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Germline variants in *HEY2* functional domains lead to congenital heart defects and thoracic aortic aneurysms

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Abstract

Purpose—In this study we aimed to establish the genetic cause of a myriad of cardiovascular defects prevalent in individuals from a genetically isolated population, who were found to share a common ancestor in 1728.

Methods—Trio genome sequencing was carried out in an index patient with critical congenital heart disease (CHD), family members had either exome or Sanger sequencing. To confirm enrichment, we performed a gene-based association test and meta-analysis in two independent validation cohorts: one with 2685 CHD cases versus 4370 controls, and the other 326 cases with familial thoracic aortic aneurysms (FTAA) and dissections versus 570 ancestry-matched controls. Functional consequences of identified variants were evaluated using expression studies.

Results—We identified a loss-of-function variant in the Notch target transcription factor-encoding gene *HEY2*. The homozygous state (n=3) causes life-threatening congenital heart defects, while 80% of heterozygous carriers (n=20) had cardiovascular defects, mainly CHD and FTAA of the ascending aorta. We confirm enrichment of rare risk variants in *HEY2* functional domains after meta-analysis (meta-SKAT p=0.018). Furthermore, we show that several identified variants lead to dysregulation of repression by HEY2.

Conclusion—A homozygous germline loss-of-function variant in *HEY2* leads to critical CHD. The majority of heterozygotes show a myriad of cardiovascular defects.

Keywords

Congenital heart defect; thoracic aortic aneurysm; cardiovascular defects; HEY2

Introduction

Affecting ~1% of all newborns, congenital heart defects (CHD) are the most common congenital anomaly worldwide.[1] Besides well-recognized non-genetic etiologies,

epidemiological studies have strongly suggested genetic factors as predominant causes of CHD.[2] Exome and genome sequencing efforts of large CHD cohorts have significantly contributed to the discovery of genetic causes.[3] Pathogenic variants within identified genes result in a broad range of cardiac phenotypes, generally with incomplete penetrance and variable phenotypic expressivity.[3] However, the large majority of CHD cases remain unexplained.

In this study we present a large family ('family 1') affected by various cardiovascular defects (CVD), including CHD and familial thoracic aortic aneurysms (FTAA) of the ascending aorta. This family is from the '*DARWIN*' population (*D*NA *A*nalysis of *R*esidents *W*ithin an *I*solate in the *N*etherlands), a genetically isolated village founded in the 14th century by 7 to 20 families.[4] Trio WGS was carried out for index patient III:12, who has severe CHD. This identified a homozygous loss-of-function variant (p.G108*) in Hairy/enhancer-of-split related with YRPW motif protein 2 (*HEY2*), a transcriptional repressor that regulates cardiogenesis in vertebrates.[5] Subsequent screening of family members with CVD revealed two pregnancies carrying homozygous fetuses which were terminated at 16 weeks because of critical CHD, as well as many heterozygous carriers with CVD. Furthermore, we demonstrate enrichment of rare, protein-altering variants in *HEY2* functional domains in CHD and familial thoracic aortic aneurysms and dissections (FTAAD) cohorts using an optimized sequence kernel association test (SKAT-O) meta-analysis, and show with luciferase assays that these variants induce altered repression of a HEY2 target gene. Together, the results demonstrate that HEY2 is required for normal cardiovascular development.

Materials and Methods

Ethics Statement

Informed consent was obtained from all subjects and/or legal guardians and the institutional review board or ethical committee approval statement as applicable. Institutional review boards and ethics committees that have approved the study include the UK Research Ethics Committee (10/H0305/83, granted by the Cambridge South Research Ethics Committee and GEN/284/12, granted by the Republic of Ireland Research Ethics Committee), the Ethics Committee Charité Berlin, Germany (EA2/131/10), the East Midland Research Ethics Committee (6721) and the Committee for the Protection of Human Subjects at the University of Texas Health Science Center at Houston, United States.

Clinical and genetic testing

Proband III:12 was selected for trio genome sequencing. Four other individuals with CHD from *DARWIN* and multiple family members had cardiac evaluation and were screened for the identified variant (p.G108*) by Sanger sequencing, while one CHD patient underwent trio genome sequencing (III:17). Additional genetic testing was performed when clinically indicated. Sanger sequencing was performed in 524 anonymous individuals from *DARWIN* to estimate the p.G108* minor allele frequency. All investigated individuals underwent clinical examination, two-dimensional echocardiography, electrocardiography,

and additional investigations if indicated. See Supplementary Materials and Methods for details. This study complies with the Declaration of Helsinki.

Linkage analysis was performed by Superlink, using a two-point method.[6] All in-laws for whom no genetic or phenotypic data were available were depicted as wildtype with unaffected phenotype, and all obligate carriers as nocall (assumption 1). The minor allele frequency was set to 0.012 (as measured in *DARWIN*), and penetrance to 10%, 85.7% and 100% for wildtype, heterozygous, and homozygous genotypes, respectively (Supplementary Materials and Methods). We repeated this analysis with phenotypes (assumption 2) and both phenotypes and genotypes (assumption 3) of in-laws depicted as unknown.

Validation studies

We screened two independent cohorts with phenotypes that were most prevalent among heterozygotes in family 1 (Table 1) for candidate variants in *HEY2*: 1) 2685 Europeans with CHD versus 4370 ancestry-matched controls, and 2) 326 European-Americans with FTAAD versus 570 ancestry-matched controls, all filtered on relatedness. Controls did not undergo cardiac screening. Exome sequencing data were available in both cohorts. All studies were approved by local institutional review boards depending on local requirements. See Supplementary Materials and Methods and Table S3 and S4 for details and phenotypes.

We aggregated rare (minor allele frequency <0.01) protein altering/truncating variants with Combined Annotation Dependent Depletion (CADD) score >20 within *HEY2*. We used SKAT-O to test for enrichment of variants in cases, correcting for sex, and used meta-SKAT to meta-analyse SKAT-O results from both cohorts.[7,8] We repeated these analyses for variants affecting the functional domains (as determined by UniProtKB, entry Q9UBP5).[9]

Furthermore, to investigate possible variant associations of lower effect sizes leading to a more complex etiology for CHD, we used genotype data +/− 1 Mb around *HEY2* from the UK Biobank, and phenotypes ‘thoracic aortic aneurysms’ (275 cases) and ‘congenital malformations of cardiac septa’ (618 cases) (see Supplementary Materials and Methods).

Functional studies

In the absence of cardiac tissue, we investigated expression of 19 suspected *HEY2* target genes (Table S2) in chorionic tissue sampled at gestational age 12 weeks and two days of a homozygous p.G108* mutant (III:22) and two age and gender-matched controls through quantitative polymerase chain reaction (qPCR). Stability of the p.G108* mutant protein was investigated with Western Blotting. We checked whether there was equal transfection by using qPCR against the ampicilin gene (part of the transfected plasmid), and against Hey2, which indicates how much Hey2 is actually expressed after transfection. We found that both plasmids were transfected equally well, and that Hey2 was expressed normally (Figure S1). The regulatory activity of six identified *Hey2* mutants on a known target gene, *Tbx2*, was measured through luciferase assays, as previously described.[10] Again qPCR was used to determine levels of expression and transfection, with all plasmids showing equal levels of expression (see Supplementary Material and Methods for details).

Data were compared by two-sided t-tests, and p-values less than 0.05/number of tests were considered statistically significant. All statistical tests were carried out using R, including the SKAT-O and metaSKAT packages.[11]

Results

Identification of a frameshift variant in HEY2

WGS in patient III-12 identified a homozygous two base pair deletion in exon 4 of *HEY2* (NM_012259.3, c.318_319delAG, p.G108*, Chr6(GRCh37):g.126075682_126075683del), leading to frameshift and predicted early stop codon (Figure 1A). III-12 has severe CVD, consisting of pulmonary artery atresia with ventricular septal defect, overriding aorta, small pulmonary arteries, right aortic arch, left sided ductus, a monocoronary and a dilated aortic root (36 mm at eleven years), necessitating surgery a few days postnatally. Five other individuals from *DARWIN* with CHD were found to be heterozygous (I:31, II:3, II:25, II:29 and III:17, Figure 1B). Additionally, fetuses from two pregnancies (III:22 and III:23), which were terminated at 16 weeks due to critical CHD, were homozygous (Figure 1C, note the severe CHD consisting of mitral atresia, double outlet right ventricle, hypoplastic left ventricle and significant pulmonary stenosis, and the non-compaction cardiomyopathy). Genealogy using historical archives showed a most recent common ancestor for all carriers in 1728. Cascade screening was performed in 23 additional family members. Altogether, there were 20 heterozygous carriers for whom clinical information was available (Table 1, detailed phenotypic information in Table S1). Homozygous p.G108* variants lead to multiple critical CVDs. In heterozygous state, an autosomal dominant inheritance pattern with varying expressivity and incomplete penetrance is observed. The p.G108* variant co-segregates with CVD with a logarithm of the odds score of 7.76 under assumption 1, 5.81 under assumption 2 and −0.81 under assumption 3 (see Methods).

Some wildtype individuals had mild cardiac defects, likely due to environmental or other genetic influences (Table 1, detailed phenotypic information in Table S1).

The p.G108* variant is rare globally (1.1×10^{-5} in Genome Aggregation Database v.2.1.1), with three heterozygous and no homozygous carriers (of any predicted loss-of-function variants in *HEY2*).[12]

Homozygous loss-of-function of HEY2 leads to reduced expression of target genes in vivo

To assess the effect of the p.G108* variant, we investigated expression of known HEY2 target genes in chorionic villi of p.G108* homozygous III:22. Of 20 selected genes, 15 were expressed in chorionic tissue. Four genes had significantly higher expression levels in III:22 compared to controls indicating loss of repressive function of HEY2 (Figure 2A and Table S2). Interestingly, HEY2 expression in III:22 was similar to controls. However, p.G108* mutant HEY2 was absent on Western Blot, and only reappeared when inhibiting the proteasome (Figure 2B and Figures S3 and S4). Nuclear localization assays confirmed this finding (Figure S5). This indicates that, while escaping nonsense-mediated decay, p.G108* mutant HEY2 is degraded by the proteasome.

HEY2 functional domains are enriched for candidate variants in patients with CHD and FTAAD

Two cohorts with phenotypes that were most prevalent within family 1 (one cohort with CHD and one with FTAAD) were screened for rare risk variants in *HEY2* (Figure 3). We observed more rare risk variants affecting functional domains required for the repressive function of HEY2 in cases (6/3011) than in controls (1/4940) (Figure 3A).[13] Only heterozygous variants were identified. Again, all FTAAD cases in whom a variant in *HEY2* was found had dilatation of the ascending aorta (Table S4). We investigated the association of *HEY2* with CVD with SKAT-O (Table 2). FTAAD cases have significantly more rare, potentially deleterious variants affecting the functional domains in HEY2 than controls (p=0.0246). This was not the case in CHD (p=0.10). We then meta-analyzed the two cohorts, given the presence of both phenotypes in family 1, and the co-occurrence of both CHD and altered aortic wall histology in *Hey2* knockout mice, which did show significant enrichment (p=0.018).[14] Therefore, we confirm enrichment of rare risk variants in *HEY2* functional domains in individuals with CVDs after meta-analysis.

Identified HEY2 variants lead to both loss and gain of function

To test the effect of *HEY2* mutants we employed a luciferase assay using the activity of a *Tbx2* enhancer sequence, known to be repressed by HEY2.[10,15,16] All investigated CHD and FTAAD mutants showed significantly higher or lower activity of the *Tbx2* enhancer sequence in comparison to wildtype HEY2 (Figure 3B and Tables S5 and S6). We conclude that all investigated variants lead to alteration, mainly reduction, of the repressive function of HEY2.

No evidence for involvement of common HEY2 risk variants

Given the incomplete penetrance of CVDs we assume a complex genetic architecture, where variants of lower effect size near *HEY2* might also influence disease risk. Therefore, we investigated the association between variants +/− 1 Mb around *HEY2* and thoracic aortic aneurysms and congenital malformations of cardiac septa from UK Biobank. However, no significant variants were identified (Supplementary Materials and Methods).

Discussion

Combining human genetics and biochemical analyses we report that complete HEY2 loss resulting from a homozygous loss-of-function variant causes critical CHD, whereas the majority of heterozygous carriers show a spectrum of CVDs. Combining results from SKAT-O analyses in two cohorts, we confirm that variants in *HEY2* functional domains increase the risk for CHD and FTAAD. We demonstrate that *HEY2* variants lead to altered repression of a target gene, likely explaining the complex phenotypes observed.

Previous studies have shown that knockout of *HEY2* in mice results in CVDs, with varying phenotypes depending on genetic background.[14,17] CVDs in mice include septal defects, cardiomyopathy, a thin-walled aorta and valve anomalies.[14,17–21] Interestingly, in mice with the same inbred background varying phenotypes are observed, suggesting that non-genetic factors are also at play.[14] The important role of *HEY2* in cardiac development is

further underscored by a study demonstrating that *HEY2*-edited human induced pluripotent stem cell-derived cardiomyocytes show dysregulation of genes involved in cardiogenesis.[22] Consequently, *HEY2* has long been a candidate gene for CHDs, but no causal variants had been identified.[23,24] Two studies did report on finding *HEY2* variants in cardiac tissue of CHD patients, but it is unclear if these variants were causative.[25,26,27] Furthermore, a variant near *HEY2* was identified as a risk factor for Brugada Syndrome, a cardiac arrhythmia disorder, through a genome-wide association study.[28]

HEY2 is required for the repression of embryonic ventricular genes in the compact wall cardiomyocytes formed during fetal ventricular development, and ventricular maturation and function.[20,22,28,29] Moreover, Hey2 suppresses atrioventricular specification of the embryonic ventricle.[10,15,30] These data suggest that abnormal ventricular patterning and developmental gene expression caused by altered HEY2 functioning could account for CHDs in humans. In our qPCR assays, we show that CHD mutants lead to upregulation of some known target genes, indicating HEY2 loss-of-function. Three of the upregulated genes have a known association with cardiovascular defects themselves: *ACTN2* is associated with cardiomyopathy with or without left ventricular non-compaction, *RBM20* with dilated cardiomyopathy and *TBX1* has been shown to be responsible for the cardiovascular defects in 22q11.2 deletion syndrome (including Tetralogy of Fallot, pulmonary atresia and atrial septal defect).[31–34]

Murine *Hey2* knockouts have thinner aortic walls, which is in line with the involvement of the gene in the regulation of vascular smooth muscle proliferation and postinjury neointimal formation.[14,35] This suggests that *HEY2* variants might lead to weakened aortic walls and thus predispose to FTAAD as observed in our study. However, screening a cohort of 225 cases with mostly non-familial thoracic aortic aneurysms (described in Supplementary Materials and Methods) did not identify potentially deleterious variants in *HEY2*, indicating that this gene is not a major genetic cause for most (non-familial) thoracic aortic aneurysms. Interestingly, luciferase assays show both loss- and gain-of-function effects of identified variants. We hypothesize that both types of mechanisms could lead to disturbance of the optimal functioning of HEY2 and lead to cardiac defects, in a similar fashion as variants that lead to loss- and gain-of-function in other cardiac transcription factors, such as *TBX5*, *TBX20* and *GATA6*.[36–38]

The luciferase assay (figure 3B) suggests that loss-of-function variants are predominantly present in individuals with CHD, whereas the two variants investigated from individuals with FTAAD were gain-of-function. Whether this association is true remains to be investigated further, as it is difficult to draw strong conclusions from a small number of variants investigated using one *in vitro* assay. Overall, we hypothesize that different variants leading to altered functioning of HEY2 can result in different phenotypes, further mediated by genetic and environmental background. Rare, moderate-effect variants perturb the range in which normal cardiovascular development can occur, allowing other, stochastic factors to become significant in causing CVD. This is in line with the observation that mice with the same variants, and even identical genetic background, develop divergent phenotypes.[14,17] We aimed to address this genetic background by examining whether variants in and around *HEY2* with smaller effect sizes influence CVD risk in UK Biobank, but found no significant

associations. However, the number of CVD cases in a population-based cohort such as UK Biobank is likely too small to detect such variants.

The presence of rare risk variants in controls in our study might indicate the absence of a genetic or environmental risk background that would have pushed the individuals towards developing CVDs. Studying larger, multi-ethnic cohorts with varying genetic or environmental backgrounds are needed to confirm the association between heterozygous variants in *HEY2* and various CVDs. Furthermore, this could help explain why individuals with the same variant develop different phenotypes, for example by combining the usual rare, exonic variant studies with polygenic scores or gene-environment interaction studies. In *DARWIN*, the contribution of *HEY2* to CVD is substantial. Yet, even in an inbred population such as *DARWIN* various causal variants might be segregating, accounting for p.G108* non-carriers in CVD cases.

We find that rare *HEY2* variants predispose to a broad spectrum of CVDs and advise clinicians to consider *HEY2* as a causative gene in unsolved CVD patients. If a potentially deleterious variant in *HEY2* is identified, clinical follow-up and genetic screening of family members is warranted, as HEY2 perturbation can lead to critical cardiac defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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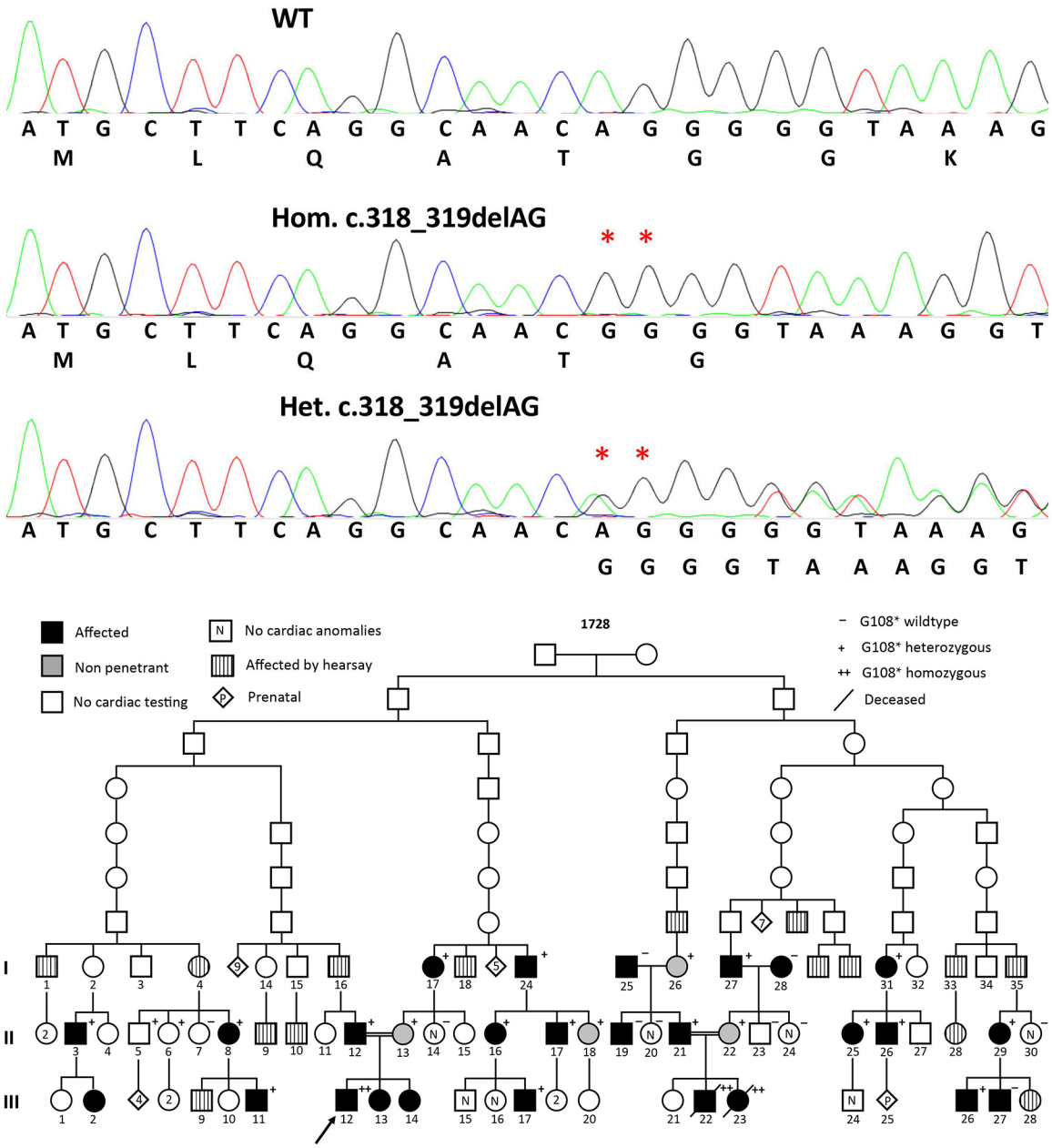


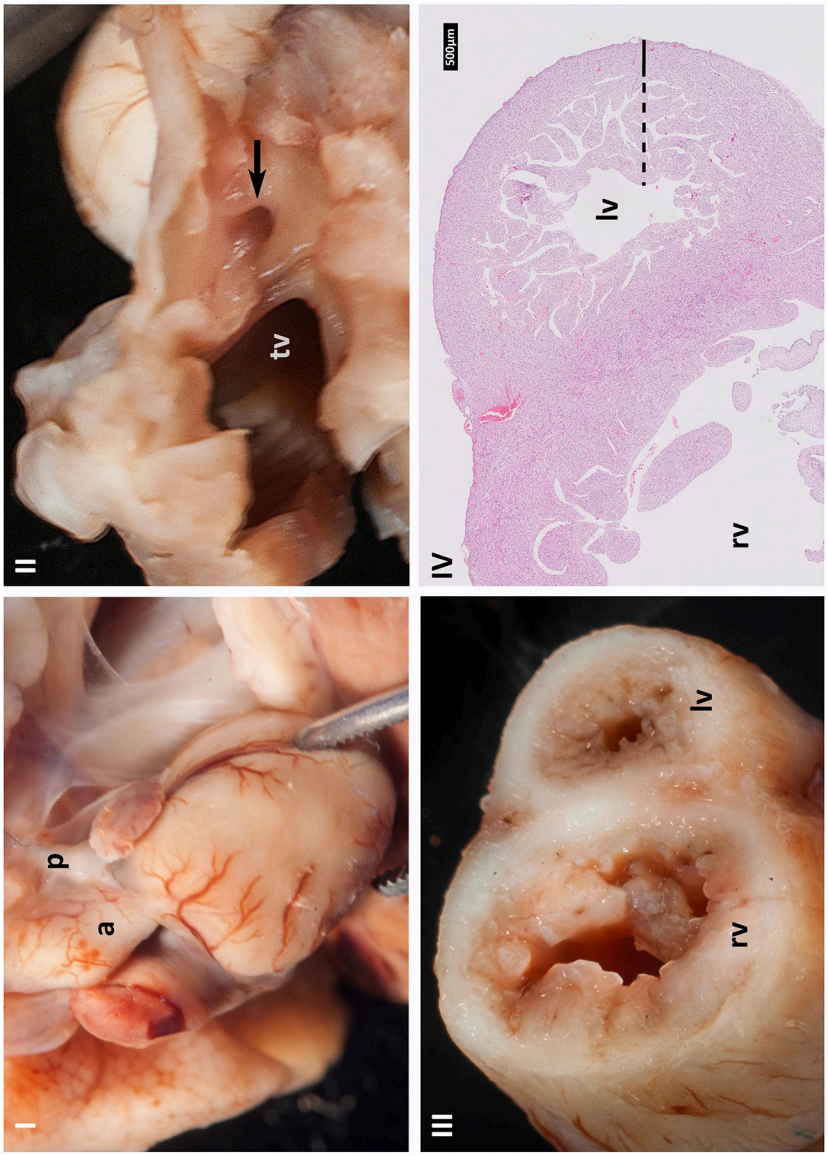
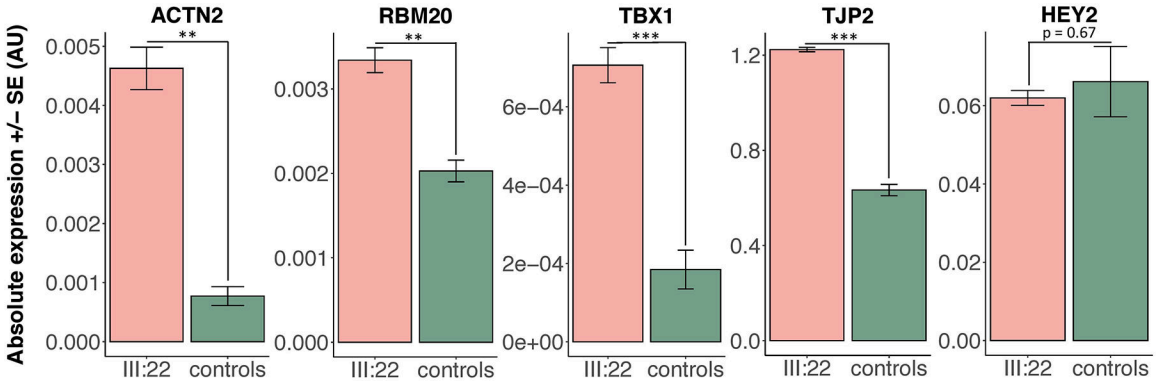


Figure 1. Identification of the p.G108* variant in *HEY2* segregating in a family from *DARWIN* with cardiac abnormalities.

A. Sanger sequencing results of a homozygous and heterozygous patient, showing two base pair deletion leading to frameshift and an early stop codon. **B.** Pedigree of family 1. Symbols: circles = females; squares = males; arrow = proband. A solid symbol can indicate various CVDs (congenital heart defect, thoracic aortic aneurysm, myocardial hypertrabeculation and/or valve abnormality). **C.** Macroscopic and light microscopic images of the heart of patient III:22, showing mitral atresia, double outlet right ventricle, hypoplastic left ventricle and significant pulmonary stenosis. **I** frontal view of the heart and great vessels, showing large aorta (a) and much smaller pulmonary trunk (p); dominant anterior located right ventricle is held between ends of tweezers. **II** Detail of the heart from above after partial removal of atrial walls. The bottom of the small left atrium is closed with only a dimple visible at the site of atretic mitral valve (indicated by black arrow); tv: wide tricuspid valve orifice. **III** Biventricular view of the heart after removal of apical parts,

showing a large dilated right ventricle (rv) and a much smaller left ventricle (lv) with small lumen. **IV** Histological image of right ventricle, ventricular septum and part of left ventricle, stained with Haematoxylin & Eosin (HE), illustrating hypertrabeculation, particularly of the left ventricular wall. Continuous line delineates compact parts and interrupted line trabecular parts of the left ventricular free wall. Scale bar represents 500 μm.



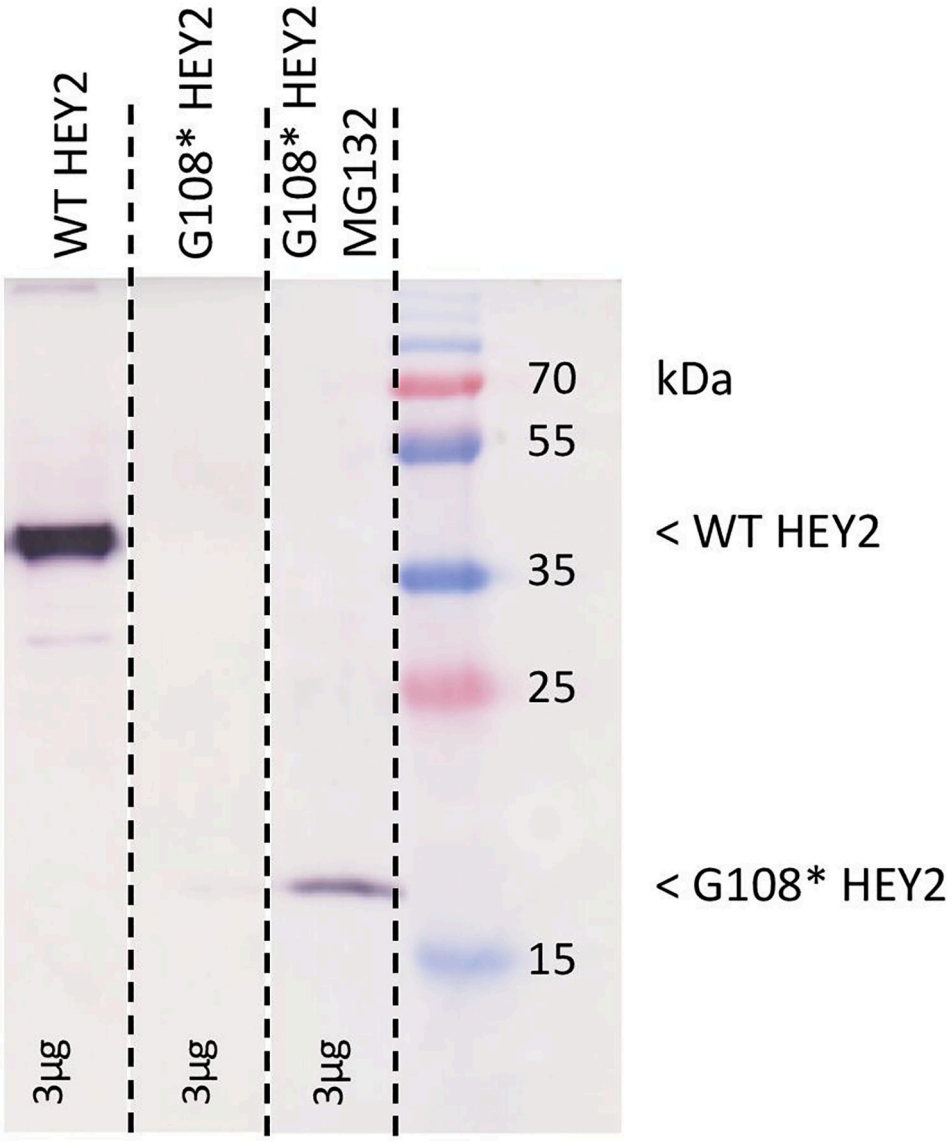


Figure 2. Expression of *HEY2* with p.G108* variant and target genes.

A. qPCR in chorionic villi from III:22 and two gender- and age matched controls. Only significantly deregulated genes and *HEY2* are shown, for other qPCR comparisons see Supplementary Table 2. **B.** Western Blot shows absent bands for HEY2 carrying a p.G108* variant ("G108* HEY2") in vitro. If the proteasome is inhibited through MG132 ("G108* HEY2 MG132"), a band appears at the height expected for a protein carrying a stop-codon at p.G108*. AU, arbitrary units; SE, standard error; WT, wildtype; **, p≤0.01; ***, p≤0.001

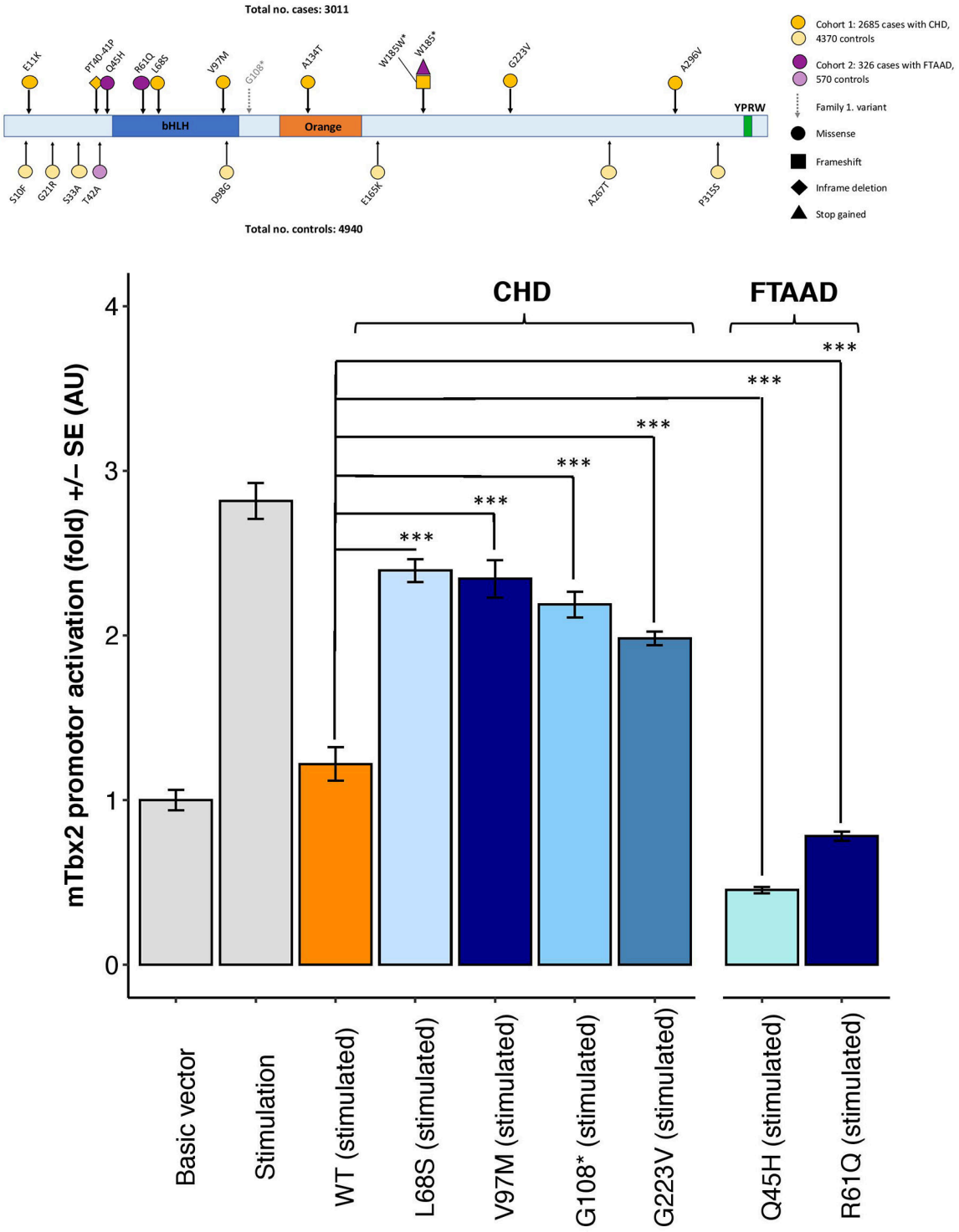


Figure 3. *HEY2* variants in all cohorts.

A. All variants in unrelated cohorts and family 1 located in the HEY2 protein. Variants observed in unrelated cases (total n = 3011) are illustrated above the schematic overview of the protein, variants observed in unrelated controls (total n = 4940) below. The grey arrow indicates the position of the p.G108* variant in family 1. **B.** Relative activation

of the *Tbx2*-luciferase assay by *HEY2* mutants found in patients with CHD and FTAAD compared to wildtype *HEY2*, investigated in murine H10 cells. The first panel shows Tbx2 activity without stimulation. A cocktail was added to stimulate expression in all other panels ('Stimulation', see Supplementary Material and Methods). Values are plotted relative to the basic vector. AU, arbitrary units; bHLH, basic helix-loop-helix; CHD, congenital heart defect; FTAAD, familial thoracic aortic aneurysms and dissections; no., number of; SE, standard error; WT, wildtype; ***, P≤0.001.

Table 1.

Cardiovascular defects in family 1 have an autosomal dominant inheritance pattern with varying expressivity and incomplete penetrance in individuals with a heterozygous p.G108* variant. Homozygous p.G108* variants lead to multiple critical cardiovascular defects.

Table 1. Overview of phenotypes present in p.G108* carriers/non-carriers in family 1.

Defect	Specific symptom	G108*/G108* (n=3)	G108*/WT (n=20)	WT/WT (n=8)
		n/total n (%)	*n/total n (%)*	*n/total n (%)*
Congenital heart disease				
	Any congenital heart disease	3/3 (100%)	8/20 (40%)	2/8 (25%)
	Right aortic arch	2/3 (67%)	4/20 (20%)	0/8 (0%)
	Atrial septal defect	1/1 (100%)[a]	4/20 (20%)	1/8 (13%)
	Ventricular septal defect	3/3 (100%)[b]	3/20 (15%)	1/8 (13%)
	Anterior and rightwards deviation of aortic root	3/3 (100%)	2/20 (10%)	0/8 (0%)
	Hypoplastic pulmonary arteries	3/3 (100%)	0/20 (0%)	0/8 (0%)
	Monocoronary	1/1 (100%)[a]	0/20 (0%)	0/8 (0%)
	Hypoplastic left ventricle	1/3 (33%)	0/20 (0%)	0/8 (0%)
	Requiring surgery	3/3 (100%)	4/20 (20%)	0/8 (0%)
Thoracic aortic aneurysm				
	Any thoracic aortic aneurysm	2/3 (67%)	7/20 (35%)	1/8 (13%)
	Aortic root (sinus of Valsalva)	1/1 (100%)[a]	7/20 (35%)	1/8 (13%)
	Ascending tubular aorta	1/3 (33%)	4/20 (20%)	1/8 (13%)
Myocardial hypertrabeculation/Non-compaction cardiomyopathy				
	Any myocardial hypertrabeculation	3/3 (100%)	5/20 (25%)	1/8 (13%)
	Non-compaction cardiomyopathy	3/3 (100%)	2/20 (10%)	0/8 (0%)
	Myocardial hypertrabeculation not meeting criteria for non-compaction cardiomyopathy	0/3 (0%)	3/20 (15%)	1/8 (13%)
Valve abnormalities				
	Any valve abnormalities	3/3 (100%)	4/20 (20%)	0/8 (0%)
	Pulmonary stenosis/atresia	3/3 (100%)†	2/20 (10%)	0/8 (0%)
	Aortic valve insufficiency	0/3 (0%)	1/20 (5%)	0/8 (0%)
	Mitral valve stenosis/atresia	1/3 (33%)	0/20 (0%)	0/8 (0%)
	Mitral valve prolapse	0/3 (0%)	1/20 (5%)	0/8 (0%)
	Dysplastic aortic valve	1/3 (33%)	0/20 (0%)	0/8 (0%)
	Dysplastic tricuspid valve	1/3 (33%)	0/20 (0%)	0/8 (0%)
	Bicuspid pulmonary valve	1/3 (33%)	1/20 (5%)	0/8 (0%)
Any cardiovascular abnormalities				
		3/3 (100%)	16/20 (80%)	4/8 (50%)

[a] could not be assessed in homozygous fetus at gestational age of 16 weeks

[b] 2/3 homozygous cases had pulmonary atresia + VSD (“extreme Fallot”)

n, number; WT, wildtype

Table 2.

SKAT-O and META-SKAT results of the CHD and the FTAAD cohort showing significant enrichment of rare, potentially deleterious variants in *HEY2* functional domains after meta-analysis.

Table 2. SKAT-O results

Cohort	All variants with MAF<1% & CADD>20				Variants with MAF<1% & CADD>20 in functional domains[a]			
	no. ***in cases***	***no.*** ***in controls***	***SKAT-O*** ***p-value***	***MetaSKAT*** ***p-value***	***no.*** ***in cases***	***no.*** ***in controls***	***SKAT-O*** ***p-value***	***MetaSKAT*** ***p-value***
2685 CHD cases, 4370 controls	8	7	0.54	0.21	4	1	0.10	0.018
326 FTAAD cases, 570 controls	3	1	0.034		2	0	0.025	

[a]including stop-gain or frameshift variants leading to loss of a functional domain

CADD, Combined Annotation Dependent Depletion PHRED-score (GRCh38-v1.5); CHD, congenital heart disease; FTAAD, familiar thoracic aortic aneurysms and dissections; MAF, minor allele frequency; no., number of