RESEARCH ARTICLE

Developmental Expression and Role of Kinesin Eg5 During *Xenopus laevis* Embryogenesis

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Background: The neural crest is a transient multipotent migratory cell population unique to vertebrates. These cells undergo an epithelial-to-mesenchymal transition and migrate extensively through the embryo. They differentiate into numerous diverse derivatives including the peripheral nervous system, melanocytes,and craniofacial cartilages. The development of the neural crest is mediated by complex interactions of multiple signals and transcription factors. The kinesin Eg5 is a plus end–directed microtubule-based motor protein that is essential for bipolar spindle formation during mitosis and meiosis, axon growth, and mammal embryonic development. **Results:** We analyzed in detail the expression pattern of *eg5* and established that it is expressed at the prospective neural fold, in the premigratory and migratory neural crest. Functional analysis revealed that in *Xenopus*, early embryogenesis *eg5* function is required during neural crest induction, specification, and maintenance. *eg5* is also required during neural crest migration and for derivatives formation. Moreover, we demonstrated a hierarchical relationship with the Indian Hedgehog signaling pathway. **Conclusions:** Our results show that *eg5* is essential for the specification and maintenance of neural crest progenitors during *Xenopus* early embryogenesis rather than cell proliferation and survival. *Developmental Dynamics 243:527–540, 2014.* © 2013 Wiley Periodicals, Inc.

Key words: neural crest; cell specification; morpholino; monastrol

INTRODUCTION

The neural crest is a transient embryonic cell population generated from the neural plate lateral borders during neurulation. The neural plate folds forming the neural tube and then the neural crest cells delaminate and migrate, reaching numerous destinations. Then, they differentiate into numerous derivatives including melanocytes, neurons, and glia of the peripheral nervous system, face cartilage, smooth muscle, and neuroendocrine cells (Gammill and Bronner-Fraser, 2003; LeDouarin and Kalcheim, 1999; Rogers et al., 2012). In humans, 47 cell types have been attributed to a neural crest origin (Vickaryous and Hall, 2006). Although many studies have dealt with the induction of neural crest precursor cells, the molecular mechanisms underlying early events such as cell fate decision in the ectoderm, cell precursor specification, and specification maintenance are not yet completely understood. In *Xenopus*, neural crest induction requires cell signals including BMP signaling and Wnt/FGF signaling (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; for a revision see Rogers et al., 2012). Besides these primary players, other cell signaling pathways have been reported to have a role in early neural crest development (Barembaum et al., 2000; Endo et al., 2002; Glavic et al., 2004; Bonano et al., 2008, Agüero et al., 2012). The actions of the different signaling pathways are integrated into the ectoderm to give neural crest cells tissue identity at the time when multiple specification transcription regulators are synchronically activated and organized into a highly complex crossregulation (Essex et al.,

1993, LaBonne and Bronner-Fraser, 2000; Mayor et al., 2000, Aybar et al., 2003; Spokony et al. 2002; Tribulo et al., 2003; Aoki et al., 2003; Honore et al., 2003, Bellmeyer et al., 2003). Molecular information on the regulatory network linking the genes expressed in the neural crest still remains to be established.

Eg5, also known as Kif11, is a plus end–directed kinesin that was originally identified in *Xenopus laevis* (Le Guellec et al., 1991). A highly conserved N-terminal motor domain places Eg5 in the kinesin-5 family of kinesin- related proteins (Lawrence et al., 2004). Proteins of this family also share a central coiled-coil domain and a C-terminal tail that bears a bimC box (Sawin et al., 1992; Kashina et al., 1996). Eg5 binds and generates forces upon microtubules as an anti-parallel bipolar homotetrameric structure having the two motor domains at opposing ends of the central stalk (Sawin et al., 1992). Current research indicates that *eg5* is required during cell division, as the mutations in this gene family cause failure in spindle assembly, maintenance of the bipolar spindle, and centrosome separation (Heck et al., 1993; Sawin et al., 1992; Blangy et al., 1995). In vertebrates, targeted disruption of *kif11* (mouse *eg5* orthologue) produced mouse embryos that are not viable and die at preimplantation stages (Castillo and Justice, 2007; Chauviere et al., 2008). These results in mammalian embryos indicate that *eg5* is essential in the first rounds of cell division and that zygotic Eg5 protein is required to continue development after morula stages. During mitosis, its activity produces microtubule cross-linking, antiparallel microtubule sliding, bipolar spindle formation, and microtubule poleward flux. It was shown that eg5 is expressed in postmitotic neurons (Ferhat et al., 1998), has an inhibitory role in redirecting axonal growth (Haque et al., 2004; Myers and Baas, 2007; Nadar et al., 2008), and enhances the efficiency of translation in mammalian cells (Bartoli et al., 2011). In those analyses of *eg5* function, genetic tools and approaches were extremely useful, but monastrol, a specific pharmacological inhibitor of kinesin Eg5 protein (Mayer et al.,

1999), has allowed a more critical analysis of Eg5 function (Kapoor et al., 2000; Kapoor and Mitchison, 2001).

Here we investigated the expression and participation of *eg5* in *Xenopus laevis* embryonic development. We took advantage of the ease with which molecular gain- and- loss-of-gene function and embryological manipulations in *Xenopus* embryos could be performed. Single and double whole mount in situ hybridization and RT-PCR showed that *eg5* is expressed in the neural crest during neurulation. Functional analysis by gene expression modifications and the use of the specific pharmacological inhibitor monastrol demonstrated that *eg5* is required for the specification, maintenance of specification, and migration of neural crest cells. Our study highlights a possible new role for *eg5* in cell fate decisions and specification rather than in cell proliferation and apoptosis regulatory events.

RESULTS

Developmental Expression of *eg5* in *Xenopus* Embryos

Although *eg5* was originally isolated from unfertilized *Xenopus laevis* eggs (Le Guellec et al., 1991), the expression pattern of *eg5* in developing embryos has not been reported for *Xenopus*. We decided to analyze and compare it by single and double whole mount in situ hybridization with specific tissue gene markers (Fig. 1). In blastula embryos (stage 8–9), *eg5* expression appeared as a faint staining in the animal hemisphere (data not shown). During gastrulation (stage 12.5), the expression of *eg5* was observed in the dorsal aspect of embryos (Fig. 1A). At the early neurula stage (stage 14), this expression occurred mostly in the anterior and lateral regions of the neural plate (Fig. 1B, arrowheads) but was not observed along the neural plate midline (Fig. 1B–D, arrow). At midneurula stages (stages 16–18), the expression increased laterally over the neural plate and the neural crest boundary (Fig. 1C, D). Sibling embryos stained with sense *eg5* probes showed no labeling (Fig. 1C, inset). Transversal section of midneurula embryos (Stage 16, Fig. 1C)

showed that *eg5* expression occurred weakly in the internal ectodermal layer corresponding to the most external neural plate border and strongly in the presumptive neural crest domain (Fig. 1F, red brackets). At stage 17, a double in situ hybridization with *eg5* and the neural crest marker *foxd3* evidenced a clear overlapping expression of both genes in the neural crest region (Fig. 1K) both in whole mount and in sectioned embryos (Fig. 1K'). Double in situ hybridization analysis of stage-17 embryos revealed that *eg5* partially overlaps its expression with *sox2* neural plate marker at the neural plate border and that its expression extends to the neural crest domain (Fig. 1L, and 1L'). Double staining of *eg5* and the epidermal marker *xk81a* showed that both genes overlap at the external boundary of the neural crest domain (Fig. 1M, M').

At stage 21, expression was detected in the head region (Fig. 1E) and in the Rohon-Beard sensory neurons, appearing as two lines along the dorsal anterior-posterior axis of embryos (arrows). *eg5* also appeared in a spotted distribution in the epidermal tissue. Transversal sections in the anterior region of the embryo showed expression of the kinesin *eg5* at the most dorsal domain of the dermatome (Fig. 1G). At stage 24, *eg5* expression was observed in the somites in a conspicuous chevron-like pattern, optical vesicles, profundal ganglion (Fig. 1H, arrow), and external epidermal layer (Fig. 1I). At tailbud stage 27, *eg5* expression was markedly evident in the branchial arches (Fig. 1J), somites, brain, and epidermis.

We also analyzed the temporal expression of *eg5* by RT-PCR at different stages. *eg5* was found to be expressed maternally (Fig. 1N,O). We observed a decrease in its expression at the initial blastula stage (stage 7) and a marked increase during the midblastula stage (stage 9), probably indicating the beginning of zygotic *eg5* expression at midblastula transition. During gastrulation (stages 10–12), *eg5* levels remained low (Fig. 1O), whereas in the neurula and tailbud stages eg5 expression continuously appeared from midneurula stages. We considered it interesting to evaluate the expression
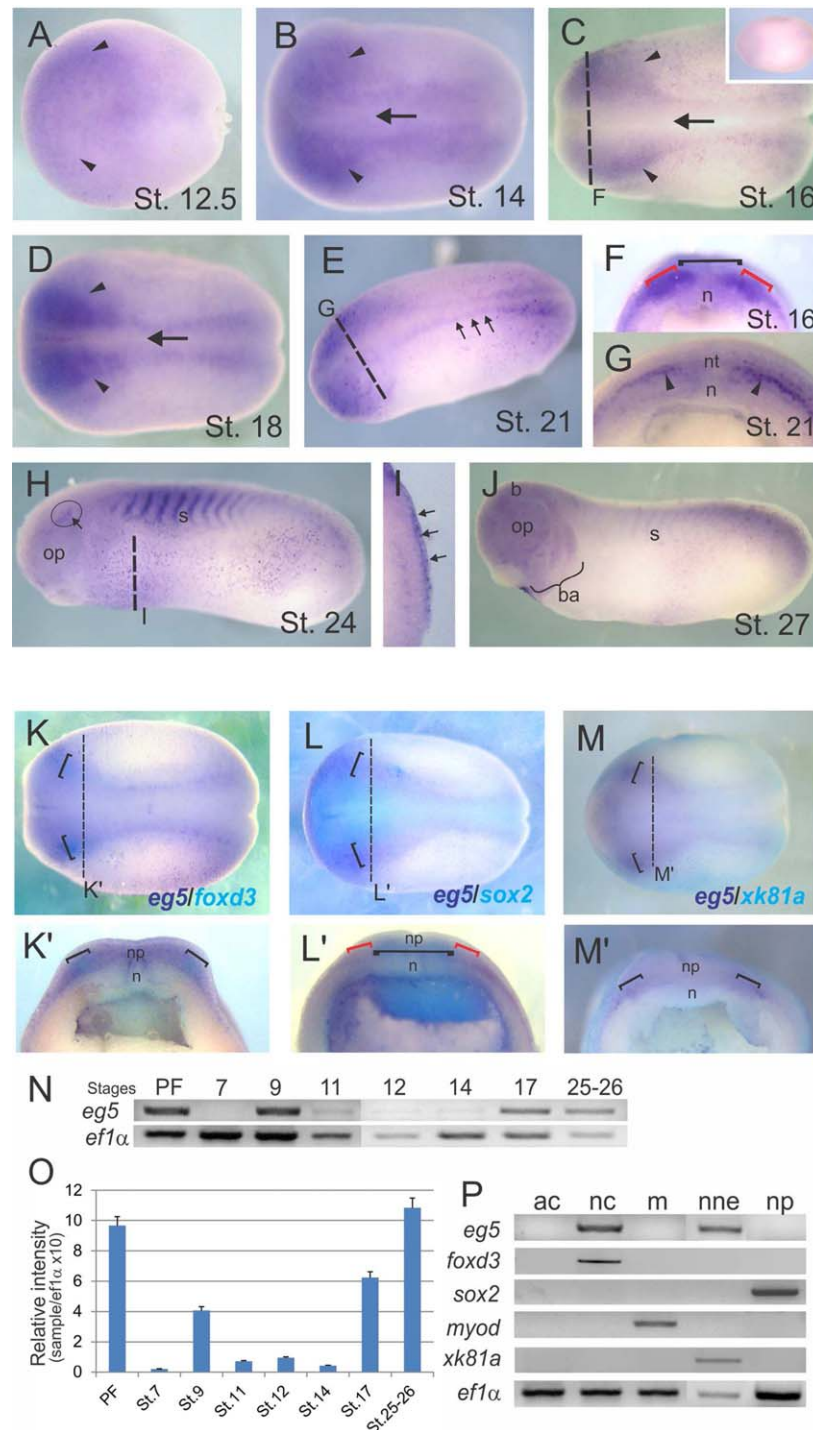
Fig. 1. *eg5* expression pattern in developing *Xenopus* embryos. Whole-mount in situ hybridization analysis of spatio-temporal *eg5* expression. **A–D:** Dorsal view, anterior on the left. **E:** Dorso-lateral view. **H, J:** Lateral view, anterior on the left, dorsal at the top. **F, G, I:** Transversal sections. Dashed lines in C, E, and H indicate the sites of transverse section shown in F, G, and I, respectively. A: *eg5* transcripts are first detected since the late gastrula stage (stage 12.5) in the dorsal region of embryos at the neural plate and neural plate borders (arrowheads). B–D: During neurulation (stages 14–18), *eg5* is expressed in the neural plate borders and in prospective neural crest (arrowheads). No expression is detected in the neural plate midline (arrow). The transversal section of a stage-16 midneurula embryo (F) shows *eg5* expression in neural plate (black bracket) and in the neural crest region (red brackets). The transversal section of stage-21 embryo (G) shows *eg5* transcripts in the dorsal region of dermatomes (arrowheads). No specific labeling is observed in the sense probe control (C, inset). E: Stage-21 embryos show *eg5* expression in the head region, Rohon-Beard sensory neurons (arrows), and the epidermis in a spotted pattern. H: Stage-24 embryos show *eg5* expression at the somites, optic vesicle, profundal ganglia primordium (encircled), and epidermis external layer (I). J: The tailbud-stage embryos show the expression at the branchial arches, somites, brain, and epidermis. n, notochord; nt, neural tube; s, somites; b, brain; ba, branchial arches; op, optic vesicle. **K–M:** Double in situ hybridizations for *eg5* (purple) and *foxd3* (turquoise), *sox2* (turquoise), and *xk81a* (turquoise). Dorsal view, anterior on the left. **K'–M':** Transverse sections of St. 15 neurula embryos displayed in K–M showing that *eg5* expression overlaps with *foxd3* in the neural crest territory (K', black brackets: *foxd3* neural crest expression), and is complementary with *sox2* and *xk81a* expressions (L', M'). L': Red brackets, neural crest region; black bracket, neural plate. M': Black brackets, neural crest region. **N–P:** RT-PCR analysis of *eg5* expression in developing embryos. O: Quantification of the gel shown in N, the results are expressed as Relative intensity (sample/*ef1α* x10). P: RT-PCR analysis of *eg5, foxd3, sox2, myod* and *xk81a* expression in neural crest, intermediate mesoderm, neural plate and non-neural ectoderm explants. Explants were dissected out from stage-14 embryos (see Experimental Procedures section). *ef1α*, loading control. np, neural plate; n, notochord; nc, neural crest; m, intermediate mesoderm; nne, nonneural ectoderm; ac, animal cap (Stage 9).

of *eg5* in different tissues dissected out from stage-14 embryos using RT-PCR analysis. The isolated regions selected were neural crest, midline neural plate, non-neural ectoderm, and intermediate mesoderm (neural crest underlying mesoderm) explants, and the homogeinity of each sample confirmed by the analysis of tissue-specific markers (Fig. 1P). The results confirmed that *eg5* was expressed in the neural crest and in the non-neural ectoderm at this stage (Fig. 1P).

eg5 Is Required for Neural Crest Initial Development

Previous studies in mouse have shown that *eg5* (*kif11*) null mutation is lethal since this gene is required during early mouse development (Castillo and Justice, 2007; Chauviere et al., 2008). The expression of *eg5* during initial *Xenopus* development strongly suggests that this gene could play a role during the initial steps of neural crest development. We investigated the requirement of *eg5* by evaluating the effects of knocking down its expression using an antisense morpholino oligonucleotide directed against the initial codon of the *eg5* transcript (*eg5MO*).

We first assessed the efficacy of *eg5MO* to block the expression of *eg5*. This morpholino oligonucleotide inhibited translation of the GFP-tagged form of *eg5* (ΔC-*eg5GPF*) in a dose-dependent manner both in vitro (Fig. 2A) and in vivo (Fig. 2B–E). ΔC-*eg5GPF*-injected embryos (Fig. 2B, B', 67%, n=12), and control morpholino (*CoMO*)- and &Dgr;*C-eg5GPF*-coinjected embryos (Fig. 2C, C', 78%, n=9) showed strong GFP fluorescence. Then we examined *eg5MO* oligonucleotide efficiency and confirmed that *eg5MO* was able to block the translation and expression of the *eg5* GFP-tagged capped mRNA in a dose-dependent manner (Fig. 2D, D' and E, E' 10 ng/embryo, 73%, n=15 and 22 ng/embryo, 77%, n=13, respectively).

To assess the requirement of *eg5* during neural crest induction, embryos were injected at the 8–16-cell stage in one dorsal blastomere with *eg5MO* (22 ng/embryo) (Fig. 2F–O) or an equivalent amount of a control morpholino (*CoMO*). The embryos injected with *CoMO* showed

normal morphology and normal expression of gene markers (Fig. 2O, 100%, n=29), while embryos injected with equivalent amounts of *eg5MO* failed to form the neural fold and showed reduced expression of *foxd3* and *snail2* expression at the injected side (Fig. 2F, 83%, n=103, and 2G, 68%, n= 94, respectively). Also the neural plate border specification marker *pax3* showed reduced expression as a consequence of the *egMO* (Fig. 2K, 58%, n=24). The neural plate marker *sox2* also appeared reduced in the injected side by the effect of *eg5MO* (Fig. 2H, 64%, n=58) and the epidermal marker *xk81a* was expanded by the *eg5MO* treatment (Fig. 2I, 63% of expansion, n=62). This latter effect was probably a consequence of the reduction in the neural plate and neural crest domains. Double in situ hybridization analysis using *sox2* and *xk81a* markers, confirmed that both territories were reduced compared to the control side of embryos (Fig. 2J, 58%, n=19). Our results show that *eg5* depletion in the ectoderm by directed microinjection of the morpholino causes a nearly specific loss of neural and neural crest progenitors, indicating that this gene is required for the early specification of neural crest cells and probably the most lateral neural plate cell population that expresses *eg5* (Fig. 1). To evaluate the specificity of the morpholino effects, we determined whether the phenotype of *eg5*-depleted embryos could be rescued by restoring the *eg5* function. We coinjected a *Xenopus tropicalis eg5* (*Xteg5*) capped mRNA that bears 6-bp mismatches within the recognition sequence of *eg5MO* in order to avoid hybridization between the morpholino and the coinjected mRNA. The *Xteg5* mRNA strongly rescued *foxd3* and *sox2* expression (Fig. 2L, 73%, n=78 and 2M, 71%, n=63). The rescue effect was specifically produced by the coinjection of *Xteg5* mRNA but not by the coinjection of unrelated mRNAs like GFP or nβGal mRNA (not shown). Thus, we concluded that *eg5MO* was able to specifically knock down *eg5* expression and therefore to abolish its activity.

Next, we decided to explore whether *eg5* might participate in the control of neural crest specification.

We overexpressed *eg5* and microinjected the in vitro transcribed and capped mRNA into the animal pole of one dorsal blastomere of 8–16-cell stage embryos. *eg5* injection led to an expansion of the neural crest territory in the treated side analyzed by the expression of *foxd3* (Fig 3A, 78%, n=120) and *snail2* (Fig. 3B, 70%, n=87). The neural plate border specification marker *pax3* also resulted increased by the *eg5* overexpression (Fig. 3F, 53%, n=17). This effect was accompanied by a concomitant expansion in *sox2* (Fig. 3C, 67%, n=74) neural plate marker and a slight reduction in *xk81a* epidermal marker (Fig. 3D, 61%, n=53). Double in situ hybridization labeling of *eg5*-injected embryos showed that both neural plate and neural crest territories resulted in being slightly expanded in the injected side (Fig. 3E, 60%, n=15). These results suggest that the expansion of the neural crest as a consequence of the overexpression of *eg5* could be produced by the transformation of the epidermis into prospective neural crest cells.

Temporal Requirements of eg5 During Development

Antisense morpholino technology can block the expression of maternal and zygotic transcripts specifically and efficiently. However, this approach is limited since it is difficult to control the timing of morpholino activity. In order to precisely control the timing of *eg5* blocking, we used the specific antagonist monastrol (Mayer et al., 1999; Maliga et al., 2002; DeBonis et al., 2003). Three periods of early development were tested for their ability to respond to Eg5 blocking. First, we explored the effect of monastrol incubation during the first stages of development. Embryos in the 16-cell stage were incubated in different monastrol concentration ranging from 50 to 750 μM. We detected no mitotic arrest by Eg5 specific inhibition at the monastrol dose (100 μM) used to carry out our neural crest specification experiments. At the dose tested 7.5 times higher (750 μM) we only detected almost 50% of mitosis arrest that stopped embryonic development (Fig. 4A). This result highlights the possibility that other molecules are

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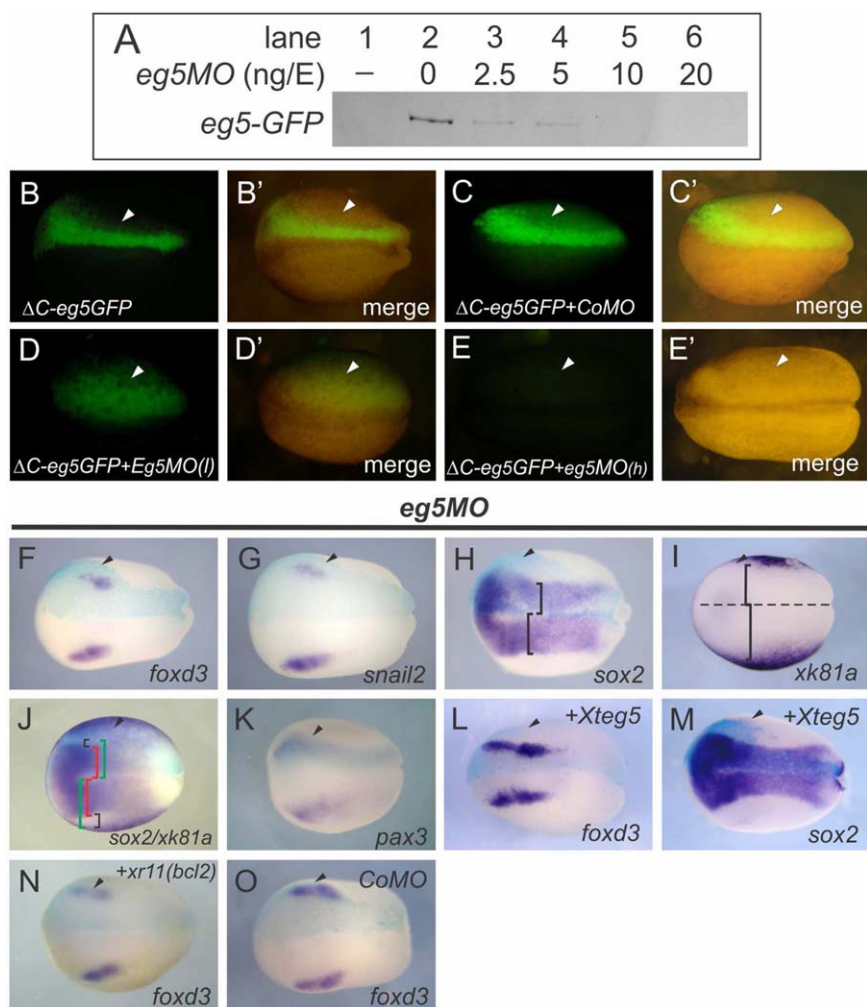
Fig. 2. *eg5* is required for early neural crest specification in *Xenopus* embryos. **A–E:** In vitro and in vivo efficiency of *eg5* antisense morpholino oligonucleotide (*eg5MO*). A: Efficiency of *eg5* antisense morpholino oligonucleotide. *eg5MO* injected and control embryos were cultured until stage 14 and homogenized for total protein extraction. Eg5-GFP protein was detected by Western blotting using an anti-green fluorescent protein (GFP) antibody. Each lane was loaded with 100 μg of total protein. *eg5MO* inhibits translation of *eg5* in a dose-dependent manner. *eg5MO* concentration is expressed in ng/embryo. B–E: Dorsal views of *Xenopus laevis* embryos under the fluorescence stereo microscope, anterior side is on the left. White arrowheads indicate the injected side. B', D', E': Fluorescence and clear field images of each embryo are shown in merged images. B, B': Embryo injected with mRNA encoding ΔC-eg5GFP (1 ng/embryo) showing GFP fluorescence on the treated side. C, C': Embryo injected with ΔC-eg5GFP mRNA (1 ng/embryo) and control antisense morpholino oligonucleotide (*CoMO*, 20 ng/embryo). D, D', E, E': Embryos injected with ΔC-eg5GFP mRNA (1 ng/embryo) and *eg5MO* (C and C', low dose (l), 10 ng/embryo; D and D', high dose (h), 22 ng/embryo. No embryo shows GFP fluorescence at a high dose of *eg5MO*. **F–O:** Analysis of *eg5MO* effects on neural crest early specification. Dorsal views of *Xenopus laevis* embryos, anterior side is on the left. Black arrowheads indicate the injected side. F, G: *eg5MO*-injected embryos show inhibition of *foxd3* and *snail2* neural crest markers, respectively. H, I: The expression of the neural plate marker *sox2* is reduced and the epidermal marker *xk81a* is expanded on the *eg5MO*-treated side. Brackets indicate the width of the neural plate (H) and the width of the neural plate plus the neural crest domain (I). J: Embryo labeled by double in situ hybridization for *sox2* and *xk81a* genes showing the reduction of prospective neural crest (black brackets) and neural plate (red brackets) domains in the *eg5MO*-injected side. Green brackets indicate neural crest and neural plate territories together. This result is identical to H and I. K: The neural plate border specification marker *pax3* show reduced expression in the *eg5MO*-injected side. L, M: Coinjection of *eg5MO* and *Xenopus tropicalis eg5* mRNA rescues *foxd3* and *sox2* expression, respectively. N: Embryo injected with *eg5MO* and *xr11*, the *Xenopus bcl2* orthologue. The antiapoptotic factor *xr11* did not rescue the expression of *foxd3* in the neural crest. O: *CoMO*-injected embryos show normal expression of *foxd3*.

taking control of the mitotic process in these segmentation developmental stages since it has been reported that *eg5* mRNA levels are narrowed down from 1-cell stage to stage 10–11 (Yanai et al., 2011). Second, we performed batch incubations of stage-11.5 and −16 embryos in NAM solution containing 100 μM monastrol. This treatment produced an almost complete depletion of the expression of *foxd3* (Fig. 4B, arrows, 90%, n=25) or *sox10* (Fig. 4D, arrow, 100%, n=22) neural crest gene markers at the two stages analyzed. No effect on the expression of mesodermal marker gene *paraxis* was observed in embryos incubated in monastrol (Fig. 4C, 95%, n=47) suggesting that effects were only on neural crest territories On the other hand, control embryos incubated in a similar amount of DMSO vehicle exhibited a normal expression of both markers (Fig. 4E and 4F; 100%, n=28 and 98%, n=24, respectively). In order to have more spatial and temporal control of Eg5 loss of function, we grafted monastrol-soaked beads into living embryos to specifically deliver the inhibitor into localized territories at different stages of development. Beads soaked with monastrol were implanted into the right prospective neural crest at stage-11.5 (Fig. 4H), or stage-17 embryos (Fig. 4J). Embryos were cultured until different stages and the expression of gene markers was analyzed by in situ hybridization. Treatment of stage-11.5 embryos with 100 μM monastrol-soaked beads markedly downregulated *foxd3* (Fig. 4H, 60%, n=35) expression in the neural crest territory. No effect on the expression of the neural crest marker *foxd3* was observed when DMSO (vehicle)-soaked beads were grafted at stage 11.5 (Fig. 4I, 79%, n=29).

The *eg5* transcripts were expressed in the premigratory and probably in the migratory neural crest (Fig. 1) and our morpholino approach showed that neural crest requires *eg5* function during the early stages of development. All these findings suggest that *eg5* might also participate in the migratory process. We blocked Eg5 function using monastrol-soaked beads grafted into the neural crest region of stage-17 embryos, when neural crest cells migration is about to begin. Our results showed that
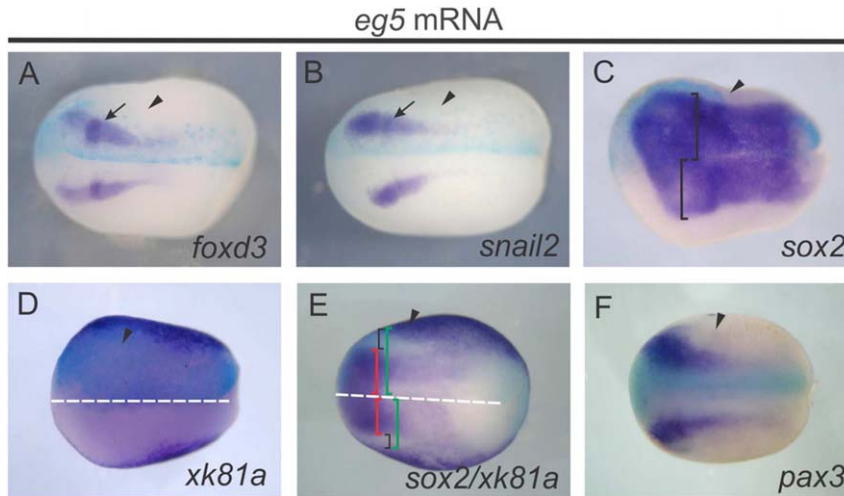
Fig. 3. Overexpression of *eg5* mRNA leads to an increase in neural crest markers. **A–F:** Dorsal views of *Xenopus laevis* embryos, anterior side is on the left. The injected side is indicated by an arrowhead. Embryos were injected into one blastomere at the 8–16-cell stage with 4 ng of *eg5* mRNA and the expression of several markers was analyzed by in situ hybridization. A–C: *eg5*-injected embryos show increased expression of *foxd3* and *snail2* and an expanded expression of neural plate marker *sox2* (C). D: *eg5* overexpression produced a slight reduction in the expression of *xk81a* epidermal marker. E: Embryo labeled by double in situ hybridization for *sox2* and *xk81a* genes showing the expansion of prospective neural crest (black brackets) and neural plate (red brackets) domains in the *eg5* mRNA-injected side. Green brackets indicate neural crest and neural plate territories together. This result is identical to C and D. Dashed line, embryo midline. F: The neural plate border specification marker *pax3* show increased expression in the *eg5* mRNA-injected side.

monastrol produced a clear inhibitory effect on neural crest cell migration since the front of migration in the treated side, revealed by *sox10* expression, migrated only a short distance (Fig. 4J, 72%, n=42) compared with the control side (Fig. 4K). Control DMSO-soaked beads produced no effect on neural crest migration (Fig. 4L, 80%, n=17).

Prospective neural crest cells receive multiple signals from different tissues (i.e., non-neural ectoderm, underlying mesoderm) during their specification. We also decided to assess the effects of monastrol on the neural crest cell population without the influence of the whole embryo environment. We specifically assessed the effects of monastrol on the specification of neural plate border explants that were dissected out from stage-11.5 embryos and incubated in 100 μM monastrol until stage 15, when they were processed for *foxd3* expression. Monastrol-treated explants showed a reduced expression of *foxd3* marker (Fig. 4O; 100 %, n=27) compared with vehicle incubated explants (Fig. 4N; 90%, n=20).

It has been suggested that during the midneurula to the late neurula stage, the neural crest cells require interaction with the mesoderm and the activity of genes and signaling molecules to maintain their specification status (Monsoro-Burq et al., 2003; Bonano et al., 2008; Steventon et al., 2009; Agüero et al., 2012). The evidence suggests that at the midneurula stage, the neural crest specification is still in progress. Taking this into consideration, we evaluated the participation of Eg5 in the maintenance of neural crest specification during midneurula stages by treating neural crest explants containing the underlying mesodermal tissue with the specific pharmacological inhibitor monastrol.

A strong expression of *sox10* was observed in neural crest explants dissected at stage 16 and fixed immediately (Fig. 4P, 95%, n=22). When equivalent stage-16 neural crest explants containing the underlying mesoderm were cultured until the equivalent of stage 22 in monastrol-containing solution, the expression of *sox10* was notoriously reduced

(Fig. 4R, 88%, n=67). Conversely, in similar neural crest explants, the expression of *sox10* was strong when they were incubated in DMSO vehicle solution (Fig. 4Q, 83%, n=37).

Thus, the pharmacological blockade by monastrol reproduced the phenotype obtained by the directed microinjection of morpholino oligonucleotide, confirming that *eg5* activity is required for the early and intermediate steps of neural crest specification.

Eg5 Is Not Required for Cell Proliferation and Apoptosis During Neural Crest Specification

It has been shown that the apoptotic process is involved in neural crest development (Hensey and Gautier, 1998; Tribulo et al., 2004) and that the experimental manipulation of some factors could affect their proliferative status (Honore et al., 2003). We observed significant changes in neural crest cell population as a consequence of the overexpression or the inhibition of *eg5* function. These changes could account for the experimental effects on the cell proliferative status or in the apoptotic process. To investigate such possibilities, we decided to analyze cell proliferation and apoptosis during *eg5* gain and loss of function.

We first microinjected embryos with *eg5* mRNA or *eg5MO* and incubated them in a cocktail containing two strong inhibitors of cell proliferation (HUA, hydroxyurea and aphidicolin) (Harris and Hartenstein, 1991; Sharpe and Goldstone, 1997; Aybar et al., 2003). We observed that the HUA mix produced no changes in the increased expression of neural crest marker *foxd3* caused by the overexpression of *eg5* mRNA (Fig. 5A, 64%, n=19). In the presence of HUA, the *eg5MO*-microinjected embryos showed the reduced *foxd3* expression produced by the specific morpholino (Fig. 5B, 78%, n=21). These results showed that the cell proliferation blockage produced no alteration in the *eg5* gain or loss of function phenotypes.

Moreover, the overexpression of *eg5* mRNA or the microinjection of *eg5MO* produced no detectable changes in cell proliferation assessed by the

detection of a phosphorylated H3 histone (Fig. 5C and I, n=22; Fig. 4D and I, n=19). We also found no significant changes in apoptotic cell death analyzed by TUNEL staining between the control side and the treated side of *eg5-* or *eg5MO*-injected embryos (Fig. 5E and J, 65%, n=20; Fig. 5F and J, 57%, n=21), or in embryos injected with *CoMO* (0% increase in TUNEL staining, n=18, not shown). Monastrol is a well-characterized specific allosteric inhibitor of Eg5 (Mayer et al., 1999), known to be selective for eg5 over other related kinesins (Mayer et al., 1999; Maliga et al., 2002; DeBonis et al., 2003). Studies using motility assays suggest that monastrol produces an eg5 state that is weakly bound to the microtubule (Crevel et al., 2004). The apoptosis status showed no differences in the apoptosis of embryos that were treated with the Eg5 specific pharmacological inhibitor monastrol (Fig. 5G and J, n=23) or the vehicle DMSO (Fig. 5H and J, n=19). To corroborate the effect on apoptosis of *eg5* gain and loss of function approaches, we assessed the activity of effector caspases 3/7 in neural crest explants lysates dissected out at stage 14. We found that eg5 overexpression or the *eg5MO* knock down, or the monastrol treatment (Fig. 5J, green bars, n=40 microinjected or incubated explants) produced no significant changes in the apoptosis levels on neural crest explants. The caspase activity of each treatment was almost the same to that of control neural crest explants (Fig. 5J, n=40 explants). In addition, in order to test whether decreased *foxd3* expression in *eg5MO*-treated side of embryos was produced by an increase in the apoptosis level, we co-injected the *Xenopus* homologue of *bcl2* (*xr11*) along *eg5MO* (Tribulo et al., 2004), (Fig. 2N). Results showed that the overexpression of the antiapoptotic factor *xr11* was not able to rescue *foxd3* expression (Fig. 2N, 73%, n=30).

Taken together, these results demonstrated that eg5 does not play a role in the control of cell proliferation or apoptosis processes and that the expansion or reduction observed in the neural crest territories after *eg5* gain- or loss-of-function could
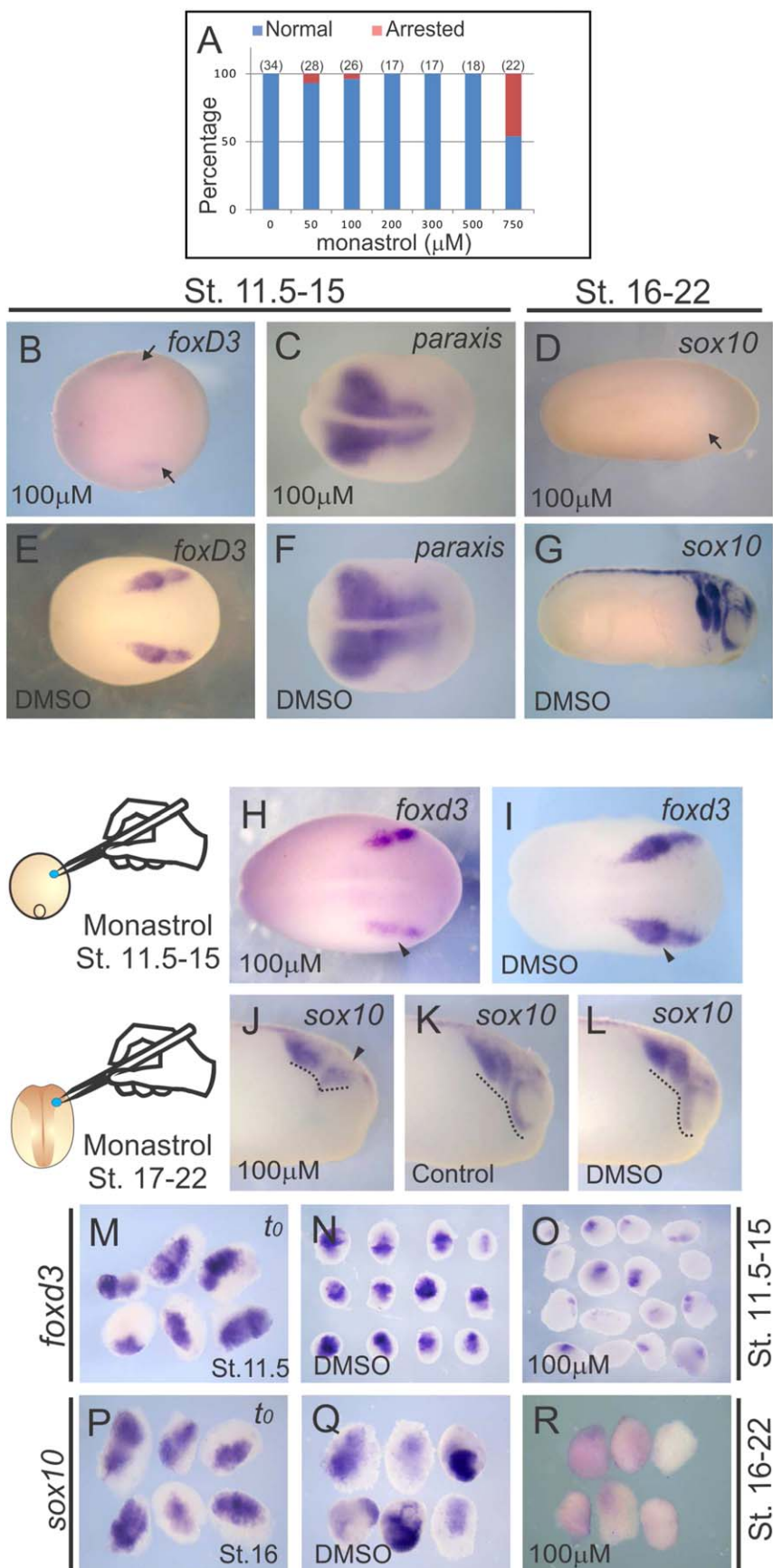
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Fig. 4.

represent cell fate decisions in the ectodermal tissue.

eg5 Is Required for Neural Crest Derivatives Development

We decided to investigate the effect of *eg5* depletion on neural crest derivatives after the injection of specific morpholino antisense oligonucleotides (Fig. 6). The injection of *CoMO* produced no effect on craniofacial cartilage size or morphology (Fig. 6A). The analysis of cranial cartilage morphology by Alcian Blue staining of *eg5MO*-injected larvae showed a severe reduction in craniofacial cartilages in the *eg5MO*-injected side (Fig. 6B, 80%, n= 54). The morphology of neural crest–derived Meckel's, ceratobranchial and ceratohyal cartilages was severely affected as these cartilages appeared markedly reduced. The effect of *eg5MO* was specifically rescued by the coinjection of *Xenopus tropicalis eg5* mRNA (Fig. 6C, 75%, n=44). The morphology of cranial cartilages derived from mesodermal tissue (e.g., infrarrostral and basihyal cartilages) was not affected by the morpholino. We also evaluated the effect of *eg5MO* on the Rohon-Beard (RB) sensory neurons, as they also arise from the neural plate border domain and require inductive mechanism as similar to neural crest. The injection of *eg5MO*

led to changes in the expression of *tbx2* marker (Takabatake et al., 2000), evidenced as a reduced number of RB neurons in the injected side (Fig. 6E, white arrow, 68%, n=50). This marker gene has also shown that cranial ganglia primordia (Schlosser and Ahrens, 2004) were also affected by *eg5MO* treatment (Fig. 6E', black arrow, 68%, n=50). Taken together, these results show that *eg5* is not only required for specific neural crest derivative development such as cartilage, but also RB neurons and cranial ganglia precursors.

Hierarchical Relationships of *eg5* in Early *Xenopus* Development

We have recently shown that the *ihh* cell signaling pathway operates in neural crest development (Agüero et al., 2012). The temporal requirements of *ihh* signaling and *eg5* together with the ability to positively regulate neural crest specification suggest their participation in the genetic cascade that specifies the neural crest territory. We decided to evaluate the epistatic relationship between *eg5* and two key members of the *Ihh* signaling pathway, the ligand *ihh* and the effector gene *gli3*. We speculated that *eg5* function could depend on the function of *ihh* signaling to promote neural crest develop-

ment. In order to test this prediction, embryos were coinjected with *ihhMO* (Agüero et al., 2012) and *eg5* mRNA, and the expression of the neural crest marker *foxD3* was analyzed. *ihhMO* produced a strong reduction in the neural crest cell population (Fig. 7C, 74%, n=27), and an almost complete rescue of *foxd3* expression in the neural crest territory was observed when *eg5* was simultaneously overexpressed (Fig. 7D, 76%, n=42). This suggests that *eg5* activity is downstream of *ihh*. The relationship between *eg5* and the Hedgehog signaling component *gli3* was also analyzed. *gli3MO* injection blocked the expression of the *foxD3* marker in the neural crest (Fig. 7E, 68%, n=47). The coinjection of *gli3MO* and *eg5* mRNA showed a reduced expression of *foxd3* marker (Fig. 7F, 73%, n=45), indicating that the effect of *gli3MO* cannot be rescued by the overexpression of *eg5*. Conversely, the effect of *eg5MO* (Fig. 7G, 64%, n=30) was rescued by the coinjection of *gli3* mRNA (Fig. 7H, 70%, n=41). Thus, these results indicate that *eg5* activity lies between *ihh* and *gli3* in the genetic cascade that specifies the neural crest in the ectoderm.

DISCUSSION

The development of the neural crest is mediated by complex interactions of multiple signals and genes (for reviews, see LaBonne and Bronner-Fraser, 2000; Mayor and Aybar, 2001; Nieto, 2001; Rogers et al., 2012). In this study, we show for the first time that the early induction, migration, and maintenance of neural crest specification require the function of kinesin Eg5, a kinesin-5 family member (Lawrence et al., 2004). The developmental spatio-temporal expression of *eg5* has not been analyzed in vertebrate animal models although it has been reported that this gene is essential during early mouse development (Castillo and Justice, 2007; Chauviere et al., 2008). The early embryonic lethality of *eg5* (*kif11*) knock-out mice precluded a detailed analysis of the role of this gene in developing embryos. It has been shown that *eg5* participates in dendrite and axon growth in neuron cultures (Yoon et al., 2005), but relatively little is

Fig. 4. Analysis of *eg5* temporal requirements in neural crest development. **A:** Evaluation of early effect of monastrol on *Xenopus* embryos development. Sixteen-cell-stage embryos were incubated in increasing concentration of monastrol. Embryos show normal segmentation up to 500 μM monastrol, while 750 μM arrested development of almost 50% of embryos. Number of embryos for each treatment are in parenthesis. **B, C, E, F, H, I:** Dorsal view of embryos. Anterior side is on the right. **D, G; J–L:** Lateral views of embryos, anterior side is on the right. B, D: Embryos incubated in NAM solution containing monastrol show an almost complete depletion of *foxd3* (arrows in B) and *sox10* (arrow in D) expression compared to DMSO-incubated embryos (B, F). C: Embryos incubated in monastrol show normal *paraxis* mesodermal marker expression, compared with sibling control (F). H, I: Embryos were grafted at stage 11.5 on the right neural fold with a monastrol-soaked bead (H) or DMSO-soaked bead (I) and fixed at stage 15. Monastrol-soaked beads grafted in stage 11.5 produced a decrease in the expression of neural crest markers *foxd3* (H) while no effect on **foxd3** was observed when control DMSO containing beads were grafted on embryos (H). Arrowhead, treated side. J: Stage-17 embryos were grafted on the right neural crest region with a 100-μM monastrol-soaked bead. Embryos were cultured until stages 21–23 and the expression pattern of *sox10* marker shows arrested neural crest cell migration and the accumulation of these cells laterally to the hindbrain. The leading edge of migration is indicated by dotted lines. K, L: Non-grafted embryos and embryos grafted with control DMSO-soaked beads show normal neural crest migration. **M–R:** Neural crest explants were dissected including the underlying mesoderm from stage-11.5 and stage-16 embryos. M, P: Groups of explants were fixed immediately (t_0) after excision at stages 11.5 or 16. Groups of explants were cultured until stage 15 (N, O) or stage 22 (Q, R) in the presence of monastrol or DMSO vehicle. O, R: The neural crest explants isolated at stage 11.5 and cultured until stage 15 or dissected at stage 16 and incubated until stage 22 in the presence of 100 μM monastrol lose *foxd3* and *sox10* expression, respectively.
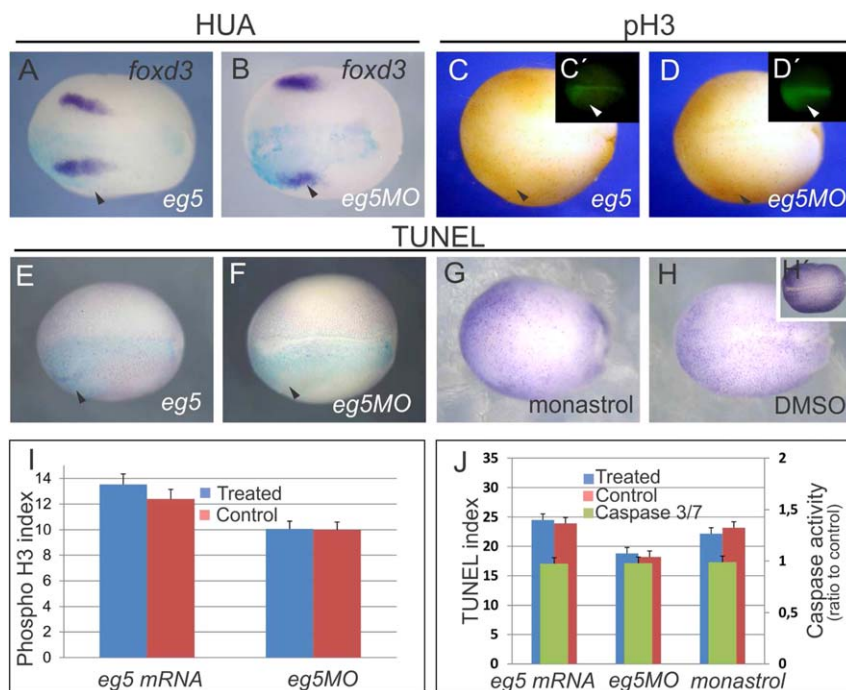
Fig. 5. *eg5* does not control cell proliferation or apoptosis during neural crest specification. **A–H:** Dorsal views of *Xenopus laevis* embryos, anterior side is on the left. The injected side is indicated by an arrowhead (A–F). Embryos were injected into one blastomere at the 8–16-cell stage with 4 ng of *eg5* mRNA (A, C, E) or 22 ng of **eg5MO** (B, D, F), incubated until stage 14 and fixed. A, B: *eg5* mRNA- or *eg5MO*-injected embryos were incubated in the presence of HUA (hydroxyurea and aphidicolin) inhibitors mix until stage 14 and the expression of *foxd3* was analyzed by in situ hybridization. *eg5* produced an expanded *foxd3* labeling (A) and *eg5MO* blocked the expression of *foxd3* marker in the neural crest (B). The presence of HUA produced no changes in the *foxd3* phenotype after *eg5* gain- or loss-of-function. Wholemount anti- phospho H3 immunohistochemistry (C, D) and TUNEL (E–H) labeling were performed as indicated in the Experimental Procedures section. **C', D':** The injected side is recognized by the fluorescence of the lineage tracer fluorescein dextran. G: Embryos were incubated in 100 μM monastrol from stage 11.5 to stage 14, fixed and processed for TUNEL. Sibling embryos were incubated in parallel in a solution containing DMSO (H). **H':** Positive control performed by DNAase treatment and subsequent TUNEL labeling. **I, J:** Quantification of phospho-H3 and TUNEL labeling for *eg5*-, *eg5MO*-microinjected embryos and monastrol treatment of embryos (see Experimental Procedures section). J, green bars: Quantification of the effector caspases 3/7 activity in neural crest explants lysates dissected out at stage 14. Caspase activity was expressed as sample:control ratio. No significant changes were observed in cell proliferation or apoptosis caused by the microinjection of *eg5* mRNA and *egMO* or by incubation in the specific inhibitor monastrol.

nance (*sox10*). It is tempting to speculate that *eg5* participates in key developmental mechanisms, but the kinesin overexpression by itself was not sufficient to induce neural crest population completely outside neural crest surroundings. The neural crest inductive process integrates many inputs and it was proposed that such a phenomenon occurs in a competence territory even though this cell population was transitorily specified into another cellular fate (Bastidas et al., 2004). This suggests that eg5 activity could facilitate essential mechanisms that lead to fate transformation of territories contiguous to the neural crest cells and that Eg5 is required for neural crest induction.

There is a possibility that these effects were produced by changes in the regulation of cell proliferation or apoptosis mechanisms. It is accepted that Eg5 participates in meiotic and mitotic processes in *Xenopus* oocytes and embryos (Houliston et al., 1994) and in neuron cell culture (Yoon et al., 2005), but the lack of effects on cell proliferation that we have observed here led us to hypothesize that Eg5 could be associated with morphogenetic aspects (i.e., participation in a particular step of a cell signaling pathway) rather than with the classic role in cell proliferation. Our results are consistent with observations previously reported that showed that neural or neural crest cell specification could proceed independently of cell proliferation (Harris and Hartenstein, 1991; Bellmeyer et al., 2003; Aybar et al., 2003; Hardcastle and Papalopulu, 2000; Schneider et al., 2010). The lack of a marked effect of *eg5* depletion on either apoptosis or cell proliferation during neural crest induction stages suggests that Eg5, like other proteins with critical mitotic functions, could also participate in alternative mechanisms (Sawin and Endow, 1993; Ferhat et al., 1998). However, several questions remain to be addressed to dissect all Eg5 functions.

During the mid neurula stage, the neural crest cells require the interaction with underlying mesoderm to maintain the specification (Agüero et al., 2012; Bonano et al., 2008; Monsoro-Burq et al., 2003; Steventon et al., 2009). Our results using
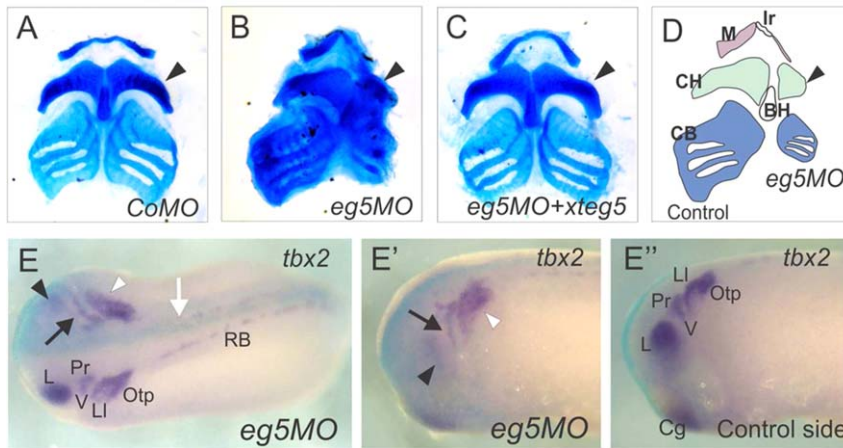
known about its participation in other developmental processes. We show here that in the earliest cell divisions, high levels of *eg5* could support the *Xenopus* embryo segmentation process. The *eg5* expression profile described is in agreement with findings reported by Yanai et al. (2011) and does not rule out a potentially redundant effect with other molecular motors to ensure the proper division in a depleted *eg5* background. Surprisingly, we found that *eg5* is particularly expressed in the neural crest prospective tissue in neurula embryos (Fig. 1), and this early expression has not been described in other animal models. The expression of *eg5* in neural

crest progenitors prompted us to examine its function during the neural crest specification of *Xenopus* embryos. In this work, we identified a novel requirement for the Eg5 protein in *Xenopus* neural crest specification using a specific morpholino antisense oligonucleotide and the specific pharmacological inhibitor monastrol (Mayer et al., 1999). The temporal analysis of *eg5* requirement showed that blocking Eg5 function reduced the expression of key developmental regulators at different stages (Figs. 2, 4) indicating that Eg5 is required first during neural plate border specification (*pax3*), neural crest specification (*snail2, foxd3*), and later during the specification mainte-

Fig. 6. *eg5* is required for the formation of neural crest derivatives formation. **A–C:** Craniofacial cartilages preparations of stage-46 tadpoles, Alcian blue staining. Ventral view, anterior side is at the top. Injected side is indicated by an arrowhead. A: Control morpholino *CoMO*-injected embryos show no effects on the treated side. B: *eg5MO* injected side of embryos (arrowhead) present a marked reduction in Meckel's, ceratohyal and ceratobranchial cartilages. C: The coinjection of *eg5MO* and *Xenopus tropicalis eg5* mRNA rescues normal cartilages morphology. **D:** Schematic representation of *eg5MO* effects on *Xenopus* head cartilages. **E, E', E":** *eg5MO*-injected embryos show altered Rohon-Beard sensory neurons and cranial ganglia precursors derivatives. Notice that *tbx2* expression pattern was altered in the injected side of embryos. M, Meckel's cartilage; CH, ceratohyal cartilage; CB, ceratobranchial cartilage; Ir, infrarostral cartilage; BH, basihyal cartilage; Pr, profoundal placode; L, lens placode; Ll, lateral line placode; V, trigeminal placode; Cg, cement gland; RB, Rohon-Beard sensory neurons. A-D, black arrowhead, injected side. E, E', white arrowhead, injected side; black arrow, cranial ganglia precursors; black arrowhead, lens placode; Otp, otic placode.

monastrol (Fig. 4) show that Eg5 activity is continuously required after initial neural crest induction and confirmed that the specification process is not yet finished during the mid-neurula stages. Many others genes and signals have been implicated in this period such as BMP/Wnt (Steventon et al., 2009), Ihh pathway (Agüero et al., 2012), and Edn1/Ednra (Bonano et al., 2008). Thus, our results suggest that *eg5* could integrate a complex regulatory network that probably coordinates the action of other neural crest maintenance factors such as *snail2* (Aybar et al., 2003) or *sox10* (Kim et al., 2003).

Total or partial loss of gene- and cell-signaling pathways activity in the early stages of development has an enormous impact to establish each neural crest derivative. BMP is required for autonomous neuron formation (Hebert et al., 2002), the Wnt signaling pathway has been implicated in melanocytes development (Dunn et al., 2000), and sensory neurons emergence (Yanfeng et al., 2003). Moreover, Edn1/Ednra signaling has been associated with melanocytes and craniofacial cartilage development

(Bonano et al., 2008), while *Ihh* participates only in craniofacial cartilage formation (Agüero et al., 2012). These data led us to figure out the effect of neural crest derivatives in an *eg5*-depleted background. First, we assessed the effect on cranial ganglia precursors (Schlosser and Ahrens, 2004) and Rohon-Beard sensory neurons. We found that these cell populations have a reduced or abnormal morphology. Neurons and glia in the cranial sensory ganglia derive from cranial neural crest cells, and the accompanying cells derive from ectodermal placodes (Baker and Bronner-Fraser, 2001). Our results indicate that *eg5* is required for the correct development of neurons derived from the neural crest or from the neurogenic placodes. A similar effect was observed in embryos lacking normal *id3* function but in this case cell proliferation was altered (Kee and Bronner-Fraser, 2005). The changes observed in cranial ganglia precursors could also be the consequence of delayed neural crest cells migration (Nishijima et al., 2000). However, an alternative explanation is that *eg5* is required per se for nerves and cranial placode
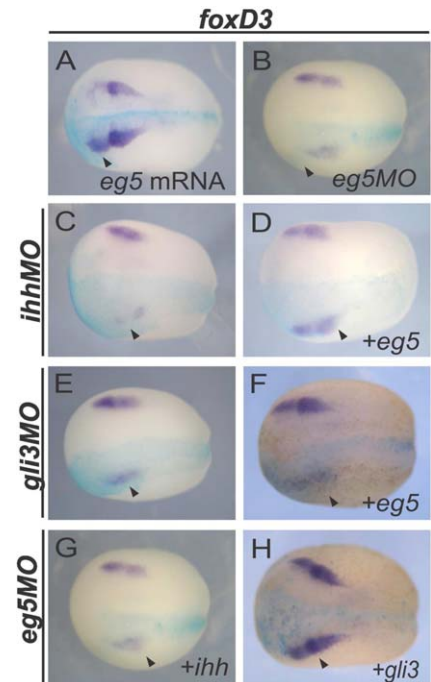
Fig. 7. Hierarchical relationships between *eg5* and *ihh* cell signaling pathway genes. **A–H:** Dorsal view of embryos. Injected side is indicated by an arrowhead. Anterior side is on the left. *eg5* mRNA-injected embryos show increased expression of neural crest marker *foxd3* (A) and *eg5MO*-injected embryos show inhibition of *foxd3* (B). C, D: *ihhMO*-injected embryos show inhibition of *foxd3* expression (C). The coinjection of *ihhMO* and *eg5* mRNA rescues *foxd3* expression on neural crest (D). E, F: *gli3MO* strongly reduces *foxd3* marker (E), and this effect is not rescued by the coinjection of *eg5* mRNA (F). G, H: The coinjection of *ihh* mRNA cannot rescue *foxd3* inhibition caused by *eg5MO* (G). The effect of *eg5MO* was rescued by the coinjection of *gli3* mRNA (H). The results suggest that *eg5* is downstream of *ihh* and upstream of *gli3* in the genetic cascade that controls neural crest specification.

growth. This explanation is in agreement with a recent finding in which mouse neuron growth was inhibited by blocking *eg5* (*kif11*) function (Nadar et al., 2012). Rohon-Beard sensory neurons were also reduced by *eg5MO* treatment suggesting that this cell population shares with neural crest the requirement for this gene probably for the specification of the neural plate border and later during the maintenance process. This result is consistent with the expression of *eg5* along these stages of development. Accordingly, it has been proposed that both cell types require similar inductive mechanisms (Olesnicky et al., 2010). On the other hand, the specific morpholino inhibitory effect on *eg5* expression produced

a severe morphological alteration in craniofacial cartilage size. This finding further emphasizes the implication of this protein as a key factor in normal craniofacial development, and that the effects of its absence are not restored by other cell signals or molecular mechanisms. This situation also suggests that early linage specification or mechanisms operate early in midneurula stages defining specific derivative progenitor cells in the neural crest (Bonano et. al., 2008, Agüero et. al., 2012, Wada et al., 2005; Schwend and Ahlgren, 2009). Taking all these findings into consideration, we could also speculate that from early stages the neural crest cell population is heterogeneous and that linage segregation mechanisms operate early to establish different neural crest derivatives.

We recently demonstrated the participation of *ihh* signaling (Agüero et. al., 2012) in neural crest initial development. The onset of *eg5* expression at the neural crest and the fact that this kinesin is required for its specification prompted us to investigate if there was a relationship between this gene and different components of the Ihh cell signaling pathway. Given that there is an increasingly large amount of Hedgehog (Hh) signaling cytoplasmatic regulators in vertebrate phyla (Hooper and Scott, 2005) and that the evolutionary history of many components has led to the duplication of some elements such as Gli proteins (Shimeld et al., 2007), it seems possible that kinesin Eg5, in the same way as other kinesin and ciliary proteins like KIF3a and the intraflagellar transport proteins, IFT (Huangfu et al., 2003), could also mediate Cos2-like functions in vertebrates, participating in both full repression and full activation of Hh responses. Our epistatic analysis indicates that *eg5* has a hierarchical relationship with members of the Ihh pathway and that *eg5* activity could be located downstream of the ligand *ihh* and upstream of the *gli3* effector gene. Although it is likely that some or all of these proteins could fulfill the biochemical role(s) of Cos2, this possibility remains to be tested. Despite the well-established functions of Eg5 during cell division, its nonmitotic functions have also been demonstrated. *KLP61F*, the *eg5 Drosophila* homologue, participates in the germ cells in microtubule-independent interactions with the fusomes (Wilson, 1999). Moreover, *eg5* is expressed in postmitotic neurons and restricts axonal growth by antagonizing the forces generated by dynein (Ferhat et al., 1998; Myers and Baas, 2007). In addition, phosphorylated Eg5 kinesin is differentially localized in the neuronal growth cone in response to particular external guiding cues (Nadar et al., 2008). In vertebrates, genes of the Hh pathway have bona fide orthologues, others appear to have no orthologues in *D. melanogaster*, some orthologues have other functions, and some other orthologues play a still unexplored role in Hh signaling (Hooper and Scott, 2005). For example, one of the newest components of Hh signaling, Missing in metastasis (MIM), is a protein that binds to actin and regulates GLI activity (Callahan et al., 2004). Rab23 is a regulator of vesicular trafficking both in flagella and cilia and is a negative regulator of Hh signaling (Eggenschwiler et al., 2001). We suggest that Eg5 generates forces that help to regulate microtubule behaviors (Ferhat et al., 1998) or provides a scaffold for the Hedgehog signaling complex within the cilia. Notwithstanding these speculations, additional studies are required to establish the nature of the molecular mechanism that involves the Eg5 kinesin activity in *Xenopus* development.

In conclusion, *eg5* requirement during *Xenopus* neural crest development provides useful data to understand the genes and signals involved in each particular neural crest developmental process. Likewise, we can conclude that *eg5* is essential during amphibian neural crest development and that its activity cannot be compensated by another molecular motor. Nevertheless, our results raise new and interesting biological questions. It will be important first to identify the mechanisms in which *eg5* is involved beyond its mitotic activity to have a fuller understanding of its role in neural crest development.

EXPERIMENTAL PROCEDURES

Isolation of *Xenopus Eg5* Homologue

Using the zebrafish *costal2* cDNA as a probe to query the NCBI sequence database, we found several interesting ESTs using the Blastn algorithm under conditions of low stringency. We fully sequenced the clone XL195f23 from the Mochii-normalized *Xenopus* early gastrula cDNA library (kindly donated by Dr. Naoto Ueno, NIBB, Okazaki, Japan). This clone encodes a new *Xenopus laevis eg5* sequence.

In order to identify *eg5* genes, we used *Xenopus laevis eg5* sequence to query the *Xenopus tropicalis* genome at JGI and Ensembl databases. *eg5* homologue sequences were initially identified using Blastn and Tblastx algorithms, and the corresponding cDNAs were reconstructed using Wise2 (EMBL) and Spidey (NCBI) algorithms. *Xenopus tropicalis eg5* gene and cDNA were deposited at NCBI under Accession numbers EF524558 and EF524557, respectively.

Embryonic Manipulation, RNA Microinjection, and Lineage Tracing

Embryos were obtained by standard procedures (Aybar et al., 2003) and staged according to Nieuwkoop and Faber series (Nieuwkoop and Faber, 1967). Tissue microdissections, mRNA in vitro synthesis, microinjection and lineage tracing were performed as previously described (Bonano et al., 2008; Agüero et al., 2012). In vitro transcribed mRNA was synthesized from *pCS2+Xleg5* and *pCMV-SPORT6-Xteg5* (kindly donated by Dr. Bruce Blumberg, UCI, CA) by standard procedures.

Monastrol and HUA Treatments

In order to perform the spatiotemporal assessment of eg5 activity, resin (Bio-Rex RG 501-8X resin, Bio-Rad, Hercules, CA) or Heparin-Sepharose (Amersham, Pittsburgh, PA) beads were soaked with 100 μM of monastrol (4-(3-Hydroxyphenyl)–6-methyl-2-thioxo-1, 2, 3, 4-tetrahydro-4H-pyrimidin-5-carboxylic Acid Ethyl Ester, Sigma-Aldrich, St. Louis, MO) prepared from a DMSO- monastrol stock solution, and grafted into embryos as previously described (Bonano et al., 2008; Agüero et al., 2012). Monastrol-soaked beads were

always grafted in the right neural fold region (stages 11.5, 14, and 16), and the final location of beads was carefully examined under a stereoscopic microscope after the in situ hybridization procedure. Vehicle-soaked beads containing the same amount of DMSO as monastrol-soaked beads were grafted as control into sibling embryos at the same position. Whole embryos or tissue explants were also incubated in 1/10 or 3/8 NAM solutions containing 100 μM monastrol. Sibling embryos or explants were incubated in NAM solutions containing an equivalent amount of DMSO vehicle. Hydroxyurea and Aphidicolin (HUA) cell proliferation blocking cocktail was assayed in control and *eg5* mRNA- or *egMO*-microinjected embryos as previously described (Harris and Hartenstein, 1991; Aybar et al., 2003). The efficiency of HUA treatment was monitored by phospho-H3 histone labeling as previously described (Aybar et al., 2003).

Morpholino Antisense Oligonucleotide and DNA Constructs

A morpholino antisense oligonucleotide (*eg5MO*) was designed and synthesized against *Xenopus laevis eg5* cDNA including the initiation start site (from −8 to +17 bp) with the sequence 5′- AGGTCACTATGTCTTC CCAGAATTC −3′ (Gene Tools, LLC). Doses of 10–22 ng/embryo were microinjected with a lineage tracer into one blastomere at 8- or 16-cell-stage embryos. A random sequence control antisense morpholino oligonucleotide (*CoMO*, 5′-CCTCTTACCTCAGTT ACAATTTATA-3′, Gene Tools, LLC) was microinjected as a control. In order to rescue *eg5* morpholino knockdown, we used in vitro transcribed *Xenopus tropicalis eg5* that bears 6 mismatches in the *Eg5MO* recognition DNA sequence. *Xenopus laevis eg5* was kindly donated by Dr. Naoto Ueno (NIBB, Okazaki, Japan) cloned in a pBSII-SK(-) plasmid between *EcoRI* and *XhoI* sites. This *eg5* cDNA was subcloned into the *pCS2+nβGal* vector after *XmaI* (5′) and *XhoI* (3′) removal of nβGal and used as a template for DNA constructs and for in vitro transcription. In order to test the efficacy of *eg5MO* in vivo, an

eg5GFP fusion construct was generated using restriction enzymes. A *HindIII* and *NcoI*- fragment containing the 5′UTR region and the N-terminal 428-amino acid residues of *eg5* was cloned directionally into the same sites of the *pCS2+EGFP* vector to produce the *Dgr;C-eg5GFP* construct.

Cartilage Staining

Alcian blue cartilage staining, specimens mounting, and photography were performed as previously described (Agüero et al., 2012).

RNA Isolation From Embryos and RT-PCR Analysis

Total RNA was isolated from whole embryos or embryonic tissues after microdissection and cDNAs were synthesized as previously described (Aybar et al., 2003; Tribulo et al., 2012). The primers designed for this study were: *eg5*: For: 5′-GAACA GTTGGCTCAGGAAGC −3′, Rev: 5′-ACAGGACTGGAGCAAGCACT −3′; *ef1α, For: 5′-CAGATTGGTGCTGGAT ATGC-3′, Rev: 5′-ACTGCCTTGAT GACTCCTAG-3′. Primers for RT-PCR analysis of sox2, foxd3, myod*, and *xk81a* were described previously (A güero et al., 2012; Tribulo et al., 2012). PCR amplification with these primers was performed over 30 cycles and PCR products were analyzed on 1.0% agarose gels. The gels were photographed and the semi-quantitative analysis was carefully performed using the ImageJ software (NIH, Bethesda, MD) as previously described (Tribulo et al., 2003). As a control, PCR was performed with RNA that had not been reverse-transcribed to check for DNA contamination. In each case, elongation factor 1α (*ef1α*) was used as a loading control.

In Situ Hybridization, Immunohistochemistry, TUNEL, and Caspase Activity Determination

Antisense probes containing Digoxigenin-11-UTP or Fluorescein-12-UTP were prepared for eg5, snail2 (Mayor et al., 1995), *foxD3* (Sasai et al., 2001), *sox2* (Dr. R.M. Grainger, personal communication), *xk81a* (Jonas et al., 1985), *paraxis*

(Agüero et al., 2012), *msx1* (Tribulo et al., 2003), *sox10* (Honore et al., 2003), *pax3* (Bang et al., 1997), *tbx2* (EST clone Xl107n21, kindly donated by Dr. Naoto Ueno) by in vitro transcription. Specimens were prepared, hybridized, and stained as previously described (Aybar et al., 2003). As routine control, sense probes were synthesized and hybridized and did not produce any staining.

Apoptosis was detected by TUNEL staining according to the procedure previously described (Tribulo et al., 2004; Bonano et al., 2008). The TUNEL index was obtained by manually counting TUNEL-positive cells/ 40,000 μm² . Rabbit Polyclonal anti-phosphohistone-3 (Upstate Biotechnology, East Syracuse, NY) was used to analyze mitotic cells according to the method previously described (Turner and Weintraub, 1994; Agüero et al., 2012). We manually counted the number of positive cells/40,000 μm² for Phospho Histone H3 labeling, which we define as the Phospho H3 index (Brenner et al., 2003). Caspases 3/7 activity was determined in neural crest explants using Apo-ONE homogeneous assay (Promega, Madison, WI) according to the manufacturer's instructions. Control and treated explants in 50 μl 0.75 NAM were added in 1:1 ratio and homogenized in the Apo-One Caspase 3/7 reagent, incubated 40 min at room temperature and fluorescence measured as arbitrary fluorescence units in a Perkin-Elmer LS55 fluorescence spectrometer (excitation wavelength 499 nm, emission wavelength 521 nm). Caspases 3/7 activity was calculated for each assay fluorescence reading minus blank and expressed as percentage of control explants fluorescence. Student's *t*-test was used to analyze the differences between each of the groups for pH3 staining, TUNEL, and Caspases 3/7 activity with respect to the corresponding control group. Differences were considered statistically significant at $*P < 0.001$.

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