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Sox10 regulates the development of neural crest-derived melanocytes in *Xenopus*

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Abstract

The transcription factors of the Sox family play important roles in diverse developmental processes. A number of genetic studies have established that Sox10 is a major regulator of neural crest formation. Here, we report the cloning and functional analysis of the *Xenopus* Sox10 gene. Sox10 mRNA accumulates during gastrulation at the lateral edges of the neural plate, in the neural crest-forming region. In this tissue, Sox10 expression is regulated by Wnt signaling and colocalizes with two major regulators of neural crest formation, Slug and Sox9. While initially expressed in neural crest cells from all axial levels, at the tailbud stage, Sox10 is downregulated in the cranial neural crest and persists mostly in neural crest cells from the trunk region. Overexpression of Sox10 causes a dramatic expansion of the Slug expression domain. We show that the C-terminal portion of Sox10 is sufficient to mediate this activity. Later during embryogenesis, Sox10-injected embryos show a massive increase in pigment cells (Trp-2-expressing cells). The responsiveness of the embryo to Sox10 overexpression by expansion of the Slug expression domain and ectopic production of Trp-2-positive cells and differentiated melanocytes is lost during gastrulation, as revealed by a hormone-inducible Sox10 construct. These results suggest that Sox10 is involved in the specification of neural crest progenitors fated to form the pigment cell lineage.

Keywords: Sox10; Slug; Sox9; Trp-2; Neural crest; Melanocytes; *Xenopus*

Introduction

The neural crest is a multipotent population of migratory cells that develops at the lateral edges of the neural plate. During neurulation, neural crest becomes progressively positioned at the dorsal-most aspect of the neural tube. In most vertebrates, neural crest arises from the entire length of the neuraxis starting at a level caudal to the prospective diencephalon. Upon neural tube closure, neural crest cells delaminate in a rostrocaudal wave and migrate to diverse locations throughout the embryo. As they reach their final locations, neural crest cells have the ability to differentiate into a large variety of cell types, including peripheral and enteric neurons and glia, smooth muscle cells, craniofacial cartilage and bone and endocrine and pigment cells. These cell types exhibit diversity along the rostrocaudal aspect of the neuraxis, such that craniofacial cartilage and bone arise from the cranial region, while sensory neurons/glia and pigment cells derive from the vagal/trunk region (reviewed in LeDouarin and Kalcheim, 1999).

Abnormal development of the neural crest and its derivatives has been associated with a large number of diseases designated under the generic term of neurocristopathies (Bolande, 1974, 1997). Defining the mechanism of the neural crest formation represents an important challenge to further our understanding of the molecular basis underlying these pathologies. While several models have been proposed to explain induction of neural crest at the neural plate border (reviewed in Knecht and Bronner-Fraser, 2002; Ay-

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bar and Mayor, 2002), the molecular regulators involved in neural crest induction and diversification remain largely unknown.

Sox proteins form a large class of transcriptional regulators characterized by the presence of an HMG-box, a sequence specific DNA binding domain (Pevney and Lovell-Badge, 1997; Wegner, 1999). Expression of these proteins in defined cell types has been implicated in the control of a variety of developmental processes, including cell fate decisions (Kamachi et al., 2000). We have recently shown that one member of this family, Sox9, is expressed in early neural crest progenitors and is required for development of skeletogenic derivatives of the cranial neural crest during *Xenopus* embryogenesis (Spokony et al., 2002). Consistent with this result, mutations in one Sox9 allele result in Campomelic Dysplasia (CD), a lethal human disorder characterized by sex reversal and severe skeletal malformations, including defects in craniofacial skeletal elements of neural crest origin (Houston et al., 1983; Wagner et al., 1994). Similarly, Sox9 heterozygous mutant mice present major skeletal defects, including a shortened jaw and cleft palate (Bi et al., 2001).

Another Sox family member, Sox10, has also been implicated in neural crest formation. During mouse embryogenesis, Sox10 is expressed in the cells of the neural crest at the time of their emergence and is detected transiently in enteric neurons and melanoblasts. Sox10 persists in the peripheral nervous system into adulthood, where it is confined to the peripheral glia (Kuhlbrodt et al., 1998; Pusch et al., 1998; Britsch et al., 2001). Mutations in one Sox10 allele have been found in patients that suffer from congenital aganglionic megacolon (Hirschsprung disease), sometimes associated with a combination of pigmentation defects and deafness (Waardenburg–Shah syndrome; Pingault et al., 1998). In the spontaneous mouse mutant *Dom*, Sox10 carries a frameshift mutation. As a result, *Dom* mice suffer from a loss of neurons and glial cells in the peripheral nervous system and melanocytes (Southard Smith et al., 1998; Herbarth et al., 1998; Kapur, 1999). In the zebrafish, the *colourless* gene encodes a Sox10 homologue and has been shown to be required for the specification of a number of nonectomesenchymal neural crest derivatives (Kelsh and Eisen, 2000; Dutton et al., 2001).

Here, we report the cloning and functional analysis of the *Xenopus* Sox10 gene during development of neural crest-derived melanocytes. Sox10 mRNA accumulates shortly after gastrulation in the neural crest-forming region. Initially, Sox10 is detected in neural crest progenitors from all axial levels. As development proceeds, Sox10 expression is downregulated in the cranial region but persists strongly in the trunk neural crest. Overexpression of Sox10 at the gastrula stage, by means of a hormone inducible construct, causes a dramatic expansion of the Slug expression domain and leads to a massive increase in differentiated melanocytes at a later stage. These results suggest that Sox10 is involved in the induction of the neural crest-derived pigment cell lineage.

Materials and methods

Isolation of Sox10 and Trp-2

Xenopus Sox10 was amplified by PCR from stage 20 cDNA using degenerate primers (forward: AA[G/A][A/C]GNCCNATGAA[T/C]GCNTT[T/C]ATG and reverse: [C/T]TT[A/G]TA[A/G]TGNGC[C/T]TGNAT) based on the published sequences of chicken (Cheng et al., 2000) and human (Pusch et al., 1998) Sox10. A 306-bp PCR product was isolated, ligated into pGEMTeasy (Promega), and sequenced by using an automated ABIPrism 373 sequencer. This PCR product was subsequently used to screen a stage 17 cDNA library (a gift of Dr. Michael King). A 3.3-kb cDNA was isolated, cloned into pBSK+, and sequenced on both strands (pBSK-Sox10). This clone contains the entire ORF of Sox10 (1341 bp), 104 bp of 5′UTR, and 1881 bp of 3′UTR. The complete sequence of *Xenopus* Sox10 has been deposited into GenBank (Accession No. AY149116). The Sox10 ORF was subcloned into the *Cla*I and *Xho*I sites of pCS2+ (Turner and Weintraub, 1994). This construct is referred as pCS2 + Sox10. This expression plasmid was sequenced and the corresponding protein monitored by using an in vitro transcription/translation coupled rabbit reticulocyte lysate system (TNT, Promega).

Xenopus Trp-2 was amplified by PCR from stage 46 cDNA using degenerate primers (forward: TA[C/T]AA[C/T]TA[C/T]TT[C/T]GTNTGG and reverse: AANGGNACCAT[A/G]TT[A/G]TA) based on the published sequences of mouse (Tsukamoto et al., 1992) and chicken (April et al., 1998) Trp-2. A 760-bp product was recovered, cloned into pGEMT, sequenced, and subsequently used to generate Northern and in situ hybridization probes and to design specific primers for RT-PCR analysis.

Sox10 deletion mutant and hormone-inducible constructs

The Sox10 deletion constructs NSox10 (amino acids 1–95), Sox10ΔC (amino acids 1–200), and ΔSox10 (amino acids 176−446) were generated by PCR using pCS2 + Sox10 as template and were cloned into the *Cla*I and *Xho*I sites of pCS2+. A Sox10-GR construct was generated by fusing the coding region of Sox10 to the human glucocorticoid receptor ligand-binding domain (GR) as described (Gammill and Sive, 1997; Tada et al., 1997). This fusion construct was subcloned into pCS2+ vector. Each expression plasmid was sequenced and the corresponding protein monitored by using an in vitro transcription/translation coupled rabbit reticulocyte lysate system in the presence of [^{35}S]methionine (SP6-TNT; Promega) and resolved on a NuPAGE BIS–Tris gel (Invitrogen).

Embryo injections and dexamethasone treatment

Xwnt-1 plasmid DNA (100 pg), GSK3β mRNA (1 ng), and Sox9 morpholino antisense (Sox9-AS, 10 ng) injections were performed as described (Saint-Jeannet et al., 1997; Spokony et al., 2002). A dominant-negative Slug construct (Δ-Slug) lacking the amino-terminal domain was generated by PCR as described (LaBonne and Bronner-Fraser, 2000) and cloned into pCS2+ vector. Capped mRNA were synthesized by using an Ambion transcription kit. Injections were performed in one blastomere at the two-cell stage. For animal explants experiments, both blastomeres of two-cell-stage embryos were injected in the animal pole region, and explants were dissected at the blastula stage and cultured in vitro. Embryos or animal explants injected with 1 ng of Sox10-GR or GR mRNA were treated at different time points with 10 μM of dexamethasone (Sigma) in NAM 0.1$\times$ (whole embryos) or NAM 0.5$\times$ (animal explants) as described (Gammill and Sive, 1997; Tada et al., 1997).

Western blot analysis

Sox10-GR-injected embryos were collected at different stages, homogenized, resolved on a NuPAGE BIS–Tris gel, and blotted onto nitrocellulose. Blots were subsequently incubated in the presence of a Sox10 polyclonal antibody (N-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 by using the SuperSignal West Femto Maximum Sensitivity Substrate from Pierce and detected by exposure onto a BioMax film (Kodak). This Sox10 antibody does not cross-react with Sox9 or Sox2 proteins (not shown). Blots were stripped according to the manufacturer recommendations (Pierce) and probed with anti-α-tubulin antibody (Sigma; 1:500 dilution).

Northern hybridization and RT-PCR analysis

RNA extraction, electrophoresis, and blotting were performed as described (Taira et al., 1992). For hybridization, Trp-2 probe was synthesized by using a DNA labeling kit (α-dCTP) from Pharmacia (Ready-To-Go). Filters were analyzed by autoradiography. The RT-PCR primers used in this study were as follow: EF-1α (forward primer: CAGATTGGTGCTGGATATGC; reverse primer: ACTGCCTTGATGACTCCTAG; 268 nt, 22 cycles) and Trp-2 (forward primer: TGTTACTTGGCATCGTTACC; reverse primer: GCACTTATCAGATTCGGGTC; 180 nt, 28 cycles).

Lineage tracing and whole-mount in situ hybridization

Embryos were coinjected with β-galactosidase mRNA (β-gal, 1 ng). At stage 17 or 32, embryos were fixed in MEMFA (Harland, 1991) and successively processed for Red-Gal (Research Organics) staining and in situ hybridization. Antisense DIG-labeled probes (Genius kit, Roche) were synthesized by using template cDNA encoding Slug (Mayor et al., 1995) and Sox9 (Spokony et al., 2002). Sox10 and Trp-2 in situ hybridization was performed by using pBSK-Sox10 and pGEMT-Trp-2 constructs as templates. Whole-mount in situ hybridization was performed as previously described (Harland, 1991). For histology, stained embryos were embedded into Paraplast+, 12 μm sections cut on a rotary microtome and counterstained with eosin.

Results

Cloning of Xenopus Sox10

A PCR product corresponding to a partial sequence of *Xenopus* Sox10 was amplified from stage 20 cDNA by using degenerate primers (Fig. 1). This product was subsequently used to screen a stage 17 cDNA library (a gift of Michael King). A 3.3-kb cDNA was isolated and sequenced. The predicted sequence of *Xenopus* Sox10 encodes a 446-amino-acid protein (Fig. 1) that shares 73% identity with mouse Sox10 (Wagner et al., 1994) and 64% identity with zebrafish Sox10 (Dutton et al., 2001). When compared with *Xenopus* Sox9 (Spokony et al., 2002), the overall amino acid identity drops to 56%. Based on the similarity of their HMG domains, Sox genes have been categorized into several subgroups (Wegner, 1999). Members of a subgroup share more than 80% amino acid identity within the HMG domain, in addition to regions of homology outside the HMG box. Sox10 proteins fall into subgroup E that also includes Sox8 (Bell et al., 2000) and Sox9 (Wagner et al., 1994; Spokony et al., 2002).

Sox10 is expressed in the developing neural crest

To determine the spatial expression pattern of Sox10, we performed whole-mount in situ hybridization on a variety of embryonic stages. Sox10 expression is first detected at the open neural plate stage (stage 13/14; Nieuwkoop and Farber, 1952) in the neural crest-forming regions (Fig. 2A). This expression pattern at the neural plate border is identical to that described for Snail (Essex et al., 1993), Slug (Mayor et al., 1995), Foxd3 (Sasai et al., 2001), and Sox9 (Spokony et al., 2002). Slug and Sox9 expression at stage 13/14 is shown for comparison (Fig. 2B and C). Tissue sections of stage 14 embryos illustrate the neural crest expression of Sox10 at the neural plate border (Fig. 2D). In *Xenopus*, the prospective neural crest is derived from the deep (lateral neural crest) and the superficial (medial neural crest) layers of the ectoderm. The cranial neural crest consists of a larger number of cells than the trunk crest, which remains, at all times, lateral to the neuraxis (Sadaghiani and Thiebaud, 1987). As the neural tube closes, Sox10 is detected in the lateral neural crest (Fig. 2E and F). At a similar stage, Sox9

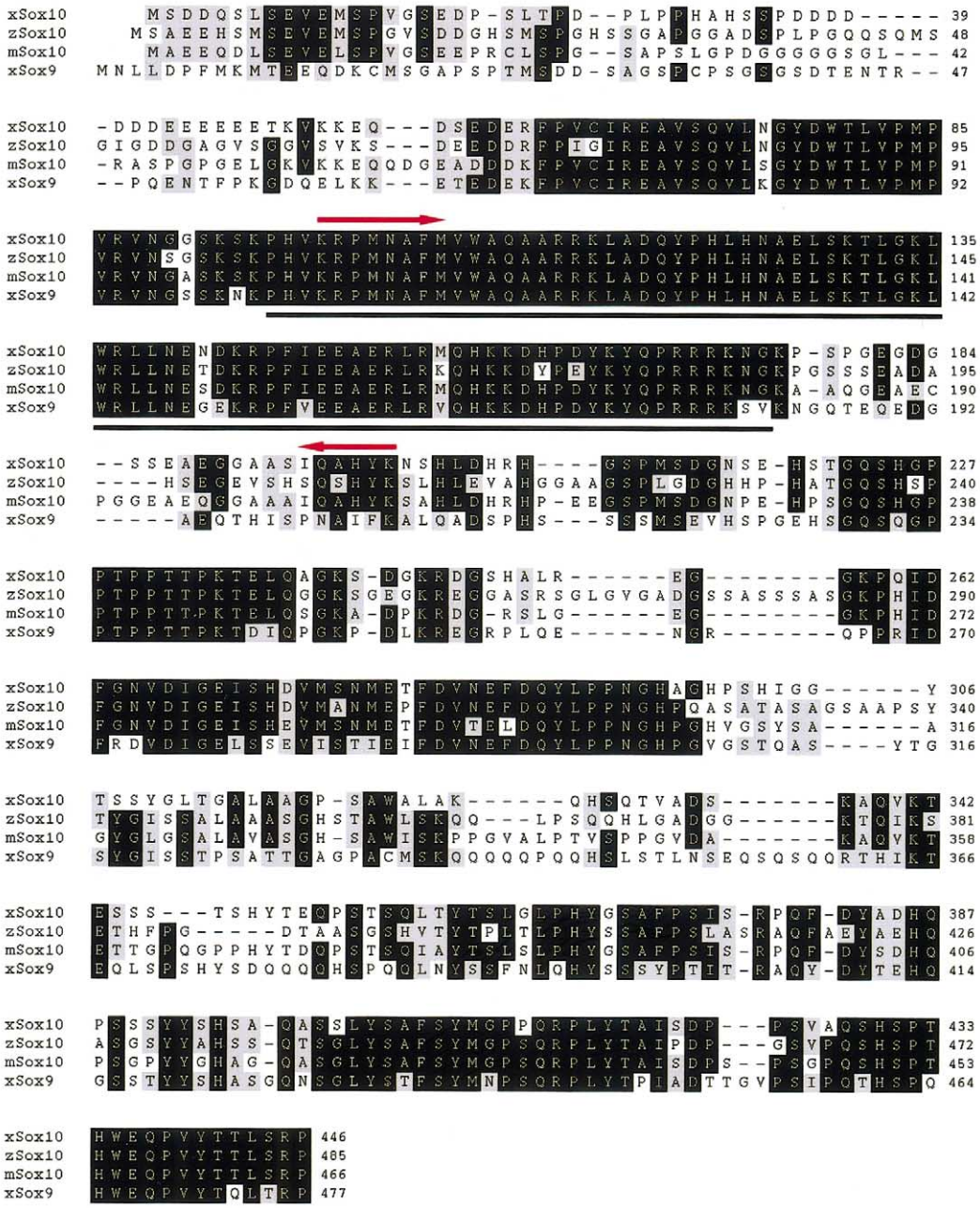

Fig. 1. Sequence and structure of *Xenopus* Sox10. Deduced amino acid sequences from *Xenopus*, mouse, zebrafish Sox10, and *Xenopus* Sox9 were aligned by using Mac Vector CustalW Alignment. Identical and similar amino acids are in black and gray boxes, respectively. The HMG box is underlined in black. The position of the degenerate primers used to isolate *Xenopus* Sox10 is indicated by red arrows.

is expressed in both the lateral and medial neural crest in addition to the presumptive otic placode (Fig. 2G and H). By stage 22, neural crest cells migrate in the cranial region and strong expression of Sox10 is detected in the individual streams of crest cells that populate the branchial arches as well as neural crest cells migrating in the frontonasal region (Fig. 2I). Sox10 is also strongly expressed at the dorsal midline, in the trunk neural crest (Fig. 2I). Around stage 25, Sox10 is detected in the otic vesicle (Fig. 2J and K) and will persist in this tissue at least until stage 40 (Fig. 2Q and R). Tissue sections of stage 25 embryos illustrate the massive expression of Sox10 in the premigratory trunk neural crest cells on top of the neural tube (Fig. 2L). Around stage 30, Sox10 expression is downregulated in the cranial neural crest of the pharyngeal arches but persists in the trunk neural crest, in the otic vesicle, and in discrete domains adjacent to the hindbrain (Fig. 2M N and P). Sox10 expression in the epithelium of the developing inner ear is consistent with its expression in other species (Bondurand et al., 1998; Southard-Smith et al., 1998; Watanabe et al., 2000;

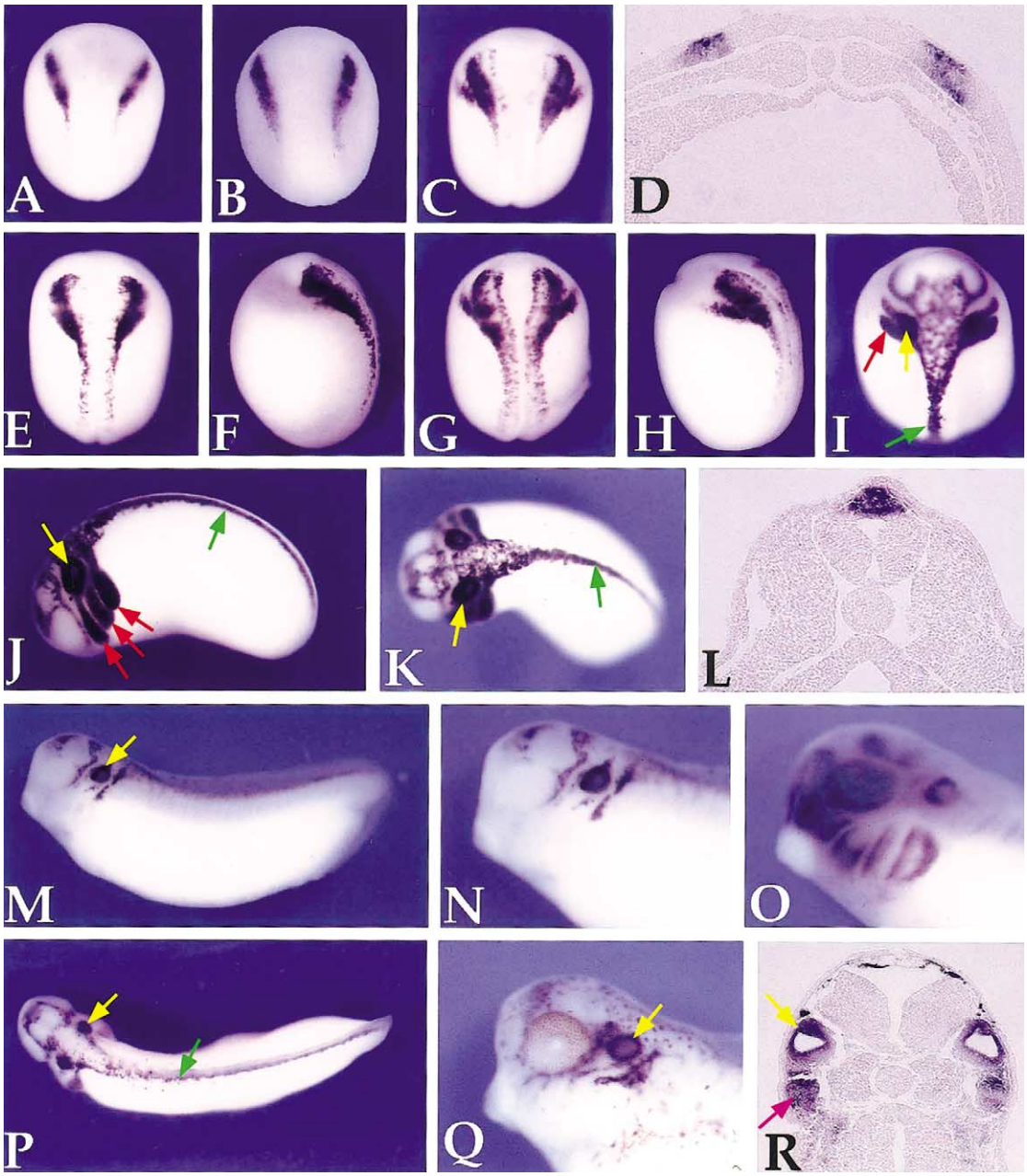

Fig. 2. Developmental expression of Sox10 by whole-mount in situ hybridization. (A) Sox10 expression at the neurula stage (stage 14) is detected at the lateral edges of the neural plate. Slug (B) and Sox9 (C) expression at a similar stage are shown for comparison. (A–C) Dorsal views, anterior to top. (D) Transverse section of a stage 14 embryo. Sox10 expression is restricted to the neural crest-forming region. (E–H) Stage 16 embryo, dorsal (E, G) or lateral (F, H) views, anterior to top. As the neural tube closes, Sox10 (E, F) remains strongly expressed in the neural crest at all axial levels. Sox9 expression (G, H) is shown for comparison. (I) At stage 22, Sox10 is detected in migrating cranial neural crest cells (red arrow) as well as in neural crest at the dorsal midline in the trunk region (green arrow). An additional domain of expression includes the otic placode (yellow arrow). Dorsal view, anterior to top. (J–L) In stage 25 embryos, Sox10 is detected in the streams of cranial neural crest that populate the branchial arches (red arrows), in the dorsal aspect of the neural tube (green arrow) and in the otic placode (yellow arrow). Lateral (J) and dorsal (K) views, anterior to left. (L) Transverse section of a stage 25 embryo, highlights Sox10 expression in neural crest progenitors at the dorsal midline. (M-P) At stage 32, Sox10 expression is downregulated in the branchial arches but persists in the otic vesicle (yellow arrow), at the dorsal midline (green arrow), in the forebrain, and in discrete domains adjacent to the hindbrain. Lateral (M) and dorsal (P) views, anterior to left. Higher magnification view of Sox10 expression in the head (N). Sox9 expression in the head of an embryo at a similar stage is shown for comparison (O). In a lateral view of an embryo at stage 35 (Q), Sox10 is detected in the forebrain, the otic vesicle (yellow arrow), a number of cranial ganglia and in differentiating melanoblasts. (R) Transverse section at the level of the hindbrain illustrates Sox10 expression in the otic vesicle (yellow arrow) and adjacent VII/IX cranial ganglion (purple arrow).

Cheng et al., 2000; Dutton et al., 2001). For comparison, at a similar stage, Sox9 is still strongly expressed in the pharyngeal arches, the otic vesicle, and the eye (Fig. 2O), but is totally absent from the trunk neural crest (Spokony et al., 2002). At the latest stage analyzed in this study (stage 40), Sox10 expression is restricted to the otic vesicle, differen-

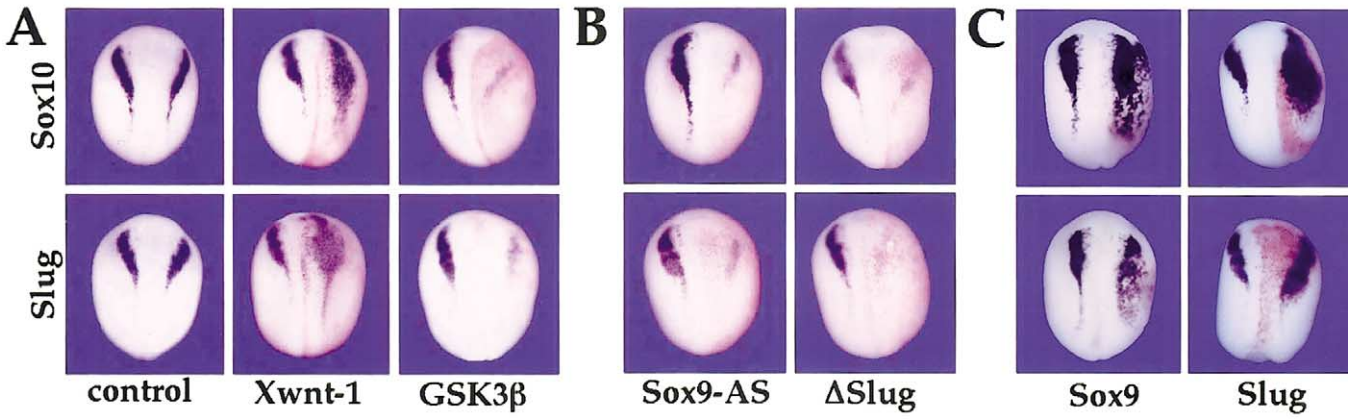

Fig. 3. Regulation of Sox10 expression in the neural crest-forming region. (A) Regulation of Sox10 and Slug expression by Wnt signaling. Injection of Xwnt-1 plasmid (100 pg) expands Slug (79%, $n = 53$) and Sox10 (82%, $n = 50$) expression domains. Conversely, overexpression of GSK3β (1 ng), known to block Wnt signaling, prevents Slug (81%, $n = 42$) and Sox10 (65%, $n = 51$) expression on the injected side. (B) Sox9 and Slug are required for Sox10 expression. Sox9-AS injection (10 ng) blocks Slug (89%, $n = 35$) and Sox10 (96%, $n = 54$) expression. Similarly, a dominant negative Slug (ΔSlug, 1 ng) blocks Slug (61%, $n = 51$) and Sox10 (56%, $n = 50$) expression. (C) Sox9 and Slug overexpression upregulate Sox10 expression. Sox9 (1 ng) overexpression leads to a limited expansion of Slug (83%, $n = 58$) and Sox10 (96%, $n = 79$) expression domains. Overexpression of Slug (0.1 ng) results in a robust expansion of Slug (90%, $n = 30$) and Sox10 (98%, $n = 44$). In all panels, early neurula stage embryos are viewed from the dorsal side, anterior to top. RNA encoding the lineage tracer β-galactosidase was coinjected to identify the injected side (red staining), the right side in all panels.

tiated pigment cells, and in several cranial ganglia (Fig. 2Q and R).

Regulation of Sox10 expression in the neural crest-forming region

Studies in the mouse (Ikeya et al., 1997; Dunn et al., 2000), frog (Saint-Jeannet et al., 1997, Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Bang et al. 1999; Deardorff et al., 2001; Tan et al., 2001), zebrafish (Dorsky et al., 1998), and chick (Garcia-Castro et al., 2002) have implicated the Wnt family of growth factors in neural crest formation (reviewed in Wu et al., 2003). Ectopic Wnt expression enhances, whereas lack of Wnt signaling inhibits neural crest formation in vivo (Saint-Jeannet et al., 1997; Labonne and Bronner-Fraser, 1998; Deardorff et al., 2001). We analyzed whether Wnt signaling also regulates Sox10 expression in the neural crest-forming region. Injection of Xwnt-1 plasmid DNA (100 pg) into one blastomere at the two-cell stage elicited ectopic expression of Sox10 at stage 17 (Fig. 3A) in more than 80% of the embryos ($n = 50$), without affecting axis formation (Saint-Jeannet et al., 1997). Conversely, injection of 1 ng of glycogen synthase kinase 3 (GSK3) mRNA, a downstream component of the canonical Wnt pathway known to antagonize Wnt signaling (He et al., 1995), blocked Sox10 expression (Fig. 3A) in 65% of the embryos ($n = 51$). These results indicate that Wnt signaling regulates Sox10 and Slug expression in a similar manner, suggesting that both transcription factors may act in the same pathway during neural crest formation.

Previous work has established that Slug and Sox9 are essential components of the signaling cascade leading to neural crest formation, as Slug or Sox9 loss-of-function leads to a specific loss of neural crest progenitors (LaBonne and Bronner-Fraser, 2000; Spokony et al., 2002). Therefore, we tested whether Sox10 expression in the neural crest-forming region is dependent on these factors. Injection of either 10 ng of Sox9-AS (Spokony et al., 2002) or 1 ng of a dominant negative Slug mRNA, ΔSlug (LaBonne and Bronner-Fraser, 2000), resulted in a severe loss of Sox10 expression on the injected side of stage 17 embryos (Fig. 3B), similar to that observed for Slug (Fig. 3B; LaBonne and Bronner-Fraser, 2000; Spokony et al., 2002). These results indicate that Sox9 and Slug, two major regulators of neural crest formation in *Xenopus*, are required for Sox10 expression at the neural plate border. Interestingly, Sox9 and Slug overexpression can upregulate Sox10 expression in the neural crest-forming region (Fig. 3C). The mutual regulation of Sox9, Slug, and Sox10 indicates that these factors may be involved in maintaining each other's expression. This type of reciprocal activation loops has also been implicated in the maintenance of Zic-r1, FoxD3, and Slug expression at the neural plate border (Sasai et al., 2001).

The C-terminal domain of Sox10 is sufficient to mediate Sox10 function

To evaluate Sox10 function during neural crest formation, we analyzed the effect of Sox10 overexpression on the formation of neural crest progenitors, as determined by expression of the early neural crest marker, Slug. Injection of 1 ng of Sox10 mRNA in one blastomere at the two-cell stage resulted in a dramatic lateral expansion of Slug expression domain (Fig. 4A and B) in approximately 60% of the injected embryos ($n = 264$). This result suggests that

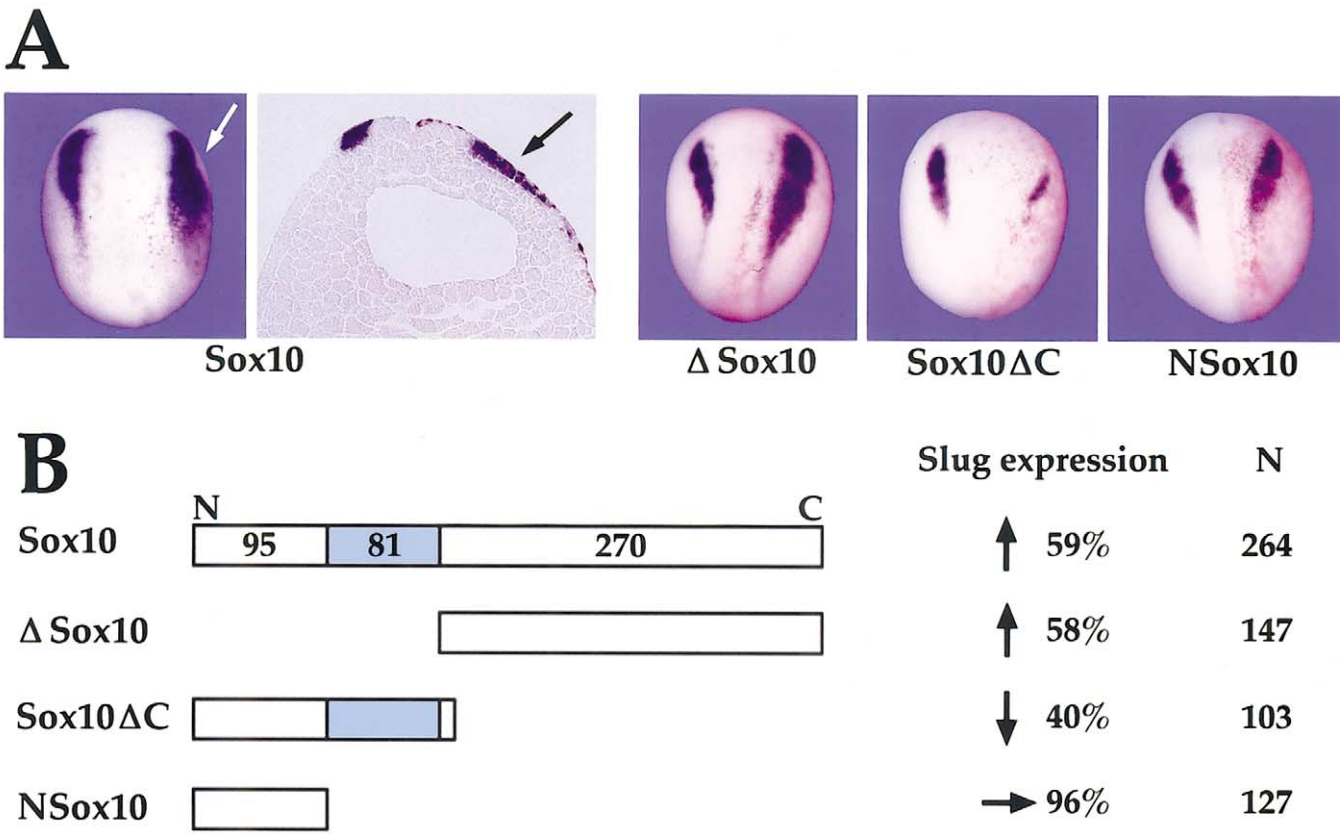

Fig. 4. The C-terminal domain of Sox10 is sufficient to induce neural crest progenitors. (A) Embryos were injected in one blastomere at the two-cell stage with mRNA encoding either wild type Sox10 (Sox10) or various Sox10 deletion constructs (ΔSox10, Sox10ΔC, or Nsox10) and analyzed for Slug expression at stage 17. Sox10 injection (1 ng) expands Slug expression domain (white arrow). A transverse section through a Sox10-injected embryo illustrates Slug expansion on the injected side (black arrow). ΔSox10-injected embryos (1 ng) display a dramatic expansion of the Slug expression domain. By contrast, injection of Sox10ΔC (2 ng) resulted in a moderate inhibition of Slug expression, whereas NSox10-injected embryos (1 ng) were unaffected. RNA encoding the lineage tracer β-galactosidase was coinjected to identify the injected side (red staining), the right side in all panels. (B) Schematic representation of wild type Sox10 and various Sox10 deletion constructs. The HMG box is depicted in blue. Quantification of the in situ hybridization results. (↑), expansion of Slug expression domain; (↓), reduction of Slug expression domain; (→), Slug expression domain is unaffected. *N*, number of cases analyzed.

Sox10 is sufficient to induce neural crest progenitors and therefore places Sox10 in the complex regulatory cascade controlling neural crest formation.

Based on this initial observation, we decided to use Sox10-mediated Slug expansion as an assay to perform a structure-function analysis of the Sox10 protein. We generated three deletion constructs, ΔSox10, Sox10ΔC, and NSox10 (Fig. 4B), and tested their ability to expand Slug expression at stage 17, after unilateral injection of the corresponding mRNA at the two-cell stage. While the N-terminal domain alone (Nsox10) had no effect on endogenous Slug expression (Fig. 4A and B), a construct lacking the C-terminal putative transcriptional activation domain of Sox10 (Sox10ΔC) led to a moderate loss of Slug expression on the injected side in 40% of embryos, possibly by acting as a dominant-negative (Fig. 4A and B). By contrast, a construct lacking the N-terminal domain and the HMG-box (ΔSox10) generated dramatic expansion of Slug expression domain, similar to wild type Sox10 overexpression, and at a similar frequency (Fig. 4A and B). These results indicate that the N-terminal domain and the HMG-box are dispensable for Sox10 activity in the context of this assay and suggest that the C-terminal domain of Sox10 carries sufficient information to mediate Sox10 function, presumably by establishing interactions with specific partner molecules (Kamachi et al., 2000).

Sox10 expression is sufficient to induce pigment cell lineage

Melanocytes represent one major derivative of the neural crest, and Sox10 has been implicated in the formation of this cell type, as mutations in Sox10 result in pigmentation defects (Pingault et al., 1998; Southard-Smith, 1998; Herbarth et al., 1998; Dutton et al., 2001). Therefore, we decided to further characterize the neural crest derivatives generated by Sox10 overexpression, by focusing primarily on the pigment cell lineage. To this end, we cloned a partial sequence of *Xenopus* tyrosinase related protein-2 (Trp-2), a pigment cell-specific enzyme involved in melanin synthesis (Tsukamoto et al., 1992). By Northern blot analysis, Trp-2 transcripts are detected around stage 30 (Fig. 5A), while the

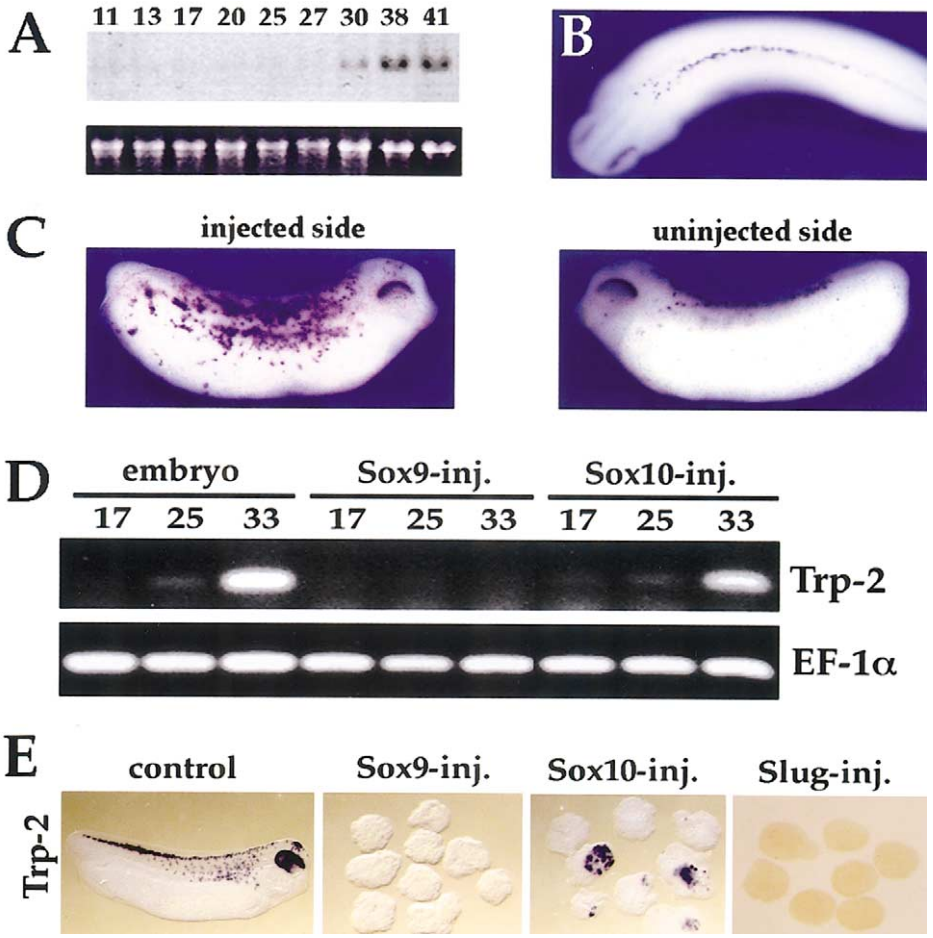

Fig. 5. Sox10 is sufficient to induce pigment cell lineage as revealed by Trp-2 expression. (A) Developmental expression of *Xenopus* Trp-2 by Northern hybridization. The stages are according to Nieuwkoop and Farber (1967). (B) By whole-mount in situ hybridization at stage 27/28, Trp-2 is detected in the developing retina and in individual cells scattered at the dorsal midline at a level posterior to the hindbrain. Dorsal view, anterior to the left. (C) An embryo injected at the two-cell stage with 1 ng of Sox10 mRNA and analyzed at stage 30 displays massive ectopic expression of Trp-2 (left panel), while the uninjected side is unperturbed (right panel). Lateral view, anterior is to the right. (D) RT-PCR analysis of animal explants derived from embryos injected at the two-cell stage with 1 ng of Sox9 or Sox10 mRNA. Only Sox10 overexpression induces strong expression of Trp-2 at stage 33. EF1-α is used as a loading control. (E) In situ hybridization of explants overexpressing Sox9, Sox10, or Slug. Induction of Trp-2 is exclusively detected in Sox10-injected animal explants at equivalent stage 32. Trp2 expression in an uninjected embryo (control) at stage 32 is shown (left panel).

first pigmented melanophores appear a few hours later around stage 33 (Nieuwkoop and Farber, 1950). By whole-mount in situ hybridization, Trp-2 is first detected in the developing eye at stage 26, in the dorsal aspect of the retina (not shown). A few hours later at stage 27/28, Trp-2 is expressed at the dorsal midline in the trunk and vagal regions of the embryo (Fig. 5B). Eventually, Trp-2 expression will persist at all sites of pigment cell differentiation (not shown).

Upon injection of 1 ng of Sox10 mRNA in one blastomere at the two-cell stage, approximately 20% of injected embryos ($n = 51$) exhibited a massive overproduction of pigment cells precursors on the injected side (Fig. 5C), as assayed by Trp-2 expression at stage 33. This effect is specific to Sox10 since neither Sox2 (Misuzeki, 1998) nor Sox9 (Spokony et al., 2002) overexpression was able to mimic this activity (data not shown). However, the transcription factor Slug has also been shown to induce ectopic pigment cells upon overexpression in the embryo (LaBonne and Bronner-Fraser, 1998). Animal explants isolated at the blastula stage from embryos overexpressing Sox10 showed strong activation of Trp-2 expression by RT-PCR and by in situ hybridization at stage 33 (Fig. 5D and E). Interestingly, animal explants derived from embryos injected with Sox9 or Slug mRNA failed to activate Trp-2 expression (Fig. 5D and E). This is consistent with previous work showing that Slug expression in animal explants is not sufficient to induce a neural crest differentiation program (LaBonne and Bronner-Fraser, 1998). Nevertheless, we cannot exclude the possibility that the differences in activity between Sox10 and other neural crest effectors (Sox9 and Slug) may be due to the production of insufficient levels of active protein. The winged-helix gene Foxd3 has been reported to induce melanocytes when expressed in animal explants (Sasai et al., 2001), suggesting that Sox10 and FoxD3 may act in the same pathway during pigment cell formation. Altogether,

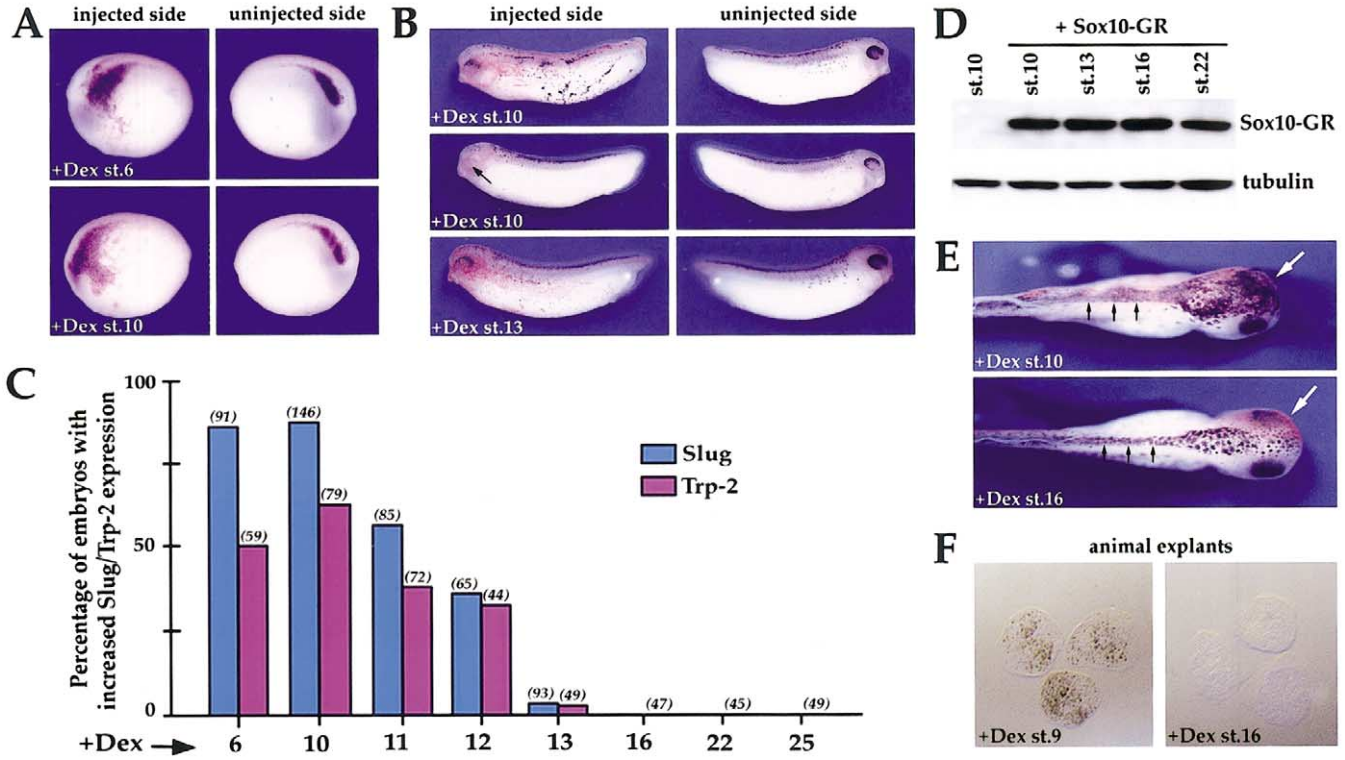

Fig. 6. Sox10 expands Slug and induces ectopic Trp2-expressing cells and melanocytes at the gastrula stage. (A) Ectopic Slug expression in embryos injected with Sox10-GR at the two-cell stage and treated with dexamethasone at stage 6 or stage 10 (left panels). Slug expression on the uninjected side is shown for comparison (right panels). (B) Ectopic Trp-2 expression in embryos injected with Sox10-GR at the two-cell stage and treated with dexamethasone at stage 10 (upper left panel). Trp-2 expression is unaffected in the neural crest lineage of embryos treated with dexamethasone at stage 13 (lower left panel). When targeted in the head region, Sox10-GR blocks Trp-2 expression in the pigmented epithelium of the retina (arrow, left panel). Trp-2 expression on the uninjected side is presented for comparison (right panels). Lateral view, anterior is to the left (left panels) or to the right (right panels). RNA encoding the lineage tracer β-galactosidase was coinjected to identify the injected side (red staining). (C) Quantification of the in situ hybridization results. The number of cases analyzed for each time point (+Dex) is indicated in parentheses. (D) Western blot analysis. Detection of Sox10-GR protein in extracts from Sox10-GR-injected embryos (+Sox10-GR) collected at different stages after injection at the two-cell stage. The fusion protein is expressed at similar levels at all stages (st 10–22). This Sox10 antibody does not allow detection of endogenous Sox10 protein (first lane). Tubulin is presented as a loading control. (E) Development of ectopic melanocytes in Sox10-GR-injected embryos. Embryos injected with Sox10-GR at the two-cell stage and treated with dexamathasone at stage 10 (upper panel, +Dex st 10) displays ectopic formation of pigment cells in the head (white arrow) and the trunk (black arrows) regions. The pattern of pigment cells is unaffected in sibling embryos treated with dexamethasone at stage 16 (lower panel, +Dex st 16). (F) Induction of differentiated melanocytes in animal explants at equivalent stage 40. Animal explants isolated at the blastula stage from embryo injected with 2 ng of Sox10-GR mRNA at the two-cell stage were treated with dexamethasone at stage 9 (+Dex st. 9) or at stage 16 (+Dex st. 16). Induction of melanocytes is only observed in animal explants treated by dexamethasone at stage 9.

these results indicate that Sox10 expression is sufficient to activate a melanocyte differentiation program in the whole embryo, as well as in animal explants.

Sox10 function at the gastrula stage regulates the generation of neural crest-derived pigment cells

The results presented here indicate that Sox10 is implicated in the formation of neural crest progenitors fated to form the pigment cell lineage. We next decided to determine the window of time during which Sox10 is functioning to generate melanocytes. To this end, we generated an inducible Sox10 construct (Sox10-GR) in which the hormone-binding domain of the human glucocorticoid receptor is fused in frame with Sox10 protein. This construct allows temporal activation of Sox10 protein by addition of dexamethasone at specific times during embryogenesis (Gammill and Sive, 1997; Tada et al., 1997). Embryos were injected in one blastomere at the two-cell stage with 1 ng of Sox10-GR mRNA and treated with dexamethasone at various stages. Addition of dexamethasone at the blastula (st. 6) and gastrula (st. 10–12) stages caused expansion of Slug expression at stage 17 (Fig. 6A and C). This result is consistent with the phenotype generated by Sox10 overexpression described earlier (Fig. 4) and suggests that this fusion construct is fully active. However, addition of dexamethasone at the end of gastrulation (st. 13) failed to expand Slug expression. In a similar manner, ectopic expression of Trp-2 at stage 32 (Fig. 6B and C) was only observed when dexamethasone was added at the blastula and gastrula stages (st. 6–12). At the neurula stage (st. 13–16) and during the phase of neural crest cell migration (st. 22–25), addition of

dexamethasone failed to generate ectopic Trp-2 cells (Fig. 6B and C). Overall, Sox10-GR is a more potent inducer of Slug and Trp-2 than wild type Sox10. As a control, injections of GR mRNA had no effect on endogenous Slug and Trp-2 expression after addition of dexamethasone (not shown).

By Western blot analysis, using a Sox10-specific antibody, we showed that the levels of Sox10-GR protein remained constant throughout development, from stage 10 to 22 (Fig. 6D). This result indicates that the lack of Slug/Trp-2-inducing activity of Sox10-GR at stage 13 and beyond is not due to a reduced accumulation of Sox10-GR protein at these later stages, further validating the temporal requirement for Sox10 function during gastrulation.

In Sox10-GR-injected embryos, the development of differentiated melanocytes, at stage 40, was also dependent on the addition of dexamethasone prior to stage 13 (Fig. 6E). In these embryos, supernumerary pigment cells were found in the head region and at the dorsal midline in the trunk (Fig. 6E), though at a lower frequency than ectopic Trp-2-expressing cells. Sox10-GR-injected embryos treated with dexamethasone at stage 16 had a normal pattern of differentiated pigment cells (Fig. 6E). Similarly, animal explants derived from Sox10-GR-injected embryos formed differentiated melanocytes only when exposed to dexamethasone before stage 13 (Fig. 6F). These results indicate that, in whole embryos and in animal explants, Sox10 has the same temporal requirement to generate differentiated melanocytes.

In contrast to these results, a reduction or a loss of pigment cells of nonneural crest origin (retinal pigmented epithelium) was observed when Sox10-GR expression was targeted in the head region (Fig. 6B, left middle panel). This result indicates that these two populations of melanoblasts behave differently in response to Sox10 overexpression. A similar difference in the way these two types of melanocytes are affected was also reported in mice embryos carrying mutation in Mitf, a downstream target of Sox10 (Nakayama et al., 1998). Alternatively, by expanding the neural crest domain, Sox10 overexpression may perturb anterior neural plate patterning, leading to an indirect effect on the pigmented epithelium of the retina.

Altogether, these results indicate that the ability of the embryo to respond to Sox10 overexpression by expansion of Slug expression domain and ectopic production of Trp-2-expressing cells and differentiated melanocytes are lost during gastrulation in whole embryo and animal explants, suggesting that Sox10 functions in the early phases of neural crest specification to generate the pigment cell lineage.

Increased pigment cells in response to Sox10 overexpression do not result from a conversion of cranial neural crest into trunk crest

The formation of supernumerary melanocytes in embryos overexpressing Sox10 could be explain by a conversion of cranial crest into trunk neural crest rather than by a de novo induction of neural crest progenitors. Previous studies have shown that Sox9 depletion in *Xenopus*, by means of a morpholino antisense oligonucleotide (Sox9-AS), results in a loss cranial neural crest (Spokony et al., 2002). Therefore, we analyzed the pattern of pigment cells in embryos overexpressing Sox10 and in which the development of cranial neural crest cells has been compromised by injection of Sox9-AS. The prediction is that, if conversion from cranial to trunk crest occurs, formation of ectopic pigment cells observed in Sox10-injected embryos, at stage 40, should be dramatically impaired in the context of Sox9-depleted embryos. Our results indicate that ectopic formation of pigment cells in Sox10-GR-injected embryos, treated with dexamethasone at stage 10, is not significantly different in the context of Sox9-depleted embryos as compared with embryos expressing normal levels of Sox9 (Table 1), suggesting that supernumerary pigment cells induced by Sox10 overexpression do not result from a conversion of cranial into trunk neural crest. This conclusion is further supported by the observation that Sox10 overexpression does not lead to noticeable craniofacial defects.

Table 1
Increased pigment cells induced by Sox10 overexpression do not result from a conversion of cranial neural crest into trunk/vagal neural crest

	Dex st. 10	*n*	Number of pigment cells		
			Normal	Increased	Reduced
Sox9-AS	−	66	82%	−	18%
Sox10-GR	−	29	100%	−	−
Sox10-GR	+	55	35%	65%	−
Sox10-GR + Sox9-AS	−	36	89%	−	11%
Sox10-GR + Sox9-AS	+	34	32%	56%	12%

Discussion

In this study, we report the cloning of the *Xenopus* Sox10 gene and describe its expression in the neural crest-forming region of the embryo. In this tissue, Sox10 expression is regulated by Wnt signaling and colocalizes with Slug and Sox9, two major regulators of neural crest formation. Sox10 overexpression at the gastrula stages, using a hormone-inducible construct, expands neural crest progenitors. Later in embryogenesis, this expansion of neural crest progenitors is correlated with a massive overproduction of pigment cells. These results indicate that Sox10 is an important component of the regulatory cascade involved in neural crest formation and support the existence of a Sox10-dependent pathway involved in the induction of neural crest progenitors fated to form melanocytes.

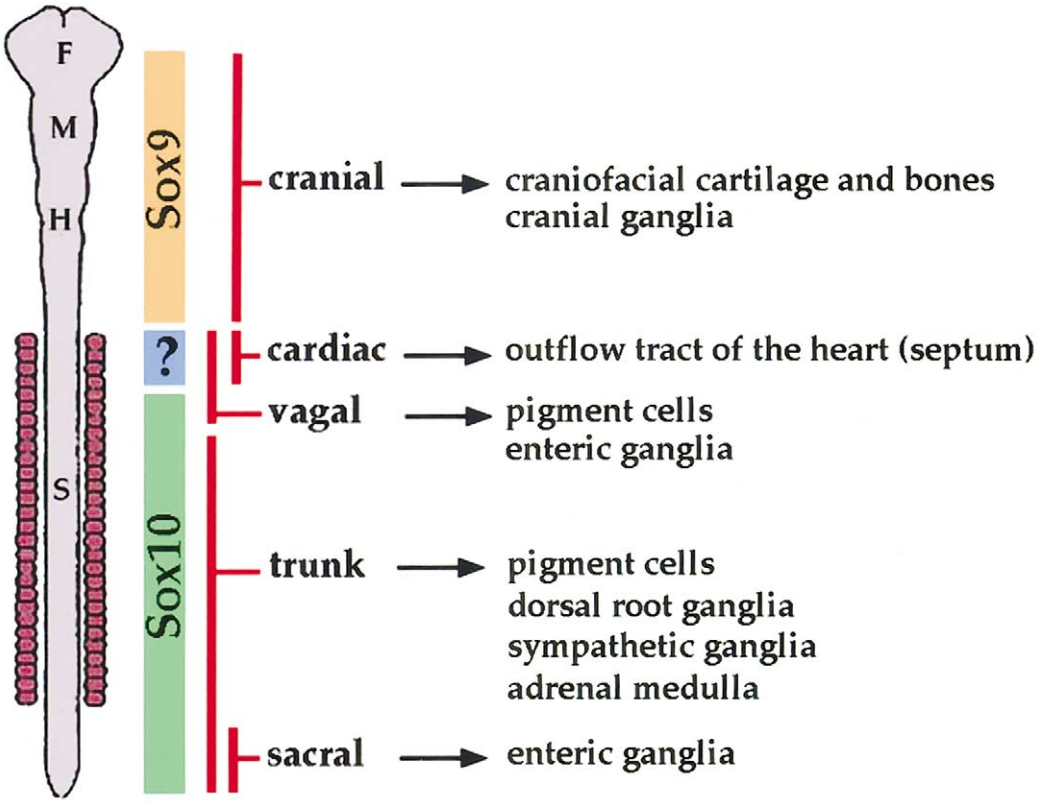

Fig. 7. Sox proteins regulate the development of neural crest derivatives from different axial levels. Sox proteins differentially expressed in neural crest progenitors are involved in the development of neural crest derivatives from different axial levels. Sox9 and Sox10, respectively, function in the formation of cranial (cartilage and bone) and trunk/vagal (pigment cells, enteric and sensory ganglia) neural crest derivatives. Whether Sox9 or another Sox family member is involved in the development of cardiac neural crest lineage remains to be determined. F, forebrain; H, hindbrain; M, midbrain; S, spinal cord.

Significance of Sox10 expression pattern for neural crest formation

Sox10 accumulates shortly after gastrulation at the lateral edges of the neural plate. In this tissue, it colocalizes with a number of early genes activated in response to neural crest-inducing signals, including Slug, Snail, Sox9, and FoxD3. Temporally, these genes precede Sox10 expression by a few hours (Mayor et al., 1995; Essex et al., 1993; Linker et al., 2000, Sasai et al., 2002, Spokony et al., 2002). A similar delay in Sox10 expression as compared with Slug was also reported in chick neural crest progenitors (Cheng et al., 2000). Following its initial activation at the neural plate border, Sox10 is broadly expressed in neural crest progenitors along the entire anteroposterior axis, similar to Sox9 (Spokony et al., 2002), with Sox10 being excluded from the medial neural crest. As neural crest cell migration is initiated, Sox10 is strongly expressed in cranial neural crest cells as they migrate into the branchial arches. This is consistent with Sox10 expression pattern in chick and human (Cheng et al., 2000; Bondurand et al., 1998) but is in contrast to mouse and zebrafish, in which Sox10 is not expressed in migrating cranial neural crest cells (Kuhlbrodt et al., 1998; Dutton et al., 2002). By the tailbud stage, Sox9 and Sox10 develop complementary and nonoverlapping expression domains. While Sox9 expression persists primarily in cranial neural crest cells of the pharyngeal arches, Sox10 expression is downregulated in the cranial region and is exclusively maintained in trunk neural crest cells at the dorsal midline of the neural tube.

Humans, mice, and zebrafish carrying mutations in Sox10 (Pingault et al., 1998; Southard-Smith, 1998; Herbarth et al., 1998; Dutton et al., 2001) suffer from defects in vagal/trunk neural crest derivatives (enteric ganglia, pigment cells, and dorsal root ganglia), but not in cranial (craniofacial cartilages and bones) or cardiac (outflow tract of the heart) neural crest derivatives (Kapur, 1999), suggesting a strict requirement for Sox10 in vagal and trunk neural crest derivatives. Consistent with this view, Sox9 depletion in *Xenopus* embryos, using morpholino antisense oligonucleotides, resulted in defects in craniofacial skeletal elements, while pigment cell lineage appeared to be largely unperturbed (Spokony et al., 2002). This interesting parallel between Sox9 and Sox10 function in the cranial and trunk/vagal neural crest, respectively, suggests that differentially expressed Sox proteins are required for the development of distinct neural crest derivatives as they emerge from different axial levels (Fig. 7). However, it is not clear whether Sox9 is also required for the development of cardiac neural

crest derivatives or whether a distinct family member is required for this lineage (Fig. 7).

Position of Sox10 in the regulatory cascade leading to neural crest formation

The Wnt signaling pathway has been implicated in neural crest formation in a number of species (reviewed in Wu et al., 2003). Sox10 is regulated by Wnts as activation of Wnt signaling expands, whereas blockage of Wnt signaling inhibits Sox10 expression in the neural crest-forming region. This result indicates that Sox10 expression is regulated by Wnt signaling and also suggests the existence of a pathway required for Sox10 activation at the neural plate border. A number of genes involved in the control of neural crest formation, including Slug, Sox9, and AP2, have also been shown to be regulated by Wnt signaling in vivo (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Deradorff et al., 2001; Luo et al., 2003), suggesting that these factors may act in the same regulatory pathway, or that they may act in parallel pathways sharing a common upstream Wnt regulator.

In *Xenopus*, a number of transcription factors have been found to be expressed in the developing neural crest, including Slug, (Mayor et al., 1995), Snail (Essex et al., 1993), Twist (Hopwood et al., 1985), Pax3 (Bang et al., 1997), Zic3 (Nakata et al., 1997), Zic5 (Nakata et al., 2000), Foxd3 (Dirksen and Jamrich, 1995; Sasai et al., 2001), Sox9 (Spokony et al., 2002), and AP2 (Luo et al., 2003). Some of these factors have also been implicated at different levels in the regulation of neural crest formation, migration, or both (Nakata et al., 1997; Nakata et al., 2000; LaBonne and Bronner-Fraser, 2000; Pohl and Knochel, 2001; Sasai et al., 2001; Spokony et al., 2002; Luo et al., 2003).

We found that loss of Slug or Sox9 function in the developing embryo prevented Sox10 expression, indicating that Slug and Sox9 are both required for Sox10 expression. This also suggests that Slug and Sox9 may act upstream of Sox10 during neural crest formation, consistent with the timing of Sox10 versus Slug/Sox9 expression at the neural plate border. However, Slug, Sox9, and Sox10 appear to be regulating each other's expression in a manner suggesting that they may be reciprocally involved in maintaining their expression, as previously described for other genes expressed at the neural plate border (Sasai et al., 2001). While it is somewhat difficult to understand the early regulation of Sox9 by Sox10, it may indicate that Sox genes may have multiple essential roles during the steps leading to development of the neural crest, initially in the specification of progenitors from different axial levels and later in the differentiation of these precursors into specific lineages. Recent studies on zebrafish Sox9 suggest that this might be the case (Yan et al., 2002; Li et al., 2002).

In all, these results indicate that Sox10 is a component of the regulatory cascade leading to neural crest formation. However, further studies will be needed to fully characterize and understand the hierarchy of the molecular regulators involved in the multiple steps leading to the generation of neural crest progenitors.

Functional domains of Sox10 protein

Sox proteins are characterized by the presence of a HMG box (DNA binding domain) encoded by a domain of about 80 amino acids and a transactivation domain at the C-terminal portion of the molecule. Sox proteins are believed to regulate their target genes through interaction with specific partner factors allowing Sox proteins to act in a cell-specific manner (Kamachi et al., 2000).

Our structure-function analysis revealed that the C-terminal domain of Sox10, in the absence of the DNA binding domain (ΔSox10 construct), is sufficient for Sox10-mediated activation of Slug. This result predicts that at least one Sox10-interacting molecule is capable of mediating Sox10 function in the absence of the HMG box. Therefore, it can be argued that the Sox10-interacting partner is sufficient to recruit ΔSox10 to the DNA, in the vicinity of Sox10-target gene, thereby allowing transcription through the unaltered Sox10 transactivation domain. By extension, we can predict that in the developing embryo Sox10 activity will be largely dependent on the availability of this putative partner molecule. Candidate partner molecules that interact with Sox10 to regulate expression of target genes include Pax3 (Bondurand et al., 2000; Potterf et al., 2000; Lang et al., 2000) and the POU-domain protein Tst-1/Oct6/SCIP (Kuhlbrodt et al., 1998). In *Xenopus*, Pax3 is expressed in the neural crest-forming region (Bang et al., 1997; 1999) and is therefore a viable candidate to play this role. Whether Pax3 or any other factors directly interact with Sox10 at the neural plate border remains to be determined. Moreover, the characterization of downstream target genes for Sox10 is critical to further our understanding of Sox10 function in the context of the developing neural crest.

Role of Sox10 in neural crest-derived melanocytes formation

Melanocytes represent one major derivative of the neural crest. These cells start as nonpigmented precursors, or melanoblasts, migrating along specific dorsolateral pathways into the epidermis. Initially, there is a difference in the production of melanoblasts between the cephalic and the trunk neural crest. In amphibian embryos, it is believed that a majority of melanoblasts are derived from the trunk and that approximately one-third of melanoblasts are contributed by the cephalic region (Model and Dalton, 1968). All the pigment cells of the body originate from the neural crest except the pigment cells of the retina, which derive from the neural epithelium of the optic vesicle.

A number of genes, including Mitf, c-kit, Pax3, Ednrb, and End3, have been implicated in the development of the melanocyte lineage (reviewed in Goding, 2000). A current

model of the transcriptional hierarchy involved in melanocyte formation placed Sox10 upstream of Mitf and c-Kit. Consistent with this model, homozygous Sox10 mutant mice are severely deficient in melanoblasts (Pingault et al., 1998; Southard-Smith, 1998; Potterf et al., 2001), and these cells failed to express markers such as Mitf, Trp-2, and c-kit (Brisch et al., 2001; Potterf et al., 2001).

Because Sox10 overexpression expands both the pan-neural crest (Slug) and a melanocyte-specific marker (Trp2), it was critical to define more precisely the window of time during which Sox10 is functioning. When Sox10-GR-injected embryos were treated with dexamethasone prior to or during gastrulation, expression of Slug and Trp-2 were greatly increased. Expansion of Slug and Trp-2 at stage 17 and stage 32, respectively, suggests that the expression of both genes have a similar requirement with regard to Sox10. Addition of dexamethasone during the phases of neural crest migration (stage 22 and beyond) was not able to induce ectopic production of Trp-2-positive cells and, therefore, rules out an involvement of Sox10 in neural crest migration.

We have shown that supernumerary pigment cells induced by Sox10 overexpression do not result from a conversion of cranial neural crest into trunk/vagal neural crest. Two pieces of evidence support this view: (1) Sox10 overexpression do not lead to craniofacial defects, and (2) in embryos lacking Sox9 protein, which are believed to be deficient in cranial neural crest (Spokony et al., 2002), ectopic formation of pigment cells by Sox10 overexpression is not significantly lower than in embryos expressing normal levels of Sox9 (Table 1). This suggests that increased pigment cells observed upon Sox10 overexpression result from the induction of ectopic trunk neural crest progenitors. Alternatively, one cannot exclude the possibility that these cells may arise from the direct conversion of epidermal ectoderm into melanocytes. However, the parallel between Slug expansion (at early stage) and ectopic production of Trp-2-positive cells/melanocytes (at later stage) favors the interpretation of a Sox10-mediated induction of crest progenitors.

Loss of Sox10 function in the mouse and zebrafish (Southard-Smith et al., 1997; Kapur et al., 1999; Sonnenberg-Reithmacher et al., 2001; Dutton et al., 2002) has been shown to result in increased apoptosis of nonectomesenchymal neural crest progenitors, supporting a role for Sox10 in neural crest survival. Our results indicate that Sox10 is also implicated in the specification of neural crest progenitors, including those fated to form the pigment cell lineage.

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