

ORIGINAL RESEARCH



Nx3 Is a Z-Disk Structural and Signaling Hub That Is Reduced in Heart Failure

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BACKGROUND: Nx3 (novex-3) is an exceptionally small isoform of the giant protein titin, whose structural and functional roles within the sarcomere remain poorly understood.

METHODS AND RESULTS: We used a comprehensive, multimodal approach to define the key properties of Nx3 in healthy and failing hearts, including its abundance relative to FLT (full-length titin), sarcomeric localization, protein interactions, and functional relevance in mouse and human cardiomyocytes under physiological and pathological conditions. Using Western blotting, quantitative polymerase chain reaction, total RNA sequencing, and ribosome profiling, we show that Nx3 is constitutively expressed from fetal development through adulthood. In adult mouse and human myocardium, Nx3 accounts for ≈20% to 25% of total titin protein, despite representing only ≈8% to 14% at the transcript level. Immunoelectron microscopy and binding studies reveal that Nx3 adopts a nonlinear configuration within the sarcomere: its N terminus is anchored at the Z-disk, although the proximal portion of its unique region encoded by *Ttn* exon 48, enriched in coiled-coil motifs, engages laterally with adjacent titin or Nx3 molecules at the Z-disk/I-band interface. Its monomeric C terminus extends toward the A-band but remains confined to the Z-/I-band region. This architecture confers enhanced stability and flexibility to the Z-disk under mechanical load. Protein interaction studies, including yeast 2-hybrid screening and coimmunoprecipitation, identified Pin1 (peptidyl-prolyl cis-trans isomerase NIMA-interacting 1) and TBC1D4 (TBC1 domain family member 4) as binding partners of the Nx3 C terminal region, suggesting participation in signaling networks regulating cardiomyocyte metabolism. Genetic ablation of Nx3 in mouse hearts and human induced pluripotent stem cell-derived cardiomyocytes indicates that, although dispensable for sarcomere assembly, Nx3 is required for optimal Z-disk organization and mechanical performance. In end-stage dilated cardiomyopathy, human hearts exhibit dysregulated Nx3 expression, with reduced protein abundance relative to nonfailing controls and focal Z-disk disruption, likely contributing to impaired contractile function.

CONCLUSIONS: Nx3 regulates Z-disk stability and modulates signaling pathways that optimize cardiac performance, and its dysregulation contributes to heart failure pathogenesis.

GRAPHIC ABSTRACT: A [graphic abstract](#) is available for this article.

Key Words: blotting, western ■ cardiomyopathies ■ heart failure ■ myocardium ■ sarcomeres

Titin, the largest human protein, is indispensable for the architecture and function of the sarcomeric contractile units in striated muscle, including myocardium.

Individual titin molecules extend from the Z-disk to the center (M-band) of the sarcomere, thereby preserving its integrity. Titin transduces mechanical stress, modulates

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Novelty and Significance

What Is Known?

- Titin is a giant sarcomeric protein that is essential for cardiac muscle structure and mechanics, but the physiological function of its much smaller Nx3 (novex-3) isoform is poorly understood.
- Nx3 is generated by alternative splicing of the *TTN* gene and has been reported in several cellular locations, but its organization within the sarcomere, molecular interactions, and functional role have remained unclear.
- Changes in full-length titin isoforms are associated with heart failure and dilated cardiomyopathy, but whether Nx3 is altered in human heart disease is unknown.

What New Information Does This Article Contribute?

- Nx3 is a constitutively expressed and relatively abundant cardiac titin isoform that adopts a distinct organization at the sarcomeric Z-disk.
- Nx3 stabilizes the Z-disk/I-band junction and serves as a signaling scaffold for Pin1 (peptidyl-prolyl cis-trans isomerase NIMA-interacting 1) and TBC1D4 (TBC1 domain family member 4), thereby linking sarcomere architecture with metabolic signaling.
- Loss of Nx3 compromises cardiomyocyte structural, electrical, and mechanical function, and Nx3 is reduced

in end-stage human dilated cardiomyopathy, identifying its dysregulation as a previously unrecognized feature of heart failure.

The function of Nx3, a short titin isoform that differs markedly from the giant titins responsible for sarcomere elasticity, has remained largely unexplored. This study establishes Nx3 as a constitutive and functionally important component of the cardiac sarcomere. We show that Nx3 has a distinct organization at the Z-disk, where it contributes to mechanical stability while also recruiting signaling proteins involved in metabolic regulation. Loss of Nx3 disrupts Z-disk integrity and impairs cardiomyocyte electrical and mechanical function without preventing sarcomere formation, revealing a specialized role in maintaining rather than assembling the contractile apparatus. The reduction of Nx3 in human dilated cardiomyopathy further links this previously understudied titin isoform to cardiac disease. Together, these findings expand the biological functions attributed to titin beyond its full-length isoforms and identify Nx3 as a molecular link between sarcomere architecture, cellular signaling, and cardiac function. This work provides a new framework for investigating how isoform-specific titin dysfunction contributes to heart failure.

Nonstandard Abbreviations and Acronyms

ACTN2	α -actinin
AMPK	AMP-activated protein kinase
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat-associated 9
DAPI	4',6-diamidino-2-phenylindole
DCM	dilated cardiomyopathy
ex48	exon 48
FLT	full-length titin
hiPSC	human induced pluripotent stem cell
ID	intercalated disk
LV	left ventricular
Nx3	novex-3
Pin1	peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
SL	sarcomere length
TBC1D4	TBC1 domain family member 4
TTNtv	titin-truncating variant
WB	Western blot
WT	wild-type
Y2H	yeast-2-hybrid

cardiomyocyte elasticity and contractility, and mediates length-dependent activation.^{1,2} Among its diverse isoforms, Nx3 (novex-3) represents a uniquely truncated variant expressed across all striated muscle types.³ Despite its likely significance, Nx3 has received scant attention.⁴ Nx3 shares an N-terminal segment with the cardiac FLTs (full-length titins) N2BA and N2B and with the skeletal muscle N2A isoform. However, the inclusion of exon 48 (ex48) in the *TTN* gene (termed the Nx3 exon) introduces a novel stop codon and polyadenylation signal, yielding the smallest titin isoform (≈ 650 kDa).³ Over 2000 amino acids derive from ex48, which encodes 6 immunoglobulin-like (Ig) domains (I18-I23; nomenclature by Bang et al³) interspersed with extensive insertion sequences (is19-is23).

First identified decades ago,³ many facets of Nx3 biology and function remain obscure. The C terminal region is proposed to bind cytoskeletal partners such as obscurin,³ yet the precise arrangement of Nx3 within the sarcomere and its contributions to assembly, stability, and mechanical performance are uncharacterized.⁴ Moreover, potential dysregulation of Nx3 in cardiomyopathy and heart failure remains largely unexplored. Although transcriptomic alterations in dilated cardiomyopathy (DCM) and arrhythmogenic right ventricular cardiomyopathy have been reported,⁵ changes in Nx3 protein abundance in failing human hearts remain

unknown compared with extensive studies of other titin isoforms.^{6–9} Beyond its sarcomeric role, Nx3 has been reported to localize to cardiomyocyte nuclei, an unexpected location for a Z-disk-associated large protein, where it is suggested to influence nuclear elasticity and promote proliferation during fetal development.¹⁰ Nx3 deletion in mice reduces embryonic cardiomyocyte proliferation and precipitates postnatal hypertrophy.¹¹ In adult mice, Nx3 deficiency induces ventricular dilation and dysfunction, implicating aberrant nuclear mechanics,¹¹ suggesting that Nx3 may exert both sarcomeric and extrasarcomeric functions.

Here, we comprehensively characterize Nx3 in the heart, with a focus on its properties in the sarcomere. We quantify its expression relative to FLT, map its precise sarcomeric localization, delineate its Z-disk interactions, and assess its functional role in human CMs under physiological and chronic disease states. Employing custom anti-Nx3 antibodies, confocal and immunoelectron microscopy, interaction assays, the murine Nx3-deletion model,¹¹ and clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat-associated 9 (CRISPR/Cas9)-mediated Nx3 knockout in human induced pluripotent stem cell-derived cardiomyocytes, we evaluate sarcomere organization, mechanical performance, and ligand binding. Furthermore, we analyze Nx3 expression in over 100 end-stage failing human heart tissues compared with nonfailing donor hearts. Our integrated approach elucidates the structural and functional significance of Nx3 in cardiac biology and demonstrates its potential involvement in heart disease.

METHODS

Data Availability

All data supporting the findings of this study are provided in the main figures and [Supplemental Material](#) or are available from the corresponding authors upon reasonable request. Detailed experimental procedures and resource information are included in the [Supplemental Material](#); see the [Major Resources Table](#) for a comprehensive list of reagents and resources. Results of the yeast-2-hybrid (Y2H) interaction screens are presented in [Tables S1 and S2](#). Analysis parameters are available upon request.

Human and Murine Heart Tissues

Left-ventricular (LV) tissue from patients undergoing transplantation for DCM and nonfailing donor hearts was obtained from the Heart and Diabetes Center NRW and the Sydney Heart Bank, as described previously.⁹ All studies had appropriate institutional ethics approval and informed consent/next-of-kin consent and conformed to the Declaration of Helsinki.¹² Patient characteristics are given in [Table S3](#) and donor characteristics in [Table S4](#). Failing hearts were genotyped using the 174-gene TruSight Cardio panel and classified as TTN-truncating-variant (TTNtv) or idiopathic DCM, with TTNtv further stratified by

I-band or A-band location ([Table S3](#)). Preenrolled hearts were included; 6 to 7 of >100 samples lacking detectable protein bands on electrophoresis were excluded. Five fetal human LVs (105–117 days postconception) were obtained with maternal consent and ethics approval (no. STUDY00000380). Murine LV samples comprised adult Nx3-deficient mice generated by CRISPR/Cas9 (20–25 weeks; N=5) and age-matched wild-type (WT) controls (N=4),¹¹ plus C57BL/6 hearts at E13, postnatal day 1, and adulthood (8–12 weeks). Animal procedures were institutionally approved; sexes were pooled, because no significant sex-related differences were detected.

hiPSC Generation and Cardiomyocyte Differentiation

Two previously characterized WT hiPSC lines^{13,14} were used under Göttingen University Ethical Board approvals (20/9/16An, 15/2/20, 7/5/24). CRISPR/Cas9-mediated Nx3 ATG knockout was generated as described^{15,16} using sgRNA 5'-AGAGGTGGTGAAGAGTACG-3' cloned into pSpCas9(BB)-2A-Puro, followed by transfection and single-clone isolation. hiPSCs were maintained feeder-free, regularly tested for mycoplasma, and differentiated on Matrigel using a small-molecule monolayer protocol.^{17–19} Beating cells appeared from day 7; hiPSC-cardiomyocytes were maintained in RPMI/B27 and replated after TrypLE dissociation on days 20 to 25. Experiments used 3 independent differentiations.

Protein, Imaging, and Biophysical Analyses

Antibodies, sources, catalog numbers, and concentrations are listed in [Table S5](#). Cardiac tissue immunofluorescence used rehydrated paraffin sections, antigen retrieval, BSA/Triton blocking, primary and secondary antibodies, optional DAPI (4',6-diamidino-2-phenylindole), and confocal microscopy. TEM and immunogold electron microscopy were performed as described^{9,20}; epitope distances and Z-disk/Z-body morphology were quantified manually with Nikon NIS, or with MATLAB (R2024b), using linear mixed-effects models for repeated image-level measurements. Proteins were analyzed by SDS-PAGE/Western blot (WB); titin was resolved on 1.8% to 2.2% agarose-stabilized acrylamide gels, transferred to polyvinylidene fluoride, detected by ECL, and quantified with ImageQuant TL, with normalization to GAPDH, total protein, or FLT as indicated. Recombinant Nx3 I21-I23 was analyzed by sedimentation-velocity analytical ultracentrifugation at 55 000 rpm and 20°C; continuous c(s) distributions were calculated with SEDFIT v18.1 (ME regularization, $P=0.68$)²¹ and scans/residuals plotted with GUSI v2.1.6.²² Full protocols, including Y2H, protein purification, circular dichroism, coimmunoprecipitation, droplet digital polymerase chain reaction/ribosomal sequencing/qRT-PCR, hiPSC imaging, and functional assays, are provided in [Supplemental Methods](#).

Statistical Analysis

GraphPad Prism v8-10 or MATLAB R2024b was used. N denotes animals, patient hearts, or independent hiPSC-cardiomyocyte differentiations; n denotes biological replicates. Unless stated otherwise, experiments included at least 3 independent biological replicates. Normality was assessed by Shapiro-Wilk testing. Normally distributed data were analyzed

by unpaired 2-tailed *t* test with Welch correction or 1-way ANOVA with Holm-Šidák post hoc testing; non-normal data by Mann-Whitney *U* test or Kruskal-Wallis with Dunn post hoc testing. Repeated measurements were analyzed by linear mixed-effects models with Bonferroni correction where appropriate. Correlations used Spearman or Pearson tests as indicated. Data are mean±SEM unless stated otherwise; 2-sided *P* < 0.05 was significant. Multiple-comparison correction was applied within individual analyses, not across the article.

RESULTS

Nx3 Protein Expression During Development of Murine and Human Hearts

Considering the limited information available on the expression levels of Nx3 titin in the heart, we first assessed its expression during fetal, neonatal, and adult cardiac development using a panel of anti-titin antibodies (Figure 1A). This panel included 2 custom-made antibodies specific to Nx3: one recognizing the unique insertion sequence is21, located between the 3 N terminal and 3 C terminal Ig domains of the Nx3 region,²⁰ and another recognizing the most C terminal Ig domain (I23) within the Nx3 region²³ (Figure 1B). During murine heart development, we observed a continuous increase in the absolute amount of Nx3 expression, from embryonic stage E13 through neonatal day 1 (D1) to adulthood (Figure 1C). Similarly, Nx3 detection by WB in human fetal (D105–117) and adult (nonfailing) LV using is21- or I23-specific antibodies indicated significantly increased Nx3 expression later in development (Figure 1D). Notably, the relatively high expression of Nx3 in adult LVs contrasts with that of the A-band titin isoform Cronos, which is abundantly expressed in fetal hearts but significantly less so in adult LVs.^{9,24} The increase in Nx3 expression during heart maturation aligns with the higher myofibril content observed at later developmental stages. We also quantified Nx3 expression relative to FLT (N2BA+N2B isoforms) by WB using antibodies against the Z-disk or Z/I junctional region of titin. Interestingly, the Nx3:FLT expression ratio remained remarkably constant from fetal development through adulthood in both murine (Figure 1E) and human hearts (Figure 1F), ranging from ≈1:5 to 1:4.

Nx3 Isoform Barely Reaches Into the I-Band but Supports a Stress-Responsive Z-Disk

Next, we determined the precise localization of Nx3 in CMs. Using confocal imaging and the I23 antibody, we identified Nx3 at 2 distinct locations in embryonic murine and fetal human heart tissue: at the Z-disk and at the intercalated disk (ID; Figure 2A). The latter was confirmed by colocalization of Nx3 with desmoplakin, a major component of desmosomes at the ID (Figure 2A):

both proteins appeared in a punctate pattern reflecting the not-yet-mature ID structure in these fetal/embryonic tissues. Immunoelectron micrographs using the I23 antibody further confirmed the staining of Z-disks and IDs in fetal mouse and human hearts (Figure 2B). Clear nuclear staining^{10,11} was not observed. The dual distribution of Nx3 at the Z-disk and ID was also noted by immunoelectron microscopy in adult murine hearts (Figure 2C). The nanogold particles marking the Nx3 (I23) position were densely aligned at the periphery of the Z-disk, although they were less dense (approximately half the density of Z-disk Nx3) and more scattered at the ID, where they marked a zone previously described as the transitional junction.²⁵ This zone is located between the terminal sarcomeres of the cardiac myofibrils and the meandering ID structure.

Focusing on the sarcomeric localization of Nx3, we compared the epitope positions of our 2 Nx3-specific antibodies with those of other sarcomere protein-directed antibodies, particularly other antititin antibodies, at a given sarcomere length (SL). In adult mouse sarcomeres, we found that the Nx3 region (is21) localizes somewhat closer to the Z-disk center than the T12 (N-terminal I-band) antibody epitope and much closer than the N2B element recognized by the anti-N2Bus antibody (Figure 2D, left). Furthermore, we determined the position of the last Ig domain within the Nx3 region (I23) relative to that of ACTN2 (α-actinin), the Z/I junction encoded by *Ttn* exon 28 (anti-S2080), and the PEVK region (Figure 2D, right). We found that the Nx3 C terminus localizes more distally to the Z-disk center than α-actinin, slightly more distal than the S2080 position but much more proximal than PEVK (Figure 2E, left). Upon sarcomere stretch, the Nx3 (I23) position at the Z-disk periphery modestly but significantly translocated away from the Z-disk center, similar to the S2080 epitope, indicating limited extensibility of the Nx3 isoform; during the stretch, the Nx3 (I23) and S2080 epitopes maintained a similar distance from one another (Figure 2E, right). We conclude that the Nx3 isoform is extensible to a minor extent but barely reaches into the I-band.

Based on the position of the Nx3 epitopes relative to other titin epitopes, the Nx3 molecule cannot be arranged in a straight configuration within the sarcomere, because its C terminus (I23) would have to extend farther into the I-band than even the N2B element. Instead, we propose a layout where the Nx3 isoform adopts an S-shaped configuration, with the N terminus anchored at the Z-disk (like the FLT isoforms), the midsection looping back to the Z-disk, and the C terminal region, composed of the Nx3 element (ex48), pointing away from the Z-disk towards the I-band (Figure 2F). This arrangement would provide sufficient length in the direction perpendicular to the myofibril axis, allowing interaction with adjacent FLT or Nx3 molecules at the Z/I junction.

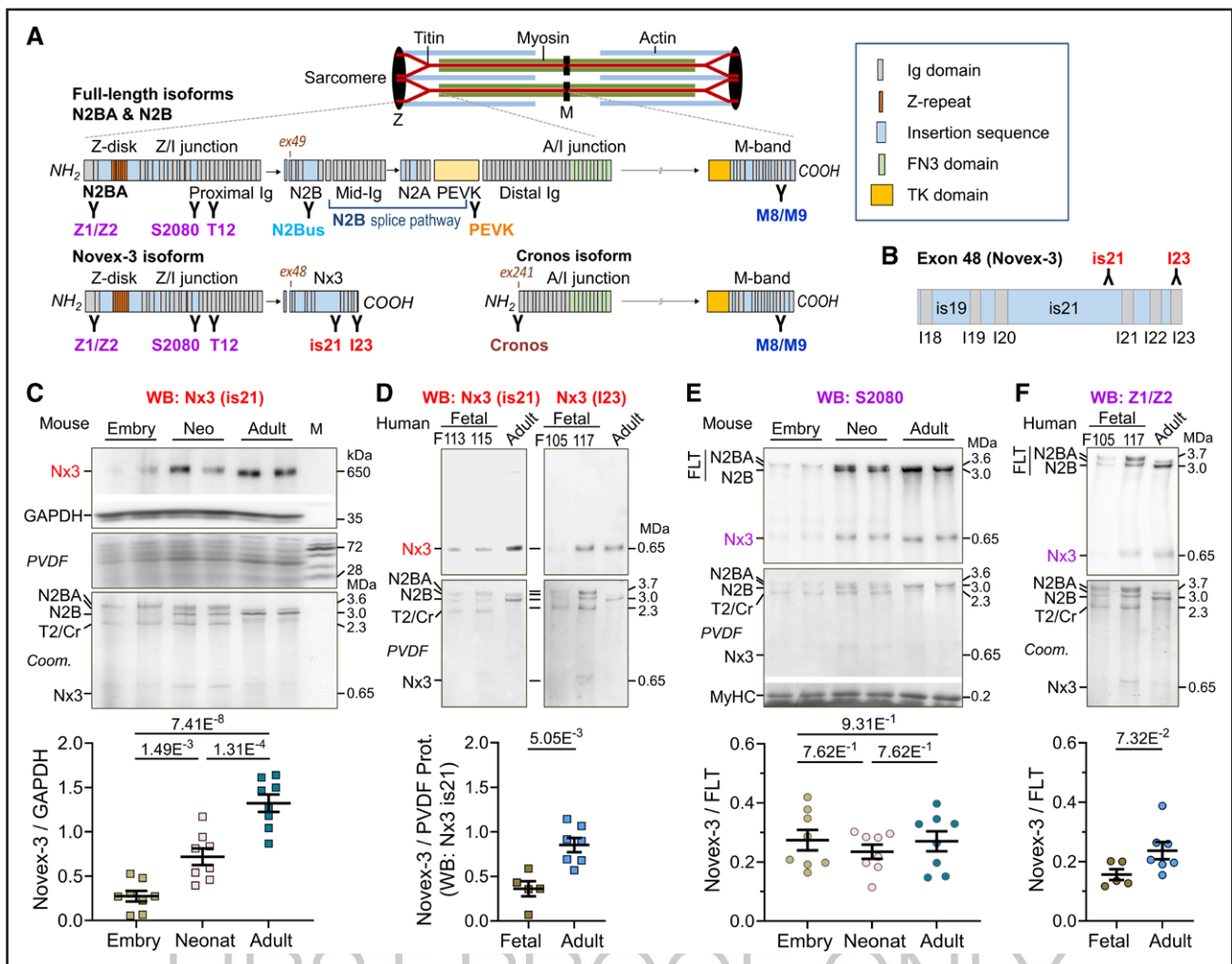


Figure 1. Nx3 (novex-3) expression during prenatal and postnatal mouse and human heart development.

A, Domain structures of full-length (N2BA and N2B), Nx3, and Cronos titin, showing the targets of 2 new Nx3-specific antibodies: insertion sequence 21 (is21) and C terminal Ig domain 23 (I23; purple). **B**, Schematic of the Nx3 region (exon 48 [ex48]). **C**, Western blot (WB) and quantification of is21-detected Nx3 normalized to GAPDH in E13, neonatal, and adult mouse hearts (2% gels for Nx3; 12.5% for GAPDH). N=8 per group. **D**, Loose titin gel WBs and quantification of Nx3 detected with is21 (left) or I23 (right) and normalized to GAPDH in fetal (days 113, 115, and 117) and adult nonfailing human hearts. N=5 fetal, 7 adult. **E**, Nx3 relative to full-length titin (FLT) in developing and adult mouse hearts, detected with the S2080 antibody (Z-/I-band junction). N=8 per group. **F**, Nx3 relative to full-length titin, detected with the Z1/Z2 antibody (N terminus), in the human samples from D. N=5 fetal, 7 adult. Data are mean $\pm$ SEM; 1-way ANOVA with Holm-Šidák correction (**C** and **E**) or Mann-Whitney *U* test (**D** and **F**). Coom. indicates Coomassie-stained loose titin gel.

To test for the possibility of such interactions, we used the region encoded by *Ttn* exons 27/28 (Z7-I1 Ig domains plus sequence insertions), which is present in all Z-disk-anchored isoforms and is known to bind to actin filaments,²⁶ as bait in a Y2H screen against a human cardiac cDNA library (Figure S1A). Among the various proteins detected as potential interacting partners, (Table S1) was the N terminus of the Nx3 element (Figure S1A). Coimmunoprecipitation of the Z7-I1 recombinant construct with this Nx3 fragment confirmed a weak direct interaction (Figure S1B). Thus, the S-shaped configuration of Nx3 presumably is stabilized by interaction with the titin Z/I junction, enhancing Z-disk stability and flexibility under mechanical stress.

Recombinant Nx3 I21-I23 Predominantly Forms Monomers in Solution

We next assessed the oligomerization capacity of Nx3 in vitro. Full-length Nx3 was not amenable to recombinant expression; therefore, we expressed the C terminal I21-I23 fragment (3 Ig domains plus linkers; Figure 3A), hypothesized to mediate homotypic interactions. Circular dichroism spectroscopy confirmed proper folding. The spectrum showed a minimum at ≈ 218 nm, consistent with β -sheet-rich structure (Figure 3B). Circular dichroism deconvolution using BeStSel (<https://bestsel.elte.hu>)²⁷ closely matched the experimental data (NRMSD=0.04419) and indicated 44.7% β -sheet (41.2% antiparallel), 0% α -helix, and 55.3% turns/unordered regions. This agrees with MESSA

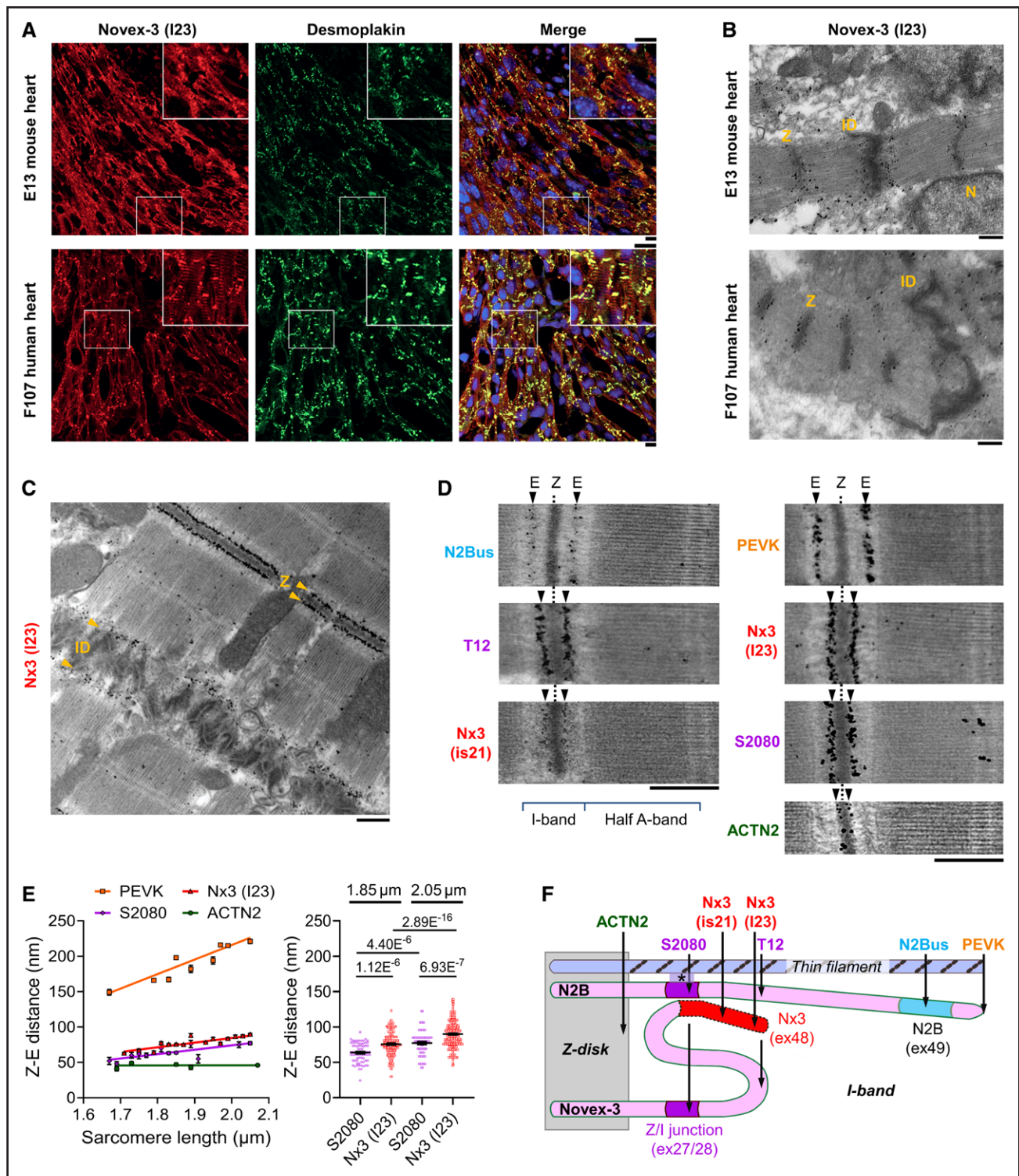


Figure 2. Nx3 (novex-3) localization in cardiomyocytes during development and in adult hearts.

A, Nx3 (I23; Alexa Fluor 555, red) localizes to sarcomeres and desmoplakin-positive intercalated disks (IDs; Alexa Fluor 488, green) in embryonic mouse (**top**) and fetal human (day 107; **bottom**) hearts. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole); scale bars, 10 μm. Representative images from ≈20 per condition are shown. **B**, Immuno-EM localization of Nx3 (I23; nanogold) at Z-disks (Z) and IDs in embryonic mouse (**top**) and fetal human (day 117; **bottom**) hearts. Scale bars, 500 nm. **C**, Immuno-EM localization of Nx3 (I23; nanogold) in adult mouse heart; scale bar, 500 nm. **D**, Positions of Nx3 epitopes (is21 and I23) relative to other titin epitopes (N2Bus, PEVK, T12, and S2080) and ACTN2 (α-actinin) in stretched mouse cardiac half-sarcomeres at identical sarcomere length (SL). Scale bars, 500 nm. **E**, Mean distance from the Z-disk center to 3 titin epitopes and ACTN2 at different SLs (**left**), and relative shift of the Nx3 I23 epitope vs S2080 at SLs of 1.85 and 2.05 μm (**right**). Data are mean±SEM; n=74 S2080- and 82 Nx3-labeled sarcomeres from N=5 mouse hearts per group; linear mixed-effects model with Bonferroni adjustment. **F**, Model of Nx3 at the Z-disk/I-band junction alongside full-length titin (N2B) and the thin filament; arrows indicate mapped epitopes. E indicates epitope; N, nucleus; and Z, Z-disk. *Actin-titin interaction.

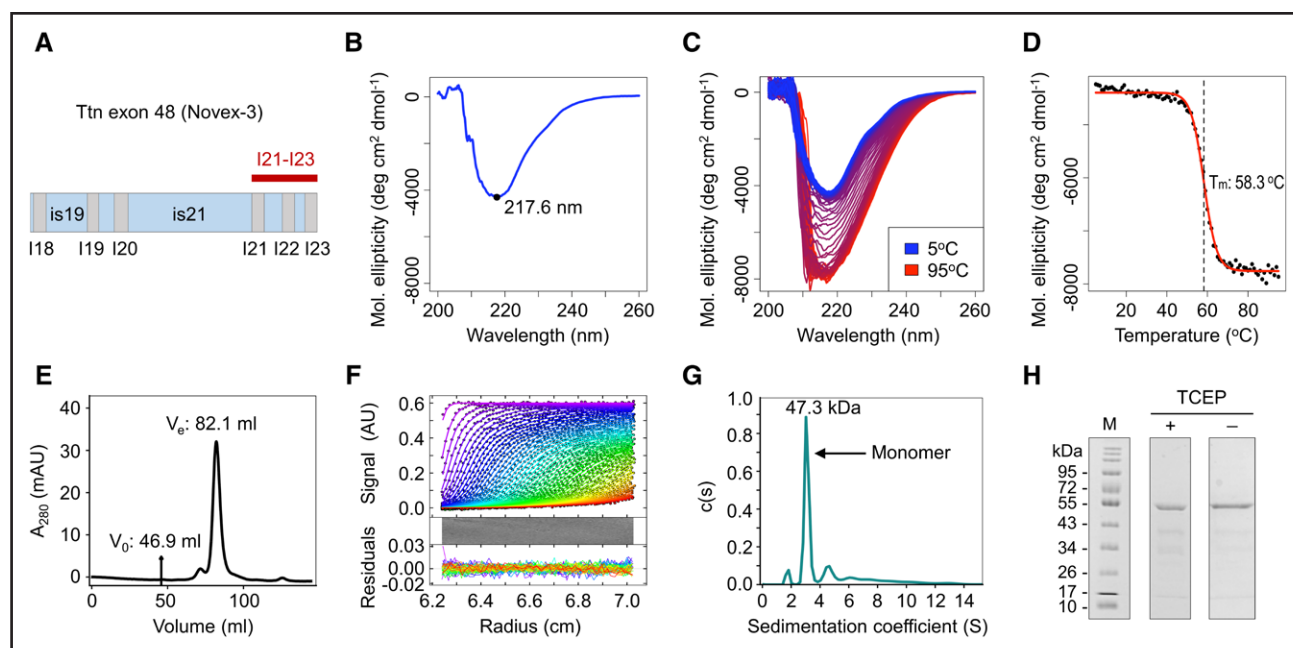


Figure 3. Circular dichroism (CD) spectroscopy and oligomeric state of Nx3 (novex-3) I21-I23.

A, Location of the recombinant C terminal Nx3 construct within the titin-truncating (TTN) exon 48 region. **B**, Far-UV CD spectrum of I21-I23. **C**, Thermal denaturation monitored by CD from 5°C (blue) to 95°C (red) in 1°C increments. **D**, Melting curve derived from ellipticity at 218 nm. Black points show experimental data and the red curve a nonlinear least-squares sigmoidal fit. **E**, Size-exclusion chromatogram of I21-I23 on a HiLoad 16/600 Superdex 200 PG column (Cytiva) equilibrated with 25 mmol/L HEPES; pH, 7.5; 100 mmol/L NaCl; and 1 mmol/L TCEP. **F**, Sedimentation-velocity analytical ultracentrifugation data. Absorbance profiles (upper) were corrected for time- and radius-invariant noise and fitted using the continuous $c(s)$ model (solid lines); residuals are shown as a bitmap (middle) and overlay (bottom). **G**, Diffusion-corrected $c(s)$ distribution. The dominant peak at 3.03 S (≈ 47.3 kDa) indicates that I21-I23 is predominantly monomeric in solution. **H**, Nonreducing SDS-PAGE of I21-I23 incubated with or without 1 mmol/L TCEP. T_m indicates melting temperature; V_0 , void volume; and V_e , elution volume.

predictions (<http://prodata.swmed.edu/MESSA>)²⁸: 43.3% β -sheet, 0% α -helix, and 56.7% coil. BeStSel fold recognition further identified Ig-like structures (eg, PDB: 1H8N, 5XMH, and 5TBD) as closest matches. Together, these results suggest that domains within I21-I23 adopt a native Ig-like fold. In addition, thermal denaturation yielded a melting temperature (T_m) of 58.3°C (Figure 3C and 3D), within the range reported for titin Ig domains (54–68°C),²⁹ confirming native-like stability.

Recombinant I21-I23 was purified to homogeneity. Size-exclusion chromatography showed a single, monodisperse species (Figure 3E). Analytical ultracentrifugation revealed a dominant peak at 47.3 kDa (Figure 3F and 3G), matching the theoretical monomer mass (48.0 kDa), demonstrating that the protein is predominantly monomeric in solution. Moreover, nonreducing SDS-PAGE, with and without 1 mmol/L TCEP, showed only monomeric species (Figure 3H), implying no intermolecular disulfide formation. Thus, I21-I23 does not oligomerize via oxidative cysteine cross-linking, suggesting that the C terminus of Nx3 remains monomeric under redox stress conditions.

C Terminal Nx3 Region Participates in Cardiomyocyte Signaling Pathways

Nx3 (ex28) is detected at the sarcomeric Z-disk/I-band interface, which may be a signaling hub in CMs.^{30–32}

Therefore, it may also bind to other sarcomere proteins and signaling molecules. To identify candidate proteins that interact with the C terminal region of Nx3, we performed 2 independent Y2H screens using Nx3 I21-I23 as bait against a human cardiac cDNA library (Figure 4A). Several potential Nx3-binding partners repeatedly appeared as prey in both Y2H screens (Table S2) but were not further pursued. However, in both screens, we identified multiple clones of Pin1 (peptidyl-prolyl isomerase NIMA-interacting 1), a multifunctional regulator of proline-directed kinases.³³

The I21-I23-Pin1 interaction was confirmed by direct Y2H assays (Figure 4B and 4C) and coimmunoprecipitation (Figure 4D), with no interaction detected in control experiments lacking Pin1 (Figure 4E). Immunoelectron microscopy of mouse LV tissue localized Pin1 to the Z-disk periphery, similar to Nx3 (is21; Figure 4F). Consistently, confocal microscopy of human nonfailing LV tissue showed Pin1 in a cross-striated pattern colocalizing with the Z-disk marker ACTN2 (Figure 4G). Pin1 also labeled the ID, as previously observed for Nx3.

Pin1 interacts with TBC1D4 (TBC1 domain family member 4/AS160),³⁴ a Rab-GTPase regulator essential for glucose and fatty acid uptake in muscle cells.^{35–37} Notably, TBC1D4 was also identified as an interactor of Nx3 I21-I23 in our Y2H screen (Table S2).

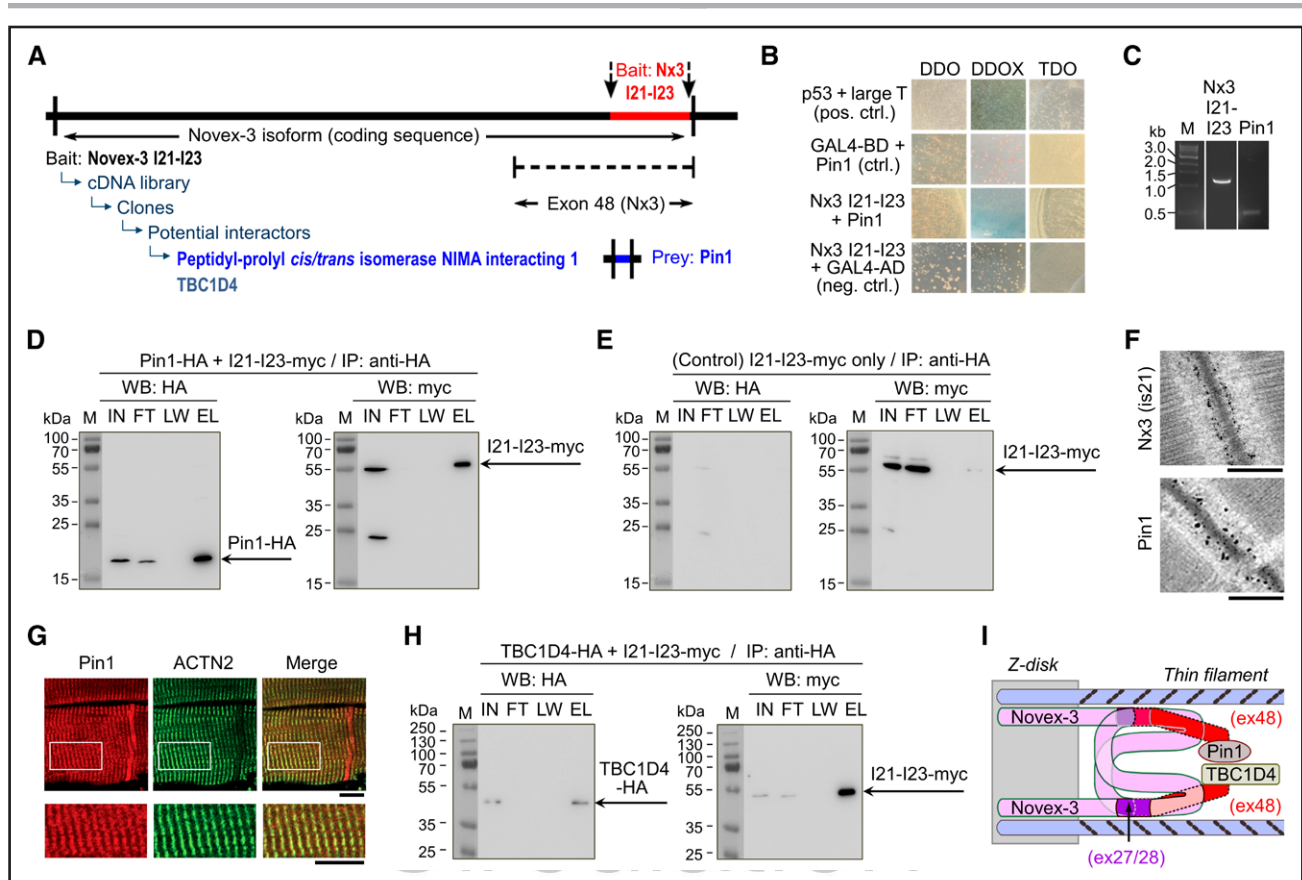


Figure 4. Novel interactors of the C-terminal Nx3 (novex-3) region.

A, Yeast 2-hybrid (Y2H) screening with Nx3 I21-I23 (Ttn ex48) as bait identified full-length Pin1 (peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; 163 amino acids) as prey; the Pin1 interactor TBC1D4 (AS160) was also identified. **B**, Direct Y2H mating assay with Nx3 I21-I23 as bait and Pin1 as prey on selective media (DDO, DDOX, and TDO). Interaction is indicated by growth on all media and blue colonies on DDOX. Controls: p53/SV40 large T antigen (positive), empty bait vector, and bait only (negative). **C**, Colony polymerase chain reaction confirming bait (Nx3 I21-I23; 1248 bp) and prey (Pin1; 504 bp) plasmids in single diploid yeast colonies; products were resolved on 1% agarose gels. **D**, Coimmunoprecipitation (co-IP) of Pin1-HA (20 kDa) with Nx3 I21-I23-myc (49 kDa) from HEK cell lysates using anti-HA agarose. Input (IN), flow-through (FT), last wash (LW), and eluate (EL) were resolved on 12.5% SDS gels; Western blot (WB) exposure, 2 minutes. **E**, Control co-IP without HA-tagged prey, showing no I21-I23-myc in the eluate. **F**, Representative immuno-EM images (~80 acquired) localizing Nx3 is21 (**left**) and Pin1 (**right**) to the Z-disk periphery in mouse heart; scale bars, 500 nm. **G**, Confocal immunofluorescence showing Pin1 (Cy3, red) colocalization with ACTN2 (α -actinin; fluorescein isothiocyanate, green) at Z-disks and Pin1 staining at intercalated disks in nonfailing human left ventricle. **Bottom**, enlarged view; scale bars, 10 μ m. **H**, Co-IP of TBC1D4-HA (40 kDa) with Nx3 I21-I23-myc (49 kDa) from HEK cell lysates using anti-HA agarose. **I**, Model showing Pin1 and TBC1D4 colocalization with the Nx3 C-terminus and potential cross-linking of 2 Nx3 molecules.

Coimmunoprecipitation confirmed this interaction (Figure 4H). Together, these findings suggest that Nx3 may act as a hub in cardiomyocyte signaling networks regulating metabolic pathways. Binding of Pin1 and TBC1D4 to the Nx3 C terminus may further stabilize this region, potentially enabling cross-linking of adjacent Nx3 molecules (Figure 4I).

Nx3 Depletion in Mouse Hearts Abolishes Pin1 and TBC1D4 Localization at Z-Disks

To elucidate the relevance of Nx3 in the sarcomeric localization of Pin1 and TBC1D4, we analyzed LV tissue from homozygous Nx3-knockout mice.¹¹ WB analysis confirmed the absence of Nx3 (Figure 5A) and revealed

a strong trend toward reduced Pin1 protein levels in knockout hearts, whereas TBC1D4 abundance remained unchanged compared with WT controls (Figure 5B and 5C). IF analysis demonstrated a marked loss of Z-disk localization of both proteins in knockout myocardium. In WT, Pin1 colocalized with Nx3 and titin Z1/Z2 in a striated pattern, although TBC1D4 likewise showed clear Z-disk localization. These Z-disk associations were largely abolished in the absence of Nx3 (Figure 5D and 5E) and were replaced by diffuse and intense spot-like IF signals, even in the extracellular space. Together, these results indicate that Nx3 is dispensable for TBC1D4 abundance and largely dispensable for Pin1 abundance but is required for the proper Z-disk targeting of both proteins.

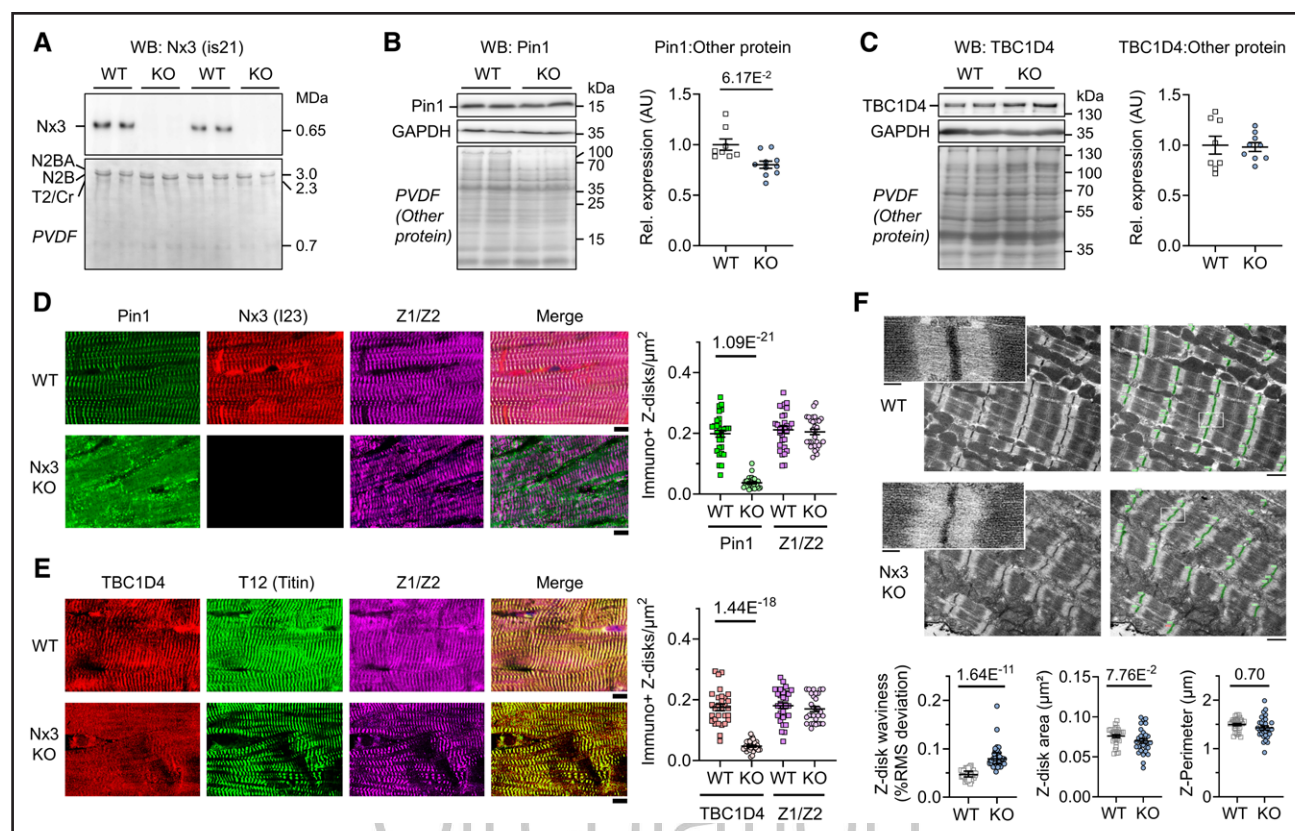


Figure 5. Altered Nx3 (novex-3) interactions and Z-disk morphology in Nx3 knockout mouse hearts.

A, Anti-Nx3 (is21) Western blot (WB) of adult left ventricular (LV) tissue from wild type (WT) and homozygous Nx3 knockout (KO) mice. **B** and **C**, WB and quantification of Pin1 (peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; **B**) and TBC1D4 (**C**) expression normalized to total cardiac protein. Data are mean±SEM; N=4 WT and 5 Nx3-KO mice. **D**, Immunofluorescence costaining of WT and Nx3-KO LV for Pin1 (Alexa Fluor 488, green), Nx3 (I23; Alexa Fluor 555, red), and titin Z1/Z2 (Alexa Fluor 647, magenta), with quantification of Pin1- and Z1/Z2-positive Z-disks (**right**). **E**, Immunofluorescence costaining for TBC1D4 (Alexa Fluor 555, red), titin T12 (Alexa Fluor 488, green), and titin Z1/Z2 (Alexa Fluor 647, magenta), with quantification of TBC1D4- and Z1/Z2-positive Z-disks per area (**right**). Merged images in (**D**) and (**E**) include DAPI (4',6-diamidino-2-phenylindole; nuclear staining; blue); scale bars, 10 μm. Data are mean±SEM; N=3 mice per genotype and n=10 averaged intensity profiles per animal. **F**, Representative transmission electron micrographs of LV tissue (from ≈50 images per group) used to quantify Z-disk alterations in Nx3-KO vs WT hearts. Z-disks were traced semiautomatically using NIS software (Nikon; **right**); scale bars, 1 μm. **Bottom**, Show Z-disk waviness (**left**; root-mean-square deviation from a fitted reference line; median and quartiles; N=3 mice per group; n=36 averaged Z-disks for WT and 32 for Nx3-KO), area (**center**), and perimeter (**right**). Area and perimeter data are mean±SEM; N=3 mice per group and n=10 averaged Z-disks per animal. All data were analyzed using linear mixed-effects models, with Bonferroni adjustment for multiple comparisons in (**D**) and (**E**).

Nx3-Knockout Models Reveal a Role in Z-Disk Stability, Formation, and Cellular Contractility

TEM of LV tissue showed largely preserved sarcomere structure but altered Z-disk morphology in Nx3-knockout hearts compared with WT (Figure 5F). Quantitative tracing of Z-disks (Figure 5F, bottom) revealed increased waviness (RMS deviation), implying reduced alignment, whereas Z-disk area and perimeter were not significantly altered in knockout myocardium. Together, these data strongly suggest that loss of Nx3 compromises Z-disk mechanical stability at the ultrastructural level.

To assess the consequences of Nx3 loss also in human CMs, we generated an Nx3 knockout in hiPSCs from a healthy donor by targeting titin ex48 using CRISPR-Cas9 (Figure 6A; Figure S2A). Knockout and

WT hiPSCs expressed comparable levels of pluripotency markers at protein and mRNA levels (Figure S2B through S2E). Both lines were differentiated into beating hiPSC-CMs using a small molecule-based monolayer protocol. Loss of Nx3 was confirmed by WB with antibodies against Z-disk titin and the Nx3 (is21) epitope, as well as the N terminus of Cronos and M-band titin; WT hiPSC-CMs and adult human LV tissue served as controls (Figure 6B). Immunostaining for Nx3 (is21), ACTN2, Z-disk titin, and M-band titin showed that knockout cells formed sarcomeres, although sarcomeric structures were occasionally reduced compared with WT (Figure 6C), suggesting modestly impaired sarcomere assembly. Consistently, SL measurements in ACTN2-stained cells revealed no significant change in mean SL but a broader distribution in Nx3-knockout cultures, including cells with abnormal or shortened SL and disorganized sarcomeres,

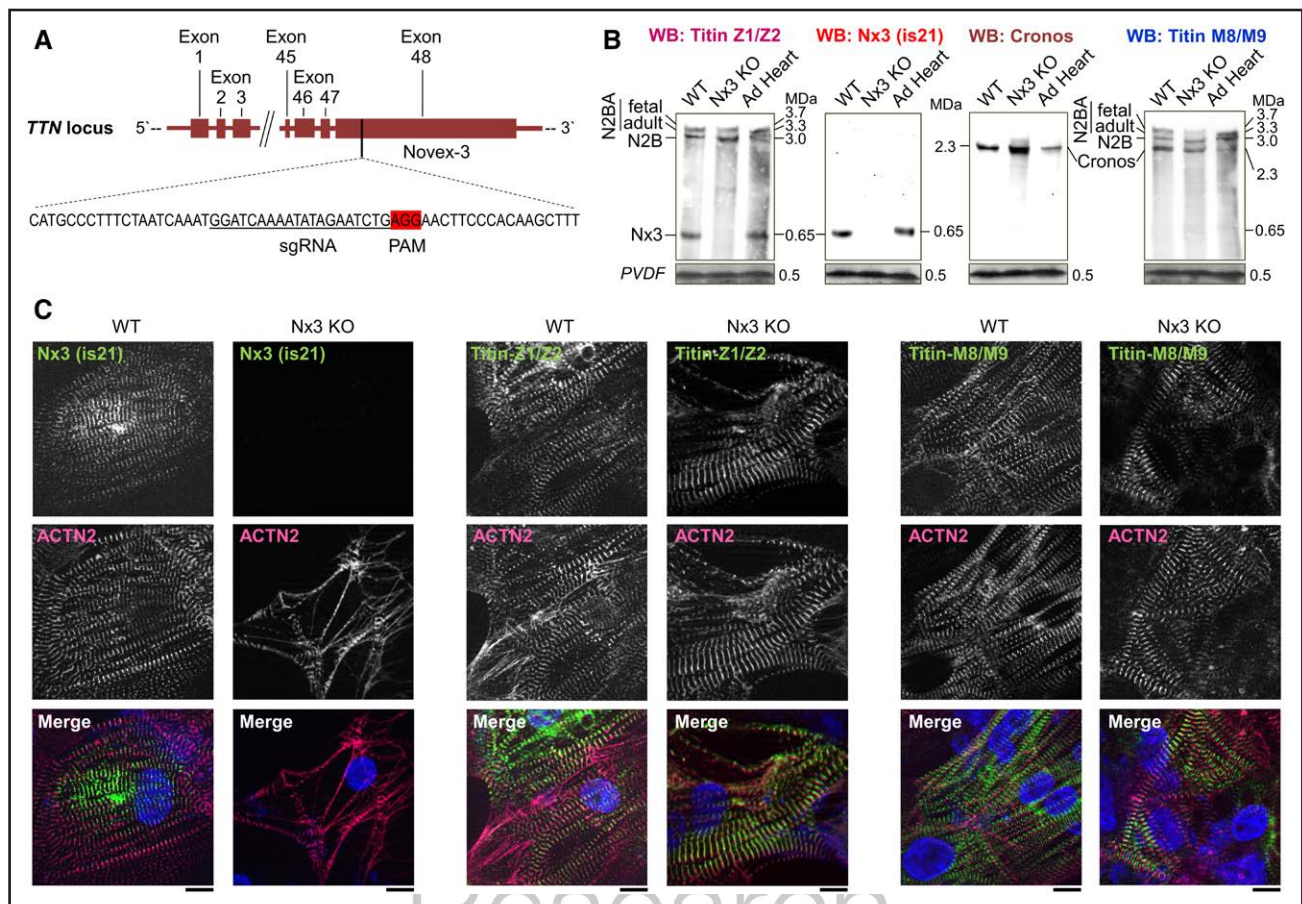


Figure 6. Cas9-targeted regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat-associated 9-engineered human titin Nx3 (novex-3)-knockout (KO)-induced pluripotent stem cell-cardiomyocytes (iPSC-CMs) can form sarcomeres.

A, Targeting of *TTN* exon 48 (ex48) with a single sgRNA to generate Nx3-KO human induced pluripotent stem cells (hiPSCs). **B**, Western blot (WB) confirmation of Nx3-KO after differentiation into cardiomyocytes (hiPSC-CMs), using antibodies against the titin Z-disk region (Z1/Z2), Nx3 (is21), Cronos, and the M-band region (M8/M9). A Coomassie-stained polyvinylidene fluoride membrane served as loading control. Compared with wild type (WT) hiPSC-CMs and adult (Ad) human donor heart, Nx3-KO cells lacked the Nx3 band and occasionally showed reduced full-length titin (N2BA and N2B), suggesting delayed or impaired sarcomere formation. **C**, Confocal immunofluorescence of WT and Nx3-KO iPSC-CMs stained for Nx3 (is21; Alexa Fluor 488, green) and ACTN2 (α -actinin; Alexa Fluor 555, red; left), titin Z1/Z2 (middle), or titin M8/M9 (right). Merged images include DAPI (4',6-diamidino-2-phenylindole)-stained nuclei (blue); scale bars, 10 μ m.

which were rare in WT (Figure 7A, middle and bottom; insets), implying increased heterogeneity.

TEM combined with semiautomated analysis of developing Z-disks (Z-bodies) demonstrated that Nx3-knockout hiPSC-CMs contained Z-bodies of significantly shorter length than WT controls (Figure 7B). In contrast, Z-body perimeter was unchanged, although Z-body density showed a trend toward reduction in Nx3-knockout cells. These findings confirm that Nx3 is not essential for sarcomerogenesis but facilitates efficient Z-disk assembly.

Electrophysiological analysis (CardioExcyte 96) revealed a significantly reduced signal amplitude (Figure 7C), prolonged field potential duration (Figure 7D), and a trend toward increased spontaneous beat rate (Figure 7E) in knockout cells. Motion-traction analysis (eg, traces in Figure 7F) further showed significantly shortened contraction duration (Figure 7G) without changes in time-to-peak shortening (Figure 7H), a significantly

increased beat rate (Figure 7I) and shorter contraction-relaxation intervals (Figure 7J), although rhythmicity remained unaffected (Figure 7K). These functional alterations likely reflect impaired intercellular junction organization and inefficient maturation in the absence of Nx3. Overall, Nx3 is dispensable for sarcomere formation but enhances Z-disk assembly and the electrical and mechanical performance of mouse and human CMs.

Nx3 Is Reduced in End-Stage Failing Human Hearts and Associates With Rare Z-Disk Defects

End-stage DCM hearts show reduced FLT but unchanged Cronos expression in the LV.⁹ Whether Nx3 is similarly affected remained unclear. We therefore analyzed LV samples from >100 orthotopic heart transplant patients with end-stage DCM (Table S3) and compared them with

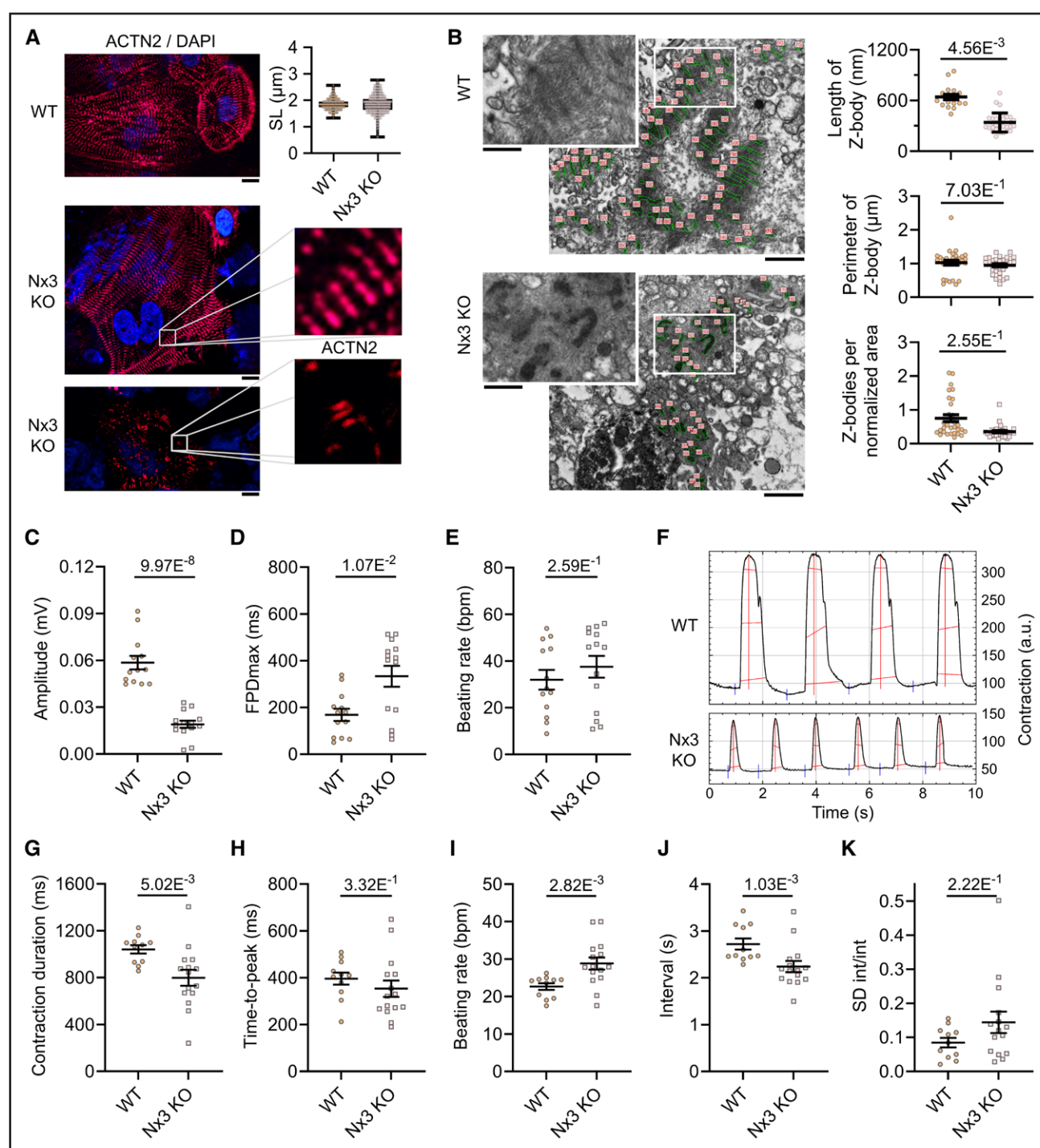


Figure 7. Nx3 (novex-3) modulates sarcomerogenesis and contractility in human induced pluripotent stem cell-cardiomyocytes (hiPSC-CMs).

A, Sarcomere length (SL) measured by confocal ACTN2 (α -actinin) staining (Cy3, red; DAPI [4',6-diamidino-2-phenylindole], blue) in wild-type (WT) and Nx3-KO hiPSC-CMs. KO cells showed unchanged median SL but greater variance due to very short sarcomeres. Box plots; $n=887$ WT and 662 KO sarcomeres; scale bars, 10 μm . **B**, Electron microscopy and quantification of Z-body length, perimeter, and density. Averaged values per differentiation: $n=17$ WT/30 KO (length) and 10 per group (perimeter/density). Linear mixed-effects model with genotype as fixed effect and differentiation as random intercept; scale bars, 2 μm (main) and 1 μm (insets). **C** through **E**, CardioExcyte 96 measurements of field potential amplitude (**C**), maximum duration (FPDmax; **D**), and beating rate (**E**); $n=13$ WT/14 KO averaged recordings; Mann-Whitney U test. **F** through **K**, High-speed motion analysis: representative traces (≈ 80 recordings; **F**), contraction duration (**G**), time-to-peak shortening (**H**), beating rate (**I**), interval duration (**J**), and interval variability (SD/interval; **K**); $n=11$ WT/15 KO averaged recordings; Welch-corrected unpaired t test (**G** through **I**) or Mann-Whitney U test (**J** and **K**). $N=3$ differentiations per group throughout. Data are mean $\pm$ SEM.

14 nonfailing donor hearts unsuitable for transplantation (Table S4). Nx3 (is21) expression was assessed by 2-phase PAGE followed by WB. Densitometry revealed a small but significant reduction in the average Nx3 content (normalized to GAPDH) in DCM versus donor hearts (Figure 8A), with no differences by sex or age (Figure S3A and S3B).

We next stratified DCM samples by the presence of a TTNtv, identified by next-generation sequencing (Table S3).⁹ TTNtv-positive patients were further subdivided into I-band (TTNtv-I) and A-band (TTNtv-A) truncations. Nx3 protein abundance was modestly but significantly reduced in TTNtv-negative (idiopathic DCM) versus donor hearts, whereas TTNtv-positive samples showed greater variability and, on average, significantly higher Nx3 expression than idiopathic DCM (Figure 8A). This increase was driven by significantly elevated, albeit heterogeneous, Nx3 protein levels in TTNtv-A but not TTNtv-I hearts (Figure 8A), possibly reflecting a compensatory response to sarcomere dysfunction caused by titin haploinsufficiency and FLT loss.

The Nx3:FLT ratio, determined from Z-disk titin (Z1/Z2) WBs, was unchanged across DCM groups and donors (Figure 8B), consistent with the concomitant reduction of both proteins in heart failure.⁹ Normalized Nx3 and FLT levels showed a weak but significant correlation (Spearman analysis) across the DCM-all cohort (Figure 8C, left), driven primarily by a mildly significant correlation within the idiopathic DCM group but not the TTNtv group (Figure 8C, right). Further correlation analyses incorporating available clinical parameters and patient metadata (Table S3) revealed a weak but significant positive correlation between left ventricular ejection fraction and Nx3 expression in the DCM cohort by Pearson (but not Spearman) analysis (Figure 8D). In TTNtv-positive patients and the TTNtv-I/-A subgroups, only nonsignificant trends toward higher left ventricular ejection fraction with increasing Nx3 were observed (Figure S3C).

At the transcript level, quantitative droplet digital polymerase chain reaction analysis of a DCM subset and donor controls showed no significant difference in Nx3:FLT mRNA ratios (Figure 8E). Notably, Nx3 accounted for $\approx 12\%$ to 14% of FLT at the transcript level but, despite considerable inter-sample variability, reached an ≈ 2 -fold higher relative abundance at the protein level (Figure 8B), suggesting that the shorter Nx3 isoform may be translated more efficiently. Reanalysis of published data sets obtained through total RNA sequencing and ribosomal sequencing from 80 human hearts (DCM, N=65; donor, N=15)³⁸ (Figure S4) supported this result: splice junction reads indicated Nx3 at 9% (RNA sequencing) versus 29% (ribosomal sequencing) of total titin (Nx3+FLT), although normalized base-level counts yielded 8% and 12%, respectively. Together, these data suggest a lower Nx3:FLT ratio at the transcript level than

the protein level, consistent with enhanced translational efficiency of Nx3.

Finally, we asked whether reduced Nx3 protein in failing DCM hearts contributes to sarcomere defects. Hearts with average, low, or very high Nx3 levels (based on the expression analysis by WB; Figure 8A) were validated by IF using the Nx3 (I23) antibody, with ACTN2 as a Z-disk control marker (Figure 8F). Confocal imaging confirmed corresponding differences in sarcomeric Nx3 signal intensity (Figure 8F, bottom). TEM of these samples showed normal sarcomeres in hearts with average or high Nx3, whereas low-Nx3 hearts exhibited rare but pronounced Z-disk abnormalities, primarily Z-disk streaming (Figure 8G). These findings suggest that substantial Nx3 loss compromises Z-disk integrity in failing human hearts and may contribute to contractile dysfunction.

DISCUSSION

This study addresses an important gap in our understanding of the multifaceted roles of titin in the heart by providing a detailed characterization of the previously neglected Nx3 isoform. We define its developmental expression profile, sarcomeric localization, protein-protein interactions, and functional relevance in healthy and diseased states. Quantitative immunoblotting reveals that Nx3 constitutes at least $\approx 20\%$ of the total titin protein pool in adult murine and human hearts. This relatively high abundance is maintained from fetal to adult stages and contrasts with the Cronos isoform, which is prominent during fetal heart development but represents only $\approx 10\%$ of FLT in the adult LV.^{9,24} Although Nx3 is dispensable for sarcomere assembly, it contributes to Z-disk stabilization in fetal and adult CMs and modulates the mechanical performance of hiPSC-CMs. Notably, we identify Nx3 at the ID, suggesting a potential role in coordinating both mechanical and electrical cardiac function. Loss of Nx3, observed in failing human hearts and recapitulated in CRISPR-Cas9-engineered mouse hearts and hiPSC-CMs, is associated with Z-disk structural abnormalities, sarcomere disorganization, and impaired contractility. These functional deficits may also reflect disrupted interactions with proteins involved in metabolic signaling.

Using microscopic imaging and biochemical interaction assays, we propose that Nx3 adopts a nonlinear configuration within the sarcomere, with its N terminus anchored at the Z-disk and its central region looping back toward the Z-disk while engaging an adjacent titin molecule. Its C terminus exists as a monomer in solution and extends toward the A-band while remaining confined to the Z-disk/I-band junction. Our data further indicate that the N-terminal region of the *Ttn*-ex48-encoded Nx3 segment associates with the Z-I junction

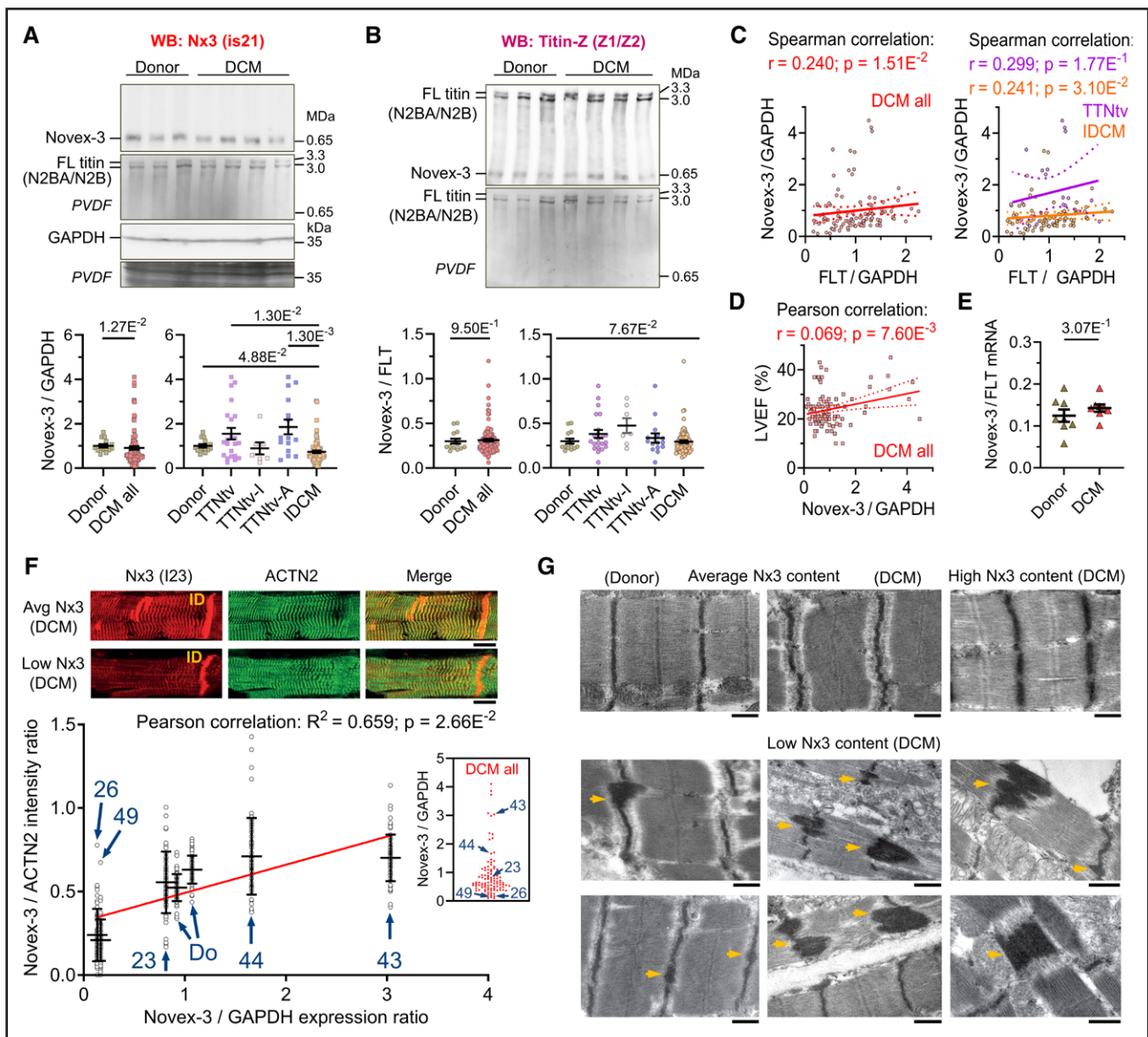


Figure 8. Nx3 (novex-3) is downregulated in end-stage dilated cardiomyopathy (DCM) left ventricles.

A, Western blot (WB) quantification of GAPDH-normalized Nx3 (is21) in nonfailing donors (N=14) and DCM hearts (N=102): TTN-truncating variant (TTNtv, N=22 (I-band, N=7; A-band, N=15), and idiopathic DCM (IDCM, N=80). **B**, Nx3/full-length (FL) titin ratio detected with Z1/Z2 in donors (N=14) and DCM hearts (N=103; TTNtv groups as in **A**; IDCM, N=81); no significant differences. **A** and **B**, Mean±SEM; Mann-Whitney *U* test (donor vs DCM) and Kruskal-Wallis test with Dunn correction (subgroups). **C**, Spearman correlation of GAPDH-normalized Nx3 with FL titin (N2BA+N2B) in all DCM (N=102; **left**), TTNtv (N=22), and IDCM (N=80; **right**) hearts. Solid and dotted curves show the fit and 95% CI. **D**, Pearson correlation of left ventricular ejection fraction with normalized Nx3 in all DCM hearts (N=102); curves as in **C**. **E**, Digital droplet polymerase chain reaction of Nx3/FLT transcript ratios in donors and DCM hearts (N=8/group); mean±SEM, unpaired *t* test. **F**, Confocal imaging of DCM hearts with high/average or low Nx3, stained for Nx3 (I23; Cy3) and ACTN2 (fluorescein isothiocyanate); scale bars, 10 μ m. **Bottom**, Nx3 intensity ratios from 60 regions/heart (mean±SD) vs WB Nx3 levels in 5 DCM and 2 representative donor (Do) hearts. The red regression line indicates a significant Pearson correlation. **G**, Representative TEM images of the hearts in **F**, showing rare Z-disk deformations in low-Nx3 DCM but normal Z-disks with average/high Nx3; scale bars, 500 nm. ID indicates intercalated disk.

of titin/Nx3 (Z7-I1 domains, encoded by *Ttn* ex27/28), a site also known to interact with actin.²⁶ Notably, this N-terminal region is predicted to form coiled-coil structures that favor dimerization,³⁹ and these motifs likely mediate binding to the Z7-I1 titin segment, which likewise contains localized coiled-coil domains. Together, this organization is poised to enhance Z-disk stability and mechanical flexibility under load.

Previous studies have suggested that Nx3 interacts with Ig domains 58 and 59 of obscurin,^{3,40} a large muscle protein implicated in heart failure.⁴¹ Accordingly, obscurin may associate with Nx3 either directly or indirectly via binding to the titin Z8-Z9 domains,⁴² which are shared between FLT and Nx3. However, we did not detect an interaction between Nx3 (I21-I23) and obscurin in Y2H assays, suggesting either the absence of a direct

interaction or a false-negative result. Obscurin has also been reported to associate with the sarcoplasmic reticulum.⁴³ Consistent with this, obscurin-knockout mice exhibit altered sarcoplasmic reticulum architecture but no sarcomeric defects,⁴⁴ indicating that obscurin is not required for sarcomere assembly and maintenance.

From an evolutionary perspective, Nx3 exhibits notable functional parallels with titin-like proteins in *Drosophila melanogaster* muscle, including the $\approx$ 500-kDa kettin isoform encoded by the *sallimus* (*Sls*) gene,⁴⁵ which stabilizes the Z-I-band region but does not associate with thick filaments.⁴⁶ In contrast, the *Drosophila* titin homologue projectin, encoded by the *bent* (*bt*) gene,⁴⁵ more closely resembles the Cronos isoform, as it associates with myosin thick filaments but not Z-disks. These analogies provide a useful framework for understanding the specialization of titin isoforms in vertebrate sarcomere organization.

Collectively, our findings suggest that Nx3 is specialized for structural stabilization at the Z-disk rather than for contributing to sarcomeric elasticity, in contrast to FLT, which functions both as a molecular spring and a ruler for thick filament assembly. However, as the most recent high-resolution cryo-electron microscopy structure of the vertebrate Z-disk does not yet include titin,⁴⁷ more complete atomic-level models will be required to definitely validate the proposed S-shaped conformation of Nx3.

Structural and functional analyses in Nx3-deficient adult mouse hearts and fetal-stage hiPSC-CMs confirm that Nx3 optimizes sarcomere organization and mechanical performance but is not essential for sarcomere assembly. Nx3-knockout hiPSC-CMs exhibit increased SL variability, compromised Z-body-to-Z-disk transition, occasional sarcomere disorganization and Z-disk loss, and impaired electrophysiological and contractile properties. These findings align with the modest cardiac dysfunction reported in the Nx3-knockout mouse model,¹¹ highlighting the role of Nx3 in fine-tuning sarcomeric structure and cardiomyocyte function. However, whereas Hashimoto et al^{10,11} reported nuclear localization of Nx3, our data support its predominant localization to the Z-disk and ID in both fetal and adult hearts. This discrepancy warrants further investigation.

Our results further implicate Nx3 in cardiomyocyte signaling through interactions with Pin1 and TBC1D4, linking it to metabolic regulation. Pin1, a phosphorylation-dependent prolyl isomerase, regulates diverse processes including insulin signaling, cardiac hypertrophy, and energy metabolism^{48–51} and also modulates the transcription factor myocyte-specific enhancer factor 2C, thereby influencing muscle differentiation and regeneration.^{52,53} This is notable given that global Nx3 knockout affects cardiac hypertrophy in adult hearts and cardiomyocyte proliferation during development, previously attributed to a nuclear role of titin.¹¹ Moreover, Pin1 interacts with

TBC1D4,³⁴ a Rab GTPase-activating protein identified here as another Nx3 binding partner. TBC1D4 plays a crucial role in glucose and fatty acid uptake,^{35–37} including in the heart. Its phosphorylation by AMPK (AMP-activated protein kinase) in response to contraction³⁶ is counter-regulated by Pin1,⁴⁹ further integrating Nx3 into this metabolic signaling network. Together, these interactions suggest that Nx3 may function as a signaling scaffold coordinating metabolic adaptation in CMs.

In end-stage failing human DCM hearts, we observed a modest but significant reduction in Nx3 protein expression (but not mRNA), alongside the previously reported loss of FLT^{9,54} and the N2BA:N2B isoform shift.^{6–8} We propose that substantial Nx3 protein loss in some failing hearts contributes to Z-disk defects and exacerbates contractile dysfunction. Alternatively, reduced Nx3 expression in certain patients may be secondary rather than causative of sarcomeric disarray. Regardless of causality, our data support the concept that Nx3 deficiency promotes sarcomeric instability and worsens mechanical and electrical dysfunction in heart failure. Clinically, the identification of Nx3 as both a structural and signaling component has several implications. Its downregulation in end-stage DCM suggests that strategies aimed at stabilizing or restoring Nx3 expression may ameliorate sarcomere dysfunction and improve cardiac performance. Future studies should address the molecular mechanisms regulating Nx3 expression, including posttranslational modifications,⁵⁵ alternative splicing,⁵ and response to mechanical stress. In addition, cardiac-specific, inducible knockout models will be essential to further define its (patho)physiological functions.

This study establishes Nx3 as a multifunctional titin isoform integral to cardiomyocyte Z-disk stability and signaling. By defining its unique structural and functional properties, we provide a framework for future investigation into titin isoform diversity and its implications for cardiac health and disease.

ARTICLE INFORMATION

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Disclosures

None.

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