

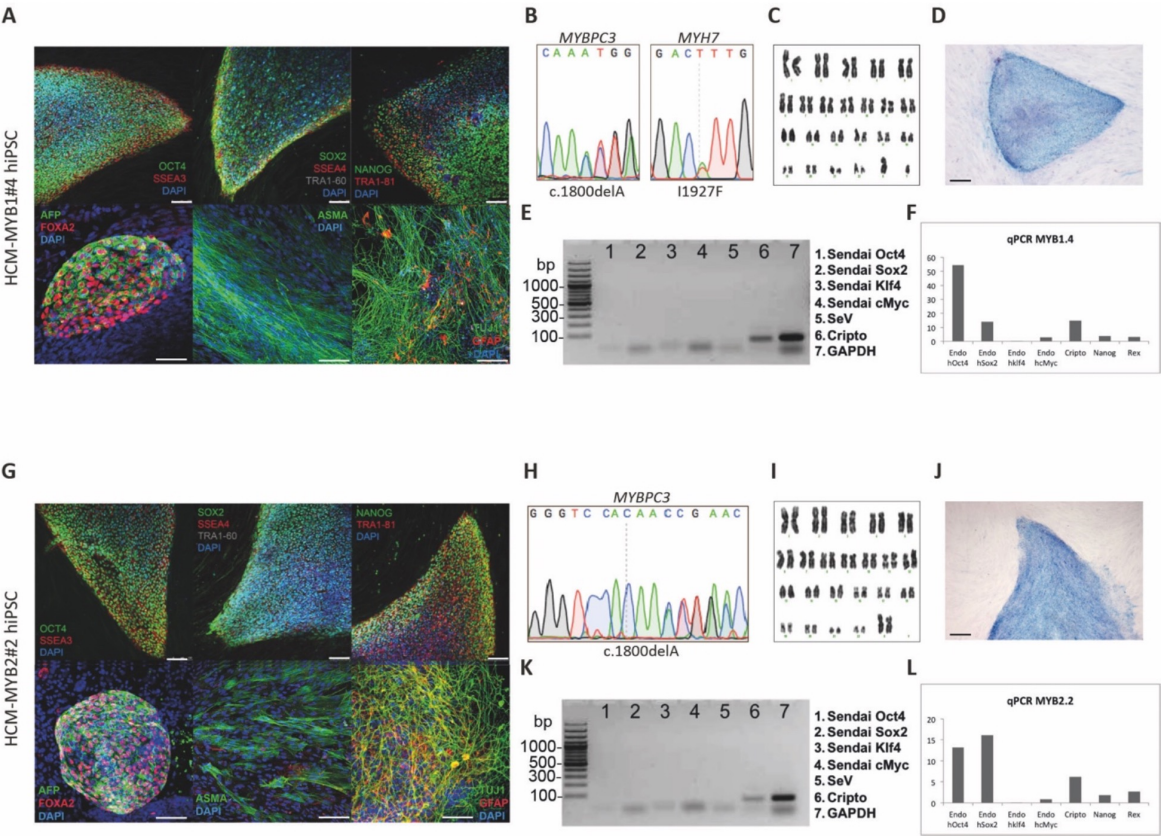
APPENDIX II

CHARACTERIZATION

MYCOPLASMA TEST

MICROSATELLITES

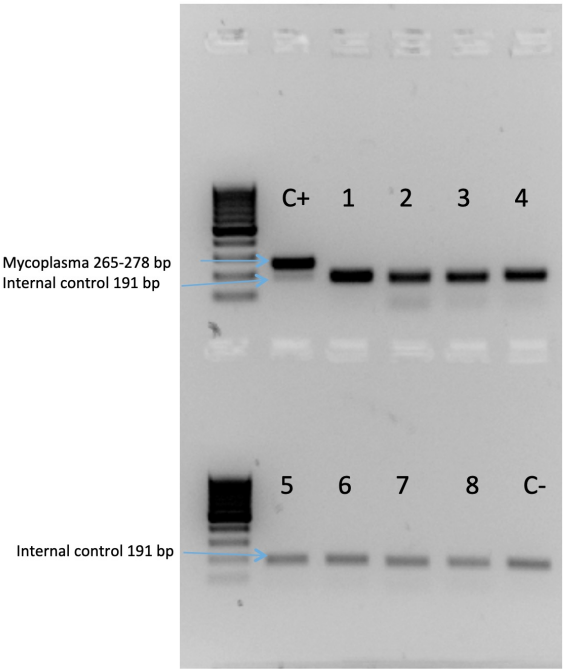
CHARACTERIZATION TESTS



Supplementary Figure S2. Characterization of patient-specific hiPSC. Patient-specific induced pluripotent stem cell lines were generated from proband III.7 (MYB1#4) (A-F) and from proband III.5 (MYB2#2) (G-L). **(A, G)** Top: immunofluorescence of hiPSC colonies showing expression of pluripotent markers including OCT4, SSEA3, SOX2, SSEA4, TRA1-60, NANOG and TRA1-81. Bottom: immunofluorescence analysis of cell derivatives differentiated *in vitro* to the three primary germ layers including endoderm (stained for α -fetoprotein (green) and FOXA2A (red)), mesoderm (stained for α -smooth muscle actin, ASMA (green)), and ectoderm (stained for TUJ1 (green) and GFAP (red)). Scale bar 100 μ m. **(B, H)** Sanger sequencing of *MYBPC3* mutant hiPSCs carrying the c.1800delA (p.K600fs) mutation and the *MYH7* missense variant I1927F in the MYB1#4 hiPSC. **(C, I)** Normal karyotype. **(D, J)** Staining for alkaline phosphatase (AP) activity. Scale bar 100 μ m. **(E, K)** RT-qPCR analysis of the endogenous expression levels of pluripotent genes and **(F, L)** the Sendai-derived reprogramming factors.

MYCOPLASMA TEST

Mycoplasma test (VenorGeM Classic kit) 17/09/2020



- 1-Myb14
- 2-|
- 3-Myb22
- 4-
- 5-
- 6-
- 7-
- 8-

P-CMR[C]

ESTUDI DE MICROSATÈL·LITS (STRs)

Mostra: Identificació ID Ambar Tipus de mostra
MYB1 FIBROBLAST 1221755 Extracte DNA

Descripció de l'estudi

L'estudi de consisteix en l'anàlisi de microsatèl·lits o "short tandem repeats" (STR) de DNA procedent determinades línies cel·lulars. El procediment seguit ha estat el següent:

- . PCR - Amplificació de 15 regions de DNA (STR- short tandem repeats) amb els Kit de Genotipat AmpFLSTR® Identifiler® Plus PCR Amplification Kit. La combinació d'aquestes 15 regions constitueix el perfil genètic (impressió digital genètica)
- . Electroforesi capil·lar – Lectura del perfil genètic de la mostra.
- . Software d'anàlisi – Assignació del perfil genètic.

Resultats

Marcadors Genètics	Localització cromosòmica	Al·lels mostra	
		1221755	
D8S1179	8	12	15
D21S11	21q11.2-q21	29	33,2
D7S820	7q11.21-22	8	10
CSF1PO	5q33.3-34	10	12
D3S1358	3p	15	16
TH01	11p15.5	7	9,3
D13S317	13q22-31	9	11
D16S539	16q24-qter	11	13
D2S1338	2q35-37.1	17	24
D19S433	19q12-13.1	13	13
VWA	12p12-pter	15	18
TPOX	2p23-2per	9	11
D18S51	18q21.3	12	16
D5S818	5q21-31	12	12
FGA	4q28	20	21
AMELOGENINA*	X: p22.1-22.3- Y: p11.2	X	Y

*Amelogenina: Resultat al·lel relatiu al sexe cromosòmic: XX (sexe femení); XY (sexe masculí)

L'Hospitalet del Llobregat, 27/07/2023

ESTUDI DE MICROSATÈL·LITS (STRs)

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MYB1.4 1221753 Extracte DNA

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Resultats

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L'Hospitalet del Llobregat, 27/07/2023

SUPPLEMENTAL INFORMATION

iPSC-based modeling of variable clinical presentation in hypertrophic cardiomyopathy

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Supplemental Information contains:

Expanded Materials & Methods

6 Supplementary Tables:

Table S1. List of the primary and secondary antibodies used for immunohistochemistry analysis

Table S2. Primers used for quantitative RT-PCR analyses

Table S3. List of oligonucleotides and ssODN donor template for correcting c.1800delA mutation in the *MYBPC3* gene

Table S4. List of sgRNA and ssODN donor template for correcting c.5779A>T (I1927F) mutation in the *MYH7* gene

Table S5. Genetic variants identified by WES in related individuals within a family with HCM.

Table S6. Description of the carriers of the family

4 Supplementary Figures:

Figure S1. Representative imaging studies and electrocardiograms of the family members

Figure S2. Characterization of patient-specific hiPSC

Figure S3. Characterization of CRISPR/Cas9 gene-edited isogenic controls hiPSCs

Figure S4. Generation and characterization of CRISPR/Cas9 gene-corrected isogenic hiPSCs

Expanded Materials & Methods

Generation and characterization of iPSC

The studies were approved by the authors' Institutional Review Board and conducted according to the Declaration of Helsinki. Patients were encoded to protect their confidentiality, and written informed consent obtained. The generation of human iPSC cells was done following a protocol approved by the Spanish competent authorities (Commission on Guarantees concerning the Donation and Use of Human Tissues and Cells of the Carlos III Health Institute). Primary cultures of dermal fibroblasts were reprogrammed to transgene-free iPSCs using the CytoTune iPSC Sendai Reprogramming Kit (Invitrogen, cat. no. A16517) following the manufacturer's instructions. A minimum of 4 independent colonies were picked based on morphology 20-30 days after initial infection, adapted to grow on Matrigel (Corning, cat. no. 356234)-coated dishes with mTeSR1 medium (Stem Cell Technologies, cat. no. 85850), and expanded by splitting 1:6 – 1:10 by dissociation with 0.5 mM EDTA (Gibco, cat. no. 15575-020) for 2 min at 37°C. Full characterization of iPSC lines included testing alkaline phosphatase activity, dilution of Sendai vector transgenes, expression of endogenous pluripotency-associated transcription factors by RT-PCR and immunofluorescence, *in vitro* differentiation towards definitive endoderm, mesoderm and neuroectoderm, and karyotyping, and were carried out as previously described²⁸. For these studies, dermal fibroblasts from two individuals from the same family, MYB1 and MYB2, both carrying the pathogenic frameshift p.Lys600Asnfs*2 variant in *MYBPC3*, were reprogrammed to generate iPSC lines MYB1#4 and MYB2#2, respectively. A control human iPSC line generated from a healthy individual (codename FiPS Ctrl2 SV4F1, registered in the National Stem Cell Bank, Carlos III Spanish National Institute of Health) was used.

Differentiation of iPSC toward cardiomyocytes

hiPSC were differentiated into cardiomyocytes in monolayer culture as previously described^{29,30}. hiPSC maintained in mTeSR1 medium on Matrigel were dissociated into single cells with Accutase (Stemcell Technologies, cat. no. 7922) at 37°C for 5 min and

seeded onto Matrigel-coated 12-well plate at a density of 1.5 million cells per well in mTeSR1 medium supplemented with 10 μ M Y-27632 (Stemcell Technologies, cat. no. 72304). Cells were maintained in mTeSR1 medium, with daily changes for 3 days. Upon reaching confluence, cells were treated with 7-12 μ M CHIR99021 (Sigma, cat. no. SML1046) in RPMI medium (Gibco, cat. no. 31870-074) supplemented with B27 lacking insulin (Gibco, cat. no. A18956-01), 1% glutamax (Gibco, cat. no. 35050038), 0.5% penicillin-streptomycin (Gibco, cat. no. 151401122), 1% nonessential amino acids (Corning, cat. no. 25-025-CI), and 0.1 mM 2-mercaptoethanol (Gibco, cat. no. 31350-10) (RPMI/B27- insulin medium) for 24 h (day 0 to day 1). After 24 h, the medium was changed to RPMI/B27-insulin and cultured for 2 additional days. On day 3 of differentiation, cells were treated with 5 μ M IWP4 (TargetMol, cat. no. T4245) in RPMI/B27-insulin medium and cultured without medium change for 2 days. Cells were maintained in RPMI supplemented with B27 (Gibco, cat. no. 17504001), 1% glutamax, 0.5% penicillin/streptomycin, 1% non-essential amino acids, and 0.1 mM 2-mercaptoethanol (RPMI/B27 medium) starting from day 7, with medium change every 2 days. Spontaneously beating cardiomyocytes were typically observed beginning on day 8 of differentiation. Beating clusters were disaggregated by incubation with 0.25% trypsin-EDTA (Gibco, cat. no. 25200-056) for 5-8 min at 37°C for subsequent characterization and *in vitro* functional studies.

Immunofluorescence analyses

Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After three washes with 1X TBS (5 min each), cells were incubated in blocking solution I (1X TBS, 0.5% Triton-X100 (Sigma, cat. no. X100-500ML) and 6% donkey serum (Chemicon, cat. no. S30-100) for 1 h at room temperature and incubated with primary antibodies diluted in blocking solution II (1X TBS, 0.1% Triton-X100 and 6% donkey serum) overnight at 4°C in agitation. The following antibodies were used: goat anti-Nanog (R&D Systems; AF1997; 1:50), mouse anti-Tra-1-81 (Merck-Millipore; MAB4381; 1:200), mouse anti-OCT4 (Santa Cruz; sc-5279; 1:30). Rat anti-SSEA-3 (Developmental Studies Hybridoma Bank (DSHB); MC-631; 1:10), rabbit anti-SOX2 (Thermo Fisher; PA1-16968; 1:100), mouse anti-SSEA-4 (Developmental Studies Hybridoma Bank (DSHB); MC-813-70;

1:100), mouse anti-Troponin T (Thermo Fisher; MS-295-P; 1:100), mouse anti-ASA (Sigma; A2172; 1:400), goat anti-FOXA2 (R&D Systems; AF2400; 1:100), mouse anti- α SMA (Sigma-Aldrich; A5228; 1:400), mouse anti-TUJ1 (Biolegend; MMS-435P; 1:500), rabbit anti-GFAP (Dako (Agilent); Z0334; 1:500). The detailed list of antibodies is presented in Suppl. Table S1. Cells were then washed 3 times with 1X TBS for 5 min each and incubated with 0.5 μ g/ml DAPI (4',6-diamino-2-phenylindole) (Invitrogen, cat. no. D21490) for 10 min at room temperature. Mouse/rat/rabbit/goat IgG isotype controls were used for validating the specificity of primary antibodies. Samples with secondary antibodies only were used as negative controls for distinguishing positive signals from the background. Images were taken using Leica SP5 Inverted confocal microscope (Leica) and analyzed using ImageJ software (National Institutes of Health, USA).

Flow cytometry analyses

Cultures at day 20 of differentiation were dissociated using 0.25% trypsin-EDTA at 37 °C for 5 min and then fixed with 4% paraformaldehyde for 20 min at room temperature. After washing with 1X saponin (Sigma, cat. no. 47036), cells were permeabilized using Cell Permeabilization Kit (Invitrogen, cat. no. GAS003) and blocked with 5% mouse serum for 15 min at room temperature. Then, cells were stained with the antibodies mouse PE-anti myosin heavy chain (MHC) (IgG2b, 1:400 BD Biosciences) and mouse Alexa Fluor 647 cardiac troponin I (cTnI) (IgG2b, 1:100, BD Biosciences). Mouse IgG2b PE (1:400 BD Biosciences) and mouse IgG2b Alexa Fluor 647 (1:100, BD Biosciences) antibodies were used as isotype controls. After incubation in the dark for 15 min at room temperature and washing twice with 1X saponin, cells were analyzed with FACS MoFlo (Beckman Coulter) and data acquisition and analysis performed by Kaluza software (Beckman Coulter). The detailed list of antibodies is shown in Suppl. Table S1.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the GeneJET RNA purification Kit (ThermoFisher, cat. no. K0731) following the manufacturer's instructions, and 1 μ g was used to synthesize cDNA using Transcriptor first-strand cDNA synthesis kit (Roche, cat. no. 04896866001), as per

the manufacturer's instructions. qRT-PCR was carried out using the 7900 HT Fast RT-PCR System (Applied Biosystems) using the following conditions: 15s at 95°C for denaturation and 1 min at 60°C for annealing and extension, for 40 cycles. All qPCR reactions were performed in triplicate. CT values were measured and transformed to relative gene expression levels by the $2^{-\Delta\Delta C_T}$ method. Human *GAPDH* was used as housekeeping gene for normalization. The sequences of the primers used are listed in Suppl. Table S2.

Morphological analyses of iPSC-CMs

Transmission electron microscopy was carried out and analyzed essentially as previously described ³⁰. For cell area analysis, beating iPSC-derived cardiac monolayers were dissociated at day 20 and seeded at low density in RPMI/B27 medium supplemented with 10 μ M Y-27632 and 10% FBS (Cytiva HyClone, cat. no. SV30160.03) for 24 h. Dissociated cardiomyocytes were seeded for 4-5 days before fixation and immunostaining for the cardiac marker Troponin T as described above. Ten randomly selected fields were imaged from each differentiated culture, and the images then analyzed and the cell area manually measured using ImageJ software (National Institutes of Health, USA), until a minimum of 100 cells per differentiated culture were scored. Depending on the cell density, this required analyzing between 2 and 6 (3.1 ± 1.1 ; mean \pm SD) images per differentiated culture. The number of nuclei were manually counted for every cell measurement.

Calcium transients imaging

At day 30 of cardiac differentiation, cardiomyocytes were dissociated with 0.25% Trypsin/EDTA and 80,000 cells were seeded on a gelatin-coated glass chamber that allows for temperature control and electrical stimulation (Cell MicroControl). Cells were maintained for 5 days, with medium change every other day. Cells were loaded with 5 μ M of Fluo-4 (AAT Bioquest, cat. no 273221-67-3) in Tyrode's solution (129mM NaCl, 5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 30mM Glucose, 25mM HEPES) containing 0.02% Pluronic F-127 (Sigma, cat. no. P2443) for 30 min. Cells were washed 3 times with Tyrode's solution and images were acquired at 60 fps in a Confocal Inverted Microscope

for 10s (Ex/Em = 490/525 nm) at a rate of 0.5 Hz. Fluorescence signals were recorded, and data analysis was performed with ImageJ with the multi kymograph plugin to produce line-based kymograph images. MATLAB (Mathworks) was used to calculate the calcium kinetic parameters.

Analysis of contraction-relaxation kinetics

Contractile kinetics analysis was performed at the indicated time-points on iPSC-derived cardiomyocytes cultured on monolayer. 8-10 days prior to contractile assessment, cells were dissociated using 0.25% trypsin-EDTA and seeded onto Matrigel-coated 96-well plates at a density of 100,000 cells per well in RPMI/B27 medium supplemented with 10 μ M Y-27632 and 10% FBS for 24 h. Cells were maintained in RPMI/B27 medium, with medium changes every other day. Contraction-relaxation movement was recorded every 56 ms for 16 seconds using an inverted microscope (Olympus CellR). During the recording process, temperature was kept at 37°C and 5% CO₂. Data was analyzed in ImageJ using the 'MuscleMotion' tool ³¹.

Metabolic analyses

The Seahorse XFe96 extracellular flux analyzer (Agilent) was used to assess respiration and acidification rates and mitochondrial function. Cardiomyocytes were seeded on Matrigel-coated assay plates 7 days before measurement at a density of 20,000 cells per XFe96 well. Undifferentiated hiPSC were seeded at low density in small clumps 3-4 days before measurement. Cells were washed twice in Agilent Seahorse XFe DMEM Basal Medium (Agilent Technologies, cat. no. 103575-100) supplemented with 2 mM glutamine, 10 mM glucose and 1 mM sodium pyruvate 1 h before the assay and for the duration of the measurement. For the standard profiling (Mito Stress test), oligomycin was injected at 1.5 μ M, FCCP at 0.5 μ M and rotenone/antimycin A were added at 0.5 μ M. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) values were normalized to the number of nuclei quantified by DAPI staining. OCR, ECAR and ATP production rates were obtained using the Seahorse Wave controller Software 2.6.1 (Agilent).

Mitochondrial DNA content

Cardiac cultures at day 30 of differentiation were collected and total RNA-free DNA was extracted using the QIAmp® DNA Mini Kit (Qiagen, cat. no. 51304) according to manufacturer's instructions. 10ng of isolated DNA were used for Real-time quantitative PCR using the 7900 HT Fast Real-Time PCR System (Applied Biosystems). The nuclear gene *ACTB* was used for normalization. Specific primers are listed in Suppl. Table S2.

Genome editing of iPSC

The CRISPR/Cas9 plasmid pSpCas9(BB)-2A-GFP (PX458), a gift from Feng Zhang (Addgene plasmid # 48138) ³², modified with the full-length pCAGGS promoter as described ³³ was used. Custom guide RNAs (gRNAs) were cloned into the BbsI sites as annealed oligonucleotides. The donor templates for homology-directed repair (HDR) were ordered as Ultramer DNA oligos of 100-120 bases (Integrated DNA Technologies). For correcting the *MYBPC3* p.Lys600Asnfs*2 variant, the day before transfection mutant iPSC were disaggregated into small clumps using 0.5 mM EDTA and 400,000 cells were seeded on Matrigel-coated 6-cm plates. The day after, cells were transfected using FuGENE HD (Promega, cat. no. E2311) and a mixture of 5 µg containing CRISPR/Cas9 plasmid and HDR donor template in 250 µl of OptiMEM (Gibco, cat. no. 31985-047). 72 hours post-transfection, cells were pre-treated for 1 hour with 10 µM Y-27632 and dissociated with Accutase for FACS-sorting based on GFP fluorescence (MoFlo XDP, Beckman Coulter). Approximately 15,000 cells were seeded in Matrigel-coated 10-cm plates containing RI-supplemented mTesR1 medium and maintained for 10-14 days until colonies were large enough to be screened. Colonies were manually picked and genotyped by restriction site polymorphism and Sanger sequencing. Colonies with the desired genotype were isolated, expanded and cryopreserved. Specific gRNA and single-stranded oligo DNA nucleotides (ssODN) for correcting the *MYBPC3* p.Lys600Asnfs*2 variant are listed in Suppl. Table S3.

For correcting the heterozygous p.Ile1927Phe variant in the *MYH7* gene, MYB1#4-iPSC were edited based on CRISPR/Cas9 ribonucleoprotein (RNP) complexes in

order to achieve a higher gene-editing efficiency. For RNP delivery, 200 pmol of annealed cr:tracrRNA (ThermoFisher, cat no. NC1690985) were mixed with 100 pmol of Cas9 nuclease (ThermoFisher, cat. no. A36499) and incubated at room temperature for 10 min to allow for RNP assembly. The assembled RNP complexes were mixed with 250,000 dissociated cells in 20 μ l P3 primary Cell solution (Lonza, cat. no. PBP3-00675) and 100 pmol of ssODN donor template. Cells were placed into the Nucleocuvette strips (Lonza) and transfected using the Amaxa 4D-Nucleofector system (Lonza) and the electroporation protocol CA137. Transfected cells were then plated onto 48-well plates containing pre-warmed mTeSR1 medium supplemented with 10 μ M Y-27632. 3-4 days post-transfection, cells were dissociated into single cells and plated at low density in 10-cm plates. Subsequent steps were carried out as described before. Specific sgRNA and ssODN are listed in Suppl. Table S4.

Whole-exome sequencing (WES)

Exome sequencing was performed and analyzed by the research pipeline at Health in Code (A Coruña, Spain) as previously described ³⁴. In brief, individual's gDNA was extracted using QIAasympphony SP® (Qiagen, cat. no. 9001297). Library preparation was performed using the SureSelectXT Library Preparation kit (Agilent, cat. no. 5190-4806) for the Illumina multiplexed sequencing paired-end. Exome capture was performed with the Agilent Clinical Research Exome V2 probe kit (Agilent, cat. no. 5190-9491) and sequenced using the Illumina HiSeq 1500 platform. Bioinformatic analysis was carried out using an in-house pipeline for the interpretation of 405 genes within a panel for global cardiovascular diseases, in accordance with best WES analysis practices. Selected variants were confirmed by Sanger sequencing. Genetic variants potentially linked to HCM pathogenesis are listed in Suppl. Table S5.

Statistical Analyses

Data are presented in text and figures as mean \pm SD. The number of independent experiments (N) and data points per experiment (n) is indicated in the figure legends. Statistical tests were performed using GraphPad Prism 8 Software (San Diego, California,

USA). Data normality was established by Shapiro-Wilk test before parametric or non-parametric tests were used. When data were normally distributed and comprised more than two experimental groups, data were first evaluated using one-way ANOVA followed by a *post-hoc* test as indicated in the figure legends, or two-tailed unpaired t-test with Welch's correction when comparing between isogenic sets. For data departing from normality or $n < 9$, we used the Mann-Whitney test when two groups were compared, or the Kruskal-Wallis followed by Dunn's multiple comparison test for comparing more than two groups. For the calculation of gene variant enrichment in HCM probands, we used genotype/phenotype information of the Health in Code cohort, as previously described ³⁵. In brief, the phenotypes of the patients were established by each center prior to the genetic studies; all of them had a suspicion/diagnosis of HCM, but symptoms were not described for all the cases. Patients' samples were referred mainly from centers from Spain (90%), followed by centers from the United Kingdom, Denmark, United States, Germany, and Argentina. The predominant ethnicity was European (more than 90% of the probands), and there were no differences between HCM probands and controls. Control data was also obtained from the gnomAD v2.1.1 database. Odds ratios were calculated with confidence intervals computed using Woolf logit, and statistical significance assessed by two-sided Fisher's exact test. The penetrance of a variant was estimated using the case-controls data according to the method described by Minikel et al. ³⁶, with the help of *alleleFrequencyApp*, a Shiny App from James Ware's laboratory at Imperial College London available at <http://cardiodb.org/allelefrequencyapp/>, and an estimated prevalence of HCM of 1:500.

Table S1. List of the primary and secondary antibodies used for immunohistochemistry analysis

Primary Antibodies	Host	Dilution	Manufacturer
Nanog (Polyclonal IgG)	Goat	1:50	R&D Systems, AF1997
Tra-1-81 (Monoclonal IgM)	Mouse	1:200	Merck-Millipore, MAB4381
OCT4 (Monoclonal IgG2b)	Mouse	1:30	Santa Cruz, sc-5279
SSEA-3 (Monoclonal IgM)	Rat	1:10	DSHB, MC-631
SOX2 (polyclonal IgG)	Rabbit	1:100	Thermo Fisher, PA1-16968
SSEA-4 (Monoclonal IgG3)	Mouse	1:100	DSHB, MC-813-70
FOXA2 (Polyclonal IgG)	Goat	1:100	R&D Systems, AF2400
α SMA (Monoclonal IgG2a)	Mouse	1:400	Sigma-Aldrich, A5228
TUJ1 (Monoclonal IgG2a)	Mouse	1:500	Biolegend, MMS-435P
GFAP (Polyclonal IgG)	Rabbit	1:500	Dako (Agilent), Z0334
Actin α -sarcomeric (Monoclonal IgM)	Mouse	1:400	Sigma-Aldrich, A2172
Troponin T (Monoclonal IgG1)	Mouse	1:100	Thermo Fisher, MS-295-P
PE-Myosin heavy chain (IgG2b)	Mouse	1:400	BD Biosciences, 564408
Alexa Fluor® 647, cardiac troponin I (IgG2b)	Mouse	1:100	BD Biosciences, 564409
PE IgG2b isotype control	Mouse	1:400	BD Biosciences, 559529
Alexa Fluor® 647 IgG2b isotype control	Mouse	1:100	BD Biosciences, 558713
Secondary Antibodies			
Alexa Fluor® 488, Mouse IgG	Goat	1:200	Jackson, 115-546-071
Cy™3, Rat IgM	Goat	1:200	Jackson, 112-165-020
Alexa Fluor® 488, Rabbit IgG	Donkey	1:200	Jackson, 711-545-152
Cy™3, Mouse IgG	Goat	1:200	Jackson, 115-165-071
DyLight™ 649, Mouse IgM	Goat	1:200	Jackson, 115-495-075
Alexa Fluor® 488, Goat IgG	Donkey	1:200	Jackson, 705-545-147
Cy™3, Mouse IgM	Donkey	1:200	Jackson, 715-165-140
Cy™3, Rabbit IgG	Donkey	1:200	Jackson, 711-165-152
Cy™3, Goat IgG	Donkey	1:200	Jackson, 705-165-147
Cy™3, Mouse IgM	Goat	1:200	Jackson, 115-165-075

Table S2. List of qRT-PCR primer sequences

Primers for gene	Forward 5'-3'	Reverse 5'-3'	Amplicon size (bp)
ND1	ATGGCCAACCTCCTACTCCTCATT	TTATGGCGTCAGCGAAGGGTTGTA	154
ACTB	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT	250
Endo hOCT4	GGAGGAAGCTGACAACAATGAAA	GGCCTGCACGAGGGTTT	64
Endo hSOX2	TGCGAGCGCTGCACAT	TCATGAGCGTCTTGGTTTCC	72
Endo KLF4	CGAACCACACAGGTGAGAA	GAGCGGGCGAATTTCAT	68
Endo hc-MYC	AGGGTCAAGTTGGACAGTGTC	TGGTGCATTTTCGGTTGTTG	61
Cripto	CGGAAGTGTGAGCACGATGT	GGGCAGCCAGGTGTCATG	66
Nanog	ACAACTGGCCGAAGAATAGCA	GGTCCCAGTCGGGTTTCC	111
Rex	CCTGCAGGCGGAAATAGAAC	GCACACATAGCCATCACATAAGG	61
Sendai OCT4	CCCGAAAGAGAAAGCGAACCAG	AATGTATCGAAGGTGCTCAA	528
Sendai SOX2	ATGCACCGCTACGACGTGAGCGC	AATGTATCGAAGGTGCTCAA	528
Sendai KLF4	TTCCTGCATGCCAGAGGAGCCC	AATGTATCGAAGGTGCTCAA	410
Sendai c-MYC	TAACTGACTAGCAGGCTTGTCG	TCCACATACAGTCCTGGATGATGATG	532
SeV	GGATCACTAGGTGATATCGAGC	ACCAGACAAGAGTTTAAGAGATATGTATC	181
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	87

Table S3. List of oligonucleotides and ssODN donor template for correcting c.1800delA mutation in the *MYBPC3* gene

Oligonucleotides	Sequence 5'-3'	sgRNA (Protospacer sequence 5'-3')
MYBPC3 px458 DelA Fw	CACCGCGTCGTCAATGGTCAGTTG	ACGTCGTCATGGTCAGTTG
MYBPC3 px458 DelA Rv	AAACCAACTGACCATTGACGACGC	
ssODN	Sequence 5'-3'	
MYBPC3 delA to WT	GGGGTGTGTGGCCAGTGGGGTCCCCTGAGCCACTGCTCCCCTGCAGGGT G CACAAAC TGACCATTGACGACGTCACACCTGCCGACGAGGCTGACTACAGC	

C>**G** → ApaLI site

Table S4. List of sgRNA and ssODN donor template for correcting c.5779A>T (I1927F) mutation in the *MYH7* gene

sgRNA	Sequence 5'-3'
MYH7 I1917 to WT	CCGTGACTTTGGCACGAAGG
ssODN	Sequence 5'-3'
MYBPC3 I1927 to WT	GCAGAGGAGCGGGCGGACATCGCCGAGTCCCAGGTCAACAAGCTGCGGGCCAAGAGCC GTGACAATTGGTACGAAGGTGGGTCCCTCTTTGGGCTTTGCTAGTCACCCCCACAG

A>T → wild type sequence; C>T → RsaI site

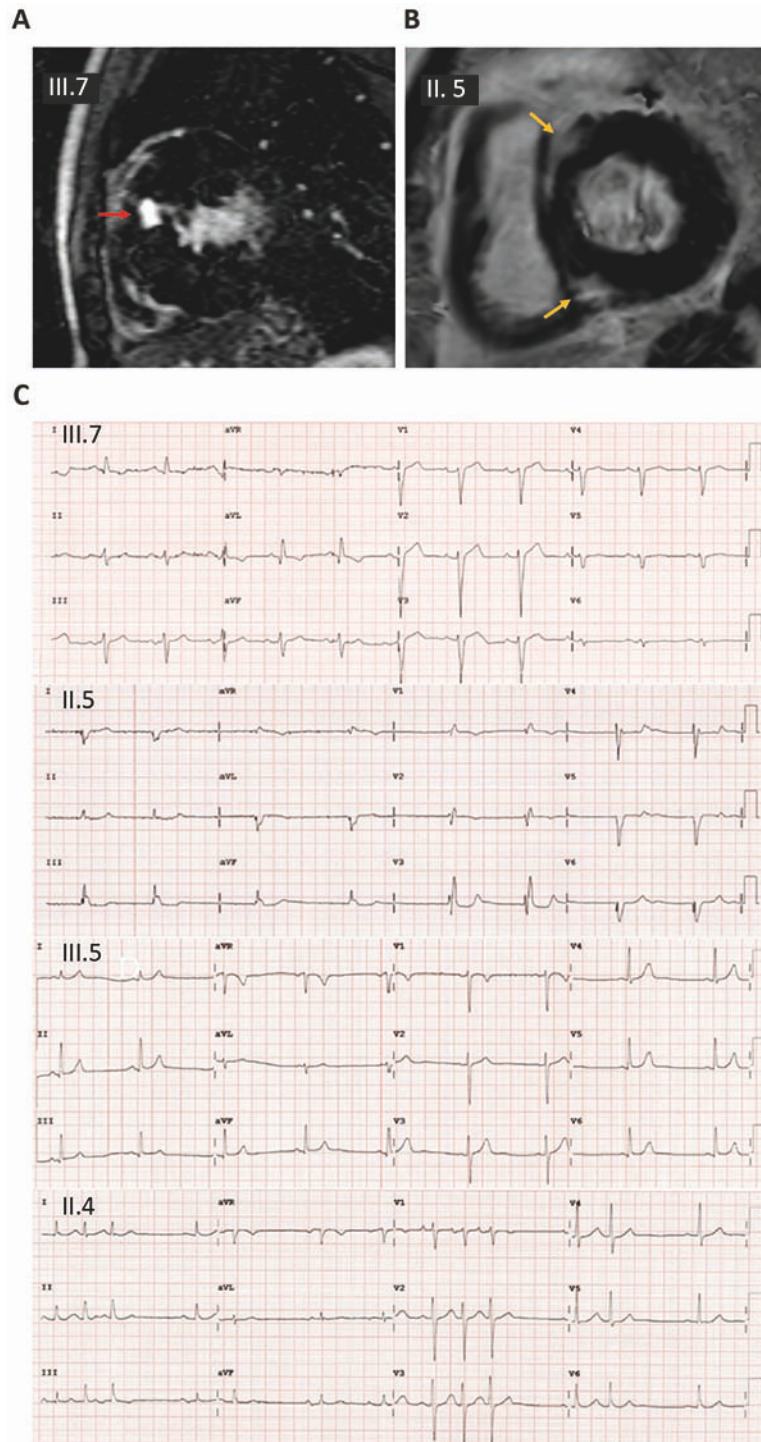
Table S5. Genetic variants identified by WES in related individuals within a family with HCM

Gene	Variant	Result	Pathogenicity	Frequency in gnomAD
Variant identified in all individuals				
MYBPC3	NP_000247.2:p.Lys600Asnfs*2 NM_000256.3:c.1800delA NC_000011.9:g.47362788delT	Heterozygosis	Pathogenic (+++)	<0,001%
Variants identified in individual III.7; MYB1				
MYH7	NP_000248.2:p.Ile1927Phe NM_000257.3:c.5779A>T NC_000014.8:g.23882979T>A	Heterozygosis	Variant of uncertain significance, possibly pathogenic	<0,005%
C10orf71	NP_001128668.1:p.Glu1092Lys NM_001135196.1:c.3274G>A NC_000010.10:g.50533864G>A	Heterozygosis	Variant of uncertain significance	<0,001%
CACNA1D	NP_000711.1:p.Ser1801Cys NM_000720.3:c.5402C>G NC_000003.11:g.53835386C>G	Heterozygosis	Variant of uncertain significance	-
LAMA4	NM_001105206.2:c.4822-9T>C NC_000006.11:g.112439110A>G	Heterozygosis	Possibly benign	<0,001%
Variants identified in individual III.5; MYB2				
C10orf71	NP_001128668.1:p.Glu1092Lys NM_001135196.1:c.3274G>A NC_000010.10:g.50533864G>A	Heterozygosis	Variant of uncertain significance	<0,001%
CREBBP	NP_004371.2:p.Arg1081Cys NM_004380.2:c.3241C>T NC_000016.9:g.3817730G>A	Heterozygosis	Variant of uncertain significance	<0,001%
LAMA4	NM_001105206.2:c.4822-9T>C NC_000006.11:g.112439110A>G	Heterozygosis	Possibly benign	<0,001%
Variants identified in individual II.5				
PTPN11	NP_002825.3:p.Val382Ile NM_002834.3:c.1144G>A NC_000012.11:g.112919929G>A	Heterozygosis	Variant of uncertain significance	<0,001%
FOXF1	NP_001442.2:p.Met158Arg NM_001451.2:c.473T>G NC_000016.9:g.86544648T>G	Heterozygosis	Variant of uncertain significance	-

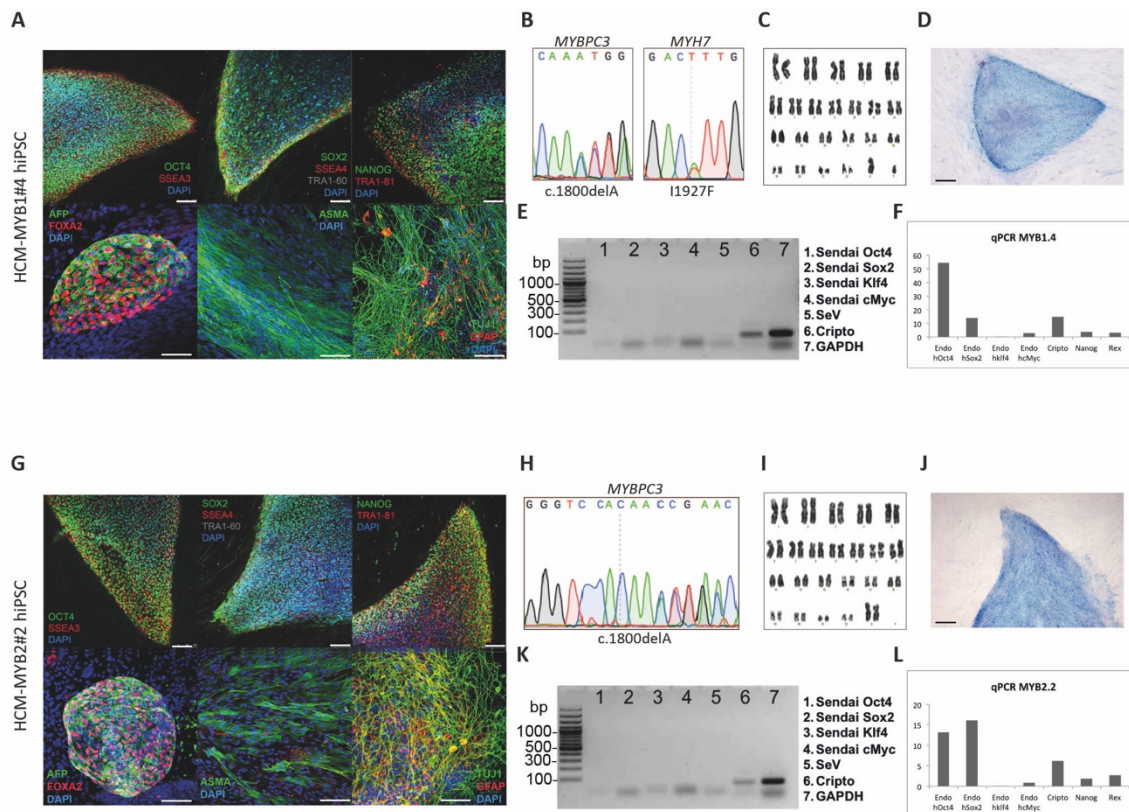
Table S6. Description of the carriers of the family.

ID	Sex	Age diagnosis	MYBPC3 variant	MYH7 variant	Maximal LVH (mm)	Obstruction	LA (mm)	NSVT	Syncope	Fibrosis (MRI)	AF	ICD / PM	HCM-Risk SCD
III.7	M	18	+	+	39	Yes	41	Yes	No	Yes	Yes	ICD	8.6%
III.5	F	41	+	-	12	Yes	29	No	No	?	No	-	1,3%
II.4	F	73	-	+	14	No	44	No	No	No	No	-	0.9%
II.5	M	60	+	-	16	No	59	Yes	No	Yes	Yes	PM	4,3%
II.10	M	52	+	-	18	No	51	No	No	Yes	Yes	-	2,1%
III.9	F	No	+	-	9	No	25	No	No	?	No	-	-
III-11	M	No	+	-	11	No	36	No	No	?	No	-	-

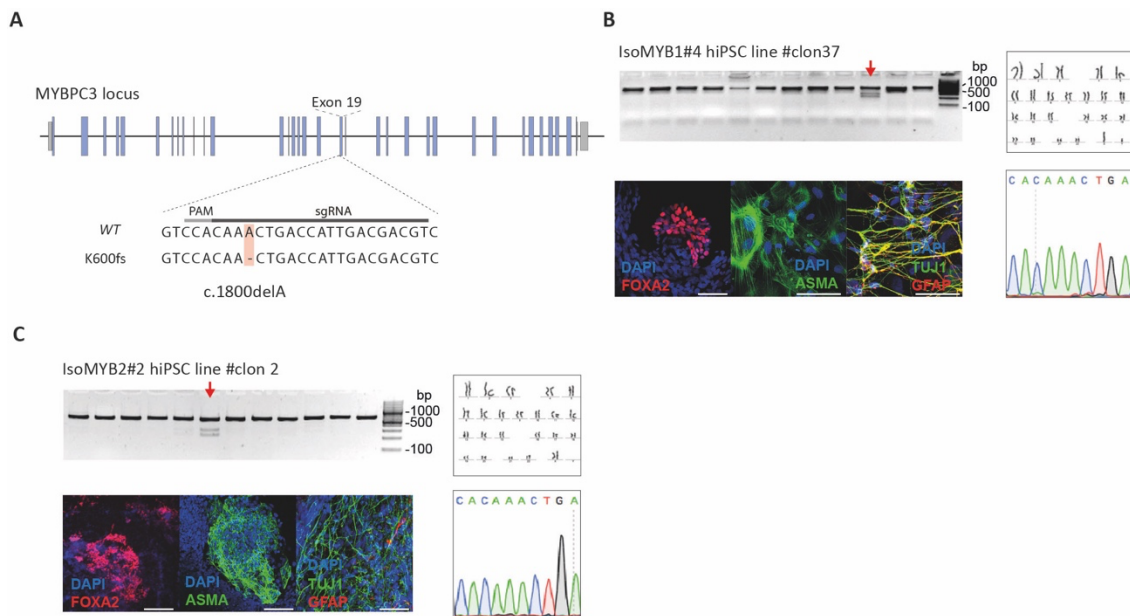
ID: Pedigree identification; M: Male; F: Female; LVH: left ventricular hypertrophy; LA: left atrium diameter; NSVT: non-sustained ventricular tachycardia in Holter-24h; MRI: magnetic resonance imaging; AF: atrial fibrillation; ICD: implantable cardiac defibrillator; PM: pacemaker; HCM-Risk SCD: maximal hypertrophic cardiomyopathy sudden cardiac risk score according to ESC HCM guidelines.



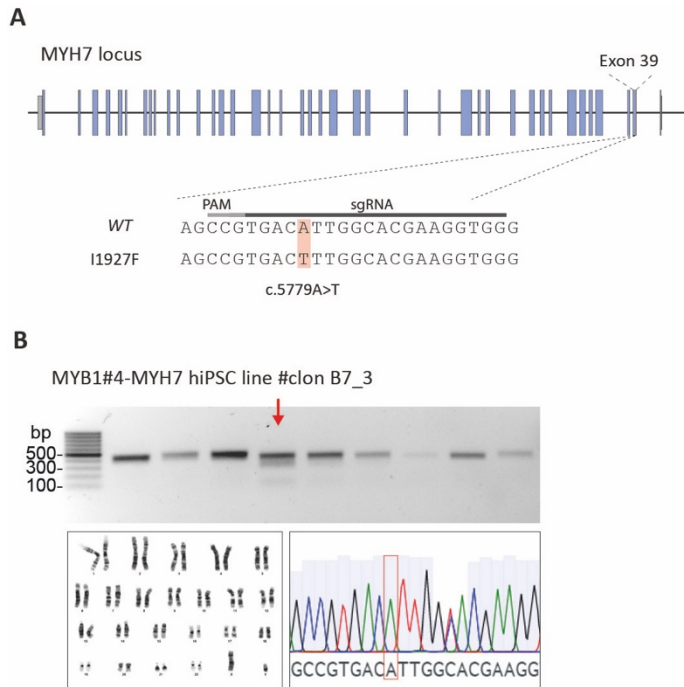
Supplementary Figure S1. Representative imaging studies and electrocardiograms of the family members. A-B) Cardiac resonance imaging (CMRI) showing fibrotic areas (arrows) of (A) proband III.7 and (B) individual II.5. C) ECGs of the family members showing: (III.7) left anterior hemiblock, poor R wave progression in precordial leads and Q waves in I and aVL; (II.5) atrial fibrillation, right bundle branch block and left posterior hemiblock; (III.5) ECGs within normal limits; (II.4) with premature atrial contractions.



Supplementary Figure S2. Characterization of patient-specific hiPSC. Patient-specific induced pluripotent stem cell lines were generated from proband III.7 (MYB1#4) (A-F) and from proband III.5 (MYB2#2) (G-L). **(A, G)** Top: immunofluorescence of hiPSC colonies showing expression of pluripotent markers including OCT4, SSEA3, SOX2, SSEA4, TRA1-60, NANOG and TRA1-81. Bottom: immunofluorescence analysis of cell derivatives differentiated *in vitro* to the three primary germ layers including endoderm (stained for α-fetoprotein (green) and FOXA2A (red)), mesoderm (stained for α-smooth muscle actin, ASMA (green)), and ectoderm (stained for TUJ1 (green) and GFAP (red)). Scale bar 100 μm. **(B, H)** Sanger sequencing of MYBPC3 mutant hiPSCs carrying the c.1800delA (p.K600fs) mutation and the MYH7 missense variant I1927F in the MYB1#4 hiPSC. **(C, I)** Normal karyotype. **(D, J)** Staining for alkaline phosphatase (AP) activity. Scale bar 100 μm. **(E, K)** RT-qPCR analysis of the endogenous expression levels of pluripotent genes and **(F, L)** the Sendai-derived reprogramming factors.



Supplementary Figure S3. Characterization of CRISPR/Cas9 gene-edited isogenic controls hiPSCs. **A)** Schematic representation of the CRISPR/Cas9 gene editing design, showing the c.1800delA mutation in the *MYBPC3* gene and the sgRNAs used in our study. **B-C)** Generation and characterization of isogenic corrected IsoMYB1#4-hiPSC line (B) and IsoMYB2#2-hiPSC line (C) showing from top left to bottom right: Molecular analysis of the gene-edited clones (indicated by red arrows) confirming the proper restriction site integration; normal karyotype; immunofluorescence analysis of cell derivatives differentiated *in vitro* to the three primary germ layers including endoderm (stained FOXA2A (red)), mesoderm (stained for α -smooth muscle actin, ASMA (green)), and ectoderm (stained for TUJ1 (green) and GFAP (red)) and Sanger sequencing confirming the correction of the pathogenic c.1800delA mutation in the *MYBPC3* gene. Scale bar 100 μ m.



Supplementary Figure S4. Generation and characterization of CRISPR/Cas9 gene-corrected isogenic hiPSCs. A) Schematic representation of the CRISPR/Cas9 gene editing design, showing the c.5779A>T (p.I1927F) mutation in the *MYH7* gene and the sgRNA used in our study. **B)** Generation and characterization of isogenic corrected MYB1#4-MYH7 hiPSC line showing: (top) molecular analysis of the gene-edited clone (indicated by red arrow) confirming the proper restriction site integration; (bottom left) normal karyotype and (bottom right) Sanger sequencing confirming the correction of the c.5779A>T (p.I1927F) mutation in the *MYH7* gene.