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The ePress version of this article published on the 30th August 2006 contains a mistake in Fig. 8A,B.

Both the final print and online versions of the article are correct.

We apologise to readers and to the authors for the mistake.

Functional analysis of Sox8 during neural crest development in *Xenopus*

Michael O'Donnell^{*,†}, Chang-Soo Hong^{*}, Xiao Huang[‡], Raymond J. Delnicki[§] and Jean-Pierre Saint-Jeannet[¶]

Among the families of transcription factors expressed at the neural plate border, Sox proteins have been shown to regulate multiple aspects of neural crest development. Sox8, Sox9 and Sox10, exhibit overlapping expression domains in neural crest progenitors, and studies in mouse suggest that Sox8 functions redundantly with Sox9 and Sox10 during neural crest development. Here, we show that in *Xenopus*, Sox8 accumulates at the lateral edges of the neural plate at the mid-gastrula stage; in contrast to its mouse and chick orthologs, Sox8 expression precedes that of Sox9 and Sox10 in neural crest progenitors. Later in development, Sox8 expression persists in migrating cranial crest cells as they populate the pharyngeal arches and in trunk neural crest cells, in a pattern that recapitulates both Sox9 and Sox10 expression domains. Although morpholino-mediated knockdown of Sox8 protein did not prevent the formation of neural crest progenitors, the timing of their induction was severely affected. This delay in neural crest specification had dramatic consequences on the development of multiple lineages of the neural crest. We demonstrate that these defects are due to the inability of neural crest cells to migrate into the periphery, rather than to a deficiency in neural crest progenitors specification and survival. These results indicate that the control of Sox8 expression at the neural plate border is a key process in initiating neural crest formation in *Xenopus*, and highlight species-specific differences in the relative importance of SoxE proteins during neural crest development.

KEY WORDS: Neural crest, Induction, Sox9, Sox10, Craniofacial, Melanocytes, *Xenopus*

INTRODUCTION

Members of the Sox family of transcription factors are important regulators of multiple developmental processes (Wegner, 1999). These proteins are characterized by the presence of a DNA-binding domain known as the HMG box. Based on their homology within and outside this domain, Sox proteins have been classified into 10 groups (A–J) (Bowles et al., 2000). Members of Sox group E include Sox8, Sox9 and Sox10; in the recent years SoxE genes have been extensively studied for their role in chondrogenesis, sex determination, pigment cell differentiation, gliogenesis and neural crest development (reviewed by de Crombrughe et al., 2001; Koopman, 2005; Wegner, 2005; Wegner and Stolt, 2005; Hong and Saint-Jeannet, 2005).

The neural crest constitutes a multipotent population of cells generated at the lateral edge of the neural plate. These cells have the remarkable ability to migrate in the embryo to give rise to a broad range of derivatives including craniofacial cartilage, pigment cells, spinal and enteric ganglia. Among the three SoxE factors expressed in the developing neural crest, the functions of Sox9 and Sox10 are best understood. Genetic studies in mouse (Mori-Akiyama et al., 2003; Akiyama et al., 2004; Herbarth et al., 1998; Southard-Smith et al., 1998; Britsch et al., 2001) and zebrafish (Yan et al., 2002; Yan et al., 2005; Dutton et al., 2001) have demonstrated that Sox9 and Sox10 have non-overlapping function in cranial/cardiac and

trunk/vagal neural crest, respectively. Similarly, gain-of-function and knockdown experiments in chick (Cheung and Briscoe, 2003; McKeown et al., 2005) and *Xenopus* (Spokony et al., 2002; Aoki et al., 2003; Honore et al., 2003; Lee et al., 2004) have established that Sox9 and Sox10 regulate neural crest precursor formation and their subsequent development along distinct lineages (reviewed by Hong and Saint-Jeannet, 2005).

The importance of Sox8 in neural crest development is not as firmly established. Sox8-deficient mouse embryos are primarily characterized by idiopathic weight reduction. These mutants are viable and do not exhibit any neural crest defects (Sock et al., 2001). The lack of a neural crest phenotype in these animals is believed to be due to the functional redundancy of Sox9 and/or Sox10, as the expression of these genes overlaps largely with that of Sox8 in neural crest progenitors and their derivatives (Sock et al., 2001). The functional compensation between SoxE proteins is non-reciprocal, as both Sox9- and Sox10-deficient mice exhibit severe developmental defects, despite continued Sox8 expression. One question is whether the redundant function of SoxE proteins is also a prevailing mechanism in other vertebrates. Here, we describe the expression and function of Sox8 during *Xenopus* neural crest formation. Unlike its chick and mouse counterparts, *Xenopus* Sox8 expression precedes that of Sox9 and Sox10 in the neural crest. The emergence of neural crest progenitors was dramatically delayed in Sox8-deficient embryos, leading to severe defects in multiple lineages of the neural crest. These results indicate that Sox8 functions in initiating neural crest formation in *Xenopus* and underscore differences in the relative importance of SoxE factors across species in the development of this important cell population.

MATERIALS AND METHODS

Isolation of *Xenopus* Sox8 and DNA constructs

Xenopus Sox8 was amplified by PCR from stage 41 cDNA using degenerate primers (F:GNCA[A/G]AA[C/T]AT[A/C/T]GA[C/T]TT and R:[A/G]AA[A/G]TANGG[A/G]TA [C/T]TG[A/G]TA) based on published

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chick (Bell et al., 2000) and mouse (Schepers et al., 2000) Sox8 sequences. The resulting 506 bp PCR product was used to screen a stage 17 lambda ZAPII cDNA library (gift from Michael King) to isolate a full-length clone. The sequence of *Xenopus* Sox8 has been submitted to GenBank (Accession Number, AY324658). Sox8 ORF was subcloned into pCS2+ expression plasmid. A mutated version of Sox8 (mSox8) was generated by PCR. In this construct, four bases (underlined) were mutated 3' to the ATG (bold) (ACCATGTTAAATATGICTTCG), within the recognition motif for Sox8 morpholino oligonucleotide (see below). These mutations did not affect the amino acid composition of *Xenopus* Sox8 protein. The inducible constructs Sox8GR, Sox9GR and Sox10GR were generated by fusing the coding region of each SoxE genes to the human glucocorticoid receptor ligand-binding domain (GR), as described (Gammill and Sive, 1997; Tada et al., 1997). All constructs were sequenced and the corresponding protein monitored using an in vitro transcription/translation coupled rabbit reticulocyte lysate system.

In vitro transcription/translation

The in vitro transcription/translation coupled rabbit reticulocyte lysate system was used according to the manufacturer recommendations (Promega) in the presence of [³⁵S] methionine and resolved on a NuPAGE BIS-Tris gel (Invitrogen). The specificity of the morpholino antisense oligonucleotide (see below) was determined by adding increasing amount of morpholino (10-1000 ng) to the in vitro transcription/translation reaction directed by Sox8, mSox8, Sox9 or Sox10 cDNAs.

Xenopus embryo injections and dexamethasone treatment

Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). Synthetic mRNAs were synthesized in vitro using the Message Machine kit (Ambion). Sox8 morpholino antisense oligonucleotide (Sox8mo, TCATGTTTCAGCATTGAGGAGCCGGG) and a five-base (underlined) mismatched Sox8 morpholino (Sox8mis, TCATCTTGA GCATTCAGGACC CCGG) were purchased from Gene Tools. Sox8 (1 ng), mSox8 (1 ng), Sox8GR (1 ng), Sox9GR (1 ng) and Sox10GR (1 ng) mRNA and morpholinos were injected in the animal pole of two-cell or eight-cell stage embryos. For animal explant experiments, both blastomeres of two-cell stage embryos were injected with SoxE-GR mRNAs in the animal pole region, explants were dissected at the late blastula stage and cultured in vitro for 4 hours in NAM 0.5× plus 10 μM of dexamethasone (Sigma) as described (Gammill and Sive, 1997; Tada et al., 1997). In some experiments, the protein synthesis inhibitor cycloheximide (10 μg/ml; Sigma) was also added to the culture medium (Gammill and Sive, 1997). Animal explants were subsequently analyzed by RT-PCR for the expression of various marker genes (see below). For the rescue experiments using SoxE-GR constructs, embryos were treated with dexamethasone at stage 10.5 and analyzed by in situ hybridization for Slug (stage 14) and Sox10 (stage 16) expression.

Lineage tracing and whole-mount in situ hybridization

Embryos were co-injected with β-gal mRNA or fluorescein lysine dextran (FLDX; *M_r* 10,000, Molecular Probes) to identify the injected side. Embryos at the appropriate stage were fixed in MEMFA and successively processed for Red-Gal (Research Organics) staining and in situ hybridization. Antisense DIG-labeled probes (Genius kit, Roche) were synthesized using template cDNA encoding Slug (Mayor et al., 1995), Snail (Essex et al., 1993), Myc (Bellmeyer et al., 2003), Sox9 (Spokony et al., 2002), Sox10 (Aoki et al., 2003), Pax3 (Bang et al., 1997), Xag1 (Sive et al., 1989), Pdx1 (Lee and Saint-Jeannet, 2003) and Trp2 (Aoki et al., 2003). Whole-mount in situ hybridization was performed as previously described (Harland, 1991). For histology, embryos were fixed in MEMFA and embedded in Paraplast+. Sections (12 μm) were cut on a rotary microtome and stained with Eosin alone or with a combination of Hematoxylin and Eosin.

Cartilage staining

Alcian Blue staining of embryos was performed as described (Berry et al., 1998; Spokony et al., 2002). Briefly, stage 45 embryos were fixed, skinned, eviscerated, dehydrated and stained in Alcian Blue for 12 hours. After several rinses in 95% ethanol, embryos were rehydrated and macerated in 2% potassium hydroxide. Specimens were then transferred successively in

20%, 40%, 60% and 80% glycerol in 2% potassium hydroxide. The ethmoidal plate was dissected out and specimens were flat mounted under a coverslip in 80% glycerol.

Proliferation assay and TUNEL staining

For phosphohistone H3 detection (Saka and Smith, 2001), Sox8mo-injected albinos embryos were fixed in MEMFA. Embryos were incubated successively in α-phosphohistone H3 antibody (Upstate Biotechnology; 1 μg/ml) and anti-rabbit IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch; 1:1000). Alkaline phosphatase activity was revealed using NBT/BCIP (Roche). TUNEL staining was carried as described (Hensy and Gautier, 1998). Sox8mo-injected albinos embryos fixed in MEMFA were rehydrated in PBT and washed in TdT buffer (Roche) for 30 minutes. End labeling was carried out overnight at room temperature in TdT buffer containing 0.5 μM DIG-dUTP and 150 U/ml TdT (Roche). Embryos were then washed for 2 hours at 65°C in PBS/1 mM EDTA. DIG was detected by anti-DIG Fab fragments conjugated to alkaline phosphatase (Roche; 1:2000) and the chromogenic reaction performed using NBT/BCIP (Roche). For proliferation assay and TUNEL staining, FLDX was used as a lineage tracer to identify the injected side.

Western blot analysis

SoxE-GR-injected embryos were collected at stage 17, homogenized, resolved on a NuPAGE BIS-Tris gel and blotted onto nitrocellulose. Blots were subsequently incubated in the presence of the α-GR polyclonal antibody (P-20, Santa Cruz Biotechnology) at a 1:100 dilution, washed and incubated with anti-goat Ig coupled to horseradish peroxidase (Santa Cruz Biotechnology; 1:60,000 dilution). The product of the reaction was revealed using the SuperSignal West Femto Maximum Sensitivity Substrate from Pierce and detected by exposure onto a BioMax film (Kodak). Blots were stripped according to the manufacturer recommendations (Pierce) and probed with anti-α-tubulin antibody (Sigma; 1:500 dilution) as a loading control.

Analysis of gene expression by RT-PCR

For each injected sample, total RNAs from 10 animal explants were extracted using RNeasy micro kit (Qiagen). Real-time RT-PCR (LightCycler, Roche) was performed using specific primer sets: Sox8 (F, AAGGTCTCTGGTGGCTGAAA; R, CACCGCCACATTTTCAGAGTA); Sox9 (Lee et al., 2004); Sox10 (F, CTGTGAACACAGCATGCAAA; R, TGGCCAACCTGACCATGTAAA); and EF1α (Lee et al., 2004). The cycle conditions were as follows: denaturation at 95°C (3 seconds), annealing at 55°C (5 seconds) and extension at 72°C (10 seconds). With the exception of EF1α, all primers were designed outside the coding region. By optimizing primers and reaction conditions, a single specific product was amplified as confirmed by melting curve analysis. Water blank and -RT reactions were also performed as negative controls. To quantify expression levels relative to control, serial dilutions of total RNA extracted from stage 22 embryos were used as concentration standards in each real-time RT-PCR reaction. In each case, EF1α was used as an internal reference (not shown), and for each histogram the values were normalized to the level of EF1α expression. The histograms presented in Fig. 6 are representative of at least three independent experiments.

RESULTS

Cloning of *Xenopus* Sox8

A 506 bp PCR product presenting high homology to chicken and mouse Sox8 was isolated using degenerate primers (see Fig. S1 in the supplementary material) and subsequently used to screen a stage 17 cDNA library. A 3 kb cDNA was recovered with an ORF encoding a 459 amino acid protein (see Fig. S1 in the supplementary material). At the amino acid level, this clone shared 69% identity with human Sox8 (Schepers et al., 2000), 74% identity with mouse Sox8 (Schepers et al., 2000) and 78% identity with chicken Sox8 (Bell et al., 2000). When compared with *Xenopus* Sox9 (Spokony et al., 2002) and Sox10 (Aoki et al., 2003; Honore et al., 2003), the overall amino acid identities dropped to 52% and 48%, respectively.

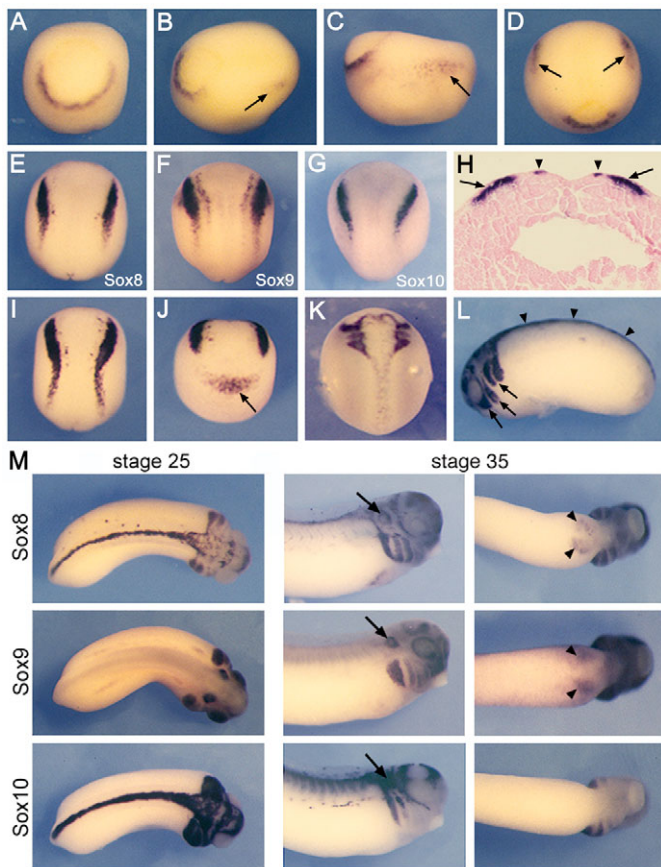


Fig. 1. Sox8 expression in neural crest progenitors and their derivatives. (A) Sox8 is first detected at the gastrula stage in a ventrolateral domain around the blastopore. At stage 11.5 (B–D) Sox8 expression around the blastopore persists and additional expression is detected lateral to the neural plate (arrows). Vegetal (A), lateral (B,C; anterior towards right) and dorsal (D; anterior towards the top) views. Comparison of Sox8 (E) Sox9 (F) and Sox10 (G) expression in sibling stage 14/15 embryos illustrates that all three genes are expressed in the presumptive neural crest. Dorsal views, anterior towards the top. (H) Section of a stage 15 embryo illustrates the expression of Sox8 in both the lateral (arrows) and the medial neural crest (arrowheads). At stage 17 (I,J), Sox8 persists in the neural crest region and is also expressed anterior to the neural plate in the prospective cement gland (arrow). As the neural tube closes (K,L), Sox8 is detected in the migrating neural crest cells in the cranial region (arrows), and the premigratory cells in the trunk neural crest (arrowheads). Dorsal view, anterior towards top (I,K); lateral view, anterior towards the left (L); cranial view (J). (M) Comparison of Sox8, Sox9 and Sox10 expression at the tailbud stages. Dorsal views of stage 25 embryos, anterior towards the right. Sox8, Sox9 and Sox10 are co-expressed in the migrating cranial neural crest. Posteriorly, although Sox8 and Sox10 are both expressed in trunk neural crest cells, Sox9 is downregulated in this cell population. At stage 35 (left panels, lateral views), Sox8 is detected in the cranial neural crest similar to Sox9; however, at this stage, Sox10 starts to be downregulated in the branchial arches. Sox8, Sox9 and Sox10 are co-expressed in the otic vesicle at this stage (arrows). Ventral views (right panels) showing colocalization of Sox8 and Sox9 in the pancreatic rudiments (arrowheads) where Sox10 is not detected.

Sox8 is expressed in neural crest progenitors

To analyze the expression of Sox8, whole-mount in situ hybridization was performed on embryos at different stages. Sox8 transcripts were first detected at the gastrula stage, in a domain

around the blastopore restricted to the ventrolateral side (Fig. 1A). This expression domain persisted after blastopore closure and into the neurula stages (Fig. 1B–D). A second domain of expression appeared at the mid-gastrula stage (stage 11.5), lateral to the prospective neural plate (Fig. 1B,C). At stage 12, this lateral expression domain increased in what will become the neural crest-forming region (Fig. 1D). At the neurula stage (stage 15), the Sox8 neural crest expression domain overlapped with that of Sox9 and Sox10 (Fig. 1E–G), and persists in both the medial and lateral neural crest throughout neurulation (Fig. 1H–J). At these stages, Sox8 was also expressed in a region anterior to the neural plate, presumably corresponding to the prospective cement gland, as confirmed by the expression of the cement gland marker Xag1 (Fig. 1J; data not shown); however, the level of expression of Sox8 in the presumptive cement gland was very variable from one batch of embryos to the next. As development proceeds, neural crest cells initiated their migration in the cranial region and strong expression of Sox8 was detected in streams of neural crest cells migrating towards the branchial arches and into the frontonasal region, as well as in the prospective trunk neural crest at the dorsal midline (Fig. 1K,L). In Fig. 1M, the expression of the three SoxE genes is analyzed and compared in stage 25 and 35 embryos. At stage 25, Sox8 expression persisted in the trunk and in migrating cranial neural crest cells and in discrete domains within the brain. This is the stage when Sox9 is downregulated in the trunk neural crest (Spokony et al., 2002). In contrast to Sox9 and Sox10, Sox8 did not appear to be expressed early on in the presumptive otic placode. Sox8 otic expression was only detected around stage 30 (not shown). At stage 35, although Sox10 expression was downregulated in the pharyngeal arches (Aoki et al., 2003), Sox8 expression was maintained in the neural crest component of the branchial arches in a pattern reminiscent to that of Sox9 (Fig. 1M; middle panels). Sox8 was also detected in the pancreatic rudiment (Fig. 1M; right panels), similar to Sox9 where it is co-expressed with Pdx1 (not shown) (Lee and Saint-Jeannet, 2003).

Fig. 2A, summarizes the onset of expression of Sox8 when compared with a number of well-characterized neural plate border genes (Meulemans and Bronner-Fraser, 2004). The initial expression of Sox8 at the neural plate border coincides with that of Snail at stage 11.5, following Pax3 expression detected as early as stage 11, but preceding Sox9 (Spokony et al., 2002), Myc (Bellmeyer et al., 2003), Slug (Mayor et al., 1995) and Foxd3 (Sasai et al., 2001). Sox10 (Aoki et al., 2003; Honore et al., 2003) expression was first detected only around stage 13.5/14 (Fig. 2B).

We conclude that Sox8 is one of the earliest genes expressed in the prospective neural crest territory. In this tissue, its expression precedes Slug, Foxd3, Sox9, Myc and Sox10. Later, Sox8 expression in neural crest derivatives and appears to recapitulate both Sox9 and Sox10 expression patterns.

Sox8 is required for the formation of neural crest progenitors

To investigate Sox8 function during early neural crest development, we performed knockdown of Sox8 protein in developing embryos using morpholino antisense oligonucleotides. A Sox8 morpholino (Sox8mo) was designed to interfere specifically with translation of Sox8 mRNA. In an in vitro transcription/translation assay (Fig. 3A), Sox8mo blocked Sox8 protein production in a concentration-dependent manner but did not interfere with the production of other SoxE proteins, Sox9 and Sox10 (Fig. 3A). Unilateral injection of Sox8mo (10 ng to 30 ng) in the animal region of two-cell stage embryos resulted in a marked decrease of Sox10 expression at stage

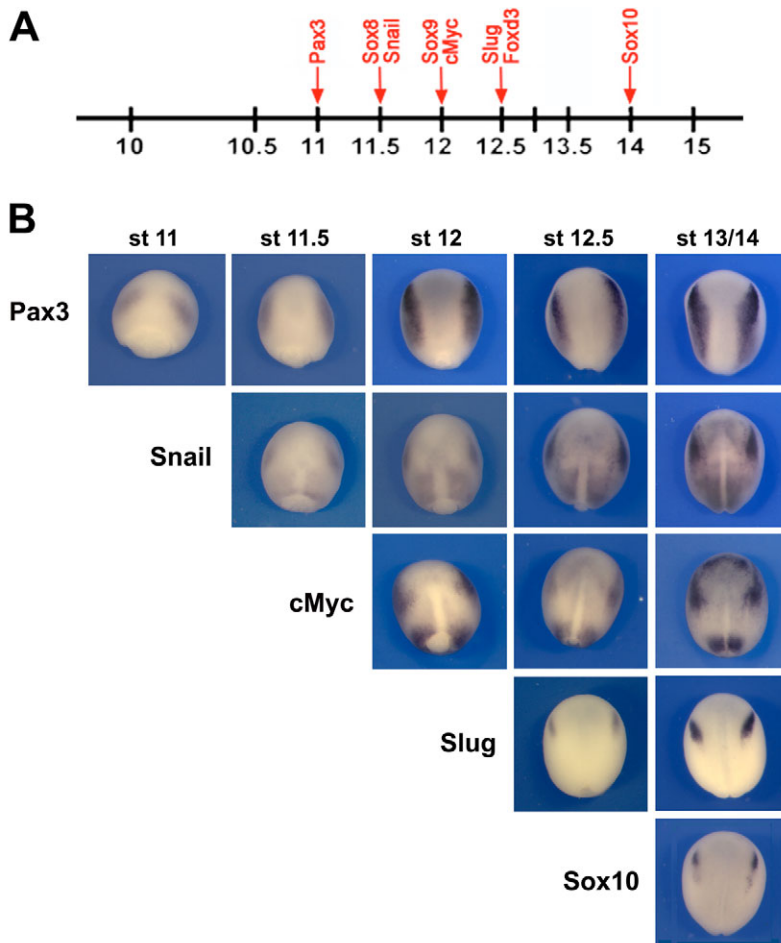


Fig. 2. Comparison of the onset expression of Sox8 with other neural plate border-specific genes.

(A) Summary of the onset of expression of Sox8 and seven other neural plate border-specific genes in *Xenopus*. The developmental stages are according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956).

(B) Developmental expression of Pax3, Snail, Myc, Slug and Sox10 from stage 11 to stage 14 by whole-mount in situ hybridization. Dorsal views, anterior towards the top.

17 in more than 80% of the embryos analyzed (Fig. 3B,C). Injection of a 5 bp mismatched morpholino (Sox8mis) at the same concentrations had no effect on Sox10 expression (Fig. 3E). Interestingly, at stage 17, the proportion of embryos with reduced Sox9 and Slug expression was much lower (40%), even for the highest dose of morpholino (Fig. 3B,C). The neural plate marker Sox2 was also only marginally expanded in 35% ($n=79$) of the embryos that received the higher dose (20 ng to 30 ng) of Sox8mo (Fig. 3B). At this stage, the overall anteroposterior patterning of these embryos was not affected, as determined by the expression of forebrain (Otx2), hindbrain (Krox20) and spinal cord (HoxB9) marker genes (not shown).

To further assess specificity, we next asked whether the phenotype of Sox8-depleted embryos could be rescued by restoring Sox8 expression. In these experiments, we injected a Sox8 mRNA (mSox8) derived from a construct carrying a 4 bp mutation within the recognition motif for Sox8mo. In an in vitro transcription/translation assay, Sox8mo failed to block translation directed by the mSox8 construct (Fig. 3D). Injection of mSox8 mRNA led to a lateral expansion of Sox10 expression domain (Fig. 3E), similar to that observed with wild-type Sox8 mRNA injections (not shown). Co-injection of mSox8 mRNA and Sox8mo in one animal dorsal blastomere at the eight-cell stage restored bilateral Sox10 expression in a large number of embryos when compared with siblings that received injection of Sox8mo alone (Fig. 3E,F).

Because Slug and Sox9 are only marginally affected in Sox8-depleted embryos when compared with Sox10, we decided to investigate whether this difference could be due to the fact that

Sox10 expression at the neural plate border is initiated several hours after Slug and Sox9, around stage 14 (Aoki et al., 2003; Honore et al., 2003) (Fig. 2A). To achieve this, we analyzed the expression of Slug and Sox9 at early time points after Sox8mo injection. We found that the onset of expression of both Slug and Sox9 was affected in a large number of Sox8mo-injected embryos analyzed at stage 12.5 or stage 14 (Fig. 4A,B), and at a similar frequency to what was observed for Sox10 at stage 17 (Fig. 4B). These observations suggested that Sox8 regulates the onset of expression of most neural crest marker genes; however, Sox8 does not appear to be required for the maintenance of the expression of these genes.

SoxE factors function redundantly at the neural plate border

To determine whether SoxE factors can function redundantly during neural crest formation in *Xenopus*, we compared the ability of Sox8, Sox9 and Sox10 to rescue the phenotype of Sox8-depleted embryos. As described earlier (Fig. 3D), injection of mSox8 mRNA was efficient at restoring Sox10 expression at stage 17 (70%, $n=50$; Fig. 5A), but was also able to restore normal levels of Slug expression at stage 14 (57%, $n=52$; Fig. 5A). Expression of Sox9 at the gastrula stage using an inducible construct (Sox9GR) was also able to rescue Slug and Sox10 expression in a large proportion of Sox8-depleted embryos (80.5%, $n=53$ and 100%, $n=66$, respectively; Fig. 5A). An inducible Sox10 (Sox10GR) shared the same ability as Sox8 and Sox9 to rescue Sox10 expression in Sox8-deficient embryos (100% rescued, $n=41$; Fig. 5A); however, Sox10GR was somewhat less

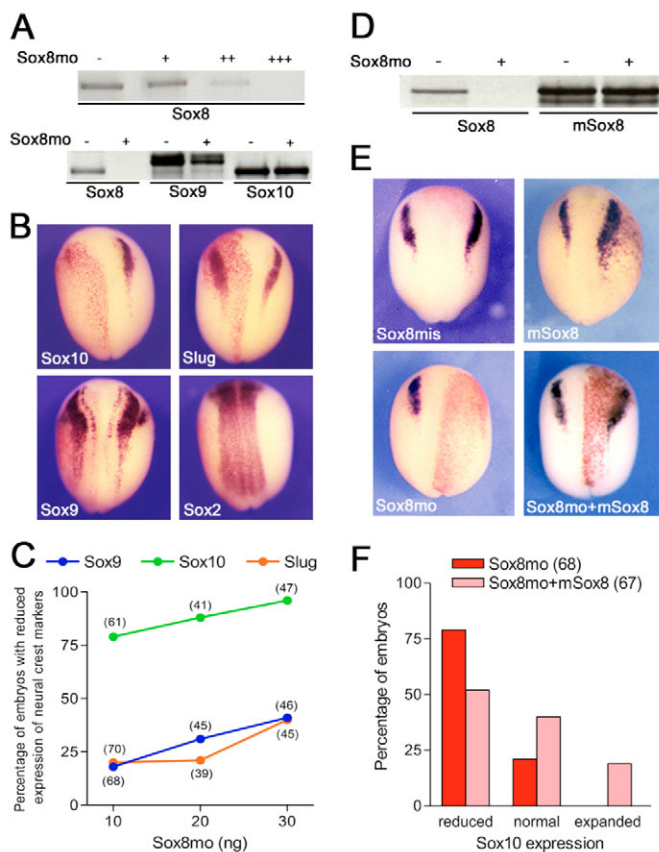


Fig. 3. Sox8-depleted embryos fail to express Sox10 at the neural plate border. (A) Increasing amounts of Sox8mo (10 ng, 100 ng and 1000 ng) blocks translation directed by Sox8 mRNA. The same morpholino (500 ng) fails to block Sox9 and Sox10 translation. (B) Embryos injected in one blastomere at the two-cell stage with 30 ng of Sox8mo exhibit reduced Sox10 expression at stage 17, while Slug, Sox9 and Sox2 expression appears largely unaffected at this stage. (C) Quantification of the in situ hybridization results. The numbers in parenthesis indicate the number of embryos analyzed. (D) Sox8mo (500 ng) does not interfere with translation of a mutated Sox8 mRNA (mSox8). (E) Rescue experiments were performed by injection of an animal dorsal blastomere at the eight-cell stage. Bilateral Sox10 expression is rescued in Sox8mo-injected embryo by co-injection of mSox8 mRNA (Sox8mo+mSox8). Single injection of mSox8 expanded the Sox10 expression domain. Injection of a 5 bp mis-matched Sox8 morpholino (Sox8mis) had no effect on Sox10 expression. (F) Quantification of the in situ hybridization results. The numbers in parentheses indicate the number of embryos analyzed. (B,E) Dorsal view, anterior is towards the top. RNA encoding the lineage tracer β -galactosidase was co-injected to identify the injected side (red staining) (left side in B and right side in E).

potent than the other two SoxE genes at rescuing Slug expression at stage 14 (16% rescued, $n=39$; Fig. 5A). Interestingly, in these experiments, although Slug expression was primarily restored within its normal domain, Sox10 rescue was always associated with ectopic expression domains lateral to the neural crest region (Fig. 5A). This more potent activation of Sox10 may suggest a direct regulation of Sox10 by all three SoxE genes. Importantly, in these experiments the SoxE-GR constructs produced similar level of proteins when determined by western blot analysis (Fig. 5B). These results indicate that the activity of all three Sox genes is largely interchangeable in this assay and suggest that SoxE factors may function redundantly at the neural plate border.

Sox8 and Sox9 directly regulates Sox10 expression

In an attempt to determine the relationship between Sox8, Sox9 and Sox10 during neural crest formation, embryos at the two-cell stage were injected in the animal pole region with inducible SoxE constructs (SoxE-GR). Animal explants were dissected at the blastula stage and cultured in vitro for 4 hours in the presence of dexamethasone and analyzed by real-time RT-PCR. Although Sox8 and Sox9 expression levels were not significantly modified upon Sox8GR, Sox9GR or Sox10GR injection, strong induction of Sox10 was observed these explants 4 hours after addition of dexamethasone (Fig. 6A). Importantly, Sox8- and Sox9- and Sox10-mediated

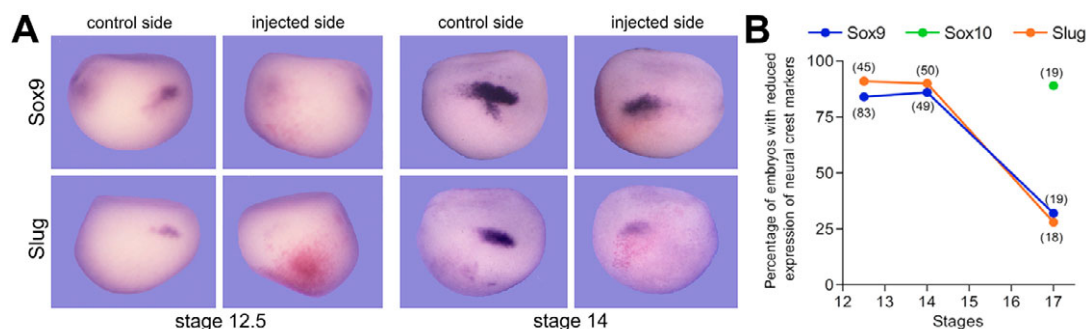


Fig. 4. Sox8 regulates the onset of expression of Slug and Sox9. (A) Embryos injected in one blastomere at the two-cell stage with 30 ng of Sox8mo exhibited reduced Sox9 and Slug expression at stage 12.5 and stage 14. Lateral view in all panels, anterior is towards the right (control side) or to the left (injected side). (B) Quantification of the in situ hybridization results. The numbers in parentheses indicate the number of embryos analyzed.

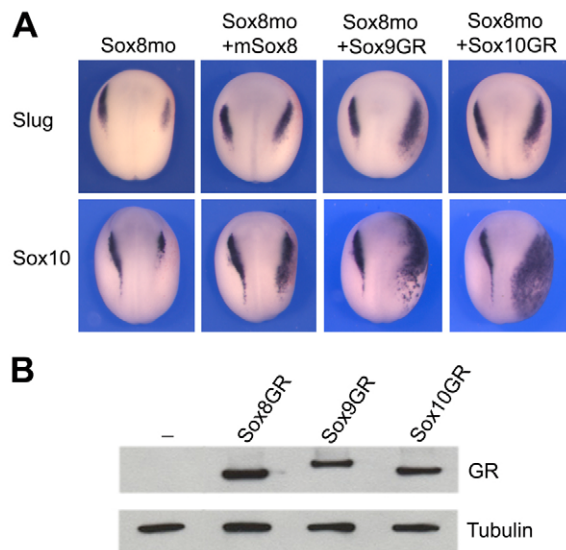


Fig. 5. SoxE factors function redundantly at the neural plate border. (A) Sox8, Sox9 or Sox10 can equally rescue Slug expression levels at stage 14 and expand Sox10 expression domain in Sox8-depleted embryos. In these experiments, Sox10 expression was evaluated using a probe against Sox10 3' UTR. (B) Western blot analysis. Detection of SoxE-GR proteins in extracts from injected embryos collected at stage 17 after injection at the two-cell stage. The fusion proteins are expressed at similar levels, as revealed with α -GR antibody. -, uninjected control embryo; α -tubulin is presented as a loading control.

induction of Sox10 was either unaffected (Sox8 and Sox10) or partially inhibited (Sox9) by the presence of the protein synthesis inhibitor cycloheximide (Fig. 6B). These results indicate that Sox8 and Sox9 can independently activate Sox10 expression directly in animal explants, providing evidence that Sox8 and Sox9 are acting upstream of Sox10 in the hierarchy of genes involved in promoting neural crest formation.

Sox8 depletion results in a severe and broad loss of neural crest derivatives

What are the consequences of the loss of Sox8 on further neural crest development? To analyze the late phenotype of Sox8-depleted embryos, Sox8mo was injected in one dorsal animal blastomere at the eight-cell stage and embryos analyzed by gross morphology at stage 30. All embryos that received injection of Sox8mo in the cranial region failed to develop well defined pharyngeal arches (Fig. 7A). The phenotype of these embryos was further analyzed by documenting the development of crest-derived skeletal elements at stage 45 (Sadaghiani and Thiebaud, 1987; Spokony et al., 2002). Alcian Blue staining revealed that all affected embryos (67% of the embryos; $n=70$) presented a severe loss or reduction of craniofacial skeletal elements (Fig. 7B). We also wished to determine whether other neural crest derivatives were affected in these embryos. Pigment cells are one of the derivatives of the trunk neural crest, and around 65% of the embryos injected with Sox8mo showed reduced Trp2-expressing cells ($n=60$) on the injected side (Fig. 7C). Another trunk neural crest derivative, the dorsal root ganglia, were undetectable in stage 42 Sox8mo-injected embryos (Fig. 7D). Several days later, at equivalent stage 47, these embryos showed reduced dorsal root ganglia when compared with sibling embryos that received

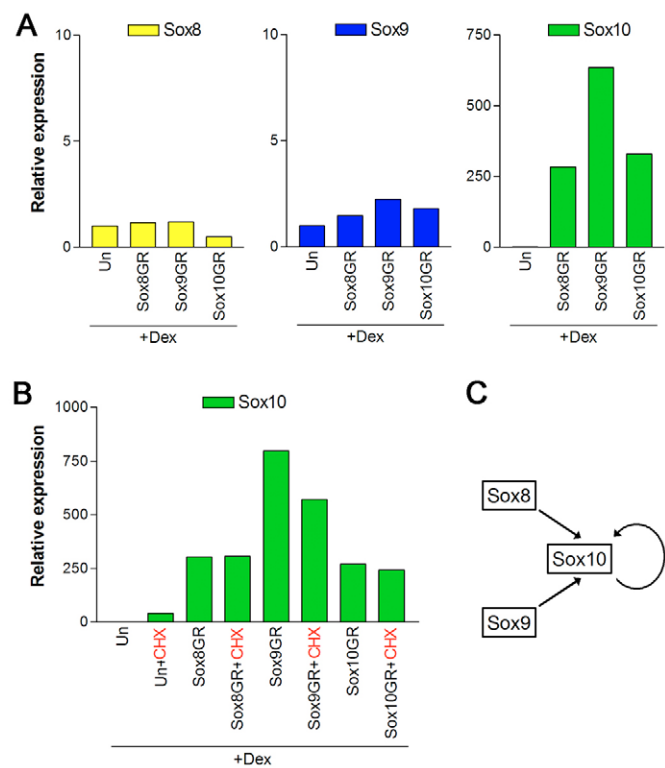


Fig. 6. Sox8 and Sox9 regulate Sox10 expression in animal explants. (A) Real-time RT-PCR of animal explants isolated from embryos injected with Sox8GR, Sox9GR or Sox10GR. The histograms indicate the relative expression levels of Sox8 (yellow), Sox9 (blue) and Sox10 (green) in animal explants collected 4 hours after dexamethasone treatment (+Dex). (B) In animal explants, Sox8GR, Sox9GR and Sox10GR strongly induce Sox10 expression after 4 hours of dexamethasone treatment (+Dex). The induction of Sox10 by Sox8GR, Sox9GR or Sox10GR occurs independently of protein synthesis (+CHX), indicating that not only can Sox10 regulate its own expression but that Sox8 and Sox9 can directly activate Sox10. (C) Hierarchy of SoxE genes activation in the developing neural crest.

injection of Sox8mis oligonucleotides (Fig. 7D). These results indicate that Sox8-deficient embryos have a broad range of defects in multiple lineages of the neural crest.

Sox8-depleted embryos have impaired neural crest cells migration

The late neural crest phenotype observed in Sox8-depleted embryos was somewhat surprising, as Sox9 and Slug expression levels appeared fairly normal at stage 17 in these embryos. To better understand the basis of these defects, we analyzed the patterns of cell division and cell death in Sox8-depleted embryos. Using an α -phosphohistone H3 antibody, no significant difference was observed in the numbers of dividing cells in regions of the neural folds that received Sox8mo when compared with the uninjected side (Fig. 8A). Similarly, no significant increase in TUNEL staining was observed in Sox8-depleted embryos at early neurula stages (Fig. 8B). The lack of an apparent effect of Sox8 depletion either on cell proliferation or on apoptosis suggested that the phenotype of Sox8-depleted embryos cannot be explained by an initial reduction in the neural crest progenitor pool or by a decrease in cell survival.

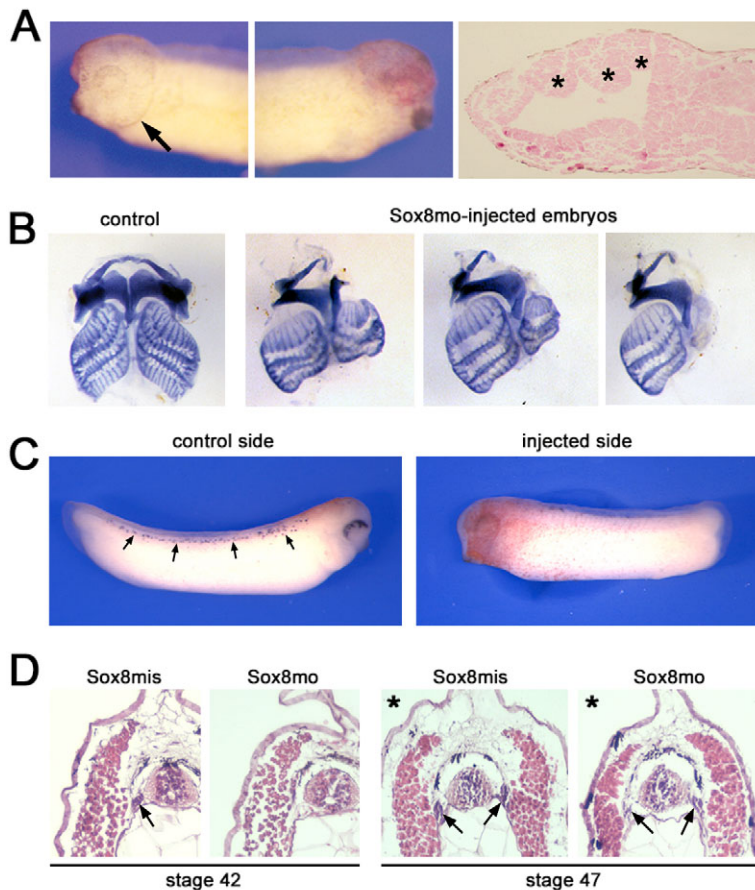


Fig. 7. Sox8-depletion leads to a broad range of defects in neural crest derivatives. (A) Embryos were co-injected in one blastomere on the dorsal side at the eight-cell stage with 20-30ng of Sox8mo and RNA encoding the lineage tracer β -galactosidase. Well-defined pharyngeal arches are absent on the injected side when compared with the control side (arrow). Longitudinal section of the embryo shown on the left panel; the asterisks indicate individual pharyngeal arches on the control side. (B) Flat-mount Alcian Blue-stained skeletal preparations from control and Sox8mo-injected embryos at stage 45. Injected side is on the right. Sox8mo-injected embryos present different levels of skeletal defects. (C) A reduced number of Trp2-positive cells is observed in embryos that received 30 ng of Sox8mo in one blastomere at the two-cell stage. The control side shows a normal pattern of Trp2-expressing cells (arrows) at stage 33. Embryo is viewed from the lateral side, anterior towards the right (left panel) or anterior towards the left (right panel). (D) Histological analysis of Sox8mo- and Sox8mis-injected embryos at stage 42 and stage 47. In each panel, an arrow indicates the position of the dorsal root ganglia. Sox8-depleted embryos (Sox8mo) have no or reduced dorsal root ganglia on the injected side when compared with sibling to Sox8mis-injected embryos. For stage 42 embryos, only the injected side is shown. In sections of stage 47 embryos, the injected side is indicated by an asterisk.

To further investigate the mechanism by which Sox8 regulates neural crest development, we analyzed the pattern of neural crest cells migration in the cranial region of Sox8-depleted embryos. Sox9 and Sox10 are both expressed in the migrating cranial neural crest cells around stage 25, and this pattern of migration was severely disrupted in Sox8mo-injected embryos (Fig. 8C). Cranial neural crest cells failed to migrate into the pharyngeal arches of Sox8mo-injected embryos and accumulated lateral to the hindbrain (Fig. 8C,D). Although at this stage embryos showed no obvious increase in cell death in the vicinity of the hindbrain, as determined by TUNEL staining (Fig. 8E), it is likely that later in development these non-migrating cells will fail to differentiate and become apoptotic. These results suggest that the timing of induction of neural crest progenitors is crucial for their subsequent migration and that any delay in this process is detrimental to their proper migration and further differentiation.

DISCUSSION

Neural crest formation is a multistep process that starts with the induction of neural crest progenitors at the neural plate border in response to signals derived from surrounding tissues (reviewed by Knecht and Bronner-Fraser, 2002; Huang and Saint-Jeannet, 2004). One of the immediate consequences of the induction of neural crest cells is the activation of a number of crest-specific genes that define the identity of these cells (reviewed by Gammill and Bronner-Fraser, 2003; Heeg-Truesdell and LaBonne, 2004; Huang and Saint-Jeannet, 2004). Among those genes, the Sox family of transcription factors has emerged as key regulator of this cell type. Work in different species has demonstrated the fundamental role of SoxE

factors (Sox8, Sox9 and Sox10) in the control of processes as diverse as specification, maintenance, delamination, migration and differentiation of neural crest cells (reviewed by Hong and Saint-Jeannet, 2005).

Although expression of all three SoxE genes is detected in neural crest progenitors at some point following neural crest induction, there are some differences in the onset and the sequence of their appearance across species. For example, in *Xenopus*, Sox8 is the first Sox family member detected in the presumptive neural crest immediately followed by Sox9 (Spokony et al., 2002) and Sox10 (Aoki et al., 2003; Honore et al., 2003). In chick and mouse embryos, Sox9 and Sox10 precede Sox8 expression in neural crest progenitors (Bell et al., 2000; Cheung and Briscoe, 2003; Cheng et al., 2000; Sock et al., 2001; Zhao et al., 1997; Kuhlbrodt et al., 1998). In zebrafish, Sox8 is never expressed in the developing neural crest (Yan et al., 2005), while the two orthologs of the tetrapod Sox9, Sox9a and Sox9b, are detected in neural crest progenitors (Chiang et al., 2001; Li et al., 2002; Yan et al., 2005) prior to Sox10 (Dutton et al., 2001). These differences in the timing of appearance of these factors may have important implications on the relative contribution of these proteins to various aspects of neural crest development in each species.

Another remarkable characteristic of Sox8 expression is its strong overlap with Sox9 and/or Sox10 in neural crest derivatives. In *Xenopus*, Sox9 and Sox10 are initially co-expressed at the neural plate border and later have a complementary expression pattern in the developing neural crest: while Sox9 expression is maintained in migrating cranial neural crest cells, Sox10 expression persists primarily in trunk neural crest cells (Spokony et al., 2002; Aoki et al.,

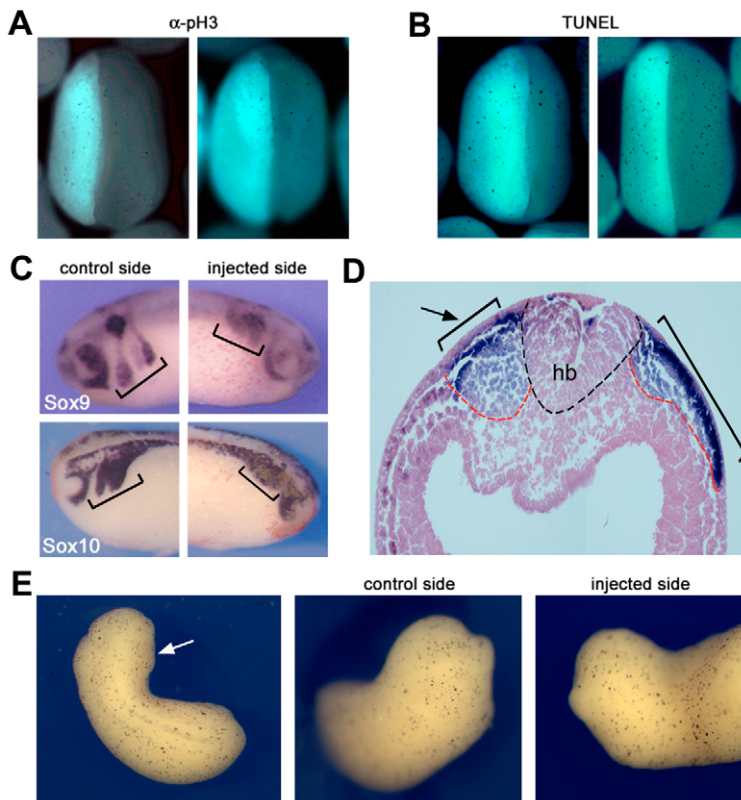


Fig. 8. Sox8mo-injected embryos have defects in neural crest migration. (A) Phosphohistone H3 immunostaining (α -pH3) shows no difference in the pattern of cell proliferation in stage 17 embryos that received unilateral injection of Sox8mo (left side; FITC label) when compared with the uninjected side. (B) TUNEL staining shows no difference in the pattern of cell death in stage 17 embryos that received unilateral injection of Sox8mo (left side; FITC label) when compared with the uninjected side. In A,B, embryos are viewed from the dorsal side, anterior towards the top. (C) The migration of cranial neural crest cells into the pharyngeal arches visualized by Sox9 and Sox10 expression is severely perturbed in Sox8-depleted embryos (brackets). These cells appear to accumulate lateral to the hindbrain. RNA encoding the lineage tracer β -galactosidase was co-injected to identify the injected side (red staining). Embryos are viewed from the lateral side, anterior towards the left (left panels) or anterior towards the right (right panels). (D) Tissue section of Sox8mo-injected embryos showing accumulation of Sox10-positive cells (small bracket; outlined in red) lateral to the hindbrain on the injected side (arrow); on the control side, Sox10-positive cells have initiated their migration (large bracket; outlined in red). The black outline indicates the position of the hindbrain (hb). (E) TUNEL staining of a stage 25 embryo that received unilateral injection of Sox8mo (left panel; dorsal view, anterior to top). The injected side (arrow) is characterized by reduced pharyngeal arches. Higher power views of the cranial regions on the injected side show no significant increase in TUNEL-positive cells (right panel; lateral view, anterior towards left, dorsal towards top) when compared with the uninjected side (middle panel; lateral view, anterior towards the right, dorsal towards the top).

2003; Honore et al., 2003). Interestingly, Sox8 appears to be the sum of Sox9 and Sox10 expression, as it is expressed in both premigratory trunk and migratory cranial neural crest. This is also the case in the mouse embryo, where Sox8 is co-expressed with Sox10 in the enteric nervous system (Kuhlbrodt et al., 1998; Sock et al., 2001) and with Sox9 in the pharyngeal arches (Wright et al., 1995; Sock et al., 2001). Because of its extensive overlap with Sox9 or Sox10, or both, compensatory mechanisms between Sox8 and Sox9/Sox10 are expected to take place during development of the neural crest.

Sox8-null mice are primarily characterized by weight reduction but do not exhibit a strong neural crest phenotype, suggesting that other SoxE proteins expressed in the neural crest are compensating for the loss of Sox8 (Sock et al., 2001). Interestingly, although inactivation of one Sox10 allele is sufficient to elicit agangliogenesis of the gut (Southard-Smith et al., 1998; Herbarth et al., 1998), homozygous Sox8 mutant mouse embryos develop normal enteric ganglia (Sock et al., 2001). Similarly, in the central nervous system, where Sox8 and Sox10 are both expressed in the oligodendrocyte lineage, a milder impact on oligodendrocyte development was observed in Sox8-deficient embryos when compared with Sox10 heterozygous animals (Stolt et al., 2004). In both cases, it has been proposed that the unilateral compensatory activity of Sox8 by Sox10 was likely to be due to unequal amounts of Sox8 and Sox10 proteins in the developing embryo (Stolt et al., 2004; Maka et al., 2005). It is believed that Sox8 acts as a modifier gene as loss of Sox8 alleles increases the penetrance and severity of the gut phenotype in Sox10 heterozygous mice (Maka et al., 2005). Double mutant analyses also indicate a cooperation between Sox8 and Sox9 in testis differentiation in the mouse (Chaboissier et al., 2004).

In *Xenopus*, the observation that Sox8-depletion leads to a delay in the emergence of neural crest progenitors suggests that Sox8 is required to initiate the specification of neural crest cells, consistent

with the timing of expression of this gene at the neural plate border (Fig. 2A). However, as neural plate border cells eventually express neural crest-specific genes such as Slug and Sox9, it is clear that Sox8 is dispensable for the maintenance of this cell population. Based on the mouse work, it is likely that Sox9 and/or Sox10 are also compensating for the loss of Sox8 in *Xenopus*. This is supported by recent results suggesting that Sox9 and Sox10 in *Xenopus* share similar properties during neural crest development (Taylor and LaBonne, 2005). Additionally, overexpression studies in the chick embryo also point to a functional equivalence of SoxE factors, based on their ability to promote ectopic neural crest formation in the neural tube (Cheung and Briscoe, 2003). In an attempt to address this redundancy issue directly, we analyzed the ability of individual SoxE factors to rescue the phenotype of Sox8-depleted embryos. We found that all three factors were as efficient at rescuing the early phenotype of Sox8mo-injected embryos, further arguing for a redundant activity of these factors at the neural plate border. Likewise, in animal explants, we found that qualitatively Sox8, Sox9 and Sox10 had a comparable ability to upregulate Sox10 expression. Recently, using a gene replacement approach, it has been shown that Sox8 can only partially rescue the neural crest phenotype of Sox10-deficient mouse embryos, arguing for an incomplete functional equivalence of Sox8 and Sox10 (Kellerer et al., 2006). These differences suggest that the extent of compensatory activity of SoxE factors is likely to be context dependent.

Because, in *Xenopus*, Sox8 is expressed earlier than Sox9 and Sox10, there is a short window of time (between stage 11.5 and stage 12) during which no other SoxE factor is expressed to compensate for the loss of Sox8; this period appears to be crucial for the timely specification of neural crest progenitors. Our results indicate that a delay in the induction of neural crest progenitors is never fully compensated for in Sox8-depleted embryos. Although these

embryos express what appears to be normal level of Slug and Sox9 at the late neurula stages, they also exhibit major defects in both cranial and trunk neural crest derivatives. The level of resolution of in situ hybridization does not allow us to determine whether the overall number of neural crest progenitors was decreased in morpholino-treated embryos. However, using an α -phosphohistone H3 antibody, no significant difference was noted in the numbers of dividing cells in regions of the neural folds that did receive Sox8mo. Similarly, no significant increase in TUNEL labeling was observed in Sox8-depleted embryos at early neurula stage. However, morpholino-treated embryos showed impaired neural crest migration, suggesting that at least the craniofacial phenotype of Sox8-depleted embryos is a direct consequence of the inability of neural crest cells to reach their proper targets in the periphery. Here, we can speculate that, because of the delay in their specification, neural crest progenitors generated in Sox8-depleted embryos did not receive all the necessary instructive cues that are crucial to their migration.

In conclusion, this study provides evidence for an early function of Sox8 in neural crest development in *Xenopus*. In this organism, the tight regulation of Sox8 expression at the neural plate border appears to be crucial to the timely specification of neural crest progenitors. Although this is in apparent contrast to the reported phenotype of Sox8-null mouse embryos in which other SoxE proteins are compensating for the loss of Sox8, these results also highlight species-specific differences in the relative contribution of SoxE proteins to the development of the neural crest. We propose that these differences are the result of divergences in the timing and sequence of expression of SoxE factors at the neural plate border, rather than the result of differences in the intrinsic activity of these factors.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/19/3817/DC1>

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