Supplementary Online Content

Tessler I, Albuisson J, Piñeiro-Sabarís R, et al. Association of the novel NOTCH pathway regulator *MIB1* gene with the development of bicuspid aortic valve. *JAMA Cardiol*. Published online July 5, 2023. doi:10.1001/jamacardio.2023.1469

This supplementary material has been provided by the authors to give readers additional information about their work.

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 in… (if present)	BAV	OMIM #	OMIM phenotype	Inheritance in humans
ACTA2	NA	611788	Aortic aneurysm, familial thoracic 6	AD
Acvr1	Mouse	NA	NA	NA
BGN	Human	300989	Meester-Loeys syndrome	NA
ELN	NA	185500	Supravalvular aortic stenosis	NA
FBN1	NA	154700	Marfan syndrome	NA
FLNA	Human	300049	Periventricular nodular heterotopia 1	XLD
Fn1	Mouse	184255	Spondylometaphyseal dysplasia, corner fracture type	NA
Gata4	Mouse	607941	Atrial septal defect 2	AD
Gata5	Mouse	617912	Congenital heart defects, multiple types, 5	NA
Gata6	Mouse	600001	Heart defects, congenital, and other congenital anomalies	AD
LOX	NA	617168	Aortic aneurysm, familial thoracic 10	AD
MAT2A	Human	607086	Aortic aneurysm, familial thoracic 1	AD
Matr3	Mouse	NA	NA	NA
MFAP5	NA	616166	Aortic aneurysm, familial thoracic 9	AD
MYH11	NA	132900	Aortic aneurysm, familial thoracic 4	AD
Nkx2.5	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
Nos3	Mouse	NA	NA	NA
NOTCH1	Human, Mouse	109730	Aortic valve disease 1	AD
PRKG1	NA	615436	Aortic aneurysm, familial thoracic 8	AD
Robo1	Mouse	NA	NA	NA
Robo2	Mouse	NA	NA	NA
ROBO4	Human	607528	Aortic valve disease 8	AD
SKI	NA	182212	Shprintzen-Goldberg craniosynostosis syndrome	AD
SKIL	NA	NA	NA	NA
SLC2A10	NA	208050	Arterial tortuosity syndrome	AR
SMAD2	NA	NA	NA	NA
Smad3	Mouse	613795	Loeys-Dietz syndrome 3	AD
SMAD6	Human	614823	Aortic valve disease 2	AD
TGFB2	Human	614816	Loeys-Dietz syndrome 4	AD
TGFB3	Human	615582	Loeys-Dietz syndrome 5	AD
TGFBR1	Human	609192 610168	Loeys-Dietz syndromes 1&2	AD
TGFBR2	Human	609192 610168	Loeys-Dietz syndromes 1&2	AD
TGFBR3	NA	NA	NA	NA
YY1AP1	Human	602531	Grange syndrome	AR

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, Mib1 K735R	tracr RNA	*Mib1* Exon15	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCG AGU CGGUGCUUU	67 bp
	crRNA	*Mib1* Exon15	GGGCAAGGUAGACGCUGCCUGUUUUAGAGCUAUGCU	36 bp
	ssODN	*Mib1* Exon15	CAAATCAGATGCAGAAGAAATATAAAACTTGCTTCCACTAGTAGTGAGAGGACATTA TCT TTACTTACTGTGTTTCTTGAAGGTTCCCAAGCGGCATCGACCTTGCCCACATCTTGCA TGTC TTGTA	127bp

(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, Mib1K735R	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, *MIb1K735R*	*Mib1K735R* WT	For: ATGTGGGCAAGGTAGACGCT	53.9ºC	497bp
		Rev: ATTAGAAGAAAACAAACGACC		
	Mib1K735R Mut	For: GGCACACGATGAAATAATCAGT	55.4ºC	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>= 40mm, child>=2 Z score; Individual patient data can be found in eTables 4 and 5

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

For each candidate gene, one variant per family was identified.

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

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

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

132 **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	-

133 [a]Found in 2 index cases.

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eTable 11: MIB1 association study by burden testing using gnomAD control population

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	CASES			GnomAD CONTROLS			
	Ethnic background	**Total alleles count**	**Variant alleles count**	**Ethnic background**	**Total alleles count**	**Variant alleles count**	**p-value**
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

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Burden analysis was performed using TRAPD which is based on allelic frequencies. The allelic frequencies presented correspond to half of genotype and disease frequencies in the context of a dominantly inherited disease as BAV.

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eTable 12. Replication cohort II – Summery 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)

148 **eTable 14. The 5 most significant SNVs in the common variants analysis**

Chromosome	RS Number	Position (GRCh37)	Location to *MIB1*	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

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eTable 15. 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 16. Results from the combination of missense *Mib1* K735R mutant alleles with Notch1 loss of function mutations

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

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

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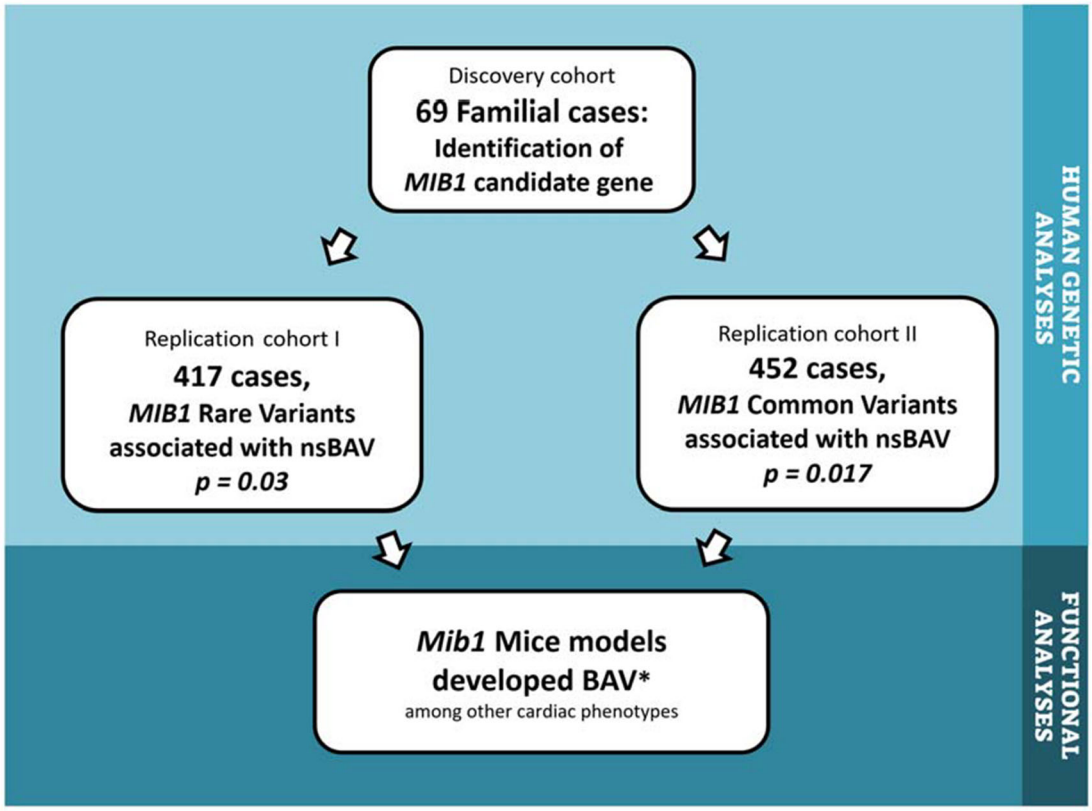
eFigure 1: A flow chart summarizing the design of the study



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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.

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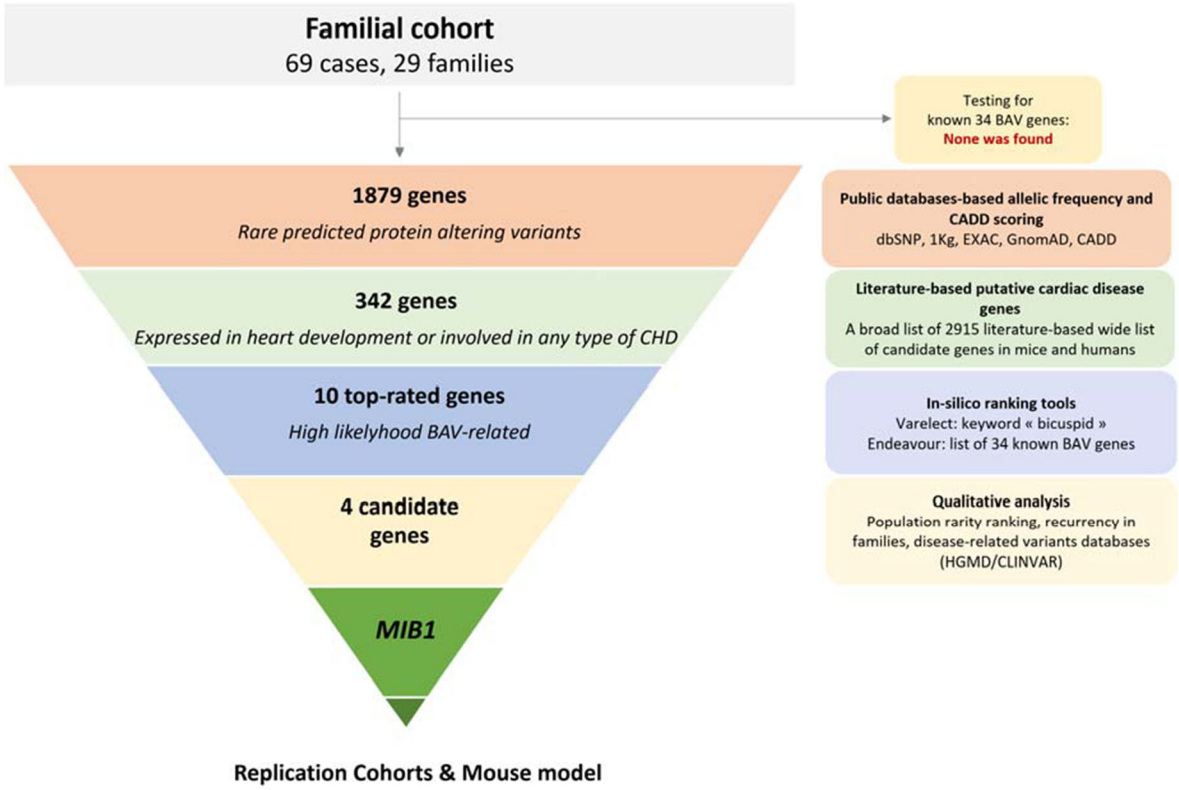
eFigure 2: Flow chart displaying the prioritization process for candidate genes and variants analysis



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(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.

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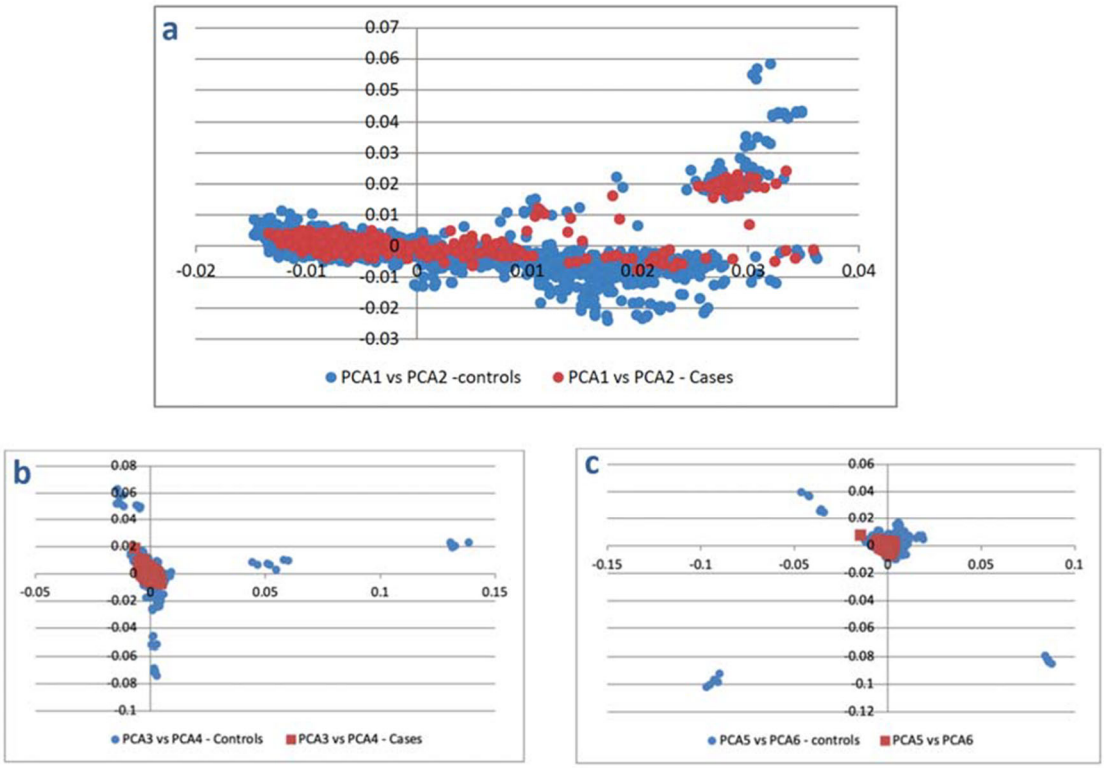
eFigure 3: Multidimensional Scaling (MDS) plot for 452 BAV cases and 1911 FHS controls

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.



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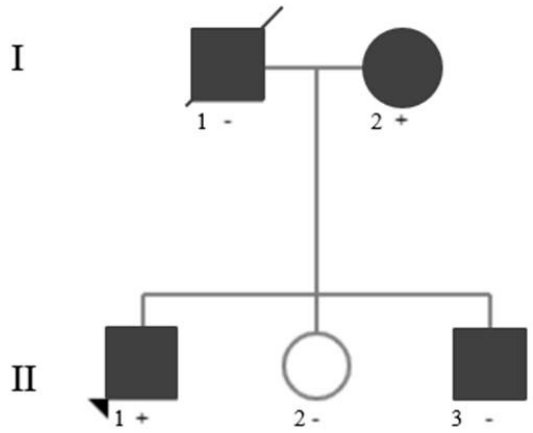
Quality control of the genotype data from each cohort was performed using Genome Studio and PLINK. We excluded individuals with low overall call rates, phenotypic and genotypic sex disparity and related individuals. We excluded SNPs with low call rates, MAF >1%, non-autosomal origin, HWE p-value<1.0E-5 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 ± S.D. *Mib1*+/+ (n=31); $Mib1^{+/+}$; $N1^{+/+}$ (n=4), $Mib1^{+/+}$;$Rbp^{+/+}$ (n=10), Non-significant differences by Chi-square. Scale bars, 100µm and 200µm.