

Supplemental Material

Functional interrogation of contextually-correct *MYH7* variants using CRaTER-flox gene editing and contractility profiling

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Conflicts of Interest

CEM is a scientific founder and equity holder in StemCardia and equity holder in Sana Biotechnology.

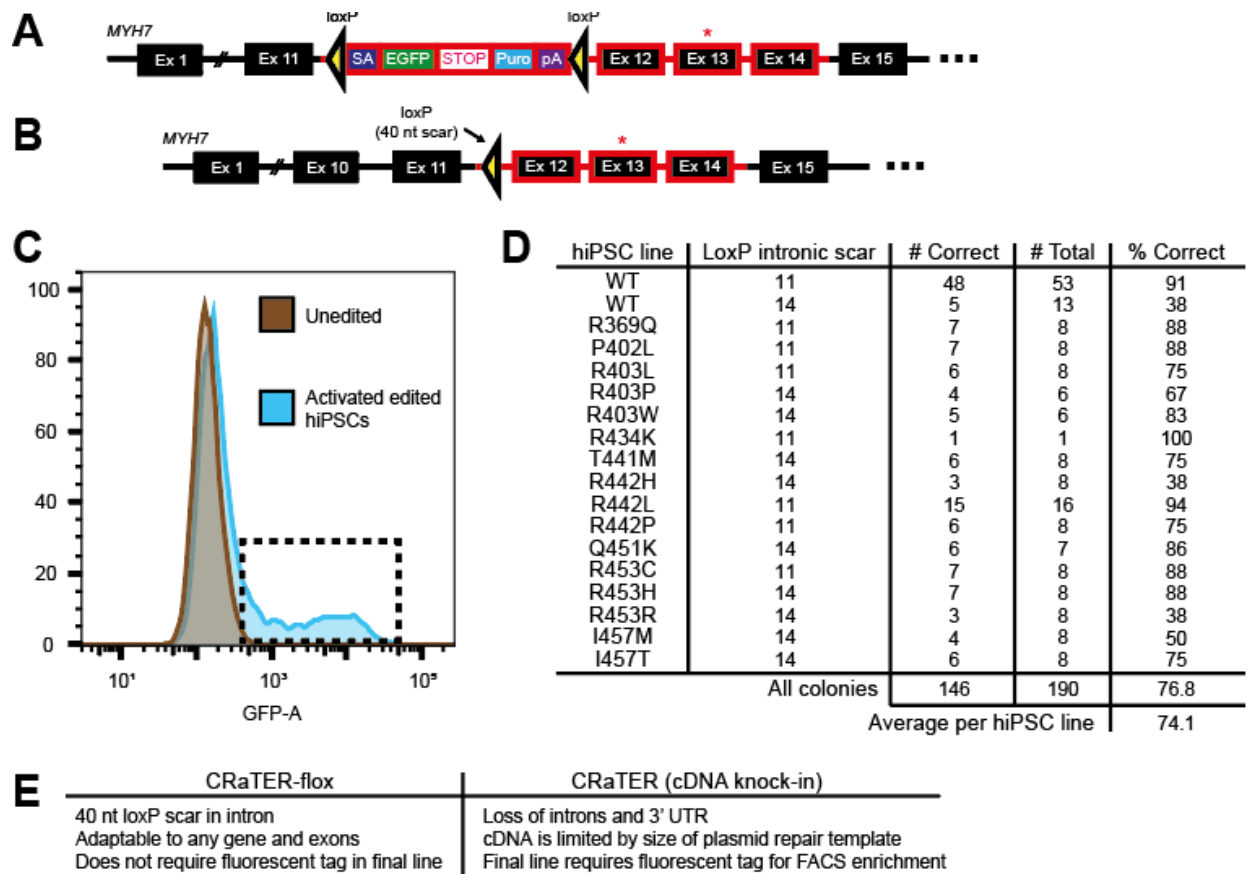


Figure S1: CRaTER-flox editing enables efficient near-scarless generation of variant hiPSCs. **(A)** Schematic of *MYH7* with donor DNA in red knocked in. **(B)** Schematic of edited *MYH7* allele after Cre recombination. **(C)** FACS enrichment of EGFP⁺ hiPSCs after transient CRISPR activation of *MYH7*. Brown: unedited WTC11 *CLYBL*^{dCas9-VPR/dCas9-VPR} *MYH7*^{WT/WT} hiPSCs; blue: WTC11 *CLYBL*^{dCas9-VPR/dCas9-VPR} *MYH7*^{EGFP-R403W/WT} hiPSCs prior to Cre recombination. Boxed region: EGFP⁺ hiPSC population with (A) genotype; sorted for subsequent Cre recombination. **(D)** CRaTER-flox editing efficiency for generation of variant hiPSC lines. **(E)** Comparison between CRaTER-flox and CRaTER cDNA knock-in gene-editing strategies.

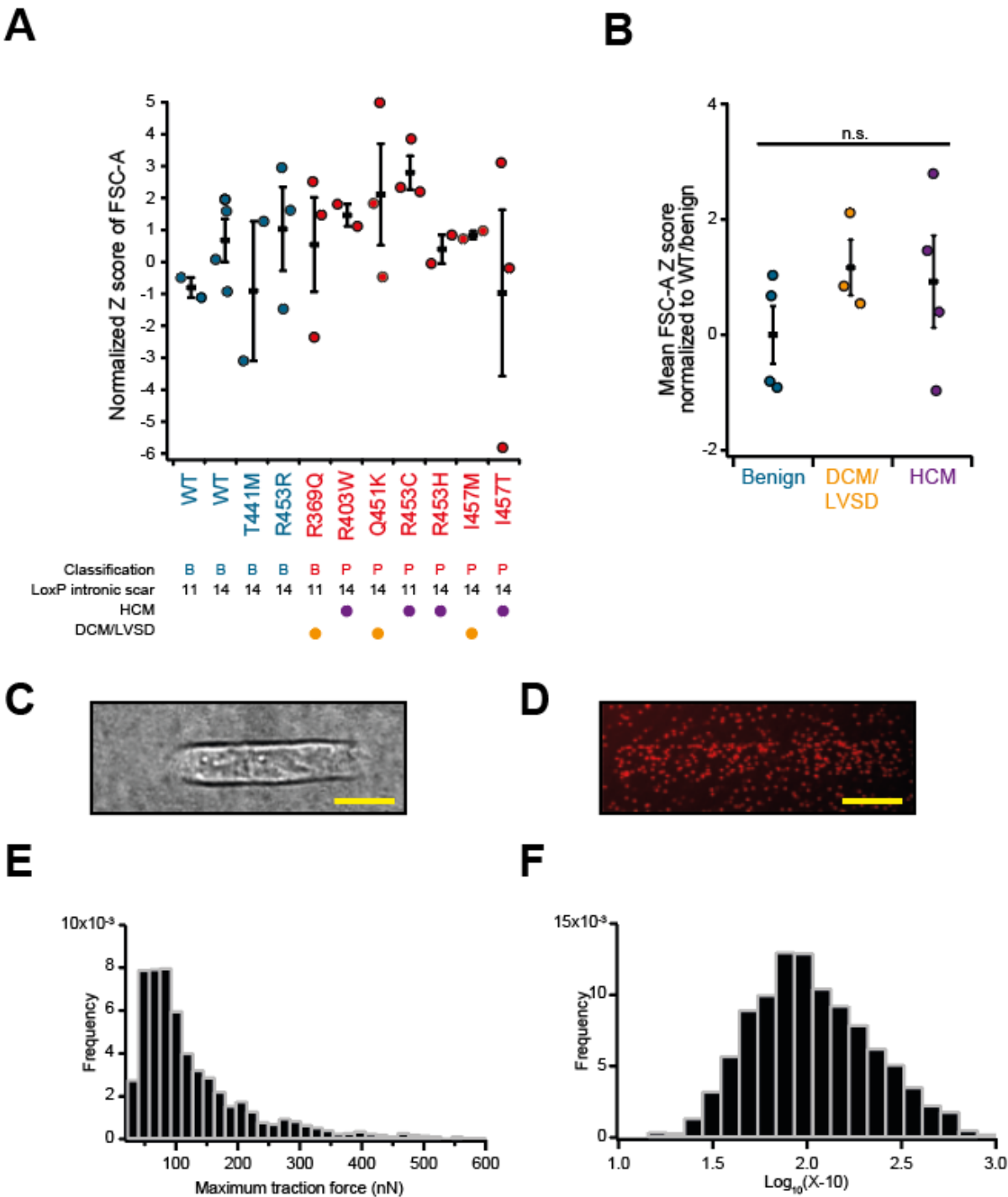


Figure S2: Phenotypic assays to interrogate *MYH7* variant effect in hiPSC-CMs. **(A)** FSC-A Z scores normalized to distribution of control means, n = 2-4 biological replicates per

33 line, $n \geq 10,000$ cells per replicate. Blue: benign/likely benign (B); red: pathogenic/likely
34 pathogenic (P). Circle: variant-associated cardiomyopathy as reported in ClinVar or (4).
35 **(B)** Mean FSC-A Z scores for benign, DCM / LV systolic dysfunction (LVSD), and HCM
36 lines; n.s.: not significant Kruskal-Wallis test at $\alpha = 0.05$. **(C)** Representative brightfield
37 image of hiPSC-CM on patterned hydrogel, 40x objective, scale bar 25 μm . **(D)** Confocal
38 microscopy image of 500 nm red fluorescent beads 5 μm below hydrogel surface
39 corresponding with (E), 40x objective, scale bar 25 μm . **(E)** Combined histogram of
40 maximum traction force values for all tested *MYH7* exon 12-14 lines, $n = 1534$ cells. **(F)**
41 Combined histogram of maximum traction force values for all tested *MYH7* exon 12-14
42 lines after $\log_{10}(X-10)$ transformation to convert to a normal distribution, $n = 1534$ cells.

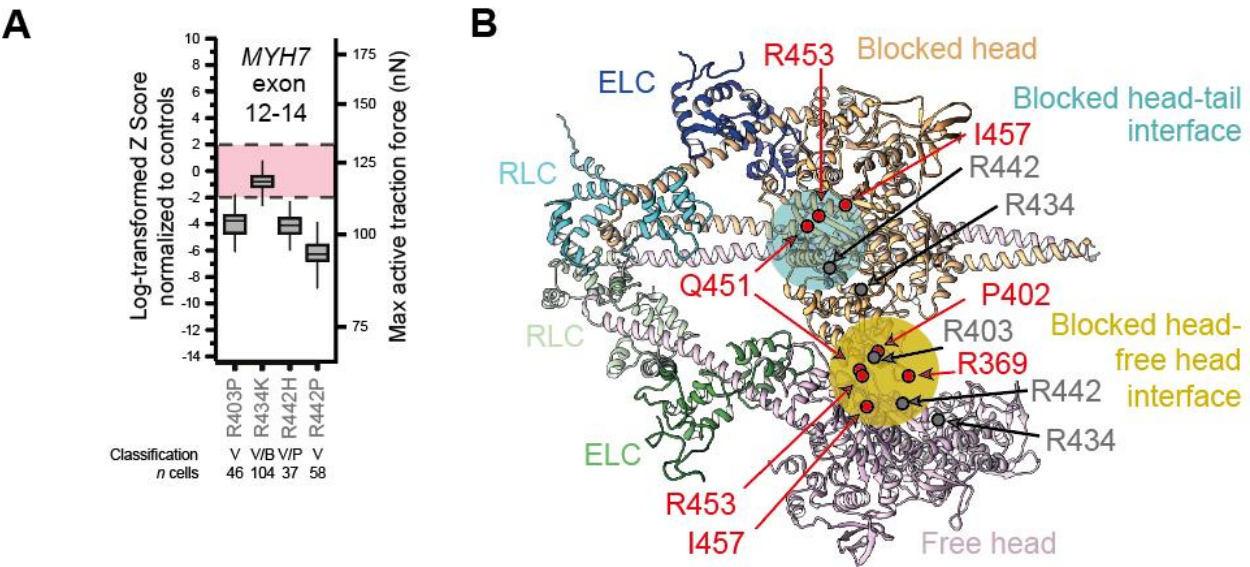


Figure S3: Functional assessment and structural contextualization of *MYH7* VUS. (A) Mechanical assessment of *MYH7* VUS in CRaTER-flox edited hiPSC-derived cardiomyocytes. Log-transformed maximum hiPSC-CM active traction force Z scores normalized to benign lines and raw force values. Box: upper and lower quartiles; midline: median; whiskers: 1.5x IQR. V: VUS; B: likely benign; P: likely pathogenic. R434K has conflicting assertions (VUS, likely benign); R442H has conflicting assertions (VUS, likely pathogenic). (B) Ribbon structure of a two-headed myosin with essential light chain (ELC) and regulatory light chain (RLC) in the interacting heads motif (IHM) conformation (Protein Database: 8ACT). Gray: VUS residues; red: pathogenic residues.

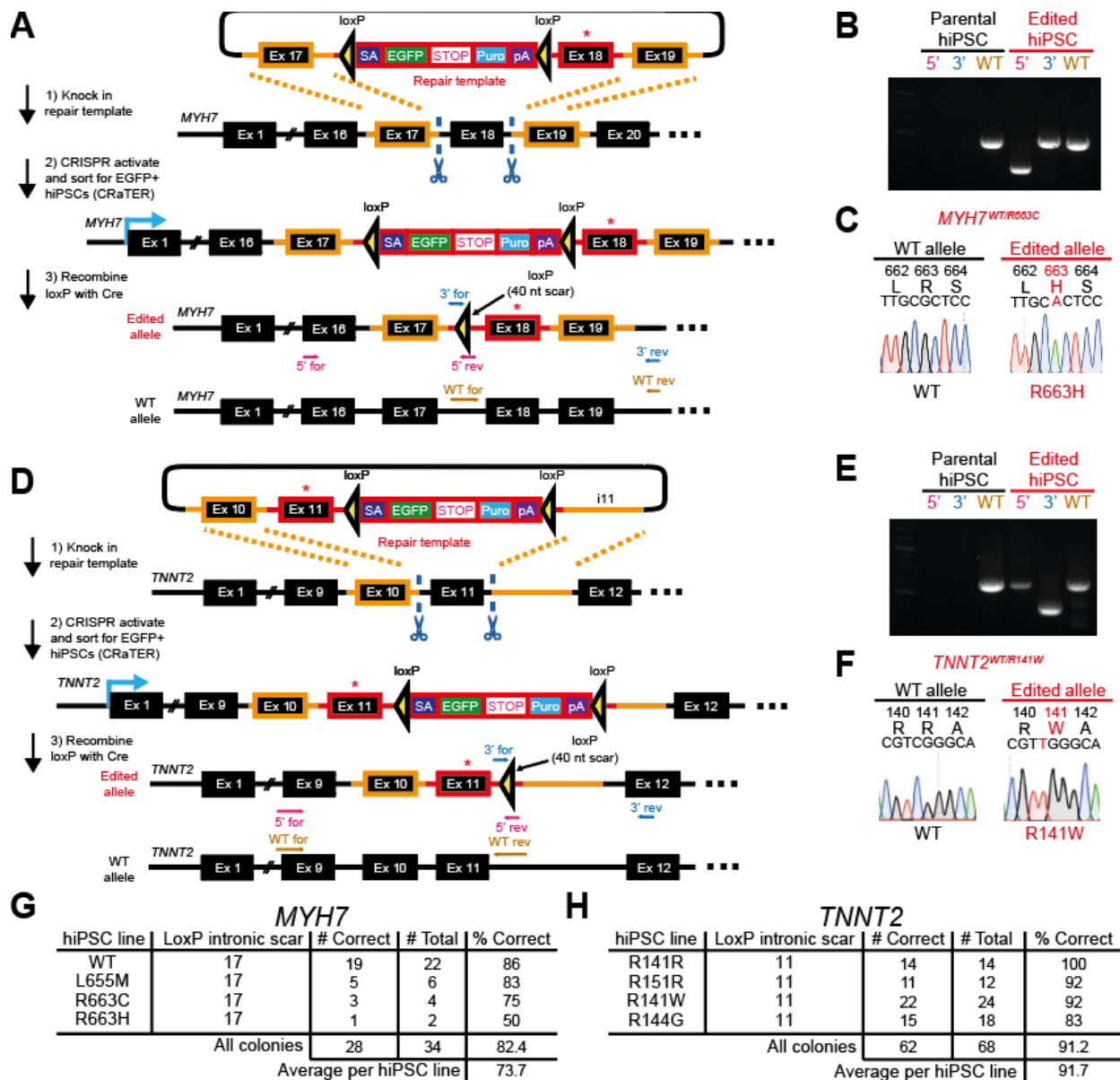


Figure S4: CRaTER-flox gene editing of *MYH7* and *TNNT2*. (A-C) *MYH7* exon 18 editing strategy. (A) Editing schematic. *variant. (B) Representative genotyping gel. Parental: WTC11 *MYH7*^{WT/WT}; edited: *MYH7*^{WT/R663H}. (C) Representative chromatograms. (D-F) *TNNT2* exon 11 editing strategy. (D) Editing schematic. (E) Representative genotyping gel. Parental: WTC11 *TNNT2*^{WT/WT}; edited: *TNNT2*^{WT/R141W}. (F) Representative

- 63 chromatogram. (**G-H**) CRaTER-flox editing efficiency for generation of *MYH7* exon 18 (G)
- 64 and *TNNT2* exon 11 (H) variant hiPSC lines.

65 *Methods*

66 Sex as a biological variable: Our study exclusively examined variants in the context of a
67 human male induced pluripotent stem cell line to ensure an isogenic background. A male
68 cell line was chosen because HCM variants are known to be more penetrant with earlier
69 onset in males compared to females.

70 hiPSC culture: hiPSCs were maintained on Matrigel-coated plastic using mTeSR Plus
71 media (STEMCELL Technologies) with the addition of Y-27632 rho kinase (ROCK)
72 inhibitor in the first 24h post-replating.

73 Generation of hiPSC line for heterozygous editing of *MYH7* intron 11-14 (“landing pad”):
74 WTC11 *CLYBL*^{dCas9-VPR-mCherry/dCas9-VPR-mCherry} hiPSCs were previously generated as
75 described in (3). To remove the mCherry fluorophore so that a red fluorophore could be
76 used in the future if desired, Cas9 protein and three sgRNAs targeting mCherry
77 (UCUGGGUGCCCUCGUAGGGG, GUCGGCGGGGUGCUUCACGU,
78 CUGCAUUACGGGGCCGUCGG) were electroporated with the Neon Electroporation
79 System (1400V, 20 ms pulse, one pulse) into WTC11 *CLYBL*^{dCas9-VPR-mCherry/dCas9-VPR-}
80 ^{mCherry} hiPSCs to delete 284 bp from the mCherry sequence. This yielded WTC11
81 *CLYBL*^{dCas9-VPR/dCas9-VPR} hiPSCs.

82 To create a heterozygous hiPSC “landing pad” line for *MYH7* introns 11-14 that
83 ensures only one *MYH7* allele can be edited, we leveraged CRaTER-flox to first destroy
84 the i11 and i14 gRNA sites on one *MYH7* allele. To achieve this, we created a plasmid
85 repair template containing a lox257-flanked cassette that includes a splice acceptor,
86 EGFP, stop codon, phosphoglycerate kinase 1 (PGK1) promoter, puromycin resistance,

87 and polyA, analogous to the Repair Template in Figure A but flanked with lox257. The
88 lox257-flanked cassette was then followed by genomic DNA consisting of *MYH7* intron
89 11 to 14 and a 5-nucleotide deletion in intron 14 that destroys the i14 gRNA site. The
90 plasmid repair template, Cas9 protein, and sgRNAs targeting *MYH7* intron 11 and 14
91 (CCAAGAAACAAGCATCACCG, TATTTGCTTAGAGCCAGCTG) were electroporated
92 with the Neon Electroporation System (1400V, 20 ms pulse, one pulse) into WTC11
93 *CLYBL*^{dCas9-VPR/dCas9-VPR} hiPSCs. After puromycin selection, cells were transfected with
94 three sgRNAs targeting 150 bp upstream of the *MYH7* transcriptional start site
95 (TCACTAGAAGCATTTCCCCC, GTCCCTAGCCGGATTAGAAA,
96 GTTATGGCATGGACTGTGCA) and FACS enriched for EGFP expression after 48-72h.
97 Enriched cells were treated with TAT-Cre recombinase (1 μ M, Excellgen #EG-1001) for
98 24h to flox out the EGFP and puromycin selection cassette. Single colonies were picked
99 and screened for the desired heterozygous genotype (one WT allele and one 5-nucleotide
100 deletion in intron 14 that destroys the i14 gRNA site), establishing a heterozygous
101 “landing pad” line that destroys the gRNAs sites for i11 and i14 on one *MYH7* allele (the
102 recombined loxP257 site destroys the i11 gRNA site). This ensures that subsequent
103 CRaTER-flox editing of *MYH7* introns 11-14 will only occur on one allele. Lox257 was
104 selected for the generation of the landing pad line so that it would not recombine with
105 wildtype loxP site, which is used for the CRaTER-flox gene editing of the variant allele.

106 CRaTER-flox gene editing: *MYH7* exon 12-14 variants with the intron 11 scar used the
107 “landing pad” hiPSC line (which ensures heterozygous gene editing), while all other
108 variant hiPSC lines (*MYH7* exon 12-14 variants with the intron 14 scar, *MYH7* exon 18
109 variants, *TNNT2* exon 11 variants) used the parental WTC11 *CLYBL*^{dCas9-VPR/dCas9-VPR}

110 hiPSC line. All gene edited lines that started with the parental WTC11 *CLYBL*^{dCas9-}
111 VPR/dCas9-VPR hiPSC line were screened to ensure the presence of one variant allele and
112 one WT allele (as demonstrated in Figure B, Figure S4 B, E). For CRaTER-flox gene
113 editing, the parental hiPSC line was electroporated with an appropriate plasmid repair
114 template (Figure A, Figure S4 A, D), Cas9, and appropriate sgRNAs (*MYH7* Exon 18:
115 CCTGTCTCCTTGGTGCATTC, GATGGACATTACCTCATCAG; *TNNT2*:
116 TACCCTGGTGTGCACGTCCG, GGAGCATGGGGGGCCTCCAT) using the same
117 electroporation settings as above. After puromycin selection, cells were electroporated
118 with sgRNAs upstream of the *MYH7* or *TNNT2* transcriptional start site (*TNNT2*:
119 CACATGGGCTTATATGGCGT, TGTTCTGTAGCCTTGTCCC) and FACS-enriched for
120 EGFP expression after 48-72h. Enriched cells were treated with TAT-Cre recombinase
121 for 24h, yielding a 40-nucleotide intronic scar (34-nucleotide LoxP sequence and 6-
122 nucleotide EcoRV restriction enzyme cloning artifact). Single colonies were picked and
123 screened for the correct genotype, establishing isogenic clonal variant hiPSC lines.

124 hiPSC-CM differentiation: hiPSCs were differentiated using established protocols via
125 timed activation and inhibition of Wnt in L-ascorbic acid, bovine serum albumin and RPMI
126 media, then maintained with RPMI cardiomyocyte media with B-27 Plus supplement
127 (Gibco). On differentiation day 20, hiPSC-CMs were metabolically purified in media
128 supplemented with lactate yet deprived of glucose or pyruvate for 5d. At differentiation
129 day 25, hiPSC-CMs were cryopreserved in Cryostor CS10 (StemCell Technologies, #07-
130 930) at 4x10⁶ cells/mL density. hiPSC-CMs were thawed at 250k cells/cm² on Matrigel
131 coated plastic and fed with DMEM cardiomyocyte media for recovery from
132 cryopreservation prior to seeding on differentiation day 30.

Forward scattering area (FSC-A): Differentiation day 30 hiPSC-CMs were seeded at 100k cells/cm² on Matrigel coated plastic and fed with DMEM cardiomyocyte media for 10 ± 2d. Cells were lifted and FSC-A was measured for single cells using flow cytometry with consistent voltage. 10,000 cells per biological replicate was collected.

Traction force microscopy (TFM): TFM polyacrylamide hydrogels were created using established protocols (5). Briefly, polydimethylsiloxane (PDMS) stamps were generated using silicon wafer patterns with 17x119x10 µm rectangles (Research Microstamps) and coated overnight with 1 mg/mL Matrigel. Patterns were imprinted onto 25 mm glass coverslips. Polyacrylamide hydrogels of physiological stiffness (10 kPa) were created with 500 nm diameter red fluorescent microbeads (Thermo Fisher, F8812) uniformly embedded. After post-cryopreservation recovery, differentiation day 30 hiPSC-CMs were seeded (100k cells, 250 µl) for 1h and then fed with DMEM cardiomyocyte media for 14 ± 7d. Cells were electrically stimulated at 1 Hz in 1x Tyrode's solution and fluorescent beads 5 µm below the hydrogel surface were imaged for 6s at 15 fps with 40x spinning disk confocal objective. Bead displacements and corresponding raw traction forces were calculated using previously published Matlab software (5).

Statistics: For FSC, maximum contraction velocity, and maximum relaxation velocity, raw values were converted to Z scores based on the distribution of mean values for control lines (N = 4-6). Mean values for benign, DCM, and HCM lines were compared by Kruskal-Wallis test with p < 0.05 considered significant, and then post hoc confirmed with 2-tailed Mann-Whitney U test with p < 0.0167 considered significant after Bonferroni correction for 3 pairwise comparisons. For maximum traction force, raw values for each cell were transformed into a normal distribution using the formula $Y = \log_{10}(X-10)$, where X is the

raw value and Y is the transformed value. The transformed distribution was not significantly different from a normal distribution based on D'Agostino-Pearson normality test for skewness and kurtosis, with $p < 0.05$ considered significant. Transformed values were converted to Z scores based on the distribution of mean values for the benign variants across all editing schemes ($N = 8$). Mean Z scores for benign, DCM, and HCM lines were compared by Kruskal-Wallis test with $p < 0.05$ considered significant, and then post hoc confirmed with 2-tailed Mann-Whitney U test with $p < 0.0167$ considered significant after Bonferroni correction for 3 pairwise comparisons.

Data availability: Values for all data points in graphs are reported in the Supporting Data Values file. TFM videos and other raw data files are available from the Authors upon request.

Author contributions: A.M. Loiben designed research experiments, conducted experiments, analyzed data, wrote the manuscript, and edited the manuscript. W-M. Chien designed research experiments, conducted experiments, analyzed data, and edited the manuscript. A. McKinstry conducted experiments and analyzed data. D. Ahmed conducted experiments and analyzed data. M.C. Childers analyzed data and edited the manuscript. M. Regnier edited the manuscript, provided supervision, and acquired funding. C.E. Murry edited the manuscript, provided supervision, and acquired funding. K-C. Yang designed the research experiments, wrote the manuscript, edited the manuscript, provided supervision, administered the project, and acquired funding.

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