

Supplementary Materials for
Truncated titin proteins and titin haploinsufficiency are targets for functional recovery in human cardiomyopathy due to *TTN* mutations

Andrey Fomin *et al.*

Corresponding author: Wolfgang A. Linke, wlinke@uni-muenster.de

Sci. Transl. Med. **13**, eabd3079 (2021)
DOI: 10.1126/scitranslmed.abd3079

The PDF file includes:

Materials and Methods
Figs. S1 to S9
Tables S1 to S5
Legends for data files S1 and S2
References (54–61)

Other Supplementary Material for this manuscript includes the following:

Data files S1 and S2

Materials and Methods

Characteristics of the DCM-patient cohort

Average patient age, LVEF, and LVEDD at the time of heart transplantation were available from patients and were similar between the IDCM and TTNtv DCM groups (Table S1). Gender was also available, along with additional information, including the age at first diagnosis, from a majority of patients. Age and gender were known for some heart donors; their mean age was not significantly different from that of the TTNtv and IDCM patients (Table S1).

Genetic screening by next-generation sequencing

Molecular genetics screening was performed at the Heart and Diabetes Center NRW in Bad Oeynhausen, University Hospital of Ruhr-University Bochum, with approval from the local ethics committee (Reg.-No. 58/2014 and 2018-330), and after oral and written informed consent obtained from all patients. DNA was isolated from white blood cells (High Pure PCR Template Preparation Kit[®], Roche Diagnostics) and prepared for cardiac gene enrichment re-sequencing on a MiSeq[®] next generation sequencing system (Illumina) according to the manufacturer's instructions (TruSight[™] Rapid Capture Sample Preparation Kit). Index patients were screened for variants in 174 genes (in two cases, only 46 genes) associated with inherited cardiac conditions, using the TruSight[™] cardio gene panel. Evidence criteria for mutation classification and sequencing were used as previously published (44, 45) and variants classified according to ACMG guidelines (46). For variant annotation, we used VariantStudio[™] v3.0 software (Illumina). Heterozygous TTNtv classified as 'likely pathogenic' were present in 22 patient hearts. *TTN* variants refer to the following databases: NCBI, NM_001267550.1; NCBI, NP_001254479.2 or UniProtKB, Q8WZ42-1.

Isolation of total RNA and quantitative real-time PCR

Total RNA was isolated from LV myocardium (~30 mg tissue/heart) using a commercial kit (RNeasy, Qiagen), as described (47). Purified total RNA was quantified photometrically at 260 nm. RNA-purity and integrity were assessed by agarose-gel electrophoresis. Reverse transcription of myocardial RNA was performed on a StepOnePlus[™] real-time PCR system (ThermoFisher Scientific), using 250 ng of total RNA and 50 units of the enzyme Superscript II (Invitrogen), following random priming with hexamers. Experiments were done with 2 µl of the reverse transcription reaction as a template. Real-time PCR data were analyzed using GAPDH, 60S ribosomal protein L32, and peptidyl-prolyl isomerase A as housekeeping genes. Measurements were performed in duplicate. Relative mRNA expression was evaluated using the comparative C_T-method ($\Delta\Delta C_T$) (48) involving the geometric mean of the housekeeping genes. Conditions for the PCR reaction were as follows: 95°C, 10 min initial denaturation, 40 cycles 60°C, 1 min/95°C, 15 s, using Maxima Probe/ROX qPCR MasterMix (ThermoFisher Scientific). The ratio of Cronos to FL-titin mRNA was calculated as: mRNA ratio = $2^{-[C_T(\text{Cronos}) - C_T(\text{FL-TTN})]}$. Primer sequences used are listed in Table S4.

Detecting Ribo-seq translation signatures specific to Cronos

To visualize active translation of Cronos, we explored human Ribo-seq data of 80 human hearts, including 65 DCM and 15 donor hearts (17). Ribo-seq was performed on an Illumina HiSeq 2500 using the TruSeq Ribo Profile (Mammalian) Library Prep Kit (Illumina), following the TruSeq Ribo Profile protocol, and producing single-end 1x51nt reads (17). Sample processing was done on large batches of a maximum of 30 samples to avoid bias. Human heart Ribo-seq libraries were sequenced to an average depth of 115M (min. 56M, max. 232M) raw reads. Ribosomal footprints were mapped to the *TTN* gene locus (hg38; Ensembl release 87) and specifically inspected for footprints around the predicted Cronos translation initiation site located upstream of meta-*TTN* exon 240. This ribosomal signature matching the Cronos N-terminus is the only part of Cronos that can be discriminated from the canonical *TTN* protein sequence.

RNA sequencing and processing of reads

RNA isolation and sequencing were performed as described previously (54). The Qiagen RNeasy Mini Kit (#74104) was used for RNA isolation of ~30 mg of (liquid nitrogen) frozen LV heart tissue. Only if the RNA integrity number, measured using the Agilent RNA 6000 Pico Kit (#5067-1513), was at least 7.8, RNA was processed for further analysis. The TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold (#RS-122-2301) was used for library preparation. The indexed libraries were enriched with seven PCR cycles, quantified using the Qubit dsDNA HS assay kit (#Q32854), and qualified with a Bioanalyzer using the HS DNA Kit (#5067-4627). The Illumina HiSeq 3000 (single-end; 50 bp) was employed for sequencing equimolar amounts of each library using sequencing-by-synthesis chemistry v4, according to the manufacturer's instructions. Sequencing resulted in an average yield of 500 Mb of sequencing data, with an average of 96% reads achieving a quality score $\geq$ Q30.

We first validated variants in the fastq files using Mutscan v1.14.0 (55) and then quality-trimmed the raw reads using Trimmomatic v0.27 (56), as described (54). For quality control, FastQC v0.11.7 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) was applied. The trimmed RNA-seq reads were mapped to the hg38 (GRCh38) reference genome sequence using STAR v2.5.1b (57). The obtained BAM files were then inspected with the integrated genome viewer software (IGV_2.7.0, Broad Institute) (58). To analyze TTNtv-transcript frequencies, the number of transcripts with or without the TTNtv was counted at the corresponding genomic position.

Protein gel electrophoresis and Western blot analysis

Snap-frozen human heart tissues, hiPSC-CMs, or hiPSC-EHM were solubilized in titin sample buffer (8 M urea, 2 M thiourea, 3% SDS, 0.05 M Tris, 10% glycerol, 75 mM DTT, 0.03% Serva Blue), for 20 min on ice. After boiling for 3 min at 96°C, the debris was spun down for 5 min at 20,000 xg and directly loaded onto gels or frozen at -80°C until use. The total protein concentration per sample was assessed using Bradford reagent (Sigma-Aldrich). For the separation of titin, protein lysates (7-30 µg) were resolved on agarose-stabilized 1.6-2.2% slab

gels overnight (20, 51). Proteins up to 250 kDa were resolved by standard SDS-PAGE (15%, 12.5%, or 10%). For simultaneous separation of titin and small proteins, we used an agarose-stabilized, 1.8% ‘stacking’ gel on top of a standard SDS-PAGE ‘running’ gel (15% 12.5%, 10%, or 6%). Electrophoresis was performed at 2 mA/plate at RT overnight. Protein bands were visualized with Coomassie brilliant blue (1610436, Sigma-Aldrich). For WBs, proteins separated by gel electrophoresis were transferred to PVDF membranes (Millipore) using the Trans-Blot® Turbo™ transfer system (Bio-Rad). After transfer, proteins on membranes were stained with Coomassie brilliant blue; these signals served as additional markers of loading on titin gels, as there are no housekeeping proteins available in this size range. Signals from HRP-conjugated secondary antibodies were visualized by chemiluminescence (Amersham ECL start Western Blotting Detection Reagent, GE Healthcare) and recorded using the ImageQuant LAS 4000 Imaging System (GE Healthcare). Signal intensity was quantified using either MultiGauge v.3.0 or ImageQuant TL v.7.1 software (GE Healthcare). Detailed information on the primary and secondary antibodies is provided in Table S5.

Quantitation of titin protein species normalized to other cardiac proteins

All 113 human DCM heart samples (91 IDCM, 22 TTNtv) and 14 donor heart samples were studied on Coomassie-stained two-phase protein gels, in order to measure the composition and expression of wt-titin species (wt-N2BA, wt-N2B, T2, wt-Cronos), also in relation to other cardiac proteins. Additionally, the titin species were measured on Coomassie-stained, 1.8% SDS-PAGE gels. All 127 heart tissues were included in the analysis. Images were recorded using the ImageQuant LAS 4000 Imaging System (GE Healthcare) and densitometry was performed using MultiGauge v.3.0 (GE Healthcare). Densitometric values were calculated for each titin species and the pool of other cardiac proteins (“other protein”, OP) between ~10 and 250 kDa. Average values were calculated for each titin species, using data from the two different gel types. Expression ratios were calculated from signals on the same gel lane.

Analysis of truncated titin and PQC marker proteins

Quantitative Western blotting was performed to detect tr-titin proteins and determine their expression in the 22 TTNtv-hearts. The following titin antibodies were used (Table S5): TTN-Z (N-terminus; against the first two titin Ig domains), I20/22 (against the first three Ig domains downstream of the PEVK segment) (59), TTN-I/A-MIR (against titin at the A-band edge), TTN-M (against Ig domains M8-M9 in M-band titin; detects wt- but not tr-titin), and Cronos (N-terminus) (15). These antibodies were purchased from Myomedix (Mannheim, Germany), except anti-I20/22 and anti-Cronos, which were made by Kaneka Eurogentec S.A. (Table S5). The anti-human Cronos antibodies have recently been verified for their high specificity against this isoform only (15). Images were recorded using the ImageQuant LAS 4000 Imaging System (GE Healthcare). Densitometric analyses of adult human heart samples and hiPSC-EHM were performed on TTN-Z, TTN-I/A-MIR, and Cronos blots, using MultiGauge v.3.0 (GE Healthcare). On TTN-Z and TTN-I/A-MIR blots, truncated titin was directly compared to FL-

titin (wt-N2BA, wt-N2B) and ratios were calculated. Similarly, tr-Cronos was related to wt-Cronos on Cronos blots. To combine the tr-Cronos and the tr-titin data, the proportion of wt-Cronos (measured on Coomassie-stained gels) was used as a normalization factor. Thus, the expression of all truncated species (tr-N2BA, tr-N2B, and tr-Cronos) could be calculated relative to all wt-species (wt-FL-titin and wt-Cronos). Loading differences between gel lanes were taken into account, as signals on WBs were indexed to the corresponding signals on Coomassie-stained PVDF membranes, or to GAPDH. For analyses in hiPSC-EHM, titin (wt or tr) protein content was also normalized to the mean content observed in vehicle-treated TTNtv lines.

Quantitative Western blotting was also performed with human heart tissues (donor, $n=9$; IDCM, $n=85$ to 89 ; TTNtv, $n=22$) to measure the expression of markers of the PQC machinery: MURF-1, SQSTM1/p62 (both after 10% SDS-PAGE), LC3B-I/-II (12.5% SDS-PAGE), and CRYAB (15% SDS-PAGE) (Table S5). Signals were normalized to GAPDH or, in the case of CRYAB, to the 'all-protein' content quantified on Coomassie-stained PVDF membranes. Moreover, pan-ubiquitination was measured by WB following 1.8% or 2.2% SDS-PAGE to resolve titin, in both human hearts and hiPSC-CMs. Loading was monitored by PVDF stain. On all immunoblots, signals were detected using horseradish peroxidase-conjugated secondary antibodies (Amersham™ ECL™ Prime; GE Healthcare) and documented using a luminescence image analyzer (LAS 4000; GE Healthcare) or chemiluminescence scanner (C-DiGit®; Li-Cor). Densitometry was performed using MultiGauge v.3.0 or ImageQuant TL v.7.1 (GE Healthcare).

Immunofluorescence microscopy of human heart tissue

Confocal microscopy was performed as described (52). Briefly, heart samples were fixed in 100 mM phosphate-buffered saline (PBS; ThermoFisher Scientific) containing 4% paraformaldehyde (PFA) and 15% saturated picric acid at 4°C overnight. Samples were dehydrated by ascending ethanol series, embedded in paraffin, and thin-sectioned (5-7 μm) using a microtome (RM 2235, Leica). Sections were rehydrated, blocked in peroxidase-containing buffer, and citrate-EGTA antigen recovery was performed. Slides were rinsed with PBS and blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) in the presence of 0.5% Triton X-100 for 60 min. Then, sections were incubated with primary antibodies, followed by fluorophore-conjugated secondary antibodies (see Table S5), at 4°C overnight. Samples were embedded in Mowiol supplemented with N-propyl-gallate to reduce bleaching. Imaging was done with a confocal laser scanning microscope (Leica TCS SP8) using a 63 $\times$ oil Plan-Apochromat objective.

Electron microscopy and immunoelectron microscopy

Human heart samples were fixed in 4% PFA, 15% saturated picric acid in 0.1 M PBS, pH 7.4, at 4°C overnight. For standard transmission EM, tissue samples were embedded in resin and processed as described (52). The area occupied by aggregates was measured in donor, IDCM, pre-Cronos and post-Cronos TTNtv-hearts on 128 electron micrographs/heart taken from 2 different heart-tissue sections. Analysis and quantitation were done using ImageSP software (TRS Systems). For immuno-EM, 50- μm -thick longitudinal sections were cut on a vibratome

(Leica VT 1000S), blocked in 20% normal goat serum (NGS, Vector Laboratories) in PBS, and incubated with Cronos antibodies diluted in PBS containing 5% NGS at 4°C overnight (Table S5). After several washes in PBS, sections were incubated with 1.4 nm gold-coupled secondary antibodies (Nanoprobes) in PBS at 4°C overnight. After extensive washing, sections were postfixed in 1% glutaraldehyde in PBS for 10 min and then reacted with HQ Silver kit (Nanoprobes). Following treatment with osmium tetroxide, sections were stained with uranyl acetate, dehydrated, and embedded in Durcupan resin (Fluka, Switzerland). Ultrathin sections were prepared (Ultracut S) and adsorbed onto glow-discharged Formvar-carbon-coated copper grids. Images were recorded under a Zeiss LEO 910 electron microscope equipped with a TRS sharp-eye CCD camera (TRS Systems).

Quantitation of sarcomere loss in TTNtv compared to IDCM hearts

The number of striation signals (sarcomere bands) within a 100- μm^2 cell area was calculated on IF images of IDCM and TTNtv hearts labeled with primary TTN-Z and fluorescent secondary antibodies. One Z-disc signal was counted as one sarcomere. A total of 300-1,500 antibody-marked striation signals were counted per heart. To allow for inter-sample comparison, we measured the IF signals at a sarcomere length of 1.8 μm , or normalized the sarcomere count/unit area to this sarcomere length. The same manual Z-disc counting procedure was also performed on electron micrographs recorded from patient heart tissues: the number of sarcomeres/100 μm^2 cell area was calculated and normalized considering a common sarcomere length of 1.8 μm .

2D culture of hiPSC-CMs

An hiPSC line was generated from a patient with DCM (*isTitin-MUC.10*, *MRIi010-A*) with a heterozygous TTNtv in exon 327 (c.70692_70693insAT; p.T23565SfsX5), as described (18). Several healthy donor hiPSC lines (*UMGi005-A*; *UMGi001-A*; *UMGi014-B*; *UMGi014-C*; *RUCDRi002-A*) were used as WT controls (49, 50). We also engineered hiPSC lines with a homozygous or heterozygous TTNtv in exon 359 (c.103103T>G; p.L34368X) by CRISPR/Cas9 gene-editing and compared them to the corresponding WT line on isogenic background. This TTNtv mimics the truncation in our TTNtv-DCM patient #21 (heterozygous M-band TTNtv).

Human iPSCs were cultured according to our published protocols (26, 49). Briefly, stem cells were seeded at a density of 1.3×10^4 cells/ cm^2 on 1:120 Matrigel-coated (Matrigel®, Corning; Geltrex®, Gibco), growth factor reduced (BD Biosciences) plates. Cells were passaged every 4-6 days with Versene solution (ThermoFisher Scientific) and cultured in StemMACS iPS-Brew XF medium (Miltenyi Biotech), supplemented with 2 μM thiazovivin (Merck Millipore) for 24 h, after passaging with a daily medium change. Cell cultures were grown in a humidified incubator at 5% CO_2 , 21% O_2 , and 37°C. Directed differentiation of the hiPSCs into hiPSC-CMs was performed via WNT-signaling modulation and subsequent metabolic selection (26, 49). Differentiated hiPSC-CMs were cultured for 1-3 months and subjected to biochemical, microscopic, and functional analyses. General viability of the cells and their spontaneous contractile activity were estimated by phase-contrast microscopy.

CRISPR/Cas9-based gene editing of hiPSCs

Genetic insertion of the M-band TTNtv c.103103T>G in the control hiPSC line *RUCDRi002-A* was achieved using ribonucleoprotein (RNP)-based CRISPR/Cas9 by targeting exon 359 of *TTN*. The guideRNA target sequence was (**PAM** in bold): 5' - ACCCATGTTCAAAAGGTTACT**GG**-3'. Genetic correction of the A-band TTNtv variant c.70692_70693insAT was performed using RNP-based CRISPR/Cas9 by targeting exon 327 of the *TTN* gene. The guideRNA target sequence was: 5' - TGGTGGCAGCAAGATATCACT**GG**-3'. For homology-directed repair, a single-stranded oligonucleotide with 45-bp homology arms was used. Briefly, hiPSC-lines *MRIi010-A* and *RUCDRi002-A* were cultured in StemFlex medium (ThermoFisher Scientific) on Matrigel-coated (growth factor reduced, BD Biosciences) plates. One hour before nucleofection, hiPSCs at a confluence of 70-80% were pretreated with 2 μ M thiazovivin (Merck Millipore), dissociated using Versene solution (ThermoFisher Scientific), and 2×10^6 hiPSCs quantified by CASY cell counter system (OMNI Life Science) were used for nucleofection. The CRISPR/Cas9 RNP complex was assembled by mixing the individual Alt-R CRISPR-Cas9 crRNA and the Alt-R CRISPR-Cas9 tracrRNA, pre-assembled at a 1:1 ratio, with the Alt-R S.p. HiFi Cas9 Nuclease 3NLS (IDT DNA Technologies) at a 1:3 molar ratio. The mix was incubated for 10 min at room temperature (RT) and diluted in nucleofector solution. Nucleofection was performed using the Amaxa 4D Nucleofector system (Lonza; program CA-137) and the P3 Primary Cell 4D-Nucleofector X kit (Lonza). Following nucleofection, hiPSCs were replated into two Matrigel-coated wells of a 6-well plate containing StemFlex medium supplemented with 2 μ M thiazovivin. After 72 h, transfected hiPSCs were analyzed for editing efficiency by Amplicon sequencing (Genewiz) and subsequently singularized using the CellenONE single cell dispenser (Scienion). Individual iPSC clones were expanded for approximately 2-3 weeks in StemFlex medium and analyzed for genetic modification by Sanger sequencing.

Verification of CRISPR/Cas9 gene editing of hiPSCs

Edited hiPSC lines *Corr-TTNtv.1*, *Corr-TTNtv.2*, and *Corr-TTNtv.3* (*MRIi010-A-1*, clone 2, 46, and 49), as well as hiPSC lines *TTN-L34368X.14* and *TTN-L34368X.20* (*RUCDRi002-A-12*, clone 14 and 20), were selected and verified for pluripotency and normal karyotype (Fig. S6, Fig. S9). To this end, cells were cultured on coverslips, fixed in Roti-Histofix 4% (Carl Roth) at RT for 20 min and blocked with 1% BSA (Sigma-Aldrich) in PBS (ThermoFisher Scientific) at 4°C overnight. For immunofluorescence, primary antibodies were applied in PBS with 1% BSA at 37°C for 1 h or at 4°C overnight (Table S5). Secondary antibodies with minimal cross-reactivity were administered in PBS with 1% BSA at RT for 1 h. For nuclear proteins, cells were permeabilized with 0.1% Triton-X 100 (Carl Roth) in staining solution, and nuclei stained with 4.8 μ M DAPI (ThermoFisher Scientific) at RT for 10 min. Samples were mounted in Fluoromount-G (ThermoFisher Scientific). Images were collected using the AxioImager M2 microscopy system and Zen 2.3 software (Zeiss).

For flow cytometry analysis, cells were dissociated into single cells, fixed in Roti-Histofix 4% at RT for 20 min, and blocked with 1% BSA in PBS at 4°C for at least 2 h. The hiPSCs were permeabilized with 0.1% Triton-X 100 in staining solution and co-incubated with fluorescence-conjugated antibodies against OCT4 and TRA-1-60 at RT for 1 h (Table S5). Nuclei were counter-stained with 8.1 μ M Hoechst 33342 (ThermoFisher Scientific). Subsequently, cells were analyzed using the LSRII flow cytometer (BD Biosciences) and BD FACSDiva software. Gating of cells was applied based on forward scatter area (FSC-A), sideward scatter area (SSC-A), and gating of intact cells based on DNA signal area and width. At least 10,000 events/sample were analyzed. For karyotyping, genomic DNA of Corr-hiPSCs was isolated using the QIAamp DNA Mini kit (Qiagen), according to the manufacturer's instructions. SNP-based human microarray was performed using Infinium Global Screening Array-24 v3.0 BeadChip and iScan array scanner (Illumina). Digital karyotypes were analyzed in GenomeStudio v2.0 software (Illumina) using the CNVpartition 3.2.0 algorithm with default settings. Copy number events were reported if larger than 3.5×10^5 bps and 1×10^6 bps for loss of heterozygosity.

Pharmacologic modulation of PQC pathways in 2D hiPSC-CMs

TTNtv- and WT-hiPSC-CMs were treated with activators or inhibitors of major PQC pathways. Care was taken to maintain the cells' spontaneous beating activity throughout the entire treatment periods. One day following the last regular renewal of full cardiac culture medium, a fresh batch of medium was added to the hiPSC-CMs, supplemented with cell-permeable drugs modulating autophagy-lysosomal pathways or proteasome-activity. The following substances were regularly used: autophagy-activator rapamycin (100 nM); autophagy-inhibitor bafilomycin A1 (10 to 50 nM); proteasome-inhibitors MG132 (500 nM to 1 μ M) or bortezomib (25 to 100 nM), as well as MG132 and bafilomycin combined. If not otherwise specified, cells were treated with a given substance for 3-5 days, while medium was replaced every 36 hours. In the control groups, only DMSO was applied to the culture medium, at a final concentration of 0.1%.

Monitoring of autophagy in 2D hiPSC-CMs

Our analysis followed recent recommendations (60). WT-hiPSC-CMs were cultured for 57 days and treated for another three days with bafilomycin A1 (50 nM). Cells were processed for IF microscopy using antibodies to SQSTM1, followed by fluorophore-conjugated secondary IgG (Table S5). Images were recorded using an LSM 700 confocal microscope and ZEN software (Carl Zeiss). Titin-antibody (TTN-M) and DAPI (nuclear) staining were performed, for comparison. WT-hiPSC-CMs were also cultured for 60 days and transduced with the BacMam gene delivery system containing an expression vector coding for pH-sensitive tagRFP-eGFP-LC3B fluorescent fusion protein (Thermo Fisher Scientific) (61). On day 65 of culture, cells were treated with autophagy-inhibitor bafilomycin A1 (50 nM), proteasome-inhibitor MG132 (1 μ M), or a combination of both, for 3 days. Fluorescence microscopy (Axiovert 40 C; software, AxioVision, Zeiss) was performed to study accumulation of LC3B-containing vesicles. Their surface area was quantified using ImageJ (NIH).

Immunofluorescence microscopy of hiPSC-CMs

Cells were washed with sterile PBS, replated onto matrix-coated chamber slides, and cultured as a monolayer until recovery. Then, they were fixed with 4% PFA in PBS for 20 min, washed, and subsequently permeabilized and blocked using 3% BSA at RT. Primary antibodies (Table S5) were applied to the cells at 4°C overnight and after washing, fluorophore-conjugated secondary antibodies were applied for two hours in a dark cabinet. Double immunostaining was performed by incubation with a suitable combination of 2 primary and 2 secondary antibodies. Specimens were washed with PBS, briefly air-dried, covered with fluid Vectashield® mounting medium with DAPI (Vector Laboratories), sealed with nail polish, and air-dried. Analysis was performed using structured illumination microscopy (Axio Observer equipped with Apotome.2; Zeiss).

Analysis of sarcomere length distribution in hiPSC-CMs

The SLs of hiPSC-CMs were quantified on IF images following co-staining with TTN-M or TTN-Z and ACTN2 antibodies. A minimum of 25 different regions-of-interest per sample were analyzed for average SL using an algorithm based on fast Fourier transform (FFT) calculation. The analysis was done with ImageJ software using built-in functions and a macro. The algorithm considers the distance between repeating segments (SL) as the wavelength of the periodic function propagating at a certain angle from the center. On the FFT processed image, all periodic wavelengths and propagation angles detectable were recorded and subsequently integrated over all directions (360°) as a radial plot. The integral FFT intensity peaks represent the most frequent SLs in the image. The analysis allowed measurements of SL (Z-disc or M-band periodicity) and SL-inhomogeneity (full width at half maximum (FWHM) of Z-/M-band periodicity distribution).

Test for integration of tr-titin protein into sarcomeres

To measure whether or not truncated titin is built into the sarcomeres of adult human TTNtv-hearts or TTNtv-hiPSC-CMs, samples were stained using TTN-Z or TTN-M antibodies and co-stained against ACTN2, followed by different fluorophore-conjugated secondary IgG. Identical staining conditions (antibody dilution and incubation times) were used throughout these experiments. Structured illumination microscopy (Axio Observer equipped with Apotome.2, Zeiss) or confocal laser scanning microscopy (Leica TCS SP8; 63× oil Plan-Apochromat objective; software: Leica Application Suite X; Version 3.1.5.16308) were employed. Automated three-channel fluorescence imaging (nuclei, TTN-Z or TTN-M, and ACTN2) was performed using manufacturer's software (AxioVision and ZEN), and care was taken to use identical settings for image recordings. Four to eight images were taken in different regions of a specimen. Image analysis was performed using ImageJ. IF-intensity profiles were calculated along the long axis of a selected myofibril, crossing a minimum of seven sarcomeres. IF-intensity values were integrated over the profile plots, separately for each antibody and myofibril. The integral value for the TTN-Z or TTN-M signal was divided by the integral value of the corresponding ACTN2 signal. Alternatively, the average maximum peak height of the regular IF signals corresponding to the sarcomeric Z-disc or M-band was calculated from the intensity profiles. Results were

similar for both approaches. Up to 40 profile plots per image were analyzed. Only IF-intensity signals recorded at a sarcomere length at or above 1.6 μm were included in the final analyses, because IF intensities dropped at shorter lengths. Average normalized intensity ratios (TTN/ACTN2) were calculated. For patient hearts, a “double quotient” of TTN-M/ACTN2 divided by TTN-Z/ACTN2 IF-intensity ratio was calculated as a final step, to further improve comparability between recordings. Comparisons were made between WT and TTNtv hiPSC-CMs or IDCM and TTNtv patient heart groups. Integration of tr-titin protein into the sarcomere was assumed to lower the number of available immunogenic epitopes for the TTN-M antibody, without changing the number of available epitopes for the ACTN2 or TTN-Z antibody. If tr-protein were built into the sarcomeres, the normalized IF intensity of the TTN-M signal in TTNtv cardiomyocytes would be reduced compared to controls.

EHM generation and drug treatment

Defined, serum-free EHM was generated from high purity (>92% α -actinin-positive) WT, TTNtv, or Corr-TTNtv(1-3) hiPSC-CMs and human foreskin fibroblasts (ATCC) at a 70:30% ratio (26). Cells were reconstituted in a mixture of pH-neutralized, medical grade, bovine collagen (0.4 mg/EHM; LLC Collagen Solutions) and concentrated serum-free medium (2 x RPMI, 8% B27 without insulin, 200 U/ml penicillin, 200 $\mu\text{g/ml}$ streptomycin). The mixture was cultured for 3 days in Iscove medium with 4% B27 without insulin, 1% non-essential amino acids, 2 mmol/l glutamine, 300 $\mu\text{mol/l}$ ascorbic acid, 100 ng/ml IGF1 (AF-100-11), 10 ng/ml FGF-2 (AF-100-18B), 5 ng/ml VEGF₁₆₅ (AF-100-20), 5 ng/ml TGF- β 1 (AF-100-21C) (growth factors obtained from Peprotech), 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin. Samples were cast in ring-shaped molds and grown on flexible holders for auxotonic loading. EHM was matured for 4 weeks and then treated with vehicle (0.1% DMSO), MG132 (500 nM), or Oleuropein (100 nM) (27) for 6 days. MG132 was used as a proteasome inhibitor, as it was better tolerated than bortezomib.

Analysis of EHM contractile function

Mechanical measurements on EHM rings were performed under isometric conditions in organ baths at 37°C in gassed (5% CO₂, 95% O₂) Tyrode's solution containing (in mmol/L): 120 NaCl, 1 MgCl₂, 0.2 CaCl₂, 5.4 KCl, 22.6 NaHCO₃, 4.2 NaH₂PO₄, 5.6 glucose, and 0.56 ascorbate. EHM were electrically stimulated at 1.5 Hz with 5 ms square pulses of 150 mA. They were mechanically stretched at intervals of 125 μm until the maximum systolic force amplitude (force of contraction; FOC) was observed according to the Frank-Starling law. Resting force and FOC were determined at increasing extracellular calcium concentrations (0.2 to 4.0 mM). The time-to-90% contraction and the time-to-50% relaxation were recorded as well. After measurements, EHM were snap frozen in liquid nitrogen and stored at -80°C until biochemical analysis.

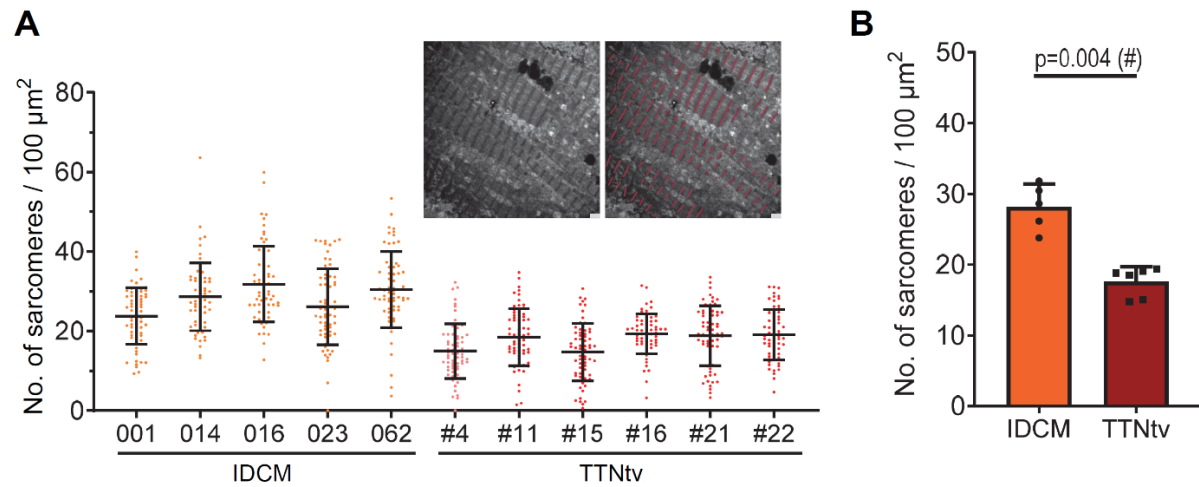


Fig. S1: Sarcomere loss in TTNtv vs. IDCM heart tissues by electron microscopic analysis.

(A) Average number of sarcomeres (normalized to a sarcomere length of 1.8 μm) within a 100- μm^2 area on electron micrographs of individual IDCM and TTNtv hearts; 65 images/heart were analyzed by counting the Z-discs. The inset images show the principle of the approach (left: raw image, right: marked image). Scale bars, 1 μm . (B) Summary of results for IDCM ($n=5$) and TTNtv ($n=6$) hearts. Data are mean $\pm$ SD. #Significance tested by unpaired, two-tailed t -test.

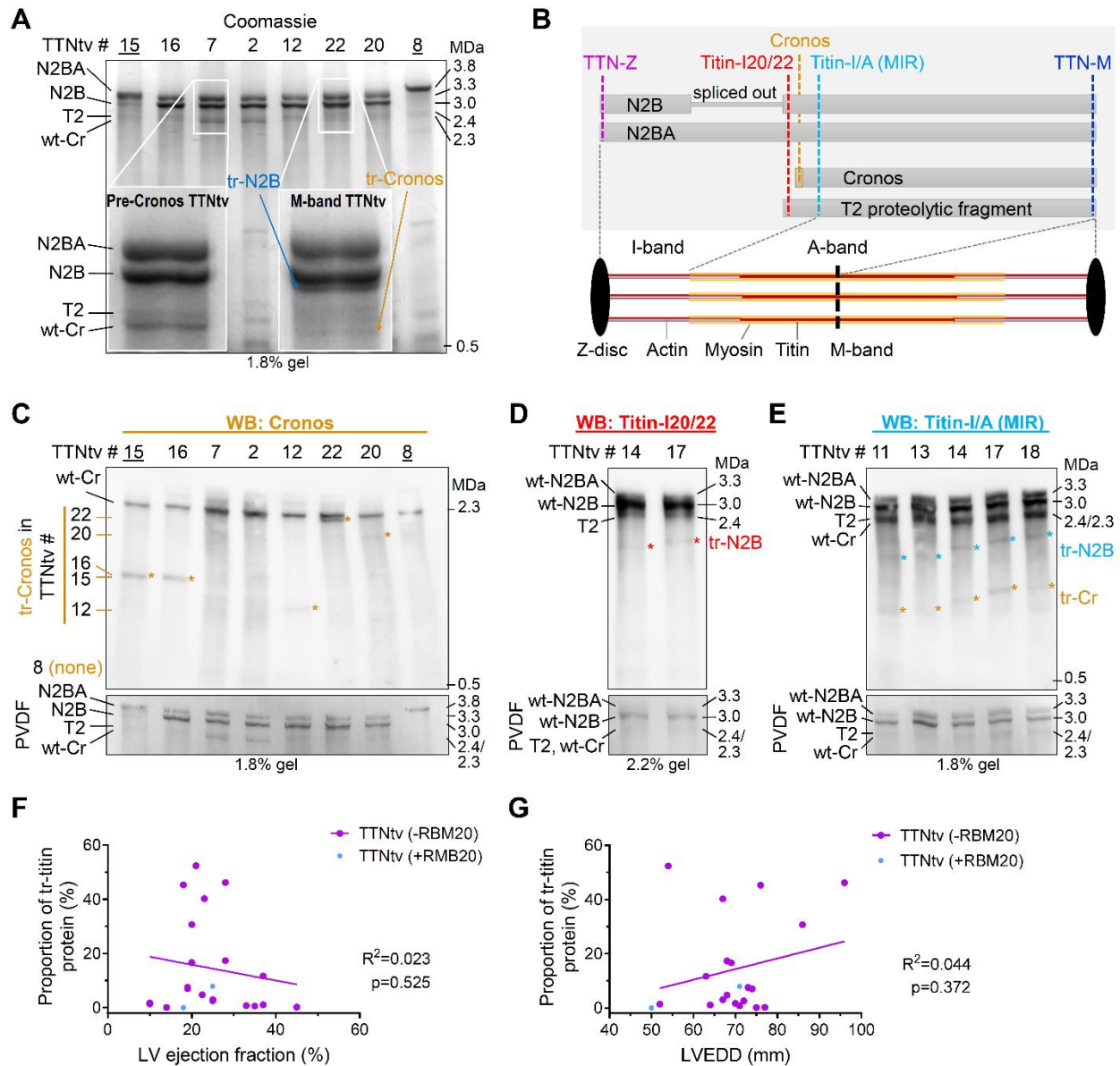


Fig. S2: Additional results of biochemical analyses of tr-titin proteins in TTNtv-hearts.

(A) Example of Coomassie-stained, two-phase SDS-PAGE gel loaded with TTNtv-DCM heart samples. tr, truncated protein; wt, wildtype protein. (B) Schematic of titin isoforms in the sarcomere and epitope positions of titin antibodies used in this study. (C) Anti-Cronos (Cr) western blot (WB), for a subset of A-band TTNtv-DCM hearts. (D) WB using Titin-I20/22 antibodies against a pre-Cronos titin epitope within distal I-band titin. (E) WB using Titin-I/A (MIR) antibodies against a post-Cronos epitope at the A-band edge. Asterisks in (C)-(E) indicate truncated protein. (F) and (G) depict analyses comparing relative tr-(N2BA+N2B+Cronos) protein content detected in TTNtv-DCM hearts ($n=22$) to left ventricular (LV) ejection fraction (F) or LV end-diastolic diameter (LVEDD) (G) of the respective patients at the time of heart transplantation. Lines are linear fits to $n=20$ data points, excluding the two hearts with additional RBM20 mutation (blue dots). Underlined numbers indicate additional RBM20 mutation.

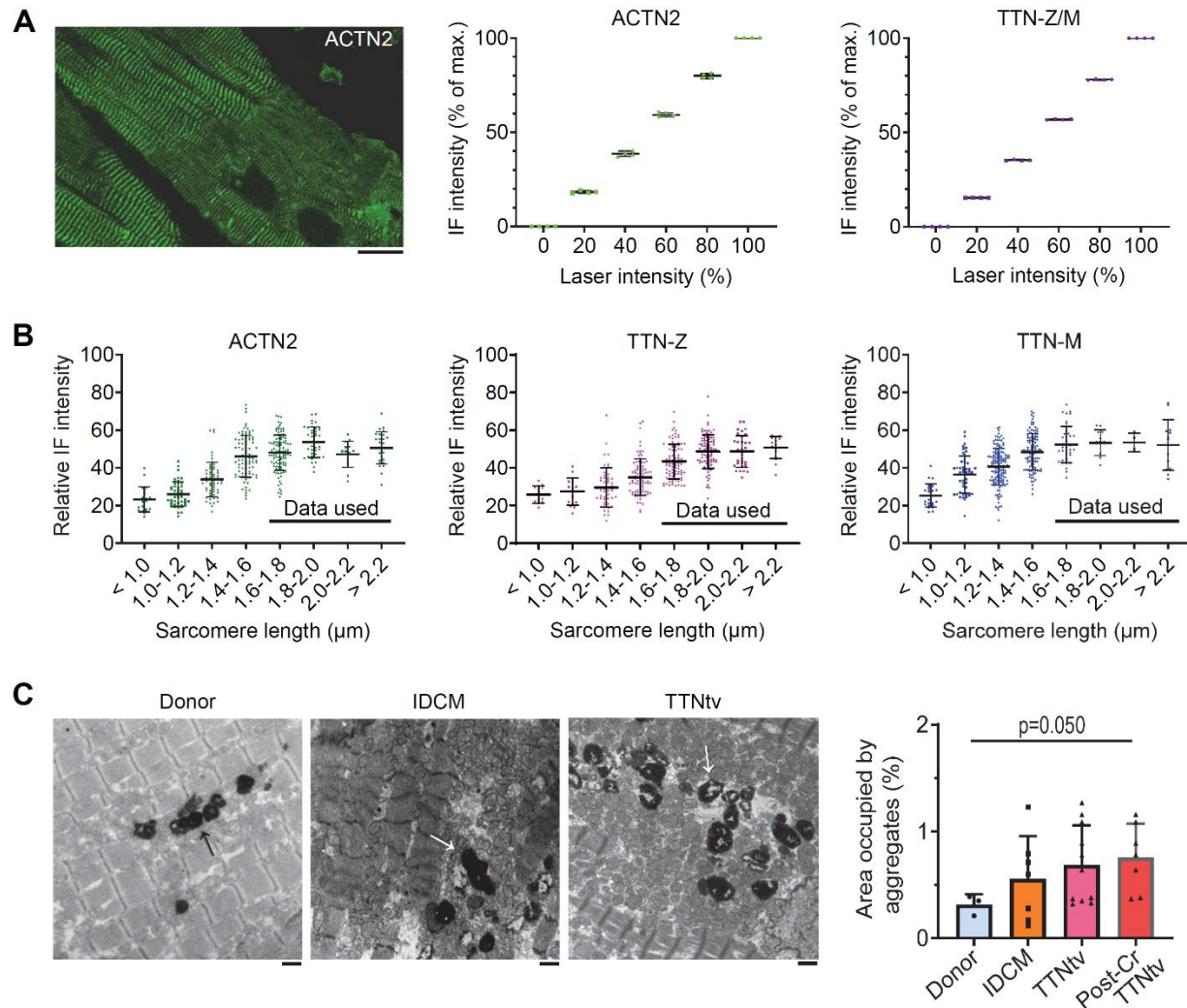


Fig. S3: Microscopic analyses of human heart tissues.

(A) Representative IF image of heart-tissue section stained against the Z-disc marker of α -actinin (ACTN2), followed by Alexa-488-conjugated secondary IgG. Scale bar, 20 μm . On similar IF images, the IF intensity was determined as a function of laser power in the confocal microscope (100%: maximal IF intensity at maximum laser power), adjusted in 20% intervals. Analysis was done using the “Rectangle” tool in ImageJ (NIH). The same analysis was done for ACTN2 (middle panel) and anti-titin (TTN-Z/M; right panel) antibodies. (B) Typical relationship between IF intensity of Z-disc or M-band peak signals on ACTN2 (left), TTN-Z (middle), and TTN-M (right) antibody-stained human heart-tissue sections and sarcomere length (SL). Each antibody was tested on a different heart. One-hundred sarcomeres per IF image were analyzed using the “Straight”-tool in ImageJ, in total 400 sarcomeres/antibody/heart, noting down the SL and the relative IF intensity of the respective antibody label. Parameters of staining and confocal microscopy settings were identical. IF intensity generally plateaued above $\sim 1.6 \mu\text{m}$ SL. (C) Representative electron micrographs of donor, IDCM, and TTNtv patient-heart tissue depicting aggregate formation (arrows); scale bars, 1 μm . The graph (right) shows average area occupied by aggregates (on 128 micrographs/heart), for donor ($n=3$), IDCM ($n=7$), and TTNtv ($n=11$) hearts, or only hearts with a post-Cronos (post-Cr) TTNtv ($n=7$). Data are mean $\pm$ SD. Significance tested by one-way ANOVA.

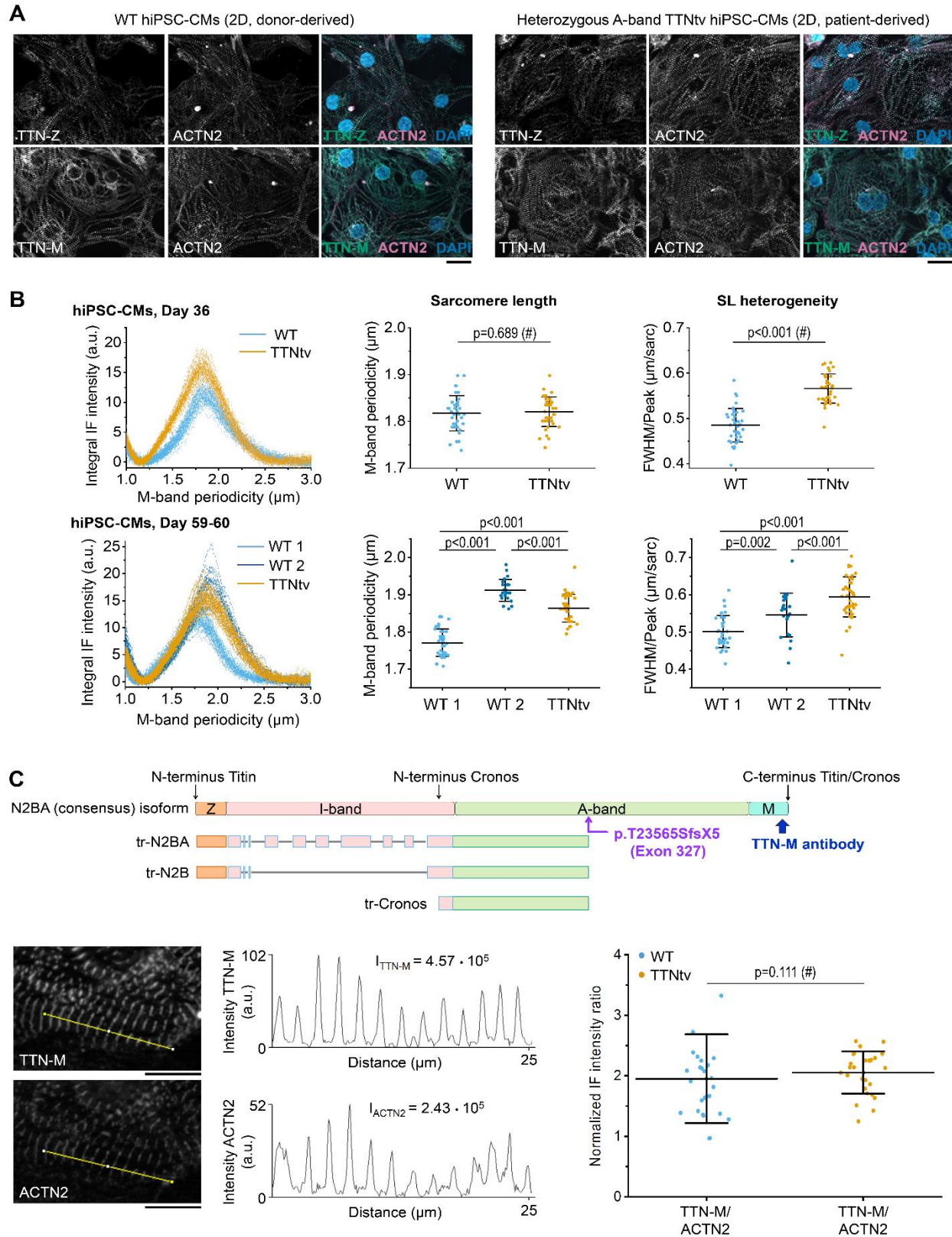


Fig. S4: Properties of hiPSC-CMs comparing patient-derived A-band (exon 327) TTNtv and donor-derived control (WT) cells.

(A) Sarcomere organization of cells (WT: *UMGi001-A*; TTNtv: *MRIi010-A*) after 36 days of cardiac differentiation. Comparative three-channel IF staining was performed: TTN-Z or TTN-M vs. α -actinin (ACTN2) antibodies (secondary antibodies were Alexa488 or Cy3-conjugated IgG); DAPI for nuclei. Scale bars, 25 μ m. (B) Sarcomere-length (SL) measurements in hiPSC-CMs at day 36 (WT: *UMGi014-C*; TTNtv: *MRIi010-A*) or day 59-60 (WT 1: *UMGi001-A*; WT 2: *RUCDRi002-A*; TTNtv: *MRIi010-A*) of cardiac differentiation, performed on TTN-M IF images. Left panels show results of fast Fourier transform (FFT) analysis, demonstrating the SL-distribution based on the periodicity of the TTN-M antibody signals. Middle panels show average SL (=M-band periodicity) at day 36 (WT, 1.82 ± 0.04 μ m; TTNtv 1.82 ± 0.03 μ m; $n=39-40$ /group) and at day 59-60 (WT 1, 1.77 ± 0.04 μ m; WT 2, 1.91 ± 0.03 μ m; TTNtv, 1.86 ± 0.04 μ m; $n=24-39$ /group). Right panels indicate SL-heterogeneity, judged from the full-width-at-half-maximum (FWHM) of FFT-curves depicted on left panels. At day 36, SL-heterogeneity was 0.48 ± 0.04 μ m/sarcomere in WT and 0.57 ± 0.03 μ m/sarcomere in TTNtv, $n=39-40$ /group; at day 59, SL-heterogeneity was 0.50 ± 0.04 μ m/sarcomere in WT 1, 0.55 ± 0.06 μ m/sarcomere in WT 2, and 0.59 ± 0.05 μ m/sarcomere in TTNtv, $n=24-39$ /group). (C) Assessment of tr-titin-protein integration into sarcomeres of (day-36) TTNtv-hiPSC-CMs. Top, titin species and TTN-M antibody epitope. Bottom panels show IF-intensity profiles taken along the axis of myofibrils on TTN-M- and ACTN2-stained images (left; scale bars, 10 μ m), from which the integral IF intensity was calculated for each antibody (middle); TTN-M signals were then normalized to ACTN2 signals (right). TTN-M/ACTN2 IF-intensity ratio was similar in WT (1.95 ± 0.73 ; *UMGi014-C*) and TTNtv (2.05 ± 0.35 ; *MRIi010-A*) cells, suggesting no incorporation of tr-titin/Cronos into TTNtv-hiPSC-CM sarcomeres. Average data are mean $\pm$ SD. Significance tested by one-way ANOVA and multi-comparison test (Holm-Sidak) or #unpaired, two-tailed, *t*-test.

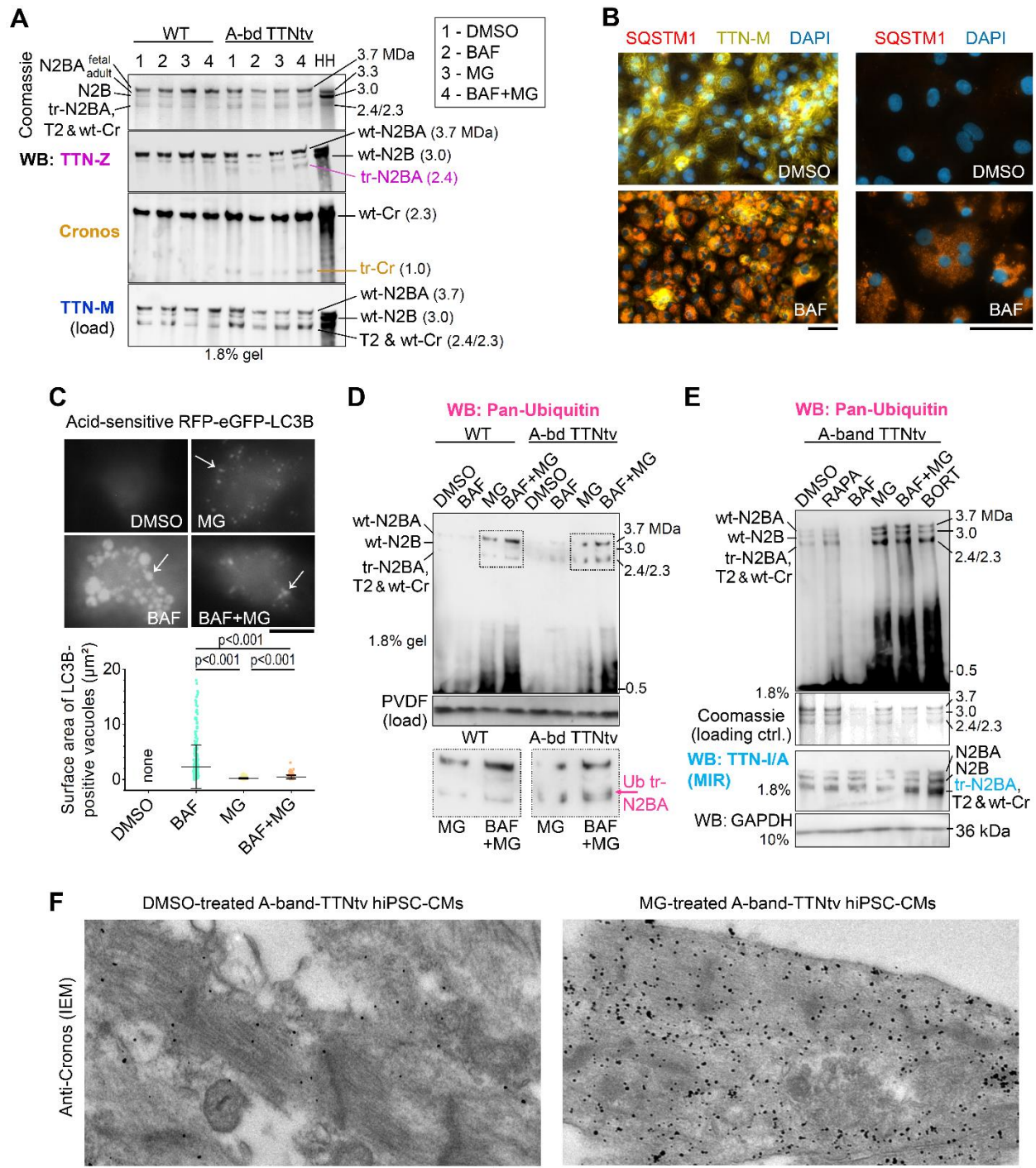


Fig. S5: Pharmacologic modulation of protein quality control pathways in hiPSC-CMs.

(A) Coomassie-stained titin gel (1.8 % SDS-PAGE, top) and Western blots (WB) using TTN-Z, Cronos, or TTN-M antibodies, which detect truncated (tr)-N2BA, tr-N2B, and tr-Cronos protein in patient-derived A-band (exon 327) TTNtv (*MRI010-A*) but not wildtype (WT) hiPSC-CMs (*UMGi001-A*); HH, adult human donor heart. Cells differentiated for 2 months were treated for three days with autophagy-inhibitor bafilomycin-A (BAF, 50 nM), proteasome-inhibitor MG132 (MG, 1 μM), or their combination; DMSO, vehicle-only control. (B) Accumulation of SQSTM1

in WT-hiPSC-CMs (*UMGi001-A*) differentiated for two months and treated for three days with BAF (10 nM), but not in those treated with DMSO only. SQSTM1-detection by immunofluorescence microscopy and co-staining against TTN-M to visualize cardiomyocytes; DAPI marks nuclei. Right images are at higher magnification and do not show the TTN-M channel. Scale bars, 50 μ m. (C) LC3B-accumulation upon pharmacologic autophagy-inhibition in WT-hiPSC-CMs (*UMGi014-B*) overexpressing pH-sensitive RFP-eGFP-LC3B fusion protein and treated as above; scale bar, 50 μ m. Arrows point to perinuclear puncta, which were enlarged in presence of BAF, as demonstrated by measuring the surface area of these LC3B-positive vacuoles (bottom). DMSO ($n=0$ puncta); BAF ($n=700$); MG ($n=779$); BAF+MG ($n=264$). Data are mean $\pm$ SD. Significance tested by one-way ANOVA and Kruskal-Wallis test. (D) Pan-ubiquitin WB (after 1.8% SDS-PAGE) of WT- (*UMGi001-A*) and A-band-TTNtv (*MRIi010-A*) hiPSC-CMs, detecting increased ubiquitination of titin and smaller cardiac proteins upon treatment with MG or BAF+MG, but not BAF alone. PVDF, Coomassie-stained blotting membrane (loading control). Boxes on bottom show enlarged gel areas, on which pan-ubiquitination of truncated (tr-) N2BA titin was evident in TTNtv-hiPSC-CMs (arrow). (E) Pan-ubiquitin WB of A-band-TTNtv hiPSC-CMs (*MRIi010-A*) treated as above, and additionally with autophagy-activator rapamycin (RAPA, 100 nM) or UPS-inhibitor bortezomib (BORT, 100 nM). Upon drug-treatment, tr-titin (N2BA) protein (~ 2.4 MDa) increased in presence of UPS-blockers, as observed on Titin-I/A (MIR) WB (bottom), and both wt- and tr-N2BA were more highly pan-ubiquitinated than in DMSO-controls. Coomassie-stained gel and GAPDH-WB are loading controls. (F) Representative immunoelectron micrographs of A-band-TTNtv hiPSC-CMs (*MRIi010-A*) before (left) and after (right) MG132-treatment, stained against Cronos (secondary antibody: nanogold-conjugated IgG). Scale bars, 0.5 μ m.

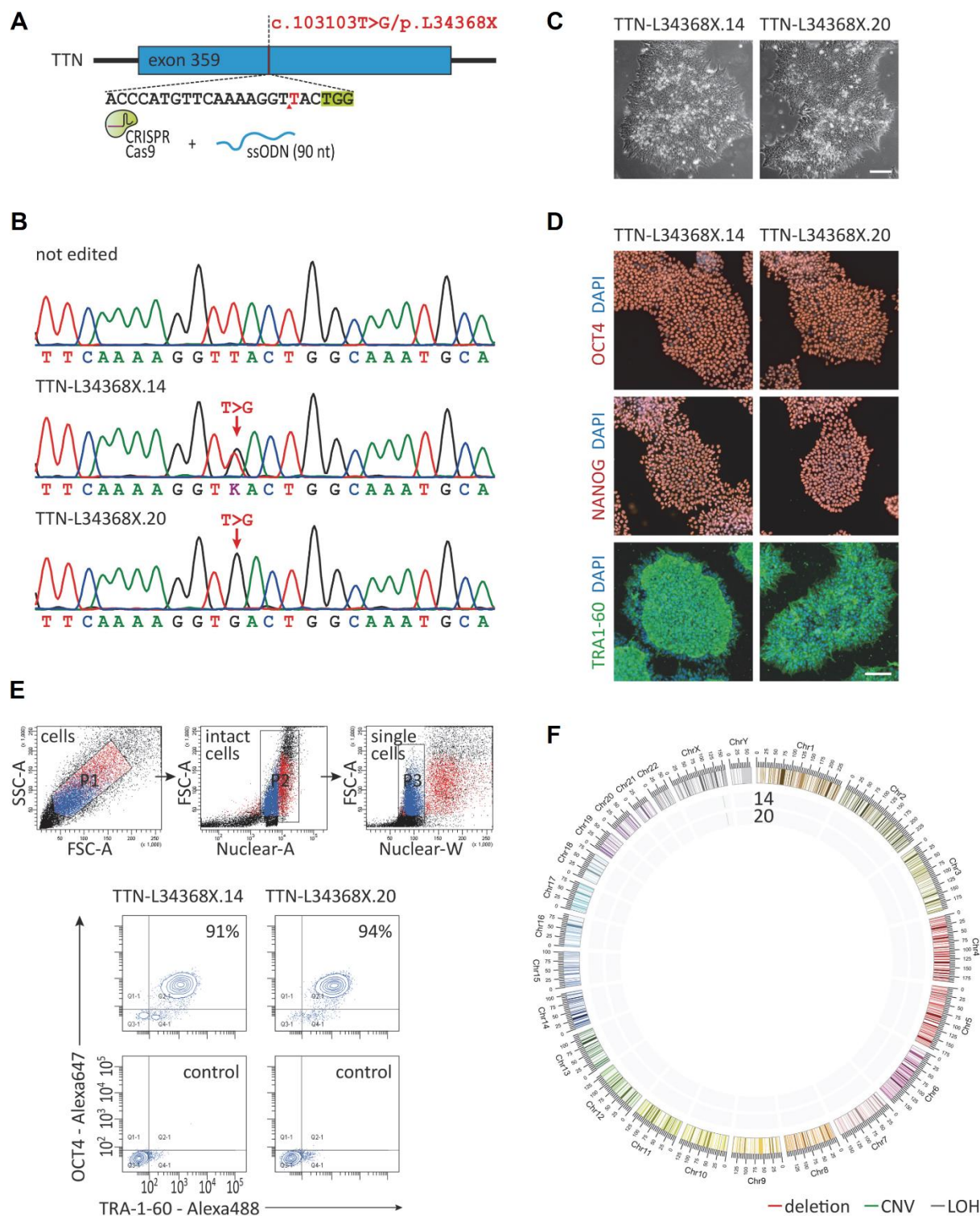


Fig. S6: Introduction of nonsense mutation (truncation) p.L34368X in *TTN* exon 359 by CRISPR/Cas9-editing of healthy hiPSCs.

(A) M-band (exon 359) *TTN*tv hiPSCs were generated by genetic insertion of *TTN* variant c.103103T>G in a control hiPSC line (*RUCDRi002-A*), using RNP-based CRISPR/Cas9 and a

single-stranded oligonucleotide (ssODN) for homology-directed repair. **(B)** Gene-editing confirmed by Sanger sequencing of genomic DNA. **(C)** Mutant hiPSCs exhibiting typical human stem cell-like morphology. Scale bar, 100 μm . **(D)** IF-staining for key pluripotency markers, OCT4, NANOG and TRA1-60, in mutant hiPSC lines. Nuclei were counter-stained with DAPI. Scale bar, 100 μm . **(E)** Purity of mutant hiPSC lines evaluated by flow cytometry analysis of pluripotency markers OCT4 and TRA1-60. The gating strategy is exemplified. Black dots represent all measured events. FSC-A, forward scatter area; SSC-A, sideward scatter area; Nuclear-A, nuclear area; Nuclear-W, nuclear width (from Hoechst staining). **(F)** ‘Digital’ karyotypes of mutant hiPSC lines (14, 20) demonstrating chromosomal stability after genome editing. CNV, copy number variation; LOH, loss of heterozygosity.

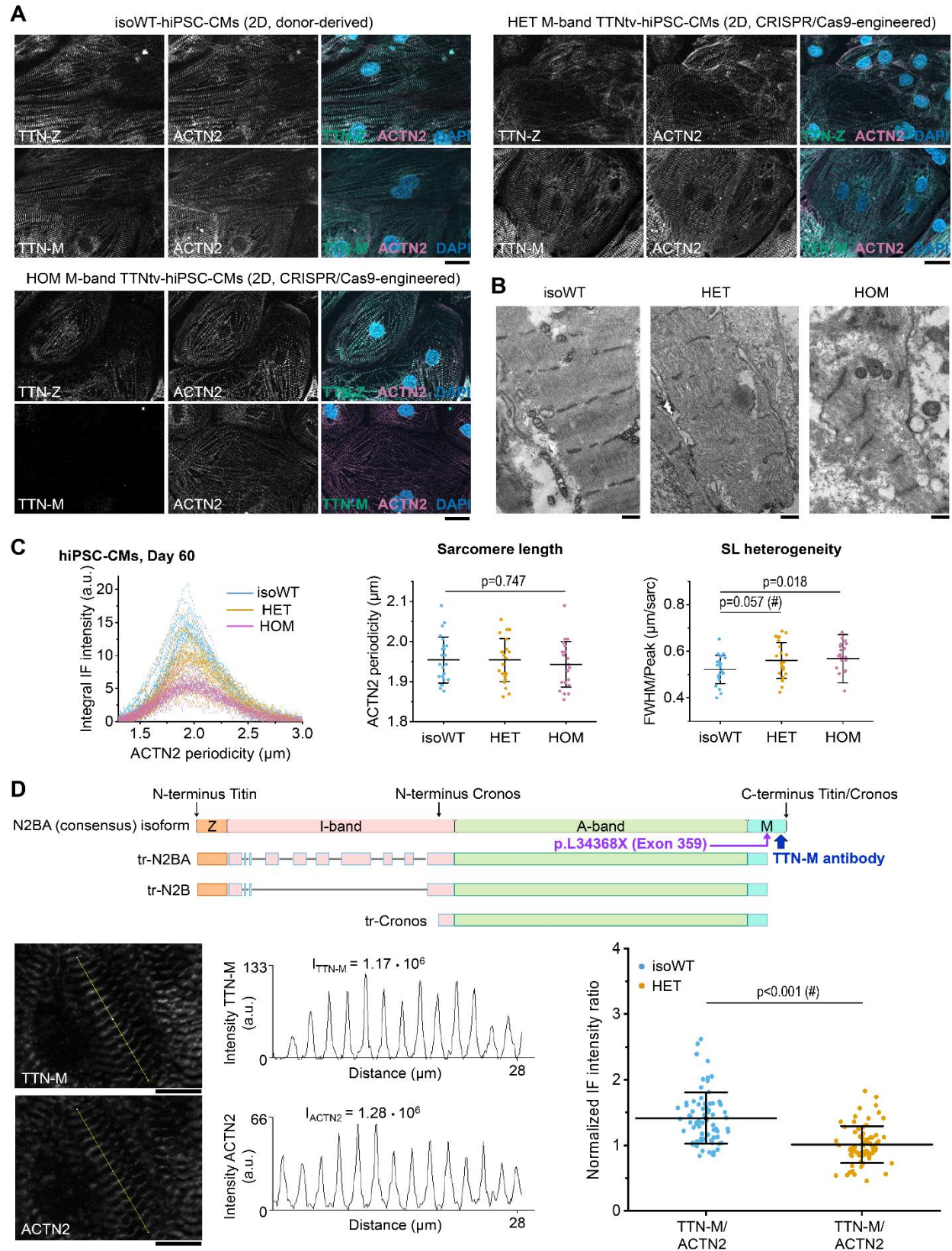


Fig. S7: Properties of hiPSC-CMs with or without CRISPR/Cas9-generated M-band TTNtv.

(A) Sarcomere organization of cells (isoWT: *RUCDRi002-A*; *TTN**tv*: *TTN-L34368X.20*) after 2 months of cardiac differentiation. Comparative three-channel IF staining was performed: TTN-Z or TTN-M vs. α -actinin (ACTN2) antibodies (secondary antibodies were Alexa488 or Cy3-conjugated IgG); DAPI for nuclei. Scale bars, 25 μ m. **(B)** Representative electron micrographs of hiPSC-CMs of the three genotypes. Scale bars, 0.5 μ m. **(C)** Sarcomere-length (SL) measurements in hiPSC-CMs at day 60 of cardiac differentiation, performed on ACTN2 IF images. Left panel shows results of fast Fourier transform (FFT) analysis, demonstrating the SL-distribution. Middle panel shows average SL (=ACTN2 periodicity), which was identical for WT (*RUCDRi002-A*) and mutant (*TTN-L34368X.20*) cells (1.95 ± 0.06 μ m/cycle in isoWT vs. 1.95 ± 0.05 μ m/cycle in HET and 1.94 ± 0.06 μ m/cycle in HOM; $n=23-24$ /group). Right panel indicates SL-heterogeneity, judged from the full-width-at-half-maximum (FWHM) of the FFT-curves depicted on the left panel. HOM-*TTN**tv*-hiPSC-CMs showed larger SL-inhomogeneity than isoWT-cells (0.52 ± 0.06 μ m/cycle in isoWT vs. 0.56 ± 0.08 μ m/cycle in HET and 0.57 ± 0.10 μ m/cycle in HOM; $n=23-24$ /group.) **(D)** Assessment of tr-titin-protein integration into sarcomeres of (day-60) HET M-band-*TTN**tv* hiPSC-CMs. Top, titin species and TTN-M antibody epitope. Bottom panels show IF-intensity profiles taken along the axis of myofibrils on TTN-M- and ACTN2-stained images (left; scale bars, 10 μ m), from which the integral IF intensity was calculated for each antibody (middle); TTN-M signals were then normalized to ACTN2 signals (right). The TTN-M/ACTN2 IF-intensity ratio was lower in HET (1.01 ± 0.28 ; $n=72$) than in isoWT (1.42 ± 0.39 ; $n=72$). Data are mean $\pm$ SD. Significance tested by one-way ANOVA, followed by multiple-comparison test, or [#]unpaired, two-tailed, *t*-test.

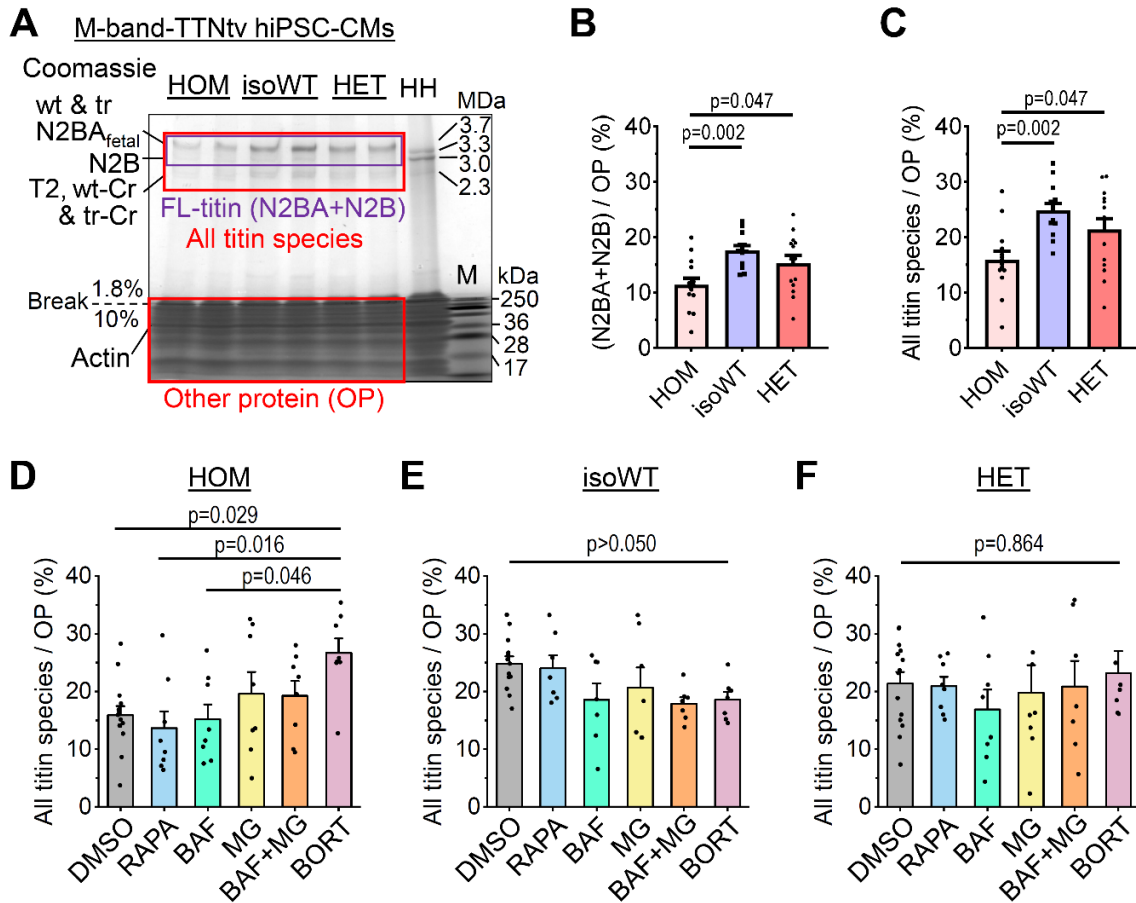


Fig. S8: Effects of CRISPR/Cas9-mediated generation of M-band-TTNtv in hiPSC-CMs on titin protein expression and role of pharmacologic manipulation of PQC pathways.

(A) Coomassie-stained two-phase gel loaded with hiPSC-CMs homozygous (HOM) or heterozygous (HET) for the M-band (exon 359) TTNtv (*TTN-L34368X.20*; *TTN-L34368X.14*); isoWT is WT on isogenic background (*RUCDRi002-A*). Two different cell batches of similar age (differentiation time, ~2 months) were loaded per genotype. HH, adult human donor heart. (B) Summary of densitometric quantitation of (N2BA+N2B)-titin expression normalized to “other protein” (OP; range, ~10-250 kDa) on Coomassie-stained two-phase gels. (C) Summary also made for all titin species (N2BA+N2B+T2+Cronos). (D)-(F) M-band-TTNtv hiPSC-CMs of the three genotypes, HOM (D), isoWT (E), and HET (F), were differentiated for ~2 months and treated for 5 days with DMSO (vehicle control), autophagy-modulators, or UPS-inhibitors, including rapamycin (RAPA, 100 nM); bafilomycin-A (BAF, 10 nM); MG132 (MG, 500 nM); BAF and MG combined; and bortezomib (BORT, 100 nM). On Coomassie-stained two-phase gels (see Fig. 6D-F), the all-titin (N2BA+N2B+T2+Cronos) to OP protein ratio was determined by densitometry. $n=13-14$ cell lysates/condition in (B), (C), and “DMSO” in (D)-(F); $n=7-8$ cell lysates/condition for drug treatments). Data are mean $\pm$ SEM. Significance tested by one-way ANOVA and multiple-comparison test.

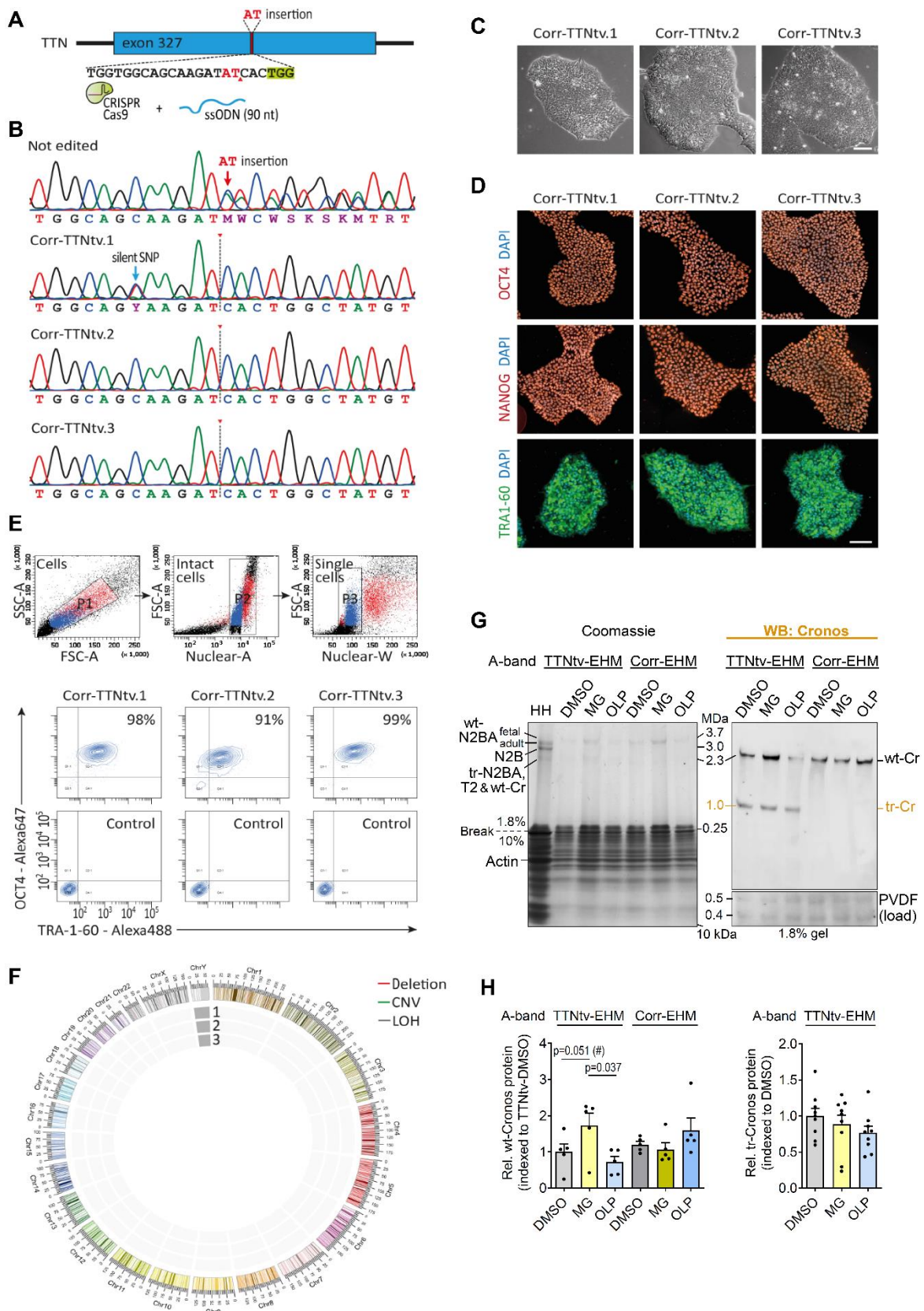


Fig. S9: Gene-correction of A-band (Ex 327) TTNtv-hiPSCs by CRISPR/Cas9-based editing.

(A) Corrected (Corr-)hiPSCs (*MRi010-A-1*, clone 2, 46, and 49) were generated from the patient-derived A-band exon-27-TTNtv line (*MRi010-A*) with a CRISPR guideRNA exclusively targeting the mutated *TTN* allele and a single-stranded oligonucleotide (ssODN) for homology-directed repair. (B) Genetic correction confirmed by Sanger sequencing of genomic DNA. Silent SNP was introduced by ssODN. (C) Corr-hiPSCs exhibiting typical human stem cell-like morphology. Scale bar, 100 μ m. (D) IF-staining for key pluripotency markers, OCT4, NANOG and TRA1-60, in Corr-hiPSC lines. Nuclei were counter-stained with DAPI. Scale bar, 100 μ m. (E) Purity of Corr-hiPSC lines evaluated by flow cytometry analysis of pluripotency markers OCT4 and TRA1-60. The gating strategy is exemplified. Black dots represent all measured events. FSC-A, forward scatter area; SSC-A, sideward scatter area; Nuclear-A, nuclear area; Nuclear-W, nuclear width (from Hoechst staining). (F) 'Digital' karyotypes of Corr-hiPSC lines (1-3) demonstrating chromosomal stability after genome editing. CNV, copy number variation; LOH, loss of heterozygosity. No CNV, LOH, or deletion was found. (G) Coomassie-stained, two-phase gel loaded with TTNtv-EHM and Corr-EHM (left), and Cronos-WB (right). EHM differentiated for 1 month from 4-week-old hiPSC-CMs was treated for 6 days with vehicle (DMSO), MG132 (MG, 500 nM), or oleuropein (OLP, 100 nM). HH, adult human donor heart. PVDF, stained blotting membrane (non-titin proteins). (H) Results of densitometric quantitation of wt-Cronos (left) and tr-Cronos (right) protein expression on WBs of TTNtv-EHM (*MRi010-A*) and Corr-EHM (*MRi010-A-1*, clone 2, 46, and 49) lysates. Data (mean $\pm$ SEM) were normalized to the expression in DMSO-treated TTNtv-EHM. $n=5-8$ /group. Significance tested by one-way ANOVA with Tukey's post-hoc test or #unpaired, two-tailed, *t*-test.

Table S1: Nomenclature of human DCM and donor hearts, information on patients, and results of assessment of heart function at time of transplantation.

Running No.	HDZ heart ID	TTNtv heart #	Age at HTx	Gender	Intervention	Age at first diagnosis and additional information	LVEDD (mm)	LVEF (%)
1	TTN001		7	w	HTx	"congenital DCM"	65	20-25
2	TTN002		63	m	HTx	First inpatient admission at HDZ at age 55: "known DCM"	57	35-40
3	TTN003		45	m	HTx	20	39	30
4	TTN004		48	w	HTx	46	60	25
5	TTN005		42	w	HTx	not known	40	28
6	TTN006/S1	12	69	w	HTx	Pacemaker implantation at age 60	71	35
7	TTN007/S2	9	49	m	HTx	not known	77	45
8	TTN008		55	m	HTx	Posterior myocardial infarction at age 38	52	25
9	TTN009		65	w	HTx	not known	79	15-20
10	TTN010		55	m	HTx	Mitral valve replacement at age 22	92-98	21
11	TTN011		61	w	HTx	First inpatient admission at HDZ at age 59	69	30
12	TTN012		66	m	HTx	Pacemaker implantation at age 52	82	18-20
13	TTN013		57	w	HTx	not known	80	25
14	TTN014		64	m	HTx	Anterior myocardial infarction at age 45	77	20
15	TTN015		46	m	HTx	not known	60	25
16	TTN016		41	w	HTx	First inpatient admission at HDZ at age 41: "Diagnosis years ago"	72	19
17	TTN017		60	w	HTx	Pacemaker implantation at age 51	52	36
18	TTN018		66	w	HTx	not known	56	37
19	TTN019		57	m	HTx	53	70	24
20	TTN020		65	m	HTx	approximately 51	80	25
21	TTN021		66	m	HTx	Exclusion of CAD at age 58	78	25
22	TTN022		9	w	HTx	not known	65	19
23	TTN023		55	m	HTx	not known	82	27
24	TTN024		70	m	HTx	not known	68	22
25	TTN025		62	w	HTx	Pacemaker implantation at age 59	67	25
26	TTN026		66	m	HTx	not known	67	28
27	TTN027		59	m	HTx	not known	75	20
28	TTN028		62	m	reHTx	HTx at age 47	56	37
29	TTN029		43	m	HTx	Condition after myocarditis at age 26	66	21
30	TTN030		54	w	HTx	Exclusion of CAD at age 54	56	37
31	TTN031		53	m	HTx	Exclusion of CAD at age 46	95	13
32	TTN032		47	m	HTx	31	72	33
33	TTN033		52	m	HTx	Condition after cardiopulm. reanim./ventricular fibrill. at age 46	66	28
34	TTN034		64	m	HTx	First inpatient admission at HDZ at age 64: "long-known DCM"	72	20
35	TTN035		27	m	HTx	Condition after myocarditis at age 21	84	20
36	TTN036		56	m	HTx	Anterior myocardial infarction at age 54	85	25
37	TTN037		65	m	HTx	not known	79	28
38	TTN038		64	m	HTx	not known	80	24
39	TTN039		43	m	HTx	AICD implantation at age 38	83	22
40	TTN040		52	m	HTx	AICD implantation at age 51	86	28
41	TTN041		51	m	HTx	AICD implantation at age 49	91	27
42	TTN042		61	m	HTx	Exclusion of CAD at age 56	63	21
43	TTN043		59	m	HTx	Heart palpitations with dyspnea at age 53	74	32
44	TTN044		36	m	HTx	not known, First admission at HDZ at age 32 (already diseased)	97	15-20
45	TTN045		66	m	HTx	Pacemaker implantation at age 56	80	21
46	TTN046		28	w	LVAD-IP	28	68	<15
47	TTN047		53	m	HTx	not known	"moderately dilated"	43
48	TTN048		65	m	HTx	Mitral valve reconstruction and ICD implantation at age 60	73	19
49	TTN049		62	m	HTx	not known	81	26
50	TTN050/S3	21	40	m	HTx	not known	67	23
51	TTN051		48	m	HTx	42	70	18
52	TTN052/S4	17	59	m	HTx	43	69	20
53	TTN053		49	m	HTx	Exclusion of CAD at age 43	66	20-25
54	TTN054		44	m	HTx	Myocarditis at age 43	53	26
55	TTN055		61	m	HTx	Implantation of CRT-ICD at age 54	76	15
56	TTN056		60	m	HTx	Exclusion of CAD at age 59	69	18
57	TTN057/S5	14	63	m	HTx	44	63	37
58	TTN058		21	m	HTx	not known	71	20
59	TTN059		15	m	HTx	not known	76	20
60	TTN060		62	w	HTx	Posterior myocardial infarction at age 42	77	18
61	TTN061		65	m	HTx	Exclusion of stenosing CAD at age 53	70	20
62	TTN062		58	w	HTx	Condition after sec. prophylactic 1-chamber ICD implant. at age 47	55	25
63	TTN063		54	m	HTx	not known	83	23
64	TTN064/S6	19	56	m	HTx	Exclusion of CAD at age 51	72	25
65	TTN065		62	m	HTx	Exclusion of stenosing CAD at age 49	63	17
66	TTN066		48	m	HTx	44	75	25
67	TTN067		54	w	HTx	not known	68	20
68	TTN068		61	m	LVAD-Tx	Myocarditis at age 52	66	13
69	TTN069		62	w	HTx	Exclusion of CAD at age 56	82	19
70	TTN070	5	41	m	HTx	not known	76	18
71	TTN071		33	m	HTx	not known	82	23
72	TTN072		62	w	HTx	52	67	15
73	TTN073		46	w	HTx	Cardiopulmonary reanimation at age 29	67	21
74	TTN074/S7	1	55	m	HTx	AICD implantation at age 50	67	25
75	TTN075		66	m	HTx	AICD implantation at age 54	80	23
76	TTN076		47	m	HTx	not known	67	21
77	TTN077		55	m	HTx	Condition after aortic valve replacement at age 35	77	20
78	TTN078		52	m	HTx	not known	70	25
79	TTN079		24	m	HTx	Condition after AICD implantation at age 20	62	25
80	TTN080		57	m	HTx	not known	72	20
81	TTN081/S8	18	51	m	HTx	/	68	28
82	TTN082/S9	6	59	m	HTx	Exclusion of CAD at age 51	71	33
83	TTN083		62	w	HTx	not known	79	35
84	TTN084		44	m	HTx	Anterior myocardial infarction at age 43	59	25
85	TTN085		33	w	HTx	not known	59	26
86	TTN086		60	m	HTx	not known	85	17
87	TTN087		24	m	HTx	not known	72	26
88	TTN088		34	m	HTx	31	74	24
89	TTN089		4	m	HTx	not known	40	40
90	TTN090		64	m	HTx	Exclusion of CAD at age 53	87	15
91	TTN091		38	m	HTx	DCM after myocarditis at about 23 of age	92	20

92	TTN092		61	m	HTx	not known	70	20
93	TTN093		62	m	HTx	Persisting atrial fibrillations since the age of 57	59	15
94	TTN094		48	w	HTx	not known	66	39
95	TTN095		42	m	HTx	AICD implantation at age 40	44	41
96	TTN096		23	w	HTx	22	92	21
97	TTN097		71	w	HTx	not known	62	20
98	TTN098		57	w	HTx	Exclusion of a coronary macroangiopathy at age 53	74	29
99	TTN099		29	m	HTx	not known	81	18
100	TTN100		41	m	HTx	not known; in-frame deletion in <i>TTN</i>	57	33
101	TTN102		56	m	HTx	Reanim. and AICD impl. after recurrent ventr. tachycardia at age 54	58	36
102	HDZ120918-1/S11	7	46	m	HTx	34	75	14
103	HDZ120918-2/S12	2	38	w	HTx	CRT-D implantation at age 32	54	21
104	HDZ120918-3/S13	13	50	m	LVAD-IP	Condition after ICD implantation/ventricular tachycardia at age 45	70	10
105	HDZ120918-4/S14	22	21	m	HTx	not known	86	20
106	HDZ120918-5/S15	20	49	m	LVAD-IP	not known	52	10
107	HDZ120918-6/S16	8	8	w	BVAD-Tx	not known	50	18
108	HDZ2021116-2/S17	4	58	m	LVAD-Tx	45	74	19
109	HDZ210617-1/S18	15	34	m	TAH-IP	Condition after exclusion of CAD at age 27	71	25
110	HDZ210617-2/S19	16	23	m	LVAD-IP	not known	68	15-20
111	tvTTN001/S20	11	69	m	LVAD-IP	Exclusion of CAD and ICD implantation at age 68	73	19
112	tvTTN002/S21	10	61	m	HTx	ICD implantation at age 57	64	37
113	tvTTN003/S22	3	29	m	LVAD-IP	Exclusion of CAD and ICD implantation at age 23	96	28

I-band TTNtv
A-band TTNtv
M-band TTNtv

Mean±SD Age of ICDM patients: **50.9±15.0 (n=91)**
Mean±SD Age of TTNtv patients: **47.7±16.1 (n=22)**

m/w (ICDM): **66/25**
m/w (TTNtv): **19/3**

Mean±SD LVEDD of ICDM patients: **70.76±12.31 (n=90)**
Mean±SD LVEDD of TTNtv patients: **69.73±10.15 (n=22)**

Mean±SD EF of ICDM patients: **24.20±6.72 (n=91)**
Mean±SD EF of TTNtv patients: **23.98±9.04 (n=22)**

Donor heart No.	Sydney Heart Bank ID	Source	Age	Gender
Donor 1	5.089 LV 10	Sydney Heart Bank	48	w
Donor 2	5.041 LV 6	Sydney Heart Bank	42	w
Donor 3	5.114 LV 1	Sydney Heart Bank	1.2	w
Donor 4	5.030 LV 6	Sydney Heart Bank		
Donor 5	19.12.02 LV 2	Sydney Heart Bank	26	m
Donor 6	10.12.00 LV 1	Sydney Heart Bank		
Donor 7		Boston MGH		
Donor 8		Boston MGH		
Donor 9		Boston MGH	67	w
Donor 10		Boston MGH		
Donor 11		Boston MGH		
Donor 12		Boston MGH		
Donor 13		Boston MGH		
Donor 14		Boston MGH	54	w

Mean±SD Age Donor (protein analysis): **39.7±23.2 (n=6)**

Donor 15 (only for qRT-PCR)	HDZ-NRW; Bad Oeynhausen	40	f
Donor 16 (only for qRT-PCR)	HDZ-NRW; Bad Oeynhausen	60	m
Donor 17 (only for qRT-PCR)	HDZ-NRW; Bad Oeynhausen	50	m
Donor 18 (only for qRT-PCR)	HDZ-NRW; Bad Oeynhausen	31	f
Donor 19 (only for qRT-PCR)	HDZ-NRW; Bad Oeynhausen	54	f
Donor 20 (only for qRT-PCR)	HDZ-NRW; Bad Oeynhausen	44	m
Donor 21 (only for qRT-PCR)	HDZ-NRW; Bad Oeynhausen	55	m
Donor 22 (only for qRT-PCR)	HDZ-NRW; Bad Oeynhausen	5	f
Donor 23 (only for qRT-PCR)	HDZ-NRW; Bad Oeynhausen	28	m
Donor 24 (only for qRT-PCR)	HDZ-NRW; Bad Oeynhausen	45	m

Mean±SD Age Donor (qRT-PCR): **41.2±16.3 (n=10)**

Mean±SD Age Donor (All): **40.6±18.4 (n=16)**

Table S2: Location of the truncating *TTN* mutation in all 22 patients with TTNtv-DCM.

TTNtv #	Gene	Nucleotide position	Peptide position	HG19 coordinate	TTN exon	PSI (%)	Sarcomere band	UniProtKB	Titin domain
		NM_001267550.1 (for all)	NP_001254479.1 (for all)					Q8WZ42-1	
1	<i>TTN</i>	c.14301C>A	p.Cys4767Ter	179602879	50	100	I-Band	4450	Ig-like 24
	<i>TTN</i>	c.11183dupG	p.Leu3729ThrfsTer9	179621019	46	4	I-Band	3412	Nx2
2	<i>TTN</i>	c.11483delA	p.Asn3828MetfsTer4	179606476	49	100	I-Band	3511	Ig-like 21
3	<i>TTN</i>	c.12438_12448delATCTCCAACC	p.Ser4147ThrfsTer20	179605511	49	100	I-Band	3830	N2Bus
4	<i>TTN</i>	c.13696C>T	p.Gln4566Ter	179604264	49	100	I-Band	4249	N2Bus
5	<i>TTN</i>	c.13838_13839delTG	p.Val4613GlyfsTer5	179604120	49	100	I-Band	4296	Ig-like 23
6	<i>TTN</i>	c.42214C>T	p.Arg14072Ter	179499294	231	100	I-Band	12431	Insertion sequence
7	<i>TTN</i>	c.42909_42910delTG	p.Cys14303TrpfsTer12	179498175	233	100	I-Band	12662	Ig-like 84
8	<i>TTN</i>	c.53743C>T	p.Arg17915Ter	179470279	280	100	A-Band	16274	FN3-18
9	<i>TTN</i>	c.56806C>T	p.Arg18936Ter	179463631	292	100	A-Band	17295	FN3-25
10	<i>TTN</i>	c.61682C>G	p.Ser20561Ter	179454770	305	100	A-Band	18920	Ig-like 107
11	<i>TTN</i>	c.65035_65036delGC	p.Ala21679LeufsTer6	179449241	312	100	A-Band	20038	FN3-45
12	<i>TTN</i>	c.66632dupA	p.Asn22211LysfsTer8	179446362	317	100	A-Band	20570	FN3-49 same as TTNtv #13
13	<i>TTN</i>	c.66632dupA	p.Asn22211LysfsTer8	179446362	317	100	A-Band	20570	FN3-49 same as TTNtv #12
14	<i>TTN</i>	c.71005A>T	p.Lys23669Ter	179439854	327	100	A-Band	22029	Ig-like 115 same as TTNtv #15 & #16
15	<i>TTN</i>	c.71005A>T	p.Lys23669Ter	179439854	327	100	A-Band	22029	Ig-like 115 same as TTNtv #14 & #16
16	<i>TTN</i>	c.71005A>T	p.Lys23669Ter	179439854	327	100	A-Band	22029	Ig-like 115 same as TTNtv #14 & #15
17	<i>TTN</i>	c.73032G>A	p.Trp24344Ter	179437827	327	100	A-Band	22703	FN3-65
18	<i>TTN</i>	c.73200T>A	p.Tyr24400Ter	179437659	327	100	A-Band	22757	FN3-65
19	<i>TTN</i>	c.84677_84678delAG	p.Glu28226ValfsTer6	179426180	327	100	A-Band	26585	FN3-93
20	<i>TTN</i>	c.98990-1G>T	(Intron?)	179403567	before 355	100	A-Band	?	?
21	<i>TTN</i>	c.103103T>G	p.Leu34368Ter	179398239	359	100	M-Band	32727	Ig-like 145
22	<i>TTN</i>	c.103360delG	p.Glu34454AsnfsTer3	179397981	359	100	M-Band	32318	Ig-like 146

Table S3: Overview of the gene variants (MAF<0.05%) identified in patients with TTNtv-DCM.[†]

Sample	Gene	Chromosome	Position	NM-number and base exchange	NP-number and protein exchange	rs-number
1	TTN	2	179602879	NM_001267550.1:c.14301C>A	NP_001254479.1:p.Cys4767Ter	n.a.
	TTN	2	179621019	NM_001267550.1:c.11183dupG	NP_001254479.1:p.Leu3729ThrfsTer9*	rs778172350;rs786205317
2	TTN	2	179606476	NM_001267550.1:c.11483delA	NP_001254479.1:p.Asn3828MetfsTer4	n.a.
	TTN	2	179665301	NM_001267550.1:c.404T>A	NP_001254479.1:p.Val135Glu	rs1332868582
	TTN	2	179421869	NM_001267550.1:c.88012C>T	NP_001254479.1:p.Pro29338Ser	rs774162189
3	TNNT2	1	201331086	NM_001276345.1:c.674G>A	NP_001263274.1:p.Arg225Lys	n.a.
	TTN	2	179605511	NM_001267550.1:c.12438_12448delATCTCCCAACC	NP_001254479.1:p.Ser4147ThrfsTer20	n.a.
4	NPPA	1	11907414	NM_006172.3:c.206A>C	NP_006163.1:p.Glu69Ala	rs376184349
	ZBTB17	1	16272649	NM_003443.2:c.535G>A	NP_003434.2:p.Gly179Ser	rs1368741903
	TTN	2	179463477	NM_001267550.1:c.56960T>C	NP_001254479.1:p.Ile18987Thr	rs373351577
	TTN	2	179604264	NM_001267550.1:c.13696C>T	NP_001254479.1:p.Gln4566Ter	rs775072385
	TMPO	12	98938242	NM_001032283.2:c.806G>A	NP_001027454.1:p.Arg269His	rs142891873
5	TTN	2	179604120	NM_001267550.1:c.13838_13839delITG	NP_001254479.1:p.Val4613GlyfsTer5	n.a.
	TTN	2	179527707	NM_001267550.1:c.36776C>T	NP_001254479.1:p.Ala12259Val	rs755562550
	CACNB2	10	18823025	NM_201596.2:c.1075G>A	NP_963890.2:p.Glu359Lys	n.a.
	SALL4	20	50407719	NM_020436.3:c.1303G>A	NP_065169.1:p.Val435Met	rs373070046
6	TTN	2	179499294	NM_001267550.1:c.42214C>T	NP_001254479.1:p.Arg14072Ter	rs794729258
	TTN	2	179647631	NM_001267550.1:c.3002T>G	NP_001254479.1:p.Met1001Arg	rs148269839
	TTN	2	179595389	NM_001267550.1:c.17871A>T	NP_001254479.1:p.Gln5957His	rs181067357
	TTN	2	179453880	NM_001267550.1:c.62572A>G	NP_001254479.1:p.Thr20858Ala	rs200689750
	TTN	2	179566921	NM_001267550.1:c.30485C>T	NP_001254479.1:p.Thr10162Met	rs200593368
7	TTN	2	179498175	NM_001267550.1:c.42909_42910delITG	NP_001254479.1:p.Cys14303TrpfsTer12	n.a.
	DSP	6	7542284	NM_004415.2:c.136G>A	NP_004406.2:p.Gly46Ser	rs371517189
8	TTN	2	179470279	NM_001267550.1:c.53743C>T	NP_001254479.1:p.Arg17915Ter	rs753333359
	AKAP9	7	91718834	NM_005751.4:c.9349C>A	NP_005742.4:p.Pro3117Thr	rs769119204
	RBM20	10	112581111	NM_001134363.1:c.2734G>C	NP_001127835.1:p.Asp912His	n.a.
	MYH6	14	23858874	NM_002471.3:c.3787G>A	NP_002462.2:p.Val1263Met	rs375819633
	MYH6	14	23866768	NM_002471.3:c.1946T>C	NP_002462.2:p.Val649Ala	rs543231422
	SNTA1	20	32005549	NM_003098.2:c.677G>A	NP_003089.1:p.Cys226Tyr	rs34479952
	KRT17	17	39777928	NM_000422.2:c.751C>T	NP_000413.1:p.Arg251Cys	rs139367104
	NOTCH1	9	139390902	NM_017617.3:c.7289G>C	NP_060087.3:p.Gly2430Ala	rs36049318
	NOTCH1	9	139404341	NM_017617.3:c.2813G>A	NP_060087.3:p.Arg938Gln	rs35962301
9	TTN	2	179413091	NM_001267550.1:c.93262T>G	NP_001254479.1:p.Phe31088Val	rs1023137329
	TTN	2	179463631	NM_001267550.1:c.56806C>T	NP_001254479.1:p.Arg18936Ter	rs72646828
	FBN2	5	127664406	NM_001999.3:c.4453G>A	NP_001990.2:p.Asp1485Asn	rs187786356
10	TTN	2	179454770	NM_001267550.1:c.61682C>G	NP_001254479.1:p.Ser20561Ter	n.a.
	DSP	6	7569429	NM_004415.2:c.1430A>G	NP_004406.2:p.His477Arg	n.a.
11	APOB	2	21230283	NM_000384.2:c.9457T>A	NP_000375.2:p.Trp3153Arg	rs761484953
	TTN	2	179413758	NM_001267550.1:c.92595A>C	NP_001254479.1:p.Leu30865Phe	rs192086736
	TTN	2	179449241	NM_001267550.1:c.65035_65036delIGC	NP_001254479.1:p.Ala21679LeufsTer6	n.a.
	DSC2	18	28660275	NM_024422.3:c.1307G>T	NP_077740.1:p.Gly436Val	rs763981974
12	TTN	2	179446362	NM_001267550.1:c.66632dupA	NP_001254479.1:p.Asn22211LysfsTer8	rs1440081449
	CACNA2D1	7	81626509	NM_000722.2:c.1648G>T	NP_000713.2:p.Asp550Tyr	rs542692632
	CBS	21	44483161	NM_001178008.1:c.856A>G	NP_001171479.1:p.Ile286Val	rs147040567
13	TTN	2	179446362	NM_001267550.1:c.66632dupA	NP_001254479.1:p.Asn22211LysfsTer8	rs1440081449
	COL5A2	2	189912958	NM_000393.3:c.3178C>T	NP_000384.2:p.Arg1060Trp	rs374549843
	RYR1	19	38937148	NM_000540.2:c.668A>G	NP_000531.2:p.His223Arg	rs766836202
	TXNRD2	22	19902736	NM_006440.3:c.591+1G>C		rs200610775
14	TTN	2	179439854	NM_001267550.1:c.71005A>T	NP_001254479.1:p.Lys23669Ter	n.a.
	TTN	2	179582922	NM_001267550.1:c.24811G>C	NP_001254479.1:p.Glu8271Gln	n.a.
15	TTN	2	179439854	NM_001267550.1:c.71005A>T	NP_001254479.1:p.Lys23669Ter	n.a.
	TTN	2	179582922	NM_001267550.1:c.24811G>C	NP_001254479.1:p.Glu8271Gln	n.a.
	RBM20	10	112570147	NM_001134363.1:c.1807G>A	NP_001127835.1:p.Gly603Arg	rs558674954
	RBM20	10	112572526	NM_001134363.1:c.2374dupG	NP_001127835.1:p.Glu792GlyfsTer9	n.a.
16	TTN	2	179439854	NM_001267550.1:c.71005A>T	NP_001254479.1:p.Lys23669Ter	n.a.
	TTN	2	179582922	NM_001267550.1:c.24811G>C	NP_001254479.1:p.Glu8271Gln	n.a.
	NOTCH1	9	139391281	NM_017617.3:c.6910T>G	NP_060087.3:p.Leu2304Val	rs749993527
	CACNB2	10	18807897	NM_201596.2:c.944G>A	NP_963890.2:p.Arg315Gln	rs1225227914
17	TTN	2	179437827	NM_001267550.1:c.73032G>A	NP_001254479.1:p.Trp24344Ter	n.a.
	MYH11	16	15812192	NM_01040114.1:c.5296G>A	NP_001035203.1:p.Val1766Ile	rs138059405
18	TTN	2	179437659	NM_001267550.1:c.73200T>A	NP_001254479.1:p.Tyr24400Ter	n.a.
19	TTN	2	179426180	NM_001267550.1:c.84677_84678delIAG	NP_001254479.1:p.Glu28226ValfsTer6	n.a.
	TTN	2	179486240	NM_001267550.1:c.45311T>C	NP_001254479.1:p.Leu15104Pro	rs778421383
20	SCN1B	19	35523524	NM_199037.3:c.133C>T	NP_950238.1:p.Arg45Cys	rs757554677
	SCO2	22	50962378	NM_001169109.1:c.463C>G	NP_001162580.1:p.Pro155Ala	rs777864630
	COL5A2	2	189904129	NM_000393.3:c.3794A>G	NP_000384.2:p.Asp1265Gly	rs200325397
	LTBP2	14	75078428	NM_000428.2:c.220G>C	NP_000419.1:p.Glu74Gln	rs79886273
21	TTN	2	179403567	NM_001267550.1:c.98990-1G>T		rs796111471
	TTN	2	179433610	NM_001267550.1:c.77249G>A	NP_001254479.1:p.Arg25750Gln	rs368038362
22	APOB	2	21237970	NM_000384.2:c.3671G>A	NP_000375.2:p.Arg1224Gln	rs53051188
	TTN	2	179398239	NM_001267550.1:c.103103T>G	NP_001254479.1:p.Leu34368Ter	n.a.
	SCN5A	3	38592336	NM_198056.2:c.5527G>T	NP_932173.1:p.Val1843Leu	n.a.
23	TTN	2	179397981	NM_001267550.1:c.103360delG	NP_001254479.1:p.Glu34454AsnfsTer3	rs760768093

Table S4: Oligonucleotides used for qPCR analysis.

mRNA	Primer forward	Primer reverse	Probe
<i>Cronos-TTN</i>	ACGCAAAGCTGTTTCTTCCC	CTTCGTAGGAGAGCTCGCAG	TGGTCTTCTGAGGCCTTTAAAGGATGTCACCGT
<i>Full-length (FL-)TTN</i>	TAGTGCCACCTCCGAGTT	CTTCGTAGGAGAGCTCGCAG	TGGTCTTCTGAGGCCTTTAAAGGATGTCACCGT
<i>RPL32 (p0255)</i> <i>RPL32 (p1056)</i>	AAGTTCATCCGGCACCAGTC or (with identical results) CTGGTCCACAACGTCAAGGA	TGGCCCTTGAATCTTCTACGA or (with identical results) AGCGATCTCGGCACAGTAAGA	CCCAGAGGCATTGACAACAGGG or (with identical results) TGGAAGTGCTGCTGATGTGCAA
<i>PPIA</i>	GCCATGGAGCGCTTTGG	CCACAGTCAGCAATGGTGATCT	TCCAGGAATGGCAAGACCAGCAAG
<i>GAPDH</i>	CTGGGCTACACTGAGCACCA	CAGCGTCAAAGGTGGAGGAG	TGGTCTCCTGACTTCAACAGCGACAC

Sequences are listed in 5'-3' direction. Probes were labeled with FAM at 5' and TAMRA at 3'.

Table S5: Antibodies used for Western blot (WB), immunofluorescence microscopy (IF), immunoelectron microscopy (IEM), and flow cytometry (FC).

Antibody	Host	Manufacturer	Cat. number	Used for	Dilution
Actinin, alpha 2 (ACTN2)	Mouse	Sigma-Aldrich	A7811	IF	1:1,000
Cronos*	Rabbit	Kaneka Eurogentec S.A.	n/a	WB IF IEM	1:4,000 1:200 1:500
α B-crystallin (CRYAB)	Rabbit	Abcam	ab76467	WB	1:1,000
GAPDH	Rabbit	Cell Signaling	2118S	WB	1:20,000
LC3B	Rabbit	Cell Signaling	2775	WB	1:2,500
MURF1, Ig-Y type	Chicken	Myomedix	n/a	WB	1:2,500
NANOG	Rabbit	ThermoFisher Scientific	PA1-097, RRID: AB_2539867	IF	1:100
OCT3/4	Goat	R and D Systems	AF1759, RRID: AB_354975	IF	1:40
OCT3/4 conjugated Alexa Fluor 647	Mouse	BD Biosciences	560329, RRID: AB_1645318	FC	1:50
SQSTM1/p62	Rabbit	Antibodies Online	ABIN6148417	WB	1:2,500
	Rabbit	Abcam	ab109012	IF	1:1,000
TRA-1-60, IgM	Mouse	Abcam	ab16288, RRID: AB_778563	IF	1:200
TRA-1-60 conjugated Alexa Fluor 488	Mouse	BD Biosciences	560173, RRID: AB_1645379	FC	1:50
TTN-Z (N-terminus)	Rabbit	Myomedix	TTN-1	WB IF	1:1,750 1:200
TTN-I20/22	Rabbit	Kaneka Eurogentec S.A	n/a	WB	1:1,000
TTN-I/A (MIR)	Rabbit	Myomedix	TTN-6	WB	1:1,750
TTN-M (C-terminus)	Rabbit	Myomedix	TTN-9	WB IF	1:1,500 1:250
Ubiquitin, pan	Mouse	Santa Cruz	Sc-8017	WB	1:100
Anti-Mouse IgG (H+L) HRP	Goat	Life Technologies	31430	WB	1:10,000
Anti-Mouse IgG (H+L) HRP	Goat	Abcam	ab6789	WB	1:10,000
Anti-Rabbit IgG (H+L) HRP	Goat	Life Technologies	31460	WB	1:10,000
Anti-Rabbit IgG (H+L) HRP	Goat	Abcam	ab6721	WB	1:10,000
Anti-Mouse IgG (H+L) HRP	Goat	Dianova	115-035-146	WB	1:5,000
Anti-Rabbit IgG (H+L) HRP	Goat	Acris	R1364HRP	WB	1:10,000
Anti-Chicken IgY (H+L) HRP	Goat	Dianova	103-035-155	WB	1:40,000
Anti-Mouse IgG (H+L) Alexa Fluor 488	Goat	Life Technologies	A11029	IF	1:1,000
Anti-Mouse IgG (H+L) Alexa Fluor 555	Goat	Life Technologies	A32727	IF	1:1,000
Anti-Mouse IgG (H+L) Alexa Fluor 633	Goat	Life Technologies	A21050	IF	1:1,000
Anti-Rabbit IgG (H+L) Alexa Fluor 488	Goat	Life Technologies	A11034	IF	1:1,000
Anti-Rabbit IgG (H+L) Alexa Fluor 555	Goat	Life Technologies	A32732	IF	1:1,000
Anti-Mouse IgG (H+L) Alexa Fluor 488	Goat	Jackson ImmunoResearch	115-546-146	IF	1:250
Anti-Rabbit IgG (H+L) Alexa Cy TM 3	Goat	Jackson ImmunoResearch	111-166-003	IF	1:250
Anti-Rabbit Nanogold [®] -IgG	Goat	Nanoprobes	#2003	IEM	1:100
Anti-Mouse IgG Alexa Fluor 488	Donkey	ThermoFisher Scientific	A-21202, RRID: AB_141607	IF	1:1,000
Anti-Goat IgG Alexa Fluor 555	Donkey	ThermoFisher Scientific	A-21432, RRID: AB_2535853	IF	1:1,000
Anti-Rabbit IgG Alexa Fluor 555	Donkey	ThermoFisher Scientific	A-31572, RRID: AB_162543	IF	1:1,000

*The human Cronos (N-terminus) antibody has been validated in Ref. 15; the epitope sequence is: SNAKLFLPSRLA.

The following source data files are included:

Data file S1 (Microsoft Excel format)

This file contains the source data (gels, blots, data, and statistics) for the main figures 1-8.

Data file S2 (Microsoft Excel format)

This file contains the source data (gels, blots, data, and statistics) for the supplementary figures S1-S9)