Development 138, 641-652 (2011) doi:10.1242/dev.054718

Neural crest stem cell multipotency requires Foxd3 to maintain neural potential and repress mesenchymal fates

Nathan A. Mundell[1,2,3] and Patricia A. Labosky[1,2,3,4,*]

SUMMARY

Neural crest (NC) progenitors generate a wide array of cell types, yet molecules controlling NC multipotency and self-renewal and factors mediating cell-intrinsic distinctions between multipotent versus fate-restricted progenitors are poorly understood. Our earlier work demonstrated that Foxd3 is required for maintenance of NC progenitors in the embryo. Here, we show that Foxd3 mediates a fate restriction choice for multipotent NC progenitors with loss of Foxd3 biasing NC toward a mesenchymal fate. Neural derivatives of NC were lost in Foxd3 mutant mouse embryos, whereas abnormally fated NC-derived vascular smooth muscle cells were ectopically located in the aorta. Cranial NC defects were associated with precocious differentiation towards osteoblast and chondrocyte cell fates, and individual mutant NC from different anteroposterior regions underwent fate changes, losing neural and increasing myofibroblast potential. Our results demonstrate that neural potential can be separated from NC multipotency by the action of a single gene, and establish novel parallels between NC and other progenitor populations that depend on this functionally conserved stem cell protein to regulate self-renewal and multipotency.

KEY WORDS: Foxd3, Neural crest, Multipotency, Mouse

INTRODUCTION

At the onset of their migration from the neural tube, neural crest (NC) cells are a heterogeneous pool of multipotent neural crest stem cells (NCSCs) and fate-restricted progenitors that give rise to a wide variety of cell types, including neurons, glia, melanocytes, vascular smooth muscle cells (VSMCs), chondrocytes and osteoblasts (Le Douarin and Kalcheim, 1999). In vitro clonal analyses and in vivo cell transplantation and labeling experiments established that NC is both multipotent and self-renewing (Baroffio et al., 1991; Bronner-Fraser and Fraser, 1988; Bronner-Fraser et al., 1980; Ito and Sieber-Blum, 1991; Sieber-Blum and Cohen, 1980; Trentin et al., 2004). NCSCs can be isolated from neural tube explants or embryonic and postnatal NC derivatives, including fetal peripheral nerves, heart, skin and the adult enteric nervous system (ENS) (reviewed in Crane and Trainor, 2006; Delfino-Machin et al., 2007; Teng and Labosky, 2006). Additionally, NCSCs can be isolated by flow cytometry based on their expression of the low-affinity nerve growth factor receptor, p75 (Stemple and Anderson, 1992). Combinatorial Wnt and bone morphogenic protein (BMP) signaling synergistically maintains NCSC multipotency in vitro by suppressing differentiation (Kleber et al., 2005), and overexpressing the HMG-box transcription factor Sox10 in NC maintains multipotency by preserving gliogenic potential and inhibiting neural differentiation (Kim et al., 2003). Despite these significant advances in characterization of NCSCs, relatively little is known about the molecules required for establishing and controlling their self-renewal and/or multipotency.

NCSCs may share common transcriptional mechanisms controlling self-renewal and multipotency with other embryonic progenitor populations. Our earlier work established that Foxd3 functions in embryonic stem cells (ESCs) and trophoblast stem cells (TSCs) to maintain self-renewal and repress differentiation, supporting the notion of a conserved role for Foxd3 in stem cell self-renewal and multipotency (Hanna et al., 2002; Liu and Labosky, 2008; Tompers et al., 2005). In the NC, Foxd3 is expressed in pre-migratory and early migrating cells, and its expression decreases as cells differentiate into most derivatives, suggesting a link between Foxd3 expression and multipotency (Labosky and Kaestner, 1998). Ectopic expression of *Foxd3* in avian NC inhibited neural differentiation (Dottori et al., 2001), supporting the hypothesis that Foxd3 largely maintains an early, uncommitted NCSC state. Loss-of-function studies in *Xenopus laevis*, zebrafish and mice demonstrate dramatic losses of distinct NC derivatives and suggest a central role for Foxd3 in early NC maintenance (Lister et al., 2006; Montero-Balaguer et al., 2006; Sasai et al., 2001; Stewart et al., 2006; Teng et al., 2008). An NC-specific deletion of murine *Foxd3* caused severe defects in most NC derivatives; however, this requirement was not identical throughout the NC: although Foxd3 is required for establishment of the ENS, it is dispensable for cardiac outflow tract septation (Teng et al., 2008). This led us to hypothesize that intrinsic differences between these overlapping NC populations might reflect divergent molecular requirements controlling multipotency and lineage commitment. However, direct roles for Foxd3 in NCSC multipotency and self-renewal, or the extent to which Foxd3 controls lineage allocation of NC has not been directly examined.

To address these unanswered questions, we combined an NC-specific deletion of *Foxd3* with in vivo lineage mapping and in vitro clonal analysis of isolated NC to demonstrate a cell-autonomous requirement for Foxd3 in NC self-renewal and multipotency. In vivo, the NC-specific deletion of *Foxd3* resulted in loss of neural derivatives of the cranial, vagal and cardiac NC, with aberrant differentiation of mesenchymal NC derivatives and

[1]Center for Stem Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232-0494, USA. [2]Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232-0494, USA. [3]Program in Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232-0494, USA. [4]Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232-0494, USA.

*Author for correspondence (trish.labosky@vanderbilt.edu)

Accepted 29 November 2010

ectopic expansion of the NC-derived VSMC domain. In single-cell analyses of multipotency, Foxd3 played a crucial role in negatively regulating myofibroblast differentiation, thereby maintaining newly generated NCSCs in an uncommitted multipotent state. We therefore provide the first genetic identification of a specific gene requirement for both self-renewal and multipotency of NC. Together with published findings, we describe a model in which Foxd3 maintains NCSCs by inhibiting non-neural differentiation, drawing important gene-regulatory parallels between disparate stem and progenitor cell populations.

MATERIALS AND METHODS

Mouse lines

The *Foxd3* null alleles *Foxd3*$^{tm1.Lby}$ and *Foxd3*$^{tm2.Lby}$ (called *Foxd3*$^{-}$ throughout) were used interchangeably in combination with *Foxd3*$^{tm3.Lby}$, the *Foxd3* conditional allele (*Foxd3*^{flox}). These alleles were described previously (Hanna et al., 2002; Teng et al., 2008). The *Wnt1-Cre* transgenic line (Danielian et al., 1998) was used to delete *Foxd3*^{flox} and to lineage-map NC-using reporter strains *Gt*(*ROSA*)*26Sor*^{tm1Sor} (called *R26R*^{lacZ}) and *Gt*(*ROSA*)*26Sor*$^{tm1(EYFP)Cos}$ (called *R26R*^{YFP}) (Soriano, 1999; Srinivas et al., 2001). The tamoxifen-inducible transgenic line (*CAGG Cre-ER*^{TM}) (Hayashi and McMahon, 2002) was used for inducible deletion of *Foxd3*. All lines are on a mixed genetic background and maintained in accordance with protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC).

Histology

Embryos were fixed in 4% paraformaldehyde (PFA) in PBS for 4 hours or overnight, and histology was performed using standard procedures (Presnell and Schreibman, 1997). Whole-mount immunostaining (Wall et al., 1992) and 5-bromo-4-chloro-3-indolyl–D-galactoside (X-gal) staining (Nagy et al., 2003) were performed as described. The following antibodies were used: chicken-anti-green fluorescent protein to detect yellow fluorescent protein (YFP) expression from the activated *R26R*^{YFP} allele (1:500, Abcam), goat-anti-Sox10 (1:20, Santa Cruz), goat-anti-SM22α (1:200, Abcam), mouse-anti-neurofilament (2H3, 1:1000, Developmental Studies Hybridoma Bank), mouse-anti-β-III tubulin (1:500, Covance, TUJ1), mouse-anti-α-smooth muscle actin (1:200, Sigma), mouse-anti-glial fibrillary acidic protein (1:200, Sigma), rabbit-anti-Foxd3 [1:500 (Tompers et al., 2005)], rabbit anti-smooth muscle myosin heavy chain (1:200, Biomedical Technologies), rabbit anti-Sox9 (1:500, Chemicon), rabbit anti-Runx2 [1:500 (Yang et al., 2004)], rabbit-anti-cleaved caspase3 (1:200, Cell Signaling), rabbit-anti-p75 (1:200, Promega), rabbit anti-peripherin (1:1000, Chemicon). Secondary antisera were purchased from Jackson ImmunoResearch and 4′,6-diamidino-2-phenylindole (DAPI) (1:5000, Molecular Probes) was used to detect nuclei. For sequential detection of Foxd3 and p75, anti-Foxd3 and Cy3-conjugated secondary antibody labeled sections were incubated in unconjugated anti-rabbit IgG (1:15) before immunodetection of p75. The Vectastain ABC Kit (Vector Laboratories) was used for colorimetric immunohistochemical experiments. Terminal deoxynucleotidyl transferase (TUNEL) analysis was performed using the In Situ Cell Death Detection Kit (Roche).

NC explant culture

Cardiac/vagal neural tube (from mid-otic placode to somite four) and trunk neural tube segments (from somites 16 to 22) of embryos 9.0-9.5 days post coitum (dpc) were isolated by microdissection and dissociated from surrounding tissues with collagenase/dispase (Roche) using procedures modified from Stemple and Anderson (Stemple and Anderson, 1992). Explants were cultured in hypoxic conditions (3-6% oxygen and 5% CO_2) (Morrison et al., 2000) in wells coated with 30 μg/ml Fibronectin (Gibco) in self-renewal medium containing: Dulbecco's modified Eagle's medium low glucose (Invitrogen), 30% neurobasal medium (Invitrogen), 15% chick embryo extract (CEE), 2% B27 (Invitrogen), 1% N2 (Invitrogen), 117 nM retinoic acid (Sigma), 50 μM β-mercaptoethanol (Sigma), 20 ng/ml insulin-like growth factor (IGF) 1 and 20 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems) using procedures modified from Morrison et al. (Morrison et al., 1999) and Stemple and Anderson (Stemple and Anderson, 1992). After 48 hours, neural tubes were physically removed. For adherent clonal cultures, NC cells were plated at low density (25 cells/cm^2) ensuring that individual cells formed spatially distinct colonies. After 6 days, medium was changed to differentiation-promoting medium (10 ng/ml bFGF and 1% CEE) for 8 days before analysis of colony composition (Morrison et al., 1999; Mosher et al., 2007). Sphere cultures were maintained 8-10 days in low-adherence dishes (Costar) undisturbed and at low density (<1 cell per 10 μl of medium) to minimize fusion, and dissociated according to Fasano et al. (Fasano et al., 2007). All statistics were mean ± s.e.m. and *P*-values calculated with Student's *t*-test.

RESULTS

Foxd3 expression is maintained in neural derivatives and downregulated in ectomesenchymal derivatives of NC

Vagal NC (from somites one to six) and cardiac NC (from the mid-otic placode to somite four) are initially overlapping cell populations that innervate the digestive tract and diaphragm, and function in cardiac development by mediating outflow tract septation, contributing VSMCs to the great vessels and generating parasympathetic cardiac ganglia (Allan and Greer, 1997; Hildreth et al., 2008; Hutson and Kirby, 2007; Young and Newgreen, 2001). Foxd3 is differentially required in these NC populations; NC-specific *Foxd3*-null embryos have no ENS, whereas cardiac outflow tract septation is overtly normal (Teng et al., 2008). This striking difference led us to examine temporal expression of Foxd3 in *Wnt1-Cre; R26R*^{YFP} lineage-traced NC from distinct axial origins: cranial, cardiac, vagal and trunk NC. Foxd3 expression overlapped with YFP-positive cells in pre-migratory NC at the dorsal-lateralmost region of the headfolds at 8.0 dpc (four-somite stage), and expression was maintained in migratory cranial NC at 8.5 dpc (see Fig. S1A,B in the supplementary material), in agreement with our previous work (Labosky and Kaestner, 1998). At vagal/cardiac somite levels, Foxd3 was co-expressed with YFP in ventral migrating NC at 8.5 dpc (Fig. 1A). Beginning at 9.0 dpc (14 somites), Foxd3 expression was extinguished in ventralmost NC cells (Fig. 1B, arrowhead) and by 9.5 dpc, Foxd3 expression was restricted to presumptive cranial and dorsal root ganglia (DRG), with no expression detected in cranial or cardiac NC mesenchyme (Fig. 1C and see Fig. S1C,D in the supplementary material). At 10.5 dpc, Foxd3 was co-expressed with Sox10, an NCSC and glial marker in NC at the periphery of vagal and trunk DRG (Fig. 1D,E). This expression pattern was similar to previously reported co-expression of p75, an NCSC and neuronal progenitor marker, and Sox10 (Sonnenberg-Riethmacher et al., 2001), that led us to examine expression of Foxd3 and p75. At 10.5-11.5 dpc, Foxd3 and p75 were co-expressed in NC at the level of the developing esophagus and trachea, and in cranial ganglia (Fig. 1F,G). Foxd3 and p75 were downregulated in cardiac NC before entry into the outflow tract (Fig. 1F) and in cranial NC-derived mesenchyme (Fig. 1G). These expression patterns indicate that Foxd3 is present in NCSCs and support a role for Foxd3 in segregation of neural, glial and ectomesenchymal lineages of the NC.

Foxd3 is necessary for diaphragm and gastrointestinal tract innervation and formation of the parasympathetic cardiac ganglia

Foxd3 NC-conditional null mice die perinatally, presumably as a result of respiratory failure (Teng et al., 2008). To determine if this lethality is associated with defects in NC-mediated diaphragm

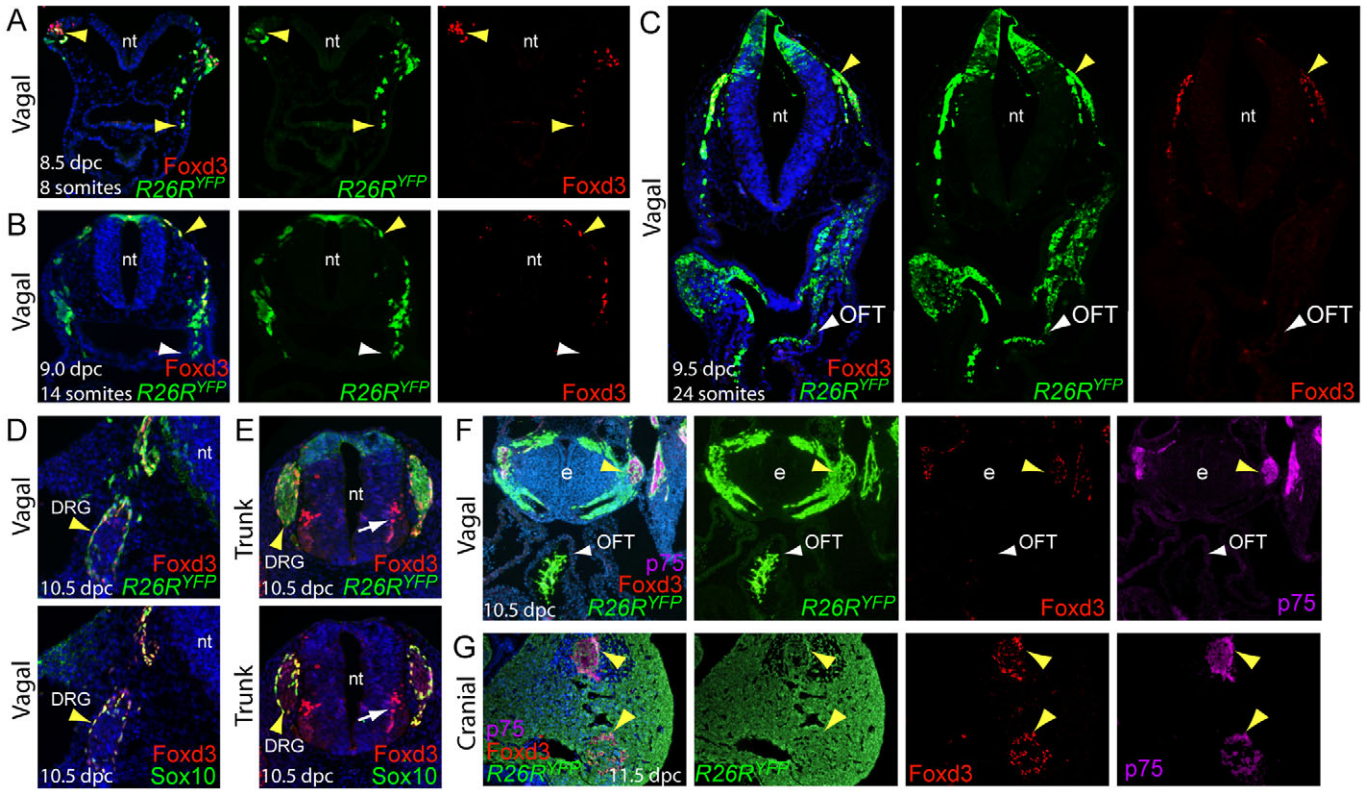

Fig. 1. Foxd3 expression is selectively maintained in neural derivatives and downregulated in mesenchymal derivatives of the NC. (**A**) Transverse sections through the vagal NC level of *Wnt1-Cre; R26R*^{YFP} (NC lineage marked) embryos shows Foxd3 (red) and YFP expression (green) at 8.5 dpc in premigratory and ventral migratory NC indicated by yellow arrowheads. (**B**,**C**) At 9.0-9.5 dpc, Foxd3 expression was maintained in dorsal migratory cells (yellow arrowheads), but was not detected in ventral NC migrating towards the heart or within the pharyngeal arch or outflow tract (white arrowheads). (**D**,**E**) NC specific co-expression of Foxd3 (red) and YFP (green, top panels) or Sox10 (green, bottom panels) in DRG at vagal (D) and trunk (E) levels. Foxd3 was also expressed in interneurons of the spinal cord (arrows in E). (**F**,**G**) Foxd3 and p75 were co-expressed in NC proximal to the esophagus (yellow arrowhead, F) and in cranial ganglia (yellow arrowheads, G). Expression was absent in cardiac and cranial NC mesenchyme. e, esophagus; nt, neural tube; OFT, outflow tract.

innervation, we evaluated NC with *Wnt1-Cre* activated *R26R*^{lacZ} and X-gal staining. In *Foxd3*$^{flox/+}$*; Wnt1-Cre* control embryos at 16.5 dpc the phrenic nerves projected to the antral right and left leaflets of the diaphragm and elongated dorsally and ventrally through the central region of the muscle (Fig. 2A, top). By contrast, only a few NC-derived cells were present in a small cluster in the left leaflet of diaphragms from NC-conditional nulls (Fig. 2A, bottom). Examination of *R26R*^{YFP} with neurofilament expression (2H3) confirmed a paucity of neurons projecting into the diaphragm in mutant embryos (Fig. 2B). Additionally, mutant vagal NC were not present in the esophagus (Fig. 2C). These data indicate a vital role for Foxd3 in development of neural derivatives of the vagal NC.

To determine if Foxd3 is required for normal differentiation of cardiac NC-derived neuronal lineages, we examined contribution of lineage-traced control (*Foxd3* heterozygous) and *Foxd3* mutant NC to the parasympathetic cardiac neurons using β-III tubulin (TUJ1) immunodetection. From 12.5-17.5 dpc, NC contributed to the dorsal cardiac ganglia in heterozygous control embryos, but in mutants, NC cells were not present in this location (n=12 mutant embryos; Fig. 2D,E). In *Foxd3* mutant embryos, NC did not contribute to parasympathetic innervation, and although non-NC neurons [presumably of placodal origin (Kirby, 1988)] were present, only rudimentary ganglia formed (Fig. 2E).

Ectopic location of NC-derived VSMCs without Foxd3

Our data suggest a model in which Foxd3 is initially expressed in all NC but then extinguished in cardiac NC before outflow tract septation, as a requisite step in the differentiation and generation of VSMCs in the great vessels. Although *Foxd3*-deficient NC mediate outflow tract septation, it was unknown whether the normal contribution of VSMC progenitors to pharyngeal arch arteries required Foxd3 expression in NC progenitors. Previous lineage-tracing studies demonstrated that cardiac NC gives rise to VSMCs in the aortic arch, but NC-derived cells do not populate the descending aorta, forming a distinct boundary with mesoderm-derived VSMCs at the junction of the left fourth and sixth arch arteries with the dorsal aorta (Jiang et al., 2000; Le Lievre and Le Douarin, 1975). To investigate if Foxd3 is required for cardiac NC to contribute appropriately to the great vessels, we compared distributions of lineage-traced control and conditional *Foxd3*-null NC in the aorta. At 16.5 dpc, NC cells populated the aortic arch of both *Foxd3* heterozygous control and mutant embryos (Fig. 3A,B). The sharp boundary between NC- and mesoderm-derived VSMC was apparent at the level of the ductus arteriosus in control embryos (Fig. 3A, arrow). However, this border was no longer respected in *Foxd3* mutant embryos and NC-derived cells were detected caudally, throughout the descending aorta, to at least the

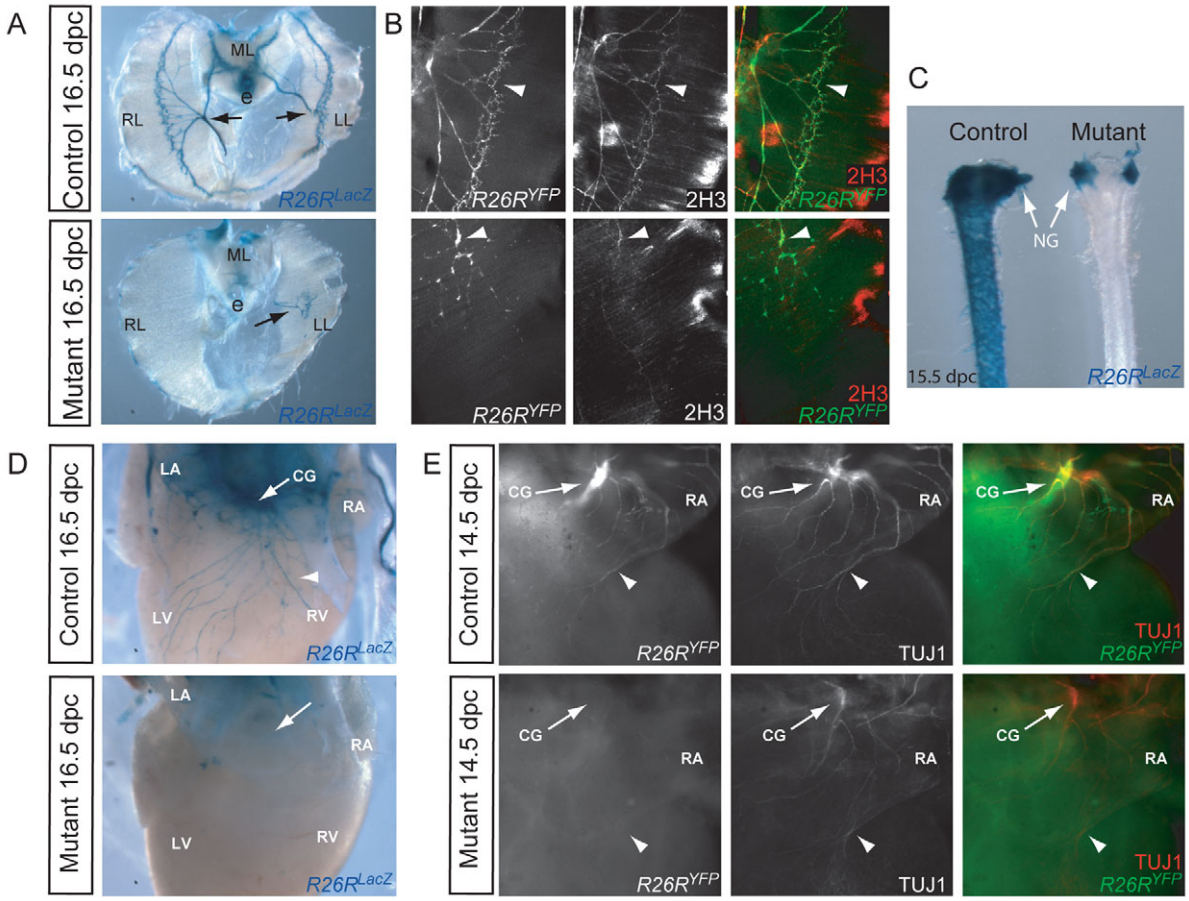

Fig. 2. Foxd3 is required for neural derivatives of the cranial, vagal and cardiac NC. (**A**) Loss of diaphragm innervation in mutant embryos. Top view of diaphragm from a lineage-mapped 16.5 dpc *Wnt1-Cre; R26R*^{lacZ} (control) embryo showed X-gal-positive cells in the phrenic nerves (arrows) that extended dorsally and ventrally to the diaphragm muscle leaflets (top panel). In mutant embryos, diaphragms were almost completely devoid of NC cells (bottom panel). A small cluster of NC cells from the left phrenic nerve (arrow) was present in five out of six mutant diaphragms examined. (**B**) Whole-mount immunohistochemistry for neurofilament (2H3) and YFP from the *R26R*^{YFP} allele identified NC-derived neurons (arrowhead) in control embryos at 16.5 dpc (top). The small number of mutant NC-derived cells were disorganized and few 2H3-positive neurons were detected (bottom). (**C**) Lineage mapping at 15.5 dpc shows NC cells did not innervate the esophagus in *Foxd3* mutant embryos (right). Nodose ganglia (arrows) were present but smaller in mutants. (**D**) Cardiac ganglia in control and mutant embryos. An NC-derived network of nerve ramifications emanated from the parasympathetic cardiac ganglia (arrow) in control X-gal stained hearts at 16.5 dpc (dorsal view), but this was missing in Foxd3 mutant embryos. Arrows indicate position of ganglia. (**E**) *Wnt1-Cre* mediated YFP expression from *R26R*^{YFP} and neuronal β-III Tubulin (TUJ1) identified neurons of NC (YFP-positive) and placodal (TUJ1-positive/YFP-negative) origin in control cardiac ganglia (arrows) at 14.5 dpc (top panels). YFP expression was not detected in mutant TUJ1-positive cardiac ganglia and neural projections (arrowheads) were greatly reduced (bottom panels). CG, cardiac ganglia; e, esophagus; LA, left atria; LL, left leaflet; LV, left ventricle; ML, middle leaflet; NG, nodose ganglia; RA, right atria; RL, right leaflet; RV, right ventricle.

level of the diaphragm. Confocal analysis of the aorta subjected to YFP immunodetection co-labeled with smooth muscle 22α (SM22α) or smooth muscle myosin heavy chain (SM-MHC) showed *Foxd3* mutant NC in the VSMC layer of the descending aorta (Fig. 3C-H). Further analysis of VSMC markers in transverse sections from lineage-traced 14.5 and 16.5 dpc embryos confirmed contribution of mutant NC to VSMCs within the tunica media of the descending aorta (see Fig. S2F-H in the supplementary material; data not shown). The relative contribution of NC cells in the descending aorta at 14.5-16.5 dpc was greater at regions proximal to the normal NC boundary (average YFP-positive vascular area=31% just caudal to the ductus arteriosus, n=4) compared with more distal regions (8.2% YFP-positive, at the diaphragm), demonstrating a rostral-to-caudal gradient of ectopic mutant NC. These data may indicate that *Foxd3* mutant cardiac NC-derived VSMCs are unable to maintain their position rostral to this border, or alternatively, other initially non-VSMC NC progenitors, presumably from vagal and/or trunk NC, may inappropriately acquire VSMC fates and aberrantly contribute to the descending aorta.

Foxd3 regulates mesenchymal differentiation in cranial NC

The ectopic location of Foxd3 mutant NC-derived VSMCs in the aorta raised the question of whether other mesenchymal NC populations were similarly affected by loss of Foxd3. Cranial NC generate both neural (cranial ganglia) and mesenchymal (facial bone and cartilage) derivatives, therefore we examined differentiation and patterning of this NC population. Analysis of TUJ1 expression (neurons) in lineage-traced embryos revealed

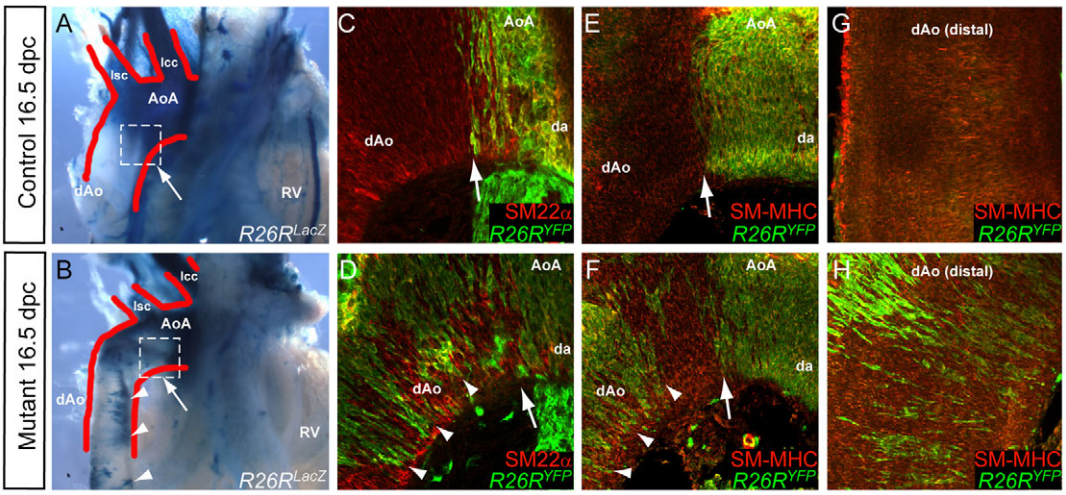

Fig. 3. Deletion of Foxd3 results in defects in location of NC-derived VSMCs. (**A**,**B**) Lineage mapping at 16.5 dpc in *Wnt1-Cre; R26R*^{lacZ} control and mutant embryos (dorsal view) showed X-gal-positive NC present in the aortic arch (outlined in red) at the normal lineage boundary at the level of the ductus arteriosus (arrow) in control embryos. By contrast, mutant NC was present throughout the descending aorta (arrowheads in B). Boxes in A,B indicate approximate fields in C-F. (**C-H**) Representative confocal images of *Wnt1-Cre; R26R*^{YFP} NC (green) in the VSMC layer of the aortic arch, determined by Z-stack analysis; SM22α (C,D) or SM-MHC (E-H) in red. Control YFP-positive cells formed a distinct border at the level of the ductus arteriosus (C,E) and were not detected in the descending aorta (G). By contrast, *Foxd3* mutant NC (YFP-positive) was located distal to the border (border marked with arrows, ectopic cells indicated with arrowheads in D and F) in the descending aorta (H). Fields in G and H depict distal regions of the aorta approximately one-third of the distance towards the diaphragm. AoA, aortic arch; da, ductus arteriosus; dAo, dorsal aorta; lcc, left common carotid; lsc, left subclavian artery; RV, right ventricle.

that, in contrast to control embryos (Fig. 4A), mutant NC showed limited contribution to cranial nerves, with co-expression of TUJ1 and YFP primarily in DRG (Fig. 4B). During early stages of endochondrial bone formation (11.5-12.5 dpc), chondrogenesis was not apparent in control embryos: Runx2, marking osteoblast progenitors, and collagen-II, a chondrocyte marker, were not detected (data not shown). Sox9 expression (marking osteo-chondroprogenitors) was only faintly detected in the mandibular component of the first pharyngeal arch at 11.5 dpc (Fig. 4C). In 12.5 dpc control embryos, Sox9 was uniformly expressed in the distal mandibular precartilage primordium (Fig. 4E-E″), and this expression domain largely overlapped with that of Runx2 (Fig. 4F-F″, arrowheads). In stark contrast, mutant NC showed precocious induction of Sox9 at 11.5 dpc (Fig. 4D). By 12.5 dpc, Sox9 was strongly expressed in the medial region of the developing mandible with lower expression at the lateral edges (Fig. 4G-G″, arrow) whereas Runx2 was highly expressed at the lateral edges (Fig. 4H-H″, arrowheads). Our results indicate that *Foxd3*-null cranial NC cells preferentially formed non-neural lineages and that differentiation of mesenchymal NC lineages was accelerated.

Maintenance of NCSC marker expression is dependent on Foxd3

The observation that Foxd3 is expressed in early, presumably multipotent, NC, and downregulated in later migratory NC, raised the hypothesis that Foxd3 is required to maintain the multipotent nature of NC. To address this in a tightly controlled manner, we turned to a culture system in which neural tube-derived NCSCs can be maintained as undifferentiated cells (Stemple and Anderson, 1992). Approximately 96% of cells actively migrating away from neural tube explants demonstrated *Wnt1-Cre*-based reporter activation (see Fig. S3 in the supplementary material). Nuclear Foxd3 expression was detected in control NC cultures but undetectable in mutant NC (Fig. 5A,B), demonstrating efficient deletion of the coding region. Heterozygous control NC cultured for 48 hours in self-renewal medium maintained expression of NCSC markers p75 and Sox10, and few cells expressed smooth muscle α-actin (SMA), a marker of myofibroblast and VSMC differentiation (Fig. 5C,E). By contrast, almost all mutant NC cells from both vagal and trunk levels were devoid of p75 [decreased by 99% in vagal and trunk NC cultures (P<0.05); Fig 5D,F and see Figs S4 and S5 in the supplementary material]. Similarly, p75 expression was reduced in vivo in fate-mapped vagal and trunk NC in mutant embryos at 9.5 dpc (see Fig. S4D,E,I,J in the supplementary material), suggesting a role for Foxd3 in establishing or maintaining p75-expressing NCSCs. Additionally, expression of Sox10, normally detected in NCSCs and differentiated glia, was reduced in mutant cultures. The few Sox10-positive cells present did not co-express p75 (Fig. 5F), suggesting differentiation towards the glial lineage. Mutant explant cultures contained increased numbers of SMA-expressing cells (Fig. 5D), demonstrating that mutant NC differentiate towards the myofibroblast lineage under conditions that normally maintain undifferentiated NCSC.

To further evaluate if Foxd3 is required to repress differentiation of individual NC cells towards multiple lineages, thereby ensuring continued multipotency, we performed in vitro culture of NC cells at clonal density (Stemple and Anderson, 1992). Individual control NC cells typically proliferated to form round, tightly packed colonies whereas the majority of mutant colonies consisted of loosely-packed fibroblast-like cells, consistent with the data above that showed a tendency for mutant NC to adopt a myofibroblast fate (Fig. 5G,H). To examine differentiation status at a molecular level, NC colonies were cultured for six days under self-renewing conditions and evaluated for expression of p75 or markers of neural

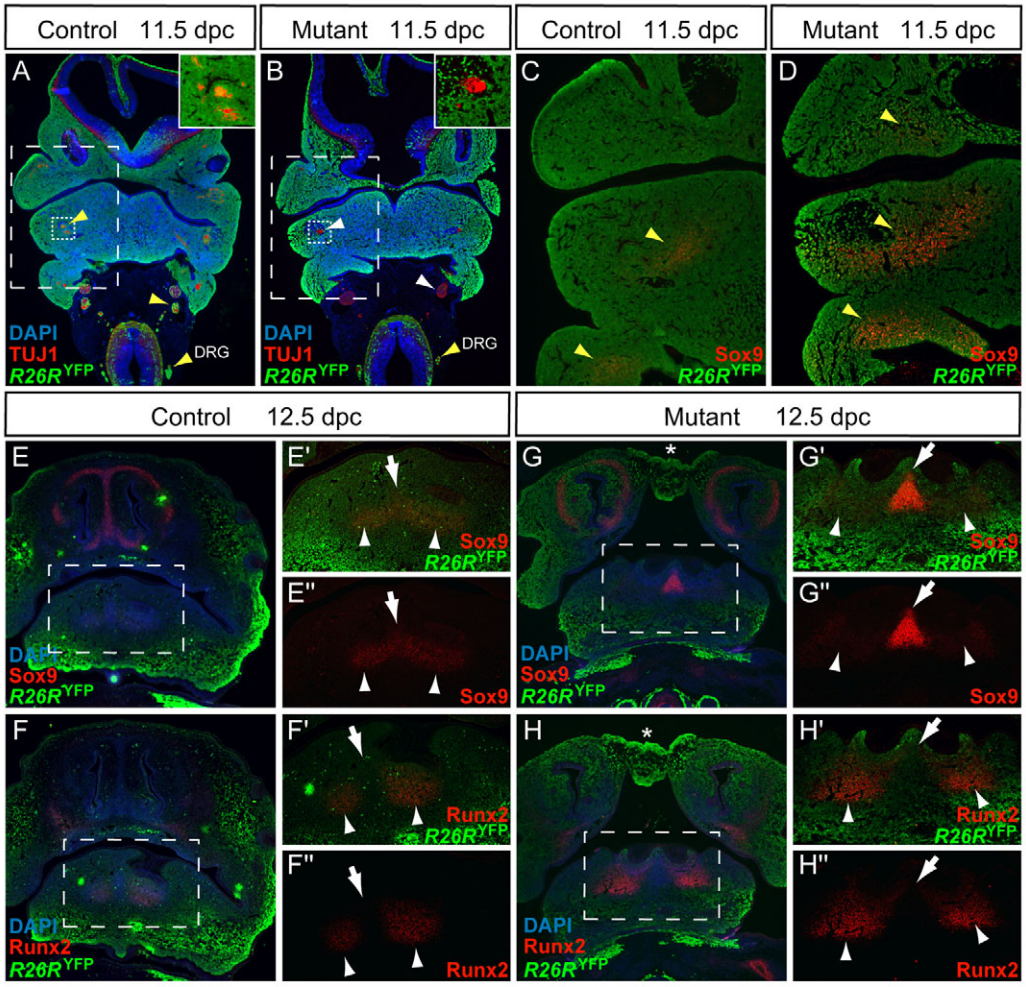

Fig. 4. Foxd3 regulates timing of mesenchymal differentiation in cranial NC. (**A-D**) In vivo characterization of cranial NC differentiation in *Wnt1-Cre; R26R*^{YFP} control and mutant embryos. TUJ1 (red) and YFP (green) expression identified NC-derived neurons (yellow arrowheads) and non-NC-derived neurons (white arrowheads) in transverse sections of 11.5 dpc embryos (A,B). Expression of Sox9 (red) in the mandible represents precocious differentiation towards mesenchymal NC fates in mutant (D) compared with control (C) embryos. (**E-H″**) Sox9 and Runx2 expression at 12.5 dpc. In near-adjacent sections through the mandible of a control embryo, Sox9 expression (E-E″) overlaps extensively with the Runx2 expression domain (arrowheads, F-F″). In *Foxd3* mutant embryos, Sox9 was strongly expressed in a wedge of cells at the center of the mandible (arrow) with low levels of expression in adjacent mesenchyme (arrowheads, G-G″). Runx2-expressing osteoblast progenitors are adjacent to cells expressing high levels of Sox9 (H-H″), indicating segregation of osteoblast and chondrocyte lineages characteristic of a later developmental stage. Images were captured at identical exposures for control and mutant sections and manipulated in a similar manner. Small boxes in A and B denote magnified region at the upper right, larger boxes denote regions shown in panels C and D. Boxes in E-H indicate regions shown in E′-H″ to the right. *, craniofacial defect.

(Peripherin), glial [glial fibrillary acidic protein (GFAP)], and myofibroblast (SMA) differentiation. Control NC colonies expressed p75 (Fig. 5I), and significant neural, glial and myofibroblast differentiation was not detected (Fig. 5K). In stark contrast to controls, clonally derived mutant NC colonies rarely expressed p75 (Fig. 3J), and although many NC colonies (60-70%) contained SMA-positive cells, the remaining non-myofibroblast colonies did not exhibit aberrant neural and glial differentiation (Fig. 5L). These data indicate that Foxd3 is required to repress myofibroblast differentiation and maintain NCSCs in an undifferentiated state.

Consistent with increased apoptosis in the neural tube of *Foxd3* NC-mutant embryos (Teng et al., 2008), the loss of p75 and Sox10 in mutant NC could reflect selective apoptosis of NCSCs. However, apoptosis was only modestly increased in *Foxd3* mutant NC in vitro, measured by cleaved-caspase3 detection or TUNEL assay (see Fig. S5 in the supplementary material). Comparison of control and mutant outgrowths showed no significant differences in initial cell numbers or migration index despite loss of p75 expression (see Fig. S4C,H in the supplementary material). In a complementary approach, we failed to detect restoration of NCSC numbers when neural tube explants were exposed to an apoptosis inhibitor [the caspase inhibitor, Z-VAD-FMK, (Maynard et al., 2000; Uesaka et al., 2007)]. Using TUNEL assays to detect caspase-dependent and independent apoptosis, Z-VAD-FMK reduced cell death in both control and mutant NC, but p75 expression was not restored (see Fig. S5 in the supplementary material). This suggests a direct cell-autonomous role for Foxd3 in maintaining NCSC multipotency, rather than simply increasing cell survival.

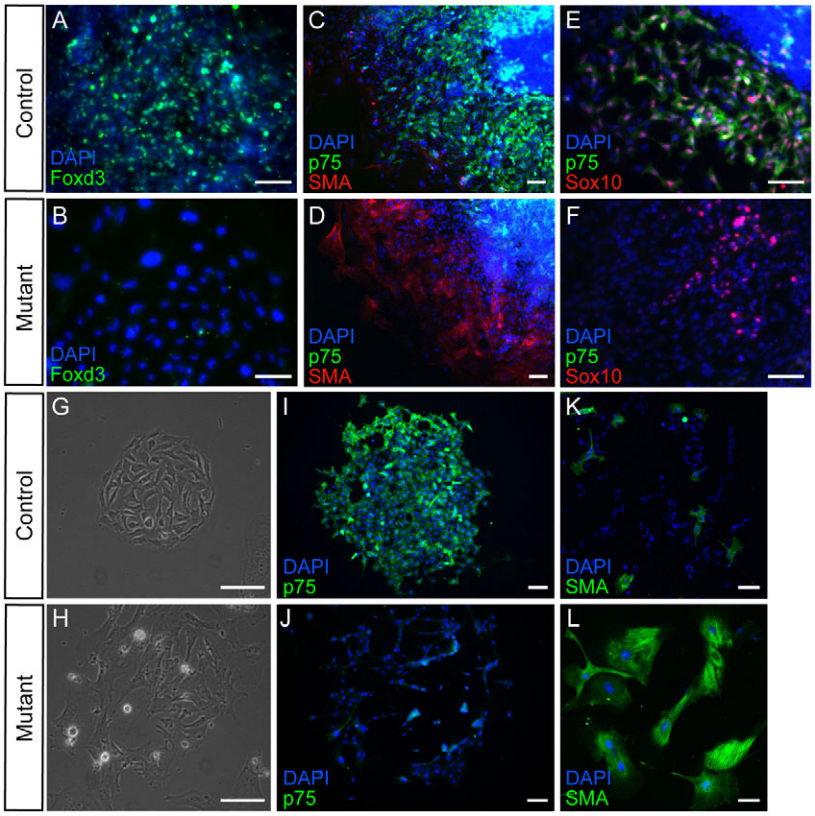

Fig. 5. Maintenance of NCSC marker expression is dependent on Foxd3. (**A**,**B**) Immunocytochemistry revealed nuclear Foxd3 (green) in the majority of control NC cells in a 48-hour vagal neural tube explant culture (A). Foxd3 expression was undetectable in a *Foxd3*$^{flox/-}$*; Wnt1-Cre* mutant neural tube outgrowth (B). (**C**,**D**) Immunofluorescence images showing p75 (green) and SMA (red) in NC explants. Most cells in control cultures were negative for SMA, with robust p75 expression. Mutant NC outgrowths had reduced p75 expression and increased numbers of SMA-expressing cells. (**E**,**F**) Immunocytochemistry for Sox10 (red) and p75 (green) in control NC cultures showed widespread co-expression of these two NCSC markers (E). Mutant outgrowths contained reduced numbers of Sox10-positive cells and p75 expression was not detected (F). (**G-L**) Secondary NC cultures at clonal density. Control colonies were typically round and contained spindle-like cells (G), maintained p75 expression (green) (I) and showed little significant differentiation into myofibroblasts (assayed by SMA expression, green) (K) after 6 days of culture. Mutant NC formed irregularly shaped colonies (H), did not maintain p75 expression (J), and the majority of colonies (approximately 70%) contained large, flattened cells expressing SMA (L). Scale bars: 100 μm.

Reduced self-renewal in Foxd3 mutant NC

Self-renewal is a defining characteristic of stem cells, and Foxd3 is crucial for this property in ESCs (Hanna et al., 2002; Liu and Labosky, 2008). Therefore, to determine if Foxd3 regulates self-renewal in NCSCs we used neurosphere culture assays to measure this crucial stem cell property. Compared with wild-type NC, *Foxd3* mutant NC generated smaller neurospheres at much lower frequency (Fig. 6A-D). Analysis of secondary neurosphere formation from dissociated individual primary neurospheres showed that *Foxd3* mutant NC was significantly impaired in self-renewal capacity (vagal NC reduced by 64%; trunk NC by 47%; Fig. 6E). To control for the smaller size of primary *Foxd3* mutant neurospheres, we also dissociated pools of primary neurospheres, plated equivalent cell numbers, and again observed similar reductions in self-renewal from mutant neurospheres (reduced 63% for vagal NC, 55% for trunk NC; Fig. 6F-H). Taken together, our findings reveal a conserved role for Foxd3 in both NC and ESCs in the maintenance of stem cell self-renewal.

Foxd3 is required for multilineage potential of NC progenitors

If Foxd3 plays a role in maintaining multipotency of NCSCs, it is possible that individual mutant NC may have a restricted ability to generate progeny towards one or more NC lineages. In vivo, *Foxd3* mutant NC gave rise to mesenchymal lineages, but showed limited capacity to generate neurons (Figs 2 and 3). Similarly, NC contributed some neurons and glia to smaller than normal DRG, but derivatives more distal to the neural tube were absent (Teng et al., 2008). To directly address whether individual NC cells have reduced lineage potential, clonally-derived colonies from vagal and trunk NC were cultured under differentiation-promoting conditions, and lineage potency measured by immunocytochemistry for differentiation markers as above and scored as described (Morrison et al., 1999) (Fig. 7A). Compared with wild-type (or heterozygous; see Fig. S6C in the supplementary material) control cultures, *Foxd3* mutant NC had significantly reduced numbers of neuron (N)-containing colonies (mutant vagal and trunk N-containing colonies reduced by 61 and 69%, $P<0.001$; see Fig. S6A,B in the supplementary material), supporting a role for Foxd3 in maintaining neural potential, consistent with in vivo results (Fig. 2). Further quantification indicated that individual mutant cells had additional defects in potency. For example, a reduced proportion of bi-potent cells produced both neurons and myofibroblasts (N+M; Fig. 7B,C). Most significantly, mutant NC rarely (1% of vagal NC, 2% of trunk NC, $P<0.001$) formed multipotent (neural/glial/myofibroblast, N+G+M) colonies compared with control NC (10% of vagal NC, 16% of trunk NC). The rare mutant multipotent colonies were significantly smaller than controls, and almost always showed a strong differentiation bias towards a single fate (N, G or M), with few cells adopting other fates. Coincident with loss of multipotency, mutant clonal cultures displayed an increased incidence of myofibroblast (M-only) colonies, but the percentage of neural or glial (N-only or G-only) colonies was unaffected. This altered spectrum of potential in control versus mutant NC was similar in vagal and trunk NC, demonstrating that loss of multipotency, and an increased bias toward the myofibroblast lineage, is an equivalently penetrant phenotype along the anteroposterior axis (Fig. 7B,C). In a complimentary approach, inducible deletion of Foxd3 after NC migrated from the neural tube resulted in reduction of Sox10 expression, and recapitulated these changes in NC potency (loss of N+G+M and bias towards M-only; see Fig. S7 in the supplementary material). In all of the above assays, *Foxd3*-heterozygous NC were similar to wild type in p75 expression, self-

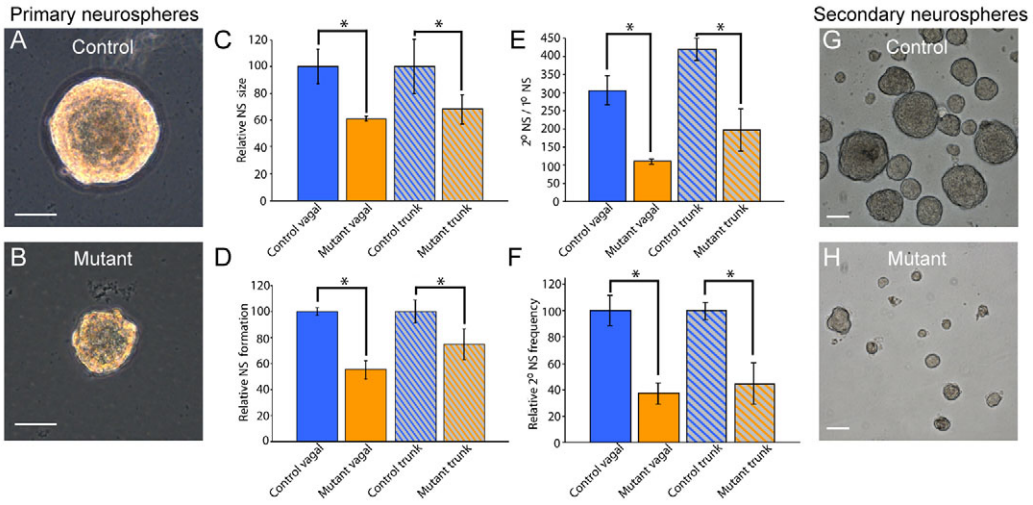

Fig. 6. Reduction of self-renewal in Foxd3 mutant NC. (**A-D**) Dissociated vagal and trunk NC were cultured at clonal density in non-adherent conditions for 10 days. Representative control (A) and mutant (B) primary NC neurospheres are shown. Quantification of relative neurosphere diameter (C) and percent of dissociated cells that formed primary neurospheres (D) demonstrated defects in *Foxd3* mutant NC. (**E-H**) Self-renewal was measured from individually isolated primary neurospheres (E) or as the relative frequency of secondary sphere formation from pooled primary neurospheres (F). Representative fields of control (G) and mutant (H) secondary neurospheres. Data represent three to six independent experiments with three technical replicates for pooled neurospheres and eight replicates for individual neurospheres for each experiment. All statistics are mean ± s.e.m.; *, $P<0.05$. Scale bars: 50 μm.

renewal and lineage composition of clonal cultures (see Fig. S5C in the supplementary material; data not shown), consistent with absence of NC defects in heterozygous embryos or mice. Together, these data clearly demonstrate that Foxd3 is required to maintain multipotency in NCSCs, but is not required for differentiation to any specific lineage.

DISCUSSION

Our findings demonstrate a cell-autonomous requirement for the transcription factor Foxd3 in maintaining both self-renewal and multipotency of NC progenitors. Foxd3 plays a pervasive role in preserving these definitive stem cell properties throughout the NC; the conditional inactivation of *Foxd3* affected cranial, cardiac, vagal and trunk domains similarly. Clonal analyses of *Foxd3* mutant NC indicated a severe reduction in self-renewal and an increased bias towards myofibroblast-directed NC, which was associated with an almost complete loss of multipotent NCSCs. Overall, neural differentiation was reduced in mutant cultures, suggesting that multipotent (N+G+M) cells and neural-containing bipotent cells (N+M or N+G) undergo a selective loss of neural potential, moving towards myofibroblast or glial fates. However, the frequency of neural-only or glial-only restricted progenitors was not significantly altered, indicating that lineage entry is not impaired by loss of Foxd3. Our current model is that Foxd3 permissively maintains multipotent NCSCs in an uncommitted state, rather than functioning as part of an instructive cue to instill neural or glial competency.

Our results support a model in which Foxd3 functions as a gatekeeper in multipotent NCSCs to repress mesenchymal lineages and preserve neural fates and multipotency (Fig. 7D). NC-specific inactivation of Foxd3 caused profound deficits in innervation of several target organs, and NC cells aberrantly adopted mesenchymal cell fates. This is consistent with the notion that Foxd3 functions in opposing programs controlling neural versus mesenchymal potential of NC. Without Foxd3, individual NC cells committed to one of the three major lineages (neural, glial or myofibroblast) may be unaffected, whereas multipotent and bi-potent progenitors destined to more distally located neural and/or glial fates are unable to repress differentiation toward mesenchymal lineages. An alternative, although not mutually exclusive, explanation is that the progressive reduction in mutant NC self-renewal we describe here may selectively exhaust the neural-containing progenitor pool, so that by the time NC reach distant locations, their overall neural potential is dramatically reduced or absent. In either of these possibilities, we suggest that Foxd3 promotes self-renewal and multipotency independent of NCSC survival mechanisms because blocking caspase activity in *Foxd3* mutant NC cultures did not restore expression of NCSC-associated proteins.

Foxd3 represses ectomesenchymal cell fates

Distinct NC cell fates are thought to arise via progressive restrictions in lineage potential before and during NC migration (Krispin et al., 2010; Young et al., 2003). Foxd3 is expressed in pre-migratory NC and is maintained in early migrating NC progenitors, but is downregulated in cranial and cardiac NC mesenchyme well before chondrocyte, osteoblast or VSMC differentiation. Our findings demonstrate an early requirement for Foxd3 in repression of ectomesenchymal fates shortly after cells leave the neural tube, before arrival at the pharyngeal arches or cardiac outflow tract. During craniofacial development, Sox9 is required for specification of bi-potent osteochondroprogenitors (Akiyama et al., 2002; Lefebvre et al., 1997; Mori-Akiyama et al., 2003) and subsequently, Runx2 acts as a determining factor for differentiation of osteoblasts (Ducy et al., 1997; Komori et al., 1997). Loss of Foxd3 in the NC resulted in ectopic activation of both Sox9 and Runx2 in cranial NC, therefore premature endochondrial bone and cartilage formation may underlie the severe craniofacial defects observed in *Foxd3* NC mutant embryos (Teng et al., 2008). In addition to controlling the timing of cranial

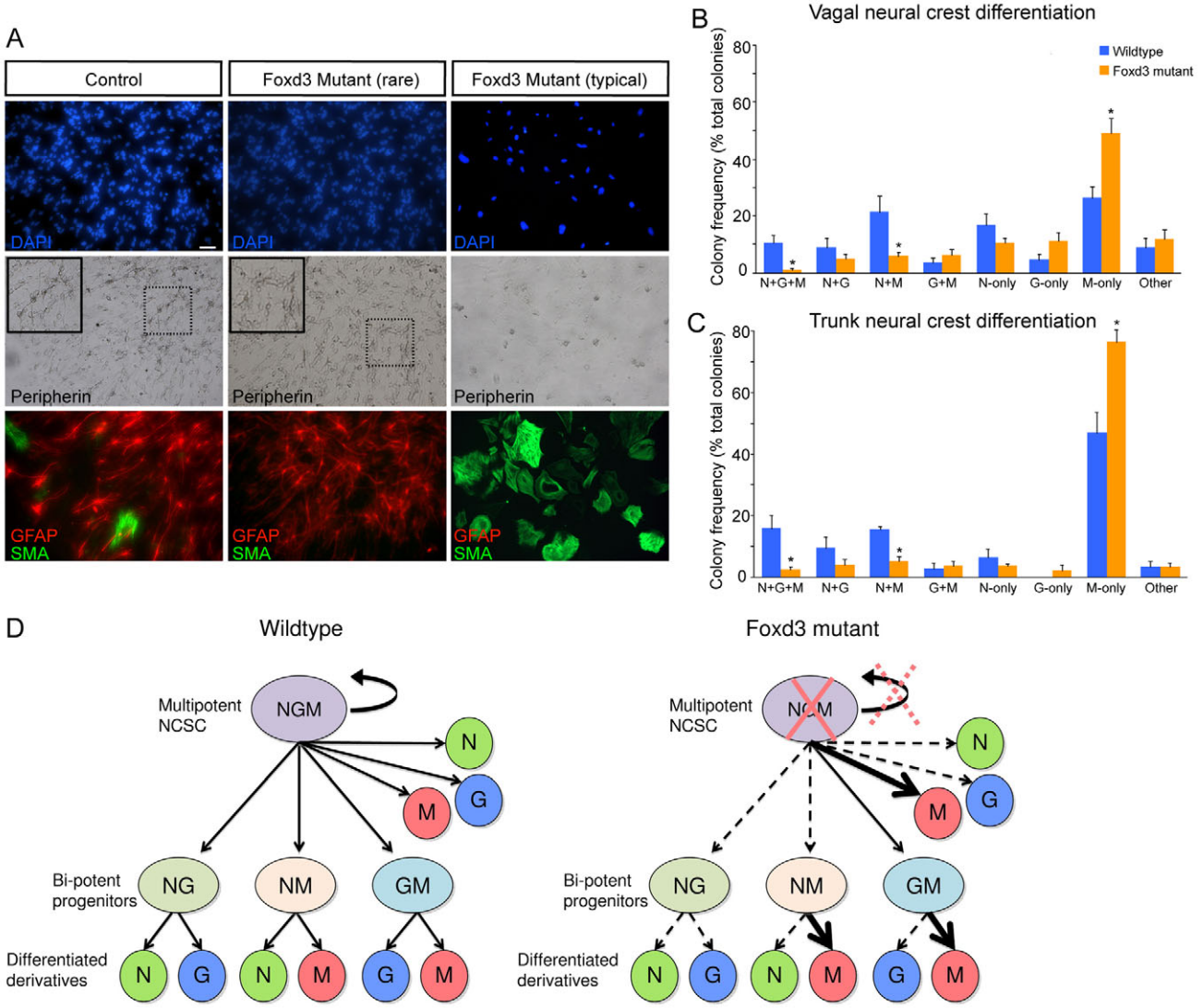

Fig. 7. Foxd3 controls multipotency of individual NC cells. (**A**) NC cells were cultured at clonal density in self-renewal medium for 6 days and then differentiation was promoted for 8 days by changing culture medium. Immunocytochemical analysis of multilineage differentiation into neurons (peripherin), glia (GFAP, red) and myofibroblasts (SMA, green). Images of a single field from a control (N+G+M) multilineage containing colony (left), a rare Foxd3 mutant (N+G) colony (center) and a more typical (M-only) mutant colony (right panels) are shown. Insets show higher magnification of Peripherin-positive neurons. (**B**,**C**) Quantification of lineage composition of single-cell derived colonies. Both vagal and trunk *Foxd3* mutant NC formed bi-potent colonies with neural potential (N+G and N+M) at reduced frequency and rarely formed multipotent colonies compared with control cultures. Mutant clonal cultures contained an increased percentage of myofibroblast-only (M-only) colonies and were unchanged from control NC in establishing restricted progenitor colonies (N-only or G-only). Data represent six independent experiments with at least three technical replicates of clonal cultures from ten mutant and six wild-type littermate control embryos. All statistics are mean ± s.e.m.; *, $P<0.05$ comparing control with mutant. (**D**) A model of Foxd3 action in multipotent NCSCs. Foxd3 is required for self-renewal of NCSCs (curved arrows) and for maintenance of the neural lineage choice (N). Without Foxd3 (right side of diagram) NCSCs have reduced self-renewal capacity (dashed X) and preferentially and precociously differentiate towards mesenchymal fates.

NC differentiation, we also provide evidence that Foxd3 is required to restrict NC progenitors from VSMC and myofibroblast fates. Normally, cardiac NC generates VSMCs in precise regions of the aortic arch and great vessels (Jiang et al., 2000; Le Lievre and Le Douarin, 1975). The contribution of NC-derived VSMCs extends from the ascending aorta caudally to the precise location where the ductus arteriosus connects to the aortic arch. Further caudally, VSMCs in the descending aorta are derived from mesoderm (Esner et al., 2006; Wasteson et al., 2008; Wiegreffe et al., 2007). Our lineage-tracing experiments showed that *Foxd3* mutant NC extended caudally well past the aortic boundary, but other NC-mesoderm boundaries in the pulmonary arteries were still respected (data not shown). In multiple locations in vivo and in vitro the neural potential of mutant NC was decreased, whereas mesenchymal potential was increased. Therefore, it is tempting to speculate that mutant NC located in the distal aorta are derived from cells that would normally give rise to neural and/or glial cells in the nearby gastrointestinal tract or sympathetic ganglia that underwent an inappropriate lineage decision to a mesenchymal fate. Lineage analysis of NC populations captured on the basis of

emigration and Foxd3 expression status could begin to define these transitional cell states in a prospective manner, and live cell imaging in the whole embryo could resolve some of these unanswered questions.

Molecular regulation of progenitor cell multipotency and self-renewal

One surprising discovery we describe here is that Foxd3 functions to preserve multi (or bi-) potency by maintaining the neural lineage choice open in multipotent NCSCs. Therefore, regulation of Foxd3 expression is important with respect to the ability of NC to interpret environmental signals effecting lineage allocation. Combinatorial Wnt and BMP signaling synergistically maintain NCSC multipotency and suppress differentiation (Kleber et al., 2005; Lee et al., 2004; Wurdak et al., 2005), and transforming growth factor (TGF) β signaling promotes non-neural lineage differentiation from NCSCs (Shah et al., 1996; Wurdak et al., 2005). Our findings here are consistent with the possibility that Foxd3 functions at a crucial early step in NCSC multipotency and self-renewal by permissively maintaining responsiveness to Wnt/BMP signaling and/or functions in opposition to TGFβ signaling to promote neural fates. In contrast to factors such as Bmi1 and Hmga2 that specifically regulate self-renewal independent of initial NC multipotency (Molofsky et al., 2003; Nishino et al., 2008), Foxd3 is required in newly generated NC for both multilineage potential and self-renewal. It is likely that these properties are intimately coupled by Foxd3; reduced self-renewal of mutant NC may be because of increased differentiation and not as a result of direct cell cycle regulation. Proliferation of *Foxd3* mutant NC is not reduced in vivo, or in *Foxd3* mutant ESCs despite diminished self-renewal and multipotency (Liu and Labosky, 2008; Teng et al., 2008).

In addition to Foxd3, the only other transcription factor definitively linked to multipotency of NC is Sox10, which maintains NCSCs by inhibiting differentiation into neural cell fates to allow for gliogenesis (Kim et al., 2003). Heterozygous loss of *Sox10* results in defects in glial and melanocyte lineages and, similarly to *Foxd3* mutant embryos, *Sox10* homozygous mutants fail to form the ENS (Southard-Smith et al., 1998). Sox10 expression is markedly reduced in both *Foxd3* mutant embryos (Teng et al., 2008) and NC cultures. Although it is possible that Foxd3 may regulate Sox10 expression and vice versa, it is also likely that they are coordinately regulated (Drerup et al., 2009). Sox10 preservation of glial fates may act in synchrony with Foxd3-mediated maintenance of neural competency within NCSCs to ensure multipotency. Future studies will address whether these two transcription factors act in a single common pathway or in parallel to regulate NCSC multipotency.

The precise molecular mechanisms by which Foxd3 controls self-renewal and multipotency are incompletely understood, but presumably involve direct regulation of gene transcription and/or regulation of chromatin modifications (Liber et al., 2010; Xu et al., 2009). Although primarily characterized as a transcriptional repressor, Foxd3 has both activator or repressor activity, depending upon the cellular context (Guo et al., 2002; Lee et al., 2006; Steiner et al., 2006; Sutton et al., 1996; Yaklichkin et al., 2007). A distinct possibility is that interactions with differentially expressed co-factors modulate Foxd3 function. Consistent with this idea, Foxd3 mediates repression of *Mitf* (thereby inhibiting melanocyte cell fate) via a protein-protein interaction with Pax3, and not by direct effects on the *Mitf cis*-regulatory sequences (Thomas and Erickson, 2009). Together with our previous findings, the model emerging is of a complex, context-dependent role for Foxd3 as a multifunctional protein regulating gene expression in distinct cellular programs. A further possibility is that Foxd3 controls multipotency and self-renewal of NC by its central participation in the overall repression of gene regulatory networks promoting NC differentiation to non-neural lineages.

Our work now adds NCSCs to ESCs and TSCs as multipotent progenitor pools with a strict requirement for Foxd3 in maintaining multipotency and self-renewal (Hanna et al., 2002; Liu and Labosky, 2008; Tompers et al., 2005). This molecular conservation of function suggests that Foxd3 may be a fundamental integrator and/or effector of the upstream pathways modulating ‘stemness’. Determining if Foxd3 regulates common target genes or signaling pathways in these divergent progenitor populations could lead to a clearer fundamental understanding of the levels of regulation involved in multipotency, and assist with development of methods for accessing control of populations for use in stem cell-based therapies; these will be important future directions.

Acknowledgements

We thank A. Frist, A. LeGrone and J. Kappa for technical assistance; E. Armour for preliminary apoptosis data; X. Yang for advice and the Runx2 antibody; and D. Bader, S. Huppert, B. Nelms, J. Saint-Jeannet, M. Southard-Smith and C. Wright for helpful discussions and crucial reading of the manuscript. The 2H3 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD. Olympus FV1000 confocal microscopy was performed in part through use of the VUMC Cell Imaging Shared Resource. This work was supported by an R01 grant from the NIH (HD36720) to P.A.L., Vanderbilt University Medical Center Academic Program Support, and predoctoral fellowships from the A.H.A. (0615209B) and NIH (NS065604) to N.A.M. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.054718/-/DC1

References

Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedl, A. and de Crombrugghe, B. (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev.* **16**, 2813-2828.

Allan, D. W. and Greer, J. J. (1997). Embryogenesis of the phrenic nerve and diaphragm in the fetal rat. *J. Comp. Neurol.* **382**, 459-468.

Baroffio, A., Dupin, E. and Le Douarin, N. M. (1991). Common precursors for neural and mesectodermal derivatives in the cephalic neural crest. *Development* **112**, 301-305.

Bronner-Fraser, M. and Fraser, S. E. (1988). Application of new technologies to studies of neural crest migration and differentiation. *Am. J. Med. Genet.* **Suppl. 4**, 23-39.

Bronner-Fraser, M., Sieber-Blum, M. and Cohen, A. M. (1980). Clonal analysis of the avian neural crest: migration and maturation of mixed neural crest clones injected into host chicken embryos. *J. Comp. Neurol.* **193**, 423-434.

Crane, J. F. and Trainor, P. A. (2006). Neural crest stem and progenitor cells. *Annu. Rev. Cell Dev. Biol.* **22**, 267-286.

Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K. and McMahon, A. P. (1998). Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr. Biol.* **8**, 1323-1326.

Delfino-Machin, M., Chipperfield, T. R., Rodrigues, F. S. and Kelsh, R. N. (2007). The proliferating field of neural crest stem cells. *Dev. Dyn.* **236**, 3242-3254.

Dottori, M., Gross, M. K., Labosky, P. and Goulding, M. (2001). The winged-helix transcription factor Foxd3 suppresses interneuron differentiation and promotes neural crest cell fate. *Development* **128**, 4127-4138.

Drerup, C. M., Wiora, H. M., Topczewski, J. and Morris, J. A. (2009). Disc1 regulates foxd3 and sox10 expression, affecting neural crest migration and differentiation. *Development* **136**, 2623-2632.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L. and Karsenty, G. (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89**, 747-754.

Esner, M., Meilhac, S. M., Relaix, F., Nicolas, J. F., Cossu, G. and Buckingham, M. E. (2006). Smooth muscle of the dorsal aorta shares a common clonal origin with skeletal muscle of the myotome. *Development* **133**, 737-749.

Fasano, C. A., Dimos, J. T., Ivanova, N. B., Lowry, N., Lemischka, I. R. and Temple, S. (2007). shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal during development. *Cell Stem Cell* **1**, 87-99.
Guo, Y., Costa, R., Ramsey, H., Starnes, T., Vance, G., Robertson, K., Kelley, M., Reinbold, R., Scholer, H. and Hromas, R. (2002). The embryonic stem cell transcription factors Oct-4 and FoxD3 interact to regulate endodermal-specific promoter expression. *Proc. Natl. Acad. Sci. USA* **99**, 3663-3667.
Hanna, L. A., Foreman, R. K., Tarasenko, I. A., Kessler, D. S. and Labosky, P. A. (2002). Requirement for Foxd3 in maintaining pluripotent cells of the early mouse embryo. *Genes Dev.* **16**, 2650-2661.
Hayashi, S. and McMahon, A. P. (2002). Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev. Biol.* **244**, 305-318.
Hildreth, V., Webb, S., Bradshaw, L., Brown, N. A., Anderson, R. H. and Henderson, D. J. (2008). Cells migrating from the neural crest contribute to the innervation of the venous pole of the heart. *J. Anat.* **212**, 1-11.
Hutson, M. R. and Kirby, M. L. (2007). Model systems for the study of heart development and disease. Cardiac neural crest and conotruncal malformations. *Semin. Cell Dev. Biol.* **18**, 101-110.
Ito, K. and Sieber-Blum, M. (1991). In vitro clonal analysis of quail cardiac neural crest development. *Dev. Biol.* **148**, 95-106.
Jiang, X., Rowitch, D. H., Soriano, P., McMahon, A. P. and Sucov, H. M. (2000). Fate of the mammalian cardiac neural crest. *Development* **127**, 1607-1616.
Kim, J., Lo, L., Dormand, E. and Anderson, D. J. (2003). SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. *Neuron* **38**, 17-31.
Kirby, M. L. (1988). Nodose placode contributes autonomic neurons to the heart in the absence of cardiac neural crest. *J. Neurosci.* **8**, 1089-1095.
Kleber, M., Lee, H. Y., Wurdak, H., Buchstaller, J., Riccomagno, M. M., Ittner, L. M., Suter, U., Epstein, D. J. and Sommer, L. (2005). Neural crest stem cell maintenance by combinatorial Wnt and BMP signaling. *J. Cell Biol.* **169**, 309-320.
Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y. H., Inada, M. et al. (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89**, 755-764.
Krispin, S., Nitzan, E., Kassem, Y. and Kalcheim, C. (2010). Evidence for a dynamic spatiotemporal fate map and early fate restrictions of premigratory avian neural crest. *Development* **137**, 585-595.
Labosky, P. A. and Kaestner, K. H. (1998). The winged helix transcription factor Hfh2 is expressed in neural crest and spinal cord during mouse development. *Mech. Dev.* **76**, 185-190.
Le Douarin, N. M. and Kalcheim, C. (1999). *The Neural Crest*. Cambridge: Cambridge University Press.
Le Lievre, C. and Le Douarin, N. (1975). Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. *J. Embryol. Exp. Morphol.* **34**, 125-154.
Lee, H. C., Huang, H. Y., Lin, C. Y., Chen, Y. H. and Tsai, H. J. (2006). Foxd3 mediates zebrafish myf5 expression during early somitogenesis. *Dev. Biol.* **290**, 359-372.
Lee, H. Y., Kleber, M., Hari, L., Brault, V., Suter, U., Taketo, M. M., Kemler, R. and Sommer, L. (2004). Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. *Science* **303**, 1020-1023.
Lefebvre, V., Huang, W., Harley, V. R., Goodfellow, P. N. and de Crombrugghe, B. (1997). SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. *Mol. Cell. Biol.* **17**, 2336-2346.
Liber, D., Domaschenz, R., Holmqvist, P. H., Mazzarella, L., Georgiou, A., Leleu, M., Fisher, A. G., Labosky, P. A. and Dillon, N. (2010). Epigenetic priming of a pre-B cell-specific enhancer through binding of Sox2 and Foxd3 at the ESC stage. *Cell Stem Cell* **7**, 114-126.
Lister, J. A., Cooper, C., Nguyen, K., Modrell, M., Grant, K. and Raible, D. W. (2006). Zebrafish Foxd3 is required for development of a subset of neural crest derivatives. *Dev. Biol.* **290**, 92-104.
Liu, Y. and Labosky, P. A. (2008). Regulation of embryonic stem cell self-renewal and pluripotency by Foxd3. *Stem Cells* **26**, 2475-2484.
Maynard, T. M., Wakamatsu, Y. and Weston, J. A. (2000). Cell interactions within nascent neural crest cell populations transiently promote death of neurogenic precursors. *Development* **127**, 4561-4572.
Molofsky, A. V., Pardal, R., Iwashita, T., Park, I. K., Clarke, M. F. and Morrison, S. J. (2003). Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature* **425**, 962-967.
Montero-Balaguer, M., Lang, M. R., Sachdev, S. W., Knappmeyer, C., Stewart, R. A., De La Guardia, A., Hatzopoulos, A. K. and Knapik, E. W. (2006). The mother superior mutation ablates foxd3 activity in neural crest progenitor cells and depletes neural crest derivatives in zebrafish. *Dev. Dyn.* **235**, 3199-3212.
Mori-Akiyama, Y., Akiyama, H., Rowitch, D. H. and de Crombrugghe, B. (2003). Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. *Proc. Natl. Acad. Sci. USA* **100**, 9360-9365.
Morrison, S. J., White, P. M., Zock, C. and Anderson, D. J. (1999). Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. *Cell* **96**, 737-749.
Morrison, S. J., Csete, M., Groves, A. K., Melega, W., Wold, B. and Anderson, D. J. (2000). Culture in reduced levels of oxygen promotes clonogenic sympathoadrenal differentiation by isolated neural crest stem cells. *J. Neurosci.* **20**, 7370-7376.
Mosher, J. T., Yeager, K. J., Kruger, G. M., Joseph, N. M., Hutchin, M. E., Dlugosz, A. A. and Morrison, S. J. (2007). Intrinsic differences among spatially distinct neural crest stem cells in terms of migratory properties, fate determination, and ability to colonize the enteric nervous system. *Dev. Biol.* **303**, 1-15.
Nagy, A., Gertsenstein, M., Vintersten, K. and Behringer, R. (2003). *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Nishino, J., Kim, I., Chada, K. and Morrison, S. J. (2008). Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf Expression. *Cell* **135**, 227-239.
Presnell, J. K. and Schreibman, M. P. (1997). *Humason's Animal Tissue Techniques*. Baltimore, MD: The Johns Hopkins University Press.
Sasai, N., Mizuseki, K. and Sasai, Y. (2001). Requirement of FoxD3-class signaling for neural crest determination in Xenopus. *Development* **128**, 2525-2536.
Shah, N. M., Groves, A. K. and Anderson, D. J. (1996). Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. *Cell* **85**, 331-343.
Sieber-Blum, M. and Cohen, A. M. (1980). Clonal analysis of quail neural crest cells: they are pluripotent and differentiate in vitro in the absence of noncrest cells. *Dev. Biol.* **80**, 96-106.
Sonnenberg-Riethmacher, E., Miehe, M., Stolt, C. C., Goerich, D. E., Wegner, M. and Riethmacher, D. (2001). Development and degeneration of dorsal root ganglia in the absence of the HMG-domain transcription factor Sox10. *Mech. Dev.* **109**, 253-265.
Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.
Southard-Smith, E. M., Kos, L. and Pavan, W. J. (1998). Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. *Nat. Genet.* **18**, 60-64.
Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M. and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev. Biol.* **1**, 4.
Steiner, A. B., Engleka, M. J., Lu, Q., Piwarzyk, E. C., Yaklichkin, S., Lefebvre, J. L., Walters, J. W., Pineda-Salgado, L., Labosky, P. A. and Kessler, D. S. (2006). FoxD3 regulation of Nodal in the Spemann organizer is essential for Xenopus dorsal mesoderm development. *Development* **133**, 4827-4838.
Stemple, D. L. and Anderson, D. J. (1992). Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell* **71**, 973-985.
Stewart, R. A., Arduini, B. L., Berghmans, S., George, R. E., Kanki, J. P., Henion, P. D. and Look, A. T. (2006). Zebrafish foxd3 is selectively required for neural crest specification, migration and survival. *Dev. Biol.* **292**, 174-188.
Sutton, J., Costa, R., Klug, M., Field, L., Xu, D., Largaespada, D. A., Fletcher, C. F., Jenkins, N. A., Copeland, N. G., Klemsz, M. et al. (1996). Genesis, a winged helix transcriptional repressor with expression restricted to embryonic stem cells. *J. Biol. Chem.* **271**, 23126-23133.
Teng, L. and Labosky, P. A. (2006). Neural crest stem cells. *Adv. Exp. Med. Biol.* **589**, 206-212.
Teng, L., Mundell, N. A., Frist, A. Y., Wang, Q. and Labosky, P. A. (2008). Requirement for Foxd3 in the maintenance of neural crest progenitors. *Development* **135**, 1615-1624.
Thomas, A. J. and Erickson, C. A. (2009). FOXD3 regulates the lineage switch between neural crest-derived glial cells and pigment cells by repressing MITF through a non-canonical mechanism. *Development* **136**, 1849-1858.
Tompers, D. M., Foreman, R. K., Wang, Q., Kumanova, M. and Labosky, P. A. (2005). Foxd3 is required in the trophoblast progenitor cell lineage of the mouse embryo. *Dev. Biol.* **285**, 126-137.
Trentin, A., Glavieux-Pardanaud, C., Le Douarin, N. M. and Dupin, E. (2004). Self-renewal capacity is a widespread property of various types of neural crest precursor cells. *Proc. Natl. Acad. Sci. USA* **101**, 4495-4500.
Uesaka, T., Jain, S., Yonemura, S., Uchiyama, Y., Milbrandt, J. and Enomoto, H. (2007). Conditional ablation of GFRalpha1 in postmigratory enteric neurons triggers unconventional neuronal death in the colon and causes a Hirschsprung's disease phenotype. *Development* **134**, 2171-2181.
Wall, N. A., Jones, C. M., Hogan, B. L. and Wright, C. V. (1992). Expression and modification of Hox 2.1 protein in mouse embryos. *Mech. Dev.* **37**, 111-120.
Wasteson, P., Johansson, B. R., Jukkola, T., Breuer, S., Akyurek, L. M., Partanen, J. and Lindahl, P. (2008). Developmental origin of smooth muscle cells in the descending aorta in mice. *Development* **135**, 1823-1832.
Wiegreffe, C., Christ, B., Huang, R. and Scaal, M. (2007). Sclerotomal origin of smooth muscle cells in the wall of the avian dorsal aorta. *Dev. Dyn.* **236**, 2578-2585.
Wurdak, H., Ittner, L. M., Lang, K. S., Leveen, P., Suter, U., Fischer, J. A., Karlsson, S., Born, W. and Sommer, L. (2005). Inactivation of TGFbeta signaling

in neural crest stem cells leads to multiple defects reminiscent of DiGeorge syndrome. *Genes Dev.* **19**, 530-535.

Xu, J., Watts, J. A., Pope, S. D., Gadue, P., Kamps, M., Plath, K., Zaret, K. S. and Smale, S. T. (2009). Transcriptional competence and the active marking of tissue-specific enhancers by defined transcription factors in embryonic and induced pluripotent stem cells. *Genes Dev.* **23**, 2824-2838.

Yaklichkin, S., Steiner, A. B., Lu, Q. and Kessler, D. S. (2007). FoxD3 and Grg4 physically interact to repress transcription and induce mesoderm in Xenopus. *J. Biol. Chem.* **282**, 2548-2557.

Yang, X., Matsuda, K., Bialek, P., Jacquot, S., Masuoka, H. C., Schinke, T., Li, L., Brancorsini, S., Sassone-Corsi, P., Townes, T. M. et al. (2004). ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. *Cell* **117**, 387-398.

Young, H. M. and Newgreen, D. (2001). Enteric neural crest-derived cells: origin, identification, migration, and differentiation. *Anat. Rec.* **262**, 1-15.

Young, H. M., Bergner, A. J. and Muller, T. (2003). Acquisition of neuronal and glial markers by neural crest-derived cells in the mouse intestine. *J. Comp. Neurol.* **456**, 1-11.