Epiblastic Cited2 deficiency results in cardiac phenotypic heterogeneity and provides a mechanism for haploinsufficiency

Simon T. MacDonald†, Simon D. Bamforth†, Chiann-Mun Chen, Cassandra R. Farthing, Angela Franklyn, Carol Broadbent, Jürgen E. Schneider, Yumiko Saga, Mark Lewandoski, and Shoumo Bhattacharya*

1Department of Cardiovascular Medicine, University of Oxford, Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Headington, Oxford OX3 7BN, UK; 2Division of Mammalian Development, National Institute of Genetics, Yata 1111, Mishima 411-8540, Japan; and 3Genetics of Vertebrate Development Section, National Cancer Institute, Frederick Cancer Research and Development Center, Box B, Building 539, Frederick, MD 21702, USA

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Cited2; Cardiac development; Phenotypic heterogeneity; Haploinsufficiency; Left–right patterning; Pitx2c

Aims Deletion of the transcription factor Cited2 causes penetrant and phenotypically heterogeneous cardiovascular and laterality defects and adrenal agenesis. Heterozygous human CITED2 mutation is associated with congenital heart disease, suggesting haploinsufficiency. Cited2 functions partly via a Nodal→Pitx2c pathway controlling left–right patterning. In this present study we investigated the primary site of Cited2 function and mechanisms of haploinsufficiency.

Methods and results A Cited2 conditional allele enabled its deletion in particular cell lineages in mouse development. A lacZ reporter cassette allowed indication of deletion. Congenic Cited2 heterozygous mice were used to investigate haploinsufficiency. Embryos were examined by magnetic resonance imaging, by sectioning and by quantitative real-time polymerase chain reaction (qRT-PCR). Epiblast-specific deletion of Cited2 using Sox2Cre recapitulated penetrant and phenotypically heterogeneous cardiovascular and laterality defects. Neural crest-specific deletion using Wnt1Cre affected cranial ganglia but not cardiac development. Mesodermal deletion with Mesp1Cre resulted in low penetrance of septal defect. Mesodermal deletion with T-Cre resulted in adrenal agenesis, but infrequent cardiac septal and laterality defects. β-Galactosidase staining and qRT-PCR demonstrated the efficiency and location of Cited2 deletion. Murine Cited2 heterozygosity is itself associated with cardiac malformation, with three of 45 embryos showing ventricular septal defect. Cited2 gene expression in E13.5 hearts was reduced 2.13-fold in Cited2+/− compared with wild-type (P = 2.62 × 10−4). The Cited2 target gene Pitx2c was reduced 1.5-fold in Cited2+/− (P = 0.038) hearts compared with wild-type, and reduced 4.9-fold in Cited2−/− hearts (P = 0.00031). Pitx2c levels were reduced two-fold (P = 0.009) in Cited2+/− embryos, in comparison with wild-type. Cited2 and Pitx2c expression were strongly correlated in wild-type and Cited2+/− hearts (Pearson rank correlation = 0.68, P = 0.0009). Cited2 expression was reduced 7474-fold in Sox2Cre deleted hearts compared with controls (P = 0.00017) and Pitx2c was reduced 3.1-fold (P = 0.013). Deletion of Cited2 with Mesp1Cre resulted in a 130-fold reduction in cardiac Cited2 expression compared with control (P = 0.0002), but Pitx2c expression was not affected.

Conclusion These results indicate that phenotypically heterogeneous and penetrant cardiac malformations in Cited2 deficiency arise from a primary requirement in epiblast derivatives for left–right patterning, with a secondary cell-autonomous role in the mesoderm. Cardiac malformation associated with Cited2 haploinsufficiency may occur by reducing expression of key Cited2 targets such as Pitx2c.

*Corresponding author. Tel: +44 1865 287771; fax: +44 1865 287742.
E-mail address: shoumo.bhattacharya@well.ox.ac.uk
† These authors contributed equally to this work.

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1. Introduction

The transcription factor CITED2 binds the histone acetyltransferase CREBBP/EP300 with high affinity, acting as a co-activator for transcription factors, such as TFAP2, LHX2, PPARA, and SMAD2/3. It also acts as a repressor of hypoxia-activated transcription. Genetic evidence indicates that Cited2 is essential for cardiac, adrenal, neural, and placental development, and also for embryonic left–right patterning. Cardiovascular malformations in mice lacking Cited2 are highly penetrant and phenotypically heterogeneous, and include atrial, atrioventricular, and ventricular septal defects (ASD, AVSD, VSD), outflow tract defects (double outlet right ventricle (DORV)), common arterial trunk (CAT), tetralogy of Fallot (TOF), transposition of great arteries (TGA), and interrupted and right-sided aortic arch. Mutation in Cited2 also results in adrenal agenesis, fusion of cranial ganglia, abnormal cardiac neural crest migration, and exencephaly.

In addition, Cited2−/− mice show left–right patterning defects characterized by right atrial and pulmonary isomerism, and abnormal ventricular topology at a lower penetration. Cardiovascular left–right patterning is created, in part, by the left-determining Nodal→Pitx2c pathway (reviewed by Hamada et al. and Ramsdell). Genetic evidence shows that Cited2 is necessary for expression of Pitx2c, Nodal, and Lefty2 in the left-lateral plate mesoderm and of Lefty1 in the prospective floor plate, and is present at the promoter of the endogenous Pitx2c gene during development. Thus, Cited2 is necessary for Nodal-activated gene transcription. The mechanism for the cardiovascular phenotypic heterogeneity and the partial penetrance of left–right patterning defect, which is observed even on a congenic Cited2-deficient background, is not clear. Importantly, loss-of-function mutations in CITED2 have been found in patients with phenotypically variable congenital heart disease. These mutations are heterozygous, suggesting haploinsufficiency, but the mechanism for this is unclear.

Cited2 is expressed before gastrulation in the visceral endoderm. Following gastrulation it is present in the newly forming mesoderm, developing blood islands, cardiac crescent, presomitic and splanchnic mesoderm, cranial neuroectoderm, and migrating neural crest before becoming ubiquitously expressed by embryonic day (E)8.5. In the heart, it is highly expressed in the AV endocardial cushions. One possible mechanism for cardiac phenotypic heterogeneity in Cited2 deficiency is that not only is it required for the earlier process of left–right patterning but also independently for the later processes of atrioventricular septation, outflow tract, and aortic arch development. Abnormal patterning of aortic arches and CAT observed in Cited2 deficiency could, for instance, arise from defective neural crest development. An alternative possibility is that an abnormality in the earlier developmental process of left–right patterning may secondarily affect the subsequent processes of atrioventricular septation, outflow tract, or aortic arch development. These different mechanisms were investigated with a conditional knockout approach. We also investigated the mechanism of haploinsufficiency using quantitative analysis of Cited2 and its target gene Pitx2c. We show that the phenotypic heterogeneity in Cited2 deficiency is because of a primary requirement in epiblast derivatives with a secondary cell-autonomous role in the mesoderm. Also, Cited2 haploinsufficiency may cause cardiac malformation by reducing expression of key Cited2 targets such as Pitx2c.

2. Methods

2.1 Molecular biology

Standard molecular biology procedures were used for plasmid constructions.

2.2 Generation of Cited2flox/flox mice

The targeting vector strategy and generation of correctly targeted embryonic stem cells with a conditional Cited2 allele (Cited2flox or Cited2flox) is shown in Supplementary material online. Methods are also provided in detail in the Supplementary data. The allele was designed such that, after successful Cre recombination, a loxP expression cassette came under the control of the endogenous Cited2 promoter. This allowed indication of efficient recombination by β-galactosidase staining.

2.3 Conditional deletion of Cited2

Cited2−/− mice (Cited2tm1Bha on a C57BL/6J background) were crossed with Sox2Cre (Tg(Sox2-cre)1Amc), Wnt1Cre (Tg(Wnt1-cre)1Rth) (both gifts from Andrew McMahon, Harvard University), Mesp1Cre (Mesp1tm2(cre)Ysa) or TcCre mice to generate males with Cited2+/−;Cre genotypes. These males were crossed to Cited2flox/flox females on a mixed 129Sv × C57BL/6J background to generate embryos with Cited2−/−;flox;Cre and Cited2−/−;flox;Cre (control) genotypes. This strategy prevented global recombination owing to Cre expression in the maternal germ-line. Cited2−/−;flox;Cre embryos lack Cited2 expression in cells expressing Cre-recombinase. Cited2−/−;flox;Cre embryos continue to express Cited2, providing littermate controls. Embryos were harvested at the indicated time points after the detection of a vaginal plug (E0.5), and genotyped using allele-specific polymerase chain reaction (PCR) (primer details are available on request). All mouse experiments conform with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.4 Analysis of embryos

Magnetic resonance imaging (MRI) was performed on a horizontal 9.4 T/21 cm VNMRS Direct DriveTM MR system (Varian Inc., Palo Alto, CA, USA). Magnetic resonance imaging (MRI) was performed on a horizontal 9.4 T/21 cm VNMRS Direct DriveTM MR system (Varian Inc., Palo Alto, CA, USA). Whole embryo sections were cut on a vibratome (Campbell and Day). Whole mount X-Gal and nuclear fast red (Sigma, St. Louis, MO, USA) staining was performed according to standard procedures.

2.5 Quantitative real time-polymerase chain reaction

Embryo hearts (E13.5) were dissected out initially in DEPC treated phosphate-buffered saline (PBS), and then finely dissected in RNA later (Ambion, Austin, TX, USA) to remove mediastinal tissue. Embryos at E8.5 were dissected free from the yolk sac in cold DEPC–PBS and flash-frozen in liquid nitrogen. RNA was isolated using a Nucleospin RNA purification kit (Clontech, Palo Alto, CA, USA), and an Archive cDNA kit (Ambion) used to synthesize complementary DNA. Quantitative RT-PCR (qRT-PCR) reactions were carried out using a Bio-Rad i-Cycler (Bio-Rad, CA, USA) and pre-optimized TaqMan® primer-probe sets from Applied Biosystems (Mus musculus assays Mm00516121_m1 (for NM_010828 Cited2), Mm00516121_m1 (for NM_010828 Cited2).
Mm00440826_m1 (for NM_001042502 Pltxz2c), and eukaryotic 18S rRNA. Expression levels were normalized to 18S rRNA using the R0 method of analysis.\textsuperscript{22, 23} Multiplex reactions were performed with 18S rRNA and the TaqMan primer-probe set for the gene of interest. Data were normalized to the level obtained for 18S RNA in each case. All reactions were performed in triplicates and, as a quality control, only samples for which the threshold cycle (Ct) replicate values were within 1 Ct value of each other were used for subsequent analysis.\textsuperscript{24} Mean values for each reaction triplicate were analysed.

2.6 Statistical analyses

We used the chi-squared test for deviation from expected Mendelian ratios (http://www.kursus.kvl.dk/shares/vetgen/_Popgen/genetik/applets/ki.htm), and calculated probability of a type I error using the CHIDIST function in Microsoft Excel (Redmond, WA, USA). For analysis of the qRT-PCR data, we used a two-tailed, two-sample heteroscedastic $t$-test assuming unequal variance.

3. Results

3.1 Deletion of \textit{Cited2} in the epiblast

To determine the role of \textit{Cited2} in the epiblast it was deleted using Sox2Cre which induces recombination in all epiblast cells by E6.5.\textsuperscript{15} Genotyping of mice at weaning showed a complete absence of \textit{Cited2}^{-/-};Sox2Cre embryos, but genotyping at E15.5 showed that these embryos were present in the expected Mendelian ratio (Supplementary material online, Table S1). This indicated that \textit{Cited2}^{-/-};Sox2Cre embryos die either in late gestation or perinatally. Two of 10 \textit{Cited2}^{-/-};Sox2Cre embryos at E15.5 had visible exencephaly (not shown). Analysis of \textit{Cited2}^{-/-};Sox2Cre embryos at E15.5 using MRI showed that they frequently had a classical cardiac laterality defect, i.e. right-atrial isomerism, associated with right-pulmonary isomerism (six of 10 embryos) (Figure 1; see also Supplementary material online, Table S2), indicating a defect in embryonic left–right patterning. VSDs were present in all embryos (see Supplementary material online, Table S2). The embryos invariably had adrenal agenesis (see Supplementary material online, Figure S2). No defects were seen in the 11 littermate control \textit{Cited2}^{+/+};Sox2Cre or in eight \textit{Cited2}^{-/-} embryos.

3.2 Deletion of \textit{Cited2} in the neural crest

To determine the requirement for \textit{Cited2} in the neural crest it was deleted using Wnt1Cre, which drives expression of Cre-recombinase in all neural crest-derived tissues, including the cardiac neural crest, from E9.\textsuperscript{16, 25, 26} There was a significant reduction in the numbers of \textit{Cited2}^{-/-};Wnt1Cre mice in comparison with \textit{Cited2}^{+/+};Wnt1Cre mice at weaning (21 of 61 vs. 40 of 61, respectively ($\chi^2 = 5.9; P < 0.015$). There was no observed reduction when genotyping was performed between E9.5 and E18.5. Examination of embryos at E15.5–E18.5 (n = 16) using MRI revealed none with heart or great vessel defects, but one embryo had exencephaly (Figure 2B). Exencephaly was not observed in \textit{Cited2}^{+/+};Wnt1Cre embryos (n = 16). Examination of cranial ganglia by anti-neurofilament staining in \textit{Cited2}^{-/-};Wnt1Cre embryos showed that 11 of 14 sets had fusions of ganglia IX and X (Figure 2E) and (F) and hypomorphic ganglia V and VII/VIII. In contrast, two of 28 sets of ganglia from \textit{Cited2}^{+/+};Wnt1Cre embryos had fusions of ganglia IX and X ($\chi^2 = 22.28; P < 0.001$). In both \textit{Cited2}^{-/-};Wnt1Cre and in \textit{Cited2}^{+/+};Wnt1Cre embryos, we observed extensive \textit{lacZ} staining in the cranial mesenchyme, and in the first and second pharyngeal arches.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Deletion of \textit{Cited2} in the epiblast: cardiac morphology. Magnetic resonance imaging of E15.5 embryos. (A–D) Transverse sections and three-dimensional (3D) reconstructions (dorsal views) of \textit{Cited2}^{-/-};Sox2Cre (control) and \textit{Cited2}^{+/+};Sox2Cre hearts. (A, B) Control normal heart showing a pectinated (P) right atrium (RA), with a systemic venous sinus (SVS), into which drains the right superior caval vein (RSCV), the left superior caval vein (LSCV) via the coronary sinus (CS) and the inferior caval vein (ICV). The left atrium (LA) is characterized by the primary atrial septum (PAS). Other structures seen are right and left ventricles (RV, LV), interventricular septum (IVS), and the mitral and tricuspid valves (MV, TV not labelled). (C, D) \textit{Cited2}^{-/-};Sox2Cre heart showing a large primum atrial septal defect (ASDP), resulting in a common atrium (A). This is pectinated on each side and has bilateral systemic venous sinuses (LSVS, RSVS), into which drains the bilateral superior and inferior caval veins. The coronary sinus is absent. These appearances indicate right atrial isomerism. A common atrioventricular valve (CAV) over-rides a ventricular septal defect (VSD). Note also that the heart is malpositioned to the right. (E–J) Coronal sections and 3D reconstructions (ventral views) of \textit{Cited2}^{-/-};Sox2Cre (control) and \textit{Cited2}^{+/+};Sox2Cre hearts. (E, F) Control normal heart where the right ventricle is dextral to the left, and gives rise to the main pulmonary trunk (PT). The left ventricle gives rise to the aorta (Ao), which arises to the left of the trachea (Tr) over the left main bronchus. (G, H) \textit{Cited2}^{-/-};Sox2Cre heart with normal ventricular topology, cardiac dextroposition, and a right-sided aortic arch arising from the right ventricle. (I, J) \textit{Cited2}^{-/-};Sox2Cre heart with abnormal ventricular topology: the right ventricle is sinusstral (and anterior) to the left ventricle. Scale bars = 500 \textmu m; axes: D, dorsal; V, ventral; R, right; L, left; A, anterior; P, posterior.}
\end{figure}
3.3 Deletion of Cited2 in the mesoderm

The role of Cited2 in the cranial, cardiac, and extraembryonic mesoderm was examined by deletion using crosses with Mesp1Cre mice, which deletes in these tissues by E7.5.17,27 As this Cre allele is a ‘knockin’ and disrupts the endogenous gene, we analysed genotypic ratios of offspring born by crossing these lines with Cited2+/− mice to determine if there were a genetic interaction. The number of Cited2+/−; Mesp1Cre+/-/KI mice at weaning was similar to Cited2+/−, Mesp1Cre+/-/KI mice indicating no significant genetic interaction (Supplementary material online, Table S3). Isolated septal defects (but no outflow or aortic arch defects) were seen in three of 18 Cited2−/−;Mesp1Cre embryos and no defects were seen in the seven Cited2+/−;Mesp1Cre or nine Cited2−/−;floxed littermate control embryos studied (Supplementary material online, Figure S3). The deletion of Cited2 using a TCre driver was then investigated. This would be expected to delete extensively in the lateral-plate, intermediate, and paraxial mesoderm and the hindgut endoderm, with earliest deletion being detected by E7.518. Deletion of Cited2 using TCre resulted in frequent adrenal agenesis (10 of 14 embryos), and infrequent cardiac septal and left–right patterning defect (three of 14 and one of 14, respectively, Figure S3). No defects were identified in the 11 Cited2−/−;TCre littermate control embryos studied.

3.4 Analysis of Cited2 recombination

3.4.1 LacZ staining

The above results indicate that Cited2 deficiency induced by Wnt1Cre, Mesp1Cre, or TCre drivers cannot explain either the phenotypic heterogeneity or penetrance of cardiac malformation and left–right patterning defects observed in the epiblast-specific deletion of Cited2. To explore the mechanism and evaluate the efficiency of recombination we examined the expression of lacZ in these different Cited2+/−;Mesp1Cre;E9.5 embryos in comparison with Cited2+/−;Mesp1Cre;E9.5 (Figure 4 and Supplementary material online, Table S4). Deletion of exon 2 by Cre-recombinase brings the lacZ cassette under control of the endogenous Cited2 promoter, so analysis of lacZ expression allowed the confirmation of Cited2 recombination in the embryonic lineages where both the Cre driver and Cited2 are expressed. In the heart, we observed staining resembling the Sox2Cre pattern using Mesp1Cre, consistent with effective recombination in the cardiogenic mesoderm.17,27 Reduced lacZ staining was observed in the outer curvature of the left ventricle suggesting that Cited2 is not highly expressed here (Figure 4). With TCre we observed complete recombination in the common atrial chamber, partial recombination in the atrioretricular canal, primitive ventricle, bulbus cordis, and outflow tract. The lateral plate mesoderm is thought to play a key role in establishing left–right patterning (reviewed in Hamada et al.10). We therefore examined the expression of lacZ in structures derived from the lateral plate mesoderm: these include the mesenchyme of the body wall (somatopleure) and of the viscera (splanchnopleure).18 In Cited2+/−;Sox2Cre embryos, strong lacZ expression was observed in the splanchnopleure, and weaker expression in the somatopleure. With TCre and Mesp1Cre, lacZ expression was detected chiefly in the posterior splanchnopleure surrounding the midgut. No lacZ staining was detected in the somatopleure.
We also examined lacZ expression at earlier developmental stages (Supplementary material online, Figure S4). At earlier stages, in Cited2\^+/\text{flox}\text{; Sox2Cre} embryos at E7.5, lacZ expression was present in all epiblast-derived tissues, including the extraembryonic mesoderm. In Cited2\^+/\text{flox}\text{; TCre} embryos, we observed lacZ staining in the extraembryonic mesoderm, amnion, and blood islands, and also to some extent in the nascent embryonic mesoderm. This became more extensive in the primitive streak and migrating embryonic mesoderm at slightly later stages. Examination of head-fold stage Cited2\^+/\text{flox}\text{; Mesp1Cre} embryos showed extensive recombination in the cardiac crescent (Supplementary material online, Figure S4).

### 3.4.2 Quantitative real-time polymerase chain reaction

To verify efficient deletion of Cited2 in the Sox2Cre and Mesp1Cre conditional knockouts at the mRNA level, and to determine the effect on the Cited2 target gene Pitx2c, qRT-PCR was performed. Cited2 expression was reduced 7474-fold \((P = 0.00017)\) in Cited2\^+/\text{flox}\text{; Sox2Cre} hearts compared with littermate controls (Figure 5A). There was also a 3.1-fold reduction in Pitx2c expression \((P = 0.013; \text{Figure 5B})\). Cited2 expression was reduced 130-fold in Cited2\^+/\text{flox}\text{; Mesp1Cre} hearts compared with littermate controls (Figure 5C). However, no reduction in Pitx2c expression was seen in these hearts (Figure 5D).

### 3.5 Cited2 haploinsufficiency in the mouse

The mutations found in human congenital heart disease patients\(^{15}\) suggested that Cited2 haploinsufficiency may occur. We tested for this in the mouse by examining the genotypic ratios of Cited2\(^{+/−}\) intercrosses where we obtained 500 wild-type and 782 Cited2\(^{+/−}\) pups (expected 855). The 8.5% reduction in Cited2\(^{+/−}\) pups was statistically significant \((\chi^2 = 18.5; P < 0.0001)\). A similar reduction was not observed however when the genotypic ratios of E13.5 or E15.5 embryos were examined (Supplementary material online, Table S5).

To determine a mechanism for reduced survival, we examined Cited2\(^{+/−}\) embryos at E15.5 by MRI (Figure 6). This was followed by histological sectioning of embryos that had either oedema or a cardiac abnormality visible on MRI. We found that three of 45 Cited2\(^{+/−}\) embryos had a clearly identifiable VSD, and one presented with DORV with hypoplastic pulmonary trunk (Figure 6). These defects were not seen in the wild-type littermate controls. Thus, heterozygosity for a Cited2 null allele is associated, at low penetrance, with cardiac malformation in the mouse, implying haploinsufficiency.

### 3.6 Quantitative real-time-polymerase chain reaction analysis of Pitx2c and Cited2 expression

To determine if haploinsufficiency in Cited2-deficient hearts is linked to abnormality in Pitx2c expression, we examined gene expression using qRT-PCR. Embryos and hearts on a C57BL/6J background, generated by backcrossing for 14 generations, were used to minimize the effects of genetic background. Hearts at E13.5 were used as this is a key time-point in cardiac septation, and embryos at E8.5 were selected as Pitx2c is expressed asymetrically in the left lateral plate mesoderm at this stage. In E13.5 hearts, Cited2 was reduced 2.13-fold in Cited2\(^{+/−}\) compared with...
wild-type ($P = 2.62 \times 10^{-6}$) (Figure 7A). We found that the Cited2 mRNA in Cited2$^{-/-}$ hearts was either undetectable or, if detectable, had values ranging between $1.45 \times 10^{-12}$ and $3.47 \times 10^{-11}$ relative to 18S (Figure 7C), likely to represent the limits of the assay. The Cited2 target gene Pitx2c was reduced 1.5-fold in Cited2$^{+/+}$ hearts and 4.7-fold ($P = 0.00031$) in Cited2$^{-/-}$ hearts compared with wild-type controls (Figure 7B and D). Cited2 and Pitx2c mRNA were strongly correlated in wild-type and Cited2$^{+/+}$ hearts (Pearson rank correlation $= 0.68$, $P =$
observations are consistent with, and fully recapitulate, those previously reported with the global deletion of Cited2. 6–9,29 They indicate that Cited2 is essential in the epiblast or its derivatives for normal embryonic left–right patterning, and for cardiac and adrenal development. The complete penetrance of the defects, and the widespread expression of lacZ in embryos containing a Cited2/lox allele and the Cre transgene also indicate that efficient recombination occurs in response to Cre expression. This was further validated by the large reduction seen in Cited2 mRNA levels in the hearts of Cited2/lox;Sox2Cre embryos. We observed reduced lacZ staining in the outer curvature of the left ventricle following conditional deletion of Cited2 with Sox2Cre (as well as with Mesp1Cre and TCre). This is consistent with the published data,4 and indicates that Cited2 is expressed at a lower level in this region of the heart.

To define the role of Cited2 in the neural crest, we deleted it with Wnt1Cre.16,30 This resulted in cranial ganglia fusions and exencephaly, but did not affect cardiac septal, outflow tract, aortic arch or adrenal development, or left–right patterning. These results indicate that although Cited2 does have a cell autonomous role in the neural crest, as evidenced by fusion of the cranial ganglia, it is not required here for outflow tract septation or aortic arch patterning.

To determine the requirement of Cited2 in the cranial, cardiac, and extraembryonic mesoderm it was deleted with Mesp1Cre, which results in extensive recombination in these tissues by E7.5.17,27 Subsequently, by E9.5, recombination is observed extensively in myocardium and endocardium including the mesenchyme of the cono-truncal and atrioventricular cushions, in the epicardium, and in the pharyngeal arch mesoderm and vascular endothelium.17,27,31 Deletion of Cited2 using Mesp1Cre resulted in low penetrance of septal defect, and no outflow tract, aortic arch, or laterality defects. These results indicate that Cited2 function in the mesoderm does not explain the phenotypically heterogeneous cardiac malformations observed in the epiblast-specific deletion. Efficient recombination at the Cited2 locus in response to Cre expression as demonstrated by lacZ staining and qRT-PCR indicated that lack of a phenotypically heterogeneous phenotype is unlikely to be owing to inefficient recombination.

As Cited2 is expressed in the lateral-plate mesoderm,8 and as its deficiency also leads to loss of Nodal-activated transcripts such as a Nodal, Lefty2, and Ptx2c in the left-lateral plate mesoderm,2,8 we investigated its role here by deletion using TCre.18 This resulted in infrequent septal and laterality defect. Deletion of Cited2 induced by TCre was predominantly in the posterior rather than the anterior extent of the lateral plate mesoderm, making this one possible mechanism for the infrequency of left–right patterning defect observed with a TCre-induced deletion. The lacZ staining pattern seen in Cited2/lox;TCre embryos was comparable with that seen with TCre × R26R lacZ embryos.19 Moreover, in the heart the staining pattern was highly reminiscent of that observed for Isl1 suggesting that TCre may also drive recombination in second heart field-derived regions of the heart. These results indicate that the penetrant and phenotypically heterogeneous cardiac malformations in Cited2 deficiency arise from a primary requirement in epiblast derivatives for left–right

Figure 6 Oedema and cardiac malformations in Cited2−/− embryos. Magnetic resonance imaging of wild-type and littermate Cited2−/− embryos at E15.5. Sagittal sections of wild-type (A) and Cited2−/− (B) embryos. Severe oedema (asterisks) is seen in the Cited2−/− embryo. (C) Transverse section of wild-type and Cited2−/− embryos. The Cited2−/− embryo has a ventricular septal defect (VSD) (D). (E, F) Three-dimensional reconstruction of the heart in the wild-type and Cited2−/− embryos shown in the previous panel. The aorta (Ao) and pulmonary artery (Pa) arise from the left ventricle and right ventricle (RV), respectively, in the wild-type embryo, but in the Cited2−/− embryo they arise from the RV creating a double-outlet right ventricle (DORV). VSD is indicated. Scale bars, 500 μm. Axes are as shown in Figure 1.

0.0009; Figure 7F). In the E8.5 embryos, we found a two-fold reduction in Ptx2c levels in Cited2−/− compared with wild-type (P = 0.009; Figure 7E).

4. Discussion

In these experiments, we used a conditional knockout approach to understand how Cited2 deficiency results in cardiac phenotypic heterogeneity. To define the role of Cited2 in the epiblast, we deleted it with Sox2Cre.15 This resulted in 100% penetrance of cardiovascular malformations and adrenal agenesis. The most common cardiac malformation was a VSD, which was present in all embryos, and was associated with DORV in some embryos. In addition we observed left–right patterning, atrial septal, outflow tract, and aortic arch defects. These
patterning with a secondary cell-autonomous role in the mesoderm for septation. A limitation of our studies, however, is that the role of Cited2 in the endoderm—which has a major role in cardiac induction—could not be established. We are currently limited here by the lack of a suitable Cre driver that expresses in the endoderm at the appropriate time-point.

Our data also show that Cited2 haploinsufficiency occurs in the mouse and is associated with cardiac malformation. Although 8.5% of Cited2 heterozygous pups are lost by weaning, consistent with the numbers of cardiac malformations observed, no deviation from expected numbers was seen during development. The mechanistic link between cardiac malformations and Cited2 haploinsufficiency is unclear, and to address this, we quantitatively examined the expression of Cited2 and its target gene Pitx2c in embryonic hearts and E8.5 embryos. Using qRT-PCR we showed that Cited2 expression is significantly reduced in Cited2 heterozygous embryonic hearts. We also showed that this was accompanied by a significant reduction in Pitx2c expression, and that the two were strongly correlated. We also observed a significant reduction in Pitx2c levels in E8.5 Cited2/-/- embryos. As the dose of Pitx2c is crucial for normal left–right patterning and cardiac development, we suggest that Pitx2c deficiency observed in Cited2 heterozygous hearts and embryos could explain, at least in part, the observed cardiac malformations.

In conclusion, these results indicate that phenotypically heterogeneous and penetrant cardiac malformations in Cited2 deficiency arise from a primary requirement in epiblast derivatives for left–right patterning with a secondary cell-autonomous role in the mesoderm for septation. Cardiac malformation associated with Cited2 haploinsufficiency may occur by reducing the expression of key Cited2 targets, such as Pitx2c from E8.5. Importantly, cardiovascular phenotypic heterogeneity is observed, for instance, with mutation in NKX2-5, 36–38 CFC 39,40 or ZIC3. 41–43 Consistent with our proposed model, these genes, like Cited2, are also known to control or modulate left–right patterning pathways. Our results support the emerging idea that genes controlling early left–right patterning are candidates for diverse forms of human congenital heart disease, even in the absence of a classical lateral defect such as isomerism.
Supplementary material

Supplementary material is available at Cardiovascular Research online.

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