recognized by the morpholino and was made by high fidelity PCR using the following primers: 5′-GAATTCATGCCGGCTGATGTGATGGAGAAGA-3′ and 5′-GAGCTCGCACTGAGGGGTCGGC-3′ (underlined, *Eco*RI and *Sac*I restriction sites, respectively). The PCR products were purified, A-tailed and cloned into pTOPO-TA (pCR II-TOPO) (Invitrogen) to produce pTOPO-*CRhairy2b* which was EcoRI/SacI-digested and ligated into a pCS2 + vector digested with *Eco*RI/*Sac*I. Both fusion constructs were sequenced on both strands at junction sites by automated DNA sequencing and used to synthesize capped mRNA by in vitro transcription (Agüero et al., 2012).

Cartilage Staining

Craniofacial cartilages were stained, and skin and muscles were manually removed and photographed as previously described (Fernandez et al., 2014).

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: *EF1α*, for: 5′-CAGATTGGTGCTGGATATGC-3′, rev: 5′-ACTGCCTTGATGACTCCTAG-3′; *Snail1*, for: 5′- GCACAATGGACTCCTTAAATTCCTG-3′, rev: 5′-GTGACCGGGTGCTCATTGTG-3′; *sox2*: for: 5′-GAGGATGGACACTTATGCCCAC-3′, rev: 5′-GGACATGCTGTAGGTAGGCGA-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). Quantitation of PCR bands was performed on 8-bit greyscale JPG files and the values were normalized to the ef1α levels from the same sample and expressed for comparison as relative intensities (sample/*ef1α*×100), as previously described (Tribulo et al., 2012; Fernandez et al., 2014). As a control, PCR was performed with RNA that had not been reverse-transcribed to check for DNA contamination.

In Situ Hybridization, TUNEL, and Immunohistochemistry

Antisense probes containing Digoxigenin-11-UTP or Fluorescein-12-UTP were prepared for *hairy* genes (Davis et al., 2001; Tsuji

et al., 2003; Nagatomo and Hashimoto, 2007), *Snail2*, *Snail1* (Aybar et al., 2003), *Foxd3* (Sasai et al., 2001), *Sox2* (R. Grainger, personal communication), *Xk81a* (Jonas et al., 1985) by in vitro transcription. Specimens were prepared, hybridized and stained as previously described (Aybar et al., 2003). Sense probes were synthesized and hybridized as negative controls. Apoptosis was detected by TUNEL staining according to the procedure previously described (Tribulo et al., 2012). For mitotic cell analysis, rabbit Polyclonal anti-phosphohistone-3 (Upstate Biotechnology, Lake Placid, NY) was used according to the method previously described (Aybar et al., 2003).

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