Truncation of Titin’s Elastic PEVK Region Leads to Cardiomyopathy With Diastolic Dysfunction

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Rationale: The giant protein titin plays key roles in myofilament assembly and determines the passive mechanical properties of the sarcomere. The cardiac titin molecule has 2 mayor elastic elements, the N2B and the PEVK region. Both have been suggested to determine the elastic properties of the heart with loss of function data only available for the N2B region.

Objective: The purpose of this study was to investigate the contribution of titin’s proline–glutamate–valine–lysine (PEVK) region to biomechanics and growth of the heart.

Methods and Results: We removed a portion of the PEVK segment (exons 219 to 225; 282 aa) that corresponds to the PEVK element of N2B titin, the main cardiac titin isoform. Adult homozygous PEVK knockout (KO) mice developed diastolic dysfunction, as determined by pressure-volume loops, echocardiography, isolated heart experiments, and muscle mechanics. Immunoelectron microscopy revealed increased strain of the N2B element, a spring region retained in the PEVK-KO. Interestingly, the PEVK-KO mice had hypertrophied hearts with an induction of the hypertrophy and fetal gene response that includes upregulation of FHL proteins. This contrasts the cardiac atrophy phenotype with decreased FHL2 levels that result from the deletion of the N2B element.

Conclusions: Titin’s PEVK region contributes to the elastic properties of the cardiac ventricle. Our findings are consistent with a model in which strain of the N2B spring element and expression of FHL proteins trigger cardiac hypertrophy. These novel findings provide a molecular basis for the future differential therapy of isolated diastolic dysfunction versus more complex cardiomyopathies. (Circ Res. 2009;105:557-564.)

Key Words: diastole ♦ connectin ♦ hypertrophy ♦ compliance ♦ FHL

Titin is the largest protein in mammals and forms a continuous elastic filament along the myofibril (reviewed in1). Because of its enormous size, titin is a prominent target for mutations that give rise to diseases such as familial dilated cardiomyopathy and muscular dystrophy.2–3 Titin’s extensible region resides in the I-band of the sarcomere and consists of immunoglobulin (Ig)-like domains arranged in tandem, the heart specific N2B element, and the proline–glutamate–valine–lysine (PEVK) element.4 The PEVK element is thought to function as a largely unfolded polypeptide that extends at low force levels and that thereby provides an important source of elasticity at physiological sarcomere lengths.5–7 Unlike the 1-exon heart specific N2B element, the titin gene contains 112 PEVK exons that are differentially expressed between muscle types.8 Of these PEVK exons, 219 to 225 are expressed in the so-called N2B titin isoform, that constitutes the dominant cardiac isoform in the left ventricle of a wide range of species, including rodents and human.9

Here we generated a mouse deficient in titin’s exons 219 to 225 that results in a deletion of the c-terminal PEVK region (282 aa) and determined its role in cardiac function using echocardiography, in vivo pressure-volume loops, isolated heart physiology, muscle mechanics, immuno-electron microscopy, and expression analysis. We investigated the hypertrophy phenotype and studied members of the four-and-a-half LIM family involved in atrophy/hypertrophy signaling—FHL1 and FHL2.10,11 Our results reveal the strong effect of the PEVK element on diastolic function but also that the role of the PEVK extends beyond that of a mechanical spring including a novel role in hypertrophy signaling.

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Non-standard Abbreviations and Acronyms

- FHL: four and half LIM domain protein
- KO: knockout
- LV: left ventricular
- MHC: myosin heavy chain
- MV DT: mitral valve deceleration time
- PEVK: proline–glutamate–valine–lysine
- SL: sarcomere length
- WT: wild-type

Methods

Generation of Titin N2B PEVK Knockout Mice
A targeting construct was assembled to replace exons 219 to 225 with a Flp recombinase target-flanked neomycin expression cassette, which was subsequently removed using the Flp deleter stain. Details on generation and genotyping of knockout (KO) mice are provided in the Online Data Supplement (available online at http://circres.ahajournals.org).

Animals were maintained on a mixed C57Bl/6×129S6 genetic background. Sex- and age-matched animals (4 months) were used for experiments. All experiments involving animals were carried out following institutional and NIH guidelines, “Using Animals in Intramural Research.”

Echocardiography and Conductance Catheter
For data acquisition with the Acuson Sequoia C512 Echocardiography imaging system and the 15-MHz linear transducer and functional calculations we followed American Society of Echocardiography guidelines. We used a microconductance pressure catheter (ARIA SPR-719; Millar-Instruments Inc) for continuous registration of left ventricular (LV) pressure/volume loops in an open-chest model. Details on the in vivo cardiac analysis are provided in the Online Data Supplement.

Isolated Heart Experiments
We determined developed and passive pressure volume relationships using the isolated heart preparation and a single beat Frank-Starling (FS) protocol. The FS protocol was run first in normal tyrode solution, followed by adrenergic stimulation (dobutamine, 0.2 μmol/L) and β adrenergic blockade (propranolol, 0.1 μmol/L) as described previously. Pressures were converted to LV wall stress (σ) using a thick-walled spherical model: σ = P/(LVw/LVw/1.05V′ + 1)^2/3 - 1], where LVw is the weight of the LV wall. This conversion normalizes for differences in LV size and wall thickness and obtained values reflect the intrinsic stress generated by the myocardium. We determined the equilibrium volume, Vequ, defined as the volume at which passive pressure is zero. Note that this is not the volume at diastasis which we cannot measure in the isolated heart setup. This causes a systematic shift of the curve (relative to the diastatic pressure-volume relation), but maintains the relative measurement of stiffness. Thus, the diastolic properties can reliably be differentiated between genotypes.

Skinned Muscle Mechanics
The mechanical properties of isolated LV wall muscle strips were analyzed as described previously. Details are provided in the Online Data Supplement.

Histology
Hearts from wild-type (WT) and KO littermates (1 year) were sectioned across the ventricles (transverse) for histological analyses. Tissues were fixed with 10% buffered formalin and embedded in paraffin. After deparaffinization in xylene, sections were rehydrated in a series of graded alcohols, rinsed in PBS, and stained with hematoxylin and eosin.

Immunoelectron Microscopy
Immunoelectron microscopy to localize titin epitopes at different sarcomere lengths has been described previously. Primary antititin ABs used were rabbit polyclonal (UC, UN, I84, MIR). Epitope distances with respect to the middle of the Z-disc were measured from scanned negatives of electron micrographs using Scion Image software (v1.6).

Expression Analysis
Protein and RNA levels were quantified as described previously. For titin transcript analysis we used an established oligonucleotide array. Details on the expression analyses are available in the Online Data Supplement.

Statistics
For statistical analysis, GraphPad prism software was used. All results are expressed as means±SEM. An unpaired 2-tailed t test was performed to assess differences between 2 groups. The significance level was P=0.05.

An expanded Materials and Methods section can be found in the Online Data Supplement.

Results

Excision of the PEVK Element in the N2B Isoform of Cardiac Titin Results in Truncated Titin Specifically Lacking the PEVK Exons
Using homologous recombination we removed the titin exons 219 to 225 (Figure 1A). Homozygous KO mice survive to adulthood and are fertile, without any obvious abnormalities. PCR, Southern, and Western blotting, as well as transcript analysis confirmed the deletion of the PEVK region (Figure 1A and B; Online Figure I). The reading frame 3′ of exon 225 is maintained in the mutant titin as indicated by the presence of the C-terminal M-band region (Online Figure ID). The deleted exons encode 282 amino acids (188 PEVK residues and an Ig domain), representing a ∼30 kDa polypeptide or ∼1% of the total titin molecule. Although we used the highest resolution gel system that exists, this small difference is below the detection limit and, thus, WT and KO titins comigrate on these gels. Quantification of titin isoforms and the cleavage product T2 normalized to myosin heavy chain (MHC) indicates no significant change in total titin levels but reduced cleavage of KO-titin (Online Figure IIA through IIC). Thus, the cleavage site that produces T2 could be masked in KO titin or, more likely, the site is localized within the PEVK region and when the site is excised a more stable titin molecule is obtained. Consistent with our recent study, the N2BA to N2B titin isoform ratio in WT hearts is ∼0.2. In response to the loss of the PEVK exons we detected a minor but significant decrease in the N2BA to N2B titin isoform ratio (Online Figure IID). In summary, we successfully generated a KO model in which all PEVK exons of the N2B cardiac titin isoform have been deleted.

The PEVK KO Leads to Diastolic Dysfunction Accompanied by Hypertrophy
The PEVK KO shows a cardiac phenotype with hypertrophy resulting in a ∼10% increase in heart to body weight ratio (Figure 1C). Lung to body weight ratio was unchanged (WT:...
Histological analysis revealed an enlarged left ventricular cavity but normal morphology of the ventricular wall (Figure 1D). We also performed echocardiography and obtained calculated LV weights that were increased in the PEVK KO mice, further establishing the phenotype of cardiac hypertrophy (Online Table I). Both diastolic and systolic LV volumes were increased after deletion of the PEVK exons indicating chamber dilation (Online Table I and Figure 2A). Diastolic function was evaluated by Doppler imaging of mitral inflow (Online Table II) and in vivo hemodynamic measurements (Figure 2). The reduced deceleration time and increased late filling velocity indicate increased LV stiffness and diastolic dysfunction (Online Table II), consistent with the increased end-diastolic pressures and the increased slope of the end-diastolic pressure volume relation (beta) as documented by conductance catheter analysis (Figure 2B). We isolated LV cardiac myocytes and found that unlike in the KO of the N2B element,13 the slack sarcomere length of cardiac myocytes was unaffected (Figure 3A). However, the cellular dimensions were increased in the KO-mice (Figure 3B through 3D), supporting the hypertrophy phenotype seen at the level of the LV with the increase in cell length consistent with the observed chamber dilation.

**Diastolic Wall Stress Is Increased in PEVK KO Hearts**

To further characterize cardiac function we performed isolated heart experiments. In KO's, the pressure-volume relationship revealed both increased diastolic pressures and an increased Vequ (here, volume at which pressure is zero), whereas contractile function was unaffected (Figure 4). To account for differences in LV wall thickness and chamber size, we converted pressures to wall stress and found that diastolic wall stress was increased in the KO (supplement Figure S3). This indicates that the diastolic pressure increase is not attributable to altered chamber geometry but is instead an intrinsic muscle property. We also measured systolic function under baseline conditions and in the presence of dobutamine or propranolol and found no significant change in the KO (Figure 4B and 4C). Heterozygote animals displayed an intermediate phenotype (Online Figure IX). Thus, results of the isolated heart studies support the echo data and establish increased diastolic LV wall stress as a major phenotype of the PEVK KO model.

**Altered Structural and Mechanical Properties of the PEVK-Deficient Sarcomere**

To investigate the mechanism underlying altered diastolic function in PEVK KO animals, we determined the mech-
ical properties of skinned cardiac LV muscle. There was no significant difference in slack sarcomere length or maximal active tension between WT and KO (Figure 5A). The total passive tension–sarcomere length (SL) relationship was significantly steeper at SL >2.05 μm in KO as compared to WT myocardium (Figure 5B). While titin-based tension was significantly increased at all SLs >2.0 μm (Figure 5C), there was no significant change in collagen-based tension (Figure 5D). Thus, increased LV passive wall stress is attributable to increased titin-based tension. A preliminary analysis of skeletal muscle did not reveal an effect on weight or passive and active properties (Online Figure X), which is likely to reflect differences between cardiac and skeletal muscle titin isoforms (with a larger PEVK segment in skeletal muscle, the relative contribution of the 7 deleted PEVK exons is considerably smaller).

To determine how loss of the PEVK element affects the cardiac sarcomeric I-band and the extensibility of the N2B element, an important remaining source of elasticity in the KO heart, we studied the ultrastructure of PEVK-deficient sarcomeres (Figure 6). As the gross sarcomere structure is unchanged in the PEVK-KO (Figure 6A), we were able to quantify the I-band epitope distances by immuno-electron microscopy using antibodies that flank the N2B and PEVK elements, as indicated in Figure 6B. Because the excision of the PEVK region in the KO eliminates the elastic region between UC and I84, these epitopes are separated in the WT, but overlap in the KO (Figure 6C). In contrast the distance between epitopes UN and UC was increased in the KO mice, indicating an augmented contribution of the N2B element. Figure 6D shows the length of the N2B element (Uc-Un epitope distance) and that of the PEVK (Uc-I84) as a function of SL. At all sarcomere lengths, the N2B element extends to a higher degree in the KO mice. For example, at a SL of 2.3 μm, the N2B element extends ~30 nm more in the KO than in the WT, accounting thereby for nearly half of the extensibility that in the WT is provided by the PEVK (with the other half provided by the 2 tandem Ig segments). The increased extension of the remaining spring elements in the PEVK KO provides an explanation for the increased passive...
tension and ensuing diastolic dysfunction of PEVK deficient cardiomyocytes.

**Hypertrophy of PEVK-KO Cardiomyocytes Is Associated With an Increased Expression of Hypertrophy and Dedifferentiation Markers and Elevated FHL1, FHL2, and αB-Crystallin Protein Levels**

We previously described a KO model of the elastic N2B element that develops cardiac atrophy. In contrast, the PEVK KO displays cardiac hypertrophy and dilation. Because expression of FHL2, a protein linked to atrophy/hypertrophy signaling, is downregulated in the N2B KO we tested the expression of FHL-proteins in the PEVK KO. Although on loss of the PEVK-region mRNA levels were increased by only ∼10% for FHL1 and FHL2 (Online Figure IV), protein levels were more than doubled (Figure 7A and 7B). Unlike FHL1, which is not expressed at high levels in the normal myocardium,20 cardiac FHL2 protein levels were sufficient to enable subcellular localization studies by immunofluorescence (Online Figure V). The comparison between WT, N2B-KO, and PEVK-KO animals confirmed the differential FHL2 expression and indicated its proper localization even with the strongly increased FHL2 levels in the PEVK-KO (Online Figure VI). FHL2 was localized at the I-band of the sarcomere with no additional signal in the cytoplasm or nucleus.

The upregulation of both FHL1 and FHL2 protein in the PEVK-KO suggests an important role of FHL-proteins in titin-based cardiac atrophy/hypertrophy signaling further supported by the increased expression of the hypertrophy markers ANP and Mapkap2 in the KO (Online Figure VI). The heat-shock-protein αB-crystallin has also been associated with the hypertrophy response21; furthermore, it acts as a chaperone and might play a role in protecting the titin filament.22 We found αB-crystallin upregulated in the PEVK KO (Figure 7B and 7C). We also included markers of the fetal gene program in our expression analysis and found that skeletal muscle actin was upregulated, accompanied by an MHC isoform shift toward the βMHC isoform (Online Figure VII).

**Discussion**

The mouse PEVK region is encoded by 97 exons (112 in human) that correspond to ∼53-kbp genomic sequence.6 Although this virtually rules out a complete PEVK KO using conventional gene targeting, alternative splicing in the mouse heart provides a titin N2B isoform that contains the 7 C-terminal PEVK exons (N2B-PEVK), which can be excised using standard gene-targeting protocols. Thus, hearts deficient in these exons express the dominant titin N2B isoform, which is devoid of PEVK sequence and an N2BA isoform.
that constitutes <20% of total titin with a reduced number of PEVK exons. Together with the published KO of the elastic N2B element this new model allows us to compare in vivo how distinct elastic elements differentially affect cardiac function and growth.

Although the 7 C-terminal PEVK exons comprise only a modest fraction of the elastic region of cardiac titin, the KO shows a diastolic dysfunction phenotype and increased titin-based passive tension. The large effect of excising a relatively small region can be explained by earlier work\(^{23}\) that showed the PEVK as a major source of elasticity toward the upper limit of the physiological sarcomere length range (tandem Ig segments are relatively inextensible at these lengths and the N2B is the other major source). Eliminating this source of extensibility in the PEVK KO results in increased extension of the N2B element (Figure 6D), explaining the increase in titin-based passive tension that we found. This increased passive tension of cardiac KO myocytes is a likely explanation for the large increase in diastolic LV wall pressure derived from the isolated heart experiments (Figure 4). The resulting diastolic dysfunction was documented by Doppler analysis (Online Table II) with a significant reduction in deceleration time (MV DT - early rapid filling phase), and a restrictive filling pattern as indicated by both reduced deceleration time and aortic ejection time. Because MV DT has been inversely correlated with LV stiffness in both animals and humans,\(^{24,25}\) the reduction in MV DT of the PEVK KO supports our ex vivo data, which indicate a diastolic phenotype. It was recently shown that the deceleration time cannot solely be ascribed to chamber stiffening but is also affected by viscosity,\(^{26}\) and future work is needed to establish an additional role of viscosity in the reduced MV DT in PEVK KO mice. In our in vivo functional analysis we found that the end-diastolic pressure and the slope of diastolic pressure volume relationship were both significantly increased in the KO heart (Figure 2). These findings are consistent with echo, isolated heart, and skinned muscle data that all indicate increased diastolic stiffness in the PEVK KO.

The N2B cardiac titin isoform is the dominant isoform in the ventricular myocardium of the mouse, where it is coexpressed with a small amount of N2BA titin (a larger and more compliant isoform more abundantly found in the atria). The N2B titin isoform has a shorter contour length than N2BA titin, and thus the fractional extension (end-to-end length divided by contour length) for a given sarcomere stretch will be higher in N2B than N2BA cardiac titin. Because titin’s force is a function of fractional extension, force will be much higher for N2B titin than N2BA titin. In the N2B KO a minor but significant increase in expression of N2BA titin is present, which we interpret as an attempt to compensate for the increased passive stiffness that results from deletion of the N2B element.\(^{13}\) In contrast we found in the PEVK KO a reduction in the N2BA/N2B ratio, which is expected to increase passive stiffness. A possible explanation for this reduction is that the mutant N2BA isoform is more vulnerable to degradation, relative to the mutant N2B isoform, because some of its PEVK exons (outside the 219 to 225 exons) are still present in the PEVK KO. We calculated the expected increase of both the PEVK excision and the isoform switch and found a predicted force increase in the KO of ≈30%, of which ≈2% can be accounted for by the isoform switch (Online Figure VIII). Thus the major reason for the passive tension increase in the PEVK KO is the excision of the PEVK region and not a reduced N2BA/N2B expression ratio.

No significant changes were present in active tension of skinned muscle (Figure 5A) or in developed pressure of the isolated heart (Figure 4B and 4C). In contrast in vivo P-V loops and echo revealed a modest reduction in the ejection fraction in KO mice (Figure 2A and Online Table I). Our interpretation is that the change in ejection fraction is likely to be a secondary effect, possibly triggered by anesthesia, and that the PEVK KO is a primary diastolic dysfunction model with secondary changes in systolic function.

Although the loss of elastic elements in both the N2B and the PEVK KO results in increased stiffness, the net effect on slack sarcomere length is significant in the former but not in the latter. In slack sarcomeres titin corresponds to a flexible chain at zero external force where the mean square end-to-end distance is a function of the contour length of the chain. A reduction in contour length (KO of N2B or PEVK) will reduce the end-to-end distance and hence slack sarcomere length. Although this holds true for the N2B KO, the sarcomere length in the PEVK KO is unchanged, possibly because of the smaller deletion as compared to the N2B KO, reducing the effect to the point where it was undetectable in our experiments.

Hearts deficient in the elastic N2B or PEVK region do not show signs of fibrosis as determined by histology (Figure 1D) and the unchanged collagen based stiffness (Figure 5D). Both models share an increased titin based stiffness that results in diastolic dysfunction. However, their deletion differentially affects cardiac growth with atrophy in the N2B KO\(^{13}\) and hypertrophy and chamber dilation in the PEVK KO. In part, this might be explained by the differential binding of proteins that relate to hypertrophy and stress signaling such as FHL1, FHL2, and αB-crystallin, which all bind the N2B element, but not the cardiac PEVK.\(^{11,27,28}\) All these proteins were upregulated in the PEVK KO with differential implications for the phenotypic differences in the N2B and PEVK KO. αB-crystallin is a small heat shock protein that acts as a chaperone to maintain the folded state of proteins. In addition to binding to the N2B element of titin, it binds the tandem Ig segment between the N2B element and the Z-disc in highly stretched sarcomeres and possibly protects titin from structural damage under conditions of increased vulnerability.\(^{28,29}\) On elimination of PEVK exons, the strain of titin’s I-band region at a given sarcomere length is increased, and the increased expression of αB-crystallin can be interpreted as a compensatory mechanism to maintain a functional I-band region.

The unexpected hypertrophy phenotype in the PEVK-KO could result both from the loss of a PEVK-dependent signal or from a mechanosignal secondary to the increased strain of the remaining I-band titin. Recent work in both skeletal and cardiac muscle has indicated a critical role for FHL1 and FHL2 in hypertrophy signaling.\(^{10,11}\) The differential regulation of FHL proteins in the PEVK- and N2B KO could thus explain their disparate trophic phenotypes\(^{13}\) (Figure 1). Only
recently has FHL1 been implied in cardiac pathology with protection of the FHL1 KO from pressure induced hypertrophy.11 Gain of function studies have shown induction of hypertrophy by FHL1 in skeletal muscle,20 and we find a similar hypertrophy phenotype with increased cardiomyocyte diameter in the PEVK KO heart. Although future work is needed to dissect the mechanosignaling pathway, we propose that the N2B region plays a critical role in the tropic response to strain based on the data presented here and published work.11,13,27 Our localization and expression studies of FHL1 and 2 suggest a model where increased strain of the N2B region in the PEVK-KO induces structural changes in the N2B region that facilitate binding of FHL proteins and the assembly of a signaling complex to induce hypertrophy (Figure 8 and Online Figure V). The N2B-KO lacks the I-band attachment sites for FHL2 and thus the basis for signalosome assembly, resulting in atrophy. We propose that the moderate changes in fetal and hypertrophy signaling (Online Figures VI and VII) are likely to reflect structural changes in the N2B region secondary to the PEVK deficiency and that these changes are translated via FHL proteins. Altered biomechanics attributable to the excision of titin’s PEVK region is thus the primary determinant of both the diastolic and the trophic PEVK KO phenotype.

In summary, we have generated a novel PEVK KO and have shown that the PEVK region is an important source of elasticity within the physiological sarcomere length range of the heart. Its absence results in diastolic dysfunction and hypertrophy accompanied by upregulation of FHL1 and FHL2. Our findings indicate that the cardiac PEVK region is required for proper ventricular filling and the regulation of cardiac muscle mass. Insights provided by the PEVK KO should aid in the design of titin-based therapies for diastolic dysfunction and more complex cardiomyopathies.

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**Disclosures**
None.

**References**
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*Generation of Titin N2B PEVK - knockout mice (Titin N2B PEVK ^{-/-})*

A targeting construct was assembled by standard procedures from a mouse genomic BAC clone (bacterial artificial chromosome library MGS1 from mouse ES cells; Genome Systems/Incyte Genomics) spanning the 5' region of the mouse titin gene. Primers were designed from sequence information available in the Celera database and Genbank (GenBank entry AL928789). A PCR based strategy was used to replace exons 219-225 (C-terminus of the PEVK region) with a FRT flanked neomycin expression cassette. All exons within the targeting vector were sequence verified. Homologous recombination in ES cells was used to derive ES cell clones (targeting frequency ~1:250) and generate titin N2B PEVK (+/neo) animals. To remove the neomycin cassette, which has the potential to influence the phenotype of targeted animals, we expressed the Flp recombinase in the germline of neo positive animals using the Flp deleter strain. Offspring lacking the neomycin cassette were used to obtain Flp negative, homozygous N2B PEVK deficient animals (N2B PEVK ^{-/-}). The excision of the N2B PEVK- exons (219-225) results in a deletion of 4298 bp genomic DNA and corresponds to 282 aa. Splicing around the N2B PEVK region proceeds normally and maintains the open reading frame as shown using antibodies against M-band titin (Supplement Figure S1).

*Genotyping*

Homologous recombination in ES cells was monitored by PCR using the primers P1: 5'-GTGGCTCACAACCATCCGTAACAAG-3' and P2: 5'-TCGACTAGAGGATCAGCTTGGGCTG-3'. For Southern blot analysis, genomic DNA was digested with HindIII overnight and probed with a PCR product generated with primer PEVK-P forward (5'-CTCTGACTTCCTGCCTGCTTTCTC-3') and PEVK-P reverse (5'-CACCAGCCCCGAGTTTATCAGTTTTTA-3') (Fig. 1A) following standard procedures. To discriminate the wildtype (+) and knockout (-) allele in offspring from heterozygous matings, the primers P3: 5'-GCTCTTCCGAAGGTCCAGAGTTCAA-3', P4: 5'-ATCGGAATGCAAAGTTCTCAATGG, and P5: 5'-GTGTTTTCACAAAGCGCACAGGAAG-3' were used. Primer locations are indicated in Fig. 1A (P1 and P2 produce the neo fragment; P2 and P4 the wildtype (+/+)) fragment; P3 and P4 the recf fragment).

*Extensor digitorum longus (EDL) skeletal muscle mechanics*

The EDL was dissected from 4 month old male mice in an oxygenated physiological solution (in mM, 145 NaCl, 2.5 KCl, 1.0 MgSO$_4$, 1.0 CaCl$_2$$\times$2H$_2$O, 1.0 HEPES, 10 glucose, and 2.5 U/l insulin, pH 7.4 at 30°C). We attached the muscles, via sutures, to an Aurora 1200A Intact Muscle Test System. We determined the optimal twitch length (L0) by incrementally lengthening the muscle until twitch force was maximal. Passive force was measured by stretching the muscle passively (passive stretch) from L0 to 20% of L0 at a rate of 10% of L0/sec, we held the muscle at the stretched length for 60 seconds and then released it to L0. Seven minutes after the stretch, we imposed a single twitch and a single 150 Hz tetanus. Measured force in mN was divided by the cross-sectional area (mm$^2$) (area determined as EDL...
mass (mg)/(L0 (mm)*1.06)) to obtain tension (mN/mm²). Twitch and tetanus force, half-
relaxation time, and time to maximum tension were determined using Aurora’s DMA software.

**Gel electrophoresis and western blot analysis**

Protein samples were prepared from left ventricles by homogenization in liquid N₂ and lysis in 8
M urea, 50% Glycerol with DTT (80 mM final) and protease inhibitors (Leupeptin, E-64, PMSF).
FHL1, FHL2, and B crystalline were separated on 12% SDS PAGE gels, titin isoforms on
SDS-agarose gels. Proteins were stained with Coomassie or transferred to nitrocellulose (titin
only) or Hybond P PVDF membranes (GE Healthcare) for subsequent Western blot analysis.
Membranes were stained with India ink for loading control and blocked with 5% skim milk in
PBS-Tween 20 (PBS-T) followed by incubation with antibodies: anti Z1-Z2 and M8-M9 antibody
or the 9D10 antibody. Vectastain avidin/ biotin –AP complex 1:10000 was used for detection.
Commercial antibodies against FHL1 (rabbit polyclonal, Aviva Systems Biology), FHL2 (mouse
monoclonal, MBL), or B crystalline (rabbit polyclonal, Calbiochem) were used according to the
manufacturer’s instructions and detected with Horseradish peroxidase-conjugated secondary
antibodies and chemiluminescence staining using ECL (Supersignal West Pico Chemiluminescent Substrate, Pierce). Quantification followed our standard protocol.

**Immunofluorescence (IF) Staining**

Preliminary experiments were carried out on adult mouse hearts that were flash frozen in liquid
nitrogen cooled Isopentane. 12 µm sections were cut with a Cryotom (Mycrom HM560) and
fixed with 4% PFA for 15 minutes. The staining procedure including the source of the antibodies
has been described previously. In addition a primary rabbit polyclonal antibody against FHL2
was used (1:200; BL455; Bethyl). Stained tissues were analyzed on a confocal scanning laser
microscope (Leica TCS Sp2, Leica, Germany) with a HCX PL APG 63X lens. Quantitative IF
analysis of FHL2 was carried out as follows. Mouse hearts were cannulated for Langedorff
Perfusion with an oxygenated 37°C Tyrode solution. A small needle was used to guide a
custom balloon into the LV and then the heart was submerged in a temperature-controlled
chamber filled with Tyrode perfusate. The balloon was connected to a servo motor for volume
control. The balloon was inflated to a baseline volume achieving a diastolic pressure of ~5
mmHg and ventricle externally paced at 200 bpm. The LV was then set at a 30% increased
volume and the heart was fixed by changing the perfusate to Tyrode with 3% PFA for 10
minutes. Hearts were removed and cryoprotected in 25% sucrose/PBS solution, frozen in
isopentane/liquid nitrogen, and sectioned in short axis slices (10-µM thick) on a cryostat and
serially mounted on positively charged slides. Slides were washed and then blocked for 2 hours
in PBS containing 1% normal donkey serum and 2% BSA. Sections were incubated overnight
in anti-FHL2 (1:200) (MBL international Corporation), washed 3 x 5 minutes in PBS and labeled
with goat anti-mouse AlexaFluor 594 (1:500) for 45 minutes. Slides were imaged using a Zeiss
LSM 510 Meta NLO confocal microscope. Special care was taken throughout this experiment
to ensure that FHL2 intensity could be quantitatively compared between groups. Not only were
the incubation times strictly controlled, but also the confocal images were all taken at the same
intensity and focal settings. Plot profiles with projections of 10 pixels wide and at least 10
sarcomeres long were taken from captured images parallel to myofibrils using ImageJ (NIH,
Bethesda MD). Each FHL2 peak was fit with a gaussian curve in fityk (Marcin Wojdyr, GPL), the
fit area was integrated and all curves were averaged to give an intensity value per I-Band for
each image.
Titin transcript analysis

An established oligonucleotide array 4 was used to monitor the expression of all 358 exons of the mouse titin gene in N2B PEVK KO mice. Briefly, RNA was isolated from left ventricle and converted to biotinylated cDNA. A 50–mer oligonucleotide array containing all of titin’s gene exons and normalization, positive, and negative controls was used. Biotinylated target was hybridized to the array and quantified with an arrayWoRxTM Auto biochip reader (Applied Precision, LLC, Issaquah, Washington).

Real time PCR

RNA from 13 ventricles was isolated and amplified with TaqMan probes for ANP, BNP, TGFβ, αMHC, βMHC, sk actin, MEF2c, MAPKAP2, and 18S RNA (for normalization) as described previously 5. The primers used were: 18S-f 5’-CGCCGCTAGAGGTGAAATTC, 18S-r 5’-TGGCGGAATGCTTTCGCTC, 18S-probe 6-FAM-5’-TGGACGCCGCGCAAGAGGAC-TAMRA, ANP-f 5’-TTCTAGGCGCAGCCCCCT, ANP-r 5’-GCAGAGCCCTCAGTTGCTT-3’, ANP-probe 6-FAM-5’-ACCCCTCCGATAGCTGCCTTGGAA-3’-TAMRA, BNP-f 5’-AGCTGCTGGAGCTGATAAGACATGCTGAAAGGAA-3’, BNP-r 5’-GTGAGGCCTTGGTCTTCAA-3’, BNP-probe 6-FAM-5’-AGCTGCTGGAGCTGATAAGACATGCTGAAAGGAA-3’, BNP-r 5’-GTGAGGCCTTGGTCTTCAA-3’, TGFβ-f 5’-CCCCTGGCTACTGCAAGTCAG-3’, TGFβ-r 5’-GTGAGGCCTTGGTCTTCAA-3’, TGFβ-probe 6-FAM-5’-CTTCTGAGCTCCGCTTGCTC-3’-TAMRA, MEF2c-f 5’-GGCTCTGTAAGTCTGAGCCGCG-3’, MEF2c-r 5’-CTTCTGTAAGTCTGAGCCGCG-3’, MEF2c-probe 6-FAM-5’-CACCGAGCACCTAACAATGCGGCC-3’-TAMRA, MAPKAP2-f 5’-GTGAGGCTATCCCCCCTCT-3’, MAPKAP2-r 5’-TACGAGTCTTCCATGCGCCGG-3’, MAPKAP2-probe 6-FAM-5’-TACGAGTCTTCCATGCGCCGG-3’, sk-actin-f 5’-AGCTGCTGGAGCTGATAAGACATGCTGAAAGGAA-3’, sk-actin-r 5’-GCCGCCGACAGCCCGCTTGGAA-3’, sk-actin-probe 6-FAM-5’-GCCGCCGACAGCCCGCTTGGAA-3’, sk-actin probe 6-FAM-5’-GCCGCCGACAGCCCGCTTGGAA-3’, αMHC-f 5’-CCTCAGTGGAGCCGGATGATGAT-3’, αMHC-probe 6-FAM-5’-TGACCCGAGGCAAGCTCTCCTACA-3’-TAMRA, βMHC-f 5’-ATGAGCGCCCGCAATGCTGCTGCTTGGAA-3’, βMHC-r 5’-CTCAGTGGAGCCGGATGATGAT-3’, βMHC-probe 6-FAM-5’-TGACCCGAGGCAAGCTCTCCTACA-3’-TAMRA. Primer Probe Sets to amplify FHL1 and FHL2 were ordered from Applied Biosystems (Mm00515772_m1 and Mm00515781_m1).

Echocardiography

For data acquisition with the Acuson Sequoia C512 Echocardiography imaging system and the 15 MHz linear transducer and functional calculations we followed American Society of Echocardiography guidelines. In the LV parasternal long axis 4-chamber view we derived fractional shortening (%FS), ejection fraction (%EF), and ventricular dimensions. In the subcostal long axis view from the left apex we derived mitral inflow and aortic ejection profiles by Doppler imaging. An extended description of the procedure has been described previously 3.
Surgical procedures and hemodynamic measurements

The Millar Conductance Catheter System was used as has been described previously. Aged matched male PEVK KO and littermates were anesthetized with urethane in saline (1000 mg/kg, IP) and chloralose in propylene glycol (50 mg/kg, IP), ventilated, and the external jugular vein cannulated for volume administration. This anesthetic technique causes minimal cardiac and vascular depression and inhibits central nervous system catecholamine outflow that may confound data interpretation. The apical portion of the heart and the inferior vena cava were exposed through a substernal-transverse incision, and a 1.4-fr Millar Conductance Catheter (Millar Corporation) was inserted into the apex of the left ventricle. Pressure-volume loops were acquired and computed as reported previously. Systolic function was quantified by LV end-systolic pressure (LVP, mmHg) and +dP/dt (mmHg/s) as an index of LV contractility. Diastolic function was measured by LV end-diastolic pressure (LVEDP, mmHg), -dP/dt (mmHg/s) and Tau (ms). Global cardiac function was quantified by the end systolic and diastolic volume (ESV and EDV, µl), ejection fraction (EF, %), and heart rate (HR, beat/min). Furthermore, during transient vena cava occlusion the diastolic stiffness was calculated from the EDPVR with beta reflecting the slope of this relationship in mmHg/µl.

Cardiomyocyte Cell Isolation

Single cardiomyocytes were isolated from PEVK KO or wt mice via retrograde perfusion through the aorta and collagen digestion as previously described (AfCS Procedure Protocol PP00000125). Briefly, hearts were removed from the mouse, cannulated and perfused with liberase blendzyme 2 (0.08 mg/ml) to break down the extra-cellular matrix. When the hearts were flaccid, they were removed from perfusion and the left ventricle was cut into small pieces. The small pieces were triturated several times. Following trituration, the cell suspension was passed through a nylon mesh filter (300 µm pore size) to remove tissue debris. Finally, the filtered cells were skinned following a previously described method using 0.3% triton X-100. These cells were then kept at 4°C until used for experiments.

Skinned muscle mechanics

LV wall muscle strips were dissected and skinned overnight at 4°C in relaxing solution containing 1% (wt/vol) Triton X-100, followed by washing with relaxing solution. The skinned muscles were mounted to a force transducer and a high-speed motor. The cross-sectional area was measured to convert force to tension. Experiments were performed at 20 - 22°C. Sarcomere length was measured by laser diffraction, and maximal active tension (pCa 4.0) was measured at a sarcomere length of 2.0 µm. Passive tension was measured during a slow stretch (10% of slack length / s) before and after extraction of myofilaments (including titin) with high salt solutions, with the extraction resistant tension assumed to be collagen-based and the extraction sensitive tension, titin-based

Calculation of the force – SL relations.

We calculated the force—SL relations of the two titin isoforms using the wormlike chain WLC equation.
\[
\frac{F_x(PL)}{k_B T} = \frac{z}{L} + \frac{1}{4(1-z/L)^2} - \frac{1}{4}
\]  \hspace{1cm} (1)

where \( F \) is force (in pN), \( k_B \) is Boltzmann’s constant, \( T \) is absolute temperature, \( PL \) and \( L \) are the persistence and contour lengths. We serially-linked three WLCs, representing the combined tandem Ig segments, the PEVK, and N2B-Ub spring elements. We assumed a PEVK contour length (CL) of 70 nm in N2B titin and 300 nm in N2BA titin; CL of tandem Ig segments were set at 200 nm in N2B titin and 300 nm in N2BA titin; CL of N2B Us was 200 nm for both isoforms. The assumed persistence lengths (PL) were 1.3 nm, 12 nm and 0.65 nm, respectively. We then calculated the force-SL relation of a single titin molecule and compared results for +/+ N2B titin and +/+ N2BA titin, -/- N2B and -/- N2BA titin and the average force-SL relation for sarcomeres that coexpress N2B and N2BA titins. For additional details on the calculation see 11.
References


Fig. S1: Verification of PEVK knockout mice

A) Genotyping results were verified by Southern blotting. The location of the probe and the HindIII restriction sites are indicated in Figure 1A).

B) Left ventricular transcripts analysis by an oligonucleotide array representing all 358 exons of the mouse titin gene. Exon 5 is an example of a constitutively expressed exon and 5MM indicates a 50-mer from exon 5 including a 5-bp mismatch as a control for hybridization stringency. Only exons 219 to 225 show a significant difference between WT and KO (n=4 in +/- or +/+).

C) Quantification of the KO-PEVK exons. The background signal in KO animals is 20-40% of the wildtype signal.

D) Western blotting with primary antibodies against N-terminus (left), PEVK region (middle) and C-terminus (right) of titin demonstrates that in KO mice, the PEVK region is deleted, and that the N- and C-termini are unaffected.
Fig. S2: Titin isoform expression. A) Agarose protein gels of WT and KO left ventricles reveal the N2BA and N2B titin isoforms, the proteolytic fragment (T2), and MHC. Quantification of titin expression normalized to MHC indicates unaltered total titin (B) but reduced levels of T2 (C) and reduced N2BA to N2B isoform ratio in the N2B-PEVK KO (D). (n=6)
**Fig. S3:** Increased diastolic wall stress ($\sigma$) in N2B PEVK KO hearts. A) Example of $\sigma$ - $V$ relation in WT (left) and KO (right). Dotted line: $V_{equ}$; open circles: developed wall stress; closed squares: diastolic wall stress. B) Diastolic wall stress at $V_{equ} +25\%$ (baseline, presence of dobutamine and presence of propranolol) was increased in KO mice ($p <0.01$). Systolic function as determined by developed wall stress at $V_{equ}$ (C ) and at $V_{equ}+25\%$ (D) is not significantly different between WT and KO animals under baseline, dobutamine, and propranolol conditions. (n=8 per genotype)
Fig. S4: RNA expression analysis of the titin binding FHL-Proteins. FHL1 and FHL2 mRNA levels were upregulated by 10 and 6%, respectively. (n=13)
Fig. S5: Subcellular localization of FHL2 is unaffected by differential protein expression between titin deficient animals. A) FHL2 (green) localizes at the I-band close to α-actinin (red) which labels the Z-disc. Nuclei are labeled with DAPI (blue). The increased or decreased FHL2 levels are contained at the I-band. While expression of FHL2 is greatly reduced in the N2B-knockout and increased in the PEVK knockout, the protein is contained at the I-band. No increased cytoplasmic or nuclear staining was detected in either genotype. B) Quantification of the FHL2 protein signal associated with the I-band documents a >75% increase (P<0.005) in the PEVK KO. (Scale bar, 10μm)
Fig. S6: Hypertrophic gene response in PEVK KO hearts. Mean values of hypertrophy markers ANP (A), BNP (B) and MAPKAP2 (C) were increased in the N2B PEVK KO mice (with ANP and MAPKAP reaching significance at p< 0.05) while TGFβ was unchanged (D). Expression data were normalized to 18S (n=6 for WT and 7 for KO).
**Fig. S7:** Fetal gene program in PEVK KO hearts. A, B) Differences in MHC isoform expression are consistent with induction of the fetal gene program (significantly reduced expression of αMHC and significantly increased expression of βMHC). C) Skeletal muscle actin is significantly upregulated in the PEVK KO, while MEF2C was unchanged (D). Expression data were normalized to 18S (n=6 for WT and 7 for KO).
Fig. S8: Predicted effect of titin isoform switch on passive force. The force SL relations of single N2B (red) and N2BA molecules (blue) were calculated by using a serially-linked wormlike-chain model assuming that in the KO (dashed lines, -/-) the whole PEVK was absent in N2B titin and that the PEVK CL of N2BA titin was reduced from 300 nm to 230 nm. Wildtype force- SL relations (+/+) are plotted as solid lines. We calculated for WT sarcomeres the average force per titin molecule (purple) assuming that the WT N2BA/WT N2B ratio is 0.25 (as per Fig. S2D) and that in KO sarcomeres the KO N2BA/ KO N2B ratio is 0.17 (S2D). The predicted force is on average 30.1 % higher in KO sarcomeres than in WT sarcomeres. In absence of an isoform switch the increase is 28.3%. Thus the effect of the isoform switch is small and accounts for only 1.8% of the force increase. The majority of the force increase is due to the excision of the PEVK from N2B titin.
**Fig. S9:** Intermediate cardiac phenotype in heterozygous animals. A) Ventricular weight to body weight ratio (VW/BW) is significantly changed in the homozygous mice and is intermediate in the heterozygous mice (n=18). B) Diastolic and developed wall stress (σ) obtained in isolated heart experiments is increased in heterozygotes and further increased in KO hearts. Only differences in diastolic wall stress reached statistical significance (n=8 per genotype). C) Quantification of Western blot data (normalized to actin) shows the intermediate phenotype in heterozygotes for FHL2 and αB-crystallin (n=4-6 per group). * = Significant with p<0.05; **= p<0.01 in ANOVA with post Hoc Tukey test.
Fig. S10: Loss of the N2B-PEVK has no major effect on skeletal muscle. A) The preliminary analysis of skeletal muscle did not show a significant effect on the weight (extensor digitorum longus weight per body weight EDLW/BW). B) Passive stress analysis at 20% stretch did not show a significant change in KO vs. WT EDL. C) Neither twitch nor tetanic response were significantly altered in skeletal muscle upon loss of the N2B-PEVK region. (n=5-6 per group).
Table S1. Echocardiography in WT vs. PEVK-KO mice

<table>
<thead>
<tr>
<th></th>
<th>IVS,d [mm]</th>
<th>LVID,d [mm]</th>
<th>LVID,s [mm]</th>
<th>Vol,d [µl]</th>
<th>Vol,s [µl]</th>
<th>Str Vol [µl]</th>
<th>LVEF [%]</th>
<th>LVWc/BW [mg/g]</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>0.78 ± 0.02</td>
<td>3.28 ± 0.20</td>
<td>2.0 ± 0.24</td>
<td>44.8 ± 7.4</td>
<td>15.3 ± 4.1</td>
<td>29.5 ± 3.9</td>
<td>67.3 ± 5.8</td>
<td>15.3 ± 4.1</td>
</tr>
<tr>
<td>KO</td>
<td>0.90 ± 0.08</td>
<td>3.84 ± 0.19*</td>
<td>2.9 ± 0.23*</td>
<td>64.8 ± 7.3*</td>
<td>36.5 ± 5.6*</td>
<td>28.3 ± 2.4</td>
<td>45.7 ± 4.7*</td>
<td>36.5 ± 5.6*</td>
</tr>
</tbody>
</table>

Continuous variables are described as mean ± SD.

IVS,d = interventricular septum in diastole; LVID,d left ventricular inner-distance diastole; LVID,s = left ventricular inner-distance systole; Vol,d = chamber volume in diastole; Vol,s = chamber volume in systole; Str Vol = stroke volume; LVEF = left ventricular ejection fraction; LVWc/BW, calculated left ventricular weight per body weight (n= 6) * P <0.05 versus PEVK+/+; ** P <0.01 versus PEVK+/+
Table S2. Doppler of WT vs. PEVK-KO mice

<table>
<thead>
<tr>
<th></th>
<th>MV DT [ms]</th>
<th>MV E [cm/s]</th>
<th>MV A [cm/s]</th>
<th>MV E/A</th>
<th>IVRT [ms]</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>27.8 ± 2.0</td>
<td>51.7 ± 8.2</td>
<td>31.4 ± 3.8</td>
<td>1.6 ± 0.1</td>
<td>19.3 ± 2.4</td>
</tr>
<tr>
<td>KO</td>
<td>21.0 ± 2.7*</td>
<td>52.6 ± 1.1</td>
<td>52.1 ± 7.4*</td>
<td>1.3 ± 0.2</td>
<td>17.3 ± 2.1</td>
</tr>
</tbody>
</table>

Continuous variables are described as mean ± SD.

MV DT = mitral valve deceleration time; MV E = early diastolic filling wave of mitral flow; MV A = late diastolic filling wave of mitral flow; MV E/A = early to late diastolic peak velocity ratio; IVRT = left ventricular isovolumic relaxation time; (n=6) * P <0.05 versus PEVK+/+