Developmental Biology 323 (2008) 114–129

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

A new role for the Endothelin-1/Endothelin-A receptor signaling during early neural crest specification

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ARTICLE INFO

Article history:
Received for publication 25 June 2008
Revised 1 August 2008
Accepted 5 August 2008
Available online 15 August 2008

Keywords:
Neural crest
Specification
Maintenance
Endothelin
Ednra
Apoptosis
Cell migration

ABSTRACT

The neural crest is induced at the border of the neural plate in a multistep process by signals emanated from the epidermis, neural plate and mesoderm. In this work we show for the first time the existence of a neural crest maintenance step which is dependent on signals released from the mesoderm. We identified Endothelin-1 (Edn1) and its receptor (Ednra) as key players of this signal and we show that Edn1/Ednra signaling is required for maintenance of the neural crest by a dual mechanism of cell specification and cell survival. We show that: (i) *Ednra* is expressed in prospective neural crest; (ii) loss-of-function experiments with antisense morpholino or with specific chemical inhibitor suppress the expression of early neural crest markers; (iii) gain-of-function experiments expand the neural crest territory; (iv) epistatic experiments show that Ednra/Edn1 is downstream of the early neural crest gene *Msx1* and upstream of the late genes *Sox9* and *Sox10*; and (v) Edn1/Ednra signaling inhibits apoptosis and controls cell specification of the neural crest. Together, our results provide insight on a new role of Edn1/Ednra cell signaling pathway during early neural crest development.

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Introduction

The neural crest is a transient embryonic structure unique to vertebrates that is generated at the lateral borders of the neural plate. Neural crest delaminates from the dorsal neural tube and migrates extensively to various parts of the embryo, where they differentiate into a wide variety of cell types, including most of the craniofacial skeleton, cartilage, neurons and glia of the peripheral nervous system, connective tissue, neuroendocrine cells, and melanocytes (Le Douarin, Kalcheim, 1999).

The induction of the neural crest integrates signals emanating from different tissues but their relationships are still poorly understood (Meulemans and Bronner-Fraser, 2004; Steventon et al., 2005). Signals from the nonneural ectoderm, the paraxial mesoderm, or both, are currently believed to be involved in the specification of the neural crest in different species (Bonstein et al., 1998; Mancilla and Mayor, 1996; Marchant et al., 1998; Monsoro-Burq et al., 2003; Selleck and Bronner-Fraser, 1995). There is increasing evidence that these tissue interactions are mediated by molecules related to the BMP family, Wnt, FGF, retinoic acid and *Notch/Delta* (reviewed in Meulemans and Bronner-Fraser, 2004; Sauka-Spengler and Bronner-Fraser, 2006; Steventon et al., 2005).

In *Xenopus* and zebrafish embryos the evidence indicates that the neural plate, neural crest and epidermis are specified by a dorso-ventral gradient of BMP activity generated by anti-BMP molecules that directly bind to BMPs. However, BMP signaling appears to be insufficient to induce the neural plate or the neural crest, and FGF, Wnt or RA are also required (reviewed in Aybar and Mayor, 2002; Meulemans and Bronner-Fraser, 2004; Steventon et al., 2005).

Endothelins and their receptors were initially known for their involvement in the regulation of blood pressure (Yanagisawa et al., 1988). Endothelins belong to a family of conserved 21-amino acid peptides that includes three members encoded by different genes (*Edn1*, *Edn2*, *Edn3*). Soon after discovery, it was found that mice carrying targeted homozygous mutations for *Endothelin receptor type A* (*Ednra*), *Endothelin-1* (*Edn1*) or *Endothelin Converting Enzyme-1* (*ECE-1*) were viable to term but die shortly after due to severe defects in the formation of neural crest derivatives (Clouthier et al., 1998; Kurihara et al., 1994; Yanagisawa et al., 1998b). It has been shown in the mouse embryo that *Edn1/Ednra* is required for the patterning process in the pharyngeal arch by controlling the expression of *Dlx6/Hand2* transcription factors (Charite et al., 2001; Thomas et al., 1998; Yanagisawa et al., 2003) through $G_{\alpha q}/G_{\alpha 11}$ proteins (Ivey et al., 2003). *Ednra* is expressed in the mouse embryo in migrating cranial neural crest cells and ectomesenchymal cells in the pharyngeal arches (Clouthier et al., 1998; Yanagisawa et al., 1998a), while its counterpart Edn1 peptide is secreted by pharyngeal arch ectoderm, core paraxial mesoderm and pharyngeal pouch

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doi:10.1016/j.ydbio.2008.08.007

endoderm (Clouthier et al., 1998; Maemura et al., 1996; Yanagisawa et al., 1998a). The expression and function of Edn1/Ednra signaling appears to be conserved in all gnathostomes since the pharmacological alteration of Ednra function in chick (Kempf et al., 1998) and rat (Spence et al., 1999) embryos reproduce the distal arch effect described for knock-out mice. In addition, the zebrafish mutation of *Edn1* gene *sucker* (*suc* or *suc/et1*) have multiple defects that are consistent with the patterning role of Edn1/Ednra signaling in the pharyngeal arch (Kimmel et al., 2001, 2003; Miller and Kimmel, 2001; Miller et al., 2000, 2003). These studies have demonstrated for the mice, chick and fish embryos that *Ednra* signaling is essential for late neural crest development.

Edn1 is synthesised as a large peptide encoded by the *Ppet-1* gene, which is converted to the active peptide Edn1 by the converting enzyme *ECE-1* (Xu et al., 1994). In order to investigate the function of Edn1/Ednra signaling pathway in *Xenopus* embryos, we have isolated the *Ednra*, *Ppet-1*, and *ECE-1* homologues. We found, unexpectedly, an early expression of *Ednra* in the neural folds from late gastrula stage onward. Gain- and loss-of-function experiments show that the Edn1/Ednra signaling is involved in the early induction of the neural crest precursors. By performing rescue experiments, we found that *Ednra* function lies downstream the transcription factor *Msx1* in the genetic cascade of neural crest induction. Furthermore, we describe a new step of neural crest development: neural crest maintenance by signals produced by the mesoderm during mid-neurula stages. We show that Edn1/Ednra signaling is required and sufficient for this neural crest maintenance step and that they control neural crest survival. Our results show a new role of Edn1/Ednra signaling in early neural crest maintenance; and we have been able to show that this step is dependent on a dual activity of Edn1/Ednra on cell survival and cell specification.

Materials and methods

Isolation of Xenopus Ednra homologues

Using the human and *Xenopus* heart *Ednra* mRNA (BC022511 and U06633, respectively) as probes, several interesting ESTs were found at the NCBI database. We fully sequenced the clone XL061f18 from the Mochii *Xenopus* tailbud library that was kindly gifted by Dr. Naoto Ueno (NIBB, Okazaki, Japan). This clone encoding *X. laevis Ednra* was deposited at NCBI Nucleotide database (DQ523688). Homology searches were also carried out in order to isolate *Xenopus laevis Preproendothelin-1* (*Ppet-1*) and *Endothelin Converting Enzyme-1* (*ECE-1*). The screening resulted in the identification of clones XL041d02, originated from a neurula-stage library and XL333c19ex from a stage 10.5 dorsal marginal zone library (NIBB, Japan). These clones were fully sequenced and deposited at NCBI Nucleotide database (EF127993 and EF127992, respectively). In order to identify and isolate unknown *Ednra* genes we used *X. laevis Ednra* sequence to query *Xenopus tropicalis* genome at JGI and Ensembl databases. *Ednra* homologue sequences were initially identified using Blastn and Tblastx algorithms, and the corresponding cDNAs reconstructed by means of Wise2 (EMBL) and Spidey (NCBI) algorithms. *Xenopus tropicalis Ednra* gene and cDNA were deposited at NCBI under Accession numbers DQ865481 and DQ865482, respectively. Alignments of amino acid sequences were done using the ClustalW algorithm. The phylogenetic tree was drawn with the Phylodendron application from data generated with ClustalW. Protein domains were analyzed using SignalP, Prosite and SOSUI systems (Hirokawa et al., 1998).

Embryonic manipulation, RNA microinjection, lineage tracing and dexamethasone induction

Embryos were obtained by standard procedures (Aybar et al., 2003) and staged according to Nieuwkoop and Faber (1967). Tissue dissections and melanocyte induction were carried out as previously described (Aybar et al., 2003; Bonstein et al., 1998; Honore et al., 2003). In order to assess Ednra activity, resin or Heparin-Sepharose beads were soaked with 5–20 μM of BQ123 or 5–20 μM Endothelin-1 (Edn1) peptide (Sigma, USA) and grafted in embryos as previously described (Honore et al., 2003). Soaked beads were implanted in the right neural fold region, and the final position of beads was carefully examined under stereoscopic microscopy after the in situ hybridization procedure. RNA microinjection, lineage tracing and dexamethasone induction was performed as described (Aybar et al., 2003).

Morpholino antisense oligonucleotide and DNA constructs

A morpholino antisense oligonucleotide (*EdnraMO*) was designed against *X. laevis Ednra* including the initiation start site (from −5 to +18 bp) with the following sequence 5′-CAAGGTGTTTCCTCCC-ATCCTGATG-3′ (Gene Tools, LLC). Doses of 10–25 ng/embryo were microinjected together with a lineage tracer into one blastomere at the 8- or 16-cell stage. A control antisense oligonucleotide composed of a random sequence (*CoMO*, Gene Tools, LLC) was injected as a control. The *Ednra* ORF from pBS was subcloned into a pCS2+ vector. For the rescue of morpholino knock-down experiments a DNA construct was prepared by changing only the codon sequence targeted by the morpholino antisense oligonucleotide. Special care was taken to mutate the sequence for one that codes for the wild type Ednra amino acid sequence. This construct, named *Ednra′*, introduced 10 mismatches in the nucleotide sequence recognized by the morpholino and was made by high fidelity PCR using the following primers: 5′-ATCGATATGGGTGGTAATACGTTAAGATTC-ACTGTG-3′ and 5′-TCTAGACTAATTGATGCTGTCCTTGTGAATAC-3′. In order to test the efficacy of *EdnraMO* in vivo, an Ednra–GFP fusion was generated by high fidelity PCR using pBS-*Ednra* as the template and the following primers: 5′-GCGCAATTAACCCTCACTAAAGGG-3′ and 5′-CCATGGCAAAGCCTATGGCTTCAGG-3′ (underlined, NcoI restriction site). A fragment containing the 5′ UTR region of *Ednra* (178 bp) and encoding 214 amino acid residues of Ednra was cloned into the TOPO-TA vector, amplified and then cloned directionally into BamHI and NcoI pCS2-EGFP vector to produce the *Ednra-GFP* construct. In vitro transcribed mRNA of this construct was coinjected with the morpholino oligonucleotides.

In vitro translation and western blotting

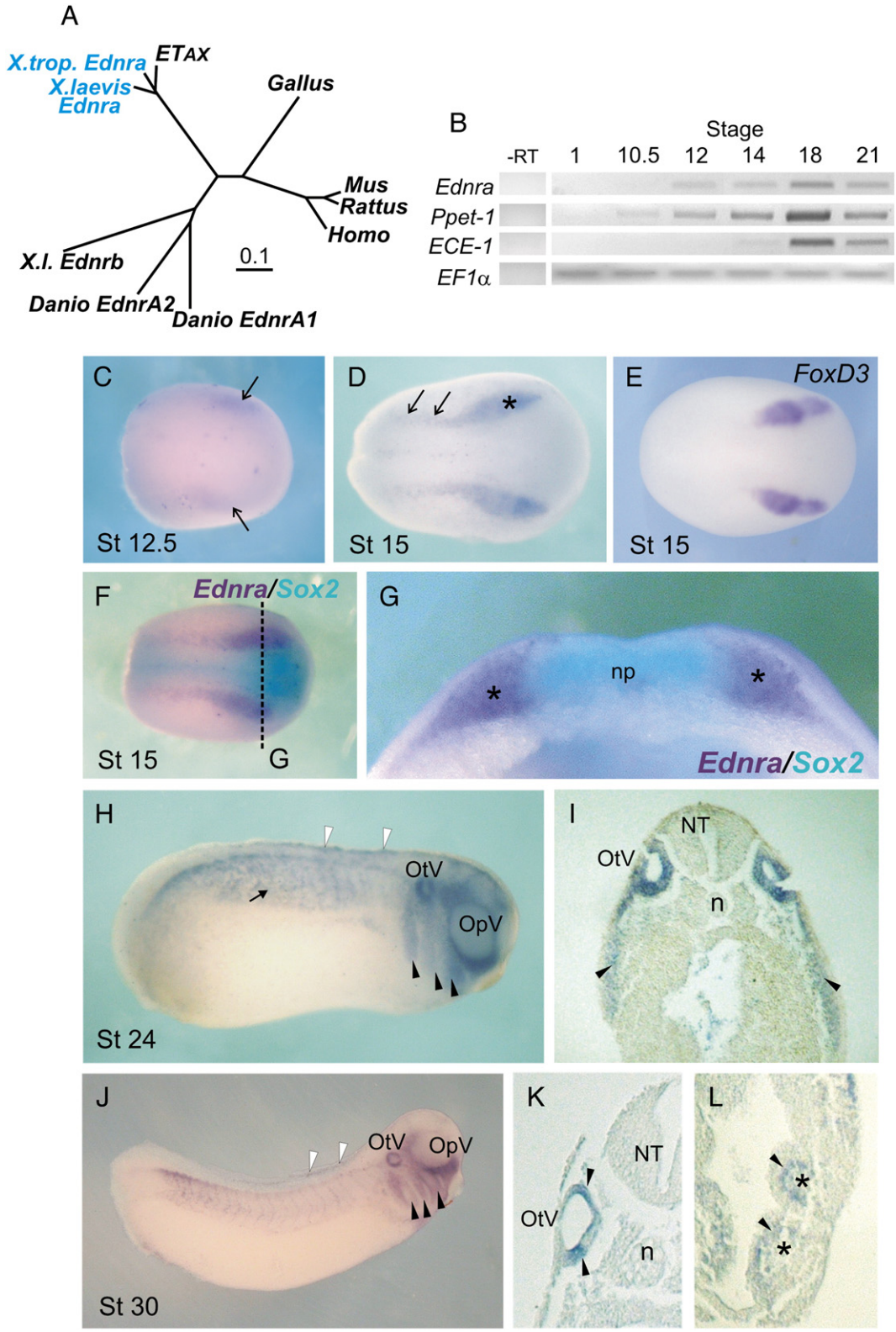
The efficacy of antisense morpholino oligonucleotide *EdnraMO*, directed against *Xenopus laevis Ednra*, was tested in vitro. *Ednra* or *Ednra′* mRNA were translated in vitro using a rabbit reticulocyte lysate (Promega, WI, USA) and the *Trascend*™ non-radioactive translation detection system (Promega) in the presence of *EdnraMO* or *CoMO*. Reactions were assembled on ice and then incubated at 37 °C for 1.5 h. Aliquots of the reactions were separated in a 7.5% Tris–HCl electrophoresis gel and blotted to a nitrocellulose membrane. The membranes were blocked and incubated with Streptavidin-AP (Sigma-Aldrich) and developed with NBT-BCIP substrate.

RNA isolation from embryos and RT-PCR analysis

Total RNA was isolated from whole embryos or embryonic tissues after microdissection and cDNA were synthesised as previously described (Aybar et al., 2003). The primers designed for this study were: *Ednra*, 5′-GCCTTTCTTATGTAGATAGTGGG-3′ and 5′-TCTAAT-GAGCGTTTTGCATGG-3′; *Pppet-1*, 5′-CCCTGGGCTGTTTATAGG-AATGTAG-3′ and 5′-GTTTCTCTTGGTCAGTGTCCTGC-3′; *ECE-1*, 5′-TGCACAAGTCTGGTGTACTGTG-3′ and 5′-CCTTTCATACAGTC-TGGCAGTG-3′. PCR amplification with these primers was performed over 29 cycles and the PCR products were analyzed on 1.5% agarose gels. As a control, PCR was performed with RNA that had not been reverse-transcribed to check for DNA contamination.

In situ hybridization, TUNEL, cartilage staining, and immunohistochemistry

Antisense probes containing Digoxigenin-11-UTP or Fluorescein-12-UTP were prepared for *Ednra* (digested with EcoRV from pBS vector, transcribed with T7), *Sox10* and *Sox9* (Honore et al., 2003), *Sox2* (Dr. RM Grainger, personal communication), cytokeratin *Xk81A* (Jonas et al., 1985; Mayor et al., 1995), *Snail2* (Mayor et al., 1995), *Snail1* (Essex et al., 1993), *FoxD3* (Sasai et al., 2001), and *Trp-2* (Aoki et al., 2003) by in vitro transcription. Specimens were prepared, hybridized, and stained as previously described (Aybar et al., 2003; Tribulo et al., 2003). Apoptosis was detected by TUNEL staining according to the procedure previously described (Hensey and Gautier, 1998; Tribulo et al., 2004). For cartilage staining, embryos were fixed



at stage 45–47 and processed as was previously described (Tribulo et al., 2004). Rabbit Polyclonal anti-phosphohistone-3 (Upstate Biotechnology) was used to analyze mitotic cells according to the method previously described (Aybar et al., 2003; Turner and Weintraub, 1994).

Results

Isolation of embryonic Xenopus laevis Ednra

A cDNA of an Endothelin receptor was previously isolated from the heart of adult *Xenopus* frogs (ET_{AX}, genbank:U06633; (Kumar et al., 1994), but there was no indication that an Endothelin receptor is expressed in *Xenopus* embryonic tissues. To clone the *X. laevis Ednra* cDNA expressed during embryonic development we performed a homology search in EST databases at the NCBI. The cDNA isolated (clone XL061f18) was 2832 bp in length and contained an open reading frame encoding a 415 amino acid residues protein (Supplemental data, Fig. S1, A, B). Sequence analysis revealed a novel *Ednra* isoform and predicted seven transmembrane domains and a topology of G protein-coupled superfamily of receptors (Fig. S1, A). *Xenopus* embryonic *Ednra* cDNA (genbank:DQ523688) shares 94% amino acid identity and 90% nucleotide identity with *Xenopus* ET_{AX}. (Kumar et al., 1994), 66% and 51% amino acid identity with *Xenopus Ednrb* and *Ednrc* (Karne et al., 1993). In order to identify unknown *Ednra* genes for the phylogenetic analysis we searched *Xenopus tropicalis* genomic databases. The gene structure comparison for *X. tropicalis* (Supplemental data, Table 1S, Fig. S1, C) and human *Ednra* (Livingston et al., 2004) revealed a high conservation of the overall 8-exon structure and exon sizes despite differences in the entire length of these genes (Supplemental data, Fig. S1C–D, Table 2S). On the basis of the data obtained from the comparison of different Ednra proteins, we built the unrooted phylogenetic tree of vertebrate Ednra that illustrates clearly the relatedness of these receptor proteins (Fig. 1A).

The expression pattern of *Ednra* was analyzed by RT-PCR (Fig. 1B) and whole-mount in situ hybridization (Figs. 1C–O). No maternal expression of *Ednra* was observed, and the transcripts were first detected by RT-PCR from stage 12 (Fig. 1B), and by in situ hybridization from the late gastrula stage restricted only to the ectoderm, in two faint patches at the anterior-lateral sides of the neural plate (Fig. 1C). At mid-neurula stage (stages 14–18) *Ednra* expression is observed in the prospective cranial and trunk neural crest cells (Figs. 1D, F) throughout neurulation, showing a similar pattern to the neural crest marker gene *FoxD3* (Fig. 1E). Double in situ hybridization showed that this expression is complementary and not overlapping with the neural plate marker *Sox2* (Figs. 1F, G). As development continues, neural crest cells initiate their migration and the expression of *Ednra* is detected in the cranial migrating neural crest, the otic placode and in the trunk region (Figs. 1K–O). We conclude that *Ednra* is expressed in the premigratory and migratory neural crest.

Edn1/Ednra signaling is required for neural crest development

Ednra expression pattern strongly suggests that Edn1/Ednra signaling is involved in neural crest development. To analyze the function of *Ednra*, we designed an antisense morpholino oligonucleotide directed against the start codon of *Ednra* (*EdnraMO*). This morpholino oligonucleotide inhibited translation of a GFP-tagged form of Ednra in a dose-dependent manner in vitro (Fig. 2A) and in vivo (Figs. 2B–I). We decided to investigate the development of some neural crest derivatives after injection of *Ednra* morpholino antisense oligonucleotides. Control side of the embryos show the normal melanocyte pattern (Fig. 2J), and normal expression of the melanocyte precursor marker *Trp2* (Fig. 2M), while in *EdnraMO*-injected embryos *Trp2* marker and melanocyte formation were inhibited (Fig. 2K, 38% of embryos with melanocytes, $n=50$; Fig. 2N, 25% of embryo with *Trp2* expression, $n=66$). A similar inhibition was observed in vitro. Melanocytes were induced in vitro by conjugating animal caps with prospective paraxial mesoderm taken from early gastrula embryos (Fig. 2O, 81%, $n=22$); while conjugates made in the presence of the Ednra-inhibitor BQ123 showed no melanocytes formation (Fig. 2P, 76%, $n=25$). Cartilage development was also analyzed. A severe loss or reduction of craniofacial cartilages was observed in the *EdnraMO*-injected side by Alcian Blue staining (Fig. 2Q, 66% of the embryos; $n=27$). The gross morphology analysis of cranial cartilages revealed that Meckel's and ceratohyal cartilages were markedly reduced, and branchial cartilage was mildly affected (Fig. 2R). These results show Edn1/Ednra signaling is required for the normal formation of neural crest derivatives such as melanocyte and cartilage.

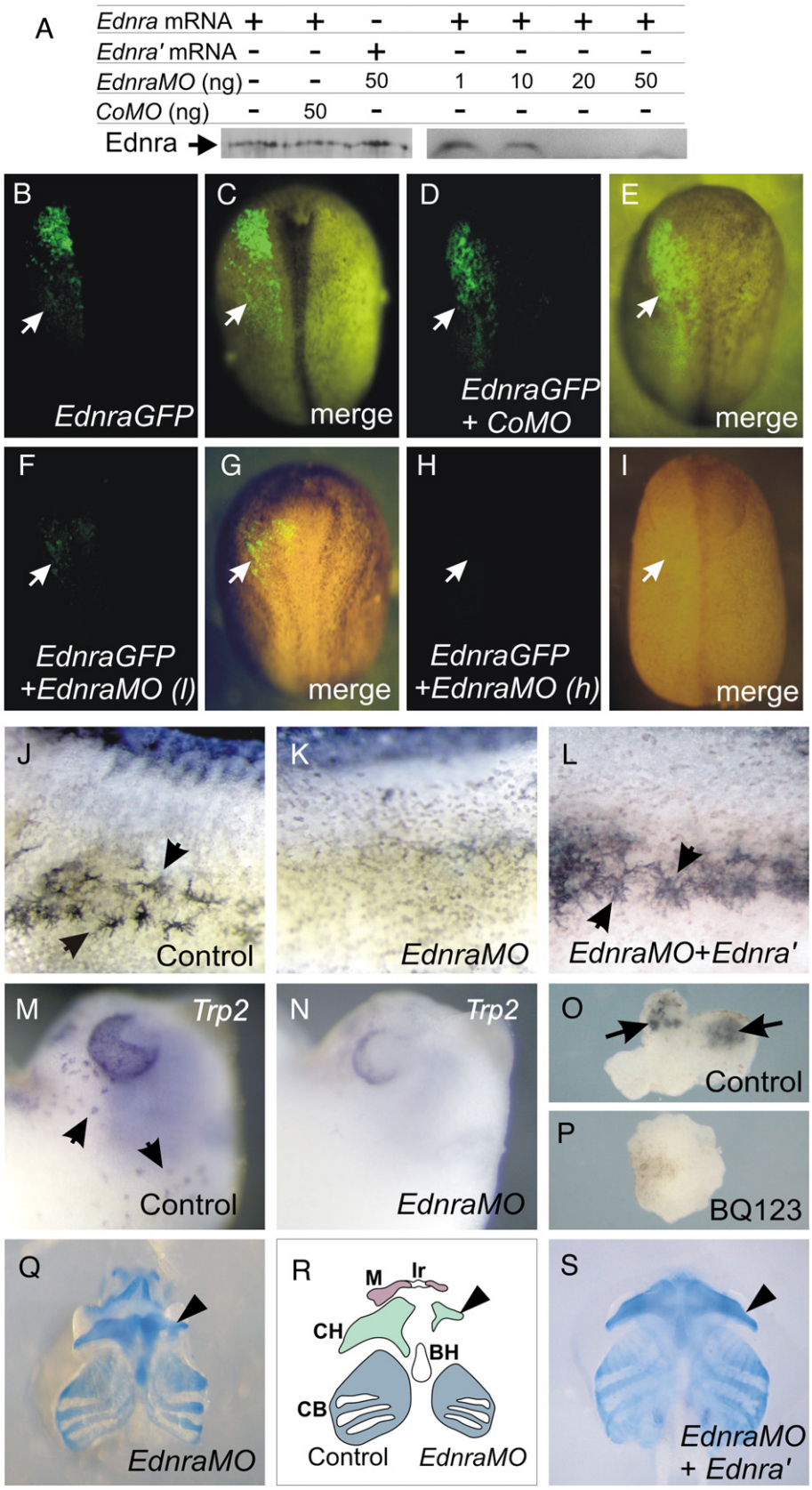
Ednra is required for the early formation of neural crest cells

The *Ednra* expression pattern and the effects of *EdnraMO* or BQ123-treatment suggest Ednra-mediated signaling is required during early neural crest development. In order to evaluate the participation of Edn1/Ednra signaling during neural crest induction, embryos were injected at the four- or eight-cell-stage in one dorsal blastomere with *EdnraMO* (25 ng/embryo) or an equivalent amount of a control morpholino (*CoMO*) (Figs. 3A–I). The embryos injected with *CoMO* showed normal morphology and expression of *Snail2* (Figs. 3F, I), while embryos injected with *EdnraMO* failed to form a neural fold and showed a clear inhibition of *Snail2*, *FoxD3* and *Snail1* expression at the injected side (Fig. 3A, 75%, $n=120$; Fig. 3B, 72%, $n=98$; *Snail1* not shown, 71%, $n=32$ of inhibition). The neural plate markers *Sox2* (Fig. 3C, 64%, $n=31$) and *Sox3* (66%, $n=45$; not shown) and the epidermal marker *XK81a* were expanded (Fig. 3D, 69% of expansion, $n=27$). The effect on neural plate and epidermal markers could be a consequence of the absence of neural crest at the border of each tissue, as no overlapping of neural plate and epidermis was observed (Fig. 3E). These results show that *Ednra* depletion leads to a specific loss of neural crest progenitors, thus suggesting that *Ednra* is involved in the early specification of the neural crest cells.

To assess the specificity of the morpholino effects, we asked whether the phenotype of *Ednra*-depleted embryos could be rescued by restoring *Ednra* function. We injected an *Ednra* construct (*Ednra′*) carrying a 10 bp mutation within the recognition motif of *EdnraMO* in order to avoid hybridization between the morpholino and the microinjected mRNA. Injection of *Ednra′* mRNA shows an expansion of *Snail2* expression in 53% ($n=51$) of the embryos (Fig. 3H),

Fig. 1. *Xenopus laevis Endothelin-1 receptor type A* features and expression pattern. (A) Molecular phylogenetic analysis of Ednra shows the relationship with *Xenopus* ET_{AX}, *Xenopus* Ednrb and with Ednra from different vertebrate species. The protein sequences of different *Ednra* were analyzed using ClustalW 1.81, and an unrooted tree was constructed by neighbour-joining analysis. *Xenopus laevis Ednrb*, *Danio rerio EdnrA1*, and *Danio rerio EdnrA2* branches are collapsed 50%. (B) RT-PCR analysis of temporal expression pattern of *Ednra*, *Ppet-1*, and *ECE-1* during different developmental stages. Total RNA isolated from stages 1, 10.5, 12, 14, 18, and 21 was isolated, retrotranscribed and amplified as described in Materials and methods. Contamination of genomic DNA was examined by experiments without RT reactions (−RT) using total RNA from stage 18 embryos. *EF1α* expression was used as loading control. (C–L) Expression pattern of *Xenopus laevis Ednra*. (C) *Ednra* transcripts are first detected from late gastrula stage (St. 12.5) in the lateral domains of neural plate (arrows). (D) During neurulation *Ednra* is expressed in the prospective cephalic (asterisk) and trunk neural crest (arrows). (E) In situ hybridization for *FoxD3*. (F) Double in situ hybridization for *Ednra* (purple) and *Sox2* (turquoise). (G) Transverse section of (F). Asterisk, prospective neural crest. (H) Tailbud-stage (St. 24) embryo showing expression in the migrating cephalic neural crest (arrowheads), trunk region (arrow), crest cells migration into dorsal fin (white arrowheads), and otic vesicle (OtV). (I) Frontal section of (H), *Ednra* expression in the otic vesicle (OtV) and the neural crest hyoid migratory stream (arrowheads). NT, neural tube; n, notochord. (J) A St. 30-embryo showing *Ednra* expression in the branchial arches (black arrowheads), cells into the dorsal fin (white arrowheads), and otic vesicle (OtV). OpV, optic vesicle. (K) Section showing *Ednra* expression in the dorsomedial and ventromedial region of the otic vesicle (arrowheads). (L) Horizontal section showing *Ednra* expression (arrowheads) in the first and second pharyngeal arches surrounding the central mesodermal core (asterisks).

evidencing that *Ednra* function was not affected by the mutation. However, when *EdnraMO* was coinjected with *Ednra′* mRNA a strong rescue in *Snail2* expression (Figs. 3G, I), and in melanocytes (Fig. 2L) and cartilage development (Fig. 2S) was observed. This effect was specifically produced by the coinjection of *Ednra′* mRNA but not by the coinjection of unrelated mRNAs like GFP mRNA (not shown). Thus, we conclude that *EdnraMO* was able to specifically knock-down *Ednra* activity.



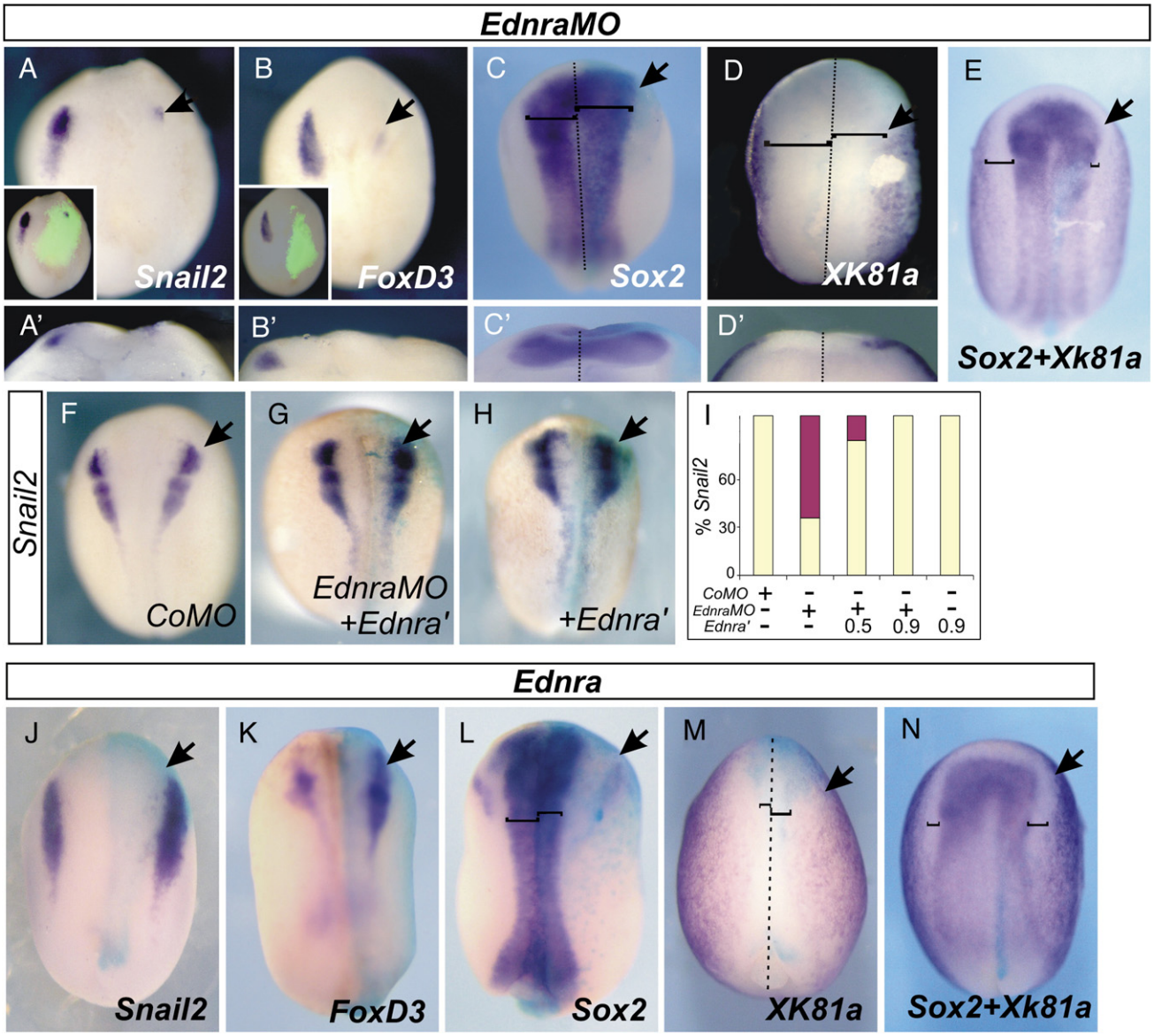


Fig. 3. Ednra is required for early neural crest specification. (A–I) Effect of EdnraMO on neural crest specification. Arrows indicate the injected side. (A'–D') Transverse sections of embryos at the level of cephalic neural crest. (A, B) *Ednra*-depleted embryos fail to express neural crest markers *Snail2* (A) and *FoxD3* (B). (A, B, insets) The injected side is recognized by the fluorescence of the lineage tracer FLDx. (C, D) Expression of neural plate marker *Sox2* and epidermal marker *XK81a* is expanded on the *EdnraMO*-injected side. (E) Embryo processed by double in situ hybridization for *Sox2* and *XK81a* genes showing the reduction of prospective neural crest domain in the injected side. (F) Injection of control morpholino (*CoMO*) showed no effect. (G) Coinjection of *EdnraMO* and *Ednra′* mRNA rescues *Snail2* expression. (H) Single injection of *Ednra′* mRNA expanded the *Snail2* expression domain. (I) Quantification of rescue experiments. Results are expressed as percentage of embryos normally expressing *Snail2* for each treatment (yellow bars), and as percentage of embryos showing reduced *Snail2* expression (purple bars). Two different concentrations of *Ednra′* mRNA were coinjected for rescue experiments (0.5 ng/embryo and 0.9 ng/embryo). (J–N) *Ednra* participates in the early neural crest specification. *Ednra*-injected embryos show increased expression of *Snail2* (J) and *FoxD3* (K). Expression of the neural plate marker *Sox2* (L) and epidermal marker *XK81a* (M) are reduced on the injected side. (N) Double in situ hybridization for *Sox2* and *XK81a* shows the enlargement of prospective neural crest territory in the injected side. Broken line, dorsal midline; brackets indicate the width of the neural plate (C, L), the width of neural crest domain (E, N) or the width of the neural plate plus the neural crest domain (D, M).

To determine whether *Ednra* might be participating in the control of neural crest specification we microinjected *Ednra* mRNA in the animal pole of a dorsal blastomere at four to eight-cell stage embryos. The injection of *Ednra* leads to an expansion of the neural crest territory analyzed by the expression of *Snail2* (66%, $n=33$), *FoxD3* (65%, $n=40$) (Figs. 3J, K), and *Snail1* (not shown, 73%, $n=41$), and a

Fig. 2. *Ednra* is required for neural crest development. (A–I) Efficiency of Ednra antisense morpholino oligonucleotide. (A) *EdnraMO* inhibits in vitro translation of Ednra in a dose-dependent fashion. Arrow indicates Ednra protein product. (B–I) *EdnraMO* inhibits in vivo expression of Ednra–GFP. (B,C) Embryos injected with mRNA encoding *Ednra–GFP* (1 ng/embryo) showing GFP fluorescence. (D,E) Embryos injected with *Ednra–GFP* mRNA (1 ng/embryo) and *CoMO* (30 ng/embryo). (F–I) Embryos injected with *Ednra–GFP* mRNA (1 ng/embryo) and *EdnraMO* (F–G, low dose, (I) 10 ng/embryo; H–I, high dose (h), 20 ng/embryo). No embryo shows GFP fluorescence at high dose. White arrows indicate the injected side. (J–S) Analysis of the formation of neural crest derivatives. Normal melanocyte formation (J, arrows) and *Trp2* expression (M, arrows). *EdnraMO*-injected embryos show inhibition in melanocyte development (K) or *Trp2* expression (N). The melanocyte formation was completely rescued by the coinjection of *EdnraMO* and Ednra′ mRNA (L). Induction of melanocytes in vitro by conjugating animal caps with prospective paraxial mesoderm cultured until the equivalent of St. 38. Control conjugates show melanocytes (O, arrows). Conjugates prepared with a 10 μM BQ123-soaked bead show no melanocyte formation (P). *EdnraMO*-injected (Q, arrowhead) embryos present a reduction of Meckel's and ceratohyal cartilages. (R) Schematic representation of *EdnraMO* effects on *Xenopus* head cartilages. M, Meckel's cartilage; Ir, infrarrostral; CH, ceratohyal; BH, basihyal; CB, ceratobranchial. (S) Normal cartilage formation was completely rescued by the coinjection of EdnraMO and Ednra′ mRNA.

reduction of *Sox2* (66%, $n=32$) and epidermal marker *XK81a* expression (60%, $n=35$, Figs. 3L–N). These observations suggest that the expansion of the neural crest as a result of the ectopic expression of *Ednra* is produced by the transformation of the epidermis and some of the neural plate region into prospective neural crest cells.

Temporal requirement of Edn1/Ednra signaling for neural crest induction

Antisense morpholino treatments are very specific for the inhibition of a particular gene. However, it is more difficult to control the time of its activity. In order to control the time of Edn1/Ednra

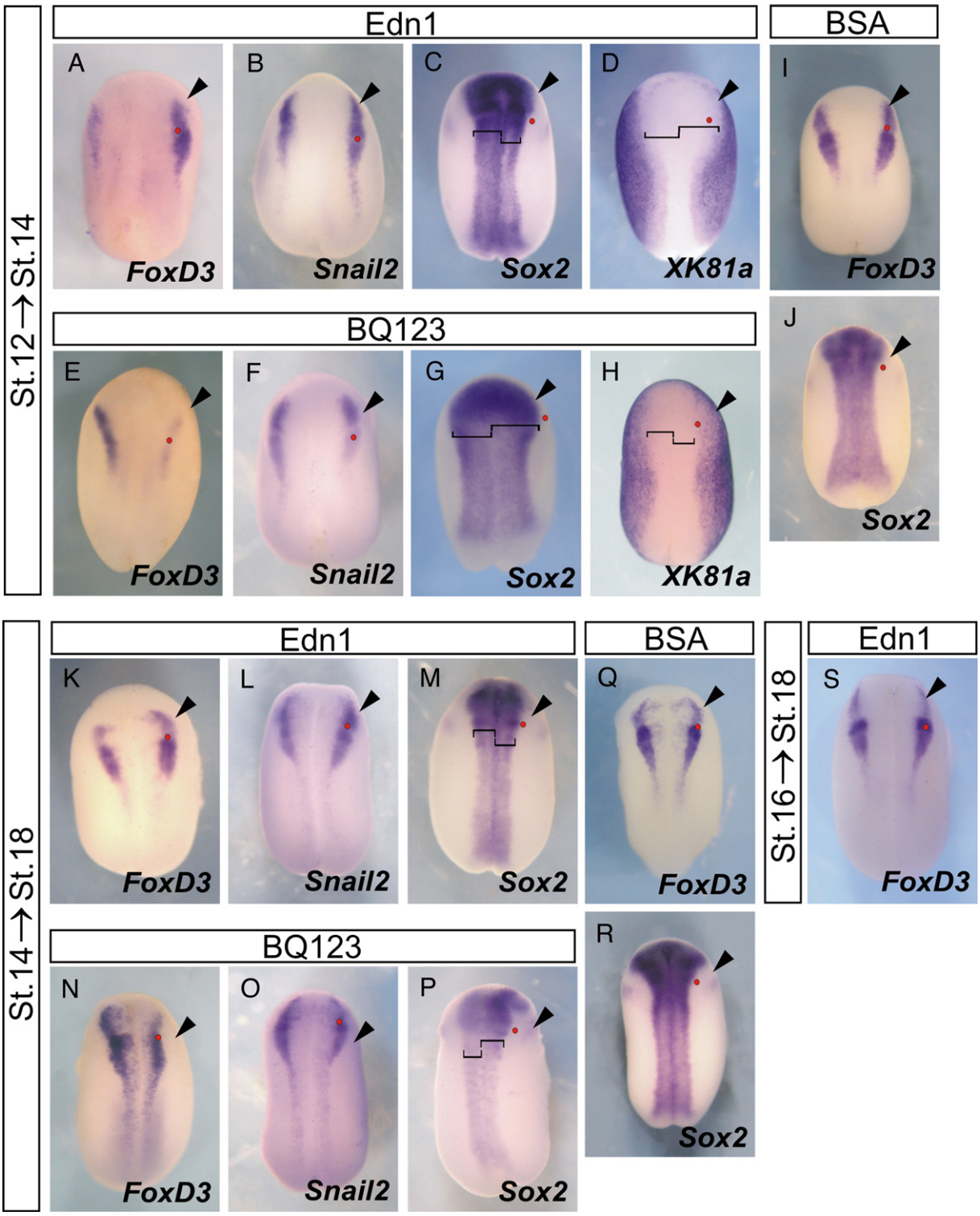


Fig. 4. Temporal requirement of Edn1/Ednra signaling for neural crest induction. Stage 12 (A–J), 14 (K–R) or 16 (S) embryos were grafted on the right neural fold with an Edn1 peptide-soaked bead or with the specific Ednra-inhibitor BQ123. Embryos were cultured until stage 14 (A–J) or 18 (K–S), when the expression of marker genes was analyzed. Arrowheads indicate the grafted side. Red circles indicate the position of Edn1- or BQ123-soaked beads. (A–D, K–M) Edn1 peptide increases the expression of neural crest markers *Snail2* (A, K) and *FoxD3* (B, L). The marker *Sox2* (C) and *XK81a* are reduced (D). (S) No changes in the expression of *FoxD3* were observed when Edn1-soaked beads were grafted into stage-16 embryos. (E–H, N–P) BQ123 leads to a reduction in the expression of neural crest markers *FoxD3* (E, N) and *Snail2* (F, O) on the treated side. BQ123 treatment produces an increase in the expression of *Sox2* (P). (I–J, Q–R) Control embryos grafted with BSA-soaked bead. No effect on the expression of neural crest (I, Q; *FoxD3*) or neural plate (J, R; *Sox2*) markers is observed.

signaling inhibition or activation we used the Edn1 ligand and the Ednra-specific antagonist BQ123. Three different stages of development were tested in their ability to respond to Edn1/Ednra signaling. Beads soaked with different amounts of Edn1 or BQ123 were implanted next to the right prospective neural crest of stage-12 (Figs. 4A–J), or stage-14 embryos (Figs. 4K–R), and beads soaked with Edn1 were implanted at stage-16 embryos (Fig. 4S). Embryos were cultured until stage 14 (Figs. 4A–H) or stage 18 (Figs. 4K–R), and the expression of different markers was analyzed by in situ hybridization. Treatment of stage 12 embryos with Edn1-soaked beads produced a moderate increase in the expression of neural crest markers *Snail2*, *FoxD3* (Figs. 4A–B, 62%, $n=34$; 65%, $n=57$; respectively) and *Snail1* (not shown, 82%, $n=28$). This effect was accompanied with a reduction in the expression of the neural plate marker *Sox2* (Fig. 4C, 57%, $n=28$) and the epidermal marker *XK81a* (Fig. 4D, 64%, $n=33$). Treatment of stage-12 embryos with 10 μM BQ123 markedly downregulated *FoxD3* (Fig. 4E, 78%, $n=32$) and *Snail2* (Fig. 4F, 77%, $n=53$) expression in the neural crest territory. The inhibitor BQ123 produced an increase on the expression of genes *Sox2* (Fig. 4G, 59%, $n=33$) and *XK81a* (Fig. 4H, 68%, $n=38$) expressed in tissues adjacent to neural crest. No effect on the expression of the neural crest marker *FoxD3* or the neural plate marker *Sox2* was observed when BSA-soaked beads were grafted (Figs. 4I, J). Thus, the pharmacological blockade by BQ123 is able to reproduce the phenotype produced by the morpholino knockdown showing that Ednra activity is required for neural crest induction.

Similar, but weaker, effects were observed when stage-14 embryos were used. Edn1-soaked beads lead to an increase in the expression of neural crest markers *FoxD3* (Fig. 4K, 64%, $n=45$), *Snail2* (Fig. 4L, 65%,

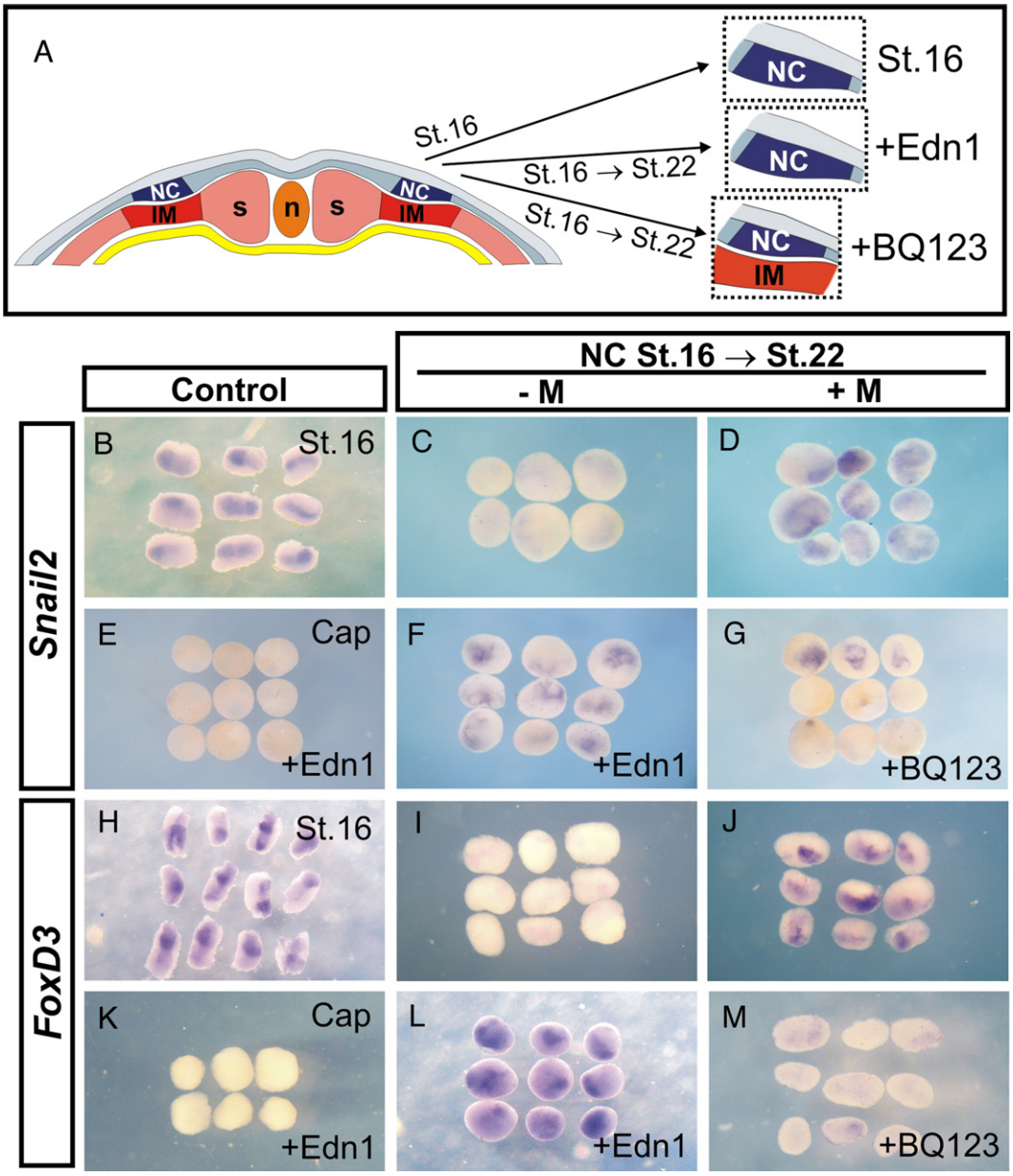


Fig. 5. Edn1/Ednra signaling is necessary for neural crest maintenance. (A) Schematic representation of transverse section mid-neurula embryos and the different neural crest explants dissected out for the experiments. Blue, neural crest; red, mesoderm: s, paraxial mesoderm; IM, intermediate mesoderm; orange, notochord; yellow, endoderm. Neural crest explants (NC) were prepared by dissection of the neural crest (−M), or by including the underlying mesoderm (+M). NC explants dissected at stage 16-embryos express *Snail2* (B), and *FoxD3* (H). NC(−M) explants dissected from stage 16-embryos and cultured until equivalent stage 22 have lost *Snail2* (C) and *FoxD3* (I) expression. NC(+M) explants taken from stage 16-embryos and cultured until stage 22, express *Snail2* (D) and *FoxD3* (J) markers. NC(−M) explants cultured in 10 μM Edn1 peptide until equivalent stage 22 show positive *Snail2* (F) and *FoxD3* (L) expression. In NC(+M) explants cultured in 10 μM BQ123 the expression of *Snail2* (G) and *FoxD3* (M) was inhibited. Control animal cap explants were cultured until equivalent stage 22 in 10 μM Edn1 peptide. No expression of *Snail2* (E) or *FoxD3* (K) was induced.

$n=55$), and *Snail1* (not shown, 68%, $n=43$); and to an inhibition in *Sox2* expression (Fig. 4M, 64%, $n=33$). Treatment with BQ123 slightly but consistently affected *FoxD3* (Fig. 4N, 71%, $n=35$) and *Snail2* (Fig. 4O, 58%, $n=32$) expression. Expression analysis of the neural plate marker *Sox2* in these embryos showed that the neural plate was expanded (Fig. 4P, 51%, $n=35$). Control beads soaked in BSA had no effect on gene expression at any stage and the embryos developed normally (Figs. 4Q, R). At this stage, the inhibition of Ednra was not able to completely downregulate the expression of the neural crest markers analyzed. When Edn1-soaked beads were implanted in the neural fold of stage-16 embryos, no changes were observed in the expression of neural crest marker *FoxD3* (Fig. 4S, 83%, $n=31$). Taken together, these experiments show that neural crest specification by Edn1/Ednra takes place between late gastrula (stage 12) and early neurula stage (stage 14), suggesting the existence of a new step of neural crest induction later that the one initially described (Mancilla and Mayor, 1996).

Edn1/Ednra signaling is necessary for the maintenance of neural crest specification

There is strong evidence that the mesoderm plays an important role in neural crest induction (Bonstein et al., 1998; Mancilla and Mayor, 1996; Marchant et al., 1998; Monsoro-Burq et al., 2003; Raven and Kloos, 1945). As our previous temporal analysis of neural crest specification by Edn1/Ednra suggest that this signaling pathway could play a late role on neural crest induction, we decided to analyze whether there was a late requirement of mesoderm for the specification of the neural crest, and if Edn1/Ednra was involved in this late step.

Snail2 and *FoxD3* are expressed in neural crest explants dissected at stage 16 and fixed immediately (Figs. 5A, B, H). However, when equivalent neural crest explants (NC−M) are cultured in vitro until the equivalent of stage 22, the expression of both neural crest markers *Snail2* (Fig. 5C) and *FoxD3* (Fig. 5I) is lost. On the contrary, when the cultured neural crest contained the adjacent mesoderm (NC+M) an almost normal expression of both markers was observed (Figs. 5D, J). This observation suggests that neural crest requires a signal from the mesoderm to maintain its specification. However, when NC+M explants are incubated with the inhibitor BQ123 a clear inhibition of neural crest markers was observed (Figs. 5G, M). Furthermore, the addition of Edn1 is sufficient to maintain neural crest markers expression in vitro in absence of mesoderm (Figs. 5F, L). However, the addition of Edn1 peptide to competent animal caps is not able to induce the expression of neural crest markers (Figs. 5E, K). Taken together, these findings indicate that Edn1 can replace the maintenance signal produced by the mesoderm and that Ednra activity is essential for neural crest maintenance by the mesoderm.

As these results suggest that an Edn1 signaling produced by the mesoderm is required for neural crest maintenance, we decided to analyze the expression of all the components required to have a fully active Edn1 signaling. We cloned the *Preproendothelin-1* (*Ppet-1*) and *Endothelin Converting Enzyme-1* (*ECE-1*) from *Xenopus laevis* which are required for Edn1 secretion and processing. Our high stringency homology search resulted in the isolation of *Ppet-1* and *ECE-1* cDNAs. *Ppet-1* shared protein sequence similarity ranging 54% to 60% among vertebrate homologues (Supplemental data, Table 3S). However, the protein sequence of Edn1 active peptide (Supplemental data, Fig. 2S) shares 100% identity with human, rat, mice, porcine, and bovine Edn1 peptides. The temporal expression profile of *Ppet-1* transcripts was analyzed by RT-PCR (Fig. 1B). Maternal expression of *Ppet-1* was not detected and the zygotic expression was first detectable at a low level at stage 10.5 and expression gradually increased up in later stages. We also analyzed by RT-PCR the expression of *Ppet-1*, *Ednra* and *ECE-1* in neural crest and mesoderm explants dissected from embryos at stage 16 (Fig. 6A). NC explants expressed *Ednra* and *ECE-1*, and the mesoderm underlying the neural crest expressed *Ppet-1* and *ECE-1*. The analysis of spatial expression pattern by whole-mount *in situ* hybridization was more difficult as *Ppet-1* and *ECE-1* are expressed at relatively low levels. We detected *Ppet-1* transcripts predominantly in the somitic and lateral mesoderm of mid-neurula embryos adjacent neural crest (Figs. 6B, B') and *ECE-1* transcripts in ectodermal and mesodermal tissues (Figs. 6C, C'). These observations allow to propose that Edn1 signal could be secreted by the mesoderm underlying the neural crest and processed by the ECE-1 produced by tissues adjacent to the neural crest cells.

Edn1/Ednra signaling controls the apoptosis in the neural crest

It has been previously proposed that the inhibition of apoptosis could be necessary for the maintenance of neural crest progenitors (Honore et al., 2003; Light et al., 2005; Tribulo et al., 2004). We therefore decided to analyze whether Edn1/Ednra participates in the maintenance of neural crest throughout the inhibition of the apoptosis. A marked increase in the number of cells stained by TUNEL was observed in the neural fold of *EdnraMO*-injected embryos (Fig. 7A, 78% of embryos with increase in TUNEL staining, $n=98$, and Supplemental Fig. S4,B), while no effect was observed when TUNEL assay was performed before the onset of Ednra expression (inset in Fig. 7A', 0%, $n=25$) or in embryos injected with *CoMO* (0% of increase in TUNEL staining, $n=55$, not shown). This increase in the number of apoptotic cell by *EdnraMO* injection was reversed by coinjection of Ednra′ mRNA (Fig S4C, 16%, $n=32$). The microinjection of *EdnraMO* produced no detectable changes in the cell proliferation status

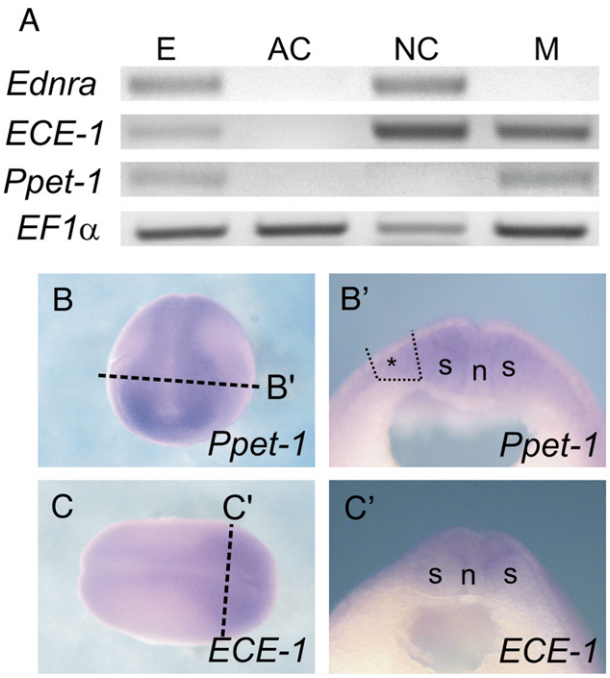


Fig. 6. Analysis of Ppet-1 and ECE-1 expression. (A) RT-PCR analysis of the expression of *Ednra*, *Ppet-1* and *ECE-1* in neural crest and mesodermal explants. Explants were dissected out from stage-16 embryos (see Materials and methods). E, stage-16 embryo; AC, animal caps; NC, neural crest explants; M, mesodermal explants. *EF1α*, loading control. (B–C) Expression pattern of *Xenopus laevis Preproendothelin-1* and *Endothelin Converting Enzyme-1*. (B) *Preproendothelin-1* (*Ppet-1*) transcripts are detected by whole-mount in situ hybridization during mid-neurula stage (St. 16) in the mesoderm. (B') Transversal section of (B), *Ppet-1* is expressed in somite (s) and lateral mesoderm (asterisk). The broken line indicates the tissues that were dissected out for the isolation of neural crest explants containing the mesoderm underlying the neural crest (see Fig. 5). (C) *Endothelin Converting Enzyme-1* (*ECE-1*) is expressed during mid-neurula stage (St. 16) in the dorsal ectoderm and mesoderm. (C') Transversal section of (C). s, somite; n, notochord.

assessed by the detection of a phosphorylated form of H3 histone (Supplemental Fig. 4S,A, A').

We next asked whether apoptosis levels in neural crest explants depends on the presence of mesodermal tissue and Edn1/Ednra signaling. We analyzed TUNEL staining in neural crest explants that were dissected and cultured as previously described. We found moderate levels of TUNEL staining in explants fixed at stage 16, immediately after dissection (Fig. 7B) that are in accordance to the pattern normally observed in vivo (Tribulo et al., 2004). A higher level of apoptotic nuclei was observed in neural crest explants (NC−M) that were cultured until stage 22 (Figs. 7D, H), whereas apoptosis remained at lower levels in the explants that contained mesoderm (Figs. 7E, H). However, when NC−M explants were cultured with Edn1, a dramatic reduction of TUNEL-positive nuclei was observed (Figs. 7F, H). In contrast, the levels of apoptosis increased in NC+M explants by incubation in BQ123 (Figs. 7G, H). As controls, animal cap explants cultured in Edn1 (Fig. 7C) or BQ123 (not shown) showed that apoptosis was not influenced by these treatments. In conclusion, these results strongly suggest that in the neural crest Edn1/Ednra signaling can act as an anti-apoptotic factor, and that mesoderm instructively signals over ectoderm to maintain neural crest survival.

The previous results suggest that Edn1/Ednra control neural crest specification by allowing the survival of the induced neural crest cells. Thus, the absence of neural crest markers after inhibition of Ednra signaling could be explained by the apoptosis of the neural crest cells. Alternatively, Ednra could control cell specification independently of cell survival. To distinguish the effects of blocking Ednra on specification from those on cell survival, we activated an inducible *Snail2* (*Snail2GR*) construct after stage 14 which affects apoptosis without any effect on neural crest specification (Tribulo et al., 2004). Alternatively, we also used a *Xenopus Bcl2* homologue (Tribulo et al., 2004) that acts as an anti-apoptotic factor in the neural crest (Tribulo et al., 2004). *Snail2GR* and *XBcl2* successfully inhibited apoptosis in neural crest explants regardless the underlying mesodermal tissue was included or not, or whether the explants were treated with Edn1 and BQ123 (Figs. 8A–F, K–N, S). Next, we analyzed the expression of neural crest marker *FoxD3* under the same conditions (Figs. 8G–J, O–R). We found in apoptosis-inhibited explants the same effects of Edn1 and BQ123 on neural crest expression markers, than when apoptosis was not inhibited. Absence of mesoderm leads to the disappearance of *FoxD3* (Figs. 8G, O), which is rescued by Edn1 treatment (Figs. 8I, Q); while neural crest maintenance by mesoderm (Figs. 8H, P) is inhibited by BQ123 treatment (Figs. 8J, R). In conclusion, the effect of Edn1/Ednr1 signaling on neural crest cell death can be dissociated from its effect on cell specification. Taken together, our results show that the Edn1/Ednra signaling plays a dual role on neural crest development: cell specification and cell survival.

Ednra is downstream Msx1 in the genetic cascade that specifies neural crest cells

Having established that Edn1/Ednra signaling is involved in the early specification of the neural crest we decided to investigate the hierarchical relationship between *Ednra* and other members of the neural crest genetic cascade. The different genes involved in neural crest development have been grouped into three categories: early neural plate border specifiers, neural crest specifiers or survival genes

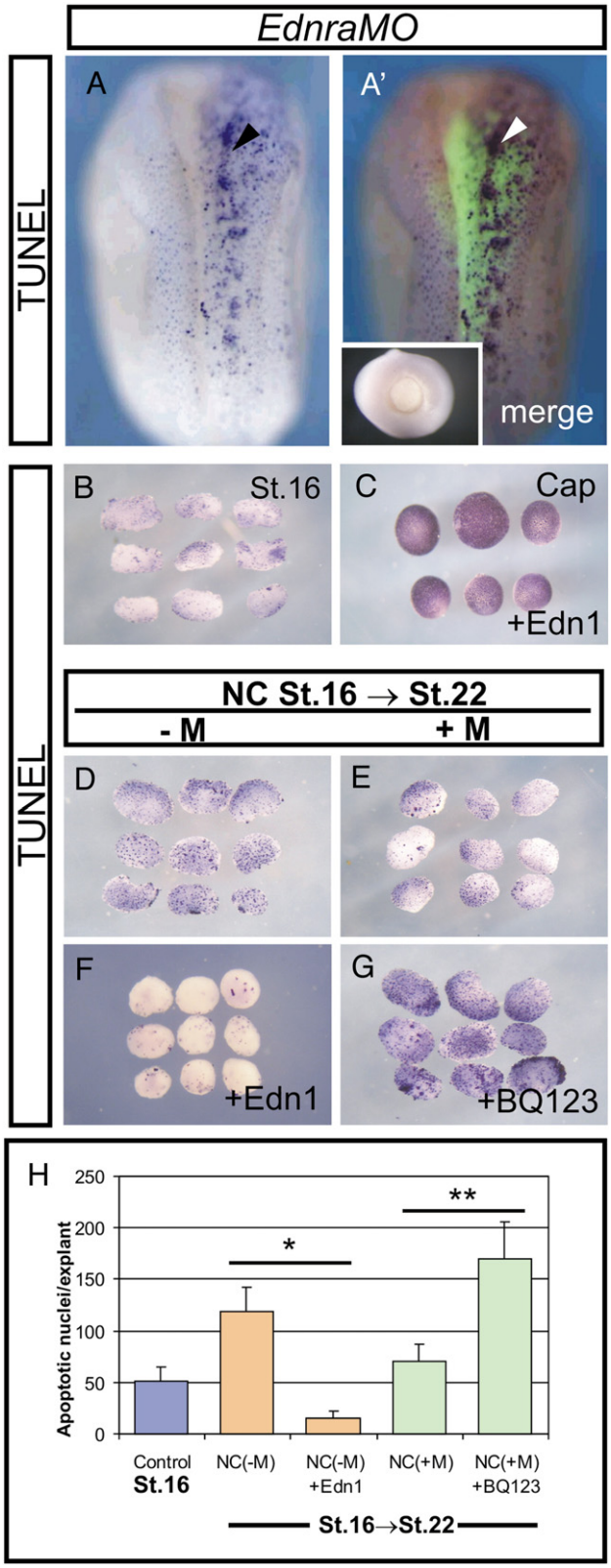


Fig. 7. Edn1/Ednra signaling controls the apoptosis in the neural crest. (A) *EdnraMO*-injected embryos show increased TUNEL staining (arrowhead, injected side; A', FLDx lineage tracer). (A', inset) *EdnraMO*-injected embryos show no increase of TUNEL labeling at stage 12, indicating that apoptosis was not induced by morpholino toxicity at stages when *Ednra* is not expressed. (B) Neural crest (NC) explants taken from stage 16-embryos and immediately fixed show moderate level of TUNEL staining. (C) Control animal cap explants cultured until equivalent stage 22 in 10 μM Edn1 show no inhibition of normal apoptosis. (D) NC(−M) explants dissected at stage 16 and cultured until stage 22 show increased apoptosis. (E) NC(+M) explants show a moderate inhibition of apoptosis. (F) NC(−M) explants treated with 10 μM Edn1 show inhibition of TUNEL staining. (G) NC(+M) explants cultured with 10 μM Ednra-specific inhibitor BQ123 show increased number of TUNEL-positive apoptotic nuclei. (H) Quantification of apoptosis in neural crest explants. Results are expressed as number of apoptotic nuclei/explant ± S.D. Control refers to the TUNEL staining of stage 16-embryos. Every condition or treatment, except NC(+M) explant, was significantly different from control explants (Student's *t*-test, $P<0.001$). (*) NC(−M)+Edn1 was statistically different from NC(−M), $P<0.001$. (**) NC(+M)+BQ123 was statistically different from NC(+M), $P<0.001$.

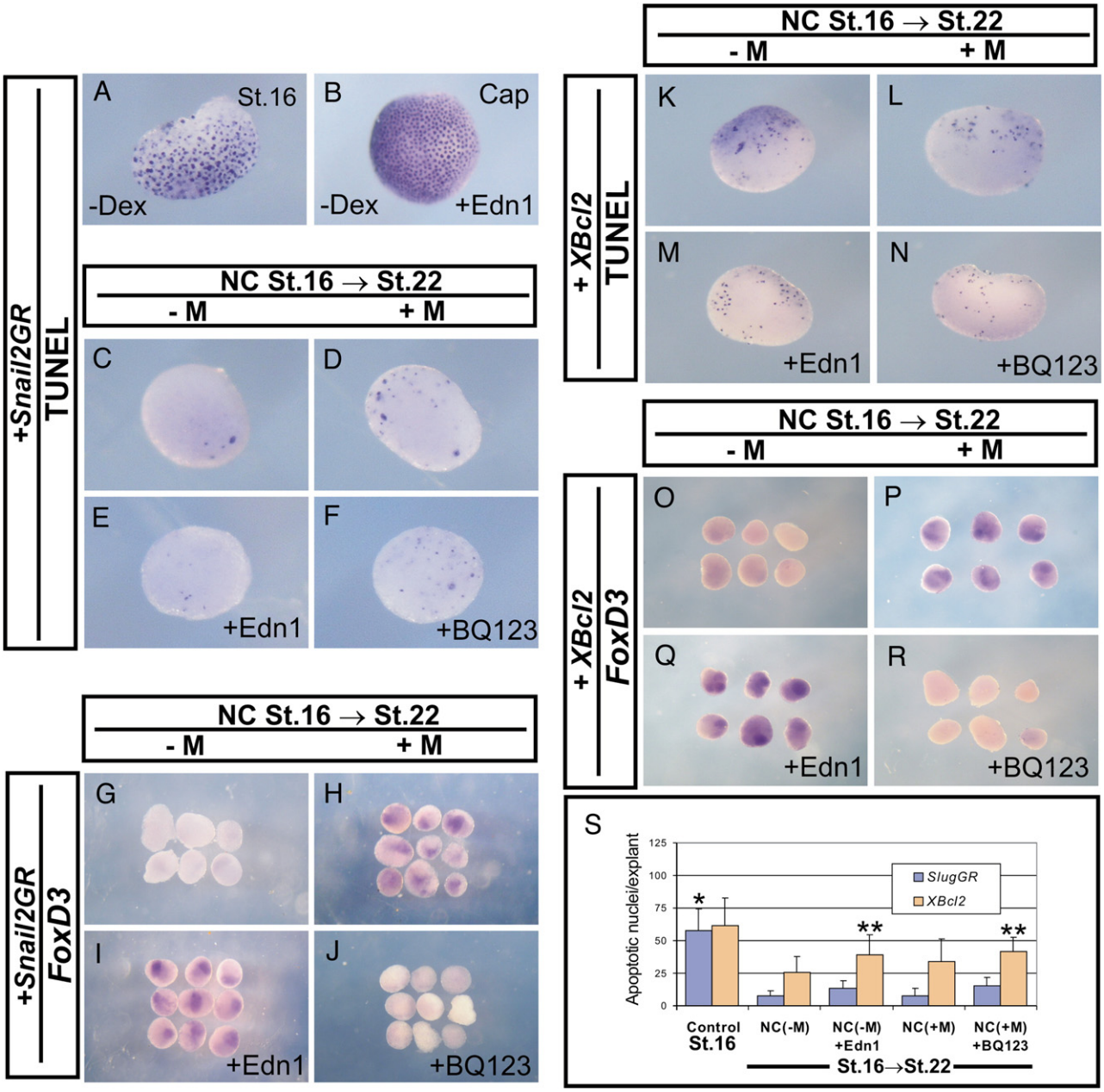


Fig. 8. The Edn1/Ednra signaling control on the maintenance of neural crest specification can be dissociated from apoptosis. (A) *Snail2GR*-injected neural crest (NC) explant dissected out and fixed at stage 16. In control explants the *Snail2GR* inducible construct was not activated. Explants show the normal apoptotic pattern. (B) Control animal cap explants cultured until equivalent stage 22 in 10 μM Edn1 show no inhibition of normal apoptosis. NC(−M) or NC(+M) explants from *Snail2GR*-injected embryos (C–J) and *XBcl2*-injected embryos (K–R) were dissected at stage 16 as indicated in Fig. 5A. *Snail2GR* construct was activated at stage 16 by adding dexamethasone (10 μM final concentration). Explants were processed for TUNEL staining (C–F, K–N) or in situ hybridization (G–J, O–R). (C–F, K–N, S) All *Snail2GR*- and *XBcl2*-injected explants show a marked inhibition of apoptosis, regardless the presence or not of mesodermal tissue into the explant, or the incubation with 10 μM Edn1 peptide or 10 μM BQ123. The expression of *FoxD3* was lost in *Snail2GR*-injected and *XBcl2*-injected (G, O; respectively) NC(−M) explants. In *Snail2GR*-injected and *XBcl2*-injected NC(+M) explants the expression of *FoxD3* marker was maintained (H, P; respectively). *Snail2GR*-injected and *XBcl2*-injected NC(−M) explants incubated in 10 μM Edn1 show the expression of *FoxD3* (I, Q; respectively). *Snail2GR*-injected and *XBcl2*-injected NC(+M) explants incubated in 10 μM BQ123 failed to express *FoxD3* marker (J, R). (S) Quantification of apoptosis in *Snail2GR* and *XBcl2*-injected neural crest explants. Results are expressed as number of apoptotic nuclei/explant ± S.D. Control refers to the TUNEL staining of NC explants taken from stage 16-embryos. Every condition or treatment in *Snail2GR*-injected explants was significantly different from control explants (*, $P<0.001$). *XBcl2*-injected NC(−M) and NC(+M) explants were statistically different from control, $P<0.001$. (**) *XBcl2*-injected NC(−M)+Edn1 and NC(+M)+BQ123 explants were statistically different from control, $P<0.01$.

and neural crest effector genes (Meulemans and Bronner-Fraser, 2004; Steventon et al., 2005). We choose *Msx1* as a neural plate border specifier and *Sox9* and *Sox10* as neural crest specifier to analyze the hierarchical position of *Ednra* in the genetic cascade. Representative examples of embryos injected with the indicated reagents are shown in Figs. 9A–O, and the percentage of effect for each experiment is shown in the graphs of Fig. 9. Injection of an *Msx1*-inducible construct, *Msx1-GR*, leads to an increase in the expression of *Ednra* (Fig. 9A, 71%, $n=41$) that correlates with the increase in *FoxD3* expression (Fig. 9B, 68%, $n=44$), while an inducible dominant negative of *Msx1*, *HDMsx1-GR*, produces a reduction in *Ednra* (Fig. 9D, 68%, $n=52$) and *FoxD3* expression (Fig. 9E, 66%, $n=47$). The inhibition of *FoxD3* (Fig. 9E) by *HDMsx1-GR* injection was almost completely rescued by the coinjection of *Ednra* (Fig. 9F, 85%, $n=59$). Furthermore, the expansion of the neural crest markers by injection of *Msx1-GR* (Fig. 9B) was reversed by coinjection with *EdnraMO* (20 ng/embryo, Fig. 9C; 78%, $n=46$). The

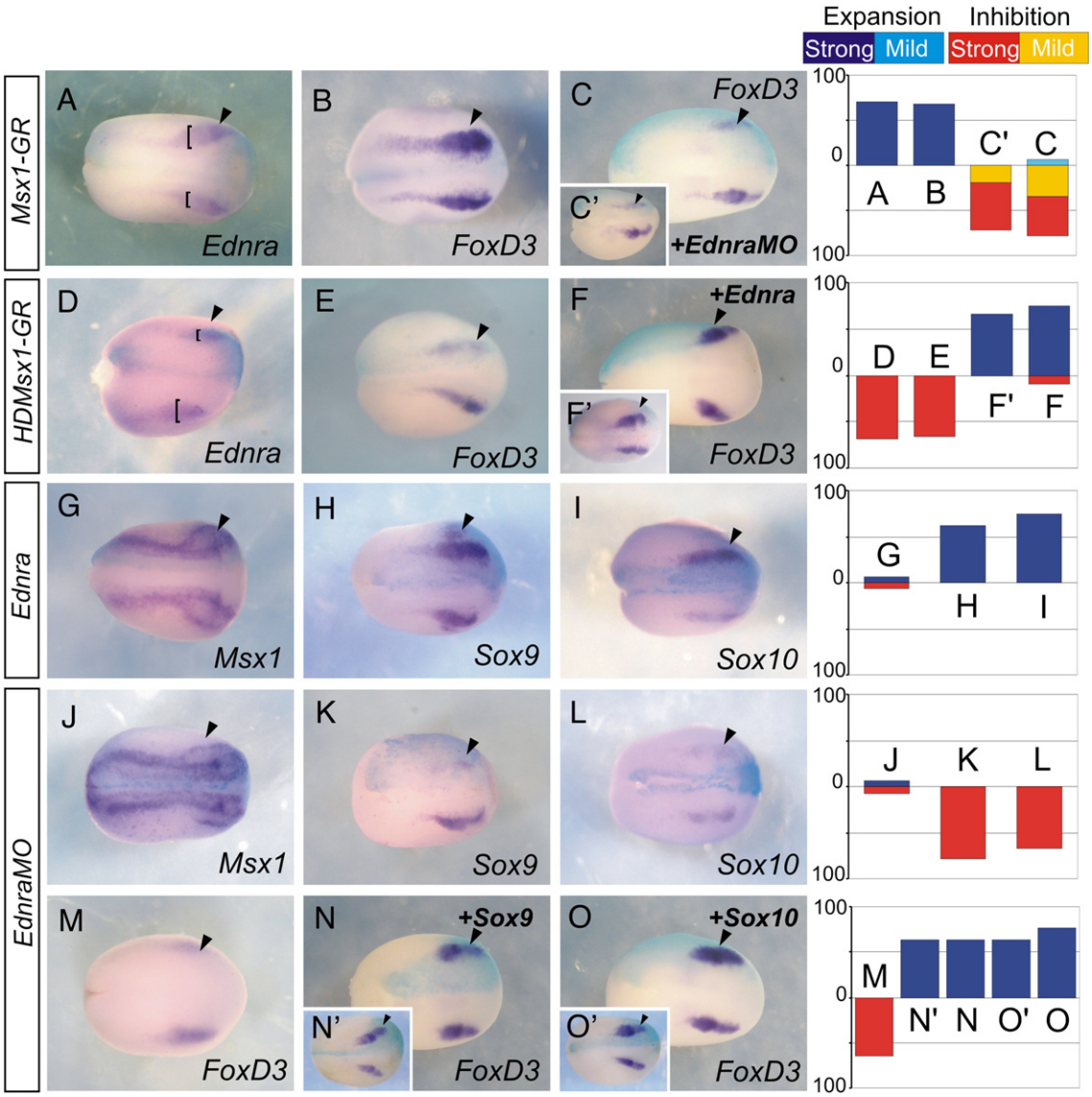


Fig. 9. *Ednra* in the genetic cascade that specifies the neural crest. *Ednra* lies downstream *Msx1* and upstream *Sox9–Sox10*. (A–O) Representative examples of embryos injected with the indicated reagents. The graphs at the right side represent the percentage of embryos with the indicated phenotypes; the letter in each column correspond to the treatment described for each figure in panels A–O. (A, B) Embryos injected with *Msx1-GR* show increased expression of *Ednra* and neural crest marker *FoxD3*. (C) *Msx1-GR* construct failed to increase the expression of *FoxD3* marker when was coinjected with *EdnraMO*. (C') *EdnraMO* leads to a reduction of *FoxD3* expression in the injected side (arrowhead). (D, E) *Msx1*-dominant negative inducible construct (*HDMsx1-GR*) inhibited the expression of *Ednra* and *FoxD3*. (F) The coinjection of dominant negative *HDMsx1-GR* construct and *Ednra* mRNA rescue the expression of *FoxD3* in the neural crest. (F') *Ednra* overexpression leads to an increased *FoxD3* expression in the injected side (arrowhead). (G) No changes in the expression of *Msx1* was observed in embryos microinjected with *Ednra* mRNA. (H, I) *Ednra* mRNA-microinjected embryos show expanded expression of *Sox9* and *Sox10* in the injected side (arrowhead). (J–L) *EdnraMO*-microinjected embryos show no effect on *Msx1* (J), but inhibition of *Sox9* (K) and *Sox10* (L) expression in the injected side (arrowhead). (M–O) *Sox9* mRNA and *Sox10* mRNA rescue *FoxD3* expression in *EdnraMO*-injected embryos. (N') Expansion of *FoxD3* in embryo injected with *Sox9*. (O') Expansion of *FoxD3* in embryo injected with *Sox9*.

expression of *Msx1* was not affected by the overexpression of *Ednra* (Fig. 9G; 94%, $n=44$) or *EdnraMO* (Fig. 9J; 87%, $n=31$). Taken together, these observations indicate that *Msx1* is upstream *Ednra* in the genetic cascade that specifies the neural crest in the ectoderm.

We then analyzed the relationship of *Ednra* with *Sox9* and *Sox10* genes, two genes that have been proposed to be downstream of the early neural crest genes *Msx1* and *Pax3* (Steventon et al., 2005). *Sox9* (Fig. 9H, 62%, $n=40$) and *Sox10* (Fig. 9I, 75%, $n=41$) expressions were increased when *Ednra* mRNA was injected in 4–8-cells stage and were reduced by the injection of *EdnraMO* (Fig. 9K, 79%, $n=61$, and Fig. 9L, 67%, $n=55$, respectively). The effect of *EdnraMO* on *FoxD3* (Fig. 9M) was rescued by the coinjection of *Sox9* (Fig. 9N, 63%, $n=51$) or *Sox10* (Fig. 9O, 77%, $n=46$). Thus, these results suggest that *Sox10* and *Sox9* activities are likely to be downstream Edn1/Ednra activity in specifying the neural crest progenitors.

Edn1/Ednra signaling is required for neural crest migration

Ednra is expressed both in the premigratory and migratory neural crest. This fact suggests Edn1/Ednra signaling might be controlling the migratory process. When Edn1/Ednra signaling was inhibited by *EdnraMO* (Fig. 10B; *Snail2* 55%, $n=50$) a clear inhibition in neural crest migration was observed. However, in order to observe this phenotype we had to analyze only the embryo that had a weak phenotype in neural crest specification, as an effect on neural crest induction could affect neural crest migration. In order to directly test neural crest migration we activated or inhibited Edn1 signaling, with Edn1 or BQ123 soaked bead respectively, at an stage where no effect on neural crest specification is observed (Fig. 4S). When Edn1 or BQ123 were added at stage 18 a clear effect in neural crest migration was observed (Fig. 10E; 61% with more migration, $n=34$; Fig. 10H;

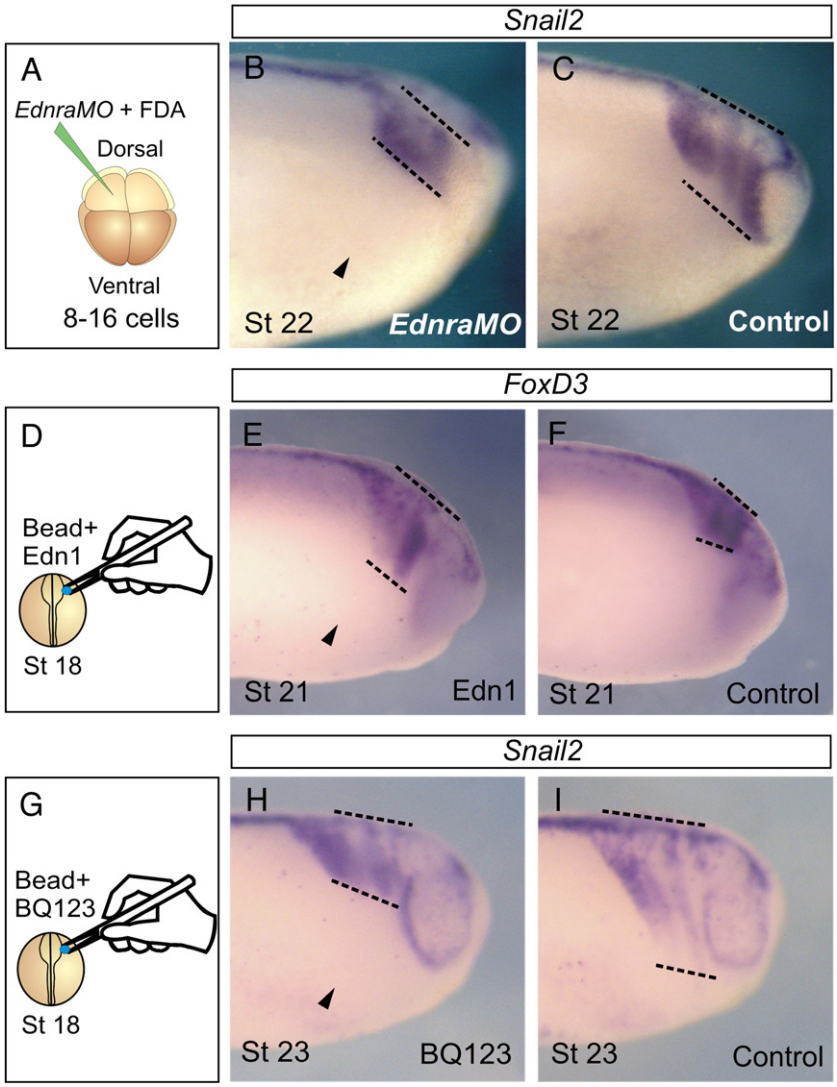


Fig. 10. Edn1/Ednra signaling is required for neural crest migration. (A–C) One dorsal blastomere of a 4–8-cell embryo was injected with 20 ng/embryos *EdnraMO* (A). *EdnraMO*-injected side (B) show arrested neural crest migration. Note *FoxD3*-expressing neural crest cells accumulated lateral to the hindbrain. (D, G) Embryos were grafted before the onset of neural crest migration (St. 18) with Edn1- or BQ123-soaked beads, fixed between St. 21–23, and the expression of *Snail2* or *FoxD3* analyzed. The midline (upper line) and the leading edge of migration are indicated by broken lines. Embryos grafted with Edn1-soaked beads show an increased neural crest migration (E). Embryos grafted with BQ123-soaked bead show inhibition of neural crest migration (H). (C, F, I) Control side showing normal neural crest migration.

69% with less migration, $n=39$). Taken together, these observations indicate that Edn1/Ednra signaling regulates the migration of neural crest cells.

Discussion

The development of neural crest is mediated by complex interactions of multiple signals and transcription factors. In this study, we show that the early induction, migration and maintenance of neural crest specification require the Edn1/Ednra signaling pathway.

We report here the isolation of the *Xenopus laevis Ednra* cDNA. Following the comparison with *Ednra* molecules from different species, this transcript has shown to be different from the Endothelin receptor (ET_{AX}) previously isolated from *Xenopus* heart (Kumar et al., 1994), and *Edrnb* or *Ednrc* (Karne et al., 1993), and probably represents a different isotype or an isoform expressed during embryonic development. In addition, we were able to identify *Xenopus tropicalis Ednra* gene from genomic sequence in order to expand our phylogenetic analysis. Either *Ednra* mRNAs sequences or gene structures for this species and human *Ednra* (Livingston et al., 2004) demonstrated to be highly conserved.

Xenopus laevis Ednra is expressed in the prospective cranial and trunk neural crest from late gastrula stage (St. 12.5). The onset of *Ednra* expression is not as early as other genes expressed in the neural crest like *Snail1* (Aybar et al., 2003; Essex et al., 1993; Linker et al., 2000; Mayor et al., 1993), *Msx1* (Suzuki et al., 1997; Tribulo et al., 2003) or *Pax3* (Bang et al., 1997), and it is occurring simultaneously to *Snail2* (Aybar et al., 2003; Mayor et al., 1995; O'Donnell et al., 2006) and *FoxD3* (O'Donnell et al., 2006; Sasai et al., 2001), and much earlier than *Sox10* (Aoki et al., 2003; Honore et al., 2003). In mouse embryos, *Ednra* is expressed by migrating cranial neural crest cells and by the cephalic neural crest-derived ectomesenchyme of the pharyngeal arches (Clouthier et al., 1998; Yanagisawa et al., 1998a). In chicken embryos, *Ednra* expression appears first in the mesectodermal cells surrounding the arteries of branchial arches (Kempf et al., 1998; Nataf et al., 1998). Recently, *Ednra* was cloned in zebrafish and the in situ hybridization analysis revealed that *Ednra* is first expressed in the neural crest cells during migration (Nair et al., 2007). The analysis of *Edn1* (*suc/et1*) expression pattern and functional studies indicate that Edn1/Ednra cell signaling pathway is involved in the pharyngeal arches formation (Miller et al., 2000, 2003). In conclusion, the early expression of *Ednra*

that we have observed in *Xenopus* neural crest have not been described in other animal models, however we cannot rule out the possibility that low levels of this receptor are expressed in other systems but are hard to detect.

The *Ednra* expression in neural crest progenitors prompted us to examine the role Edn1/Ednra signaling pathway plays in the neural crest specification in *Xenopus* embryos. *Ednra* knockdown using a specific morpholino oligonucleotide or the pharmacological inactivation of Ednra produced depletion of neural crest precursors. On the contrary, the overexpression of *Ednra* or the Edn1-peptide ligand produced an increase in the neural crest cell population. In our experiments, the changes in neural crest territory are accompanied by compensatory expansion or reduction in the neural plate and the epidermal domains. This suggests that *Ednra* activity lead to a fate transformation of cells contiguous to the neural crest that is consistent with the idea of neural crest competence territory (Bastidas et al., 2004).

To further understand the participation of Edn1/Ednra signaling in neural crest development, we examined the temporal requirement of this signaling pathway during neural crest specification. It has been established for *Xenopus* embryos that ectoderm is competent to form neural crest cells from stages 10–12, and that neural crest cells are specified from stage 13, although a loss of *Snail2* signal was reported at stage 17 (Mancilla and Mayor, 1996). Our in vivo results, using Edn1- or BQ123 soaked bead grafted at stage 14, show that the activity of Edn1/Ednra signaling is continuously required and suggest that the specification process is still not finished. A possible explanation to these observations is that neural crest explants dissected at stage 13 (Mancilla and Mayor, 1996) received an initial priming of Edn1 signal enough to maintain some *Snail2* expression. In our study, the use of an Ednra blocking reagent like BQ123 revealed this requirement of Edn1 as a maintenance signal.

It is known that mesodermal tissue from the dorsolateral marginal zone (DLMZ) is required for the induction of *Xenopus* neural crest (Bonstein et al., 1998; Mancilla and Mayor, 1996; Marchant et al., 1998), although the involution of this tissue is dispensable for zebrafish neural crest induction (Ragland, Raible, 2004). Here, we show that lateral mesodermal tissue is required for the maintenance of the neural crest specification and for cell survival during mid-neurula stages, and that it might be the source of Edn1 signal. *Ednra* lies downstream *Msx1* at earlier stages and in the mid-neurula stages is required for cell survival as was demonstrated in vivo and in vitro. The participation of Edn1/Ednra signaling in the control of apoptosis during neural crest development could be interpreted on the basis of its requirement for the expression of *Snail2* and *Sox10*, two genes that function as survival factors in the neural crest (Honore et al., 2003; Tribulo et al., 2004). Probably, a cascade *Edn1–Ednra–Snail2* might function to control cell survival in the neural crest. Alternatively, the activity of *Ednra* might activate a *Snail2*-independent intracellular cascade to repress the apoptotic machinery. However, this aspect of Edn1/Ednra signaling during embryonic development still remains to be unraveled.

The maintenance of neural crest precursors could occur by keeping them from cell death or by maintaining their specification state as well. Our results favor a double role for Edn1/Ednra signaling in controlling both processes during mid- to late neurula stages. We were able to dissociate the effects of inhibition of Edn1/Ednra signaling on apoptosis from cell specification through the over-expresion of a *Snail2*-inducible construct and expression of *XBcl2*. *Snail2* is unable to induce the expression of specification markers during mid-neurula stages, and has demonstrated to act as an anti-apoptotic factor both in vivo as in neural crest explants (Tribulo et al., 2004; this work). When *Snail2GR*- or *XBcl2*-injected neural crest explants were cultured from stage 16 until stage 22 in the absence of mesoderm or when Edn1/Ednra pathway was inhibited, *FoxD3* and *Snail2* markers were lost, and this was not due to a cell death process that eliminated the neural crest cells from the explant. Our findings emphasize the role of the mesoderm and indicate that Edn1/Ednra signaling pathway controls cell survival and maintenance of specification processes independently. However, this does not rule out the possibility that both processes are simultaneously occurring in vivo, or that for neural crest cells is necessary to survive first and then to maintain its specification status. Thus, our results suggest that Edn1/Ednra signaling controls a complex regulatory network that probably coordinates the action of other neural crest maintenance factors such as *Id3* (Light et al., 2005) or *Sox10* (Kim et al., 2003; Paratore et al., 2002).

Ednra is expressed continuously during stages 18–23 by the migrating population of neural crest cells in *Xenopus* embryos. This feature also is evident in mouse embryos, although *Ednra*-null mice do not have defects on cephalic and cardiac neural crest migration (Clouthier et al., 1998, 2003; Yanagisawa et al., 1998a). Chick embryos do not express *Ednra* in the migrating neural crest cells (Kempf et al., 1998; Nataf et al., 1998), and it has been demonstrated that Edn1 exclusively controls postmigratory neural crest development in the zebrafish embryo (Kimmel et al., 2003; Miller and Kimmel, 2001; Miller et al., 2000, 2003). Here we showed that Edn1/Ednra signaling is required for neural crest migration.

The temporal appearance of *Ednra* expression suggests that it could be controlled by genes expressed earlier in the neural crest. As *Msx1* is one of the earliest transcription factors in the genetic cascade of neural crest specification (Monsoro-Burq et al., 2005; Tribulo et al., 2003) we investigated the hierarchical relationship between these two genes. It has been established for the developing branchial arch of mouse embryos that *HAND* genes are mediators of Edn1/Ednra signaling and control the expression of *Msx1* (Thomas et al., 1998) probably by its binding to a selective enhancer that control first branchial arch expression (MacKenzie et al., 1997). Our findings show that the relationship of both genes is reverse during *Xenopus* neural crest initial specification, since the activation of an *Msx1* inducible construct (*Msx1-GR*) at the initial moment of neural crest specification leads to an increase in neural crest markers (Tribulo et al., 2003; this work) and *Ednra* expression. Furthermore, *Ednra* is able to rescue the dominant negative of *Msx1* (*HDMsx1-GR*), and *EdnraMO* blocks the

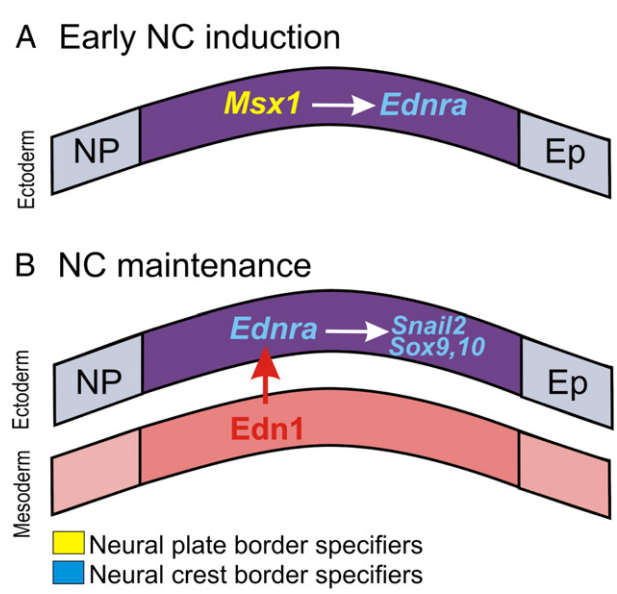


Fig. 11. Model for the early role of Edn1/Ednra signaling in *Xenopus* embryo neural crest specification. (A) During early neural crest induction (stages 12–14, gastrula-early neurula), *Msx1* is expressed in the neural fold and works upstream of *Ednra*; while *Ednra* is upstream of *Snail2*, *Sox9* and *Sox10*. (B) During mid-neurula to late neurula stages, the activity of Edn1/Ednra signaling is required for neural crest maintenance and for cell survival. The intermediate mesoderm is the source of Edn1.

Msx1-induced neural crest expansion. Altogether, these epistatic experiments indicate that *Msx1* lays upstream *Ednra* in the neural crest specification cascade. However, overexpression of *Msx1* do not induce ectopic expression of *Ednra* or general neural crest markers far from the neural fold region (Tribulo et al., 2003), or within the neural plate or the epidermis indicating that additional factors are required for neural crest specification. In addition to show that *Msx1* works upstream of *Ednra*, we also present evidence that indicates that *Sox9* and *Sox10* are downstream of Edn1/Ednra signaling. Taken together the following model of neural crest development can be proposed (Fig. 11). The inductive signals (BMP, Wnts, FGF and RA) transform the ectoderm into neural plate border, which expresses the Neural Plate Border Specifier Genes (e.g. *Msx1;* see Meulemans and Bronner-Fraser, 2004; Steventon et al., 2005). These genes control the expression of the Neural Crest Specifiers (e.g. *Snail2*, *FoxD3*, *Sox9* and *Sox10*), including Ednra. Once the Ednra gene is expressed, the neural crest can respond to Edn1 secreted by the mesoderm underlying the neural crest. The activation of the Edn1/Ednra pathway is required for the specification of the neural crest and for the maintenance of several Neural Crest Specifier Genes.

Acknowledgments

We thank N. Ueno for the EST clones, Y. Sasai, R. Grainger, J.-P. Saint-Jeannet, and T. Sargent for other reagents used in this research. We specially thank N. LeDouarin for discussion and helpful comments. This investigation was supported by grants from ANPCyT-Foncyt (PICT10623), CIUNT-Foncyt (PICTO UNT 367), CONICET (PIP 6278) CIUNT (26/D307) and the Millennium Program (ICM P02-050) to MJA, and by grants from Howard Hughes Medical Institute, MRC, BBSRC and the Millennium Program to RM.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.08.007.

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Supplemental data

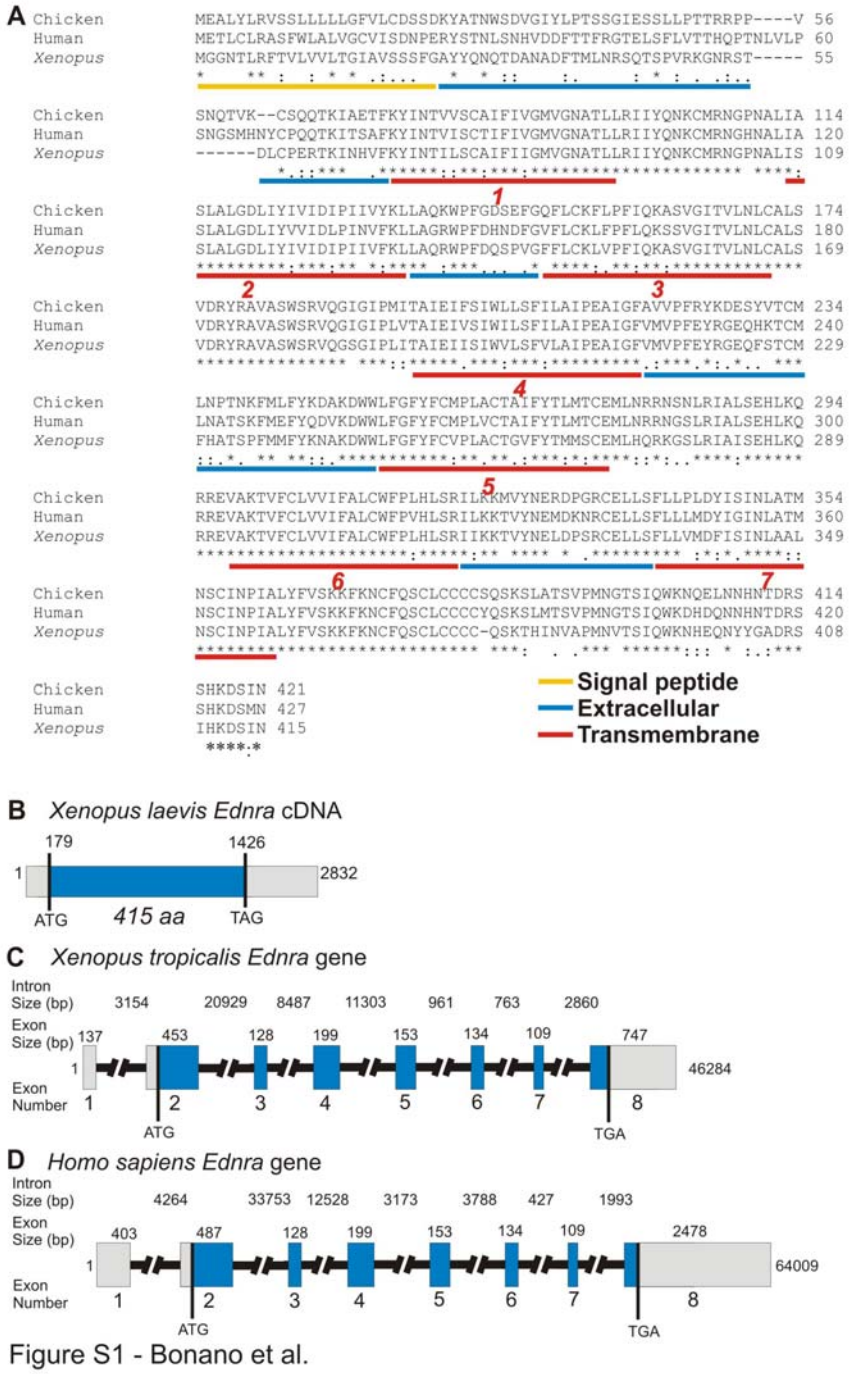

Figure S1 - Bonano et al.

Fig. S1. Alignment of Ednra protein sequences

(A) Amino acid sequences from *Xenopus laevis* (DQ523688), chicken (AF472618) and human *Ednra* (BC022511) were aligned using ClustalW algorithm. Asterisk means identical residues in all aligned sequences, colon means conserved substitutions, and period means semi-conserved substitutions. Signal peptide is underlined in yellow. The topology of Ednra was predicted using SOSUI software. The extracellular domains are underlined in blue, and transmembrane domains are numbered and underlined in red. (B) Schematic diagram of *X. laevis Endothelin-1 receptor type A* cDNA isolated in this work. (C-D) Comparison of genomic structure of *X. tropicalis* (B), zebrafish (C), and human (D) *Ednra* genes. *Xenopus laevis, tropicalis* and zebrafish sequences were obtained or deduced in this work using Wise2 (EBI) and Spidey (NCBI) algorithms; human sequence was obtained from accession AY422989. Exons are depicted schematically as boxes, and introns as lines. The number and size of each exon or intron are indicated. The blue filled boxes represent coding sequences, and the grey portions of the exons identify UTRs.

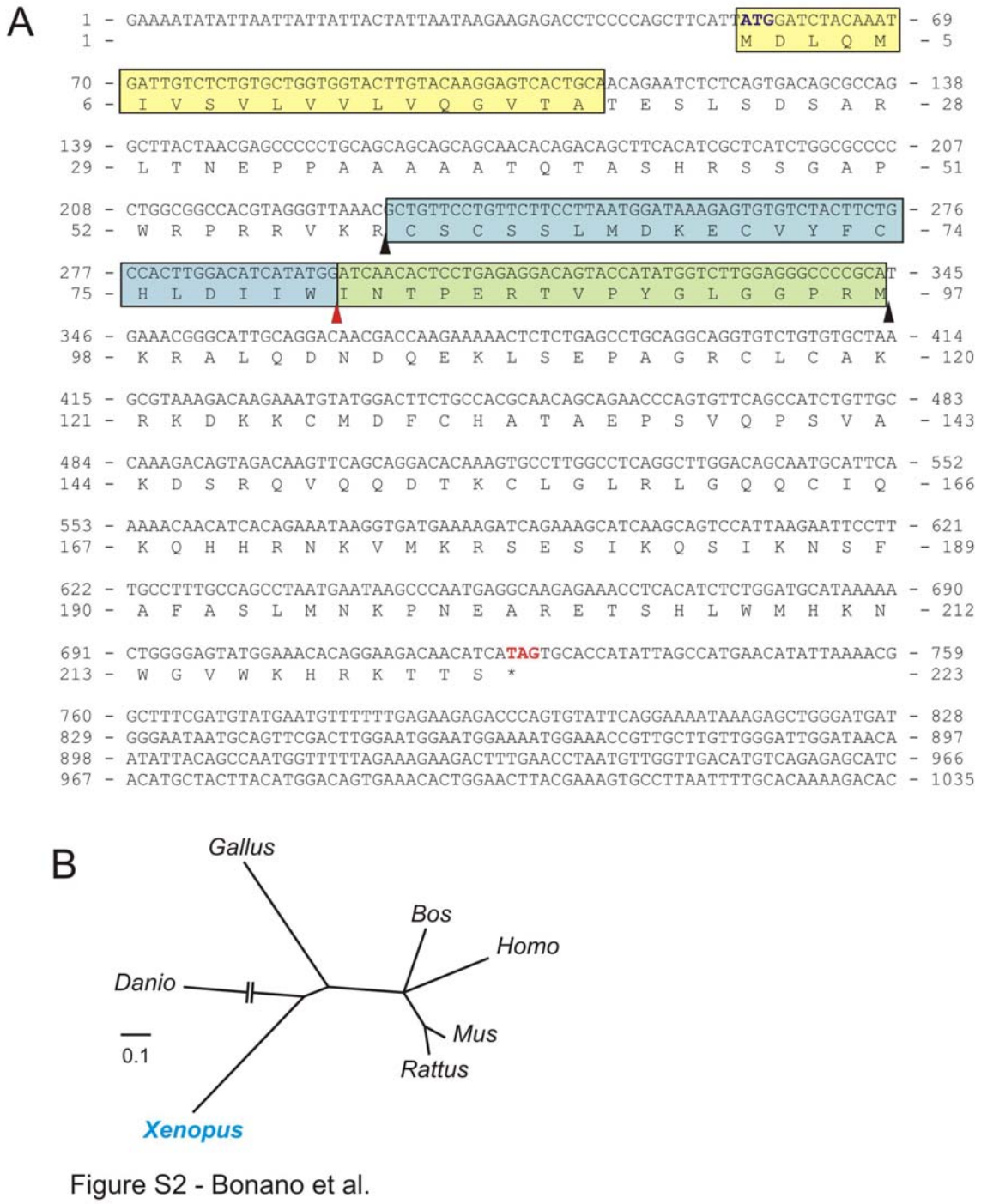

Figure S2 - Bonano et al.

Fig. S2. *Xenopus laevis Preproendothelin-1* cDNA and protein

(A) The *Xenopus laevis Preproendothelin-1 (PPET-1,* Accession EF127993*)* cDNA was isolated by a high stringency homology search in expressed sequence tags (ESTs) databases at the NCBI. The *in silico* screening resulted in the identification of one clone (XL041d02) originated from a neurula- stage library (NIBB, Japan). Start and stop codons are indicated in blue and red, respectively. The protein sequence analysis predicted a putative signal peptide from residues 1-19 (yellow box), and the presence of Big Endothelin-1 peptide from residues 60-97, that includes the active Endothelin-1 peptide (Edn1, light blue box). Putative Furin-like endopeptidase cleavage sites are indicated by black arrowheads and the Endothelin Converting Enzyme-1 (ECE-1) cleavage site by a red arrowhead. The protein sequence of Edn1 active peptide (light blue box) shares 100% identity with human, rat, mice, and bovine Edn1. (B) Molecular phylogenetic analysis of Preproendothelin-1. The protein sequences of different PPET-1 were analyzed using ClustalW 1.81, and an unrooted tree was constructed by neighbour-joining analysis. *Danio* PPET-1 branch is collapsed 50%.

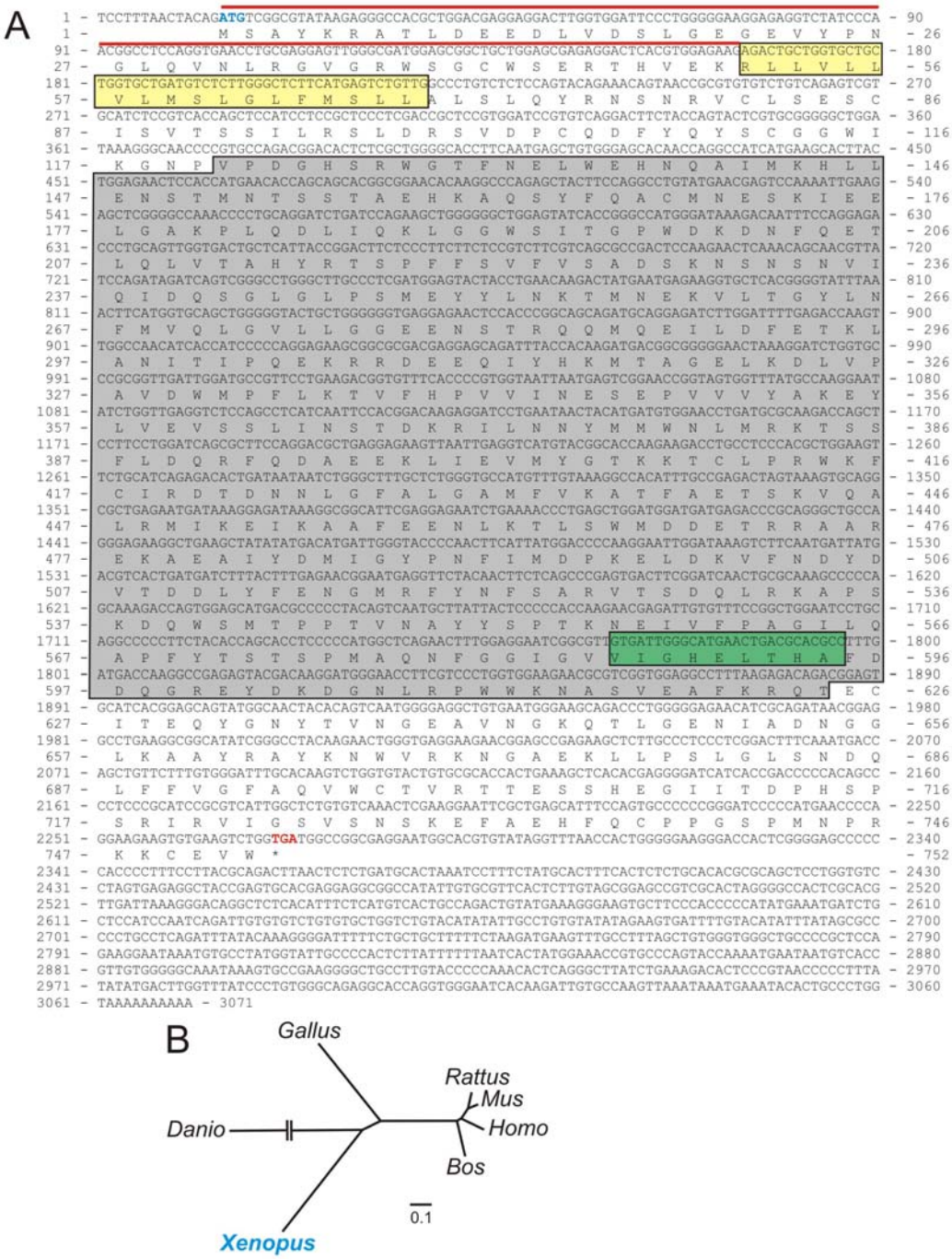

Figure S3 - Bonano et al.

Fig. S3. ***Xenopus laevis Endothelin Converting Enzyme-1*** **cDNA and protein**

(A) The *Xenopus laevis Endothelin Converting Enzyme-1 (ECE-1*, Accession EF127992*)* cDNA was isolated by a high stringency homology search in expressed sequence tags (ESTs) databases at the NCBI. The *in silico* screening resulted in the identification of one clone (XL333c19ex) originated from a stage 10.5 dorsal marginal zone library (NIBB, Japan). Start and stop codons are indicated in blue and red, respectively. The protein sequence analysis using the PHD software predicted a putative transmembrane domain from residues 51-68 (yellow box), the intracellular domain from residues 1-50 (red line) and the extracellular region from residues 69-752. The Prosite algorithm predicted a neutral zinc-binding region signature present in metallopeptidases from residues 586-595 (green box). NCBI software RSPBLAST and CDART identified a conserved Peptidase M13-like domain from residues 121-624 (grey box). (B) Molecular phylogenetic analysis of Endothelin Converting Enzyme-1 (ECE-1). The protein sequences of different ECE-1 were analyzed using ClustalW 1.81, and an unrooted tree was constructed by neighbour-joining analysis. *Danio* ECE-1 branch is collapsed 50%.

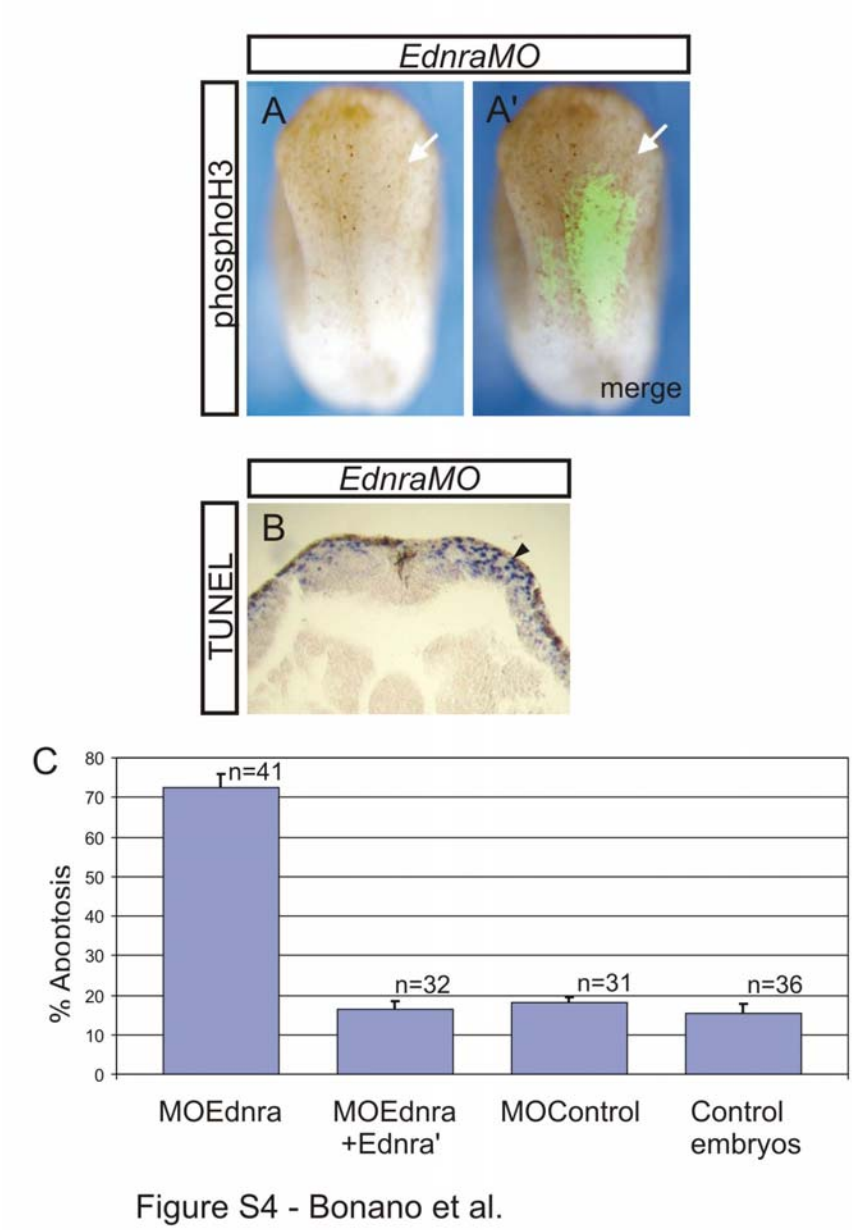

Fig. S4. (A, A') *EdnraMO* injection produces no changes in cell proliferation. The mitotic nuclei were visualized by whole-mount immunostaining using anti- Phospho-Histone H3 antibody. White arrows indicate the injected side. (B) Transverse histological section of a TUNEL stained *EdnraMO*- injected embryo. Arrowhead indicates the injected side. Apoptotic nuclei are found in the superficial tissues and in the deep layer of the ectoderm. (C) The injection of *EndraMO* (20 ng) and *Ednra'* mRNA (1 ng) was able to reduce the apoptosis in the injected side of stage 16 embryos.

Table S1. Exon–intron boundaries of the *Xenopus tropicalis Ednra* gene

Exon	*Genomic coordinates*	*Length*	*3' Splice acceptor site*	*5' Splice donor site*
Exon 1	*1-137*	*137*		..TCATTACATG**gt**atgttaa..
Exon 2	*3565-4017*	*453*	..atgtttt**ag**GGTGATCAAC..	..TGTCTTCAAG**gt**aagacact..
Exon 3	*24946-25073*	*128*	..tctctct**ag**CTGCTTGCCC..	..GTGTGGACAG**gt**aaagactg..
Exon 4	*33560-33758*	*199*	..gctttcc**ag**GTACCGAGCA..	..ATTTATGATG**gt**aggtttat..
Exon 5	*40561-40713*	*153*	..tctttgc**ag**TTCTACAAAG..	..CCTGAAACAG**gt**aaataacg..
Exon 6	*41674-41807*	*134*	..ttactgc**ag**CGCCGTGAAG..	..AGCTCCTCAG**gt**aaggtttc..
Exon 7	*42570-42678*	*109*	..attcttt**ag**CTTTTTGTTG..	..CTGCTTCCAG**gt**aagtagac..
Exon 8	*45538-46284*	*747*	..ttgtttc**ag**TCATGCCTTT..	

Xenopus tropicalis Ednra gene was deposited under accession DQ865481. Prediction and assembling were performed using Wise2 (EBI), and Spidey (NCBI) algorithms. Exons are numbered beginning at the 5'-end of the gene. Exon sequences are in uppercase letters and intron sequences in lowercase letters. Conserved 5'- spliced donors sites (gt) and 3'-spliced acceptor sites (ag) are shown in bold and underlined. The alignment of the assembled *X. tropicalis Ednra* cDNA with the genomic sequence revealed that *X. tropicalis Ednra* has 8 exons distributed over 46284 bp encoding 414 amino acid residues. The introns range in size from 763 bp to 20.9 Kb and the initiator methionine located to nucleotides 3634-3636 of exon 2, while the stop codon located to nucleotides 45673-45675 of exon 8.

Table S2. Ednra sequence identity and similarity

		Identity					
		X. laevis	*X. trop.*	Mouse	Human	Zebrafish	Chicken
Similarity	*X. laevis*		93	79	70	66	71
	X. tropicalis	96		73	70	67	71
	Mouse	89	84		91	73	77
	Human	81	81	96		72	76
	Zebrafish	77	78	84	84		71
	Chicken	81	81	84	83	81	

Sequence identity and similarity for pairwise comparison between six *Ednra* sequences from *Xenopus laevis* (DQ532688), *Xenopus tropicalis* (DQ865481), mouse (BC008277), human (BC022511), zebrafish (DQ917438), and chicken (AF472618). Numbers express percentages. Diagonal shaded boxes indicate sequence comparisons between the same sequences, and therefore represent 100% identity. Sequence comparisons were performed using BLAST algorithm.

Table S3. Preproendothelin-1 sequence identity and similarity

		Identity					
		X. laevis	Human	Rat	Mouse	Chicken	Zebrafish
Similarity	*X. laevis*		41	47	43	44	45
	Human	54		68	69	46	39
	Rat	60	75		91	55	63
	Mouse	57	77	91		71	66
	Chicken	60	60	70	56		54
	Zebrafish	57	58	45	45	36	

Sequence identity and similarity for pairwise comparison between six *Preproendothelin-1* sequences from *Xenopus laevis* (EF127993), human (NM_001955), rat (NM_012548), mouse (NM_010104), chicken (XM_418943.2), and zebrafish (AF281858). Numbers express percentages. Diagonal shaded boxes indicate sequence comparisons between the same sequences, and therefore represent 100% identity. Sequence comparisons were performed using BLAST algorithm.

Table S4. Endothelin Converting Enzyme-1 sequence identity and similarity

		Identity					
		X. laevis	Human	Rat	Mouse	Chicken	Zebrafish
Similarity	*X. laevis*		77	77	77	77	64
	Human	88		95	95	94	64
	Rat	88	98		97	94	63
	Mouse	88	98	99		80	64
	Chicken	86	90	90	90		65
	Zebrafish	82	81	81	81	80	

Sequence identity and similarity for pairwise comparison between six *Endothelin Converting Enzyme-1* sequences from *Xenopus laevis* (EF127992), human (BC126257), rat (NM_053596), mouse (NM_199307), chicken (NM_204717), and zebrafish (XM_689595). Numbers express percentages. Diagonal shaded boxes indicate sequence comparisons between the same sequences, and therefore represent 100% identity. Sequence comparisons were performed using BLAST algorithm.