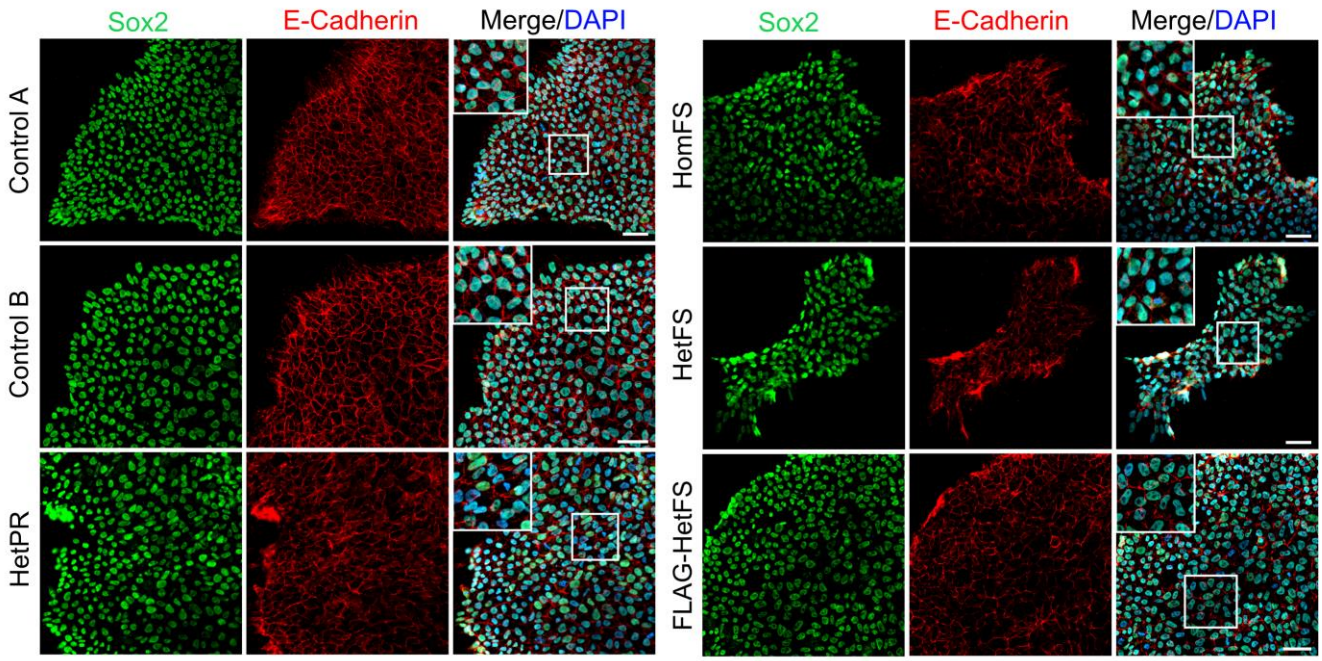
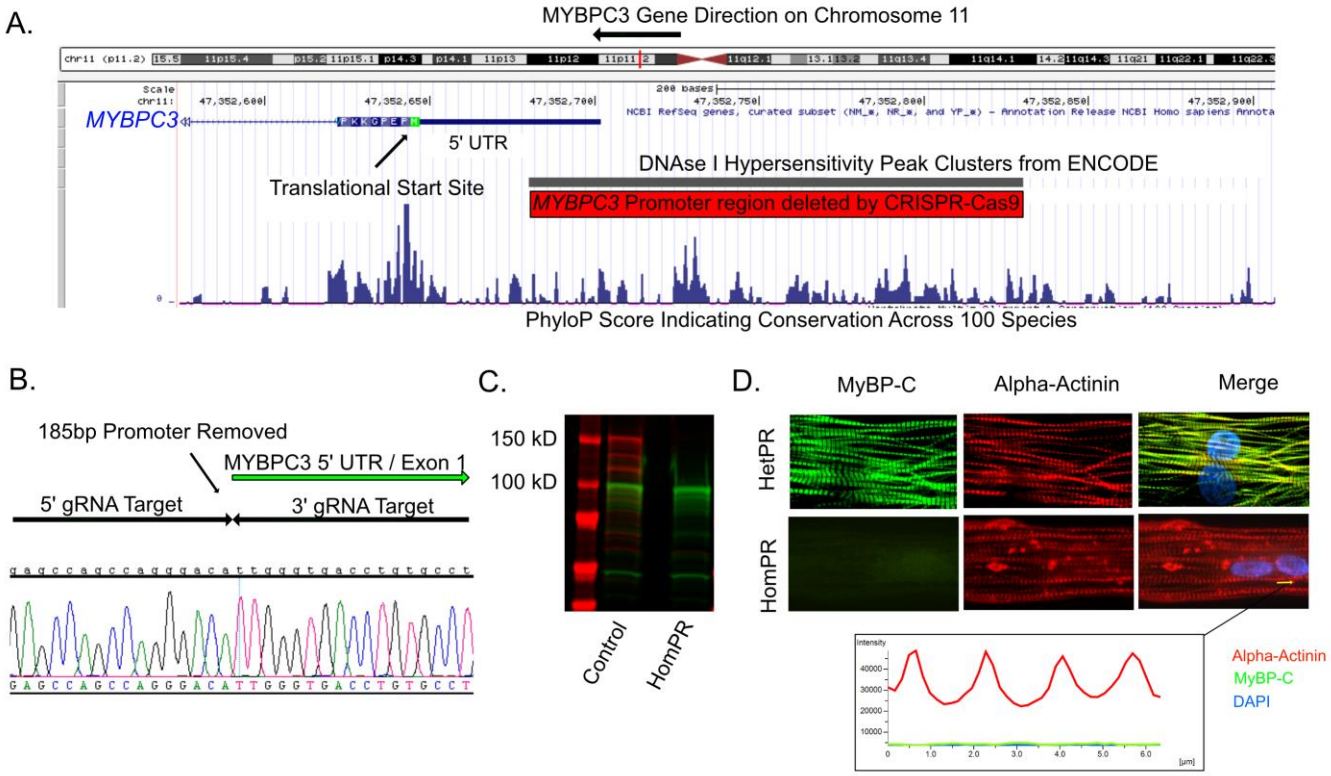
Supplemental Data

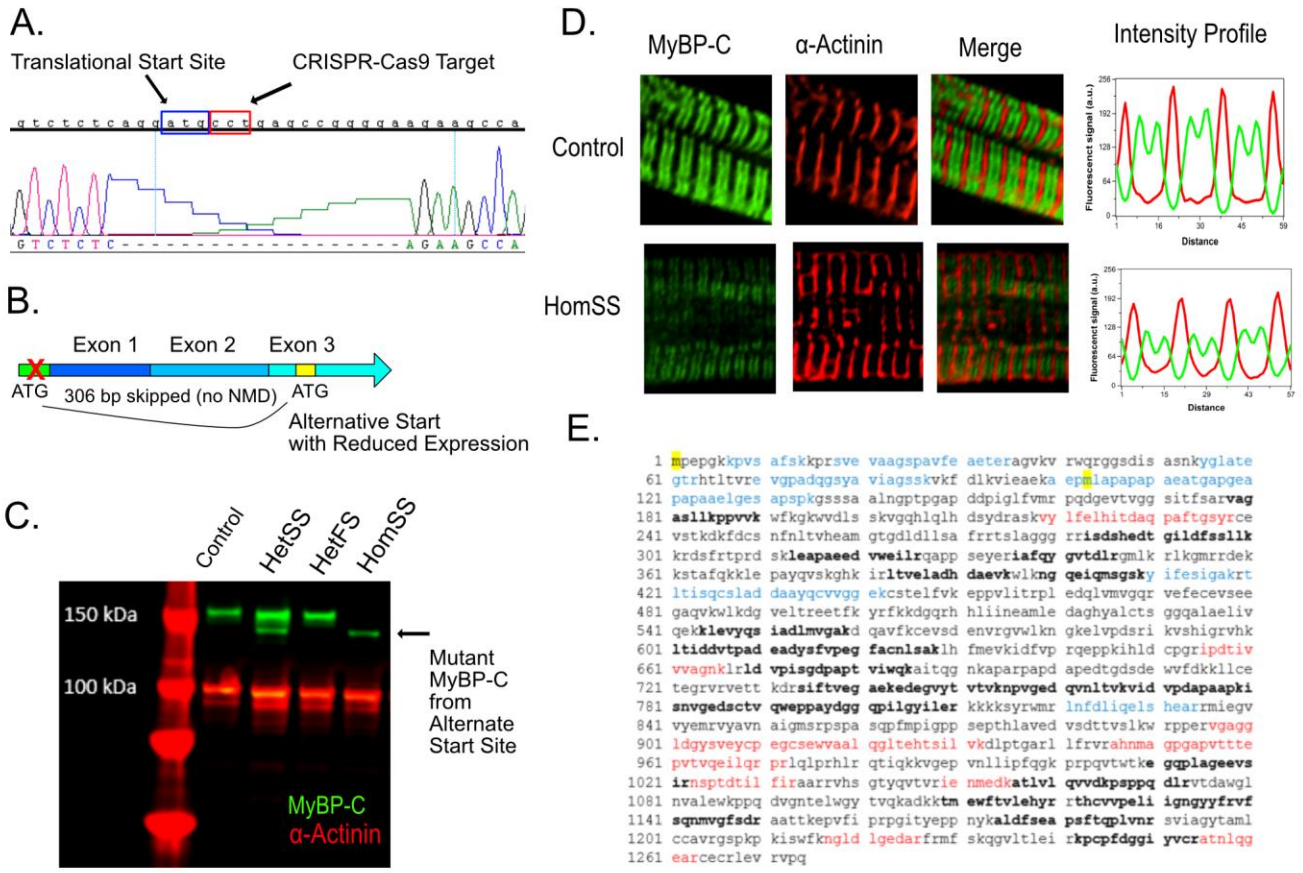
Effects of *MYBPC3* Loss of Function Mutations Preceding Hypertrophic Cardiomyopathy



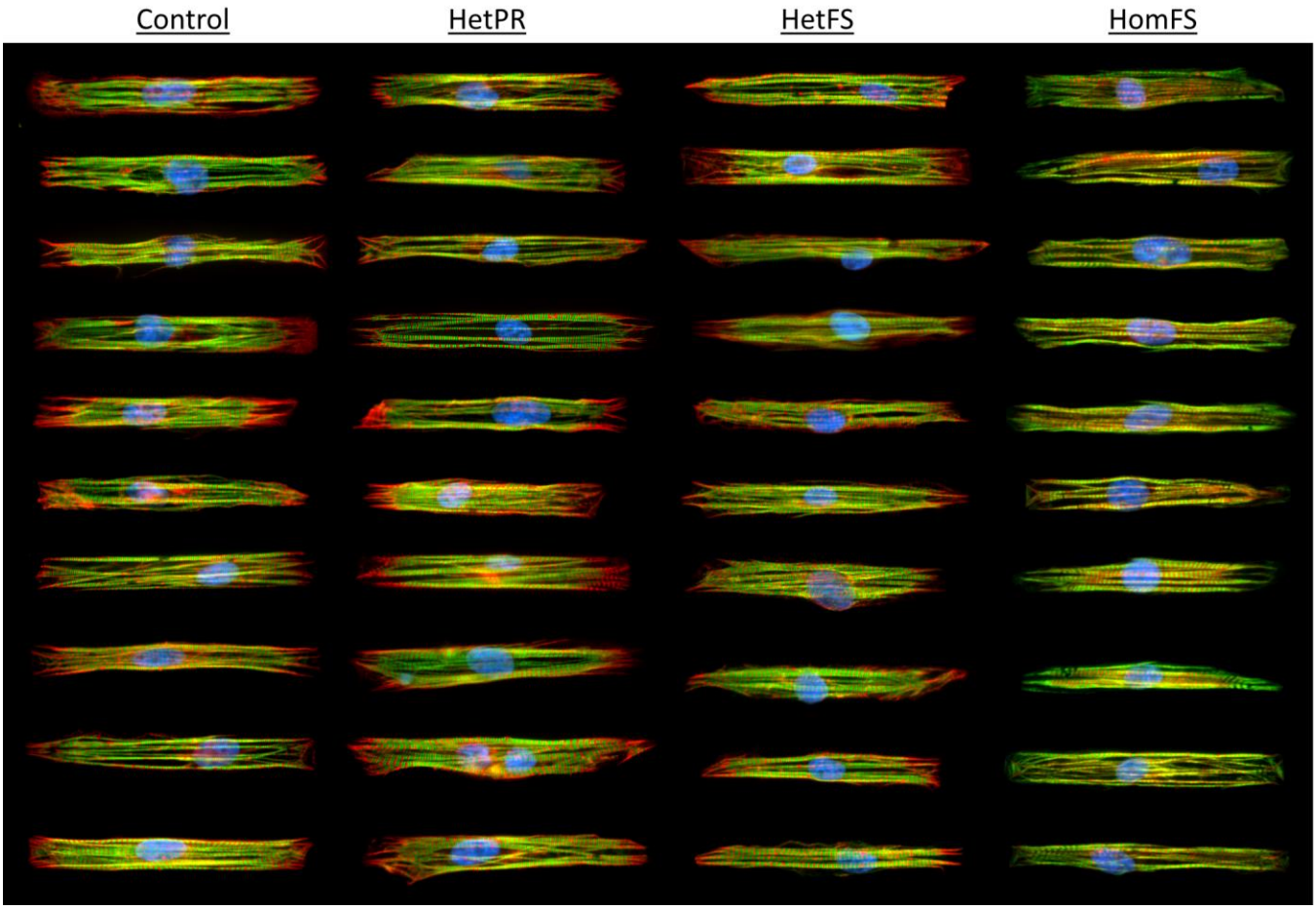
Supplemental Figure 1. Demonstration of Pluripotency in Genome-Edited iPSC Lines. Immunofluorescent staining demonstrates robust expression of pluripotency markers in gene-edited iPSC lines. Control A is the original iPSC line (DF19-9-11). Control B is a non-targeted control line derived from the same gene-editing experiment as HetFS and HomFS, but no mutations were verified at the gRNA targeting site. iPSC clones were co-stained for both Sox2 and E-Cadherin, demonstrating uniform staining throughout the iPSC colonies in all genome-edited iPSC lines.



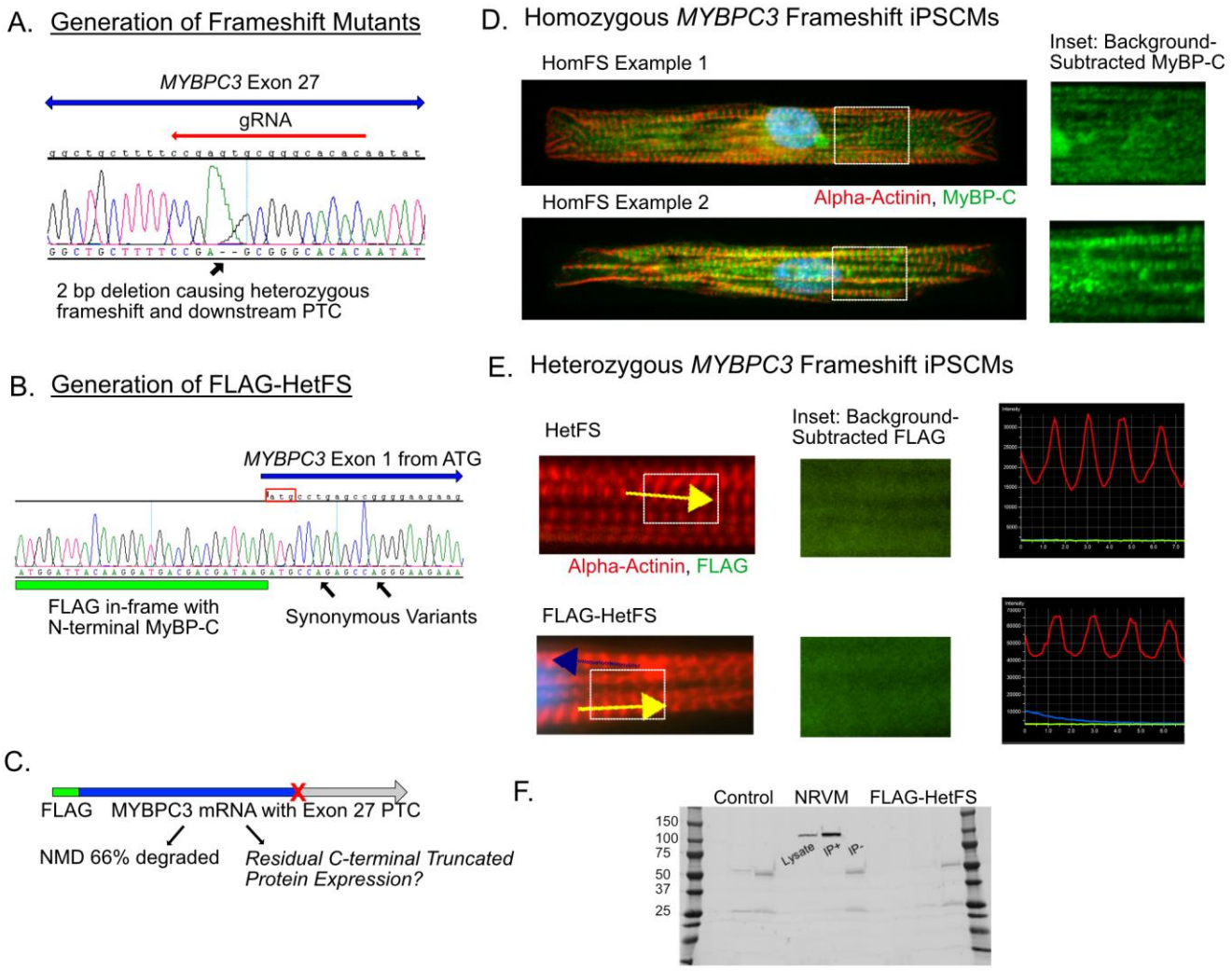
Supplemental Figure 2. Generation and Validation of Heterozygous and Homozygous *MYBPC3* Promoter Deletion iPSC Lines. The probable promoter region of *MYBPC3* was determined through identification of the DNAse I sensitive region 5' of the gene using the UCSD genome browser. The phyloP score, indicative of sequence conservation across 100 species, was used as a second confirmation of the probable location of the promoter. Guide RNAs targeting both the 5' and 3' ends of the region designated by the red bar were selected and co-transfected with Cas9 to delete the entire promoter. **B.** Sanger sequencing of gel-extracted PCR products confirmed the 185-bp promoter deletion for both heterozygous (HetPR) and homozygous (HomPR) iPSC lines. **C.** Western blot showing lack of MyBP-C expression (full length MyBP-C at ~150 kD) in the homozygous promoter deletion line HomPR (red=anti-MyBP-C polyclonal, anti-C0-M domains[1]; green=anti-alpha-actinin). **D.** Immunofluorescence imaging of MyBP-C and alpha-actinin demonstrates absence of MyBP-C in the HomPR iPSCMs, validating a complete lack of MyBP-C expression from promoter-deleted alleles. The inset shows an intensity profile of the immunofluorescence along a myofibrillar bundle, demonstrating no significant fluorescence signal for MyBP-C. Heterozygous promoter deletion (HetPR) iPSCMs have no apparent loss of MyBP-C localization by immunofluorescence.



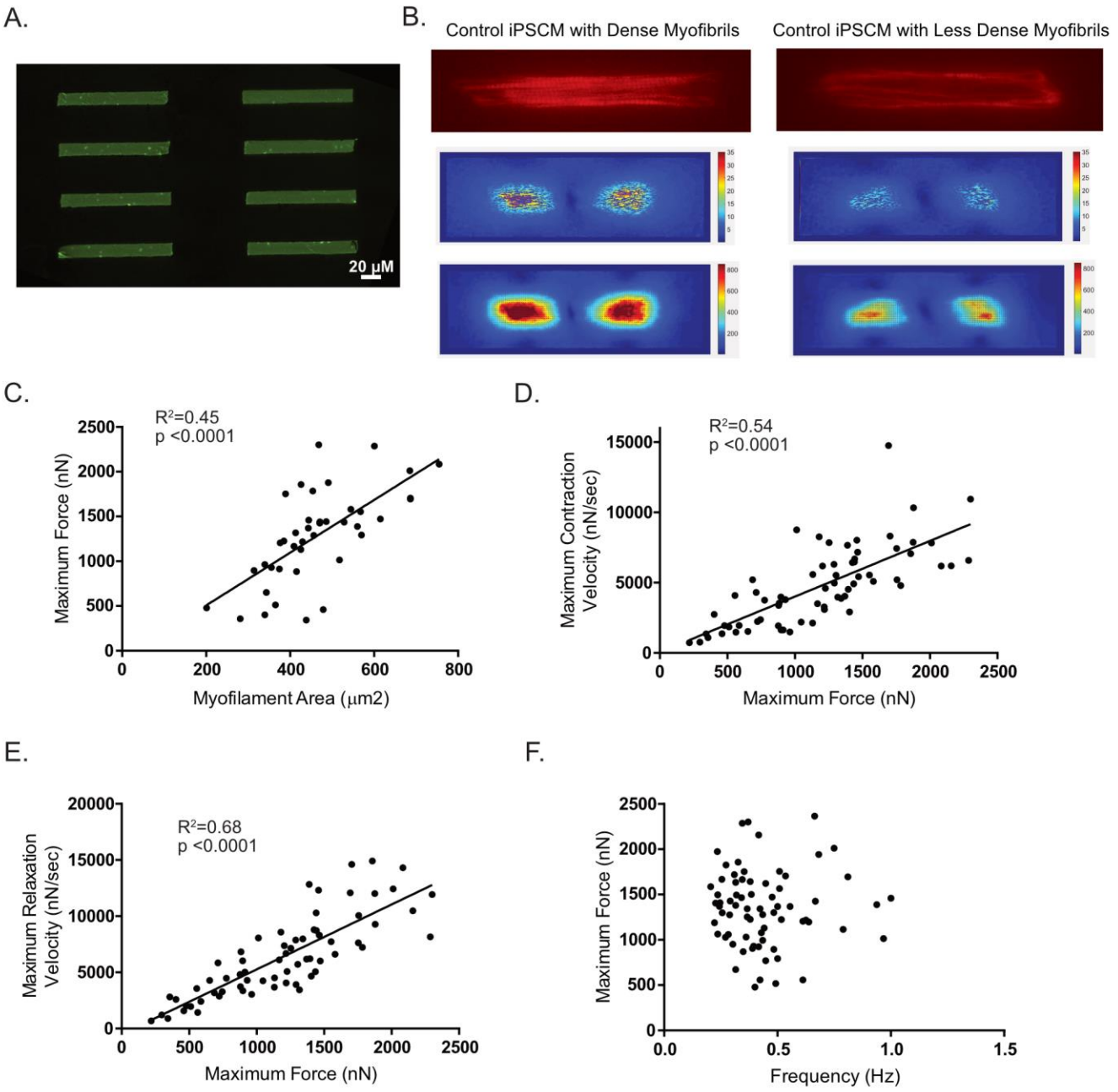
Supplemental Figure 3. Generation of a Translational Start Site Disrupted *MYBPC3* iPSC Line That Expresses Low-Level Mutant MyBP-C from an Alternative Start Site. A. The *MYBPC3* translational start site was targeted using a single guide RNA binding in proximity to the ATG. Sanger sequencing of TOPO-cloned PCR product from a successfully targeted clone demonstrates deletion of the translational start site in the heterozygous line used for subsequent studies (HetSS). **B.** Although loss of expression from start site deleted mRNA was predicted, mutant MyBP-C from an alternative downstream start site was detected at low-level in this iPSCM line. The diagram depicts the observed expression pattern from this clone – lack of full length MyBP-C from start site mutant alleles but a low level of expression of MyBP-C from an alternative start site 306 base pairs downstream. **C.** Western blot detection of mutant MyBP-C (~130 kD) at low level of expression compared to the wild-type expression in the HetSS line, with absent wild-type and exclusive mutant MyBP-C in the HomSS line (MyBP-C detected with a polyclonal antibody that detects anti-C0-M domains[1]). **D.** The HomSS line shows a low level of normally-localized MyBP-C compared to control iPSCMs. The N-terminal-shortened mutant MyBP-C retains actin and myosin binding regions. **E.** Mass spectroscopy of the full length (top band in panel C) and alternative start site mutant MyBP-C (bottom band in panel C) gel bands extracted from a protein gel from the HetSS line demonstrates only peptides from the full length protein in the N-terminal region (blue = peptides identified in the full length band only; red = peptides identified in the N-terminal truncated band only; bold = peptides identified in both bands; yellow highlighted = start site and alternative start site).



Supplemental Figure 4. Myofibrillar Alignment and Density are Similar Across Otherwise Isogenic Mutant *MYBPC3* iPSCMs. Ten examples of micropatterned iPSCMs from gene-edited lines used for assessment of myofibrillar alignment and quantification of myofibrillar number in Figure 2. All iPSCM lines exhibit robust myofibrillar alignment in response to the geometric cues of the micropatterns. All lines also exhibit variability in myofibrillar density among individual cells without an apparent increase in myofibril density in *MYBPC3* mutant iPSCMs (as quantified in Figure 2). For heterozygous iPSCMs: Green = MyBP-C (anti-C0-M domains), red = alpha-actinin, blue = DAPI. For the homozygous frameshift line (HomFS), an antibody for TNNT2 was used instead of MyBP-C for assessment of myofibrils since wild-type MyBP-C was absent. Note that one each of the control and HetPR iPSCMs in this figure are the same as in Figure 2C.

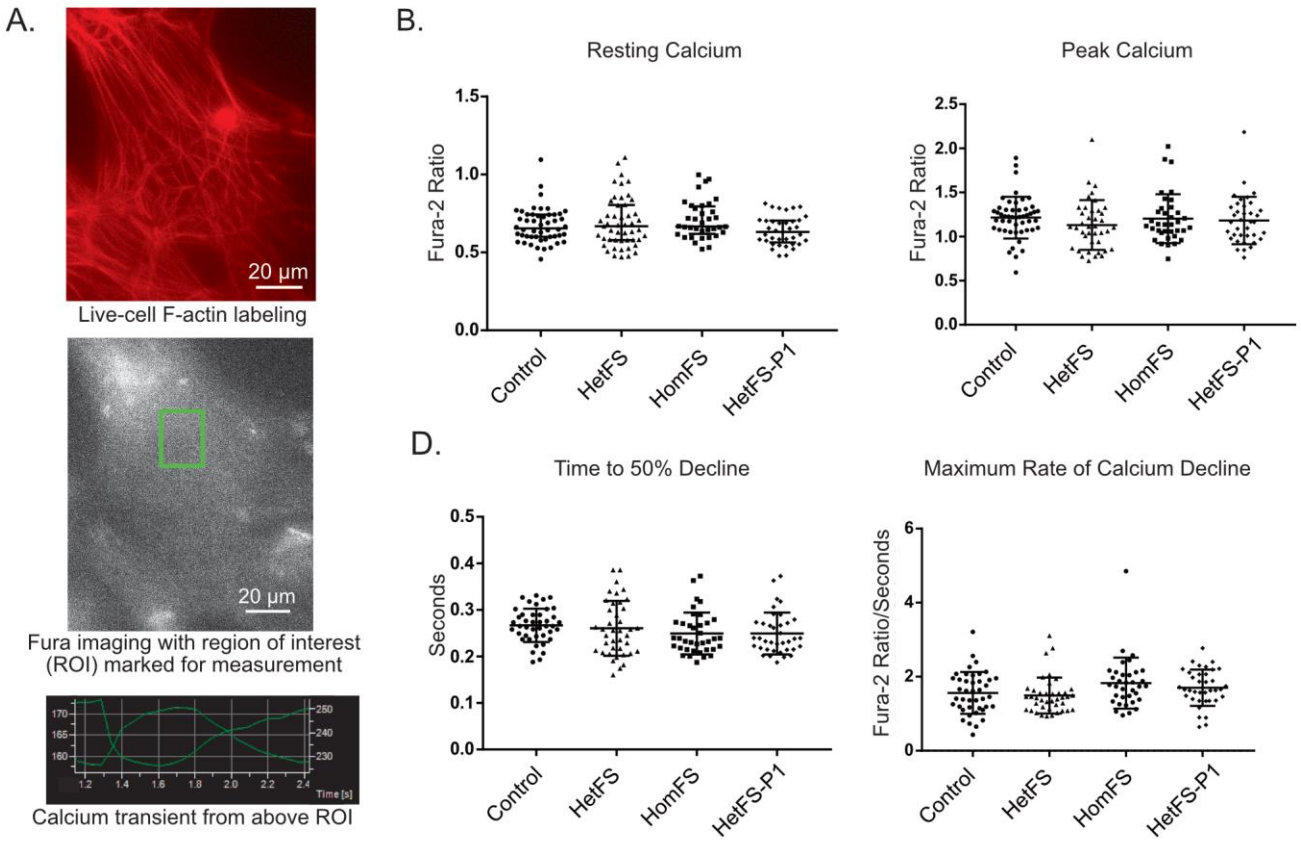


Supplemental Figure 5. Generation of Frameshift Mutant *MYBPC3* iPSCMs. A. The HetFS and HomFS iPSC lines were generated using a guide RNA targeting exon 27 to create frame shift mutations causing downstream premature termination codons, thus rendering the mutant allele mRNA susceptible to NMD and also creating a possibility of C-terminal truncated MyBP-C. **B.** A second round of gene-editing was performed on the HetFS line to generate an N-terminal FLAG epitope tagged line by using a guide RNA targeting exon 1, introducing an N-terminal FLAG sequence previously shown to be robustly expressed with *MYBPC3*.[2] Synonymous variants were introduced into the FLAG oligonucleotide repair template used to generate this line, to prevent CAS9 cleavage of successfully targeted and repaired clones. Sanger sequencing confirms a successfully targeted clone from gel-extracted PCR product. **C.** Diagram depicts the purpose of the FLAG-tagged HetCT line – to test whether the residual mutant mRNA not degraded by NMD is sufficient to cause a detectable level of C-terminal truncated MyBP-C expression. **D.** In the homozygous frameshift iPSCMs (HomFS), no significant MyBP-C was detected using the same microscopy settings as in control and heterozygous mutant iPSCMs (as in Figure 2C), consistent with <0.5% MyBP-C present by mass spectroscopy (Figure 1B). However, with background subtraction as shown here, it was possible to detect a low-level signal that localizes to myofibrils using an MyBP-C antibody (anti-C0-M domains), indicating the presence of very low-level MyBP-C in the homozygous line. **E.** No truncated mutant MyBP-C is detected in the FLAG-labeled heterozygous frameshift mutant iPSCMs by immunofluorescence, even with background subtraction and signal amplification. No truncated mutant MyBP-C is detected by immunoprecipitation of MyBP-C using an antibody to FLAG, with the positive control being FLAG-labeled C-terminal truncated MyBP-C overexpressed in neonatal rat ventricular myocytes using an adenovirus construct.



Supplemental Figure 6. Micropatterning and Traction Force Method Development. A. Polyacrylamide gels were functionalized with hydroxyl groups by addition of bis-acrylamide as developed by Grevesse, et al. to enable adherence of matrix proteins by direct microcontact printing.[3, 4] Verification of precise protein transfer is demonstrated here following microcontact printing with fluorescently-labeled gelatin onto the polyacrylamide gel substrate. **B.** Since we initially found substantial variability in contractile force among micropatterned control iPSCMs, we performed live-cell staining with an F-actin dye. Shown here are two examples of control iPSCMs with different densities of myofibrils. The iPSCM with denser myofibrils (top panel) imposes greater traction stress on the underlying gel substrates (middle panel shows fluorescent beads displacement measurements; bottom panel shows calculated traction force heat map (dark red regions are areas of high force development at ends of the cell, contracting toward the center). **C.** The amount of myofibrils per iPSCM, measured here as myofibrillar area by F-actin staining, correlated with the force development of the iPSCMs. **D-E.** In control iPSCMs, the maximum contraction velocity and maximum

relaxation velocity were correlated with the maximum force development. Therefore, the maximum contraction and relaxation velocities were normalized to maximum force for comparisons across mutant iPSCM lines. **F.** Spontaneous contraction frequency of the iPSCMs did not correlate with maximum force development.



Supplemental Figure 7. MYBPC3 Mutations do not Perturb Calcium Handling Dynamics in iPSCMs. A. Calcium dynamics were quantified using Fura-2 for mutant and control iPSCMs cultured as small multicellular 2D tissues on polyacrylamide gels. To ensure consistency across calcium measurements in iPSCMs, live-cell F-actin imaging was first performed to identify iPSCMs exhibiting robust contractions, and regions of interest that contained >80% density of myofibrils were selected for quantification. 360/380 fluorescence measurements were performed for these regions with high myofibrillar density to achieve high signal:noise. **B-E.** Resting calcium, peak calcium, time to 50% decline, and max rate of calcium decline were all similar across the *MYBPC3* mutant and control iPSCMs.

Original Cell Line	Line Name	Location Targeted	gRNAs	gRNA 2	Genotype	Number Alleles	Mutation Effect
DF19-9-11	Control A	NA	NA	NA	Control	NA	NA
DF19-9-11	Control B	Exon 27	gcctgttaccaccacggagc	NA	Non-targeted control	NA	NA
DF19-9-11	HetPR	promoter	ggagccagccagggacaatg	gggcacaggtcacccaaaga	Whole promoter deletion	Heterozygous	Loss of expression from one allele
DF19-9-11	HomPR	promoter	ggagccagccagggacaatg	gggcacaggtcacccaaaga	Whole promoter deletion	Homozygous	Loss of expression from both alleles
DF19-9-11	HetSS	Exon 1	gctggcttcttccccggctc	NA	Translational start site deletion	Heterozygous	mRNA from one allele has lack of normal translational start site. Reduced expression from 3' alternative start site. No premature termination codon so mutant mRNA not subject to NMD.
DF19-9-11	HomSS	Exon 1	gctggcttcttccccggctc	NA	Deletion immediately 5' of translational start site on both alleles.	Homozygous	Predicted to lack expression completely, but demonstrated to express a low level of MyBP-C from translational re-initiation at the next downstream in-frame ATG in exon 3.
DF19-9-11	HetFS	Exon 27	gcatattgtgtgcccgcact	NA	2bp deletion in exon 27	Heterozygous	Frame-shift mutation leading to premature termination codon on one allele
DF19-9-11	HomFS	Exon 27	gcctgttaccaccacggagc	NA	8bp deletion and 4bp insertion in exon 27	Homozygous	Frame-shift mutation leading to premature termination codon on both alleles
DF19-9-11	FHetCT	Exon 1 & 27	gctggcttcttccccggctc	NA	Flag in phase with exon 27 mutation in HetCT	Heterozygous	Flag epitope tag added in-frame and on same allele as the exon 27 mutation in the HetFS line
HCM-Leiden 1	HetFS-P1	Exon 24	NA	NA	c.2373insG	Heterozygous	Frame-shift mutation leading to premature termination codon on one allele
HCM-Leiden 2	HetFS-P2	Exon 24	NA	NA	c.2373insG	Heterozygous	Frame-shift mutation leading to premature termination codon on one allele

Supplemental Table 1. iPSC Lines

Gene	FOR primer	REV primer	Region Amplified
MYBPC3	gctcccaccgtacaatttgc	ccccaattgtagacaccccc	Promoter
MYBPC3	gtccctctttgggtgacctg	agaactccctcctagccctg	Exon 1
MYBPC3	atactggtgaaggacctgcc	gagcttgggtactgtggctt	Exon 27

Supplemental Table 2. Primer Sequences

Expanded Mass Spectroscopy Methods

Prior to mass spectroscopy, pelleted iPSCMs were thawed and solubilized in 75 µl 0.1% RapiGest SF Surfactant (Waters Corporation) at 50°C for 1 hour. The samples were reduced by adding 5 µl of 0.1M dithiothreitol and heating (100°C, 10 min), then alkylated with 10.4 µl of 100 mM iodoacetamide in 50 mM ammonium bicarbonate with incubation in the dark (22°C, 30 min). The proteins were digested into tryptic peptides with the addition of 25 µl of 0.2 µg/µl trypsin (Promega) and incubating (37°C, 18 hours). The samples were dried down in a speed vacuum device. 100 µl of 7% formic acid in 50 mM ammonium bicarbonate was added to deactivate the trypsin and cleave the RapiGest and heated (37°C, 1 hour). The sample was dried down again. 100 µl of 0.1% trifluoroacetic acid was added and heated (37°C, 1 hour) to cleave the RapiGest again. The resultant peptides were dried down and reconstituted for a final time in 60 µl of 0.1% trifluoroacetic acid. The tubes were then centrifuged at 14,000 rpms for 5 minutes to pellet the surfactant. The top 55 µl of solution was transferred into a mass spectrometry (MS) analysis vial.

Peptides were separated by liquid chromatography and their abundances were measured by mass spectrometry. A 20 µl of each sample was injected onto an Acquity UPLC HSS T3 column (100 Å, 1.8 µm, 1 × 150 mm) (Waters Corporation) attached to a UltiMate 3000 ultra-high pressure liquid chromatography (UHPLC) system (Dionex). The UHPLC effluent was directly infused into a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer through an electrospray ionization source (Thermo Fisher Scientific). Data were collected in data dependent MS/MS mode with the top five most abundant ions being selected for fragmentation. The resultant .RAW files containing the mass spectra were processed using the SEQUEST algorithm in the Proteome Discoverer 2.2 (PD 2.2) software package (Thermo Fisher Scientific). The MS/MS spectra were matched to theoretical spectra generated using the human proteome database (downloaded from UniProt 2/15). Variable mass changes accounting for the loss of methionine from the N-terminus of each protein with the addition of acetylation (-89.16 Da), the carbamidomethylation of cysteine (57.02 Da), oxidation of methionine and proline (M, P; 15.99 Da: M; 32.00 Da) and phosphorylation serine, threonine and

tyrosine (79.98 Da) were added. The area of the LCMS peak for each peptide detected in the MS/MS spectra were determined using PD 2.2. The Minora Feature Detector was enabled to allow for quantification of LC peak areas for peaks lacking MS/MS spectra in a .RAW files but having the same exact mass, charge state, elution time, and isotope pattern as those detected in other .RAW files. MyBP-C relative abundance was quantified by using the average of the counts for the 3 most abundant MyBP-C peptides ([R].LTVELADHDAEVK.[W], [K].VIDVPDAPAAPK.[I], [R].IPDTIVVVAGNK.[L]) normalized to the average of the counts for the 3 most abundant myosin peptides shared between the alpha and beta isoforms ([K].DTQIQLDDAVR.[A], [K].EALISQLTR.[G], [R].AQLEFNQIK.[A]). Using the most abundant peptides for both MyBP-C and myosin resulted in the expected molar ratio of ~0.10 in control samples, similar to our previous report from human heart tissue.[5]

Protein synthesis and degradation were quantified by determining the incorporation of stable isotope labeled amino acids into proteins using mass isotopomer analysis.[6] Cryopreserved iPSCMs from 3 different batches for each of the lines tested (control, HetFS-P1, HetFS-P2) were thawed and pooled together for each line at the beginning of the experiment to synchronize all subsequent time points. After combining iPSCMs for each line from thawing, iPSCMs were resuspended in glucose-free RPMI with B27 supplement with an oxidative phosphorylation supplement to minimize the possibility of non-cardiomyocyte growth during the experiment (consisting of galactose, lactate, glutamax, and pyruvate to achieve final media concentrations of 4 mM, 4 mM, 2 mM, and 0.5 mM, respectively). 24 hours after plating, the cells were rinsed with PBS, and the media was changed to identical media except without the 2% FBS present in the replating media and with addition of deuterated leucine (L-Leucine-5,5,5-d3, Sigma) at an equivalent concentration of standard RPMI (0.05 mg/ml) to attain a final 50:50 ratio of labeled to native leucine isotopes. The media was subsequently changed every 48 hours. Duplicate samples were collected at each of the designated time points for each cell line, rinsed with PBS containing protease inhibitors (Roche), and frozen at -80 C until processing. The samples were prepared and analyzed by LCMS. The abundance of each mass isotopomer in the LCMS peaks of interest

was determined using Pinpoint Software (Thermo Fisher Scientific) and distribution analysis was performed using Excel (Microsoft). The MyBP-C M+3 and M+0 (unlabeled) isotopomers were then quantified for each time point.

Absolute isotopmomer abundances of interest were normalized using the summed isotopomer abundance determined from 10 histone peptides present in each sample. For MyBP-C synthesis quantification, the abundances of the M+3 isotopomer at T=0 in each sample was subtracted from all time points and this corrected abundance was used to approximate protein synthesis in each sample. For MyBP-C degradation quantification, the M+3 values were used to correct the M+0 (unlabeled) values to account for the 50% D_3-leucine label. The corrected M+0 values were then used to approximate MyBP-C decay in each sample. The synthesis data were scaled so that the total amount of protein at T=0 was equal for each of the duplicate sample sets. The decay data were scaled so that the total amount of protein at T=12 hours was equal for each of the duplicate sample sets. The decay data was quantified beginning at T=12 hours following labeling since MyBP-C levels decreased acutely following withdrawal of 2% serum in the replating media despite myosin remaining relatively constant – this relative decrease was similar in control (70%) versus HCM iPSCMs (83% in HetFS-P1, 62% in HetFS-P2), indicating a mutation-independent susceptibility to degradation with serum withdrawal.

Supplemental Material References

1. Lee K, Harris SP, Sadayappan S, Craig R. Orientation of myosin binding protein c in the cardiac muscle sarcomere determined by domain-specific immuno-em. *J Mol Biol.* 2015;427:274-286. doi: 210.1016/j.jmb.2014.1010.1023. Epub 2014 Nov 1016.
2. Glazier AA, Hafeez N, Mellacheruvu D, Basrur V, Nesvizhskii AI, Lee LM, Shao H, Tang V, Yob JM, Gestwicki JE, Helms AS, Day SM. Hsc70 is a chaperone for wild-type and mutant cardiac myosin binding protein c. *JCI Insight.* 2018;3(11).99319. doi: 99310.91172/jci.insight.99319. eCollection 92018 Jun 99317.
3. Grevesse T, Versaevel M, Circelli G, Desprez S, Gabriele S. A simple route to functionalize polyacrylamide hydrogels for the independent tuning of mechanotransduction cues. *Lab Chip.* 2013;13:777-780. doi: 710.1039/c1032lc41168g. Epub 42013 Jan 41118.
4. Grevesse T, Versaevel M, Gabriele S. Preparation of hydroxy-paam hydrogels for decoupling the effects of mechanotransduction cues. *J Vis Exp.* 2014;(90).10.3791/51010.
5. O'Leary TS, Snyder J, Sadayappan S, Day SM, Previs MJ. Mybpc3 truncation mutations enhance actomyosin contractile mechanics in human hypertrophic cardiomyopathy. *J Mol Cell Cardiol.* 2019;127:165-173.:10.1016/j.yjmcc.2018.1012.1003. Epub 2018 Dec 1011.
6. Hellerstein MK, Neese RA. Mass isotopomer distribution analysis: A technique for measuring biosynthesis and turnover of polymers. *Am J Physiol.* 1992;263:E988-1001. doi: 1010.1152/ajpendo.1992.1263.1005.E1988.