Circulation Research

ORIGINAL RESEARCH

iPSC-Based Modeling of Variable Clinical Presentation in Hypertrophic Cardiomyopathy

Rubén Escribá, José M. Larrañaga-Moreira, Yvonne Richaud-Patin, Léa Pourchet, Ioannis Lazis, Senda Jiménez-Delgado, Alba Morillas-García, Martín Ortiz-Genga, Juan Pablo Ochoa, David Carreras, Guillermo Javier Pérez, José Luis de la Pompa, Ramón Brugada, Lorenzo Monserrat, Roberto Barriales-Villa, Angel Raya

BACKGROUND: Hypertrophic cardiomyopathy (HCM) is the most common inherited cardiac disease and a frequent cause of heart failure and sudden cardiac death. Our understanding of the genetic bases and pathogenic mechanisms underlying HCM has improved significantly in the recent past, but the combined effect of various pathogenic gene variants and the influence of genetic modifiers in disease manifestation are very poorly understood. Here, we set out to investigate genotype-phenotype relationships in 2 siblings with an extensive family history of HCM, both carrying a pathogenic truncating variant in the *MYBPC3* gene (p.Lys600Asnfs*2), but who exhibited highly divergent clinical manifestations.

METHODS: We used a combination of induced pluripotent stem cell (iPSC)-based disease modeling and CRISPR (clustered regularly interspersed short palindromic repeats)/Cas9 (CRISPR-associated protein 9)-mediated genome editing to generate patient-specific cardiomyocytes (iPSC-CMs) and isogenic controls lacking the pathogenic *MYBPC3* variant.

RESULTS: Mutant iPSC-CMs developed impaired mitochondrial bioenergetics, which was dependent on the presence of the mutation. Moreover, we could detect altered excitation-contraction coupling in iPSC-CMs from the severely affected individual. The pathogenic *MYBPC3* variant was found to be necessary, but not sufficient, to induce iPSC-CM hyperexcitability, suggesting the presence of additional genetic modifiers. Whole-exome sequencing of the mutant carriers identified a variant of unknown significance in the *MYH7* gene (p.Ile1927Phe) uniquely present in the individual with severe HCM. We finally assessed the pathogenicity of this variant of unknown significance by functionally evaluating iPSC-CMs after editing the variant.

CONCLUSIONS: Our results indicate that the p.Ile1927Phe variant of unknown significance in *MYH7* can be considered as a modifier of HCM expressivity when found in combination with truncating variants in *MYBPC3*. Overall, our studies show that iPSC-based modeling of clinically discordant subjects provides a unique platform to functionally assess the effect of genetic modifiers.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: energy metabolism ■ mutation ■ phenotype ■ sibling ■ studies

In This Issue, see p 105

Hypertrophic cardiomyopathy (HCM) is the most common inherited cardiac disease.[1] With an estimated prevalence of 1 in 500 people,[2] it is a frequent cause of heart failure and sudden cardiac death in the young population.[3,4] HCM is a complex condition defined by heterogeneous clinical manifestations, ranging from severely affected to asymptomatic individuals.[5] During the past 2 decades, genetic testing has identified a plethora of pathogenic variants associated to HCM, mainly in genes encoding for sarcomere proteins.[6] Among them, mutations in *MYBPC3*, encoding myosin-binding protein C, and in *MYH7*, encoding cardiac β-myosin heavy chain, are the most frequent.[5,7,8] However, incomplete penetrance of pathogenic genetic variants and variable expressivity further underscore the strong influence of genetic and environmental modifiers of HCM progression.[9–11]

Correspondence to: Angel Raya, MD, PhD, Regenerative Medicine Program, Institut d'Investigació Biomèdica de Bellvitge (IDIBELL), 08908 L'Hospitalet de Llobregat, Barcelona, Spain. Email araya@idibell.cat

Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.122.321951.
For Sources of Funding and Disclosures, see page 118.

Novelty and Significance

What Is Known?

- Genetic testing has identified a plethora of gene mutations causing familial hypertrophic cardiomyopathy, but phenotype-genotype relationships are still poorly understood.
- Genetic association studies allow researchers and geneticists to assign pathogenic risk to genetic variants, but they are typically underpowered for variants of low prevalence.
- Induced pluripotent stem cell (iPSC)-based disease modeling, in combination with CRISPR/Cas9-mediated genome editing, emerge as powerful approaches to assess pathogenicity of disease-causing mutations.

What New Information Does This Article Contribute?

- We studied clinically discordant siblings sharing a pathogenic mutation in *MYBPC3* and generated isogenic pairs of iPSC-derived cardiomyocytes (iPSC-CMs).
- We identified disease-related phenotypes dependent on the *MYBPC3* mutation (mitochondrial dysfunction) and others for which the mutation was necessary, but not sufficient (CM hyperexcitability and hypercontractility).
- The search for additional genetic modifiers identified a variant of unknown significance in the *MYH7* gene, the pathogenicity which could be functionally evaluated after editing the variant in iPSC-CMs.

Our study aimed to investigate the relationship between genetic variations and the clinical presentation of HCM in 2 siblings who carried a pathogenic truncating variant in the *MYBPC3* gene but showed different clinical manifestations. We generated patient-specific iPSC-CMs from the siblings and used CRISPR/Cas9 genome editing to create isogenic control iPSC-CMs without the pathogenic variant. iPSC-CMs carrying the *MYBPC3* mutation exhibited impaired mitochondrial bioenergetics, and altered excitation-contraction coupling was observed in iPSC-CMs from the severely affected individual. However, the pathogenic *MYBPC3* mutation alone was not sufficient to induce hyperexcitability in iPSC-CMs, suggesting the presence of additional genetic modifiers. Whole-exome sequencing revealed a variant of unknown significance in the *MYH7* gene uniquely present in the severely affected individual. Functional evaluation of iPSC-CMs after editing the *MYH7* variant confirmed its pathogenicity and its role as a modifier of HCM expressivity when combined with truncating variants in *MYBPC3*. Our study illustrates the usefulness of iPSC-based modeling of clinically discordant subjects to identify and functionally assess the effect of genetic modifiers in cardiomyopathies.

Nonstandard Abbreviations and Acronyms

cTnI	cardiac troponin I
cTnT	cardiac troponin T
HCM	hypertrophic cardiomyopathy
iPSC	induced pluripotent stem cell
MHC	myosin heavy chain
VUS	variant of unknown significance

Advances in human induced pluripotent stem cell (iPSC) technology have aided disease modeling of several genetic cardiomyopathies, usually by investigating known pathogenic genetic variants in a patient-specific manner.[12–15] The use of directed genome editing techniques to generate gene-corrected isogenic control iPSC lines has further improved researchers' ability to study genotype-phenotype correlations in HCM.[16] However, dissecting the effect of genetic modifiers of cardiomyopathies is still limited by their complex genetic etiology.[17,18] In the case of HCM associated to *MYBPC3* gene variants, several efforts at modeling the disease using iPSC-derived cardiomyocytes (iPSC-CMs) have been reported, although their results have not always been consistent. Whereas some studies have found clear signs of HCM such as increased iPSC-CM cell area,[19–21] and various degrees of alterations in calcium handling and/or contractility,[15,20–24] others did not find any disease-relevant phenotype in iPSC-CMs carrying *MYBPC3* variants.[25] These seemingly conflicting results have been attributed to differences in the specific type of *MYBPC3* variant, the stage of differentiation/maturation of iPSC-CMs analyzed in the different studies, and the sensitivity of the analytical methodology employed, and underscore the importance of including isogenic iPSC control lines in the comparisons.[26]

For the current studies, we took advantage of iPSC-based disease modeling to tease apart the contribution of genetic and environmental factors to disease onset/progression.[27] We generated iPSC from 2 siblings with a family history of HCM who shared a pathogenic variant in the *MYBPC3* gene, but exhibited highly divergent clinical manifestations (severe versus mild HCM). iPSC-CM representing these individuals also displayed disease-related phenotypes of divergent severity in vitro, which strongly suggested the presence of underlying genetic modifiers. Whole-exome sequencing identified a variant

of unknown significance (VUS) in the *MYH7* gene present exclusively in the patient with severe HCM. The use of CRISPR/Cas9-mediated directed genome editing and functional phenotyping of resulting iPSC-CM allowed ascribing pathogenicity to the *MYH7* variant, at least when combined with truncating *MYBPC3* mutations. Our study provides a valuable platform to functionally assess causality and ascribing pathogenicity to genetic variants in the context of familial cardiomyopathies.

METHODS

Data Availability

All data generated or analyzed during this study are included in this published article (and its Supplemental Material). The sequencing data generated in the study have been deposited in the European Variation Archive at EMBL-EBI under accession number PRJEB61786 (https://www.ebi.ac.uk/eva/?eva-study=PRJEB61786). The iPSC lines generated are being banked and will be available through the Spanish National Stem Cell Bank-Carlos III Spanish National Institute of Health, in compliance with the informed consent signed by the patients.

Detailed description of materials and methods can be found in the Supplemental Material. Please see the Major Resources Table in the Supplemental Material.

RESULTS

Modeling HCM Phenotypic Divergence

The index case in our studies (individual III.7 in Figure 1A) is a male patient diagnosed with obstructive HCM at 18 years of age. Cardiac magnetic resonance imaging revealed a maximal left ventricular hypertrophy of 39 mm with moderate fibrosis in septal segments (Figure 1B; Figure S1A). A cardiac defibrillator was implanted during follow-up after a sudden death episode.

Genetic testing of known variants using the Sequenom MassArray platform detected a truncating variant in the *MYBPC3* gene (g12413delA, c.1800delA, p.Lys600Asnfs*2, *MYBPC3*^{K600fs}) in heterozygosis that was considered pathogenic according to the recommendations of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Clinical and genetic evaluation of family members unveiled that the father was a carrier of the *MYBPC3*^{K600fs} variant and had nonobstructive HCM with left ventricular hypertrophy of 16 mm and fibrosis in the septal insertion points in cardiac magnetic resonance imaging (Figure 1C; Figure S1B and S1C). The sister of the proband also carried the *MYBPC3*^{K600fs} variant but was asymptomatic at the time of the original study and she only developed a borderline phenotype with septal left ventricular hypertrophy of 12 mm and mild inducible obstructive gradient in echocardiogram (Figure 1D) at 42 years of age. The clinical description of the family is summarized in Table S6.

Variable disease expressivity is not uncommon in familial HCM, even within members of the same family, and it is thought to arise from genetic, epigenetic, and/or environmental factors that modify disease progression and clinical outcome.[5] To gain further insights into this phenomenon, we chose the proband (individual III.7 in Figure 1A, codenamed MYB1) and his sister (individual III.5 in Figure 1A, codenamed MYB2) as representative examples of severe and mild cases, respectively,

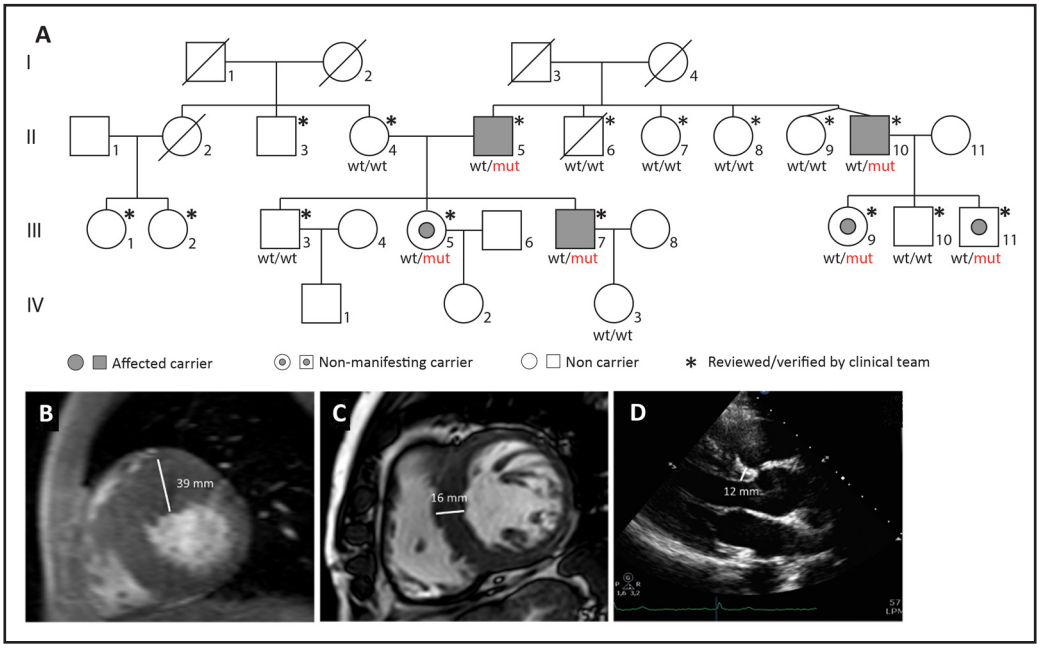

Figure 1. Description of a familial case of hypertrophic cardiomyopathy (HCM).
A, Pedigree of the HCM family. **B** and **C**, cardiac magnetic resonance imaging (CMRI) sections showing mid short-axis views from the proband III.7 (**B**) and his father II.5 (**C**), both carrying the *MYBPC3*^{K600fs} variant. **D**, Echocardiogram parasternal long-axis of the proband's sister III.5.

of familial HCM associated to *MYBPC3* gene variants. After obtaining appropriate informed consent, primary cultures of dermal fibroblasts from skin biopsies were reprogrammed to iPSCs using nonintegrative Sendai virus vectors.[28] The derived iPSC lines MYB1#4 and MYB2#2 were thoroughly characterized for pluripotency and were confirmed to carry the *MYBPC3*^{K600fs} variant in heterozygosity as expected (Figure S2A through S2L). We also included in our analyses an iPSC line previously generated from a healthy individual as an unrelated control. iPSC were differentiated into cardiomyocytes (iPSC-CMs) following a robust monolayer protocol based on small molecule modulation of the WNT signaling pathway.[29] Differentiation efficiency was similar for all iPSC lines and consistently yielded iPSC-CM cultures of purity >80%, as evaluated by coexpression of MHC (myosin heavy chain) and cTnI (cardiac troponin I) by flow cytometry (Figure 2A), and of cTnT (cardiac troponin T) and α-sarcomeric actin by immunostaining (Figure 2B).

Since myofibrillar disarray is a hallmark of HCM observed in CMs from patients and mouse models, albeit conflicting results having been reported in iPSC-CMs,[16,24] we first explored whether the sarcomere organization was affected in iPSC-CM carrying the *MYBPC3*^{K600fs} variant. Cardiomyocytes stained for the sarcomeric proteins cTnT and α-sarcomeric actin showed well aligned

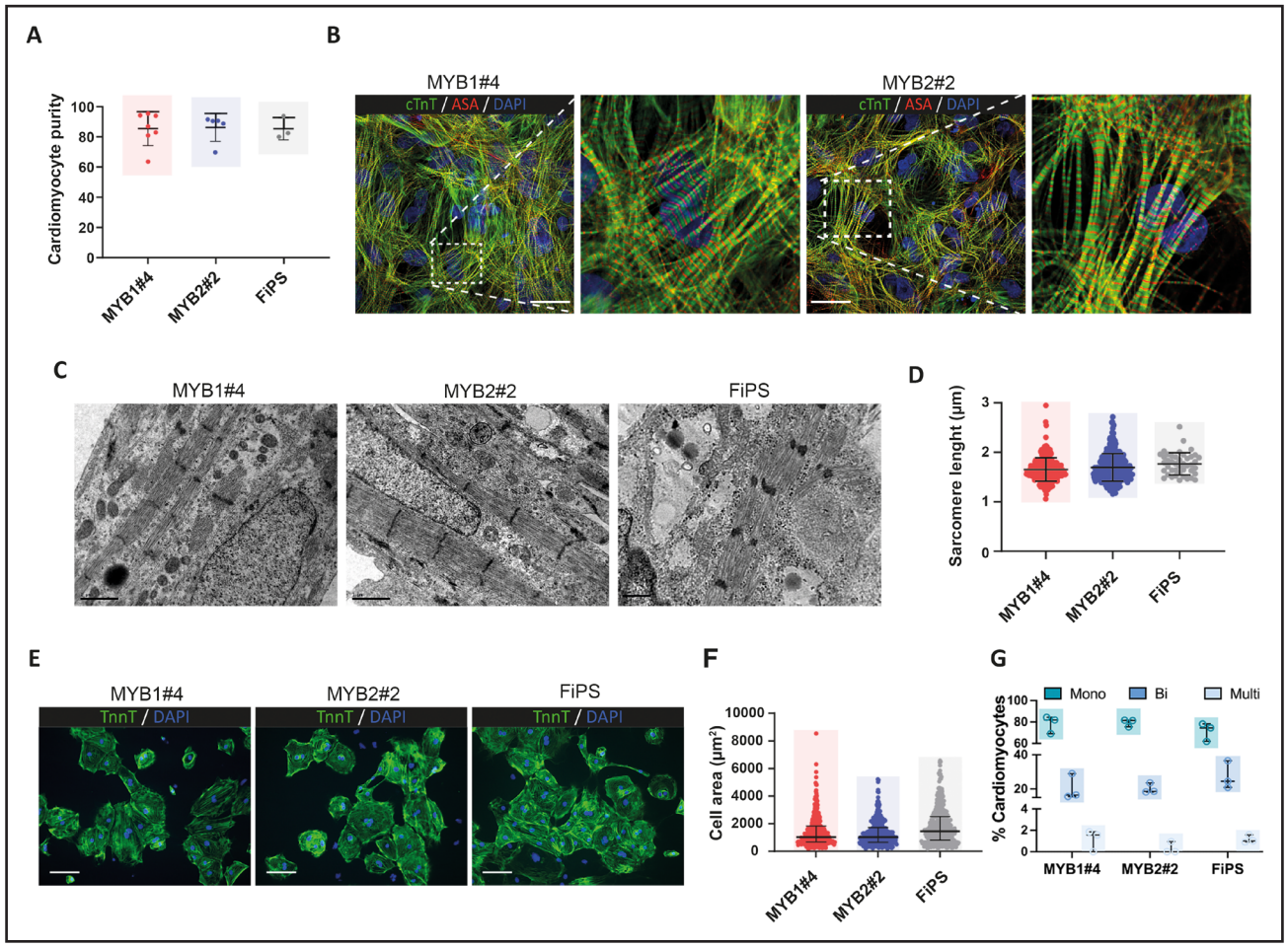

Figure 2. Generation and characterization of hypertrophic cardiomyopathy (HCM) human-induced pluripotent stem cell-cardiomyocytes (hiPSC-CMs).

A, Cardiac differentiation cultures are composed off >80% cardiomyocytes. Data from MYB1#4 (*N*=7), MYB2#2 (*N*=5), and FiPS (*N*=3). Individual data plotted, along with mean±SD. Nonparametric Kruskal-Wallis test followed by Dunn multiple comparison test. **B**, Representative immunostaining of mutant hiPSC-CMs staining positive for cTnT (cardiac troponin T) and α-sarcomeric actin (ASA), showing well aligned sarcomeres. Scale bar, 200 µm. **C**, Ultrastructural analysis of HCM mutants and controls hiPSC-CMs. Representative TEM images of cardiomyocytes after 20 days in culture. Scale bar, 1 µm. **D**, Quantification of sarcomere length. *n*=266 (MYB1#4), 324 (MYB2#2), and 43 (FiPS) sarcomeres studied. Individual data plotted, along with mean±SD. One-way ANOVA with Dunnett correction for multiple comparison test. **E**, Representative immunofluorescence images stained for cTnT from mutants and controls hiPSC-CMs. Scale bar 100 µm. **F**, Morphological analysis of single hiPSC-CMs showing quantification of cell area; *n*=362 (MYB1#4), 351 (MYB2#2) and 335 (FiPS) cells from *N*=3 independent differentiations. Individual data plotted, along with mean±SD. One-way ANOVA with Dunnett's correction for multiple comparison test. **G**, Percentage of mononucleated (mono), binucleated (bi), and multinucleated (multi) cardiomyocytes measured in *N*=3 independent differentiations. Individual data plotted, along with whisker plots. Kruskal-Wallis nonparametric test for multiple comparisons with Dunn correction between each group.

sarcomeres with no apparent structural disarray for either of the HCM mutant lines (Figure 2B). Additionally, transmission electron microscopy showed that both mutant iPSC-CMs as well as control iPSC-CMs formed organized and regular myofibrils with defined Z bands (Figure 2C) and sarcomeres of comparable lengths (Figure 2D). Similarly, we could not observe significant differences in cell size and multinucleation levels between MYB1#4 and MYB2#2 when compared with control iPSC-CMs (Figure 2E through 2G). Our results so far show that iPSC-CMs carrying the *MYBPC3*^{K600fs} variant do not recapitulate in vitro some hallmarks of HCM, such as cellular hypertrophy, increased multinucleation, and myofibrillar disarray. These results are consistent with several previous reports,[22,23,25] and most likely reflect the relative immaturity of iPSC-CMs.[30]

HCM-Related Phenotypes Caused by *MYBPC3* Gene Variant

To better ascertain genotype-phenotype relationships in our studies, we corrected the *MYBPC3*^{K600fs} variant in both MYB1#4 and MYB2#2 iPSC lines using CRISPR/Cas9 technology, generating the isogenic control iPSC lines isoMYB1#4 and isoMYB2#2, respectively (Figure S3A through S3C). HCM-causing gene variants that affect myofilament proteins deregulate ATP utilization at the sarcomere level, increasing the energetic cost of contraction.[31,32] Although whether mitochondrial energy depletion is cause or consequence of myocardial dysfunction is still a matter of debate, there is evidence in support of reduced cardiac bioenergetic performance in otherwise nonmanifesting carriers of sarcomeric mutations.[33] Moreover, mitochondrial dysfunction has more recently been shown to influence the severity of HCM-related phenotypes in iPSC-CM carrying pathogenic variants in sarcomeric genes.[34] For these reasons, we next analyzed mitochondrial metabolism in iPSC-CM carrying the *MYBPC3*^{K600fs} variant and isogenic controls using the Seahorse extracellular flux analyzer, which determines oxygen consumption rate and extracellular acidification rate upon sequential addition of electron transport chain inhibitors (Figure 3A). We could not detect differences in mitochondrial content, baseline oxygen consumption rate and extracellular acidification rate, or mitochondrial ATP production rate among the iPSC-CM tested (Figure 3B through 3E). However, maximal respiration and spare capacity were reduced in iPSC-CMs carrying the *MYBPC3*^{K600fs} variant compared with their isogenic controls and with unrelated control iPSC-CMs (Figure 3F and 3G). These results suggest that the *MYBPC3* variant forces mitochondria to function at an increased capacity to meet the elevated energetic demands of mutant CMs. It is interesting to note that a comparable degree of mitochondrial dysfunction was detected in iPSC-CMs from both *MYBPC3*^{K600fs} variant carriers, including the asymptomatic individual MYB2#2, suggesting that impaired bioenergetics could be an early pathogenic event in HCM, lending support to the energy depletion model as a common mechanism that could potentially trigger disease progression.[31]

The *MYBPC3*^{K600fs} Variant Is Not Sufficient to Explain Altered CM Excitation/Contraction

Calcium plays a key role in regulating the excitation-contraction coupling in CMs.[35] Given that alterations in intracellular calcium levels are commonly associated with HCM and can contribute to early preclinical manifestations,[15,36,37] we next sought to investigate whether iPSC-CMs from the severely affected patient and asymptomatic individual showed altered Ca^{2+} handling. For this purpose, we first recorded Ca^{2+} transients with the fluorescent Ca^{2+} indicator Fluo-4, using monolayers of iPSC-CM at 5 weeks of differentiation that were electrically paced at 0.5 Hz during imaging to avoid variation due to spontaneous beating (Figure 4A). We found that the amplitude of Ca^{2+} transients was reduced in MYB1#4-CMs when compared with MYB2#2-CMs or with isogenic controls (Figure 4B). Moreover, the kinetics of Ca^{2+} transients were significantly faster in MYB1#4-CMs compared with the other samples, exhibiting shorter times to both reaching maximum amplitude (Figure 4C) and to decaying to 50% peak (Figure 4D) and to 90% peak (Figure 4E). These findings demonstrate that iPSC-CMs from the severely affected individual show abnormal Ca^{2+} handling in vitro, for which the presence of the *MYBPC3*^{K600fs} variant is necessary, inasmuch as isogenic gene-corrected iPSC-CMs showed normal Ca^{2+} transients. However, the fact that iPSC-CM from the asymptomatic individual, also carrying the *MYBPC3*^{K600fs} variant, did not show abnormal Ca^{2+} handling, is a strong indication that this gene variant, while necessary, is not sufficient to cause the phenotype in our in vitro system.

We also analyzed the contraction kinetics of iPSC-CM monolayers using high-speed imaging and pixel-based motion analysis (Figure 4F and 4G). Examinaton of spontaneously contracting iPSC-CM monolayers at 30 days of differentiation did not reveal significant differences in beating rates or overall contraction or relaxation times among samples (Figure 4H, 4I, 4K, 4L). However, iPSC-CMs from the severely affected patient displayed a significantly shorter time to maximum contraction compared with their isogenic controls or with iPSC-CMs from the asymptomatic individual MYB2 (Figure 4J). These results are in line with our previous findings on Ca^{2+} handling and further support the notion that the *MYBPC3*^{K600fs} variant is necessary, but not sufficient, to induce measurable alterations in excitation-contraction coupling in iPSC-CMs in vitro. Moreover, these findings suggest the possibility that genetic modifiers are

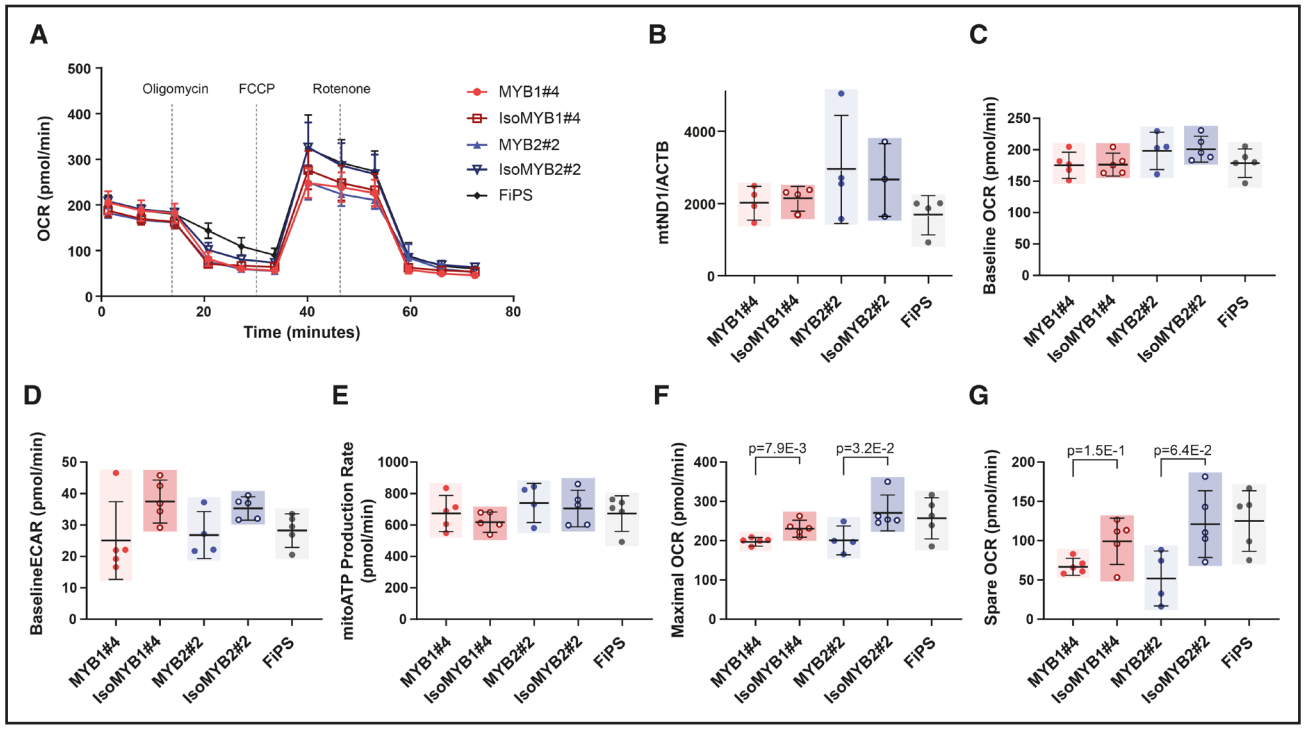

Figure 3. Bioenergetic analysis of human-induced pluripotent stem cell-cardiomyocytes (hiPSC-CMs) from *MYBPC3* mutants and isogenic controls.

A, Mitochondrial respiration rates in cardiac monolayers after the sequential addition of modulators of the respiratory chain using the Seahorse Extracellular Flux Analyzer. Data represent mean±SD. **B**, qPCR analysis showing mitochondrial (ND1):nuclear (ACTB) DNA ratio (*N*=4 for MYB1#4, IsoMYB1#4, and Iso MYB2#2, and *N*=3 for MYB2#2). **C** through **G**, Bioenergetics profile quantified for baseline OCR (**C**), ECAR (**D**), mitochondrial ATP production rate (**E**), maximal (**F**), and spare (**G**) capacity. Data were generated from *N*=5 independent differentiation batches for MYB1#4, IsoMYB1#4, and IsoMYB2#2, and from *N*=4 independent differentiation batches for MYB2#2. Individual data plotted, along with mean±SD. Mann-Whitney *U* test was used to compare isogenic pairs.

present in the genome of the severely affected patient MYB1 that cooperate with the *MYBPC3*^{K600fs} variant to bring about the hyperexcitability phenotype identified in iPSC-CMs.

The *MYH7* p.Ile1927Phe Can Be Considered a Modifier in the Context of the *MYBPC3*^{K600fs} Variant

To search for genetic modifiers that could potentiate the pathogenicity of the *MYBPC3*^{K600fs} variant in the severely affected patient MYB1, we sequenced his exome, along with those of his sister and their father (Figure 5A). Among a panel of 405 genes associated with cardiovascular diseases, we identified 2 additional rare, nonsynonymous, exonic variants that were specific to the proband and not present in the other individuals and were categorized as variants of unknown significance (VUS; Figure 5B; Table S5). The first one resulted in a heterozygous serine-to-cysteine substitution (p.Ser1801Cys) in the Calcium Voltage-Gated Channel Subunit Alpha1 D (encoded by the *CACNA1D* gene), not previously described in the general or HCM population cohorts, suggesting that this variant may not be implicated in HCM. The second variant affected *MYH7* and consisted on a previously described heterozygous isoleucine-to-phenylalanine substitution at position 1927 (p.Ile1927Phe). This residue is localized at the C-terminal end within the light meromyosin (LMM) domain of the protein. Although not a highly conserved amino acid, some *in-silico* algorithms assigned a pathogenic score to this substitution (Figure 5C). Several variants affecting the LMM domain have been associated with HCM.[38] However, a significant proportion of individuals carrying variants in the LMM region do not develop HCM, suggesting incomplete penetrance.

The *MYH7* p.Ile1927Phe is a very rare variant identified in gnomAD in 13 out of 282,718 alleles (MAF 0.005). This variant was classified as VUS in 1 patient in ClinVar and reported in the literature in a 36-year-old male with myopathy and no evidence of cardiac involvement.[39] In our laboratory, we have found it in 13 unrelated proband cases, 9 of them with HCM, but cosegregation studies in those families are still missing, thus complicating variant interpretation and resulting in the *MYH7* p.Ile1927Phe variant being currently classified as a VUS. Extending the genetic study confirmed the presence of the *MYH7* p.Ile1927Phe variant in the proband's mother (Figure 5A), who showed mild left ventricular hypertrophy with a septum of 14 mm

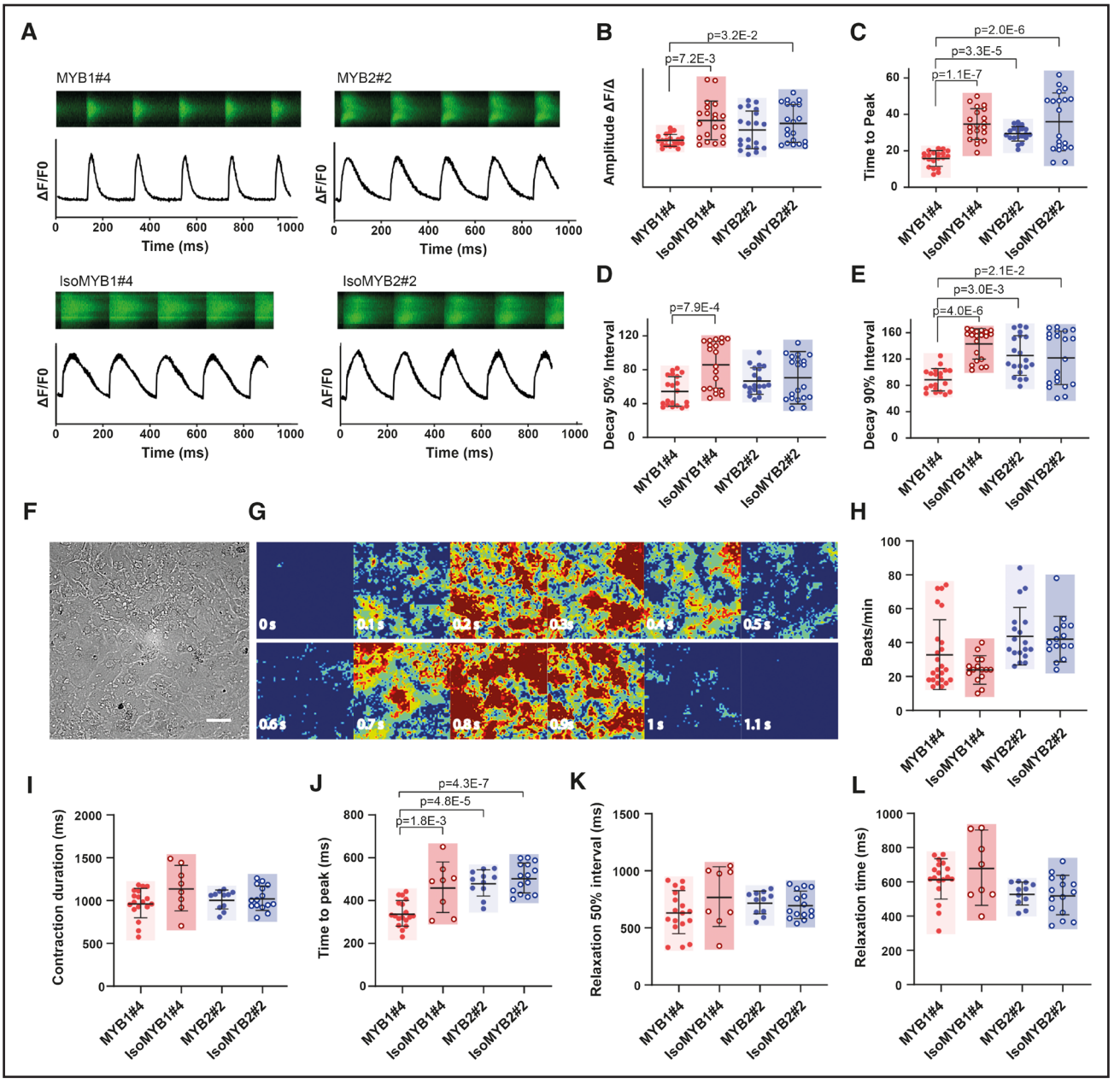

Figure 4. Calcium handling and contractile dynamics of hypertrophic cardiomyopathy (HCM) mutants and isogenic controls in induced human-pluripotent stem cell (hiPSC)-derived cardiac monolayers.

A, Representative traces and line-scan images of Fluo-4 Ca^{2+} transients from electrically paced (0.5 Hz) hiPSC-derived cardiac monolayers. **B** through **E**, Quantification of calcium handling properties in HCM mutants and isogenic corrected hiPSC-CMs showing peak amplitudes (**B**), time to reach calcium peaks (**C**), calcium decay at 50% interval (**D**) and 90% interval (**E**). Individual data plotted, along with mean±SD. *n*=20 cells in all groups. For each group in **A** through **E**, data were generated from *N*=2 independent differentiation batches and analyzed using Kruskal-Wallis nonparametric test for multiple comparisons with Dunn correction between each group. **F**, Representative bright-field image of a hiPSC-CM monolayer. Scale bar 100 µm. **G**, Vector-based contractile heatmap showing a homogeneous cardiac contraction and relaxation during a single beating event in a hiPSC-CM monolayer. **H**, Spontaneous beating rate at day 20 of cardiac differentiation. **I** through **L**, Analysis of contraction parameters from HCM mutants and isogenic edited hiPSC-CM monolayers at 5 weeks post-induction of cardiac differentiation showing quantification of contraction duration (**I**), time to maximum contraction (**J**), relaxation at 50% interval (**K**), and relaxation time (**L**). For each group in **H** through **L**, data were generated from *N*=5 independent differentiation batches for MYB1#4, *N*=4 independent differentiation batches for IsoMYB2#2, and *N*=3 independent differentiation batches for IsoMYB1#4 and MYB2#2. Individual data plotted, along with mean±SD. Data were analyzed with 1-way ANOVA with Tukey multiple comparison post hoc test.

and no fibrosis in cardiac magnetic resonance imaging (Figure 5D), all of which could be explained by arterial hypertension.

Evaluating rare and low-penetrance genetic variants by genome population studies or co-segregation analysis is frequently insufficient to produce robust evidence

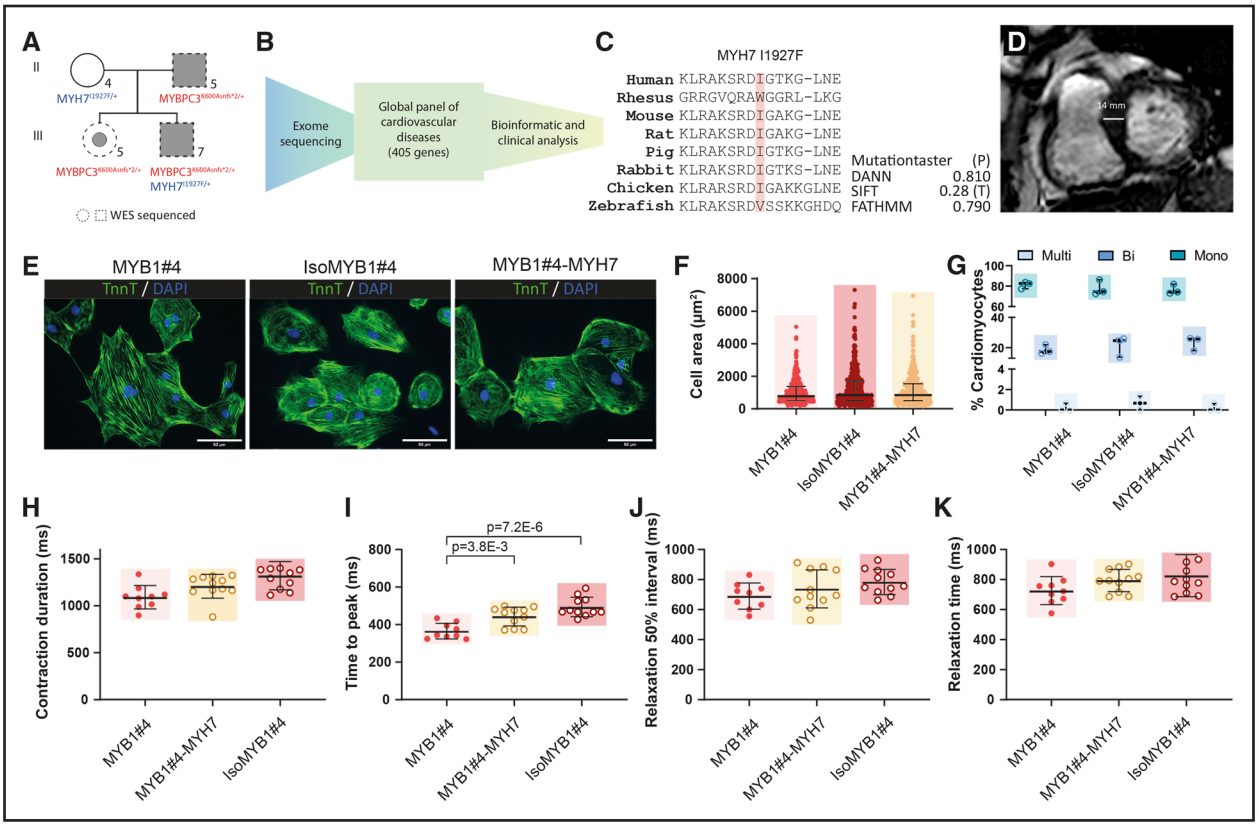

Figure 5. Identification and functional assessment of genetic modifiers of hypertrophic cardiomyopathy (HCM) associated to *MYBPC3* variant.

A, Pedigree of a subset of the HCM family under study, showing individuals subjected to whole-exome sequencing. **B**, Workflow for the identification and analysis of genetic variants following a cardiac-enriched gene panel. **C**, *MYH7* p.Ile1927Phe genetic variant identified in the symptomatic individual III.5 showing amino acid conservation across species. Variant of interest is highlighted in red. Prediction analysis of the effect of the amino acid change on protein function using Mutationtaster, DANN, SIFT and FATHMM damaging algorithms. P, pathogenic; T, tolerated. **D**, Cardiac magnetic resonance imaging (CMRI) section showing mid short-axis view from the proband's mother II.4, carrying the *MYH7* p.Ile1927Phe genetic variant. **E**, Representative immunofluorescence images stained for cTnT (cardiac troponin T) from double mutant MYB1#4 and isogenic single mutants human-induced pluripotent stem cell-cardiomyocytes (hiPSC-CMs). Scale bar 50 µm. **F)** Morphological analysis of single hiPSC-CMs showing quantification of cell area; *n*=428 (MYB1#4), 457 (IsoMYB1#4), and 452 (MYB1#4-MYH7) cells from *N*=3 independent differentiations. Individual data plotted, along with mean±SD. One-way ANOVA with Dunnett's correction for multiple comparison test. **G**, Percentage of mononucleated (mono), binucleated (bi), and multinucleated (multi) cardiomyocytes measured in *N*=3 independent differentiations. Individual data plotted, along with whisker plots. Kruskal-Wallis nonparametric test for multiple comparisons with Dunn's correction between each group. **H** through **K**, Analysis of contraction parameters from double mutant MYB1#4 and isogenic single mutants hiPSC-CMs monolayers at 5 weeks post-induction of cardiac differentiation showing quantification of contraction duration (**H**), time to maximum contraction (**I**), relaxation at 50% interval (**J**), and relaxation time (**K**). For each group in (**H**–**K**), data were generated from *N*=3 independent differentiation batches. Individual data plotted, along with mean±SD. Data were analyzed with 1-way ANOVA with Tukey multiple comparison post hoc test.

for variant interpretation. To directly assess pathogenicity of the *MYH7* p.Ile1927Phe variant, we used CRISPR/Cas9 technology to generate an additional isogenic control of MYB1#4 iPSC in which the *MYH7* variant was edited to the reference sequence, thus yielding the MYB1#4-*MYH7* iPSC line (Figure S4A and S4B). As expected, MYB1#4-*MYH7* iPSC-CMs were indistinguishable in terms of cell morphology, cell area, and percentage of multinucleation from the parental MYB1#4 iPSC-CMs or the isogenic isoMYB1#4 iPSC-CMs (Figure 5E through 5G). Also as expected, contractility analyses of cardiac monolayers did not reveal significant differences in the overall duration of the contraction or relaxation phases among samples (Figure 5H, 5J, 5K). Critically, however, the hypercontractility phenotype found in MYB1#4 iPSC-CMs as an abnormally short time to maximum contraction was rescued in both MYB1#4-*MYH7* iPSC-CMs and isoMYB1#4 iPSC-CMs, which were in turn not different from one another (Figure 5I). Taken together, these results indicate that the presence of both the *MYBPC3* and the *MYH7* variants in the severely affected MYB1#4 individual account for the hypercontractility phenotype identified in MYB1#4 iPSC-CMs.

To investigate whether the presence of the *MYH7* p.Ile1927Phe variant modifies the risk of suffering HCM at the population level, we analyzed genomic data from 30,271 consecutive probands with different inherited

Table 1. Association Between MYH7 p.Ile1972Phe Variant and Clinical Diagnosis of HCM

	Health in Code cohort		gnomAD cohort
Clinical diagnosis	HCM	Control	Control
MYH7 variant			
Reference	11 446	16 726	64 515
p.Ile1927Phe	9 (0.078%)	4 (0.023%)	8 (0.012%)
	Odds ratio	3.29	6.30
	95% CI	(1.01–10.68)	(2.50–15.90)
Significance level		P=4.7×10^{-2}	P=3.1×10^{-4}
Estimated penetrance		0.66%	1.3%
	95% CI	(0.13–3.2)	(0.34–4.8)

Number of alleles detected in the indicated cohorts. gnomAD cohort: European non-Finish population of the gnomAD v2.1.1 database. Statistical significance assessed by 2-sided Fisher exact test. Estimated penetrance calculated as described by Minikel et al.[40] HCM indicates hypertrophic cardiomyopathy.

cardiac conditions that were sequenced in Health in Code laboratory with a capture library including the *MYH7* gene (Table 1). The *MYH7* p.Ile1927Phe variant was identified in 9 out of 11,446 probands with a diagnosis of HCM, being statistically significantly enriched in this phenotype compared to probands of the same cohort without evidence of cardiomyopathy (4 out of 16726; 0.023%, P=4.7×10^{-2}), and also when compared with external controls of the gnomAD European non-Finish population (8/64515 individuals; 0.012%, P=3.1×10^{-4}). Although the variant is associated with HCM, the estimated penetrance would be very low, ranging between 0.66% and 1.3% depending on the use of internal (Health in Code) or external (gnomAD) data, respectively (Table 1). Based on our cumulative evidence, we propose that the *MYH7* p.Ile1927Phe variant can be considered as a modifier of HCM expressivity when found in combination with truncating variants in *MYBPC3*, and possibly with pathogenic variants in other sarcomeric genes as well.

DISCUSSION

In the present work, we were able to study several related individuals with a strong family history of HCM. The clinical heterogeneity frequently observed in HCM patients was also present in this family, and genetic testing and clinical assessment identified 2 siblings carrying the pathogenic *MYBPC3*^{K600fs} variant inherited from their father, but who presented with highly discordant clinical phenotypes. From a clinical point of view, patients of HCM associated to mutations in *MYBPC3* have a later disease onset compared with individuals carrying mutations in other relevant sarcomeric genes, such as *MYH7* or genes coding for thin filament proteins, with a mean age at diagnosis around the fourth decade of life.[7,41] The sister of the proband in this work was initially described as a healthy carrier at the time of the familial study, when she was 27 years old. However, during subsequent clinical evaluations, she developed a borderline phenotype with septal left ventricular hypertrophy of 12 mm in echocardiogram, by 42 years of age. It is not uncommon that index cases present with a more severe phenotype, as was the case in our HCM family. Sources of such phenotypic differences can be attributed to several factors, including environmental, epigenetic, and genetic modifiers. Gender, in particular, has been shown to be critical in explaining some of the differences in clinical presentation seen among patients with HCM, although it is still unknown whether these findings reflect a lack of gender-specific criteria for HCM diagnosis, the influence of sex hormones or sex-specific differences in cardiac physiology.[42] Here, we sought to use iPSC-based modeling to address whether and to what extent genetic modifiers could explain phenotypic diversity between these 2 siblings.

iPSC lines representing the severely affected HCM patient MYB1#4 and his asymptomatic sister MYB2#2 could be differentiated toward CMs with comparable efficiencies, which were also similar to those of control iPSC from a healthy individual. Moreover, we could not detect in those iPSC-CMs the morphological or structural abnormalities typically associated to HCM, such as increase in cell area or multinucleation index, or appearance of sarcomere disarray. These results are consistent with findings in mouse models of cardiac-specific *Mybpc3* mutation,[43,44] as well as with most recent modeling efforts using iPSC-CMs,[22,23,25,45] which have been explained by the fact that immature CMs are capable of maintaining normal levels of MyBP-C protein through compensatory reduction in MyBP-C degradation.[20,23,25] Despite the absence of overt hypertrophy-related morphological alterations, iPSC-CMs from both MYB1#4 and MYB2#2 individuals carrying the *MYBPC3*^{K600fs} variant developed mitochondrial bioenergetics alterations. The use of isogenic control iPSC-CMs in parallel allowed us to unambiguously ascribe such alterations to the presence of the *MYBPC3*^{K600fs} variant. Several previous studies have described the appearance of energetic and metabolic imbalances in iPSC-CMs carrying sarcomeric gene variants,[16,24,46,47] thus lending support to the energy depletion model that posits that energy depletion due to inefficient ATP utilization underlies HCM.[31] However, since iPSC-CMs in those studies displayed overt structural abnormalities and sarcomere disarray, it is unclear whether energy depletion was cause or consequence of HCM phenotypes. Our findings in iPSC-CMs from *MYBPC3*^{K600fs} variant carriers uncover signs of mitochondrial dysfunction in the absence of HCM-associated phenotypes, indicating that impaired bioenergetics could be an early pathogenic event in HCM. These observations are well in line with results from clinical studies showing that asymptomatic carriers of sarcomeric mutations do present bioenergetics deficits.[33]

Previous studies investigating the functionality of iPSC-CMs carrying heterozygous *MYBPC3* variants

have yielded conflicting results, some reporting abnormal calcium handling,[20] altered CM contractility,[22] or both,[15,21,48] but also alterations in calcium transients with normal contractility,[23] abnormal contractility with normal calcium handling,[24] and absence of alterations in either.[25] These discrepancies may be explained by patient-specific differences in genotype, protocol- or laboratory-specific differences in the phenotype of iPSC-CM, and differences in analytical methodology, and underscore the importance of including isogenic iPSC control lines and standardizing CM differentiation and functional testing in disease modeling efforts.[26] Our analysis of iPSC-CMs from the severely affected HCM patient MYB1#4 identified abnormally fast calcium transients and hypercontractility, both of which depended on the presence of the *MYBPC3*^{K600fs} variant. However, iPSC-CMs from the asymptomatic sibling MYB2#2, also carrying this variant, showed normal calcium handling and contractility, strongly suggesting that the *MYBPC3*^{K600fs} variant, while necessary, is not sufficient to induce functional abnormalities in iPSC-CMs in vitro.

These results prompted the search for potential genetic modifiers of HCM expressivity present in the severely affected individual. Whole-exome sequencing identified dozens of variants in probands MYB1#4 and MYB2#2 and their father, most of which shared among the 3 individuals and frequently found in the general population. We selected the *MYH7* p.Ile1927Phe variant for further characterization because it was specific to the severely affected HCM patient MYB1#4, and it implied the second-most commonly mutated gene in HCM after *MYBPC3*.[5] This variant localizes to the C-terminal end within the repetitive heptad sequence in the LMM domain, essential for thick filament assembly. According to the ClinVar database, missense variants surrounding codon 1927 in *MYH7* are mostly interpreted as VUS with no assertion criteria provided, likely due to isolated reported cases and lack of segregation and functional studies.

To address the contribution of the *MYH7* p.Ile1927Phe variant to the phenotypes observed, we used CRISPR/Cas9-mediated genome editing to generate yet another isogenic control iPSC line. In this way, we could compare in parallel iPSC-CMs carrying both the *MYBPC3*^{K600fs} and the *MYH7* p.Ile1927Phe variants (parental MYB1#4 cells), only the *MYBPC3*^{K600fs} variant (MYB1#4-*MYH7* cells), and only the *MYH7* p.Ile1927Phe variant (isoMYB1#4). These analyses allowed us to investigate the effect of the *MYH7* p.Ile1927Phe variant when occurring in combination with truncating variants in *MYBPC3*. Since we did not experimentally test the effect of the *MYH7* p.Ile1927Phe variant in genomic contexts other than that of the study proband's, we cannot formally rule out that this variant alone could be pathogenic on its own in other genomic backgrounds. However, functional and case-controls data from this study suggest that this is a variant of low penetrance, probably insufficient to be considered a classical monogenic disease-causing variant, but having a modifier effect when associated with pathogenic sarcomeric mutations. Moreover, the *MYH7* p.Ile1927Phe variant could even be considered a risk factor for developing HCM when using the concept of risk-allele put forward for other diseases such as long QT syndrome.[49] Advances in high-throughput DNA sequencing have revolutionized genetic screening and the ability to analyze genetic variation in healthy and diseased individuals.[50] Therefore, a host of novel genetic variants has been associated to HCM, often with insufficient evidence.[51,52] This ever-increasing collection of sequence changes has raised important challenges in variant interpretation.[53] For numerous rare novel variants, there is not sufficient information to ascertain pathogenicity and they are thus classified as VUS, leading to uncertainty in patients and their relatives.[9,54] Accurately determining the pathogenicity of genetic variants is a requirement for effective precision medicine of cardiomyopathies, especially when the presence of double or multiple variants can greatly modulate disease onset and clinical outcomes.[8,55,56] The Wu laboratory pioneered the combination of iPSC-based modeling and CRISPR/Cas9-mediated genome editing for evaluating pathogenicity of HCM-related VUS.[48] Our work takes this notion 1 step further and show that the inclusion of clinically discordant relatives sharing a pathogenic variant in the study is a valuable strategy to identify genetic modifiers of HCM expressivity and, at the same time, causally assess their pathogenicity.

ARTICLE INFORMATION

Received September 6, 2022; revision received May 25, 2023; accepted June 1, 2023.

Affiliations

Regenerative Medicine Program, Institut d'Investigació Biomèdica de Bellvitge - IDIBELL, L'Hospitalet de Llobregat, Spain (R.E., Y.R.-P., L.P., I.L., S.J.-D., A.M.-G., A.R.). Program for Clinical Translation of Regenerative Medicine in Catalonia – P-[CMRC], L'Hospitalet de Llobregat, Spain (R.E., Y.R.-P., L.P., I.L., S.J.-D., A.M.-G., A.R.). Center for Networked Biomedical Research on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Barcelona, Spain (R.E., Y.R.-P., L.P., A.R.). Unidad de Cardiopatías Familiares, Servicio de Cardiología, Complexo Hospitalario Universitario de A Coruña, Servizo Galego de Saúde (SERGAS) (J.M.L.-M., R.B.-V.). Instituto de Investigación Biomédica de A Coruña (INIBIC), Universidade da Coruña, A Coruña, Spain (J.M.L.-M., M.O.-G., J.P.O., R.B.-V.). Health in Code S.L., Scientific Department, A Coruña, Spain (J.P.O., L.M.). Cardiovascular Genetics Center, Biomedical Research Institute of Girona, Spain (D.C., G.J.P., R.B.). Department of Medical Sciences, Universitat de Girona, Spain (D.C., G.J.P., R.B.). Centro de Investigación Biomédica en Red de Enfermedades Cardiovasculares (CIBERCV), Spain (G.J.P., J.L.d.l.P., R.B., R.B.-V.). Intercellular Signalling in Cardiovascular Development & Disease Laboratory, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain (J.L.d.l.P.). Hospital Josep Trueta, Girona, Spain (R.B.). Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain (A.R.).

Acknowledgments

The authors are indebted to the patients, nonmanifesting carriers, and healthy individuals who agreed to participate in this study. We would like to thank the Histology and Bioimaging Platforms of IDIBELL Scientific and Technical Services for their support. The authors also thank Dr Loris Mularoni for his help depositing the sequencing data generated in the study.

Sources of Funding

The funding for this research was provided by the Spanish Ministry of Science and Innovation-MCIN (grants PID2021-123925OB-I00, PID2019-104776RB-I00, CB06/01/1056, and CB16/11/00399 financed by MCIN/AEI/10.13039/501100011033), AGAUR (2021-SGR-974), Fundació La Marató de TV3 (201534-30), Fundación BBVA (BIO14_298), Fundació Obra Social la Caixa, and CERCA Program/ Generalitat de Catalunya. The CNIC is supported by the Instituto de Salud Carlos III (ISCIII), the MCIN, and the Pro CNIC Foundation. I. Lazis was partially supported by a predoctoral fellowship from MCIN (PRE2019-087901).

Disclosures

None.

Supplemental Material

Expanded Materials and Methods
Tables S1–S6
Figures S1–S4
References 29,40, and 57–63

REFERENCES

1. Sabater-Molina M, Perez-Sanchez I, Hernandez Del Rincon JP, Gimeno JR. Genetics of hypertrophic cardiomyopathy: a review of current state. *Clin Genet*. 2018;93:3–14. doi: 10.1111/cge.13027
2. Semsarian C, Ingles J, Maron MS, Maron BJ. New perspectives on the prevalence of hypertrophic cardiomyopathy. *J Am Coll Cardiol*. 2015;65:1249–1254. doi: 10.1016/j.jacc.2015.01.019
3. Elliott PM, Anastasakis A, Borger MA, Borggrefe M, Cecchi F, Charron P, Hagege AA, Lafont A, Limongelli G, Mahrholdt H, et al; Authors/Task Force members. 2014 ESC guidelines on diagnosis and management of hypertrophic cardiomyopathy: the task force for the diagnosis and management of hypertrophic cardiomyopathy of the European Society of Cardiology (ESC). *Eur Heart J*. 2014;35:2733–2779. doi: 10.1093/eurheartj/ehu284
4. Ho CY, Day SM, Ashley EA, Michels M, Pereira AC, Jacoby D, Cirino AL, Fox JC, Lakdawala NK, Ware JS, et al. Genotype and lifetime burden of disease in hypertrophic cardiomyopathy: insights from the sarcomeric human cardiomyopathy registry (share). *Circulation*. 2018;138:1387–1398. doi: 10.1161/CIRCULATIONAHA.117.033200
5. Marian AJ, Braunwald E. Hypertrophic cardiomyopathy: genetics, pathogenesis, clinical manifestations, diagnosis, and therapy. *Circ Res*. 2017;121:749–770. doi: 10.1161/CIRCRESAHA.117.311059
6. Maron BJ, Maron MS, Semsarian C. Genetics of hypertrophic cardiomyopathy after 20 years: clinical perspectives. *J Am Coll Cardiol*. 2012;60:705–715. doi: 10.1016/j.jacc.2012.02.068
7. Lakdawala NK, Olivotto I, Day SM, Han L, Ashley EA, Michels M, Ingles J, Semsarian C, Jacoby D, Jefferies JL, et al. Associations between female sex, sarcomere variants, and clinical outcomes in hypertrophic cardiomyopathy. *Circ Genom Precis Med*. 2021;14:e003062. doi: 10.1161/CIRCGEN.120.003062
8. Alfares AA, Kelly MA, McDermott G, Funke BH, Lebo MS, Baxter SB, Shen J, McLaughlin HM, Clark EH, Babb LJ, et al. Results of clinical genetic testing of 2,912 probands with hypertrophic cardiomyopathy: expanded panels offer limited additional sensitivity. *Genet Med*. 2015;17:880–888. doi: 10.1038/gim.2014.205
9. Mazzarotto F, Olivotto I, Boschi B, Girolami F, Poggesi C, Barton PJR, Walsh R. Contemporary insights into the genetics of hypertrophic cardiomyopathy: toward a new era in clinical testing?. *J Am Heart Assoc*. 2020;9:e015473. doi: 10.1161/JAHA.119.015473
10. Repetti GG, Kim Y, Pereira AC, Ingles J, Russell MW, Lakdawala NK, Ho CY, Day S, Semsarian C, McDonough B, et al. Discordant clinical features of identical hypertrophic cardiomyopathy twins. *Proc Natl Acad Sci U S A*. 2021;118:e2021717118. doi: 10.1073/pnas.2021717118
11. Helms AS, Day SM. Hypertrophic cardiomyopathy: single gene disease or complex trait?. *Eur Heart J*. 2016;37:1823–1825. doi: 10.1093/eurheartj/ehv562
12. Itzhaki I, Maizels L, Huber I, Zwi-Dantsis L, Caspi O, Winterstern A, Feldman O, Gepstein A, Arbel G, Hammerman H, et al. Modelling the long qt syndrome with induced pluripotent stem cells. *Nature*. 2011;471:225–229. doi: 10.1038/nature09747
13. Sun N, Yazawa M, Liu J, Han L, Sanchez-Freire V, Abilez OJ, Navarrete EG, Hu S, Wang L, Lee A, et al. Patient-specific induced pluripotent stem cells as a model for familial dilated cardiomyopathy. *Sci Transl Med*. 2012;4:130ra147.
14. Kodo K, Ong SG, Jahanbani F, Termglinchan V, Hirono K, InanlooRahatloo K, Ebert AD, Shukla P, Abilez OJ, Churko JM, et al. Ipsc-derived cardiomyocytes reveal abnormal tgf-beta signalling in left ventricular non-compaction cardiomyopathy. *Nat Cell Biol*. 2016;18:1031–1042. doi: 10.1038/ncb3411
15. Wu H, Yang H, Rhee JW, Zhang JZ, Lam CK, Sallam K, Chang ACY, Ma N, Lee J, Zhang H, et al. Modelling diastolic dysfunction in induced pluripotent stem cell-derived cardiomyocytes from hypertrophic cardiomyopathy patients. *Eur Heart J*. 2019;40:3685–3695. doi: 10.1093/eurheartj/ehz326
16. Mosqueira D, Mannhardt I, Bhagwan JR, Lis-Slimak K, Katili P, Scott E, Hassan M, Prondzynski M, Harmer SC, Tinker A, et al. Crispr/cas9 editing in human pluripotent stem cell-cardiomyocytes highlights arrhythmias, hypocontractility, and energy depletion as potential therapeutic targets for hypertrophic cardiomyopathy. *Eur Heart J*. 2018;39:3879–3892. doi: 10.1093/eurheartj/ehy249
17. Gifford CA, Ranade SS, Samarakoon R, Salunga HT, de Soysa TY, Huang Y, Zhou P, Elfenbein A, Wyman SK, Bui YK, et al. Oligogenic inheritance of a human heart disease involving a genetic modifier. *Science*. 2019;364:865–870. doi: 10.1126/science.aat5056
18. Guo H, Liu L, Nishiga M, Cong L, Wu JC. Deciphering pathogenicity of variants of uncertain significance with crispr-edited ipscs. *Trends Genet*. 2021;37:1109–1123. doi: 10.1016/j.tig.2021.08.009
19. Dambrot C, Braam SR, Tertoolen LG, Birket M, Atsma DE, Mummery CL. Serum supplemented culture medium masks hypertrophic phenotypes in human pluripotent stem cell derived cardiomyocytes. *J Cell Mol Med*. 2014;18:1509–1518. doi: 10.1111/jcmm.12356
20. Monteiro da Rocha A, Guerrero-Serna G, Helms A, Luzod C, Mironov S, Russell M, Jalife J, Day SM, Smith GD, Herron TJ. Deficient cmybp-c protein expression during cardiomyocyte differentiation underlies human hypertrophic cardiomyopathy cellular phenotypes in disease specific human es cell derived cardiomyocytes. *J Mol Cell Cardiol*. 2016;99:197–206. doi: 10.1016/j.yjmcc.2016.09.004
21. Ramachandra CJA, Kp MMJ, Chua J, Hernandez-Resendiz S, Liehn EA, Knoll R, Gan LM, Michaelsson E, Jonsson MKB, Ryden-Markinhuhta K, et al. Inhibiting cardiac myeloperoxidase alleviates the relaxation defect in hypertrophic cardiomyocytes. *Cardiovasc Res*. 2022;118:517–530. doi: 10.1093/cvr/cvab077
22. Birket MJ, Ribeiro MC, Kosmidis G, Ward D, Leitoguinho AR, van de Pol V, Dambrot C, Devalla HD, Davis RP, Mastroberardino PG, et al. Contractile defect caused by mutation in mybpc3 revealed under conditions optimized for human psc-cardiomyocyte function. *Cell Rep*. 2015;13:733–745. doi: 10.1016/j.celrep.2015.09.025
23. Seeger T, Shrestha R, Lam CK, Chen C, McKeithan WL, Lau E, Wnorowski A, McMullen G, Greenhaw M, Lee J, et al. A premature termination codon mutation in mybpc3 causes hypertrophic cardiomyopathy via chronic activation of nonsense-mediated decay. *Circulation*. 2019;139:799–811. doi: 10.1161/CIRCULATIONAHA.118.034624
24. Cohn R, Thakar K, Lowe A, Ladha FA, Pettinato AM, Romano R, Meredith E, Chen YS, Atamanuk K, Huey BD, et al. A contraction stress model of hypertrophic cardiomyopathy due to sarcomere mutations. *Stem Cell Rep*. 2019;12:71–83. doi: 10.1016/j.stemcr.2018.11.015
25. Helms AS, Tang VT, O'Leary TS, Friedline S, Wauchope M, Arora A, Wasserman AH, Smith ED, Lee LM, Wen XW, et al. Effects of mybpc3 loss-of-function mutations preceding hypertrophic cardiomyopathy. *JCI Insight*. 2020;5:e133782. doi: 10.1172/jci.insight.133782
26. Li J, Feng X, Wei X. Modeling hypertrophic cardiomyopathy with human cardiomyocytes derived from induced pluripotent stem cells. *Stem Cell Res Ther*. 2022;13:232. doi: 10.1186/s13287-022-02905-0
27. Musunuru K, Sheikh F, Gupta RM, Houser SR, Maher KO, Milan DJ, Terzic A, Wu JC; American Heart Association Council on Functional Genomics and Translational Biology; Council on Cardiovascular Disease in the Young; and Council on Cardiovascular and Stroke Nursing. Induced pluripotent stem cells for cardiovascular disease modeling and precision medicine: a scientific statement from the american heart association. *Circ Genom Precis Med*. 2018;11:e000043. doi: 10.1161/HCG.0000000000000043
28. Ban H, Nishishita N, Fusaki N, Tabata T, Saeki K, Shikamura M, Takada N, Inoue M, Hasegawa M, Kawamata S, et al. Efficient generation of transgene-free human induced pluripotent stem cells (ipscs) by temperature-sensitive sendai virus vectors. *Proc Natl Acad Sci U S A*. 2011;108:14234–14239. doi: 10.1073/pnas.1103509108
29. Lian X, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, Hsiao C, Kamp TJ, Palecek SP. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating wnt/beta-catenin signaling under fully defined conditions. *Nat Protoc*. 2013;8:162–175. doi: 10.1038/nprot.2012.150
30. Tadevosyan K, Iglesias-Garcia O, Mazo MM, Prosper F, Raya A. Engineering and assessing cardiac tissue complexity. *Int J Mol Sci* . 2021;22:1479. doi: 10.3390/ijms22031479

31. Ashrafian H, Redwood C, Blair E, Watkins H. Hypertrophic cardiomyopathy:A paradigm for myocardial energy depletion. *Trends Genet.* 2003;19:263–268. doi: 10.1016/S0168-9525(03)00081-7
32. van der Velden J, Tocchetti CG, Varricchi G, Bianco A, Sequeira V, Hilfiker-Kleiner D, Hamdani N, Leite-Moreira AF, Mayr M, Falcao-Pires I, et al. Metabolic changes in hypertrophic cardiomyopathies: scientific update from the working group of myocardial function of the european society of cardiology. *Cardiovasc Res.* 2018;114:1273–1280. doi: 10.1093/cvr/cvy147
33. Crilley JG, Boehm EA, Blair E, Rajagopalan B, Blamire AM, Styles P, McKenna WJ, Ostman-Smith I, Clarke K, Watkins H. Hypertrophic cardiomyopathy due to sarcomeric gene mutations is characterized by impaired energy metabolism irrespective of the degree of hypertrophy. *J Am Coll Cardiol.* 2003;41:1776–1782. doi: 10.1016/s0735-1097(02)03009-7
34. Kargaran PK, Evans JM, Bodbin SE, Smith JGW, Nelson TJ, Denning C, Mosqueira D. Mitochondrial DNA: hotspot for potential gene modifiers regulating hypertrophic cardiomyopathy. *J Clin Med.* 2020;9:2349. doi: 10.3390/jcm9082349
35. Bers DM. Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol.* 2008;70:23–49. doi: 10.1146/annurev.physiol.70.113006.100455
36. Michels M, Soliman OI, Kofflard MJ, Hoedemaekers YM, Dooijes D, Majoor-Krakauer D, ten Cate FJ. Diastolic abnormalities as the first feature of hypertrophic cardiomyopathy in dutch myosin-binding protein c founder mutations. *JACC Cardiovasc Imaging.* 2009;2:58–64. doi: 10.1016/j.jcmg.2008.08.003
37. Liu W, Sun D, Yang J. Diastolic dysfunction of hypertrophic cardiomyopathy genotype-positive subjects without hypertrophy is detected by tissue doppler imaging: a systematic review and meta-analysis. *J Ultrasound Med.* 2017;36:2093–2103. doi: 10.1002/jum.14250
38. Homburger JR, Green EM, Caleshu C, Sunitha MS, Taylor RE, Ruppel KM, Metpally RP, Colan SD, Michels M, Day SM, et al. Multidimensional structure-function relationships in human beta-cardiac myosin from population-scale genetic variation. *Proc Natl Acad Sci U S A.* 2016;113:6701–6706. doi: 10.1073/pnas.1606950113
39. Fiorillo C, Astrea G, Savarese M, Cassandrini D, Brisca G, Trucco F, Pedemonte M, Trovato R, Ruggiero L, Vercelli L, et al; Italian Network on Congenital Myopathies. Italian Network on Congenital M. Myh7-related myopathies: clinical, histopathological and imaging findings in a cohort of italian patients. *Orphanet J Rare Dis.* 2016;11:91. doi: 10.1186/s13023-016-0476-1
40. Minikel EV, Vallabh SM, Lek M, Estrada K, Samocha KE, Sathirapongsasuti JF, McLean CY, Tung JY, Yu LP, Gambetti P, et al. Quantifying prion disease penetrance using large population control cohorts. *Sci Transl Med.* 2016;8:322ra329. doi: 10.1126/scitranslmed.aad5169
41. Sedaghat-Hamedani F, Kayvanpour E, Tugrul OF, Lai A, Amr A, Haas J, Proctor T, Ehlermann P, Jensen K, Katus HA, et al. Clinical outcomes associated with sarcomere mutations in hypertrophic cardiomyopathy: a meta-analysis on 7675 individuals. *Clin Res Cardiol.* 2018;107:30–41. doi: 10.1007/s00392-017-1155-5
42. Butters A, Lakdawala NK, Ingles J. Sex differences in hypertrophic cardiomyopathy: interaction with genetics and environment. *Curr Heart Fail Rep.* 2021;18:264–273. doi: 10.1007/s11897-021-00526-x
43. Harris SP, Bartley CR, Hacker TA, McDonald KS, Douglas PS, Greaser ML, Powers PA, Moss RL. Hypertrophic cardiomyopathy in cardiac myosin binding protein-c knockout mice. *Circ Res.* 2002;90:594–601. doi: 10.1161/01.res.0000012222.70819.64
44. Pohlmann L, Kroger I, Vignier N, Schlossarek S, Kramer E, Coirault C, Sultan KR, El-Armouche A, Winegrad S, Eschenhagen T, et al. Cardiac myosin-binding protein c is required for complete relaxation in intact myocytes. *Circ Res.* 2007;101:928–938. doi: 10.1161/CIRCRESAHA.107.158774
45. Ma Z, Huebsch N, Koo S, Mandegar MA, Siemons B, Boggess S, Conklin BR, Grigoropoulos CP, Healy KE. Contractile deficits in engineered cardiac microtissues as a result of mybpc3 deficiency and mechanical overload. *Nat Biomed Eng.* 2018;2:955–967. doi: 10.1038/s41551-018-0280-4
46. Bhagwan JR, Mosqueira D, Chairez-Cantu K, Mannhardt I, Bodbin SE, Bakar M, Smith JGW, Denning C. Isogenic models of hypertrophic cardiomyopathy unveil differential phenotypes and mechanism-driven therapeutics. *J Mol Cell Cardiol.* 2020;145:43–53. doi: 10.1016/j.yjmcc.2020.06.003
47. Toepfer CN, Garfinkel AC, Venturini G, Wakimoto H, Repetti G, Alamo L, Sharma A, Agarwal R, Ewoldt JF, Cloonan P, et al. Myosin sequestration regulates sarcomere function, cardiomyocyte energetics, and metabolism, informing the pathogenesis of hypertrophic cardiomyopathy. *Circulation.* 2020;141:828–842. doi: 10.1161/CIRCULATIONAHA.119.042339
48. Ma N, Zhang JZ, Itzhaki I, Zhang SL, Chen H, Haddad F, Kitani T, Wilson KD, Tian L, Shrestha R, et al. Determining the pathogenicity of a genomic variant of uncertain significance using crispr/cas9 and human-induced pluripotent stem cells. *Circulation.* 2018;138:2666–2681. doi: 10.1161/CIRCULATIONAHA.117.032273
49. Giudicessi JR, Roden DM, Wilde AAM, Ackerman MJ. Classification and reporting of potentially proarrhythmic common genetic variation in long qt syndrome genetic testing. *Circulation.* 2018;137:619–630. doi: 10.1161/CIRCULATIONAHA.117.030142
50. Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, O'Donnell-Luria AH, Ware JS, Hill AJ, Cummings BB, et al; Exome Aggregation Consortium. Analysis of protein-coding genetic variation in 60,706 humans. *Nature.* 2016;536:285–291. doi: 10.1038/nature19057
51. Ingles J, Goldstein J, Thaxton C, Caleshu C, Corty EW, Crowley SB, Dougherty K, Harrison SM, McGlaughon J, Milko LV, et al. Evaluating the clinical validity of hypertrophic cardiomyopathy genes. *Circ Genom Precis Med.* 2019;12:e002460. doi: 10.1161/CIRCGEN.119.002460
52. Walsh R, Buchan R, Wilk A, John S, Felkin LE, Thomson KL, Chiaw TH, Loong CCW, Pua CJ, Raphael C, et al. Defining the genetic architecture of hypertrophic cardiomyopathy: re-evaluating the role of non-sarcomeric genes. *Eur Heart J.* 2017;38:3461–3468. doi: 10.1093/eurheartj/ehw603
53. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, et al; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the american college of medical genetics and genomics and the association for molecular pathology. *Genet Med.* 2015;17:405–424. doi: 10.1038/gim.2015.30
54. Manrai AK, Funke BH, Rehm HL, Olesen MS, Maron BA, Szolovits P, Margulies DM, Loscalzo J, Kohane IS. Genetic misdiagnoses and the potential for health disparities. *N Engl J Med.* 2016;375:655–665. doi: 10.1056/NEJMsa1507092
55. Girolami F, Ho CY, Semsarian C, Baldi M, Will ML, Baldini K, Torricelli F, Yeates L, Cecchi F, Ackerman MJ, et al. Clinical features and outcome of hypertrophic cardiomyopathy associated with triple sarcomere protein gene mutations. *J Am Coll Cardiol.* 2010;55:1444–1453. doi: 10.1016/j.jacc.2009.11.062
56. Ingles J, Doolan A, Chiu C, Seidman J, Seidman C, Semsarian C. Compound and double mutations in patients with hypertrophic cardiomyopathy: implications for genetic testing and counselling. *J Med Genet.* 2005;42:e59. doi: 10.1136/jmg.2005.033886
57. Carrio M, Mazuelas H, Richaud-Patin Y, Gel B, Terribas E, Rosas I, Jimenez-Delgado S, Biayna J, Vendredy L, Blanco I, et al. Reprogramming captures the genetic and tumorigenic properties of neurofibromatosis type 1 plexiform neurofibromas. *Stem Cell Rep.* 2019;12:411–426. doi: 10.1016/j.stemcr.2019.01.001
58. Valls-Margarit M, Iglesias-Garcia O, Di Guglielmo C, Sarlabous L, Tadevosyan K, Paoli R, Comelles J, Blanco-Almazan D, Jimenez-Delgado S, Castillo-Fernandez O, et al. Engineered macroscale cardiac constructs elicit human myocardial tissue-like functionality. *Stem Cell Rep.* 2019;13:207–220. doi: 10.1016/j.stemcr.2019.05.024
59. Sala L, van Meer BJ, Tertoolen LGJ, Bakkers J, Bellin M, Davis RP, Denning C, Dieben MAE, Eschenhagen T, Giacomelli E, et al. Musclemotion: a versatile open software tool to quantify cardiomyocyte and cardiac muscle contraction in vitro and in vivo. *Circ Res.* 2018;122:e5–e16. doi:10.1161/CIRCRESAHA.117.312067
60. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the crispr-cas9 system. *Nat Protoc.* 2013;8:2281–2308. doi: 10.1038/nprot.2013.143
61. Calatayud C, Carola G, Fernandez-Carasa I, Valtorta M, Jimenez-Delgado S, Diaz M, Soriano-Fradera J, Cappelletti G, Garcia-Sancho J, Raya A, et al. Crispr/cas9-mediated generation of a tyrosine hydroxylase reporter ipsc line for live imaging and isolation of dopaminergic neurons. *Sci Rep.* 2019;9:6811. doi: 10.1038/s41598-019-43080-2
62. Garcia-Aznar JM, Ramirez N, De Una D, Santiago E, Monserrat L. Whole exome sequencing provides the correct diagnosis in a case of osteopathia striata with cranial sclerosis: case report of a novel frameshift mutation in amer1. *J Pediatr Genet.* 2021;10:139–146. doi: 10.1055/s-0040-1710058
63. Ochoa JP, Sabater-Molina M, Garcia-Pinilla JM, Mogensen J, Restrepo-Cordoba A, Palomino-Doza J, Villacorta E, Martinez-Moreno M, Ramos-Maqueda J, Zorio E, et al. Formin homology 2 domain containing 3 (fhod3) is a genetic basis for hypertrophic cardiomyopathy. J *Am Coll Cardiol.* 2018;72:2457–2467. doi: 10.1016/j.jacc.2018.10.001