# The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner

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## Summary

**The basic helix-loop-helix transcription factors Hand1 and Hand2 display dynamic and spatially restricted expression patterns in the developing heart. Mice that lack Hand2 die at embryonic day 10.5 from right ventricular hypoplasia and vascular defects, whereas mice that lack Hand1 die at embryonic day 8.5 from placental and extra-embryonic abnormalities that preclude analysis of its potential role in later stages of heart development. To determine the cardiac functions of Hand1, we generated mice harboring a conditional *Hand1*-null allele and excised the gene by cardiac-specific expression of Cre recombinase. Embryos homozygous for the cardiac *Hand1* gene deletion displayed defects in the left ventricle and endocardial cushions, and exhibited dysregulated ventricular gene expression. However, these embryos survived until the perinatal period when they died from a spectrum of cardiac abnormalities. Creation of *Hand1/2* double mutant mice revealed gene dose-sensitive functions of Hand transcription factors in the control of cardiac morphogenesis and ventricular gene expression. These findings demonstrate that Hand factors play pivotal and partially redundant roles in cardiac morphogenesis, cardiomyocyte differentiation and cardiac-specific transcription.**

Key words: Mouse, Hand1, Hand2, Cardiac ventricles

## Introduction

Cardiac malformations resulting from abnormalities in development of the embryonic heart represent the most common form of birth defects and the most prevalent cause of miscarriages in humans (Hoffman and Kaplan, 2002). There has been substantial progress in defining the morphogenic events involved in heart formation and in identifying cardiac developmental control genes (reviewed by Fishman and Chien, 1997; Harvey, 2002; McFadden and Olson, 2002; Olson and Schneider, 2003). However, there are major gaps in understanding the interconnections between cardiogenic transcription factors and their downstream effector genes that mediate cardiac myogenesis, morphogenesis and function.

Heart development begins when mesodermal cells in a region of the embryo known as the cardiac crescent become instructed to adopt a cardiac fate in response to signals from adjacent tissues (Marvin et al., 2001; Schneider and Mercola, 2001; Schultheiss et al., 1997; Tzahor and Lassar, 2001) (reviewed by Olson, 2002). Cardiac precursors proliferate and migrate to the embryonic midline to form a linear heart tube that is segmentally patterned along its anteroposterior axis into regions ultimately giving rise to the atrial and ventricular chambers. Rightward looping of the linear heart tube followed by balloon-like growth of the outer curvatures of the ventricular segments generates the right and left ventricular chambers (Christoffels et al., 2000; Moorman et al., 2000). Notably, each cardiac chamber possesses distinct physiological functions and patterns of gene expression.

Recent studies have revealed two populations of cardiac precursor cells that contribute to different parts of the heart. The primary heart field is thought to give rise to the atrial chambers and left ventricular region. A second cardiogenic region, known as the anterior or secondary heart field, lies anterior and dorsal to the linear heart tube. Cells from this region are added to the developing heart tube and give rise to the outflow tract and right ventricular region (Mjaatvedt et al., 2001; Waldo et al., 2001) (reviewed by Kelly and Buckingham,

2002) (see also Cai et al., 2003). The existence of these two distinct populations of cardiac progenitors provides a potential explanation for many cardiac abnormalities in humans and model organisms in which specific segments of the heart are underdeveloped or deleted, leaving the remainder of the heart unaffected.

Several classes of transcription factors have been implicated in cardiac morphogenesis and gene regulation (reviewed by Bruneau, 2002; Firulli and Thattaliyath, 2002). Hand1 and Hand2 (also called eHAND/Thing-1/Hxt and dHAND/Thing-2/Hed, respectively) are basic helix-loop-helix (bHLH) transcription factors that display complimentary and overlapping expression patterns in the developing heart (Cross et al., 1995; Hollenberg et al., 1995; Cserjesi et al., 1995; Srivastava et al., 1995). In mice, *Hand2* is expressed throughout the linear heart tube. Thereafter, its expression is highest in the developing right ventricle (RV), with lower levels of expression in the atrial and left ventricular chambers (Thomas et al., 1998). Targeted mutation of *Hand2* in mice results in lethality at embryonic day 10.5 (E10.5) from right ventricular hypoplasia and vascular malformations (Srivastava et al., 1997; Yamagishi et al., 2000). By contrast, *Hand1* is expressed in segments of the linear heart tube destined to form the conotruncus and left ventricle (LV). At the onset of cardiac looping, *Hand1* expression becomes localized primarily to the outer curvature of the LV and outflow tract, with lower expression along the outer curvature of the developing RV (Biben and Harvey, 1997; Thomas et al., 1998). Mice lacking Hand1 die at E8-8.5 from severe placental and extra-embryonic defects, reflecting a role of Hand1 in trophoblast differentiation, and complicating analysis of potential cardiac functions (Firulli et al., 1998; Riley et al., 1998). Nevertheless, tetraploid aggregation experiments with wild-type and *Hand1* null embryonic stem (ES) cells have shown that mutant ES cells fail to contribute to the LV of chimeric mouse embryos. Such embryos survive until E10.5 when they exhibit abnormalities in cardiac looping (Riley et al., 1998; Riley et al., 2000). Interpretation of the phenotype of such chimeric embryos is complicated by possible extra-cardiac functions of Hand1 as well as the variable contribution of *Hand1* null cells to extra-embryonic tissues.

Studies in chick and zebrafish embryos have also revealed potential functions of Hand genes in cardiac development. Exposure of chick embryos to antisense oligonucleotides for Hand1 and Hand2 together, but not separately, perturbs heart development at the looping stage, suggesting that these factors act redundantly (Srivastava et al., 1995). The zebrafish genome appears to encode only a single Hand gene, most closely related to *Hand2*, and mutations in this gene in *hands off* mutants, result in a dramatic reduction in ventricular precursors (Yelon et al., 2000). This phenotype is more severe than that observed in *Hand2* knockout mice, possibly because of the lack of compensatory activity of a second *Hand* gene in this organism.

To determine the function of *Hand1* in mouse heart development without complications from early lethality owing to extra-cardiac functions, we generated mice harboring a conditional *Hand1* allele flanked by Cre recombinase loxP recognition sites, and deleted the gene specifically in the developing heart using a cardiac-specific α-myosin heavy chain (αMHC) promoter, or the *Nkx2.5* cardiac enhancer to express Cre recombinase (Cre). Mice lacking myocardial expression of *Hand1* survive until the perinatal period when they succumb to a spectrum of congenital heart defects that reflect abnormalities in ventricular growth and maturation. In addition, combination of the conditional *Hand1* mutation with a *Hand2* loss-of-function mutation revealed dose-sensitive effects on heart development. These results identify novel functions of Hand1 in heart development and demonstrate that Hand factors play crucial and partially redundant roles in cardiac growth, morphogenesis and gene expression.

## Materials and methods

### Gene targeting

A previously characterized *Hand1* genomic clone (Firulli et al., 1998), was used to generate the *Hand1loxP* targeting vector. A 2.9 kb fragment extending upstream from the *Hand1* 5′-UTR (long arm of homology) was amplified by high-fidelity PCR (Stratagene *Pfu* Turbo) and digested with *Xho*I. An *Eco*RI restriction site was engineered into the *Hand1* 5′UTR to facilitate genotyping by Southern blotting. This fragment was ligated into the pDelboy targeting backbone upstream of the FRT-flanked neomycin resistance cassette. A *Cla*I-*Kpn*I linked fragment containing the first exon of *Hand1*, extending from the *Hand1* 5′-UTR to the intron, was amplified using high fidelity PCR and cloned into pDelboy-LA. Finally, the short arm of homology, extending from the intron to a 3′ *Sal*I site, was PCR amplified and cloned into *Sal*I-*Eco*RI digested pDelboy-LAeH upstream of the thymidine kinase negative selection cassette. Integrity of the targeting vector was confirmed by restriction mapping and DNA sequencing. PCR primer sequences are available upon request.

The completed *Hand1*$^{NEO\text{-}loxP}$ targeting vector was linearized with *Not*I and electroporated into SM-1 ES cells. Following positive-negative selection with G418 and FIAU, resistant colonies were screened by Southern analysis of *Eco*RI digested genomic DNA using a probe (Fig. 1B) from the 3′ flanking region. Recombination of the 5′ arm was confirmed by *Eco*RI-*Kpn*I double digestion of genomic DNA, and Southern blotting with the short arm of homology. Three correctly targeted clones (clones E12, C12 and C5) were expanded and injected into C57BL/6 blastocysts, and transferred into the uteri of pseudopregnant females. Chimeric males were bred onto a C57BL/6 or Black/Swiss background for germline transmission. Males from clone E12 transmitted the targeted allele through the germline, therefore mice derived from this line were used in all analyses.

Heterozygous *Hand1*$^{NEO\text{-}loxP}$ mice were intercrossed with *hACTB::FLPe* transgenic mice (Rodriguez et al., 2000) in order to remove the neomycin resistance cassette in the germline. Removal of the neomycin cassette was confirmed by Southern blotting using *Eco*RI-digested genomic DNA (Fig. 1B). The *Hand1*$^{loxP}$ allele was bred to homozygosity, which did not affect viability or fertility of these mice, suggesting that the FRT and loxP sites in the 5′-UTR did not significantly alter expression from the targeted locus.

### Generation of *Nkx2.5::Cre* mice

A 2.5 kb fragment containing the *Nkx2.5* basal promoter and cardiac enhancer (Lien et al., 1999) was cloned upstream of the NLS-Cre expression cassette (gift of J. Herz). This vector was linearized using *Not*I and injected into fertilized oocytes as previously described (McFadden et al., 2000). Founder transgenic mice were genotyped by hybridization of *Eco*RV digested tail DNA to a *Cre* cDNA probe. Three transgenic lines were obtained and intercrossed with *ROSA26R* indicator mice (Soriano, 1999) in order to assess transgene expression and Cre-mediated recombination. Transgenic line Nk9 exhibited the earliest and most efficient recombination, and was used in all subsequent experiments.

### PCR genotyping

Tail and yolk sac DNA was isolated as previously described (McFadden et al., 2000). PCR reactions were used to detect Cre transgenes, and *Hand1* and *Hand2* knockout loci (Firulli et al., 1998; Srivastava et al., 1997). Briefly, 1 μl of tail or yolk sac DNA was used a template in 25 μl PCR reactions using Promega *Taq* polymerase and 4 mM $MgCl_2$. Thermal cycle reactions were as follows: 2 minutes at 95°C, 30 cycles of 30 seconds at 95°C, 30 seconds at 55°C, 45 seconds at 72°C and a final 5 minute extension at 72°C. Reactions were visualized on 1% agarose gels in TAE. Primer sequences are available upon request.

### RT-PCR

Left ventricles from E9.5 embryos were dissected and immediately frozen and stored in liquid nitrogen until embryo and yolk sac DNA was isolated and genotyped. Left ventricular tissue from eight mutant hearts was pooled and total RNA was isolated using Trizol reagent and standard protocols. Total LV RNA (150 ng) was used as a template for first strand cDNA synthesis using the Superscript first strand synthesis kit from Invitrogen. Five percent of the cDNA synthesis reaction was used as template for PCR reactions using Promega *Taq* polymerase to detect *Hand1* transcripts. Transcripts for hypoxanthine phosphoribosyl transferase (HPRT) were detected as a control. Thermal cycles were as follows: 94°C for 2 minutes, 28 cycles of 94°C 30 for seconds, 52°C for 30 seconds, 72°C for 30 seconds and a final 5 minute extension at 72°C. Reactions were visualized on 1% agarose gels in TAE. Primer sequences are available upon request.

### Histology

Embryos were harvested from timed matings and fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). Following fixation, embryos were rinsed in PBS then dehydrated through graded ethanols and embedded in paraffin wax as previously described (Moller and Moller, 1994). Histological sections were cut and stained with Hematoxylin and Eosin, or nuclear Fast Red as previously described (Moller and Moller, 1994).

### In situ hybridization

Section in situ hybridization was performed as described (Shelton et al., 2000). Whole-mount in situ hybridization was performed as previously described (Riddle et al., 1993). Plasmids for in situ probes have been previously described and were linearized and transcribed as follows: *ANF*, *Xho*I and T7 (Miller-Hance et al., 1993); connexin 40, *Asp*718 and T3 (Haefliger et al., 1992); *Tbx5*, *Spe*I and T7 (Bruneau et al., 1999); and *mlc2V*, *Bam*HI and T7 (O'Brien et al., 1993). The coding regions of *cited1* and *cited2* were amplified as *Cla*I-*Eco*RI fragments and subcloned into pBSK. Both plasmids were linearized with *Xho*I and transcribed with T3 RNA polymerase. *Hand2*- and *Hand1*-coding regions were amplified as *Eco*RI-*Xba*I fragments and cloned into pBSK. Both plasmids were linearized with *Eco*RI and transcribed with T7.

### β-Galactosidase staining

Embryos from timed matings were harvested and pre-fixed for 1-3 hours in 2% paraformaldehyde, 0.25% glutaraldehyde in PBS. Staining for β-gal activity was performed as previously described (McFadden et al., 2000).

### TUNEL and immunohistochemistry

TUNEL staining was performed on paraffin wax embedded sections from E10.5 and E13.5 according to the Promega Fluorescein Apoptosis detection kit.

Embryos were harvested at E11.5 and fixed overnight in 4% paraformaldehyde in PBS. Embryos were rinsed in PBS and equilibrated into 10% sucrose for 2 hours, followed by 30% sucrose overnight at 4°C. Embryos were transferred into freezing medium and frozen in isopentane and liquid nitrogen. Blocks were equilibrated to –20°C and serially sectioned. Sections were stored at –80°C until antibody staining. Antibody staining was performed as described (Frey et al., 2000). Primary anti-phospho histone H3 antibody was diluted 1:200 in 1% BSA in PBS.

## Results

### Generation of floxed *Hand1* alleles

To create a conditional *Hand1*-null allele, we flanked the first exon of the mouse *Hand1* gene with loxP sites by homologous recombination in ES cells (Fig. 1A). Our targeting strategy introduced a neomycin resistance cassette into the 5′-untranslated region of *Hand1*. Chimeric male mice generated from targeted ES cells transmitted the mutant allele through the germline yielding mice heterozygous for this $Hand1^{neo\text{-}loxP}$ allele. We bred heterozygous $Hand1^{neo\text{-}loxP/+}$ mice to mice expressing the FLPe recombinase in the male germline (Rodriguez et al., 2000) in order to remove the neomycin resistance cassette. F1 progeny from these matings were genotyped by Southern blotting to detect the recombined allele ($Hand1^{loxP}$) (Fig. 1A,B).

In order to determine if the $Hand1^{loxP}$ allele might function as a hypomorphic allele because of reduced expression, we bred mice heterozygous for this allele with mice heterozygous for the *Hand1*-null allele, referred to as $Hand1^{lacZ}$, described previously (Firulli et al., 1998). Adult trans-heterozygous $Hand1^{lacZ/loxP}$ mice bearing the two mutant alleles were overtly normal and fertile, suggesting that expression from the $Hand1^{loxP}$ allele was not significantly reduced. Homozygous $Hand1^{loxP/loxP}$ mice were also phenotypically normal and fertile, and were used in subsequent breedings.

### Early embryonic recombination in α*MHC::Cre* mice

In order to delete *Hand1* specifically in the heart, we crossed $Hand1^{loxP/loxP}$ females to $Hand1^{lacZ/+}$ heterozygous male mice harboring a transgene that expresses *Cre* under the control of the α-*myosin heavy chain* (α*MHC*) promoter. These mice have been reported to express *Cre* in the embryonic and adult myocardium (Agah et al., 1997; Gaussin et al., 2002). In order to define precisely the onset of Cre-mediated recombination during embryogenesis, we intercrossed α*MHC::Cre* transgenic mice to *ROSA26R* indicator mice, which harbor a conditional *lacZ* allele that requires Cre-mediated recombination for expression (Soriano, 1999). β-Galactosidase (β-gal) activity from the *ROSA26* locus was detected as early as E8.5 and by E9.0 expression was detected throughout the embryonic myocardium as well as in the outflow tract (Fig. 1C). At E11.5, both atrial and ventricular myocardium expressed high levels of *lacZ*. We did not detect *lacZ* expression elsewhere in these embryos.

To confirm that the $Hand1^{loxP}$ allele was efficiently recombined in vivo, we analyzed *Hand1* expression in α*MHC::Cre;* $Hand1^{loxP/lacZ}$ embryos at E10.5 by whole-mount in situ hybridization. As shown in Fig. 1D, *Hand1* transcripts were specifically absent from the embryonic heart of these embryos, whereas expression of *Hand1* was not affected in the branchial arches and lateral mesoderm. The absence of *Hand1* transcripts in the LV by E9.5 was also confirmed by RT-PCR of cardiac RNA from α*MHC::Cre;* $Hand1^{loxP/lacZ}$ embryos (Fig. 1E). These results demonstrated that efficient cardiac-specific removal of *Hand1* transcripts occurred before E9.5.

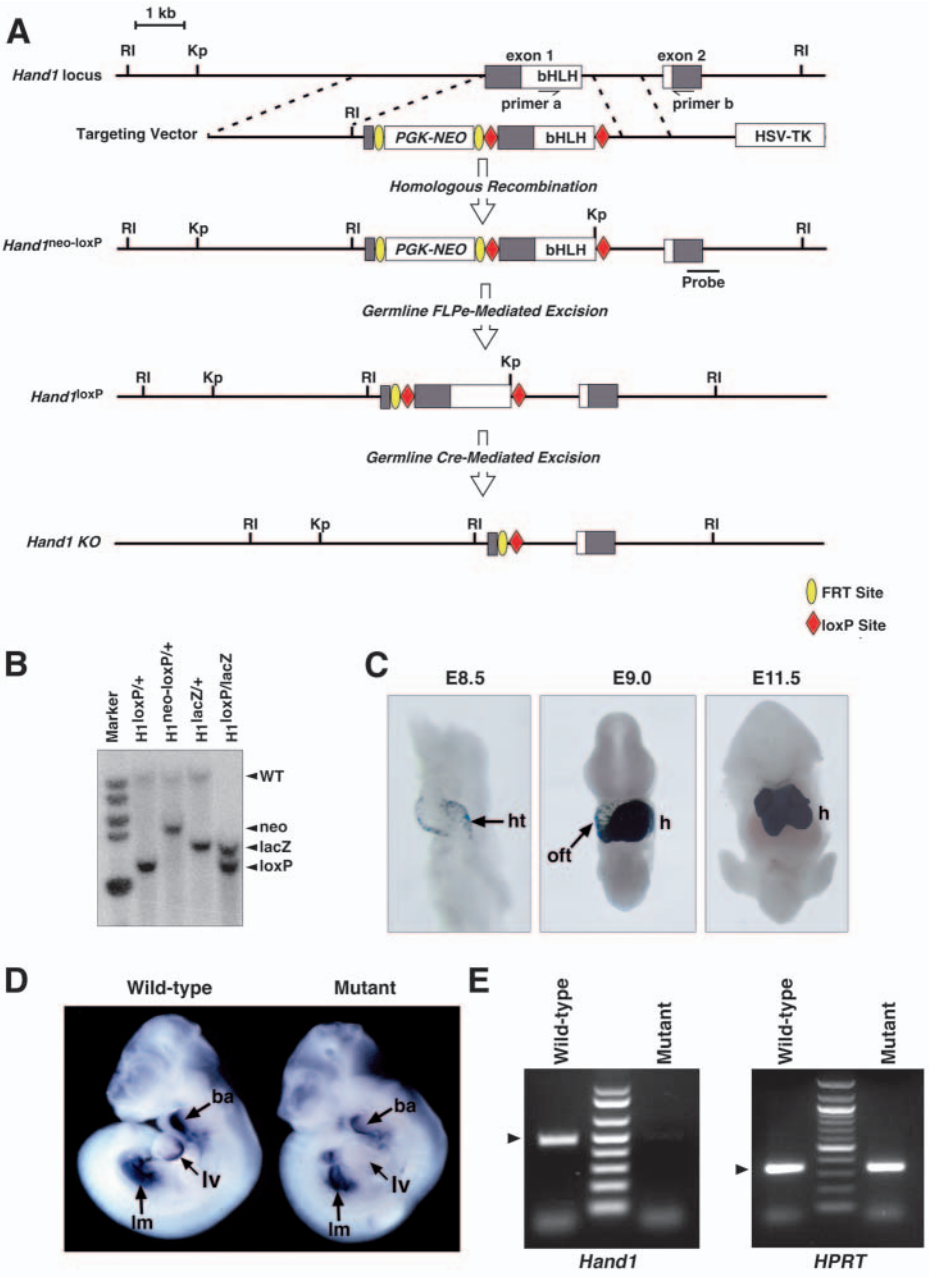


**Fig. 1.** Cardiac-specific Cre-mediated recombination of *Hand1*. (A) LoxP sites were inserted into the 5′-UTR and intron to flank the first exon of *Hand1*. The structure of the *Hand1* genomic locus, the targeting vector and the targeted allele are shown. The neomycin resistance cassette was removed in the mouse germline by breeding heterozygous mice to *hACTB::FLPe* transgenic mice. Cardiac-specific excision of exon 1 was achieved by breeding $Hand1^{loxP/loxP}$ mice to $Hand1^{LacZ/+}$ heterozygous mice harboring a transgene that expresses Cre under the control of *αMHC* promoter or *Nkx2.5* cardiac enhancer. Positions of the probe used for Southern analysis and the primers (a and b) used for RT-PCR are shown. Coding regions are shown in white and non-coding regions are shown in gray. (B) Detection of all four *Hand1* alleles by Southern blot analysis of *Eco*RI-digested genomic DNA, using the 3′ probe shown in A. (C) *αMHC::Cre* transgenic mice were intercrossed to *ROSA26R* indicator mice to determine the temporal and tissue specificity of recombination. Whole-mount photographs of β-gal stained embryos are shown. h, heart; ht, heart tube; oft, outflow tract. (D) Whole-mount in situ hybridization to *Hand1* transcripts at E10.5 in wild-type and $Hand1^{lacZ/loxP}$; *αMHC::Cre* (mutant) embryos. ba, branchial arch; lv, left ventricle; lm, lateral mesoderm. (E) Detection of *Hand1* and *Hprt* transcripts in RNA from the left ventricles of wild-type and $Hand1^{lacZ/loxP}$; *αMHC::Cre* (mutant) embryos at E9.5. Size markers are in the middle lane. *Hand1* transcripts were undetectable in the mutant. Transcripts for *Hprt* were detected as a control for RNA integrity and loading.

## Congenital heart defects resulting from cardiac deletion of *Hand1*

Genotyping of litters from intercrosses of $Hand1^{loxP/loxP}$ to *αMHC::Cre;* $Hand1^{lacZ/+}$ mice revealed that offspring with the *αMHC::Cre;* $Hand1^{loxP/lacZ}$ genotype were born at Mendelian ratios (data not shown), but the majority became cyanotic and died within 3 days of birth. This suggested that loss of Hand1 in the embryonic myocardium resulted in perinatal lethality. Newborn mice of the *αMHC::Cre*; $Hand1^{loxP/lacZ}$ genotype nursed normally. At postnatal day 10 (P10), only 4% of offspring had the *αMHC::Cre;* $Hand1^{loxP/lacZ}$ genotype (Table 1). Only three mice of the *αMHC::Cre;* $Hand1^{loxP/lacZ}$ genotype survived to adulthood (representing less than 2% of offspring from the above intercross, or less than 1/10 of the predicted number of such mice).

Histological sections of P1-2 hearts revealed a spectrum of congenital heart defects in *αMHC::Cre;* $Hand1^{loxP/lacZ}$ mice (Fig. 2A), including membranous ventricular septal defects (VSDs) (Fig. 2A, part b), overriding aorta (Fig. 2A, part d), hyperplastic atrioventricular (AV) valves (Fig. 2A, parts c,f), and double outlet right ventricle (data not shown). All mice of this genotype displayed valve defects and 90% had VSDs. A somewhat smaller fraction of these mice had outflow tract defects.

To determine the time of onset of cardiac malformations in *Hand1* mutants, we harvested embryos from timed matings and analyzed cardiac morphology by histological sectioning (Fig. 2B). The left ventricular chamber of the mutants was reduced in size at E11.5 and 13.5 (Fig. 2B); however, chamber size appeared to recover by birth (Fig. 2A). Abnormalities in the early ventricular septum were noted as early as E10.5 (data not shown), and were obvious by E11.5 (Fig. 2B, compare a with

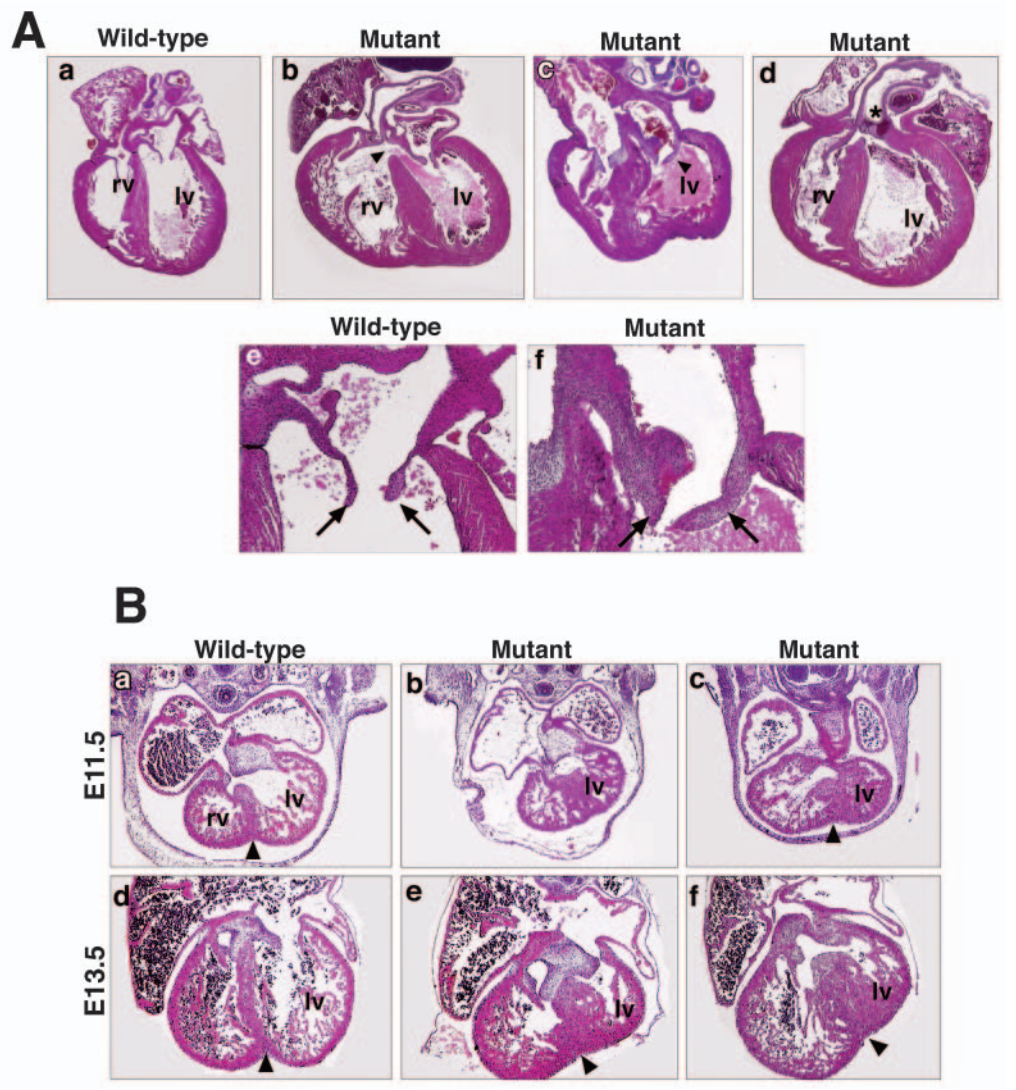


**Fig. 2.** Histology of *Hand1* mutant hearts. (A) Hearts from wild-type (a) and *Hand1* $^{lacZ/loxP}$; *αMHC::Cre* (mutant) (b-d) neonates were sectioned and stained with Hematoxylin and Eosin. A mutant heart with a membranous VSD (black arrowhead) is shown in b. A mutant heart with thickened AV valves is shown in c. A mutant heart with overriding aorta (asterisk) is shown in d. Higher magnification of the AV valves from wild type (e) and mutant (f) hearts. Arrows indicate the leaflets of the valves. (B) Hearts from wild-type (a,d) and *Hand1*$^{lacZ/loxP}$; *αMHC::Cre* (mutant) (b,c,e,f) embryos at E11.5 (a-c) and E13.5 (d-f) were sectioned and stained with Hematoxylin and Eosin. The mutant hearts show poorly organized ventricular septa and left ventricular hypoplasia. Immature endocardial cushions are also seen in mutant hearts at E13.5. Arrowheads indicate the interventricular septum.

b and c). At all developmental stages, the muscular ventricular septum of the mutant appeared thickened and disorganized. The embryonic AV endocardial cushions were also hyperplastic and, accordingly, the neonatal AV valves were thickened relative to those of wild-type hearts (Fig. 2A, compare e with f). Analysis of cell proliferation at E11.5 by staining with anti-phospho histone H3 antibody or BrdU labeling failed to reveal differences in the number of proliferating cells (data not shown). There was also no difference in the number of apoptotic cells observed by TUNEL assay in wild-type and *Hand1* mutant hearts at E9.5 or E11.5 (data not shown). These results suggest dysregulated cell proliferation or death after E10.5 is not responsible for the endocardial cushion defects observed in *Hand1* mutant embryos.

## Cardiac defects generated with an Nkx2.5::Cre transgene

Expression of *Hand1* is initially detected in the cardiac crescent at E7.75 (Cserjesi et al., 1995). Because the *αMHC::Cre* transgene does not direct high levels of *Cre* expression until after E8.5, it is possible that *Hand1* has an important role earlier in cardiac development, in which case the cardiac phenotype we observed in *αMHC::Cre; Hand1*$^{loxP/lacZ}$ mice could reflect the incomplete deletion of *Hand1* during early cardiogenesis. In an effort to eliminate cardiac *Hand1* expression at an earlier stage, we expressed Cre recombinase fused to a nuclear localization signal (NLS) under control of *Nkx2.5* regulatory sequences, which direct expression throughout the heart tube from the onset of cardiac commitment (Lien et al., 1999; Reecy et al., 1999). By E8.5, activity of these Nkx2.5 regulatory sequences is restricted to the developing ventricles and outflow tract. These sequences also direct expression in the thyroid primordium, and regions of the pharynx where *Hand1* is not expressed.

**Table 1. Genotypes of offspring at P10 from intercrosses of *Hand1*$^{loxP/loxP}$ to *αMHC::Cre*; *Hand1*$^{lacZ/+}$ mice**

| | KO/+ | Cardiac KO/KO | +/+ | Cardiac KO/+ |
|---|---|---|---|---|
| Number | 56 | 7 | 66 | 51 |
| Percent | 31 | 4 | 37 | 28 |

The *Hand1*$^{loxP/lacZ}$ genotype is referred to as KO/+, and this genotype with the *αMHC::Cre* transgene as cardiac KO/KO. Mice heterozygous for the *Hand1*$^{loxP}$ allele are referred to as +/+, and this genotype with the *αMHC::Cre* transgene as cardiac KO/+.

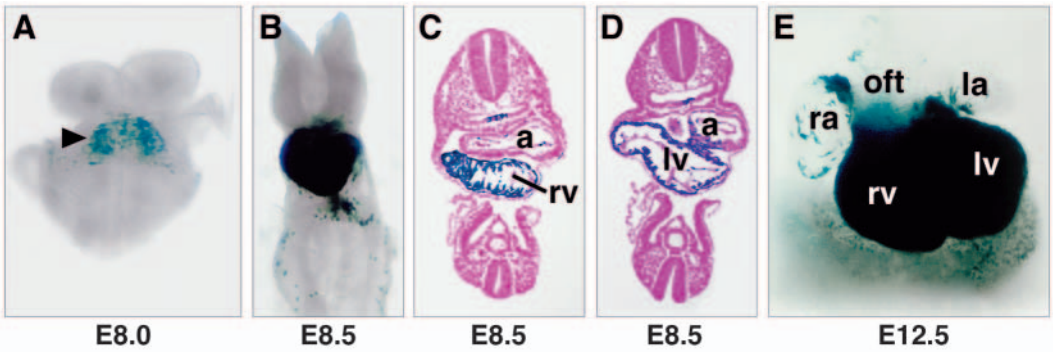


**Fig. 3.** Generation of *Nkx2.5::Cre* mice. (A,B) X-gal stained embryos showing staining in the linear heart tube (arrowhead) at E8.0 (A) and throughout the ventricular myocardium at E8.5 (B). (C,D) Nuclear Fast Red counterstained serial sections through the embryo in B. There are high levels of β-gal staining throughout the ventricular myocardium and in a subset of endocardial cells. (E) X-gal stained E12.5 embryo showing efficient ventricular recombination and minimal recombination in the outflow tract (oft) and atria (a). lv, left ventricle; rv, right ventricle; la, left atria; ra, right atria.

We generated six *Nkx2.5::Cre* transgenic lines, and crossed three lines into the *ROSA26R* heterozygous background to examine the efficiency and tissue-specificity of Cre-mediated recombination. All three lines exhibited a similar pattern of β-gal expression, which included heart, pharynx and a subset of cells within the liver (data not shown). We used a transgenic line (line 9) that directed the highest levels of recombination in the heart for all subsequent experiments. Efficient recombination was detected within the myocardium of *Nkx2.5::Cre* transgenic mice at the linear heart tube stage, and recombination occurred throughout the heart tube by E8.5 (Fig. 3A,B). Serial sections through stained E8.5 embryos revealed that the majority of cells in the right and left ventricular myocardium underwent recombination. At E10.5, the majority of cells within the LV were β-gal positive; however, some cells failed to express *lacZ*, presumably owing to a lack of *Cre* expression (data not shown). This may reflect downregulation of the *Nkx2.5* cardiac enhancer in the LV at later stages of cardiac development (Lien et al., 1999) or mosaicism of transgene expression. At E12.5, high efficiency of Cre-mediated recombination was observed in the RV and LV. Interestingly, the outflow tract failed to undergo recombination (Fig. 3E), which may reflect contribution of a secondary heart field not derived from the cardiac crescent to the outflow tract myocardium (Kelly et al., 2001; Waldo et al., 2001).

We generated and analyzed *Nkx2.5::Cre; Hand1*$^{loxP/lacZ}$ mice. Like mutant mice bearing the α*MHC::Cre* transgene, these mice were viable until 2-4 days after birth, at which point they became cyanotic and died. We did not observe any *Nkx2.5::Cre; Hand1*$^{loxP/lacZ}$ mice at adulthood. However, we cannot rule out that a small fraction of such mice might survive, as was seen with the α*MHC::Cre; Hand1*$^{loxP/lacZ}$ genotype. Histological sectioning of mutants revealed similar congenital heart defects to those observed in α*MHC::Cre; Hand1*$^{loxP/lacZ}$ embryos (data not shown). This result suggested that removal of *Hand1* transcripts before E8.5 did not cause embryonic lethality or exacerbate the phenotype observed in α*MHC::Cre; Hand1*$^{loxP/lacZ}$ embryos. Unless otherwise specified, we therefore used *Nkx2.5::Cre*

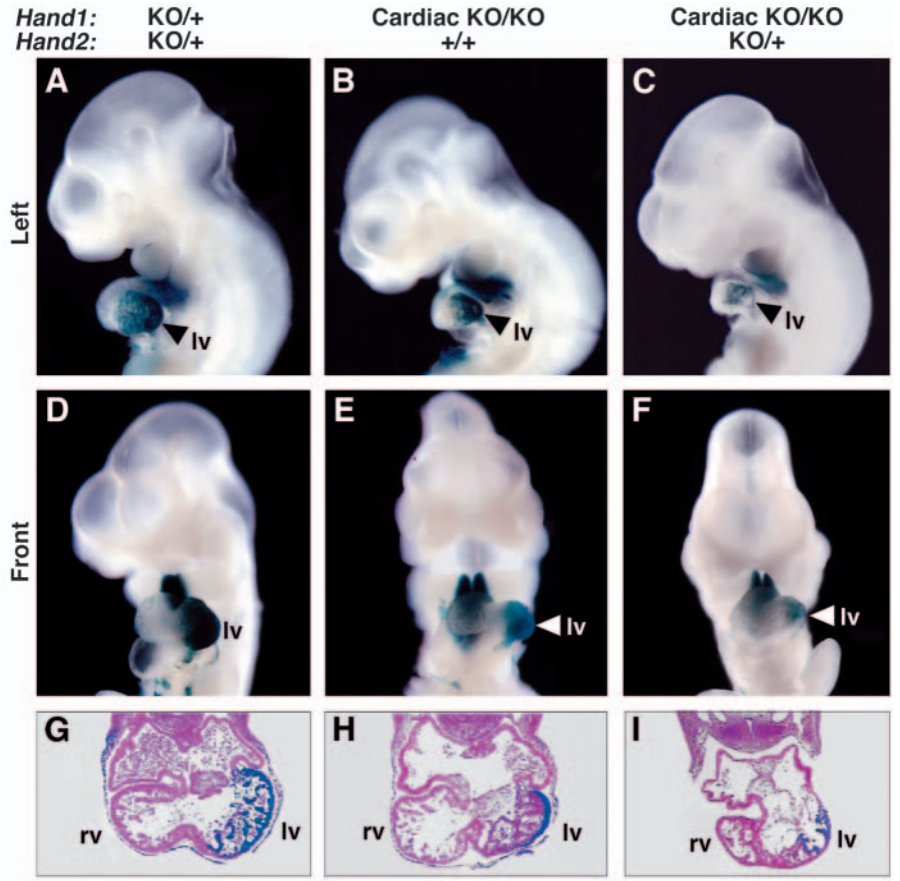


**Fig. 4.** Abnormal cardiac morphogenesis in E10.5 embryos with compound mutations in *Hand1* and *Hand2*. (A-F) E10.5 embryos stained for *lacZ* activity expressed from the *Hand1*$^{lacZ}$ allele. Left lateral view (A-C); frontal view (D-F). (G-I) Nuclear Fast Red counterstained transverse sections of the hearts of embryos in A-F. Genotypes of embryos are shown above each set of panels. There is severe reduction in *Hand1*-expressing cells in the LV and the hypoplastic ventricular chambers in H and I, and an absence of the interventricular groove and ventricular septation in I. lv, left ventricle; rv, right ventricle.

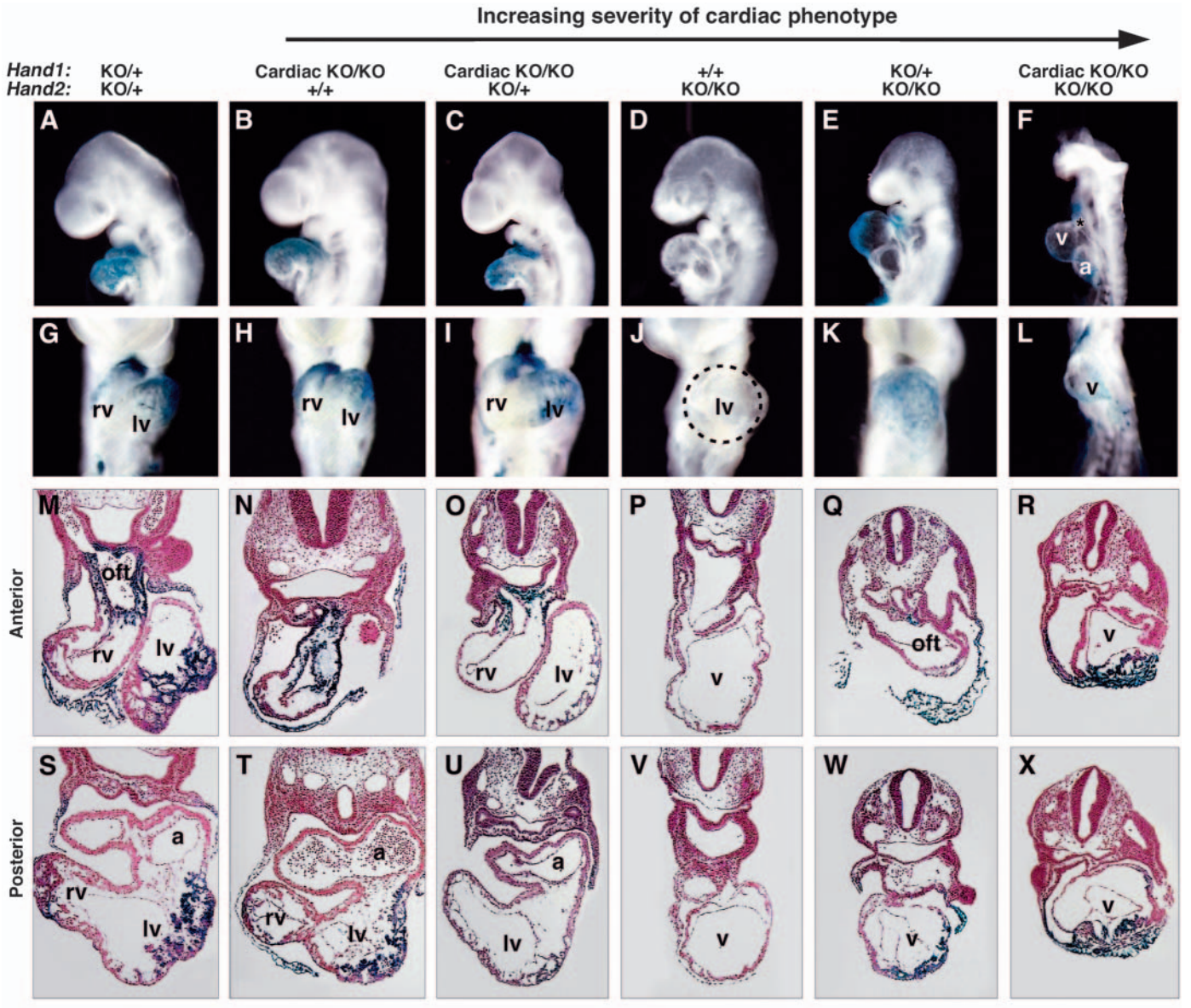


**Fig. 5.** Abnormal cardiac morphogenesis in E9.0 embryos with compound mutations in *Hand1* and *Hand2*. (A-L) E9.0 embryos stained for *lacZ* activity expressed from the *Hand1*$^{lacZ}$ allele. Left lateral view (A-F); frontal view of the heart (G-L). a, atrium; lv, left ventricle; rv, right ventricle; v, ventricular chamber. The asterisk in F shows the thin outflow tract present in the *Hand1* cardiac-KO/KO; *Hand2* KO/KO embryos. (M-X) Nuclear Fast Red counterstained transverse sections of embryos in A-L at anterior (M-R) and middle (S-X) levels of the heart. Genotypes of embryos are shown above each set of panels. Cardiac abnormalities increase in severity from left to right panels.

for the remainder of our analyses. Hereafter, we refer to the *Hand1*$^{loxP}$ allele in the presence of the *Nkx2.5::Cre* transgene as *Hand1* cardiac-KO and the *Hand1*$^{lacZ}$ allele as *Hand1* KO.

## Dose-sensitive requirements of *Hand1* and *Hand2* for left ventricular growth

We next addressed the possibility that *Hand1* and *Hand2* act in a functionally redundant fashion during cardiac development. In contrast to *Hand1*, which is expressed specifically in the developing LV and conotruncus, *Hand2* is expressed throughout the atrial and ventricular myocardium with highest levels of expression in the RV (Thomas et al., 1998). In *Hand2* mutant embryos, the RV is hypoplastic, but the LV forms, albeit with fewer trabeculations (Srivastava et al., 1995). Because of their overlapping expression in the LV, it is possible that *Hand2* may compensate for loss of *Hand1* in this region of the developing heart. To address this possibility, we reduced the level of *Hand2* expression by generating *Hand1* cardiac-KO/KO; *Hand2* KO/+ embryos from timed matings. Whereas *Hand1* cardiac-KO/KO mice survived until birth and *Hand2* KO/+ mice are normal, no embryos of the combined genotype were observed in litters harvested after E10.5. At E10.5, *Hand1* cardiac-KO/KO*; Hand2* KO/+ embryos were observed at Mendelian ratios, but appeared slightly delayed relative to *Hand1* cardiac-KO/KO littermates. Expression of *lacZ* from the *Hand1*$^{lacZ}$ allele was decreased in the LVs of *Hand1* cardiac-KO/KO embryos, and was further decreased in *Hand1* cardiac-KO/KO*; Hand2* KO/+ embryos, suggesting that expansion of the left ventricular chamber was

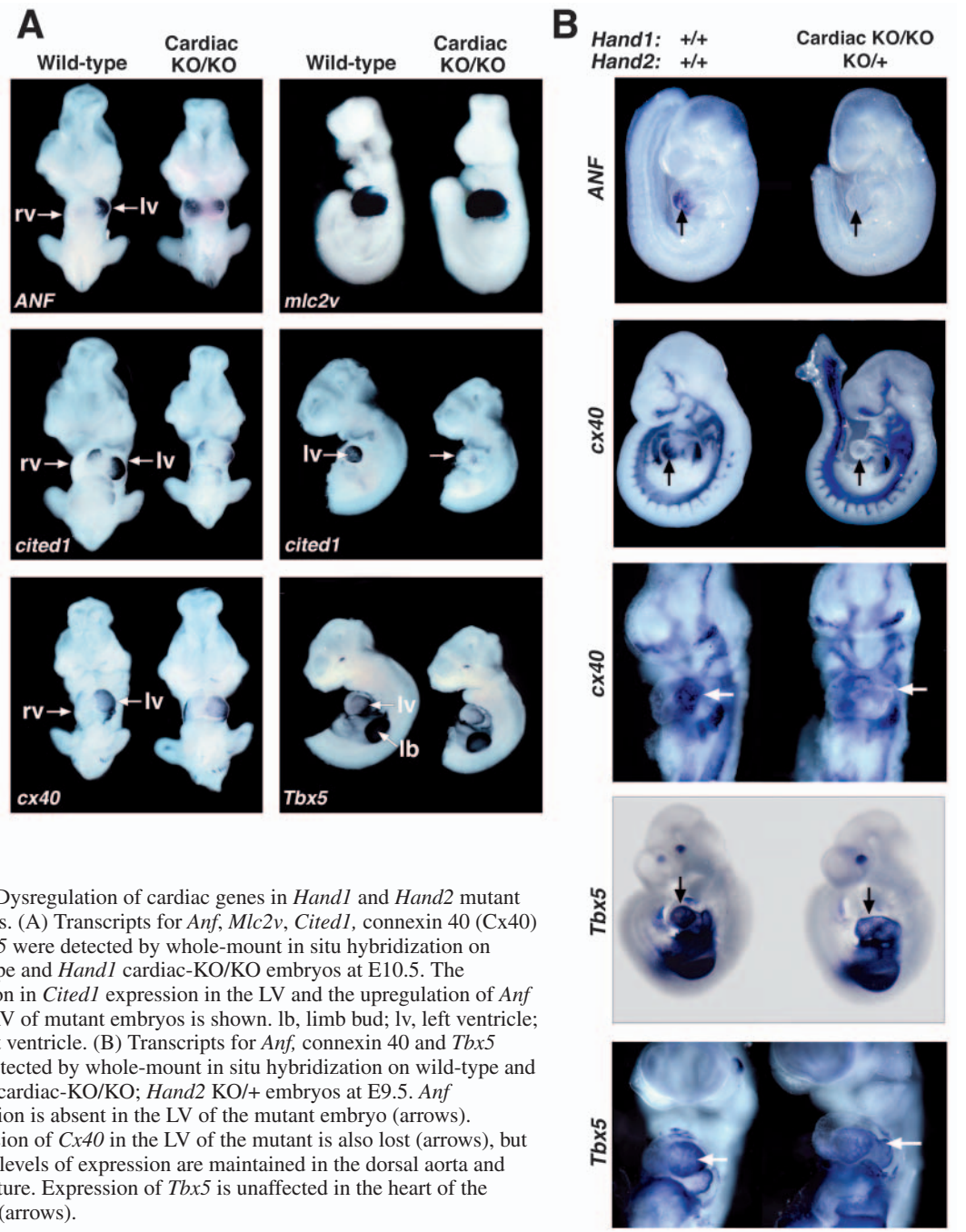


**Fig. 6.** Dysregulation of cardiac genes in *Hand1* and *Hand2* mutant embryos. (A) Transcripts for *Anf*, *Mlc2v*, *Cited1,* connexin 40 (Cx40) and*Tbx5* were detected by whole-mount in situ hybridization on wild-type and *Hand1* cardiac-KO/KO embryos at E10.5. The reduction in *Cited1* expression in the LV and the upregulation of *Anf* in the RV of mutant embryos is shown. lb, limb bud; lv, left ventricle; rv, right ventricle. (B) Transcripts for *Anf,* connexin 40 and *Tbx5* were detected by whole-mount in situ hybridization on wild-type and *Hand1* cardiac-KO/KO; *Hand2* KO/+ embryos at E9.5. *Anf* expression is absent in the LV of the mutant embryo (arrows). Expression of *Cx40* in the LV of the mutant is also lost (arrows), but normal levels of expression are maintained in the dorsal aorta and vasculature. Expression of *Tbx5* is unaffected in the heart of the mutant (arrows).

perturbed by the loss of Hand genes in a dose-sensitive manner (Fig. 4). Histological sectioning of *Hand1* cardiac-KO/KO; *Hand2* KO/+ embryos revealed a thin and poorly trabeculated myocardium (Fig. 4I). Thus, removal of one *Hand2* allele in the absence of *Hand1* generates an embryonic lethal defect in cardiac growth.

We also tested whether removal of one *Hand1* allele would exacerbate the *Hand2* null phenotype. Indeed, *Hand1* KO/+; *Hand2* KO/KO embryos were morphologically delayed relative to *Hand2* KO/KO embryos and died at E9.5, approximately 1 day earlier than *Hand2* KO/KO embryos (Fig. 5E,K). Histological analysis of *Hand1* KO/+; *Hand2* KO/KO embryos showed evidence of only a single ventricle with a poorly developed myocardium, as is characteristic of *Hand2* KO/KO embryos (Fig. 5Q,W). However, a prominent difference between *Hand1* cardiac-KO/+; *Hand2* KO/KO embryos and *Hand2* KO/KO embryos was the diminished size of the ventricular lumen (Fig. 5, compare V and W) owing to an increase in the amount of cardiac jelly, a dense extracellular matrix between the endocardium and myocardium.

Embryos homozygous for both mutant alleles (*Hand1* cardiac-KO/KO; *Hand2* KO/KO) displayed the most severe phenotype and did not survive beyond E9.0. The outflow tract and the atrial chamber were appropriately situated in these mutant embryos, indicating that looping of the heart tube had occurred (Fig. 5F), but there was only a single immature

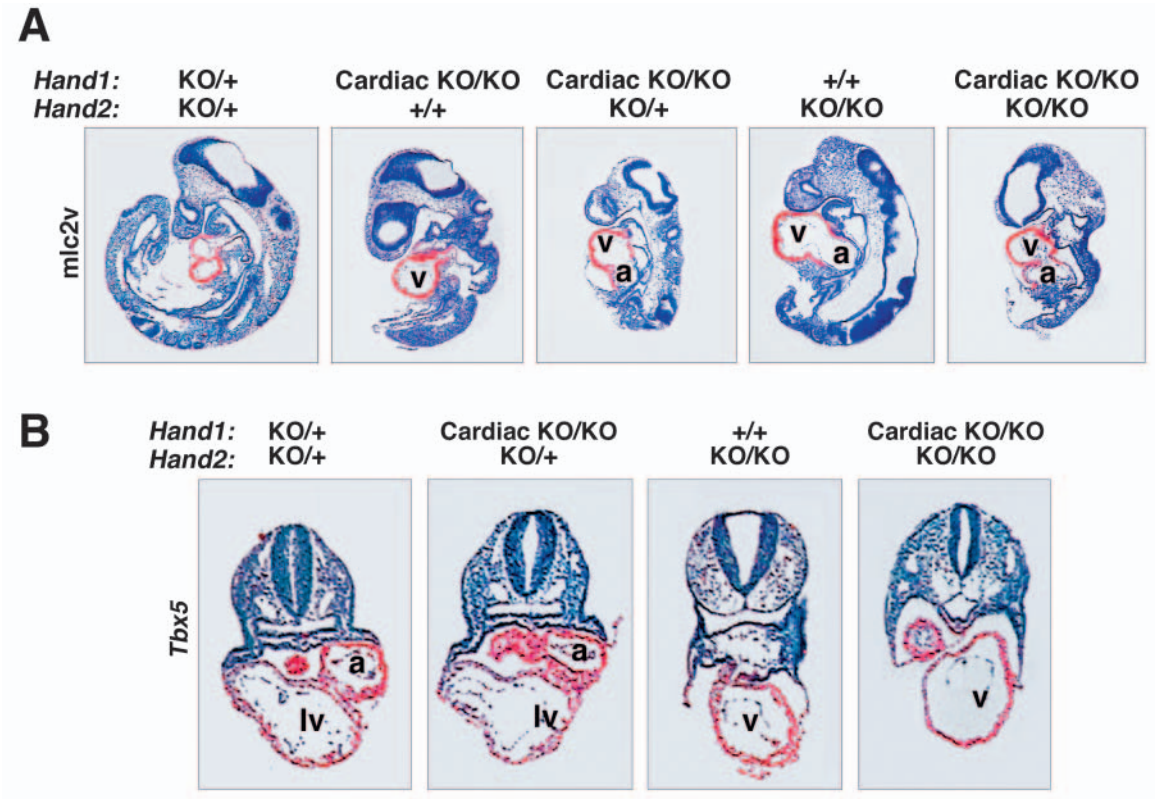


**Fig. 7.** Expression of *Mlc2v* and *Tbx5* in embryos with compound mutations in *Hand1* and *Hand2*. Expression of (A) *Mlc2v* and (B) *Tbx5* was examined by in situ hybridization to transverse or sagittal sections of E9.0 embryos of the indicated genotypes. Silver grains are pseudocolored in red. a, atrium; lv, left ventricle; v, ventricular chamber.

ventricle and a common atrial chamber. Histological analysis of the double mutants hearts showed that some segments of the myocardial wall of the ventricular chamber presented an abnormal cellular morphology resembling mesenchymal cells rather than cardiomyocytes (data not shown). In addition, the trabeculae were poorly developed and the lumen of the ventricular chamber was abnormally narrow (Fig. 5R,X).

## Dysregulation of ventricular gene expression in the absence of Hand genes

Several genes are expressed specifically along the outer curvature of the embryonic ventricles in patterns partially overlapping that of *Hand1*. Upregulation of these genes is thought to reflect the expansion of the chamber myocardium from the ventral surface of the more primitive linear heart tube myocardium (Christoffels et al., 2000). *Nkx2.5*-null embryos die at E10.5 from LV defects and fail to express *Hand1* in the heart, which has led to the suggestion that Hand1 might act as an important downstream mediator of Nkx2.5 function during cardiac morphogenesis (Biben and Harvey, 1997; Lyons et al., 1995; Tanaka et al., 1999). Thus, we examined expression of potential Nkx2.5 target genes and other markers of chamber myocardium in Hand mutant embryos to determine whether they were sensitive to the level of Hand gene expression.

Atrial natriuretic factor (*Anf*; *Nppa* – Mouse Genome Informatics) is expressed in the embryonic LV in a pattern similar to that of *Hand1* and is downregulated in the hearts of *Nkx2.5*-null embryos (Lyons et al., 1995). Expression of *ANF* in the LV was slightly reduced in hearts from *Hand1* cardiac-KO/KO embryos, but was dramatically upregulated in the RV of these mutant embryos (Fig. 6A). As *Hand1* is not expressed in the RV of wild-type embryos, we believe the upregulation of *Anf* expression in the RV of the mutant may reflect a secondary response, perhaps to cardiac stress. Expression of *Anf* was completely absent in the ventricles of both *Hand1* cardiac-KO/KO; *Hand2* KO/+ (Fig. 6B) and *Hand1* cardiac-KO/KO; *Hand2* KO/KO (data not shown) embryos. Thus, left ventricular expression of *Anf* displayed a dose-dependency on the level of *Hand1/2* expression, but was not absolutely dependent on Hand1.

Expression of ventricular myosin light chain 2 (*Mlc2v*; *Myl2* – Mouse Genome Informatics) has also been reported to be downregulated or absent in *Nkx2.5*-null hearts (Lyons et al., 1995; Tanaka et al., 1999). However, we detected no change in *Mlc2v* expression in *Hand1* cardiac-KO/KO embryos (Fig. 6A). *Mlc2v* transcripts were also detected in the ventricular remnant of *Hand1* cardiac-KO/KO; *Hand2* KO/KO double mutant embryos (Fig. 7A). We conclude that specification of ventricular cells occurs in the absence of Hand1 and Hand2, but ventricular expansion is perturbed. The expression of *Anf* and *Mlc2v* in *Hand1* cardiac-KO/KO embryos also indicates that Nkx2.5 regulates these genes independently of Hand1.

The transcriptional co-activator Cited1 is expressed in a pattern that overlaps almost perfectly with that of *Hand1* in the developing heart (Dunwoodie et al., 1998). Moreover, mice lacking the closely related factor Cited2 display congenital heart malformations similar to those we observed in *Hand1* mutants (Bamforth et al., 2001). Interestingly, expression of

*Cited1* was significantly downregulated in *Hand1* mutant hearts (Fig. 6A). Expression of *Cited2*, which is broader than that of *Cited1* during embryogenesis, was similar in wild-type and mutant hearts (data not shown).

Connexin 40 (*Cx40*; *Gja5* – Mouse Genome Informatics), connexin 43 (*Cx43*; *Gja1* – Mouse Genome Informatics), sarcoplasmic reticulum $Ca^{2+}$ ATPase (*Serca2a*) and *Tbx5* also show LV-restricted expression patterns. The expression of *Serca2a* was unaffected in *Hand1* cardiac-KO/KO; *Hand2* KO/+ embryos (data not shown). By contrast, expression of *Cx40* was slightly decreased in *Hand1* cardiac KO/KO embryos and was completely absent from the ventricular myocardium in *Hand1* cardiac-KO/KO; *Hand2* KO/+ embryos (Fig. 6A,B). It is notable in this regard that mice lacking *Tbx5* also fail to express *Anf* and *Cx40* (Bruneau et al., 2001). Therefore, to determine if *Hand1* and *Hand2* might act genetically upstream of *Tbx5*, we examined expression of *Tbx5* in embryos lacking combinations of the Hand genes and found it to be unaffected (Fig. 6, Fig. 7B). We conclude that Hand1 and Hand2 regulate *Cx40* and *Anf* expression through mechanisms independent of *Tbx5* transcription.

## Discussion

The Hand genes have been shown to display dynamic and highly specific expression patterns during heart development in mouse, chick, frog and zebrafish embryos (Cserjesi et al., 1995; Srivastava et al., 1995; Angelo et al., 2000; Yelon et al., 2000). In the mouse, *Hand2* is required for right ventricular development (Srivastava et al., 1997), but potential cardiac functions of *Hand1* have remained elusive owing to its required role in the placenta, which causes early embryonic lethality of *Hand1* null embryos (Firulli et al., 1998; Riley et al., 1998). The findings of the present study reveal specific functions of *Hand1* in growth and maturation of the heart, distinct from those of *Hand2*. In addition, analysis of *Hand1/2* compound mutant mice demonstrates that Hand transcription factors act in a gene dose-sensitive manner to regulate expansion of the ventricular chambers and expression of specific sets of cardiac genes during embryonic development. A model for the roles of mouse Hand genes in ventricular development is shown in Fig. 8.

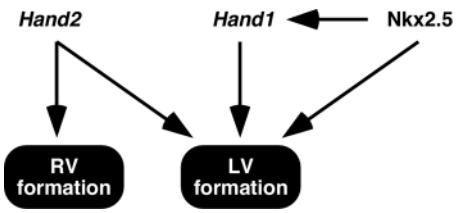


**Fig. 8.** The roles of Hand genes in ventricular development. Knockout mice lacking *Hand2* have demonstrated the essential role of this gene in formation of the RV. Knockout mice lacking *Nkx2.5* show a loss of the LV and downregulation of *Hand1*. Knockout mice lacking *Hand1* show relatively minor abnormalities in the LV, suggesting that the loss of *Hand1* alone is insufficient to account for the more severe LV defects in *Nkx2.5* mutant embryos. Knockout mice lacking both *Hand1* and *Hand2* show severe ventricular hypoplasia, suggesting redundant functions of the Hand genes in the developing LV.

### Ventricular septal defects in *Hand1* cardiac KO mice

Cardiac-specific deletion of *Hand1* resulted in a spectrum of heart abnormalities leading to death in the perinatal period. The most common defects observed in embryos and neonates were VSDs, AV valve abnormalities, and outflow tract abnormalities. VSDs are the most prevalent congenital heart defect in humans (Hoffman, 1995), and have been observed in a variety of mouse mutants (Srivastava, 2001). Regions of the AV cushions contribute to both the atrial and ventricular septa, and alterations in AV cushion remodeling result in septal defects involving the membranous ventricular septum (Bartram et al., 2001; Bamforth et al., 2001; Eisenberg and Markwald, 1995). It is therefore likely that defects in AV cushion remodeling contribute, at least in part, to the VSDs observed in *Hand1* mutants. However, in addition to AV cushion abnormalities, *Hand1* cardiac KO mice exhibit a thickened, disorganized muscular septum at all stages of embryonic development. This and the fact that we occasionally observe defects in the muscular ventricular septum suggest that growth of septal myocytes or positioning of the interventricular septum are abnormal in *Hand1* mutants.

*Hand1* expression is excluded from all but a small subpopulation of septal myocytes, suggesting that it plays a non-cell autonomous role in definition of the septal boundary, or that septal defects are secondary to abnormal growth and morphogenesis of the LV. A recent study provides evidence that Hand1 is indeed an important regulator of the interventricular boundary (Togi et al., 2004). Using homologous recombination, *Hand1* was targeted to the *Mlc2v* locus, which is expressed throughout the ventricular and septal myocardium. *Hand1* knock-in mice died at midgestation and completely lacked an interventricular septum. Thus, overexpression of Hand1 eliminates the interventricular septum, while cardiac deletion of Hand1 causes expansion of this region of the heart.

### Valve defects resulting from cardiac *Hand1* deletion

Embryos that lack cardiac expression of *Hand1* also displayed abnormally thickened AV valves and hyperplastic endocardial cushions. The endocardial cushion malformations in these mutant mice are interesting because the *αMHC::Cre* transgene does not direct *Cre* expression in the endocardial cushions or cardiac valves (Agah et al., 1997; Gaussin et al., 2002). This suggests that Hand1 regulates a myocardium-derived signal that controls endocardial cushion morphogenesis. Crosstalk between the myocardium and endocardium is well documented and has been shown to involve BMP- and TGFβ-mediated signals (Brown et al., 1999; Eisenberg and Markwald, 1995; Gaussin et al., 2002; Kim et al., 2001). Mice that lack TGFβ2 or Smad6, a BMP effector, also exhibit hyperplastic cardiac valves (Galvin et al., 2000; Bartram et al., 2001). However, *Bmp2*, *Bmp4*, *Smad6*, *Smad7* and *Tgfb* expression is unaltered in *Hand1* mutants (data not shown), suggesting that Hand1 regulates other signaling pathways. It should also be noted that early myocardial function profoundly influences endocardial cushion development (Bartman et al., 2004). Thus, abnormalities in cardiac contractility or morphogenesis in embryos lacking cardiac *Hand1* expression could indirectly influence the formation of cardiac cushions.

## Redundancy of mammalian Hand genes

The cardiac phenotype resulting from cardiac deletion of *Hand1* was much less severe than that of *Hand2* mutant embryos in which the entire right ventricular region of the heart is absent (Srivastava et al., 1997). These differences in ventricular phenotypes are likely to reflect important distinctions in the expression patterns of *Hand1* and *Hand2*. *Hand1* is expressed specifically in the outer curvatures of the embryonic LV, RV and outflow tract. By contrast, *Hand2* is expressed throughout the left and right ventricular chambers, although its expression is highest in the RV (Srivastava et al., 1997; Biben and Harvey, 1997). Therefore, in the absence of *Hand1*, residual *Hand2* expression in the LV and outflow tract may partially compensate for the loss of *Hand1*. By contrast, in the absence of *Hand2* there is a more complete lack of Hand factors in the presumptive RV (Srivastava et al., 1997).

The genome of the zebrafish encodes a single Hand gene, and loss-of-function mutations (*hands off*) result in a near complete absence of ventricular precursors. This has led to the speculation that mouse *Hand1* and *Hand2* act redundantly during ventricular differentiation (Yelon et al., 2000). Our results are consistent with this notion. Removal of one copy of the *Hand2* gene in the setting of cardiac-specific deletion of *Hand1* exacerbated the *Hand1* cardiac phenotype and resulted in embryonic lethality at midgestation; removal of both *Hand2* genes caused severe ventricular hypoplasia and lethality at yet an earlier developmental stage. However, the cardiac phenotype of our *Hand1* cardiac KO/KO; *Hand2* KO/KO is less severe than that of zebrafish *hands off* mutants. Although these results must be interpreted in light of the technical limitations of Cre-mediated gene deletion, this may also suggest that mammalian Hand genes have acquired unique functions during the evolution of the four-chambered heart.

## Hand genes regulate ventricular expansion

The morphogenesis of the single-chamber embryonic heart into the adult four-chamber heart is crucially dependent on the expansion, or 'ballooning' of the outer curvature of the right and left ventricular chambers (Christoffels et al., 2000). Expression of *Hand1* along the outer curvature of the LV and outflow tract is consistent with a role in the expansion of chamber myocardium. The expression patterns of several genes mark the expansion of this chamber myocardium from the outer curvatures of the ventricles (Christoffels et al., 2000). We unexpectedly found that ventricular expansion and gene expression was strictly dependent on the gene dose of *Hand1* and *Hand2*. In *Hand1* cardiac KO/KO; *Hand2* KO/+ embryos, expression of connexin 40 and *Anf*, both of which mark the outer curvature of the LV, was abolished. In addition, the ventricles failed to expand morphologically, and the embryos died at midgestation. Ventricular fate is specified in these embryos as evidenced by robust *Mlc2v* expression; in addition, the expression of *Tbx5* indicates that cardiac chamber specification occurred. These findings suggest that the ventricular expansion program is abnormal in these embryos. In addition, *Hand1* cardiac KO/KO; *Hand2* KO/KO embryos display an even more severe ventricular phenotype, forming only a single immature ventricle presenting abnormal cellular morphology in some segments of the ventricular myocardium. The exquisite sensitivity of *Hand1* mutant hearts to *Hand2* gene dose underscores the crucial role of these genes during ventricular expansion.

What mechanism(s) might account for the severe ventricular hypoplasia seen in mice lacking cardiac expression of *Hand1* and *Hand2*? Because we did not detect significant differences in apoptosis or proliferation of ventricular myocytes in wild-type and double *Hand* mutant embryos, it is unlikely that abnormalities in these events account for the severe deficiency of ventricular myocytes in the mutant. Therefore, we speculate that the absence of both Hand genes results in a deficiency in specification of cardiac myocytes at an early stage of cardiogenesis. A similar mechanism has been proposed to account for the lack of cardiomyocytes in the *hands off* mutant (Yelon et al., 2000).

The expression pattern of *Hand1* in the developing heart is nearly identical to that of *Cited1*, which encodes a transcriptional co-activator (Biben and Harvey, 1997; Dunwoodie et al., 1998). The downregulation of Cited1 in *Hand1* mutant embryos suggests that *Hand1* acts upstream of *Cited1* during cardiac development. It is notable, in this regard, that cited1 and Hand1 are also co-expressed in trophoblastic tissues of the placenta, and mice lacking either gene display lethal defects in placental development (Rodriguez et al., 2004; Riley et al., 1998; Firulli et al., 1998). Analysis of heart morphology has not been described in *Cited1* mutants (Rodriguez et al., 2004), but mice lacking *Cited2* display numerous congenital heart defects that overlap with those observed in *Hand1* cardiac KO animals (Bamforth et al., 2001). However, we detected no difference in *Cited2* expression between wild-type and *Hand1* mutant mice (data not shown). It remains possible that subtle cardiac defects are indeed present in *Cited1*-null mice, or differences in genetic background between *Hand1* and *Cited1* mutant mouse lines account for the lack of obvious cardiac defects in *Cited1*-null mice. Regardless, it is likely that downstream targets in addition to *Cited1* contribute to the heart defects observed in *Hand1* cardiac KO animals.

## The relationship between Hand1 and Nkx2.5

In mice lacking *Nkx2.5,* the left ventricular chamber fails to expand following cardiac looping, and expression of several markers of cardiac differentiation is reduced throughout the remaining myocardium (Lyons et al., 1995; Tanaka et al., 1999; Yamagishi et al., 2001). Interestingly, *Hand1* expression is abolished in the hearts of *Nkx2.5* mutant embryos (Biben and Harvey, 1997). Therefore, it has been proposed that loss of Hand1 contributes to abnormal cardiac morphogenesis of *Nkx2.5* mutant hearts (see Fig. 8). The data presented here suggest that lack of Hand1 is not solely responsible for left ventricular hypoplasia in *Nkx2.5*-null mice. First, loss of *Hand1* expression in the early heart tube results in only a modest and transient decrease in size of the left ventricular chamber in the embryo, much less severe than in *Nkx2.5* mutants. Second, in contrast to *Nkx2.5* null mice, *Anf* and *Mlc2v* are expressed at high levels in the LV of *Hand1* mutants. Both *Anf* and *Mlc2v* are also normally expressed in $Hand1^{lacZ/lacZ}$ null mice (Firulli et al., 1998), demonstrating that expression of these markers in *Hand1* conditional knockout mice is not due to inefficient or delayed excision of *Hand1*. In addition, markers of the left ventricular chamber, including *Tbx5* and *Cx40*, are expressed normally in the absence of Hand1.

We previously showed that mice deficient in Nkx2.5 and Hand2 formed only a single cardiac chamber, molecularly defined as the atrium (Yamagishi et al., 2001). By contrast, in the absence of cardiac Hand expression, ventricular cardiomyocytes are evident as shown by expression of *Mlc2v*, however ballooning of the ventricular chamber is abrogated. The less severe phenotype of the *Hand1/Hand2* mutant again suggests that *Nkx2.5* regulates genes in addition to *Hand1* and that the lack of *Hand1* expression is only partially responsible for cardiac defects and embryonic lethality observed in *Nkx2.5*-null mice.

### Contributions of primary and secondary heart fields to the developing heart

It is interesting to consider the roles of Hand1 and Hand2 in the context of the contributions of the primary and secondary heart fields to the developing heart. The primary heart field is believed to give rise to the atria and left ventricular chambers, while the secondary (or anterior) heart field contributes primarily to the outflow tract and right ventricular region of the heart (reviewed by Kelly et al., 2001; Kelly and Buckingham, 2002; Abu-Issa et al., 2004) (see also Cai et al., 2003). The absence of Hand2 results in the deletion of the right ventricular regions of the heart (Srivastava et al., 1997), suggesting that it is an essential component of the pathway for development of the secondary heart field. The cardiac phenotype of *Nkx2.5* mutant embryos is complementary to that of *Hand2* mutants, i.e. a lack of the left ventricular region (Lyons et al., 1995; Tanaka et al., 1999). Consistent with the idea that Hand2 and Nkx2.5 regulate growth of complementary regions of the heart, mice lacking both genes form only a primitive cardiac rudiment (Yamagishi et al., 2001). It is notable that the zebrafish heart has only a single ventricle and no evidence of a secondary heart field. The presence of only a single Hand gene in that organism raises the possibility that a primordial Hand gene may have been duplicated during evolution such that each Hand gene fell under separate temporal and spatial control as a means of generating two heart fields that contribute to the different ventricular chambers.

### Implications

The types of cardiac abnormalities observed in mice lacking either Hand1 or Hand2 are reminiscent of congenital heart defects in humans. Most congenital heart defects in humans that have been linked to mutations in specific genes represent haploinsufficiency often influenced by genetic or environmental modifiers. By contrast, most cardiac defects studied in the mouse arise owing to homozygous gene deletion. Given the diversity of cardiac abnormalities that can result from *Hand* gene mutations (ventricular hypoplasia, VSDs, valve defects, outflow tract abnormalities), we anticipate that the Hand genes will prove to be crucial for heart development and congenital heart disease in humans.

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