Essential roles of the bHLH transcription factor Hrt2 in repression of atrial gene expression and maintenance of postnatal cardiac function

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The basic helix–loop–helix transcriptional repressor Hairy-related transcription factor 2 (Hrt2) is expressed in ventricular, but not atrial, cardiomyocytes, and in endothelial and vascular smooth muscle cells. Mice homozygous for a null mutation of *Hrt2* die perinatally from a spectrum of cardiac abnormalities, raising questions about the specific functions of this transcriptional regulator in individual cardiac cell lineages. Using a conditional *Hrt2* null allele, we show that cardiomyocyte-specific deletion of *Hrt2* in mice results in ectopic activation of atrial genes in ventricular myocardium with an associated impairment of cardiac contractility and a unique distortion in morphology of the right ventricular chamber. Consistent with the atrialization of ventricular gene expression in *Hrt2* mutant mice, forced expression of *Hrt2* in atrial cardiomyocytes is sufficient to repress atrial cardiac genes. These findings reveal a ventricular myocardial cell-autonomous function for Hrt2 in the suppression of atrial cell identity and the maintenance of postnatal cardiac function.

congenital heart disease | heart development | heart failure

Formation of the four-chambered vertebrate heart requires complex morphogenetic events and interactions among diverse cell types with specialized functions. Atrial and ventricular cardiomyocytes, for example, display distinct gene expression patterns, contractile properties, and hormonal responses required for coordinated cardiac contractility. Endothelial cells give rise to the endocardium and cardiac valves, and smooth muscle cells contribute to the coronary arteries and inflow and outflow vasculature. Abnormalities in the developmental events associated with the differentiation, growth, migration, or cell–cell interactions of these different cardiac cell types result in congenital heart disease, the most common human birth defect (1–4).

The Hairy-related transcription factor (Hrt) family of basic helix–loop–helix (bHLH) proteins, also referred to as Hey, Hesr, CHF, and HERP (5–8), consists of three members, Hrt1, Hrt2, and Hrt3 (9). These proteins share homology in their bHLH regions, which mediate DNA binding and dimerization, as well as in an Orange domain of unknown function and a unique C-terminal YXXW-TE(I/V)GAF domain. Similar functional domains are contained in the Hairy/Enhancer of Split (HES) proteins (10). During embryogenesis, *Hrt2* is expressed in the ventricular myocardium, but not in the atrial myocardium, and in the cardiac outflow tract and aortic arch arteries (5, 9). Hrt proteins function as transcriptional repressors downstream of Notch signaling, which regulates binary cell fate decisions during development. Upon activation by ligands such as Delta or Jagged on the surfaces of adjacent cells, the intracellular domain of the Notch receptor is cleaved and translocated to the nucleus where it cooperates with CSL/RBP-Jκ to stimulate transcription of *Hrt* genes (11, 12). In addition to binding to an E-box DNA sequence motif, Hrt proteins physically interact with GATA factors and repress the transcriptional activity of GATA-dependent genes (13). Hrt proteins also dampen Notch-dependent activation of their own genes independent of DNA binding (11, 14). The repressive influence of the Hrt2 protein may result in part from an interaction with the mSin3, N-CoR, and HDAC1 corepressor complex (15).

Mice lacking *Hrt1* are normal, whereas homozygous deletion of *Hrt2* in mice results in a remarkably variable spectrum of cardiovascular defects, including ventricular septal defects (VSDs), valvular defects, postnatal cardiac hypertrophy, cardiomyopathy, and vascular abnormalities (16–20). Misexpression studies have also suggested a role of Hrt1 and Hrt2 in boundary formation within the atrioventricular canal (21). Because *Hrt2* is expressed in cardiomyocytes, vascular smooth muscle cells, and endothelial cells, it is unclear which of these abnormalities reflect cell-autonomous functions of Hrt2 in one particular cell type. They may also be caused by secondary consequences of the loss of Hrt2 function in one cell type that indirectly affects another. The issue of cell autonomy is especially complex in the heart in which a defect in one cell type can have profound effects on growth and morphogenesis of other cardiac structures.

Here, we show that cardiomyocyte-specific deletion of *Hrt2* results in ectopic activation of atrial genes in ventricular myocardium accompanied by contractile dysfunction and a unique distortion of right ventricular morphology. Our results indicate that Hrt2 acts in the ventricular myocardium to repress atrial gene expression, thereby functioning as a key regulator of cardiac cell identity and function.

Results

Targeting the Mouse *Hrt2* Gene. To create a conditional *Hrt2* null allele, we introduced *LoxP* sites into introns 1 and 3 of the mouse *Hrt2* gene by homologous recombination in ES cells (Fig. 1*A*). Deletion of the region of the gene between the two *LoxP* sites eliminates amino acids 29–82, which includes all of the basic and most of the helix–loop–helix region, and splicing of exon 1 to exon 4 alters the reading frame of the remainder of the transcript. The deleted gene therefore does not encode a functional

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Abbreviations: Hrt, Hairy-related transcription factor; VSD, ventricular septal defect; LVIDs, systolic left ventricular internal diameter; LVIDd, diastolic left ventricular internal diameter; FS, fractional shortening; RV, right ventricle; LV, left ventricle; Sln, sarcolipin; Mlc, myosin light chain; ANF, atrial natriuretic factor; E(*n*), embryonic day.

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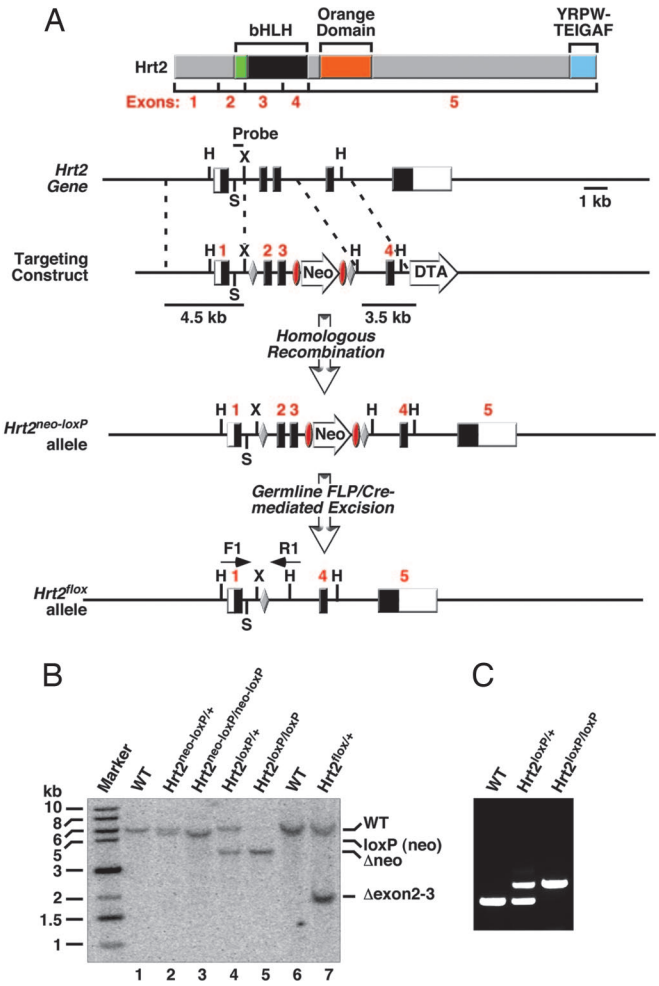

Fig. 1. Targeting the *Hrt2* locus. (*A*) The structure of the mouse Hrt2 protein, denoting position of the exons, is shown at the top, along with the genomic locus, targeting vector, and targeted allele. Lox P sites were inserted into introns 1 and 3 of the *Hrt2* gene, along with a neomycin resistance cassette flanked by FRT sites in intron 3. The neomycin resistance cassette was removed in the mouse germ line by breeding heterozygous mice to *hACTB::FLPe* transgenic mice. Conditional deletion of exons 2 and 3 was achieved by breeding $Hrt2^{loxP/loxP}$ to $Hrt2^{loxP/+}$ mice harboring transgenes that expressed Cre recombinase tissue specifically. Position of the probe used for Southern analysis is shown. bHLH, basic helix–loop–helix. (*B*) Southern blot analysis of various *Hrt2* alleles. Genomic DNA isolated from mice of the indicated genotypes was digested with HindIII and analyzed by Southern blot with the SalI/XhoI probe. (*C*) PCR strategy for genotyping various Hrt2 alleles. Primers flanking the 5′ loxP site are labeled in *A*. Genotypes are shown on the top.

protein. A neomycin resistance cassette flanked by sites for Flp recombinase was incorporated into intron 3. Chimeric mice obtained by blastocyst injection of ES cells heterozygous for the targeted *Hrt2* allele transmitted the mutant allele through the germ line, yielding mice heterozygous for this $Hrt2^{neo\text{-}loxP}$ allele. Breeding of these mice to mice expressing the FLPe recombinase in the male germ line allowed for the removal of the neomycin resistance cassette, creating the $Hrt2^{loxP}$ allele (Fig. 1*B*). A PCR strategy was also designed to genotype the various *Hrt2* alleles (Fig. 1*C*). Homozygous $Hrt2^{loxP}$ mice were phenotypically normal, demonstrating that the $Hrt2^{loxP}$ allele did not function as a hypomorphic allele.

We also created a null *Hrt2* allele by replacing exons 1–4 with a lacZ-neo cassette, referred to as $Hrt2^{KO}$ [supporting information (SI) Fig. 7]. Mice homozygous for this mutant allele, in either an isogenic 129 or a mixed 129/C57Bl6 background, died in the neonatal period, and all showed VSDs, which is consistent with studies by other groups (16–19).

Cardiac Defects Resulting from Myocardial Deletion of *Hrt2*. To analyze the cardiomyocyte-specific functions of Hrt2 and their involvement in the phenotypes in *Hrt2* null mice, we bred $Hrt2^{loxP/loxP}$ female mice to $Hrt2^{KO/+}$ mice harboring an *Nkx2–5-Cre recombinase* (*Cre*) transgene, which is highly specific for cardiomyocytes (22). In contrast to $Hrt2^{KO/KO}$ mice, $Hrt2^{loxP/KO}$;*Nkx2–5-Cre* mice with a cardiomyocyte-specific *Hrt2* deletion are viable to adulthood. They did not have significant valve defects or myocardial hypertrophy (Fig. 2), and the heart weight/body weight ratios were normal (data not shown). $Hrt2^{KO/KO}$ mice displayed a complete penetrance of VSDs, whereas we observed no VSDs in six adult $Hrt2^{loxP/KO}$;*Nkx2–5-Cre* mice. However, the hearts of adult $Hrt2^{loxP/KO}$;*Nkx2–5-Cre* mice were grossly enlarged with an aberrant architecture of the ventricular chambers in which the base of the dilated right ventricle (RV) merged with the interventricular septum above the apex of the heart (Fig. 2). Histological analysis of $Hrt2^{loxP/KO}$;*Nkx2–5-Cre* embryos at various stages revealed that this morphological defect was apparent by embryonic day (E) 13.5 and persisted in adult animals. A small fraction (2/7) of $Hrt2^{loxP/KO}$;*Nkx2–5-Cre* embryos showed VSDs or the delay of ventricular septal formation at E13.5–17.5 (Fig. 2).

The rarity of VSDs observed in $Hrt2^{loxP/KO}$;*Nkx2–5-Cre* mice suggested that cell populations other than cardiomyocytes contributed to the formation of VSDs observed in all $Hrt2^{KO/KO}$ mice. Consistent with this hypothesis, we observed that deletion of *Hrt2* with an *SM22-Cre* transgene, which is expressed in the heart until E10.5 and in a subset of arterial smooth muscle cells thereafter (23), resulted in VSDs and partial perinatal lethality, whereas deletion of *Hrt2* in the endothelium with *Tie2-Cre* did not evoke detectable defects of cardiac structure (Table 1 and data not shown). Thus, it seems likely that the VSDs of $Hrt2^{KO/KO}$ mice arise as a result of the deletion of *Hrt2* in smooth muscle cells or, possibly, from the combined deletion in smooth and cardiac muscle cells. Detailed comparison of the phenotypes in various conditional mutant mice will be described elsewhere.

Contractile Dysfunction Resulting from Cardiac Deletion of *Hrt2*. $Hrt2^{KO/KO}$ mice showed perinatal lethality in either an isogenic 129 or a mixed background, preventing the evaluation of cardiac function at adult stages. To study the effects of myocardial-specific *Hrt2* deletion on contractile function, we performed echocardiography on $Hrt2^{loxP/KO}$;*Nkx2–5-Cre* mice (hereafter referred to as $Hrt2^{CKO}$ mice) at 6 weeks of age (Fig. 3*A*). Myocardial deletion of *Hrt2* caused an increase in the systolic left ventricular internal diameter (LVIDs) and a corresponding deterioration in cardiac contractility, as indicated by decreased fractional shortening (FS) (Fig. 3*B*). The diastolic left ventricular internal diameter (LVIDd) did not change appreciably in mutant mice. Because the decrease in FS primarily reflects an increase in LVIDs, rather than an increase in both LVIDs and LVIDd as is associated with general dilative remodeling, the cardiac dysfunction in $Hrt2^{CKO}$ mice is likely to arise from contractile dysfunction.

Ectopic Activation of Atrial Genes in Ventricular Myocardium of $Hrt2^{CKO}$ Mice. In an effort to determine the molecular basis of cardiac dysfunction in $Hrt2^{CKO}$ mice, we compared the pattern of gene expression in wild-type and mutant hearts at 6 weeks of age by microarray analysis. Notably, atrial-enriched regulatory and structural genes, such as those encoding sarcolipin (Sln), myosin light chain (Mlc) 1a (*Myl4*), and Mlc2a (*Myl7*) were up-regulated in the ventricles of $Hrt2^{CKO}$ mice (Fig. 4*A*). Quantitative real-time PCR demonstrated that expression of all three genes increased >10-fold compared with wild-type littermates (Fig.

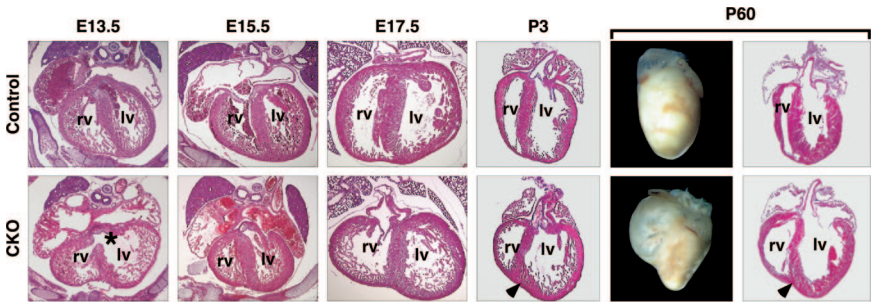

Fig. 2. Cardiac defects resulting from myocardial deletion of *Hrt2*. Whole-mount and H&E sections of hearts of wild-type and $Hrt2^{CKO}$ mice at various embryonic and postnatal (P) time points. Note the distention of the RV in the whole mount and the abnormal right ventricular chamber morphology in the H&E section of the mutant. Also note a VSD observed in one $Hrt2^{CKO}$ embryo at E13.5 denoted by *. The arrowhead indicates the base of the interventricular septum, which is shifted upward and toward the RV in the hearts of $Hrt2^{CKO}$ mice.

4*B*). In contrast, mRNA levels of *GATA4* and α-myosin heavy chain (Fig. 4*B*), *Mlc1v*, *Mlc2v*, and *Cx40* (data not shown), and phospholamban (SI Fig. 8) did not significantly change in the ventricles of $Hrt2^{CKO}$ mice. In addition, the hypertrophic markers b-type natriuretic peptide, α-skeletal actin (*Acta1*), and β-myosin heavy chain were unchanged in $Hrt2^{CKO}$ mice (data not shown).

We next examined the tissue distribution of the dysregulated genes by *in situ* hybridization. The expression of *Mlc1a* and *Mlc2a* was expanded in the ventricles of E17.5 $Hrt2^{CKO}$ mouse embryos, particularly in the interventricular septum and compact myocardium of both the LVs and RVs (Fig. 5). Wild-type mice expressed these genes predominantly in the developing atria, whereas low levels of expression were observed in the ventricular trabeculation. Expanded expression of *Mlc1a* and *Mlc2a* was also observed in the ventricles of E15.5 $Hrt2^{CKO}$ embryos (data not shown). The expression of *Sln* was observed ectopically in the compact myocardium of the ventricles of E17.5 $Hrt2^{CKO}$ mouse embryos (Fig. 5), whereas this transcript is virtually absent from ventricles of wild-type mice.

We also found the expression of atrial natriuretic factor (ANF) and *Tbx5* to be significantly increased in the left ventricles (LVs) of $Hrt2^{CKO}$ mice (Fig. 5). *ANF* and *Tbx5* normally display expression in the atria and trabecular cells of the LV in wild-type embryos. Because *Cx40* is also expressed predominantly in the atria and trabeculation of the LV, we examined the expression of this gene by *in situ* hybridization at E17.5 and quantitative RT-PCR in adult ventricular tissue. *Cx40* did not show an increase in expression in the ventricles (data not shown), suggesting that Hrt2 regulates a subset of atrial-enriched target genes.

Repression of Atrial Genes by Hrt2. To determine whether Hrt2 was sufficient to repress endogenous atrial gene expression, we infected atrial and ventricular cardiomyocytes with adenovirus expressing Myc-Hrt2 or GFP as a control (Fig. 6). Because *Sln*, *Mlc1a*, and *Mlc2a* were expressed at low levels in ventricular myocytes, it was difficult to measure further reduction of their expression by Hrt2. However, in atrial cardiomyocytes, the expression levels of *Sln*, *Mlc1a*, and *Mlc2a* all were repressed by adenovirus-mediated Hrt2 expression. Furthermore, a reduction in expression of *ANF* and *Tbx5* was apparent upon Hrt2 overexpression in both atrial and ventricular cardiomyocytes (data not shown). Importantly, *Cx40* did not display an alteration of expression levels in cardiomyocytes infected with myc-Hrt2 adenovirus, further demonstrating the specificity of Hrt2 target genes (Fig. 6). These observations are consistent with the increase in atrial gene expression observed in the $Hrt2^{CKO}$ mice and suggest that Hrt2 is both necessary and sufficient to limit the expression of atrial genes.

Discussion

Global deletion of *Hrt2* in mice results in myriad cardiac defects, which have been difficult to interpret because of the expression of *Hrt2* in numerous cell types (5, 7, 9), including cardiomyocytes, smooth muscle cells, and endothelial cells, all of which are required for cardiac development and function. Through the conditional deletion of *Hrt2* in each of these lineages, our results uncover a myocardial cell-autonomous function of Hrt2 in repression of atrial gene expression in the ventricular myocardium and maintenance of normal function of the adult heart. Misregulation of atrial genes in the ventricle likely contributes to the abnormalities in cardiac contractility resulting from cardiac-specific deletion of Hrt2.

Repression of Atrial Gene Expression by Hrt2. Based on the upregulation of multiple atrial genes including *Mlc1a*, *Mlc2a*, *Sln*, *ANF*, and *Tbx5* in ventricular cardiomyocytes of mice lacking cardiac expression of *Hrt2*, we conclude that Hrt2 is required to maintain ventricular identity and that activation of atrial genes represents a default gene program resulting from the absence of Hrt2-dependent repression. The atrialization of ventricular gene expression is likely to alter contractile properties that are required for ventricular function. For example, sarcomeric incorporation of atrial-specific MLCs in the ventricles of *RXRα* or *Mlc2v* null mice leads to a reduced LV ejection fraction (24, 25). *Sln* encodes an atrial-specific inhibitor of the cardiac sarcoplasmic reticulum Ca^{2+} ATPase SERCA2a and suppresses Ca^{2+} uptake into the sarcoplasmic reticulum (26). Notably, overexpression of *Sln* in the ventricle in transgenic mice leads to reduced cardiac contractility and heart failure (27–29). Misregulation of these genes has also been associated with human cardiac pathologies. For example, SLN levels have been shown to be reduced in patients with atrial fibrillation (30), and MLC1a and

Table 1. Consequences of conditional *Hrt2* deletion

Cre transgene	Cell type of deletion	Cardiac phenotype	Viability
Nkx2–5-Cre	Cardiomyocytes	RV dilation and contractile dysfunction	Viable
SM22-Cre	Smooth and cardiac muscle	RV dilation and contractile dysfunction; VSDs	Perinatal lethal
Tie2-Cre	Endothelial cells	Normal	Viable

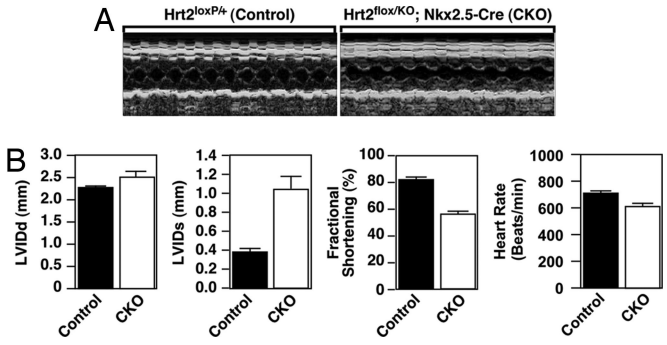

Fig. 3. Functional defects resulting from myocardial deletion of *Hrt2*. (*A*) Representative M-mode images of control mice or *Hrt2*^{CKO} mice at 6 weeks of age demonstrate an increase in LVIDs, which results in a decrease in cardiac function. (*B*) Bar graph representations of LVIDd, LVIDs, FS, and heart rate indicate that cardiac removal of Hrt2 reduces systolic function and attenuates cardiac contractility.

MLC2a up-regulation has been observed in human cardiomyopathies (31, 32). Thus, the up-regulation of these atrial genes, and *Sln* in particular, in ventricular myocardium could contribute to the functional defects observed in the *Hrt2*^{CKO} mice. Atrialization of gene expression in the ventricle may also play a role in the distended RV phenotype observed in the hearts of *Hrt2*^{CKO} mice. It is interesting to note that retrovirus-mediated misexpression of *Tbx5* in the presumptive RV results in a shift of the interventricular septum to the right and a distended and hypoplastic RV (33). The abnormal RV phenotype accompanying the ectopic expression of atrial-enriched genes, especially *Tbx5* in the LV of *Hrt2*^{CKO} mice, may suggest a role for Hrt2 in the specification or maturation of the RV and LV. It is curious that Hrt1, which shares a high structural similarity to Hrt2, is expressed in atrial myocytes (9), but appears not to repress endogenous atrial gene expression. Mice homozygous for *Hrt1* deletion are viable and have not been reported to display an increase in atrial gene expression, as might be expected (34, 35). One possibility is that ventricle-restricted corepressors, such as the homeodomain protein Irx4, which has been implicated in suppression of atrial gene expression in ventricular myocardium,

A

Gene	Fold-Change (MicroArray)
Sarcolipin	8.7
Mlc2a	5.6
Mlc1a	4.9
Tbx5	2.2
ANF	1.6

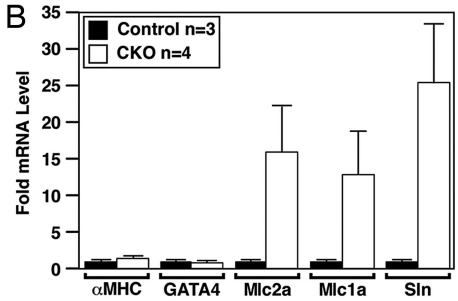

Fig. 4. Ectopic activation of atrial genes in ventricles of *Hrt2* mutant mice. (*A*) Microarray analysis showing the fold up-regulation of atrial genes in the ventricles of *Hrt2*^{CKO} mice at 6 weeks of age. (*B*) Quantitative RT-PCR analysis showing up-regulation of the atrial-specific genes *Mlc1a*, *Mlc2a*, and *Sln* in ventricles of 6-week-old *Hrt2*^{CKO} mice. The expression of *GATA4* and *αMHC* are not altered. The error bars represent standard deviation.

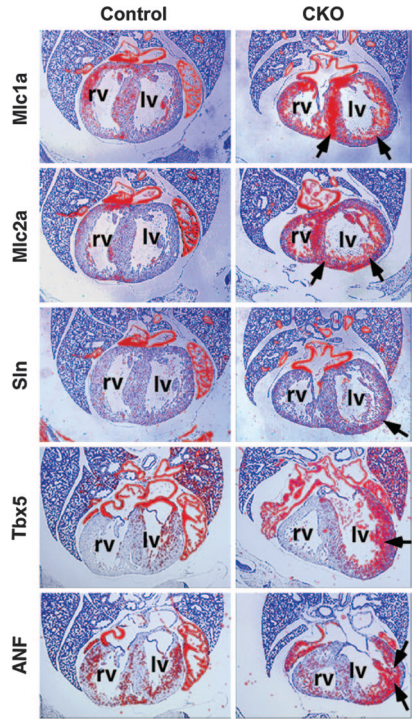

Fig. 5. Expression of atrial genes in *Hrt2* mutant mouse embryos. *In situ* hybridization of E17.5 hearts showing the ectopic expression of *Mlc1a*, *Mlc2a*, *Sln*, *ANF*, and *Tbx5* in ventricles of *Hrt2*^{CKO} mice (*Right*) compared with control mice (*Left*). Arrows depict ectopic expression in the ventricle.

mediate the negative influence of Hrt2 on atrial-specific genes (36–40). Alternatively, amino acid differences between Hrt1 and Hrt2 might confer unique functions to the proteins, possibly allowing the association of Hrt2 with specific corepressors or other transcriptional regulators.

The configuration of transcription factor binding sites in atrial-specific genes may also confer Hrt2-specific responsiveness. The *ANF* promoter, for example, contains a 17-bp element that seems to impart intrinsic repressive activity specifically in postnatal ventricular cardiomyocytes (41). Mutation of this element in the *ANF* promoter abolishes atrial specificity and results in robust expression in the ventricles of transgenic *Xenopus* embryos (42). It is interesting to note that sequences upstream of the *Sln*, *Mlc1a*, and *Mlc2a* genes contain a similar combination of cis elements, including conserved binding sites for GATA factors, which serve as sensitive targets for repression by Hrt2 (13).

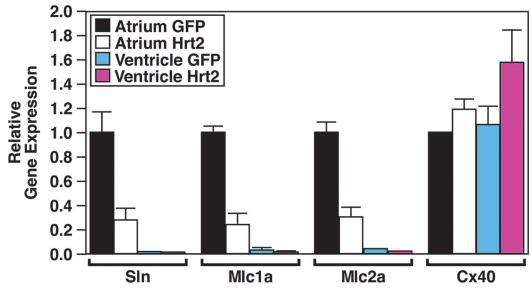

Fig. 6. Hrt2 activity is sufficient to repress atrial genes in cardiomyocytes. Quantitative RT-PCR analysis shows repression of atrial-specific genes in rat neonatal atrial and ventricle cardiomyocytes infected with adenovirus expressing *Myc-Hrt2*. The error bars represent standard deviation.

Regulation of Cell Fate Decisions by Hrt Proteins and Notch Signaling. Hrt proteins function as targets of Notch signaling, which regulates binary cell fate decisions during embryogenesis (43). The role of Hrt2 in repression of atrial gene expression in ventricular cardiomyocytes is reminiscent of its proposed role in determining arterial versus venous identity, as shown in zebrafish lacking expression of *gridlock*, the orthologue of *Hrt2*, which display the inappropriate development of venous instead of arterial endothelial cells from vascular precursors (44–46). However, mutations in genes encoding Notch family members or their ligands have not, to our knowledge, been reported to induce atrial gene expression in the ventricular myocardium. Thus, it remains to be determined whether repression of the atrial gene program by Hrt2 reflects a role of Notch signaling in this process or occurs through a Notch-independent mechanism. In this regard, the recent demonstration that members of the *Hrt*/*Hey* family of genes act downstream of the BMP–Smad pathway (47) raises the possibility that other signaling pathways may also use these factors to regulate downstream target genes.

Conclusions and Implications. Although we have focused here on the consequences of cardiac deletion of *Hrt2*, the conditional allele of the *Hrt2* gene will make it possible to determine the role of *Hrt2* in a temporal and tissue-specific manner and uncover possible functional redundancies with other members of the *Hrt* family. In this regard, mice lacking *Hrt1* are viable and do not display obvious phenotypic abnormalities, whereas *Hrt1*/*Hrt2* double mutant mice die during embryogenesis from global vascular deficiencies (34, 35). Combining the conditional *Hrt2* allele with an *Hrt1* null allele should permit the identification of additional cellular processes that rely on the expression of these genes in specific cell types. In the future, it will be interesting to determine whether mutations in *Hrt2* contribute to congenital heart defects and whether Hrt2 modulates cardiac contractility in humans as predicted from the phenotype of *Hrt2* mutant mice.

Addendum. After completion of this work, a complementary study (48) showed that global deletion of *Hrt2* in mice also resulted in up-regulation of atrial genes in the ventricular myocardium. Cardiac-specific expression of *Hrt2* from a transgene was sufficient to normalize gene expression in the ventricular chambers, consistent with our conclusion that *Hrt2* acts in a ventricular myocyte autonomous manner to repress the atrial gene program.

Materials and Methods

Gene Targeting. Details of gene targeting and generation of mutant mice are described in *SI Text*.

Conditional Gene Deletion with Cre Transgenic Mice. Heterozygous $Hrt2^{neo\text{-}loxP}$ mice were intercrossed with *hACTB*::*FLPe* transgenic mice to remove the neomycin resistance cassette in the germ line (49). The following Cre transgenic mice were used to delete the conditional *Hrt2* null allele: *Nkx2.5-Cre* (22), *SM22-Cre* (23), and *Tie2-Cre* (50).

Histology and *in Situ* Hybridization. Histological analyses and *in situ* hybridization were performed by standard procedures as described (9). Details are described in *SI Text*.

Echocardiography. Cardiac function and heart dimensions were evaluated by 2D echocardiography in conscious mice using a Vingmed System (GE Vingmed Ultrasound, Horten, Norway) and an 11.5-MHz linear array transducer. The data were analyzed by a single observer blinded to mouse genotype.

Cardiomyocyte Cell Culture and Adenovirus Infections. Primary rat cardiomyocytes were prepared as described (51), except that the atrial cardiomyocytes were isolated along with ventricle cardiomyocytes. Forty-eight hours after plating, cells were infected with adenovirus for 3 h in 10% FBS containing media at 50 multiplicities of infection. After an additional 48 h, the cells were harvested, and RNA was isolated for RT-PCR. *GFP*- and *Myc-Hrt2*-expressing adenoviruses have been described (13, 52).

RNA Purification, Microarray Analysis, and Real-Time PCR. Total RNA was purified from mouse ventricular tissues and cultured cardiomyocytes by using TRIzol (Invitrogen, Carlsbad, CA) as described (13). Microarray analysis was performed with a Mouse Genome 430 2.0 array (Affymetrix, Santa Clara, CA). Real-time PCR was performed with the 7000 Sequence Detection System (Applied Biosystems, Foster City, CA).

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1. Harvey RP (2002) *Nat Rev Genet* 3:544–556.
2. Srivastava D, Olson EN (2000) *Nature* 407:221–226.
3. Olson EN (2006) *Science* 313:1922–1927.
4. Fishman MC, Chien KR (1997) *Development (Cambridge, UK)* 124:2099–2117.
5. Leimeister C, Externbrink A, Klamt B, Gessler M (1999) *Mech Dev* 85:173–177.
6. Kokubo H, Lun Y, Johnson RL (1999) *Biochem Biophys Res Commun* 260:459–465.
7. Chin MT, Maemura K, Fukumoto S, Jain MK, Layne MD, Watanabe M, Hsieh CM, Lee ME (2000) *J Biol Chem* 275:6381–6387.
8. Iso T, Sartorelli V, Chung G, Shichinohe T, Kedes L, Hamamori Y (2001) *Mol Cell Biol* 21:6071–6079.
9. Nakagawa O, Nakagawa M, Richardson JA, Olson EN, Srivastava D (1999) *Dev Biol* 216:72–84.
10. Kageyama R, Ohtsuka T, Hatakeyama J, Ohsawa R (2005) *Exp Cell Res* 306:343–348.
11. Nakagawa O, McFadden DG, Nakagawa M, Yanagisawa H, Hu T, Srivastava D, Olson EN (2000) *Proc Natl Acad Sci USA* 97:13655–13660.
12. Maier MM, Gessler M (2000) *Biochem Biophys Res Commun* 275:652–660.
13. Kathiriya IS, King IN, Murakami M, Nakagawa M, Astle JM, Gardner KA, Gerard RD, Olson EN, Srivastava D, Nakagawa O (2004) *J Biol Chem* 279:54937–54943.
14. King IN, Kathiriya IS, Murakami M, Nakagawa M, Gardner KA, Srivastava D, Nakagawa O (2006) *Biochem Biophys Res Commun* 345:446–452.
15. Iso T, Sartorelli V, Poizat C, Iezzi S, Wu HY, Chung G, Kedes L, Hamamori Y (2001) *Mol Cell Biol* 21:6080–6089.
16. Donovan J, Kordylewska A, Jan YN, Utset MF (2002) *Curr Biol* 12:1605–1610.
17. Gessler M, Knobeloch KP, Helisch A, Amann K, Schumacher N, Rohde E, Fischer A, Leimeister C (2002) *Curr Biol* 12:1601–1604.
18. Kokubo H, Miyagawa-Tomita S, Tomimatsu H, Nakashima Y, Nakazawa M, Saga Y, Johnson RL (2004) *Circ Res* 95:540–547.
19. Sakata Y, Kamei CN, Nakagami H, Bronson R, Liao JK, Chin MT (2002) *Proc Natl Acad Sci USA* 99:16197–16202.
20. Sakata Y, Koibuchi N, Xiang F, Youngblood JM, Kamei CN, Chin MT (2006) *J Mol Cell Cardiol* 40:267–273.
21. Kokubo H, Tomita-Miyagawa S, Hamada Y, Saga Y (2007) *Development (Cambridge, UK)* 134:747–755.
22. McFadden DG, Barbosa AC, Richardson JA, Schneider MD, Srivastava D, Olson EN (2005) *Development (Cambridge, UK)* 132:189–201.
23. Holtwick R, Gotthardt M, Skryabin B, Steinmetz M, Potthast R, Zetsche B, Hammer RE, Herz J, Kuhn M (2002) *Proc Natl Acad Sci USA* 99:7142–7147.
24. Dyson E, Sucov HM, Kubalak SW, Schmid-Schonbein GW, DeLano FA, Evans RM, Ross J, Jr, Chien KR (1995) *Proc Natl Acad Sci USA* 92:7386–7390.
25. Chen J, Kubalak SW, Minamisawa S, Price RL, Becker KD, Hickey R, Ross J, Jr, Chien KR (1998) *J Biol Chem* 273:1252–1256.
26. MacLennan DH, Asahi M, Tupling AR (2003) *Ann NY Acad Sci* 986:472–480.

27. Asahi M, Otsu K, Nakayama H, Hikoso S, Takeda T, Gramolini AO, Trivieri MG, Oudit GY, Morita T, Kusakari Y, *et al.* (2004) *Proc Natl Acad Sci USA* 101:9199–9204.
28. Babu GJ, Bhupathy P, Petrashevskaya NN, Wang H, Raman S, Wheeler D, Jagatheesan G, Wieczorek D, Schwartz A, Janssen PM, *et al.* (2006) *J Biol Chem* 281:3972–3979.
29. Gramolini AO, Trivieri MG, Oudit GY, Kislinger T, Li W, Patel MM, Emili A, Kranias EG, Backx PH, Maclennan DH (2006) *Proc Natl Acad Sci USA* 103:2446–2451.
30. Uemura N, Ohkusa T, Hamano K, Nakagome M, Hori H, Shimizu M, Matsuzaki M, Mochizuki S, Minamisawa S, Ishikawa Y (2004) *Eur J Clin Invest* 34:723–730.
31. Trahair T, Yeoh T, Cartmill T, Keogh A, Spratt P, Chang V, dos Remedios CG, Gunning P (1993) *J Mol Cell Cardiol* 25:577–585.
32. Lim DS, Roberts R, Marian AJ (2001) *J Am Coll Cardiol* 38:1175–1180.
33. Takeuchi JK, Ohgi M, Koshiba-Takeuchi K, Shiratori H, Sakaki I, Ogura K, Saijoh Y, Ogura T (2003) *Development (Cambridge, UK)* 130:5953–5964.
34. Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M (2004) *Genes Dev* 18:901–911.
35. Kokubo H, Miyagawa-Tomita S, Nakazawa M, Saga Y, Johnson RL (2005) *Dev Biol* 278:301–309.
36. Bao ZZ, Bruneau BG, Seidman JG, Seidman CE, Cepko CL (1999) *Science* 283:1161–1164.
37. Garriock RJ, Vokes SA, Small EM, Larson R, Krieg PA (2001) *Dev Genes Evol* 211:257–260.
38. Houweling AC, Dildrop R, Peters T, Mummenhoff J, Moorman AF, Ruther U, Christoffels VM (2001) *Mech Dev* 107:169–174.
39. Bruneau BG, Bao ZZ, Fatkin D, Xavier-Neto J, Georgakopoulos D, Maguire CT, Berul CI, Kass DA, Kuroski-de Bold ML, de Bold AJ, *et al.* (2001) *Mol Cell Biol* 21:1730–1736.
40. Wang GF, Nikovits W, Jr, Bao ZZ, Stockdale FE (2001) *J Biol Chem* 276:28835–28841.
41. Durocher D, Chen CY, Ardati A, Schwartz RJ, Nemer M (1996) *Mol Cell Biol* 16:4648–4655.
42. Small EM, Krieg PA (2003) *Dev Biol* 261:116–131.
43. Lai EC (2004) *Development (Cambridge, UK)* 131:965–973.
44. Zhong TP, Rosenberg M, Mohideen MA, Weinstein B, Fishman MC (2000) *Science* 287:1820–1824.
45. Zhong TP, Childs S, Leu JP, Fishman MC (2001) *Nature* 414:216–220.
46. Weinstein BM, Stemple DL, Driever W, Fishman MC (1995) *Nat Med* 1:1143–1147.
47. Korchynskyi O, Dechering KJ, Sijbers AM, Olijve W, ten Dijke P (2003) *J Bone Miner Res* 18:1177–1185.
48. Koibuchi N, Chin MT (2007) *Circ Res* 100:850–855.
49. Rodriguez CI, Buchholz F, Galloway J, Sequerra R, Kasper J, Ayala R, Stewart AF, Dymecki SM (2000) *Nat Genet* 25:139–140.
50. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M (2001) *Dev Biol* 230:230–242.
51. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN (1998) *Cell* 93:215–228.
52. Rybkin II, Markham DW, Yan Z, Bassel-Duby R, Williams RS, Olson EN (2003) *J Biol Chem* 278:15927–15934.