

SUPPLEMENTAL MATERIALS

Novex-3 is a Z-disk structural and signaling hub that is reduced in heart failure

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Expanded Materials & Methods

Reproducibility and rigor

All experiments were performed in at least three independent biological replicates unless otherwise stated. For qRT-PCR and ddPCR analyses, each sample was measured in technical duplicates or triplicates, and ddPCR experiments were performed in at least two independent runs. Ribosome profiling (Ribo-seq) analyses represent a secondary analysis of previously published datasets.³⁸ Cardiomyocyte contractility measurements were conducted using three independent differentiations. Protein-based experiments were performed using two independently purified protein batches, unless stated otherwise. For immunofluorescence studies, image acquisition and analysis were carried out in a non-blinded manner. Fields of view were selected at random, and identical acquisition settings were applied uniformly across all samples. Representative images and traces were selected after quantitative analysis from samples with values close to the group mean for the corresponding quantified parameter. When image parameters were not quantified, the total number of images acquired for the respective condition is indicated in the corresponding figure legend.

Predefined inclusion and exclusion criteria were applied throughout the study. In yeast two-hybrid (Y2H) experiments (**Table S1**, **Table S2**), clones that were not in-frame were excluded. In co-immunoprecipitation (Co-IP) assays, samples exhibiting insufficient protein expression were excluded based on predefined criteria. To ensure robustness of interaction data, positive Y2H interactions were usually retested in independent mating assays.

To promote transparency and reproducibility, all software parameters used in data processing and analysis are available from the corresponding authors upon request.

Human heart tissues

LV tissue from DCM transplant recipients were obtained from the biobank of the Heart and Diabetes Center NRW (Bad Oeynhausen, Germany), as previously described.⁹ All living patients provided written informed consent for research and anonymous publication of their explanted myocardial tissue. The study was approved by the ethics committee of Ruhr University Bochum, registry Nos. 13/2009, 21/2013, 58/2014). Nonfailing donor heart tissue was obtained from the same source and the Sydney heart bank (University of Sydney, Australia), with informed consent from next of kin and approval by the University of Sydney Human Research Ethics Committee (HREC 2012/2814), in accordance with Australian National Health Medical Research Council guidelines. The study conforms to the Declaration of Helsinki.¹² Sex, age, and left ventricular (LV) ejection fraction (LVEF) of DCM patients are provided in **Table S3**; sex and age of nonfailing donors are listed in **Table S4**.

Adult LV tissue from nonfailing donors (rejected for technical reasons), DCM explants, and human fetal hearts was snap-frozen in liquid nitrogen and stored at -80°C. Failing hearts were genotyped using a next-generation sequencing panel covering 174 cardiac disease genes (TruSight™ Cardio, Illumina, San Diego, CA, USA).⁹ Samples were classified by the presence or absence of titin-truncating variants (TTNtv vs. IDCM)⁹ and further stratified by mutation location (I-band vs. A-band TTNtv; **Table S3**). Hearts of all participants pre-enrolled⁹ were included. Gel electrophoretic analysis of tissue samples occasionally did not reveal protein bands and those hearts (6-7 out of >100) were therefore excluded from subsequent

analysis. Randomization was not performed but hearts were assigned to different subgroups according to sequencing results (IDCM, TTNtv, TTNtv-A/-I,) and health status (failing, donor); investigators were not blinded to this information.

Tissues from five fetal (105-117 days post-conception) human hearts (LV) were obtained from the Birth Defects Research Laboratory at the University of Washington (Seattle, USA), with ethics board approval (#STUDY00000380) and maternal written consent. This study was performed in accordance with ethical and legal guidelines of the University Hospital Münster.

Novex-3 knockout mouse tissue

Novex-3-deficient mice on a C57BL/6N background were generated using CRISPR/Cas9 genome editing, as previously described.¹¹ Briefly, guide RNAs targeting the N-terminal region of the Nx3-specific exon were designed and validated in vitro, and the most efficient guide was selected. This guide RNA and Cas9 protein were introduced into fertilized zygotes by electroporation, followed by transfer into pseudopregnant females to obtain founder animals. Genotyping identified a line carrying a 5-bp-frameshift deletion resulting in a premature stop codon, and germline transmission was confirmed to establish a stable knockout line. Predicted off-target sites were analyzed, and no mutations were detected.

All animal experiments were conducted in accordance with ARRIVE 2.0 guidelines and approved by the Institutional Animal Care and Use Committee at Kawasaki Medical School. Mice were housed under specific pathogen-free conditions with a 12-hour light/dark cycle, controlled temperature and humidity, and ad libitum access to food and water. Adult mice (20-25 weeks old) of both sexes were used. Homozygous KO mice and WT littermate controls were analyzed (KO: N=5; WT: N=4), with a single animal defined as the experimental unit.

Animals were allocated to experimental groups based on genotype. Randomization was not performed, as group assignment was inherently determined by genotype and animal availability. To minimize bias, all experiments were conducted using standardized protocols, and investigators were blinded to genotype during outcome assessment and data analysis.

No animals were excluded from the analysis. Predefined inclusion criteria included successful genotyping and absence of overt health abnormalities. As no statistically significant sex-related differences were observed, data from males and females were pooled. Sample size was determined based on prior studies and feasibility; no formal power calculation was performed. All procedures were carried out to minimize animal suffering. Animals were monitored regularly, and euthanasia was performed using approved humane methods in accordance with institutional guidelines.

Additional murine tissues used for developmental analyses

In addition, tissues were collected from C57BL/6 mice (Janvier Labs, Le Genest-Saint-Isle, France), including adult (8-12 weeks), neonatal (postnatal day 1), and embryonic (E13) hearts. Data from males and females were pooled. All procedures complied with institutional guidelines and were approved by the Animal Care and Use Committees of Ruhr-University Bochum and the University of Münster.

Antibodies

Primary and secondary antibodies used for immunofluorescence (IF), immunoelectron microscopy (IEM), and WB are listed in **Table S5**. Titin-specific antibodies were used to distinguish the different titin isoforms, as follows (**Fig. 1A**): The titin-Z (Z1/Z2) antibody targets Z-disk titin, thereby recognizing the N-terminus of the isoforms N2B, N2BA, and Nx3 (fetal and adult), but not Cronos. Anti-titin S2080 and T12 antibodies recognize titin's Z-disk/I-band junction and also recognize all isoforms but Cronos. Anti-Nx3 is21²⁰ and anti-I23²³ recognize only the Nx3 isoform, and another recognizing the most C-terminal Ig domain (I23) within the Nx3 region. Anti-Cronos detects the extreme N-terminal sequence of Cronos and is specific to this isoform. Anti-N2Bus and anti-PEVK are directed against the large unique sequences in cardiac I-band titin and detect N2B and N2BA isoforms, but not Nx3 and Cronos. Anti-M8/M9 targets the C-terminal titin domains at the M-band, thus recognizing N2B, N2BA, and Cronos but not Nx3. With the exception of T12 (kindly provided by Dieter Fürst, Bonn, Germany), anti-titin antibodies were obtained from Myomedix (S. Labeit; Neckargemünd, Germany) or custom-made by Eurogentec (Seraing, Belgium). All antibodies were previously validated.

Yeast-two-hybrid (Y2H) screening

As described previously,^{56,57} bait plasmids were transformed into *Saccharomyces cerevisiae* strain AH109 by electroporation. A 50-ml YPD culture was inoculated from a frozen glycerol stock and grown for 2 days at 30°C with shaking (200 rpm). At an OD₅₅₀ of 0.6-0.8, cells were harvested (3,000 rpm, 5 min, 4°C), washed twice with ice-cold double-distilled water (2,500 rpm, 5 min, 4°C), and resuspended in ice-cold 1 M sorbitol. Following a final centrifugation step (2,000 rpm), the cell pellet was resuspended in 200-400 µl of 1 M sorbitol, depending on pellet size. For transformation, 3 µl of plasmid DNA were mixed with 40 µl of competent yeast cells on ice and transferred to an electroporation cuvette. Electroporation was performed at 1,600 V, immediately followed by the addition of 1 ml of 1 M sorbitol. Cells were transferred to a sterile microcentrifuge (Eppendorf) tube, recovered for 2 h at 30°C with shaking (150 rpm), pelleted (1,000 rpm, 2 min, room temperature), suspended in 100 µl of TE buffer, and plated onto SD/-Trp agar. Plates were incubated at 30°C for 3 days.

Mating of bait and prey. A fresh, large colony of the bait strain was inoculated into 50 ml of SD/-Trp liquid medium and grown overnight at 30°C with shaking (200 rpm) until an OD₅₅₀ of 0.8 was reached. Cells were harvested by centrifugation (2,500 rpm, 5 min), the supernatant was removed, and the pellet was resuspended in 5 ml SD/-Trp. The thawed library strain was mixed with the bait strain in a sterile 2-l flask. The library vial was rinsed twice with 1 ml 2xYPD, and the rinses were added to the culture together with 45 ml 2xYPD. The mating culture was incubated for 20 h at 30°C with shaking (40 rpm). A sample was examined microscopically to confirm zygote formation. Cells were collected by centrifugation (2,500 rpm, 10 min), the flask was rinsed with 45 ml 0.5xYPD, and the rinse was used to resuspend the pellet. After a second centrifugation step, cells were resuspended in 10 ml 0.5xYPD, and the total suspension volume was recorded. Serial dilutions (1:10, 1:100, 1:1,000, and 1:10,000) of the mated culture were plated (100 µl each) onto 100-mm agar plates containing SD/-Trp, SD/-Leu, or SD/-Trp/-Leu medium. The remaining culture was plated onto 150-mm TDO agar plates supplemented with 3-AT. Plates were incubated at 30°C for 6-10 days. The number of screened clones and

mating efficiency were subsequently calculated using equations (1)-(3).

$$\text{Colony forming units (cfu)/ml} = z \cdot 10 \cdot d \quad (1)$$

z = no. of clones on plate, d = dilution factor

$$\text{No. of screened clones} = \text{cfu/ml (diploids)} \cdot V \quad (2)$$

V = resuspension volume

$$\text{Mating efficiency (\%)} = \frac{\text{No. of cfu/ml of diploids}}{\text{No. of cfu/ml of limiting partner}} \cdot 100 \quad (3)$$

The limiting partner can be either the prey library or the bait strain and is determined by the lower number of cfu/ml on SD/-Leu (prey) or SD/-Trp (bait), respectively. The number of screened clones should be at least 1 million diploids to ensure a high probability of detecting genuine interactions. A mating efficiency >2 % was considered successful.

For the Identification of cDNA inserts, plasmids from diploid yeast cells were rescued by use of the Zymoprep™ Yeast Plasmid Miniprep II Kit (Zymo Research, Irvine, USA). Isolated plasmids were transformed into *E. coli* via heat shock and the bacteria plated onto Luria Broth agar plates (Sigma-Aldrich) supplemented with ampicillin to select for the library plasmid instead of the bait plasmid. After overnight incubation at 37°C, single *E. coli* clones were inoculated into 8 ml Lysogeny Broth (LB) medium (Sigma-Aldrich) and again incubated overnight at 37°C under constant shaking at 180 rpm. Plasmids were isolated and subjected to restriction and sequencing analysis. The sequencing results were analyzed using the BLAST algorithm to identify cDNA inserts. Plasmids with inserts that were not in the correct reading frame were excluded from further analysis. Primers are listed in **Table S6**.

Positive interactions were retested in independent mating assays to confirm reproducibility. For the direct mating assay (to directly analyze interactions between putative binding partners identified in the initial Y2H screen), a single clone of the bait strain AH109 and a single clone of Y187 with the gene of interest cloned into the vector backbone pGADT7 were combined in 500 µl 2xYPD. The sample was incubated at 30°C under constant shaking at 200 rpm for 20 h. The mating culture was plated at 1:10 or 1:100 dilutions onto the following agar plates: DDO; DDOX; TDO.

The plates were incubated at 30°C for 2-3 days. In case of interaction between the two fusion proteins, growth on all three agar plates was detected, as were blue colonies on DDOX media. To ensure correct mating and the presence of both plasmids in the colonies, a colony PCR was performed. A single diploid colony from the DDO plate was resuspended in 50 µl of 20 mM NaOH and incubated at 95°C for 45 min. The sample was spun down and 2 µl of the supernatant were used as PCR template in a 50 µl vent-reaction.

Cloning and protein production of Nx3 I21-I23

A synthetic, codon-optimized gene encoding Novex-3 I21-23 (UniProtKB Q8WZ42-6, residues 5180-5604) was obtained commercially (Eurofins Genomics, DE) and cloned into pETM-41 (EMBL vector collection) via KpnI and NcoI sites. This vector introduces an N-terminal His₆-tag, maltose-binding protein (MBP), and a TEV protease cleavage site. The construct was verified by sequencing (GENEWIZ, DE). It codes for the following peptide sequence:

> Novex-3 His₆-I21-I23

His₆-

EMPPSFIEPLTKRKVYENTTLGFIVEVEGLPVPGVKWYRNKSLLEPDERIKMERVGNVCSLEISNIQKG
EGGEYMCHAVNIIGEAKSFANVDIMPQEERVVALPPPVTHQHVMEFDLEHTTSSRTPSPQEIVLEVEL
SEKDVKEFEKQVKIVTVPEFTPDHKSMIVSLDVLFPNFVDPNMDSREGEDKELKIDLEVFEPPRFIM
PICDFKIPENSDAVFKCSVIGIPTPEVKWYKEYMCIEPDNIKYVISEEKGSHTLKIRNVCLSDSATYRCR
AVNCVGEAICRGFLTMDGSEIFAVIAKKSKVTLSSLMEELVLKSNYTDSFFEFQVVEGPPRFIKGISDCY
APIGTAAYFQCLVRGSPRPTVYWKDGKLVQGRRTVEESGTGFHNLFITSLVKSDEGEYRCVATNK
SGMAESFAALTLT.

The underlined sequences mark the start of each Ig domain, which exhibits the canonical features of the long N-conserved type.⁵⁸ These domains typically begin with the motif ExPPxP, where the Ex residues stabilize the loop cluster at the N-terminal pole of the Ig fold.⁵⁹

Novex-3 I21-I23 was expressed in *E. coli* Rosetta II cells (Merck, DE) grown at 37°C in Luria-Bertani broth supplemented with kanamycin (100 µg/ml) and chloramphenicol (33 µg/ml) to OD₆₀₀ = 0.6. Expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cultures were grown overnight at 18°C. Cells were harvested by centrifugation (5,422 × g, 30 min, 4°C) and resuspended in 25 mM HEPES, 300 mM NaCl, 1 mM DTT, pH 7.5, supplemented with protease inhibitors (cOmplete™ Mini EDTA-free, Roche) and 20 µg/ml DNase I. After sonication, insoluble material was removed by centrifugation. The supernatant was purified by Ni²⁺-affinity chromatography (5 ml HisTrap HP, Cytiva). Tags were removed by overnight TEV-protease digestion, followed by subtractive Ni²⁺-affinity chromatography using the same column. Final purification was performed by size-exclusion chromatography (HiLoad 16/600 Superdex® 200 PG, Cytiva) in 25 mM HEPES pH 7.5, 100 mM NaCl, and 1 mM TCEP on an ÄKTA pure system. Purified protein was used fresh and kept on ice or under cold conditions throughout handling and storage. Protein identity was confirmed by mass spectrometric analysis of SDS-PAGE-excised bands.

Non-reducing SDS-PAGE

To assess intermolecular disulfide formation upon oxidation, purified protein was split into two fractions: one buffer-exchanged into 25 mM HEPES pH 7.5, 100 mM NaCl, and the other maintained in the same buffer supplemented with 1 mM TCEP. Samples were incubated at 4°C for 1 week to allow disulfide bond formation. After incubation, samples at 0.061, 0.122, and 0.183 mmol were analyzed by non-reducing SDS-PAGE using Laemmli buffer without reducing agents. Gels were stained with Coomassie blue and inspected for high-molecular-mass species indicative of disulfide-linked oligomers.

Circular Dichroism (CD)

Freshly purified I21-23 was buffer-exchanged into 20 mM Na₂HPO₄ pH 7.5 and adjusted to 0.59 mg/ml (A₂₈₀). CD spectra were recorded at 20°C from 200-300 nm in a 1 mm quartz cuvette (100-QS, Hellma Analytics, DE) using a J-815 spectrometer (Jasco, USA). Spectra represent the average of ten scans (100 nm/min). Data were converted to molar ellipticity ([θ] = mdeg/(10 × c × l); where c is concentration in mol/L and l is path length in cm), and secondary structure content was determined by spectral deconvolution using BeStSel (<https://bestsel.elte.hu>), optimized for β-sheet-rich proteins.²⁷ Fit quality was assessed by NRMSD (values < 0.05 indicate excellent agreement). BeStSel results were compared with

sequence-based predictions from MESSA (<http://prodata.swmed.edu/MESSA>) and fold recognition was performed against the CATH database.

Thermal stability was assessed by CD from 200-260 nm over 5-95°C in 1°C increments (1 min equilibration per step). The denaturation curve was monitored at 218 nm, and the melting temperature (T_m) determined from the first derivative. This structure-verified protein was used for analytical ultracentrifugation.

Analytical ultracentrifugation

Following expression and purification of Nx3 I21-I23 construct, AUC sedimentation velocity experiments were performed on an Optima XL-A (Beckman Coulter, USA) using absorbance optics at 55,000 rpm and 20°C in 12 mm double-sector titanium centerpieces with sapphire windows (Nanolytics, DE). Samples (350 μ l) were measured against buffer (360 μ l), and the rotor was equilibrated at 20°C for 1 h prior to analysis. Continuous $c(s)$ distributions were calculated with SEDFIT v18.1 (www.analyticalultracentrifugation.com) using ME regularization ($P = 0.68$).²¹ Protein density (1.3559 g/ml) was derived from the amino acid sequence (MM 47,986 g/mol); buffer density (1.00473 g/ml) and viscosity (1.0395 cP) were measured at 20°C using a Density Meter DMA 5000 M and a Lovis 2000 ME microviscometer (Anton Paar, DE), respectively. Radial scans and residuals were plotted with GUSI v2.1.6.22.

Co-immunoprecipitation assays

Novex-3 I21-I23 or the canonical titin-exon 48 junctional segment (ex48 N-terminus) were cloned into the mammalian expression vector pCMV-Myc (plus 2 bp for frame correction), the putative interactors Pin1, TBC1D4, and titin Z7-I1 into the Δ pcDNA3.1-HA expression vector. Plasmids were verified by restriction digestion and DNA sequencing. Observed fragment sizes following separation on 1% agarose gels revealed the expected values: pCMV-Myc $\times$ Nx3 I21-I23 (BglII/NotI: 3774 bp, 1244 bp); pCMV-Myc $\times$ Novex-3 N-terminus (PvuI/XhoI: 2796 bp, 1868 bp, 896 bp); pcDNA3.1-HA $\times$ Pin1 (BamHI/EcoRI: 5480 bp, 495 bp); pcDNA3.1-HA $\times$ TBC1D4 (PstI: 4085 bp, 1546 bp, 929 bp); and pcDNA3.1-HA $\times$ Z7-I1 (PvuII: 2992 bp, 2193 bp, 1096 bp, 1071 bp). Purified plasmid DNA was quantified and confirmed for purity (A_{260}/A_{280}). Primers and vector backbones/inserts are listed in **Table S6** and **Table S7**, respectively.

Recombinant proteins were transiently expressed in HEK cells using established protocols.⁵⁷ Cells were harvested 48 h post-transfection (via Lipofectamine), and whole-cell lysates were analyzed by SDS-PAGE and Western blotting, confirming robust expression of myc- and HA-tagged proteins at the expected molecular weights. The following constructs were expressed:

> Novex-3 ***myc***-I21-I23

MASMQKLISEEDLVYENTTLGFIVEVEGLPVPGVKWYRNKSLLEPDERIKMERVGNVCSLEISNIQKG
EGGEYMCHAVNIIGEAKSFANVDIMPQEERVALPPVTHQHVMEFDLEHTTSSRTPSPQEIVLEVEL
SEKDVKEFEKQVKIVTVPEFTPDHKSMIVSLDVLFPNFVDPNMDSREGEDKELKIDLEVFMPPRFIM
PICDFKIPENSDAVFKCSVIGIPTPEVKWYKEYMCIEPDNIKYVISEEKGSHTLKIRNVCLSDSATYRCR
AVNCVGEAICRGFLTMGDSEIFAIAKKSKVTLSLMEELVLKSNYTDSEFFQVVEGPPRFIKGISDCY
APIGTAAYFQCLVRGSPRPTVYWKDGKLVQGRRTVEESGTGFHNLFITSLVKSDEGEYRCVATNK
SGMAESFAALT

> ***myc***-Novex-3 junction to TTN exon 48 / N-terminus

MASMQKLISEEDLLMAMEARIPAMVEARHIEFRKHIDIKVLEKKRAMFECEVSEPDITVQWMKDDQ
ELQITDRIKIQKEKYVHRLIPSTRMSDAGKYTVVAGGNVSTAKLFVEGRDVRIRSIKKEVQVIEKQRAV
VEFEVNEDDVAHWYKDGIEINFQVQERHKYVVERRIHRMFISETRQSDAGEYTFVAGNRSSVTLY

VNAPEPPQVLQELQPVTVQSGKPARFCAVISGRPQPKISWYKEEQLLSTGFKCKFLHDGQEYTLILLIE
 AFPEDAAYVTCEAKNDYGVATTSSASLSVEVPEVVSPPDQEMPVYPPAITPLQDVTVTSEGQPARFQCR
 VSGTDLKVSWSYKDKKIKPSRFFRMTQFEDTYQLEIAEAYPEDEGTYTFVASNAVGVSSSTANLSLE
 APESILHERIEQEIEMEMKELFSEGESEHSEDRDADFSDSEDIDHKSMAAKRYASRISSTSSWPEYF
 KPSFTQKLTFKYVLEGEPPVFTCRLIACPTPEMTWFHNNRPIPTGLRRIKAESDLHHSSSLEIKRVQ
 DRDSGSYRLLAINSEGSAESTASLLVIQKGQDEKYLEFLKRAERTHENVEALVERGEDRIKVDLRFTG
 SPFNRLEVPRPRGRSRHDKIH

> Pin1-**HA**

MADEEKLPPEGWEKMRSSGRVYFNFHITNASQWERPSGNSSSGGKNGQGEPARVRCSHLLVKH
 SQSRPSSWRQEKITRTKEEALELINGYIQKIKSGEEDFESLASQFSDCSSAKARGDLGAFSRGQM
 KPFDASFAALRTGEMSGPVFTDSGIHILRTE**YPYDVDPDYA**

> TBC1D4-**HA**

MLTLKQAFSTAAALQSAKTQIKLCEACPMHSLHKLCEGIEGLYPRAKLVIQRHLSLTDNEQADIFER
 VQKMKPVSDQEENELVILHLRQLCEAKQKTHVHIGEGPSTISNSTIPENATSSGRFKLDILKNKAKRSL
 TSSLENIFSRGANRMRGRLGSVDSFERSNSLASEKDYSPPGDSPPGTPPASPPSSAWQTFPEEDSDS
 PQFRRAHTFSHPPSSSTKRKLNLQDGRAQGVRSPLLRQSSEQCSNLSSVRRMYKESNSSSSSLPSL
 HTSFSAPSFTAPSFLKSFYQNSGRLLSPQYENEISDGEGRKRTSSTCSNESLSVGGTSVTPRRISWRQ
 RIFLRVASPMNKS**YPYDVDPDYA**

> Titin Z7-I1-**HA**

MVEHQVKPMFVEKLKNVNIKEGSQLEMKVRATGNPNPDIVWLKNSDIIVPHKYPKIRIEGTGGEAALKI
 DSTVSQDSAWYTATAINKAGRDTRCKVNVEVEFAEPEPERKLIIPRGTYRAKEIAAPELEPLHLRYGQ
 EQWEEGDLYDKEKQKQPFKKKLTSLRLKRFPAHFECRLTPIGDPTMVVEWLHDGKPLEAANRLR
 MINEFGYCSLDYGVAYSRSRSGIITCRATNKYGTDHTSATLIVKDEKSLVEESQLPEGRKGLQRIEELER
 MAHEGALTGVTTDQKEKQKPDIVLYPEPVRVLEGETARFRCRVTGYPQPKVNWYLNGLIRKSKRFR
 VRYDGIHYLDIVDCKSYDTGEVKVTAENPEGVIEHKVKLEIQQREDFRSVLRRAPEPRPEFHVHEPGK
 LQFEVQKVDRPVDTTETKEVVKLKRAERITHEKVPEESEELRSKFKRRTEEGYEAITAVELKSRKKD
 ESYEELLRKTKDELLHWTKELEEEKKALAEEGKITIPTFKPKDKIELSPSMEAPKIFERIQSQTGQGSD
 AHFRVRVVGKPDPECEWYKNGVKIERSDRIYWYWPEDNVCELVIDVTAEDSASIMVKAINIAGETSS
 HAFLLVQEF**YPYDVDPDYA**

Co-immunoprecipitation assays were performed using the Pierce HA Tag IP/Co-IP Kit (Thermo Fisher Scientific, Waltham, USA), which employs anti-HA agarose beads. HA-tagged bait protein lysates were incubated with the beads under constant end-over-end rotation at 4 rpm for 4 h to overnight at 4°C. After removal of unbound proteins by pulse centrifugation (10 s), prey protein lysates were added for 2-6 h at 4°C. Beads were washed three times with ice-cold PBS, and bound proteins were eluted with 2x non-reducing sample buffer (provided with the kit) at 95°C for 5 min, followed by brief centrifugation. For SDS-PAGE analysis, 3 µl of 0.5 M DTT were added to the eluates. Control experiments with bait alone confirmed minimal nonspecific binding. Experiments were performed using independently prepared lysates and repeated at least twice (in triplicate for Pin1-HA:Nx3-myc-I21-I23 and Z7-I1-HA:Nx3-myc-N-term and in duplicate for TBC1D4-HA:Nx3-myc-I21-I23). Samples with insufficient protein expression were excluded based on predefined criteria.

Isolation of total human heart RNA, cDNA synthesis, and droplet digital PCR (ddPCR)

Total RNA was isolated from approximately 30 mg LV myocardial tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions as described previously.⁶⁰ Reverse transcription was performed with 250 ng of RNA using Superscript II (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, RNA was incubated with random primers (Thermo Fisher Scientific) and desoxynucleoside triphosphates at 65°C for 5 min. After adding 5x First Strand Buffer, dithiothreitol and RNaseOUT (Thermo Fisher Scientific), samples were incubated at 25°C for 2 min before 200 U of SuperScript II were added.

Subsequently, samples were incubated at 25°C for 10 min and at 42°C for 50 min. The reaction was inactivated at 70°C for 15 min.

For quantification of FLT and Nx3 transcripts in human hearts, ddPCR on a QX200 ddPCR System (Bio-Rad, Hercules, CA, USA) was performed, using the following primers (5'→3'): FLT forward: TAGTGCCACCTCCGAGTT; reverse: CTTCGTAGGAGAGCTCGCAG; probe: TCTTCTGAGGCCTTTAAAGGATGTCACCGT; Nx3 forward: TTGAGAGAATTACAACCTCTCTGCTTC; reverse: TGTCACCTTCATAAACTTCTTGAGATTC; probe: ACGCCCACAACGTTGCCACCTTC. Probes were labelled with FAM (FLT) or HEX (Nx3) at 5' and Black Hole Quencher-1 at 3'.

For PCR, cDNA samples were diluted 1:10 in H₂O and mixed with FLT primer/probe mix (FAM), Nx3 primer/probe mix (HEX), and 2× ddPCR Supermix for Probes (No dUTP; Bio-Rad) according to the manufacturer's instructions. Reactions were loaded into the middle row of a DG8 Cartridge (Bio-Rad), and 70 µL of Droplet Generation Oil for Probes (Bio-Rad) was added to the bottom wells. Cartridges were sealed with a gasket and placed in the QX200 Droplet Generator, after which the generated droplets were transferred to a 96-well PCR plate. Thermocycling was performed with an initial enzyme activation at 95°C for 10 min, followed by 40 cycles of 94°C for 30 s and 60°C for 1 min, and a final enzyme inactivation step at 98°C for 10 min. Droplets were then read on the Bio-Rad QX200 ddPCR System, and data were analyzed with QuantaSoft. Thresholds were manually set to 4,000 for FAM and 2,000 for HEX. Target concentrations were reported as copies per microliter of the final ddPCR reaction. Each sample was analyzed in technical duplicates or triplicates, and experiments were performed at least twice independently. The Nx3/FLT ratio was calculated by dividing the Nx3 concentration by the FLT concentration for each sample.

Total RNA and ribosome profiling to detect translation signatures specific to Novex-3

We analyzed publicly available human heart ribosome profiling (Ribo-seq) datasets to detect translation events specific to the Nx3 isoform. The analysis represents a secondary analysis of previously published datasets.³⁸ The dataset consisted of 80 samples (65 from patients with dilated cardiomyopathy (DCM) and 15 from non-failing donor hearts) originally generated by some authors of our group and described in detail elsewhere.³⁸

Prior to mapping, ribosome profiling reads were processed to remove residual adapter sequences and to filter out reads mapping to mitochondrial RNA, ribosomal RNA and transfer RNA sequences. Adapter clipping and quality trimming were performed using Trimmomatic v0.27.⁶¹ Processed reads from both total RNA-seq and Ribo-seq libraries were aligned to the *Homo sapiens* reference transcriptome (Ensembl release v87) using STAR v2.5.2b.⁶² We employed total RNA-seq instead of poly(A)⁺ RNA-seq for *TTN* isoform quantification, as the latter is prone to 3'-end bias that could skew isoform abundance estimates. Mapping parameters were adjusted to account for read type: for total RNA-seq, we allowed a maximum of six mismatches per read, while for Ribo-seq we allowed up to two mismatches. To accommodate variable ribosome footprint lengths, we set the STAR parameter `--seedSearchStartLmaxOverLread` to 0.5, ensuring the seed search started at half the read length regardless of footprint size. To visualize *TTN* translation events, two representative BAM files were loaded into the Integrative Genomics Viewer (IGV) v2.7.0.⁶³

After mapping ribosome-protected fragments to the human *TTN* locus, we calculated the median read density per base for all exons shared by the three isoforms (N2BA+N2B+Novex-3), for exons shared by two isoforms (N2BA+N2B), and for exons unique to Novex-3. Using these values, we computed the ratio of Novex-3 versus N2BA+N2B+Novex-3 read densities for both RNA-seq and Ribo-seq.

We further compared the fraction of reads spanning splice junctions between the Novex-3 exon and the upstream exon shared by all three isoforms (N2BA+N2B+Novex-3), and the junction connecting the two main isoforms (N2BA+N2B) while skipping the Novex-3 exon.

Generation, gene editing, and differentiation of hiPSCs

All protocols for studies with hiPSCs were approved by the Göttingen University Ethical Board (20/9/16An, 15/2/20, and 7/5/24). We used two previously characterized WT hiPSC lines,^{13,14} generously provided by Joseph C. Wu (Stanford University, CA, USA). Informed consent was obtained from all participants, and research was performed in accordance with relevant guidelines and regulations. CRISPR/Cas9-mediated gene editing was performed as described.^{15,16} Briefly, ATG knock-out of Novex-3 was achieved by annealing oligonucleotides encoding the sgRNA sequence (5'-AGAGGTGGTGGGAAGAGTACG-3'), subcloning them into the pSpCas9(BB)-2A-Puro plasmid, followed by hiPSC transfection and subsequent single-clone isolation.

For CM differentiation, hiPSCs were cultured to 80-90% confluence on Matrigel-coated plates in chemically defined E8 medium.¹⁷ Differentiation into hiPSC-CMs was induced using a small molecule-based monolayer protocol, as described.^{18,19} Spontaneously contracting CMs were observed from day 7 of differentiation. Cells were maintained in RPMI medium supplemented with B27 (both from Life Technologies). On days 20-25 of differentiation, hiPSC-CMs were dissociated with TrypLE (Life Technologies) and replated for subsequent experimental analyses. hiPSCs were regularly tested for mycoplasma contamination and maintained under feeder-free conditions. Experiments were performed using three independent differentiations of hiPSC lines.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of hiPSC-CMs

The protocol for qRT-PCR was established previously.⁶⁴ Human iPSCs were passaged three days prior to experiment on Matrigel-coated plates. Cells were subsequently dissociated using 0.5 mM EDTA in PBS and centrifuged at 200 x g for 3 minutes. The supernatant was removed, and the cell pellet was flash-frozen and stored in liquid nitrogen until further processing. Total RNA was isolated using a miRNeasy kit (Qiagen, Germany) according to the manufacturer's instructions. Reverse transcription was performed using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, USA) following the manufacturer's protocol. qRT-PCR was carried out using a TaqMan Fast Advanced Master Mix and predesigned TaqMan primers (Thermo Fisher Scientific, USA) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) and StepOne software. Relative mRNA expression levels were normalized to the 18S rRNA housekeeping control for each reaction. Experiments were performed using at least two independent hiPSC differentiations, with technical replicates for each sample.

Immunofluorescence staining and confocal microscopy of hiPSC-CMs and hiPSCs

Human iPSC-CMs and established hiPSCs were seeded onto Matrigel-coated 12-mm or 10-mm glass coverslips (Eppendorf, USA) 3-4 days prior to experiments. Cells were fixed at room temperature with 4% paraformaldehyde (ThermoFisher Scientific, USA) in PBS (Gibco, USA) for 20 min, washed with PBS, and permeabilized (hiPSC-CMs: 1 h in 0.2% Triton X-100; hiPSCs: 20 min in 0.3% Triton X-100; Carl Roth, Germany). Samples were blocked for 1 h at room temperature in 5% bovine serum albumin (BSA; Sigma-Aldrich, Germany) in PBS.

Primary antibodies were applied in 3% BSA-PBS overnight at 4°C, using established protocols.⁹ For hiPSC-CMs, antibodies included rabbit anti-Nx3 (is21) and mouse anti- α -actinin (Sigma-Aldrich, Germany), or alternatively rabbit anti-Titin Z1/Z2 or M8/M9 (**Table S5**). For hiPSCs, stem cell markers were detected using Oct3/4 (POU5F1, R&D Biosystems, USA) and podocalyxin (PODXL; TRA-1-81, Stemgent Pelobiotec, Germany). After washing with 0.2% Tween-20 in PBS, secondary antibodies were applied in 3% BSA-PBS for 1.5 h. hiPSC-CMs were stained with either Alexa Fluor 488 goat anti-rabbit A-11034 combined with Alexa Fluor 555 goat anti-mouse A-21424, or Alexa Fluor 488 goat anti-mouse A-11029 combined with Alexa Fluor 555 goat anti-rabbit A-21429 (all ThermoFisher Scientific/Invitrogen), together with DAPI. hiPSCs were stained with Alexa Fluor 568 donkey anti-goat IgG (Thermo Fisher Scientific) and Alexa Fluor 488 rat anti-mouse IgM (Biolegend, USA), also combined with DAPI.

Coverslips were mounted using Fluoromount-G medium (ThermoFisher Scientific) and imaged on a confocal microscope (Carl Zeiss LSM 510 Meta or LSM 710, Göttingen, Germany) with a 63× Plan Apochromat oil immersion objective using ZEN software (Carl Zeiss). Image acquisition and analysis were performed non-blinded. Fields of view were randomly selected, and identical acquisition settings were used across all conditions.

Nanon CardioExcyte 96 functional measurements

Human iPSC-CMs on days 16-18 of differentiation were washed with PBS and dissociated using TrypLE (Life Technologies). After spinning iPSC-CMs for 5 min at 200 x g, 37°C, cells were resuspended in culture medium supplemented with 5% FBS and seeded on Nanion CardioExcyte 96 Plates (Nanon Technologies, Germany) at a density of 50×10^3 cells per well. Cells were allowed to recover from the passage for 5-7 days. Plates were recorded using a Nanion CardioExcyte 96 instrument with temperature control (Nanon Technologies, Germany). Data were acquired and analyzed via CardioExcyte Control software.

Contractility analysis

Human iPSC-CMs were plated onto Matrigel-coated 10 mm coverslips. Following four days of culture, movies were recorded under a 40x objective using an Olympus microscope equipped with a high-performance Basler acA2000-50gm camera and Pylon viewer software (Basler AG, Germany). Cellular motion was analyzed using the automated imaging platform, MuscleMotion.^{15,65} Measurements were performed using at least two independent differentiations of hiPSC-CMs.

Cell lines

HEK293 cells were obtained from DSMZ (ACC 305). Cell line identity was authenticated by the supplier, and cells were regularly tested for mycoplasma contamination.

Immunofluorescence of cardiac tissue sections

Paraffin-embedded sections were rehydrated by two xylene washes followed by graded ethanol (100-40%). After two washes in distilled water, sections were incubated in peroxidase block buffer (15 min), washed, and boiled in blocking buffer (40 mM Tris-HCl, 1.2 mM EDTA). Sections were transferred to phosphate-buffered saline (PBS) and blocked in 5% bovine serum albumin (BSA)/0.2% Triton X-100. After washing, primary antibodies (in 0.5% BSA-PBS/0.2% Triton X-100) were applied with shaking, followed by three PBS washes (10 min each) and incubation with secondary antibodies. Sections were washed, mounted in Mowiol, and in some cases stained with Hoechst 33342 (DAPI) to visualize nuclei. Samples were stored at 4°C (light-protected) until imaging on a Nikon Eclipse Ti2 confocal microscope. Image acquisition and analysis were performed in a blinded manner. Fields of view were selected randomly, and standardized acquisition settings were applied uniformly to all samples.

Transmission electron microscopy, immunoelectron microscopy, and image analysis

TEM and immunoelectron microscopy of immunogold-labeled ultrathin sections were performed as described previously,²⁰ using a LEO EM910 electron microscope (Carl Zeiss, Oberkochen, Germany). Images were acquired with a TRS sharpeye CCD Camera (Troendle, Moorenweis, Germany), and all analyses were performed using predefined parameters.^{9,20} Images of hiPSC-CMs and mouse or human heart tissue were analyzed by manual measurements (distance of antibody epitopes to the Z-disk center), semi-automated Nikon NIS software (Z-disk density, area, and perimeter), or custom MATLAB scripts (R2024b, MathWorks) for Z-body length and Z-disk waviness. For waviness analysis, images were spatially calibrated using the A-band length (1.6 μm) as an internal reference. Z-disks were segmented after Otsu thresholding, and waviness was quantified as the root mean square (RMS) deviation from the fitted principal axis. Z-body length was determined by tracing the longitudinal axis of each clearly identifiable Z-body; curved structures were measured using polylines following their visible contour. Image calibration was based on the corresponding scale bar, and the mean Z-body length per image was used for statistical analysis. To account for repeated image-level measurements from the same animal or hiPSC-CM differentiation, data were tested for normality and analyzed using linear mixed-effects models (fixed effect: genotype; random intercept: animal or differentiation).

SDS-PAGE and Western blot

Proteins were separated by SDS-PAGE after mixing samples 1:1 with Laemmli buffer and denaturation at 95°C. For cell- or tissue-containing samples, benzonase (0.2 ml / 600 μl buffer) was added to remove genomic DNA. Electrophoresis was run at 30 mA/gel, and gels were stained with Coomassie if required. For titin analysis, 1.8% or 2% acrylamide gels stabilized with agarose were used. Tissue samples were solubilized in titin buffer with benzonase, incubated on ice (30 min), boiled (95°C, 3 min), centrifuged (14,000 rpm), and the supernatant loaded or stored at -80°C.

For WB, proteins were transferred to methanol-activated PVDF membranes using a Trans-Blot system (7-15 min, up to 2.5 A, 25 V). Membranes were blocked in 3% BSA/TBST and incubated with primary antibodies (3% BSA, 4°C, shaking), washed (3x TBST), and incubated with secondary antibodies. After further washes, signals were detected by chemiluminescence (ECL) using an ImageQuant system (GE Healthcare, Chalfont St. Giles, UK). Densitometry was performed with ImageQuant TL software; signals were normalized to GAPDH or total protein (Coomassie-stained gels/PVDF); where indicated, Nx3 signals were normalized to FLT protein. SDS-PAGE/WB experiments were performed at least twice independently.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v.8, 9, or 10 (GraphPad Software, Inc., USA) or custom MATLAB script (R2024b, MathWorks). The number of animals, patient hearts, or independent hiPSC-CM differentiations is denoted as N, whereas biological replicates are denoted as n. Unless otherwise stated, all experiments were performed using at least three independent biological replicates. Data normality was assessed throughout using the Shapiro-Wilk test. Normally distributed data were analyzed using unpaired two-tailed t-tests with Welch's correction for two-group comparisons or one-way ANOVA followed by Holm-Šídák's post hoc multiple-comparison testing for comparisons involving >2 groups. Non-normally distributed data were analyzed using the Mann-Whitney test for two-group comparisons or the Kruskal-Wallis test followed by Dunn's post hoc multiple-comparison test for comparisons involving >2 groups. To account for repeated measurements from the same animal or hiPSC-CM differentiation, linear mixed-effects models were used, with Bonferroni correction applied for multiple comparisons where appropriate. Correlations were assessed using Spearman or Person correlation analyses, as indicated. Unless stated otherwise, data are presented as mean $\pm$ standard error of the mean (SEM). A two-sided $p < 0.05$ was considered statistically significant. Multiple-comparison corrections were applied within individual analyses where appropriate; no experiment-wide or manuscript-wide correction across all statistical tests was performed.

Supplementary Tables

Table S1: Full list of potential interactors detected by Y2H screen using Ex27/28 as bait.

Clone No.	Results of blast analysis
Mitochondrial proteins and genomic sequences	
1	Homo sapiens mitochondrion, complete genome
10	Homo sapiens mitochondrion, complete genome
30	Homo sapiens mitochondrion, complete genome
70	Homo sapiens calsequestrin 2 (cardiac muscle), (CASQ2)
72	Homo sapiens mitochondrion, complete genome
75	Homo sapiens mitochondrion, complete genome
88	Homo sapiens NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16kDa (NDUFB5)
92	Homo sapiens NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16kDa (NDUFB5)
98	Homo sapiens isocitrate dehydrogenase 2 (NADP+), mitochondrial (IDH2)
138	Homo sapiens mitochondrion, complete genome
154	Homo sapiens mitochondrion, complete genome
171	Homo sapiens mitochondrion, complete genome
172	Homo sapiens ATP synthase, H ⁺ transporting, mitochondrial Fo complex, subunit F6 (ATP5J)
176	Homo sapiens succinate dehydrogenase complex, subunit A, flavoprotein (Fp), (SDHA)
183	Homo sapiens cytochrome c oxidase subunit VIc (COX6C)
196	Homo sapiens mitochondrion, complete genome
225	Homo sapiens mitochondrion, complete genome
233	Homo sapiens NAD(P)H dehydrogenase, quinone 2 (NQO2)
235	Homo sapiens peroxiredoxin 5 (PRDX5)
Non-coding genomic sequences / Open reading frames	
5	Homo sapiens chromosome 17 open reading frame 51 (C17orf51)
14	Homo sapiens chromosome X genomic contig, GRCh37.p2 reference primary assembly
17	Homo sapiens chromosome 18 genomic contig, alternate assembly
85	Homo sapiens chromosome 15, alternate assembly CHM1_1.1

164	Homo sapiens chromosome 19 genomic contig, GRCh37.p2 reference primary assembly
202	Homo sapiens chromosome 6, alternate assembly CHM1_1.1
205	Homo sapiens chromosome 12 genomic contig, alternate assembly
234	Homo sapiens chromosome 3 genomic contig, GRCh37.p2 reference primary assembly
Plasma membrane	
27	Homo sapiens transmembrane 9 superfamily protein member 4 (TM9SF4)
73	Homo sapiens voltage-dependent anion channel 3 (VDAC3), tv 1
74	Homo sapiens cadherin 2, type 1, N-cadherin (neuronal) (CDH2)
76	Homo sapiens CD63 molecule (CD63), tv 2
Nucleus	
11	Homo sapiens polymerase (DNA directed), delta 2, regulatory subunit 50kDa (POLD2), tv 2
15	Homo sapiens SNRPN upstream reading frame (SNURF), tv 1
24	Homo sapiens anaphase promoting complex subunit 13 (ANAPC13), tv 1
180	Homo sapiens eukaryotic translation initiation factor 3, subunit H (EIF3H)
Sarcomeric A-band/M-band	
12	Homo sapiens myosin binding protein C, cardiac (MYBPC3)
13	Homo sapiens myosin, light chain 12A, regulatory, non-sarcomeric (MYL12A)
60	Homo sapiens myosin, heavy chain 7, cardiac muscle, beta (MYH7)
67	Homo sapiens myosin, light chain 2, regulatory, cardiac, slow (MYL2)
87	Homo sapiens myosin, heavy chain 7, cardiac muscle, beta (MYH7)
Other localizations	
3	Homo sapiens ubiquitin-conjugating enzyme E2A (RAD6 homolog) (UBE2A), tv 1
16	Homo sapiens prosaposin (PSAP), tv 3
18	Homo sapiens sorbin and SH3 domain containing 2 (SORBS2), tv 6
21	Homo sapiens cardiomyopathy associated 5 (CMYA5)
49	Homo sapiens lectin, galactoside-binding, soluble, 3 binding protein (LGALS3BP)

51	Homo sapiens creatine kinase, muscle (CKM)
52	Homo sapiens natriuretic peptide precursor A (NPPA)
68	Homo sapiens proteasome (prosome, macropain) 26S subunit, ATPase, 6 (PSMC6)
69	Homo sapiens natriuretic peptide precursor A (NPPA)
78	Homo sapiens ATPase family, AAA domain containing 1 (ATAD1)
79	Homo sapiens claudin domain containing 1 (CLDND1), tv 7
80	Homo sapiens SAR1 homolog A (S. cerevisiae) (SAR1A), tv 1
83	Homo sapiens SAR1 homolog A (S. cerevisiae) (SAR1A), tv 1
86	Homo sapiens tubulin, alpha 8 (TUBA8), tv 1
94	Homo sapiens DEAD (Asp-Glu-Ala-Asp) box helicase 5 (DDX5)
118	Homo sapiens copine IV (CPNE4)
124	Homo sapiens titin (TTN), tv novex-3
175	Homo sapiens PDZ and LIM domain 5 (PDLIM5), tv 5
?	Homo sapiens formin homology 2 domain containing 3 (FHOD3), tv 3
206	Homo sapiens TAP binding protein (tapasin) (TAPBP), tv 3
209	Homo sapiens GRB2-associated binding protein 2 (GAB2), tv 2
221	Homo sapiens BCL2-associated athanogene 5 (BAG5), tv 1
212	Homo sapiens cardiomyopathy associated 5 (CMYA5)
315	Homo sapiens actin, alpha, cardiac muscle 1

Table S2: Complete list of in-frame cDNAs from Y2H screens using Nx3 I21-I23 as bait.

cDNA insert	Full name	Screen 1 (44 clones)	Screen 2 (245 clones)
ACTB	Actin, cytoplasmic 1	-	1x
ACTC1	Actin, alpha cardiac muscle 1	-	2x
ARHGAP5-AS	ARHGAP5 antisense RNA 1	-	3x
BCR	Breakpoint cluster region protein	-	1x
BRD7	Bromodomain-containing protein 7	-	3x
CCAR2	Cell cycle and apoptosis regulator protein 2	-	1x
COX4I1	Cytochrome c oxidase subunit 4 isoform 1	-	5x
DCTN1	Dynactin subunit 1	-	1x
DDX50	ATP-dependent RNA helicase DDX50	-	2x
FILIP1	Filamin A interacting protein 1	1x	-
GFAP	Glial fibrillary acidic protein	-	3x
GGA1	ADP-ribosylation factor-binding protein GGA1	-	1x
KANSL3	KAT8 regulatory NSL complex subunit 3	-	1x
LMF2	Lipase maturation factor 2	-	2x
MAP3K2	Mitogen-activated protein kinase kinase kinase 2	1x	1x
MYBPC3	Myosin-binding protein C, cardiac type	-	1x
MYH7	Myosin heavy chain 7	2x	2x
MYOM1	Myomesin 1	2x	4x
MYOM2	Myomesin 2	-	1x
NEXN	Nexilin	2x	1x
PDE4DIP	Myomegalin	-	1x

PGM1	Phosphoglucomutase-1	-	1x
PIN1	Peptidylprolyl cis/trans isomerase NIMA interacting 1	3x	5x
POLI	DNA polymerase iota	-	1x
POLR2B	RNA polymerase II polypeptide B	1x	-
RBFOX1	RNA binding protein, fox-1 homolog 1	-	3x
RGPD5/6	RNABP2-like and GRIP domain-containing protein 5/6	-	1x
RPL31	60S ribosomal protein L31	-	2x
SDC2	Syndecan-2	-	3x
SEPT7	Septin-7	-	1x
SPTAN1	Spectrin alpha chain, non-erythrocytic 1	-	1x
SRSF11	Serine/arginine-rich splicing factor 11	-	1x
TAX1BP1	Tax1-binding protein 1	-	1x
TBC1D4	TBC1 domain family member 4	1x	-
TNNI3	Troponin I, cardiac muscle	-	5x
TNNT2	Troponin T, cardiac muscle	-	1x
TTN	Titin	2x	3x
UBC	Polyubiquitin C	1x	11x
UBE2L3	Ubiquitin-conjugation enzyme E2 L3	-	1x
UBE2S	Ubiquitin-conjugation enzyme E2S	1x	-
XAF1	XIAP associated factor 1	-	1x
ZCCHC4	Zinc finger, CCH domain containing 4	1x	-
Out-of-frame/non-coding		26x	166x

Table S3: Human hearts from patients with DCM.

Running No.	No. TTNtv hearts	Age**	Sex	LVEF (%)
1	4	58	m	19
2	15	34	m	25
3	16	23	m	17,5
4	7	46	m	14
5	2	38	f	21
6	13	50	m	10
7	22	21	m	20
8	20	49	m	10
9	8	8	f	18
10		7	f	22,5
11		63	m	37,5
12		45	m	30
13		48	f	25
14	12	69	f	35
15	9	49	m	45
16		55	m	25
17		65	f	17,5
18		55	m	21
19		61	f	30
20		66	m	19
21		57	f	25
22		64	m	20
23		46	m	25
24		41	f	19
25		60	f	36
26		66	f	37
27		57	m	24
28		65	m	25
29		66	m	25
30*		9	f	19
31		55	m	27
32		70	m	22
33		62	f	25
34		66	m	28
35		59	m	20
36		62	m	37
37		43	m	21
38		54	f	37
39		53	m	13
40		52	m	28

41		64	m	20
42		27	m	20
43		56	m	25
44		65	m	28
45		64	m	24
46		43	m	22
47		52	m	28
48		51	m	27
49		61	m	21
50*		59	m	32
51*		36	m	17,5
52		66	m	21
53		28	f	15
54		53	m	43
55		65	m	19
56		62	m	26
57	21	40	m	23
58*		48	m	18
59	17	59	m	20
60		49	m	22,5
61		44	m	26
62		61	m	15
63		60	m	18
64	14	63	m	37
65		21	m	20
66		15	m	20
67		62	f	18
68		65	m	20
69		58	f	25
70		54	m	23
71	19	56	m	25
72		62	m	17
73		48	m	25
74		54	f	20
75		61	m	13
76		62	f	19
77	5	41	m	18
78		33	m	23
79		62	f	15
80*		46	f	21
81	1	55	m	25
82		66	m	23
83		47	m	21

84*		55	m	20
85		52	m	25
86		24	m	25
87		57	m	20
88	18	51	m	28
89	6	59	m	33
90		62	f	35
91		44	m	25
92		33	f	26
93		60	m	17
94		24	m	26
95		34	m	24
96		4	m	40
97		64	m	15
98		38	m	20
99		61	m	20
100		62	m	15
101		42	m	41
102		23	f	21
103		71	f	20
104*		57	f	29
105*		29	m	18
106		41	m	33
107	11	69	m	19
108	10	61	m	37
109	3	29	m	28

Mean $\pm$ SEM Age of all DCM patients: 50.2 ± 1.5 (n = 109)

Mean $\pm$ SEM Age of IDCM patients: 51.0 ± 1.6 (n = 87)

Mean $\pm$ SEM Age of TTNtv patients: 46.7 ± 3.4 (n = 22)

Male (m)/Female (f): IDCM 64/23; TTNtv 19/3

Mean $\pm$ SEM LVEF of all DCM patients: 23.8 ± 0.7 (n = 109)

Mean $\pm$ SEM LVEF of IDCM patients: 23.8 ± 0.7 (n = 87)

Mean $\pm$ SEM LVEF of TTNtv patients: 24.0 ± 1.9 (n = 22)

* IDCM samples used for ddPCR

** At time of explantation

A-Band truncation

I-band truncation

Table S4: Human nonfailing donor hearts.

Running No. and internal code	ID	Source	Age	Sex
1. Do1	SHB 5.089 LV 10	Sydney Heart Bank	48	f
2. Do2	SHB 5.041 LV 6	Sydney Heart Bank	42	f
3. Do3	SHB 5.114 LV 4	Sydney Heart Bank	1.2	f
4. Do5	SHB 5.102 LV	Sydney Heart Bank	4.2	m
5. Do6	SHB 3.145 LV	Sydney Heart Bank	39	m
6. Do8	SHB 3.085 LV	Sydney Heart Bank	53	m
7. NF01 (Do9)		HDC*	28	m
8. NF02 (Do11)		HDC*	45	m
9. NF03 (Do12)		HDC*	60	m
10. NF04 (Do14)		HDC*	40	f
11. NF05 (Gö1)		HDC*	50	m
12. NF06 (Gö2)		HDC*	31	f
13. NF07 (Gö3)		HDC*	54	f
14. NF08 (Gö4)		HDC*	44	m

Mean $\pm$ SEM Age Donor: 38.5 ± 4.7 (n = 14)

*Heart and Diabetes Center Bad Oeynhausen, Germany (samples also used for ddPCR)

Table S5: Antibodies and staining kits

Antibody	mc/pc	Working concentration	Source	Cat.-No.
Primary antibodies				
Mouse anti- α -Actinin (ACTN2; EA53)	mc	N.A. [1:400] (IF)	Sigma Aldrich, St. Louis, USA	A7811
Mouse anti-Desmoplakin	mc	N.A. [1:5] (IF)	Progen, Heidelberg, DE	610035
Rabbit anti-GAPDH (14C10)	mc	N.A. [1:2,000] (WB)	Cell Signaling, Danvers, USA	2118
Rat anti-HA (3F10)	mc	0.025 μ g/mL (WB) [1:4,000]	Roche, Penzberg, DE	11867423001
Mouse anti-His ₆ (4E3D10H2/E3)	mc	0.20 μ g/mL (WB) [1:5,000]	Thermo Fisher Scientific, Waltham, USA	MA1-135
Mouse anti-myc (9E10)	mc	0.67 μ g/mL (WB) [1:1,500]	Abcam, Cambridge, UK	ab32
Rabbit anti-Pin1	pc	3.4 μ g/mL (IF) [1:200]; 1.36 μ g/mL (IEM) [1:500]	Thermo Fisher Scientific, Waltham, USA	PA5-29675
Mouse anti-Pin1	mc	0.2 μ g/mL (WB) [1:1,000]; 1.0 μ g/mL (IF) [1:200]	Santa Cruz, USA	sc-46660
Rabbit anti-TBC1D4 (AS160)	mc	N.A. [1:1,000] (WB)	Cell Signaling, Danvers, USA	2670
Mouse anti-TBC1D4 (AS160)	mc	5.0 μ g/mL (IF) [1:200]	Proteintech, Planegg-Martinsried, DE	68063-1-Ig
Rabbit anti-TTN-Z1/Z2	pc	N.A. [1:10,000] (WB); [1:200] (IF)	Myomedix, Neckargemünd, DE	TTN-1
Chicken anti-TTN-Z1/Z2	pc	N.A. [1:200,] (IF)	Kind gift of Siegfried Labeit, Mannheim, DE	—
Rabbit anti-TTN-S2080	pc	N.A. [1:500] (WB)	Eurogentec, Seraing, BE	Custom-made
Mouse anti-T12 (Titin)	mc	N.A. [1:1,000] (WB); [1:100] (IHC)	Kind gift of Dieter Fürst, Bonn, DE	—
Rabbit anti-TTN-N2Bus	pc	N.A. [1:200] (IEM)	Eurogentec, Seraing, BE	Custom-made
Rabbit anti-is21 (Nx3)	pc	N.A. [1:1,000] (WB); [1:200] (IHC)	Eurogentec, Seraing, BE	Custom-made
Rabbit anti-T59 (Nx3)	pc	N.A. [1:1,000] (IEM)	Eurogentec, Seraing, BE	Custom-made
Rabbit anti-TTN-PEVK	pc	N.A. [1:100] (IEM)	Myomedix, Neckargemünd, DE	TTN-5
Rabbit anti-TTN-Cronos	pc	N.A. [1:2,000] (WB)	Eurogentec, Seraing, BE	Custom-made
Rabbit anti-TTN-M8/M9	pc	N.A. [1:200] (IF)	Myomedix, Neckargemünd, DE	TTN-9
Goat anti-Oct-3/4 (POU5F1)	pc	0.20 μ g/mL (IF) [1:1,000]	R&D Systems, Minneapolis, USA	AF1759
Mouse anti-TRA-1-81 (PODXL)	mc	N.A. [1:2,000] (IF)	Stemgent, Cambridge, USA	09-011

Antibody	mc/pc	Working concentration	Source	Cat.-No.
Secondary antibodies				
Goat anti-Chicken IgY Alexa Fluor 647	pc	N.A. [1:200] (IF)	Jackson ImmunoResearch, Cambridge, UK	103-605-155
Goat anti-rabbit IgG Cy3	pc	10 µg/mL (IF) [1:200]	Invitrogen, Grand Island, USA	A10520
Goat anti-mouse IgG FITC	pc	N.A. [1:200] (IF)	Rockland, Gilbertsville, USA	210-1204
Goat anti-rabbit Alexa Fluor 488	pc	10 µg/mL (IF) [1:200]	Thermo Fisher Scientific, Waltham, USA	A-11034
Goat anti-mouse Alexa Fluor 488	pc	10 µg/mL (IF) [1:200]	Thermo Fisher Scientific, Waltham, USA	A-11029
Goat anti-rabbit Alexa Fluor 555	pc	10 µg/mL (IF) [1:200]	Thermo Fisher Scientific, Waltham, USA	A-21429
Goat anti-mouse Alexa Fluor 555	pc	10 µg/mL (IF) [1:200]	Thermo Fisher Scientific, Waltham, USA	A-21424
Goat anti-mouse HRP IgG	pc	N.A. [1:10,000] (WB)	Dianova, Hamburg, DE	115-035-003
Goat anti-rabbit HRP IgG	pc	0.2 µg/mL (WB) [1:10,000]	OriGene Technologies, Rockville, USA	R1364HRP
Goat anti-rat HRP IgG	pc	N.A. [1:15,000] (WB)	Dianova, Hamburg, DE	112-035-003
Dyes				
DAPI	—	N.A. [1:500] (fluorescence)	Thermo Fisher Scientific, Waltham, USA	D1306

Footnote. Working concentrations are reported in µg/mL where the stock antibody concentration was specified by the manufacturer. For antibodies for which a mass concentration was unavailable or not applicable, including custom-made and kindly provided antibodies, the experimentally used dilution is reported in brackets. WB, Western blot; IF, immunofluorescence; IHC, immunohistochemistry; IEM, immunoelectron microscopy; mc, monoclonal; pc, polyclonal; HRP, horseradish peroxidase; N.A., not applicable.

Table S6: PCR and sequencing primers

PCR primer	Length (bp)	Sequence	Annealing temp.
Z711_for_Bam	36	CAT GGATCC ATGGTGGAAACATCAGGTAAAACCGATG	55 °C
Z711_rev_Eco	37	CAT GAATTC TTGGACAAGTAAGAATGCGTGACTGGAG	55 °C
Z711-koz-for	30	CAT GAGCTC GGATCCGACATGGTGGAAACAT	60 °C
Nx3_for_EcoRI	39	CAT GAATTC ATGGTGGAAAGCTCGTCATATAGAATTTAGG	60 °C
Nx3_CMV_for	27	CAT GAATTC CCCGCCATGGTGGAAAGCTC	58 °C
Nx3_rev_XhoI	34	CAT CTCGAG CCTGTAAAGGGAGAGCCAGTAAAC	60 °C
m2402_SacII_for	30	TC ACCGCG GAAAATCCTGAAGGTGTGATAG	60 °C
m2402_HpaI_rev	35	TC AGTTAACT CCTTGGTCCAGTGGAGAAGTTCATC	60 °C
m996_PvuII_A_rev	30	TT CCAGCTG GGAACCTTCCTTTATATTGAC	60 °C
m996_PvuII_B_for	30	T CCAGCTG GAAATGAAAGTCAGAGCTACG	60 °C
m996_PvuII_B_rev	32	G GAATCGAT TTTAAGGGCAGCTTCTCCCTTGG	60 °C
Nx3-1576-EcoRV-r	27	G GATATC ACGGCACAGAAGCGGGCAGG	60 °C
Nx3-2051A-NheI-f	33	GTT GCTAGCA ATGCTGTAGGCCAAGTATCAAGC	60 °C
Nx3-2051B-EcoRV-f	23	CGT GATATC CGGAAGACCACAGC	60 °C
Nx3-2051B-NheI-r	27	ATT GCTAGCA ACAAACGTGTAAGTTCC	60 °C
2123_for	30	CAT GTCGAC CCGTATATGAAAACACAACAC	58 °C
2123_rev	29	CAT GCGGCCGCT TAAAGTTAAGGTGAGTGC	58 °C
CMV_2123_for	27	CC GAGATCT GTGTATATGAAAACACAA	60 °C
CMV_2123_rev	29	CAT GCGGCCGCT TAAAGTTAAGGTGAGTGC	60 °C
db_Pin1_for	27	CAT GAATTC ATGGCGGACGAGGAGAAG	56 °C
db_Pin1_rev	27	CAT CTCGAGT CACTCAGTGCGGAGGAT	56 °C
HA_Pin1_for	27	CAT GGATCC ATGGCGGACGAGGAGAAG	59 °C
HA_Pin1_rev	27	CCT GAATTC CTCAGTGCGGAGGATGAT	59 °C
HA_TBC_for	27	CAT GGATCC ATGCTGACTCTGAAACAG	66 °C
HA_TBC_rev	27	CAT GAATTC CAGATTTGTTTCATGGGAGA	66 °C

Restriction sites introduced as sequence overhangs are highlighted in bold.

Sequencing primer	Length (bp)	Sequence
Nx3-sequ-for	19	GCGATGCAGGAGAATACAC
Nx3-sequ-rev	20	TTCAGGCCAGGAGCTTGTAG
Z711-seq-for	20	AGAGCAATGGGAAGAAGGTG
Z711-seq-rev	20	CTCTTCTGTTCTGCGCTTGA
Y2H_seq_for1 (Nx3)	21	GGTAGATCATTGAGCTGAGAG
Y2H_seq_rev1 (Nx3)	20	GGGCATGTGGATTTGCACA
Y2H_seq_for2 (Nx3)	22	GCACATAAAGTCCAGTGTTCC
Y2H_seq_rev2 (Nx3)	21	GCTAACTCTTCTTCCTCATCC
Y2H_seq_for3 (Nx3)	20	GCTGCTTTCTCTCAAACCTC
Y2H_seq_rev3 (Nx3)	20	TGTTCCCTTTCTGTGCGTC
Y2H_seq_for4 (Nx3)	20	CATTCATGCCTCTGGAGAAG
Y2H_seq_rev4 (Nx3)	20	TCAGTGGTATGTGCCTCATC
Y2H_seq_for5 (Nx3)	21	GACAGTTCCCGAATTTACTCC
Y2H_seq_rev5 (Nx3)	20	TTTGTCTTCTCCCTCCCTTG
pPROEX (HTa)_for	18	AGCGGATAACAATTTACAC
pPROEX (HTa)_rev	21	ATCTGTATCAGGCTGAAAATC
Yeast2 (pGADT7)	17	TACCACTACAATGGATG
Yeast3 (pGBKT7)	17	CTTTAAATTTGTATAC
Yeast4 (pGADT7)	18	GAAATTGAGATGGTGCAC
T7 for (Δ pCDNA 3.1 HA)	20	TAATACGACTCACTATAGGG
BGH rev (Δ pCDNA 3.1 HA)	18	TAGAAGGCACAGTCGAGG
PEN1 532F (pCMV-Myc)	17	AGGCGTGACGGTGGGA
M13 reverse (pCMV-Myc)	17	CAGGAAACAGCTATGAC

All primers ordered from Eurofins, Ebersberg, Germany.

Table S7: Vector backbones and inserts

Vector	Source
pGBKT7	Clontech Laboratories, Mountain View, USA
pGADT7	Clontech Laboratories, Mountain View, USA
Δ pCDNA 3.1 HA	Custom-made
pCMV-Myc	Clontech Laboratories, Mountain View, USA
pPROEX HTa	Thermo Scientific, Waltham, USA

DNA fragment	Sequence (source)
Z7-I1	GenBank: X90568.1; bp 4642 - 6501
Novex-3 N-terminus	NCBI Reference Sequence: NM_133379.4; bp 9382 - 11155
Novex-3 I21-I23	NCBI Reference Sequence: NM_133379.4; bp 15805 - 17040
Pin1	NCBI Reference Sequence: NM_006221.3; bp 139 – 6 30
TBC1D4	NCBI Reference Sequence: NM_001286658.2, bp 1689 - 2738

Supplementary Figures

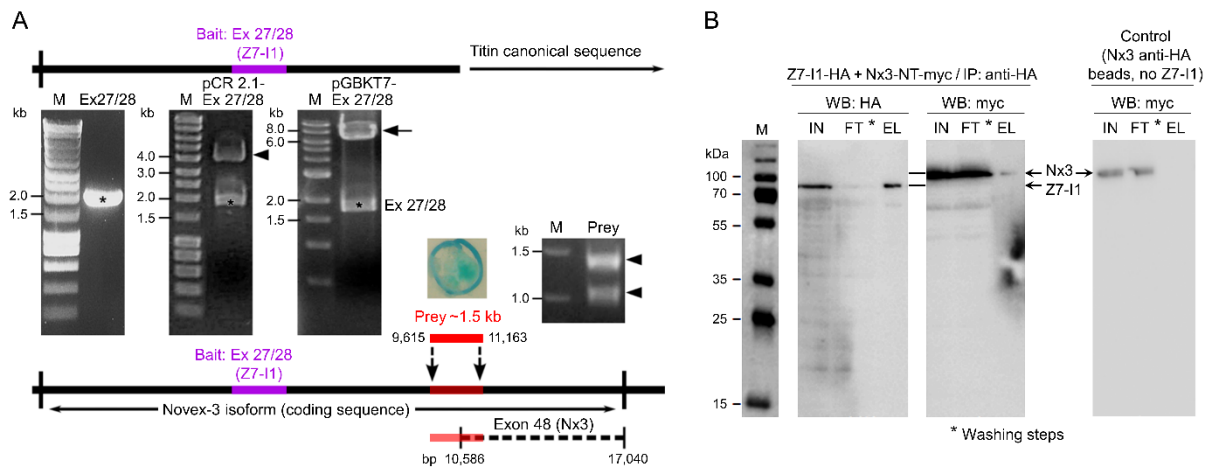


Figure S1: N-terminus of Novex-3 detected as a ligand of titin Ex27/28 by Y2H screen. A, Bait and prey constructs in Y2H screen, and confirmation by PCR. **B,** Co-immunoprecipitation assay to test for interaction between Z7-I1 titin fragment and the Nx3 N-terminus. Input (IN), flow-through (FT), and eluate (EL) fractions were separated on 12.5 % SDS gels and analyzed by Western blot (WB) for the presence of HA-/myc-tagged protein in the different fractions. Exposure time, 2 min. M, marker.

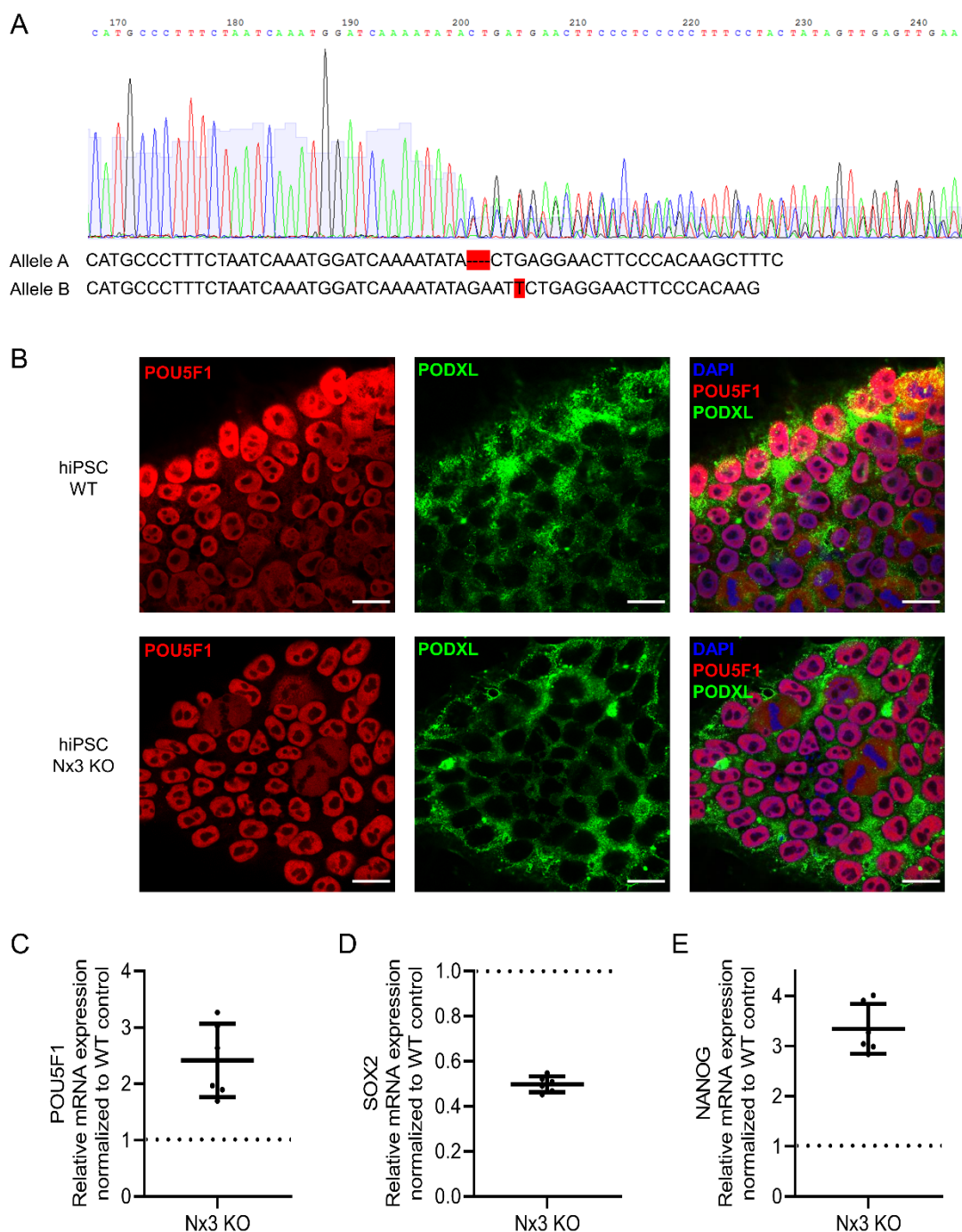


Figure S2: Characterization of CRISPR-Cas edited Nx3 KO iPSCs versus WT controls. **A**, Chromatogram of genomic DNA extracted from iPSCs of the selected Nx3 KO iPSC clone. Detected INDELs (4 bp deletion, 1 bp insertion) in respective alleles are shown. **B**, Immunohistochemistry for pluripotency markers POU5F1 (Oct 3/4) and PODXL (TRA-1-81) in CRISPR-Cas edited Nx3 KO iPSCs versus WT control. Representative confocal images are shown; scale bars, 20 μ m. **C-E**, Quantification of relative mRNA expression levels normalized to WT control levels for the pluripotency markers (**C**) POU5F1, (**D**) SOX2, and (**E**) NANOG in Nx3 KO iPSCs using quantitative real-time PCR. Data are mean $\pm$ SEM; N=2 independent experiments were performed with n=3 technical replicates per experiment. $p=0.065$, $p=0.003$ and $p=0.0001$ for POU5F1, SOX2, and NANOG respectively, by Mann Whitney test.

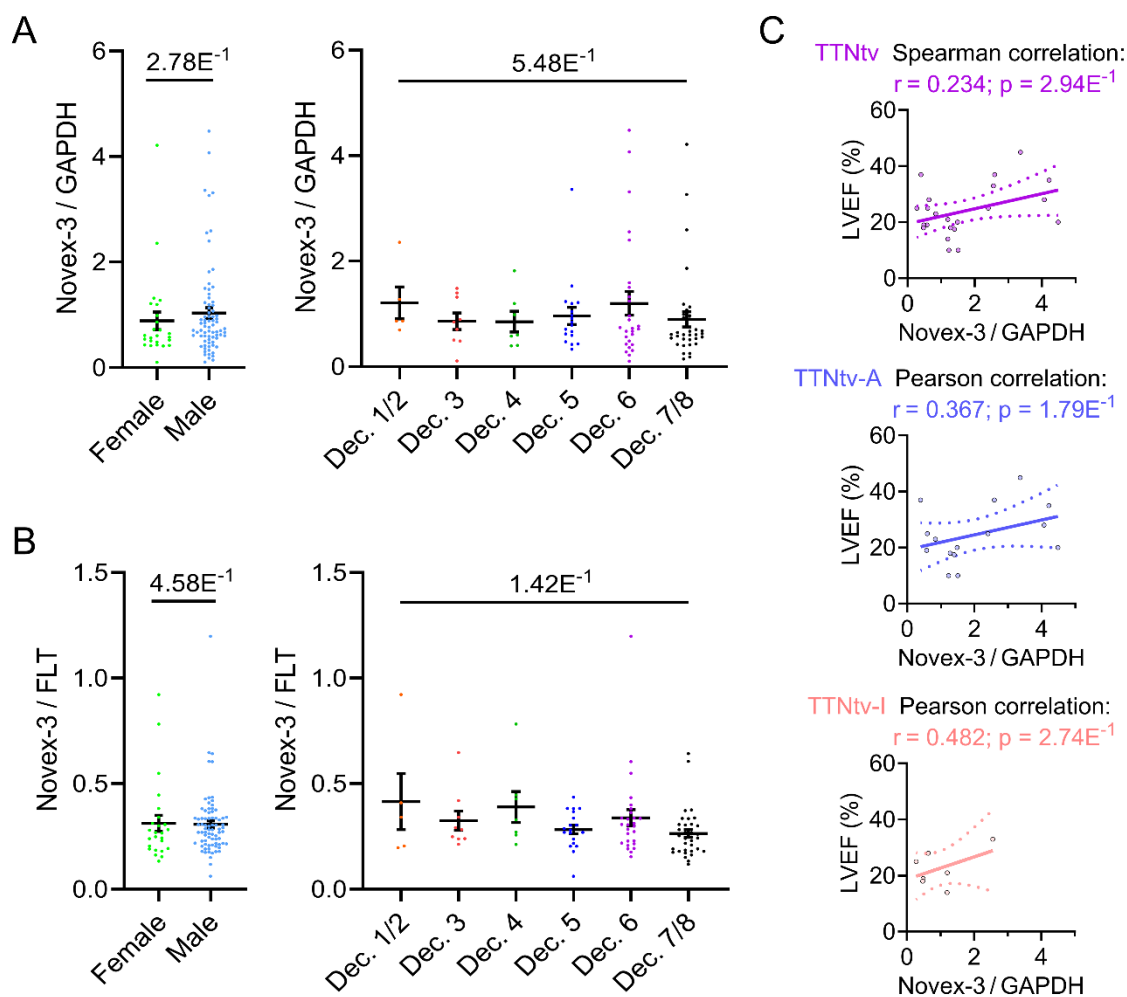


Figure S3: Expression of Novex-3 in adult human hearts: additional analysis. **A**, Expression of Novex-3 relative to GAPDH in adult endstage failing DCM human LV tissue, differentiated according to sex (left) and decade (Dec.) of life (right). Analysis of Western blots using Nx3 (is21) / GAPDH antibodies. N=76 (male). N=25 (female); analysis according to age; minimum N=5 (Dec. 1/2), maximum N=35 (Dec. 7/8). **B**, Expression of Novex-3 relative to full-length titin (FLT) in adult endstage failing DCM human LV tissue, differentiated according to sex (left) and decade of life (right). Analysis of Western blots using Titin-Z (Z1/Z2) antibodies. N=77 (male), N=25 (female); analysis according to age; minimum N=5 (Dec. 1/2), maximum N=35 (Dec. 7/8). Graphs in (A) and (B) show mean $\pm$ SEM. Statistical testing by Mann Whitney test (left panels) and Kruskal-Wallis test (right panels). **C**, Correlation of LV ejection fraction (LVEF) with normalized Nx3 protein expression in DCM patients with titin-truncating variants (TTNtv): all TTNtv-positive patients (N=22; top), A-band TTNtv (N=15; middle), and I-band TTNtv (N=7; bottom). Solid lines indicate linear regressions and dotted lines the 95% confidence interval; statistical results of Pearson or Spearman correlation analyses are indicated.

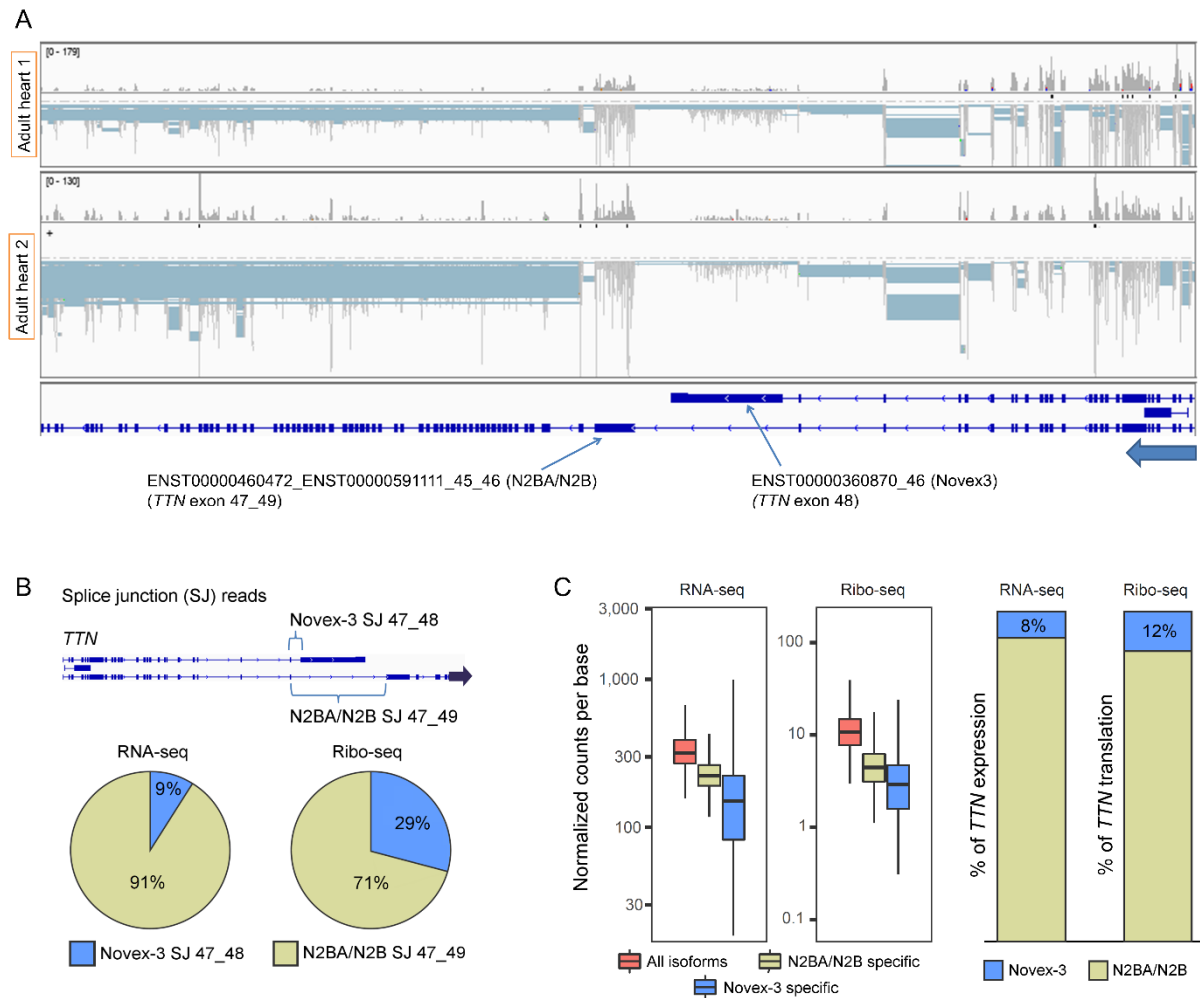


Figure S4: Nx3 expression and translation in adult human hearts by RNA-sequencing and ribosomal-sequencing. **A**, Ribosomal-sequencing coverage profile in two adult human hearts (examples). **B-C**, Expression and translation of Novex-3 versus full-length titin (N2BA/N2B) by RNA-sequencing (seq) and ribosomal (Ribo-)seq, analyzed in a cohort of 80 human hearts (65 DCM, 15 donor; re-analysis of data measured by van Heesch et al. (2019).¹ **B**, Quantification based on splice junction (SJ) reads; **C**, Quantification based on normalized read counts per base.

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Mouse Models

Strain	Vendor or Source	Background Strain	Sex	Other Information (e.g. breeding scheme for F1, F2...if applicable)	Persistent ID / URL
<i>C57BL/6JRj</i>	Janvier Labs (Le Genest-Saint-Isle, France)	C57BL/6JRj	F + M pooled	This strain was purchased to study fetal, neonatal, and adult hearts of wildtype animals	https://janvier-labs.com/en/fiche_produit/2_c57bl-6j_mouse/
<i>C57BL/6N_</i> <i>CRISPR-</i> <i>Novex-3^{-/-}</i>	Kawasaki Medical School (Japan)	C57BL/6NCrl	F + M pooled	This strain was used for mouse breeding to generate global Novex-3 knockout animals (wildtype & homozygous deletion)	https://www.criver.com/products-services/find-model/c57bl6-mouse?region=23

Mouse Models Used in This Study

Strain	Vendor or Source	Background Strain	Sex	Other Information (e.g. breeding scheme for F1, F2...if applicable)	Persistent ID / URL
<i>C57BL/6N_</i> <i>CRISPR-</i> <i>Novex-3^{-/-}</i>	Kawasaki Medical School (Japan)	C57BL/6NCrl	F + M pooled	This strain was used for mouse breeding to generate global Novex-3 knockout animals (wildtype and homozygous deletion); Male parent: CRISPR-edited founder; Female parent: pseudopregnant recipient	https://www.criver.com/products-services/find-model/c57bl6-mouse?region=23

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (not required)	Persistent ID / URL
α -Actinin (ACTN2)	Sigma-Aldrich	A7811	N.A. [1:400] (IF)		https://www.sigmaaldrich.com/DE/de/products/protein-biology/antibodies
Desmoplakin	Progen	610035	N.A. [1:5] (IF)		https://www.progen.com/products/antibodies/
GAPDH	Cell Signaling	2118	N.A. [1:2,000] (WB)		https://www.cellsignal.com
HA tag	Roche	11867423001	0.025 μ g/mL (WB) [1:4,000]		https://www.roche.com/topic/antibodies
His6	Thermo Fisher Scientific	MA1-135	0.20 μ g/mL (WB) [1:5,000]		https://www.thermofisher.com/de/de/home/life-science/antibodies.html
Myc tag	Abcam	ab32	0.67 μ g/mL (WB) [1:1,500]		https://www.abcam.com/en-us/products/primary-antibodies
Pin1	Thermo Fisher Scientific	PA5-29675	3.4 μ g/mL (IF) [1:200]; 1.36 μ g/mL (IEM) [1:500]		https://www.thermofisher.com/de/de/home/life-science/antibodies.html
Pin1	Santa Cruz	sc-46660	0.2 μ g/mL (WB) [1:1,000]; 1.0 μ g/mL (IF)		https://www.scbt.com/browse/antibodies

DOI [to be added]

			[1:200]		
TBC1D4 (AS160)	Cell Signaling	2670	N.A. [1:1,000] (WB)		https://www.cellsignal.com
TBC1D4 (AS160)	Proteintech	68063-1-Ig	5.0 µg/mL (IF) [1:200]		https://www.ptglab.com
Titin Z1/Z2	Myomedix	TTN-1	N.A. [1:10,000] (WB); [1:200] (IF)		http://www.myomedix.com/
Titin Z1/Z2	Myomedix	Custom	N.A. [1:200,] (IF)		http://www.myomedix.com/
Titin S2080	Eurogentec	Custom	N.A. [1:500] (WB)		https://www.eurogentec.com/en/catalog/c64~antibodies
Titin T12	Gift (Dieter Fürst)	N/A	N.A. [1:1,000] (WB); [1:100] (IHC)		Not available
Titin N2Bus	Eurogentec	Custom	N.A. [1:200] (IEM)		https://www.eurogentec.com/en/catalog/c64~antibodies
Nx3 (is21)	Eurogentec	Custom	N.A. [1:1,000] (WB); [1:200] (IHC)		https://www.eurogentec.com/en/catalog/c64~antibodies
Nx3 (T59)	Eurogentec	Custom	N.A. [1:1,000] (IEM)		https://www.eurogentec.com/en/catalog/c64~antibodies
Titin PEVK	Myomedix	TTN-5	N.A. [1:100] (IEM)		http://www.myomedix.com/
Titin Cronos	Eurogentec	Custom	N.A. [1:2,000] (WB)		https://www.eurogentec.com/en/catalog/c64~antibodies
Titin M8/M9	Myomedix	TTN-9	N.A. [1:200] (IF)		http://www.myomedix.com/
Oct3/4 (POU5F1)	R&D Systems	AF1759	0.20 µg/mL (IF) [1:1,000]		https://www.rndsystems.com/
TRA-1-81 (PODXL)	Stemgent	09-011	N.A. [1:2,000] (IF)		https://www.reprocell.com/stemgent

Footnote. Working concentrations are reported in µg/mL where the stock antibody concentration was specified by the manufacturer. For antibodies for which a mass concentration was unavailable or not applicable, including custom-made and kindly provided antibodies, the experimentally used dilution is reported in brackets. WB, Western blot; IF, immunofluorescence; IHC, immunohistochemistry; IEM, immunoelectron microscopy; mc, monoclonal; pc, polyclonal; HRP, horseradish peroxidase; N.A., not applicable.

DNA/cDNA Clones

Clone Name	Sequence	Source / Repository	Persistent ID / URL
Novex-3 I21-I23 (His6-MBP tagged)	Provided in Methods	Eurofins Genomics; cloned into pETM-41	https://eurofinsgenomics.eu
Novex-3 junction (exon 48 N-term)	Provided in Methods	In-house cloning (pCMV-Myc)	Not available
Pin1-HA	Provided in Methods	In-house cloning (pcDNA3.1-HA)	Not available
TBC1D4-HA	Provided in Methods	In-house cloning	Not available

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
HEK293 cells	DSMZ (ACC 305)	Unknown	https://www.dsmz.de
Human iPSCs (WT lines)	Provided by Joseph C. Wu (Stanford University)	Unknown	Not available
hiPSC-derived cardiomyocytes CRISPR edited Nx3 ^{-/-}	Derived in-house	Unknown	Not available

Data & Code Availability

Description	Source / Repository	Persistent ID / URL
Ribosome profiling datasets (Ribo-seq)	Previously published dataset	https://doi.org/10.1016/j.cell.2019.05.010
RNA-seq and analysis parameters	Previously published dataset	https://doi.org/10.1016/j.cell.2019.05.010
Image analysis pipelines (MATLAB, NIS)	Available upon request	https://de.mathworks.com/products/matlab.html https://www.microscope.healthcare.nikon.com/de_EU/products/software/nis-elements

Other

Description	Source / Repository	Persistent ID / URL
TruSight Cardio sequencing panel	Illumina	https://www.illumina.com
QX200 ddPCR System	Bio-Rad	https://www.bio-rad.com/
Optima XL-A ultracentrifuge	Beckman Coulter	https://www.beckman.de/
J-815 CD spectrometer	Jasco	https://www.jasco.de/
CardioExcyte 96 system	Nanon Technologies	https://www.nanion.de/
Confocal microscopes (LSM 510/710)	Carl Zeiss	https://www.zeiss.com/
Confocal microscopes (Eclipse Ti2)	Nikon	https://www.microscope.healthcare.nikon.com/
Transmission electron microscope EM 910	Carl Zeiss	https://www.zeiss.com/

ARRIVE GUIDELINES

The ARRIVE guidelines (<https://arriveguidelines.org/>) are a checklist of recommendations to improve the reporting of research involving animals. Key elements of the study design should be included below to better enable readers to scrutinize the research adequately, evaluate its methodological rigor, and reproduce the methods or findings.

Study Design

Groups	Sex	Age	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)	Other description
1. Mouse Wildtype (Control)	M	20-25-weeks	4	0	Yes	
2. Mouse Nx3-KO	M + F	20-25-weeks	5	0	Yes	
3. Wildtype mouse hearts used for developmental Nx3 expression and localization studies	M + F	Embryonic (E13); Postnatal (Day 1); Adult (8-12 weeks)	8/group	0	Yes/No	

Sample Size

Sample size was determined based on prior studies and feasibility; no formal power calculation was performed.

Inclusion Criteria

Predefined inclusion criteria included successful genotyping and absence of overt health abnormalities.

Exclusion Criteria

No animals were excluded

Randomization

Randomization was not performed, as group assignment was inherently determined by genotype and animal availability.

Blinding

Investigators were blinded to genotype during outcome assessment and data analysis. Image acquisition and analysis involving mouse heart tissue were carried out in a non-blinded manner.