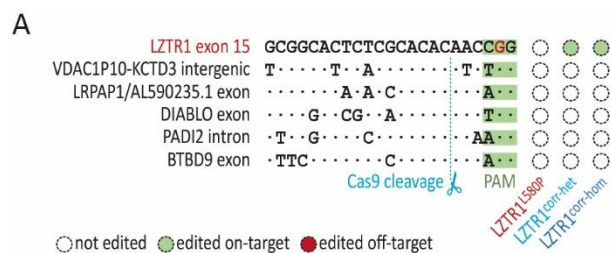


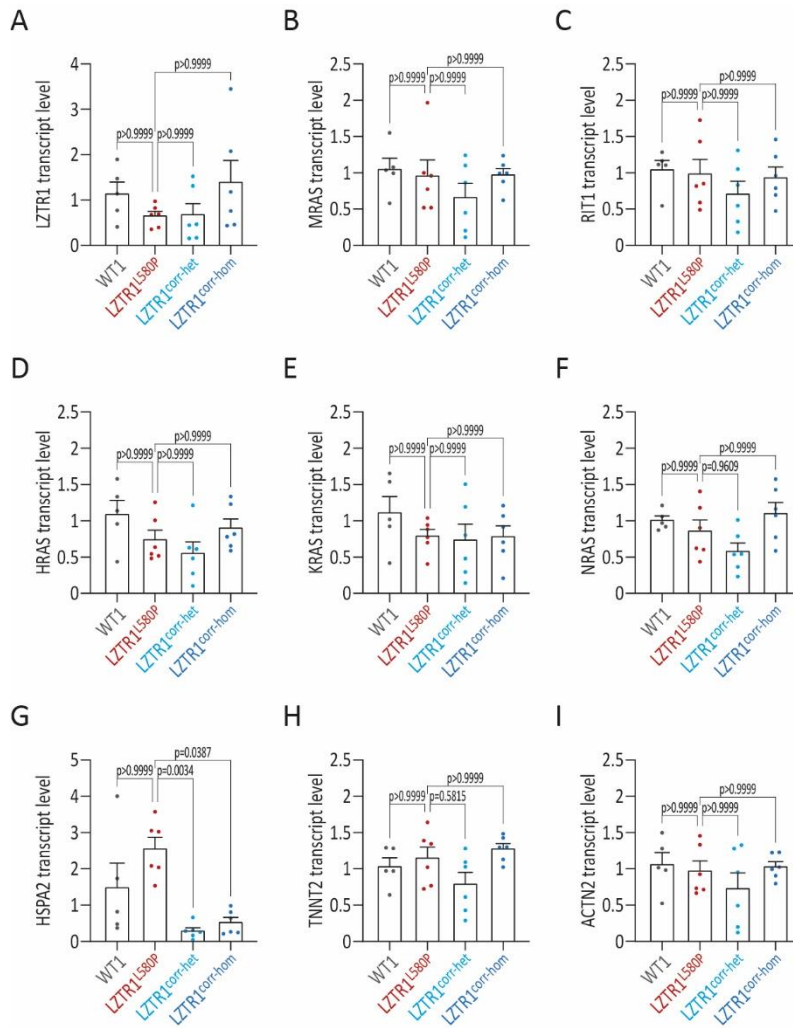
## **Supplemental information**

### **Mutation-induced LZTR1 polymerization provokes cardiac pathology in recessive Noonan syndrome**

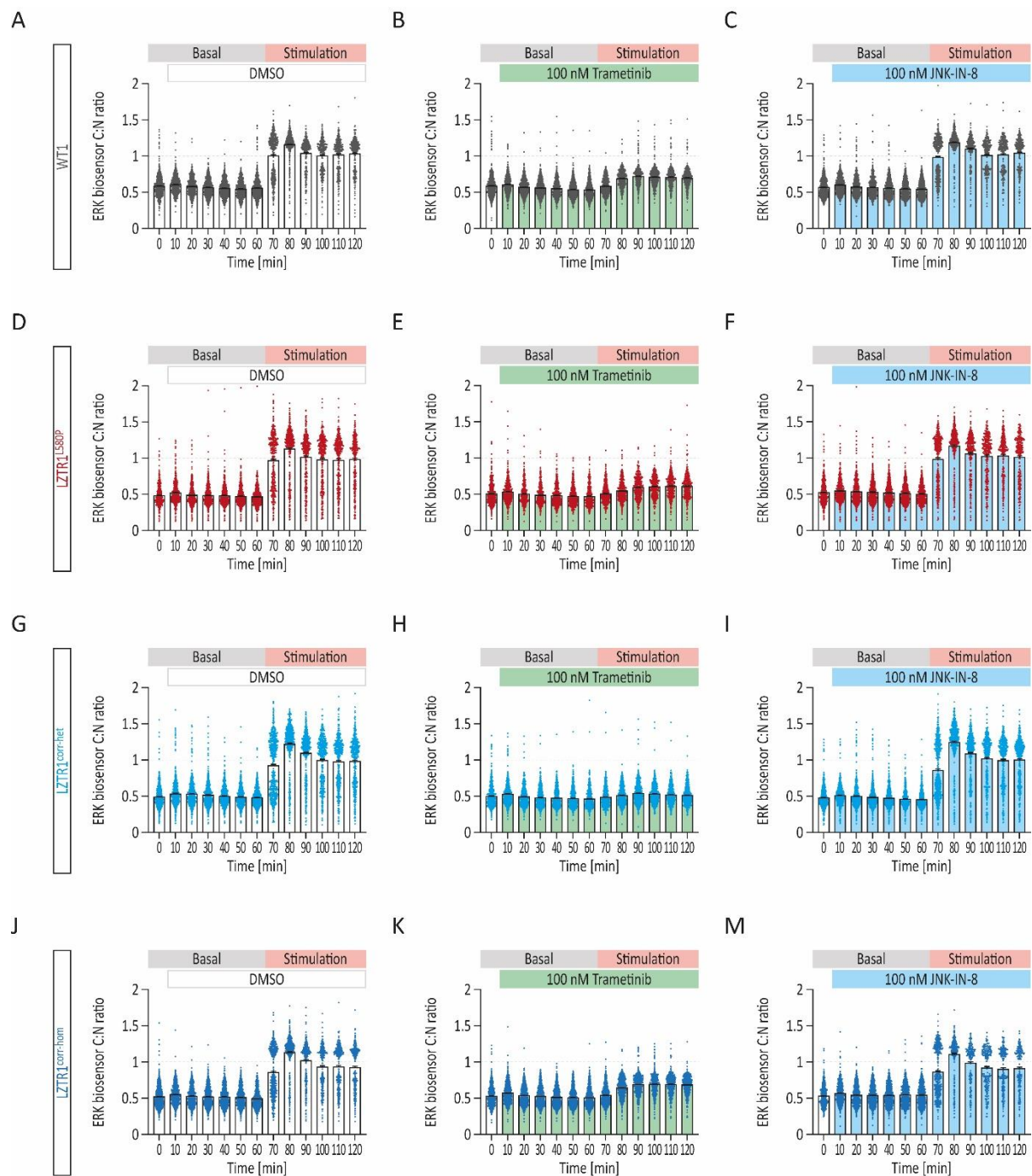
**Alexandra Viktoria Busley, Óscar Gutiérrez-Gutiérrez, Elke Hammer, Fabian Koitka, Amin Mirzaiebadizi, Martin Steinegger, Constantin Pape, Linda Böhmer, Henning Schroeder, Mandy Kleinsorge, Melanie Engler, Ion Cristian Cirstea, Lothar Gremer, Dieter Willbold, Janine Altmüller, Felix Marbach, Gerd Hasenfuss, Wolfram-Hubertus Zimmermann, Mohammad Reza Ahmadian, Bernd Wollnik, and Lukas Cyganek**



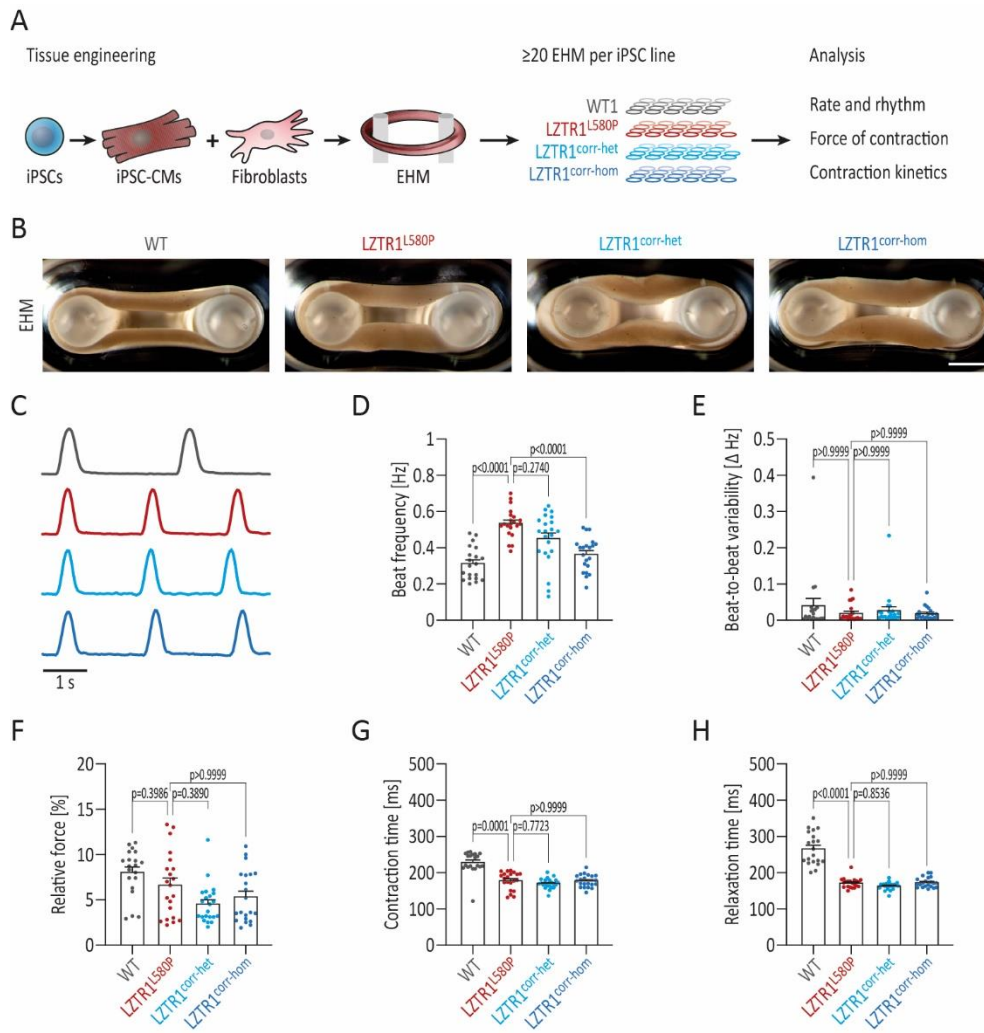
**Figure S1: Off-target screening in CRISPR/Cas9-edited iPSCs; Related to Figure 1. (A)** Sanger sequencing of the top five predicted off-target regions, ranked by the CFD off-target score using CRISPOR, revealed no off-target editing of CRISPR/Cas9 in CRISPR-corrected iPSCs compared to the patient-derived cells.



**Figure S2: Homozygous *LZTR1*<sup>L580P</sup> shows no upregulation of RAS GTPases at transcriptional level; Related to Figure 2.** (A-I) Quantitative gene expression analysis of *LZTR1* (A), of *LZTR1* substrates *MRAS* (B), *RIT1* (C), *HRAS* (D), *KRAS* (E), and *NRAS* (F), of *HSPA2* (G), and of cardiac-specific genes *TNNT2* (H), and *ACTN2* (I) in WT, the patient-specific, and the two CRISPR-corrected iPSC-CMs at day 60 of differentiation, assessed by real-time polymerase chain reaction, revealed no expression differences at transcriptional level across all iPSC lines; samples were analyzed in duplicates and data were normalized to *GAPDH* expression and WT controls; n=5-6 independent differentiations per iPSC line. Data were analyzed by nonparametric Kruskal-Wallis test with Dunn correction and are presented as mean  $\pm$  SEM (A-I).

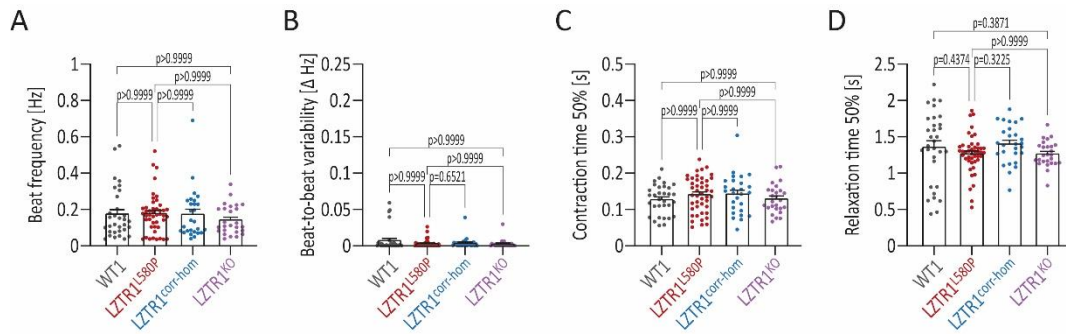


**Figure S3: Biosensor-based analysis of ERK signaling dynamics in real time; Related to Figure 3.** (A-M) Quantitative analysis of ERK biosensor cytosol/nucleus (C:N) ratio in WT (A-C), the patient-specific (D-F), and the two CRISPR-corrected (G-M) biosensor-transduced iPSC-CMs treated with MEK inhibitor trametinib, with JNK inhibitor JNK-IN-8, or with DMSO for 60 minutes, before stimulation with serum for another 60 minutes; n=2 independent differentiations per iPSC line with n=4-5 individual wells per condition.



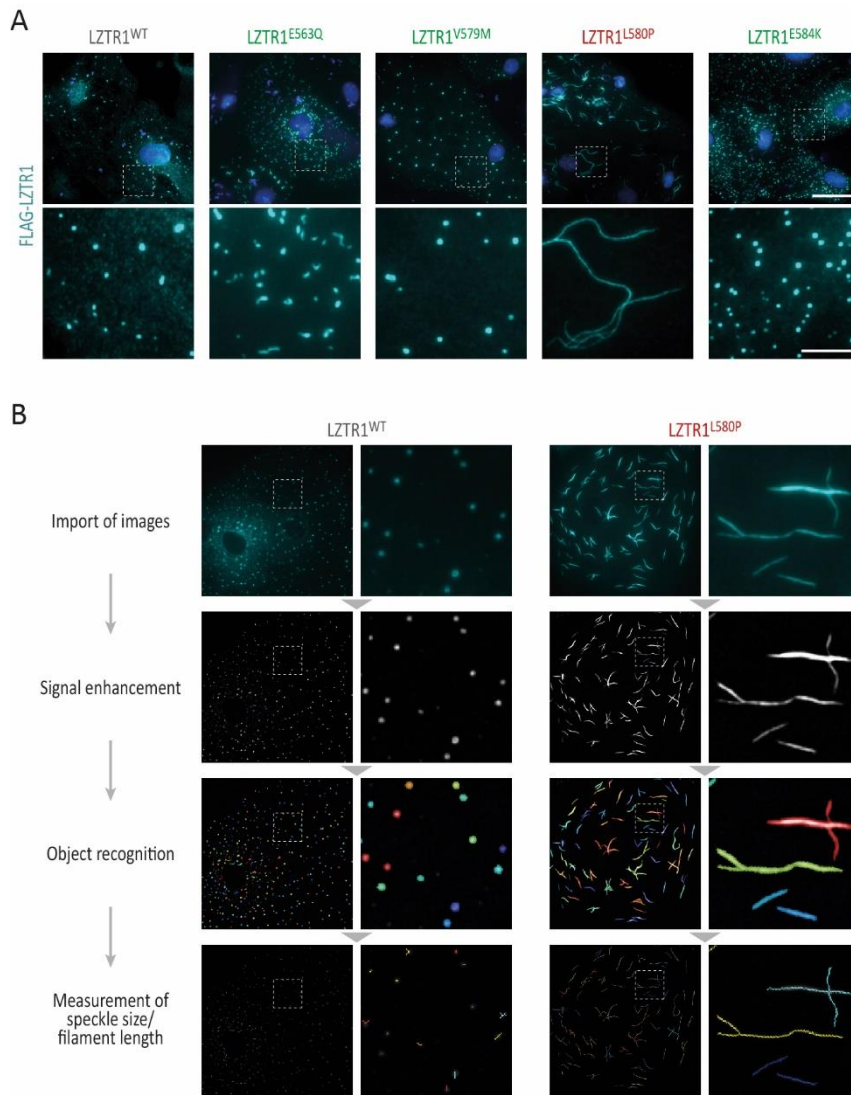
**Figure S4: Homozygous *LZTR1*<sup>L580P</sup> does not compromise contractile function of EHMs; Related to Figure 4.** (A) Depiction of the experimental design: the WT, the patient-specific, and the two CRISPR-corrected iPSC lines were differentiated into ventricular iPSC-CMs and casted at day 30 of differentiation together with fibroblasts in a collagen matrix for generation of EHMs. Tissues were analyzed for rhythmogenicity and contractile parameters by optical recordings at 5-6 weeks post-casting; n=20-22 EHMs from 3 individual differentiations per iPSC line. (B) Representative microscopic images of generated EHMs 6 weeks post-casting showing comparable tissue morphologies; scale bar: 1 mm. (C) Exemplary contraction traces from optical recordings of EHMs 6 weeks post-casting; peak amplitudes were normalized. (D) Quantitative analysis of the beating frequency of spontaneously contracting EHMs displayed minor differences in patient-derived tissues. (E) Quantitative measurement of the beat-to-beat variability of spontaneously contracting EHMs showed equal beating regularities across all tissues. (F) Quantitative analysis of the force of contraction, assessed by measuring the relative deflection of flexible poles, identified no significant differences across all iPSC lines. (G-H)

Quantitative analysis of the contraction kinetics revealed longer contraction times (G) and relaxation times (H) in WT compared to patient's and CRISPR-corrected EHMs. Data were analyzed by nonparametric Kruskal-Wallis test with Dunn correction and are presented as mean  $\pm$  SEM (D-H).



**Figure S5: Homozygous *LZTR1*<sup>L580P</sup> shows unchanged contractile properties in 2D; Related to Figure 4. (A-D) Quantitative analysis of beating frequency (A), beat-to-beat variability (B), contraction time (C), and relaxation time (D) in WT, the patient-specific, the homozygous CRISPR-corrected, and *LZTR1*<sup>KO</sup> iPSC-CMs at day 60 of differentiation, assessed by video-based contractility analysis in monolayer cultures, revealed no significant differences in contractile function across all iPSC lines; n=3-9 independent differentiations per iPSC line. Data were analyzed by nonparametric Kruskal-Wallis test with Dunn correction and are presented as mean  $\pm$  SEM (A-D).**

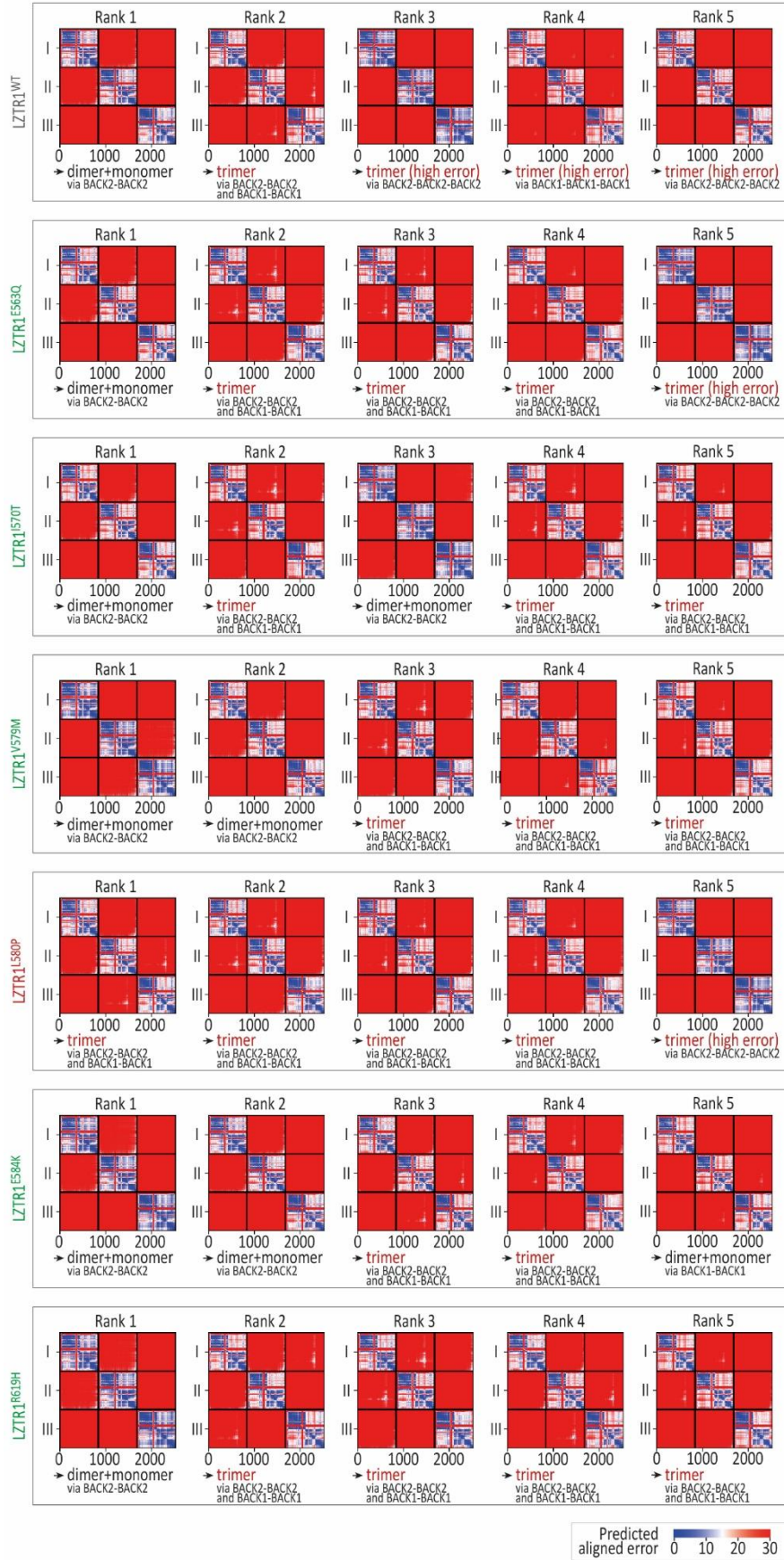




**Figure S6: Unique *LZTR1*<sup>L580P</sup>-induced polymerization of LZTR1 complexes; Related to Figure 5. (A)** Representative images of WT iPSC-CMs at day 60 of differentiation after single plasmid transfection stained for FLAG-tagged LZTR1 confirmed that only LZTR1<sup>L580P</sup> forms large filaments, whereas LZTR1<sup>WT</sup> and the other variants present a speckle-like pattern; nuclei were counter-stained with Hoechst 33342 (blue); scale bars: 20  $\mu$ m in upper panel, 5  $\mu$ m in lower panel. **(B)** Customized CellProfiler pipeline for recognition and quantification of speckle size and filament length in iPSC-CMs with ectopic expression of LZTR1 variants.



A



**Figure S7: Computational prediction for LZTR1 interactions via ColabFold; Related to Figure 6.** (A) The five predicted models for each LZTR1 variant were ranked according to the predicted template modeling score and interactions between the chains were inspected through the predicted alignment error generated by AlphaFold-multimer.

**Table S1: Clinical characterization of the affected patient; Related to Figure 1.**

<b>Cardiac findings</b>
Mild left ventricular hypertrophy
Prolonged QT interval
Stress-induced cardiac arrhythmias
Pericardial effusion
<b>Facial characteristics</b>
Down-slanting palpebral fissures
Mild bilateral ptosis
Triangular facial contour
Curly hair
Low posterior hairline
High-arched palate
<b>Physical characteristics</b>
Marfanoid habitus: height 186 cm (75th-90th percentile) weight 56 kg (3th percentile)
Pronounced pectus excavatum
Scoliosis
Stretch marks on lower back
Clinodactyly
<b>Additional findings</b>
Mild bilateral sensorineural hearing loss

**Table S2: Primer sequences used for PCR and real-time PCR; Related to STAR methods.**

<b>Gene (gDNA)</b>	<b>Primer</b>
LZTR1 Ex15	CGAGGCCTTGTTTCCTACCTA / GAGGGGCTCACAGTGGTG
Off-target 1	GGTTCAGAAGCACTCATCTCC / AAGCCATCAACCCGAAACAA
Off-target 2	ATGGATCCTGACTGCAACCC / TCTGGGCAGTCTGTGTCTTT
Off-target 3	GATGCCACAATAACCGCTCC / TGAGGAGACGTGGAGAGGAG
Off-target 4	AGTAAGGCGTTTGAGTCCCA / AAGAGGCACATGGATGAGGG
Off-target 5	AACACACTGGGGAAGGAAGT / GAGCTGCTTCCTATCCCCTC
<b>Gene (cDNA)</b>	<b>Primer</b>
ACTN2	GCCAGAGAGAAGGATGCAATCAC / AAGCATGGGAACCTGGAATCAA
GAPDH	GGAGCGAGATCCCTCCAAAAT / GGCTGTTGTCATACTTCTCATGG
HRAS	ACGCACTGTGGAATCTCGGCAG / TCACGCACCAACGTGTAGAAGG
HSPA2	GACCAAGGACAATAACCTGCTGG / GGCGTCAATGTCTGAAGGTAACC
KRAS	AGTGCCTTGACGATACAG / GCATCATCAACACCCTGTCTT
LZTR1	GAGCCAACCTCAAGGAGCACT / CAATGTCCACTGGCTGGTCC
MRAS	CCACCATTGAAGACTCCTACCTG / ACGGAGTAGACGATGAGGAAGC
NRAS	GGCAATCCCATAACAACCCTGAG / GAAACCTCAGCCAAGACCAGAC
RIT1	TTCATCAGCCACCGATTCCC / GCAGGCTCATCATCAATACGG
TNNT2	ACAGAGCGGAAAAGTGGGAAG / TCGTTGATCCTGTTTCGGAGA

**Table S3: Plasmids used in this study; Related to STAR methods.**

<b>Plasmid</b>	<b>Source</b>
pcDNA3-HA-LZTR1-WT	modified from RRID:Addgene_13512
pcDNA3-FLAG-LZTR1-WT	modified from pcDNA3-HA-LZTR1-WT
pcDNA3-HA-LZTR1-E563Q	modified from pcDNA3-HA-LZTR1-WT
pcDNA3-FLAG-LZTR1-E563Q	modified from pcDNA3-FLAG-LZTR1-WT
pcDNA3-HA-LZTR1-I570T	modified from pcDNA3-HA-LZTR1-WT
pcDNA3-HA-LZTR1-V579M	modified from pcDNA3-HA-LZTR1-WT
pcDNA3-FLAG-LZTR1-V579M	modified from pcDNA3-FLAG-LZTR1-WT
pcDNA3-HA-LZTR1-L580P	modified from pcDNA3-HA-LZTR1-WT
pcDNA3-FLAG-LZTR1- L580P	modified from pcDNA3-FLAG-LZTR1-WT
pcDNA3-HA-LZTR1-E584K	modified from pcDNA3-HA-LZTR1-WT
pcDNA3-FLAG-LZTR1-E584K	modified from pcDNA3-FLAG-LZTR1-WT
pcDNA3-HA-LZTR1-R619H	modified from pcDNA3-HA-LZTR1-WT
pcDNA3-HA-LZTR1- $\Delta$ BTB2-BACK2	modified from pcDNA3-HA-LZTR1-WT
pLentiPGK Puro DEST ERKKTRClover	RRID:Addgene_90227
pMD2.G	RRID:Addgene_12259
psPAX2	RRID:Addgene_12260
pcDNA3.1-LZTR1-Myc-6xHis	Jens Kroll (Heidelberg University and German Cancer Research Center)
pcDNA3.1-LZTR1-L580P-Myc-6xHis	modified from pcDNA3.1-LZTR1-Myc-6xHis