Published in final edited form as:

Genesis. 2011 January ; 49(1): 10–23. doi:10.1002/dvg.20686.

Functional Interaction Between *Foxd3* and *Pax3* in Cardiac Neural Crest Development

Brian L. Nelms, Elise R. Pfaltzgraff, and Patricia A. Labosky*

Vanderbilt University Medical Center, Department of Cell and Developmental Biology, Center for Stem Cell Biology, Program in Developmental Biology, Nashville, Tennessee

Summary

The transcription factors *Foxd3* and *Pax3* are important early regulators of neural crest (NC) progenitor cell properties. Homozygous mutations of *Pax3* or a homozygous NC-specific deletion of *Foxd3* cause marked defects in most NC derivatives, but neither loss of both *Foxd3* alleles nor loss of one *Pax3* allele alone greatly affects overall development of cardiac NC derivatives. In contrast, compound mutant embryos homozygous for a NC-specific *Foxd3* mutation and heterozygous for *Pax3* have fully penetrant persistent truncus arteriosus, severe thymus hypoplasia, and midgestation lethality. *Foxd3*; *Pax3* compound mutant embryos have increased cell death in the neural folds and a drastic early reduction of NC cells, with an almost complete absence of NC caudal to the first pharyngeal arch. The genetic interaction between these genes implicates gene dosage-sensitive roles for *Foxd3* and *Pax3* in cardiac NC progenitors. *Foxd3* and *Pax3* act together to affect survival and maintenance of cardiac NC progenitors, and loss of these progenitors catastrophically affects key aspects of later cardiovascular development.

Keywords

truncus arteriosus; velocardiofacial syndrome; transcription factors; mouse; progenitors

INTRODUCTION

In vertebrates, the neural crest (NC) is critical for the coordinated development of multiple systems. NC cells (NCCs) form peripheral sensory and autonomic nervous system derivatives, facial skeleton, connective tissue, melanocytes, and smooth muscle of the great arteries of the heart. The cardiac NC is a subset of the NC that, in addition to contributing vascular smooth muscle and pericytes to the outflow tract (OFT) and great vessels, also forms the mesenchyme of the third pharyngeal pouch critical for signaling to the adjacent ectoderm and subsequent normal thymus development (Bockman and Kirby, 1989). Cardiac NC ablation in both chicken and mouse embryos revealed that cardiac NC is required for development of the conotruncus, aortic arch arteries, and thymus (Kirby, 1990; Porras and Brown, 2008). In humans, abnormalities in these cells are often at the root of many cardiovascular defects, including persistent truncus arteriosus (PTA), a large, single great vessel instead of the normally distinct pulmonary trunk and ascending aorta exiting the ventricles. PTA is a severe, life-threatening birth defect on its own (Bando *et al.*, 1996); it is also one of the features of velocardiofacial syndrome (VCFS), which can include cleft

palate, craniofacial abnormalities, and thymus defects, all tissues with NC contribution. Prevalence of VCFS is approximately 1 of 2,000 live births, making it one of the most common syndromic birth defects (Shprintzen, 2008). Some of the genetic causes for human congenital heart defects (CHDs) and later cardiovascular disease have been identified (Pierpont *et al.*, 2007), but, in many cases, the presence of unknown genetic modifiers contributes considerable variability to CHD severity, and some CHDs manifest as part of a cohort of phenotypes including noncardiac defects, as in VCFS. Understanding molecular regulation of cardiac NC development is critical for a thorough understanding of the etiology of many human CHDs, cardiovascular disease, and multiorgan syndromes such as VCFS. Our data here suggest the involvement and genetic interaction of *Foxd3* and *Pax3* in control of cardiac NC development, particularly on early NC progenitor cells.

Foxd3 is a winged-helix transcription factor required for maintenance of multipotency and self-renewal of embryonic stem (ES) cells and trophoblast stem cells in culture (Hanna *et al.*, 2002; Liu and Labosky, 2008; Tompers *et al.*, 2005), and *Foxd3* null mutant embryos do not survive past early gastrulation (Hanna *et al.*, 2002). *Foxd3* is one of the initial markers of the NC lineage (Labosky and Kaestner, 1998), and when removed from the NC, most NC progenitors and derivatives are markedly affected, both *in vivo* and in explant cultures (Teng *et al.*, 2008; Mundell and Labosky, manuscript Submitted). In these NC-specific *Foxd3* mutant embryos, many of the bones and cartilage of the developing craniofacial skeleton are absent or markedly malformed, and the face does not fuse at the midline. The peripheral nervous system and enteric nervous system (ENS) are almost completely absent, and dorsal root ganglia are reduced in size, all because of defective maintenance of the NC progenitor pool within the premigratory and early migratory NC (Teng *et al.*, 2008). Surprisingly, the reduction in cardiac NCCs reaching the OFT in these mutants does not disrupt cardiac development, and these mutants have a low occurrence of heart defects (Teng *et al.*, 2008).

Like *Foxd3*, *Pax3* is a transcription factor critical for early NC development and NC-derived stem cell maintenance. In addition to its role in NC induction (Sato *et al.*, 2005), *Pax3* is required for maintenance and expansion of early NC progenitors and is required to maintain melanocyte stem cells (Lang *et al.*, 2005). *Pax3* homozygous-null embryos have a drastic early reduction in NC progenitors and profound defects in OFT septation (Chan *et al.*, 2004; Morgan *et al.*, 2008). Although *Foxd3* clearly plays a paramount role in multiple progenitor lineages, little is known about the genetic and molecular pathways in which *Foxd3* acts, or the factors interacting with *Foxd3*. Here, we describe a genetic interaction between *Foxd3* and *Pax3* with direct functional significance for development of the cardiac NC. *Foxd3* NC mutants have subtle cardiac defects, but introducing one mutant *Pax3* allele into this genetic background causes a 100% penetrant PTA. This striking complete penetrance of PTA is only observed in a handful of mouse models (Choi and Klingensmith, 2009; Choudhary *et al.*, 2006; Jiang *et al.*, 2002; Richarte *et al.*, 2007; Stankunas *et al.*, 2008), and with respect to the cardiac NC subset of NCCs, approximates an NC ablation model (Porrás and Brown, 2008). The PTA observed here in this compound deletion of *Foxd3* and *Pax3* together is caused by a severe reduction of presumptive NCCs entering the second and third pharyngeal arches, preceded by a wave of cell death in the presumptive NC. Our results suggest a profound requirement for these two transcription factors in NC progenitor maintenance.

RESULTS

***Pax3*^{Cre}-Mediated Deletion of *Foxd3* From the NC Results in Craniofacial Defects and Embryonic Lethality**

Cardiac NCCs migrate along the third, fourth, and sixth pharyngeal arch arteries, facilitating remodeling of these arteries and septation of the OFT into the distinct aortic and pulmonary arteries (Snider *et al.*, 2007). A *Wnt1-Cre*-mediated NC deletion of *Foxd3* results in severe

defects in almost all NC lineages, but cardiac NC derivatives are minimally affected, and most *Wnt1-Cre; Foxd3^{fllox/-}* mutant embryos survive until birth (Teng *et al.*, 2008). (Mice and their genotypes, MGI allele names, and references are in Table 1; breeding schemes are in Supplemental Fig. 1.) This minimal cardiac phenotype is the result of a small remaining population of cardiac NCCs sufficient for cardiac NC function in the majority of *Wnt1-Cre; Foxd3^{fllox/-}* mutant embryos. To test if this somewhat surprising result was due to an early population of NCCs that did not yet express *Wnt1-Cre* and, therefore, maintained *Foxd3* expression, an alternative, earlier NC-expressed Cre recombinase, *Pax3^{Cre}*, was used. This *Pax3Cre* allele was generated by insertion of a *Cre* cassette into the *Pax3* locus, resulting in a null allele (Engleka *et al.*, 2005). *Pax3* is expressed in the dorsal neural tube, early NC, and presomitic mesoderm. The effect of *Pax3^{Cre}* in deleting *Foxd3* in this mouse model is limited to the NC, because *Foxd3* is not expressed in the presomitic mesoderm of the mouse embryo (Labosky and Kaestner, 1998; Tompers *et al.*, 2005). *Foxd3* is expressed in murine premigratory NC starting slightly before 8.5 days post coitum (Labosky and Kaestner, 1998). *Foxd3* is maintained in migratory NCCs as they leave the neural tube, but its expression is extinguished in many NCCs as they begin to differentiate toward other lineages (see Supplemental Fig. 2) and, by 10.5 dpc, is only maintained in ENS precursors, future spinal ganglia and sympathetic ganglia, and is not expressed in cells entering the developing OFT (Labosky and Kaestner, 1998; Mundell and Labosky, manuscript submitted).

Although *Pax3^{Cre/+}; Foxd3^{fllox/+}* control embryos had no discernible defects, *Pax3^{Cre/+}; Foxd3^{fllox/-}* compound mutant embryos had a fully penetrant cleft face phenotype from 11.5 dpc and onward (Fig. 1a compared with 1b) and loss of NCCs in the ENS (Supplemental Fig. 3), indicating similar loss of cranial and trunk NC derivatives compared with the *Wnt1-Cre*-driven deletion of *Foxd3* (Teng *et al.*, 2008). Control embryos (*Pax3^{Cre/+}; Foxd3^{fllox/+}*) at these stages had a 100% survival rate (Table 2). In contrast, littermate compound mutant embryos (*Pax3^{Cre/+}; Foxd3^{fllox/-}*) died at midgestation as early as 11.5 dpc, with the majority (71%) dead by 13.5 dpc, and 100% by 14.5 dpc (Table 2). Only compound *Pax3; Foxd3* mutants exhibited midgestation lethality or craniofacial defects. Much like *Wnt1-Cre; Foxd3^{fllox/-}* mutant embryos, *Foxd3* protein was efficiently deleted to levels beyond detection within the NC by 9.5 dpc (Supplemental Fig. 2).

***Pax3^{Cre}*-Mediated Deletion of *Foxd3* From the NC Results in PTA**

Cardiac NCCs migrate ventrally from dorsal neural tube regions corresponding to rhombomeres 6, 7, and 8, and travel along the pharyngeal arch arteries, facilitating remodeling of these arteries. A wedge of cardiac NCCs continues migration, forming a septation complex dividing the common truncus into the dorsal aorta and the pulmonary trunk. All control embryo OFTs at 12.5 and 13.5 dpc had clearly septated into aortic and pulmonary arteries (Figs. 1c, 2a, and 3a). In contrast, a failure to septate the OFT, resulting in PTA, was first evident in compound mutants at 12.5 dpc, when all wild-type and littermate control embryos had completed septation. All compound mutant embryos examined at 12.5 and 13.5 dpc had an unseptated truncus (Figs. 1d, 2b, and 3b). Therefore, in stark contrast to *Wnt1-Cre; Foxd3^{fllox/-}* mutant embryos, all *Pax3^{Cre/+}; Foxd3^{fllox/-}* compound mutant embryos had a 100% penetrant PTA ($n = 25$).

Ink Fills, Resin Casts, and Histological Analysis Highlight the Cardiac NC Phenotype

Ink injections and resin casts of control ($n = 6$ and 2, respectively) and mutant ($n = 6$ and 2, respectively) littermate hearts confirmed PTA in mutants and revealed truncated pulmonary arteries at 13.5 dpc (Fig. 1e compared with 1f). It is unclear whether this was a defect in the pulmonary arteries or a filling artifact because of obstructed fluid flow through the abnormal OFT morphology and lack of a ductus arteriosus. Otherwise, the patterning of the aortic arch

arteries was generally unperturbed. Morphological and histological analyses and whole-mount immunohistochemistry for CD31/PECAM to examine endothelial cells revealed no differences (Supplemental Fig. 4).

Lineage Labeling Shows Reduced NC Contribution to the OFT of the Heart

Pax3 and *Foxd3* are both expressed in premigratory and early migrating cardiac NC (Goulding *et al.*, 1991; Serbedzija and McMahon, 1997). However, as migration continues and cardiac NCCs enter the OFT, expression of these genes is lost (Epstein *et al.*, 2000; Labosky and Kaestner, 1998; Mundell and Labosky, manuscript submitted). To follow the fate of NCCs and progeny in control and mutant embryos, the *R-6R^{lacZ}* reporter allele was used with *Pax3^{Cre}* (Engleka *et al.*, 2005; Jiang *et al.*, 2002). This combination indelibly marks the entire lineage of Pax3-expressing cells, regardless of current Pax3 expression status. We used this approach to lineage map Pax3-expressing NC in embryos with a *Pax3^{Cre}*-driven deletion of *Foxd3* compared directly with embryos with one wild-type *Foxd3* allele. *Pax3^{Cre/+}; Foxd3^{flox/+}; R26R^{lacZ/+}* control and *Pax3^{Cre/+}; Foxd3^{flox/-}; R26R^{lacZ/+}* compound mutant embryos were compared to assess defects in mutant NC migration and expansion. In control embryonic OFTs at 12.5 and 13.5 dpc, we observed lineage-labeled X-gal-positive cells in sections (arrowhead in Fig. 2a, *n* = 3) and in whole-mount embryos (Fig. 3a) as a wedge of cells in the cardiac OFT cushions of the conotruncus. Embryos without either the *Pax3^{Cre}* or *R26R^{lacZ}* alleles had no X-gal-positive cells (Supplemental Fig. 5). Lineage-labeled cells were present in compound mutant embryonic OFTs throughout the anterior-to-posterior axial levels of the conotruncus, but the population of lineage-labeled NCCs was drastically reduced along the entire axis (Fig. 2a compared with 2b, and data not shown). Whole-mount lineage labeling of 13.5 dpc compound mutant hearts also revealed mislocalized Pax3-lineage marked cells. A few labeled cells were seen in the right ventricle of control embryos (Fig. 3a), but we observed a larger cluster of cells in compound mutants (Fig. 3b) that extended to the dorsal side of the heart (Fig. 3d). The size of this patch of X-gal-positive cells was variable but typically within the same location, and fell within a spectrum of largest (Fig. 3b) to smallest (Supplemental Fig. 3b), but was always observed (*n* = 5, three of these were large and two were small). In contrast, the smaller patch of cells in controls was not always seen (Fig. 3a, two of five samples). In sections of compound mutant embryos, aberrantly localized labeled cells were observed near or in myocardial walls (arrows in Fig. 2d). Experimental pursuit of this observation was beyond the scope of our work here, but it is possible that appearance of these cells could be an artifact of ectopic Cre expression or related to non-NC Pax3 expression.

Pax3^{Cre/+}; Foxd3^{flox/-} Mutant Embryos Also Have Thymus Defects

NCCs contribute to mesenchyme surrounding the developing thymus (Bockman and Kirby, 1989; Le Lievre and Le Douarin, 1975). *Wnt1-Cre; Foxd3^{flox/-}* mutant embryos had severe defects in most of the NC, with the exception of the cardiac NC; they also had only subtle thymic defects with occasional undescended and asymmetric thymic lobes (Teng *et al.*, 2008; unpublished data). We observed no thymic defects in *Pax3^{Sp/+}* or *Pax3^{Cre/+}* heterozygous embryos, consistent with previous studies (Conway *et al.*, 1997; Griffith *et al.*, 2009). However, in *Pax3^{Cre/+}; Foxd3^{flox/-}* embryos, the thymus was severely reduced in size compared with controls and lacked the outer layer of surrounding NCCs at 12.5 dpc (*n* = 4, Fig. 2e,f). Serial sections were examined to exclude the possibility of an undescended thymus. The remaining mutant thymic rudiments at 12.5 dpc did not always descend as far as control thymus, but there was never evidence of a normal undescended thymus (data not shown). By 13.5 dpc, the thymus was clearly visible in whole-mount dissections of controls (Fig. 3a; see also Supplemental Fig. 3a), but was not detected in whole-mount dissections (*n* = 5) of mutant embryos (Fig. 3a,b; see also Supplemental Fig. 3). *Pax3^{Cre/+}; Foxd3^{flox/-}*

compound mutants, in addition to marked loss of the thymus, appear to have an irregular morphology of the trachea and esophagus, areas normally invested with NC (Fig. 2e,f).

***Pax3*^{Cre}-Mediated Deletion of *Foxd3* From the NC Results in an Early Loss of NC Progenitors Caudal to the First Pharyngeal Arch**

To determine whether the reduced populations of *Pax3*-lineage presumptive NCCs in cardiac NC-derived structures were a result of an earlier loss of progenitor NCCs, we used the same fate-mapping strategy to examine embryos at earlier developmental stages. Although *Pax3* expression begins by 8.5 dpc, we observed no detectable levels of β -galactosidase activity in either control or mutant embryos at 8.5 dpc (data not shown), most likely because of a delay in Cre activation of the *R26R* reporter gene. However, at 9.5 dpc, we saw robust X-gal staining, indicating Cre activity from the *Pax3* locus in control embryos (Fig. 4a,c, $n = 4$). In these control embryos, presumptive NCCs filled the first three pharyngeal arches and contributed to the presumptive cranial skeleton. In *Pax3; Foxd3* compound mutant embryos (Fig. 4b,d), we observed a decrease in lineage-labeled NCCs at 9.5 dpc ($n = 2$). The extent of migration of *Pax3*-lineage cells into the first pharyngeal arch was comparable with controls, but the intensity of X-gal staining was reduced, suggesting fewer NCCs were present. Strikingly, there was no detectable X-gal staining caudal to the first pharyngeal arch of mutant embryos. This was in contrast to the reduction of NCCs seen in the *Wnt1-Cre*-driven deletion of *Foxd3*, where reduced numbers of presumptive NC lineage-positive cells were located in all pharyngeal arches at 9.5 dpc (Teng *et al.*, 2008). The apparent lack of *Pax3*-lineage-positive cells in the second and third pharyngeal arches, despite a significant population within the first pharyngeal arch, suggests that *Pax3* and *Foxd3* are required together to confer migratory competence to NC progenitors or preferentially control the initial expansion or survival of progenitors that later enter the second and third pharyngeal arches.

Cell Death Within the Early, Premigratory NC Progenitor Pool Is Increased in Compound Mutants

To determine whether the drastic reduction in NCCs was the result of cell death of premigratory or migratory NC progenitors, we performed a LysoTracker assay to visualize dying cells. At 8.5 dpc, mutant embryos ($n = 4$) had a distinct stripe of LysoTracker-positive cells along the lateral edge of the neural folds (corresponding to the origin of premigratory NCCs) that was not present in controls ($n = 3$) (Fig. 5a–f). After 9.0 dpc, we detected no discernable differences in pattern or intensity of LysoTracker staining (Fig. 5g–i).

Loss of One Functional Allele of *Pax3* Combined With NC Deletion of *Foxd3* Is Responsible for PTA and Lethality

At least two plausible hypotheses exist for the marked difference in phenotype between *Wnt1-Cre; Foxd3*^{flox/-} embryos and *Pax3*^{Cre/+}; *Foxd3*^{flox/-} embryos. One hypothesis is that subtle differences in the spatiotemporal expression of Cre when driven by *Wnt1* versus *Pax3* regulatory sequences or differences in Cre efficiency cause differential deletion of *Foxd3*.

This could result in a population of compensatory *Foxd3*-expressing cells in *Wnt1-Cre*-mediated *Foxd3* mutants not present when *Pax3*^{Cre/+} is used. An alternative hypothesis is that the dosage of *Pax3* in a *Foxd3*-conditional null background is critical for NC survival and, therefore, OFT septation, and loss of one allele of *Pax3* combined with NC-specific loss of *Foxd3* causes the more severe phenotype.

To distinguish between these hypotheses, mice were bred to introduce the *Pax3*^{Sp} or *Pax3*^{Sp-d} mutant alleles into the *Wnt1-Cre; Foxd3*^{flox/-} background (Supplementary Fig. 1). *Pax3*^{Sp} is a null allele encoding a truncated Pax3 protein (Chalepakakis *et al.*, 1994).

Homozygous *Pax3^{Sp/Sp}* mutants die at midgestation, with a reduced cardiac NC progenitor pool and PTA (Conway *et al.*, 2000; Epstein *et al.*, 2000). *Pax3^{Sp-d}* is a hypomorphic allele encoding a missense mutation in the Pax3 DNA binding domain (Underhill *et al.*, 1995; Vogan *et al.*, 1993). Homozygous *Pax3^{Sp-d/Sp-d}* mutants have mild NC defects compared with *Pax3^{Sp/Sp}* embryos and no PTA (Franz, 1993), surviving until birth when they die from neural tube defects. Neither allele causes discernable cardiac defects when heterozygous. We compared control *Wnt1-Cre; Foxd3^{lox/-}* embryos with *Wnt1-Cre*-driven *Foxd3* mutant embryos containing either of these mutant *Pax3* alleles (*Pax3^{Sp/+}; Wnt1-Cre; Foxd3^{lox/-}* and *Pax3^{Sp-d/+}; Wnt1-Cre; Foxd3^{lox/-}* embryos). When the *Wnt1-Cre* transgene was used to delete *Foxd3* in NCCs, additional loss of one functional allele of *Pax3* resulted in midgestational embryonic lethality and 100% penetrant PTA, mimicking the *Pax3^{Cre/+}; Foxd3^{lox/-}* compound mutant phenotype (Table 3). Both *Spotch* and *Spotch-delayed* alleles gave the same phenotype. These results supported the second hypothesis above: reductions in *Pax3* gene dosage are responsible for midgestation lethality and PTA in conjunction with loss of *Foxd3* in the NC. This experiment also eliminated the possibility of differences in genetic background between *Wnt1-Cre* and *Pax3^{Cre}* strains being responsible for phenotypic differences: *Pax3^{Sp/+}; Wnt1-Cre; Foxd3^{lox/-}* compound mutant embryos with PTA and early lethality were on identical genetic backgrounds with littermate controls (*Wnt1-Cre; Foxd3^{lox/-}*) that did not display early lethality or PTA.

DISCUSSION

Our data show that *Foxd3* and *Pax3* interact genetically during development of the NC, specifically affecting the cardiac NC and the thymus beyond defects seen in either mutant alone, revealing previously unappreciated roles for these two proteins. *Foxd3* NC homozygous mutants do not have severe heart or thymus defects, and *Pax3* heterozygous or homozygous null mutants do not have severe thymus hypoplasia, but compound mutants had both severe heart defects and thymus hypoplasia, and at greater penetrance. Although these defects approximate those in human VCFS, *Foxd3* and *Pax3* are not initiators of VCFS (neither gene is located on mouse chromosome 16, syntenic to human chromosome 22 (Galili *et al.*, 1997)), but our work here suggests that *Foxd3* and *Pax3* may serve as effectors in the same gene regulatory networks. Alternatively, with respect to the cardiac NC, the *Foxd3/Pax3* compound mutants may more closely approximate a NC ablation model, suggesting that these two genes may be involved in the master control of earlier steps in pathways affecting cardiac NC development, including expansion, survival, or maintenance of a cardiac NC progenitor pool, and in fact, our lineage-labeling experiments (Figs. 2–4) support this notion.

In the process of OFT septation, the combined loss of both alleles of *Foxd3* and one allele of *Pax3* is similar to loss of both alleles of *Pax3*. These mutants do not display neural tube defects characteristic of *Pax3^{-/-}* mutants, but the expression domain of *Foxd3* does not overlap with *Pax3* in the non-NC dorsal neural tube. This suggests that in the NC, *Foxd3* and *Pax3* play a similar role in mediating progenitor fate, or that *Pax3* may be acting through *Foxd3*. Indeed, a reduction in *Foxd3* expression in *Pax3^{Sp/Sp}* mutants suggests that the latter is true (Dottori *et al.*, 2001): *Foxd3* is genetically downstream of *Pax3*. Work in other vertebrates demonstrated a similar relationship between *Foxd3* and *Pax3*; in *Xenopus laevis* NC induction, *Pax3*, together with *Zic1*, induces early NC genes, including *Foxd3* (Sato *et al.*, 2005).

This genetic interaction may reflect a physical interaction, and if the interaction were direct, the two transcription factors could be acting at shared targets or at the protein–protein level; ectopic expression of *Foxd3* in an avian melanoma cell line results in *Foxd3* repressing melanin synthesis by directly binding *Pax3* protein and sequestering it, preventing *Pax3*

binding and activation of the *Mitf* promoter (Thomas and Erickson, 2009). However, Pax3 and Foxd3 could just as likely be acting through separate, parallel pathways. There are a number of possibilities for the molecular mechanism of this genetic interaction that remain to be addressed in future studies. Regardless, the function of Pax3 on transcription figures to be important for the genetic interaction we observe, considering that there was no difference in the genetic interaction we defined here between the null *Pax3^{Sp}* and the DNA-binding domain mutant *Pax3^{Sp-d}* alleles.

Use of the classic *Pax3^{Sp}* and *Pax3^{Sp-d}* alleles confirms that we have uncovered a true genetic interaction rather than differences between *Wnt1-Cre* and *Pax3^{Cre}* expression domains, despite the fact that there are known differences in Cre expression in these two mouse lines (Engleka *et al.*, 2005; Jiang *et al.*, 2000). Pax3 is expressed before Wnt1 in the NC (Fenby *et al.*, 2008; Osorio *et al.*, 2009), and Pax3 binds directly to the *Wnt1* promoter to activate *Wnt1* transcription (Fenby *et al.*, 2008), suggesting it is upstream. *Wnt1-Cre* is more commonly used for NC-specific deletion, but will act on cells within the developing roof plate and midbrain and does not label NCCs from the posterior-most levels of the NT (Jiang *et al.*, 2000). *Pax3^{Cre}* has a broader expression domain than *Wnt1-Cre*, active in presomitic mesoderm and the NC, but also in cells originating from the non-NC dorsal NT. To our knowledge, no direct comparison between *Wnt1-Cre* and *Pax3^{Cre}* in the NC has been published, but a few studies have used both with no differences. A conditional knockout of *Myocardin* using *Wnt1-Cre* or *Pax3^{Cre}* separately resulted in no significant phenotypic differences (Huang *et al.*, 2008), and a conditional deletion of *Connexin43* with *Wnt1-Cre* versus *Pax3^{Cre}* resulted in a more severe phenotype with *Pax3^{Cre}*, but these defects were related to Pax3 expression in the non-NC dorsal neural tube (Kretz *et al.*, 2006; Liu *et al.*, 2006). Therefore, our work represents the first example of a genetic interaction discovered with this approach.

Another hypothesis is that Pax3 and Foxd3 pathways are not intertwined, and mutations in both are merely two separate NC disruptions converging on the same readout. However, other than a reduction in melanocytes resulting in a belly spot, there is no evidence of functional defects in heterozygous *Pax3* mutants or double-heterozygous *Pax3^{+/-}; Foxd3^{+/-}* mice. In a *Wnt1-Cre; PUATK* mouse NC ablation model, severity of ablation was controlled by the number of gancyclovir injections (Porrás and Brown, 2008), and the greater the extent of NC ablation, the higher the percentage of embryos with PTA. The 100% penetrant PTA we observed, coupled with the drastic reduction of lineage-labeled NCCs, suggests that with respect to the cardiac NC, the compound mutants here approximate an NC ablation model. This may represent a difference from single homozygous mutations in *Pax3* or *Foxd3* resulting in NC deficiency but with enough NCCs remaining to facilitate at least partial thymic and/or cardiovascular development. Whether the genetic interaction we describe represents the action of Foxd3 and Pax3 in the same pathway, converging pathways, or whether they are completely separate insults that together reduce the population of NCCs below levels critical for OFT and thymus development, our results indicate Foxd3 and Pax3 together may be master control genes for the early cardiac NC.

METHODS

Mouse Strains and Breeding

Mus musculus were used for all experiments; these mice were of a mixed genetic background derived from C57BL6, SvEv129S6, and CD-1 strains. Genetically manipulated mice described here are compared with control littermates. Because of the need for several different alleles in each mouse line generated, the intermediate mouse strains are generally on an outbred, mixed genetic background. All mouse strains (Table 1) used are previously described and listed here: *Foxd3⁻* refers to *Foxd3^{tm2Lby}* (Hanna *et al.*, 2002) in all

experiments except those using the $R26R^{YFP}$ reporter allele $Gt(ROSA)26Sor^{tm1(EYFP)Cos}$ (Srinivas *et al.*, 2001), in which case $Foxd3^{-}$ refers to $Foxd3^{tm1Lby}$ (Hanna *et al.*, 2002). $Foxd3^{flox}$ is $Foxd3^{tm3Lby}$ (Teng *et al.*, 2008), $Pax3^{Cre}$ is $Pax3^{tm1(Cre)Joe}$ (Engleka *et al.*, 2005), and $Wnt1-Cre$ is $Tg(Wnt1-Cre)11Rth$ (Danielian *et al.*, 1998). $Pax3^{Sp}$ and $Pax3^{Sp-d}$ refer to the $Pax3$ alleles *Splotch* and *Splotch-delayed* (Dickie, 1964), and $R26R^{lacZ}$ is $Gt(ROSA)26Sor^{tm1Sor}$ (Soriano, 1999); these last three lines were obtained from The Jackson Laboratory (Bar Harbor, ME). Breeding details are shown in Supplemental Figure 1. $Pax3^{Cre/+}; Foxd3^{flox/-}$ compound mutant embryos were generated from $Foxd3^{flox/flox}$ crossed to $Pax3^{Cre/+}; Foxd3^{+/-}$ mice. Matings were also carried out with $Foxd3^{flox/flox}; R26R^{lacZ/lacZ}$ dams for lineage mapping of Cre-expressing cells. To obtain $Pax3^{Splotch/+}; Wnt1-Cre; Foxd3^{flox/-}$ embryos and littermate controls, $Pax3^{Splotch/+}; Wnt1-Cre; Foxd3^{-/+}$ male mice were mated to $Foxd3^{flox/flox}$ dams. The same breeding scheme was used with $Pax3^{Splotch-delayed}$ in place of $Pax3^{Splotch}$ to generate the equivalent littermate mutants and controls. Genotyping of mice and embryos was performed with polymerase chain reaction (PCR) as previously described (Danielian *et al.*, 1998; Engleka *et al.*, 2005; Hanna *et al.*, 2002; Teng *et al.*, 2008). All mice were maintained and handled in accordance with Association for Assessment and Accreditation of Laboratory Animal Care standards as approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee (IACUC).

Embryo Dissections

For timed matings, females were checked daily for presence of a vaginal plug; noon on the day of an observed plug was considered 0.5 dpc. Embryos were harvested around noon of the day of dissection, so that these embryos would correspond to $x.5$ dpc (x = number of days after plug observed). Embryos were dissected from the uterus and surrounding deciduum into phosphate buffered saline (PBS) plus 0.1% bovine serum albumin (BSA) and immediately examined for signs of early lethality, including pale tissue, blood pooling, lack of heartbeat, decreased size, and in cases of embryonic lethality occurring presumably more than a day before dissection, gross tissue death and reabsorption of the embryo.

Visualization of Whole-Mount Cardiac Morphology

For cardiovascular analysis, embryos from 10.5 to 13.5 dpc were dissected in PBS, and the chest cavities opened to reveal the embryonic heart. For ink injections, a 1:10 dilution of Super Black India Ink (Speed-ball) in PBS was injected into the ventricles using a 1.0 mm outer diameter/0.78 mm inner diameter borosilicate glass pipette needle (pulled with a Kopf Vertical Pipette Puller). For resin casts, Mercor II resin containing methyl methacrylate was mixed with red dye and catalyst (Ladd Research Industries, Williston, VT) and immediately injected using needles as above until resin was visualized in the heart, OFT, and great vessels. The resin was hardened overnight at 4°C, samples macerated at 50°C with maceration solution containing potassium hydroxide (Polysciences, Inc., Warrington, PA) and washed in water for visualization and storage.

Histological and Whole-Mount Embryo Labeling

Embryos were prepared for histology, and histology was performed by standard procedures (Nagy *et al.*, 2003; Presnell and Schreiber, 1997) and carried out on 7- μ M paraffin sections on SuperFrost-Plus slides (Fisher, Fair Lawn, NJ). 5-Bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) reactions were performed following standard techniques (Nagy *et al.*, 2003). For histological sections of X-gal-labeled embryos, samples were processed using Histoclear in place of Xylenes and sections counterstained with eosin Y. Antibodies used on histological sections were rabbit anti-Foxd3 (Washington Biotechnology, Simpsonville, MD) at 1:500 and chicken anti-GFP (Abcam, Cambridge, MA) at 1:500. Secondary antibodies used were donkey anti-rabbit Cy3 (1:500; Jackson ImmunoResearch, West

Grove, PA) and donkey anti-chicken biotin (1:500; Jackson ImmunoResearch) amplified with horse radish peroxidase (HRP)-conjugated streptavidin (Vector RTU Kit) plus tyramide-Alexa488 (1:400; Invitrogen, Carlsbad, CA), respectively. Antibodies used for whole-mount PECAM staining were rat anti-PECAM-1/CD31 (3:500; BD Pharmingen, Sparks, MD) and a biotinylated donkey anti-rat secondary (1:1,000, Jackson ImmunoResearch) in combination with VectaStain ABC (Vector Laboratories, Burlingame, CA). Immunohistochemistry for Foxd3 on sections from 9.5 dpc mutant and control embryos was performed as previously described (Tompers et al., 2005). Whole embryo images were captured with a Q-Imaging Micropublisher camera and a Nikon SMZ-U stereomicroscope; sections were imaged with a Q-Imaging Retiga 2000r camera on a Zeiss Axioskop2 Plus or a Nikon Eclipse E600 microscope. For whole-mount cell death visualization, embryos were dissected in Hanks balanced salt solution and incubated in 2- μ M LysoTracker (Molecular Probes) for either 1 h (9.5 dpc and above) or 15–20 min (8.5 dpc) at 37°C with gentle agitation. Embryos were then washed and fixed overnight in 4% paraformaldehyde (PFA) in PBS, washed in PBS, partially dehydrated in 50% methanol in PBS, and imaged with a Leica MZ16 FA stereoscope equipped with fluorescence and a Q-Imaging Retiga 4000r camera.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful for assistance from Alison LeGrone in maintaining, weaning, and genotyping mice, and from Audrey Frist with histology and genotyping embryos. They thank the past and current members of the Labosky Lab and the laboratories of Drs. Mark Magnuson and Stacey Huppert for thoughtful discussion and use of equipment. They also thank Dr. Lu Teng for preliminary data on this project, and Dr. Christopher Brown and Nathan Mundell for guidance on ink injections, resin casting, and discussion. They also thank Dr. Jonathan Epstein for *Pax3*^{Cre/+} mice and Dr. H. Scott Baldwin and Jennifer Plank for critical reading and discussion of the manuscript.

Contract grant sponsor: NIH, Contract grant numbers: R01 HD036720 (to P.A.L.), T32 HD007043 (to B.L.N.), and T32 HD007502 (to E.R.P.), Contract grant sponsor: American Heart Association, Contract grant number: Postdoctoral Fellowship 0725615B (to B.L.N)

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FIG. 1. *Pax3^{Cre/+}; Foxd3^{flox/-}* compound mutant embryos have craniofacial defects and persistent truncus arteriosus. (a,b) Frontal views of littermate control and compound mutant embryos at 12.5 dpc highlight craniofacial defects in the compound mutants. (a) In controls, the two sides of the face have met at the midline and fused. (b) The *Pax3^{Cre/+}; Foxd3^{flox/-}* compound mutant embryo has a characteristic cleft face. (c,d) Higher magnification ventral views of the developing hearts of the same embryos shown in (a) and (b), after dissection of body cavity and injection of India ink into the right ventricle. (c) In control embryos, the common truncus was septated to form the ascending aorta and pulmonary trunk. (d) In *Pax3^{Cre/+}; Foxd3^{flox/-}* embryos, the developing truncus arteriosus is not septated and

persists as a single tube with less leftward rotation than that of the control pulmonary trunk as it exits the right ventricle. **(e,f)** Microresin casts of OFTs from 13.5 dpc control and mutant embryos. **(e)** *Pax3^{Cre/+}; Foxd3^{flox/+}* control embryos have normal OFT morphology. **(f)** Mutant embryos lack the ductus arteriosus, but have normal patterning of the aortic arch arteries. The two pulmonary arteries emanating from the pulmonary trunk appear stunted with attenuated branching (arrowhead). **(g,h)** Outline trace of resin casts from **(e)** and **(f)**, respectively. A, atria; AA, ascending aorta; BCA, brachiocephalic artery; CFD, craniofacial defect; DA, ductus arteriosus; LCA, left carotid artery; LSA, left subclavian artery; LV, left ventricle; PA, pulmonary artery; PT, pulmonary trunk; PTA, persistent truncus arteriosus; RV, right ventricle.

FIG. 2.

Lineage analysis of NC in control and compound mutant embryos reveals a reduction in the population of *Pax3*-lineage NCCs migrating into the OFT cushions and aortic arch arteries. (a–d) Transverse sections of X-gal-stained hearts from 12.5 dpc littermate control embryos (a,c) and compound mutant embryos (b,d). Sections (a) and (b), and (c) and (d) are at the same levels. (a) In controls, *Pax3*-lineage-labeled cells enter the OFT cushions as a large wedge in the pulmonary trunk and the inner layer of the ascending aorta (arrowhead). The majority of cells along the aortic arch are labeled. The ascending aorta and pulmonary trunk are clearly septated. (b) In mutants, the aortic arch (AoA) is mostly devoid of labeled cells. The truncus arteriosus has some X-gal-positive cells present, but remains a single vessel

with no septation between the ascending aorta and pulmonary trunk (PTA). (c) In controls, *Pax3*-lineage-labeled cells are only rarely detected in the ventricles or elsewhere within the heart. (d) In mutants, labeled cells are found ectopically within the ventricles and sometimes in the atria, indicative of abnormal *Pax3*-lineage contribution and migratory behavior (arrows). (e,f) X-gal-labeled transverse sections of 12.5 dpc *Pax3*^{Cre/+}; *Foxd3*^{flax/-}; *R26R*^{LacZ/+} littermate control and compound mutant embryos. (e) Developing thymic primordia (asterisks) contain an outer layer of *Pax3*-lineage-labeled cells in controls. (f) Compound mutant thymic primordia are drastically reduced in size (asterisks) and devoid of *Pax3*-lineage-labeled cells. The trachea and esophagus from mutant embryos have a significant loss of surrounding labeled cells, and morphology is abnormal; the two structures are not as distinctly separated and appear less compact. A, atria; AA, ascending aorta; AoA, aortic arch artery; E, esophagus; PT, pulmonary trunk; PTA, persistent truncus arteriosus; T, trachea; V, ventricle.

Control: *Foxd3* NC het. **Mutant: *Foxd3* NC null**
Pax3^{Cre/+}; *Foxd3*^{flox/+}; *R26R*^{lacZ/+} *Pax3*^{Cre/+}; *Foxd3*^{flox/-}; *R26R*^{lacZ/+}

FIG. 3.

Whole-mount lineage-labeling analysis reveals disruption of cardiac NCCs caused by lack of *Foxd3* and reduced *Pax3* gene dosage. Whole-mount X-gal labeling of 13.5 dpc control (a,c) and compound mutant (b,d) littermate embryos. (a,b) Ventral views of dissected and stained hearts. (a) Labeled NCCs are present in the pulmonary trunk and ascending aorta of *Pax3*^{Cre/+}; *Foxd3*^{flox/+} control hearts. The thymus (circled with a dashed line) is present in controls and robustly labeled. (b) In hearts from compound mutant embryos, the number of X-gal-positive cells in the truncus arteriosus is drastically reduced compared with the septated truncus of control hearts, and a large mass of labeled cells is present in the right ventricle. A thymus is not present in mutant embryos at 13.5 dpc. (c,d) Dorsal views of dissected and stained hearts. (d) The same mass of cells seen in (b) can be seen from the

dorsal side of the heart. (Note: the large mass of blue labeled cells in the top left corner of (d) is the descending aorta, projecting out perpendicularly from the plane of the image. This structure is labeled with X-gal staining from *Pax3*-lineage cells that are not of NC origin in both mutants and controls; in the control embryo shown in (c), the aorta had been dissected away.) A, atrium; AA, ascending aorta; LV, left ventricle; PT, pulmonary trunk; PTA, persistent truncus arteriosus; RV, right ventricle.

FIG. 4.

Compound mutant embryos have a drastic early reduction in NCCs. Littermate control (**a,c**) and compound mutant (**b,d**) whole-mount embryos at 9.5 dpc labeled by X-gal staining to trace *Pax3*-lineage cells. In controls, blue *Pax3*-lineage cells fill the first three pharyngeal arches (PA1, PA2, and PA3) and more of the developing skull. In littermate compound mutant embryos, only PA1 contains *Pax3*-lineage cells; PA2 and PA3 appear devoid of *Pax3*-lineage cells. Both embryos are at identical developmental stages, each with 26 somites. (c) and (d) are enlarged regions from (a) and (b), respectively. PA, pharyngeal arch; ss, somite stage.

FIG. 5.

Compound mutant *Pax3*; *Foxd3* embryos have greatly increased cell death in the neural folds at 8.5 dpc. Littermate control and compound mutant whole-mount embryos at 8.5 dpc (**a–f**), 9.5 dpc (**g,h**), and 10.0 dpc (**i**), treated with LysoTracker to monitor cell death. (**a–c**) and (**g–i**) are lateral views, and (**d–f**) are dorsal views of the same embryos from (**a–c**). The embryos in (**a,d**) and (**b,e**) each have 10 somites, whereas (**c,f**) has 11 somites. (**g**) and (**h**) are both ~ 26 somites, (**i**) is ~ 30 somites. PA, pharyngeal arch; ss, somite stage.

Table 1Summary of Alleles Used^a

Allele	Type of allele	Full name	MGI	Reference
<i>Foxd3^{fllox}</i>	Conditional	<i>Foxd3^{tm3Lby}</i>	1347473	Teng <i>et al.</i> (2008)
<i>Foxd3⁻</i>	Deletion (by GFP insertion)	<i>Foxd3^{tm2Lby}</i>	1347473	Hanna <i>et al.</i> (2002)
<i>Pax3^{Cre}</i>	Insertion	<i>Pax3^{tm1(cre)Joe}</i>	97487	Engleka <i>et al.</i> (2005)
<i>Pax3^{Sp}</i>	Spontaneous splice-site mutation (null)	<i>Pax3^{Splootch}</i>	97487	Chalepakos <i>et al.</i> (1994)
<i>Pax3^{Sp-d}</i>	Missense mutation	<i>Pax3^{Splootch-delayed}</i>	97487	Vogan <i>et al.</i> (1993)
<i>Wnt1-Cre</i>	Transgene	<i>Tg(Wnt1-Cre)11Rth</i>	98953	Danielian <i>et al.</i> (1998)
<i>R26R^{lacZ}</i>	Reporter	<i>Gt(ROSA)26Sor^{tm1Sor}</i>	104735	Soriano (1999)

^aDetails are in Methods section.

Table 2

Pax3^{Cre/+}; Foxd3^{flox/+} Compound Mutant Embryos Die at Midgestation

	Control (<i>Pax3</i> ^{Cre/+} ; <i>Foxd3</i> ^{flox/+})			Mutant (<i>Pax3</i> ^{Cre/+} ; <i>Foxd3</i> ^{flox/-})		
	Alive/total	% Alive	PTA	Alive/total	% Alive	PTA
10.5 dpc	14/14	100	N/A	16/16	100	N/A
11.5 dpc	26/26	100	N/A	17/25	68	N/A
12.5 dpc	32/33	97	0%	24/40	60	100%
13.5 dpc	13/13	100	0%	4/14	29	100%
≥ 14.5 dpc	12/12	100	0%	0/11	0	100%

Deletion of *Foxd3* from the NC in a *Pax3* heterozygous null (*Pax3*^{Cre/+}) background resulted in embryonic lethality beginning at 11.5 dpc; all embryos were dead by 14.5 dpc. Control embryos did not exhibit embryonic lethality with the exception of one 12.5 dpc embryo that most likely died because of an unrelated cause; this embryo did not have a craniofacial or OFT defect. All mutant embryos examined had a PTA. N/A, not applicable.

Table 3

Pax3^{Sp/+}; Wnt1-Cre; Foxd3^{flox/-} Compound Mutant Embryos Die at Midgestation

		Mutant			
Control Wnt1-Cre; Foxd3 ^{flox/-}		Wnt1-Cre; Pax3 ^{Sp/+} ; Foxd3 ^{flox/-}	Wnt1-Cre; Pax3 ^{Sp^{del}/+} ; Foxd3 ^{flox/-}		
Alive/total	% Alive	Alive/total	% Alive	Alive/total	% Alive
11.5 dpc	8/8 100	2/3 67	2/2 100	2/2	100
12.5 dpc	10/10 100	3/5 60	3/6 50	3/6	50
13.5 dpc	7/7 100	1/7 14	1/6 17	1/6	17
≥ 14.5 dpc	19/19 100	0/13 0	0/18 0	0/18	0

Deletion of *Foxd3* from the NC in two independent *Pax3* heterozygous mutant backgrounds resulted in midgestational lethality and a 100% penetrant persistent truncus arteriosus, independent of *Cre* line. All mutant embryos examined had a PTA.