A Human Hereditary Cardiomyopathy Shares a Genetic Substrate With Bicuspid Aortic Valve

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BACKGROUND: The complex genetics underlying human cardiac disease is evidenced by its heterogenous manifestation, multigenic basis, and sporadic occurrence. These features have hampered disease modeling and mechanistic understanding. Here, we show that 2 structural cardiac diseases, left ventricular noncompaction (LVNC) and bicuspid aortic valve, can be caused by a set of inherited heterozygous gene mutations affecting the NOTCH ligand regulator MIB1 (MINDBOMB1) and cosegregating genes.

METHODS: We used CRISPR-Cas9 gene editing to generate mice harboring a nonsense or a missense MIB1 mutation that are both found in LVNC families. We also generated mice separately carrying these MIB1 mutations plus 5 additional cosegregating variants in the ASXL3, APCDD1, TMX3, CEP192, and BCL7A genes identified in these LVNC families by whole exome sequencing. Histological, developmental, and functional analyses of these mouse models were carried out by echocardiography and cardiac magnetic resonance imaging, together with gene expression profiling by RNA sequencing of both selected engineered mouse models and human induced pluripotent stem cell–derived cardiomyocytes. Potential biochemical interactions were assayed in vitro by coimmunoprecipitation and Western blot.

RESULTS: Mice homozygous for the MIB1 nonsense mutation did not survive, and the mutation caused LVNC only in heteroallelic combination with a conditional allele inactivated in the myocardium. The heterozygous MIB1 missense allele leads to bicuspid aortic valve in a NOTCH-sensitized genetic background. These data suggest that development of LVNC is influenced by genetic modifiers present in affected families, whereas valve defects are highly sensitive to NOTCH haploinsufficiency. Whole exome sequencing of LVNC families revealed single-nucleotide gene variants of ASXL3, APCDD1, TMX3, CEP192, and BCL7A cosegregating with the MIB1 mutations and LVNC. In experiments with mice harboring the orthologous variants on the corresponding Mib1 backgrounds, triple heterozygous Mib1 Apcdd1 Asxl3 mice showed LVNC, whereas quadruple heterozygous Mib1 Cep192 Tmx3 Bcl7a mice developed bicuspid aortic valve and other valve-associated defects. Biochemical analysis suggested interactions between CEP192, BCL7A, and NOTCH. Gene expression profiling of mutant mouse hearts and human induced pluripotent stem cell–derived cardiomyocytes revealed increased cardiomyocyte proliferation and defective morphological and metabolic maturation.

CONCLUSIONS: These findings reveal a shared genetic substrate underlying LVNC and bicuspid aortic valve in which MIB1-NOTCH variants play a crucial role in heterozygous combination with cosegregating genetic modifiers.

Key Words: bicuspid aortic valve ■ cardiomyopathy ■ genetic modifiers ■ left ventricular non compaction ■ MIB1 ■ NOTCH ■ valves
The genetic basis of cardiovascular disease is poorly understood, and it is only with the advent of new high-throughput DNA sequencing techniques that a picture has begun to emerge of its complexity and the difficulty of establishing direct genotype-phenotype correlations. Cardiomyopathies were initially thought to be monogenic disorders; however, phenotypic expression and penetrance have been found to be affected by variable presentation within a family with the same mutation, the influence of multiple genetic variants and their epistatic relationships, and epigenetic and environmental factors.

Left ventricular noncompaction (LVNC) is the third most common cardiomyopathy (prevalence, 0.05%4) and is characterized by the presence of excessive trabeculae with deep recesses in the left ventricle.5,6 Trabeculae are endocardial cell–covered cardiomyocyte bundles in the vertebrate ventricle that facilitate oxygen and nutrient exchange.7 As development proceeds, the outer compact myocardium layer expands by proliferation, contributing to the integration of trabeculae in the ventricular wall through the poorly understood process of compaction,8,9 which coincides with the invasion of the myocardium by the coronary vasculature.8,10 Familial LVNC has been attributed to defective ventricular maturation and compaction in utero5,11 and is characterized by a dilated left ventricle and associated systolic dysfunction.5 In addition, zones of fibrotic tissue related to disease severity might be dispersed on the endocardial surfaces.12 One of the echocardiographic diagnosis criteria for LVNC is a ratio of noncompacted to compacted myocardium of ≥2 at end-systole.13,14 Clinical presentation of LVNC ranges from asymptomatic cases to severe heart failure requiring heart transplantation.15–17

LVNC is genetically heterogeneous, with a predominantly autosomal-dominant inheritance pattern,18 and has been linked to sarcomere gene mutations, particularly in MYH7.19,20 Other mutations implicated in LVNC affect genes encoding the scaffold protein α-dystrobrevin and

**Clinical Perspective**

**What Is New?**
- Heterozygous mutations in the NOTCH regulator MIB1 lead to human left ventricular noncompaction (LVNC), but cause LVNC or bicuspid aortic valve (BAV) in a NOTCH-sensitized mouse genetic background.
- Whole exome sequencing of LVNC families has identified heterozygous missense mutations in 5 genes, cosegregating with MIB1 and LVNC. The corresponding mouse models show LVNC or BAV in a NOTCH-sensitized genetic background.
- Gene profiling shows increased cardiomyocyte proliferation and defective morphological and metabolic maturation in mouse hearts and human induced pluripotent stem cell–derived cardiomyocytes. Biochemistry suggests a direct interaction between NOTCH and some of the identified gene products.
- These data support a shared genetic basis for LVNC and BAV with MIB1-NOTCH playing a crucial role.

**What Are the Clinical Implications?**
- Novel insights into the genetic basis and oligogenic nature of LVNC.
- Identification of heterozygous mutations leading to LVNC (MIB1 ASXL3 APCDD1) or BAV (MIB1 CEP192 TMX3; BCL7A) may allow expansion of the genetic testing panel repertoire for better diagnosis and stratification of patients with LVNC and BAV.
- Whether patients with LVNC have a higher prevalence of BAV remains uncertain, and further evaluation is required.

**Nonstandard Abbreviations and Acronyms**

| Acronym | Description |
|---------|-------------|
| APCDD1  | APC down-regulated 1 |
| ASXL3   | ASXL transcriptional regulator 3 |
| BAV     | bicuspid aortic valve |
| BCL7A   | BAF chromatin remodeling complex subunit BCL7A |
| CEP192  | centrosomal protein 192 |
| co-IP   | coimmunoprecipitation |
| CT      | C-terminal fragment |
| E       | embryonic day |
| EMT     | EPITHELIAL_TO_MESENCHYMAL_TRANSITION |
| GATK    | Genome Analysis Toolkit |
| GERP    | genomic evolutionary rate profiling |
| hiPSC   | human induced pluripotent stem cell |
| hiPSC-CM| human induced pluripotent stem cell–derived cardiomyocyte |
| iPSC    | induced pluripotent stem cell |
| LVNC    | left ventricular noncompaction |
| MIB1    | MIB E3 ubiquitin protein ligase 1 |
| N1ICD   | NOTCH1 intracellular domain |
| NOTCH1  | NOTCH receptor 1 |
| PCR     | polymerase chain reaction |
| RNA-seq | RNA sequencing |
| RBPJ    | recombining binding protein suppressor of hairless |
| SNP     | single nucleotide polymorphism |
| ssODN   | single-stranded donor oligonucleotides |
| TMX3    | thioredoxin related transmembrane protein 3 |
| Tnnt2   | cardiac troponin T |
| WB      | Western blot |
| WES     | whole exome sequencing |
the nuclear protein lamin A/C.\textsuperscript{21,22} The proposed developmental origin of LVNC has prompted suggestions of the involvement of genetic alterations of the signals and transcription factors that regulate cardiovascular development.\textsuperscript{23–25} A key mediator of cell fate specification and tissue patterning in metazoans is the highly conserved signaling pathway NOTCH,\textsuperscript{26,27} and NOTCH signaling disruption in humans leads to developmental abnormalities affecting the heart and vessels.\textsuperscript{28–31} Studies in targeted mutant mice have shown that NOTCH is crucial for the endocardium-to-myocardium signaling processes that govern cardiac valve and ventricle development and have shed light on the disease mechanisms associated with NOTCH dysfunction (see reviews\textsuperscript{32,33}). We showed that LVNC in mice and humans can be caused by mutations in the ubiquitin ligase MIB1 (MIB E3 ubiquitin protein ligase 1),\textsuperscript{34} which is required for NOTCH ligand endocytosis and signaling activation.\textsuperscript{35}

Here, we used CRISPR-Cas9 gene editing and whole exome sequencing (WES) to study LVNC inheritance in 2 large families carrying \textit{MIB1}-inactivating mutations.\textsuperscript{34} The \textit{Mib1}^R530X nonsense mutation causes LVNC in mice in a heteroallelic combination with a conditional \textit{Mib1}^fox allele, whereas the \textit{Mib1}^V943F missense allele leads to bicuspid aortic valve (BAV) in a NOTCH-sensitized genetic background. We identified single-nucleotide variants in the \textit{MIB1} mutations and LVNC in our pedigrees. Triple heterozygous \textit{Mib1}^R530X/+, \textit{Mib1}^V943F/+, and \textit{R26MIB1WT}/+ (see “Generation of New Mouse Lines”) mutant mice develop features of LVNC, whereas quadruple heterozygous \textit{Mib1}^R530X/\textit{Cep192}^V943F/\textit{Bcl7A}^flox/fox mice develop BAV and valve-associated defects. Coimmunoprecipitation (co-IP) analysis confirmed interaction among \textit{CEP192} (centrosomal protein 192), and \textit{BCL7A} (BAF chromatin remodelling complex subunit BCL7A) genes that cosegregate with the \textit{Mib1} mutations and LVNC in our pedigrees. Triple heterozygous \textit{Mib1}^R530X/\textit{Apccdd1}^Asxl3/fox mutant mice develop features of LVNC, whereas quadruple heterozygous \textit{Mib1}^R530X/\textit{Cep192}^V943F/\textit{Bcl7A}^flox/fox mice develop BAV and valve-associated defects. Coimmunoprecipitation (co-IP) analysis confirmed interaction among \textit{CEP192} (centrosomal protein 192), \textit{BCL7A} (BAF chromatin remodelling complex subunit BCL7A), and NOTCH protein products. Genes profiling of mouse hearts and human induced pluripotent stem cell (hiPSC)–derived cardiomyocytes (hiPSC-CM) revealed a common defect in metabolism and the CRISPOR-TEFOR online tool.\textsuperscript{42} The \textit{Cas9} mRNA and single guide RNAs (sgRNAs) templates were amplified by polymerase chain reaction (PCR) with the addition of the SP6 and T7 promoters, respectively, and assembled in the pX330 plasmid.\textsuperscript{43} \textit{Cas9} mRNA was transcribed using the \textit{mMESSAGE mMACHINE SP6 Transcription Kit} (Invitrogen, AM1340), sgRNAs were transcribed with the Megashortscript T7 transcription Kit (Invitrogen, AM1354), and all species were purified on NucAway Spin Columns (Ambion, AM10070). The final concentration of components was 30 pmol/μL sgRNA, 30 ng/μL \textit{Cas9} mRNA, and 30 or 10 ng/μL ssODN, as detailed in Table S1, sheets 1 and 2. The sgRNA and ssODN sequences used are listed in Table S1, sheet 1. Reagents were microinjected into 1-cell fertilized C57BL/6 mouse embryos.\textsuperscript{44} 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.

**Methods**

**Data Availability**

The authors declare that all data that support the findings of this study are available within the article and its Supplemental Material. The data, analytical methods, and study materials will be available to other researchers for purposes of reproducing the results or replicating the procedure. The RNA sequencing (RNA-seq) data are deposited in the NCBI GEO database under accession number GSE185395. The UK Biobank data for this study are available within the article and its Supplemental Material. The data, analytical methods, and study materials will be available to other researchers for purposes of reproducing the results or replicating the procedure. The RNA sequencing (RNA-seq) data are deposited in the NCBI GEO database under accession number GSE185395. The UK Biobank data for this study are available within the article and its Supplemental Material. The data, analytical methods, and study materials will be available to other researchers for purposes of reproducing the results or replicating the procedure. The RNA sequencing (RNA-seq) data are deposited in the NCBI GEO database under accession number GSE185395. The UK Biobank data for this study are available within the article and its Supplemental Material. The data, analytical methods, and study materials will be available to other researchers for purposes of reproducing the results or replicating the procedure. The RNA sequencing (RNA-seq) data are deposited in the NCBI GEO database under accession number GSE185395. The UK Biobank data for this study are available within the article and its Supplemental Material.

**Ethics and DNA collection**

Clinical evaluations and genetic studies were performed in accordance with the principles of the Helsinki Declaration, and after informed consent of participating subjects for inclusion according to the protocol approved by the Ethics Committee of Clinical Research from the Hospital Universitario Virgen de la Arrixaca (218/C/2020). All patients underwent a clinical evaluation, including an ECG and 2-dimensional and Doppler echocardiography. A pedigree was drawn for each patient, and first-degree relatives were screened with the same protocol. Blood samples were taken for genetic analysis, and all patients and their relatives gave written informed consent. Genomic DNA was obtained from 1-mL blood samples extracted in EDTA using the DNAEasy Blood & Tissue Kit (Qiagen, 69506).

**Mice**

Established mouse strains used in this study were \textit{Ttnnt}^\textit{2Tn} (\textit{Ttnnt}: cardiac troponin T),\textsuperscript{36} \textit{Myh6}^\textit{Cre},\textsuperscript{37} \textit{Nkx2.5}^\textit{Cre},\textsuperscript{38} \textit{Mib1}^\textit{fox},\textsuperscript{39} and \textit{Notch1}^\textit{fox}.\textsuperscript{40} The following new mouse lines were generated as part of this study: \textit{Mib1}R530X/+, \textit{Mib1}V943F/+, \textit{R26Mib1}WT/+ (see “Generation of New Mouse Lines”), Genotyping details will be provided on request.

Animal studies were approved by the Centro Nacional de Investigaciones Cardiovasculares Animal Experimentation Ethics Committee and by the Community of Madrid (Ref. PROEX 155/7/20). All animal procedures conformed to EU Directive 2010/63/EU and Recommendation 2007/526/EC on the protection of animals used for experimental and other scientific purposes, enacted in Spanish law under Real Decreto 1201/2005.

**Generation of New Mouse Lines**

To generate the \textit{Mib1}^R530X/+ and \textit{Mib1}^V943F/+ lines, complementary single-stranded oligodeoxynucleotides (ssODNs) were designed as custom synthetic genes (Megamer single-stranded Gene Fragments, IDT) including these point mutations. sgRNAs sequences were selected using Breaking-Cas\textsuperscript{41} and the CRISPOR-TEFOR online tool.\textsuperscript{42} The \textit{Cas9} mRNA and single guide RNAs (sgRNAs) templates were amplified by polymerase chain reaction (PCR) with the addition of the SP6 and T7 promoters, respectively, and assembled in the pX330 plasmid.\textsuperscript{43} \textit{Cas9} mRNA was transcribed using the \textit{mMESSAGE mMACHINE SP6 Transcription Kit} (Invitrogen, AM1340), sgRNAs were transcribed with the Megashortscript T7 transcription Kit (Invitrogen, AM1354), and all species were purified on NucAway Spin Columns (Ambion, AM10070). The final concentration of components was 30 pmol/μL sgRNA, 30 ng/μL \textit{Cas9} mRNA, and 30 or 10 ng/μL ssODN, as detailed in Table S1, sheets 1 and 2. The sgRNA and ssODN sequences used are listed in Table S1, sheet 1. Reagents were microinjected into 1-cell fertilized C57BL/6 mouse embryos.\textsuperscript{44} 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.

Triple mutant mice were obtained by microinjecting the editing reagents for each combination into zygotes obtained from crosses between \textit{Mib1} mutant males (\textit{Mib1}^V943F/\textit{V943F} or \textit{Mib1}^R530X/+).
and C57Bl/6CrI females, with synthetic crisprRNA (crRNA) and trans-activating crRNA (tracrRNA) incubated with Cas9 protein and ssODN at the concentrations indicated in Table S1, sheet 3. Complementary and asymmetric ssODNs (Table S1, sheet 1) were designed according to published guidelines as custom synthetic genes (Megamer single-stranded Gene Fragments, IDT). Founders were identified by PCR and confirmed by Sanger sequencing. Single and multiple mutants were obtained for all microinjections except for the second Cep192+Tmx3 experiment, in which no pup was born. In a second experiment with these reagents, we obtained 1 triple heterozygote founder (Mib1V943F/+ Cep192215250M/+ Tmx3-204F19114/+ ) out of 4 survivors (25%). In the Bcl7a microinjection experiment, with only 1 crRNA and 1 ssODN carrying the intron variants, we obtained 13 founders with both intronic mutations out of 21 pups (61.9%).

For the polymorphisms found in the R530X family, 2 triple heterozygotes (Mib1R530X/+ Axl3M1361V/+ Apcc1V150I/+ ) were obtained out of 16 pups (12.5%) (Table S1, sheet 3). Founders were crossed with C57Bl/6 mice to dilute possible off-target effects of CRISPR-Cas9 editing. The crosses also allowed us to determine whether the mutations were introduced in cis or trans heterozygosity. Thus, the generation of single mutants was a rare event because most of the animals were either triple heterozygotes or WT, indicating cis heterozygosity (Table S2, sheet 4). Generation of the cis triple heterozygote was further confirmed by comparing the number of mice with cosegregating alleles versus the number with mixed markers, which showed that all gene pairs were linked. With the set of mutations associated with the MIB1V943F family, we obtained similar results, although cosegregation was less complete (Table S2, sheet 4). When comparing pairs of genes, no significant difference between mutant and WT alleles was found. In addition, despite the distance between Tmx3 and Mib1 (almost 80 Mb), the 3 genes cosegregated. Primers for genotyping and expression analyses are provided in Table S3.

Transgenic R26MIB1V943F/+ and R26MIB1V943F/+ mice were generated by homologous recombination in mouse embryonic stem cells. The HA-MIB1WT-IRES-eGFP and HA-MIB1V943F-IRESeGFP constructs were obtained from pCDNA3.1-HA-MIB1WT or V943F-IRESeGFP cloned into pBGI (Addgene, plasmid 15037) and loxP-flanked. PKG-NeoSTOP-MIB1WT-eGFP or MIB1V943F-eGFP expression cassettes were cloned into a modified version of the pROSA26-1 plasmid (Figure S1A). Gene targeting of these 2 constructs was performed in G4 mouse embryonic stem cells and confirmed by Southern blotting with external 5’ and 3’ hybridization probes (Figure S1B). Mice were generated by injecting targeted cells into B6CRL blastocytes to generate chimeras that were then analyzed for germline transmission. The selected animals were backcrossed to the C57BL/6 background.

LVNC Clinical Phenotype Evaluation

We included patients with an echocardiographic diagnosis of LVNC, defined by the presence of at least 3 prominent trabeculations in the left ventricle and a ratio of noncompacted to compacted segment >2.0 at end-diastole.46,47

Exome Sequencing and Data Analysis

DNA was sequenced on Illumina HiSeq2500 or Illumina HiSeq3000 platforms. Variant discovery was performed using Genome Analysis Toolkit (GATK) Best Practices Workflows for germline short variants version 3.7 and bundle reference files for genome version b37/GRCh37. For data pre-processing, quality trimming and adaptor removal were performed using Trimmomatic 0.38 in paired mode (ILLUMINACLIP:TruSeq3-PE.fa:2:30:10). Low quality bases from the beginning and end of the read pair were deleted if the score was <3 (LEADING:3 TRAILING:3) or if the average quality was <15 using a sliding window of size 4 for the whole read (SLIDINGWINDOW:4:15). The read pair was discarded if the posttrimming length was <36 bp (MINLEN:36). Reads were mapped to GRCh37/b37 human genome using BWA mem version 0.7.10-r789 with shorter split hits set as secondary (-M).46 Reads originating from the same DNA fragment (duplicates) during library construction were identified with Picard v1.97 (MarkDuplicates). For reads obtained from HiSeq 2500 runs (unpatterned flowcell), the maximum offset between 2 duplicated clusters parameter (OPTICAL_DUPLICATE_PIXEL_DISTANCE) was set to 100, and reads obtained from HiSeq3000 (patterned flowcell) were set to 2500. The base quality score of bases was recalibrated to better account for systematic errors using the BaseRecalibrator tool in GATK 3.7. The following databases of known polymorphic sites (-knownSites) were used: 1000G_phase1.snps.high_confidence.b37.vcf.gz; Mills_and_1000G_gold_standard.indels.b37.vcf.gz and dbnsnp_137.b37.vcf.gz. For each sample, intermediate gVCF files with single nucleotide polymorphisms (SNPs) and Indels calls were created for each sample independently using GATK HaplotypeCaller, restricting calling regions to the enrichment targeted regions (L). "SureSelect Human All EXon V6" target intervals were downloaded from Agilent (https://www.agilent.com).

Joint genotyping from gVCF files was performed using the GenotypeGVCFs tool in GATK 3.7. Variants were annotated using the VEP (Variant Effect Predictor) in ensembl-tools version 84 with the plugin modules LoFtool and CADD (version 1.3) in offline cache version 90.51 For each putative variant, a variant quality score log-odds (VOSLOD) score was calculated using the VariantRecalibrator and ApplyRecalibration tools in GATK 3.7. SNPs and Indels were treated separately. For SNPs (~mode SNP), QualByDepth (QD), RMSMappingQuality (MQ), MappingQualityRankSumTest (MORankSum), ReadPosRankSumTest (ReadPosRankSum), FisherStrand (FS), and StrandOddsRatio (SOR) covariates were annotated. In addition, hapmap_3.3.b37.vcf.gz was set as both a truth and a training set (prior 15.0). 1000G_omni2.5.b37.vcf.gz and 1000G_phase1.snps.high_confidence.b37.vcf.gz were set as training sets (prior 12.0 and 10.0, respectively), dbnsnp_137.b37.vcf.gz was set as a known set (prior 2.0). For Indels (~mode INDEL), the max number of Gaussians for the positive model (~maxGaussians) was set to 4. QualByDepth (QD), FisherStrand (FS), StrandOddsRatio (SOR), MappingQualityRankSumTest (MORankSum), and ReadPosRankSumTest (ReadPosRankSum) covariates were annotated.

Mills_and_1000G_gold_standard.indels.b37.vcf.gz was set both as a truth and a training set (prior 12), and dbnsnp_137.b37.vcf.gz was set as a known set (prior 2.0). In both cases, the truth sensitivity level was set to 75 (~ts_filter_level) to achieve a Ti/Tv ratio close to the expected value for WES (2.8). Because family pedigree data were insufficiently sensitive to allow dismissal of Mendelian inconsistencies as presumable
false positives, we did not use VQSLOD for filtering (although we did use it as for accuracy guidance). The total number of variants identified and proportion of them present in the Single Nucleotide Polymorphism Database (dbSNP) as a readout of homogeneity and quality are shown in Table S4.

Cosegregating Variants Filtering

Our workflow is summarized in Figure S2. After obtaining the raw reads and filtering out the low-quality sequences, we mapped the reads to the reference human genome version GRCh37. These mapped reads were handled according to GATK best practices. For the MIB1R530X family, of 134 variants present in an autosomal dominant pattern cosegregating with LVNC, we had data for all the family members of 106 SNPs. These 106 variants affected 21 unique genomic positions, and filtering out low impact and untranslated region (UTR)-affecting polymorphisms, 15 candidates were further examined. In the MIB1V943F family, we found 1963 variants inherited in an autosomal dominant fashion, of which 565 were sequenced in all samples. Of the 77 unique positions affected, 32 were not variants of low impact or affecting UTRs. Candidate variants were selected through 3 approaches. First, we performed filtering on the basis of variants. We used databases such as ClinVar to determine if the identified candidate variants were already described in congenital heart disease, but found none already related to LVNC except MIB1R530X and MIB1V943F. We also examined the prevalence of the polymorphisms and their presence in the homologous condition in gnomAD, although they were not excluding criteria, given how rare MIB1 mutations are and our hypothesis of the cosegregation of additional mutations. The highest frequency of the selected variants was 0.21 for TMX3F191X (Figure S3C). Another criterion was their predicted functional effect, based on the nature of the mutations, how deleterious were they predicted to be, using CADD (ex past cutoff value: 0.771), PolyPhen (0), and genomic evolutionary rate profiling (GERP) (-3.92), or the potentially affected domain (based on PFAM, PROSITE, etc). These criteria did not allow us to exclude any variant, but were useful to prioritize them for the next 2 approaches. Second, we used a gene-centered strategy. We searched several gene databases for information about the relationship of the variants to congenital heart disease, but found none already related to LVNC except MIB1R530X and MIB1V943F. To assess the consequences of the MIB1R530X and MIB1V943F mutations previously identified in LVNC families, we generated mice harboring the orthologous nonsense and missense variants by CRISPR-Cas9 gene editing (Figure S1A and S1B, Table S1, sheets 1 and 2). Homozygous Mib1R530X mutants died at embryonic day (E) 11 (Table S2, sheet 1) with severe growth retardation and defective heart looping (Figure 1A through 1A’’), reduced NOTCH1 activity (Figure 1B through 1C’’), and impaired trabeculation and ventricular patterning (Figure S4C through S4J). Quantitative PCR analysis showed a severely reduced Mib1 transcription in E10.5 homozygous Mib1R530X mutants (Figure S4K). Heterozygous Mib1R530X/+ mice were born at the expected Mendelian ratio and did not show LVNC (Table S2, sheet 1, Figure S5A and S5B).

During compaction, MIB1 regulates myocardial NOTCH-ligand signaling to the endocardium. To specifically abrogate MIB1 in the myocardium, we generated Mib1R530X/+;Tnnt2Cre mice. These mice developed severe LVNC by E16.5 (Figure 1D through 1E’’; Figure S5C and S5D), with a 45% thinning of the compact myocardium, deep endocardial recesses, a reduced compact-to-trabecular myocardium ratio (Figure 1E’’), and attenuated NOTCH1 activity (Figure 1C’’ and 1F through 1G’’). Only 5% of Mib1R530X/+/;Tnnt2Cre mice reached adulthood (Table S2, sheet 2), suggesting that the combination of myocardial MIB1 abrogation with global MIB1 haploinsufficiency compromises viability (P<0.0001, 2-tailed binomial test). Indeed, Western blot (WB) analysis revealed a drastically reduced MIB1 expression in Mib1R530X/+/;Tnnt2Cre hearts (Figure S5E).

Ultrasound analysis of surviving adult mice revealed the

TMX3 Variants Analysis

The identified TMX3 mutation (c.637+16_367+17del) affects intron 9 of the main isoform (TMX3-201), but also generates a nonsense mutation at Phe191 (TTT) in isoform TMX3-202, located in exon 8. This is equivalent to exon 8 and part of intron 9 in TMX3-201, because alternative splicing discards exon 6. The TMX3-201 nonsense mutation deletes Ts 2 and 3 from the codon, creating a stop codon through combination with the As in the subsequent Asp192 codon (AAC). The remaining protein coding isoform, TMX3-204, consists of the first 8 exons of the main isoform with an alternative 3’ end fragment including part of TMX3-201 intron 8. This third isoform encodes a VIFKI sequence in a fragment of intron 8, as well as including a Phe191 (TTT) followed by Lys192 (AAA). The c.579+8_c.579+9_del deletion, analogous to that generating TMX3-202F191X, would therefore cause a similar nonsense mutation in TMX3204 (Figure 3E). Although the DNA sequence is conserved at that amino acid position, the mouse sequence contains a previous stop codon (Q189K). Mouse homologs of the TMX3-202 and TMX3-204 isoforms have not been annotated. To determine if the mouse heart expresses a Tmx3-204 isoform, we designed 2 primer pairs for reverse transcription-PCR. One forward primer (long) binds to exon 6; the other (short) is homologous to a sequence in exon 7. The shared reverse primer binds to the putative 3’UTR, located in intron 8 of the main isoform, and allowed us to amplify Tmx3-204 alone. Reverse transcription-PCR with RNA from whole E14.5 hearts showed transcription of this mRNA species in the mouse (Figure S3F).

Additional methods are detailed in the Supplemental Material.

RESULTS

Mib1R530X and Mib1V943F Mutations Cause LVNC and BAV

To assess the consequences of the MIB1R530X and MIB1V943F mutations previously identified in LVNC families, we generated mice harboring the orthologous nonsense and missense variants by CRISPR-Cas9 gene editing (Figure S1A and S1B, Table S1, sheets 1 and 2). Homozygous Mib1R530X mutants died at embryonic day (E) 11 (Table S2, sheet 1) with severe growth retardation and defective heart looping (Figure 1A through 1A’’'), re-
Figure 1. Mib1<sup>R530X</sup> and Mib1<sup>V943F</sup> mutations cause LVNC or BAV in a sensitized NOTCH1-deficient genetic background. 
A. Whole mounts of E10.5 control (left) and Mib1<sup>R530X/R530X</sup> mouse embryos (right). High-magnification views of the boxed areas show a lateral aspect of a control looped heart (A') and a dysmorphic unlooped mutant heart (A''). B through C', E10.5 control and Mib1<sup>R530X/R530X</sup> hearts immunostained for N1ICD (red), Tnnt2 (green), and IB4 (isoelectin B4; white). Nuclei are counterstained with DAPI. Arrows point to positive nuclei. Scale bars, 100 μm (B and C) and 50 μm (B' and C'). C''. Left. Quantification of N1ICD staining. Data are mean±SD (n=3 sections from 5 control and n=3 sections from 4 mutant embryos, ****P<0.0001 by Student t-test). D and E, E16.5 control (D through D'') and Mib1<sup>R530X</sup>/<sup>Flox</sup>-Tnnt2<sup>Cre</sup> (E through E'') transverse heart sections immunostained for Tnnt2 (red), IB4 (white), and emcn (endomucin, green). (Continued)
presence of trabeculations and a significant reduction in ejection fraction and fractional shortening, impairing cardiac function (Figure 1H, 1I, and 1K'). In addition, color Doppler profiling detected regurgitation through the aortic valve (Figure 1K), indicating heart failure (Figure 1H). We generated 2 transgenic lines bearing a Rosa26-floxNeoSTOPflox-MIB1WT-EGFP or a Rosa26-floxNeoSTOPflox-MIB1V943F-EGFP expression cassette, resulting in conditional expression of a wild-type or a mutant (MIB1V943F) MIB1 (Figure S1A and S1B). To monitor transgene expression, we crossed these lines with mice harboring the Nkx2.5Cre driver line, which is active in cardiac progenitors from E7.5CRE-mediated removal of the floxed NeoSTOP sequences resulted in Rosa26-driven MIB1V943F-EGFP expression in the E9.0 heart (Figure S1C). To examine the effect of MIB1V943F expression on compaction, we used the MYH6Cre driver, expressed in the developing chamber myocardium from E10.5 onwards. The hearts of mice expressing wild-type MIB1 in the myocardium were normal at E15.5, whereas mice expressing MIB1V943F had thinner compact myocardium and persistent trabeculae compatible with LVNC (Figure S1D through S1F). These results indicate that the MIB1R530X and MIB1V943F mutations impair both chamber and valve development, leading to LVNC and BAV in specific genetic configurations.

**MIB1 Is Required for Myocardial Differentiation and Metabolic Maturation**

To gain mechanistic insight, we performed RNA-seq on E15.5 Mib1R530X/flox;Tnnt2Cre and control ventricles followed by gene set enrichment analysis against “HALLMARK” gene sets (Figure 2A, Table S6, sheets 1 through 6). "EPITHELIAL_TO_MESENCHYMAL_TRANSITION" (EMT) was the highest positively enriched gene set in Mib1R530X/flox;Tnnt2Cre mutants (Figure 2A and 2B, Table S5, sheet 1). We generated 2 transgenic lines bearing a Rosa26-floxNeoSTOPflox-MIB1WT-EGFP or a Rosa26-floxNeoSTOPflox-MIB1V943F-EGFP expression cassette, resulting in conditional expression of a wild-type or a mutant (MIB1V943F) MIB1 (Figure S1A and S1B). To monitor transgene expression, we crossed these lines with mice harboring the Nkx2.5Cre driver line, which is active in cardiac progenitors from E7.5CRE-mediated removal of the floxed NeoSTOP sequences resulted in Rosa26-driven MIB1V943F-EGFP expression in the E9.0 heart (Figure S1C). To examine the effect of MIB1V943F expression on compaction, we used the MYH6Cre driver, expressed in the developing chamber myocardium from E10.5 onwards. The hearts of mice expressing wild-type MIB1 in the myocardium were normal at E15.5, whereas mice expressing MIB1V943F had thinner compact myocardium and persistent trabeculae compatible with LVNC (Figure S1D through S1F). These results indicate that the MIB1R530X and MIB1V943F mutations impair both chamber and valve development, leading to LVNC and BAV in specific genetic configurations.
Figure 2. Defective cardiomyocyte differentiation and metabolic maturation in Mib<sup>1</sup>R530X/flox;Tnnt2<sup>Cre<sup>+/-</sup></sup> mice and MIB1R530X/+ hiPSC-derived cardiomyocytes.

Gene set enrichment analysis (GSEA) of Mib<sup>1</sup>R530X/flox;Tnnt2<sup>Cre<sup>+/-</sup></sup> and control expression profiles. **A**, Bar plot representing enrichment data for 25 gene sets at false discovery rate (FDR q-value) <0.25. Of these, 15 had an FDR q-value <0.05. Scale bar indicates the Normalized Enrichment Score (NES) from –4 to 4. Positive and negative scores were found for 16 and 9 gene sets, respectively. **B**, Gene enrichment profiles for “HALLMARK” gene sets “EPITHELIAL_MESENCHYMAL_TRANSITION” (FDR q-value=0; NES=2.54), “TGF_BETA_SIGNALING” (FDR q-value=0; NES=2), “HYPOXIA” (FDR q-value=0; NES=2.15), and “OXIDATIVE_PHOSPHORYLATION” (FDR q-value=0; NES=2.88). (Continued)
**Figure 2 Continued.** C. Cardiomyocytes derived from control, MIB1\(^{R530X/+}\), edited MIB1\(^{R530X/+}\), MIB1\(^{R530X/+}\), edited MIB1\(^{V943F/+}\) hiPSCs after 20 days of differentiation. Cells were stained with antibodies against BrdU (white), Tnnt2 (red), and Tnni3 (green) and counterstained with DAPI (top and bottom rows), or DAPI and CellMask (blue and white, middle row). The top row shows increased BrdU staining in cells derived from the patients; the middle row shows less multinucleated mature cardiomyocytes in the patients’ cardiomyocytes (arrowheads); the bottom row shows predominant Tnni3 staining in the nuclei of hiPSC-CMs. D. Quantification of BrdU incorporation, percentage of multinucleation, and percentage of nuclear Tnni3 (see Supplemental Material). Data are mean±SD (n=5 or 6 proliferation; n=9–12 multinucleation/maturation). ***P<0.005 and ****P<0.001, 1-way ANOVA, Tukey multiple comparisons tests. E, GSEA of R530X_d10 versus R530X_ED_d10 expression profiles. Bar plot representing enrichment data for 29 gene sets at FDR q-value <0.05. Of these, 16 had FDR q-value <0.05. Scale bar indicates NES from –3 to 3. Positive and negative scores were found for 20 and 9 gene sets, respectively. F, Gene enrichment profiles for “HALLMARK” gene sets ‘EPITHELIAL_MESENCHYMAL_TRANSITION’ (FDR q-value = 0; NES=2.46), ‘HYPOXIA’ (FDR q-value = 6.13E-04; NES=1.95), ‘G2M_CHECKPOINT’ (FDR q-value = 0; NES=2.39), ‘E2F_TARGETS’ (FDR q-value = 0; NES=2.10), and ‘MYOGENESIS’ (FDR q-value = 0; NES=2.31). FDR indicates false discovery rate; hiPSC, human induced pluripotent stem cell; hiPSC-CM, hiPSC-derived cardiomyocytes; NES, normalized enrichment score; Tnnt2, cardiac troponin T; and Tnni3, cardiac troponin I.

Table S6, sheets 1 and 3). Related to this pathway are “TGF_BETAL_SIGNALING”, “APICAL_JUNCTION”, and “MITOTIC_SPINDLE” (Figure 2A and 2B, Table S6, sheets 1 and 4), suggesting activation of cell migratory processes involving oriented cell division as those occurring in cardiomyocytes during chamber development. 

Prominent cellular stress responses such as “HYPOXIA”, “TNFA_SIGNALING_VIA_NFKB”, “UV_RESPONSE_DN”, “INFLAMMATORY_RESPONSE”, and “IL2_STAT5_SIGNALING”, were also enriched positively and might involve “APOPTOSIS” as a final common pathway (Figure 2A and 2B, Table S6, sheets 1 and 5). Conversely, the most negatively enriched pathway was “OXIDATIVE_PHOSPHORYLATION” (Figure 2A and 2B, Table S5, sheets 2 and 6). Related to this pathway are “FATTY_ACID_METABOLISM” and “ROS_PRODUCTION” (Figure 2A), suggesting defective cardiac metabolic maturation. Pathways involved in protein homeostasis such as “MYC_TARGET_V1”, “MYC_TARGET_V2”, and “PROTEIN_SECRETION” were also enriched in Mib1\(^{R530X/}\);Tnnt2\(^{Cre}\) mutants (Figure 2A, Table S6, sheets 1 and 2).

**hiPSC-CMs Show Maturation Impairment and Enhanced Proliferation**

To determine the cellular phenotype of disease-affect ed cardiomyocytes, we generated hiPSCs by retroviral transduction of reprogramming factors in skin fibroblasts from control and MIB1-/- patients; the patients’ hiPSC-CMs (Figure 2C and 2D). In addition, MIB1-mutant hiPSC-CMs showed reduced multinucleation and a predominately nuclear, rather than cytosolic, distribution of Tnni3 (cardiac troponin I) (Figure 2C and 2D), an indication of immaturity. 

We performed RNA-seq in mutant (MIB1\(^{R530X/+}\), named R530X) and gene-edited (R530X_ED) patient hiPSC lines at day 10 of cardiomyocyte differentiation (Figure 2E). Enrichment against “HALLMARK” gene sets revealed “EMT” as the most positively enriched gene set (Figure 2E and 2F, Table S7, sheets 1 and 3), consistent with in vivo findings in mice. The cellular stress pathways “UV_RESPONSE_DN”, “HYPOXIA”, and “UV_RESPONSE_UP” (Figure 2E, Table S7, sheets 1 and 4) were also enriched. Enrichment of “G2M_CHECKPOINT” (Figure 2E and 2F, Table S6, sheets 1 and 5) and “E2F_Targets” (Figure 2E and 2F, Table S7, sheets 1 and 6), supports deregulation of cell cycle progression and the observed increased in proliferation of R530X hiPSC-CMs (Figure 2C and 2D). MIB1\(^{R530X/+}\) hiPSC-CMs displayed altered protein and lipid metabolisms reflected in “MTORC1_SIGNALING”, “PI3K_AKT_MTOR_SIGNALING”, “MYOGENESIS” (Figure 2E and 2F, Table S7, sheets 1 and 7), and “CHOLESTEROL_HOMEO STASIS”, “ADIPOGENESIS”, “BILE_ACID_METABOLISM”, “XENOBIOTIC_METABOLISM” and “PEROXSOME” (Figure 2E, Table S7, sheet 1), respectively. Conversely, downregulation of several immunohematological processes (Figure 2E, Table S7, sheet 2) suggests impaired processes of innateimmunological recognition.

**Multiple Genetic Variants Cosegregate With MIB1 and LVNC**

Individuals heterozygous for the MIB1\(^{R530X}\) and MIB1\(^{V943F}\) mutations in our pedigrees show fully penetrant LVNC (Table S8) with characteristic heart dilatation and trabeculations (Figure 3A). The absence of disease in mice heterozygous for these Mib1 mutations prompted us to perform a WES of these families. We hypothesized that additional genetic variants may cosegregate with the MIB1 mutations and contribute to the manifestation of LVNC.
Figure 3. Identification of variants segregating with MIB1 mutations in human LVNC families by whole exome sequencing.

A, CMRI heart sections showing short axis (sa) views of the right and left ventricles of a control individual (II:10) and patients II:2 and I:5, carrying the MIB1 R530X/+ and MIB1 V943F/+ mutations, respectively. Arrowheads point to trabeculae. Scale bar, 50 mm. B, Genetic variants associated with the MIB1 R530X/+ and MIB1 V943F/+ mutations. Corresponding reference SNP (rs) report references are shown in parentheses. C, MIB1 R530X/+ pedigree. Note the perfect cosegregation of the MIB1 R530X/+ mutation with the newly identified variants and LVNC. D, MIB1 V943F/+ pedigree. Note the cosegregation of the MIB1 V943F/+ mutation with the newly identified variants and LVNC. Members of each generation are indicated numerically. Squares, males; circles, females. Asterisks indicate the individuals from which hiPSCs were generated. CMRI indicates cardiac magnetic resonance imaging; hiPSC, human induced pluripotent stem cell; HCM, hypertrophic cardiomyopathy; lv, left ventricle; LVNC, left ventricular noncompaction; rv, right ventricle; and SNP, single nucleotide polymorphism.
the LVNC phenotype in a heterozygous setting. Briefly, variants were filtered using several quality parameters and inheritance assumptions on the basis of LVNC affection in the family. Filtered variants (15 in MIB1\textsuperscript{R30X} family and 32 in MIB1\textsuperscript{V43F} family) were analyzed focusing on variant, gene, or haplotype characteristics. Our workflow is summarized in Figure S2, and a detailed description of the filtering process can be found in the Methods.

We identified 2 candidate missense variants in the MIB1\textsuperscript{R30X} family (Figure 3B), occurring in heterozygosis in all affected family members and in none of the healthy relatives (Figure 3C). rs3748415 is a valine-to-isoleucine substitution at position 150 (V150I) in APCDD1, a WNT signaling inhibitor.\textsuperscript{70} It is interesting that WNT and NOTCH have been shown to genetically interact during cardiac development.\textsuperscript{71} The second genetic variant, rs181303838, is a methionine-to-valine substitution in position 1415 (M1415V) in ASXL3, a transcription factor involved in heart development and homeostasis.\textsuperscript{72} Both APCDD1 and ASXL3 are physically close to MIB1 in chromosome 18, increasing the chances of cosegregation (Figure S6A).

The mutation found in APCDD1 alters Val150, which is conserved between zebrafish and humans (Figure S6B) and predicted to be in the top 2.4% of deleterious variants in the genome (CADD Phred 16.26). Met1415 in ASXL3 is a rare variant (minor allele frequency 0.0004467) conserved in all eutherians examined (Figure S6B). Its hydrophobicity is maintained in Xenopus laevis’s Leu1368 (Figure S6B). The APCDD-1\textsuperscript{V150I} mutation was predicted to be more deleterious than ASXL3\textsuperscript{M1415V} (PolyPhen scores of 0.555 and 0, respectively) and to be more evolutionarily conserved (CADD Phred, 16.26 and 8.681; and GERP, 1.79 and −1.58). Nevertheless, APCDD1\textsuperscript{V150I} was more prevalent in human populations (minor allele frequency 0.1611; Figure S6C). Both genes are expressed during mouse ventricular and cardiac valve morphogenesis (E12.5−E16.5). Quantitative PCR of whole hearts from C57Bl/6 embryos from E10.5 to E16.5 demonstrated a significant decline in transcription (Figure S3D). The mutated phenylalanine residue in TMX3\textsuperscript{F191X} is subjected to some selective pressure (GERP score: 0.63, Figure S3C), and substitution occurs at a frequency that could fit in LVNC prevalence in combination with any of the other candidate mutations found in the MIB1\textsuperscript{V43F} family (Figure S3E).

Reverse transcription-PCR analysis at E14.5 confirmed that the TMX3 is expressed in the mouse heart (Figure S3F; see Methods). All these variants, although predicted as benign or of uncertain significance by their effect or prevalence (Figures S3 and S6), are not present simultaneously with their respective MIB1 variants in any gnomAD HGDP or 1000 genomes\textsuperscript{77} individual (Tables S9 and S10).

**Heterozygous Combination of MIB1 Mutations With the ASXL3 and APCDD1 or With the CEP192, TMX3, and BCL7a Variants Causes LVNC or BAV in Mice**

To determine whether the newly identified candidate variants contribute to the autosomal dominant inheritance pattern of LVNC in our pedigrees, we used CRISPR-Cas9 gene editing to generate triple (Mib1\textsuperscript{R30X}/+ Asxl3\textsuperscript{M1361V}/+ Apcc1\textsuperscript{V150I}/+) and quadruple (Mib1\textsuperscript{V43F}/+ Cep1\textsuperscript{192T1522M}/+ Tmx3\textsuperscript{F191X}/+ Bcl7a\textsuperscript{A56G,S64V}/+) heterozygous mutant mice using gene editing (Figure S7). CRISPR reagents were microinjected into zygotes.
of crosses between *Mib1* mutant males (*Mib1*<sup>V943F/V943F</sup> or *Mib1*<sup>R530X/−</sup>) and C57BL/6J females (Table S1, sheet 3). We aimed to introduce the new variants in cis with those in *Mib1* because the genes involved are linked, so this would yield a higher frequency of multiple heterozygotes. Founders were identified by PCR and confirmed by Sanger sequencing (Figure S7). For the variants identified in the MIB1<sup>R530X</sup> family, we obtained 2 triple heterozygotes (*Mib1*<sup>R530X/+</sup> *Asxl3*<sup>M1361V/+</sup> *Apcdd1*<sup>V150I/+</sup>) out of 16 (12.5%) animals (Table S1, sheet 3). For those of the MIB1<sup>V943F</sup> family variants, we obtained 1 triple heterozygote founder (*Mib1*<sup>V943F/+</sup> *Cep192*<sup>T1522M/+</sup> *Tmx3*<sup>F191X/+</sup>) out of 4 survivors (25%). In the Bcl7a microinjection, we obtained 13 founders out of 21 pups (61.9%) with both intronic mutations (Table S1, sheet 3). The founders generated were crossed with C57BL/6J mice to dilute any possible off-target effect of CRISPR-Cas9 gene edition. These crosses also allowed us to determine if the mutations were introduced in cis or in trans heterozygosity. Most of the animals were either triple heterozygotes or wild-type, indicating cis heterozygosity. For details, see the Supplemental Material.

Triple heterozygous *Mib1*<sup>R530X/+</sup> *Asxl3*<sup>M1361V/+</sup> *Apcdd1*<sup>V150I/+</sup> mice (abbreviated as *Thet*) reached adulthood and were fertile. At E16.5, the hearts of these mice displayed thinner ventricles and larger but thinner trabeculae than control counterparts, and also had a significantly reduced a compact myocardium:trabeculae displayed thinner ventricles and larger but thinner trabeculae (Figure 4D through 4H). These elevated parameters are compatible with the pathogenesis of high-output heart failure. 78

We performed RNA-seq at E15.5, followed by gene set enrichment analysis, and identified “DNA_REPAIR” as the most enriched pathway, followed by “MYC_TARGETS_V1” and “E2F_TARGETS” (Figure 4I and 4J, Table S11, sheets 1 through 3), reflecting dysregulation of cell cycle checkpoint controls (G2/M and S phases). We also found enriched the “P53_PATHWAY”, an effector of cellular stress pathways including “ROS_PATHWAY”, “UNFOLDED PROTEIN_RESPONSE”, and “UV_RESPONSE (_UP_ and _DN)” that may act upstream of cell-cycle checkpoints and involve “APOPTOSIS” as an outcome (Figure 4I, Table S11, sheet 1). Enrichment of the “ALLOGRAFT_REJECTION” and “INTERFERON_ALPHA_RESPONSE” terms (Figure 4I, Table S11, sheet 1), may reflect activation of innate immunity pathways. “MTORC1_SIGNALING” enrichment and “PROTEIN_SECRETION” depletion suggest that protein homeostasis is likely altered in *Thet* mice (Figure 4I, Table S11, sheets 1 and 6). Given that APCDD1 is a direct WNT signaling target,70 the enrichment of “WNT_BETA_CATENIN_SIGNALING” (Figure 4I and 4J, Table S11, sheets 1 and 4), is consistent with WNT and NOTCH pathways playing opposite roles during cardiogenesis. “EMT” enrichment (Figure 4I and 4J, Table S11, sheets 1 and 5) is consistent with an increase in cell migratory processes. Defective ventricular development in *Thet* mutants is associated with increased proliferative and migratory processes, defective cellular stress responses, and protein homeostasis, indicative of a maturation defect.

After including our published *Mib1*<sup>flox/flox</sup>;<*Tnt2*<sup>Cre</sup> mice gene profile,34 we performed a comparative analysis of all the gene set enrichment analyses made in this study. We observed that the *Mib1*<sup>R530X/flox</sup>;<*Tnt2*<sup>Cre</sup> signature was more closely related to those of the *Mib1*<sup>flox/flox</sup>;*Tnt2*<sup>Cre</sup> mice and *R530X*<sup>d10</sup> iPSCs than to that of *Thet* mice (Figure S8). Considering all the pathway overlaps, we found that contrasts showing the strongest enrichments (P<0.05), were *Mib1*<sup>R530X/flox</sup>;<*Tnt2*<sup>Cre</sup> versus control and *R530X*<sup>d10</sup> versus *R530X*<sup>d10</sup> iPSCs (Figure S8). Shared pathways included “EMT”, “TGF_BETA_SIGNALING”, “ESTROGEN_RESPONSE_EARLY”, “TNFA_SIGNALING VIA_NFKB”, and “IL2_STA5_SIGNALING” (Figure S8). These pathways were more enriched, with a level of significance at q-value<0.05 or q-value<0.25) in *Mib1*<sup>R530X/flox</sup>;<*Tnt2*<sup>Cre</sup> and *R530X*<sup>d10</sup> versus *R530X*<sup>d10</sup> iPSCs (Figure S8). Depleted pathways (at q-value<0.05 or q-value<0.25) involved immune-hematological processes (Figure S8). However, these pathways were either moderately enriched or unchanged in *Thet* mice (Figure S8). Collectively, the RNA-seq data suggest that defective ventricular chamber maturation in *Mib1* mutants is caused by aberrant proliferative, migratory, fibrotic, and inflammatory pathway gene activation. Acting alongside these ‘primary’ pathways are ‘secondary’ pathways, which are enriched or depleted depending on the specific genotype.

Triple heterozygous *Mib1*<sup>V943F/+</sup> *Cep192*<sup>T1522M/+</sup> *Tmx3*<sup>F191X/+</sup> mice showed significantly more penetrant valve abnormalities at E16.5, including BAV (Figure 4K through 4N and 4S), as well as defects in both the muscular and the membranous ventricular septum (Table S5, sheet 3). Quadruple heterozygous mutants harboring the Bcl7a variants also had BAV, as well as coronary artery defects (Figure 4O through 4S, Table S5, sheet 3). We examined the UK Biobank in search of BAV or aortopathy cases with *Mib1* mutations. We found that in the WES cohort of 454 756 individuals, 1408 heterozygous participants carried 533 rare missense *Mib1* variants (0.3%; 1 in 323), and 3028 participants carried 533 rare missense *Mib1* variants (0.2%; 1 in 853). Eighty-five heterozygous missense variant carriers of 3028 heterozygote individuals were diagnosed with aortic valve disease (0.03%; 1 in 36). Thirty-six heterozygous predicted loss of function variant carriers...
Figure 4. Mib1R530X/+ Asxl3M1361V/+ Apccdd1V150I/+ and Mib1V943F/+ Cep192T1522M/+ Tmx3F191X/+;Bcl7aAG,GA/+ compound heterozygous mice show LVNC and BAV.

A and B, Transverse heart sections from E16.5 control and Mib1R530X/+ Asxl3M1361V/+ Apccdd1V150I/+ embryos stained with antibodies to Tnnt2 (green) and isolectin B4 (white), and counterstained with DAPI (blue). General views of left and right ventricle (A and B) and high-magnification views of boxed areas (A', A'', B', and B''), showing reduced compact myocardium thickness in triple heterozygous embryos. Scale bars, 500 μm (A and B) and 100 μm (magnifications).

C, Quantification of compact myocardium (CM) thickness and the compact-to-trabecular myocardium area ratio in both ventricles. Data are mean±SD (n=3 sections from 11 and 1 section from 2 WT and n=3 sections from 11 and 1 section from 3 mutant embryos, *P<0.05 and **P<0.005, by Student t-test).

D through G, CMRI images of adult (10-month-old) control (Continued)
Molecular Interactions Among CEP192, BCL7A, and NOTCH

Our results support a genetic interaction between MIB1 and the genes identified in our LVNC family exome: ASXL3, APCDD1, CEP192, TMX3, and BCL7A. To determine if the corresponding proteins interacted in vitro, we conducted co-IP experiments in HEK293T cells. On the basis of the nature of the proteins involved, we cotransfected MIB1, N1ICD (NOTCH1 intracellular domain), or RBPJ (recombinating binding protein suppressor of hairless) in pairwise combinations with the candidate interacting proteins. Thus, according to the exome data from the MIB1 R530X family, we cotransfected HEK293T cells with FLAG-ASXL3 plus N1ICD-myc, RBPJ-GFP, or RBP-myc expression vectors, or with APCDD1FLAG plus MIB1-HA or N1ICD-myc expression vectors. For the MIB1 V943F family, we cotransfected HEK293T cells with CEPI92-GFP plus MIB1-HA or N1ICD-myc; with TMX3-HA plus N1ICD-myc or RBPJ-GFP; or with BCL7a-FLAG plus N1ICD-myc or RBPJ-GFP. Co-IP experiments with N1ICD showed that it is the CT CEP192 region. Conversely, immunoprecipitation with anti-FLAG and WB with anti-myc detected a ~150-KDa band corresponding to BCL7A, and the reciprocal experiment detected both the reciprocal interaction with CEP192 and MIB1 (Figure S9B), despite the association of MIB1 with centrosomal proteins.79,80 In the case of BCL7A, co-IP with anti-myc antibody and WB with anti-FLAG detected a 50-KDa band corresponding to BCL7A, and the reciprocal experiment detected the 70-KDa band corresponding to N1ICD (Figure 5C). Because BCL7A is a transcription factor, we tested whether it interacted with the NOTCH effector transcription factor RBRJ cotransfecting cells with RBRJ-GFP and BCL7A-FLAG. Co-IP with anti-GFP antibody and WB with anti-FLAG detected a 50-KDa band corresponding to BCL7A, and the reciprocal experiment detected the 100-KDa band corresponding to RBRJ-GFP (Figure 5D). These results support direct molecular interaction among CEP192, BCL7A, and N1ICD proteins.

DISCUSSION

Defining the genetic basis of cardiac disease has proved more challenging than anticipated, despite the technological advances in genomic analysis of the last decade.81 The likely reason is that coding and noncoding DNA variants appear to make similar contributions to cardiac disease,82 implying that progress toward precision cardiovascular medicine will require experimental testing of the influence of multiple potential regulatory and coding sequence variants.83 Even in severe cardiac diseases that manifest in children or young adults (ie, congenital heart disease and cardiomyopathies) and may appear to have a single Mendelian genetic basis, phenotypic expression is influenced by multiple DNA variants, suggesting that these diseases are oligogenic84 or polygenic.85 This implies that the combined action of genetic modifiers can have a major effect on the expression of a cardiac disease phenotype,86 making it difficult to define its genetic pathogenesis.

Our data show that mice harboring the ortholog MIB1 R530X and MIB1 V943F mutations that cause LVNC of 1408 heterozygote individuals were diagnosed with aortic valve disease (0.3%; 1 in 39). Assessments of the burden of aortic valve disease in MIB1 variant carriers were not significant (P > 0.05).
in heterozygous human carriers develop LVNC when cardiac MIB1 is depleted, or BAV on a MIB1 heterozygous and NOTCH-sensitized genetic background. The recessive nature of the LVNC phenotype in mice contrasts with its dominant character in humans, suggesting the existence of additional contributing genes (modifiers) that interact with MIB1 to cause LVNC in a dominant fashion in humans. Moreover, the BAV phenotype of mice heterozygous for the Mib1 V943F or Mib1 R530X mutations on a NOTCH-sensitized genetic background suggests that valve morphogenesis is strictly sensitive to the NOTCH-MIB1 dosage and that its haploinsufficiency leads to BAV. The high dosage requirement for NOTCH-MIB1 function is also supported by the observation that only 5% of mice with cardiac MIB1 depletion (Mib1 R530X/flox;Tnnt2 CRE) survived embryogenesis, instead of the Mendelian 12.5%.

The MIB1 V943F mutation is deleterious in cellular assays and in zebrafish embryos, and our results show that mice expressing the MIB1 V943F protein in the embryonic myocardium display LVNC features. In our search for additional genetic variants that contribute to LVNC, WES of families carrying the MIB1 R530X and MIB1 V943F mutations identified a set of missense variants in ASXL3, APCDD1, CEP192, TMX3, and BCL7A that cosegregate with the MIB1 mutations and LVNC, suggesting a genetic interaction between MIB1 and these modifier genes during cardiogenesis. With the exception of BCL7A, these genes are located on chromosome 18 close to MIB1, increasing the probability of cosegregation, and the exome data reveal a perfect cosegregation of these variants with MIB1 mutations and LVNC. It remains to be determined if MIB1 and these newly identified candidates are coordinately regulated during cardiac development.

Generation of the corresponding mouse models revealed that triple heterozygous Mib1 R530X/+ Asx-1 M1361V/+; Apcdd1 V150I/+ mice (Thet mice) showed features of LVNC, whereas quadruple heterozygous Mib1 V943F/+ Cep192 T1522M/+; TMX3 F191X/+; Bcl7a AG,GA/+ mice showed a variety of valve-related defects, including BAV. Thus, WES of our LVNC pedigrees allowed us to identify new genes contributing to LVNC or BAV. These results suggest that 2 congenital structural heart abnormalities, LVNC and BAV, share a genetic substrate in which MIB1 mutations disrupt a major developmental pathway regulating ventricular and valve development. MIB1 mutations can thus lead to LVNC or BAV depending on their combination with a set of mutations in modifier genes that influence disease phenotype. In other words,
MIB1-NOTCH signaling plays a major role in ventricular and valve development, and its genetic disruption can lead to human LVNC or BAV in the presence of a certain genetic substrate, produced by alterations in a set of modifier genes (Figure 5E). The causal implication of NOTCH1 mutations in BAV has been described in humans91 and mice.99 Likewise, MIB1 inactivation causes LVNC in both humans and mice34 and also BAV and valve dysmorphology in mice,8990 although its implication in human LVNC has not been described so far.

Although patients heterozygous for the MIB1V943F/+ CEP192T1522M/+ TMX3F191X/+; BCL7AAGGA/+ variants show LVNC and not BAV, mice carrying the 4 ortholog mutations have BAV but no LVNC, emphasizing the strong influence of the genetic background on disease phenotype and the sensitive response of inbred mouse strains (C57BL/6J) in the manifestation of cardiac phenotypes. Developmental marker analysis revealed defective chamber patterning and maturation in mice deficient for MIB1 in the myocardium, indicating the developmental origin of LVNC. Gene profiling of Mib1R530X/+; Tnnt2Cre mice, Mib1R530X/+; Asx13M1361V/+; Apccdf1V150I/+; triple heterozygous mice, and Mib1R530X/- patient-derived hiPSCs revealed shared defects in myocardial differentiation, and metabolic maturation together with increased cardiomyocyte proliferation, processes that must be tightly coordinated to achieve full cardiomyocyte maturation.91 Mechanistically, abnormal activation of the TGFβ pathway is a consistent finding across the different Mib1 genotypes, and TGFβ signaling has been related to pro-EMT and pro-antiproliferative pathways.92 Reinforcing this notion, persistent cardiomyocyte proliferation has been reported in mice with myocardial Mib1 inactivation,1034 whereas blunted proliferation was found in a hiPSC model of LVNC with perturbed TGFβ signalling.67 Immunoprecipitation assays between NOTCH pathway elements and the proteins identified by WES suggested direct biochemical interaction of N1ICD/BRBJ with CEP192 and BCL7A. The lack of positive evidence about the other proteins may be a result of low expression or defective protein modification or folding, and thus possible interactions cannot be discarded. In addition, the genetic interaction between MIB1 and APCDD1 is worth exploring on the basis of the interplay between NOTCH and WNT in different cardiac settings.7193

Previous studies have suggested the oligogenic nature of LVNC.2834 Our study is the first in which exome data from 2 large pedigrees reveal LVNC resulting from cosegregation of mutations in a critical gene with mutations in a set of linked modifier genes. Modeling of these mutations in mice suggests that ventricle and valve morphogenesis share, at least in part, a common genetic substrate and developmental pathway. Extrapolation of our mouse model’s data to humans would imply that patients with LVNC harboring MIB1 mutations should also be screened for valve abnormalities, and vice versa. Our study demonstrates the power of combining WES with humanized animal models generated through careful gene editing to provide mechanistic insight into complex diseases and the contribution to disease phenotype of coding and noncoding genetic variants identified through massive sequencing.

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