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NOTCH Pathway Regulator MIB1: A Novel Gene for Bicuspid Aortic Valve

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Abstract

Importance: Non-syndromic bicuspid aortic valve (nsBAV) is the most common congenital heart malformation. BAV has a heritable component, yet only a few causative genes have been identified. Understanding BAV genetics is a key point in developing personalized medicine.

Objective: This study aims at identifying new genes for nsBAV.

Design and setting: A comprehensive multi-center genetic analysis, based on (i) candidate gene prioritization in a familial cohort, (2) rare variants association study in an additional replication cohort and (3) common variants association study in a third, large cohort. Further validation was done in *in-vivo* mice models.

Participants: Three cohorts of BAV patients (N=938) were utilized for the study: The discovery cohort is a large cohort of inherited cases (n=69) from 28 pedigrees of French and Israeli origin. Replication cohort I for rare variants included unrelated sporadic cases from various European

ancestries (n=417). Replication cohort II is a second validation cohort for common variants in unrelated sporadic cases from Europe and the U.S. (n=452).

Main outcomes and measures: We aimed to identify a candidate gene for BAV through analysis of familial cases exome sequencing and gene prioritization tools. We then sought for rare and predicted deleterious variants in replication cohort I and genetic association. We further investigated the association of common variants with BAV in replication cohort II. *In-vivo* phenotyping of mice showed BAV associated with deficient *MIB1* function.

Results: We identified *MIB1*, an E3-ubiquitin ligase essential for NOTCH-signal activation during heart development, as a novel nsBAV gene. In ~1.3% of nsBAV index-cases from Discovery and Replication I cohorts, we detected rare *MIB1* variants which were predicted to be damaging, and were significantly enriched compared to population-based controls (p=0.03). In Replication II cohort, we identified *MIB1* risk haplotypes associated with nsBAV (p=0.017). Two genetically modified mice models carrying *Mib1* variants identified in our cohort showed BAV on a Notch1 sensitized genetic background.

Conclusions and Relevance: This study identified *MIB1* as a nsBAV gene and a potential target for future diagnostic and therapeutic intervention. This underscores the crucial role of the NOTCH pathway in the pathophysiology of BAV.

INTRODUCTION

Bicuspid aortic valve (BAV) is a heritable condition, affecting 1%–2% of the population^{1,2} and is the most common congenital heart defect (CHD). BAV is heritable, with first-degree family members having a 10-fold increased risk of being affected^{3,7–10}. When seen in families, BAV mostly presents as a dominant inherited trait with incomplete penetrance and a higher prevalence in males (sex ratio of 3:1)^{3–7}. BAV genetics is complex and can be caused by a combination of Mendelian, oligogenic, and polygenic inheritance⁸. Several candidate genes, often identified in animal models, were reported^{9–13}. Yet only few genes were shown to cause non-syndromic BAV (nsBAV) in humans^{14,15}. These include *NOTCH1*, *GATA6*, and *SMAD6*, each accounting for 1%–3% of cases^{3,14,16}.

The NOTCH pathway, an evolutionarily conserved cell-cell communication signaling pathway involved in multiple developmental processes¹⁷, is known to be related to BAV. NOTCH signaling regulates aortic valve morphogenesis and its disruption causes aortic valve diseases in both humans and mice^{18–20}. MIB1 (MINDBOMB1) is an essential E3-ubiquitin ligase that induces NOTCH ligand ubiquitination and endocytosis, an essential downstream pathway activation step²¹.

In this study we identified *MIB1* as a novel gene for nsBAV. We combined three human genetic approaches and animal model functional studies. These results support an involvement of human *MIB1* deleterious variants, and more broadly NOTCH signaling alterations in human aortic valve dysmorphology.

METHODS

The study was approved by each institution's review board according to local regulations. Animal studies were approved by the CNIC Animal Experimentation Ethics Committee and the Madrid Community (Ref. PROEX 118/15). All animal procedures conformed to E.U. Directive 2010/63EU and Recommendation 2007/526/E.C. regarding protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

A flow chart summarizing the design of the study is presented in eFigure 1.

Study cohort

The cohort's composition is presented in Table 1.

Discovery cohort: In the discovery cohort we included a large familial cohort from two tertiary medical centers. Index-cases were included if they had BAV and if one or more relatives had BAV or thoracic aortic aneurysm (TAA). All patients underwent a comprehensive clinical examination including personal medical and family histories. Inclusion was based on BAV presence as shown by echocardiography, MRI, or cardiac surgery. Dysmorphic features were evaluated medically, and syndromic conditions were excluded. Patients under the age of 18 were included only as part of a family, not as index-cases.

Replication cohort I: Patients whose relatives were unaffected (a negative BAV or TAA test) or unavailable were classified as sporadic cases and were included in the cohort.I, along with additional cases from the MIBAVA-Leducq consortium. In the analysis of this cohort, we also included the index-cases from the Discovery cohort.

Replication cohort II included 452 BAV cases of European ancestry from Mass General Brigham Hospital and 1849 ethnically-matched controls from the Framingham Heart study⁹.

Whole exome and candidate gene sequencing

Discovery cohort: DNA was extracted from the peripheral blood or saliva of study participants using standard protocols. Whole exome sequencing was performed at the Genomics Core Facility in the Imagine Institute²². DNA extraction using the QIAmp Blood DNA Mini Kit was followed by exome capture using the Agilent SureSelect Human All Exon Kit and sequencing on an Illumina HiSeq2500. The mean depth of coverage obtained for each sample was >150×, with >97% of the exome covered at least 30×.

Replication cohort I: Sanger sequencing of the 21 exons and flanking intronic sequences of *MIB1* (NM_020774) was performed on the sporadic Israeli and French cohort. Sequences were amplified by PCR with specific primers, sequenced using BigDye Terminator v3.1 cycle sequencing kits, and run on an ABI Prism 3730XL DNA Analyzer (Life Technologies, Foster City, CA). DNA variants were identified using Sequencher software.

MIBAVA-Leducq: Whole blood-derived gDNA of patients was used for whole-exome sequencing with the Nimblegen SeqCap Exome Enrichment Kit (Roche). Samples were $2 \times 100\text{bp}$ pair-end sequenced on a HiSeq1500 instrument (Illumina). Raw data were processed using an in-house developed pipeline²³, followed by variant calling with the Genome Analysis Toolkit Unified Genotyper²⁴. Variants were annotated and filtered with VariantDB, an in-house developed tool²⁵.

All rare variants of *MIB1* identified by WES were confirmed by targeted Sanger sequencing. When samples and phenotypes from relatives were available, segregation analysis of the selected variants was performed by Sanger sequencing as described above.

Exome data analyses and prioritization in Discovery cohort

A filtering pipeline was systematically applied for exome sequencing data²². Variant calling was performed with the GATK Unified Genotyper (<https://www.broadinstitute.org/gatk/>) based on the ENSEMBL 72 database, using standard parameters. Variants that did not pass the quality filters were excluded (read depth $<10\times$ and/or Phred score <30). Only rare variants (MAF $<0.1\%$ according to gnomAD v2.1.1) with a CADD score above 20 (using the CADD v1.6 framework²⁶), and with coding impact were retained for any further analysis.

We assembled a list of 34 known BAV genes by thorough and critical literature review, identifying the most robust genes associated with syndromic and nsBAV (in humans and/or mice, eTable 1). We aim to exclude cases with predicted deleterious variants in these 34 known genes. The remaining genes carrying variants in the Discovery cohort were then subjected to a prioritization and ranking process, aiming to identify leading candidate gene for further investigation via validation cohorts and functional work based on their roles in heart development pathophysiology (detailed in eFigure 2, eTable 1, 2).^{24,25,26,63} The resulting candidate genes were prioritized by in-silico bioinformatics tools (VarElect and Endeavour), based on pathobiology, relevant pathways, and genetic and biological interactions. The top-rated genes were chosen for further qualitative analyses according to (1) *Biological relevance to BAV* – genes were prioritized if known to have a role in the valve development; (2) *Frequency* – the number of families and family members sharing pathogenic variants in the gene; and (3) *Variant “weight”* – according to the variant type and its predicted deleteriousness. The overall process yielded four final candidate genes (eFigure 2).

Rare variants burden testing

Burden testing was performed for *MIB1* rare variants with coding impact in index-cases from the Discovery cohort and Replication cohort I (n=446) against gnomAD v2.1.1 publicly available controls using the TRAPD software package²⁷. Only gnomAD variants passing filters from exome data were retained, using the same filtering process as in the discovery step (MAF $<0.1\%$, CADD score >20 , read depth $\geq 10\times$). Similarly, burden testing was performed on presumably benign, silent variants used as internal method control. Owing to the various ethnic backgrounds of cases, testing was performed in all cases vs all gnomAD controls, as well as by ethnically matched sub-populations. A 2×2 contingency table was constructed for each of the groups described above. A one-sided Fisher’s exact test

was used to estimate the association p-values. AlphaFold model of MIB1 with BAV variants mapped was constructed to demonstrate the sites of the identified variants^{28,29}.

Common SNPs genotyping data analyses in Replication cohort II

Cases of the second Replication cohort were recruited from Mass General Brigham, Boston, MA, United States⁹. Genotypes were generated using the Omni2.5 chip for cases and the HumanOmni5.0 chip for controls (obtained from the Framingham Heart Study). The Illumina Omni2.5 chip is a subset of the Omni5.0 chip, with a similar set of primers. In the Q.C. process, only markers with MAF>1% were included, and populations were stratified by principal components-based filtering (eFigure 3). After merging cases and controls, we had 452 BAV cases and 1834 white controls with a set of 24 single-nucleotide polymorphisms (SNPs) in MIB1 [(uc002ktp.3) hg19 chr18:19,284,918–19,450,912 region +/-100kb] common to both Illumina arrays⁹ which were included for final analysis.

PLINK (version 1.9) was used for SNP association tests in an additive logistic regression model for BAV cases and controls. The false discovery rate (FDR) method was applied for multiple comparison correction. The coefficient of linkage disequilibrium (D') between SNPs was calculated using LDlink³⁰. PHASE (version 2.1)³¹ was used for haplotype reconstruction and for performing association tests between haplotypes and BAV status. We used a permutation test with 1000 repeats to test for differences in haplotype frequencies between BAV cases and controls, each time shuffling the case/control status of individuals.

Mice

Generation of Mib1^{K735R} mouse line.—CRISPR RNA (crRNA) sequences were designed using the CRISPOR-TEFOR online tool (<http://crispor.tefor.net/crispor.py>). The annealed two-part synthetic crRNA (Alt-RR CRISPR-Cas9 crRNA, 2 nmol, Integrated DNA Technologies, IDT) and tracrRNA (Alt-RR CRISPR-Cas9 tracrRNA, 5 nmol, IDT, 1072532) molecules were diluted in microinjection buffer (1 mM Tris HCl, pH 7.5; 0.1 mM EDTA) and incubated with Sp Cas9 nuclease (IDT, 1081058). To generate the Mib1K735R line, a complementary and asymmetric single-stranded oligodeoxynucleotides (ssODNs) was designed³² as custom synthetic genes (Megamer™ single-stranded Gene Fragments, IDT) introducing this point mutation. The final concentration of components was 0.61 pmol/μl of crRNA and tracrRNA, 30 ng/μl of Cas9 protein and 10 ng/μl of ssODN. Microinjections were performed at one-cell stage fertilized C57BL/6 mouse embryos³³. Pups were screened for the targeted mutation or insertion by PCR analysis and sequencing, and the selected founders were backcrossed to the C57BL/6 background. Detailed information about the microinjection reagents, summary of results, genotyping and tissue processing^{34,35} is given in eTable 3. We have described recently the generation of the mice harboring the Mib1^{V943F} mutation³⁶. The Notch1^{KO}³⁷ (denote as Notch1⁻) and Rbp^{KO}³⁸ (denote as Rbp⁻) mutant lines were used for genetic sensitization studies with Mib1 alleles.

RESULTS

A flow chart summarizing the study design and main results is displayed in eFigure 1.

Inherited nsBAV is a highly heterogeneous trait

Sixty-nine familial cases from 29 pedigrees from various ethnic backgrounds were included in our Discovery cohort, dedicated to new candidate gene identification (Table 1). Detailed description of the cohort demographics and clinical characteristics can be found in eTables 4, 5, 6. In most families, two members were affected (18 families, 62%); ten families had three affected members (34.5%), and one family had four (3.5%). BAV cases were predominantly male (46/69, 66%).

After conducting exome sequencing, we applied a step-by-step variant- and gene-filtering strategy detailed in the eFigure 2. We retained only genes harboring rare and predicted damaging protein-altering variants, with minor allele frequency (MAF) < 1%, and combined annotation dependent depletion (CADD) score > 20.

First, we excluded known or suspected syndromic and non-syndromic BAV genes (34 genes, including NOTCH1, ROBO4 and SMAD6; eTable 1). We then excluded genes that are not expressed during cardiac development or involved in CHD (eTable 2)^{32–34}. Then, we used in-silico gene prioritization tools (Endeavour and VarElect^{35,36}, eTable 7) to further prune the remaining genes. The top 10 ranked genes from each tool were chosen for further qualitative analysis based on population rarity, gene recurrence in families, known or potential impact of each variant. Ultimately, we identified 4 candidate genes (eTable 8).

MIB1 was identified as the leading candidate gene. In addition to the genetic finding (Figure 1), our selection was based on literature data: (i) *MIB1* role in NOTCH pathway; (ii) mouse models of *Mib1* inactivation have BAV^{18,37}; (iii) the identified variant was previously found in various CHD phenotypes including left ventricular non-compaction (LVNC)^{38–40}. The index case in our pedigree carrying the *MIB1* variant had a complex phenotype combining BAV with a myocardial crypt. Myocardial crypts have been reported in increased prevalence among carriers of cardiomyopathy mutations with otherwise normal phenotype³⁹.

Segregation analysis in this pedigree was complex as both parents of the index-case had BAV (eFigure 4). The index-case (III-1) and his affected mother (II-2) both harbored the *MIB1* V943F variant. The affected father (II-1) was deceased at the time of the study. The index-case's affected brother (III-3) did not harbor the variant.

Rare variant association study demonstrates association between nsBAV and rare *MIB* protein-altering variants

We assembled Replication Cohort I, including nsBAV sporadic cases (n=417) from European, Canadian, and Israeli backgrounds (Table 1, eTables 4, 9) and performed next Generation Sequencing or Sanger sequencing targeting *MIB1*. Using the same variant-filtering criteria as in Discovery Cohort, we identified seven additional rare, predicted deleterious, protein-altering *MIB1* variants in eight cases: three nonsense variants and four missense variants, of which one was found twice (eTable 10). Using Genomic Evolutionary Rate Profiling (GERP)⁴⁰, all the identified variants were predicted to be under a selective constraint with high positive scores (4.27–5.64). Segregation analysis could be done for the p.D380N missense variant, which was confirmed *de novo* (parents were healthy and did not harbor the mutation). In an *in-vitro* model, we previously showed that this variant induced

impaired interaction between MIB1 and its ligand JAG1, leading to defective NOTCH pathway activation⁴¹.

The cumulative frequency of rare and predicted deleterious variants in sporadic BAV cases was 1.3% compared to 0.2% for silent variants. In order to determine if sporadic BAV cases present enrichment for rare and predicted deleterious variants, variant association study was performed using TRAPD (Testing Rare Variants using Public Data) burden testing²⁷ against either all controls or ethnically-matched controls from gnomAD (Genome Aggregation Database). Enrichment analysis demonstrated association for all cases vs. all gnomAD controls ($p=0.03$) and the Israeli cases vs Ashkenazi Jewish and NFE controls ($p=0.03$). In contrast, silent variants were not enriched in our cases ($p=0.99$ and 0.95 respectively), eTable 11.

Common variant association study demonstrates association of *MIB1* locus with sporadic nsBAV

Replication Cohort II includes 452 BAV cases from the Mass General Brigham and Women's Hospital and 1834 controls from the Framingham Heart Study. Demographics and clinical data of BAV cases and controls is detailed in eTable 12. We considered common SNPs at the *MIB1* locus (Figure 2). Using a logistic regression model with a false discovery rate (FDR) correction for multiple comparisons, we detected an association for five noncoding SNPs (eTable 13), four of them within coding regions or introns (Figure 2a). Linkage disequilibrium (L.D.) assessment demonstrated that that all five SNPs were part of a delineated L.D. block, with D' (coefficient of L.D.) of 1, indicating that the SNPs are in high L.D. (Figure 2b). Two blocks were apparent from the L.D. analysis: one with SNPs 1,2, and 5 (rs7241299, rs79023008, rs11083391) and the other with SNPs 3 and 4 (rs1893384, rs3017041). Haplotype reconstruction allowed us to identify significant differences in haplotype frequencies between cases and controls, identifying two risk haplotypes and one protective haplotype ($p=0.017$, permutation test, eTable 14). This was further supported by permutation tests in a randomly shuffled cohort of cases and controls, which resulted in non-significant differences ($p=0.94$).

Mice harboring the identified *Mib1* missense variants develop BAV

We have introduced the *Mib1* p.K735R and the *Mib1* p.V943F missense variants³⁶ into the mouse genome using CRISPR-Cas9 genomic edition. Previously, only loss-of-function (LOF) studies have been performed in mice based on conditional *Mib1* inactivation in the heart^{19,42}. Genetic studies have suggested a dominant requirement of NOTCH signaling for aortic valve development, thus, NOTCH1 haploinsufficiency might predispose to cardiac outflow tract (OFT) abnormalities, including BAV⁴³. We found that mice heterozygous or homozygous for the *Mib1*^{K735R} variant ($n=41$ and $n=28$, respectively), or the *Mib1*^{V943F} variant ($n=50$ and $n=24$, respectively) displayed normal aortic valves (Figure 3A[a,d] and 3C; eFigure 5a,b,g show quantification of background controls). To test the sensitivity of the BAV phenotype to NOTCH gene dosage, we introduced *Rbp* or *Notch1* loss-of-function alleles^{37,38} into the *Mib1*^{V943F} and *Mib1*^{K735R} backgrounds. *Notch1*^{+/-} mice alone showed only a 9% BAV penetrance ($n=11$; P 0.05 by Chi-square; Figure 3B [e,f] and 3C; eTable 15). In contrast, the combination of missense *Mib1* mutant alleles with *Notch1* or *Rbp*

loss of function mutations revealed that *Mib1*^{K735R/+};*Notch1*^{+/-} double heterozygous mice developed BAV and associated valve defects with 44% penetrance (n=9; *P* 0.0001 by Chi-square; Figure 3B [g,h] and 3C; eTable 15). All *Mib1*^{K735R/+};*Notch1*^{+/-} mice show 100% VSD and 44% BAV, thus, all BAV is accompanied by VSD and there is no BAV without VSD in these double heterozygous mice. *Rbp*^{KO/+} mice do not show BAV (n=10; Figure 3B [i,j] and 3C). *Mib1*^{V943F/+};*Rbp*^{+/-} mice developed BAV with 6% frequency (n=20; Figure 3B [k,l] and 3C; eFigure 5e,f,g, show background controls). These results indicated that NOTCH signaling attenuation in a double heterozygous *Mib1* and *Notch1* mutant background leads to BAV and associated valve defects in mice.

DISCUSSION

Using both human genetics and functional assays, we identified *MIB1* as a novel gene for nsBAV in humans, with variants present in around 1.3% of BAV index cases in our cohorts. Our study implemented complementary human genetics that allow the identification of *MIB1*, and were further supported by functional approaches (eFigure 1): (1) The initial strategy was based on exome sequencing of a large BAV familial cohort (the discovery cohort), in which we identified a *MIB1* germline mutation (p.V943F) previously shown to cause LVNC¹⁵. (2) We used a first replication cohort of unrelated BAV cases, finding 7 additional rare variants with high predicted pathogenicity. (3) A rare variant association study with burden testing³⁸ showed enrichment in *MIB1* variants among the BAV cohort. (4) A common variant association study identified association identified risk haplotypes in an additional independent cohort of 452 sporadic BAV cases compared to 1849 ethnically matched controls. (5) We finally constructed functional models to confirm our findings, demonstrating that two genetically modified animal models, carrying the identified *Mib1* missense variants in double heterozygous combination with *Notch1* or *Rbp* deficiency cause BAV. These findings are entirely consistent with a mouse model of *Mib1* inactivation in which mice developed cardiac abnormalities, including BAV¹⁹.

Very low penetrance and oligogenic architecture are distinct frameworks to interpret inheritance that is neither purely Mendelian, nor polygenic.³⁻⁵ Our data demonstrate rare variants are associated with BAV, which evokes oligogenic inheritance involving *MIB1*. An oligogenic pattern has been demonstrated in mouse models with BAV^{3,9,44-47}. In the Slit/Robo pathway, the generation of double mutants' progenies is necessary to increase the penetrance of BAV⁴⁶. Genetic interaction between *NOS3* and *NOTCH1* was also demonstrated to increase BAV penetrance⁴⁸. A similar oligogenic dose effect has been suggested for NOTCH pathway mutations in outflow tract syndromes such as Alagille⁴⁹. Our mouse data show that the *Mib1*^{V943F} and *Mib1*^{K735R} variants do not cause BAV in heterozygous or homozygous condition, but rather when combined with *Notch1* or *Rbp* heterozygous loss-of-function mutations, indicating that the BAV phenotype is very sensitive to the combined insufficiency of NOTCH pathway genes (Figure 4), at least in mice. Our data are in full agreement with the results of a report showing that double heterozygous *Mib1*^{V943F/+};*Notch1*^{KO/+} mice show highly significant BAV.³⁶ Previously, our group and others demonstrated that deleterious *MIB1* variants associated with LVNC^{50,51}, atrial and ventricular septal defects, and patent ductus arteriosus (PDA)⁵². A complex phenotype of BAV and cardiac muscle malformation, as found in one case in the Discovery cohort,

has also been described^{53,54}. This phenotypic overlap between CHDs, including BAV and other left heart anomalies, is common: *GATA4*, *GATA5*, *GATA6*, *ROBO4*, and *TBX20* show patterns of inheritance in multiple heart defects (such as ASD, VSD, BAV, PDA, and mitral valve anomalies)^{55–62}. The phenotypic overlap between LVNC and valve anomalies was also observed in a mouse model of *Mib1* inactivation⁵⁰, as well as in a diversity of phenotypes induced by mutations in other BAV-related genes. Genetic sensitization experiments, in which mutations in various genes are combined to uncover a dose-sensitive mutant phenotype as found in our mice model, are typical of complex functional studies and are essential to identify the full implication of a given signaling pathway (i.e., NOTCH) in a developmental process or disease⁶³.

Ligand ubiquitination by MIB1 is essential for NOTCH pathway activation, although the downstream mechanisms are not fully understood. The key notion is that ubiquitination in the signaling cell of the ligand bound to the extracellular domain of NOTCH drives its endocytosis⁶⁴, eliciting in the receiving cell further NOTCH receptor processing and signaling activation (see Figure 5 for details).⁶⁵

Other promising candidates were identified during our analysis (eTable 7). One of the leading candidates is JAG1, another NOTCH1 pathway gene and a crucial substrate of MIB1 during cardiogenesis^{19,66}. Interestingly, both Dll4 and Jag1 are expressed in valve endocardium during early valve development (E9.5), whereas only Jag1 is expressed in aortic valve endocardium at later stages (E12.5 onwards)^{19,73}. These three ligands depend on MIB1 for their normal function as NOTCH signaling activating ligands⁶⁷ (Figure 3). In our discovery cohort, we identified rare and predicted deleterious missense variants in JAG1 in two pedigrees (eTable 7). Previous mice models of cardiac-specific JAG1 mutant resulted in BAV in 7 out of 15 mice¹⁹. Further studies and collaboration may facilitate the discovery of its role in BAV.

Several limitations to our study should be considered. By using a referral center-based cohort, there is a possibility for bias as the hospital clinic patients are characterized by a more severe form of disease compared to the general BAV population. The prevalence of *Mib1* mutation in the general BAV patient population may be lower than in our cohort. In both replication cohorts, we used public databases for controls, and these lacks validated phenotyping and may also include BAV cases, as in the general population. Finally, here we studied only one gene in the NOTCH pathway, but further investigation of the entire pathway is warranted, as several genes of the pathway are involved in valve development in mice⁷⁴.

In conclusion, our approach combining various human analyses and models and *in-vivo* functional studies reveals the involvement of *MIB1* in the development of nsBAV, highlighting the NOTCH pathway as a significant contributor to nsBAV inheritance and pathophysiology. This work also underscores the need for further investigation of NOTCH pathway components as additional candidate genes for nsBAV and as a future therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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KEY POINTS**Question:**

What is the genetic cause for BAV development?

Findings:

By integrating independent human genetic approaches, starting with familial segregation, followed by rare and common variants analyses, we identified *MIB1*, an essential regulator of NOTCH ligands signaling, as a new gene for BAV. These data were further supported by mouse models: Mice carrying the identified human *MIB1* mutations demonstrated BAV, associating MIB1 with BAV.

Meaning:

Our findings underscore the role of *MIB1* in the pathophysiology of BAV and identify the NOTCH pathway as a potential target in the management of BAV.

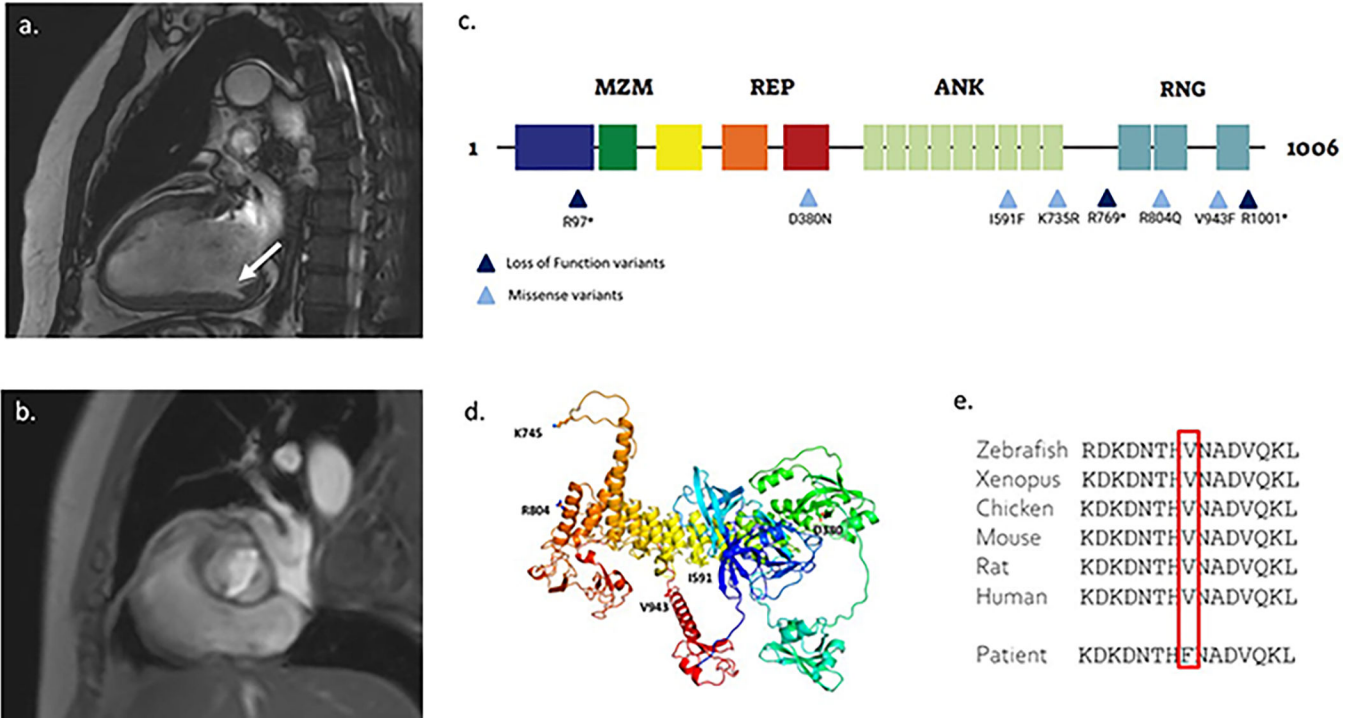


Figure 1: MIB1 rare variants in nsBAV.

(a) Cardiac MRI of a case with the *MIB1* p.V943F variant and a combined valve and muscle phenotype, sagittal section; showing a myocardial crypt in the left ventricle free wall. (b) sagittal section of the same study; showing bicuspid aortic valve; (c) A graphical representation of the MIB1 domain organization, and the location of the identified *MIB1* variants. MZM: Mib-Herc2 domain 1 + ZZ finger domain + Mib-Herc2 domain 2. REP: Mib Repeats 1 & 2. ANK: Ankyrin repeats 1–9. RNG: Ring domains 1–3; (d) An AlphaFold model of MIB1 with BAV mutations mapped. The model is rendered as a cartoon (colored on a rainbow scale from blue at the N-terminus to red at C-terminus) with sites of BAV mutations indicated and rendered in ball and stick format; (e) A phylogenetic analysis showing high inter-species conservation of the amino acid.

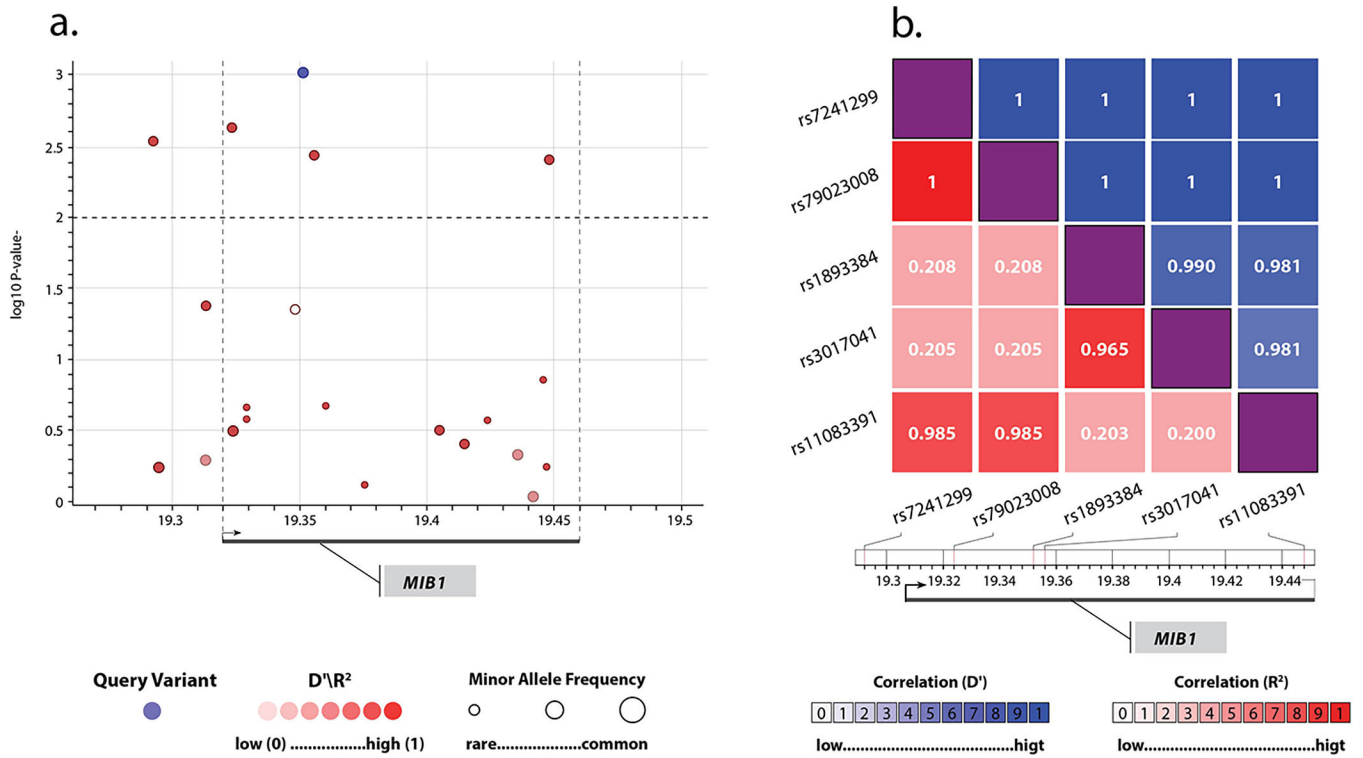


Figure 2: MIB1 common SNPs are associated with BAV

(a) SNPs in the *MIB1* locus. The x-axis represents the chromosomal coordinates; the y-axis represents the $-\log_{10}$ p-value (left) and the combined recombination rate from HapMap (right). The locus spans 166Kb from chr18:19,284,918 to chr18:19,450,912 in GRCh37/hg19. Each point represents a SNP and is colored on the basis of D' in relation to the most significant SNP, colored in blue. The dashed horizontal line marks the critical p-value of 0.01, which was set by the FDR multiple comparisons analysis⁶⁸; (b) A heatmap of pairwise linkage disequilibrium statistics for the statistically significant SNPs. The x and y dimensions represent the five significant SNPs identified, demonstrating L.D.' approaching 1 for all 5, implying high linkage disequilibrium (L.D.).

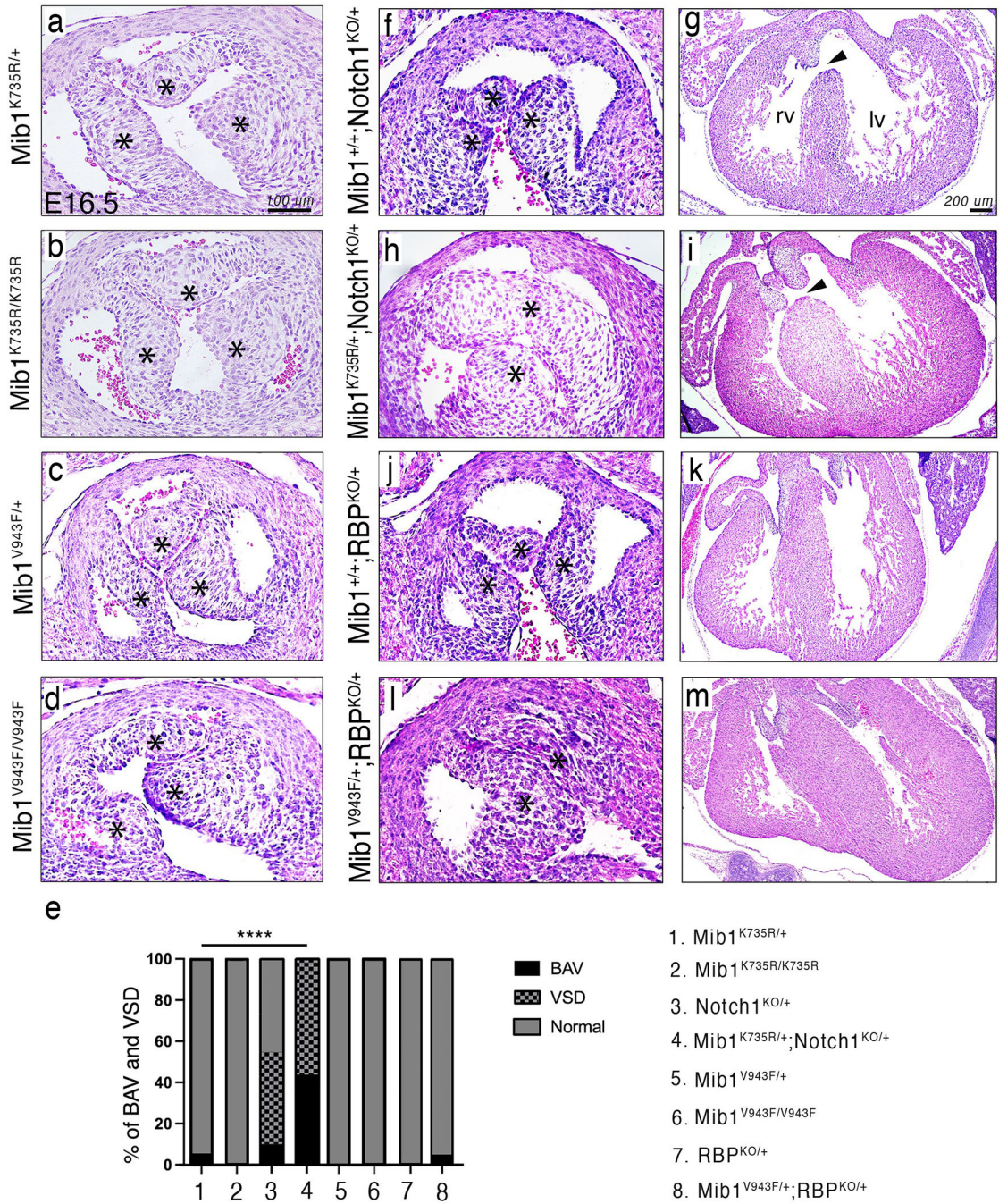


Figure 3: Heterozygous *Mib1*^{K735R} and *Mib1*^{V943F} variants cause BAV in a NOTCH-sensitized mouse genetic background.

Panel A. H&E staining of aortic valves from E16.5 *Mib1*^{K735R/+} (a), *Mib1*^{K735R/K735R} (b), *Mib1*^{V943F/+} (c) and *Mib1*^{V943F/V943F} mice (d) showing normal tricuspid morphology (asterisks). **Panel B.** Aortic valves and transverse heart sections of E16.5 *Mib1*^{+/+};*Notch1*^{KO/+} (e,f), *Mib1*^{K735R/+};*Notch1*^{+/-} (g,h), *Mib1*^{+/+};*Rbp*^{+/-} (i,j), and *Mib1*^{V943F/+};*Rbp*^{+/-} (k,l) embryos. Bicuspid aortic valves are observed in the double heterozygotes (g,k). Note also the defective membranous ventricular septum, which is

defective in $\text{Notch1}^{\text{KO/+}};\text{Mib1}^{+/+}$ (e,f) and $\text{Mib1}^{\text{K735R/+}};\text{Notch1}^{\text{KO/+}}$ hearts (g,h). **Panel C.** Percent of mice manifesting bicuspid aortic valve (BAV) and ventricular septal defect (VSD) phenotypes according to Mib1 variant and Notch1 and Rbp sensitization: $\text{Mib1}^{\text{K735R/+}}$ (n=41), $\text{Mib1}^{\text{K735R/K735R}}$ (n=28), $\text{Mib1}^{\text{V943F/+}}$ (n=50), $\text{Mib1}^{\text{V943F/V943F}}$ (n=24), $\text{Mib1}^{+/+}; \text{Notch1}^{+/-}$ (n=11), $\text{Mib1}^{\text{K735R/+}}; \text{Notch1}^{+/-}$ (n=9), $\text{Mib1}^{+/+}; \text{Rbp}^{+/-}$ (n=10), and $\text{Mib1}^{\text{V943F/+}};\text{Rbp}^{+/-}$ (n=20).

Abbreviations: lv, left ventricle; rv, right ventricle. Scale bars, 100 μm for aortic valve sections and 200 μm for transverse heart sections.

**** P 0.0001 by Chi-square.

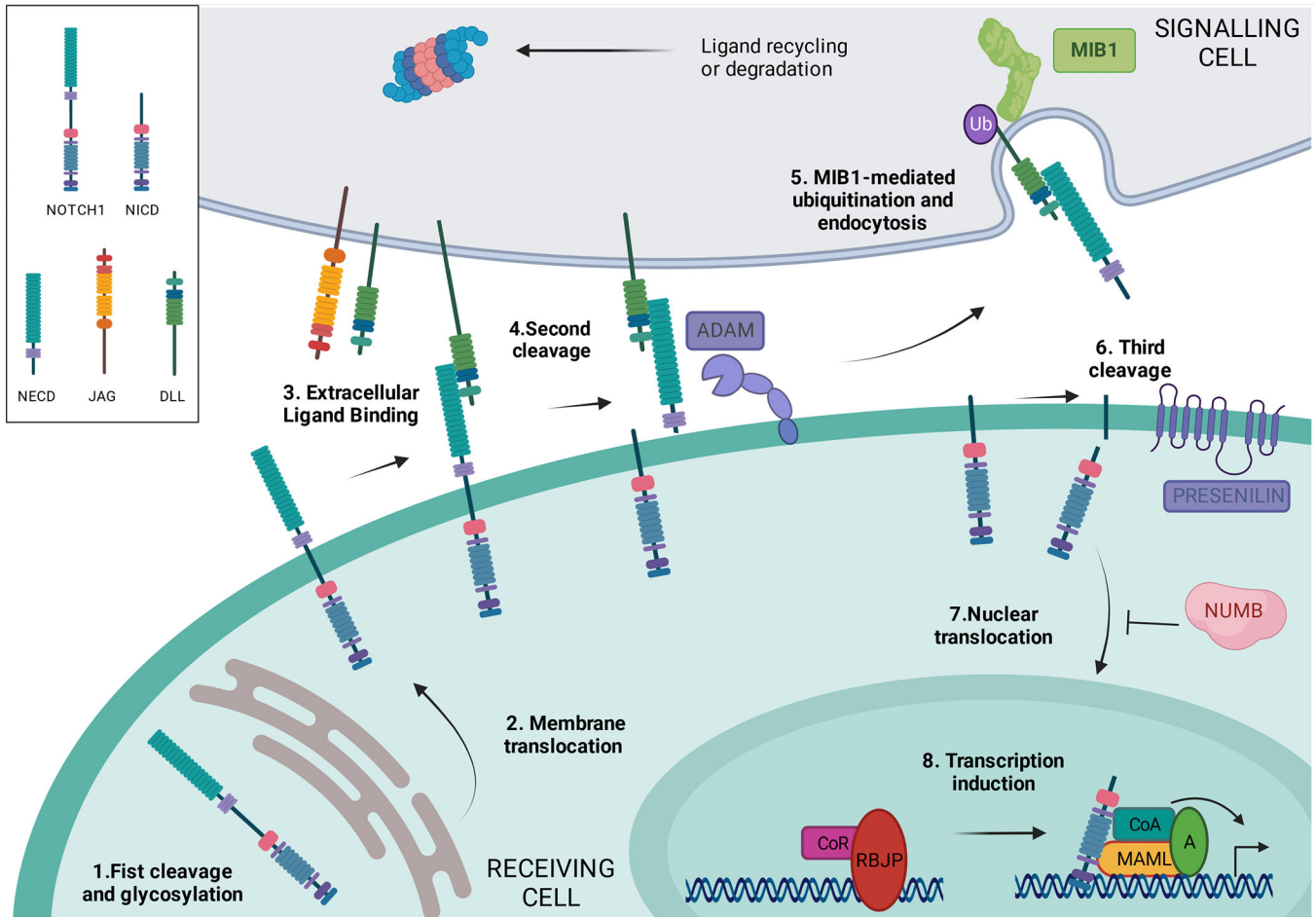


Figure 4: Schematic presentation of the main players in the NOTCH pathway

NOTCH is a local signaling mechanism in which cells are in a close position. Ligand-receptor interaction leads to a series of cleavage events that ultimately lead to the generation of the NOTCH intracellular domain (NICD), which is able to activate gene expression when bound to the appropriate factors. MIB1 and the DELTA and JAGGED ligands are expressed in the signaling cell (grey). The receiving cell (green) expresses receptors from the NOTCH family that are cleaved and glycosylated in the Golgi apparatus (Step 1). Once at the membrane (Step 2), NOTCH receptor binds to the ligands expressed in a neighboring cell (Step 3). After this interaction, the exposed S2 cleavage site in membrane-bound NOTCH is recognized by ADAM metalloproteinases and cleaved (Step 4), while MIB1 ubiquitinates the intracellular domain of the ligand in the signaling cell (Step 5), eliciting ligand-receptor complex endocytosis and degradation or recycling. This induces the γ -secretase cleavage (Step 6) that releases NICD, which is able to translocate to the nucleus (Step 7). Once in the nucleus, NICD binds to the repressor RBPJ/RBP/CSL/Su(H), releasing its co-repressors and recruiting coactivators as MAML, leading to the activation of a tissue-specific transcriptional program (Step 8). MIB1 is essential for NOTCH pathway activation. The figure was created using 'biorender' (<https://www.biorender.com>).

Table 1:

a. nsBAV discovery cohort and replication cohorts, N= 938 BAV cases.

Cohort	N	Center	City, Country	BAV cases, n (families)	Sex, % males (n)	Age, years, mean±SD
Discovery cohort	N = 69, Familial cases	APHP-Hôpital Européen Georges Pompidou	Paris, France	46 (18)	77.4% (127)	51 (±15.5)
		Hadassah Medical Center	Jerusalem, Israel	23 (11)	69% (40)	44.8 ±18.8
Replication cohort I	N= 417 Cases*	MIBAVA-Leducq	Europe*, Canada, United States	195 ¹	-	52.2 ±11.8
		APHP-Hôpital Européen Georges Pompidou	Paris, France	164	65.2% (30)	39 (± 16.5)
		Hadassah Medical Center	Jerusalem, Israel	58	73.9% (17)	40 ±22.7
		Genome Aggregation Database	Mixed	-	-	-
Replication cohort II	N = 62,874 Controls					
	N = 452 Cases	Mass General Brigham Hospital	Boston, MA, United States	452	74.3% (336)	54.4 ±11.8
	N = 1834 Controls	The database of Genotypes and Phenotypes	Framingham Heart Study, United States	-	54.4% (713)	-