Ventricular septal defect and cardiomyopathy in mice lacking the transcription factor CHF1/Hey2

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Ventricular septal defects are common in human infants, but the genetic programs that control ventricular septation are poorly understood. Here we report that mice with a targeted disruption of the cardiovascular basic helix–loop–helix factor (*CHF*)*1*/*Hey2* gene show isolated ventricular septal defects. These defects result primarily in failure to thrive. Mice often succumbed within the first 3 wk after birth and showed pulmonary and liver congestion. The penetrance of this phenotype varied, depending on genetic background, suggesting the presence of modifier genes. Expression patterns of other cardiac-specific genes were not affected. Of the few animals on a mixed genetic background that survived to adulthood, most developed a cardiomyopathy but did not have ventricular septal defects. Our results indicate that CHF1 plays an important role in regulation of ventricular septation in mammalian heart development and is important for normal myocardial contractility. These mice provide a useful model for the study of the ontogeny and natural history of ventricular septal defects and cardiomyopathy.

Ventricular septal defects (VSD) are the most common congenital cardiac defects in human infants, if bicuspid aortic valve is excluded (1). The ontogeny of atrioventricular septation is complex (2). Multiple primordia contribute to the formation of the interatrial and interventricular septa, including endocardial cushion tissue and myocardium. The muscular portion of the interventricular septum arises from ingrowth and folding of the myocardial wall of the developing ventricle, whereas the membranous portion of the interventricular septum is derived primarily from endocardial cushion tissue. The genetics of ventricular septation are poorly understood, and in human populations, no single gene disorder primarily causing isolated VSD exists. Isolated VSD can occur in patients heterozygous for mutations in Nkx2.5 (3) or Tbx5 (4); however, this phenotype is less common. In mice, a number of gene disruptions have been shown to cause VSDs, but all of these occur in the context of anomalies involving other cardiac structures such as the semilunar valves, ventricular outflow, compaction of myocardium, etc. (5–11). To our knowledge, no genes in mice or humans have been linked primarily to isolated ventricular septal defects without other associated anatomic findings.

Members of the cardiovascular basic helix–loop–helix factor (CHF) family of hairy-related transcription factors (also called gridlock, Hey, Hesr, Hrt, and HERP) have been suggested to play important roles in cardiovascular development (12–17). In zebrafish, the gridlock mutation is associated with coarctation of the aorta (13). Loss-of-function and gain-of-function studies using morpholino-antisense oligonucleotides or microinjected RNA showed that gridlock expression favors the development of arteries instead of veins from undifferentiated vascular precursors (18). CHF1 (also called Hesr2, Hey2, Hrt2, and HERP1, henceforth to be referred to as CHF1) is expressed in both the developing ventricle and vasculature and has been shown to function as a transcriptional repressor (12, 19, 20). To date, genetic evidence for a critical function of CHF1 or any of its relatives in mammalian cardiovascular development has not been reported. This study aimed to address this question by creating mice containing a targeted deletion in the *CHF1/Hey2* gene. Here we report that mice deficient in CHF1 show isolated VSDs and will provide a model system to study the ontogeny and natural history of VSDs.

Congestive heart failure is a growing public health concern, because the incidence and prevalence continue to rise worldwide (21). In mice, numerous structural genes have been associated with dilated cardiomyopathy (22–25). The transcriptional cascades that are important for normal myocardial contractility have been only partially characterized. Irx4, expressed specifically in the ventricle, has been linked to contractile dysfunction in adult mice containing a targeted disruption of the gene (26). cAMP-response element-binding protein (CREB), an important transcription factor in response to cAMP, has also been linked to contractile dysfunction in transgenic mice that overexpress a dominant negative isoform (27). Here we report that the mice lacking CHF1 who survive to adulthood are also prone to the development of a cardiomyopathy in the absence of VSDs, which suggests that these mice have an intrinsic myopathy.

Methods

Cloning of the Gene and Generation of CHF1 Knockout Mice. We screened a commercially available λ FIX II 129/SvJ mouse genomic DNA library (Stratagene) with the mouse cDNA for CHF1 (12). We constructed a targeting vector designed to replace the 3-kb fragment containing the proximal promoter, exons 1 and 2 and the majority of exon 3, with the neomycin resistance gene. Electroporation of embryonic stem (ES) cells, isolation of neomycin-resistant colonies, and initial Southern blot screening were performed commercially on a fee-for-service basis (DNX, Cranbury, NJ). Clones containing the targeted mutation were injected into C57BL/6 blastocysts to generate chimeric mice by the core transgenic facility of Brigham and Women's Hospital. Male chimeras were crossbred with C57BL/6 or 129/SvJ females, and agouti offspring were genotyped by Southern blotting and PCR.

Histological Analysis of Mouse Tissue. Mouse tissue was fixed in Bouin's fixative. Tissues were embedded in paraffin and sectioned at 6 μm by standard methods. Tissues were stained with hematoxylin/eosin by standard methods.

RNA Preparation, RT-PCR, and Northern Blot Analysis. RNA was prepared with the RNeasy kit (Qiagen, Germantown, MD). Tissues were homogenized in RNeasy homogenization buffer and purified on RNeasy columns according to the manufacturer's instructions. For RT-PCR analyses, cDNA was synthesized from 1 μg of total RNA of embryonic day (E)18.5 mouse cardiac ventricles with an oligo-dT primer by Advantage RT for PCR kit (BD Biosciences, Palo Alto, CA). One-thirtieth of the single-strand cDNA products were used for each PCR amplification with 27–35 cycles. Primer sets were designed from mouse cDNA sequences as follows: 5′-gacaactacctctcagattatggc-3′ and 5′-cgggagcatgggaaaagc-3′ for CHF1/Hey2, 5′-catgaagagagct-

Abbreviations: CHF1, cardiovascular basic helix–loop–helix factor 1; VSD, ventricular septal defect; E*n*, embryonic day *n*.

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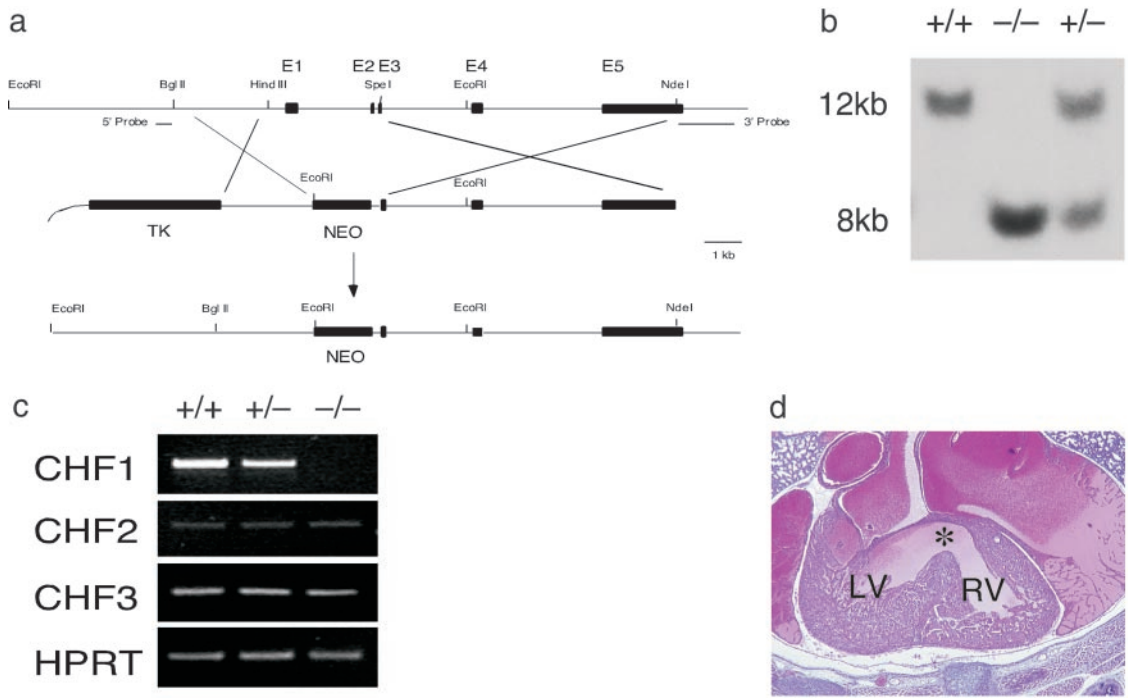

Fig. 1. Targeted disruption of the CHF1/Hey2 gene. (*a*) The gene for CHF1 consists of five exons within ≈15 kb of genomic DNA. The targeting construct will remove 551 bp of upstream promoter, exons 1 and 2, and the majority of exon 3. These exons encode the N terminus, the basic region, and the majority of the helix–loop–helix domain. (*b*) Southern blot showing DNA fragments generated after digestion of wild-type and knockout genomic DNA and probing with the 5′ probe. (*c*) RT-PCR showing the absence of CHF1 mRNA expression and no compensatory increase of CHF2 and CHF3 expression in cardiac ventricles of E18.5 knockout mice. (*d*) Ventricular septal defect in E18.5 embryo.

cacc-3′ and 5′-aatgtgtccgaggccac-3′ for CHF2/Hey1, 5′-atggacccatcgatgtgg-3′ and 5′-aaaggccagggcactgg-3′ for CHF3/HeyL, 5′-ctcgaagtgttggatacagg-3′ and 5′-tggcctataggctcatagtg-3′ for HPRT, 5′-ctgctgcagaggttattcctcg-3′ and 5,-ggaagagtgagcg-gcgcatcaagg-3 for α cardiac myosin heavy chain (αMHC), 5′-tgcaaaggctccaggtctgagggc-3′ and 5′-gccaacaccaacctgtccaagttc-3′ for β cardiac MHC (βMHC), 5′-tgttcctcacgatgtttggg-3′ and 5′-ctcagtccttctcttctccg-3′ for myosin light chain 2v (MLC2v), 5′-tgcagaaggcagtggagctggacaaagcc-3′ and 5′-ttgcacttgtagcgacg-gttctggaaccag-3′ for Nkx2.5/Csx, 5′-cactatgggcacagcagctcc-3′ and 5′-ttgcagctggcctgcgatgtc-3′ for GATA4, and 5′-gccctgagtct-gaggacaag-3′ and 5′-atcgtgttcttgctgccag-3′ for myocyte enhancer factor (MEF)2C. Northern blotting and hybridization were performed by using adult heart RNA, as described (12). Probes for CHF1, Nkx2.5, MLC2v, Tbx5, and dHAND have been described (12, 28–31). The probe for atrial natriuretic peptide (ANP) was generated by RT-PCR from mouse RNA with the oligonucleotide primers 5′-gatctgatggatttcaagaac-3′ and 5′-gctctgggctccaatcctgtc-3′. The probe for CHF1 spans nucleotides 76–1164 of the cDNA (GenBank accession no. AF173902) and contains the entire coding sequence, so that any potential protein-encoding transcripts will be detected.

Mouse Echocardiography. CHF1 knockout and wild-type mice were anesthetized with i.p. pentobarbital (50 mg/kg mouse body weight), which induced anesthesia with spontaneous respiration. Echocardiography was performed with a commercially available system (Sonos 4500; Hewlett–Packard) and a 6- to 15-MHz transducer. To assess the degree of anesthesia, heart rate was monitored before echocardiographic measurement. A heart rate of >500 was considered appropriate.

Results

Targeted Disruption of the CHF1/Hey2 Locus Results in VSDs. To determine whether CHF1 plays an important role in cardiovascular development, the *CHF1/Hey2* gene was cloned, mapped, and disrupted in ES cells. The gene consists of five exons within ≈15 kb of DNA, in agreement with published reports (19, 32). Targeted disruption was accomplished by replacement of a 3-kb genomic fragment with a neomycin-resistance gene (Fig. 1 *a* and *b*). This deletion removes the 551-bp proximal promoter region, exons 1 and 2 and the majority of exon 3, encoding amino acids 1–75, which includes the initiation codon and the majority of the basic helix–loop–helix domain. This strategy led to the complete absence of RNA derived from the coding sequence (Figs. 1*c* and 3*b*). After blastocyst injection and germline transmission, $CHF1^{+/-}$ mice were intercrossed to generate $CHF1^{-/-}$ mice. Of 196 neonatal pups studied, the number of homozygous knockout mice present at birth did not differ significantly from the expected Mendelian ratio (Table 1).

Wild-type, heterozygous, and knockout mice were not grossly distinguishable from each other at birth. Histological analysis of wild-type and knockout mice demonstrated the presence of VSDs consistently, in the absence of other morphological findings (Figs. 1*d* and 2*a*). VSDs were present in eight of nine E18.5 and neonatal knockout hearts from 129/SvJ mice that were serially sectioned. On a mixed background, four of six mice had a VSD. The VSDs were striking in that the membranous septum appeared to be completely absent. Other structures, such as

Table 1. Genotypes of CHF1 knockout mice at birth and 3 wk

Background	Age	(+/+)	(+/−)	(−/−)	Total	χ^2
Mixed	Birth	46	111	39	196	P = NS
Mixed	3 wk	57	117	22	196	$P < 0.001$
129/SvJ	Birth	54	117	46	217	P = NS
129/SvJ	3 wk	83	174	5	262	$P < 0.001$

NS, not significant.

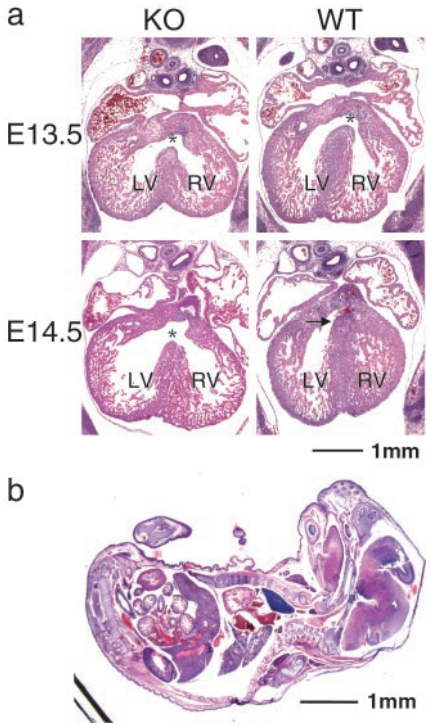

Fig. 2. CHF1 knockout mice have ventricular septal defects but no other organ pathology. (*a*) Transverse section through thorax of knockout and wild-type mouse at E13.5 and E14.5. KO, knockout; WT, wild type; LV, left ventricle; RV, right ventricle. *, VSD. Note that ventricular septation was completed in E14.5 wild-type mouse (arrow). (*b*) Sagittal section of E18.5 knockout mouse. Morphological structure of brain, lung, liver, kidney, intestines, and other organs appeared normal.

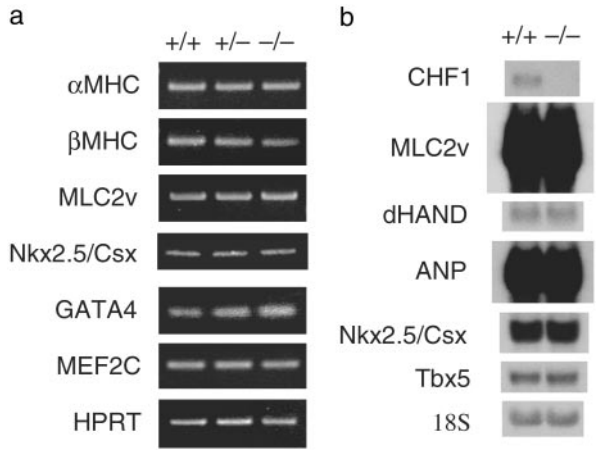

Fig. 3. CHF1 knockout mice have no obvious defect in cardiac gene expression. (*a*) RT-PCR analysis for the E18.5 cardiac ventricle RNA. (*b*) Northern blotting for adult heart RNA. Note that expression of the cardiac specific transcription factors and contractile proteins was normal in knockout mice.

semilunar valves, atrioventricular valves, and aortic and pulmonary vessels, appeared normal. In addition, the myocardium appeared to be normal in thickness and trabeculation, with normal coronary arteries. The aorta itself showed no evidence of coarctation along its length. The absence of other cardiac anomalies indicates that the developmental defect occurs late during cardiac morphogenesis and does not affect looping or alignment of the cardiac chambers and great vessels.

Analysis of cardiac-specific gene expression showed no perturbation in the expression of αMHC, βMHC, MLC2v, Nkx2.5/Csx, GATA4, MEF2C, dHAND, ANP, and Tbx5 (Fig. 3). These findings suggest that the cardiac myocyte differentiation program is not perturbed. Analysis of stage-specific embryos demonstrated the failure of the interventricular septum to close by E14.5, whereas it had closed in the wild-type animals (Fig. 2*a*). These findings indicate that the defects seen result from failure of septal morphogenesis rather than inappropriate apoptotic cell death after septation.

In contrast to other knockout mice that show VSDs, the phenotype of the CHF1 knockout mouse is notable for the relative paucity of associated anatomical findings. The presence of an isolated VSD in the absence of other disorders of alignment or valvular malformation closely mimics the most common septal defects seen in human disease.

Mice Lacking CHF1 Show Failure to Thrive. After birth, many of the homozygous knockout mice developed a profound wasting syndrome, resulting in death before weaning (Table 1, Fig. 4*a*). To assess the effect of genetic background, mice were also inbred into the 129/SvJ strain. Postnatal mortality was more severe, with fewer pups surviving to weaning (Table 1). The timing of death was variable, with most deaths occurring within the first week after birth. All of the dead animals that were recovered after 2 days of life showed severe growth retardation compared with littermates. To determine the cause of death in the knockout mice, we performed necropsies on recovered bodies. The dying pups usually showed milk in the stomach, despite their profound wasting syndrome. Gut, thymus, and spleen morphology and other organs appeared normal (Fig. 2*b* and data not shown). Most pups showed severe pulmonary and liver congestion, suggestive of heart failure (Fig. 4 *c* and *d*).

In the dying mice, VSDs were present, as expected (Fig. 4*b*). Large VSDs are known to cause failure to thrive in human infants (33). In the absence of any additional organ pathology, it is likely that VSDs present in these mice are responsible for their failure to thrive. Prenatally, the fetal lungs have high pulmonary vascular resistance, so that septation defects will not lead to significant left-to-right shunting. After birth, as the lungs inflate and pulmonary resistance falls, left-to-right shunting will be significant, leading to poor functional capacity and probably poor feeding on that basis. These mice may therefore serve as a useful model for studying both the ontogeny and the natural history of large VSDs. Some important discrepancies exist, however, between this mouse model and human disease, particularly the mortality seen in mice. Potential reasons for this discrepancy are discussed below.

Mice Lacking CHF1 Develop Cardiomyopathy. On a mixed genetic background, knockout mice were sometimes able to survive to adulthood. Some of these mice were runted, whereas others were normal in size. In contrast, on a 129/SvJ background, the few mice that survived to weaning did not live long into adulthood. To assess the cardiac function of surviving mice, echocardiography was performed. At ≈12 wk of age, the majority of the surviving mice on the mixed background began to develop systolic contractile dysfunction with preserved left ventricular wall thickness (Table 2). By 40 wk, 8 of 11 knockout mice developed cardiomyopathy (data not shown). In addition, they developed subendocardial calcification (Table 2 and Fig. 4 *e* and *f*), which is sometimes associated with cardiomyopathy in mice and humans (34–36). These findings suggest that CHF1 is also required for normal myocardial contractility. Surprisingly, of the eight knockout mice with cardiomyopathy studied, none had demonstrable VSDs, either by echocardiography or by dissection and histopathology (data not shown).

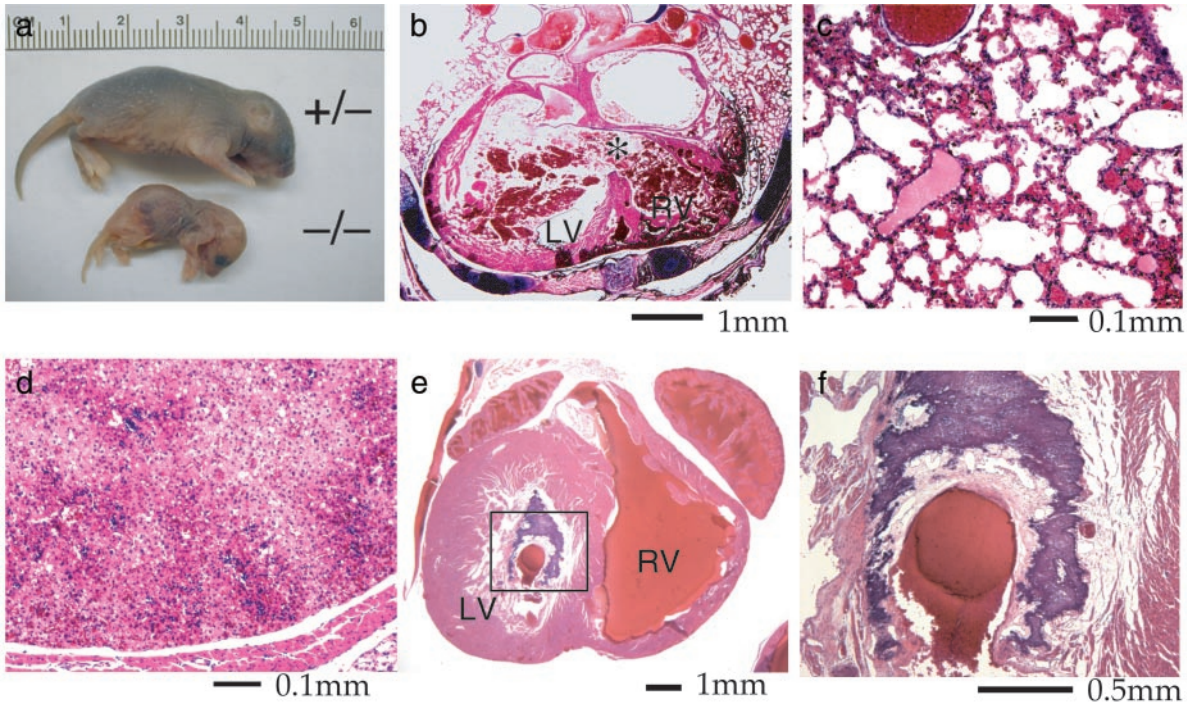

Fig. 4. CHF1-deficient mice develop failure to thrive. (*a*) A 4-day-old knockout mouse, compared with a heterozygous littermate. Wild-type and heterozygotes from the same litter were indistinguishable. (*b–d*) A 5-day-old runted mouse was found dead. Sections stained with hematoxylin/eosin showed a large VSD (indicated by *, *b*), pulmonary congestion (*c*), and liver congestion (*d*), suggesting the presence of heart failure in association with a VSD. (*e*) Pathology of a knockout mouse that survived into adulthood but died at 12 weeks of age. Note the presence of biventricular enlargement, suggesting the presence of cardiomyopathy. (*f*) Higher-power view of the same knockout heart showing endocardial calcification and interstitial fibrosis. RV, right ventricle; LV, left ventricle.

Discussion

The hairy-related transcription factors comprise a large family of diverse proteins involved in many aspects of development (37). The CHF/Hey subfamily has been postulated to be involved in cardiovascular development, based on expression pattern, loss of function in zebrafish, and transcriptional regulation (12–16, 38). CHF1, in particular, has been implicated in the development of the ventricle, vasculature, somites, and retina (12, 13, 15, 16, 18, 39–41). Our work describes a knockout of a member of the CHF/Hey subfamily of hairy-related transcription factors in mammals and demonstrates an essential role in cardiac septation and myocardial function. No obvious anatomical defects were seen in the vasculature (data not shown), in contrast with the gridlock phenotype seen in zebrafish (13). The reasons for this disparity are unclear but may be due to redundancy of Hey genes in mice. In addition, no obvious defect in somitogenesis or retinal development was seen (data not shown).

Table 2. Echocardiography of CHF1 knockout mice

	Wild type	CHF1 −/−	*P* value
Number	9	7	–
Age, weeks	17.9 ± 1.2	17.9 ± 0.63	NS
HR, bpm	613 ± 97	600 ± 62	NS
WThd, mm	1.01 ± 0.79	1.03 ± 0.94	NS
LVDs, mm	1.81 ± 0.44	3.20 ± 0.96	<0.01
LVDd, mm	3.21 ± 0.47	4.07 ± 0.86	<0.05
FS	0.44 ± 0.12	0.22 ± 0.14	<0.01
Calc (N/Mi/Mo/S)	7/2/0/0	0/0/3/4	<0.01

Results were expressed as mean value ± SD, if applicable. Statistical analysis was performed by using an unpaired Student's *t* test for age, HR, WThd, LVDs, and FS, and a Mann–Whitney U test for calcification. A *P* value of <0.05 was considered statistically significant. Abbreviations: HR, heart rate; bpm, beats per minute; WThd, diastolic wall thickness; LVDs, systolic left ventricular dimension; LVDd, diastolic left ventricular dimension; FS, fractional shortening; Calc, grade of subendocardial calcification; N/Mi/Mo/S, none/mild/moderate/severe; NS, not significant.

One caveat to our study is that only one targeted clone was incorporated into the germline. We cannot rule out the possibility of additional mutations within this one ES cell clone that may result in the phenotype. In addition, the retained neomycin resistance cassette in the targeted allele may affect transcription of neighboring genes. While this work was in revision, however, two other groups independently reported targeting the same locus using different constructs and different ES cell lines and generated overlapping phenotypes. In one case, the predominant feature is cardiomyopathy, but ventricular septal defects are also seen (42), whereas in the other case, VSD is associated with pulmonary valve stenosis and tricuspid atresia (43). These reported findings make a spurious result unlikely; however, they also require explanation of the variations in phenotype seen. We have looked specifically for valvular lesions in our mice and have not found them to date. These discrepancies in phenotype may be secondary to differences in genetic background. They also suggest that CHF1/Hey2 may have a broader role in cardiovascular development and endocardial cushion remodeling that is unmasked in varying genetic contexts.

The mechanism by which membranous ventricular septal defects arise is not known. Environmental influences, such as alcohol exposure, are known to play a role (44). No other genes have been shown to affect the ventricular septum exclusively, but many, such as NFATc (5, 6), FOG-2 (7, 8), endothelin-1 (45), Tbx5 (4), Nkx2.5 (46), BMP6 and BMP7 (10), RXRα (47), and Tll1 (48), have been shown to affect ventricular septation as part of a larger constellation of congenital cardiac abnormalities. In

all cases, homozygous deletion of these factors leads to embryonic lethality. CHF1-deficient mice, in contrast, have a more restricted phenotype where VSD is the predominant anatomical feature. CHF1 may therefore be an important downstream effector of septation. Further investigation of upstream regulators and downstream target genes of CHF1 will provide insights into the molecular mechanisms of ventricular septation. At the tissue level, the anatomic defect is likely due to failure of the fused endocardial cushions to extend inferiorly to the muscular component of the interventricular septum. Our previous work (12) demonstrated expression of CHF1 in the ventricular myocardium at E8.5 persisting through adulthood, and our unpublished data demonstrate expression in the endocardial cushions, both consistent with a role in ventricular septation.

Clinically, isolated VSDs can lead to failure to thrive. To date, no mouse model to study the genes and environmental influences that contribute to the development of VSD has been developed. The CHF1/Hey2-deficient mice also exhibit failure to thrive, as is seen in human infants. Although we favor a causal relationship between VSD and the failure-to-thrive syndrome, other mechanisms that contribute to this syndrome cannot be excluded. The variable penetrance seen suggests the presence of modifier genes, consistent with a multigenic etiology. Our work provides a knockout mouse model of isolated ventricular septal defect and a system for dissecting further the genetic and environmental pathways that control ventricular septation. Of note, the human gene has been mapped to 6q22.2–6q22.33, a locus that has not yet been associated with congenital heart disorders.

One important disparity between this mouse model and VSDs seen in human infants is the high mortality of mice compared with patients. In human patients, the spectrum of isolated VSD disease is quite variable, by anatomic location and by size. In our mouse model, the VSDs are localized to the perimembranous region and generally are large. A greater percentage of mice, therefore, would be predicted to have significant left-to-right shunting. We also believe that the discrepancy in mortality most likely reflects the extreme sensitivity of mice to left-to-right shunting, given their high heart rates of 600. In other mouse models of predominant left-to-right shunting, such as patent ductus arteriosis (PDA), for example, neonatal lethality is also seen (49). PDA is well tolerated in human infants, in contrast. Another potential exacerbating factor is that CHF1 knockout mice have an intrinsic myopathy, which would likely increase mortality, because the ventricle is unable to compensate for the shunt associated with a VSD.

The development of cardiomyopathy in these mice suggests a link between CHF1-dependent transcriptional regulation and myocardial function, especially in the absence of VSDs. Although it remains possible that VSDs were present in these mice earlier in life but closed spontaneously, and that this may have contributed to their decrease in myocardial function, we favor a primary effect on myocardial function. In mice, the majority of the genes associated with the development of dilated cardiomyopathy are cytoplasmic and structural (22–25). Other transcription factors implicated in the development of a cardiomyopathy in mice are the *Iroquois*-related factor Irx4 (26), the cAMP response element-binding protein CREB (27), and the serum response factor SRF (50). Our findings raise the possibility that CHF1 regulates a critical transcriptional cascade for myocardial contractile function. In the future, it will be essential to determine how CHF1 transcriptional programs overlap with those of other transcription factors to regulate expression of downstream target genes that affect myocardial contractility.

Our study links the transcription factor CHF1 to the development of the interventricular septum and to cardiac function. The transcriptional networks that regulate these processes have not been extensively characterized. Our mouse model provides an entry point for elucidating the etiology of these complex processes and will allow further progress in understanding the molecular mechanisms of ventricular septation and cardiac contractility.

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