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# Zebrafish Foxd3 is required for development of a subset of neural crest derivatives

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**Abstract**

*foxd3* encodes a winged helix/forkhead class transcription factor expressed in the premigratory neural crest cells of many vertebrates. We have investigated the function of this gene in zebrafish neural crest by a loss of function approach using antisense morpholino oligonucleotides and immunostaining for Foxd3 protein. Knockdown of Foxd3 expression produces deficits in several differentiated neural crest derivatives, including jaw cartilage, peripheral neurons, and glia, and iridophore pigment cells. Other derivatives, such as melanophore and xanthophore pigment cells are not affected. Reduction in the expression of several lineage-specific markers becomes evident soon after the onset of neural crest migration, suggesting that Foxd3 knockdown affects these lineages at early stages in their development. In contrast, analysis of the expression of early neural crest markers indicates little effect on neural crest induction or initial emigration. Finally, cell transplantation suggests that with respect to dorsal root ganglia neurons the Foxd3 requirement is cell autonomous, although Foxd3 itself is not detectable in differentiated DRG neurons. These results suggest that in zebrafish Foxd3 may not be required for induction of neural crest identity but is necessary for the differentiation of a subset of neural crest cell fates, perhaps in precursors of particular neural crest lineages.


*Keywords:* Zebrafish; Neural crest; *foxd3*; Winged helix; Morpholino; *sox10*; *sna2*; *dlx2*; *ngn1*; *phox2b*

## Introduction

The neural crest is a population of migratory, ectodermally derived cells considered to be a key innovation in the evolutionary history of vertebrates (Northcutt and Gans, 1983; Shimeld and Holland, 2000). Several genes, many of which encode regulatory transcription factors, have been identified that are expressed at the neural plate border from which the neural crest arises, and later in neural crest itself. Some of these, such as *snail*, *zic*, and *pax3/7*, are present in this border region in one or more deuterostome invertebrates (reviewed in Meulemans and Bronner-Fraser, 2004; Meulemans and Bronner-Fraser, 2005). Others are not, however, raising the possibility that, during evolution, recruitment of one or more additional genes to this domain of expression was a key to establishing the full set of properties of this tissue.

One such gene, *foxd3* (formerly *HFH2*/*Genesis*/*CWH-3*/*fkd6*) belongs to the forkhead box or winged helix family of transcription factors, which comprises over 180 members in 17 subgroups in over a dozen metazoan species ((Carlsson and Mahlapuu, 2002; Lehmann et al., 2003); www.biology.pomona.edu/fox.html). It was first identified as a gene expressed in embryonic stem cells and embryonal carcinomas (Clevidence et al., 1993; Sutton et al., 1996) and later found to have highly conserved expression in premigratory neural crest, as well as neural crest derivatives, of various classes of vertebrates (Hromas et al., 1999; Kelsh et al., 2000a; Kos et al., 2001; Labosky and Kaestner, 1998; Odenthal and Nüsslein-Volhard, 1998; Pohl and Knöchel, 2001; Sasai et al., 2001; Yamagata and Noda, 1998). In contrast, the *foxd* subgroup genes of the cephalochordate *amphioxus* (Yu et al., 2002) and the urochor-

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date *ciona* (Imai et al., 2002) are not expressed at the neural plate border. Therefore, Foxd3 function in neural crest may be closely linked to the special properties and behaviors of these cells, such as pluripotentiality, epithelial–mesenchymal transformation, and migration.

In *Xenopus* embryos, Foxd3 overexpression has been reported variously to inhibit or promote neural crest formation as measured by marker gene expression. The discrepancy in these studies may be attributable in part to differences in timing and dose of Foxd3 mRNA injected: earlier injection/higher dose expanded neural plate at the expense of neural crest (Pohl and Knöchel, 2001), while later injection/lower dose induced markers of both populations (Sasai et al., 2001). Misexpression by electroporation into the chick neural tube also induces the neural crest marker HNK-1 (Dottori et al., 2001; Kos et al., 2001) and in one report a corresponding increase in delaminating and emigrating cells (Dottori et al., 2001). At later stages, forced Foxd3 expression appears to push cells to adopt the glial fate and inhibit melanocyte and dorsal interneuron fates (Dottori et al., 2001; Kos et al., 2001), while antisense knockdown of Foxd3 protein expression had the opposite effect of an increase in melanocytes in culture and in cells on melanocyte-specific migration pathways in vivo (Kos et al., 2001). While these results are intriguing, it is still not entirely clear from these various studies the extent to which Foxd3 is necessary or sufficient for establishing neural crest identity, if it is required for the specification of glial or other neural crest lineages, or if it in fact functions reiteratively during development, in a manner analogous to, or perhaps integrated with, the Wnt signaling pathway (Lewis et al., 2004). Inactivation of the mouse Foxd3 gene has been minimally informative to date in this regard because null homozygotes display early lethality (Hanna et al., 2002), and a conditional knockout has not yet been reported.

To address these questions, we have investigated the function of zebrafish *foxd3* during embryogenesis and neural crest development, by means of morpholino antisense oligonucleotide-mediated knockdown of Foxd3 expression. By immunostaining for Foxd3 protein, we have verified that translation can be blocked from the earliest onset of Foxd3 expression. Elimination of Foxd3 activity results in reduction or loss of neural crest derivatives, including jaw structures, glia, dorsal root ganglia neurons, enteric neurons, and iridophore pigment cells. Marker gene analysis indicates that neural crest induction per se is relatively unaffected, but later lineage-specific markers are reduced or lost. A comparison of the cell types affected by Foxd3 knockdown with the expression profile of Foxd3 protein, coupled with cell transplant experiments, suggests that Foxd3 may be required both in precursors of particular lineages, such as dorsal root ganglia neurons, and later in the differentiated end products of other lineages, such as peripheral glia.

## Materials and methods

### Fish stocks

Adult fish of the *AB strain, as well as the transgenic reporter strain *foxd3:GFP* (*zFoxd3*GFP; Gilmour et al., 2002), were maintained on a 14-h/10-h light/dark cycle at 28.5°C. Embryos were staged according to Kimmel et al. (1995). In some experiments, phenylthiocarbamide (Sigma) was added to the embryo medium at a final concentration of 0.2 mM to inhibit melanin synthesis.

### BamHI

The *foxd3* coding sequence was amplified from plasmid DNA using the following primers: forward, 5′ -CGG GAT CCA TGA CCC TGT CTG GAG GC-3′ ; reverse, 5′ -GCT CTA GAT CAT TGA GAA GGC CAT TTC-3′ . Following digestion with BamHI and Xba I, the product was ligated into a variant of the maltose binding protein expression vector pMAL-c2E (New England Biolabs) to which a TEV cleavage site had been added. The resulting plasmid pMBP-TEV-foxd3 was transformed into the strain BL21 (Stratagene). Cultures were grown in rich broth supplemented with glucose to an OD600 of 0.5 whereupon protein expression was induced for 1 h with the addition of IPTG to a final concentration of 50 nM. Cells were collected by centrifugation, lysed with a French press, and the soluble fraction was bound to amylose resin (New England Biolabs). Protein was eluted with maltose-containing buffer, and fractions were collected, dialyzed, and concentrated using 10 kDa cut-off microcentrifuge columns (Amicon). Fusion protein thus purified was used to immunize rabbits (R&R Rabbitry, Stanwood, WA).

### In vitro translations, immunoprecipitations and immunoblotting

The full-length *foxd3* cDNA was subcloned into pCS2 + MT vector for expression of Foxd3 protein with N-terminal myc-tags. In vitro transcription of *myc-foxd3* and *myc-foxd3mut* encoding a DNA-binding mutant of Foxd3 (Pohl and Knöchel, 2001) was performed using the SP6 mMessage mMachine kit (Ambion). Coupled in vitro transcription/translation of *myc-foxd3* cDNA was performed using the TnT SP6 Quick Coupled Transcription/Translation System (Promega, Madison, WI) according to the manufacturer's directions. Protein was synthesized in the presence of Transcend tRNA (Promega) to incorporate biotin label. For immunoprecipitation reactions, in vitro translated Myc-Foxd3 was diluted in Rubenfeld Lysis Buffer (20 mM Tris, pH 8, 140 mM NaCl, 1 mM EGTA, 50 mM NaF, 1mM $Na_3VO_4$, 10% glycerol, 1.5 mM $MgCl_2$, 1%Triton X-100) and incubated with anti-Foxd3 or anti-Myc antibodies (Cell Signaling Technology, Inc., Beverly, MA) overnight at 4°C with constant mixing. Protein/antibody complexes were precipitated with protein A agarose (Sigma) followed by extensive washing in phosphate-buffered saline (PBS). Myc-Foxd3 was eluted in 1X Laemmli Sample Buffer and separated by SDS-PAGE on 10% Tris–Glycine polyacrylamide gels. After protein transfer to PVDF membrane (Biorad), the membrane was blocked and incubated with HRP–streptavidin Conjugate (ZyMax Grade, Zymed Laboratories, San Francisco, CA) according to the manufacturer's recommendations. Following several washes in Tris-buffered saline containing 0.1% Tween, signal was visualized with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford IL) followed by exposure to Kodak Biomax MR film.

### Morpholino injections

Embryos were obtained by natural matings and injected at the 1–4-cell stage using a gas-driven microinjection apparatus (ASI). A morpholino antisense oligonucleotide was designed against the 5′ untranslated region of the *foxd3* messenger RNA (Genbank accession number AF052249) with the following sequence: (nt 160–184), 5′ -TGC TGC TGG AGC AAC CCA AGG TAA G-3′ . A control version was synthesized with 5 mismatches, indicated in lower case: 5′ –TGC TaC TGa AGa AAC aCA AGa TAA G-3′ . An injection volume of approximately 1 nl was used at the concentrations indicated. Morpholino oligonucleotides against *neurogenin1* and *gdnf* have been described previously (Cornell and Eisen, 2002; Shepherd et al., 2001).

### Alcian blue staining and iridophore counts

Following overnight fixation in 4% paraformaldehyde and several water washes, jaw cartilages were stained overnight with a solution of 0.01% Alcian