Combined Loss of Hey1 and HeyL Causes Congenital Heart Defects Because of Impaired Epithelial to Mesenchymal Transition

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Abstract—Congenital heart defects affect almost 1% of human newborns. Recently, mutations in Notch ligands and receptors have been found to cause a variety of heart defects in rodents and humans. However, the molecular effects downstream of Notch are still poorly understood. Here we report that combined inactivation of Hey1 and HeyL, two primary target genes of Notch, causes severe heart malformations, including membranous ventricular septal defects and dysplastic atrioventricular and pulmonary valves. These defects lead to congestive cardiac failure with high lethality. We found both genes to be coexpressed with Notch1, Notch2 and the Notch ligand Jagged1 in the endocardium of the atrioventricular canal, representing the primary source of mesenchymal cells forming membranous septum and valves. Atrioventricular explants from Hey1/HeyL deficient mice exhibited impaired epithelial to mesenchymal transition. Although epithelial to mesenchymal transition was initiated regularly, full transformation into mesenchymal cells failed. This was accompanied by reduced levels of matrix metalloproteinase-2 expression and reduced cell density in endocardial cushions in vivo. We further show that loss of Hey2 leads to very similar deficiencies, whereas a Notch1 null mutation completely abolishes epithelial to mesenchymal transition. Thus, the Hey gene family shows overlap in controlling Notch induced endocardial epithelial to mesenchymal transition, a process critical for valve and septum formation. (Circ Res. 2007;100:856-863.)

Key Words: Hey1 ▪ HeyL ▪ Hey2 ▪ Notch ▪ epithelial to mesenchymal transition

Congenital heart defects (CHD) are the most common human congenital malformations, present in ∼1% of all life births.1 In most cases the underlying reason remains elusive. During the last decade examples of genetic alterations that may lead to CHD have been identified, but the molecular mechanisms are still poorly understood. Recently NOTCH1 mutations have been found to cause aortic valve disease.2 In addition, mutations of the Notch ligand Jagged1 (JAG1) are responsible for most cases of Alagille syndrome, affecting development of the heart and other organs.3,4 Specific missense JAG1 mutations can further cause complex congenital cardiac defects like tetralogy of Fallot.5

Notch signaling is an evolutionarily conserved intercellular signaling pathway involved in cell fate specification.6 It is implicated in a vast array of developmental processes, and diverse developmental defects are because of improper Notch activity.7 After binding to a δ-like (DLL1, DLL3 and DLL4) or Serrate (Jag1, Jag2) ligand, the Notch transmembrane receptor undergoes a complex cleavage process, which releases its intracellular domain (NICD). The NICD then translocates into the nucleus, where it activates a transcription factor called RBPJκ (also known as CBF1, Su[H], or Rbpsuh).8 The mechanisms by which disturbance of Notch signaling leads to cardiac malformations are still puzzling, because effects downstream of NICD/RBPJκ are poorly understood. Established target genes of the Notch cascade are the Hes and Hey gene families, the latter also known as Hesr, Herp, Hrt, Chf or gridlock.9 We and others have shown before that loss of Hey2 in mice causes CHDs, including ventricular septum defects, persistent foramen ovale, atrioventricular valve defects, pulmonary stenosis and even tetralogy of Fallot.10–13 It is not clear, however, which cellular process during heart development is disturbed in this case. Interestingly, Hey1−/− mice are healthy without any apparent pathological findings. There is partial redundancy between Hey1 and Hey2 however, because Hey1/2 double knockout embryos die during midgestation because of severe vascular problems, thus preventing analysis of cardiogenesis.14

In this study, we addressed the function of HeyL, the third Hey gene family member in cardiac morphogenesis. HeyL is...
a bona fide Notch target\textsuperscript{15,16} that is dynamically expressed during embryogenesis.\textsuperscript{17–19} Until now the function of this gene was largely unknown. Here we report that loss of \textit{HeyL} does not seem to significantly interfere with normal embryonic development. However, during cardiogenesis \textit{HeyL} cooperates with \textit{Hey1} to permit epithelial to mesenchymal transition of endocardial cells to promote endocardial cushion development. As a consequence, a combined \textit{Hey1/L} loss causes CHDs leading to cardiac failure shortly after birth.

Materials and Methods

An expanded Materials and Methods section is provided in the online data supplement available at http://circres.ahajournals.org.

Mice

Based on the known gene structure of \textit{HeyL},\textsuperscript{20} a gene targeting vector was constructed to replace exons 2 to 4 and parts of intron 4 by a lacZ-PGKneo cassette. \textit{Heyl} and \textit{Notch1} KO strains have been described.\textsuperscript{14,21}

Gene Expression Analysis

Protocols for mRNA in situ hybridization, \textgreek{b} galactosidase staining, RNA isolation and real-time RT-PCR have been described.\textsuperscript{10,14,22}

Atrioventricular Canal Explant Assay

Atrioventricular (AV) explants were derived from E9.5 mouse embryos as described.\textsuperscript{23,24}

MRI

Embryos were fixed in 4\% paraformaldehyde containing 2 mmol/L gadolinium-diethylenetriamine pentaacetic anhydride and imaging was performed on a Bruker Avance 500 spectrometer (11.7 Tesla; 500 MHz).

Protein Interaction Analysis

Coimmunoprecipitation was performed as described.\textsuperscript{25} A doxycycline inducible HEK293 cell line was established by successive lentiviral transduction of the pWHE13426 transcriptional regulator as cline inducible HEK293 cell line was established by successive

Results

\textit{HeyL}\textsuperscript{−/−} Mice Do Not Show Obvious Defects

To determine the function of \textit{HeyL} in vivo, we generated a mutant allele by homologous recombination in ES cells. The targeting vector replaces exons 2 to 4, encoding the basic helix-loop-helix domain, with an in-frame lacZ-neo cassette (Figure 1\textsuperscript{A}). After blastocyst injection and germline transmission heterozygous mice were intercrossed to generate \textit{HeyL}\textsuperscript{−/−} mice. No \textit{HeyL} mRNA expression was detectable in these embryos (Figure 1\textsuperscript{B}, C) confirming a null mutation. Mutant embryos expressed the \textgreek{b} galactosidase reporter gene in a pattern that is very similar to that published for endogenous \textit{HeyL} mRNA (Figure 1\textsuperscript{D}–\textsuperscript{F}).\textsuperscript{17,19} Especially expression in spinal nerves, dorsal root ganglia, somites and arteries was predominant. Homozygous mice exhibited a more intensive \textgreek{b} galactosidase staining because of dosage effects.

Of 132 pups genotyped at weaning, we obtained 30 wild-type, 63 heterozygous and 39 homozygous mice, which fits the expected Mendelian ratio. \textit{HeyL}\textsuperscript{−/−} mice were grossly normal and fertile. It had been shown previously that the phenotype and survival rate of \textit{Hey2}\textsuperscript{−/−} mice strongly de-
mice and mice lacking three of the four alleles were fertile and did not exhibit any pathological changes. From matings of Hey1−/−/HeyL−/− with Hey1−/−/HeyL+/− we obtained DKO neonatal pups in the expected Mendelian ratio, however at weaning Hey1/L DKO mice were significantly underrepresented. Interestingly, survival of DKO embryos was dependent on the genetic background. When DKO mice were generated with HeyL mice crossed only twice with C57BL/6 mice (F2 generation), 89% (57/64) of DKO offspring survived until weaning. However, in the F9 backcross generation less than 5% of the DKO animals survived the first 3 weeks after birth. DKO animals of the F9 generation appeared normal at birth, but failed to thrive and died within the first few days after birth.

**Congenital Heart Defects in Hey1/L DKO**

Gross morphological and histological analysis of neonatal Hey1/L DKO mice revealed no major changes in the nervous system, kidney, digestive tract and skeleton (data not shown). However, the hearts showed a slight biventricular enlargement. On histological sections we found a high incidence of ventricular septal defects (VSD) in hearts of DKO newborns (21% in F2, 82% in F9 generation). Notably, in all cases the membranous septum was completely missing, whereas the muscular septum appeared unaffected (Figure 2). We next analyzed embryonic hearts at stages E10.5 to E18.5 by serial section histology (Figure 3). Hearts from Hey1/L DKO embryos were indistinguishable from controls at E10.5 to E13.5. However, 20 of 25 (80%) Hey1/L DKO embryos of the F9 generation failed to close the ventricular septum after E13.5, although this was never observed in control littermates. Again, the muscular septum was normal, but the membranous part was missing. On histological sections myocardium, endocardium and trabeculation appeared unaffected in DKO animals; however the AV valves were often dysplastic when compared with controls (13% in F2, 53% in F9 generation). This was already seen at E15.5 (Figure 3E and 3H) and even more pronounced in hearts of newborns (Figure 2E and 2G). Furthermore, we found severely thickened pulmonary valves in DKO newborns, which are prone to blood flow obstruction and insufficiency (Figure 2F). Such dysplastic valves often lead to regurgitation of blood and cardiac dysfunction. Indeed, postmortem examination revealed ventricular hypertrophy in DKO mice suggesting cardiac insufficiency and failure (Figure 2D).

To better evaluate the 3 dimensional aspect of these heart defects we performed MRI on embryos at stage E15.5. MRI has been shown to provide excellent spatial information about complex cardiac malformations even in mouse embryos.28 Serial 2D magnetic resonance images allowed us to identify all cardiac chambers, septa and valves, as well as the VSD in three anatomical planes (Figure 4). 3D reconstructions showed a large membranous VSD located directly below the atrioventricular plane, whereas other cardiac structures or associated great blood vessels were not affected (Figure 4G, I). Next we compared the cardiac defects of Hey1/L DKO embryos with those seen in Hey2−/− mice.10 Interestingly, we found a very similar type of VSD in 6/6 Hey2−/− mice, suggesting a common route of pathogenesis (Figure 4C, F, H, J).

To address the molecular mechanism underlying the heart malformations of Hey1/L DKO mice, expression of cardiogenic genes was analyzed by mRNA in situ hybridization. Anf (Nppa), Carp (cardiac ankyrin repeat domain1, Ankrd1) and Bnp (brain natriuretic peptide, Nppb) signals were correctly localized in cardiac myocytes and absent from the atrioventricular cushion
in controls and Hey1/L DKO. Bmp2, a marker of the AV canal, was detected in the AV region, whereas Hey2 mRNA was found in ventricular myocardium and outflow tract (supplemental Figure I in the online data supplement available at http://circres.ahajournals.org). In addition, we could not detect significant changes of Anf, Carp, Gata4, Gata6 and Hey2 mRNA expression in E9.5 hearts with quantitative real-time RT-PCR (data not shown). This suggests that cardiomyocyte differentiation is not perturbed by Hey1/L loss. Furthermore, mRNA expression patterns of Sox9, Has2, Msx2, Fog2, Runx2 and Tgf-b2, which play crucial roles for valve development were unaltered (data not shown).

**Notch1, 2, and Hey1, 2, and L Coexpression in AV Endocardial Cells**

According to previous HeyL expression pattern reports, the phenotype of Hey1/L DKO mice would be difficult to explain, as HeyL mRNA had not been found in the developing heart. On the other hand, Hey1 was published to be exclusively transcribed in the atria of the embryonic heart. Therefore we repeated Hey1, Hey2 and HeyL as well as Notch1, Notch2 and Jagged1 expression analyses with an emphasis on the embryonic heart. Surprisingly, we found HeyL to be coexpressed with Notch1, Notch2, the Notch ligand Jagged1 and the other two Hey genes in AV cushions at embryonic stages E9.5 to E12.5 (Figure 5 and data not shown). Robust expression of the Notch signaling members was seen in endocardial cells of the AV canal, which are characterized by VE-Cadherin and mesenchymal cells with Snai1 or Has2 expression. Weaker signals were seen in the underlying mesenchyme. Most mesenchymal cells, which abundantly express Snai1 (Snail) or Has2 (hyaluronate synthase 2), almost completely lack expression of Notch pathway members.

The mechanism how Hey transcription factors act at the molecular level is still controversial. One possibility is the formation of homodimers or Hey/Hes heterodimers in the presence of DNA. We therefore tested protein interactions of HeyL by coimmunoprecipitation from HEK293T cell

**Figure 5. Expression of Notch signaling members in the AV cushion.** A, HE staining of an embryonic heart at E11.5 showing right and left ventricle (rv, lv), interventricular septum (s) and atrioventricular cushion (av). B, Magnification of the AV cushion which consists of an outer endocardial cell layer (arrowheads) and mesenchymal cells (m) surrounded by extracellular matrix. C–K, RNA in situ hybridization showing endocardial cells stained with VE-Cadherin (C) and mesenchymal cells with Snai1 or Has2 expression (D and E). F–K, Jagged1, Notch1, Notch2 and the Hey genes are all coexpressed in the endocardium and weaker in the underlying mesenchyme.
lysates and from inducible stable cell lines expressing HA-Hey1 and FLAG-HeyL at low levels. HeyL was able to efficiently form homodimers, but also interacted strongly with Hey1 and Hey2 (supplemental Figure II). Only endocardial cells of the AV canal coexpress both, Hey1 and HeyL and the two proteins can interact on the protein level. This implies that the primary cause of heart defects in Hey1/L DKO mice must be located in the endocardial cells of the AV canal.

Impaired EMT in Hey1/L DKO and Hey2−/− Hearts

To form the cardiac septum and valve structures, endocardial cells of the AV canal undergo a process of epithelial to mesenchymal transition (EMT).23 During EMT endocardial cells lose their epithelial gene expression, polarity and cell-cell adhesion, delaminate from the endocardial sheet and become migratory to invade the initially acellular cardiac jelly as mesenchymal cells. These cells remodel the primordial extracellular matrix (ECM) and form the endocardial cushions required for septum and AV valve development.32 Intriguingly, these exact two structures are disturbed in Hey1/L DKO embryos.

To test whether defective EMT underlies heart malformation in Hey1/L DKO embryos, we used the Markwald and Runyan explant model based on AV cushion tissue from E9.5 mouse embryos cultured on collagen gels.23 Wild-type as well as Hey1/L het/het, KO/het and het/KO explants allowed the emigration and scattering of elongated, spindle-like, mesenchymal cells, which migrated through the collagen gel (Figure 6A), consistent with EMT. A minority of migrating cells showed a round morphology. These rounded cells underwent loss of epithelial cell-cell cohesion, but failed to convert to mesenchymal state, thus using an poorly defined, amoeboid-type of cell movement, which has also been referred to as “transitional or intermediate phenotype”.33 In comparison to controls, Hey1/L DKO explants showed only a slightly reduced total number of outgrowing cells, but a strong reduction of fully transformed, elongated mesenchymal cells (Figure 6B).

For a more detailed analysis of endocardial EMT we used time-lapse video microscopy. In control cultures endothelial cells rapidly migrated of the explant and most of these cells immediately transformed into mesenchymal cells. Hey1/L DKO explants behaved similar to controls during the first hours of culture. However, most of the outgrowing cells moved in a round shape and did not fully transform into mesenchymal cells (Figure 7 and online data supplement available at http://circres.ahajournals.org).

To find out whether the observed EMT defects in explant cultures reflect EMT defects in vivo, we quantified the cell density of AV cushions in Hey1/L mutant embryos at stage E11.5. This revealed no alterations of cushion size, but a 31% reduction (P=0.02) of cell density compared with littermate controls (Figure 8A, B), corroborating the in vitro results. Furthermore, cell density in the outflow tract cushions was also significantly reduced (37%, P=0.03), suggesting that EMT is disturbed in vivo.

Recently, Timmerman et al showed that Notch1 and RBPJk are required for EMT, as Notch1−/− and RBPJk−/− embryos lack mesenchymal cells in their atrioventricular cushions, and in AV explant cultures only few cells delaminate from the explant and these fail to transform.34 When we repeated these experiments with Notch1−/− hearts, we observed very similar results (Figure 6). Time-lapse microscopy revealed that cardiomyocytes in these explant were contracting for at least 48 hours, excluding an intrinsic cardiomyocyte defect (Figure 7 and online data supplement). Compared with Notch1−/− hearts, the phenotype observed in Hey1/L DKO is less severe, suggesting that loss of Hey1 and HeyL resembles a hypomorphic Notch1 mutation in the endocardium.

Because a combined loss of Hey1 and HeyL does not fully reproduce the loss of Notch1 or RBPJk, it is obvious that they do not represent the only Notch transducers. Interestingly, Hey2−/− mice also exhibit membranous VSDs and dysplastic AV valves.10−13 Similar to Hey1/L DKO explants, Hey2−/− AV explants exhibited strongly reduced transformation of epithelial cells into spindle-shaped mesenchymal cells (Figure 6A). A minority migrating cells (24%) transformed into mesenchymal cells, whereas the majority of outgrowing cells remained nontransformed (Figure 6B). Time-lapse video
microscopy showed a regular onset of EMT, similar to wild-type controls, but after 12 hours the majority of out-growing cells remained rounded (Figure 7 and online data supplement).

Decreased Mmp2 Expression in Notch1 and Hey Deficient AV Cushions

The migration of Hey1/L or Hey2 deficient endocardial cells is reminiscent of amoeboïd cell migration with poorly developed cell-matrix contacts and an impaired capability to remodel extracellular matrix. It had been demonstrated before that inhibition of proteases can change cell migration from a mesenchymal to an amoeboïd behavior in some tumor cell lines.\(^{35}\) For endocardial cushion EMT matrix metalloproteinase-2 (Mmp2) has been identified as an essential component.\(^{36}\) When we analyzed Mmp2 expression in the AV canal of Notch1\(^{-/-}\) embryos, we found a severe reduction of mRNA for this key enzyme (5.9-fold; \(P<0.01\)). Although the EMT defects in Hey1/L and Hey2 deficient hearts are less severe, Mmp2 levels were still significantly reduced (2.1 and 5.4 fold; \(P<0.05\)) (Figure 8C).

It is not known how Mmp2 expression is regulated during endocardial EMT. In tumor cells, Mmp2 can be induced by the zinc finger transcription factor Snai1.\(^{38}\) We found Snai1 expression to be reduced in Notch1/L and Hey2 deficient AV canals (50%, 67% and 48%) compared with wild type littermates (data not shown), although probability values did not reach significance levels (\(P<0.1\)). Thus, mutant endocardial cells in the AV cushion region apparently fail to fully induce a mesenchymal gene expression program.

Discussion

In this study, we report a new mechanism by which disturbance of Notch signaling can lead to fatal congenital heart disease. HeyL, hitherto only noted as a Notch target gene with unknown function, cooperates with Hey1 to regulate endocardial EMT essential in septum and valve development.

In humans specific mutations of NOTCH1 or JAG1 can cause a wide spectrum of CHDs.\(^{2-5}\) However, the underlying genetic processes downstream of Notch are unknown. Functional analysis is cumbersome because mice with a global loss of Notch1 or its transducer RBPJ\(\kappa\) die early during development with vascular and other defects preventing an
in-depth examination of heart development. In this regard, a thorough investigation of the prime Notch target genes is of great importance. Our previous work demonstrated that a combined Hey1/2 deletion mimicks a Notch1 or RBPJk loss in the vascular system, but does not interfere with eg, somitogenesis. On the other hand, Hes7 deficiency recapitulates the somitogenesis defects seen in Notch1 mutants, but does not interfere with angiogenesis. Thus, a selected inactivation of Hey or Hes genes can be key to a better understanding of cellular and molecular outputs of Notch signaling in a particular organ, without affecting the development of other tissues.

In the developing heart Notch is mainly active in the endocardium and outflow tract, as judged by expression pattern analyses. We here show that all three Hey genes are coexpressed with Jag1, Notch1 and Notch2 in the AV endocardium, suggesting a potential cooperation in transducing Notch signaling in this cell type. There is a close interplay between endocardium and myocardium as both influence each other, eg, by secretion of signaling molecules. At around E9.5 a subset of endocardial cells in the AV canal reacts to signals from the myocardium and undergoes EMT to invade the cardiac jelly and form the AV cushions, which contribute to heart septation and AV valve formation. Time-lapse imaging showed, that initial delamination and single cell migration occurs at an almost normal rate in Hey1/L DKO AV explants, but full transformation into mesenchymal cells is strongly reduced. Thus, deletion of Hey1/L leads to a partial inhibition of EMT. Rather than acquiring and maintaining mesenchymal cell elongation, upregulating of ECM-degrading proteases including Mmp2 and ECM remodelling, the cells persisted in a mobile, amoeboid type state, reminiscent of undifferentiated circulating stem cells or leukocytes. Thus, Hey genes induce or maintain the mesenchymal state rather than contributing to the loss of epithelial differentiation.

The Hey1/L DKO phenotype in the heart does not completely recapitulate a Notch1 loss. We rather suggest that all three Hey genes together redundantly transduce the Notch signal, and a certain threshold is needed to initiate EMT and even more to fully execute it. There is precedence for a mutual compensation, in this case of Hey1 and Hey2, in angiogenesis, where the defect is only seen in double knock-out embryos, while an intact copy of one of the two genes suffices for normal blood vessel development. In addition, all three Hey proteins can efficiently form heterodimers with each other. Given the strong sequence similarity, especially in the bHLH regions, it is conceivable that Hey proteins can functionally replace other coexpressing cells. To a certain extent the output of Notch signaling may depend of the total amount of partially redundant Hey proteins. To formally prove such a scenario, endothelial specific triple knockout and compound heterozygous mice may be needed in future studies. This would also reveal whether Hey proteins are the only relevant transducers of Notch signals at this stage of cardiac development.

Our observations clearly suggest that Mmp2 is a target of the Notch-Hey axis as it is decreased in the AV region of Notch1 and—to a lesser extent—Hey deficient embryos. In this regard it is noteworthy, that silencing of Notch1 in pancreatic cancer cells inhibited invasion by downregulation of Mmp9. EMT of the endocardium is strictly dependent on Mmp2 activity. We propose that the delay or loss of mesenchymal cell functions together with a lack in Mmp2 upregulation may underlie the defective capability to remodel the primordial ECM in the cushion in sufficient temporal coordination with other developmental steps of the heart, and, thus, represents the cellular mechanism of septum and valve defect.

In conclusion, the Hey1/L DKO or Hey2−/− phenotype is milder than that observed in Notch1−/− or RBPJk−/− hearts. Taking into account the phenotypic variability seen in inbred and mixed genetic backgrounds it is conceivable that such a hypomorphic phenotype may well be compatible with life in humans. Impaired Notch signaling at various levels may thus contribute to a larger fraction of congenital heart diseases than previously envisaged.

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Disclosures

None.

References


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**Material and Methods**

Generation of HeyL knockout mice

Based on the known gene structure of HeyL, a gene targeting vector was constructed to replace exons 2 to 4 and parts of intron 4 by a lacZ-PGKneo cassette in E14.1 ES cells. The lacZ gene was fused in-frame with the HeyL coding sequence at the start of exon 2.

Homology arms were 1.5 kb and 8 kb. Two correctly targeted ES cell lines were identified by PCR and Southern blot analysis and used to generate chimeric mice. After germine transmission the floxed PGKneo cassette was removed by crossing with CMV-cre transgenic mice.

Mouse breeding and genotyping

HeyL\(^{+/−}\) mice were interbred with C57BL/6 mice for either two or nine generations and subsequently crossed with HeyL\(^{−/−}\) mice (backcrossed with C57BL/6 for nine generations). HeyL status was tested by PCR using primers Z3L (atcggtgcgggcctcttcgctatta), mHeyLex2lb (ggatcctcagctctgagaaa) and HeyLex2r (ttgtgcacactcacccctct), which yielded products of 123 bp (wild type) and 210 bp (lacZ knock-in allele).

Histological analysis

Tissues were dissected in PBS, fixed in 4% paraformaldehyd overnight and embedded in paraffin. Sections were stained with haemalaun and eosine or Alcian Blue for routine analysis.

AV canal explant assay

The AV canal was dissected, cut longitudinally and cultured on top of drained type I collagen gels (1.5 mg/ml; BD Biosciences). After 48 hours explants were fixed with 2% PFA and stained with 10 µg/ml Phalloidin-FITC (Sigma) and DAPI. About 75% of more than 300
atrioventricular explants analysed firmly attached to the matrix and spontaneously started contracting. There were no differences between wild type and mutant explants in terms of attachment and survival rates. Images were obtained using confocal laser-scanning microscopy (Leica, TCS-SP2/AOBS). For time-lapse video microscopy AV explants were cultured under an inverted microscope. Pictures were taken every 4 minutes and converted to QuickTime movies.

Magnetic resonance imaging
E15.5 embryos were fixed in 4% paraformaldehyd containing 2 mM gadolinium-diethylenetriamine pentaacetic anhydride and were individually embedded in 2ml syringes using 1.5% agarose. MRI measurements were performed on a Bruker Avance 500 spectrometer (11.7 T, 500 MHz), equipped with a gradient unit with 0,6T/m, and a quadrature-driven birdcage coil with an inner diameter of 20 mm (Rapid Biomedical, Rimpar, Germany). A 3-D FLASH sequence (echo time 5.9 ms, 30 degree hermite excitation pulse, repetition time 50 ms, sweepwidth 40 kHz) was used. The field of view was 13 x 10 x 10 mm, with a matrix size of 648 x 256 x 256, resulting in a nominal resolution of 20 x 39 x 39 µm. The total experimental time was less than 4h including a four time averaging. Data sets were reconstructed and zero filled to a matrix size of 648 x 512 x 512, leading to an image isotropic voxel size of 20 x 20 x 20 µm.

The 3-D reconstruction and the surface view were accomplished using a commercial software package (Amira 3.1, Mercury Computer Systems GmbH). In the 3-D reconstruction the interior is shown in purple, myocardium in green, aorta in red, pulmonary artery in blue, ductus arterious in yellow and veins in light blue.

**Online Figure 1.** Expression of several cardiac marker genes is unaffected in Hey1/L mutant embryos. At E9.5 *Anf*, *Bmp2* and *Hey2* expression in Hey1/L DKO embryos (D-F) is
indistinguishable from controls (A-C). *Anf* marks myocardium of the atrium (a) and primitive ventricle (v), *Bmp2* is restricted to the atrioventricular canal (av), while *Hey2* defines ventricle and outflow tract. Note that there is expression in the endocardium of the AV canal. (G-L) Sections through a heart at E11.5 stained with antisense probes against *Anf, Carp,* and *Bnp.* The same expression is observed in controls and *Hey1/L* DKO.

**Online Figure 2.** Co-immunoprecipitation of FLAG-tagged HeyL with HA-tagged Hey1, Hey2 or HeyL from lysates of transiently transfected HEK293T cells (A). (B) Co-immunoprecipitation of FLAG-HeyL and HA-Hey1 from two independent clones of low expressing, doxycycline (Dox) inducible stable cell lines.

**Movie 1 of a wild type AV explant.**

**Movie 2 of a *Hey1/L* deficient explant.**

**Movie 3 of a *Notch1*−/− explant.**

**Movie 4 of a *Hey2*−/− explant.**