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RESEARCH ARTICLE

Identification and Functional Analysis of *CITED2* Mutations in Patients With Congenital Heart Defects

Silke Sperling,[1]* Christina H. Grimm,[1] Ilona Dunkel,[1] Siegrun Mebus,[2] Hans-Peter Sperling,[2] Arno Ebner,[3] Raffaello Galli,[1] Hans Lehrach,[1] Christoph Fusch,[3] Felix Berger,[2] and Stefanie Hammer[1]

[1]Max Planck Institute for Molecular Genetics, Berlin, Germany; [2]German Heart Center Berlin, Berlin, Germany; [3]Department of Pediatrics, Ernst-Moritz-Arndt University, Greifswald, Germany

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Recent reports have demonstrated that mice lacking the transcription factor Cited2 die in utero showing various cardiac malformations. We present for the first time functionally relevant mutations of *CITED2* in patients with congenital heart defects (CHDs). *CITED2* encodes a CREBBP/EP300 interacting transcriptional modulator of HIF1A and TFAP2. To study the potential impact of sequence variations in *CITED2* for CHDs in humans, we screened a cohort of 392 well-characterized patients and 192 control individuals using DHPLC, sequencing, and Amplifluor™ genotyping techniques. We identified 15 *CITED2* nucleotide alterations. Seven of these alterations were found only in CHD patients and were not detected in controls, including three mutations leading to alterations of the amino acid sequence (p.Ser170_Gly178del, p.Gly178_Ser179ins9, and p.Ser198_Gly199del). All three of these amino acid changing mutations cluster in the serine-glycine-rich junction of the protein, to which no functionality had heretofore been assigned. Here we show that these mutations significantly reduce the capacity of CITED2 to transrepress HIF1A, and that the p.Ser170_Gly178del mutation significantly diminishes TFAP2C coactivation. This reveals a modifying role for the serine-glycine-rich region in CITED2 function. In summary, the observation of these mutations in patients with septal defects indicates that *CITED2* has a causative impact in the development of CHD in humans. Hum Mutat 26(6), 575–582, 2005. **© 2005 Wiley-Liss, Inc.**

KEY WORDS: CITED2; congenital heart defects; CHD; septal defects; arterial malrotation

INTRODUCTION

Congenital heart defects (CHDs) account for the largest number of birth defects in humans, with an incidence of about eight per 1,000 live births and are the leading noninfectious cause of mortality in newborns. Although major insights into the cardiac developmental process have been gained in studies of animal models, such as mice, chicken, and zebrafish, little is known about the genetic basis in humans. The overwhelming majority of congenital heart malformations do not segregate in Mendelian ratios, although they show familial aggregation, which suggests that genetic factors play a role in their development. Almost 30% of major cardiac malformations are associated with additional developmental abnormalities and result from a recognized chromosomal anomaly or occur as part of a syndrome. Primarily based on knowledge gained from model organisms, disease genes have been identified in a few syndromes, familial nonsyndromic conditions, and very few sporadic cases. The identified disease genes point to a key role of transcription factors in the process of cardiac maldevelopment. It has been demonstrated that TBX5 (MIM# 601620) mutations are frequent causes of Holt-Oram syndrome (MIM# 142900) [Basson et al., 1997; Li et al., 1997] and mutations in the transcriptional coactivators CREBBP (MIM# 600140) and EP300 (MIM# 602700) are associated with cardiac malformations in Rubinstein-Taybi syndrome (MIM# 180849) [Petrij et al., 1995; Roelfsema et al., 2005]. Moreover, causative gene defects have been described for nonsyndromic congenital heart malformations, e.g., mutations in the cardiac transcription factors NKX2.5 (MIM# 600584) and GATA4 (MIM# 600576) [Schott et al., 1998; Garg et al., 2003; Pizzuti et al., 2003; Reamon-Buettner and Borlak, 2004; Ware et al., 2004; Ching et al., 2005]. The heterogeneity of CHDs associated with single-gene defects in patients, and the broad phenotype spectrum seen in mouse models point to a complex genetic network with modifier genes, genetic polymorphisms, and the influence of environmental factors [Bamford et al., 2000; Srivastava, 2001; Solloway and Harvey, 2003; Olson, 2004]. For example, human mutations in the cardiac homeobox protein NKX2.5 cause a diverse set of congenital heart malformations that include septal defects, cardiomyopathy, outflow tract defects, hypoplastic left heart, and associated arrhythmias. Here we present an analysis of the *CITED2* (MIM# 602937) gene in a patient cohort representing

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*Correspondence to: Silke Sperling, Max Planck Institute for Molecular Genetics, Ihnestr. 73, 14195 Berlin, Germany.
E-mail: sperling@molgen.mpg.de

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a broad phenotype spectrum, and show for the first time its impact as a disease gene for CHDs in humans.

CITED2 is an ubiquitously expressed hypoxia-inducible transcriptional cofactor and interacts with high affinity with CREBBP and EP300 [Bhattacharya et al., 1999; Leung et al., 1999; Freedman et al., 2003]. This binding competitively inhibits the interaction between EP300 and the transcription factor HIF1A (MIM# 603348), blocking transcriptional activation by HIF1A. Further, CITED2 coactivates TFAP2 isoforms (MIM#s 107580, 601601, 601602) and both can be detected together at the *Pitx2c* (MIM# 601542) promoter in embryonic mouse hearts. This suggests that CITED2 plays a role in left–right patterning through the Nodal-PITX2C pathway [Braganca et al., 2003; Bamforth et al., 2004; Weninger et al., 2005]. CITED2 is essential for normal development of the heart, as evidenced by the fact that mice lacking *Cited2* die in utero showing various cardiac malformations including atrial and ventricular septal defects, right-sided aortic arches, double-outlet right ventricle, common arterial trunk and overriding aorta [Bamforth et al., 2001, 2004; Weninger et al., 2005]. Deficiencies in TFAP2 coactivation have been suggested to cause laterality defects in *Cited2*−/− mice, but also dysregulation of hypoxia-activated gene transcription may account for the cardiac malformations seen in *Cited2*−/− embryos [Yin et al., 2002].

In the present study we performed a mutation screen of the *CITED2* gene in a cohort of patients with well-characterized phenotypes of sporadic nonsyndromic CHD [Kaynak et al., 2003]. Novel *CITED2* mutations were identified and their functional significance was investigated by transactivation and subcellular-localization assays. The results indicate that *CITED2* has a causative impact on the development of CHD in humans.

MATERIALS AND METHODS

Patient Samples

Patient blood samples were obtained from the German Heart Center after ethics approval was granted by the institutional review committee and informed consent was obtained from the patients or their parents. Phenotypic information was documented in detail in a cardiovascular genetic database established at the Max-Planck-Institute for Molecular Genetics [Seelow et al., 2004]. Genomic DNA was prepared from the blood samples by standard procedures, and DNA was purified when necessary using DNA Cleanup (Qiagen, Hilden, Germany; www.qiagen.com). Control DNA samples were obtained from the Community and Molecular Medicine Newborn Survey (University Hospital Greifswald, Greifswald, Germany).

Mutation Detection

Both exons of the human *CITED2* gene, including the entire 5′ and 3′ untranslated regions (GenBank: NM_006079.3), were amplified by PCR using the primers described in Table 1A. All PCR reactions were performed using 50 ng of genomic DNA, 200 μM of dNTPs and 500 nM of primer. PCR products were denatured for 10 min at 95°C and subjected to denaturing HPLC (DHPLC) analysis on the automated WAVE^{TM} nucleic acid fragment analysis system (Transgenomic, San Jose, CA; www.transgenomic.com) as described previously [Eng et al., 2001]. The fragments were eluted with temperatures calculated by the DHPLC melt program for the successful resolution of heteroduplexes (http://insertion.stanford.edu/melt.html) [Jones et al., 1999]. Samples with double- or triple-peaked DHPLC chromatograms were purified using Qiagen PCR purification and sequenced by the Services in Molecular Biology Company (Berlin, Germany).

Amplifluor^{TM} Allele-Specific PCR

The Amplifluor^{TM} genotyping assay based on PCR amplification in the presence of tailed allele-specific primers, a common reverse primer, and universal fluorescence labeled Amplifluor^{TM} primers (Serologicals, Temecula, CA; www.serologicals.com) was performed as described previously [Myakishev et al., 2001; Rickert et al., 2004]. The primers designed by the Amplifluor^{TM} assay architect software (www.assayarchitect.com) are listed in Table 1B. The 5-μl amplification reactions contained 25 nM of FAM- and JOE-labeled Amplifluor^{TM} primers, 25 nM of tailed allele-specific primers, 375 nM of reverse primer, 1 × reaction buffer (Serologicals), 0.2 mM of dNTPs, 0.25 U of HotStar Taq Polymerase (Qiagen), and 20 ng of genomic DNA. The amplification profiles were as follows: 96°C, 10 min; (95°C, 30 sec; 56°C, 30 sec; 72°C, 40 sec) × 45 cycles; 72°C, 3 min. The amplification signals were analyzed via endpoint measurement using the ABI Prism 7900HT system (Applied Biosystems, Darmstadt, Germany; www.appliedbiosystems.com).

Haplotype Analysis

Haplotype structure was determined using HAPLOVIEW [Barrett et al., 2005]. Linkage disequilibrium (LD) was calculated as D′ values using an expectation-maximization (EM) algorithm, and haplotype frequencies comparing the patient and the control cohort were analyzed by χ^2 tests.

Plasmids

The open reading frames of wild-type (wt) and mutant *CITED2* were amplified by PCR from genomic DNA and cloned into pcDNA3.1(+) (Invitrogen, Karlsruhe, Germany; www.invitrogen.com) to obtain expression vectors for CITED2-wt and the CITED2 mutants. The resulting clones were verified by sequencing. To create N- and C-terminal GFP-CITED2 fusion proteins, the open reading frames were amplified by PCR from the plasmid DNA and cloned in frame into pEGFP-N1 and pEGFP-C1 (BD Biosciences, Palo Alto, CA). The HIF1A reporter system pGal4-HIF1A and pGal4-Luc was described previously and kindly provided by L.E. Huang (NCI, Bethesda, MD) [Huang et al., 1998]. The pGal4-HIF1A plasmid contains the Gal4-DNA binding domain fused to the C-terminal transactivation domain of HIF1A, which is stable under nonhypoxic conditions. In pGal4-Luc the Luciferase reporter gene is under the control of Gal4-DNA binding sites. The TFAP2 responsive reporter plasmid pAP2-Blue harboring a Luciferase reporter gene under the control of three TFAP2 response elements, and the human TFAP2C expression plasmid pRSV-TFAP2C were a kind gift from Helen Hurst (Hammersmith Hospital, London, UK) [Bosher et al., 1996; Bamforth et al., 2001].

Transcriptional Assay

HepG2 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Cells were seeded into 96-well plates and on the next day at 60% confluency were transfected using Fugene6 (Roche, Mannheim, Germany; www.roche-applied-science.com) according to the manufacturer's instructions. Wt and mutant *CITED2* constructs or empty vector were cotransfected together with pGal4-HIF1A and pGal4-Luc or pRSV-TFAP2C and pAP2-Blue, respectively. In all wells a pRL-TK Luciferase vector (Promega, Mannheim, Germany; www.prome

TABLE 1. Primers Used for CITED2 Amplification and Allele-Specific PCR

Name	Sequence
A: Primers for amplification of *CITED2* exons	
exon1_F1	GCTCATTGTTGGCAGCTGC
exon1_R1	TTCGCCTCACGCTCTTCCTC
exon2_F1	ATCTGCCCTTTTCACTTCCAG
exon2_R1	GGAGTTGTTAAACCTGGCCG
exon2_F2	TGTGAACGGAGGGCACCCC
exon2_R2	CGAGCTGCTGCCAGAGCCG
exon2_F3	ACCAGATGAACGGGACAAAC
exon2_R3	CGGTCCAAACCCATTTCTAT
exon2_F4	GCCCAATGTCATAGACACTG
exon2_R4	ATTCACGCCGAAGAAGTTG
exon2_F5	GGCGAAAGAAATCAAACCC
exon2_R5	AATGTCAAGGCTACAAAAACGA
exon2_F6	CTGCCACTTTTTTTTCCTGTTT
exon2_R6	AAAATGAAGCGAGATGGCAGT
exon2_F7	TAGTTGGTTGCATGAACTTC
exon2_R7	AACTATTAGCACAGTGTCAAA
exon2_F8	GTCAGTGGCAAACATTTCACAGA
exon2_R8	TGTTCAACTCAAAGACGGGG
B: Primers for Amplifluor™ allele-specific PCR	
c.−91_A_green_r	GAAGGTGACCAAGTTCATGCTTTCAGCAGCACATAGAGGGGAT
c.−91_G_red_r	GAAGGTCGGAGTCAACGGATTAGCAGCACATAGAGGGGAC
c.−91_com_f	CGCTTTGCACGCCAGGAA
c.−81_T_green_r	GAAGGTGACCAAGTTCATGCTTGACCGGCTCAGCAGCACA
c.−81_C_red_r	GAAGGTCGGAGTCAACGGATTACCGGCTCAGCAGCACG
c.−81_com_f	CGCTTTGCACGCCAGGAA
c.115_117_CAC_green_r	GAAGGTGACCAAGTTCATGCTTCTGCTGCTGCTGGTGGT
c.115_117_delCAC_red_r	GAAGGTCGGAGTCAACGGATTGCTGCTGCTGCTGGTGAT
c.115_117_com_f	ATGGGCATGGGGCAGTT
c.1040_T_green_f	GAAGGTGACCAAGTTCATGCTTCCTTGACATTCACCCACCTCT
c.1040_C_red_f	GAAGGTCGGAGTCAACGGATTCTTGACATTCACCCACCTCC
c.1040_com_r	CAACGAAAAAGACCAAGTTAGCTA
c.1268_G_green_r	GAAGGTGACCAAGTTCATGCTAAGCGAGATGGCAGTTTGC
c.1268_A_red_r	GAAGGTCGGAGTCAACGGATTTGAAGCGAGATGGCAGTTTGT
c.1268_com_f	GGAAAAATTGCATTAGTTGGTTGCAT
rs1131400_C_green_r	GAAGGTGACCAAGTTCATGCTAAGCGCCCGTGGTTCATG
rs1131400_A_red_r	GAAGGTCGGAGTCAACGGATTAAGCGCCCGTGGTTCATT
rs1131400_com_f	GACTGGAAATGGCAGACCATAT
rs1131431_C_green_r	GAAGGTGACCAAGTTCATGCTGTGCAGTAATATCTGCCCTTCG
rs1131431_T_red_r	GAAGGTCGGAGTCAACGGATTGTGCAGTAATATCTGCCCTTCAA
rs1131431_com_f	GGAAAAATTGCATTAGTTGGTTGCAT
rs1804687_C_green_f	GAAGGTGACCAAGTTCATGCTCCGGTCCTGGACGCGACCA
rs1804687_G_red_f	GAAGGTCGGAGTCAACGGATTCCGGTCCTGGACGCGACGA
rs1804687_com_r	CTCGGAGGACTGGGCTGGCAA
rs2001409_T_green_f	GAAGGTGACCAAGTTCATGCTTCCTCGGTCTTCGGAGCAGAAT
rs2001409_A_red_f	GAAGGTCGGAGTCAACGGATTCCTCGGTCTTCGGAGCAGAAA
rs2001409_com_r	AAGAGCCCCAGCCAGCTT
rs4177_TTT_green_f	GAAGGTGACCAAGTTCATGCTGTCAGTGGCAAACATTTCACAGATTT
rs4177_delTTT_red_f	GAAGGTCGGAGTCAACGGATTGTCAGTGGCAAACATTTCACAGATTA
rs4177_com_r	ACAGTGTCAAAAATGTTGAAGACAGA

ga.com) was cotransfected to control for transfection efficiency. Subsequently, 40 hr after transfection the cells were washed with phosphate-buffered saline (PBS) and lysed in 50 µl of passive lysis buffer (Promega). Firefly and *Renilla* Luciferase activities were measured using the Dual-Luciferase-Reporter Assay System (Promega) in a Centro LB960 luminometer (Berthold, Bad Wildbad, Germany; www.berthold.com). Firefly Luciferase activities were normalized to *Renilla* Luciferase activity and the results for the samples transfected with CITED2-wt construct were set to 100%. The results shown represent a minimum of three independent experiments performed in at least triplicates.

Immunofluorescence and Subcellular Localization

HepG2 and HEK293 cells were seeded onto glass coverslips 6 hr prior to transfection at about 60% confluency. GFP-CITED2 expression constructs containing wt and mutant *CITED2* were transfected using Fugene6 (Roche) according to the manufacturer's instructions. Then 48 hr after transfection, the cells were fixed in 4% paraformaldehyde/PBS at RT for 15 min, washed in PBS, and mounted with Vectashield containing DAPI (Vector Laboratories, Inc., Burlingame, CA; www.vectorlabs.com). The cells were analyzed by fluorescence microscopy.

RESULTS

Phenotypes of the Analyzed CHD Patient Cohort

CITED2 mutation analysis was performed using DHPLC analysis in a cohort of 392 unrelated nonsyndromic patients who showed a broad spectrum of CHDs. The cardiac phenotypes of the analyzed patients are described in Table 2. To allow a more detailed visualization of the panel of analyzed CHD phenotypes in the overall patient cohort, as well as their association with the

observed genotypes, we have set up a freely accessible interactive Web supplement using the database front-end d-matrix applied to our cardiovascular genetics database (http://dmatrix.molgen.mpg.de/SV) [Seelow et al., 2004]. A further 192 individuals from the Greifswald Newborn Survey served as controls.

Identification of *CITED2* Mutations

From a total of 392 patient samples, we identified five sequence variations that were already listed in dbSNP (NCBI), as well as 10 novel *CITED2* nucleotide alterations (three amino acid deletions, one amino acid insertion, one amino acid substitution, one silent nucleotide alteration, two nucleotide substitutions in the 5′UTR, and two alterations in the 3′UTR). The localization of these sequence variations and the predicted effects on the CITED2 amino acid sequence, as well as the frequencies detected in CHD patients and controls, are shown in Table 3. Seven of the novel sequence variations were detected only in CHD patients and not in controls, namely c.−91G>A, and c.−81T>C in the untranslated 5′ region; c.456C>T, c.508_534del27 (p.Ser170_Gly178del), c.534_535ins27 (p.Gly178_Ser179ins9), and c.592_597delAGCGGC (p.Ser198_Gly199del) in the coding region; and c.1268A>G in the untranslated 3′ region (nomenclature based on GenBank NM_006079.3, *CITED2* cDNA, with +1 corresponding to A of the initiation codon; www.hgvs.org/mutnomen/). All patient-exclusive mutations that alter the amino acid sequence of CITED2 (p.Ser170_Gly178del, p.Gly178_Ser179ins9, and p.Ser198_Gly199del) cluster in the serine-glycine-rich junction [Leung et al., 1999] of the protein (Fig. 1).

The phenotype characteristics of patients with potential disease-causing mutations are shown in Table 4. We observed amino acid altering mutations in one patient with a sinus venosus atrial septal defect and abnormal pulmonary venous return to the right atria, one patient with an atrial septal defect of the secundum type (ASDII), and one patient with a perimembranous ventricular septal defect (VSD). Furthermore, we found non-amino acid altering mutations in two patients with tetralogy of Fallot; one patient with situs inversus totalis, transposition of the great arteries, and perimembranous VSD; one patient with perimembranous VSD and ASDII; and one patient with perimembranous VSD and right ventricular outflow tract stenosis.

TABLE 2. Congenital Heart Defects of Analyzed Patients

Cardiovascular anomalies	Patients genotyped (N = 392)
Situs inversus totalis	5 (1.3%)
Dextrocardia	2 (0.5%)
D-transposition of the great arteries	21 (5.4%)
Right aortic arch	30 (7.7%)
Secundum atrial septal defect	129 (32.9%)
Sinus venosus atrial septal defect	26 (6.6%)
Partial anomalous pulmonary venous return	11 (2.8%)
Perimembranous ventricular septal defect	180 (45.9%)
Incomplete atrioventricular septal defect	3 (0.8%)
Complete atrioventricular septal defect	16 (4.1%)
Tetralogy of Fallot	46 (11.7%)
Hypoplastic left heart syndrome	2 (0.5%)
Pulmonary atresia	14 (3.6%)
Pulmonary stenosis	56 (14.3%)
Double inlet left ventricle	1 (0.3%)
Double outlet right ventricle	13 (3.3%)
Left superior vena cava	29 (7.4%)
Aortic isthmus stenosis	19 (4.8%)

Influence of CITED2 Mutations on TFAP2 Transactivation and HIF1A Transrepression

To assess the functional significance of the amino acid altering CITED2 mutations found in CHD patients, we tested their influence on reporter gene transactivation and repression. To date, no functionality has been assigned to the serine-glycine-rich junction of the protein harboring the observed mutations. Previous reports showed that CITED2 acts as a binding partner and transcriptional coactivator of TFAP2 [Bamforth et al., 2001], and interacts with the histone acetylases CREBBP/EP300 via its C-terminus. The latter leads to a transcriptional repression of HIF1A due to overlapping binding sites [Bhattacharya et al., 1999]. In our experiments CITED2-wt coactivated the TFAP2C-mediated stimulation of a TFAP2-reporter construct, as described previously [Bamforth et al., 2001]. However, the p.Ser170_Gly178del mutant

TABLE 3. Localization and Frequencies of *CITED2* Variants

				Patients			Controls		
dbSNP	Position	Nucleotide variation[a]	Amino acid variation	Mut chr	Total chr	Mut allele freq	Mut chr	Total chr	Mut allele freq
	Exon1 (5′UTR)	**c.−91G>A[b]**		**2**	**332**	**0.0060**	**0**	**368**	**0.0000**
	Exon1 (5′UTR)	**c.−81T>C[b]**		**1**	**362**	**0.0028**	**0**	**368**	**0.0000**
rs1804687	Exon1 (5′UTR)	c.−52G>C		15	352	0.0426	16	332	0.0482
rs2001409	Exon1 (5′UTR)	c.−24A>T		65	358	0.1816	65	356	0.1826
rs1131400	Exon2	c.21C>A	p.Ala7Ala	75	372	0.2016	67	382	0.1754
	Exon2	**c.115_117delCAC**	**p.His39del**	**2**	**728**	**0.0027**	**2**	**388**	**0.0052**
	Exon2	**c.456C>T[b]**	**p.His52His**	**1**	**686**	**0.0015**	**0**	**382**	**0.0000**
	Exon2	**c.479A>T**	**p.His160Leu**	**1**	**702**	**0.0014**	**1**	**382**	**0.0026**
	Exon2	**c.508_534del27[b]**	**p.Ser170_Gly178del[c]**	**1**	**702**	**0.0014**	**0**	**382**	**0.0000**
	Exon2	**c.534_535ins27[b]**	**p.Gly178_Ser179ins9[c]**	**1**	**702**	**0.0014**	**0**	**382**	**0.0000**
	Exon2	**c.592_597delAGCGGC[b]**	**p.Ser198_Gly199del[c]**	**1**	**648**	**0.0015**	**0**	**378**	**0.0000**
	Exon2 (3′UTR)	**c.1040C>T**		**13**	**372**	**0.0349**	**10**	**374**	**0.0267**
rs1131431	Exon2 (3′UTR)	c.1248C>T		55	374	0.1471	61	382	0.1597
	Exon2 (3′UTR)	**c.1268A>G[b]**		**1**	**372**	**0.0027**	**0**	**382**	**0.0000**
rs4177	Exon2 (3′UTR)	c.1497_1499delTTT		55	350	0.1571	59	378	0.1561

[a]Systematic nomenclature for SNPs (www.hgvs.org) based on GenBank NM_006079.3 (*CITED2* cDNA) and counting +1 as A of the initiation codon. Novel sequence variations are in bold.
[b]Sequence variations not found in the control cohort.
[c]Patient-exclusive CITED2 protein mutations altering transcriptional properties. Mut, mutant; chr, chromosome; freq, frequency.

showed significantly reduced costimulation capacity compared to wt, and reached only half-maximal coactivation (Fig. 2A). All other CITED2 mutants coactivated TFAP2C to the same extent as the CITED2-wt. Next, we confirmed with a HIF1A responsive reporter system [Huang et al., 1998] that CITED2-wt is an efficient repressor of HIF1A transcriptional activation independently of hypoxia [Bhattacharya et al., 1999]. Again, the p.Ser170_Gly178del mutant displayed a significant loss of activity, as it was only able to repress HIF1A with about 60% efficiency compared to wt. Moreover, in the HIF1A reporter system, the mutations p.Ser198_Gly199del and p.Gly178_Ser179ins9 also significantly affected the activity of CITED2, revealing only about 75% repressive activity compared to wt. In contrast, the p.His39del mutation and the p.His160Leu amino acid substitution, which had also been found in controls, did not alter CITED2 mediated HIF1A repression significantly (Fig. 2B). These results show that deletions or insertions within the serine-glycine-rich junction modulate CITED2 signal transduction and point to a causative impact of CITED2 on the development of congenital heart diseases in human.

Influence of CITED2 Mutations on Subcellular Localization

To further evaluate whether the functional changes are due to altered subcellular localization of the protein, transient transfections were carried out using N- and C-terminal GFP fusion constructs of mutant and wt CITED2, followed by fluorescence microscopy. CITED2-wt was detected mainly in the nucleus and to a lesser extent in the cytoplasm of HEK and HepG2 cells. However, none of the CITED2 mutations altered cellular localization or expression of the protein (Fig. 3 and data not shown). Thus, the diminished TFAP2 coactivation and HIF1A repression of the CITED2 mutants are not caused by an incorrect localization of the protein.

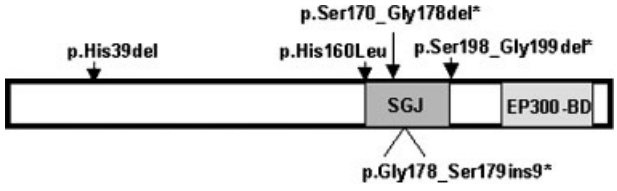

FIGURE 1. **Position of mutations in the CITED2 protein observed in CHD patients. The discovered mutations cluster mainly in the serine-glycine rich junction (SGJ; p.Ser161_Gly199), which therefore represents a hotspot for mutations in the protein. The position of the EP300 binding domain (EP300-BD; p.Asp224_Phe255) is indicated. Mutations found only in CHD patients and not in the control cohort are marked with*.**

Haplotype Analysis of the *CITED2* Gene

Finally, to evaluate the polymorphisms observed in *CITED2*, we calculated the Hardy-Weinberg equilibrium using multiple tests and a two-sided significance level of 5%. None of the genotyped polymorphisms showed a significant deviation from the Hardy-Weinberg equilibrium. For the haplotype analysis there were 359 individuals available with successful genotyping for each of the six *CITED2* polymorphisms and a minor allele frequency of >1%. The EM algorithm showed that three out of 32 possible haplotypes exceeded a frequency of 1%, and these accounted for 98.6% of the chromosomes within our samples, with the main haplotype G-A-C-C-C-TTT showing a frequency of 65.4% (Table 5). An allelic association between the different loci (represented as D′ values) is indicated in Figure 4. This analysis suggests the existence of one single haplotype block [Gabriel et al., 2002], and we identified rs1131400 and rs1131431 as haplotype tag (ht) SNPs, which are sufficient for determining the corresponding haplotype. However, none of the common haplotypes showed an association with CHD when patients and control individuals were compared (data not shown).

DISCUSSION

Previous reports of mice lacking *Cited2* suggested that it plays a direct role in the development of the AV canal and cardiac septa, and that it is required for the normal establishment of the left–right axis. *Cited2*−/− embryos show a variety of cardiac malformations, including atrial and ventricular septal defects, abnormal heart looping with overriding aorta, and outflow tract abnormalities [Bamforth et al., 2001, 2004; Weninger et al., 2005]. In the present study we analyzed 392 patients with a broad range of CHDs, and discovered seven potential disease-causing mutations in eight patients. These mutations, which were exclusively observed in the patient cohort and not found in 192 control individuals, give rise to cardiac septal defects as well as outflow tract abnormalities associated with malrotation of the great arteries. This reflects the range of defects observed in *Cited2*−/− embryos.

Three of these *CITED2* mutations (p.Ser170_Gly178del, p.Gly178_Ser179ins9, and p.Ser198_Gly199del) alter the amino acid sequence and cluster in the serine-glycine rich junction of the protein, which therefore represents a potential hotspot for mutations in CITED2. Our further analysis of these mutations using reporter-gene assays revealed their functional implications

TABLE 4. ***CITED2* Mutations Identified among 392 Patients with Congenital Heart Disease**

Nucleotide variation	Amino acid variation	# Patients	Type of congenital heart defect
c.−91G>A		2	Tetralogy of Fallot Perimembranous ventricular septal defect and secundum atrial septal defect
c.−81T>C		1	Situs inversus totalis, transposition of the great arteries and perimembranous ventricular septal defect
c.456C>T	p.His52His	1	Perimembranous ventricular septal defect and right ventricular outflow tract obstruction
c.508_534del27	p.Ser170_Gly178del	1	Perimembranous ventricular septal defect
c.534_535ins27	p.Gly178_Ser179ins9	1	Secundum atrial septal defect
c.592_597delAGCGGC	p.Ser198_Gly199del	1	Sinus venosus atrial septal defect, abnormal pulmonary venous return to the right atria
c.1268A>G		1	Tetralogy of Fallot

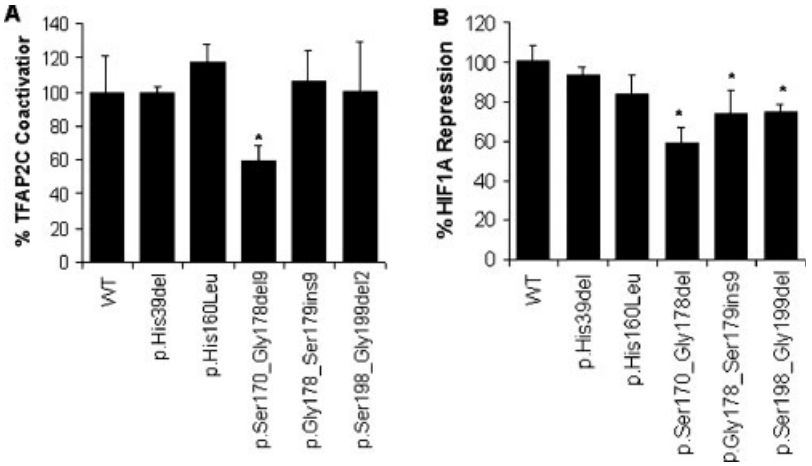

FIGURE 2. **Transcriptional modulation of CITED2 variants. Luciferase activity of a TFAP2C-stimulated TFAP2-reporter construct (A) and a Gal4-HIF1A stimulated Gal4-reporter construct (B) cotransfected with CITED2-wt or mutant constructs as indicated. The specific CITED2 construct used is shown below each bar. Luciferase activities were measured and the mean fold-coactivation/repression as compared to wt is expressed as a percentage. Each bar represents a minimum of three independent experiments performed in at least triplicates (* significantly different from wt, $P < 0.05$).**

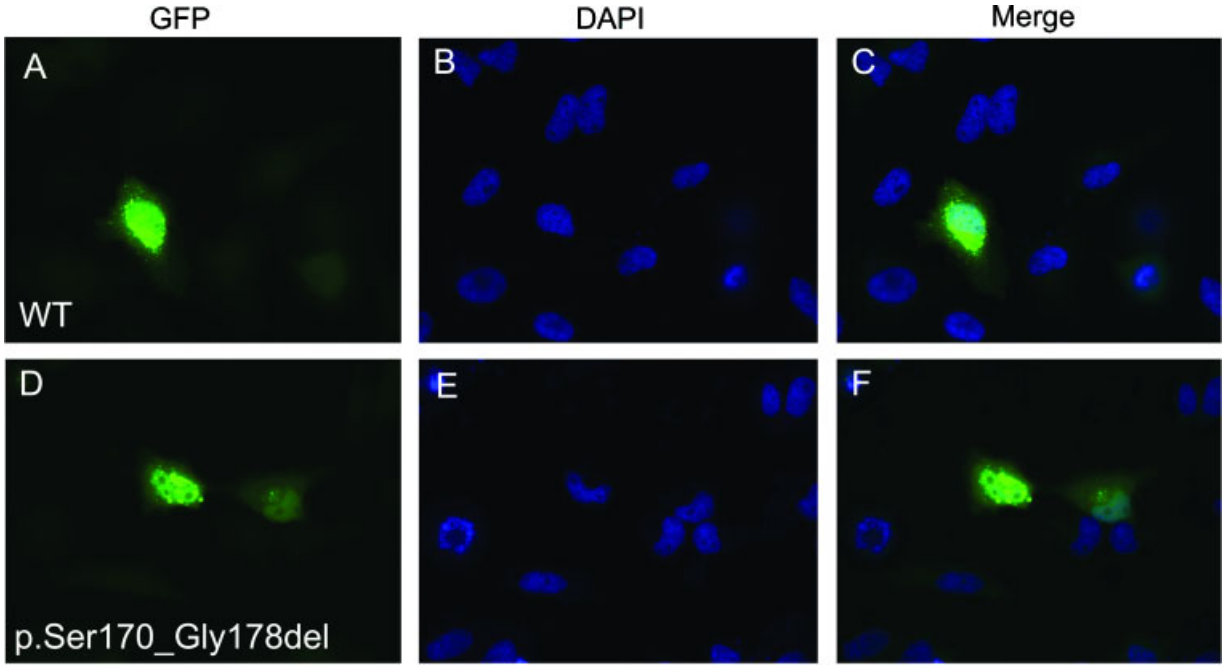

FIGURE 3. **Subcellular localization of CITED2. For CITED2-wt and the p.Ser170_Gly178del mutant fluorescence of GFP-fusion proteins (A and D) and DAPI staining (B and E) are shown individually and merged (C and F).**

TABLE 5. ***CITED2* Haplotypes With Estimated Frequencies >1%**

No.	Haplotype[a]	Frequency (%)
1	G-A-**C**-C-**C**-TTT	65.4
2	G-T-**A**-C-**C**-TTT	18.3
3	G-A-**C**-C-T-delTTT	14.9

[a]**Haplotypes are designated with the SNPs in the following order: rs1804687-rs2001409-rs1131400-c.1040C>T-rs1131431-rs4177. htSNPs are in bold.**

(e.g., all three mutations lead to a significant loss in HIF1A transcriptional repressive capacity of CITED2). Moreover, we observed a significantly diminished TFAP2C coactivation of the p.Ser170_Gly178del mutant. These findings indicate a modifying role for the serine-glycine rich junction in CITED2 function, to which no functionality had been assigned to date. One might speculate that variations in this region cause conformational changes, altering the ability of the EP300 binding domain to interact with CREBBP and EP300 or to recruit other cofactors.

This suggests that the detected CITED2 mutations are potential risk factors for CHD and account for ~2% (8/392) of our patient cohort of sporadic CHD cases. The broad phenotypical spectrum of heart defects seen in *Cited2*−/− mice, as well as in our patients, points to other, potentially relevant but currently unknown modifying factors. In the future it will be of interest to evaluate the functionality of the serine-glycine-rich junction of CITED2 and the non-amino acid altering mutations observed in the coding region, the 5′UTR and 3′UTR. To gain insights into the

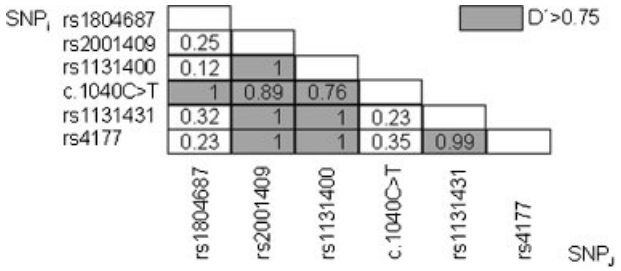

FIGURE 4. **Pairwise allelic association of SNPs in *CITED2* as measured by D′ (numbers). Only SNPs with minor allele frequencies >1% are included.**

inheritance of the mutations, it would be useful to analyze family triplets, which unfortunately were not available for the present study.

Finally, in a comparison of patients and controls we did not observe any significant differences in allele frequencies of the common variants, in accordance with a previous study [Volcik et al., 2004]. Haplotype analysis showed that only three out of 32 possible haplotypes accounted for at least 98.6% of the investigated chromosomes, which suggests the existence of only one haplotype block (Table 5; Fig. 4). Therefore, two htSNPs were extracted that are sufficient for haplotype determination.

In summary, we present the first evidence that CITED2 is a disease-causing gene for congenital heart malformations (particularly septal defects and malrotations of the great arteries) in humans.

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