A familial congenital heart disease with a possible multigenic origin involving a mutation in BMPR1A

The genetics of many congenital heart diseases (CHDs) can only unsatisfactorily be explained by known chromosomal or Mendelian syndromes. Here, we present sequencing data of a family with a potentially multigenic origin of CHD. Twelve of nineteen family members carry a familial mutation [NM_004329.2:c.1328 G>A (p.R443H)] which encodes a predicted deleterious variant of BMPR1A. This mutation co-segregates with a linkage region on chromosome 1 that associates with the emergence of severe CHDs including Ebstein’s anomaly, atrioventricular septal defect, and others. We show that the continuous overexpression of the zebrafish homologous mutation bmpr1aa.R438H within endocardium causes a reduced AV valve area, a downregulation of Wnt/β-catenin signalling at the AV canal, and growth of additional tissue mass in adult zebrafish hearts. This finding opens the possibility of testing genetic interactions between BMPR1A and other candidate genes within linkage region 1 which may provide a first step towards unravelling more complex genetic patterns in cardiovascular disease aetiology.

Congenital heart diseases (CHDs) are the most common organ malformations and affect 1% of newborns1,2. Due to recent improvements in the treatment of CHDs, increasing numbers of patients reach a reproductive age. This has raised renewed interest in understanding the molecular causes of CHDs with the aim of improving diagnostic or therapeutic tools. Although a variety of genes has been implicated in the development of CHDs, only a minority of these diseases is caused by monogenic mutations3. Hence, one of the most urgent challenges in cardiovascular disease aetiology is a better understanding of more complex genetic traits leading to CHDs.

A large proportion of all CHDs affect the formation of atrioventricular (AV) valves. In higher vertebrates, the endocardial cushions are precursors of AV valves, cardiac septa, and parts of the cardiac outflow tract. The atrioventricular endocardial cushions are formed by endocardial cells of the atrioventricular canal (AVC) that hypertrophy and migrate into the extracellular matrix in between the inner endocardial and the outer myocardial layer of the heart tube4. This process is known as endothelial-mesenchymal transition (endoMT). Afterwards the endocardial cushions located in the AVC form the atrioventricular valves5. Defective development of the endocardial cushions can lead to CHDs including atrial, ventricular, and atrioventricular septal defects in mice6,7.

The zebrafish is an excellent vertebrate model for functional studies of valve leaflet morphogenesis8. The zebrafish and human genome share a high degree of similarity with 69% of protein-coding zebrafish genes being related to genes found in humans9. Hence, the analysis of human congenital defects is feasible in this animal model. In contrast to human anatomy, the zebrafish heart consists of only one atrium and ventricle. These two
cardiac chambers are separated by an AV valve. During zebrafish cardiac valve development, cardiac cushions elongate and form paired primitive bicuspid valve leaflets, which protrude from either side of the AVC into the lumen10,11. Within three months, the initially bicuspid valves transform into quadricuspid structures12.

The bone morphogenetic protein (BMP) pathway plays an important role in the development of embryonic heart valves7,13,14. BMPs are involved in the development of endocardial cushions via endoMT, the maturation of the tissue surrounding the AV valves, and the septation of heart cavities14. In mice, expression of BMP-Receptor 1A (BMPR1A, also known as ALK3) is required in both endocardium and myocardium to ensure the correct development of endocardial cushions15,16.

There are numerous case reports about patients with BMPR1A mutations and cardiac septal defects. These defects often occur in the context of deletion syndromes and are in combination with mental retardation, facial dysmorphism, or juvenile polyposis syndrome (JPS)17–20. In addition, isolated BMPR1A mutations have been reported to associate with cardiac malformations and occurrence of JPS. Several missense mutations of BMPR1A are associated with the emergence of ventricular septal defects and Ebstein's anomaly21. Mutations in the BMP pathway have also been connected to non-syndromic CHDs. D’Allessandro et al. described three rare mutations of BMPR1A (p.R478H, p.D429V, and p.P481S) and the concomitant occurrence of atrioventricular septal defects22.

The involvement of BMPR1A in the development of Ebstein's anomaly has also been shown in animal studies. Mice with a conditional knockout of BMPR1A in the AV canal displayed a malformation of the tricuspid valve and a disruption of the annulus fibrosus with a consecutive ventricular preexcitation, both which are characteristics of Ebstein's anomaly23.

Although numerous reports of patients with BMPR1A mutations and associated CHDs exist, a clear causal connection has not yet been demonstrated in functional studies. Since chromosomal and Mendelian syndromes explain only 20% of the cases24, also more complex genetic processes may have an important influence on the development of CHD.

In 1997, we described a family with multiple cardiac cushion defects (e.g. Ebstein's anomaly, atrioventricular septal defect, and aortic stenosis)25. Within four generations, at least 13 family members were affected. Here we present the results of next generation sequencing of this family. Using an adult zebrafish model, we provide a detailed functional analysis of a candidate mutation in BMPR1A. Our results indicate a more complex genetic trait involved in CHD.

**Results**

**Phenotype and genotype data of a large pedigree with CHDs.** Since the first description by Schunkert et al. of a family as “a large pedigree with valvuloseptal defects”26, two more family members have been identified with 13 of 19 family members suffering from CHDs (Fig. 1). No extracardiac anomalies were reported, apart from a single patient that anamnestically suffered from “severe malformations” before he died during the first days after birth (no medical records available). The main phenotypes were characterized by atrioventricular septal defects (AVSD) (n = 3), an atrial septal defect (ASD) (n = 1), a ventricular septal defect (VSD) (n = 1), Ebstein's anomalies (n = 4), Wolff-Parkinson-White (WPW) syndromes (n = 3), cleft mitral valves (n = 3), and right bundle branch blocks (RBBB) (n = 3) (see Supplementary Table S1 for a summary of all available clinical features and patient details).

The family was first characterized by short tandem repeat (STR)-based linkage analysis26. Although this led to the discovery of a linked region on chromosome 1, the signal did not yield genome-wide significance based...
.registry describing an association to the Hereditary cancer-predisposing syndrome 32. Subsequent validation of a missense mutation which produces a dominant-negative BMPR1A 34. Finally, we also tested whether the variant occurs significantly more frequently in family members suffering from CHD when compared to unaffected family members carried the variant, it was also present in three unaffected family members (Fig. 1; No. 1, 12, and 16). This variant in other family members revealed, that the co-segregation is not perfect: although all affected family members carried the linkage region, which suggests reduced penetrance, a multifactorial inheritance, and/or the contribution of environmental factors.

After the identification of potential disease-associated loci by linkage analysis, we screened the chromosome 1 region in more detail with the aim to identify candidates for disease-causing variants. However, a clear pathogenic variant was not identified within the coding regions of genes in the linkage region on chromosome 1. Due to limited DNA availability, not all family members were sequenced.

One variant that strongly co-segregated with linkage region 1 was within the BMPRIA locus on chromosome 10 (hg19 genomic position: chr10:g.88681438 G>A). This variant causes a predicted deleterious AA-change [NM_004329.2:c.1328 G > A(p.(R443H)] as indicated by multiple functional prediction tools including SIFT27, PolyPhen228, MutationTaster229, CADD30, and DANN31. So far, this variant has only been reported in the ClinVar registry describing an association to the Hereditary cancer-predisposing syndrome32. Subsequent validation of this variant in other family members revealed, that the co-segregation is not perfect: although all affected family members carried the variant, it was also present in three unaffected family members (Fig. 1; No. 1, 12, and 16). However, the variant occurs significantly more frequently in family members suffering from CHD when compared to their unaffected relatives (p = 0.044, Fisher's exact test).

Because BMPRIA<sup>p.R443H</sup> (chromosome 10) and the linkage region on chromosome 1 showed a strong co-segregation within the family, we next tested whether a chromosomal translocation was present. However, this possibility was excluded by Fluorescence in situ hybridization (FISH) analysis.

The human BMPRIA<sup>p.R443H</sup> mutation complements the loss of zebrafish Bmpr1aa/Bmpr1ab receptors and hence encodes a functional receptor variant. To test whether the human gene encoding BMPRIA<sup>p.R443H</sup> is functional, we performed a complementation assay based on mRNA injections into zebrafish <i>bmpr1a</i> double morphants. Loss of the two Bmpr1a proteins in zebrafish causes severe dorsalization defects that can partially be rescued by the injection of human BMPRIA<sup>WT</sup> mRNA33. In addition to using human BMPRIA<sup>WT</sup> and BMPRIA<sup>p.R443H</sup> mRNAs, we also injected BMPRIA<sup>p.L342R</sup> (“Linkspoot”) mRNA that contains a missense mutation which produces a dominant-negative BMPRIA<sup>p.R443C</sup> that harbours a mutation, which is associated with the occurrence of juvenile polyposis syndrome (JPS)35. As BMPRIA<sup>p.R443C</sup> (referred to as JPS variant) affects the same residue as the mutation found in the reported family, we used this mutant variant to elucidate possible residue-specific effects on the BMPRIA1 mutant protein (see Supplementary Table S2 for a list of all BMPRIA variants used in this study; Supplementary Fig. S2 shows a sequence alignment of human BMPRIA1 with zebrafish Bmpr1aa).
For complementation assays, we co-injected BMPR1A mRNAs together with antisense oligonucleotide morpholinos (MO) against bmpr1aa and bmpr1ab. At 24hpf, zebrafish embryos were classified into one of four different dorsalization classes that were categorized from C1 to C4 with ascending severity as previously described\(^3^6\) (Fig. 3A).

As previously shown, co-injection of BMPR1A\(^{p.L342R}\) (“Linkspoot”) mRNA together with bmpr1aa/ab MOs did not rescue the morphant phenotype\(^3^\). In comparison, all other mRNAs showed a statistically significant rescue (Fig. 3C). Hence, contrary to our expectations based on the functional prediction tools used in this study, human BMPR1A\(^{p.R443H}\) and BMPR1A\(^{p.R443C}\) mRNA (JPS variant) receptor variants are functional in zebrafish and functionally complement the Bmpr1aa/ab knockdown-associated dorsalization phenotypes in zebrafish embryos. This finding provided further evidence for a genetic pattern of inheritance associated with the CHDs that is more complex than initially assumed.

The diameter of zebrafish embryonic AV valves is not affected by pan-endothelial/endo-cardial overexpression of a bmpr1aa\(^{p.R438H}\) mutant. Bmp signalling has an essential role during valve development in mice, where it controls endoMT\(^1^6\). In zebrafish, bmp4 is highly expressed at the cardiac cushions and Bmp receptors including Bmpr1aa are expressed within the early endocardium\(^1^7\). To elucidate...
whether a zebrafish Bmpr1aa<sup>R438H</sup> variant corresponding to the human BMPR1A p.R443H variant exerts some dominant-negative or gain-of function activity which may not be detected during dorsoventral pattern formation of the zebrafish embryo, we next analysed its activity during zebrafish cardiac valve leaflet formation. To this end, we generated two stable transgenic lines of zebrafish for Gal4-dependent overexpression of Bmpr1aa<sup>R438H</sup> variants [Tg(UAS:bmpr1aa<sup>R438H</sup>_IRES_EGFP)] or Bmpr1aa<sup>WT</sup> variants [Tg(UAS:bmpr1aa<sup>WT</sup>_IRES_EGFP)]. We used these stable transgenic lines in combination with the pan-endothelial activator line Tg(fli1a:Gal4FF)<sup>ubs3</sup> to drive expression specifically within endocardium/endothelium. We validated that expression from these transgenic overexpression lines was detectable within endothelium, by performing whole mount in situ hybridizations using an EGFP probe against the bicistronic bmpr1aa<sup>WT</sup>_IRES_EGFP mRNAs (Supplementary Fig. S3).

Functional studies of early cardiac development revealed that three independent stable transgenic lines carrying the mutation (alleles numbers md60, md61, and md66) were phenotypically not distinguishable. Similarly, two stable transgenic lines with the WT version of the receptor gene (allele numbers md65 and md67) did not show any phenotypes.

In functional tests, we compared valve size and morphology in bmpr1aa<sup>R438H</sup> or bmpr1aa<sup>WT</sup>-overexpressing zebrafish embryos at 120hpf. At that stage, the AVC diameter was marked by the transgenic Wnt signalling reporter line Tg(7xTCF-Xia:Sia:NLS-mCherry)<sup>i5</sup> which strongly labels valve leaflets<sup>39</sup> (Fig. 4A). The maximum AVC diameter was measured from edge to edge of the labelled valve leaflets (Fig. 4B). However, the AVC diameters of embryos expressing either of the two bmpr1aa variants did not significantly differ (bmpr1aa<sup>R438H</sup>: 60.2 ± 15.1 μm, n = 8; bmpr1aa<sup>WT</sup>: 74.1 ± 8.4 μm, n = 6; two-sided student’s t-test, p = 0.066, Fig. 4F). Obvious morphological changes due to the overexpression of bmpr1aa<sup>R438H</sup> were not detectable (Fig. 4D,E). Hence, the endocardial/endothelial overexpression of bmpr1aa<sup>R438H</sup> does not obviously interfere with growth or morphology of zebrafish AV valves during embryogenesis.

**Figure 4.** Pan-endothelial expression of a zebrafish Bmpr1aa<sup>R438H</sup> mutant protein does not affect embryonic valve morphogenesis. (A–E) Shown are reconstructions of confocal z-stack images of the zebrafish embryonic atrioventricular canal (AVC) region. (A) Embryonic cardiac ventricle in a Tg(fli1a:Gal4FF)<sup>ubs3</sup>, Tg(UAS:bmpr1aa<sup>WT</sup>_IRES_EGFP)<sup>md65</sup>, Tg(7xTCF-Xia:Sia:NLS-mCherry)<sup>i5</sup> zebrafish at 120hpf. The AVC region is outlined. Scale bar = 20 μm. (B) AVC region in a Tg(fli1a:Gal4FF)<sup>ubs3</sup>, Tg(UAS:bmpr1aa<sup>WT</sup>_IRES_EGFP)<sup>md65</sup>, Tg(7xTCF-Xia:Sia:NLS-mCherry)<sup>i5</sup> or (C) Tg(fli1a:Gal4FF)<sup>ubs3</sup>, Tg(UAS:bmpr1aa<sup>R438H</sup>_IRES_EGFP)<sup>md60</sup>, Tg(7xTCF-Xia:Sia:NLS-mCherry)<sup>i5</sup> zebrafish at 120hpf. Indicated is the diameter of the AVC (d). Scale bar = 10 μm. (D,D′) Embryonic valve formation in the WT zebrafish embryo at 72hpf. Embryos that show a normal double-layered leaflet morphology with active Wnt signalling marked by Tg(7xTCF-Xia:Sia:NLS-mCherry)<sup>i5</sup> (arrow). Cell membranes of luminal cells are marked by immuno-labelling against ALCAM (cells marked by asterisks). (E,E′) Similarly, valvulogenesis is not affected in Tg(fli1a:Gal4FF)<sup>ubs3</sup>, Tg(UAS:bmpr1aa<sup>R438H</sup>_IRES_EGFP)<sup>md60</sup>, Tg(7xTCF-Xia:Sia:NLS-mCherry)<sup>i5</sup> embryos that show a normal double-layered leaflet morphology with Wnt signalling in abluminal cells (arrow) and ALCAM-positive luminal cells (asterisks). Scale bars = 20 μm. (F) The diameter of the AVC in zebrafish embryos with pan-endothelial overexpression of bmpr1aa<sup>R438H</sup> does not significantly differ from that upon bmpr1aa<sup>WT</sup> overexpression [total number of embryos analysed: bmpr1aa<sup>WT</sup>; n = 6; bmpr1aa<sup>R438H</sup>; n = 8; two-sided student’s t-test, p = 0.066].
The ectopic tissue growths formed oblong or circular shapes in a size range of 75–150 µm in length. This finding provides further evidence for an effect of the bmpr1aaWT variant on cardiac morphology. During zebrafish embryonic cardiac valve leaflet morphogenesis, the Wnt reporter is expressed on the abluminal side of forming valve leaflet10,11. Strikingly, we found that adult fish overexpressing bmpr1apR438Hp variant caused a reduced AV valve size in adult zebrafish that was significantly reduced in animals with a continuous endothelial-specific overexpression of the bmpr1aapR438H variant when compared to the control fish that were overexpressing bmpr1aaWT (bmpr1apR438Hp, 94.638.6 µm², n = 13; bmpr1aapR438Hp, 136.352.7 µm², n = 12; ANCOVA; F(1, 22) = 10.73; ***p = 0.003). Since valve morphogenesis is affected by the size of the developing larvae41, an analysis of covariance (ANCOVA) was used, which statistically removes the effects of fish length on AV valve area using linear regression before performing a standard ANOVA. The AV valve measurement technique is further explained in Supplementary Fig. S4.

Canonical Wnt signalling acts as a mitogenic trigger of cushion mesenchyme proliferation following endoMT54 and the inhibition of Wnt/β-catenin signalling induces a lack of endothelial cushion tissue55. During zebrafish embryonic cardiac valve leaflet morphogenesis, the Wnt reporter is expressed on the abluminal side of the forming valve leaflet9,11. Strikingly, we found that adult fish overexpressing bmpr1aapR438Hp variant within endothium displayed a severe reduction of Tg(7xTCF-Xia.Sia.NLS-mCherry)ia5 reporter expression in comparison to fish overexpressing bmpr1aaWT (Supplementary Fig. S4). This finding is indicative of a decreased Wnt signalling activity within adult AV valves upon bmpr1aapR438Hp overexpression. Taken together, these findings demonstrate that the pan-endothelial overexpression of bmpr1aapR438Hp causes a reduced AV valve size in adult zebrafish that may be triggered by a downregulation of Wnt/β-catenin signalling.

**Ectopic valvular tissue mass occurs in adult zebrafish with an endocardial overexpression of the bmpr1aapR438Hp variant.** To characterize AV valve morphology in adult zebrafish with an endocardial overexpression of the bmpr1aapR438Hp variant, we performed an electron-microscopic analysis. We found that three of eight analysed hearts had some growth of ectopic tissue mass on AV valve leaflets (Fig. 6). In comparison, none of seven zebrafish hearts overexpressing bmpr1aaWT displayed any such tissue growth (p = 0.200, fisher’s exact test). The ectopic tissue growths formed oblong or circular shapes in a size range of 75–150 µm in length. This finding provides further evidence for an effect of the bmpr1aapR438Hp variant on cardiac morphology.

**Discussion**

As the genetics of most CHDs is unsatisfactorily explained by monogenic inheritance, more complex inheritance patterns gain importance. Here, we report a family with an exceptionally high percentage of affected members (68%, or 13 out of 19) and a potentially multigenic inheritance of CHDs. Every affected family member for which complete sequencing data is available carries both a BMPR1A missense mutation and a defined linkage region on chromosome 1 (Fig. 1). Therefore, an interaction of the BMPR1A mutation with another genetic entity within the linkage region on Chr.1 may be causative for the diseases.

BMPR1A plays a crucial role during gastrulation and differentiation of mesodermal cells33. As Bmpr1a knockout mice die by day 10 of development44, a complete loss of BMPR1A would presumably result in human neonatal death as well. Since embryonic development is not affected in the presented family, it appears unlikely that the BMPR1A apR438H mutation causes a complete loss-of-function of BMPR1A signalling. This is in agreement...
with our finding that the injection of human BMPR1A<sup>p.R443H</sup> mRNA into zebrafish at the one cell stage complements the knockdown of zebrafish <i>bmpr1aa</i>. Currently, we cannot entirely exclude the possibility that the expression of human BMPR1A<sup>p.R443H</sup> exerts a weak effect on zebrafish embryonic development. The lack of obvious embryonic or cardiac defects upon injection of BMPR1A<sup>p.R443H</sup> mRNA into zebrafish provides further evidence for a more complex trait involving the BMPR1A mutation together with another modifier in causing cardiac defects. However, as family members are heterozygous carriers, the BMPR1A<sup>p.R443H</sup> mutation may also cause a (semi-) dominant effect as assayed in transgenic zebrafish. Although continuous pan-endothelial overexpression of <i>bmpr1aap</i><sup>p.R438H</sup> does not affect embryonic valve development, adult zebrafish hearts are affected. Here, the endothelial overexpression of <i>bmpr1aap</i><sup>p.R438H</sup> leads to a clear reduction of the AV valve area when compared to the

**Figure 6.** Ectopic valvular tissue growths occur in adult zebrafish overexpressing the <i>bmpr1aap</i><sup>p.R438H</sup> variant within endocardium. (A,B) Electron microscopic images of adult zebrafish hearts with (A) Tg(fl1a:Gal4FF)<sup>ub56</sup>; Tg(UAS:<i>bmpr1aaWT</i> IRES_EGFP)<sup>md65</sup>; Tg(7xTCF-Xia.Sia:NLS-mCherry)<sup>ia5</sup> or (B) Tg(5xTL:Gal4FF)<sup>ub56</sup>; Tg(UAS:<i>bmpr1aap</i><sup>p.R438H</sup> IRES_EGFP)<sup>md65</sup>; Tg(7xTCF-Xia.Sia:NLS-mCherry)<sup>ia5</sup>. M = myocardium. Scale bar = 100 µm. (A′,B′) Magnified view of the atrioventricular valve. L = valve leaflet. Scale bar = 20 µm. (A) Adult zebrafish hearts with endothelial overexpression of <i>bmpr1aawt</i> do not have any obvious morphological changes. (B′) 3 out of 8 analysed adult zebrafish hearts overexpressing <i>bmpr1aap</i><sup>p.R438H</sup> have ectopic valvular tissue growth at the atrioventricular valve (arrows). (C) Numbers of zebrafish hearts overexpressing either <i>bmpr1aawt</i> or <i>bmpr1aap</i><sup>p.R438H</sup> with ectopic valvular tissue growth. None out of 7 analysed zebrafish hearts overexpressing <i>bmpr1aawt</i> had ectopic valvular tissue growths (p = 0.200, fisher's exact test).
overexpression of bmp1aap

Strikingly, adult fish overexpressing bmp1aapR438H in endocardials display a severe reduction of Tg(gfp;Cas.1.xa.5-NLS-mCherry)142 reporter expression, which is indicative of decreased Wnt signalling activity within adult AV valves. In mice, canonical Wnt signalling acts as a mitogenic trigger of cushion mesenchyme proliferation following endoMT42. Indeed, injection of apc or dkk-1 mRNA, both encoding inhibitors of Wnt/β-catenin signalling, induces a complete lack of embryonic endocardial cushion tissue in zebrafish. Correspondingly, zebrafish with a constitutively active Wnt/β-catenin signalling have massively expanded atrioventricular endocardial cushions43,45. Similar studies in chicken revealed an increased AVC cell number due to Wnt overexpression46. Several lines of evidence suggest that in mice, endoMT within the AVC is accompanied by an upregulation of Wnt/β-catenin signalling46. The expression of the mesenchymal cell marker oSMa is induced by TGFβ32 signalling and TGFβ32-induced endoMT depends on Wnt/β-catenin signalling47. Because of the known role of Wnt signalling in cardiac valve development, it is tempting to speculate that the reduced valvular Wnt signalling observed in adult bmp1aapR438H-overexpressing zebrafish may be directly related to the reduced AV valve area. Although the exact mechanism of Wnt signalling activation within cardiac valves is currently unknown, it may be a TGF-β/Wnt cross-talk, that occurs by reciprocally-regulated ligand production, synergistically regulated shared target genes, or by cytoplasmatic protein interactions46. It is unclear why the reduction of the Wnt/β-catenin signalling is not apparent in the embryonic heart. Potentially, bmp1aapR438H-overexpression may only exert a long term and weak effect on this pathway or Wnt/β-catenin signalling is more sensitive at later stages of development.

We observed that 3 out of 8 analysed adult zebrafish overexpressing bmp1aapR438H in endocardials develop ectopic valvular tissue mass. The occurrence of these morphological defects in the adult bmp1aapR438H mutant is not statistically significant due to the small sample size (p = 0.200, fisher's exact test). However, the growth of ectopic valvular tissue mass indicates a tendency of the bmp1aapR438H allele to cause defective valve leaflets, something never observed among the control population of animals. Hence, the long-term endocardial overexpression of bmp1aapR438H in zebrafish did not cause the same severe cardiac defects that occur within the described family25. This provides additional evidence for a potentially combinatorial origin of this severe form of CHD. Similar to the human condition that is associated with the BMPRApR438H allele, continuous long-term expression of zebrafish bmp1aapR438H alone was not sufficient to cause severe morphological cardiac defects beyond size differences of the cardiac valve leaflets and growths of ectopic valvular tissue mass. This finding lends additional significance to a potentially multigenic combinatorial origin in the aetiology of these inherited CHDs. To elucidate the potential involvement of genetic modifiers within a defined genomic interval on chromosome 1 that co-segregates with the BMPRApR438H mutation in affected family members, a functional characterization of candidate genes is required.

Among the genes homologous to those present within the interval on human chromosome 1, one gene, GIPC PDZ domain containing family, member 2 (gipc2), is a particularly strong candidate that may have a synergistic effect together with BMPRApR438H in the occurrence of CHDs due to its expression within the zebrafish heart47 and its interaction with TGFβR3 in regulating endoMT48. This TGFβ receptor enhances both BMPRA and BMP1B signalling49. Such a signalling crosstalk may explain a possible genetic interaction between GIPC2 and BMPRApR438H in the occurrence of CHDs. In preliminary own knockdown experiments in zebrafish, we have tested several candidate genes from within the 5 Mb interval, including gipc2, nexn, eld1, and fahp1, and found that gipc2 showed the strongest phenotype (unpublished own data). However, more substantial functional studies are required to test a potential genetic interaction between any of these candidate genes and bmp1aap during cardiac valve development.

Whole genome sequencing of patient material did not reveal any coding sequence mutations in GIPC2 or any other genes within the linkage interval on chromosome 1. Nevertheless, changes in regulatory elements or non-coding RNAs may cause alterations in gene expression that were not detected in the patient material. In addition, structural variants could be present in the linkage region, although no copy number variations (CNVs) were detected.

Taken together, we conclude that BMPRApR438H is a functional receptor variant that leads to a downregulation of Wnt/β-catenin signalling, a reduced AV valve size, and ectopic valvular tissue mass in zebrafish. It is therefore a strong candidate for playing a key role in the development of the reported congenital heart defects. A complex inheritance pattern with an interaction of BMPRA and modifiers such as GIPC2 in the aetiology of these CHDs seems plausible but needs to be functionally tested in further investigations.

Materials and Methods

Sequencing. SNP markers were genotyped with the “Genome-Wide Human SNP Array 6.0” by Thermo Fisher Scientific (former Affymetrix). Linkage analysis was performed using the LINKADATAGEN52 and MERLIN53 software. Affected individual 8 was exome sequenced at the Helmholtz Zentrum Munich. Family members 13, 15, 17 were whole-genome sequenced by Complete Genomics (Mountain View, USA) using their proprietary software. Affected family members 4 and 22 were whole-genome sequenced by Centogene (Rostock, Germany). We confirm that all methods were carried out in accordance with relevant guidelines and regulations. Moreover, we confirm that all experimental protocols were approved by a named institutional ethics committee (Licence-No.: Neuangr 2/042 from March 7th, 2002, University of Regensburg). Informed consent was obtained from all subjects or from a parent.

Cloning. Plasmids were generated using the Tol2kit and the Gateway system by Invitrogen. Expression clones used for RNA transcription contain a CMV/SP6 promoter. Those used to generate stable transgenic lines
contain a UAS Promoter. The inserted BMPR1A (human) ORF sequence corresponds to the Ensembl Transcript ID ENST00000372037.7. The bmpr1aa (zebrafish) ORF sequence corresponds to nucleotides 456 to 2039 of the Genbank sequence BC115245.1 (http://www.ncbi.nlm.nih.gov/entrez). Mutagenesis was performed using the QuikChange II XL Site-Directed Mutagenesis Kit. Supplementary Table S3 summarizes the generated expression clones.

Zebras begin with standard laboratory procedures. Handling of zebrafish was done in compliance with German, Berlin and Lower-Saxony state law and carefully monitored by the local authority for animal protection [Landesamt für Gesundheit und Soziales (Berlin, Germany) and Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Oldenburg, Germany)]. Breeding and harvesting of fish eggs were authorized by the State Office of Consumer protection and food safety Lower-Saxony (Oldenburg, Germany) (Licence-No.: 33.19-42502-04-Demal; 06.01.2016). The generation of transgenic lines was authorized by the State Office of Health and Social Issues (LaGeSo Berlin, Germany) (Licence-No.: Reg0254/12) and by the State Office of consumer protection and food safety Lower-Saxony (Oldenburg, Germany) (Licence-No.: 33.12-42502-04-15/2012). mRNA and MO injections were performed using AB, TüLF, and WIK wildtype strains. To generate transgenic lines, AB, TüLF, and WIK wildtype strains were outcrossed with Tg(fli1a:Gal4FF)ubs3 or Tg(kdr:EGFP)md60. These transgenic lines later were outcrossed with Tg(7xTCF-Xla:Stam:nlsCherry)md59. mRNA rescue injections. mRNA in vitro transcription was performed using a SP6 polymerase. Zebrafish embryos were injected at 1-cell stage with 1 nl mRNA (20 ng/ul) and 1 nl morpholinol oligo mix [bmpr1aa MO (4 mM), bmpr1ab MO1 (4 mM), bmpr1ab MO3 (4 mM)] in Danieau's Solution. To prevent cell death by p53 activation due to high MO concentrations, we co-injected p53 MO (tp53 MO4) Moz were obtained from Gene Tools and are summarized in Supplementary Table S4. Following the injections, embryos were incubated in eggwater for 24 hours classified by the severity of their state of dorsalization. Some embryos of each clump remained uninjecteled and were used as negative control. Clutches with over 25% dead embryos 24hpf in the negative control were sorted out and not analysed. Only clutches with at least n ≥ 15 (or n ≥ 10 in negative control) were analysed. The experiment was performed at least 4 times with each mRNA variant. The mRNA variants were blinded before injection and unblinded after dorsalization analysis.

As statistical analysis, the mean percentage of dead and C4-malformed embryos were compared between the 4 groups of MO/mRNA co-injection and a group with MO injection only. For this purpose, data was transformed using an arcsine square root transformation. Homoscedasticity and normal distribution were ensured using Levene's test and the Shapiro-Wilk test, respectively. Data was weighted with the square of the number of embryos in the respective clump. Afterwards the groups were compared using a two-sided student's t-test and the data was corrected using Bonferroni-Holms procedure.

Generation of transgenic lines. The mutation c.G1313A (p.R438H) was introduced in the zebrafish bmpr1aa gene and cloned into a Tol2 vector using the Gateway/Tol2 kit. Constructs were injected into 1-cell stage zebrafish embryos to generate the transgenic lines Tg(UAS:bmpr1aaWT_IRES_EGFP)md65, md67 or Tg(UAS:bmpr1aaR438H_IRES_EGFP)md60. The founders of these transgenic lines were used to raise stable generations, which were outcrossed with Tg(fli1a:GAL4FF)ubs3 and Tg(7xTCF-Xla:Sia-NLS-mCherry)md65. As some transgenic embryos showed a mosaic endothelial expression pattern of GFP, only embryos with strong expression were selected and raised for a later inspection of AV valve morphology.

Whole-mount in situ hybridization. DIG labeled probe for egfp was generated as previously described. Whole-mount in situ hybridization experiments were performed as previously described. Images were recorded on a stereomicroscope (Leica M165 FC) with an EOS 5 D Mark III (Canon) camera and processed using Adobe Illustrator (Adobe Systems).

PTU treatment and fixation. Tg(fli1a:Gal4FF)ubs3, Tg(UAS:bmpr1aaWT_IRES_EGFP)/Tg(UAS:bmpr1aaR438H_IRES_EGFP), Tg(7xTCF-Xla:Sia-NLS-mCherry)md65 embryos were treated with PTU (Sigma) at 24hpf, anaesthetized with 3-aminobenzoic acid ethyl ester (Tricaine) (Sigma) and fixed in 4% PFA at 120dpf.

Adult heart extraction. Adult heart extraction was performed according to the published protocol. After extraction of the heart, the atrium was carefully severed from the ventricle exposing the AV valve. Hearts were embedded in 1% low-melting agarose in glass-bottom dishes with the AV valve facing down and imaged.

Immunohistochemistry. Whole-mount antibody stainings of zebrafish embryos were performed as previously described. The following antibodies were used: mouse anti–ALCAM/Dm-GRASP/Neurolin (1:200; Developmental Studies Hybridoma Bank). Nuclear stainings were performed using 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). We used secondary antibodies conjugated to Alexa-561 or Alexa-647 (Life Technologies) at 1:200.

Preparation and fixation of adult hearts for scanning electron microscopy (SEM). Adult fishes were anaesthetized and transferred to a Petri dish filled with cool Locke's solution (6 °C). The bottom of the Petri dish was covered with a layer of wax. Insect needles were inserted into the mouths, caudal trunks and pectoral fins of the fishes to physically fix them to the bottom of the Petri dish. The pericardial cavity was then opened using microsurgical scissors and the still beating hearts were perfused with Locke's solution (via a micropipette inserted into the sinus venosus) until all visible signs of blood were removed from the heart and bulbus arteriosus. To fix
hearts in a general dilation, final perfusion was carried out with a calcium-free Locke's solution of 20 mmol/l manganese chloride\(^{25}\). MnCl\(_2\) causes a cardiac arrest in a general dilation by calcium channel blocking. After cardiac arrest, the hearts were externally rinsed with a 25% solution of glutaraldehyde to achieve a rapid pre-fixation of the specimens.\(^{26}\) Final fixation of the specimens was carried out in a 20% solution of glutaraldehyde followed by post-fixation in Bouin's solution according to established protocols\(^{27}\). The fixed specimens were dehydrated in the usual manner and dried by the critical point method. The dried specimens were mounted on aluminum tabs with conducting silver and their ventricles were opened by removal of their ventral myocardial walls using electrolytically sharpened tungsten needles. Specimens were sputter-coated with platinum-palladium (Leica EM ACE 200).

**Image acquisition.** Confocal images were obtained using a Leica TCS SP8 confocal laser microscope with 20x and 40x magnification for adult and embryonic heart valves, respectively. 3D projections and oblique slices of confocal images were generated using Imaris (Bitplane). Electron microscopy (SEM) images were obtained using a Zeiss Ultra plus field emission scanning electron microscope. Images were processed using Adobe Illustrator CC2015 (Adobe Systems).

**Quantification of valve parameters and statistical analysis.** Diameter and area of AV valves/AVCs were measured using Fiji\(^{28}\). Embryonic AVC diameter was compared using a two-sided student's t-test. Homoscedasticity and normal distribution were ensured using Levene's test and the Shapiro–Wiatak test, respectively. Standard deviation is reported as measure of variability. Adult valve sizes were determined by the measurement of the area bounded by the AV valve annulus. This valve annulus surrounds the edge of the valve leaflets marked by TCF expression and occurs in the confocal images as a black ring. The AV valve measurement technique is shown in detail in Supplementary Fig. S4. The individual measurement data is shown in Supplementary Table S13. For statistical comparison of this adult valve area, analysis of covariance (ANCOVA) was used, which uses linear regression to statistically remove the effects of fish length as covariate before performing a standard ANOVA. Means of AV valve area are reported adjusted for the covariate zebrafish length using ANCOVA.

**Data Availability** Data generated or analysed during zebrafish experiments are included in this published article (and its Supplementary Information files). Sequencing data and all microscopic images are available from the corresponding authors on reasonable request.

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Author Contributions
T.D., B.R., I.B., H.S., Z.A., J.E. and S.A.-S. contributed to the conception of the project. B.R. and I.B. collected the family member sequencing data and performed biocomputational analysis. S.A.-S., J.E., T.D., M.H. and Z.A. took part in the experimental design. T.D., M.H., D.D. and J.M. collected the experimental data. T.D., M.H., Z.A., J.E. and S.A.-S. interpreted and analysed the data. T.D., M.H., B.R., H.R., Z.A., H.S., J.E. and S.A.-S. wrote the manuscript and revised the latest version of the manuscript.

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