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Requirement of *FoxD3*-class signaling for neural crest determination in *Xenopus*

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SUMMARY

Fox factors (winged-helix transcription factors) play important roles in early embryonic patterning. We show here that *FoxD3* (*Forkhead 6*) regulates neural crest determination in *Xenopus* embryos. Expression of *FoxD3* in the presumptive neural crest region starts at the late gastrula stage in a manner similar to that of *Slug*, and overlaps with that of *Zic-r1*. When overexpressed in the embryo and in ectodermal explants, FoxD3 induces expression of neural crest markers. Attenuation of FoxD3-related signaling by a dominant-negative *FoxD3* construct (*FoxD3delN*) inhibits neural crest differentiation in vivo without suppressing the CNS marker *Sox2*. Interestingly, these loss-of-function phenotypes are reversed by coinjecting *Slug*. In animal cap explants, neural crest differentiation induced by Slug and Wnt3a is also inhibited by *FoxD3delN* but not by a dominant-negative form of *XBF2*. Loss-of-function studies using dominant-negative forms of *FoxD3* and *Slug* indicate that Slug induction by Zic factors requires FoxD3-related signaling, and that *FoxD3* and *Slug* have different requirements in inducing downstream neural crest markers. These data demonstrate that *FoxD3* (or its closely related factor) is an essential upstream regulator of neural crest determination.

Key words: *FoxD3*, *Slug*, Neural crest, Dominant-negative mutant, *Xenopus*

INTRODUCTION

Neural crest cells originate from the ectoderm at the junction of the prospective neural plate and the prospective epidermis (Le Douarin and Kalcheim, 1999; Mayor et al., 1999). These unique cells are characterized by their extensive migration and ability to generate a large spectrum of cell types (Selleck et al., 1993; Bronner-Fraser, 1994; LaBonne and Bronner-Fraser, 1999). The derivatives of the neural crest include neurons and Schwann cells in the peripheral nervous system, adrenal medulla cells, pigment cells, facial cartilage cells and smooth muscle cells.

In *Xenopus*, the earliest gene markers of prospective neural crest are two genes encoding Zinc-finger transcription factors related to *Drosophila snail*, *Xenopus Snail* and *Slug*, which start to be expressed by the late gastrula stage (Essex et al., 1993; Mayor et al., 1995; reviewed in Mayor et al., 1999). Although *Slug* is suggested to function in specification and migration of neural crest cells (Nieto et al., 1994; Carl et al., 1999; LaBonne and Bronner-Fraser, 2000), relatively little is understood about the molecular mechanisms underlying neural crest determination in the early ectoderm.

Molecular embryological studies have indicated that several genes may be involved in neural crest determination. The neural inducers Noggin (Lamb et al., 1993) and Chordin (Chd; Sasai et al., 1995) do not induce neural crest cells when overexpressed alone in the *Xenopus* animal cap assay. In contrast, when Wnt or FGF acts in concert with Noggin or Chd, *Slug* is efficiently induced in the animal cap ectoderm (McGrew et al., 1995; Mayor et al., 1995; Sasai et al., 1996; Mayor et al., 1997; LaBonne and Bronner-Fraser, 1998). However, it remains to be elucidated how Wnt or FGF signals cooperate with anti-BMP signaling. Overexpression of transcription factors such as Zic and SoxD induce neural crest cells in the animal cap (Nakata et al., 1997; Nakata et al., 1998; Mizuseki et al., 1998a; Mizuseki et al., 1998b). Also, Sox2 overexpression together with FGF treatment induces neural crest markers and melanophores (Mizuseki et al., 1998a). However, as these transcription factors induce gene markers of both the central nervous system (CNS) and the neural crest, it is not clear whether their role in neural crest formation is direct or indirect.

Formation of the neural crest can be induced also by juxtaposing presumptive neural plate and epidermal tissues both in vitro and in vivo (Moury and Jacobson, 1990; Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995; Liem et al., 1995; Mancilla and Mayor, 1996). This raises the possibility that the neural crest formation is dependent on interactions between neural and non-neural tissues. However, zebrafish studies have suggested that a BMP gradient, which

patterns tissues along the dorsoventral axis during gastrulation, plays a crucial role in the formation and positioning of the neural crest (Nguyen et al., 1998; Barth et al., 1999). A *Xenopus* study (Marchant et al., 1998) also supports this mechanism.

To further understand the molecular regulation of neural crest formation, we investigated the role of the neural crest-specific winged-helix transcription factor *FoxD3* (formerly *Forkhead 6*) in *Xenopus*. *FoxD3* expression starts in the presumptive neural crest at the late gastrula stage, as early as that of *Slug*. Gain-of-function and loss-of function studies showed that FoxD3 acts as an essential upstream regulator of neural crest determination in complex regulatory pathways together with Slug and Zic factors.

MATERIALS AND METHODS

Isolation of *XFD6/FoxD3*

To search for genes activated by neural inducers plus FGF, a differential screen was performed as follows. 50 ng of *sog* (fly *Chd*; Holley et al., 1995) mRNA (tester) or water (driver) were injected into 4 animal blastomeres of 8-cell *Xenopus* embryos. Animal caps were dissected at stage 10.25 and cultured in 1× LCMR supplemented with 0.2% of BSA (Mizuseki et al., 1998a). 50 μg/ml human recombinant bFGF (Promega) was added to the culture medium of *sog*-injected caps. Animal caps were harvested for RNA isolation at stage 13. Tester-specific and control probes were prepared by subtracting (driver from tester) and (driver from driver), respectively. cDNA subtraction was performed by using a PCR-select subtraction kit (Clontech). 437 positive clones that gave tester-specific hybridization signals were obtained from screening 2×10^4 pfu of a stage 13 *Xenopus* neural plate library. One clone (99A; GenBank accession no. AB014611) expressed in the neural crest regions turned out to be a homologue of zebrafish *Forkhead 6*. In addition, *Zic-r1* (Mizuseki et al., 1998a), *Zic2* (Brewster et al., 1998), *Sox2* (Mizuseki et al., 1998a), *Six3* (Zhou et al., 2000), and *XFD12″/XFLIP/FoxD5c* (Sölter et al., 1999; Fetka et al., 2000) were also isolated. These genes were induced by Chd+FGF in the animal cap as expected from the cloning strategy.

Embryonic manipulation and in situ hybridization

Staging of embryos was done according to the normal table of Nieuwkoop and Faber. Synthetic RNA was injected using a fine glass capillary and a pneumonic pressure injector (Narishige) in 1× Barth's solution, and then embryos were transferred into 0.1× Barth's solution (Gurdon, 1976) until further manipulation or harvesting. RNA was injected into all four animal blastomeres of the 8-cell embryo unless stated otherwise. All the injection experiments were carried out at least twice and gave reproducible results. The total amount of injected mRNA was kept constant by adding neutral *GFP* mRNA when necessary.

For animal cap assays, animal caps were excised at stage 10.5 and cultured in 1× LCMR supplemented with 0.2% BSA until the required stage. Regarding *FoxD3* injection, we noticed that *FoxD3*-induced mesodermal differentiation in the animal caps was dependent on the dose and the stage of animal cap preparation. *FoxD3*-injected animal caps (100 pg/cap) contained a small amount of *MyoD*-positive tissues when caps were prepared from stage 9 embryos, while caps from stage 10.5 embryos did not express any of the mesodermal markers tested (Fig. 3G and not shown). It has also been noted that when a several-times higher dose of *FoxD3* mRNA is injected, more efficient mesodermal induction is observed in blastula animal caps (Dan Kessler, personal communication) but not in gastrula caps (not shown). Therefore, in this study we selected conditions that do not induce mesodermal differentiation.

Whole-mount in situ hybridization was performed as described previously (Chitnis et al., 1995) with minor modifications (Sive et al., 1998). For double in situ hybridization, one DIG-labelled probe was stained with BCIP (light blue; Promega) and the other fluorescein-labelled probe was stained with BM purple (indigo; Boehringer Mannheim).

Plasmid construction

The entire coding region of 99A was subcloned into pCS2 vector at the *Eco*RI and *Xho*I sites (pCS2-*FoxD3*). To generate the *FoxD3delN* construct, the carboxyl-terminal part of FoxD3 (amino acid residues 195-371) was amplified by PCR and subcloned into pCS2-NLS. *XBF2delN* (amino acid residues 169-345) was constructed in a similar way and used as a specificity control. To generate a *FoxD3-VP16* construct, the DNA-binding domain of *FoxD3* (amino acid residues 85-194) was fused to the *VP16* activation domain (Sadowski et al., 1988) and subcloned into pCS2 vector at the *Stu*I site. *pCS2-FoxD3delN-GR* was constructed by fusing the human glucocorticoid receptor ligand-binding domain to the FoxD3 carboxyl-terminal domain (Kolm and Sive, 1995). A *FoxA4* (amino acid residues 110-219) *-VP16* construct was similarly generated, and used as a control for specificity. *FoxA4*, formerly called *XFKH1/HNF3β/pintallavis/XFD1* (Dirksen and Jamrich, 1992; Ruiz i Altaba and Jessell, 1992; Knöchel et al., 1992), shares 60% amino acid identity with FoxD3 in the DNA-binding domain. *FoxA4-VP16* is active and mimics wild-type *HNF3β* (Pani et al., 1992) in inducing target genes. For instance, *FoxA4-VP16* induced the floor-plate marker *Kielin* in the animal cap when coinjected with *Chd* (Matsui et al., 2000, and data not shown). To generate a *FoxD3-EnR* construct, the DNA-binding domain of FoxD3 was fused to the *Drosophila engrailed* repressor domain (*EnR*; Conlon et al., 1996), and was subcloned into pCS2 vector at the *Cla*I site. The entire coding regions of *Slug* and *Wnt3a* were amplified by RT-PCR from stage17 embryo cDNA and subcloned into pCS2 vector. For mRNA injection, the plasmids were linearized with *Sac*II (*FoxD3-EnR*) or *Not*I (the other constructs), and transcribed with SP6 polymerase (mMessage mMachine, Ambion).

RT-PCR analysis

RT-PCR was performed as described previously (Kengaku and Okamoto, 1995; Mizuseki et al., 1998a; Kuo et al., 1998; Nakata et al., 1998; Kishi et al., 2000). The other primers used in this study were as follows: *Zic-r1* (Mizuseki et al., 1998a) (forward primer; ATGAACATGGCTGCCCACCAT, reverse primer; CACTCTGATG-TGGTTGATCAG; 282 bp, 25 cycles), *Ets-1* (Meyer et al., 1997) (forward primer; GAGGGCTAAAGAAATAACATGCTC, reverse primer; CATAGACTTTTACAAGAAGGC; 231 bp, 28 cycles), *Ephrin-B2* (Smith et al., 1997) (forward primer; AGGACTGCAGAG-GGTGTATTCTGC, reverse primer; TTTTTAGGCATAACGAGCC-ACTTC; 210bp, 28cycles), *FoxD3* (forward primer; TCTCTGGGG-CAATCACACTC, reverse primer; GTACATTTGTTGATAAAGGG; 278 bp, 28 cycles), and *SoxD* (Mizuseki et al., 1998b) (forward primer; ACCAGGAGCTCTATGGGTACC, reverse primer; CTA-GATTCTCAAGTCAGTAGA; 240 bp, 28 cycles).

RESULTS

Isolation of *Xenopus FoxD3*

We previously reported a systematic differential hybridization screen for downstream genes of the neural inducer *Chd* (Mizuseki et al., 1998a). Although the screen identified several early regulators of neural differentiation, including *Zic*, *Sox2* and *SoxD*, we failed to isolate region-specific neural genes by this strategy (Mizuseki et al., 1998a; Mizuseki et al., 1998b). One possible reason is that Chd induces in the animal cap mainly archencephalic tissues, which remain immature until

late stages (Sasai et al., 1995). In contrast, the combination of Chd and FGF promotes differentiation of more mature cells with various regional markers, such as the floor plate, neural crest and posterior neural markers (Sasai et al., 1996). Therefore, we attempted to isolate genes activated by Chd and FGF in the animal cap ectoderm during the early phase of differentiation.

Chd/FGF-treated animal caps and control caps were prepared at early gastrula stages and harvested at the late gastrula stage equivalent as described in Materials and Methods. A differential screen was performed on 2×10^4 pfu of a *Xenopus* neural plate cDNA library (stage 13) by using RNAs from treated and control animal caps as probes. We identified 437 clones expressed preferentially in Chd/FGF-treated caps. Among them, two clones encoded *Fox*-class transcription factors. One is expressed in the CNS midline and turned out to be *FoxD5* (formerly called *XFD-12/XFLIP*; King and Moore, 1994; Sölter et al., 1999; Fetka et al., 2000). The other is a *Xenopus* homologue of zebrafish *forkhead 6* (Scheucher et al., 1995; Odenthal and Nüsslein-Volhard, 1998; Kelsh et al., 2000; now renamed *FoxD3*; Kaestner et al., 2000). Because of its intriguing expression in the neural crest, *FoxD3* was chosen for further investigation.

Spatial and temporal distribution of *Xenopus FoxD3*

FoxD3 expression starts at stage 10.25 on the dorsal surface of the gastrula (posterior neuroectoderm region). Fig. 1A shows *FoxD3* expression at stage 11. *FoxD3* is not expressed in the involuting marginal zone located just above the dorsal lip (indicated by the arrowhead) at this stage. By stages 11.5-12, *FoxD3* expression in the posterior neuroectoderm is gradually fading, while it becomes detectable in the dorsal mesoderm (Fig. 1C and data not shown). By stage 12.5, the presumptive cephalic neural crest regions start to express *FoxD3* (Fig. 1E). The onset and the spatial distribution of *FoxD3* in the neural crest regions are similar to those of *Slug* (Fig. 1F). Double in situ hybridization (Fig. 1I,J) with *Sox2* (light blue; a CNS-specific marker at this stage) shows that the expression patterns of *FoxD3* and *Slug* (indigo) in the ectoderm are indistinguishable in terms of positioning relative to the *Sox2* distribution. *FoxD3* expression (indigo; Fig. 1K) is located in the lateral and posterior part of the Zic-r1-positive area (light blue). Histological analyses (Fig. 1L) showed *FoxD3* expression in the ectoderm adjacent to the neural plate (arrowhead and np) and in the paraxial mesoderm (arrow). At late tailbud stages, *FoxD3* is also expressed in the trunk neural crest and in the migrating cephalic neural crest cells (data not shown).

FoxD3 expression largely overlaps with *Slug* expression, and demarcates the neural crest lineage in the ectoderm as early as the late gastrula stage.

Regulation of *FoxD3* expression in the animal cap ectoderm

To understand the molecular basis of *FoxD3* expression, we performed RNA microinjection studies by using animal cap explants. Wnt signals (Wnt3a, Wnt8 and Wnt1) have been implicated in neural crest formation (Mayor et al., 1995; Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998). In mice, *Wnt1* and *Wnt3a* are expressed in the dorsal roof of the neural tube (Wolda et al., 1993; McGrew et al., 1997), and are essential for proper neural crest development and dorsal CNS specification (Ikeya et al., 1997). Overexpression of Chd and Wnt3a efficiently induced *FoxD3* and *Slug* (Fig. 2G,H; 86%, *n*=44 and 90%, *n*=41, respectively) whereas little induction was observed after injection of *Chd* or *Wnt3a* mRNA alone (Fig. 2C-F; *n*=30 each). Overexpression of Slug and Wnt3a, but not Slug alone, activated *FoxD3* and *Slug* transcription (Fig. 2I-L).

As shown above, Wnt requires additional signals (e.g., Chd) to initiate neural crest differentiation in the ectodermal explant. In contrast, *Zic-r1* injection by itself was sufficient to induce *FoxD3* and *Slug* transcription (Fig. 2M,N; 97%, *n*=38 and 94%, *n*=36, respectively). Since the onset of *Zic* expression occurs before that of *FoxD3* in the neural crest regions (Mizuseki et

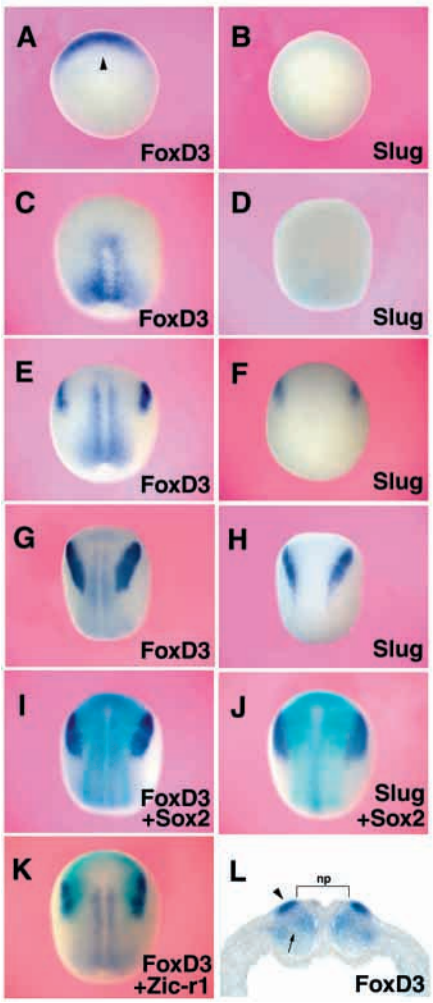


Fig. 1. Expression of *FoxD3* in early *Xenopus* embryos and comparison to that of *Slug*. (A-H) Spatial and temporal expression of *FoxD3* (A,C,E,G) and *Slug* (B,D,F,H) analyzed by whole-mount in situ hybridization. (A,B) Early gastrula stage 11 (vegetal view), arrowhead indicates dorsal lip; (C,D) mid-gastrula stage 12; (E,F) late gastrula stage 12.5; and (G,H) mid-neurula stage 16. (I-K) Double-labeled in situ hybridization. (I) *Sox2* (light blue) and *FoxD3* (indigo), (J) *Sox2* (light blue) and *Slug* (indigo), (K) *Zic-r1* (light blue) and *FoxD3* (indigo). (C-K) Dorsal views. (L) Histological analysis of *FoxD3* distribution at mid-neurula stage. np, neural plate.

al., 1998a; Nakata et al., 1998), Zic factors appear to be good candidates for activators of *FoxD3*.

Induction of neural crest and neural markers by overexpression of *FoxD3*

The intriguing expression pattern of *FoxD3* in the presumptive neural crest regions led us to test the effects of FoxD3 on neural crest differentiation. We injected *FoxD3* mRNA into two left animal blastomeres of 8-cell embryos and analyzed them at the tailbud stage (Fig. 3). *FoxD3* overexpression caused ectopic expression of neural crest markers such as *Slug* (40%, *n*=58; Fig. 3A), endogenous *FoxD3* (40%, *n*=43; Fig. 3B), *Ets-1* (55%, *n*=88; Fig. 3C) and *AP-2* (Saint-Jeannet et al., 1997) (43%, *n*=61; data not shown). We next tested whether FoxD3 overexpression induced ectopic expression of the CNS marker *Sox2*. *Sox2* was also induced by *FoxD3* injection in ectopic positions (60%, *n*=34) (Fig. 3D). Thus, overexpression of FoxD3 induces neural crest and neural markers in vivo.

To determine whether *FoxD3* can induce neural crest cells in the isolated animal cap explant, *FoxD3* mRNA was injected into 4 animal blastomeres (25 pg/cell) of 8-cell embryos, and animal caps were excised from stage 10.5 gastrula embryos. In this case, animal caps were prepared from embryos from an albino mother and a pigmented father, so that pigments would be derived only from zygotic synthesis (Mizuseki et al., 1998a). After 2 days inculture in vitro, *FoxD3*-injected caps contained a significant number of melanophores (Fig. 3F; 65%, *n*=33), in contrast to control caps (0%, *n*=25; Fig. 3E; inset, a sibling embryo), showing that FoxD3 induces production of a mature type of neural crest derivative.

We then tested whether *FoxD3* can induce early neural crest markers in the animal cap using RT-PCR (Fig. 3G). After 1 day in culture, injection of *FoxD3* induced *Slug*, *Twist*, endogenous *FoxD3*, *Zic* factors (*Zic-r1*, *Zic2*; neural crest and dorsal CNS markers at the neurula stage), *Ets-1* (a late neural crest marker) and *Ephrin-B2* (a late neural crest marker; Smith et al., 1997; data not shown) in the animal cap. These results demonstrate that *FoxD3* can initiate neural crest differentiation in the ectodermal explant. *FoxD3* did not induce the mesodermal marker *M-actin* (Fig. 3G) or the neural inducers *Noggin* or *Chd* (data not shown) under these conditions (see Materials and Methods).

FoxD3 overexpression also induced the panneural markers *Sox2*, *NCAM*, *SoxD* and *Xngnr-1* (primary neurons), and suppressed the epidermal marker *Keratin* (Fig. 3G). Expression of regional markers such as *Otx2* (forebrain), *En2* (midbrain-hindbrain border), *Krox20* (hindbrain), *Xlhbox1* (anterior spinal cord), *HoxB9* (posterior spinal cord), *Pax3* (dorsal CNS) and *Pax6* (forebrain and ventral CNS) was also observed in *FoxD3*-injected caps (data not shown). These results show that *FoxD3* induce neural crest and neural differentiation in the isolated ectodermal explant.

Previous studies have reported several Fox family factors expressed in early *Xenopus* neuroectoderm such as *FoxA4* (*HNF3β*), *XBF1* (a homologue of mouse *FoxG1*) and *XBF2* (a homologue of mouse *FoxD1*; Kaufmann and Knöchel, 1996; Bourguignon and Papalopulu, 1998; Mariani and Harland, 1998; Gómez-Skarmeta et al., 1999). It has been shown by RNA microinjection that *XBF1* and *XBF2* possess strong neuralizing activities as does *FoxD3* (Bourguignon and Papalopulu, 1998; Mariani and Harland, 1998). Taken together

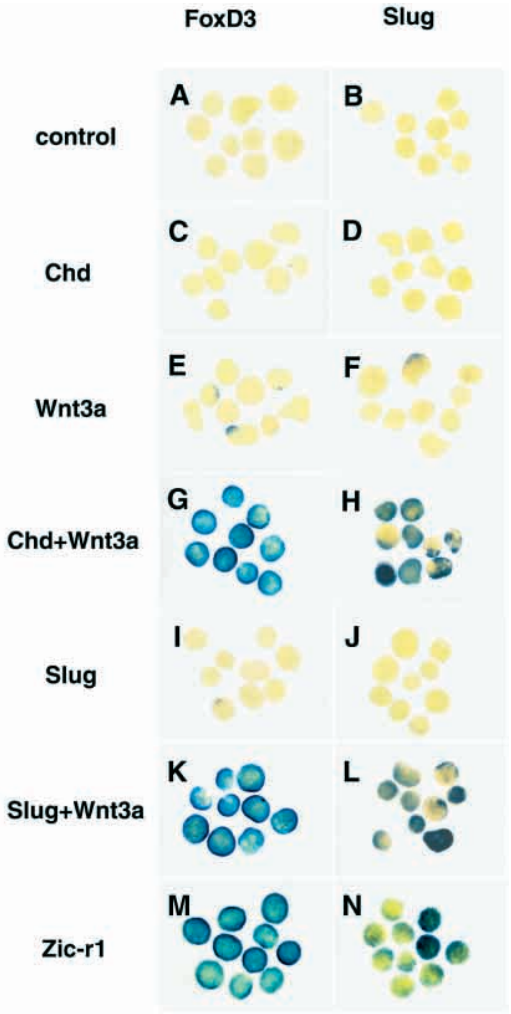


Fig. 2. Regulation of *FoxD3* expression in animal cap assay. Animal caps were prepared from stage 10.5 embryos that had been injected with (A,B) control mRNA (100 pg), (C,D) *Chd* mRNA (50 pg), (E,F) *Wnt3a* mRNA (50 pg), (G,H) *Chd* and *Wnt3a* mRNAs, (I,J) *Slug* mRNA (50 pg), (K,L) *Slug* and *Wnt3a* mRNAs, and (M,N) *Zic-r1* mRNA (100 pg). The animal caps were harvested at stage 17 and analyzed with *FoxD3* probe (A,C,E,G,I,K,M) or *Slug* 3′UTR probe (B,D,F,H,J,L,N).

with our results, these observations raise two crucial questions in terms of specificity. First, does FoxD3 induce neural crest markers by acting on its own target genes, or by acting on the target genes of related *Fox* genes such as *XBF2* (FoxD3 has the highest homology to XBF2 in the DNA-binding domain among the *Fox* family factors; 89%)? Second, does FoxD3 induce neural crest differentiation primarily by acting in the neural crest precursors, or secondarily by promoting ectopic formation of CNS tissues (as indicated by ectopic *Sox2* induction)? The latter possibility is suggested by the finding that neural plate tissues induce neural crest formation when juxtaposed with epidermal tissues (Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996).

To start addressing these questions, we first examined the effect of *XBF2* overexpression in the animal cap explant. A previous study showed that *XBF2* overexpression in vivo induces ectopic neural differentiation without activating *Slug*

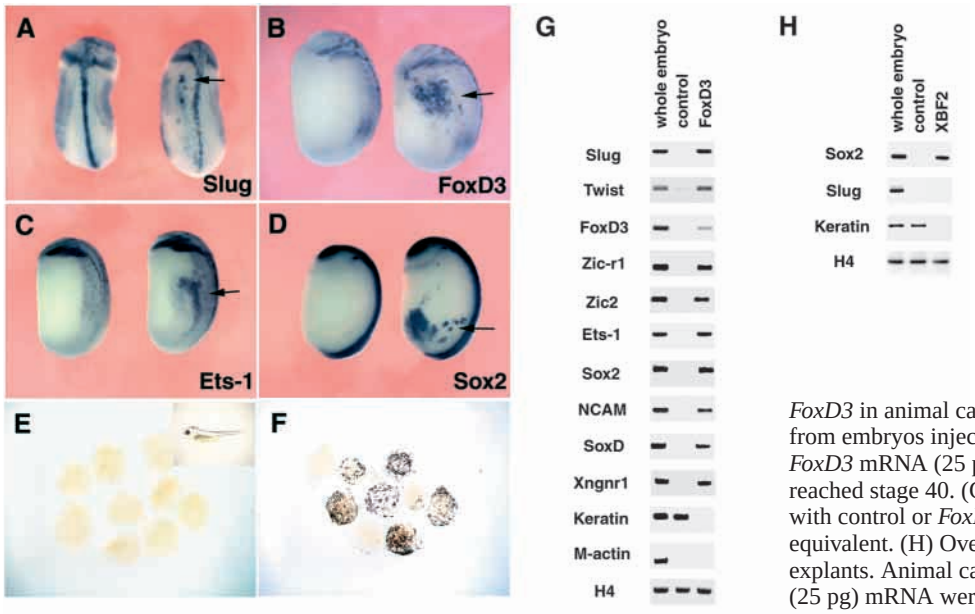


Fig. 3. Overexpression of *FoxD3* promotes neural crest differentiation as well as neuronal differentiation in vivo and in vitro. (A-D) *FoxD3* mRNA (100pg) was injected into the left animal blastomeres at the 8-cell stage. Embryos were harvested at stage 23 and subjected to whole-mount in situ hybridization with (A) *Slug*, (B) *FoxD3*, (C) *Ets-1*, and (D) *Sox2* probes. Ectopic expression is shown by arrows. (E-G) Overexpression of *FoxD3* in animal cap explants. Animal caps were prepared from embryos injected with (E) control mRNA (25 pg) or (F) *FoxD3* mRNA (25 pg), and harvested when siblings (E inset) reached stage 40. (G) RT-PCR analysis. Animal caps injected with control or *FoxD3* mRNA were harvested at stage 17 equivalent. (H) Overexpression of *XBF2* in animal cap explants. Animal caps injected with control (25 pg) or *XBF2* (25 pg) mRNA were analyzed as in G.

transcription (Mariani and Harland, 1998). Consistent with the previous in vivo data, overexpression of *XBF2* in the animal cap induces the CNS marker *Sox2*, but not the neural crest marker *Slug* (Fig. 3H). This suggests some specificity for the role of *FoxD3* in neural crest differentiation. To further understand the specific roles of *FoxD3* in neural crest formation, we performed detailed dominant-negative studies.

A specific dominant-negative mutant, *FoxD3delN*, suppresses neural crest differentiation but not neural plate development in vivo

We generated a candidate for a dominant-negative *FoxD3* by deleting the amino-terminal domain including the conserved DNA binding domain (*FoxD3delN*; Fig. 4A); a similar strategy has been successfully used to generate dominant-negative constructs of *Sox2* and *SoxD* (Kishi et al., 2000; Mizuseki et al., 1998b). Coexpression of *FoxD3delN* in the animal cap suppressed *Sox2* and *Slug* induction by *FoxD3* (Fig. 4B lanes 3 and 4) and this suppression was rescued by increasing wild-type *FoxD3* (lane 5). In contrast, *FoxD3delN* did not suppress *Sox2* induction by *XBF2* (lane 6) even at a high dose (lane 7). In a reverse experiment, a similar specificity was observed with *XBF2delN* (XBF2 lacking the DNA-binding domain) and *FoxD3* (lanes 8-12). These results indicate that *FoxD3delN* works as a specific dominant-negative *FoxD3* construct which antagonizes the activity of *FoxD3* but not that of *XBF2*. This is consistent with the fact that *FoxD3* does not share significant homology with other *FoxD* subfamily factors such as *XBF2* in the carboxyl-terminal domain. However, despite the specificity shown above, the possibility that *FoxD3delN* may also interfere other *FoxD3*-related factors (including unidentified ones) cannot be excluded. Therefore, in the context of this work, a signaling activity that is disturbed by *FoxD3delN* is termed an activity of '*FoxD3*-related signaling'.

FoxD3delN was injected into the right animal blastomeres of 8-cell embryos and the effects on differentiation markers were examined at the neural plate stage. The neural crest markers *Slug*, *FoxD3*, *Twist* and *Ets-1* were strongly suppressed by *FoxD3delN* injection (reduced in 87% of 47 embryos, 64% of 33, 82% of 38, 78% of 23, respectively; Fig. 5A,B and data not shown). Suppression of the neural crest markers by *FoxD3delN* suggests that *FoxD3* (or its closely related factor) is essential for neural crest differentiation. The CNS marker *Sox2* was not suppressed in the neural plate but rather induced in the regions of injected embryos usually fated to be neural crest (79%, *n*=53; Fig. 5C). *FoxD3delN* did not significantly affect the expression of the epidermal marker *Keratin* or the mesodermal marker *MyoD* (*n*=25) (data not shown). The finding that the CNS marker was not suppressed by the dominant-negative *FoxD3* supports the idea that *FoxD3* plays a role primarily in neural crest differentiation and argues against the alternative possibility of secondary effects due to perturbation of the CNS determination.

FoxD3delN suppressed not only late neural crest markers such as *Twist*, but also early neural crest-specific transcription factor gene, *Slug*. This led us to test whether injection of *Slug* may reverse the phenotypes caused by *FoxD3delN* (Fig. 5D-O). Injection of *Slug* alone moderately upregulated expression of the neural crest markers *Slug*, *FoxD3* and *Twist* (Fig. 5D,G,J). Coinjection of *FoxD3delN* and *Slug* reversed the suppressing effects of *FoxD3delN* on the neural crest markers (Fig. 5E,F, *Slug*, 81%, *n*=37; panels H,I, *FoxD3*, 83%, *n*=40; panels K,L, *Twist*, 74%, *n*=42), and inhibited ectopic expression of *Sox2* (panels N,O, 75%, *n*=40). These data suggest a critical role for *Slug* in *FoxD3* signaling during neural crest determination.

FoxD3 is required for neural crest differentiation in the animal cap explant

To further examine the direct involvement of *FoxD3* in neural crest determination, we analyzed effects of *FoxD3delN* by using an animal cap assay in which neural crest forms in the absence of CNS tissues. A previous study showed that overexpression of Slug and Wnt induces neural crest markers without activating the neural plate marker *Sox2* (LaBonne and Bronner-Fraser, 1998). Consistent with that report, coinjection

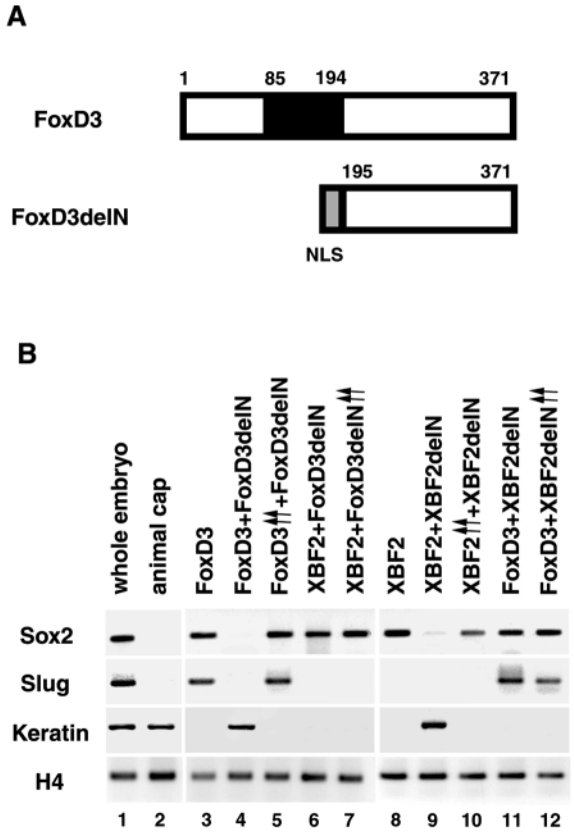
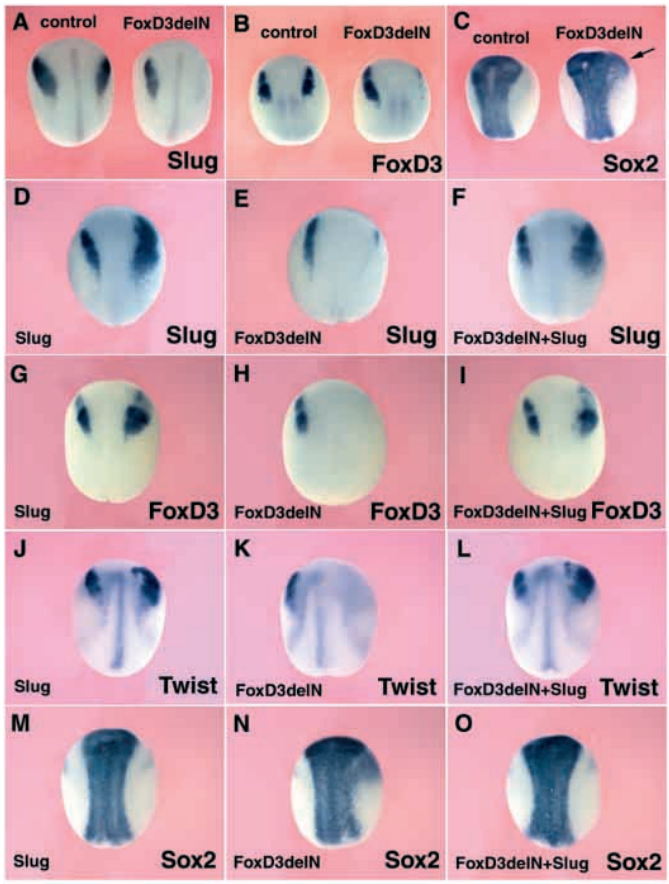


Fig. 4. Generation of a dominant-negative *FoxD3* construct. (A) A dominant-negative *FoxD3* construct, *FoxD3delN*. Amino acid residue numbers are indicated above. NLS (grey box), SV40 nuclear localization signal; black box, DNA-binding domain. (B) Animal caps were prepared from embryos injected with control RNA (150 pg), *FoxD3* (25 pg; lane 3), *FoxD3* (25 pg) + *FoxD3delN* (50 pg) (lane 4), *FoxD3* (100 pg) + *FoxD3delN* (50 pg) (lane 5), *XBF2* (25 pg) + *FoxD3delN* (50 pg) (lane 6), or *XBF2* (25 pg) + *FoxD3delN* (100 pg) (lane 7) mRNAs. As a specificity control, *XBF2delN* was also constructed (see Materials and Methods). Animal caps were prepared from embryos injected with *XBF2* (25 pg) (lane 8), *XBF2* (25 pg) + *XBF2delN* (50 pg) (lane 9), *XBF2* (100 pg) + *XBF2delN* (50 pg) (lane 10), *FoxD3* (25 pg) + *XBF2delN* (50 pg) (lane 11), or *FoxD3* (25 pg) + *XBF2delN* (100 pg) (lane 12) mRNAs. They were harvested at stage17 equivalent and analyzed by RT-PCR.

of *Slug* and *Wnt3a* mRNAs induced the neural crest markers *Slug*, *FoxD3* and *Ets-1* without inducing the CNS marker *Sox2* or the mesodermal marker *M-actin* (Fig. 5P, lanes 2 and 3). *Slug* + *Wnt3a* did not induce expression of *XBF1*, *XBF2* or *FoxD5* in the animal cap (data not shown). Induction of the neural crest markers by *Slug* and *Wnt3a* was clearly opposed by coinjection of *FoxD3delN* (lane 4) but not by the control *XBF2delN* (lane 5). These results show that the requirement for *FoxD3* in neural crest differentiation is independent of the presence of CNS tissues, and strongly support the idea that *FoxD3* is involved directly as a key regulatory factor in neural crest differentiation.

Differential requirements for *Slug* and *FoxD3* in neural crest development

The present study (Fig. 5) and previous reports (LaBonne and



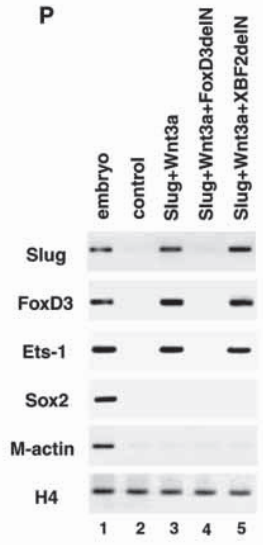


Fig. 5. *FoxD3* is required for neural crest development both in vivo and in animal caps. (A-C) Injection of *FoxD3delN* mRNA (50 pg) into two right blastomeres at the 8-cell stage suppressed *Slug* (A), and endogenous *FoxD3* (detected with 3′UTR probe) (B). *Sox2* was induced in the expected neural crest region (shown by an arrow in C) at stage 16. (D-O) Co-injection of *FoxD3delN* and *Slug* rescues the expression of neural crest markers. Injection of *Slug* mRNA (100 pg) into two right blastomeres at 8-cell stage moderately expands the expression of endogenous *Slug* (detected with 3′UTR probe) (D), *FoxD3* (G) and *Twist* (J), but not *Sox2* (M). Injection of *FoxD3delN* suppressed expression of the neural crest markers (E,H,K), while co-injection of *FoxD3delN* and *Slug* rescued their expression (F,I,L). Expansion of *Sox2* caused by *FoxD3delN* was suppressed by coinjecting *Slug* (N,O). (P) Animal caps were prepared from embryos injected with *Slug* (50 pg) + *Wnt3a* (50 pg; lane 3), *Slug* (50 pg) + *Wnt3a* (50 pg) + *FoxD3delN* (100 pg; lane 4), and *Slug* (50 pg) + *Wnt3a* (50 pg) + *XBF2delN* (100 pg; lane 5) mRNAs. They were harvested at stage17 equivalent and analyzed by RT-PCR.

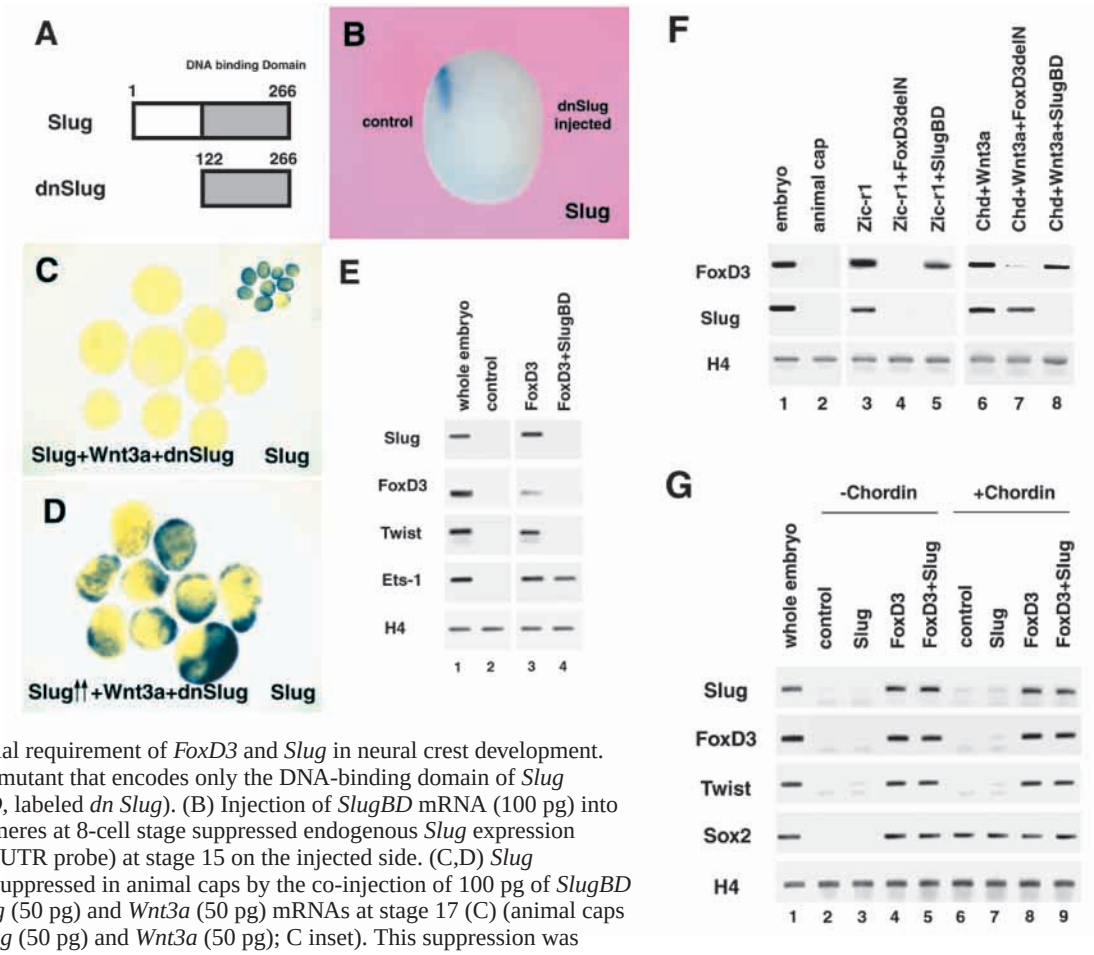


Fig. 6. Differential requirement of *FoxD3* and *Slug* in neural crest development. (A) A truncated mutant that encodes only the DNA-binding domain of *Slug* (bottom; *SlugBD*, labeled *dn Slug*). (B) Injection of *SlugBD* mRNA (100 pg) into two right blastomeres at 8-cell stage suppressed endogenous *Slug* expression (detected with 3′UTR probe) at stage 15 on the injected side. (C,D) *Slug* expression was suppressed in animal caps by the co-injection of 100 pg of *SlugBD* mRNA with *Slug* (50 pg) and *Wnt3a* (50 pg) mRNAs at stage 17 (C) (animal caps injected with *Slug* (50 pg) and *Wnt3a* (50 pg); C inset). This suppression was reversed by increasing wild-type *Slug* mRNA to 200 pg (D). (E) Effects of *SlugBD* on neural crest markers in *FoxD3*-injected animal caps. Control mRNA, *FoxD3* mRNA (25 pg), or combination of *FoxD3* (25 pg) and *SlugBD* (100 pg) mRNAs were injected into animal blastomeres in 8-cell stage embryos. Animal caps were prepared at stage 10.5 and harvested at stage 21 for RT-PCR analysis. (F) Animal caps were prepared from embryos injected with *Zic-r1* (100 pg; lane 3), *Zic-r1* + *FoxD3delN* (100 pg; lane 4), *Zic-r1* + *SlugBD* (100 pg; lane 5), *Chd* (50 pg) + *Wnt3a* (50 pg; lane 6), *Chd* + *Wnt3a* + *FoxD3delN* (lane 7), or *Chd* + *Wnt3a* + *SlugBD* (lane 8) mRNAs. Animal caps were harvested at stage 14 for RT-PCR analysis. (G) Animal caps were prepared at stage 10.5 from embryos injected with control (100 pg; lanes 2 and 6), *Slug* (50 pg; lanes 3 and 7), *FoxD3* (25 pg; lanes 4 and 8), or *FoxD3* + *Slug* (lanes 5 and 9) mRNAs, with (lanes 6-9) or without (lanes 2-5) *Chordin* (50 pg) mRNA.

Bronner-Fraser, 2000) have demonstrated that two early neural crest-specific transcription factors, *FoxD3* and *Slug*, are required for neural crest development. These two genes are expressed in an overlapping manner (Fig. 1) and are regulated by similar upstream genes (Fig. 2). Therefore, we next examined whether *FoxD3* and *Slug* function in the same pathway or have distinct roles in neural crest determination.

A recent report using dominant-negative constructs has shown that *Slug* function is required for expression of *Slug* and *Twist* and for proper migration of neural crest cells (LaBonne and Bronner-Fraser, 2000). A dominant-negative *Slug* construct (dnSlug) lacking the amino-terminal domain (*SlugBD*; Fig. 6A) was generated in accordance with that report, and used to analyze the requirement for Slug in FoxD3 signaling. Consistent with the previous report, *SlugBD* overexpression in the right half of the embryo inhibited endogenous *Slug* expression on the ipsilateral side (88%. *n*=32; Fig. 6B). *SlugBD* injection also suppressed other neural crest markers such as *FoxD3* (81%, *n*=21), *Twist* (86%, *n*=21) and *Ets-1* (100%, *n*=21), but not *Sox2* (*n*=18; not shown). Injection of *SlugBD* also inhibited *Slug* induction by *Slug* and *Wnt3a* in the animal cap (no *Slug* induction, *n*=32; Fig. 6C; inset, *Slug* and *Wnt3a* only, *Slug* induction 95%, *n*=21). This inhibition was rescued by increasing the amount of wild-type *Slug* (*Slug* induction 80%, *n*=40; Fig. 6D), showing that the dominant-negative effect was specific to *Slug* (or *Snail*-related factors; Essex et al., 1993).

Injection of *SlugBD* completely suppressed the induction of *Slug*, *FoxD3* and *Twist* by *FoxD3* in the animal cap (Fig. 6E lanes 3,4). In contrast, induction of *Ets-1*, a late neural crest marker, was not strongly affected by *SlugBD* (lanes 3,4). These results suggest the following relationship between *FoxD3* and *Slug* functions. First, *Slug*-related activity is essential for induction or maintenance of *Slug* and *FoxD3* in *FoxD3*-injected caps. Second, *Slug* is essential for *FoxD3* to induce *Twist*. Third, *FoxD3* can induce *Ets-1* without *Slug* activity in the animal cap, indicating that *Twist* and *Ets-1* are controlled in a different manner by *Slug* and by *FoxD3*. In the embryo, however, *SlugBD* injection does suppress both *Twist* and *Ets-1* as discussed above. One explanation for this discrepancy is

that the *SlugBD*-resistant portion of *Ets-1* expression is irrelevant to its neural crest expression. This seems unlikely to be the case, as *Ets-1* expression in the ectoderm is neural crest-specific during early tailbud stages (Meyer et al, 1997; we have also confirmed it ourselves). Another interpretation, which we think is more likely, is that suppression of *Ets-1* by *SlugBD* in vivo is caused secondarily by downregulation of the upstream regulator *FoxD3*.

Next we further examined the mutual requirements for *FoxD3* and *Slug* in the animal cap assay (Fig. 6F). First, we overexpressed *Zic-r1* with *FoxD3delN* or *SlugBD* in animal caps, and harvested the caps for RT-PCR analysis at the early neurula stage. Induction of *FoxD3* and *Slug* by *Zic-r1* was completely suppressed by *FoxD3delN* (Fig. 6F lanes 3,4). In contrast, *SlugBD* strongly suppressed induction of *Slug* itself but not of *FoxD3* (lane 5). Similar results were obtained in induction experiments using *Zic2* and *Zic3* (data not shown). These results suggest that induction of *Slug* by *Zic* factors is dependent on *FoxD3* signaling while induction of *FoxD3* does not rely on *Slug*.

As *FoxD3* and *Slug* are also induced by a combination of the extracellular signals Chd and Wnt (Fig. 2G), we next tested the requirements for *FoxD3* and *Slug* in this induction system. As in the experiment examining induction by *Zic* (Fig. 6F lane 5), *SlugBD* did not strongly inhibit *FoxD3* induction by *Chd+Wnt3a* (lane 8). Notably, a clear difference was found between the effects of *FoxD3delN* on the induction of *Slug* by *Zic* and by *Chd+Wnt3a* (compare lanes 4 and 7). *Slug* induction by *Chd+Wnt3a* was not affected by *FoxD3delN* (lane 7), suggesting that *Slug* induction by *Chd+Wnt3a* mainly utilizes signaling pathways other than that involving *Zic* and *FoxD3*.

The data above indicate a close relationship between *FoxD3* and *Slug* in early determination steps of neural crest development. This led us to test whether coinjection of *FoxD3* and *Slug* exerts synergistic effects on neural crest determination. First, we coinjected *FoxD3* and *Slug* into embryos and analyzed possible cooperative effects. We did not observe remarkable synergism beyond additive effects in vivo (data not shown). To further test possible cooperativity of *FoxD3* and *Slug*, we performed detailed animal cap experiments. As shown in Fig. 6G, coinjection of *FoxD3* and *Slug* (lanes 5,9) did not show significant effects on neural crest and neural markers as compared to *FoxD3* injection alone, regardless of the absence (lanes 2-5) or the presence (lanes 6-9) of the neural inducer Chordin. Thus, at least in these gain-of-function studies, *Slug* does not seem to be a limiting factor, and endogenous *Slug* induced by *FoxD3* is likely to be sufficient to initiate neural crest differentiation.

FoxD3 and *XBF2*, two transcriptional repressors, show distinct effects on *BMP4* suppression and *Slug* induction

Studies with reporter assays have shown that *XBF2* is a transcriptional repressor. A chimeric construct of the *XBF2* DNA-binding domain and the *En* repressor domain mimics the neuralizing activity of *XBF2*, while the chimeric construct of the *XBF2* DNA-binding domain and the *VP16* activation domain functions as a dominant-negative *XBF2* (Mariani and Harland, 1998). Therefore, we tested whether the same principle was applicable to *FoxD3*. Injection of the *FoxD3-En*^{R} construct caused neural differentiation of animal caps without inducing the mesodermal marker *M-actin* (Fig. 7A). In contrast, *FoxD3-VP16* suppressed the neuralizing activity of wild-type *FoxD3* and restored *Keratin* expression (lane 4). This suppression could be reversed by increasing the amount of wild-type mRNA (lane 5), indicating that the suppression was due to attenuation of *FoxD3*-related signaling. The dominant-negative effects were not observed with the *VP16*-fused *FoxA4* (*XFKH1*) construct (lane 6). These results indicate that *FoxD3*, like *XBF2*, is a transcriptional repressor.

Finally, we attempted to investigate the mode of action of *FoxD3* in neural crest induction. One model for the action of *XBF2* has been proposed by Mariani and Harland (Mariani and Harland, 1998). According to this model, *XBF2* suppresses *BMP4* expression in the ectoderm and promotes neural differentiation. We therefore tested a similar mechanism in neural crest induction by *FoxD3* by using animal cap assays. Animal caps injected with control *GFP*, *FoxA4* or *FoxD3delN* mRNA expressed *BMP4* strongly, but not *Sox2* or *Slug* (Fig. 7B-G and data not shown). Injection of *FoxD3*, *XBF2* or *dominant-negative BMP receptor* mRNA suppressed *BMP4* expression and induced *Sox2* expression (Fig. 7H,I,K,L,N,O). Suppression of *BMP4* by blocking BMP signaling is consistent with previous reports. Interestingly, at the doses that gave similar levels of *BMP4* suppression, only *FoxD3* injection induced *Slug* expression in the caps (Fig. 7J). Neither *XBF1* nor *XBF2* induced *Slug* at any of doses tested (25-100 pg/cell; Fig. 7M and data not shown). These observations indicate that induction of the neural crest marker by *FoxD3* cannot be simply explained by suppression of *BMP4* alone and involves distinct additional pathways.

DISCUSSION

FoxD3-related signaling is required for neural crest development

Fox family genes have been shown to play essential roles in the formation of specific ectodermal regions of vertebarte embyos. For instance, targeted disruption of mouse *BF-1* (*Foxg1*) causes a dramatical reduction of the telencephalon (Xuan et al., 1995). An essential role for *Foxe3* in mouse lens development has recently been reported (Blixt et al., 2000). In these cases, specification of the primordial cells of the telecephalon and lens occurs, but their proliferation is much reduced (Xuan et al., 1995; Blixt, 2000). A recent study in *Xenopus* suggests that *FoxG1* exerts distinct effects on determination and proliferation of CNS tissues depending on the dose (Hardcastle and Papalopulu, 2000).

Our present study demonstrates a role of *FoxD3*-related function in ‘determination’ of the neural crest. Attenuation of *FoxD3*-related function by *FoxD3delN* suppresses not only late neural crest markers but also the early neural crest markers *Slug* and *FoxD3* in vivo (Fig. 5), suggesting that initial specification of the neural crest is disturbed. Furthermore, *FoxD3*, which is expressed in early neural crest primordia, is sufficient for neural crest differentiation of the ectoderm (Fig. 3).

FoxD3 homologues have also been isolated from zebrafish, chicken and mice, and their neural crest expression is found to be conserved across species (Freyaldenhoven et al., 1997; Odenthal and Nüsslein-Volhard, 1998; Labosky and Kaestner,

1998; Yamagata and Noda, 1998). Future gene targeting studies, especially conditional disruption, may provide complementary data to the present work on the role of *FoxD3* in neural crest development.

Possible dual roles of *FoxD3*-related signaling in neural and neural crest differentiation

As discussed above, our results on the role of FoxD3-related signaling in neural crest differentiation are consistent in both overexpression and dominant-negative studies. Interestingly, the data on the CNS marker present an apparently puzzling situation since both gain-of-function and loss-of-function phenotypes involve upregulation of *Sox2* in the embryo (Figs 3D and 5C). Overexpression of wild-type FoxD3 induces ectopic *Sox2* expression in vivo (Fig. 3D). In contrrast, suppression of the neural crest markers by *FoxD3delN* is accompanied by expansion of *Sox2* (Fig. 5C). However, there are some qualitative differences between these two cases. In the FoxD3 overexpression study, ectopic *Sox2* expression can be induced at any injected region of the ectoderm, including ventral and posterior areas separated from the CNS (Fig. 3D). This is a distinct contrast to the dominant-negative data, in which *FoxD3delN*-induced *Sox2* expression is only seen in the cephalic neural crest region and is always contiguous to the endogenous *Sox2* expression in the neural plate (Fig. 5C). Another major difference is that *Sox2* induction in the animal cap assay is seen with wild-type *FoxD3*, but not with *FoxD3delN* (Fig. 3G and data not shown).

One interpretation of these results is that *FoxD3* has dual roles depending on the time of action and the region of ectoderm. During early gastrulation, *FoxD3* is expressed in posterior neuroectoderm (Fig. 1A) and the ability of *FoxD3* to induce neural differentiation is likely to be relevant to this expression. Suppression of *BMP4* expression by *FoxD3* (Fig. 7H) explains its neuralizing activity at least in part, as attenuation of BMP signaling by organizer factors has been shown to induce differentiation of the neuroectoderm in early gastrula embryos (Sasai et al., 1995; Piccolo et al., 1996; Zimmerman et al., 1996). Although *FoxD3delN* does not cause suppression of *Sox2* in the neural plate (Fig. 5C), this may be explained by some compensatory mechanisms, as a number of related Fox genes are expressed in the CNS (Kaufmann and Knöchel, 1996; Bourguignon and Papalopulu, 1998; Mariani and Harland, 1998; Gómez-Skarmeta et al., 1999). This idea is supported by our observation that injection of *FoxD3-VP16*, a less specific dominant-negative *FoxD3* that interferes with both *FoxD3* and *XBF2*, causes suppression of both *Slug* and *Sox2* in vivo (data not shown).

During late gastrulation, *FoxD3* expression is downregulated in the neural plate primodia and becomes apparent in the neural crest primordia (Fig. 1E). This second phase expression is consistent with the neural crest-inducing activity of *FoxD3*. *FoxD3* is the only *Fox* family gene so far reported to be expressed abundantly in the neural crest primordia of *Xenopus* gastrulae. (One paper reported a faint and transient expression of *XBF2* in the crest primordia of *Xenopus* neurulae while another paper reported no *XBF2* expression in the same region; Gómez-Skarmeta et al., 1999; Mariani and Harland, 1998) The expansion of *Sox2* by *FoxD3delN* at the cost of neural crest markers can be interpreted as a conversion of ectodermal fate from neural crest into a CNS type. According to this interpretation, in the absence of FoxD3-related signaling, ectodermal cells flanking the neural plate cannot differentiate into the neural crest but rather adopt a default CNS fate.

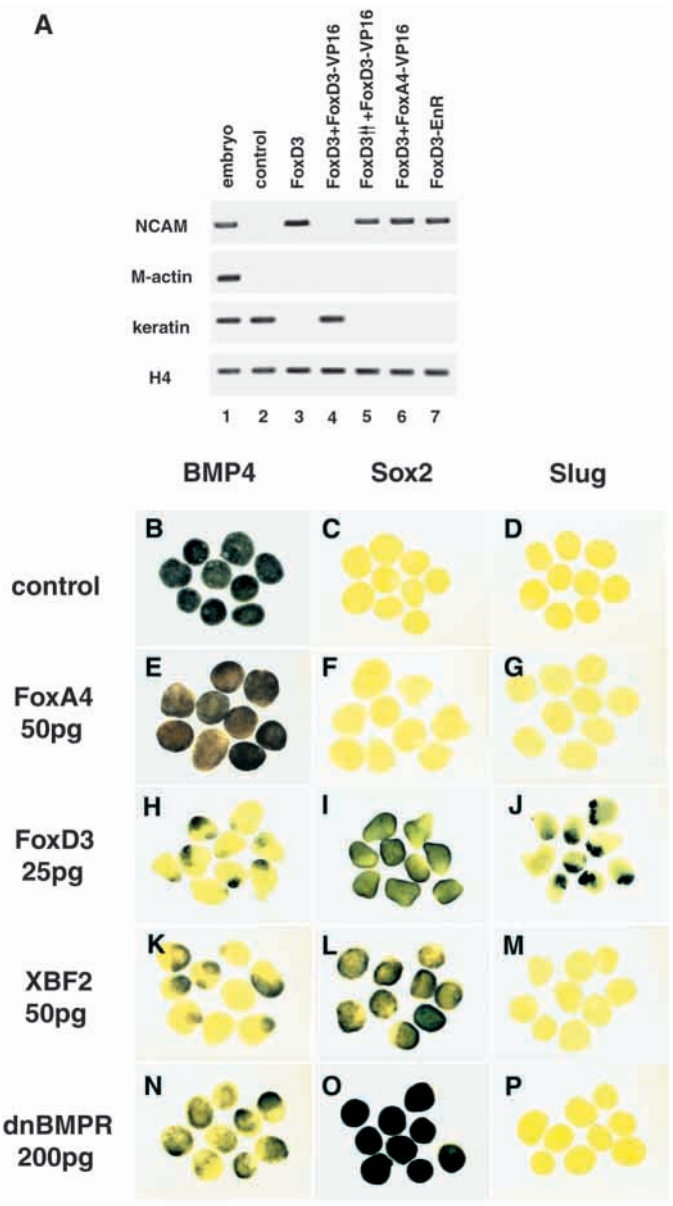


Fig. 7. *FoxD3* and *XBF2* show distinct transcriptional regulation in *Slug* induction. (A) *FoxD3* is a transcriptional repressor. Animal caps were prepared from embryos injected with *FoxD3* (25 pg; lane 3), *FoxD3* (25 pg) + *FoxD3-VP16* (50 pg; lane 4), *FoxD3* (75 pg) + *FoxD3-VP16* (50 pg; lane 5), *FoxD3* (25 pg) + *FoxA4-VP16* (100 pg; lane 6), and *FoxD3-EnR* (50 pg; lane 7) mRNAs. Animal caps were harvested at stage 17 and analyzed by RT-PCR. (B-P) Animal caps were prepared from embryos injected with control (50 pg; B-D), *FoxA4* (50 pg; E-G), *FoxD3* (25 pg; H-J), *XBF2* (50 pg; K-M) or *dnBMPR* (200 pg; N-P) mRNAs, and harvested at stage 17 equivalent. Animal caps were analyzed by in situ hybridization with *BMP4* (B,E,H,K,N), *Sox2* (C,F,I,L,O), and *Slug* (D,G,J,M,P) probes. Injection of *FoxA4*, *FoxD3*, *XBF2* and *dnBMPR* mRNA suppressed *BMP* expression and induced *Sox2* expression. In contrast, *FoxD3* induced *Slug* expression in the animal cap while neither *FoxA4*, *XBF2* nor *dnBMPR* did.

Consistently, epidermal *Keratin* expression is not significantly affected by *FoxD3delN* injection in vivo (data not shown). A similar observation of neural crest-CNS conversion was reported in a previous loss-of-function study of *Slug* (LaBonne and Bronner-Fraser, 2000). Therefore, it seems that early signaling involving *FoxD3* and *Slug* in the neural crest primordia is essential for the segregation of neural crest and CNS fates in the dorsal ectoderm.

We have attempted to establish the existence of stage-specific 'dual roles' of *FoxD3* by using inducible forms of FoxD3-fused with GR (Kolm and Sive, 1995). Unfortunately, we have not yet succeeded in generating such constructs because, for unknown reasons, all the GR-fused FoxD3 constructs we made exhibit high background activity in the absence of the activator ligand Dex. Future studies using transgenic frog techniques may resolve this problem. We also tried to understand the temporal requirement of FoxD3 signaling by using inducible GR-fused dominant-negative *FoxD3* (*FoxD3delN-GR*). This construct works fine, and neural crest markers are suppressed in the embryo injected with *FoxD3delN-GR* only when Dex is added to culture medium (our unpublished observations). In this case, injected embryos treated with Dex from stage 9 on and from stage 12 on exhibit similar extents of neural crest marker suppression, suggesting that *FoxD3* function from the late gastrula stage is essential for neural crest formation.

A model for the roles of *FoxD3*, *Slug* and *Zic* in neural crest development

To understand the molecular cascade in neural crest differentiation, we have studied transcriptional regulations involving *FoxD3* and *Slug*. Our working model of the relationships of *FoxD3*, *Slug* and *Zic* is as follows. *Zic* factors such as *Zic-r1* are induced widely in dorsal ectoderm (presumptive neural plate and neural crest regions) by neural inducers at the early gastrula stage. By the late gastrula stage, dorsoventral patterning in the dorsal ectoderm becomes evident. *Zic* expression is suppressed in the medial portion of the neuroectoderm, and *FoxD3* and *Slug* are induced in the presumptive neural crest regions. Animal cap studies showed that *Zic* factors induce both *FoxD3* and *Slug* in distinct fashions. *Slug* induction by *Zic* is dependent on *FoxD3* signaling (Fig. 6F lane 4) whereas *FoxD3* induction is largely independent of *Slug* activity (lane 5). In contrast, *Slug* induction by *Chd+Wnt* is not blocked by *FoxD3delN* (lane 8), suggesting that *Chd+Wnt* signaling involves a *FoxD3*/*Zic*-independent pathway. It remains to be clarified whether *FoxD3* induction is totally dependent on *Zic* signaling or not. The answer to this question should be attainable once an appropriate dominant-negative *Zic* becomes available.

Once *FoxD3* and *Slug* are induced, mutual activation loops of *Zic-FoxD3* and *FoxD3-Slug* (with *Wnts*) play essential roles in maintenance of these factors (Figs 2M, 3G, 5E). These 'self-activating' circuits are likely to support expression of these three factors in the neural crest regions. RNA injection studies also show that *Zic-r1* induces *Slug* while *Slug+Wnt3a* activates *Zic-r1* in the cap (Fig. 2N and data not shown). However, these inductions are not primary events because both are blocked by *FoxD3delN* (Fig. 6F lane 4 and data not shown).

Although the model above explains a majority of the data from this study, additional genes are thought to be involved. For instance, (1) as both *FoxD3* and *Slug* are shown to be repressors (this study and LaBonne and Bronner-Fraser, 2000), their ability to activate theoretically requires unknown intermediate factors. (2) The neural crest primodia express two closely related *Snail* family factors (*Slug* and *Snail*), which are suggested to play somewhat redundant roles (discussed by LaBonne and Bronner-Fraser, 2000). It remains to be clarified whether these two factors have distinct functions in the initiation and maintenance phases. (3) The in vivo expression of the upstream gene *Zic* is broader than that of the downstream gene *FoxD3* (Fig. 1K), suggesting that yet unidentified factor(s) should provide additional positional information. Also, it is important to understand in the future how FGFs, Wnts and Pax3 (Bang et al., 1999) signals are integrated in our model. (4) It remains to be elucidated whether the effects of dominant-negative *Slug* (*SlugBD*) on neural crest migration (Nieto, 1994; Carl et al, 1999; LaBonne and Bronner-Fraser, 2000) are dependent on FoxD3-related signaling or not.

Conclusion

This study provides the first evidence for the requirement of *FoxD3*-related signaling in neural crest formation. In addition, it has been shown that *FoxD3* overexpression is sufficient to initiate neural crest differentiation in the embryonic ectoderm. *FoxD3* is required for induction of *Slug* by *Zic*, while *Slug* is not needed for *FoxD3* induction. Mutual activation of *Zic*, *FoxD3* and *Slug* may be involved in their own maintenance. In addition to these mutual activation loops, *FoxD3* and *Slug* utilize distinct pathways in activating specific downstream genes, such as *Ets-1* and *Twist*. Collectively, *FoxD3* and *Slug* (or their closely related factors) are essential regulators of early neural crest differentiation, which work in concert and in partially non-overlapping pathways.

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Note added in proof

Soon after our revised manuscript was submitted, a study in the chick appeared that also indicated positive roles of FoxD3 in neural crest development (Kos et al., 2001).

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