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A loss-of-function mutation in the binding domain of HAND1 predicts hypoplasia of the human hearts

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Hypoplasia of the human heart is the most severe form of congenital heart disease (CHD) and usually lethal during early infancy. It is a leading cause of neonatal loss, especially in infants diagnosed with hypoplastic left heart syndrome (HLHS), a condition where the left side of the heart including the aorta, aortic valve, left ventricle (LV) and mitral valve are underdeveloped. The molecular causes of HLHS are unclear, but the basic helix–loop–helix (bHLH) transcription factor heart and neural crest derivatives expressed 1 (Hand1), may be a candidate culprit for this condition. The absence of Hand1 in mice resulted in the failure of rightward looping of the heart tube, a severely hypoplastic LV and outflow tract abnormalities. Nonetheless, no *HAND1* mutations associated with human CHD have been reported so far. We sequenced the human *HAND1* gene in heart tissues derived from 31 unrelated patients diagnosed with hypoplastic hearts. We detected in 24 of 31 hypoplastic ventricles, a common frameshift mutation (A126fs) in the bHLH domain, which is necessary for DNA binding and combinatorial interactions. The resulting mutant protein, unlike wild-type (wt) HAND1, was unable to modulate transcription of reporter constructs containing specific DNA-binding sites. Thus, in hypoplastic human hearts HAND1 function is impaired.

INTRODUCTION

Mortality because of heart malformations in congenital heart disease (CHD) is estimated to be one-tenth of all infant deaths worldwide (1). A serious form of CHD and a leading cause of neonatal loss is the hypoplastic left heart syndrome (HLHS), a condition where the left side of the heart including the aorta, aortic valve, left ventricle (LV) and mitral valve are underdeveloped. Usually HLHS is fatal within the first 2 weeks of life, if not surgically corrected. The molecular causes resulting in HLHS are unclear, but the transcription factor heart and neural crest derivatives expressed 1 (Hand1) may be a candidate culprit for this condition. The absence of Hand1 in mice resulted in the failure of rightward looping of the heart tube, a severely hypoplastic LV and outflow tract abnormalities (2,3). This observation prompted us to investigate the human *HAND1* gene in heart tissues derived from 31 patients diagnosed with hypoplastic hearts. There were no previous reports of *HAND1* mutations associated with human CHD.

Hand1 is a member of the class B (tissue-specific) basic helix–loop–helix (bHLH) transcription factors (reviewed in Ref. 4). The bHLH domain is a DNA binding and dimerization motif that is comprised of a short stretch of basic amino acids followed by an amphipathic α helix, a loop and an additional α helix. Hand1 heterodimerizes with class A (widely expressed) E-factor, e.g. TCF3 (=E2A, E12/E47), or with the most closely related Hand2. *In vitro* studies demonstrate that Hand1 can activate and also repress transcription depending on the target sequence (consensus E-box or degenerate Thing1/D-box) and dimerization partner (5). We show here that in hypoplastic human hearts HAND1 function is impaired.

RESULTS

Sequence analysis of *HAND1* in hypoplastic human ventricles revealed a common frameshift mutation affecting the bHLH domain

To determine whether *HAND1* has a role in hypoplasia of the human heart, we investigated 31 hypoplastic hearts (24 left and 7 right hypoplastic hearts; see Tables 1 and 2). Genetic analysis of blood samples may not be informative for

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Table 1. Summary of cardiac malformations of analyzed hypoplastic hearts from the Leipzig collection

No.	Cardiac malformations	Hyp. hearts
1	Hypoplastic left ventricle	20
2	Hypoplastic left atrium	2
3	Hypoplasia of the right positioned left ventricle	1
4	Cortriloculare biatriatum (=single ventricle)	4
5	Hypoplastic mitral valve	4
6	Mitral valve aplasia	1
7	Mitral valve atresia	8
8	Mitral valve stenosis	4
9	Hypoplastic aorta	2
10	Hypoplastic aortic arch	1
11	Right-sided aortic arch (=right aortic arch)	1
12	Interrupted aortic arch (IAA)	1
13	Atresia of the ascending aorta	1
14	Hypoplastic ascending aorta	10
15	Aortic valve atresia	14
16	Aortic valve stenosis	1
17	Aortic isthmus stenosis	1
18	Preductal aortic isthmus stenosis	8
19	Hypoplastic right ventricle	6
20	Tricuspid aplasia	1
21	Tricuspid atresia	4
22	Tricuspid stenosis	2
23	Pulmonary atresia	1
24	Pulmonary valve stenosis	3
25	Hypoplastic pulmonary trunk	2
26	Persistent left superior vena cava draining into the coronary sinus	1
27	Persistent left superior and inferior vena cava draining into the left atrium	1
28	Persistent left superior vena cava connected to the major pulmonary veins and draining into vena brachiocephalica	1
29	Complete transposition of the great arteries	2
30	Transposition of the great arteries	2
31	Dysphagia lusoria (aortic root anomaly)	3
32	Common atrioventricular canal	1
33	ASD (persistent foramen secundum)	1
34	ASD (septum primum defect)	3
35	ASD (septum secundum defect)	1
36	VSD (muscular)	3
37	VSD (subaortic)	9
38	Double-outlet right ventricle (DORV)	3
39	Patent ductus arteriosus (PDA)	22
40	Closed ductus arteriosus	1
41	Patent foramen ovale (PFO)	25
42	Right displacement of the ventricular septum	1
43	Strand-like obliteration of the pulmonary veins	1
44	Ventricular inversion (position of ventricles reversed)	1
45	Drainage of the pulmonary vein into the V. cordis magna	1
46	Isomerism of right atrial appendages	1

mutations associated with cardiac malformations, owing to the effect of HAND1 in placentation causing early embryonic lethality (2,3). In this study cohort, an array of 46 different cardiac malformations was represented (Table 1), and patients had an average of 6 (range 3–11) malformations (Table 2).

We identified a diagnostic frameshift mutation consisting of a deletion of a G nucleotide at position 376 (Fig. 1A), affecting the amino acid sequence starting from Alanine 126 in the bHLH domain. This mutation (c.376delG, A126fs) was detected in 24 of 31 hypoplastic left or right ventricles (RV) by direct DNA sequencing, and confirmed by independent polymerase chain reaction (PCR) reactions (data not shown), by cloning of the PCR products (Fig. 1B) and by PCR-RFLP analysis with *Nae*I (Fig. 1C). While A126fs was detected primarily in hypoplastic ventricles, strikingly, we identified nine patients to carry this mutation in both hypoplastic and non-hypoplastic ventricles of the same diseased heart. This finding is consistent with newly emerging concepts of heart development as will be discussed later on. We also identified additional but infrequent sequence alterations leading to amino acid change (nonsynonymous change), in hypoplastic and otherwise malformed hearts (Fig. 1D and Table 2), but only A126fs, appeared as a predictor mutation for the hypoplastic condition. The reason for the high frequency of this particular mutation in samples from unrelated patients is presently unknown, but it does not seem to be related to a specific at-risk DNA sequence context. The same sequence context (CGGC) is present eight other times in the *HAND1* gene, but no mutations were observed at those sites in our analysis.

The hypoplastic hearts studied here are further examples from the Leipzig collection and different from those reported previously (6). But since the malformed hearts in the Leipzig collection were conserved in formalin, there is a concern that DNA fragmentation might produce PCR artifacts such as allele scrambling. Thus, we carried out additional experiments on tissues derived from normal formalin-fixed ($n = 10$) hearts from the same collection and normal frozen ($n = 5$) hearts, as well as blood samples ($n = 100$) from healthy volunteers. Frozen human hypoplastic hearts were not available. Notably, the *HAND1* sequence alterations (Fig. 1D and Table 2) were absent in control group (data not shown), confirming that formalin fixation did not induce artificial sequence alterations, consistent with a previous investigation from our group regarding specificity of findings in archived DNA (7). But more importantly, when our first cohort of 68 formalin-fixed hearts, which were also from the same heart collection (i.e. treated and stored in same manner), but selected for septation defects was studied, none except 1 with severe malformations carried the *HAND1* frameshift mutation. Furthermore, the mutations we observed in diseased hearts appear to cluster in two specific areas of the protein, one surrounding the histidine-rich region in the amino-terminal and the other corresponding to the bHLH domain. Overall, our results demonstrate a pivotal role of *HAND1* mutations in CHD.

A126fs frameshift mutation results in a truncated protein that is unable to modulate transcription of reporter constructs containing specific DNA-binding sites

To address the functional consequences of the A126fs, we developed a yeast-based assay, drawing upon our previous work on NKX2-5 and p53 (8,9). HAND1 and its class A bHLH partners E12 and E47 were cloned into yeast-inducible expression vectors. Yeast reporter strains were developed placing D-box (CGTCTG) and E-box (CATCTG) response elements (REs) upstream of the luciferase gene.

The yeast-based system has been chosen for this study because it provides a defined experimental set-up to address functional properties of sequence-specific transcription

Table 2. Spectrum of *HAND1* mutations in ventricles of patients with hypoplastic hearts

Patient	Age	Hyp. heart	Total defects	Types of cardiac malformations[a]													Detected mutations		
				1–4	5–8	9–18	19	20–22	23–25	26–30	31	32–37	38	39–40	41	42–46	Hypoplastic ventricle	Non-hypoplastic ventricle	Both ventricles
A11	3 months	Left	8	1	5				24, 25	30		37		40	41		L28H, A126fs	N2D, L28H	L28H
A75	13 days	Left	9	4	6				24, 25	30	31	34			41	45	L28H, A126fs		
A82	2 months	Left	11	3	7	11			24	27		32, 34, 37	38			42, 44	A126fs		
B02	4 days	Left	6	1	5	15, 18								39	41		A126fs	A126fs	A126fs
B05	6 days	Left	6	1	8	14, 15, 18									41			P16S, L28H, K96E	
B08	4 days	Left	6	1		13, 15					31			39	41		A126fs	L150P	
B09	2 days	Left	6	1		14, 15						36		39	41			H10Y	
B10	Newborn	Left	6	1	5	10, 15								39	41		L28H		
B11	11 days	Left	6	1	8	14, 15								39	41		L28H, E116V, A126fs, L138P	F56L, A126fs	A126fs
B14	6 days	Left	7	1	7	12						36, 37		39	41		N2D, L28H, A126fs	A126fs	A126fs
B15	Newborn	Left	5	1		14, 15								39	41		E119V, A126fs	K96E	
B16	Newborn	Left	8	2, 4	7	14, 15, 18								39	41		L28H		
B22	3 days	Left	7	4	7	9, 15					31	33		39			L28H, A126fs	L28H, I133V	L28H
B23	6 weeks	Left	6	1, 2	7							37	38		41		A26fs, L28H, N2D	R84L, L138I, I143V	
B26	6 months	Left	5	1	7								38	39	41		A126fs	A126fs	A126fs
B29	5 weeks	Left	4	1	8	15, 18											E116V, A126fs	P16S, L28H	
B34	?	Left	6	1	7	14, 15								39	41		A126fs	P18H, M22T	
B35	3 days	Left	6	1		14, 15, 18								39	41		F56L, E116V, A126fs, L138P	K129 N	
C02	Newborn	Left	3	1		16, 17											A67V, A126fs	A126fs	
C11	3 days	Left	5	1	7									39	41	43	L28H		
C12	2 weeks	Left	5	1		14, 15								39	41		A126fs		
C25	13 h	Left	7	1	5	18						36, 37		39	41		E116V, A126fs, L138P	F56L	
C38	2 days	Left	6	1	8	14, 15								39	41		A126fs	A126fs	A126fs
C64	11 months	Left	4	1						28		35			41		A126fs	L3P	
A17	8 months	Right	4				19	21						39	41		A126fs	N2D	
A37	6 days	Right	7	4		18		20, 22		29				39	41	46	N2D, L28H, F56L, A126fs, L138P, E119G	A126fs	A126fs
A80	6 weeks	Right	7			14, 18	19			29		37		39	41		L28H		
B25	4 years	Right	6				19	21		26		34, 37		39			A126fs	H13Y, Q80R	
B31	?	Right	5				19	21				37		39	41		A126fs	A126fs	A126fs
C18	5 days	Right	3				19	22	23								E116V, L138P	L28H	
C56	7 days	Right	6			9	19	21				37		39	41		A126fs	L28H, A126fs	A126fs

[a]1–4 (hypoplastic left ventricle or left atrium, single ventricle); 5–8 (mitral valve defects), 9–18 (aorta, aortic valve defects); 19 (hypoplastic right ventricle); 20–22 (tricuspid valve defects); 23–25 (pulmonary valve defects); 26–30 (defects of the great vessels); 31 (dysphagia lusoria); 32–37 (septal defects); 38 (DORV); 39–40 (patent or closed ductus arterosius); 41 (patent foramen ovale); 42–46 (other malformations) (See Table 1).

factors expressed at variable levels in isogenic conditions. As yeast does not have homologs for HAND1 or E12/47, these transcription factors can be expressed in isolation in strains engineered to contain specific cognate REs upstream of a minimal promoter and a reporter gene. Exploiting appropriate selection markers and finely tunable expression systems, it is then possible to co-express interacting partners and measure the impact on transactivation. In our previous study, where the yeast reporter strains contained REs derived from the human ANF promoter, we have shown that the assay could reveal even subtle defects caused by *NKX2-5* mutations and for the cases where comparisons were possible with published data, the transactivation data in yeast matched results obtained in mammalian cells (8,10).

First, we established that the assay could assess HAND1-dependent transcriptional effects. Expression of wt or the A126fs mutant HAND1 alone did not result in transcriptional modulation of the reporters containing the D-box nor the E-box (Fig. 1E). On the contrary, expression of E12 or E47 could lead to transactivation of the reporter containing the E-box, but not the D-box RE (Fig. 1E). Co-expression of wt HAND1 and its class A partners led to the transactivation at the D-box, but to repression at the E-box RE. Comparable results were obtained with transfected JEG-3 mouse trophoblastic tumor cells (5). Next, we examined the functional consequences of A126fs mutation and compared results with a mutation in the basic region of the bHLH domain, K96E (Fig. 1D). Contrary to wt HAND1, the A126fs mutant co-expressed with E12(E47) was unable to stimulate the D-box- nor to repress the E-box-containing promoters. Instead, the K96E mutant was fully capable of repressing the E-box promoter, but activation of the D-box reporter was nearly abolished. Hence, K96E could be considered as a separation-of-function allele, whereas A126fs appeared as a loss-of-function allele.

The A126fs frameshift mutation would result in premature truncation at amino acid 137 (Fig. 2A) with a 12 amino-acid fragment specific of the mutant protein, which is predicted to have very limited α-helical content, unlike the corresponding wt sequence (http://ca.expasy.org/tools/#secondary). The mobility and expression levels of HAND1 wt and A126fs protein assayed by western blot (Fig. 2B) were consistent with the predicted loss of ∼9 kDa molecular weight and showed a nearly 2.5-fold reduction in mutant protein amount. This difference could result from reduced mRNA stability because of nonsense-mediated decay, as supported by relative mRNA quantification of the wt and A126fs transcripts at basal levels and 6 h after repression of the inducible *GAL1* promoter (Fig. 2C). Hence, the phenotypic impact of A126fs mutant protein could also be in part related to its reduced expression level.

DISCUSSION

Notably, hypoplastic LV is a cardiac abnormality observed in *Hand1*-null mice (2). *Hand1* is expressed in distinct regions of the linear heart tube, and at post-looping it becomes localized specifically in the outer curvature of the presumptive LV and developing outflow tract, and also at lower levels in the outer curvature of the RV. In *Hand1*-null embryonic stem cell-derived cardiomyocytes, there was significantly elevated cardiomyocyte differentiation, with an apparent default mesoderm pathway to a cardiomyocyte fate, to foster hypoplasia (11). Here, we report a frequent frameshift mutation in the bHLH domain of HAND1 that predicts hypoplastic human hearts. We provide a conclusive evidence for A126fs to be a loss-of-function mutation incapable of functionally interacting with its class A partners, to either stimulate the D-box- or to repress the E-box-containing promoters. Consequently, A126fs derails the cardiac development program. It is of considerable importance that none of our patients' tissues were homozygous for the mutation, consistent with the genetic studies in mice where *Hand1* knock-out results in embryonic lethality. In fact, the specific presence of the mutation in hypoplastic ventricles as well as the mutant allele proportion in the sequencing electropherograms (Fig. 1A) are both consistent observations with a *de novo* origin during heart development.

It should be noted, however, that A126fs was detected in 24 of 31 hypoplastic left or RVs, but in 8 of 24 cases it was also present in the non-hypoplastic ventricles of the same heart. This pattern could be explained by an origin of the mutant allele in precursor cells of the primary heart field. From studies in mice, it was postulated that the conditional heart-specific *Hand1* mutant embryos with LV hypoplasia could be the result of a proliferation defect in the primary heart field lineage (12). Recent studies have revealed two distinct sources of cardiac progenitor cells that contribute to different parts of the heart (see review, Ref. 12). Tracing of β-galactosidase labeled mouse cells and their descendants in the heart showed that one lineage (primary heart field) contributes to both ventricles, the atrioventricular (AV) canal and both atria, whereas the other lineage (anterior or secondary heart field) contributes to the outflow tract and all other heart regions, except the LV. Both lineages are present in the RV, AV canal and atria, where there is considerable overlap, although in some regions clonal domains of the first or second lineages seem to be complementary. The existence of these two distinct populations of cardiac progenitors may explain cardiac abnormalities in which specific segments of the heart are underdeveloped, while the remainder of the heart remains unaffected. The clonal domains can also explain why we were able to detect in a few non-hypoplastic RVs the presence of A126fs by direct sequencing (Table 2). Therefore, the identification of A126fs in non-hypoplastic ventricles is likely to be traced back to cells from the primary heart field precursor, a finding consistent with lineage-tracing studies done in mice (12). However, recent studies in mice using different approaches show even broader contribution of the secondary heart field to the heart (reviewed in ref. 13). Although some results varied, these studies demonstrate that the secondary heart field contributes extensively not only to the outflow tract and RV, but also to the interventricular septum. Nonetheless, we detected a frameshift mutation affecting bHLH of HAND1 that was highly associated with hypoplastic condition, but definitive studies, including the generation of cardiac-specific knock-in animal models, are necessary to determine its exact origin and pathogenesis at the organ level.

Furthermore, we cannot discount the possibility that besides A126fs, mutations in other cardiac transcription factor genes

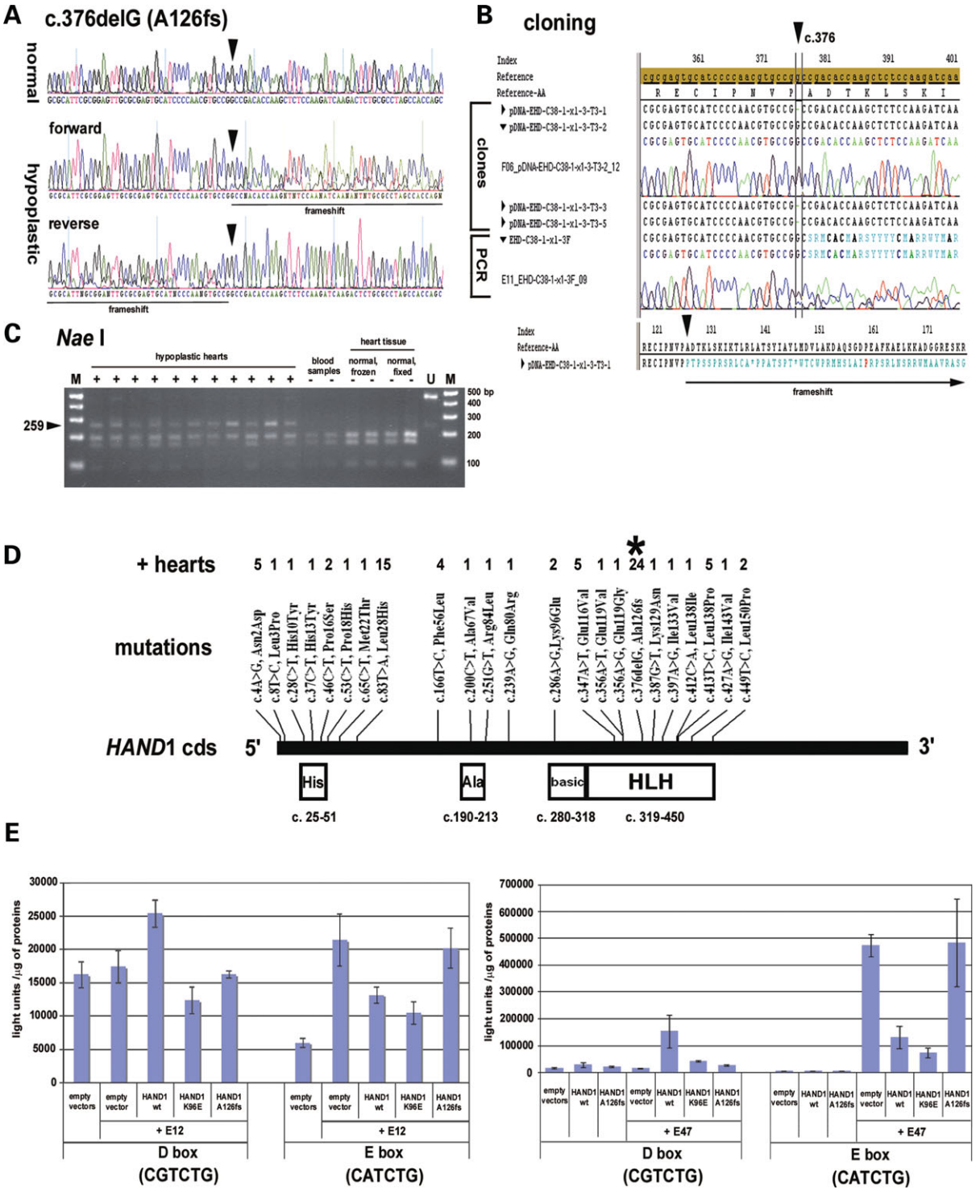

Figure 1. Genetic analysis of *HAND1* in hypoplastic human hearts. (**A**) Sequence electropherograms from a normal, fixed heart exhibiting the reference sequence, and from a hypoplastic heart with A126fs in forward and reverse directions directly from PCR products, or (**B**), after cloning (shown are results for patient C38). (**C**) Example of PCR-RFLP assay with *Nae*I digestion used as an additional confirmatory test for the presence of mutations. A 455 bp fragment was amplified using forward-5′tcggttccggcctcgcgctgtcatc3′ and reverse-5′ctccctttccgcttgctctcacg3′ primers (see undigested fragment, U). A126fs mutation results in loss of a *Nae*I site and the consequent appearance of a 259 bp fragment in positive hypoplastic hearts, but not in controls consisting of blood samples, normal frozen or fixed heart tissues. (**D**) A total of 23 sequence alterations were detected; their distribution along *HAND1* coding sequence (cds) and corresponding HAND1 domains and frequencies are shown. A126fs is designated by asterisk, detected in 24 hypoplastic ventricles. (**E**) Transactivation capacity of wt and mutant HAND1. D- and E-box yeast reporter strains were transformed with inducible expression vector coding the class A bHLH E12 (A) or E47 (B), along with a plasmid expressing wild-type or mutant HAND1. Purified transformants were grown for 24 h on selective, liquid medium containing 0.1% galactose to achieve high level of expression of the transcription factors. Luciferase activity was measured from total protein extracts and normalized to a unit of soluble proteins. Presented are the average light units and the standard deviations for three independent experiments.

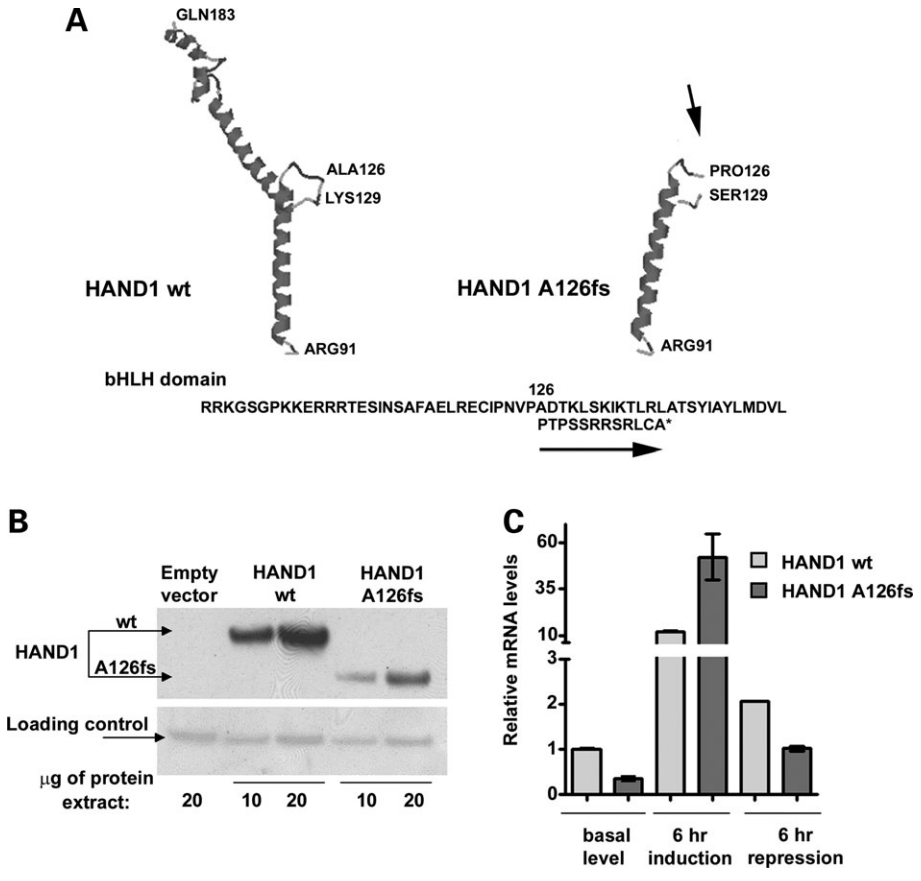

Figure 2. The A126fs mutation results in premature protein truncation. (**A**) Prediction of the effect of A126fs on the secondary structure of the protein. A model for HAND1 bHLH domain (ModBase, http://modbase.compbio.ucsf.edu/modbase-cgi/search_form.cgi) was used as template and the model was mutated by replacement of 12 amino acid sequence (aa 126–137, PTPSSRRSRLCA*) unique to the mutant protein that precedes the premature stop codon. Protein modeling was carried out using Swiss-PdbViewer (http:// expasy.org/spdbv/) and Protein Explorer (http://proteinexplorer.org). Ala126 falls within the loop of the bHLH domain; secondary structure will be disrupted resulting from the frameshift mutation. (**B**) Western blot analysis of A126fs mobility and expression level. Total protein extracts were prepared from yeast cells transformed with the HAND1 wt and A126fs (or empty) expression vectors after growth of the cells for 24 h in 0.1% galactose to achieve steady-state high level of expression. Different amount of proteins were loaded on a 4–12% acrylamide gel as indicated. Immunodetection was performed using antibodies directed against the chimeric amino terminal transactivation domain (sc-126 and sc-98). An antibody directed against yeast tubulin (sc-26147) was used as loading control. Bands were quantified using Image Quant software. (**C**) mRNA measurement by real-time PCR. Total RNA was extracted from yeast cells transformed with the inducible HAND1 expression vectors. Cells were cultured in glucose to achieve low basal level of HAND1 expression, in 0.1% galactose for 6 h to induce expression at high levels, and then after shifting the cultures in glucose for six additional hours to repress the *GAL1* promoter and estimate the rate of decrease of both mRNAs toward the basal level. Presented are the relative HAND1 mRNA expression and the variation for two biological replicates, each assessed by three technical replicates in a SYBRGreen assay. A minimal *cyc1* promoter:reporter construct was used as control reference. HAND1 primers were directed at an amino-terminal region that is common to wt and A126fs.

may have contributed to the hypoplastic condition. However, it is important to note that many patients in our cohort did not only suffer from hypoplasia of the heart, but presented other cardiac abnormalities, notably septation defects (see Tables 1 and 2). Our efforts to amplify other cardiac transcription factor genes, e.g. *MEF2C, HEY2, NKX2-5, TBX5* and *HAND2*, from the hypoplastic ventricles have not yet been successful. Indeed, in our previous studies on the Leipzig collection of malformed hearts, the amplification of a whole cardiac transcription factor gene for genetic analysis took us several years to complete. Nonetheless, in spite of this difficulty in using archived DNA, the Leipzig collection is a rare source of pathologically well-characterized malformed hearts obtained over many decades, and a valuable material for the study of cardiac-specific genetic changes in the whole explanted hearts.

We have obtained, however, partial data (24 of 31 samples comprising 21 left and 3 right hypoplastic ventricles) on exon 4 of *GATA4*, allowing analysis of mutations especially those affecting the C-terminal zinc finger of GATA4 (unpublished results). We found three *GATA4* mutations in diseased heart sections from 17 patients with left hypoplastic ventricles as follows: 7 with R266X, 7 with C292R, 2 with R266X+C292R and 1 with C271*R*+C292R. Among these 17 left hypoplastic ventricles with *GATA4* mutations, 13 had A126fs also and 6 of those diseased hearts also exhibited atrial (ASDs) or ventricular septal defects (VSDs). In the past, we and others linked *GATA4* mutations to septation defects of the heart (14–18), and we view those findings as being compatible with the role of *GATA4* in heart development.

Interestingly, mutations R266X (c.796C>T) and C292R (c.874T>C), which are likely to affect the function of the

crucial C-terminal zinc finger of GATA4 (see review, ref. 19), were identified previously in the septal defect groups, in which C292R was a frequent mutation in VSDs, whereas R266X was identified in an atrioventricular septal defect patient (14). Notably, only seven patients in that cohort were further diagnosed with hypoplasia of the LV. This observation argues against a direct causative role of the *GATA4* mutations in HLHS, and there is no molecular basis so far described for a crosstalk of *HAND1* with *GATA4* in ventricular maturation. Furthermore, *GATA4* mutations were identified in familial cases of cardiac septal defects without hypoplasia (15,17,18). At the same time, it is also of considerable importance to note that *GATA4* expression seems to be required for morphogenesis of the RV (20). By using Cre/loxP technology, *Gata4* was selectively inactivated within the myocardium at early as well as late stages of heart development. At both times, it was demonstrated that *Gata4* was an essential regulator of cardiomyocyte proliferation. Early in heart formation, myocardial inactivation of *Gata4* resulted in hypoplasia of the RV, which was associated with a downregulation of *Hand2* expression, and a decrease in cardiomyocyte proliferation that was more severe in the RV than in the LV. Thus, we cannot exclude that the *GATA4* mutations identified by us—and the emerging evidence for somatic mosaicism in our heart samples—may have contributed to the complex morphological heart defects, including the right hypoplastic condition.

The precise function of Hand1 is unclear, but studies in animal models suggest roles in multiple gene programs, and the mechanism of regulation is complex (21). Hand1 is essential in both placentation and cardiac morphogenesis (2,3), as well as in dorso-ventral patterning and interventricular septum formation in the embryonic heart (22). Cardiac-specific deletion of *Hand1* in the developing heart to circumvent early embryonic lethality caused by extraembryonic defects resulted in a spectrum of heart abnormalities including membranous VSDs, overriding aorta, hyperplastic AV valves and double-outlet RV (23). A similar spectrum of morphological abnormalities was observed in our patient cohort with defective *HAND1*. Recently, it was demonstrated that Hand1 could directly activate cardiac *Anf* promoter, making it the first known Hand1 transcriptional target (24). The action of *Hand1* did not require heterodimerization with class A or DNA binding through E-box elements. Instead, Hand1 was recruited to the promoter via physical interaction with Mef2 proteins, resulting in synergistic activation of Mef2-dependent promoters. In the light of this finding, A126fs and the other mutations affecting the bHLH domain of HAND1 observed in malformed hearts could impact on HAND1–MEF2 interactions as well, leading to cardiac malformations in CHD.

MATERIALS AND METHODS

Heart and blood samples

We investigated a total of 31 hypoplastic hearts (24 left and 7 right hypoplastic hearts; see Tables 1 and 2). These were an additional group within the Leipzig collection of formalin-fixed malformed hearts that also contains a group of hearts with septal defects described previously (6). As controls, we analyzed DNA from ten formalin-fixed heart tissues from the same collection, five normal frozen hearts, as well as blood samples of 100 unrelated healthy Caucasian individuals.

Genomic DNA isolation, mutation detection

Genomic DNA isolation and mutation detection have been described previously (6). The human *HAND1* gene was mapped to chromosome 5q32, contains two exons spanning 3.3 kb and codes for a protein of 215 amino acids. In searching for *HAND1* mutations associated with the hypoplastic condition, we PCR-amplified fragments within the exons allowing entire coverage of the coding region of the gene, except for 31 nucleotides (c.541–571), from DNA isolated from hypoplastic as well as non-hypoplastic sections of the ventricles of each patient's heart, followed by DNA sequencing. Materials used in this study were obtained in accordance with an approved protocol. JB has obtained approval to conduct genetic studies involving human materials from the Medical School of Hannover. The formalin-fixed hearts were collected more than 40 years ago and cadavers were donated voluntarily by patient's relatives. Sequence variations were verified by independent PCR, double-strand sequencing, PCR-RFLP or cloning of heterozygous genotypes followed by subsequent sequencing of clones, allowing the identification of variant alleles.

Functional assays of mutations, protein and RNA expression analyses

The wt *HAND1* cDNA was cloned in the pTSG-TAD yeast expression vector by a RT-PCR-based approach, starting from total RNA extracted from normal human heart tissue, as previously described (8). pTSG-TAD is a centromeric *TRP1*-selective yeast expression vector containing the inducible *GAL1* promoter and an acidic transactivation domain (TAD) derived from the human transcription factor p53. Full-length *HAND1* cDNA was inserted in the vector obtaining an in-frame Nter fusion with the TAD. *HAND1* mutations were constructed using pTSG-TAD-HAND1 as a template and site-directed mutagenesis, following the manufacturer's protocol (Invitrogen, Karlsruhe, Germany). Wild-type cDNAs for the class A E12 and E47 transcription factors were a generous gift of Dr. C. Murre, UCSD. The cDNAs were cloned in the pUSG-TAD yeast expression vector, which is equivalent to the pTSG-TAD except that it contains the *URA3* selection marker, enabling combined selection of HAND1 and E12 or E47 expression vectors in yeast. All clones were analyzed by restriction digestion and confirmed by DNA sequencing.

HAND1 yeast reporter strains were constructed starting from the yLFM-ICORE and following the *delitto-perfetto in vivo* mutagenesis protocol (25) and as previously described (8). The yLFM-ICORE strain contains the luciferase cDNA integrated at the *ADE2* locus on chromosome XV downstream a minimal promoter derived from the *CYC1* gene. The ICORE cassette is located 5′ of the minimal promoter and enables high-efficiency targeting of the locus by oligonucleotides

that contain desired RE sequences. With this approach we constructed strains containing D-box (CGTCTG; yLFM-Dbox) and E-box (CATCTG; yLFM-Ebox) REs (5) and followed the induction of the luciferase reporter upon expression of HAND1 and/or interacting partners. Details on strains construction and complete vectors and primers sequences are available upon request. Yeast reporter strains were transformed with the expression vector following a standard LiAc-based method (9).

Luciferase assays were performed using protein extracts obtained from mechanical lysis of yeast transformant cells cultured in liquid media containing 0.1% galactose to achieve high level of expression of HAND1 proteins and E47/E12. Protein extracts were quantified using the BCA assay (Pierce Biotechnology, Milan, Italy) and luciferase activity was measured using a multilable Mithras LB940 plate reader (Berthold technologies, Milan, Italy) and the BrightGlo assay reagent (Promega, Milan, Italy), as described previously (9). Steady-state HAND1 wt and mutant protein expression at high levels were examined by western blot. Soluble protein fractions of yeast transformants were prepared, after 24 h growth in the same culture conditions used for the luciferase assays, by mechanical cell disruption in CCLR buffer (Promega) and 0.5 mm acid-washed glass beads (Sigma-Aldrich, Milan, Italy) and clearing by centrifugation at >13 K rpm at 4°C. Extracts were quantified (Bradford assay, Bio-Rad, Milan, Italy) and loaded on 4–12% NuPage Precast acrylamide gels (Invitrogen, Milan, Italy). Electrophoresis was performed at 200 mV constant voltage, as indicated by the manufacturer's protocol, followed by semidry protein transfer (Owl Separation Systems, NH, USA) onto a PVDF membrane (Invitrogen) at constant current (1 mA/cm^2). Immunodetection was performed using the ECL plus kit (Amersham, Milan, Italy), primary antibodies sc-126 and sc-98 (Santa Cruz Biotechnology, Milan, Italy), directed against the TAD and sc-26147, directed against tubulin, as normalizing control, and appropriate secondary antibodies. Bands were quantified using Image Quant software. Relative expression of HAND1 wt and A126fs transcripts was followed using RT-PCR in real time. Total yeast RNA was obtained using the RNAeasy extraction kit (Promega) from HAND1 transformant cells grown in repressive or inducing culture media, as indicated in Figure 2. cDNAs were then prepared starting from 500 ng of total RNA using Random Hexamer Primers and the First Strand synthesis kit (Applied Biosystems, CA, USA). PCR efficiency was assessed by amplifying serial cDNA dilutions. A common Nter region for HAND1 wt and A126fs was amplified using primers L1: TCATCAGGAAAGGCCCTACT and R1: TCTTGGGTCCTGAGCCTTT and followed in real time using the SYBR® Green PCR Master Mix (Applied Biosystems) in a 384-well plate format on a 7900 real-time PCR system (Applied Biosystems). As the strain used for this experiment was isogenic with yLFM-Dbox except that it did not contain the HAND1 RE, the luciferase cDNA under the miminal *CYC1* promoter could be used as control reference (primers F:TGACAAGGATGGATGGCTACAT; R: TTTCCGGTAAGACCTTTCGGTA). Relative HAND1 mRNAs expression was calculated using the comparative CT method.

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