Requirement for Foxd3 in the maintenance of neural crest progenitors

Lu Teng1,*,†, Nathan A. Mundell2,A,†, Audrey Y. Frist3,4, Qiaohong Wang1 and Patricia A. Labosky1,2,3,4,‡

Understanding the molecular mechanisms of stem cell maintenance is crucial for the ultimate goal of manipulating stem cells for the treatment of disease. Foxd3 is required early in mouse embryogenesis; Foxd3–/– embryos fail around the time of implantation, cells of the inner cell mass cannot be maintained in vitro, and blastocyst-derived stem cell lines cannot be established. Here, we report that Foxd3 is required for maintenance of the multipotent mammalian neural crest. Using tissue-specific deletion of Foxd3 in the neural crest, we show that Foxd3flox/–; Wnt1-Cre mice die perinatally with a catastrophic loss of neural crest-derived structures. Cranial neural crest tissues are either missing or severely reduced in size, the peripheral nervous system consists of reduced dorsal root ganglia and cranial nerves, and the entire gastrointestinal tract is devoid of neural crest derivatives. These results demonstrate a global role for this transcriptional repressor in all aspects of neural crest maintenance along the anterior-posterior axis, and establish an unprecedented molecular link between multiple divergent progenitor lineages of the mammalian embryo.

KEY WORDS: Neural crest, Foxd3, Mouse embryo, Stem cell maintenance

INTRODUCTION

Neural crest (NC) cells are multipotent progenitors that possess the unique ability to contribute to a variety of disparate adult derivatives: the entire peripheral and enteric nervous systems (PNS and ENS), bones and connective tissue of the head and neck, and the outflow tract of the developing heart, among others. NC cells emigrate from the dorsal neural tube during early embryogenesis and migrate to final sites of differentiation. Their fate depends on their rostral-caudal position of origin in the neural tube, their route of migration and final destination (reviewed by Le Douarin and Kalcheim, 1999). Many of the cellular mechanisms crucial to the development of an embryo are represented within this extraordinary tissue: specification of cell type, maintenance of multipotency of progenitor cells, directed migration of cells to their final destination and terminal differentiation of cells. Members of the PDGF, BMP and Wnt families of secreted molecules are important for the initial specification and subsequent maintenance of the NC (reviewed by Steventon et al., 2005). Many transcription factors are activated in NC in response to these signaling cascades; these include Foxd3, Sox8, 9 and 10 (Cheung and Briscoe, 2003; Southard-Smith et al., 1998), Ap2 (Tcfap2a – Mouse Genome Informatics) (Schorle et al., 1996) and Pax3 (Epstein et al., 1991). It is not yet clear how these factors work together to induce and then maintain this unique cell type.

Foxd3 encodes a transcriptional repressor of the winged helix or Forkhead family of proteins (Labosky and Kaestner, 1998; Sutton et al., 1996). This large family of proteins is characterized by a 100 amino acid DNA-binding domain and transcriptional activation or repression domains (reviewed by Wijchers et al., 2006). Foxd3 was first characterized by its expression in embryonic stem (ES) cells and multipotent cells of the NC (Labosky and Kaestner, 1998). Our previous work demonstrated that Foxd3 is required early in mouse embryogenesis; Foxd3–/– embryos fail around the time of implantation, cells of the inner cell mass cannot be maintained in vitro, and ES cell and trophoblast stem cell lines cannot be established (Hanna et al., 2002; Tompers et al., 2005). In vivo, the pool of both embryonic and trophoblast progenitors is not maintained without Foxd3; embryonic progenitor cells die, while trophoblast progenitors give rise to an excess of trophoblast giant cells at the expense of the remaining trophoblast lineage. However, the ultimate effect in both cases is that the progenitor pool is not maintained and the cell lineage is lost.

Foxd3 is one of the earliest molecular markers of the NC lineage, it is expressed in many organisms in premigratory and migrating NC and expression is downregulated as the cells differentiate into most derivatives (Dottori et al., 2001; Labosky and Kaestner, 1998). Ectopic expression of Foxd3 in chick neural tube and Xenopus embryos can specify NC cell fate as measured by upregulation of HNK1 and Slug (Dottori et al., 2001; Kos et al., 2001; Sasai et al., 2001). In addition, maintained expression of Foxd3 in migrating NC interferes with differentiation and retains NC in an undifferentiated state, evidence that points to a potential role for Foxd3 in the control of stem cell self-renewal (Dottori et al., 2001). Morpholino knockdown of zebrafish foxd3 and mutations that affect Foxd3 expression in the zebrafish NC have deleterious effects on maintenance of the NC (Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006).

Because the deletion of Foxd3 results in embryonic lethality around the time of implantation, we employed Cre-loxP technology to delete the Foxd3 coding region specifically in NC. Here we demonstrate that without this transcriptional regulator the NC lineage fails to be maintained, resulting in catastrophic loss of most NC derivatives. Mutant mice perish at birth with a cleft face, the PNS is severely reduced and the ENS is not formed. Surprisingly, although the cardiac NC is greatly reduced early in embryogenesis, by midgestation most cardiac NC derivatives are normal, with subtle defects in a small percentage of mutant embryos. We show that the mechanism of cellular loss of most of the NC is by a failure to maintain the progenitor pool, as much of the premigratory and early

1Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6058, USA. †Department of Pharmacology.
2Department of Cell and Developmental Biology, Center for Stem Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232-0494, USA.
*Present address: Children’s National Medical Center, Washington DC. 20010-2970, USA
†These authors contributed equally to this work
‡Author for correspondence (e-mail: trish.labosky@vanderbilt.edu)

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migrating NC undergoes cell death. Cells that survive are able to migrate and differentiate normally but cannot completely compensate for the early loss of progenitors. Our data demonstrate that Foxd3 is required for maintenance of NC progenitor cells and, taken together with our previous work, place Foxd3 in the unique role of maintaining the undifferentiated multipotent state of three completely divergent progenitor populations of the early mammalian embryo.

MATERIALS AND METHODS

Construction of Foxd3 floxP targeting vector

A 1.2 kb fragment of the Foxd3 locus was inserted into the EcoRI site of pPNT4 downstream of the Frt-flanked Neo gene to serve as the 3’ homology arm. An oligonucleotide containing the loxP recognition sequence (AACAAGTCGTAACGATCACATATAGAAGTTAT) was inserted 5’ of the Foxd3 coding region. Upstream, a 4.2 kb fragment of the Foxd3 locus was inserted to complete the 5’ homology arm to generate the final targeting vector shown in Fig. 1A.

ES cell electroeporation and generation of mice

TL1 ES cells were electroporated as described (Tompers and Labosky, 2004). ES cell DNA was analyzed by Southern blot as shown in Fig. 1A, B. Lines were screened by PCR for the presence of the 5’ loxP site. We identified three correctly recombined cell lines out of 337 (0.9% frequency). Chimeras were generated by blastocyst injection and offspring of chimeras were genotyped to identify heterozygous founders. Two independent cell lines transmitted through the germ line and because both showed the identical phenotype we restricted our studies to one cell line. Mice were genotyped either by Southern blot or PCR with the following primers: 5’-CGGCTTCTTCTTTGGAAGA-3’ and 5’-ACATATCGCTGGCGCT-3’ to give a 130 bp band for the wild-type allele and a 220 bp band for the floxed allele.

Mouse strains

Fodx3−/− is a null mutation of Foxd3 generated previously (Hanna et al., 2002) and is referred to as Foxd3 throughout. Wnt1-Cre mice were a generous gift of Drs. A. McMahon and D. Rowitch (Harvard University, Cambridge, MA). In the reporter strain Git/Rosa26SorFls (referred to as ROSA26R), Cre expression results in removal of a loxP-flanked DNA segment that prevents expression of a lacZ gene. After Cre activation, lacZ is detected in cells/tissues where Cre is expressed. The transgenic line ElaCre [Tg(ElaCre-C5379Lmgd, from The Jackson Laboratories] carries a Cre transgene under control of the adenosinovirus Ela promoter that targets expression of Cre to the early mouse embryo and acts as a ubiquitous deleter strain for our purposes. The presence of Cre was detected with primers 5’TGTAGATGTTGCAAGAACCC−3’ and 5’-CCATGAGTGAACGAACTGG-3’, producing a band of 400 bp. All mouse strains were maintained on an outbred mixed genetic background that was primarily CD-1 and C57BL/6. Animals were maintained in accordance with the rules of the University of Pennsylvania and Vanderbilt University Institutional Animal Care and Use Committee (IACUC).

Histology and whole-mount in situ hybridization

Histology was performed using standard procedures (Presnell and Schreibman, 1997). For whole-mount LysoTracker staining, embryos were dissected in Hanks buffer, and incubated in 5 μM LysoTracker (Molecular Probes) at 37°C for 1 hour. Embryos were then washed in Hanks buffer and fixed in 4% paraformaldehyde (PFA) in PBS overnight, washed in PBS and dehydrated in methanol before imaging with a fluorescent stereoscope. TUNEL assay was performed in paraffin sections using the In Situ Cell Death Detection Kit (Roche). Whole-mount immunostaining was performed as described (Wall et al., 1992). Skeletal preparations and 3-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) staining followed standard techniques (Nagy et al., 2003). For histological analysis, paraffin sections (7–12 μm) were counterstained with Eosin or Nuclear Fast Red.

The following antibodies were used: rabbit anti-Foxd3 at 1:1000 (Tompers et al., 2005); mouse anti-PGP9.5 at 1:100 (Biogenesis); mouse anti-neurofilament (2H3 monoclonal) at 1:1000 (Developmental Studies Hybridoma Bank); mouse anti-β III tubulin (TuJ1; Tubb3) at 1:100 (Chemicon); rabbit anti-phosphohistone H3 at 1:200 (Upstate Biotechnology); goat anti-Fabp7 (also known as B-FABP) at 1:20 (R&D Systems); goat anti-rabbit Cy3, goat anti-mouse Cy3 and donkey anti-goat Cy2 (Jackson ImmunoResearch Laboratories). The Vectastain ABC Kit (Vector Laboratories) was used to detect the non-fluorescent secondary antibodies and DAPI (1:5000, Molecular Probes) was used to highlight nuclei.

Whole-mount in situ hybridization was performed using Costar 12-well inserts following standard protocols (Hanna et al., 2002). Probes for Crabp1 (Stoner and Gudas, 1989), Dlx1 (Dolle et al., 1992), Max1 (Satokata and Maas, 1994), Pdgfa (Mercola et al., 1990), Pdgfc (Ding et al., 2000) and Sox10 (Southard-Smith et al., 1998) were as described previously. Digoxigenin-labeled RNA probes were prepared using reagents from Roche Molecular Biochemicals.

Corrosion casting was performed as described (Feiner et al., 2001) by injecting the left ventricle of embryonic or newborn hearts first with PBS and then with polymer containing red pigment (Polysciences #07349) using gentle pressure. Polymer containing blue pigment was injected into the right ventricle to monitor for possible ventricular septal defects. The casts were cured overnight at 4°C and surrounding tissue was macerated (maceration solution: Polysciences #07359) at 50°C for 6–8 hours.

RESULTS

Conditional deletion of the Foxd3 locus in NC

A targeting vector was designed so that after homologous recombination in ES cells, the Foxd3 coding region (Fig. 1A, purple box) would be flanked with loxP sequences (Fig. 1A, orange triangles). Correctly targeted ES cells were used to generate mice carrying a Foxd3 flox allele. Mice carrying two Foxd3 alleles (Foxd3 flox/flox mice) appeared normal and healthy. To test whether Cre-mediated deletion of the Foxd3 locus produced a null allele efficiently, we crossed Foxd3 flox/flox mice with Foxd3 flox/+; Ela-Cre mice. Timed pregnant females from this cross were dissected at 6.5 days post-coitum (dpc) and 25% of the resulting embryos had a phenotype identical to the original loss-of-function allele (data not shown). There was no difference in the results obtained with or without the neo selection cassette (data not shown).

To selectively delete Foxd3 in the NC, we used the well-characterized Wnt1-Cre transgenic mouse line in which Cre recombinase is expressed in migratory NC starting at 8.0–8.5 dpc (Danielian et al., 1998). We used reciprocal matings between Foxd3 flox/+; Wnt1-Cre mice to obtain Foxd3 flox/+; Wnt1-Cre (mutant) embryos. At the four-somite stage (~8.0 dpc), Foxd3 protein was detected in migrating NC in the head folds (Fig. 1D). By contrast, we saw a severe reduction in Foxd3 protein in head folds of mutant embryos, detecting only one or two cells that were weakly positive for Foxd3 protein expression (Fig. 1E, arrow). By 9.5 dpc, no Foxd3 protein was detected in the mutant dorsal neural tube or migratory path of NC (Fig. 1, compare F with G). Foxd3 expression was not maintained in the facial mesenchyme at 13.5 dpc; expression was limited to forming cranial ganglia (Fig. 1H) and this expression was missing in mutant embryos. In the ENS, Foxd3 protein was expressed in the gut coils at 13.5 dpc and was missing in mutant embryos (Fig. 1J–K). These results demonstrate that Foxd3 is deleted specifically in the NC by Wnt1-Cre.

Maintenance of cranial NC is dependent on Foxd3

Initially, litters were allowed to progress to term and no mutant mice were found at weaning (21 days). However, we found one dead mutant newborn pup (Fig. 2A) and began monitoring litters before the mice were born (Table 1). Although the majority of 18.5 dpc littermates appeared normal and could survive being delivered by cesarean section, the mutant mice attempted to breathe several
times but then expired. Table 1 summarizes the number of offspring and embryos from both Foxd3flx/++;Wnt1-Cre and Foxd3flx/flx;Wnt1-Cre crosses. Embryos were found in the expected ratios at all times in development and no mutant mice survived more than a few hours.

All mutant mice had a severe cleft face and palate incompatible with survival and their inability to breathe was presumably the cause of their death immediately after birth. These defects occurred with 100% penetrance, although the width of the cleft sometimes varied in size. Nostrils and whisker pads were present but the facial midline never fused and the eyelids were partially open. Much of the skull vault is derived from cranial NC so we analyzed skeletons using Alcian Blue and Alizarin Red staining. Comparison of the control and mutant skulls in Fig. 2B revealed that the frontalnasal bone and nasal cartilage were missing in the mutants and the interparietal, parietal and basal occipital bones were greatly reduced in size. The maxilla and premaxilla were shortened and the mandible was shortened and thickened. These cranial defects were obvious at midgestation stages (Fig. 2C).

To monitor NC cells and their derivatives, we introduced the ROSA26R (R26R) conditional reporter allele into the mutant background (Soriano, 1999). Upon expression of Cre protein, all cells and their descendants transcribe the lacZ gene, and therefore NC cell fate can be monitored directly with and without Foxd3 function. Note that we are comparing Foxd3flx/flx;Wnt1-Cre;R26R (mutant) embryos with Foxd3flx/flx;Wnt1-Cre;R26R (control) embryos so that the Cre protein will be recombining two sets of loxP sites; one to delete the Foxd3 coding region and one to delete the stop sequence in the ROSA26 locus. β-gal-positive tissue was observed in the frontalnasal mesenchyme in both control and mutant embryos, indicating that NC cells contribute to these tissues in the absence of Foxd3 (Fig. 2D and see Fig. S3 in the supplementary material).

Pharyngeal arch defects in Foxd3 mutants

At 9.5 dpc, a comparison of lineage-mapped embryos revealed that NC cells have migrated into pharyngeal arches (PAs) 1 and 2 in both Foxd3 mutants and controls (Fig. 3A). However, by 10.5 dpc, morphological differences in the PAs were apparent by scanning electron microscopy (Fig. 3B). The maxillary and the mandibular prominences of PA1 were present but reduced in size. There was a striking decrease of NC in PAs 3 and 4. Immunohistochemistry for neurofilament protein showed that all cranial ganglia and nerves are present but smaller than normal. Some cranial nerves were slightly misdirected (the glossopharyngeal nerve in Fig. 3C) or had not extended as far as in the control (the facial and vestibulocochlear nerves). Spinal nerves along the trunk of the mutant embryos were thinner. Examining expression of Sox10, a marker of early NC, revealed reduced expression in the developing trigeminal, facial and vestibulocochlear ganglia and no expression in the glossopharyngeal and vagus ganglia. Sox10 expression was also reduced along the trunk of the embryo (Fig. 3D). Expression of other NC-specific genes in the PAs uniformly showed reduced expression in PAs 1 and 2 (see Fig. S1 in the supplementary material). Together, these data...
suggest that the initial specification of NC occurs in Foxd3 mutants, but there is an overall failure to maintain NC progenitors as demonstrated by a loss of PA-derived structures and a concomitant decrease in expression of NC-specific genes.

**Reduction of peripheral nervous system and loss of enteric nervous system in Foxd3 mutants**

NC cells migrate into the limbs during development giving rise to neurons, Schwann cells and terminal glia cells. X-Gal lineage staining highlighted the cutaneous nerves and the developing sciatic nerve in a hindlimb from a 14.5 dpc control embryo (Fig. 4A). All of these NC-derived cells were missing in the Foxd3 mutant embryos (Fig. 4B). A dorsal view of the same pair of embryos revealed the segmented pattern of spinal nerves exiting the dorsal root ganglia (DRG) and projecting ventrally in the control (Fig. 4C); the spinal nerves were absent in mutant embryos (Fig. 4D).

NC cells that migrate ventrally from the dorsal neural tube contribute to the ENS: the neurons and glia that innervate the entire gastrointestinal tract. Using the ROSA26R reporter allele, NC contribution to the ENS is easily visualized. In control midgestation embryos (14.5 dpc), the intestines were ensheathed in a plexus of NC-derived β-gal-positive cells, whereas in a Foxd3 mutant littermate the gut was completely lacking these cells (Fig. 4E,F). When the gastrointestinal tract was dissected out of 17.5 dpc embryos (Fig. 2A).

### Table 1. Genotypes of offspring and embryos from representative Foxd3<sup>fl/+</sup> × Foxd3<sup>+/–</sup>; Wnt1-Cre and Foxd3<sup>fl/fl</sup> × Foxd3<sup>+/–</sup>; Wnt1-Cre crosses

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>8.5 dpc</th>
<th>9.5 dpc</th>
<th>10.5 dpc</th>
<th>12.5 dpc</th>
<th>Newborn</th>
<th>Total</th>
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</thead>
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<tr>
<td>Foxd3&lt;sup&gt;fl/+&lt;/sup&gt;; Wnt1-Cre (mutants)</td>
<td>12.5%</td>
<td>12.5%</td>
<td>25%</td>
<td>12.5%</td>
<td>12.5%</td>
<td>25%</td>
</tr>
<tr>
<td>Foxd3&lt;sup&gt;fl/fl&lt;/sup&gt;; Wnt1-Cre</td>
<td>0</td>
<td>1 (9%)</td>
<td>2 (19%)</td>
<td>2 (19%)</td>
<td>0</td>
<td>2 (19%)</td>
</tr>
<tr>
<td>Foxd3&lt;sup&gt;+/–&lt;/sup&gt;; Wnt1-Cre</td>
<td>0</td>
<td>3 (25%)</td>
<td>2 (19%)</td>
<td>2 (19%)</td>
<td>0</td>
<td>2 (19%)</td>
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<tr>
<td>Foxd3&lt;sup&gt;fl/fl&lt;/sup&gt; or Foxd3&lt;sup&gt;+/–&lt;/sup&gt;; Wnt1-Cre</td>
<td>0</td>
<td>3 (25%)</td>
<td>2 (19%)</td>
<td>2 (19%)</td>
<td>0</td>
<td>2 (19%)</td>
</tr>
<tr>
<td>Foxd3&lt;sup&gt;+/–&lt;/sup&gt;; Wnt1-Cre</td>
<td>0</td>
<td>3 (25%)</td>
<td>2 (19%)</td>
<td>2 (19%)</td>
<td>0</td>
<td>2 (19%)</td>
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Percentages are rounded to the nearest whole number.

*One newborn pup was found dead in the third litter (Fig. 2A).
embryos, the control sample was surrounded by β-gal-positive cells along the entire anterior to posterior axis, whereas the mutant sample had no NC-derived cells around the outside of the gut tube (Fig. 4G,H). Expression of PGP9.5 (Uchl1 – Mouse Genome Informatics) was readily detectable in neurons of the ENS in control embryos, whereas there were no PGP9.5-positive cells in Foxd3 mutant embryos (Fig. 4I,J). Similar results were obtained with βIII tubulin antiserum used to detect neurons, and with Fabp7 antiserum used to detect glia (data not shown).

**Foxd3 mutants show subtle cardiac neural crest defects**

NC cells from the mid-otic placode to somite 4 migrate through rhombomeres 6-8 into PAs 3, 4 and 6, contributing to the aortic arch arteries and the aortcopulmonary septum of the cardiac outflow tract. In control embryos, NC cells entered PAs 3, 4 and 6 (Fig. 3A) and the developing heart field by 9.5 dpc (Fig. 5A, arrows) and continued migrating at 10.5 dpc (Fig. 5B). We observed a striking decrease in the cardiac NC of Foxd3 mutant embryos. Fewer cells entered the heart field at 9.5 dpc (Fig. 5C). At 10.5 dpc, there was a paucity of NC in mutant PAs (Fig. 5D, E, arrow) and a greatly reduced number of cells entering the heart (Fig. 5D, arrowhead).

To further investigate development of the cardiac NC, we examined patterning of vascular NC derivatives using corrosion casting (Fig. 5E-H). We observed normal patterning of the aortic arch in the majority of Foxd3 mutants (13/17, 76%) between 15.5 dpc and birth. However, in three out of 17 Foxd3 mutant embryos examined, we observed a duplication of the left common carotid artery (Fig. 5G, G'). One mutant embryo had severe cardiac NC defects,
including type A2 persistent truncus arteriosus (PTA) in which the septation of the outflow tract did not occur and the ductus arteriosus was absent (Fig. 5H,H’). We examined the NC lineage in both control and mutant embryos with the \textit{ROSA26R} reporter allele and saw no differences in NC contribution to the cardiovascular system at 17.5 dpc (\(n=7\) mutants; see Fig. S2 in the supplementary material). Histological analysis of eight mutant and control littermates was performed and no septal defects were observed (see Fig. S2 in the supplementary material); however, while in the process of performing resin casts, 1/17 (the sample shown in Fig. 5H) displayed a ventricular septal defect.

NC cell migration, differentiation, death and proliferation

NC must maintain a delicate balance of proliferation and migration, and timing of differentiation is crucial for their final tissue contributions in the adult. Lineage tracing revealed that DRG were formed in \textit{Foxd3} mutants (Fig. 6A,B) but were smaller than normal. The size of the DRG varies with the angle of section, but in whole-mount, mutant DRG were consistently smaller than controls (Fig. 3A). However, while the process of performing resin casts, 1/17 (the sample shown in Fig. 5H) displayed a ventricular septal defect.

Terminal differentiation of NC occurs as cells reach their final destination. We detected expression of neural and glial markers in control and mutant DRG. At 9.5 and 14.5 dpc, the neural marker \(\beta^\text{III}tubulin\) and the glial marker \textit{Fabp7} were expressed in both control and mutant DRG (Fig. 6E-H and data not shown).

We examined cell death in control and \textit{Foxd3} mutant embryos and detected an increase in TUNEL-positive apoptotic cells in the dorsal spinal cord in the mutant, whereas there were few, if any, TUNEL-positive cells in the spinal cord of control embryos (Fig. 6I,J). Confirming this with LysoTracker Red dye, at 9.0 dpc mutant embryos showed increased apoptosis in the hindbrain, and by 10.5 dpc apoptosis was more pronounced in the posterior tail of the embryo (Fig. 6K-N). The absence of apoptosis in distal PAs of mutant embryos at 10.5 dpc (Fig. 6M) is likely to be due to the large deficit in NC cell numbers (note morphology of PAs in Fig. 6M and lineage label in Fig. 3B).

To more closely examine the curious recovery of most of the cardiac derivate, we analyzed cell proliferation in the cardiac NC using a combination of lineage labeling and immunohistochemistry for phosphorylated histone H3 (pH3), a marker for cells in mitosis. Septation is complete by 13.0 dpc so we chose to examine embryos at 12.5 dpc (Hiruma et al., 2002). Representative sections of 12.5 dpc control and mutant embryos are shown in Fig. 6O-R, clearly demonstrating the paucity of NC present in the outflow tract at the most rostral and caudal levels. The volume of the outflow tract in control and mutant embryos was calculated as 4 mm\(^3\) and 0.7 mm\(^3\), respectively. The proportion of pH3-positive NC cells was the
same in controls and mutants (for details, see Fig. S3 in the supplementary material). These results suggest that although loss of Foxd3 reduces the number of cardiac NC, these cells are still able to pattern the outflow tract and give rise to smooth muscle cells.

**DISCUSSION**

**NC specification**

Previous data from several laboratories have demonstrated that ectopic expression of Foxd3 in the neural tube can change the fate of the neuroepithelium into NC (Cheung et al., 2005; Dottori et al., 2001; Kos et al., 2001; Sasai et al., 2001). However, our loss-of-function analysis suggests that Foxd3 is not required for the initial specification of the NC. In Foxd3flox/–; Wnt1-Cre mutant embryos, we do not detect cells expressing Foxd3 at the earliest stages of NC specification (Fig. 1D-G), yet there is still NC present (Fig. 3). There remains the possibility that the Wnt1-Cre transgene does not produce Cre protein early enough and there might be a few Foxd3-expressing cells present, but if so, our data show that they are not maintained. It is more likely that the role of Foxd3 is in maintaining the NC, not in specifying this cell type.

Our findings do not completely agree with those of other model systems, highlighting differences between *Xenopus*, chick, zebrafish and mouse NC. Ectopic expression of a dominant-negative FoxD3 in *Xenopus* embryos suppresses NC specification as measured by the loss of NC marker expression (Sasai et al., 2001). Electroporation of antisense oligonucleotides specific to Foxd3 in the chick bias the differentiation of NC towards the melanocyte lineage (Kos et al., 2001). There are three recent reports of mutations in the zebrafish *foxd3* locus or knockdown of *foxd3* using morpholinos (Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006). These groups reported that NC was specified normally with a general downregulation of NC markers similar to what we have observed. Craniofacial defects were highlighted by jaw abnormalities in the fish, whereas defects in the mandible of our mice were minor. All three groups demonstrated a complete loss of DRG, whereas we see smaller DRG that contain neurons and glia. Similar to what we observe, in all cases the zebrafish ENS was lost and increased cell death in the NC lineage was observed along with defects in migration. Zebrafish are an ideal system to monitor changes in different types of NC melanocytes and all three groups described lineage-specific differences in these derivatives. For example, in the *mother superior* mutation, which is a mutation somewhere outside the coding region that affects only NC expression of *foxd3*, melanophores are delayed in their development but then recover, whereas iridophores remain greatly reduced (Montero-Balaguer et al., 2006). Zebrafish cardiac NC contributes primarily to the myocardial cell lineage (Li et al., 2003). Despite this, only one group reported a slight edema in the heart in *foxd3* mutants (Montero-Balaguer et al., 2006).
NC patterning

We did not detect anterior-posterior patterning defects in NC of Foxd3 mutants, although caudal NC was most severely affected. All mutant embryos had severe facial clefting and were missing many NC-derived facial bones. However, the mandible was present in all mutants examined. It was shortened and thickened, similar to Dlx5/6−/− mice (Acampora et al., 1999), but its presence demonstrates that Foxd3−/− NC can differentiate into cartilage and bone and overt cranial patterning is unaltered. Similarly, trunk NC cells were present in reduced numbers, but those cells migrated away from the neural tube normally and formed small DRG that contained both neurons and glia.

By contrast, the vagal and sacral NC destined to contribute to the ENS did not migrate to the gut. There are several possibilities for the loss of this sub-lineage of NC. One hypothesis is that there may be expression of closely related Fox genes in the cranial and cardiac NC, but no such compensating family members expressed in the trunk NC caudal to somite 7. A second hypothesis is that ENS progenitors underwent selective cell death in the dorsal neural tube, whereas NC cells destined to populate the DRG were maintained. There are no molecular markers to distinguish these lineages prior to migration so we cannot test this hypothesis directly. A third possibility is that NC cells that underwent apoptosis in the dorsal neural tube were unspecified and the selective loss of the ENS NC is because this lineage must migrate farther than others. Therefore, a general loss in progenitor cell number might affect the ENS NC more severely. A fourth possibility is that Foxd3−/− NC might not be able to enter the foregut (Fig. 6, compare C with D). It has been demonstrated that vagal and sacral NC, unlike the rest of the trunk NC, possess a unique ability to enter the gut mesentery (Serbedzija et al., 1991). Recent studies suggest this difference might be at least partially explained by the expression of axon guidance molecules Slit and Robo on vagal NC (De Bellard et al., 2003). In contrast to our results, mice lacking members of the glial cell line-derived neurotrophic factor signaling pathway have enteric neurons in the anterior ENS (Cacalano et al., 1998; Pichel et al., 1996; Sanchez et al., 1996), whereas mutations in endothelin 3 or endothelin receptor type B cause the loss of ENS only in the distal colon (Baynash et al., 1994; Hosoda et al., 1994). In Phox2b−/− embryos, there is an initial migration of vagal NC into the proximal gut mesentery, but these progenitors are not maintained, leaving the entire gut without innervation (Pattyn et al., 1999). In fact, the only other mouse model lacking the entire ENS is Sox10DoxDox (Kapur, 1999; Southard-Smith et al., 1998) and it is interesting to note that Sox10 is one of the genes severely downregulated in Foxd3−/− mutant embryos (Fig. 3C, arrowheads).

One final hypothesis, not mutually exclusive to those suggested above, is that although Foxd3 appears to be equally expressed throughout the early premigratory crest, it might be differentially required in specific subpopulations of the crest. Our surprising results showing the minimal phenotype of the mutant cardiac NC supports this last hypothesis. The cardiac crest normally migrates into PAs 3, 4 and 6, eventually contributing to the cardiac outflow tract and the aortopulmonary septum. There is a striking reduction of cardiac NC in PAs 3, 4 and 6 in Foxd3−/− mutant embryos compared with controls (Fig. 3A and Fig. 5A-D). However, this greatly reduced population of cardiac NC migrates into the heart field and even though there are far fewer cells (mutants contained 18% of the volume of NC of controls by lineage label), this reduced number can effectively separte the outflow tract in most cases. We did not observe alterations from the wild-type pattern of proliferation as monitored by phosphorylated histone H3 at 9.5 dpc. This suggests that the extent of migration of the cardiac NC, rather than the number of cardiac NC cells, is crucial for their ability to effectively separte the outflow tract. One of our future goals is to understand molecular differences between the cardiac NC, which can overcome the loss of Foxd3, and the rest of the NC lineage that is dependent on this transcription factor.

Conservation of Foxd3 function in progenitor cells

Development of all embryonic tissues relies on progenitor cells that are able to self-renew and differentiate appropriately. Our initial description of the Foxd3−/− phenotype demonstrated that this protein is required for maintenance of epiblast cells and therefore required for establishing ES cells (Hanna et al., 2002). Foxd3 is similarly required in the trophectoderm lineage (Tompers et al., 2005), a lineage completely disparrate from the embryonic one primarily affected in the null mutation. Therefore, the NC is the third multipotent lineage that requires Foxd3 for maintenance of progenitor cells, making an unprecedented molecular connection between these three distinct progenitor populations. These lineages are embryonically derived and we have yet to demonstrate that Foxd3 can play a similar role in adult-derived stem cells. However, our results support the premise that certain self-renewal mechanisms are likely to be conserved in very different progenitor lineages. There are several other proteins known to regulate multiple progenitor lineages. Bmi1 and other polycomb proteins repress genes that induce cell death in the hematopoietic lineages, neural stem cells, NC stem cells and cancer stem cells (reviewed by Pardal et al., 2005). Most recently, the transcription factor Zfx has been shown to be required for self-renewal of ES cells and maintenance of adult hematopoietic stem cells (Galan-Caridad et al., 2007). The proto-oncogene c-Myc has a well-documented role in cell proliferation; recent work in Xenopus suggests it might be involved in NC formation, whereas genetic studies in the mouse show that a NC deletion of Myc results in a relatively mild phenotype (Bellmeyer et al., 2003; Wei et al., 2007). In addition to its role in NC development, expression of Myc along with several other ‘stem cell proteins’ is sufficient to change the fate of somatic cells into pluripotent stem cells (Takahashi and Yamanaka, 2006). Finally, Sox2 is required in ES cells, TS cells and embryonic neural lineages (Avilion et al., 2003; Graham et al., 2003). Sox2 is also expressed in early migrating NC, in Schwann cells (Larysa Pevny, personal communication) and in the postnatal ENS (Vohra et al., 2006), all regions competent to give rise to NC stem cells. The role of Sox2 in the NC has not been investigated, and it is tempting to speculate that there might be a genetic interaction between Foxd3 and Sox2.

It is not clear how Foxd3 functions to maintain these progenitor pools. The protein can function as either an activator or a repressor in different contexts; in Xenopus mesoderm induction, FoxD3 recruits Groucho4 to repress target genes (Steiner et al., 2006; Yaklikkin et al., 2007), whereas in zebrafish somite maturation, Foxd3 activates myf5 expression directly (Lee et al., 2006). We cannot rule out the possibility that Foxd3 might behave similarly to Foxa proteins in modifying chromatin structure to allow access of other co-repressors or activators to target genes (Cirillo et al., 2002). Our major challenges ahead are to identify Foxd3 target genes and to determine the signaling pathways in which the protein functions.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/9/1615/DC1

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