Cited2 is required both for heart morphogenesis and establishment of the left-right axis in mouse development

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Summary

Establishment of the left-right axis is a fundamental process of vertebrate embryogenesis. Failure to develop left-right asymmetry leads to incorrect positioning and morphogenesis of numerous internal organs, and is proposed to underlie the etiology of several common cardiac malformations. The transcriptional modulator Cited2 is essential for embryonic development: Cited2-null embryos die during gestation with profound developmental abnormalities, including cardiac malformations, exencephaly and adrenal agenesis. Cited2 is also required for normal establishment of the left-right axis; we demonstrate that abnormal heart looping and right atrial and pulmonary isomerism are consistent features of the left-right-patterning defect. We show by gene expression analysis that Cited2 acts upstream of Nodal, Lefty2 and Pitx2 in the lateral mesoderm, and of Left1 in the presumptive floor plate.

Although abnormal left-right patterning has a major impact on the cardiac phenotype in Cited2-null embryos, laterality defects are only observed in a proportion of these embryos. We have therefore used a combination of high-resolution imaging and three-dimensional (3D) modeling to systematically document the full spectrum of Cited2-associated cardiac defects. Previous studies have focused on the role of Cited2 in cardiac neural crest cell development, as Cited2 can bind the transcription factor Tfsp2, and thus affect the expression of Erbb3 in neural crest cells. However, we have identified Cited2-associated cardiac defects that cannot be explained by laterality or neural crest abnormalities. In particular, muscular ventricular septal defects and reduced cell density in the atrioventricular (AV) endocardial cushions are evident in Cited2-null embryos. As we found that Cited2 expression tightly correlated with these sites, we believe that Cited2 plays a direct role in development of the AV canal and cardiac septa. We therefore propose that, in addition to the previously described reduction of cardiac neural crest cells, two other distinct mechanisms contribute to the spectrum of complex cardiac defects in Cited2-null mice: disruption of normal left-right patterning and direct loss of Cited2 expression in cardiac tissues.

Key words: Cited2, Left-right axis, Heart development, Mouse

Introduction

Cardiac morphogenesis understandably relies on genes that function within the heart, but many genes extrinsic to the heart also affect its development. Genes required to establish the left-right axis of the embryo indirectly affect heart morphogenesis. They do this by ultimately affecting the expression of the Pitx2 gene (Burdine and Schier, 2000; Franco and Campione, 2003; Hamada et al., 2002). It is the Pitx2c isoform that is central to the establishment of laterality; it is expressed in left lateral plate mesoderm (LPM), and later expression in the left side of the heart is considered to extend from this (Campione et al., 2001). Pitx2c is a Nodal-responsive gene and thus its expression in the left LPM occurs as a direct consequence of Nodal signaling in the tissue. Nodal expression in the left LPM, establishes the left side of the embryo, and so genes required to initiate, maintain and restrict the expression of Nodal are all required for correct Pitx2c expression.

The Cited2 gene affects cardiac morphogenesis and was identified from studies of differential gene expression in the early embryo (Dunwoodie et al., 1998), cytokine function (Sun et al., 1998) and transcriptional regulation during hypoxia (Bhattacharya et al., 1999). Cited2 expression is widespread during early mouse and chicken development (Dunwoodie et al., 1998; Schlange et al., 2000). Furthermore, expression of Cited2 can be activated by factors, including low oxygen tension, cytokines, lipopolysacharide and shear stress (Bhattacharya et al., 1999; Sun et al., 1998; Yokota et al.,
Development cells and yet others concordant with a laterality defect, others with a reduction in neural crest display a spectrum of cardiovascular defects: some consistent within cardiac tissue to affect morphogenesis. There were, however, two defects that did not seem to relate to abnormalities in laterality or CNC cells: reduced cell density in the A V endocardial cushions and muscular ventricular septal defects (Bamforth et al., 2001; Martinez Barbera et al., 2002; Yin et al., 2002). We determined the developmental origins of the cardiac defects by examining cardiac morphogenesis in detail at a number of embryonic stages, and determined the expression pattern of Cited2 in relation to heart development.

Cited2-null embryos display numerous developmental defects (Bamforth et al., 2001; Martinez Barbera et al., 2002; Yin et al., 2002). Cardiac defects previously reported in Cited2-null embryos include: double outlet right ventricle (DORV); atrial septal defect (ASD); ventricular septal defect (VSD); overriding aorta; persistent truncus arteriosus (PTA); and pulmonary artery stenosis. Previously, these defects had only been considered in light of Tafap2 and Hif1α, through It inhibits the transcriptional activity of Hif1α, through intrinsic acetyltransferase and E4 ubiquitin ligase activity (Chen and La Thangue, 2001; Grossman et al., 2003). It inhibits the transcriptional activity of Hif1α, through competition for overlapping binding sites on CBP/p300 (De Guzman et al., 2004; Freedman et al., 2003). This may represent a paradigm for Cited2 function, as it also appears to displace Ets1 from CBP/p300 (Yokota et al., 2003). In addition, Cited2 interacts directly with the transcription factors Lhx2 and Tafap2 (Bamforth et al., 2001; Glenn and Maurer, 1999).

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We observed that the heart loops abnormally in a proportion of Cited2-null embryos, and that these embryos have right atrial isomerism (RAI). In these embryos, Nodal, Lefty2 and Pitx2c are not expressed in lateral mesoderm, and Lefty1 expression in the prospective floor plate (PFP) is abnormal. This shows that abnormal left-right patterning has a major impact on the cardiac phenotype in Cited2-null embryos. In addition, we performed a detailed analysis of the endpoint cardiac phenotypes using episcopic fluorescence image capturing (EFIC) and three-dimensional (3D) modeling. A number of abnormalities were identified in Cited2-null embryos that lacked a laterality defect, and some of these were consistent with a reduction in cardiac neural crest (CNC) cells. There were, however, two defects that did not seem to relate to abnormalities in laterality or CNC cells: reduced cell density in the AV endocardial cushions and muscular ventricular septal defects (VSD). These defects did, however, directly correlate with Cited2 expression and this suggests that Cited2 also plays a direct role in cardiac tissue.

In summary, we demonstrate that Cited2-null embryos display a spectrum of cardiovascular defects: some consistent with a laterality defect, others with a reduction in neural crest cells and yet others concordant with Cited2 acting directly within cardiac tissue to affect morphogenesis.

Materials and methods

Mouse lines and genotyping

The Cited2-lacZ mouse line (Barbera et al., 2002) is maintained on a C57BL/6;129 hybrid genetic background following three backcrosses onto C57BL/6. Mice heterozygous for this Cited2 targeted allele (Cited2+/−) were intercrossed to produce Cited2 wild-type (Cited2+/+), heterozygous (Cited2/+−) and homozygous null (Cited2−/−) embryos for analysis. Cited2 heterozygous mice were also crossed with mice homozygous for the MLC3F-nlacZ-2E transgene, which is maintained on a SJL.C57BL/6 hybrid genetic background (Kelly et al., 1995).

Morphology, histology, RNA in situ hybridization and X-gal staining

EFIC was used for high resolution morphological studies (Weninger and Mohun, 2002). Histology, RNA in situ hybridization and X-gal staining were performed as described (Kauffman, 1992; Hogan et al., 1994; Harrison et al., 1995). cDNA probe details are as follows: Cited2 (Dunwoodie et al., 1998); Nodal, Lefty1/2 and Pitx2c probes were a gift from Christine Biben and Richard Harvey.

Determination of cell density in endocardial cushions

Cell density of the AV cushions was measured in three Cited2 wild-type and three Cited2-null embryos at 10.5 dpc. Wax sections of each embryo were cut at a 45° angle (midway between frontal and transverse) and every section collected. The number of cells in the central section plus two before and two after the central section were counted. Cushion area was measured using Image J software v1.240 (NIH), and the results expressed as density of cells per mm² cushion. Cell density was compared using analysis of variance.

Scanning electron microscopy (SEM)

Following incubation in Karnovsky’s fixative (2% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M sodium phosphate buffer at pH 7.2), SEM was performed (Webb et al., 1998).

Results

Cited2 is expressed in the heart and elsewhere in the embryo

The cardiovascular defects previously identified in Cited2-null embryos have been ascribed to a reduction in CNC cells or impaired Hif1α activity (Bamforth et al., 2001; Martinez Barbera et al., 2002; Yin et al., 2002). Cited2 was expressed in a number of sites within the embryo that could impact on the development of the heart (Fig. 1). Cited2 at 7.5 dpc was expressed in the ventral node, predominantly in the caudal lip but also in the depression of the node (Fig. 1A-C) (Dunwoodie et al., 1998). Expression was readily detected in 8 dpc embryos in the cardiac crescent, anterior lateral mesoderm and in trunk paraxial mesoderm (Fig. 1C-F). At 9.25 dpc, expression was elevated in dorsal neuroectoderm at the forebrain-midbrain boundary, and in the hindbrain (Fig. 1G,H). Expression was also readily detected in branchial arches 1 to 3, and in somites. At this stage, in the heart, Cited2 expression was widespread: elevated levels of expression were identified in the aortic sac, in the myocardium adjacent to the endocardial cushions of the outflow tract and the AV canal, and in the presumptive atria and sinus venosus (Fig. 1G,I-K). At 10.5 dpc, elevated Cited2 expression was detected in branchial arch 4, the outflow tract and the AV canal (Fig. 1L-N). At 13.5 dpc Cited2 expression was largely restricted to the outflow and inflow regions of the heart, AV cushion, septum primum and the tip of the muscular ventricular septum (Fig. 1O-Q). With respect to expression in the heart, Cited2 is found predominantly in the myocardium; however, some endocardial cells, and cells within the endocardial cushion, also express the gene.

Abnormal heart looping and atrial isomerism are features of the Cited2 null phenotype

Cited2-null embryos die from 14.5 dpc (Table 1). Examination of Cited2-null hearts between 9.5 and 12.5 dpc, revealed that
Fig. 1. *Cited2* is differentially expressed in the heart and elsewhere during development. (A) Anterior view of a late headfold stage embryo (7.5 dpc) showing expression in the ventral node (arrow), cardiac crescent and blood islands. (B) Ventral view of a flatmounted four-somite stage embryo (8 dpc) showing expression in the cardiac crescent and in the caudal lip and pit of the node (arrow). (C) Ventral view of a flatmounted six-somite stage embryo (8 dpc) showing expression in the cardiac crescent, anterior lateral mesoderm (black arrow), paraxial mesoderm (white arrow) and node. (D) Anterior view of a six-somite stage embryo showing elevated expression in the cardiac crescent, anterior lateral mesoderm and trunk paraxial mesoderm. (E,F) Transverse sections through D at the levels indicated showing elevated expression in the cardiac mesoderm (arrow) and low-level expression throughout. (G) Lateral and dorsal view of 21-somite stage embryo (9.5 dpc) showing elevated expression in the forebrain-midbrain boundary, branchial arches 1 and 2, heart, and somites. (H) Dorsal view of the embryo in G showing expression in the hindbrain (rhombomeres 3, 5, 6, 7) and adjacent paraxial mesoderm. (I-K) Frontal sections through a 21-somite stage embryo at the levels indicated in G. (I) Expression is elevated in the ventricular myocardium and trabeculae of the outflow tract and presumptive right ventricle; (J) in the myocardium and, to a lesser extent, the endocardium and endocardial cushion cells in the outflow tract and atrioventricular canal; and (K) in the aortic sac, presumptive atria, sinus venosus (inflow tract). (L) Lateral view of 40-somite stage embryo (10.5 dpc) showing expression in the outflow tract, atrioventricular canal (black arrow), branchial arch 4 (white arrow), central nervous system, somites and limbs. (M,N) Sections through a 40-somite stage embryo at the levels indicated in L, showing: expression in the (M) outflow tract and, to a lesser extent, in the compact myocardium and trabeculae of the ventricles; and expression in the (N) myocardium adjacent to the endocardial cushion of the atrioventricular canal. (O) Ventral and (P) dorsal views of a heart dissected from an embryo at 13.5 dpc showing expression predominantly in the outflow tract (O) and inflow region (P). (Q) Frontal section through a dissected heart at the same stage as shown in O,P showing expression in the septum primum (black arrow), around the vena cava (asterisk) and in the endocardial cushions of the atrioventricular canal (white arrow). *Cited2* expression was assayed by detecting β-galactosidase activity from the *Cited2-lacZ* allele in embryos heterozygous (A,B,G,H,L,O,P) or homozygous (I,J,K,M,N,Q) for the allele, or via *Cited2* RNA hybridization (C-F). The colorimetric reaction used to identify *Cited2* transcripts was extended for the embryo in D, in order to reveal low levels of *Cited2* expression. cc, cardiac crescent; cc, cardiac crescent; oft, outflow tract; ift, inflow tract; v, ventricle; avc, atrioventricular canal; as, aortic sac. Scale bar: 135 μm in A,B; 260 μm in C,D,H; 35 μm in E,F; 280 μm in G; 50 μm in I-K; 550 μm L; 94 μm in M,N; 390 μm O,P; 310 μm Q.
Fig. 2. *Cited2*-null hearts display cardiac looping defects and atrial isomerism. (A-C) Scanning electron micrographs showing ventral views of hearts at 9.5 dpc (23 somites). The head, tail and the pericardium were removed. (A) *Cited2* wild-type heart showing a normal D (rightward) loop. (B) *Cited2*-null heart showing an abnormal D loop. The left ventricle is ventrally displaced and occupies a more central position than normal. (C) *Cited2*-null heart showing an abnormal L (leftward) loop. (D-F) Right atrial isomerism is observed in *Cited2*-null embryos with abnormal heart loops. Ventral views of hearts from 10.5 dpc show expression of the \(\text{MLC3F-nlacZ-2E}\) transgene. (D) A *Cited2* wild-type heart shows a normal D (rightward) loop and asymmetric \(\text{MLC3F-nlacZ-2E}\) transgene. (E) A *Cited2*-null heart shows a normal D loop and asymmetric \(\text{MLC3F-nlacZ-2E}\) transgene. (F) A *Cited2*-null heart shows an L (leftward) loop and strong bilateral expression of the \(\text{MLC3F-nlacZ-2E}\) transgene, indicating right atrial isomerism. Arrows indicate orientation of the outflow tract. A, atrium; R L, right-left axis. Scale bar: 100 \(\mu\)m.

looping was abnormal (Fig. 2). All *Cited2* wild-type \((n=167)\) and heterozygous \((n=203)\) embryos had normal rightward (D) looping hearts (Fig. 2A). By contrast, almost half \((52/115)\) of all *Cited2*-null embryos showed abnormalities in heart looping (Fig. 2B,C): 12 had abnormal D-looped hearts, while 40 had leftward (L) looped hearts. In most L-looped hearts, the ventricles were positioned on the left side of the midline of the embryo, while in abnormal D-looped hearts, both ventricles were on the right side of the midline. These looping defects indicated that specification of the left-right axis might not have occurred correctly in the *Cited2*-null embryos. To address this, we used the \(\text{MLC3F}\) enhancer transgene that marks the right atrium (Franco et al., 2001; Kelly et al., 1998). At 10.5 dpc, all *Cited2* wild-type \((n=69; \text{Fig. 2D})\) and heterozygous embryos \((n=61; \text{data not shown})\) showed characteristic transgene expression in the right atrium, as did *Cited2*-null embryos with correctly looped hearts \((n=13; \text{Fig. 2E})\). By contrast, RAI, detected by bilateral expression of the \(\text{MLC3F}\) enhancer transgene, was apparent in all *Cited2*-null embryos with an abnormally looped heart \((n=15)\) for abnormal L-loops and \(n=6\) for abnormal D-loops; Fig. 2F). These results demonstrate that the cardiac looping defects prevalent in *Cited2*-null embryos are concordant with RAI. In all *Cited2* null-embryos with RAI \((n=14)\), we found right pulmonary isomerism (RPI): four lung lobes on both sides, as opposed to the normal arrangement with one lung lobe on the left and four on the right. In contrast to the thoracic isomerism, there was incomplete penetration of abdominal isomerism: the stomach instead of lying normally on the left, was in the midline or on the right hand side \((n=8/14)\).

**Determinants of laterality are abnormally expressed in a proportion of Cited2-null embryos**

In order to examine the relationship of *Cited2* with other genes involved in specifying left-right identity, we examined *Nodal* expression. *Nodal* was asymmetrically expressed in the left LPM in *Cited2* wild-type \((n=6)\) and heterozygous \((n=12)\) embryos, and bilaterally expressed within the node (Fig. 3A,B). By contrast, two out of six *Cited2*-null embryos lacked *Nodal* expression in the left LPM (Fig. 3C). However, *Nodal* expression within the node was comparable with that in the controls. This suggests that *Cited2* is required for initiation or maintenance of *Nodal* expression in the LPM, but not within the node.

**Table 1. Genotype of mice resulting from Cited2 heterozygous intercross on a mixed C57BL6:129 genetic background**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Genotype</th>
<th>(C_{\text{Citex}})</th>
<th>(C_{\text{Cited2}})</th>
<th>(C_{\text{Cited2}})</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5 dpc</td>
<td>68</td>
<td>184</td>
<td>100</td>
<td>0.0379</td>
<td></td>
</tr>
<tr>
<td>9.5 dpc</td>
<td>36</td>
<td>80</td>
<td>29</td>
<td>0.3283</td>
<td></td>
</tr>
<tr>
<td>10.5 dpc</td>
<td>36</td>
<td>84</td>
<td>31</td>
<td>0.3255</td>
<td></td>
</tr>
<tr>
<td>11.5 dpc</td>
<td>30</td>
<td>76</td>
<td>28</td>
<td>0.2897</td>
<td></td>
</tr>
<tr>
<td>12.5 dpc</td>
<td>14</td>
<td>23</td>
<td>10</td>
<td>0.7039</td>
<td></td>
</tr>
<tr>
<td>13.5 dpc</td>
<td>13</td>
<td>22</td>
<td>10</td>
<td>0.8097</td>
<td></td>
</tr>
<tr>
<td>14.5 dpc</td>
<td>106</td>
<td>159</td>
<td>85 (+6 dead)</td>
<td>0.0657</td>
<td></td>
</tr>
<tr>
<td>15.5 dpc</td>
<td>14</td>
<td>19</td>
<td>8 (+5 dead)</td>
<td>0.3724</td>
<td></td>
</tr>
<tr>
<td>16.5 dpc</td>
<td>5</td>
<td>12</td>
<td>8 (+1 dead)</td>
<td>0.6839</td>
<td></td>
</tr>
<tr>
<td>17.5 dpc</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>0.6065</td>
<td></td>
</tr>
<tr>
<td>PD21</td>
<td>402</td>
<td>615</td>
<td>0</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Ratios of genotypes were tested for goodness of fit to expected Mendelian segregation (1:2:1) by \(\chi^2\) analysis, calculated with two degrees of freedom. \(P\) value calculated at 14.5, 15.5 and 16.5 dpc for living embryos only.

dpc, days post coitum; PD, postnatal day.

In order to examine the relationship of *Cited2* with other genes involved in specifying left-right identity, we examined *Nodal* expression. *Nodal* was asymmetrically expressed in the left LPM in *Cited2* wild-type \((n=6)\) and heterozygous \((n=12)\) embryos, and bilaterally expressed within the node (Fig. 3A,B). By contrast, two out of six *Cited2*-null embryos lacked *Nodal* expression in the left LPM (Fig. 3C). However, *Nodal* expression within the node was comparable with that in the controls. This suggests that *Cited2* is required for initiation or maintenance of *Nodal* expression in the LPM, but not within the node.

**Lefty1** and **Lefty2** interact with *Nodal* and its co-receptors (cripto and cryptic) to block *Nodal* signaling (Chen and Shen, 2004; Cheng et al., 2004; Hamada et al., 2002). *Lefty2* is expressed in the left LPM and is a direct target of *Nodal* (Meno et al., 1997; Meno et al., 1996; Saijoh et al., 1999; Saijoh et al., 2000). *Lefty1* is expressed in the left side of the PFP but, unlike *Nodal*, *Lefty2* and *Pitx2c*, its expression is independent of *Foxh1*. Despite this, the reliance of *Lefty1* expression on *Cryptic* suggests that *Nodal* and/or Gdf1 are important for *Lefty1* expression (Hamada et al., 2002; Yan et al., 1999). As loss of *Nodal* expression in the LPM is a feature of the *Cited2*-null phenotype, we would expect a proportion of *Cited2*-null embryos to lack *Lefty1* and *Lefty2* expression. *Lefty1* and *Lefty2* were expressed normally in *Cited2* wild-type \((n=13)\), heterozygous \((n=17)\) and some *Cited2*-null \((n=5)\) embryos (Fig. 3D,E,G,H). However, a proportion of *Cited2*-null embryos \((n=4)\) lacked *Lefty2*, and expression of *Lefty1* was limited to and maintained in the posterior PFP (Fig. 3F). Expression of *Shh* is normal in the midline of all *Cited2*-null embryos, precluding the possibility that the embryonic midline is compromised (data not shown). Persistent posterior
expression of Lefty1 in the PFP has not been previously reported in laterality mutants; this demonstrates that Cited2 affects the establishment of laterality differently to any known gene.

Pitx2c is initially expressed in the left LPM, and then at later stages expression persists on the left side of various LPM-derived tissues such as the sinus venosus, common atrial chamber, cardinal vein, vitelline vein, septum transversum and foregut (Yoshioka et al., 1998). In all Cited2 wild-type (n=6) and heterozygous (n=37) embryos examined, the normal pattern of Pitx2c expression was observed in the left LPM and later the left horn of the sinus venosus (Fig. 3J,K). However, in four out of 12 Cited2-null embryos, Pitx2c expression was entirely absent from this left lateral mesoderm (Fig. 3L). This indicates that Cited2 acts upstream of Pitx2c expression in lateral mesoderm and is consistent with the fact that Pitx2c is not expressed because of an absence of Nodal expression in the lateral mesoderm.

In summary, one-third of Cited2-null embryos showed loss of Nodal, Lefty2 and Pitx2c expression in left LPM, and loss of Lefty1 expression in the anterior PFP; this is consistent with the proportions of null embryos, which had RAI (Table 2).

Pitx2c expression is Nodal responsive, its expression relies on Foxh1-binding sites in the left side-specific enhancer (ASE), and expression in the heart requires its earlier expression in the lateral mesoderm (Shiratori et al., 2001). Despite the fact that Pitx2c expression in the heart has never been shown to be uncoupled from its expression in the lateral mesoderm, we examined Pitx2c expression in Cited2-null hearts at 13.5 and 14.5 dpc to explore this possibility. Pitx2c expression was readily detected in hearts dissected from Cited2 wild-type (n=4), heterozygous (n=6) and

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**Fig. 3. Cited2-null embryos have abnormal expression of left sided determinants.** RNA in situ hybridization of embryos and hearts hybridized with probes that recognize Nodal (A–C), both Lefty1 and Lefty2 (D–I), and Pitx2 (J–R) transcripts. Nodal is expressed in the left lateral mesoderm (arrow) and node of six- to seven-somite stage Cited2 wild-type (A) and heterozygous (B), but not in LPM of Cited2-null (C) embryos. (D–I) A combined Lefty1/2 probe shows that Lefty1 and Lefty2 are expressed in the left prospective floor plate of the neural tube (white arrow), and left LPM (black arrow) respectively, in seven- to nine-somite stage Cited2-null (D,G) and heterozygous (E,H) embryos. In Cited2-null embryos (E,I), Lefty2 expression is not detected, whereas the expression of Lefty1 is restricted to the posterior floor plate. (J–L) Pitx2 is expressed in left lateral mesoderm (arrow) of 12- to 14-somite stage Cited2 wild-type (J) and heterozygous (K), but not Cited2-null (L) embryos. (M–R) At 13.5 dpc, Pitx2 is expressed in the ventral myocardium of the right ventricle, throughout the left atrium and in ventrally viewed veins of Cited2 wild-type embryos (M,P) and in null embryos that lack a laterality defect (N,Q). Pitx2 is not expressed in the hearts of Cited2-null embryos that have a right isomeric phenotype (O,R). In some Cited2-null hearts, a novel domain of Pitx2 expression was observed in dorsal ventricular cells (Q; arrow). Ventral view (A–C,J–O), lateral view (D–F), posterior view (G–I), dorsal view (P–R). Scale bar: 235 μm in A–C; 175 μm in D–I; 365 μm in J–L; 335 μm in M–R.
null (n=6) embryos that lacked laterality defects (Fig. 3M,N,P,Q; data not shown). By contrast, expression could not be detected in the hearts of Cited2-null embryos with right isomerism (n=7; Fig. 3O,R). Intriguingly, some Cited2-null hearts displayed a novel expression domain on the dorsal ventricular surface irrespective of their laterality phenotype (n=4/13; Fig. 3Q; data not shown).

Cited2-null embryos develop a spectrum of severe cardiovascular defects

Some cardiovascular defects that occur in Cited2-null embryos have previously been reported (Bamforth et al., 2001; Yin et al., 2002). Yet, the full spectrum of defects, their incidence and association to one another has remained unexplored. As Cited2-null embryos die late in gestation (Table 1) we examined a total of 28 Cited2-null embryos on a mixed C57Bl6;129 genetic background between 14.5 and 15.5 dpc using high-resolution images obtained from EFIC and 3D models (Weninger and Mohun, 2002). This revealed that Cited2-null embryos showed a broad range of cardiac, vascular and other defects, and were classified into four groups based on the predominant cardiac defect (Table 2). The major defect of group 1 embryos is right isomerism, the other abnormalities being largely attributable to the patterning defects that isomerism would impart. The incidence of isomerism in 14.5-15.5 dpc hearts is consistent with our finding that ~40% of embryos examined between 8.5 and 12.5 dpc have a laterality defect. As embryos in groups 2 to 4 do not show isomerism, the spectrum of cardiovascular defects they exhibit are likely to result from other factors, such as the reduction of CNC cells, or the lack of Cited2 expression in cardiac tissues.

Cardiovascular defects associated with right isomerism

Nine out of 28 embryos (group 1; Table 2) demonstrated RPI and RAI as a component of a complex cardiac phenotype (compare Fig. 4A-C with 4D-F, Fig. 5A,C with 5B,D). Three-dimensional reconstruction of these Cited2-null hearts shows symmetrical arrangement of the atria, superior vena cavae and pulmonary veins (Fig. 6; Fig. 7A,B). A single common atrium is apparent with rudimentary ridges protruding into the lumen from the dorsal side. Both atrial appendages resemble the appearance of the normal right atrium (Figs 6-8). The superior vena cavae and inferior vena cava, along with multiple small mediastinal veins and a common pulmonary vein, drain into the central atrial area, which is bordered by the rudimentary ridges (Fig. 4B,G,H; Fig. 6C). In three embryos, an extra vessel (possibly a persisting right umbilical vein) enters the central atrial compartment from the caudal aspect (Fig. 6C').

In summary, nine out of 28 Cited2-null embryos examined have a right isomeric pulmonary and cardiac phenotype in combination with defects of the AV junction, ventricular septum, outflow tract and aortic arches. Each lesion (except for the muscular VSD) has previously been reported in association with aberrant left-right embryonic patterning (see Maclean and Dunwoodie, 2004; Schneider and Brueckner, 2000).

Cardiovascular defects in the absence of isomerism

Isomerism was not evident in the 15/28 Cited2-null embryos (Table 2). Group 2 embryos exhibited similar defects as those in group 1 but lack right isomerism. Group 3 defects were restricted to outflow tract malformations (DORV, TGA and overriding aorta) frequently in combination with muscular VSDs. Three Cited2-null embryos had multiple VSDs only (group 4), and a further four had a normal cardiac phenotype.

Endocardial cushions have reduced cell density in Cited2-null hearts

Cited2 is expressed in myocardium adjacent to endocardial cushions of the outflow tract and AV canal, and in some endocardial and cushion cells (Fig. 11J,N,Q). In addition, defects in endocardial cushions were detected at 14.5 dpc in Cited2-null embryos (Fig. 4G; Fig. 8A,B,D,D'). Therefore, we

| Table 2. Phenotypic classification of Cited2-null embryos |
|----------------|----------------|----------------|----------------|
| Group 1 (n=9) | Group 2 (n=4)  | Group 3 (n=8)  | Group 4 (n=3)  |
| Right pulmonary isomerism | 9 | 4, foramen primum |
| Right atrial isomerism | 9 | 4, common AV canal |
| ASD | 9, common atrium | 4, common AV canal, DILV |
| AV junction | 9, common AV canal | 4, membranous, muscular |
| VSD | 9, membranous, muscular | 6, membranous, muscular |
| Outflow tract | 9, DORV, TGA | 4, DORV, PTA |
| Pharyngeal arch arteries | 9, RERSA, IAA type B | 4, RERSA, IAA type B |

A total of 28 embryos were analysed by EFIC and scored for phenotype of the cardiovascular system and lungs. Four embryos showed normal cardiovascular and pulmonary structure and the remaining 24 were classified into four groups by severity of phenotype: ASD, atrial septal defect; AV, atrioventricular; VSD, ventricular septal defects; DILV, double inlet left ventricle; DORV, double outlet left ventricle; TGA, transposition of the great arteries; PTA, persistent truncus arteriosus; OA, overriding aorta; RERSA, retro-esophageal right subclavian artery; IAA, interrupted aortic arch.
examined formation of endocardial cushions of the AV canal at 10.5 dpc (Fig. 9). The average cell density was significantly reduced ($P<0.0001$) in Cited2-null hearts (1.83 $\times$ 10$^{-3}$ cells/µm$^2$±0.18 $\times$ 10$^{-3}$) compared with that in wild-type hearts (2.68 $\times$ 10$^{-3}$ cells/µm$^2$±0.15 $\times$ 10$^{-3}$) (compare Fig. 9E with 9F-H). Furthermore, the area of the endocardial cushions appeared to be reduced in the Cited2-null hearts, this is despite the fact that the overall sizes of the hearts compared were comparable.

Discussion

Here, we present a description of the full spectrum of cardiac defects that occur when the Cited2 gene is absent during development. This analysis has revealed that right isomerism is a feature of the Cited2-null phenotype, and thus has lead to the finding that Cited2 is required for correct specification of the left-right axis. In addition, the previously undefined expression profile of Cited2, in the developing heart and in tissues relevant to heart development, has been revealed. Collectively, these data demonstrate that the spectrum of cardiac defects is consistent with Cited2 functioning at a number of levels to impact on heart development; these include a role for Cited2 in establishment of the left-right axis, both directly in cardiac tissue and, as previously suggested, in cardiac neural crest cells.

A range of cardiac defects is observed in Cited2-null embryos. This variability in expressivity/penetrance of the phenotype is probably due to the effect(s) that polymorphism of a modifier(s) has on Cited2 function. For example, the incidence of right isomerism occurs in about 33% of Cited2-null hearts (1.83 $\times$ 10$^{-3}$ cells/µm$^2$±0.18 $\times$ 10$^{-3}$) compared with that in wild-type hearts (2.68 $\times$ 10$^{-3}$ cells/µm$^2$±0.15 $\times$ 10$^{-3}$) (compare Fig. 9E with 9F-H). Furthermore, the area of the endocardial cushions appeared to be reduced in the Cited2-null hearts, this is despite the fact that the overall sizes of the hearts compared were comparable.

Cited2 and the establishment of laterality

Establishment of left-right patterning can be divided into three phases: an initial break in embryonic symmetry through cilia-dependent processes originating in the node; establishment of asymmetric gene expression in lateral mesoderm; and transfer of positional information to developing organs. The Nodal signaling pathway, which is central to this process in the lateral mesoderm, is an important predictor of the phenotype (Collignon et al., 1996; Lowe et al., 1996). For example, left-sided expression results in situs solitus, right-sided expression in situs inversus, bilateral expression in left isomerism and absent expression in right isomerism.

The laterality defect in Cited2-null embryos is characterized by right atrial and RPI (100% concordant), and 50% of these individuals have altered abdominal situs. In addition, right atrial isomerism always occurs where the heart is abnormally looped. As Cited2 is expressed in and around the ventral node, it is possible that Cited2 is required for cilia-dependent processes or for the relay of the asymmetric signal to the lateral mesoderm. Mutation in Dnah5, Dnah11/Lrd, Hfh4/Foxj1 results in immotile cilia of the node, random expression of Nodal and the complete spectrum of laterality phenotypes: situs solitus, situs inversus, left and right isomerism (Brody et al., 2000; Chen et al., 1998; Collignon et al., 1996; Lowe et al., 1996; Meno et al., 1996; Oh and Li, 2002; Olbrich et al., 2000).
Moreover, mutations in genes that abrogate all nodal cilia (Kif3a, Kif3b, polaris, Wim) or just the sensory cilia (polycistin 2) result in absent, or bilateral Nodal expression that would result in situs inversus and left isomerism (Huangfu et al., 2003; Marszalek et al., 1999; Morgan et al., 1998; Moyer et al., 1994; Murcia et al., 2000; Nonaka et al., 1998; Pennekamp et al., 2002; Takeda et al., 1999; Wu et al., 2000). As Cited2-null embryos exhibit only situs solitus or right isomerism, it is unlikely that this gene is required only for cilia-dependent processes of the node and the breaking of embryonic symmetry.

Consistent with Nodal acting as a left-sided determinant, we have shown that complete absence of Nodal expression in lateral mesoderm is predictive of the right isomeric phenotype in Cited2-null embryos. This lack of Nodal suggests that Cited2 is required for either the initiation or maintenance of Nodal expression, and thus requires Cited2 to be expressed in lateral mesoderm. Appropriately, we have demonstrated low-level bilateral expression of Cited2 in mouse LPM and we suggest that it acts in this tissue to initiate or maintain Nodal expression.
It is not clear how loss of Cited2 might affect Nodal expression. Cited2 binds CBP/p300, Tfap2 and Lhx2/3, and through its interaction with CBP/p300 affects the transcriptional activity of Hif1 and Ets1 (Bamforth et al., 2001; Braganca et al., 2002; De Guzman et al., 2004; Freedman et al., 2003; Glenn and Maurer, 1999; Yahata et al., 2000; Yokota et al., 2003). Investigation of the known Cited2-binding proteins sheds no light on its function, as mutations in these genes are not associated with laterality defects (Barton et al., 1998; Goodman and Smolik, 2000; Iyer et al., 1998; Ryan et al., 1998; Schorle et al., 1996; Shikama et al., 2003; Zhang et al., 1996).

The expression of Lefty1 and Lefty2 is abnormal in a proportion of Cited2-null embryos. Consistent with the loss of Nodal expression in the left LPM, Lefty2 expression was not detected. In addition, Lefty1 expression was largely not detected in the left PFP as the loss of Nodal expression in the left LPM might predict. Intriguingly, however, Lefty1 expression was readily detected in the posterior PFP. Regulatory elements directing anterior and posterior expression of Lefty1 and Lefty2 have been identified (Saijoh et al., 1999), and so it is possible that Cited2, directly or indirectly, acts through an enhancer that controls the anterior, but not posterior, expression of Lefty1.

A proportion of Cited2-null embryos do not express Pitx2c in left lateral mesoderm, or in right isomeric hearts at later
stages of development. This may simply reflect the fact that Pitx2c expression requires Nodal signaling, and that Nodal is not expressed in Cited2-null embryos with laterality defects. This assumes that Pitx2c expression in the heart requires prior expression of Pitx2c in the lateral mesoderm. No evidence contradictory to this exists, and the fact that expression of Pitx2c in the anterior lateral mesoderm at 8.5 dpc is consistent with the sites of Pitx2c expression in the heart at later stages (13.5-14.5 dpc) supports this hypothesis (Campione et al., 2001). In addition, there are no cases in which Pitx2c expression is observed in the heart in the absence of prior expression in the anterior lateral mesoderm.

It is possible that Pitx2c expression in the heart has little to do with prior gene activation by Nodal, and that Cited2 might have a more direct role in its expression. This hypothesis is supported by the overlapping expression patterns of Cited2 and Pitx2c; both are expressed in the primary atrial septum, pulmonary veins and superior vena cava. In addition, they are expressed in the atrial myocardium, AV canals and outflow tract, although Pitx2c is only expressed on the left side of these structures (Campione et al., 2001; Franco and Campione, 2003; Franco et al., 2000). Furthermore, embryos null for either Cited2 or Pitx2c develop similar cardiac defects (Franco and Campione, 2003; Liu et al., 2001).

A very recent report proposes that Cited2 acts directly upstream of Pitx2c, and that the severe and complex heart defects apparent in Cited2-null embryos, that lack a laterality defect, occur because Cited2 is required to directly activate Pitx2c expression in the heart (Bamforth et al., 2004). This hypothesis seems unlikely for the following reasons. First, Bamforth et al. (Bamforth et al., 2004) show that the activity of the Pitx2c P1 promoter can be increased by about 1.5-fold following co-transfection of Cited2 and the A and C isoforms of transcription factor Tfap2 (in the hepatoma cell line Hep3B cells). However, the degree of transactivation is slight in comparison with a 25-fold increase in activation of the same promoter fragment through the Nodal-responsive Fast1/Foxh1 binding sites (Shiratori et al., 2001). This suggests that if Cited2-Tfap2a/c activates Pitx2c expression in the heart, it does so at relatively low levels and thus is not a major determinant of Pitx2c expression.

In addition, if the Cited2-Tfap2a/c complex activates Pitx2c in the heart, one would expect in Cited2-null embryos where laterality is normally established (and Pitx2c is expressed in left lateral mesoderm) that Pitx2c expression would be greatly reduced or absent in the heart. Evidence supporting this was not provided by Bamforth et al. (Bamforth et al., 2004); three Cited2-null hearts of a mixed genetic background (C57BL6/J;129Sv) were examined for Pitx2c expression, one showed reduced expression; however, the laterality status of these hearts was not determined. By contrast, we find that Pitx2c expression in the heart is coupled with its expression in lateral mesoderm: Cited2-null embryos with right isomerism lack Pitx2c expression in the left lateral mesoderm and in the heart; those without a laterality defect express Pitx2c in both left lateral mesoderm and the heart. Our data thus argues against Cited2 playing a direct and major role in the expression of Pitx2c in the heart, and instead supports the hypothesis that loss of Pitx2c in the heart occurs only when this gene is not expressed in the left lateral mesoderm at an earlier developmental stage. Importantly, the Cited2-null embryos, which express Pitx2c in the heart, still develop a complex array of cardiac defects, indicating that Cited2 expression is required in the heart for normal development and that it has Pitx2c-independent functions.

**Cited2, heterotaxia and congenital heart disease**

Our studies of the Cited2-null phenotype in the mouse demonstrate that mutation of a single gene can result in a remarkably wide spectrum of cardiac malformations. To date, no mutations have been identified in CITED2 in human disorders; however, the right isomeric phenotype of Cited2-null embryos resembles that seen in some individuals with heterotaxia and, in particular, asplenia (Ivemark) syndrome (OMIM 208530) (Ivemark, 1955). In humans, heterotaxia is a clinically and genetically heterogeneous disorder that is associated with a broad range of cardiac malformations. It occurs in approximately one in 10,000 live born infants or 1%
of children with congenital heart disease, and common cardiac manifestations are complex, including TGA, DORV or AV septal defects (Hutchins et al., 1983; Rose et al., 1975; Rutenberg et al., 1964; Van Mierop and Wiglesworth, 1962). Thus, the Cited2-null embryo provides a useful model with which to investigating the etiology of heterotaxia in humans. Furthermore, as the Cited2 mutation results in a spectrum of phenotypes that vary widely in their severity, it raises the possibility that many less severe congenital heart defects share a common cause with each other and with heterotaxia.

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