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Gene Replacement Strategies to Test the Functional Redundancy of Basic Helix–Loop–Helix Transcription Factor

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Abstract

Basic helix–loop–helix (bHLH) transcription factors control developmental decisions for a wide range of embryonic cell types. Hand1 and Hand2 are closely related bHLH proteins that control cardiac, craniofacial, and limb development. Within the developing heart, *Hand1* expression becomes restricted predominantly to the left ventricle, whereas *Hand2* becomes restricted predominantly to the left ventricle, for which findings have shown each Hand factor to be necessary for normal chamber formation. Forced overexpression of Hand1 throughout the early developing heart induces abnormal interventricular septal development, with resulting pathogenesis of congenital heart defects. To investigate the potential transcriptional mechanisms involved in heart morphogenesis by Hand2, this study used a replacement targeting approach to knock *Hand2* into the *Hand1* locus and ectopically express one copy of *Hand2* within the endogenous *Hand1* expression domain in the developing hearts of transgenic mice. The findings show that high-percentage $Hand1^{Hand2}$ chimeras die at birth and exhibit a range of congenital heart defects. These findings suggest that Hand factors may act via unique transcriptional mechanisms mediated by bHLH factor partner choice, supporting the notion that alterations of Hand factor stoichiometry may be as deleterious to normal heart morphogenesis as Hand factor loss of function.

Keywords

bHLH transcription factors; Chimeras; Congenital heart defects

Proper development of all multicellular organisms requires the spatial and temporal coordination of numerous transcriptional pathways and integrated extracellular signaling cues. Developing embryos comprise a complex and dynamic environment in which cells are typically exposed to a great deal of overlapping and transient transcriptional information, growth factors, and signaling pathways that may serve synergistic, parallel, and/or antagonistic roles. A key question is how cells within such an environment respond to these competing influences to enact appropriate cell fate specification and differentiation programs.

Congenital heart defects affect 1% of live births [21] and frequently require intervention for the treatment of childhood heart failure in an attempt to prevent neonatal death [6, 13]. Although the deleterious consequences of such cardiac malformations usually are evident only after birth, the underlying causes of these defects frequently involve deregulation of

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events within the transcriptional programs that control cardiac specification, differentiation, and morphogenesis in utero.

Hand1 and *Hand2* are evolutionarily conserved basic helix–loop–helix (bHLH) transcription factors. The first class B genes of the bHLH superfamily identified to play a role in cardiogenesis were the *Hand* transcription factors *Hand1* (previously termed *eHAND*, *Hxt*, *Thing1*) and *Hand2* (previously termed *dHAND*, *Hed*, *Thing2*).

Hand factors show high amino acid identity between species, suggesting conserved biologic function [6, 8, 9, 14, 15, 39], whereas functional studies have shown that Hand1 and Hand2 exhibit broad dimerization profiles [17]. *Hand1* and *Hand2* are evolutionarily conserved basic transcription factors that exhibit dynamic and partially overlapping spatiotemporal expression patterns during cardiovascular development. Cardiac expression of *Hand1* and *Hand2* is initiated after cell specification (E7.0 in mice; HH stage 8 in chick; day 21 in human). Although mammalian *Hand1* and *Hand2* are initially coexpressed, during morphogenesis and asymmetric looping of the early embryonic heart tube, *Hand1* becomes predominantly restricted to the left ventricle, whereas *Hand2* becomes predominantly restricted to the right ventricle [16, 39]. However, both genes remain coexpressed in the embryonic aortic sac, the great vessels that exit the heart, and the nascent interventricular septum that eventually septates the left and right ventricular chambers [6, 15].

As cardiogenesis progresses, *Hand* gene expression is progressively downregulated [9, 39]. A study in human patients showed that *HAND* genes may be expressed at very low levels in the adult human heart but can be reexpressed during heart disease [30, 35] and significantly upregulated in response to cardiac hypertrophy [43]. Recently, a functional genetic study identified HAND1 mutations in septation defects within tissue samples from human heart patient samples [31].

Transgenic analysis of *Hand* factors shows that both are required for normal cardiovascular development. Both systemic *Hand1*- and *Hand2*-targeted knock-out mice exhibit heart morphologic abnormalities [16, 33, 40]. *Hand2* nulls are embryonically lethal and die between E9.5 and E10.5 due to cardiac and vascular defects [40, 48]. Morphologic analysis of *Hand2* nulls shows that the region of the looping heart tube destined to become the right ventricle is missing. Although the morphologic phenotype could be the result of a looping defect, it likely is not based on the finding that *Hand2* expression tracks with alterations in sidedness putting it downstream of right-left polarity signals [44].

Molecular analysis in mice shows that cardiac specification occurs because cardiac-restricted molecular markers are normally expressed [40]. However, expression of the chamber-restricted marker natriuretic peptide precursor type A (*Nppa*) [20] is downregulated in *Hand2* nulls, and this reduction is via a direct transcriptional effect [42].

In contrast, *Hand1* does not transcriptionally influence *Nppa* expression, suggesting that *Hand1* and *Hand2* may not be functionally redundant during cardiogenesis. Although the *Hand1* null cardiovascular phenotype is difficult to analyze due to the extraembryonic defects and early E8.5 to E9.5 in utero lethality [16, 33], the cardiac lineage is specified, and cell differentiation (assessed via expression of cardiac-specific structural genes *Mlc2a* and *Mlc2v*) occurs in Hand1 nulls [16]. Tetraploid rescue of *Hand1* null embryos has suggested a looping defect [33] Combined, these data suggest that like *Hand2*, the function of *Hand1* during cardiogenesis most likely is to regulate heart morphogenesis and is not required during early cardiac lineage commitment or initial cell differentiation. In support of this, when a conditional *Hand1*-null allele was deleted in only the cardiomyocytes lineage, perinatal *Hand1* conditional mutants displayed defects in the left ventricle and endocardial cushions and exhibited dysregulated ventricular gene expression [28]. Moreover, creation of

Hand1/2 double-mutant mice showed gene dose-sensitive functions of Hand transcription factors in the control of cardiac morphogenesis and ventricular gene expression [28].

Although these results must be interpreted in light of the technical limitations of Cre-mediated gene deletion, these data do suggest that mammalian *Hand* genes may play both overlapping and unique cardiac functions during evolution [28]. Additionally, when the *Hand1* cDNA was knocked into the *Mlc2v* locus and *Hand1* was robustly overexpressed ectopically throughout the developing left and right ventricles, septation defects resulted [46].

To understand further the mechanisms whereby HAND factors regulate heart formation during cardiovascular morphogenesis and to test the functional redundancy of *Hand1* and *Hand2* directly, we substituted *Hand2* for *Hand1* by knocking *Hand2* into the *Hand1* locus ($Hd1^{Hd2}$). Remarkably, our results show that high-percentage $Hd1^{Hd2}$ chimeras die at birth and exhibit a range of congenital heart defects (CHDs). The observed phenotypes occur specifically where endogenous *Hand1* is expressed during normal heart development. These findings show that *Hand1* and *Hand2* convey unique transcriptional regulation during cardiogenesis and suggest that Hand factor partner choice is critical for normal cardiac morphogenesis.

Materials and Methods

Gene Targeting

We used a replacement targeting approach to knock a *Hand2* genomic NotI-BamHI fragment containing both exons and lacking the transcriptional start site into the *Hand1* locus using our previously published targeting strategy [16]. The *Hand2* fragment was cloned downstream of the *Hand1* transcriptional initiation codon contained in the 3.0-kb BstEII *Hand1* 5′ targeting arm, and an additional EcoRI site was engineered to create a unique restriction fragment length polymorphism (RFLP) site, enabling us to detect a 3.9-kb EcoRI fragment when a 3′ external HindIII-BssHII probe is used, as previously described [16]. All cloning junctions within the $Hand1^{Hand2}$ ($Hd1^{Hd2}$) targeting vector were confirmed by DNA sequencing, and the vector was linearized with NotI, before electroporation into 129SvJ mouse embryonic stem (ES) cells by the Indiana University PUI (IUPUI) ES Cell and Transgenic Core Facility. Genomic DNA was isolated from ES clones that survived positive-negative selection using previously established protocols [16, 34]. Southern hybridization on EcoRI-digested DNA confirmed homologous recombination at a frequency of 1:30 in the 60 ES clones analyzed via both the internal and the 3′ external HindIII-BssHII probes previously described [16].

F_0 Chimera Production and Analysis

Two correctly targeted ES clones were identified, and both independent clones were subsequently implanted into pseudopregnant wild-type C57BL/6 host blastocysts and into foster female mice to obtain F_0 chimeric embryos sacrificed at E10.5 and newborn stages using standard protocols [16, 34]. To obtain primarily high-percentage early embryonic chimeras, 10 to 14 targeted $Hd1^{Hd2}$ ES cells were injected per blastocyst. Southern analysis of genomic DNA extracted from embryo yolk sacs and newborn tails was used to estimate the percentage of chimerism by comparing the molar ratio of the wild-type and knock-in $Hd1^{Hd2}$ mutant bands via a personal FX phosphoimager (BioRad, Hercules, CA). The animal use protocols were approved by the Institutional Animal Care and Use Committee at IUPUI.

Histologic, Immunohistochemical, and Gene Expression Analysis

Tissue isolation, 4% paraformaldehyde fixation, processing, paraffin embedding, hematoxylin and eosin (H&E) staining, and immunohistochemical detection of α-smooth muscle actin (αSMA) (1:5,000 dilution αSMA; Sigma, St. Louis, MO) were performed as described [34, 38]. Sections (3 individual embryos/newborns of each genotype) were cut at 10-μm thickness and counterstained. Immunologic reactions were visualized by use of a Vector ABC kit (Sigma, St. Louis, MO) and a peroxidase-diaminobenzidine reaction. The sections were counterstained with hematoxylin and mounted on glass slides. Negative controls were obtained by substituting the primary antibody with serum. In situ hybridization using published *Hand1* and *Hand2* [47], *Tbx20* and *Nppa* [20], and *Ncx1* [24] cDNA probes was performed as previously described [5, 38]. Both sense and antisense S^{35}-uridine triphosphate (UTP)-labeled probes were used, and specific signal was observed only with hybridization of the antisense probe in serial sections within at least three independent embryos/newborns of each genotype.

Results

Forced Expression of a *Hand2* Within a *Hand1* Locus Can Result in Neonatal Lethality

We used a replacement targeting approach to knock *Hand2* into the *Hand1* locus, generating a $Hd1^{Hd2}$ knock-in allele (Fig. 1). The generation of heterozygous $Hd1^{Hd2}$ ES cells allowed us to test whether *Hand1* and *Hand2* are interchangeable in regions of endogenous *Hand1* expression. After injection of two separately targeted ES cell lines, approximately 55% of the chimeras ($n = 14$) were stillborn. Genotyping showed that only the high-percentage chimeras were nonviable. Significantly, approximately 60% of the dead chimeras showed more than 95% chimerism based on the equal molar ratio of the wild-type and mutant bands observed via Southern analysis (Fig. 1), and all stillborn chimeras contained more than 70% chimerism (6 of the 6 high-percentage chimeras were stillborn).

Although we cannot be certain that the percentage contribution of the $Hd1^{Hd2}$ ES cells is similar between the hearts and the tails, the consistency of the observed phenotypes is highly suggestive that this is the case. Phenotypic examination of the chimeric pups showed generalized whole-body edema, most severe in stillborn high-percentage chimeric pups #22 and #41, whereas viable low-percentage pup #10 appeared grossly unaffected (Fig. 1). Closer examination also showed a high incidence of polydactyly in the stillborn high-percentage chimeras (5 of the 6 high-percentage chimeras showing an extra digit on all four limbs were stillborn). An ectopic digit was present on the posterior side of the limb (Fig. 1), in a position usually occupied by the zone of polarizing activity (ZPA; [45]) and opposite the thumb/big toe. Of the surviving low- and medium-percentage chimeras, one was small and frail (~50 to 60% by coat color), but the remaining ones (~15 to 50% by coat color) were phenotypically normal and fertile but had yet to transmit the $Hd1^{Hd2}$ allele germ line (26 litters).

High-Percentage $Hand1^{Hand2}$ Chimerism Results in Congenital Heart Defects

Pathologic examination of the stillborn high-percentage chimeric neonatal $Hd1^{Hd2}$ pups showed severe dilated cardiomyopathy (both ventricles and atria, Fig. 2a), suggesting that cardiac failure is most likely the cause of postnatal death. Significantly, the high-percentage chimeric pups showed signs of congenital double-outlet right ventricle (DORV) heart defect, in which the aorta and pulmonary trunk are located side by side and exit the right ventricle, compared with the normal arrangement of out-flow tract (OFT) vessels in which the aorta exits the left ventricle and the pulmonary trunk exits the right ventricle (Fig. 2b, c).

Histologic examination showed that although αSMA expression was unperturbed, the *Hd1*^{Hd2} chimeric OFT vessel walls were hyperplastic, and the lamella organization of the smooth muscle surrounding the aorta, pulmonary artery, and ductus arteriosus was disrupted (Fig. 2e). Although the high-percentage *Hd1*^{Hd2} placenta appeared unaffected, their newborn livers were enlarged and engorged with fetal blood, supporting the implication of hemodynamic overload and heart failure [7].

Detailed histologic analysis of a stillborn high-percentage chimeric neonatal *Hd1*^{Hd2} cardiovascular system confirmed severe cardiac malformations (Fig. 3), specifically DORV with concomitant interventricular septal defects (VSDs) and patent ductus arteriosus (PDA) in all the stillborns (6 of the 6 high-percentage chimeras were stillborn). Sections showed that all the dead *Hd1*^{Hd2} neonates show VSDs, that their hearts had a thinned myocardium, and that the myocardial architecture was extensively disorganized (Fig. 3b–f). In high-percentage chimeric hearts, both multiple muscular and obligatory large perimembraneous VSDs were present (Fig. 3b–e), but in the lower-percentage *Hd1*^{Hd2} hearts, only an isolated muscular VSD was observed at the base of the heart (Fig. 3f). The most severely affected chimera (#41) showed that the entire septum was hypoplastic with multiple VSDs (Fig. 3c). Furthermore, the high-percentage chimeric *Hd1*^{Hd2} coronary vascular system was affected as the coronaries were dilated and lacked complete supporting muscle layer (because αSMA expression is irregular; Fig. 3d), further suggesting heart failure as the cause of neonatal *Hd1*^{Hd2} lethality.

Significantly, all the dead chimeras also exhibited hypoplastic/misshapen OFT valve leaflets and PDA ductus arteriosus (Fig. 3). Collectively, in the absence of any other obvious defects (apart from polydactyly), the replacement of one allele of *Hand1* for one expressing *Hand2* in regions expressing *Hand1* was sufficient to cause the DORV, VSD, and PDA congenital defects which undoubtedly would have caused hemodynamic distress that resulted in neonatal respiratory failure [7].

Ectopic *Hand2* Expression Within the *Hand1* Locus Results in Upregulation of *Nppa*

Analysis of the cardiac gene expression via in situ hybridization in newborn offspring showed upregulation of *Nppa* expression in *Hd1*^{Hd2} hearts (Fig. 4). *Nppa* is secreted by cardiac myocytes [10], is one of the first hallmarks of chamber formation [20], has been implicated in the control of extracellular fluid volume and electrolyte homeostasis, and is one of the most commonly used molecular markers of cardiac failure [2, 22, 49].

Significantly, *Nppa* was upregulated in both viable medium-percentage (~40 to 60%) and stillborn high-percentage (~60%+) chimeric pup hearts compared with age-matched littermate control subjects. Whereas *Nppa* was significantly upregulated in medium-percentage *Hd1*^{Hd2} chimeric atrial and ventricular cardiomyocytes (Fig. 4b), it was expressed in all the high-percentage chimeric cardiomyocytes, indicating complete heart failure (Fig. 4c). As expected, expression of sodium calcium exchanger-1 (*Ncx1*) also was upregulated in high-percentage chimeric hearts (data not shown), as *Ncx1* is known to be upregulated during heart failure [29]. However, expression of *Hand1*, *Hand2*, and another chamber-restricted gene *T-box20* [20] was unaffected in newborns (data not shown).

Although *Hand1* and *Hand2* are normally downregulated during embryogenesis by E13.5 and undetectable in newborns [6, 15, 16, 47], we did not detect any prolonged ectopic *Hand2* expression in stillborn knock-in mutant hearts. Similarly, chamber-restricted gene *Tbx20* also was not ectopically misexpressed, suggesting that *Nppa* and *Ncx1* upregulation are indicative of heart failure rather than chamber identity/morphologic abnormalities.

Replacement of Hand1 with Hand2 Disrupts Embryonic Cardiac Morphogenesis and Heart Looping

Given that *Hand1* cardiac expression is downregulated between E11.5 and E13.5 [15, 16] and that it is uncertain whether early morphologic and molecular events mediated by the $Hd1^{Hd2}$ allele can result in defects that became deleterious at later developmental stages in our high-percentage chimeras, we reinjected our $Hd1^{Hd2}$ targeted ES cells. This enabled us to collect F_0 chimeric embryos at E10.5 when both *Hand1* and *Hand2* were both robustly expressed in the heart [6, 15, 47].

After identifying their genotypes via Southern analysis of yolk sacs, we recovered several high-percentage E10.5 $Hd1^{Hd2}$ chimeras ($n = 4$) that exhibited cardiac looping anomalies (Fig. 5). The $Hd1^{Hd2}$ cardiac tube appears angular and loops in an anterior-to-posterior manner rather than right to left (Fig. 5b, c), as is seen in age-matched control littermates. Clearly visible in these high-percentage chimeras were regular heartbeats indicating that these mice likely would survive to later stages of development. Based on phenotypic observations from newborn high-percentage chimeras (Figs. 1, 2 and 3), in which severe VSD and OFT abnormalities were observed, these morphologic abnormalities observed in $Hd1^{Hd2}$ high-percentage chimeras at earlier time points were consistent with the VSD and OFT defects observed in the nonviable $Hd1^{Hd2}$ neonates. Moreover, if the known temporal-spatial expression of *Hand1* in cardiac development (E7.0–E13.5) is taken into account, the severe neonatal phenotypes observed are likely due to molecular events occurring in the early embryonic period during initial heart remodeling and formation of a four-chambered heart.

To confirm ectopic expression of *Hand2* within regions of the developing heart that exclusively express *Hand1*, we used in situ hybridization analysis (Fig. 5d, e). *Hand1* is normally expressed within the developing left embryonic ventricle and OFT, whereas *Hand2* expression is normally localized largely to the developing right embryonic ventricle and OFT [15, 16]. As expected, endogenous *Hand1* mRNA expression is restricted to the E10.5 left ventricle of mutant $Hd1^{Hd2}$ high-percentage chimeras (Fig. 5d) and indistinguishable from that of nonchimeric control litter-mates (data not shown). As predicted, endogenous *Hand2* mRNA expression is detected in the right ventricle and OFT, but knock-in *Hand2* mRNA is also ectopically expressed at this point in the E10.5 mutant $Hd1^{Hd2}$ high-percentage chimera left ventricle (Fig. 5e), where *Hand1* is now aberrantly coexpressed within these knock-in mutants.

Importantly, it should be noted that the expression levels of endogenous *Hand2* in the right ventricle and transgenic *Hand2* in the left ventricle were similar because only a single copy of *Hand2* was knocked in using our replacement targeting approach (Fig. 1). The findings of both morphologic defects and ectopic pharmacologically relevant *Hand2* expression within the $Hd1^{Hd2}$ left ventricle suggest that the observed neonatal high-percentage $Hd1^{Hd2}$ lethal phenotypes are the result of substituting one allele of *Hand1* for one transiently coexpressed *Hand2* allele during early chamber morphogenesis.

Discussion

We and others previously demonstrated a critical role for Hand factors in the development of limb and heart, and in the regulation of chamber morphogenesis [16, 28, 33, 40, 46]. The results of this study extend those findings and lead to the unexpected conclusion that the activity of Hand factors is mediated by partner choice, regulated stoichiometry, and restricted expression patterns. Hand2, which shares high amino acid identity with Hand1, is incapable of replacing *Hand1* during limb bud patterning and cardiac chamber morphogenesis. Moreover, *alterations of Hand factor* stoichiometry may be as deleterious to

normal heart morphogenesis (as well as limb formation) as *Hand* factor loss of function. Our results suggest that the molecular mechanisms by which *Hand* factors function during development are more complicated than traditional models of bHLH protein function predict.

To test the functional redundancy of *Hand1* and *Hand2* directly, we substituted *Hand2* for *Hand1* by knocking *Hand2* into the *Hand1* locus ($Hd1^{Hd2}$). High-percentage chimeric $Hd1^{Hd2}$ pups die at birth and exhibit polydactyly, OFT misalignment defects, and VSDs, indicating that *Hand1* and *Hand2* are not functionally redundant, and more importantly, that 50% substitution of ectopic *Hand2* for *Hand1* expression in *Hand1*-expressing cells is sufficient to alter cardiomyocyte morphology and limb patterning. The cardiovascular VSD and DORV defects undoubtedly cause hemodynamic distress and resultant neonatal respiratory failure, and in the absence of other obvious defects (apart from polydactyly), we suggest that the replacement of one allele of *Hand1* for one expressing *Hand2* in regions expressing *Hand1* is sufficient to cause lethal CHDs. The presence of polydactyly and the cardiovascular $Hd1^{Hd2}$ phenotypes in tissues in which *Hand1* is expressed strongly argues that ectopic expression of *Hand2* affects only tissues that normally express *Hand1*.

Moreover, because this mouse model expresses *Hand2* at physiologically relevant levels (50% of total *Hand1* expression) from endogenous transcriptional regulatory elements in the *Hand1* locus, the total *Hand* expression should be equal to endogenous *Hand1*. Thus, any resultant phenotypes should be only in tissues that express *Hand1*. In fact, this is what was observed. Phenotypes are evident in the developing limbs [12]. Furthermore, the dilated ventricles, VSDs, and OFT defects all are associated with sites of *Hand1* heart expression [14]. Given that *Hand1* heterozygous mice are viable, a 50% reduction in expression of *Hand1* is obviously not a lethal condition. Mechanistically, this could result from *Hand1* and *Hand2* not being redundant or from ectopic *Hand2* affecting another bHLH factors function.

Hand factors, like other tissue-specific (class B) bHLH proteins, are thought to act primarily as heterodimers with widely expressed E-proteins [26]. However, Hand factors also can form homodimers themselves as well as hetero-dimers with all members of the *Twist* class of bHLH factors and with bHLH proteins in the *HES*-related transcription factor family [1, 17–19]. Mice null for *Hey2* display membranous VSDs that result in the majority of null mice succumbing to heart failure as neonates [36]. We have shown that Hand dimerization can be regulated in part by phosphorylation of key residues found in helix-I of the bHLH domain and that hypophosphorylation and phosphorylation mimic mutations, resulting in distinct phenotypic limb outcomes when expressed in vivo [18]. Thus an alteration of dimerization affinities can modulate Hand factor function, and the chimeric phenotypes observed may represent the combined effects of diminished expression levels and a shift of the phosphorylation state of helix-I due to distorted dimer partner choice.

The ectopic digit is present on the posterior side of the $Hd1^{Hd2}$ high-percentage chimeric limbs in a region adjacent to the zone of polarizing activity (ZPA [45]) and opposite the thumb/big toe. The ZPA is a group of mesenchymal cells producing a gradient of Sonic hedgehog (*Shh*) at the posterior limb margin that controls digit identities along the anteroposterior (thumb to little finger) limb axis [32, 45, 50]. This is significant because the developing limb is a site of *Hand1* expression, and *Hand2* (along with *5′-HoxD*) expression within the ZPA itself controls *Shh* activation [4]. Furthermore, high-level transgenic misexpression of *Hand2* throughout the anterior compartment of the limb bud induces ectopic *Shh* expression, with resulting preaxial polydactyly and mirror image duplications of posterior digits [27]. Given that conditional inactivation of *Shh* at specific time points during limb morphogenesis has shown that *Shh* functions early and transiently in the specification of digit identities [51], these data demonstrate that Hand2 is unable to substitute for Hand1

expression during limb organogenesis. Further analysis is required to determine whether *Shh*, 5′-HoxD, or both are ectopically expressed in the early embryonic $Hd1^{Hd2}$ high-percentage chimeric limbs or whether ectopic *Hand2* expression within the endogenous *Hand1* expression domain alters the temporal exposure of the developing limb to *Shh* activity.

The DORV heart defect is associated with both separate and combined deficiencies in the cardiac neural crest, left-right specification, abnormalities in looping during cardiac remodeling [3, 23, 25], and cardiomyocyte morphogenesis. Pathogenesis of VSDs can exhibit different etiologies that result in similar structural anomalies. Because *Hand1* and *Hand2* are both expressed in the early OFT and because DORV/looping defects are observed in $Hd1^{Hd2}$ chimeras, we must consider that the underlying cardiomyocytes may influence the adjacent endocardial cells and that this region of the embryonic heart is considered to be a contiguous signaling center in which each lineage relies on its neighbors for normal morphogenesis.

We hypothesize that $Hd1^{Hd2}$ DORV and associated cardiovascular anomalies are not due to neural crest abnormalities because we observe comparable αSMA–positive neural crest-derived cells surrounding the OFT vessels as well as normal development of other *Hand1*-expressing neural crest–derived structures, such as the cranial ganglia and thymus (data not shown). Analysis of *Hand2* nulls shows that the neural crest-derived components of the branchial arch are present, suggesting that normal migration of the neural crest occurs even in the absence of *Hand2* [15]. Thymic gland aplasia usually results when neural crest deficiencies are present, as observed in DiGeorge/CATCH-22 [37, 41]. We suggest heart-looping abnormalities (secondary to poor cardiac function, morphogenesis, or both) as the likely cause of the cardiac alignment defects and of the DORV defects seen in $Hd1^{Hd2}$ chimeras (the presence of muscular VSDs underscores a cardiomyocyte origin). If the processes of heart looping and remodeling are compromised, the apposition of the great vessels and ventricles is disturbed, resulting in DORV and VSDs. Indeed, VSDs are the most prevalent CHDs in humans [7, 21].

The ventricular septum forms when the trabeculae condense at the interventricular groove and when the medial walls of the expanding ventricles fuse together and grow inward. The poor development of the muscular septum in the $Hd1^{Hd2}$ chimeras could be accounted for by the lack of ventricular wall expansion. The septum is the thickest portion of the ventricle in normal newborns. In $Hd1^{Hd2}$ mutants, the septum is thin-walled with poor contribution from the compact zone of the ventricle. Regions of the atrioventricular (AV) cushion contribute to both the atrial and ventricular septa, and alterations in AV cushion remodeling result in septal defects involving the membranous ventricular septum [11]. It is likely that defects in AV cushion remodeling, a lack of fusion, or both also could contribute to the observed membranous VSDs observed in $Hd1^{Hd2}$ chimeras.

In addition to AV cushion abnormalities, mutant mice exhibit a disorganized muscular septum and muscular VSDs, suggesting that growth of septal myocytes, elevated cell death, or positioning of the interventricular septum could underlie the $Hd1^{Hd2}$ knock-in defects. In situ data show that *Hand1* expression is excluded from all but a small subpopulation of septal myocytes, signifying 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 left ventricle. Evidence that *Hand1* is indeed an important regulator of the inter-ventricular boundary is observed in $Mlc2v^{Hand1}$ mice that die at midgestation lacking a septum [46].

Summary and Future Directions

The results of the aforementioned *Hd1*^{Hd2} knock-in chimeric experiments suggest that *Hand* factors are not functionally redundant and may have regulated dimerization. The survival of patients with CHDs, treated or untreated, is expected to increase, requiring the training of more cardiologists to manage moderate and complex congenital lesions [21]. Further basic developmental biologic studies are needed to help define the pathogenesis of VSDs, one of the most frequent CHDs. Hopefully, these studies will someday identify common underlying pathologic pathways and lineages that enable researchers and clinicians to design more focused interventions. Gaining insight into the early molecular mechanisms regulating cardiogenesis will undoubtedly provide a greater understanding of cardiac development as well as the genetic and cellular insults that result in CHDs affecting human newborns.

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References

1. Barnes RM, Firulli AB. A Twist of insight, the role of Twist family bHLH factors in development. Int J Dev Biol. 2009; 53:909–924. [PubMed: 19378251]
2. Bettencourt PM. Clinical usefulness of B-type natriuretic peptide measurement: present and future perspectives. Heart. 2005; 91:1489–1494. [PubMed: 16230457]
3. Bouman HG, Broekhuizen ML, Baasten AM, Gittenberger-De Groot AC, Wenink AC. Spectrum of looping disturbances in stage 34 chicken hearts after retinoic acid treatment. Anatomical Record. 1995; 243:101–108. [PubMed: 8540624]
4. Capellini TD, Di Giacomo G, Salsi V, Brendolan A, Ferretti E, Srivastava D, Zappavigna V, Selleri L. Pbx1/Pbx2 requirement for distal limb patterning is mediated by the hierarchical control of *Hox* gene spatial distribution and Shh expression. Development. 2006; 133:2263–2273. [PubMed: 16672333]
5. Conway, SJ. In situ hybridization of cell and tissue sections. Humana Press Inc; Totowa: 1996.
6. Conway SJ, Firulli AB. A bHLH code for cardiac morphogenesis. Pediatr Cardiol. 2009 [Epub ahead of print].
7. Conway SJ, Kruzynska-Frejtag A, Kneer PL, Machnicki M, Koushik SV. What cardiovascular defect does my prenatal mouse mutant have, and why? Genesis. 2003; 35:1–21. [PubMed: 12481294]
8. Cross JC, Flannery ML, Blanar MA, Steingrimsson E, Jenkins NA, Copeland NG, Rutter WJ, Werb Z. *Hxt* encodes a basic helix–loop–helix transcription factor that regulates trophoblast cell development. Development. 1995; 121:2513–2523. [PubMed: 7671815]
9. Cserjesi P, Brown D, Lyons GE, Olson EN. Expression of the novel basic helix–loop–helix gene eHAND in neural crest derivatives and extraembryonic membranes during mouse development. Dev Biol. 1995; 170:664–678. [PubMed: 7649392]
10. de Bold A. Atrial natriuretic factor: a hormone produced by the heart. Science. 1985; 230:767–770. [PubMed: 2932797]
11. Eisenberg LM, Markwald RR. Cellular recruitment and the development of the myocardium. Dev Biol. 2004; 274:225–232. [PubMed: 15385154]
12. Fernandez-Teran M, Piedra ME, Rodriguez-Rey JC, Talamillo A, Ros MA. Expression and regulation of eHAND during limb development. Dev Dyn. 2003; 226:690–701. [PubMed: 12666206]
13. Field LJ, Shou W, Caldwell RL. 2008 Riley Heart Center symposium on cardiac development: growth and morphogenesis of the ventricular wall. Pediatr Cardiol. 2009; 30:577–579. [PubMed: 19340479]

14. Firulli AB. A HANDful of questions: the molecular biology of the HAND subclass of basic helix–loop–helix transcription factors. Gene. 2003; 312C:27–40. [PubMed: 12909338]
15. Firulli AB, Conway SJ. Combinatorial transcriptional interaction within the cardiac neural crest: a pair of HANDs in heart formation. Birth Defects Research, Part C. Embryo Today. 2004; 72:151–161. [PubMed: 15269889]
16. Firulli AB, McFadden DG, Lin Q, Srivastava D, Olson EN. Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. Nat Genet. 1998; 18:266–270. [PubMed: 9500550]
17. Firulli BA, Hadzic DB, McDaid JR, Firulli AB. The basic helix–loop–helix transcription factors dHAND and eHAND exhibit dimerization characteristics that suggest complex regulation of function. J Biol Chem. 2000; 275:33567–33573. [PubMed: 10924525]
18. Firulli B, Howard MJ, McDaid JR, McIlreavey L, Dionne KM, Centonze V, Cserjesi P, Virshup DMa, Firulli AB. PKA, PKC, and the protein phosphatase 2A influence HAND factor function: a mechanism for tissue-specific transcriptional regulation. Mol Cell. 2003; 12:1225–1237. [PubMed: 14636580]
19. Firulli BA, Krawchuk D, Centonze VE, Virshup DE, Conway SJ, Cserjesi P, Laufer E, Firulli AB. Altered *Twist1* and *Hand2* dimerization is associated with Saethre-Chotzen syndrome and limb abnormalities. Nat Genet. 2005; 37:373–381. [PubMed: 15735646]
20. Habets PEMH, Moorman AFM, Clout DEW, van Roon MA, Lingbeek M, van Lohuizen M, Campione M, Christoffels VM. Cooperative action of *Tbx2* and *Nkx2.5* inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation. Genes Dev. 2002; 16:1234–1246. [PubMed: 12023302]
21. Hoffman JI, Kaplan S, Liberthson RR. Prevalence of congential heart disease. Am Heart J. 2004; 147:425–439. [PubMed: 14999190]
22. Horsthuis T, Houweling AC, Habets PEMH, de Lange FJ, el Azzouzi H, Clout DEW, Moorman AFM, Christoffels VM. Distinct regulation of developmental and heart disease-induced atrial natriuretic factor expression by two separate distal sequences. Circ Res. 2008; 102:849–859. [PubMed: 18276916]
23. Kirby ML, Gale TF, Stewart DE. Neural crest cells contribute to normal aorticopulmonary septation. Science. 1983; 220:1059–1061. [PubMed: 6844926]
24. Koushik SV, Wang J, Rogers R, Moskophidis D, Lambert NA, Creazzo TL, Conway SJ. Targeted inactivation of the sodium-calcium exchanger (*Ncx1*) results in the lack of a heartbeat and abnormal myofibrillar organization. FASEB J. 2001; 15:1209–1211. [PubMed: 11344090]
25. Lin CR, Kioussi C, O'Connell S, Briata P, Szeto D, Liu F, Izpisua-Belmonte JC, Rosenfeld MG. *Pitx2* regulates lung asymmetry, cardiac positioning, and pituitary and tooth morphogenesis. Nature. 1999; 401:279–282. [PubMed: 10499586]
26. Massari ME, Murre C. Helix–loop–helix proteins: regulators of transcription in eucaryotic organisms. Mol Cell Biol. 2000; 20:429–440. [PubMed: 10611221]
27. McFadden DG, McAnally J, Richardson JA, Charite' J, Olson EN. Misexpression of dHAND induces ectopic digits in the developing limb bud in the absence of direct DNA binding. Development. 2002; 129:3077–3088. [PubMed: 12070084]
28. McFadden DG, Barbosa AC, Richardson JA, Schneider MD, Srivastava D, Olson EN. The *Hand1* and *Hand2* transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner. Development. 2005; 132:189–201. [PubMed: 15576406]
29. Menick DR, Renaud L, Buchholz A, Muller JG, Zhou H, Kappler CS, Kubalak SW, Conway SJ, Xu L. Regulation of *Ncx1* gene expression in the normal and hypertrophic heart. Ann N Y Acad Sci. 2007; 1099:195–203. [PubMed: 17446459]
30. Natarajan A, Yamagishi H, Ahmad F, Li D, Roberts R, Matsuoka R, Hill S, Srivastava D. Human eHAND, but not dHAND, is downregulated in cardiomyopathies. J Mol Cellular Cardiol. 2001; 33:1607–1614. [PubMed: 11549340]
31. Reamon-Buettner SM, Ciribilli Y, Traverso I, Kuhls B, Inga A, Borlak J. A functional genetic study identifies HAND1 mutations in septation defects of the human heart. Hum Mol Genet. 2009; 18:3567–3578. [PubMed: 19586923]

32. Riddle RD, Johnson RL, Laufer E, Tabin C. Sonic hedgehog mediates the polarizing activity of the ZPA. Cell. 1993; 75:1401–1416. [PubMed: 8269518]
33. Riley P, Anson-Cartwright L, Cross JC. The *Hand1* bHLH transcription factor is essential for placentation and cardiac morphogenesis. Nat Genet. 1998; 18:271–275. [PubMed: 9500551]
34. Rios H, Koushik SV, Wang H, Wang J, Zhou HM, Lindsley A, Rogers R, Chen Z, Maeda M, Kruzynska-Frejtag A, Feng JQ, Conway SJ. Periostin null mice exhibit dwarfism, incisor enamel defects, and an early-onset periodontal disease-like phenotype. Mol Cellular Biol. 2005; 25:11131–11144. [PubMed: 16314533]
35. Ritter O, Haase H, Schulte HD, Lange PE, Morano I. Remodeling of the hyperophied human myocardium by cardiac bHLH transcription factors. J Cellular Biochem. 1999; 74:551–561. [PubMed: 10440925]
36. Sakata Y, Kamei CN, Nakagami H, Bronson R, Liao JK, Chin MT. Ventricular septal defect and cardiomyopathy in mice lacking the transcription factor CHF1/Hey2. Proc Natl Acad Sci USA. 2002; 99:16197–16202. [PubMed: 12454287]
37. Snider P, Olaopa M, Firulli AB, Conway SJ. Cardiovascular development and the colonizing cardiac neural crest lineage. Sci World J. 2007; 7:1090–1113.
38. Snider P, Hinton RB, Moreno-Rodriguez RA, Wang J, Rogers R, Lindsley A, Li F, Ingram DA, Menick D, Field L, Firulli AB, Molkentin JD, Markwald R, Conway SJ. Periostin is required for maturation and extracellular matrix stabilization of noncardiomyocyte lineages of the heart. Circ Res. 2008; 102:752–760. [PubMed: 18296617]
39. Srivastava D, Cserjesi P, Olson EN. A subclass of bHLH proteins required for cardiac morphogenesis. Science. 1995; 270:1995–1999. [PubMed: 8533092]
40. Srivastava D, Thomas T, Lin Q, Kirby ML, Brown D, Olson EN. Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nat Genet. 1997; 16:154–160. [PubMed: 9171826]
41. Stalmans I, Lambrechts D, De Smet F, Jansen S, Wang J, Maity S, Kneer P, von der Ohe M, Swillen A, Maes C, Gewillig M, Molin DGM, Hellings P, Boetel T, Haardt M, Compernolle V, Dewerchin M, Plaisance S, Vlietinck R, Emanuel B, Gittenberger-de Groot AC, Scambler P, Morrow B, Driscol DA, Moons L, Esguerra CV, Carmeliet G, Behn-Krappa A, Devriendt K, Collen D, Conway SJ, Carmeliet P. VEGF: a modifier of the del22q11 (DiGeorge) syndrome? Nat Med. 2003; 9:173–182. [PubMed: 12539040]
42. Thattaliyath BD, Firulli BA, Firulli AB. The basic-helix–loop–helix transcription factor *HAND2* directly regulates transcription of the atrial naturetic peptide gene. J Mol Cell Cardiol. 2002; 34:1325–1344. [PubMed: 12392993]
43. Thattaliyath BD, Livi CB, Steinhelper ME, Toney GM, Firulli AB. *HAND1* and *HAND2* are expressed in the adult rodent heart and are modulated during cardiac hypertrophy. Biochem Biophys Res Com. 2002; 297:870–875. [PubMed: 12359233]
44. Thomas T, Yamagishi H, Overbeek PA, Olson EN, Srivastava D. The bHLH factors, dHAND and eHAND, specify pulmonary and systemic cardiac ventricles independent of left-right sidedness. Dev Biol. 1998; 196:228–236. [PubMed: 9576835]
45. Tickle C. The number of polarizing region cells required to specify additional digits in the developing chick wing. Nature. 1981; 289:295–298. [PubMed: 7453825]
46. Togi K, Kawamoto T, Yamauchi R, Yoshida Y, Kita T, Tanaka M. Role of *Hand1*/eHAND in the dorsoventral patterning and interventricular septum formation in the embryonic heart. Mol Cell Biol. 2004; 24:4627–4635. [PubMed: 15143159]
47. Vincentz JW, Barnes RM, Rodgers R, Firulli BA, Conway SJ, Firulli AB. An absence of *Twist1* results in aberrant cardiac neural crest morphogenesis. Dev Biol. 2008; 320:131–139. [PubMed: 18539270]
48. Yamagishi H, Olson EN, Srivastava D. The basic helix–loop–helix transcription factor, dHAND, is required for vascular development. J Clin Invest. 2000; 105:261–270. [PubMed: 10675351]
49. Yasue H, Yoshimura M, Sumida H, Kikuta K, Kugiyama K, Jougasaki M, Ogawa H, Okumura K, Mukoyama M, Nakao K. Localization and mechanism of secretion of B-type natriuretic peptide in comparison with those of A-type natriuretic peptide in normal subjects and patients with heart failure. Circulation. 1994; 90:195–203. [PubMed: 8025996]

50. Zeller R, Lopez-Rios J, Zuniga A. Vertebrate limb bud development: moving towards integrative analysis of organogenesis. Nat Rev Genet. 2009; 10:845–858. [PubMed: 19920852]

51. Zhu J, Nakamura E, Nguyen M-T, Bao X, Akiyama H, Mackem S. Uncoupling sonic hedgehog control of pattern and expansion of the developing limb bud. Dev Cell. 2008; 14:624–632. [PubMed: 18410737]

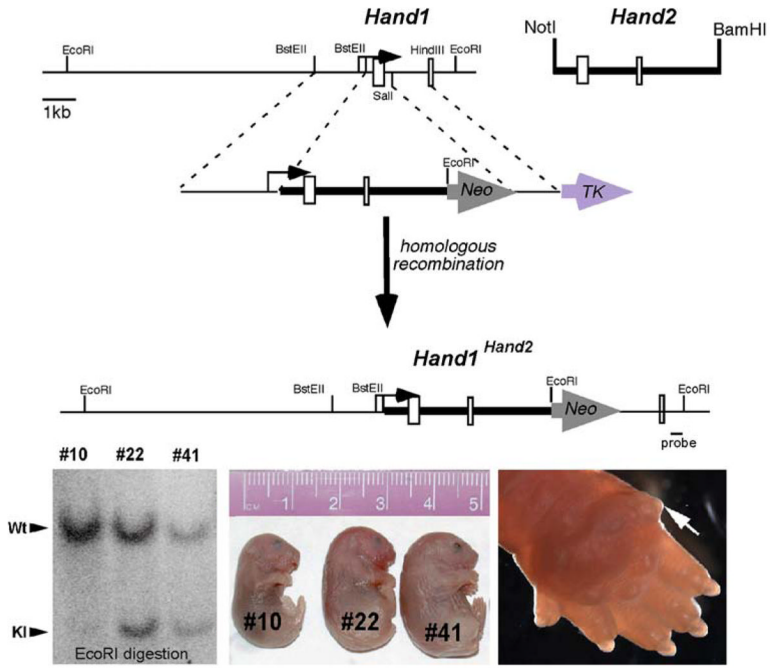

Fig. 1.

Analysis of *Hand1*^{Hand2} chimeric mice. (*upper panel*). The schematic depicts the targeting strategy used. A *Hand2* genomic NotI-BamHI fragment containing both exons and lacking the transcriptional start site was cloned downstream of the *Hand1* transcriptional start site contained in the *Hand1* 5′ targeting arm. An additional EcoRI site creates an RFLP, enabling detection of a 3.9-kb EcoRI fragment when a 3′ external probe (indicated) is used (*lower right panel*). Southern analysis demonstrates chimerism via comparison of the molar ratio of the wild-type (*Wt*) and knock-in (*KI*) mutant bands in three newborn F_0 age-matched mutant littermate chimeras. Chimera #10 contains less than 15% chimerism control (#10) whereas chimeras #22 and #41 show greater than 95% chimerism (*lower middle panel*). Stillborn high-percentage *Hand1*^{Hand2} (*Hd1*^{Hd2}) chimeras (#22 and #41) exhibit wholesale edema compared with viable low-percentage unaffected littermates (#10) (*lower left panel*). Closer examination shows polydactyly in high-percentage *Hd1*^{Hd2} chimeras (ectopic posterior digit indicated by the *white arrow*)

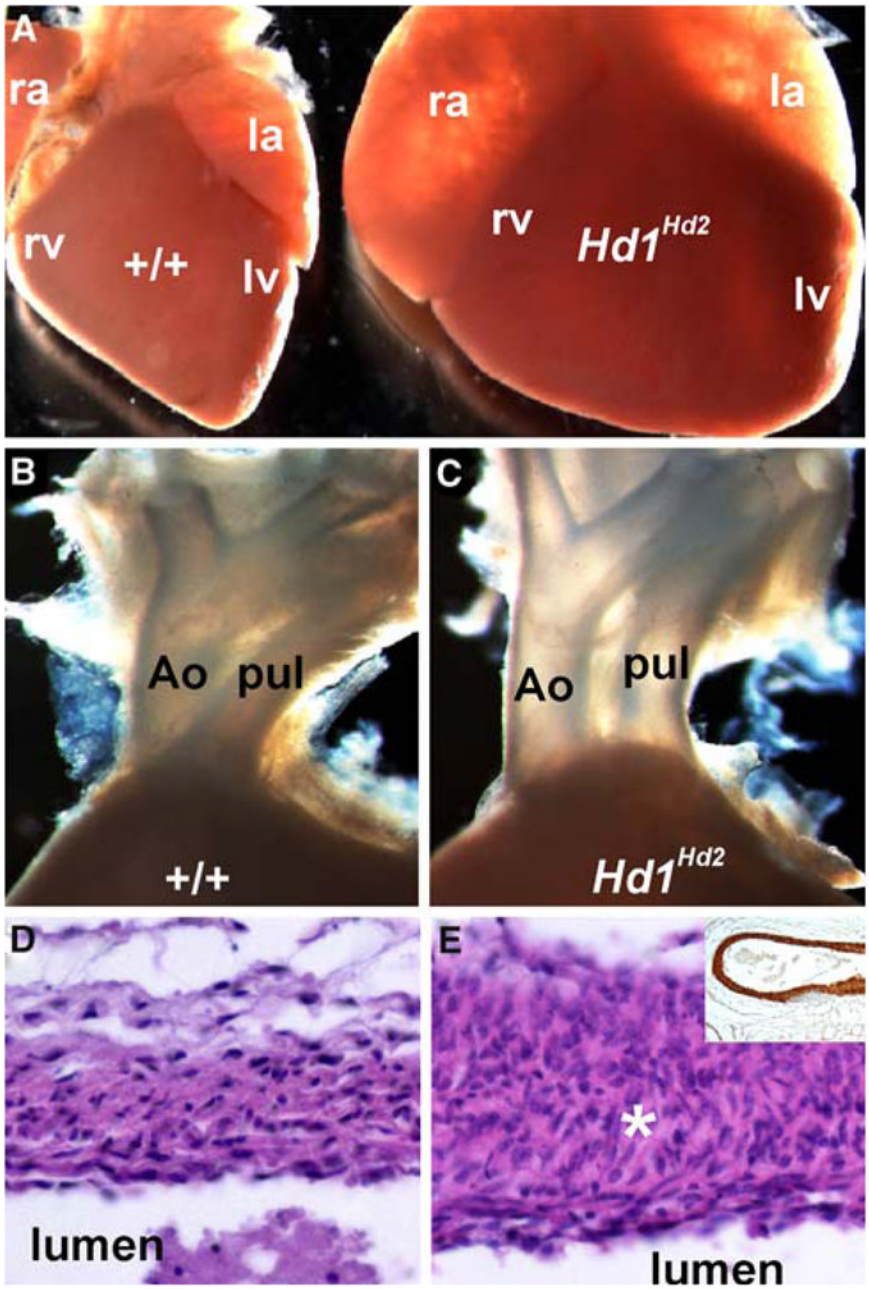

Fig. 2.

Cardiac defects in the high-percentage *Hand1*^{Hand2} chimeric newborns. **a–c** Wild-type (+/+) and stillborn high-percentage *Hd1*^{Hd2} chimeric age-matched littermate hearts. Note the dilation of both atria and ventricles and the rounded apex at the base of the heart compared with controls. Closer examination shows that high-percentage chimeras exhibit double-outlet right ventricle (DORV) as both the aorta (*Ao*) and pulmonary trunk (*pul*) exit the right ventricle (**c**) compared with wild-type littermates (**b**). **d, e** Hematoxylin and eosin (H&E) sections through the wild-type (**d**) and *Hd1*^{Hd2} chimeric aortic arch show hyperplasia of the smooth muscle layer (*asterisk*) around the outflow tract (OFT) vessels. Note however, that there is appropriate α-smooth muscle actin (αSMA) staining of the *Hd1*^{Hd2} chimeric

vasculature (shown in the *inset* in **e**). *ra* right atria, *rv* right ventricle, *la* left atria, *lv* left ventricle

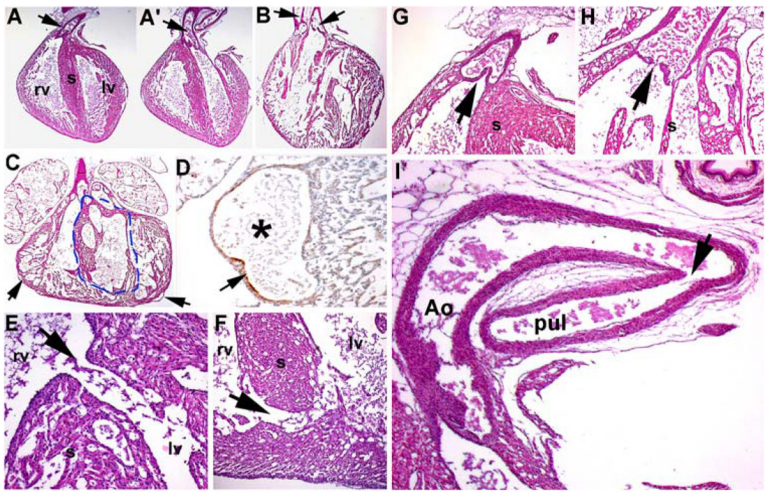

Fig. 3.

Histologic examination of cardiovascular defects in *Hand1*^{Hand2} newborns. **a, a′** Wild-type heart depicting the exit of the pulmonary trunk from the right ventricle (*arrow* in **a**) and exit of the aorta from the left ventricle (*arrow* in **a′**). **b** Stillborn high-percentage *Hd1*^{Hd2} chimeric heart exhibiting double-outlet right ventricle (DORV) as both outflow tract (OFT) vessels exit the right ventricle (*arrows*), as well as mild hypoplasia of the ventricles, thin myocardial walls, and abnormal perforated septum resulting in both membranous and muscular interventricular septal defects (VSDs). **c, d** Additional example of high-percentage *Hd1*^{Hd2} chimeric heart illustrating ventricular septal hypoplasia (indicated via the *dotted line*) and dilated coronaries (*arrows* in **c**). High-power view of fluid-filled mutant coronary artery (*asterisk* in **d**) and incomplete α-smooth muscle actin (αSMA) expression in the supporting muscle layer of the dilated coronaries (*arrow* in **d**). **e, f** Histologic sectioning showing associated perimembranous VSDs (high-percentage chimera shown in panel **b**) and muscular VSDs (low-percentage chimera). **g, h** *Hd1*^{Hd2} chimeric valve leaflets are hypoplastic and misshapen (chimera in panel **b**) compared with wild-type littermates. **i** Stillborn high-percentage *Hd1*^{Hd2} chimeras ($n = 6$) all exhibiting patent ductus arteriosus as the ductus remains open (*arrow* in **i**) between the descending aorta (*Ao*) and the pulmonary trunk (*pul*), resulting in a high-volume burden on the neonatal lungs and respiratory failure. *rv* right ventricle, *lv* left ventricle

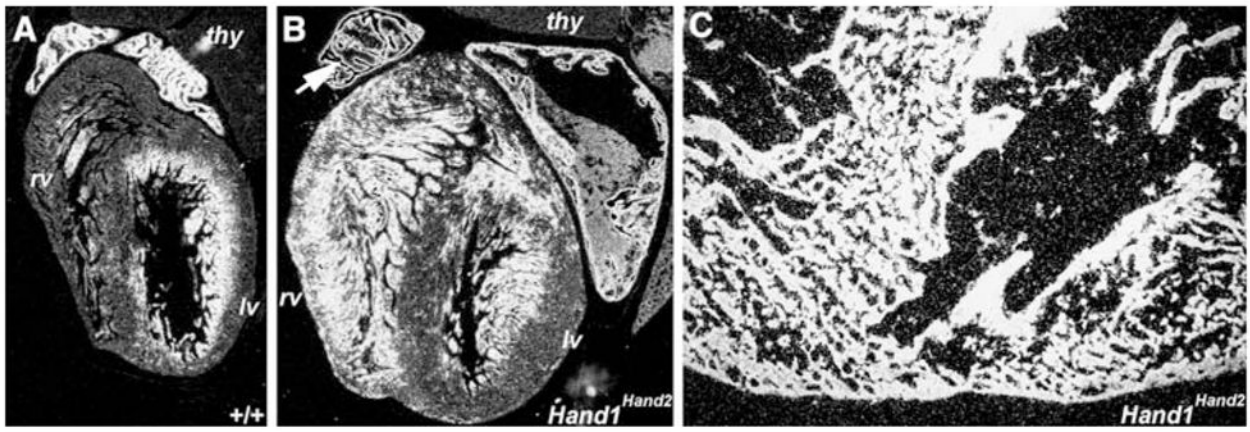

Fig. 4.

Natriuretic peptide precursor type A (*Nppa*) is upregulated in $Hand1^{Hand2}$ newborn hearts. **a, b** According to S^{35} in situ hybridization analysis, *Nppa* mRNA expression is significantly and ectopically upregulated in F_0 medium-percentage (~40 to 60%) chimeric newborn $Hd1^{Hd2}$ hearts compared with wild-type (+/+) age-matched littermate hearts. Note that in mutant hearts (**b**), upregulation of *Nppa* is clearly detectable in the atria and ventricles (but not in the thymus [thy]), whereas in normal hearts (**a**), expression usually is confined to the ventricular trabecular region and the atria. High-level *Nppa* expression in the chimeric right atria has a burnt-in signal and at this point appears black in a dark field image (*arrow* in **b**). **c** In a high-power view of stillborn high-percentage $Hd1^{Hd2}$ chimeras, *Nppa* is abnormally ubiquitously expressed in all cardiomyocytes in the dilated mutant ventricles. All in situs were probed with the same S^{35} probe and exposed for an equivalent exposure time. *rv* right ventricle, *lv* left ventricle

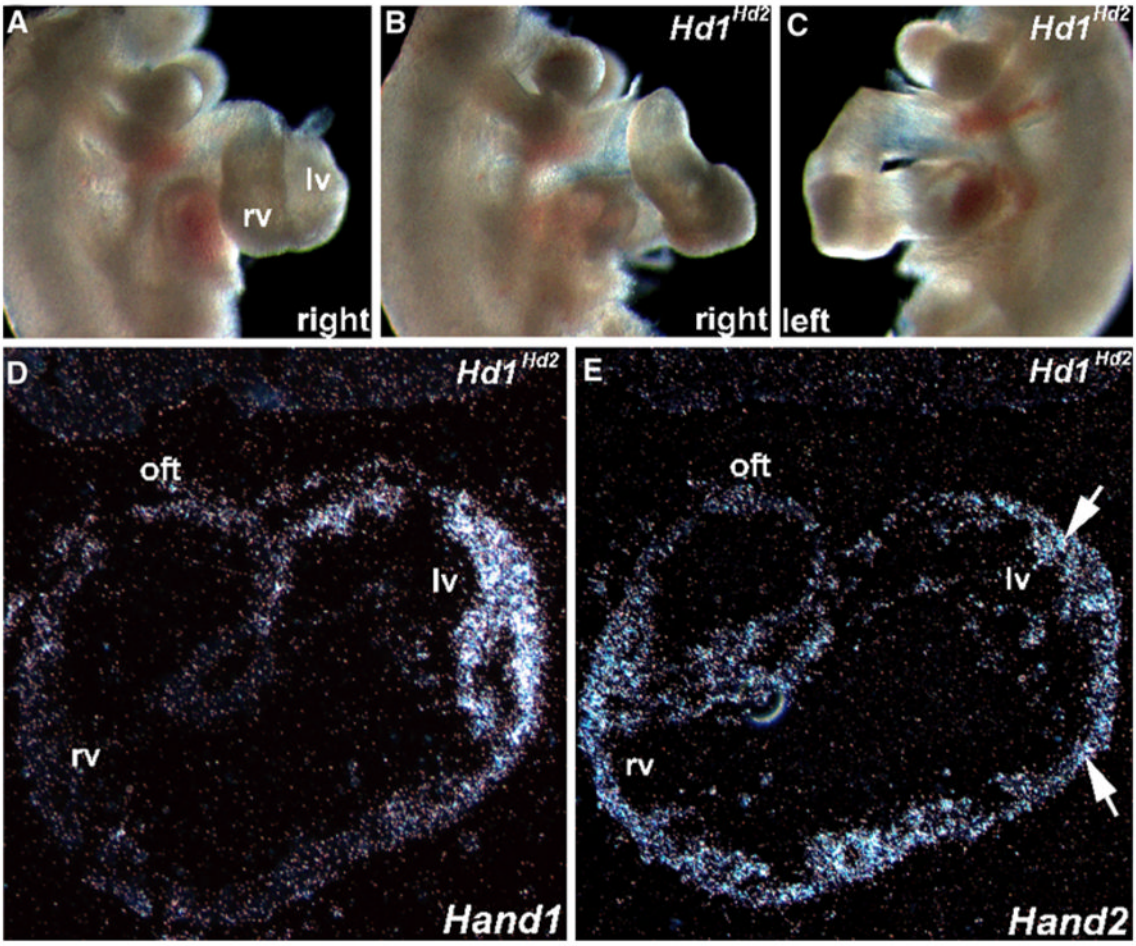

Fig. 5.

In utero analysis of E10.5 high-percentage *Hand1*^{Hand2} chimeric embryos. **a–c** Whole-embryo right-sided view of a normal E10.5 (**a**) and both right- (**b**) and left- (**c**) sided views of age-matched littermate F_0 *Hd1*^{Hd2} high-percentage chimeric embryos. Note that the chimeric heart is "unlooped" and farther away from the body of the normal littermate (i.e., both inflow and outflow tract [OFT] are elongated). Otherwise, the chimeric embryos are comparable in size, shape, and structure with their nonchimeric littermates. Also, the chimeric hearts beat at rates comparable with that of control siblings. **d, e** Using S^{35} in situ hybridization analysis, sections of the chimeric embryo heart (shown in **b** and **c**) were probed with *Hand1* (**d**) and *Hand2* (**e**) cDNA probes. Note that *Hand1* mRNA is appropriately expressed in the left ventricle but that *Hand2* mRNA is both appropriately expressed in the right ventricle and ectopically expressed in the left ventricle (*arrows* in **e**). *rv* right ventricle, *lv* left ventricle, *oft* outflow tract