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Research Letter In-Press Preview **Cardiology** **Genetics**

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.

Main Text

A major limitation of genetic testing is the frequent reporting of variants of unknown significance (VUS). Pathogenic variants in myosin heavy chain 7 (*MYH7*) cause hypertrophic cardiomyopathy (HCM) or dilated cardiomyopathy (DCM). While accurate variant interpretation can assist with diagnosis and screening of at-risk relatives, ~85% of *MYH7* missense variants are VUS. Here, we describe a functional assay that predicts *MYH7* variant effects in the context of a human sarcomere (1). Assays measuring cell size and contractility (2) have been reported but not rigorously tested with a panel of *MYH7* benign and pathogenic variants to determine their accuracy and utility for variant classification.

While human induced pluripotent stem cells (hiPSCs) and their derivatives can determine variant effect in physiologically-relevant cells, a major challenge is the generation of sufficient variant hiPSC lines to establish ranges for normal and disease phenotypes. To address this, we adapted the gene-editing strategy CRISPRa On-Target Editing Retrieval (CRaTER) (3) by combining it with the Cre-lox system, hereafter referred to as CRaTER-flox, to efficiently introduce variants near-scarlessly into hiPSCs. Using WTC11 hiPSCs, we replaced *MYH7* intron 11 through intron 14 genomic DNA, a pathogenic variant hotspot, on one allele with a repair template using standard CRISPR/Cas9 gene editing (Figure A). The repair template contained a loxP-flanked region expressing EGFP followed by *MYH7* intron 11 through intron 14 genomic DNA with a single nucleotide variant (SNV) of interest. Correctly edited hiPSCs were CRaTER-enriched by transiently activating *MYH7* and flow-sorting for $EGFP^+$ hiPSCs (Figure S1, A-C) (3). Cre recombination removed the floxed region, leaving the desired heterozygous

SNV and a 40-nucleotide loxP intronic scar. We generated two benign editing control lines (with loxP in intron 11 or 14), two benign variant lines (T441M, R453R), four pathogenic/likely pathogenic (P/LP) DCM- or LV systolic dysfunction (LVSD)-associated variant lines (R369Q, P402L, Q451K, I457M) (4), and six P/LP HCM-associated variant lines (R403L, R403W, R442L, R453C, R453H, I457T) (Figure B-C). 76.8% of these hiPSC colonies were correctly gene edited near-scarlessly with CRaTER-flox (Figure D; Figure S1D), improving upon the prior CRaTER approach (Figure S1E) (3).

We first assessed cell size as estimated by forward scattering area (FSC-A) with flow cytometry as a pathogenicity marker. We measured FSC-A of hiPSC-derived cardiomyocytes (hiPSC-CMs) and calculated Z scores based on mean FSC-A values across all benign lines (Figure S2A). While hiPSC-CMs expressing DCM or HCM variants were larger compared to hiPSC-CMs expressing benign variants, neither was statistically significant (Figure S2B), indicating that FSC-A was unable to reliably discriminate pathogenic from benign variants.

Next, we assessed whether contractile function measured by traction force microscopy (TFM) (5) can accurately determine variant effect. hiPSC-CMs were matured seven days on micropatterned hydrogels with physiological stiffness to promote adult-like morphology. Paced single-cell twitch force and contractile kinetics were derived from the displacement of fluorescent beads embedded in the hydrogel (Figure S2, C-D) (5). Maximum twitch forces were converted to a normal distribution with a logarithmic transformation (Figure S2, E-F), and Z scores were calculated based on the distribution of benign line means. All benign variants mean force Z scores were within the normal range ($\pm$2 Z scores), while all pathogenic variants were outside the normal range,

demonstrating this assay's ability to discriminate pathogenic from benign variants (Figure E-F). Furthermore, all HCM-associated pathogenic variants were hypercontractile with faster contraction and relaxation velocity, while all DCM/LVSD-associated pathogenic variants were hypocontractile with a trend toward slower contraction and relaxation velocity (Figure G-I), suggesting TFM may predict variant-specific cardiomyopathy.

We used the TFM assay to determine the effect of four *MYH7* VUS (Figure S3A). Cardiomyocytes expressing R434K had normal contractile function while cardiomyocytes expressing R403P, R442H, or R442P had reduced contractile function, suggesting the latter three may be pathogenic. The functionally abnormal variants are in the blocked head/tail (R442) and blocked head/free head (R403) interfaces, which may affect the stability of the *MYH7* interacting heads motif (Figure S3B). The R434K VUS with a conservative side chain substitution is located away from the interacting interfaces.

Next, we used CRaTER-flox to introduce variants in exon 18 of *MYH7* (Figure S4, A-C) and exon 11 of troponin T (*TNNT2)* (Figure S4, D-F), a cardiomyopathy-associated gene with many splicing isoforms, with similar editing efficiency (Figure D; Figure S4, G-H). The TFM assay accurately discriminated pathogenic from benign variants in these exons. The HCM-associated variants were hypercontractile while the DCM/LVSD-associated variants were hypocontractile (Figure F), mirroring results of *MYH7* exons 12-14 variants. Across all *MYH7* variants studied, all six benign variants were in the normal range while all 13 pathogenic variants were outside the normal range, demonstrating the robustness of this assay in discriminating benign from pathogenic variants. In addition to successfully editing other genomic loci, this near-scarless gene-editing strategy enables the study of genes with multiple splicing isoforms.

Overall, we applied CRaTER-flox, a method that efficiently introduces variants near scarlessly into hiPSCs, enabling the functional interrogation of variants in physiologically-relevant cell types with contextually-correct genetic background. We interrogated the functional consequences of a panel of benign and pathogenic *MYH7* variants with a cardiomyocyte contractility assay to assess *MYH7* variant effect. This assay could be adapted and validated to clinically classify *MYH7* variants.

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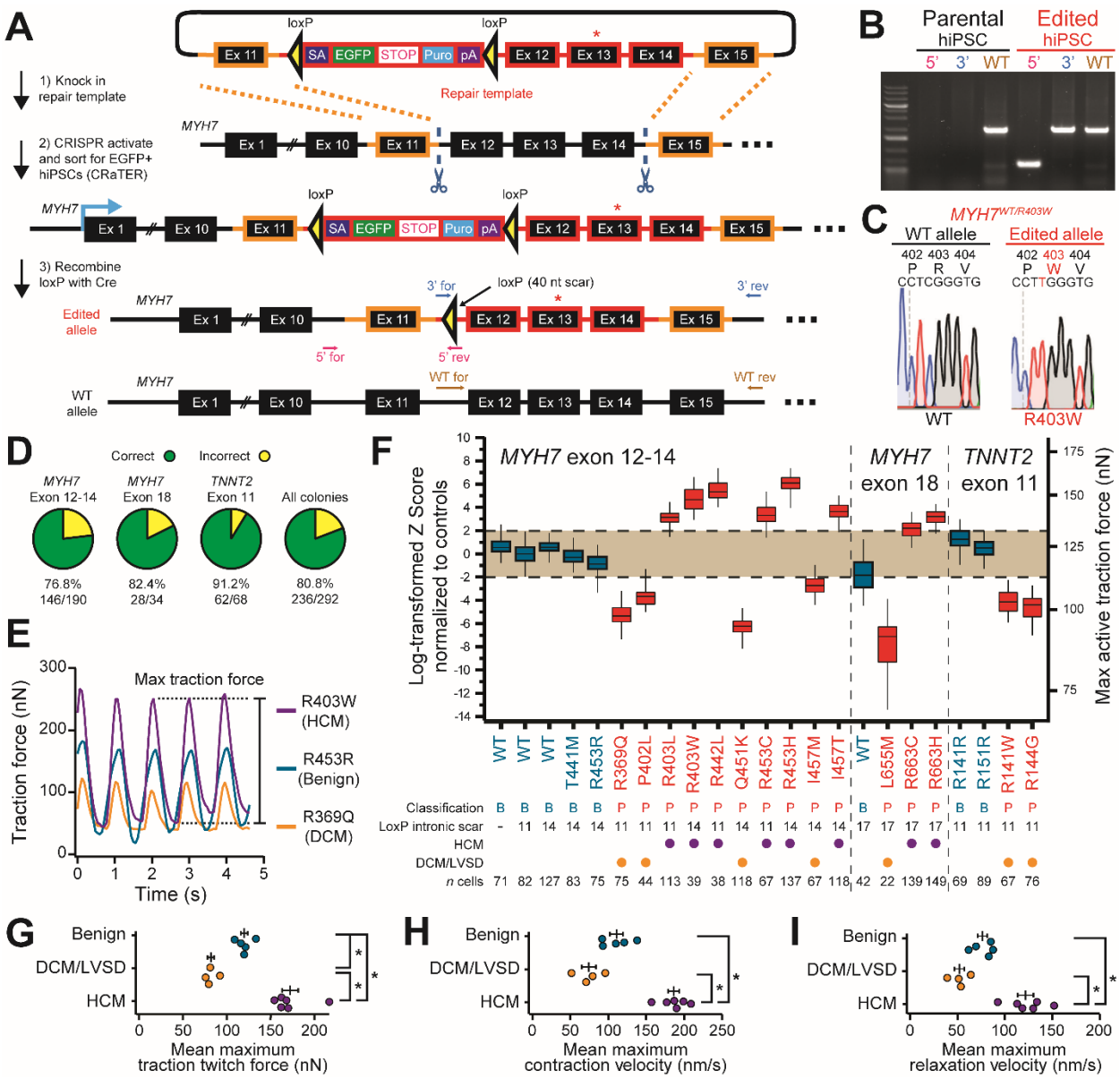

Figure Legend

CRaTER-flox efficiently gene edits variants near scarlessly into hiPSCs to enable accurate assessment of variant effect. (**A**) CRaTER-flox gene-editing approach. *variant. (**B**) Representative genotyping gel using primers indicated in (A). Parental: WTC11 *MYH7*$^{WT/WT}$; edited: WTC11 *MYH7*$^{WT/R403W}$. (**C**) Representative sequencing chromatograms. (**D**) CRaTER-flox editing efficiency to generate variant hiPSC lines. (**E**)

Representative hiPSC-CM force curves as measured with traction force microscopy. (**F**) Log-transformed maximum hiPSC-CM active traction force Z scores normalized to benign lines and raw force values. Blue: benign/likely benign (B); red: pathogenic/likely pathogenic (P) variant. Box: upper and lower quartiles; midline: median; whiskers: 1.5x IQR. Circle: variant-associated cardiomyopathy reported in ClinVar or (4). (**G-I**) Mean maximum *MYH7* exon 12-14 hiPSC-CM active traction forces (G), maximum traction velocity (H), and maximum relaxation velocity (I). Kruskal-Wallis test, $p<0.05$; post hoc 2-tailed Mann-Whitney *U* test with Bonferroni correction, $^*p<0.0167$. (G-I) error bars indicate SEM.