

Supplemental Material

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eTables

eTable 1: List of 34 genes known or suspected to cause syndromic and non-syndromic BAV
 OMIM: Online Mendelian Inheritance in Man; AV, aortic valve; AD, autosomal dominant;
 AR, autosomal recessive; NA, not applicable

Gene	BAV in... (if present)	OMIM #	OMIM phenotype	Inheritance in humans
<i>ACTA2</i>	NA	611788	Aortic aneurysm, familial thoracic 6	AD
<i>ACVRI</i>	Mouse	NA	NA	NA
<i>BGN</i>	Human	300989	Meester-Loeys syndrome	NA
<i>ELN</i>	NA	185500	Supravalvular aortic stenosis	NA
<i>FBN1</i>	NA	154700	Marfan syndrome	NA
<i>FLNA</i>	Human	300049	Periventricular nodular heterotopia 1	XLD
<i>FNI</i>	Mouse	184255	Spondylometaphyseal dysplasia, corner fracture type	NA
<i>GATA4</i>	Mouse	607941	Atrial septal defect 2	AD
<i>GATA5</i>	Mouse	617912	Congenital heart defects, multiple types, 5	NA
<i>GATA6</i>	Mouse	600001	Heart defects, congenital, and other congenital anomalies	AD
<i>LOX</i>	NA	617168	Aortic aneurysm, familial thoracic 10	AD
<i>MAT2A</i>	Human	607086	Aortic aneurysm, familial thoracic 1	AD
<i>MATR3</i>	Mouse	NA	NA	NA
<i>MFAP5</i>	NA	616166	Aortic aneurysm, familial thoracic 9	AD
<i>MYH11</i>	NA	132900	Aortic aneurysm, familial thoracic 4	AD
<i>NKX2.5</i>	Mouse	108900	Atrial septal defect 7, with or without AV conduction defects	AD
		217095	Conotruncal heart malformations, variable	AD
		614435	Hypoplastic left heart syndrome 2	AD
		187500	Tetralogy of Fallot	AD
		614432	Ventricular septal defect 3	AD
<i>NOS3</i>	Mouse	NA	NA	NA
<i>NOTCH1</i>	Human, Mouse	109730	Aortic valve disease 1	AD
<i>PRKG1</i>	NA	615436	Aortic aneurysm, familial thoracic 8	AD
<i>ROBO1</i>	Mouse	NA	NA	NA
<i>ROBO2</i>	Mouse	NA	NA	NA
<i>ROBO4</i>	Human	607528	Aortic valve disease 8	AD
<i>SKI</i>	NA	182212	Shprintzen-Goldberg craniosynostosis syndrome	AD
<i>SKIL</i>	NA	NA	NA	NA
<i>SLC2A10</i>	NA	208050	Arterial tortuosity syndrome	AR
<i>SMAD2</i>	NA	NA	NA	NA
<i>SMAD3</i>	Mouse	613795	Loeys-Dietz syndrome 3	AD
<i>SMAD6</i>	Human	614823	Aortic valve disease 2	AD
<i>TGFB2</i>	Human	614816	Loeys-Dietz syndrome 4	AD
<i>TGFB3</i>	Human	615582	Loeys-Dietz syndrome 5	AD
<i>TGFBR1</i>	Human	609192 610168	Loeys-Dietz syndromes 1&2	AD
<i>TGFBR2</i>	Human	609192 610168	Loeys-Dietz syndromes 1&2	AD
<i>TGFBR3</i>	NA	NA	NA	NA
<i>YY1AP1</i>	Human	602531	Grange syndrome	AR

eTable 2 <<see separate Excel file>>: **A list of known candidate genes**, including: (a) genes expressed in cardiac development of the outflow tract (1845 genes); (b) human paralogs of genes with mouse CHD phenotypes (671 genes); (c) known CHD genes in humans (85 genes); and (d) genes expressed in the mouse heart (314 genes)

eTable 3: Detailed information about the microinjection reagents and genotyping of the mice model

(a) CRISPR-Cas9

Organism and Genetic line	Type	Genomic target	Sequence (5' - 3')	bp
Mouse, <i>Mib1K735R</i>	tracrRNA	<i>Mib1</i> Exon15	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCCGAGU CGGUGCUUU	67 bp
	crRNA	<i>Mib1</i> Exon15	GGGCAAGGUAGACGCUGCCUGUUUAGAGCUAUGCU	36 bp
	ssODN	<i>Mib1</i> Exon15	CAAATCAGATGCAGAAGAAATATAAACTTGCTTCCACTAGTAGTGAGAGGACATTATCT TTACTTACTGTGTTTCTTGAAGGTTCCCAAGCGGCATCGACCTTGCCACATCTTGCATGTC TTGTA	127 bp

(b) Microinjection summary

Organism and Genetic line	[Cas9 protein] (ng/uL)	[crRNA] (ng/μL)	[ssODN] (ng/μL)	Viable E2C embryos	Transferred embryos	Females	Born	Weaned	Mutant	Founders
Mouse, <i>Mib1K735R</i>	20	0.61	10	100	100	4	24	22	4	1

(c) Primers Genotyping

Organism and Genetic line	Gene	Primer sequence (5' - 3')	Annealing temperature	Amplicon (bp)
Mouse, <i>Mib1K735R</i>	<i>Mib1K735R</i> WT	For: ATGTGGGCAAGGTAGACGCT	53.9°C	497bp
		Rev: ATTAGAAGAAAACAAACGACC		
	<i>Mib1K735R</i> Mut	For: GGCACACGATGAAATAATCAGT	55.4°C	329bp
		Rev: TTCTTGAAGGTTCCCAAGCG		

eTable 4: Phenotyping of French and Israeli cases from Discovery and Replication I cohorts

	French cases (n=210)	Israeli cases (n=81)
Valve morphology		
Type I (R-L)	130 (61.9%)	38 (53.5%)
Type II (R-Non)	21 (10.0%)	14 (19.7%)
Type III (L-Non)	0 (0.0%)	6 (8.45%)
Type 0 (unicuspid)	19 (9.0%)	12 (16.9%)
Two raphes	5 (2.4%)	1 (1.4%)
Aortic disease		
Aortic dilatation*	117 (55.7%)	29 (41.1%)
Aortic coarctation	28 (13.3%)	7 (8.6%)

Data is presented in n (%), unless other specified; *Dilatation of the ascending aorta: adults \geq 40mm, child \geq 2 Z score; Individual patient data can be found in eTables 4 and 5

eTable 5 <<see separate Excel file>>: Individual data for French cohort main demographics and clinical characteristics

eTable 6 <<see separate Excel file>>: Individual data for Israeli cohort main demographics and clinical characteristics

eTable 7 <<see separate Excel file>>: Input for in-silico analyses - candidate genes to prioritize and seeds gene required for Endeavour

eTable 8: Candidate genes identified after multi-step variant and gene filtering. Details are provided on global population frequencies, CADD pathogenicity scores, and corresponding pedigrees. All variants are described according to the HGVS nomenclature.

		HGVS Coding variant	HGVS nomenclature	gnomAD v2.1.1 frequency	CADD score v1.6
	<i>MIB1</i> NM_020774	c.2827G>T	p.V943F	0.00016	23.2
CHD genes	<i>JAG1</i> NM_000214	c.2884A>G	p.T962A	0.00008354	25.9
	<i>JAG1</i> NM_000214	c.925G>C	p.G309R	0.000003978	24
	<i>NCOR2</i> NM_006312	c.3698C>T	p.T1233M	0.0005768	25.8
Cardiac expressed Genes	<i>NID2</i> NM_007361	c.3979C>T	p.H1327Y	0.0005162	22.9
	<i>NID2</i> NM_007361	c.3248C>T	p.A1083V	0.0008252	25.6

Each candidate was identified in a single family.

CHD: congenital heart defect; HGVS, Human Genome Variation Society.

eTable 9: MIBAVA-Leducq cohort (belonging to Replication Cohort I) characteristics

Variable	Status
Age	52.18 years \pm 11.84 years (SD)
Sex	35% Male (70/195) 9%Female (17/195) 56% unknown (112/195)
Family history of BAV and/or Thoracic aorta aneurism	4% Positive (8/195) 35% Negative (70/195) 61% Unknown (121/195)

eTable 10: MIB1 identified coding variants

Variant class	Coding variant	Proteic variant	gnomAD Frequency (v2.1.1)	Ethnic background	CADD score (v1.6)	Domain
Missense	c.1138G>A	p.D380N	0	NFE	26	REP1
	c.1771A>T	p.I591F	0.00013	NFE	22.8	ANK
	c.2204A>G	p.K735R	0.000008	NFE	24	ANK
	c.2411G>A ^a	p.R804Q	0.00033	NFE	23.1	RING
	c.2827G>T	p.V943F	0.00016	ASJ	23.2	RING
Loss of function	c.289C>T	p.R97*	0.00005	ASJ	36	-
	c.2305C>T	p.R769*	0.00004	NFE	39	-
	c.3001C>T	p.R1001*	0.00004	NFE	38	-

eTable 11: MIB1 association study by burden testing using non-ethnically and ethnically matched gnomAD control population

	CASES			GnomAD CONTROLS			p-value
	Ethnic background	Total alleles count	Variant allele count	Ethnic background	Total allele count	Variant allele count	
Rare protein altering variants	NFE+ASJ	892	9	any	251,496	1230	0.03
	NFE+ASJ	892	9	NFE+ASJ	123,850	579	0.03
Synonymous variants	NFE+ASJ	892	1	any	251,496	1446	0.99
	NFE+ASJ	892	1	NFE+ASJ	123,850	414	0.95

Burden analysis was performed on rare, protein-altering variants on one hand, and on silent, synonymous variants on the other hand.

eTable 12a: Replication cohort II – Summary of demographics and clinical data of BAV cases

	BAV cases (n=452)
Sex (male)	74.3% (336/452)
Age (years, mean±SD)	54.4±11.8
Valve morphology	
Type I (R-L)	50.1% (230/452)
Type II (R-Non)	13.7% (62/452)
Type III (L-Non)	2.2% (10/452)
Unknown	33.2% (150/452)
Aortic disease	
Aortic coarctation	1.1% (5/452)
Aortic aneurysm	38.3% (173/452)
Aortic dissection	0.7% (3/452)

eTable 12b <<see separate Excel file>>: Replication cohort III – individuals demographics and clinical data

eTable 13: The five most significant SNPs in the common variants analysis

Chromosome	RS Number	Position (GRCh37)	Location to <i>MIB1</i>	Mean Allele Frequency	P-value
18	rs7241299	19292517	Upstream	9%	0.00283
18	rs79023008	19323399	Intron 2	9%	0.00236
18	rs1893384	19351344	Intron 3	31%	0.00095
18	rs3017041	19355507	Intron 4	31%	0.00366
18	rs11083391	19447927	3'UTR	9%	0.00401

eTable 14: Risk haplotypes at the MIB1 locus: a comparison between BAV cases and controls

index	haplotype	Total freq.	Total S.E	Controls freq.	Controls S.E.	Cases freq.	Cases S.E.
1	00000	68%	0.000358	69%	0.000442	64%	0.00019
2	00001	0%	0	0%	0	0%	0
3	00010	1%	0.000339	1%	0.000423	0%	0.000129
4	00100	0%	0.00014	0%	0.000174	0%	0.000129
5	00110	22%	0.000255	21%	0.000319	24%	0.000129
6	01000	0%	0.000284	0%	0.000355	0%	0.000141
7	01010	0%	0.00028	0%	0.000348	0%	0
8	01110	0%	0.000061	0%	0.000076	0%	0
9	10000	0%	0	0%	0	0%	0
10	11011	0%	0.000144	0%	0.000179	0%	0
11	11101	0%	0.000123	0%	0.000154	0%	0.000003
12	11110	0%	0	0%	0	0%	0
13	11111	8%	0.000208	8%	0.000259	11%	0.000003

The table summarizes the estimated sample haplotype frequencies using PHASE. "Total freq": estimated haplotype frequencies for the whole sample; "Total S.E.": estimated standard errors for these frequencies. Additional columns present estimated haplotype frequencies and their standard deviations in cases and controls. Haplotype SNPs are presented in Fig. 3.

eTable 15: Results from the combination of missense Mib1 K735R mutant alleles with Notch1 loss of function mutations

Genotypes	TAV	BAV	No VSD	VSD	Total
<i>Mib1</i> ^{+/+} ; <i>Notch1</i> ^{+/+}	4	0	0	0	4
	100%	0%	0%	0%	100%
<i>Mib1</i> ^{KR/+} ; <i>Notch1</i> ^{+/+}	11	0	11	0	11
	100%	0%	100%	0%	100%
<i>Mib1</i> ^{+/+} ; <i>Notch1</i> ^{KO/+}	10	1	6	5	11
	91%	9%	55%	45%	100%
<i>Mib1</i> ^{KR/+} ; <i>Notch1</i> ^{KO/+}	5	4	0	9	9
	56%	44%	0%	100%	100%

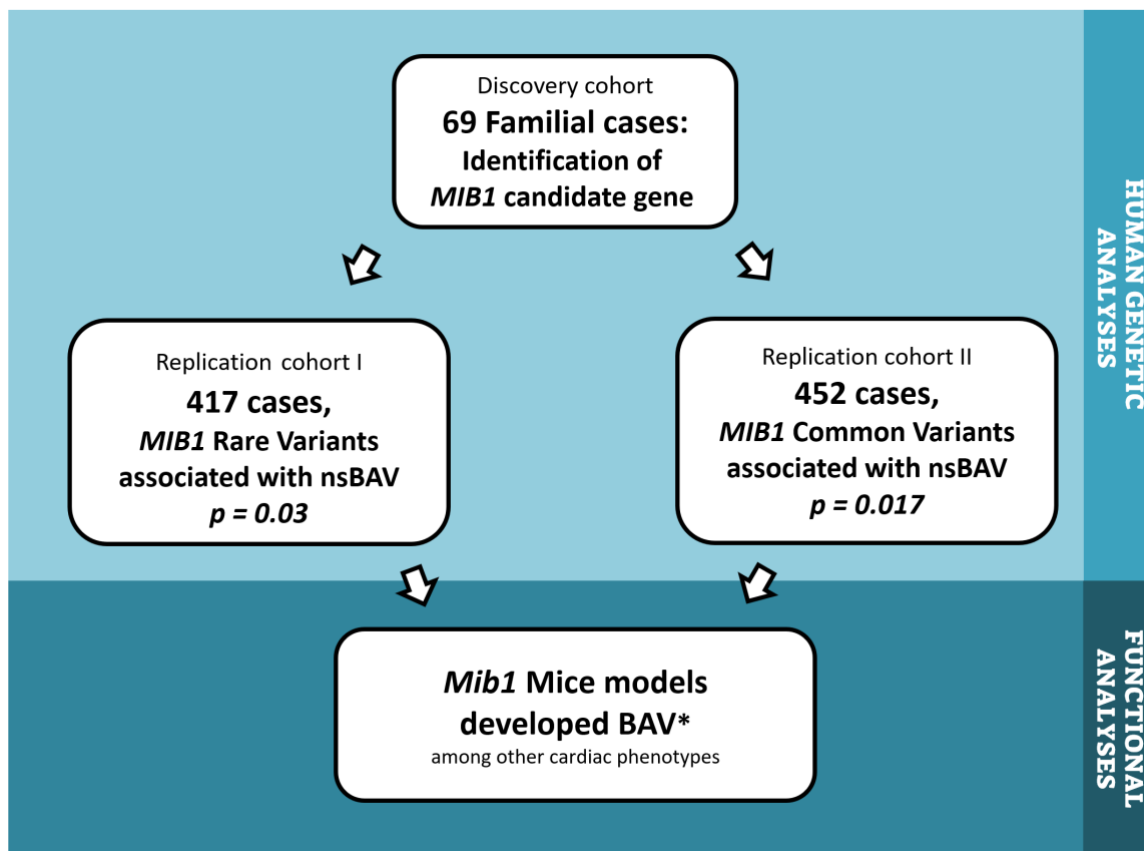
Genotypes	TAV	BAV	No VSD	VSD	Total
<i>Mib1</i> ^{+/+} ; <i>RBP</i> ^{+/+}	10	0	10	0	10
	100%	0%	100%	0%	100%
<i>Mib1</i> ^{+/+} ; <i>RBP</i> ^{KO/+}	10	0	10	0	10
	100%	0%	0%	0%	100%
<i>Mib1</i> ^{VF/+} ; <i>RBP</i> ^{+/+}	13	0	12	1	13
	100%	0%	92%	8%	100%
<i>Mib1</i> ^{VF/+} ; <i>RBP</i> ^{KO/+}	19	1	20	0	20
	95%	5%	100%	0%	100%

Genotypes	TAV	BAV	No VSD	VSD	Total
<i>Mib1</i> ^{+/+}	30	1	31	0	31
	97%	3%	100%	0%	100%
<i>Mib1</i> ^{KR/+}	38	3	41	0	41
	93%	7%	100%	0%	100%
<i>Mib1</i> ^{KR/KR}	28	0	28	0	28
	100%	0%	100%	0%	100%
<i>Mib1</i> ^{VF/+}	50	0	50	0	50
	100%	0%	100%	0%	100%
<i>Mib1</i> ^{VF/VF}	24	0	24	0	24
	100%	0%	100%	0%	100%

TAV: Tricuspid aortic valve; BAV: Bicuspid aortic valve; VSD: Ventricular septal defect.

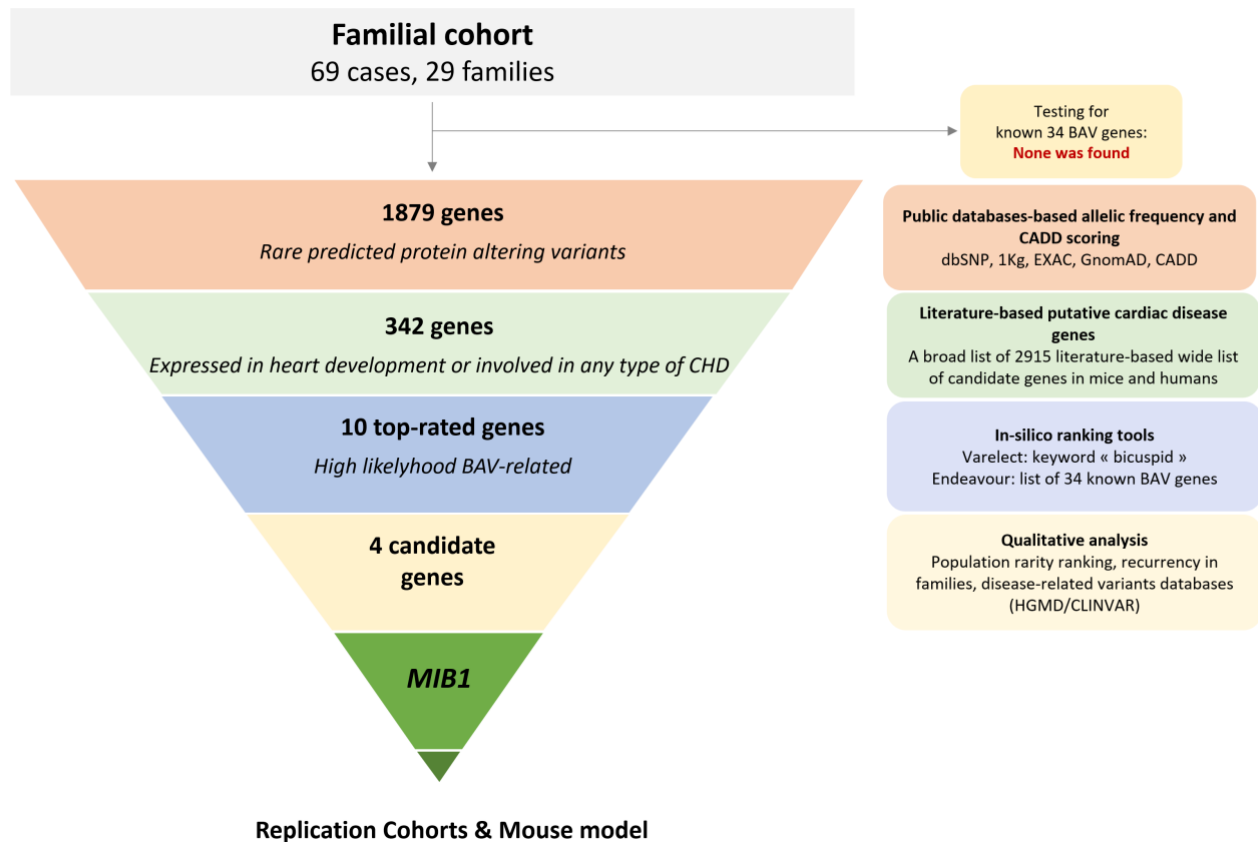
eFigures

eFigure 1: A flow chart summarizing the design of the study



The human genetics data sources include a discovery cohort of familial cases and two additional independent cohorts. We validated the association via functional models: *in-vivo* mouse models. *Mice models of the identified *MIB1* variants in the human cohort on a sensitized background.

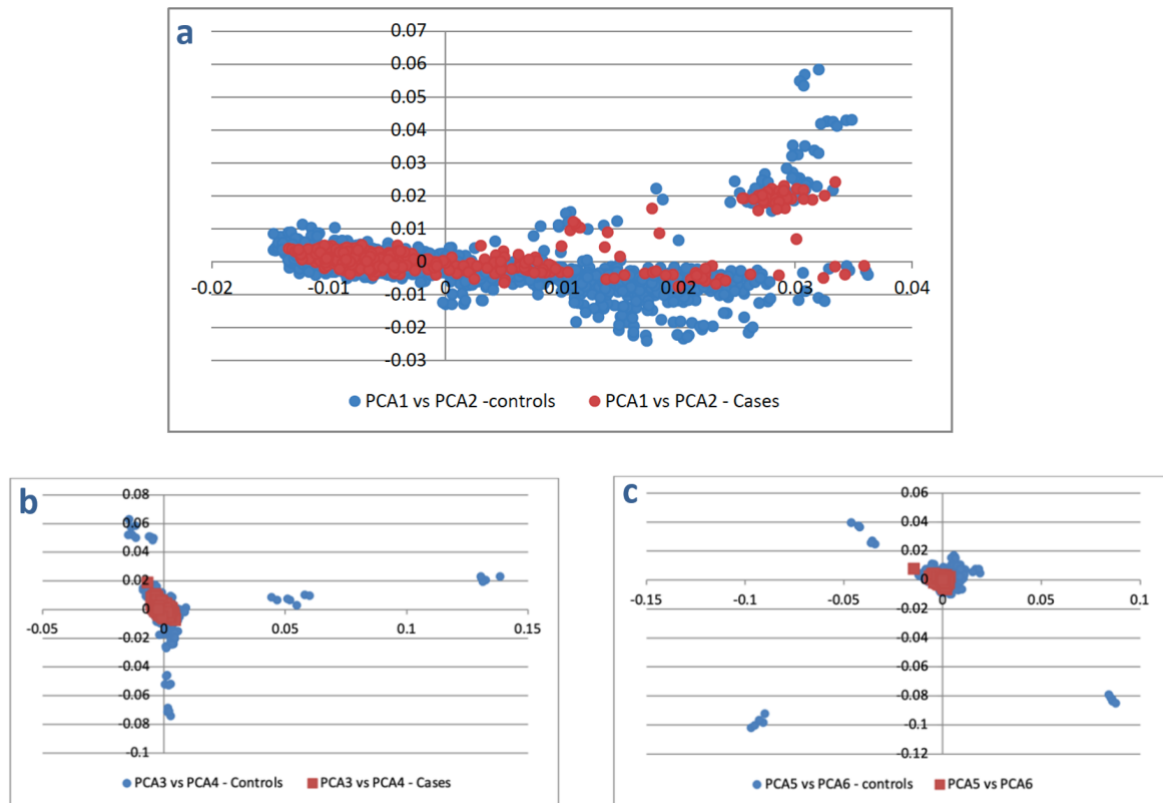
eFigure 2: Flow chart displaying the prioritization process for candidate genes and variants analysis



(a) 1879 genes with rare variants were selected in our discovery cohort based on variant frequency (MAF < 1%), annotation (missense, canonical splice-site, frameshift, indels and nonsense variants), and predicted pathogenicity (CADD score >20). (b) From literature data, two lists of candidate genes were used, based on their roles in heart development pathophysiology: genes involved in human congenital heart disease (CHD) and genes expressed in the developing mouse heart; (c) Crossing the gene list in a with the list in b resulted, respectively, in 246 and 96 common genes. (d) Gene prioritization tools (VarElect and Endeavour) were used independently to rank these lists of genes. Varelect [keyword was “bicuspid”], and Endeavour training list of genes was the 34 BAV genes involved in humans and mice (Table S12). The top 10 ranked genes from each tool were chosen for further analysis. (e) Subsequent gene lists were analyzed by (1) *Rarity* – ranking variants by lowest allelic frequencies in GnomAD, (2) *Recurrence* – the number of families sharing pathogenic variants in the gene; and (3) *Variant "weight"* – according to the variant type, its known pathogenicity using HGMD and CLINVAR disease databases and the predicted severity of pathogenicity. The overall process yielded four final candidate genes, from which *MIB1* was identified as the leading candidate: in addition to its role in NOTCH pathway, the identified variant that was published as causal in a family of LVNC.

eFigure 3: Multidimensional Scaling (MDS) plot for 452 BAV cases and 1911 FHS controls

Red dots: cases; Blue dots: controls; We identified 77 outliers in the control group that were excluded, therefore, 452 cases and 1834 controls were included for final analysis.

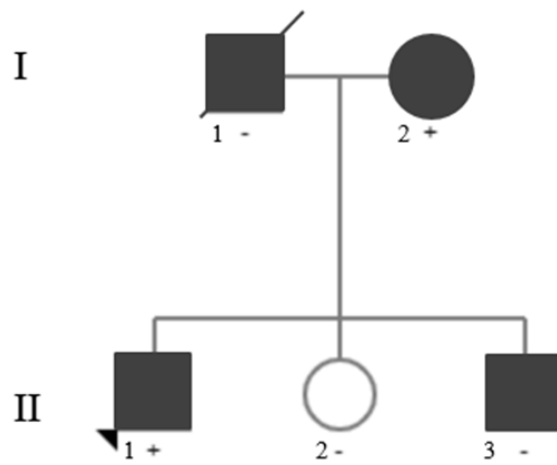


Quality control of the genotype data from each cohort was performed using Genome Studio and PLINK. We excluded individuals with low overall call rates, phenotypic and genotypic sex disparity and related individuals. We excluded SNPs with low call rates, MAF >1%, non-autosomal origin, HWE p-value < 1.0E-5 and heterozygote excess.

We performed principal components-based (PCA) filtering for population stratification. We calculated 10 PCAs for the merged data and then used an MDS-plot (multi-dimensional-scale) to identify clusters and outliers. We did not detect any clusters in the merged population. We identified 77 outliers only in the control group and excluded them by using cutoff Eigen-values (-0.02 to +0.02) on Y axis which calculated by using IBS (identical-by-state) distance for each pair of individuals (Figures 2A, 2B, 2C). No BAV case outliers were identified. An additive logistic regression model was performed for association analysis adjusted for sex and 10 PCA using PLINK.

After merging cases and controls and further quality controls, we used 452 BAV cases and 1834 white controls with a set of 1,355,128 single-nucleotide polymorphisms (SNPs) common to both Illumina arrays. From the 1,355,128 SNPs analyzed after QC for our association analysis, 66 SNPs in MIB1 (uc002ktp.3) hg19 chr18:19,284,918-19,450,912 region +/- 100kb were identified.”

eFigure 4: Pedigree of family BAV-003 with MIB1 p.V943F variant and bicuspid aortic valve (BAV) phenotype



Circles indicate females, squares indicate males. The proband (III-1) is indicated by a black arrowhead. Black filled symbols: bicuspid aortic valve. White filled symbols: tricuspid aortic valve. Genetic status: (+), p.V943F carrier patient; (-), non-carrier patient. In this pedigree, both parents have BAV.

eFigure 5: Histological analysis of *Mib1*^{+/+}, *Mib1*^{+/+};*Notch1*^{+/+} and *Mib1*^{+/+};*Rbp*^{+/+} control mice. H&E staining. Aortic valves (a,c,e) and heart sections at E16.5. (b,d,f). The asterisk indicates the positions of the leaflets. (g) Quantification of valve defects. Data are expressed as mean \pm S.D. *Mib1*^{+/+} (n=31); *Mib1*^{+/+}; *Notch1*^{+/+} (n=4), *Mib1*^{+/+};*RBP*^{+/+} (n=10), Non-significant differences by Chi-square. Scale bars, 100 μ m and 200 μ m.

