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An Absence of *Twist1* Results in Aberrant Cardiac Neural Crest Morphogenesis

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Abstract

The basic helix-loop-helix transcription factor Twist1 plays an essential role in mesenchymal cell populations during embryonic development and in pathological disease. Remodeling of the cardiac outflow track (OFT) into the functionally separate aortic arch and pulmonary trunk is dependent upon the dynamic, coordinated contribution of multiple mesenchymal cell populations. Here, we report that *Twist1*$^{-/-}$ mice exhibit OFTs that contain amorphic cellular nodules within their OFT endocardial cushions. The nodular mesenchyme expresses the related bHLH factors *Hand1* and *Hand2*, but reduced levels of the normal cushion marker Periostin. Lineage mapping confirms that nodule cells are exclusively of cardiac neural crest origin (cNCC), and are not ectopic cardiomyocytes or smooth muscle cells. These studies also reveal a delay in cNCC colonization of the OFT cushions. Furthermore, these mapping studies uncover nodules in the pharyngeal arches, and identify *Twist1*$^{-/-}$ neural crest cell defects within the dorsal neural tube, which exhibits an expanded domain of *Wnt1-Cre*-lineage marked cells. Together, these data support a model where Twist1 is required both for proper cNCC delamination, and for emigration from the dorsal neural tube and along cNCC migration pathways. Within the *Twist1*$^{-/-}$ neural crest cell populations that do emigrate to the OFT, a *Hand*-expressing subpopulation displays defective maturation, tracking, and, presumably, cell-cell adhesion, further compromising cNCC morphogenesis.

Keywords

Twist1; heart development; neural crest; Outflow track; mouse

Introduction

Development of the OFT from a simple tube to the highly structured junction between the ventricles and great arteries entails an array of tightly orchestrated morphological modifications. Reflective of its inherent developmental complexity and indispensability for proper cardiac function, abnormalities of the OFT and the tissues that govern its morphogenesis account for roughly one third of congenital heart defects (Clark, 1996; Ferencz et al., 1985; Rothenberg et al., 2003). During OFT development, mesenchymal cells derived from disparate sources accumulate within the OFT cushions, the progenitors of the OFT septum and valves.

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Integral to our understanding of the etiology of cardiac OFT defects is a clarification of the molecular mechanisms which enable these mesenchymal cell populations to emigrate into the lumen of the cardiac jelly of the OFT cushions, and which govern their differentiation upon arrival.

The OFT originates as a cardiomyocyte-lined vascular channel through which blood exits the primitive ventricle and fills the aortic sac (AS). First, the OFT cushions, which initially form as a deposit of extracellular matrix termed cardiac jelly, intervenes the conotruncal myocardium and the endocardium. The cardiac jelly is then progressively invaded by mesenchymal cells, some of which are cxardiac neural crest cells (cNCCs) (Snider et al., 2007; Stoller and Epstein, 2005), and the remainder of which are endocardial cells that have undergone epithelial-to-mesenchymal transition (EMT). The OFT cushions ultimately divide the OFT into two separate vessels, the aorta and the pulmonary trunk, and contribute to the subvalvular component of the interventricular septum (IVS). This process of septation establishes the division between the systemic and pulmonary circulation (Conway et al., 1997; Jiang et al., 2000).

The cNCC are essential for the patterning of the pharyngeal arch arteries (PAAs) as they are remodeled to form the aortic arch and contribute directly to the smooth muscle component of these tissues (Creazzo et al., 1998; Hutson and Kirby, 2003). Furthermore, proper cNCC contribution to and behavior within the OFT cushions is required for their morphogenesis into the progenitors of the conotruncal septum (Jiang et al., 2000; Qayyum et al., 2001). Additionally, the cNCCs ventral to the pharyngeal pouch form the OFT septum, a wedge that divides the aortic sac into the proximal origin of the aorta and pulmonary trunk (Waldo et al., 1998). Thus, septation and alignment of the developing cardiac OFT are contingent upon the contribution of cNCCs.

A diverse array of transcription factors is employed to mediate the local morphogenetic cues and guide the developing OFT through its intermediate stages (Firulli and Thattaliyath, 2002). Studies indicate that basic helix–loop–helix (bHLH) transcription factors contribute to a number of these processes (Firulli, 2003). bHLH factors conform to an evolutionarily conserved secondary structure, comprised of a DNA-binding and dimerization motif containing a short stretch of basic amino acids, followed by a pair of amphipathic α-helices intervened by a loop of varying length (for review, see Massari and Murre, 2000). These α-helices mediate protein-protein interactions between bHLH factors, enabling them to bind DNA, via their basic domains, at canonical consensus sites termed E-boxes (CANNTG) and to modulate transcription as either homo- or heterodimers.

Members of the Twist sub-class of bHLH transcription factors have been shown to exhibit broad dimer-partner choices. An evolutionarily conserved phosphoregulatory circuit modulates, in part, formation of these transcriptional complexes (Firulli et al., 2003; Firulli et al., 2005; Firulli et al., 2007). *Twist1* displays a broad mesodermal domain of expression (Fuchtbauer, 1995; Glackin et al., 1994; Stoetzel et al., 1995), is essential for the development of multiple organ systems, and has been correlated with EMT during tumor cell metastasis (Yang et al., 2004). Within the developing mammalian heart, *Twist1* is expressed in the endocardium of the AV canal, where it has been proposed to induce EMT (Ma et al., 2005).

The Twist1-related factors Hand1 and Hand2 are also required for heart development. Knockout studies show that *Hand2* is necessary for the formation of the presumptive right ventricle and PAAs (Srivastava et al., 1997). Conditional ablation of *Hand1* within the myocardial lineage confirmed its contribution to the development of the left ventricle, and revealed a role for *Hand1* during AV valvulogenesis (McFadden et al., 2005).

Here, we demonstrate that *Twist1* is expressed within components of the OFT and its progenitors in a manner partially overlapping the expression domains of its putative dimerization partners. We further demonstrate that cNCC derivatives are abnormal in *Twist1*$^{-/-}$ mutants, and that loss of *Twist1* function causes delayed and diminished cNCC emigration to, and aberrant cNCC morphology within the developing OFT. Importantly, we note that these defects are restricted to the subpopulation of cNCCs, which express the potential Twist1 dimer partners *Hand1* and *Hand2*. Finally, we correlate these cNCC defects with an expansion of *Wnt1*-marked cells within the dorsal neural tube of *Twist1*$^{-/-}$ embryos that suggests a trapping of NCC cells resulting in thickened neural tubes as compared to wild-type. As a whole, these data provide insight into the transcriptional regulation of cNCC development and novel understanding of the cellular processes underlying NCC and OFT morphogenesis.

Materials and Methods

Mouse strains

The targeting and PCR-based genotyping strategies for the *Twist1*^{tm1Bhr}, *Hand1*^{tm1Eno} and *Hand2*^{tm1Eno} null alleles have been previously described (Chen and Behringer, 1995; Firulli et al., 1998; Srivastava et al., 1997). For cell lineage trace analyses, either the *Wnt1-Cre* (Danielian et al., 1998) or *R26R* allele (Soriano, 1999) were introduced into the *Twist1* mutant background to generate <*Cre*$^{tg(+)}$;*Twist1*$^{+/-}$> and <*R26R*$^{+/-}$;*Twist1*$^{+/-}$> offspring, respectively. These mice were subsequently intercrossed to generate embryos for analysis.

Histology

Embryos (E9.5 – E11.5) were fixed in 4% paraformaldehyde, dehydrated through a methanol gradient and embedded in paraffin. Embryos were sectioned at 7μm unless otherwise noted. Nuclear Fast Red (Vector Laboratories) staining was performed as per manufacturer's instructions. Hematoxylin and Eosin (H&E) stain was performed exactly as described (Conway et. al. 2000). Propidium iodide (PI) staining was performed using 50μg/mL PI in 2X SSC. A minimum of 3 viable embryos (assayed via the presence of a heart beat) per genotype were used for these and all subsequent analyses.

In situ hybridization

Digoxygenin labeled section *in situ* hybridizations were carried out using established protocols on 10μm paraffin sections (Chen et al., 2004; Nagy, 2003) using T7, T3 or SP6 polymerases (Promega) and DIG-Labeling Mix (Roche). Sense and antisense digoxygenin-labeled riboprobes were transcribed for *Hand1* and *Hand2, PlexinA2* (provided by Jonathan Epstein), *Twist1*, *Periostin, Msx2, Wnt1, Pax3, Shh* (provided by Simon Conway).

X-gal staining

X-gal staining of embryos for β-galactosidase activity was performed essentially as previously described (Firulli et al., 1998).

BrdU, TUNEL, and immunohistochemistry

Pregnant mothers were injected intraperitonially with 100mg/g body weight of BrdU (Zymed Laboratories, Inc.) 1 hour prior to embryo dissection and processing as described above. BrdU immunodetection was carried out exactly as described (Conway et al., 2000). TUNEL assays were performed using Apoptag Cell Death Detection Kit (Chemicon) as per manufacturer's instruction. αSMA and Periostin antibody immunodetection was carried out exactly as described (Rios et al., 2005).

Cell counts and statistics

Cell counts were preformed manually from 7μm sections of *Twist1* mutants and WT littermates immediately caudal to the otic placodes. Sections were nuclear stained with either propidium iodide or hematoxylin. The exact dorsoventral midline was demarcated in these NTs, and cells in both the dorsal and ventral domains were counted. To account for variance in total cell number due to embryo stage, the number of cells in each *Twist1* mutant NT was normalized against a somite-matched WT littermate. Statistical significance was confirmed via one tailed student's t-Test assuming equal variances.

Results

Twist1 is required for normal mesenchymal cell contribution to and behavior within the PAs and the OFT cushions

To assess a potential requirement for *Twist1* in heart development, we performed histological analyses of *Twist1*$^{-/-}$ mutants at E11.5. *Twist1*$^{-/-}$ mice die at E11.5 and exhibit exencephaly and hypoplastic limb buds (Chen and Behringer, 1995). However, although cardiac expression of *Twist1* has recently been reported (Ma et al., 2005), no cardiac phenotypes have been described in these mutants. Examination of H&E stained transverse sections revealed that portions of the mesenchyme within the *Twist1*$^{-/-}$ mutant OFT cushion are abnormally condensed into nodules of cells (compare Fig. 1C, D). These nodules are confined to the cNCC populated truncal cushions and are absent from the EMT-derived conal cushions. Myocardium in *Twist1*$^{-/-}$ mice is not significantly different from that of wild-type littermates (Fig. 1).

We further examined the caudal PA mesenchyme. Proximal to the OFT, the PA mesenchyme appears abnormally condensed when compared with wild-type embryos (Fig. 1E, F, asterisk). PA mesenchyme within *Twist1*$^{-/-}$ embryos appears more dense and irregular than wild-type tissues and has a grossly similar appearance to the nodule clusters that appear in the OFT.

Additionally, we examined the mesenchyme of the AV cushions, which is derived solely from an endocardial lineage. Gross comparison of AV cushions of wild-type and *Twist1*$^{-/-}$ mice show no significant differences in structure (data not shown). Given that the novel defects observed in *Twist1*$^{-/-}$ embryos are more pronounced in the truncal OFT cushions and PAs; not the AV cushions, this suggests that endocardium does not require *Twist1* for EMT and normal AV cushion formation, and implicates normal behavior of the mesenchyme present in the PAs that populates the OFT as likely to be Twist1-dependent. We noted no observable myocardial or endocardial phenotypes within the hearts of *Twist1*$^{-/-}$ embryos (data not shown). We therefore initiated molecular characterization of these mesenchymal cell populations in wild-type and in *Twist1*$^{-/-}$ mice.

Twist1, Hand1 and Hand2 are expressed within tissues that contribute to the OFT in partially overlapping patterns

Given its established role in NCC function and EMT, we investigated the potential for Twist1-mediated regulation of OFT cushion development. We sought to create a detailed profile of *Twist1* expression relative to that of *Hand1* and *Hand2* within the developing OFT cushions and its progenitor cell populations. We performed DIG-labeled *in situ* hybridization for *Twist1*, *Hand1* and *Hand2* upon adjacent sections of wild-type embryos at E9.5 and E11.5 (Fig. 2A–F). We observed that *Twist1*, *Hand1* and *Hand2* are coexpressed in the body wall overlying the pericardial cavity at all three developmental stages, while both *Twist1* and *Hand2*, but not *Hand1* are expressed within the endocardium. As previously reported, *Hand1* and *Hand2* are expressed in myocardial cell populations (white arrows, Fig. 2B, C), most strongly at the truncus *Twist1* expression is not detectable within myocardium (Fig. 2A, D).

Twist1 displays a broad expression domain, including the mesenchymal cells occupying all pharyngeal arches (PAs) as well as the dorsal aorta (Fig. 2A). In the PAs more proximal to the OFT, staining is weaker, reflecting a reduction either in *Twist1* expression levels, or in number of *Twist1* expressing cells. At E9.5, it is undetectable at the distal lip of the truncus, which, at this stage, would include myocardium derived from the second heart field, endothelium, and cNCC-derived mesenchyme migrating into the lumen of the OFT cushions (Fig. 2A). *Hand2* is also expressed in the ventral PA mesenchyme, overlapping with the domains of low-level *Twist1* expression (Fig. 2A, B). Additionally, *Hand1* and *Hand2* are expressed strongly within the distal lip of the truncus (Fig. 2B, C), cells in which *Twist1* expression is absent (Fig. 2A). However, at E11.5 *Twist1* expression is strongly detected uniformly throughout the mesenchymal cells of the OFT cushions (Fig, 2D, white arrowhead), ostensibly representing a mixed populace of both cNCC and endocardial-derived cells. These cells do not express *Hand2* at detectable levels (Fig. 2E), but some of these cells do express *Hand1* (Fig. 2F). Given that *Twist1*$^{-/-}$ OFTs display both normal and abnormally condensed mesenchymal cell populations, and both *Hand1* and *Hand2* are tightly spatiotemporally regulated in these affected cells, we assessed a potential aberrant expression of these two genes within the OFTs of *Twist1*$^{-/-}$ mice.

Abnormal mesenchyme within the OFT cushions of Twist1$^{-/-}$ mice expresses *Hand1* and *Hand2*

As the majority of the mesenchymal cells within the PAs that emigrate into the OFT and differentiate into smooth muscle are cNCC derivatives, we predict that *Twist1* is necessary for proper cNCC development within the OFT. *Hand1* and *Hand2* are post-migratory markers of cNCCs (Srivastava et. al. 1997) and interestingly, are also expressed within the cNCCs in a pattern partially overlapping that of *Twist1* (Fig. 2). mRNA expression analysis shows that nodular cells within the OFT cushions of *Twist1*$^{-/-}$ mice express both *Hand1* and *Hand2*, whereas the non-nodular mesenchyme expresses less of each Hand-factor (Fig. 3E, F). To validate this observation, we took advantage of our *Hand1*^{lacZ} mice, crossing this allele onto the *Twist1*$^{-/-}$ background. Given that Twist1 and Hand factors can form transcriptional complexes and that in the developing limb genetic interactions between *Twist1* and *Hand2* are evident (Firulli et al., 2003; Firulli et al., 2005; Firulli et al., 2007), we carefully looked for changes in the *Twist1*$^{-/-}$ OFT phenotype when presented on a *Hand1* haploinsufficient background. In all embryos examined (8 total), no phenotypic differences were observed (compare Fig 1D with Fig. 3D, E). Thus, we conclude that relative gene dosages of *Hand1* and *Twist1* are not critical for OFT development. Analysis of *Hand1*^{lacZ} expression shows that the nodular cells within the *Twist1*$^{-/-}$ mice express *Hand1* whereas, in wild-type mice, these *Hand1*^{lacZ} expressing cells are evenly intermixed with a population of *Hand1*^{lacZ} negative mesenchymal cells, validating *in situ* analysis (Fig. 3C–F). *In situ* hybridization of the cNCC marker *PlexinA2* confirms that at this level of the OFT the majority of the cells are migratory cNCC (data not shown). Together, these data show that the nodular mesenchymal cells within the OFT of the *Twist1*$^{-/-}$ embryos robustly express both the post-migratory NCC markers *Hand1* and *Hand2*. Coupled with the observation that the unaffected mesenchyme within the OFT of these mutants expresses *Hand1* and *Hand2* at lower levels or, more likely, within fewer cells, these data indicate that *Hand1* and *Hand2* expressing cells are selectively affected by a loss of *Twist1*.

We next sought to determine whether these mesenchymal morphology defects were associated with cell migration defects. We therefore examined the behavior of the *Hand1*^{lacZ}-expressing mesenchymal subpopulation at E10.5. At this stage, we observed a noticeable decrease in the number of *Hand1*^{lacZ}-expressing cells that have moved into the OFT (black arrowheads, Fig. 3G, H) and ventral pharynx (white arrowheads, Fig. 3I, J), suggesting a delay in cNCC migration into these structures. Taken together, these data support that a novel cardiac defect

is present in *Twist1*$^{-/-}$ mice that effects spatiotemporal mesenchymal-cell migration. Moreover, the defective mesenchymal cell population is marked by the expression of *Hand1* and *Hand2*.

Defective mesenchyme in the *Twist1*$^{-/-}$ OFT is not composed cardiac or smooth muscle and exhibits a lack of mesenchymal maturation

cNCC-derived ectomesenchyme localizes to the PAs and OFT cushions, a portion of which ultimately differentiates and contributes to the vascular smooth muscle of the mature aortic arch (Creazzo et al., 1998; Firulli and Conway, 2004). As histological analyses indicate that mesenchymal components of both the PAs and OFT cushions are abnormal in *Twist1*$^{-/-}$ mutant embryos, we employed immunohistochemistry to look at the smooth and early cardiac muscle marker SM-α-Actin (Smart et al., 2002), and the mesenchymal maturation marker and a putative downstream target of *Twist1*, Periostin (Lindsley et al., 2007) to assess whether the defective mesenchyme within the *Twist1*$^{-/-}$ OFTs contain ectopic cardiac muscle and/or are able to mature and to ultimately differentiate. Results show that although SM-α-Actin is robustly expressed within the cardiomyocytes of the *Twist1*$^{-/-}$ OFT and more weakly within the ostensibly normal OFT cushion mesenchyme, it is undetectable within the abnormal *Twist1*$^{-/-}$ mesenchymal condensations (arrowhead, Fig. 4A) indicating that nodules are not likely to be composed of ectopic cardiomyocytes or differentiated smooth muscle. Periostin, which is broadly expressed within the OFT cushions during cushion maturation, is reduced within the OFT nodules (arrowhead, Fig. 4B) supporting the idea that these nodular cells are molecularly abnormal. Therefore, these data suggest that the nodular cells within the *Twist1*$^{-/-}$ OFT are not ectopic cardiomyocytes, but, rather, immature cNCCs.

Wnt1-Cre lineage mapping indicates that cNCC cells have defective migration

Wnt1-Cre-mediated recombination of the *R26R* allele marks all NCCs from the point at which they delaminate from the neural tube and initiate migration throughout the embryo (Chai et al., 2000). Taking advantage of real-time expression of *Hand1*^{lacZ} within the pathologically defective mesenchymal cells of the *Twist1*$^{-/-}$ OFT cushions allows us to examine these *Hand1*^{lacZ} expressing cells in the context of all cNCCs within the OFT. *Wnt1-Cre*-mediated *R26R* expression in wild-type and *Twist1*$^{-/-}$ mice was first examined at E11.5 (Fig. 5A–H). In the wild-type embryo, the OFT cushions have segregated into the conotruncal ridges that will ultimately fuse to form the septum dividing the aorta and pulmonary trunk (arrows in Fig. 5A). In the *Twist1*$^{-/-}$ mutant, these ridges are poorly defined (arrow in Fig. 5B). The aberrant morphology of this cushion is consistent with other models of cNCC dysfunction (Epstein et al., 2000; Kaartinen et al., 2004), although the early embryonic lethality of *Twist1*$^{-/-}$ mutants precludes characterization of potential persistent truncus arteriosis (PTA) or double outlet right ventricle (DORV) defects. Additionally, satellite clusters of cNCCs are evident proximal to the RV (white arrowheads in Fig. 5B) and within the pericardium (arrowheads in Fig. 5B) in *Twist1*$^{-/-}$ but not wild-type embryos, suggesting that cNCC migration paths are altered in the absence of *Twist1*. These path-finding defects are consistent with previous studies of *Twist*-deficient cranial NCCs (Soo et al., 2002). Further histological analyses of these embryos reveal that, within the OFT cushions, both the abnormally condensed and phenotypically normal mesenchyme are of cNCC origin (arrowheads in Fig. 5C, D). Given that the large majority of *Hand1*^{LacZ} positive cells contribute to the nodules within the OFT cushions, whereas the phenotypically normal cushion mesenchyme is largely *Hand1*^{LacZ} negative but still of cNCC origin, it appears likely that Hand factor-expression is defining a subpopulation of cNCC that are sensitive to Twist1 loss-of-function.

We further verified NCC migration defects, examining *Wnt1-Cre;R26R*-expressing cNCCs at E9.5 (Fig. 5I, J). At this stage, clusters of lacZ-positive cells were evident within the pericardium and endocardium of the *Twist1*$^{-/-}$ mutants (data not shown), confirming cNCC

path-finding defects. Additionally, cNCC emigration was delayed in entering the OFT cardiac jelly (compare position of arrows, Fig. 5I, J). Together, these data support the hypothesis that *Hand*-factor expression delineates a specific subpopulation of cNCCs, which are defective in their maturation, emigration, path-finding, and, presumably, adhesion, in the absence of *Twist1* expression.

Additional histological analyses of cNCC lineage mapping in both wild-type and *Twist1*$^{-/-}$ mice confirms that cNCCs are also present as nodular condensations within the PAs (Fig. 5E–H) and that these nodular cNCCs appear to emigrate as such into the OFTs of *Twist1*$^{-/-}$ mice. This suggested to us that these condensations might appear more proximal to the neural tube. In order to further explore the etiology of these Twist1-mediated OFT defects, we next examined cNCCs proximal to the neural tube to pinpoint the source of the nodule formation and defective migration behavior.

Loss of Twist1 results in an expansion of NCC ventrally along the neural tube

In order to better define the origins of observed cNCC defects in *Twist1*$^{-/-}$ embryos, we examined E9.5–E11.5 wild-type and *Twist1*$^{-/-}$ neural tubes marked via the *Wnt1-Cre;R26R* alleles (Fig. 6). As expected, at E11.5 expression of *Wnt1-Cre* marks the dorsal lip of the neural tube corresponding to the domain from which the cNCCs have undergone EMT and emigrated towards the PAs (Fig. 6A). Surprisingly, analysis of transverse sections shows a clear ventral expansion of lacZ-positive cells in the *Twist1*$^{-/-}$ neural tubes (compare white bars in Fig. 6A, B). This expansion is less apparent in the more caudal neural tube (data not shown), consistent with the increasingly pronounced defects within the more rostral neural tube of *Twist1*$^{-/-}$ mutants (Chen and Behringer, 1995). At E9.5, dorsoventral expansion is less apparent; however, the *Wnt1-Cre;R26R* domain is larger owing to an obviously thicker neural tube in the *Twist1*$^{-/-}$ mutant (compare Fig. 6C, D). These results support the idea that cNCC delamination from the neural tube may be inhibited/delayed in *Twist1*$^{-/-}$ mutants, and as embryonic development proceeds, cNCC lineage mapping renders this altered delamination more apparent.

To determine whether this expansion of the *Wnt1* lineage represents a dorsalization of the *Twist1*$^{-/-}$ neural tube, we examined mRNA expression of *Wnt1*, *Wnt3a* (Ikeya et al., 1997), *Shh* (Munsterberg et al., 1995), *Pax3* (Conway et al., 1997) and *Msx2* (Kwang et al., 2002), which delimit specific dorsal/ventral domains of the developing neural tube. Although expression of these markers appears unaffected in regards to dorsal-ventral neural tube patterning within the *Twist1*$^{-/-}$, expression of these markers is, as a whole, more robust, reflecting the increase in neural tube thickness in *Twist1*$^{-/-}$ embryos when compared to wild-type controls (Fig. 6E–J and data not shown).

To determine if the observed neural tube thickening was a result of decreased cell death or increased cell proliferation, we performed both TUNEL and BrdU and analyses upon *Twist1*$^{-/-}$ mutants at E9.5 (Fig. 7). Extensive mesenchymal apoptosis has been reported in *Twist1*$^{-/-}$ embryos within the cranial mesenchyme, first branchial arch and the somite at E9.5; however, apoptosis in the dorsal neural tube was not reported (Ota et al., 2004). We observe no significant differences in the number of apoptotic cells within the neural tubes of wild-type and *Twist1*$^{-/-}$ embryos (Fig. 7A, B). Similarly, the number of BrdU positive nuclei is not significantly different between wild-type and *Twist1*$^{-/-}$ embryos, indicating that increased proliferation is not likely involved in the expansion of *Twist1*$^{-/-}$ neural tubes (Fig. 7C–E). To validate that the total number of cells within the neural tube is altered in the absence of *Twist1*, transverse sections of wild-type and *Twist1*$^{-/-}$ embryos at E10.0 were nuclear stained (Fig. 7F, G). The neural tubes were divided along the dorsoventral midline (white lines, Fig. 7F, G), and the number of nuclei manually counted. Quantification of total cell number with the neural tube shows a nearly 2-fold increase in the number of cells within the *Twist1*$^{-/-}$ dorsal

neural tube, whereas ventral cell counts are similar between wild-type and *Twist1*$^{-/-}$ embryos (Fig. 7H). The increased cell number in the absence of decreased cell death or increased proliferation within the dorsal neural tubes of E9.5 *Twist1*$^{-/-}$ mice, in conjunction with the expanded domain of *Wnt1-Cre* marked cells within the neural tube observed at later developmental stages, is consistent with the hypothesis that NCC become trapped within the neural tube of *Twist1*$^{-/-}$ mice, which results in poor delamination, altered cell adhesion, and delayed cNCC emigration.

Discussion

Twist1 is necessary for cNCC migration, maturation and morphology

We have shown that mice null for *Twist1*, in addition to exencephaly and hypoplastic limbs, also exhibit cardiac OFT defects that result from defects in cNCC emigration, maturation, and, presumably, cell adhesion. These phenotypes can be traced back to the cNCC origins at the dorsal neural tube where an expansion of *Wnt1-Cre* expressing cells is observed, suggesting that the cNCCs may be partially trapped from emigrating out of the neural tube, and those that do escape exhibit adhesion, emigration and maturation defects. These phenotypes are summarized in Figure 8.

Morphologically affected cells within the OFTs of *Twist1*$^{-/-}$ mutants selectively express *Hand1* and *Hand2* at E11.5. Given the dynamic expression profiles of these two genes described in Figure 2, we may interpret these results to indicate that *Hand*-factors are broadly expressed in cNCCs occupying the PAs and dorsal lip of the OFT at E9.5. As development proceeds, the domains of *Hand1* and *Hand2* are restricted, and persist solely within a subset of these cells in the OFT at E11.5. It is these cells, which appear to be most affected by a loss of *Twist1* at this stage. Alternatively, *Hand*-factor expression may be restricted to early-migrating cNCCs, the progression of which appears delayed in *Twist1*$^{-/-}$ mutants. As these cNCCs are presumably intermixed with ostensibly unaffected late-migrating cNCCs within the E11.5 OFT, this would explain the difference in phenotype evident between Hand-factor expressing and non-expressing cells. Differentiation between these two possibilities would necessitate detailed lineage trace analyses employing NCC-specific *Hand1*- or *Hand2-Cre* alleles. In either case, these observations raise the intriguing possibility that *Hand*-factors ultimately mark a distinct subpopulation of cNCCs, which may contribute to specific cardiac tissues as the OFT matures.

Mesenchymal cell invasion of OFT cushions in *Twist1*$^{-/-}$ mutants is delayed. As *Twist1*$^{-/-}$ cNCCs within the PAs is abnormal and condensed, the observed delay of mesenchymal cell invasion of the cushions may reflect altered cNCC adhesive properties impeding cell emigration. The presence of *Wnt1-Cre* marked cells in ectopic locations within the heart further shows that *Twist1*$^{-/-}$ cNCC cells have compromised ability to accurately track to their intended destinations. These migration phenotypes are consistent with the defects in cranial NCC migration that were previously observed in relation to the exencephalic phenotype (Chen and Behringer, 1995; O'Rourke and Tam, 2002).

Twist1$^{-/-}$ defective mesenchymal cells within the OFT fails to upregulate a marker of maturation Periostin and are not ectopic cardiomyocytes. Previous studies have reported that cranial NCCs fail to differentiate to osteogenic and myogenic lineages in *Twist1*$^{-/-}$ mutants (Soo et al., 2002). Our data suggest that cNCCs also require *Twist1* function to progress through their developmental programs.

In contrast, the AV cushions, which are derived solely from endocardium that has undergone EMT, appear normal in *Twist1*$^{-/-}$ mutants, and express at least two expected mesenchymal markers, *Periostin* and *Tbx20* in a manner indistinguishable from wild-type mice (data not

shown). This further supports the conclusion that *Twist1*$^{-/-}$ mutant cardiac defects are cNCC in origin and that, although Twist1 has been shown to be a key player in mediating EMT, its absence is not required for the EMT of endocardial cells. Perhaps functional redundancy or synergy with *Hand2* or another Twist family member that is expressed in the endocardium is at play. *Hand1* seems unlikely to play such a role as it is not expressed at detectable levels in endocardium (Fig. 2C, F) and its haploinsufficiency does not noticeably alter the *Twist1*$^{-/-}$ cNCC phenotype. We have, however, previously shown genetic and functional interactions between *Twist1* and *Hand2* during limb development and perturbation of this antagonistic relationship can cause SCS (Firulli et al., 2005). It would be interesting to look for genetic interactions between *Twist1* and *Hand2*; however *Hand2* null mice die at E9.5 (Srivastava et al., 1997), precluding analysis without employing a conditional *Hand2* allele.

cNCCs fail to segregate normally in *Twist1*$^{-/-}$ embryos. Previous chimeric analyses demonstrate that *Twist1*$^{-/-}$ cells within the head mesenchyme fail to segregate normally with wild-type mesenchymal cells (Chen and Behringer, 1995). It has been proposed that these abnormal cells are cranial NCCs that have failed to assume a mesenchymal character despite their ability to emigrate from the neural tube. These data echo the abnormal cell interactions we observe in the PA mesenchyme and OFT. At the level of the neural tube corresponding to the presumptive cNCCs, it is unclear whether the cNCCs delaminate as nodules. However, within the head, we observe the cranial NCCs delaminating from the dorsal lip as nodules directly proximal to the neural tube (data not shown). We feel it is likely that the delaminating cNCCs exhibit similar behavior. As a whole, these observations raise the intriguing possibility that the potential adhesion, emigration, tracking, and maturation defects of *Twist1*$^{-/-}$ cNCCs can be attributed to abnormal delamination from the dorsal neural tube, although we cannot discount the possibility that these cNCCs have been mis-specified to a neuronal cell fate. Importantly, *Twist1* expression is not detected within NC stem cells or the dorsal neural tube (Fuchtbauer, 1995; Glackin et al., 1994; Stoetzel et al., 1995, our data; Wolf et al., 1991), but it has been proposed that ECM plays a significant role in cell recognition, initiation and regulation of NCC migration (Coles et al., 2007; Lofberg et al., 1985). Given that Twist1 has been reported to regulate a number of ECM genes in both development and cancer, this supports the hypothesis that the initial effects of the *Twist1*$^{-/-}$ NCC phenotype are non-cell autonomous.

Twist1 function has been implicated in establishing a boundary between cranial NCCs and mesoderm during formation of the coronal suture of the skull vault (Merrill et al., 2006). The data presented here are consistent with a role for *Twist1* function in boundary formation. Normal OFT cushion mesenchyme comprises an integrated population of cNCC and endocardially-derived cells. Integration of these distinct cell populations into a unified structure presumably entails modulation of cell adhesion programs. Our findings suggest that *Twist1* is critical for cNCC emigration and, most likely, in regulation of cell adhesion, influencing the manner in which cNCCs interact with the cell populations they encounter, such as the endocardial-derived OFT cushion cells which have undergone EMT and moved into the cardiac jelly.

Loss of *Twist1* function causes an expansion of the *Wnt1-Cre* lineage in the neural tube in the absence of defective dorsoventral patterning

We demonstrate that the cNCC defects characteristic of *Twist1*$^{-/-}$ mutants are associated with a pronounced expansion of the *Wnt1*-expressing lineage within the dorsal neural tube. The expansion of the dorsal neural tube may cause the cNCCs migration and morphogenetic phenotypes seen in these mutants. However, it is possible that this dorsal neural tube phenotype does not cause the observed cNCC phenotypes, but rather, both phenotypes reflective of a common, non-autonomous requirement of *Twist1* to regulate a process such as cell-cell adhesion. Alternatively, we cannot rule out that the cNCC and dorsal neural tube phenotypes

are unrelated. The relationship between the expansion of the dorsal neural tube and cNCC phenotypes could be clarified by spatiotemporally ablating *Twist1* function employing the recently described conditional allele bred to Cre alleles specific for the neural crest, such as *Wnt1-Cre* (Danielian et al., 1998) or *P3Pro-Cre* (Li et al., 2000), or the lateral mesenchyme, such as *T-cre* (Perantoni et al., 2005).

Following the *Twist1*$^{-/-}$ cNCC defects back to the neural tube, we observed an expansion of *Wnt1-Cre* marked cells that is not associated with increased cell proliferation or decreased cell death. This increase in cell number without change in proliferation or death suggests that delaminating NCCs become trapped in the neural tube. Similar trapping of NCCs without changes in dorsoventral patterning is observed in *Wnt1/Wnt3a* double knockout mice (Ikeya et al., 1997), which is interesting as *Twist1* expression is known to be regulated by Wnt signaling (Howe et al., 2003; Reinhold et al., 2006). Previous studies have implicated Twist1 function in the regulation of dorsoventral patterning in the brain (Soo et al., 2002). *Twist1*$^{-/-}$ mutants display a loss of dorsal markers in the forebrain and expanded or ectopic ventral marker expression in the midbrain and hindbrain (Soo et al., 2002). These observations are reflective of the exencephaly characteristic of *Twist1*$^{-/-}$ mutants (Chen and Behringer, 1995). *In situ* hybridization marker analyses within the neural tube shows increased expression of these markers reflective of the thickened-distorted neural tubes. As the expansion of dorsal neural tube cells as well as NCC lineage-marked cells correlates with decreased cNCC emigration into the OFT, and *Twist1* expression is not detectable within the neural tube, we conclude that cNCC trapping in the neural tube is the result of a non-cell autonomous mechanism, most likely the regulation of microenvironment ECM. *Twist1* is robustly expressed in the surrounding non-NCC mesenchymal cell populations proximal to the dorsal neural tube (Fuchtbauer, 1995; Glackin et al., 1994; Stoetzel et al., 1995, our data; Wolf et al., 1991), and these cells are likely the indirect source of the putative trapping defect. Moreover, Twist1 is known to regulate the expression of ECM genes, such as *N-cadherin*, in coordination with its role in EMT (Yang et al., 2004). Interfering antibodies to N-CAM and N-cadherin within the dorsal neural tube have been shown to result in grossly distorted neural tubes, ectopic migration, and abnormal adhesion (Bronner-Fraser et al., 1992) Interestingly, the effects of these antibodies are temporally dependent. Given that NCCs leave the neural tube in waves during early embryogenesis (Artinger and Bronner-Fraser, 1992; Loring and Erickson, 1987; Serbedzija et al., 1994), we hypothesize that Twist1 is necessary for early NCC emigration owing to its regulation of ECM components and cell adhesion molecules. Thus, in the absence of *Twist1*, cNCCs are temporally delayed in entering the OFT and cannot track or adhere properly. Later waves of NCC emigration, such as those, which populate more caudal structures, one would predict, would be less sensitive to a Twist1 early function. Phenotypic observations of *Twist1*$^{-/-}$ mice support this idea. Additionally, in chick-quail transplant experiments, it has been shown that transplanted quail NCCs from non-cNCC locations cannot normally contribute to the OFT. In addition to later stage phenotypes such as PTA, these abnormal chimeric OFTs display mesenchymal nodules (Kirby, 1989). This classic study put forth the idea that local environment is critical for normal cNCC behavior and strongly suggests that loss of Twist1 in the local environment surrounding the dorsal neural tube disrupts NCC positional information necessary for normal function.

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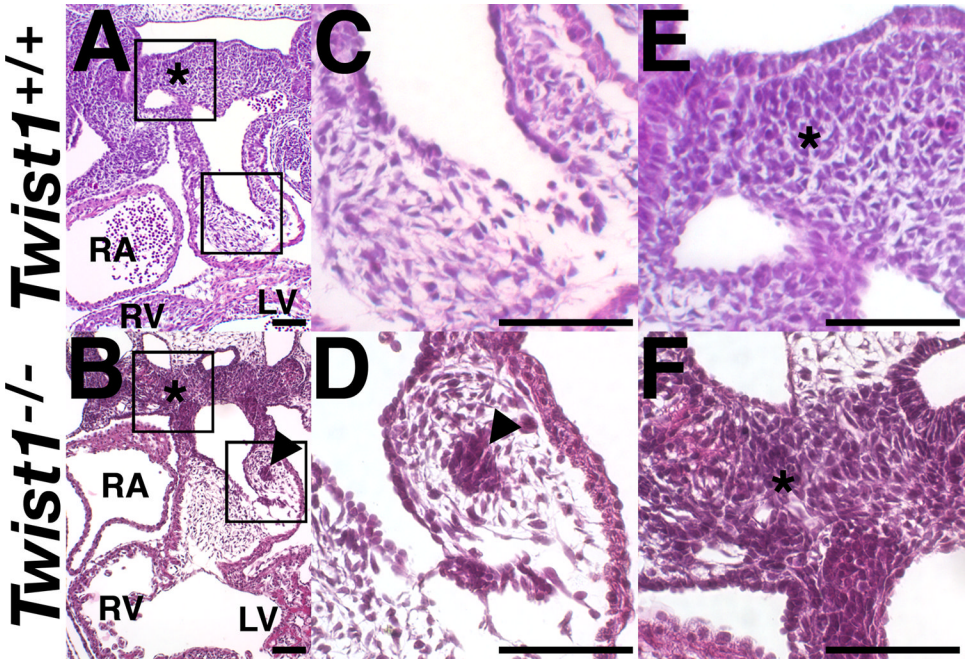

Figure 1. Defects of the OFT cushions and PAs, but not AV cushions in *Twist1*$^{-/-}$ mutant embryos

(A–F) H&E stained transverse sections of somite-matched E11.5 embryos. (C, F) High power magnification of the insets in A and B. Arrowheads denote mesenchymal nodules. Asterisks denote pharyngeal mesenchyme. LV, left ventricle; RA, right atrium; RV, right ventricle. Scale bars = 100μm.

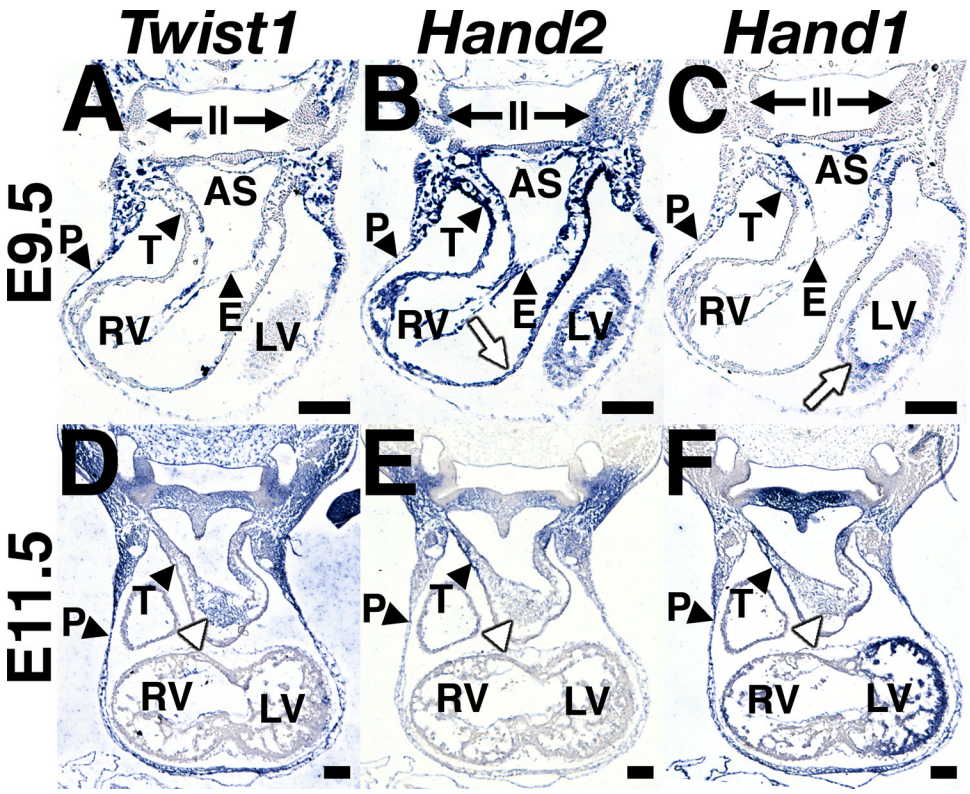

Figure 2. *Twist1* and *Hand* gene expression is partially overlapping in the OFT

(A–F) DIG-labeled *in situ* hybridization upon serial transverse sections of E9.5 (A–C) and E11.5 (D–F) embryos showing *Twist1* (A, D), *Hand2* (B, E) and *Hand1* (C, F) expression. II, second pharyngeal arch; AS, aortic sac; E, endocardium; LV, left ventricle; P, pericardium; RA, right atrium; RV right ventricle; T, truncus; white arrows, myocardium; white arrowhead, OFT cushion mesenchyme. Scale bars = 100μm.

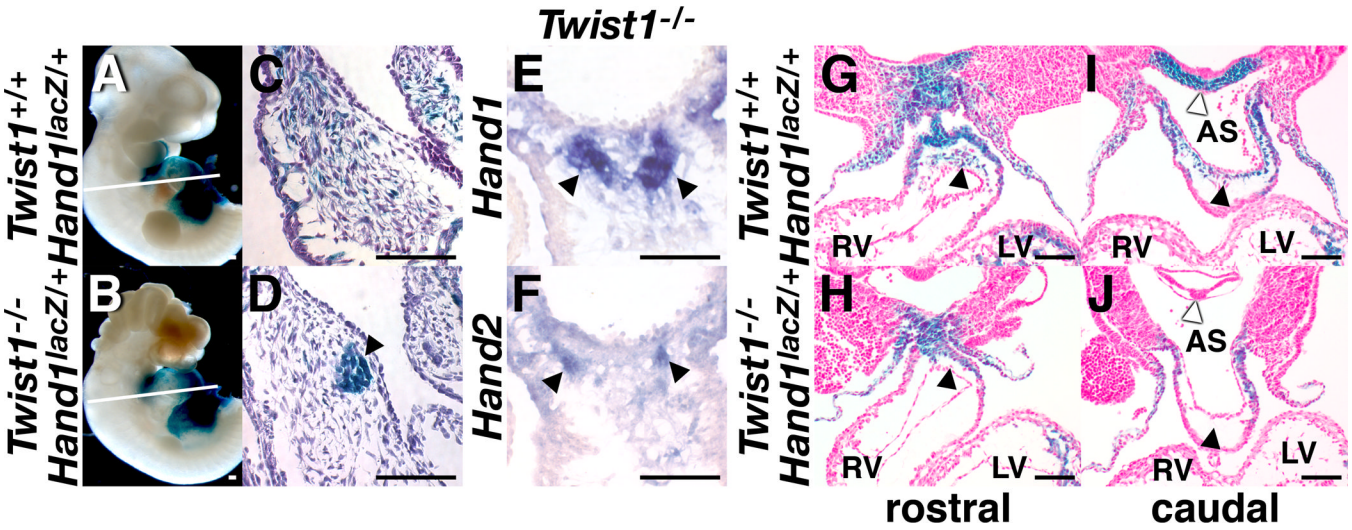

Figure 3. Defective Hand-factor-expressing mesenchyme in *Twist1*$^{-/-}$ mutants

Whole mount (A, B) and transverse sections (C, D) of X-gal-stained, somite-matched *Twist1*$^{+/+}$;*Hand1*^{lacZ} and *Twist1*$^{-/-}$;*Hand1*^{lacZ} embryos at E11.5 showing lacZ expression localized to the mesenchymal nodules (black arrowheads) of the *Twist1*$^{-/-}$ OFT. White lines in A and B denote plane of section. (E, F) DIG-labeled *in situ* hybridization upon serial transverse sections of somite-matched E11.5 embryos showing *Hand1* (G) and *Hand2* (H) expression in the mesenchymal nodules (black arrowheads) of the *Twist1*$^{-/-}$ OFT. (G–J) transverse sections of X-gal-stained Twist1+/+;*Hand1*^{lacZ} and *Twist1*$^{-/-}$;*Hand1*^{lacZ} embryos at E10.5 showing an absence of lacZ expressing cells in the OFT (black arrowheads) and pharyngeal mesenchyme (white arrowheads) of *Twist1*$^{-/-}$ mutants. AS, aortic sac; LV, left ventricle; RV, right ventricle.

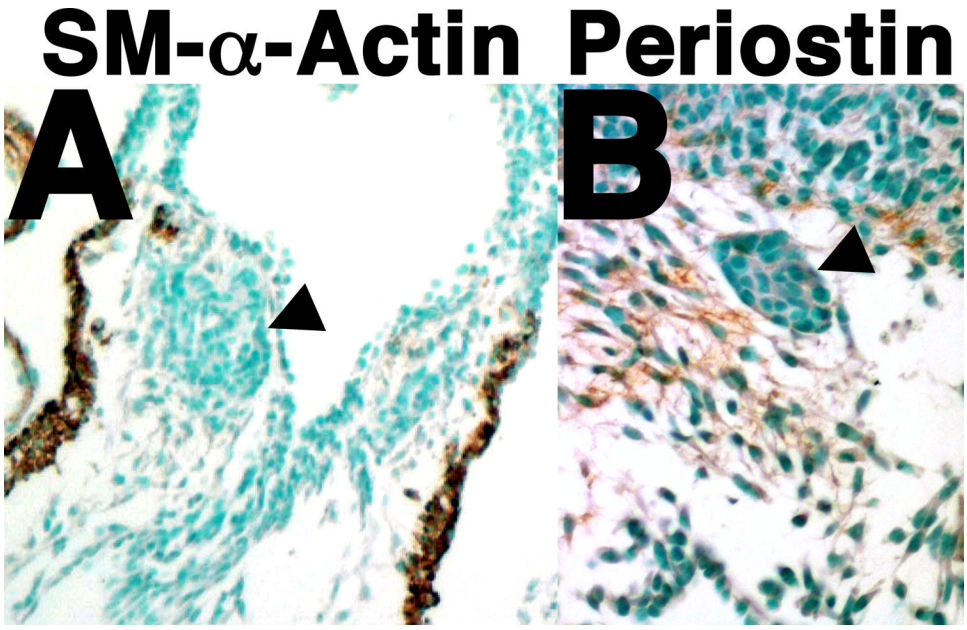

Figure 4. Defects of mesenchymal cell maturation in *Twist1*$^{-/-}$ mutant embryos

Immunohistochemical staining on transverse sections of E11.5 *Twist1*$^{-/-}$ embryos show an absence of SM-α-actin (A) and periostin (B) protein within the abnormal OFT mesenchymal nodules.

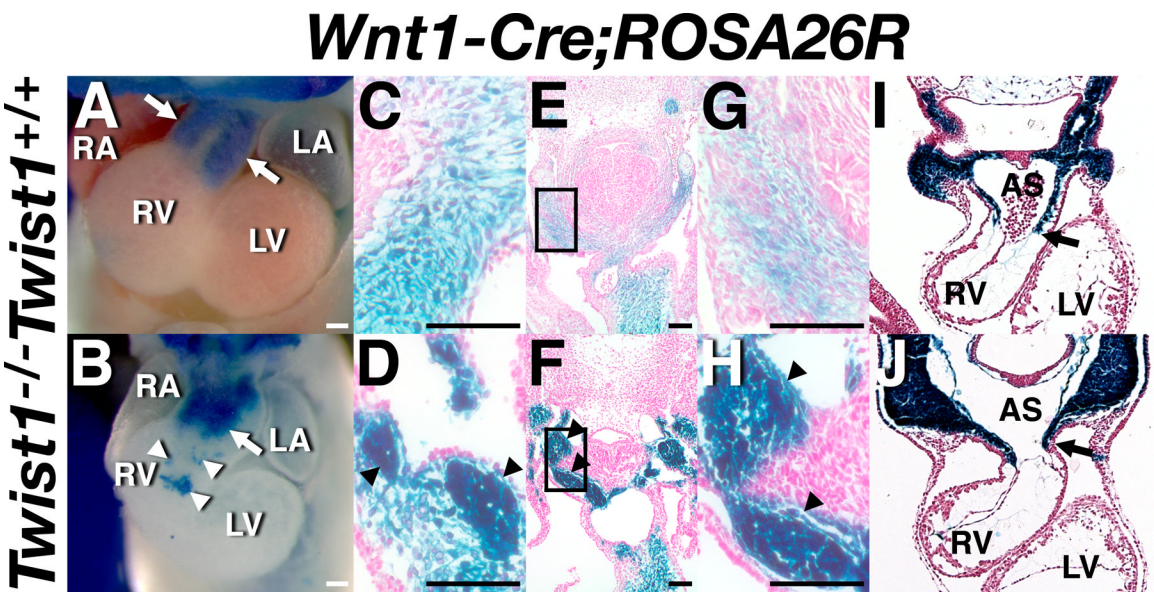

Figure 5. Defective cNCC mesenchyme in *Twist1*$^{-/-}$ mutants

Whole mount (A, B) and transverse sections (C–J) of X-gal-stained, somite-matched *Twist1*$^{+/+}$;*Wnt1-Cre(+)*;*R26R* (C, E, G, I) and *Twist1*$^{-/-}$;*Wnt1-Cre(+)*;*R26R* (D, F ,H, J) embryos at E11.5 (A-H) and E9.5 (I, J). White arrows in A and B denote the OFT cushions. White arrowheads in A and B denote mis-emigrating cNCCs. LacZ expression is apparent in the mesenchymal nodules of the *Twist1*$^{-/-}$ OFT and PAs as well as the NCC-derived truncal endocardial cushions (black arrowheads in D, F, H). AS, aortic sac; LA, left atrium; LV, left ventricle; RA, right atrium; RV right ventricle; Scale bars = 100μm.

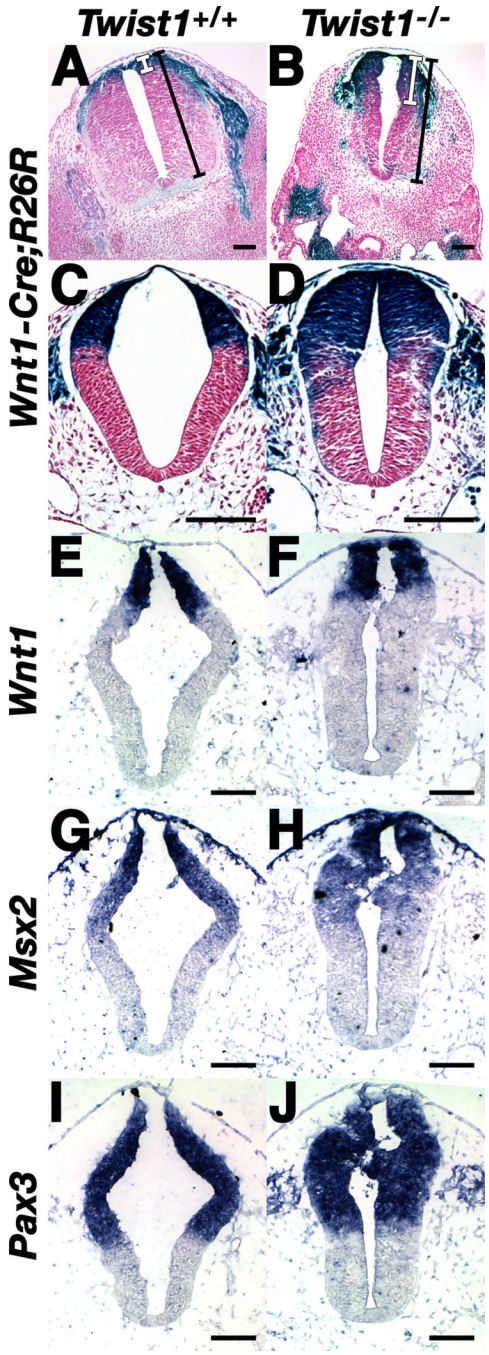

Figure 6. Expansion of the *Wnt1-Cre*-expressing lineage in the rostral neural tube *Twist1*$^{-/-}$ mutants in the absence of DV patterning defects

(A, B) Transverse sections of X-gal-stained, somite-matched *Twist1*$^{+/+}$;*Wnt1-Cre(+);R26R* and *Twist1*$^{-/-}$;*Wnt1-Cre(+);R26R* embryos at E11.5 (A, B) and E9.5 (C, D) showing expansion of lacZ expressing domain (white bars) in the dorsal neural tube (compare to total dorsoventral width of neural tube, black bars). E9.5 dorsal Wnt1-Cre expansion is less apparent in contrast to increased thickness of the neural tube (compare C & D). (E–J) DIG-labeled *in situ* hybridization upon transverse sections of somite-matched E9.5 embryos showing *Wnt1*, *Msx2*, and *Pax3* expression in wild-type (E,G, I) in *Twist1*$^{-/-}$ mutants (F, H, J). Scale bars = 100μm.

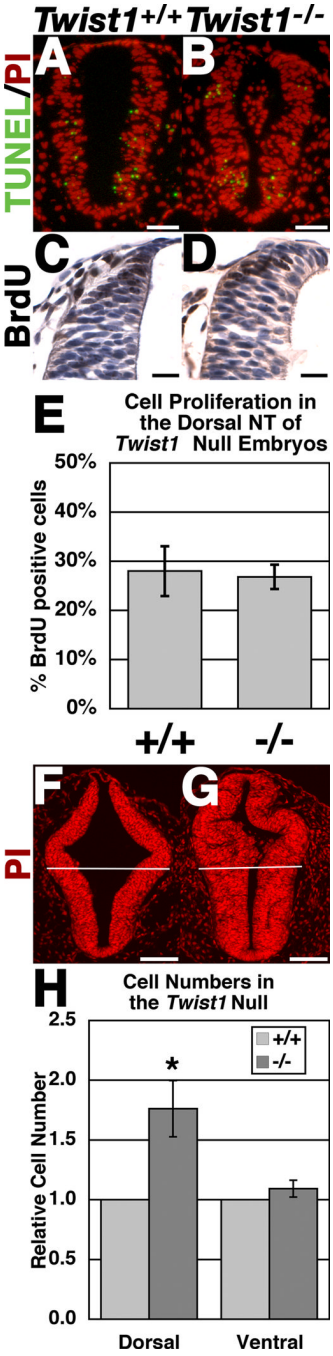

Figure 7. Cell death and cell proliferation is unaltered but cell number is increased within the dorsal neural tube of *Twist1*$^{-/-}$ mutant embryos

(A, B) TUNEL green and propidium iodide (PI; red) staining of somite-matched E9.5 wild-type (A) and *Twist1*$^{-/-}$ (B) neural tubes. (C-E) α-BrdU immunohistochemical staining on transverse sections of somite-matched E9.5 wild-type (C) and *Twist1*$^{+/+}$ embryos (D) quantified and represented as a percentage of the total number of cells within the dorsal neural tube (E). (F-H) 10μM sections of somite-matched wild-type (F) and *Twist1*$^{-/-}$ (G) neural tubes axially matched immediately caudal to the otic placodes stained with propidium iodide (PI; red). Neural tubes were bisected along the dorsoventral midline (white lines). Total cell counts

were made of dorsal vs. ventral neural tubes of each respective genotype and represented graphically (H). Scale bars (A, B) = 100μm, (C, D) = 20μm.

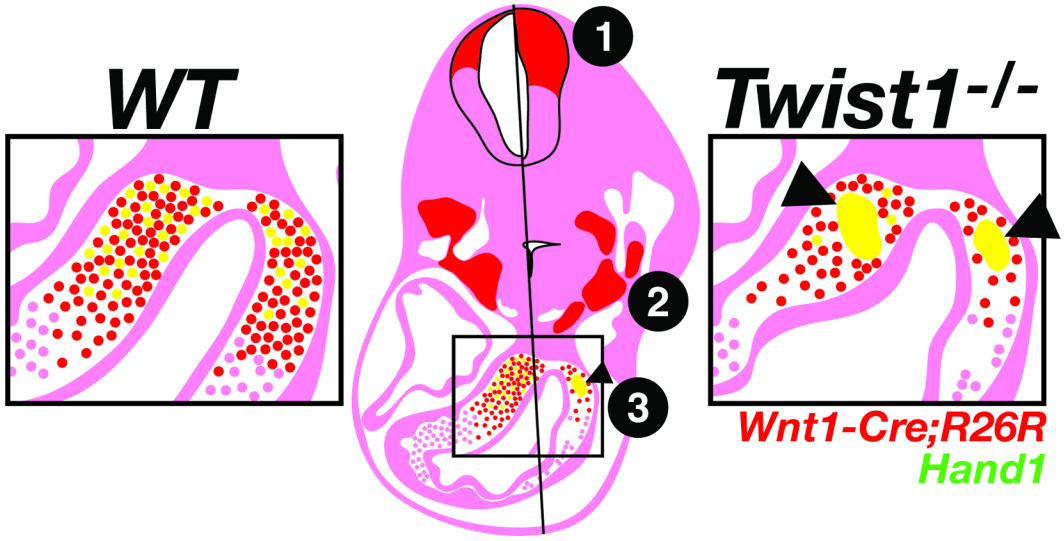

Figure 8. Schematic representation of the NCC phenotypes associated with a loss of *Twist1*

Phenotypes are depicted for the ***Twist1***$^{-/-}$ (left) compared to the wild-type (right) at E11.5. 1) Denotes expansion of the *Wnt1-Cre* lineage (red) in the dorsal neural tube. 2) Denotes abnormal condensation of the mesenchyme within the pharyngeal arches. 3) Denotes aggregation of the *Hand1*-expressing subpopulation of cNCCs (yellow) in the OFT cushions.