Shortening of the Elastic Tandem Immunoglobulin Segment of Titin Leads to Diastolic Dysfunction

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Background—Diastolic dysfunction is a poorly understood but clinically pervasive syndrome that is characterized by increased diastolic stiffness. Titin is the main determinant of cellular passive stiffness. However, the physiological role that the tandem immunoglobulin (Ig) segment of titin plays in stiffness generation and whether shortening this segment is sufficient to cause diastolic dysfunction need to be established.

Methods and Results—We generated a mouse model in which 9 Ig-like domains (Ig3–Ig11) were deleted from the proximal tandem Ig segment of the spring region of titin (IG KO). Exon microarray analysis revealed no adaptations in titin splicing, whereas novel phospho-specific antibodies did not detect changes in titin phosphorylation. Passive myocyte stiffness was increased in the IG KO, and immunoelectron microscopy revealed increased extension of the remaining titin spring segments as the sole likely underlying mechanism. Diastolic stiffness was increased at the tissue and organ levels, with no consistent changes in extracellular matrix composition or extracellular matrix–based passive stiffness, supporting a titin-based mechanism for in vivo diastolic dysfunction. Additionally, IG KO mice have a reduced exercise tolerance, a phenotype often associated with diastolic dysfunction.

Conclusions—Increased titin-based passive stiffness is sufficient to cause diastolic dysfunction with exercise intolerance. (Circulation. 2013;128:19-28.)

Key Words: elasticity ■ exercise ■ extracellular matrix ■ hypertrophy ■ vascular stiffness

Although much research has been focused on left ventricular (LV) systolic function, understanding normal and pathological diastolic function also is of great clinical significance.1-4 It has been hypothesized that the giant myoflament titin plays an important role in diastolic function.1,3,5,6 Titin spans from the Z disk to the M band of the sarcomere and has an extensible I-band region that functions as a molecular spring that largely defines cardiomyocyte passive stiffness.7 Alteration in titin isoform expression is a mechanism that changes the extensibility of the titin I band and modulates the passive stiffness of titin in health8-10 and disease.11,12 The extensible I-band region of titin comprises the N2B and PEVK segments, along with proximal and distal tandem immunoglobulin (Ig) segments composed of serially linked Ig-like domains.13 Mouse models absent of either the N2B or PEVK segment have previously been created and show increased passive stiffness.14,15 However, even though the extension of the tandem Ig segment dominates the elasticity of titin at physiological sarcomere lengths (SL),1,16 its in vivo physiological roles have not been addressed. Hence, we made a genetic model that has a shortened tandem Ig segment and evaluated how this alters diastolic function of the heart. Unlike the N2B and PEVK segments, the tandem Ig segment that was removed has no known phosphorylation sites in cardiac muscle.17-20 Thus, shortening of the tandem Ig segment is expected to give rise to a pure model of mechanical stiffness increase that will make it possible to test the effect of an increase in titin-based stiffness on diastolic function.

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The mouse model is deficient in titin exons 30 through 38, which deletes 9 of the 15 Ig domains in titin (Ig3–Ig11) from the proximal I-band segment (Figure 1A), a model that is referred to as the IG KO. The IG KO model can be viewed as a mechanical analog of the increased titin-based stiffness that is known to occur in patients with heart failure with preserved ejection fraction (HFpEF)21 and thus might be beneficial in elucidating disease mechanisms in HFpEF. We studied passive stiffness over a wide range of increasing...
physiological complexity, including the cardiomyocyte, muscle, and the ex vivo and in vivo LV chamber, and we assessed additional adaptations in titin, other sarcomeric proteins, and the extracellular matrix (ECM). Because of the association with HFpEF, we also evaluated whether stiffer titin altered cardiac hypertrophy by evaluating trophicity and hypertrophic signaling and whether stiffer titin reduced exercise tolerance using treadmill and volunteer running wheel exercise.

### Methods

An expanded Methods section is available in the online-only Data Supplement.

#### Generation of Mice Expressing Shorter Titin Ig Segment

A targeting construct was assembled to replace titin exons 30 through 38 (encoding Ig3–Ig11) with a floxed neomycin expression cassette, which was subsequently removed (Figure IA in the online-only Data Supplement). Mice were bred on a C57BL/6 background for 8 generations, and only male mice were studied. Animal experiments were approved by the University of Arizona Institutional Animal Care and Use Committee and followed the US National Institutes of Health *Using Animals in Intramural Research* guidelines for animal use.

#### Protein Expression, Phosphorylation, and Gene Expression

Titin and sarcomeric protein expression analysis was performed with standard SDS-PAGE methods. Phosphorylation was studied with ProQ diamond staining and phospho-specific antibodies. Quantitative real-time polymerase chain reaction was used to study gene expression.

#### Immunoelectron Microscopy and Histology

Immunoelectron microscopy was used to measure titin segment extension. Picrosirius red staining was used to measure the collagen volume fraction in LV cross sections.

#### Cardiac Mechanics

Using previously published techniques, we measured passive stress-SL relationships in skinned LV cardiomyocytes and muscle strips. LV diastolic wall stress-volume and wall stress-SL relationships were determined via an isolated heart method.

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**Figure 1.** Basic characterization of the immunoglobulin knockout (IG KO) mouse model. A, Location of immunoglobulin (Ig) 3 to Ig11 (deleted in the IG KO) in the spring region of titin (Ig domains are indicated by the rectangular red structures). B, Polymerase chain reaction products showing differential gene expression from wild-type (WT), heterozygous (HET), and homozygous IG KO mice. C, Titin exon microarray analysis shows that titin exon expression changes only in the 9 deleted exons. D, Titin protein analysis (1% agarose gel). Top, The shortened titin (IG KO) has a higher mobility compared with the WT titin bands, and a doublet can be seen in the HET mice. Bottom, Quantitative analysis shows that titin isoform expression is unchanged (n=6). E, Titin phosphorylation is unchanged in the IG KO. Top, Protein kinase (PK) A back-phosphorylation and phospho-specific pS26 and pS170 antibodies to PKC Western blotting examples. Bottom, Quantification showing unchanged phosphorylation levels in the IG KO (n=4). Box denotes 25th and 75th percentiles; whiskers show minimum and maximum. See Figure VII in the online-only Data Supplement for dot plots. AR indicates autoradiography; CB, Coomassie blue; MHC, myosin heavy chain; and Ponceau S.
**In Vivo Characterization**

In vivo pressure-volume measurements were obtained with an admittance-based system in anesthetized mice. Exercise tolerance was evaluated with the use of both treadmill and voluntary running wheel tests.

**Statistics**

The Mann–Whitney (Wilcoxon rank-sum) test was used to assess differences between genotypes. Results are shown as means±SEM, with values of *P*<0.05 taken as significant.

**Results**

**Generation of a Mouse Model With a Shortened Proximal Tandem Ig Segment (IG KO)**

Exons 30 through 38 were constitutively deleted from the mouse titin gene and replaced with a neomycin-resistant cassette that was subsequently removed (Figure IA in the online-only Data Supplement). These exons code for 9 Ig-like domains (Ig3–Ig11) that are part of the proximal tandem Ig segment (Figure 1A). Homozygous IG KO mice are fertile and survive to old age. Our custom titin exon microarray further validated that a loss of expression of exons 30 through 38 occurred without adaptive changes in splicing elsewhere in titin (Figure 1C). We studied whether shortening of the proximal Ig segment affects titin protein expression and posttranslational modifications on titin. The deleted 9 Ig-like domains comprise 783 amino acids, representing an 88-kDa polypeptide. With the use of a 1% agarose gel electrophoresis system, the mutant titin is resolved as a slightly higher-mobility band compared with wild-type (WT) titin (Figure 1D). Changes in isoform splicing and phosphorylation can also modulate post-translational modifications on titin, and we evaluated whether such compensatory changes occurred in the IG KO mice. Quantification of titin isoform expression (N2BA:N2B ratio), titin cleavage product (T2), and ratio of total titin to myosin heavy chain indicates no changes in titin expression in the IG KO mice at the studied ages (3–12 months; Figure 1D, bottom, for results from 3-month-old mice). Protein kinase (PK) A back-phosphorylation assays and Western blots using antibodies to the PKC phosphorylation sites in the PEVK region (Figure 1E), along with ProQ Diamond staining, revealed no change in posttranslational modifications of titin (Figure 1B and IC in the online-only Data Supplement). Similarly, quantification of myosin isoform ratios, along with thin and thick filament protein expression (cMyBP-C, desmin, actin, troponin T, tropomyosin, troponin I, MLC2v) and phosphorylation, revealed no changes (Figure 1B and IC in the online-only Data Supplement). In summary, we made an IG KO mouse that expresses a shortened proximal tandem Ig segment with only 6 of the 15 proximal Ig domains in WT titin without changes elsewhere in the titin molecule or in other any of the other sarcomeric proteins analyzed.

**Cellular Stiffness**

To investigate the effect of shortening the proximal tandem Ig segment on passive stiffness, we performed experiments on LV skinned cardiac myocytes. Stretch-hold-release protocols on passive skinned cardiomyocytes (Figure 2A, inset, shows representative results) revealed that peak and steady-state stress are increased in the IG KO by 60% and 65%, respectively (Figure 2B and 2C). Passive stress measured during the stretch was converted to stiffness (slope of stress-SL relationship); stiffness was increased in the IG KO at all SLs (Figure 2D). The increase was 26±21% at 2.0 μm and 95±22% at 2.3 μm (average increase in the SL range of 2.0–2.3 μm, 63%). We also performed a dynamic stiffness analysis using small-amplitude sinusoidal length oscillations to determine the elastic and viscous moduli at multiple SL (see Methods in the online-only Data Supplement for details). The elastic moduli were several-fold larger than the viscous moduli in both genotypes (Figure 2E and 2F). The elastic moduli were higher in the IG KO cells than in WT cells, and the mean value across the frequency range that we probed was increased >85% at each of the 4 SLs studied (Figure 2E). In contrast, the viscous moduli were increased 50% at short SLs but >85% at SLs >2.2 μm (Figure 2F). These myocyte data indicate that shortening the proximal tandem Ig segment of titin greatly increases cellular passive stiffness mainly as a result of increases in elastic stiffness.

**Immunoelectron Microscopy**

To examine the effect of reducing the length of the proximal tandem Ig segment on the extensibility of the remaining I-band segments, we performed immunoelectron microscopy. The antibodies used to demarcate the ends of the spring segments are shown in Figure 3A, along with a schematic of the titin I-band region. Examples of labeled sarcomeres are in Figure 3B, and scattergrams of the distances from the mid Z disk to epitopes are shown as a function of SL in Figure 3C and 3D. A large decrease in the end-to-end length of the proximal tandem Ig segment length in the IG KO across the measured SL range is readily apparent. At an SL of 2.3 μm, extension of the proximal tandem Ig segment was reduced by 60% in the IG KO; the remaining I-band segments extended to a higher degree. Extension of the PEVK and N2B segments increased, with the largest increase in the N2B segment (Figure 3E and 3F). The difference between the N2B and 2C). Passive stress measured during the stretch was converted to stiffness (slope of stress-SL relationship); stiffness was increased in the IG KO at all SLs at 2.0 μm and 95±22% at 2.3 μm (average increase in the SL range of 2.0–2.3 μm, 63%). We also performed a dynamic stiffness analysis using small-amplitude sinusoidal length oscillations to determine the elastic and viscous moduli at multiple SL (see Methods in the online-only Data Supplement for details). The elastic moduli were several-fold larger than the viscous moduli in both genotypes (Figure 2E and 2F). The elastic moduli were higher in the IG KO cells than in WT cells, and the mean value across the frequency range that we probed was increased >85% at each of the 4 SLs studied (Figure 2E). In contrast, the viscous moduli were increased 50% at short SLs but >85% at SLs >2.2 μm (Figure 2F). These myocyte data indicate that shortening the proximal tandem Ig segment of titin greatly increases cellular passive stiffness mainly as a result of increases in elastic stiffness.

**Integrative Physiology and Diastolic Dysfunction**

Myocardial passive stiffness is determined by titin and the ECM. To study whether the extracellular stiffness adapts in response to the increased cellular passive stiffness of the IG KO, we studied skinned myocardial muscle. We...
Titin-based stiffness of skinned myocardium, calculated from the extraction sensitive stress, was significantly increased in the IG KO tissues at SLs of ≥2.05 μm (data not shown), similar to the increase measured in cells. The ECM-based stress was not significantly different in the IG KO (Figure 4A). Importantly, ECM-based stiffness, calculated from the extraction-insensitive stress-SL relation, was not different in the IG KO compared with the WT (Figure 4B). Quantitative real-time polymerase chain reaction revealed a significant decrease in the expressions of collagen Iα1 and IIIα1 at 3 months, which normalized at 12 months. 

Dynamic stiffness analysis using small-amplitude sinusoidal length oscillations to determine elastic (E) and viscous (F) moduli of the myocytes. Elastic stiffness is increased at all SLs >2.0 μm, but viscous properties are increased only at SLs ≥2.2 μm (n=6). Box denotes 25th and 75th percentiles; whiskers show minimum and maximum. See Figure VIII in the online-only Data Supplement for dot plots. *P<0.05; **P<0.01.

Figure 2. Cardiomyocyte mechanical properties in relaxing solution. A, Passive tension is higher in the immunoglobulin knockout (IG KO) cell (n=5 for wild-type [WT] and IG KO). Inset, Representative stretch-hold-release experiment. From these experiments, both peak stress (B) and steady-state stress (C) are increased in the IG KO cells. D, Passive stiffness measured during the ramp stretch is also increased in the IG KO mouse at sarcomere lengths (SLs) >2.0 μm.

Figure 3. Titin I-band segment extension. A, Antibodies were used to demarcate the tandem immunoglobulin (Ig), N2B, and PEVK segments of titin, and (B) labeled skinned fibers from 6 wild-type (WT) and immunoglobulin knockout (IG KO) cardiac sarcomeres were studied using immunoelectron microscopy. Distances from the Z disk to epitope were calculated for WT (C) and KO (D) sarcomeres across a range of sarcomere lengths (SLs). Black lines indicate the lengths of N2B and proximal (Prox) Ig segments. E, Segment extension estimated via monoexponential fits indicates that the proximal Ig segment is shortened by nearly 30 nm at 2.3 μm and that the extension of the remaining I-band segments is increased with N2B segment extension dominating (F).
months (Figure II A–II C in the online-only Data Supplement). Expressions of matrix metalloproteinases, tissue inhibitors of metalloproteinase, and lysyl oxidase were unchanged (except for a decrease in tissue inhibitor of metalloproteinase-2 at 3 months), suggestive of similar rates of ECM turnover and crosslinking (Figure IID–IIH in the online-only Data Supplement). Picrosirius red staining of hearts suggested no differences in the collagen volume fraction (Figure 4C and part I in Figure II in the online-only Data Supplement). Thus, the ECM is unlikely to change the stiffness in IG KO cardiac muscle compared with WT, and only titin-based stiffness is increased.

To examine whether the increased titin stiffness manifests itself in the intact LV, we used an ex vivo isolated heart system to determine the diastolic stress-SL relationship of the LV.14–16 The baseline function of the WT and IG KO hearts was similar, including their developed wall stress and relaxation parameters (Table I in the online-only Data Supplement). The diastolic stress-volume relationship calculated with a spherical LV model showed an increase in the IG KO (Figure II IC–II ID in the online-only Data Supplement). To exclude possible differences in SL, we determined the diastolic SL of circumferentially aligned midmyocardial fibers of hearts chemically fixed at known volumes.16 The SL-volume relationship obtained (Figure II IE in the online-only Data Supplement) was used to convert the diastolic stress-volume relations into diastolic stress-SL relations (Figure 4D), and a significant 78% increase in stiffness derived from the diastolic stress-SL relationship was revealed (Figure 4E). Finally, we also performed an in-vivo pressure-volume analysis with inferior vena cava occlusion to determine diastolic stiffness (see Methods for details and Table I in the online-only Data Supplement for baseline functional measurements). The end-diastolic wall stress-volume relationship was significantly increased by 36% in the IG KO mice (Figure 4F and Table I in the online-only Data Supplement). Thus, IG KO mice have increased diastolic stiffness from the cell to the in vivo LV chamber levels.

Exercise intolerance is considered a global manifestation of diastolic dysfunction. Accordingly, we investigated exercise tolerance in 3-month-old WT and IG KO mice. Running mice on a treadmill at progressively higher speeds (see Methods for details) revealed a ≈24% reduced exercise tolerance in IG KO mice (Figure 5A). Similarly, using a voluntary running wheel exercise test, we found that IG KO mice had a 33% decrease in distance obtained compared with WT (Figure 5B).

Cardiac Trophicity
Diastolic dysfunction has been linked to changes in LV trophicity.1 Therefore, we determined the LV weight (LVW; in milligrams) and the ratio of LVW to tibia length (in millimeters) in neonatal mice (5 days old), young adult mice (3 months old), and middle-aged mice (12 months old; Table II in the online-only Data Supplement). We found smaller ratios of LVW to tibia length in the IG KO mice at 5 days, no difference at 3 months, but a significantly larger value in IG KO mice at 12 months (Figure 6A). These results indicate that the IG KO mice have alterations in trophicity compared with WT and exhibit age-dependent cardiac hypertrophy.

We next studied the expression of cardiac failure and hypertrophic markers in 3- and 12-month-old IG KO and WT mice. Although gene products for atrial natriuretic peptide, brain natriuretic peptide, mitogen-activated protein kinase-activated protein kinase 2, skeletal actin, α-myosin heavy chain, and β-myosin heavy chain are expected to increase in failure or hypertrophy,14 we found no change in most markers, with significant decreases in skeletal actin at 3 months.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Integrative physiology and diastolic dysfunction at 3 months. A, Extracellular matrix (ECM)–based stress–sarcomere length relationships are unchanged in the immunoglobulin knockout (IG KO; n=8, wild type [WT], ○; n=10, IG KO, ■). ECM-based stiffness (B) and collagen volume fraction by Picrosirius red staining (n=6; C) are not changed. Ex vivo isolated heart data provide wall stress–sarcomere length relationships (D) that show increased stiffness in the IG KO at physiological sarcomere lengths (2.0–2.3 μm; n=12). E. In vivo end-diastolic wall stress-volume relationship (EDSVR) exhibits a 37% increase in stiffness (n=10; F). Horizontal lines denote means±SEM, *P<0.05.
and atrial natriuretic peptide at 3 months (Figure IV in the online-only Data Supplement). Considering that several of the binding proteins of titin have been linked to hypertrophy signaling, we measured the expression of titin-binding proteins. Proteins that bind to the M-line and Z-disk regions of titin showed no changes between WT and IG KO hearts (Figure VA in the online-only Data Supplement). Four-and-half LIM protein (FHL) 1, FHL2, and cardiac ankyrin repeat protein (CARP), proteins that interact with the titin extensible I-band region, had several-fold increased expression levels in the IG KO at both 3 and 12 months, but only FHL1 increased continuously with age (Figure 6B and 6C). Because FHL2 was recently implicated in the suppression of hypertrophy, we also investigated the calcineurin/nuclear factor of activated T cell pathway, which might be a downstream target; however, we found no clear correlation between these markers and the hypertrophy observed (Figure VB–VF in the online-only Data Supplement).

**Age-Related Diastolic Dysfunction**

Because diastolic dysfunction is observed disproportionately in elderly patients, we also evaluated exercise tolerance in 12-month-old WT and IG KO mice using voluntary wheel running. IG KO mice showed a 48% decrease in running distance (Figure 5C), much larger than that in the young mice (above), suggesting that increasing age exacerbates exercise intolerance. Echocardiography in these mice revealed a significant increase in left atrial diameter in IG KO mice that was consistent with postmortem increases in left atrial mass, as well as a trend (P=0.14) to shorter E-wave deceleration time (Figure 7A–7C and Table III in the online-only Data Supplement). Pressure-volume studies revealed normal systolic function but significant increases in the end-diastolic pressure-volume and end-diastolic stress-volume relationships in KO mice, consistent with a stiffer LV chamber (Figure 7E and 7F and Table III in the online-only Data Supplement).

**Discussion**

Diastolic dysfunction occurs in ≈50% of heart failure cases and is characterized by increased stiffening of the LV despite seemingly normal systolic function. This study focused on a novel titin model that has a shortened proximal tandem Ig segment obtained by deleting domains Ig3 through Ig11 (IG KO). Studies from myocytes to the LV chamber levels showed that the primary phenotype of the model is increased diastolic stiffness. Titin exon expression analysis and protein studies did not detect compensatory changes in isoform expression or phosphorylation of titin. Immunoelectron microscopy revealed that the titin spring segments that remain in the IG KO extend to a higher degree than in WT mice, providing an explanation for the stiffness increase in IG KO mice. IG KO mice exhibited age-dependent LV hypertrophy,
and importantly, IG KO mice exhibited exercise intolerance and diastolic dysfunction that were exacerbated with age, phenotypes that are associated with diastolic dysfunction in patients.

### Passive Stiffness of Cardiac Myocytes

The IG KO model was made to study the role of titin-based stiffness in diastolic function and to establish whether an increase in titin-based stiffness per se is sufficient to cause diastolic dysfunction. We targeted the tandem Ig segment for deletion because it dominates the extensibility of titin in the physiological SL range. This is different from the 2 existing spring segment deletion models: the N2B KO, in which the N2B segment has been deleted, and the PEVK KO, in which the full PEVK segment of the N2B cardiac titin isoform has been deleted. Both segments dominate the extensibility of titin toward the upper limit of the physiological SL range and beyond. The earlier models also eliminate known phosphorylation sites that alter passive stiffness, PKA, PKG, extracellular regulated kinase 2, and Ca(2+)/calmodulin-dependent protein kinase II. Phosphorylation sites are deleted in the N2B KO, and PKCα and Ca(2+)/calmodulin-dependent protein kinase II sites are deleted in the PEVK KO, but no known phosphorylation sites have been detected in these Ig segments in cardiac tissue. Additionally, changes in titin isoform expression take place in the N2B KO and PEVK KO models with increased expression of the compliant N2BA isoform in the N2B KO (which compensates for the stiffness increase caused by the N2B segment deletion), and an opposite expression change occurs in the PEVK KO (which exacerbates the passive stiffness increase that is attributable to the PEVK deletion). In contrast, titin isoform expression is not altered in the IG KO (Figure 1D). Although it is unclear what underlies these differences between the models, it seems unlikely that the titin-based stiffness increase drives changes in isoform expression; given that all models have increased stiffness, a change in titin expression in the IG KO would also be expected. It is fortuitous that changes in phosphorylation and isoform splicing are absent in the IG KO, making it possible to attribute the phenotypes directly to the shortened titin.

Passive stiffness of skinned cardiac myocytes at physiological SLs (≈1.9–2.3 μm) is known to be primarily titin based; therefore, the increased passive stiffness in the IG KO (Figure 2D) is likely attributable to titin. The only difference in titin that was found via immunoelectron microscopy in the IG KO (in addition to the absence of Ig3–Ig11) is the higher extension of the distal tandem Ig, N2B, and PEVK spring segments (Figure 3E and 3F). Whether this increased extension explains the measured stiffness increase can be assessed from a worm-like chain model with tandem Ig, the N2B, and PEVK segments represented by a distinct worm-like chain with contour length and persistence length determined from single-molecule experiments, the force-SL relation can be calculated for WT and IG KO molecules. Calculations show that the shortened tandem Ig segment and the increased strain of the remaining spring segments in the IG KO result in higher forces in the IG KO molecule than in the WT molecule (Figure VI in the online-only Data Supplement), and the degree of increase is similar to that measured. Thus, the passive stiffness increase in IG KO myocytes can be explained by increased extension of the spring segments of titin.

Although the main difference in the passive myocyte properties of IG KO mice is their elastic stiffness, the viscous...
moduli were also found to be increased (Figure 2F). Various sources could underlie this increase (eg, weak cross-bridge interaction, friction between thin and thick filaments), but none naturally explains why viscosity is increased only at long SLs. Instead, the SL dependence suggests that Ig domain unfolding may play a role. Ig domains form β-barrel structures that resist unfolding under physiological conditions, which minimizes energy loss during repeated loading cycles.38 Of the proximal and distal segments that are expressed in the N2B isoform, the proximal Ig domains have a lower stability (lower unfolding force and higher unfolding rate).39-42 Therefore, we removed them to minimize Ig domain unfolding events. However, the increased passive forces in the IG KO will increase the likelihood of unfolding (unfolding rates are force dependent),43 especially at long SLs where the force increase is the highest. Thus, increased viscosity at long SLs in the IG KO may be attributable to increased Ig domain unfolding.

**Ventricular Diastolic Dysfunction**

Ex vivo isolated heart experiments and in vivo pressure-volume analysis revealed steeper diastolic wall stress-volume relationships in 3-month-old IG KO and higher stiffness values derived from these relationships. We expected that the IG KO mouse would also exhibit increased end-diastolic pressures but found only a trend toward an increase (Table II in the online-only Data Supplement), which is a limitation of this study. (It is possible that the use of anesthesia during in vivo characterization influenced end-diastolic pressures.) We also found a significant reduction in mRNA expression levels of collagen Iα1 and Iα11α1 at 3 months (Figure II A–IIC), which might reflect an attempt to compensate for increased titin-based passive stiffness in IG KO. However, these mRNA changes appear not to cause functional effects because no differences were found in the ECM-based passive stiffness or collagen-volume fraction between IG KO and WT mice (Figure 4A–4C). Except for a decrease in tissue inhibitor of metalloproteinase-2 expression at 3 months, this is supported by the absence of expression differences in matrix metalloproteinases, tissue inhibitors of metalloproteinase, and lysyl oxidase (Figure IID–IHH in the online-only Data Supplement).

The summation of these results suggests that the ECM contributes little to the increased diastolic wall stiffness of IG KO mice; instead, the increase is likely to reflect increased myocyte stiffness. The magnitude of the stiffness increase was less in the in vivo study (37%) than in the cell and muscle study (>85%), and various factors might be involved in this difference (eg, anesthesia effects). An interesting possibility to consider is the reduced sarcomere strain amplitude in the IG KO that was found in the ex vivo isolated heart experiments (Figure III E in the online-only Data Supplement). Although we used a single SL-LV volume relationship to derive diastolic stress-SL relations in both genotypes (Figure 4D), separate fits would result in steeper stress-SL relationships in the IG KO (relative to WT). Thus, the single SL-LV volume relationship could reduce the stiffness difference between WT and KO hearts and might explain why the in vivo stiffness difference is only 37%. In summary, elevated diastolic stiffness is consistently found at all levels in the IG KO, with increased titin stiffness being the likely mechanistic basis.

Ventricular hypertrophy is also associated with diastolic dysfunction and HFpEF.21,22,44 After birth, IG KO mice begin with atrophic LVWs, but as they age, LVW increases more than in WT, resulting in significantly increased LVW and ratio of LVW to tibia length at 1 year (Figure 6A and Table II in the online-only Data Supplement). A recent hypothesis suggests that, in response to increased N2B strain, FHL1 expression and binding to the N2B segment increase, causing increased anchoring and signaling of members of the mitogen-activated protein kinase hypertrophy pathway.33 The hypothesis is in agreement with results in the PEVK KO, which has increased N2B strain and is hypertrophied,14 and with the N2B KO, in which the strain sensing mechanism is absent and the LV is atrophied.15 We observed an increase in FHL1 expression that intensified with age (Figure 6B and 6C). CARP and FHL2 are also upregulated in the IG KO (Figure 6B and 6C). CARP is upregulated in a wide range of diseases and conditions,45-47 including in the N2B KO, which has atrophy,15 and is thus unlikely to be a primary factor in the cardiac hypertrophy phenotype of the IG KO. FHL2 is of interest because it has recently been shown to suppress the calcineurin and nuclear factor of activated T cell hypertrophy pathway.25,26 This pathway does not appear to be altered in the IG KO because the expression of calcineurin and nuclear factor of activated T cells, along with downstream regulator of calcineurin (RCAN1.4) signaling proteins, revealed no consistent change in the IG KO (Figure VB–VF in the online-only Data Supplement). Given that FHL1 is thought to induce hypertrophy35 and the IG KO model shows increased N2B strain (Figure 2F), it is tempting to speculate that titin signaling is involved in the hypertrophy, but the mechanism requires additional study, and it remains to be explained why FHL1 is upregulated at a young age when the LV is atrophied or normal. Investigation of fetal genes commonly associated with failure and hypertrophy revealed no increases (Figure IV in the online-only Data Supplement), which might be explained by the fact that these mice are not exhibiting a failure phenotype.

Exercise intolerance is a hallmark feature of HFpEF.4,21,44 Although sedentary IG KO mice appear normal, reduced involuntary treadmill running tolerance and voluntary running wheel activity (Figure 5A and 5B) indicate that their exercise tolerance is compromised. The exercise tolerance and diastolic phenotype of 12-month-old animals undergoing exercise testing are even more compelling. Compared with 3-month-old mice, 12-month-old IG KO mice showed a greater reduction (48% versus 33% at 3 months) in voluntary wheel running (Figure 5B and 5C). Various factors may reduce exercise capacity. A decrease in systolic function is unlikely to have contributed to the decreased exercise tolerance because the IG KO mice appear to have normal systolic function (Table I in the online-only Data Supplement). It is likely that increased titin-based myocardial stiffness is a contributing factor because it will limit the ability to enhance ventricular filling by limiting cardiac reserve.6,44-48 Additional evidence supporting a diastolic stiffness phenotype is revealed in the IG KO mice, as evidenced by left atrial dilation and hypertrophy (Figure 7A and 7B).21 Echocardiography and in vivo pressure-volume analysis indicate increased diastolic stiffness (Figure 7E and 7F) and provide further support that LV dysfunction is present in IG KO mice. Our study suggests that diastolic dysfunction
and exercise intolerance are present in IG KO mice, which are similar to conditions observed in HFP EF patients.23,49

Conclusions

Our work on IG KO mice shows that shortening the tandem Ig segment leads to a primary diastolic dysfunction phenotype with increased LV stiffness, age-dependent hypertrophy, and exercise intolerance. The characteristics of the IG KO overlap with those of HFP EF patients, in whom increased titin-based stiffness has also been reported and diastolic stiffness is increased,21 and thus may elucidate the role of titin in diastolic dysfunction. Although the cause of increased titin-based stiffness is distinct from HFP EF patients (hypophosphorylation of titin),21 the functional effects that ensue might be similar, with increased strain of the spring elements of titin increasing diastolic stiffness and triggering hypertrophy. Thus, our novel mouse model with a titin-based primary diastolic phenotype might be of great use for elucidating mechanisms that contribute to HFP EF.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

To study the role of increased titin-based passive stiffness in diastolic dysfunction, we studied a new mouse model in which the elastic I band of titin has been shortened as a mechanical analog of increased titin-based stiffness in heart failure with preserved ejection fraction patients. The model displays increased diastolic stiffness, hypertrophy, and exercise intolerance, all common pathological findings in patients with heart failure with preserved ejection fraction. We propose that titin is a possible therapeutic target for ameliorating diastolic stiffening in heart failure with preserved ejection fraction.
Shortening of the Elastic Tandem Immunoglobulin Segment of Titin Leads to Diastolic Dysfunction

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SUPPLEMENTAL MATERIALS

Shortening of the Elastic Tandem Immunoglobulin Segment of Titin Leads to Diastolic Dysfunction

Short Title: Stiffer titin leads to diastolic dysfunction

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SUPPLEMENTAL METHODS

GENERATION OF MICE EXPRESSING SHORTER TITIN IG SEGMENT
The IG KO was developed as a model of passive stiffness increase that does not remove any known phosphorylation sites\(^1-3\). The two tandem Ig segments in titin expressed in the dominant N2B isoform are the proximal and distal segments. The proximal Ig domains have a lower stability (reduced unfolding force and increased unfolding rate)\(^4-7\) and we therefore chose to remove these to minimize Ig domain unfolding events in the mutant protein.

Targeting vector construction. The knockout targeting vector was constructed using the recombineering technique described by Liu et al\(^8\). A 17,778 bp genomic DNA fragment (position chr2:76,734,958 - 76,752,735; Mouse Feb 2006 Assembly) containing exon 19-41 of the gene was retrieved from BAC clone RP23-134B3. A fragment of 6027 bps containing exons 30-38 was replaced by a floxed PGK-neo (PL452) cassette. The 5' homologous arm was 8395 bp and the 3' homologous arm was 3356 bps long.

ES cell targeting and screening. The targeting vector was linearized with Not1 and electroporated into D1 ES cells which were derived from F1 hybrid blastocyst of 129S6 x C57BL/6J by the Gene Targeting & Transgenic Facility at University of Connecticut Health Center. 192 G418 resistant ES colonies were isolated and screened for homologous recombination by nested PCR using primers outside the construct paired with primers inside the neo cassette. Primer sequences were as follows: 5' arm forward primers: TtnIg Scr F1 (5'-TCATGATCCGCGAAGCCTT-3') and TtnIG Scr F2 (5'-GCACTTCCTGCTATCTTTGCT-3'). Reverse primers: Neo scr R1 (5'-GGACGTAAAACCTCTTCTCAG-3') and Neo scr R2 (5'-ATGATCGGAATTGGGCTGCA-3'); 3' arm forward primers: Neo scr F3 (5'-TTCTGAGGGCGGAAAGACCA-3') and Neo scr F4, (5'-CGAAGTTATAGGTCCCTCGA-3'); Reverse primers: TtnIg Scr R3 (5'-TGCAGTTACACCAGATGAG-3') and TtnIG Scr R4 (5'-TCAGGCACACTACTGATT-3'); 6 clones PCR positive for both arms were expanded for generation of chimeric mice.

Chimera generation and mice genotype. The ES cells from two clones (1F5 and 1B9) were aggregated with 8-cell embryos of CD1 strain. The aggregated embryos were transferred to pseudopregnant recipients and allowed to develop to term. Chimeric mice were identified by coat color and offspring from two chimeras from each line were tested for germline transmission of the targeted allele. The neo cassette was removed by mating the chimeras with a Cre deleter strain (129S1-Hprt\((\text{cre})\)Mnn Stock Number 004302, Jackson Laboratory, Bar Harbor, ME). The F1 pups with neo cassette removed were genotyped by PCR using primer set Tnlg gtF P2 (5'-CACTAGCAGGACATGTGC-3') and Tnlg gtR P3 (5'-GCAGTACCCCATATGCAG-3'). The PCR product is 268 bp for the IG KO allele. Mice were subsequently bred on a C57BL/6 background (Stock Number 000664, Jackson Laboratory) for 8 generations. Template DNA for genotyping was digested from tail tips using Tail Lysis Buffer (0.1mM Tris pH 8.8, 5mM EDTA, 0.2M NaCl, 0.2% SDS) and 0.4 mg/mL proteinase K (Worthington Biochemical Corporation) at 55°C overnight. To discriminate wildtype and knockout alleles, the primers Tnlg gtF P1 (5'-GAACGGTGTTGAGATCAAGT-3') and Tnlg gtR P3 were used for WT and Tnlg gtF P2 and Tnlg gtR P3 were used for the IG KO; the PCR product sizes are 319bp for the WT and 268 bp for the IG KO alleles. The reaction was carried out for 32 cycles (94°C 20s, 55°C 30s and 72°C 30s) followed by one cycle of 72°C for 5 min. 1 µL of the template was amplified using GoTaq Green Master Mix (Promega) in a 20 µL PCR reaction. The heterozygous mice produce litters at Mendelian ratios and breeding was performed with both heterozygous and homozygous (WT/WT or IG KO/IG KO) breeding schemes. Regular backcrossing of homozygous breeders to BL6 lines was performed to eliminate genetic drift.
TITIN GENE EXPRESSION MICROARRAY
We evaluated titin mRNA expression of 3 month old male WT and IG KO hearts using our custom exon microarray as previously described\(^9\). Briefly, the mouse titin exon probes (50 mer oligonucleotides) were spotted in triplicate on glass slides (Corning Ultra GAPS, Dow Corning, Corning NY). LV lateral wall tissues were dissected and stored in Ambion RNA later (Invitrogen, Grand Island NY) to preserve RNA. RNA was then isolated using the Qiagen RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia CA) and amplified using the SenseAmp kit (Generisphere, Hatfield PA) and Superscript III reverse transcriptase enzyme (Invitrogen). Reverse transcription and dye coupling (using Alexa Flour 555 and Alexa Flour 647) was performed using the SuperScript Plus Indirect cDNA Labeling System (Invitrogen) to obtain labeled cDNA. Labeling with Alexa Flours 555 and 647 was alternated between genotypes to eliminate any dye-specific effects. cDNA concentration was evaluated using a Nanodrop system (ThermoScientific, Waltham MA) and 750ng of cDNA from one mouse of each genotype was hybridized on individual slides using SlideHyb Buffer #1 (Ambion) for 16 hours at 42°C after which slides were scanned at 595nm and 685nm with an ArrayWoRx scanner (Applied Precision, Issaquah WA). Spot finding was performed with SoftWoRx Tracker (Applied Precision) and analysis completed in CARMA\(^1^0\). CARMA is an analysis package for the R-statistics environment that calculates Loess normalization\(^1^0\) and detects relative changes in the fluorescence of a probe; these changes are reported as a log-fold-difference that reflects the ratio of IG KO expression per exon vs. WT. A positive fold-change indicates a higher relative expression in the WT, while a negative fold change indicates a decrease (or loss) of expression in the IG KO. A 2-fold change was considered the threshold for differential expression.

QUANTIFICATION OF PROTEIN EXPRESSION
Flash-frozen left ventricular (LV) tissues were prepared as previously described\(^9, 11, 12\). Briefly, (LV) tissues were snap frozen in liquid nitrogen and solubilized between glass pestles cooled in liquid nitrogen. Tissues were primed at -20°C for a minimum of 20 min, then suspended in 50% urea buffer ([in mol/L] 8 Urea, 2 Thiourea, 0.05 Tris-HCl, 0.075 Dithiothreitol with 3% SDS and 0.03% Bromophenol blue pH 6.8) and 50% glycerol protease inhibitors ([in mmol/L] 0.04 E64, 0.16 Leupeptin and 0.2 PMSF) at 60°C for 10 min, then at room temperature (RT), centrifuged, aliquoted, flash frozen in liquid nitrogen and stored at -80°C.

Titin isoform analysis was performed as previously described\(^1^2\) with LV samples (n=6) from each genotype at both 3 mo and 12 mo of age. Briefly, solubilized samples were electrophoresed on 1% agarose gels using a vertical SDS-agarose gel system (Hoefer). Gels were run at 15mA per gel for 3 h and 20 min, then stained using Coomassie brilliant blue (Acros organics), scanned using a commercial scanner (Epson 800, Epson Corporation, Long Beach CA) and analyzed using One-Dscan (Scanalytics Inc, Rockville MD). Each sample was loaded in a range of five volumes and the integrated optical density (OD) of titin and MHC were determined as a function of volume. The slope of the linear relationship was obtained for each protein to quantify expression ratios.

Titin phosphorylation levels were quantified via back-phosphorylation assays in LVs from 3 mo WT and IG KO for PKA and western blotting for PKC as previously described\(^1^3, 1^4\). Briefly, to determine PKA phosphorylation levels, fresh left ventricular tissues from WT and IG KO mice (n=5) were obtained and skinned for 24 h in a BES buffered relaxing solution ([in mmol/L] 10 BES, 10 EGTA, 6.56 MgCl$_2$, 5.88 Na-ATP, 1 DTT, 46.35 potassium-propionate, 15 creatine phosphate) with 1% triton X-100 and protease inhibitors ([in mmol/L] 0.1 E64, 0.4 Leupeptin and 0.5 PMSF). LV skinned fibers 2 mm in length, 0.5 mm in diameter were dissected. The fibers were incubated

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with 1 U/μL of protein kinase A (PKA) catalytic subunit from bovine heart (Sigma) in a BES buffered relaxing solution. 20 μCi of [γ-32P]ATP stock solution, specific activity 3,000 Ci/mmol (PerkinElmer) were added and the fibers incubated 1 h at RT. The reaction was stopped by adding solubilization buffer (in mol/L: 6 urea, 2 thiourea, 0.058 DTT, 0.0385 Tris HCl, and 2.3% SDS and 0.02% bromophenol blue, pH 6.8). The solubilized samples were electrophoresed on a 2-7% gradient SDS-PAGE, and the gels were subsequently Coomassie blue stained, dried, and exposed to BioMax MS film (Kodak, Rochester NY). The dried gels and the autoradiography films were scanned using an Epson Expression 800 scanner. The images were analyzed with One-Dscan software to obtain the integrated optical density. The titin integrated OD from the autoradiograph was normalized to that of the Coomassie blue stained gels to normalize for protein loading.

For PKC phosphorylation, 6 solubilized samples from both 3 mo old WT and IG KO LVs were run on a 0.8% agarose gel in a vertical gel electrophoresis chamber. Gels run at 15mA per gel for 3 h and 20 min were then transferred onto PVDF membranes (Immobilon-FL, Millipore) using a semi-dry transfer unit (Trans-Blot Cell, Bio-Rad, Hercules CA). Blots were stained with Ponceau S (Sigma) to visualize the total protein transferred and gels were stained with coomassie stain to confirm efficient transfer and both blots and gels scanned to confirm transfer efficiency. Blots were then probed with phosphor-specific rabbit polyclonal antibodies against phosphorylated S26 and S170 of the PEVK element sequence of the titin N2B isoform

Myosin isoform analysis was performed using 7% acrylamide gels as previously described. Briefly, 6 solubilized samples from 3 mo and 12 mo old LVs were loaded onto vertical gel electrophoresis chambers with a 4% acrylamide stacking layer and 7% acrylamide resolving layer. Gels were run at 275V for 24 h at 12ºC. Gels were stained with Coomassie brilliant blue because it binds stoichiometrically to proteins, has a wider dynamic range and a wider linear relationship between quantity of protein and staining intensity vs. silver stain. Gels were then scanned and analyzed using One-Dscan software. Each sample was loaded one time, alternating WT and IG KO samples.

Thin and thick filament regulatory proteins expression and phosphorylation were analyzed as previously described. Briefly, 5 samples from 3 mo WT and IG KO hearts were loaded on 2-7% gradient SDS-PAGE gels and run using 60V for 1 h followed immediately by running of 90V for 115 min. Gels were then fixed in 50% methanol, 10% acetic acid overnight then stained with Pro-Q Diamond Phosphoprotein Gel stain (Invitrogen), destained with 20% acetonitrile, 50 mmol/L sodium acetate pH 4, and scanned with an excitation laser at 532 nm and 560 nm long pass emission filter using a Typhoon 9400 scanner (Amersham Biosciences). Gels were then stained with Coomassie brilliant blue, scanned, and analyzed using One-Dscan for protein content. Each sample was loaded one time, alternating WT and IG KO samples.

Evaluation of titin-based signaling domains was also done using western blotting techniques as previously shown. Solubilized samples from 3 mo and 12mo old WT and IG KO LVs were loaded on 12% SDS-PAGE acrylamide gels and electrophoresed at 100V for 2 h. Gels were then transferred on to PVDF membranes using a semi-dry transfer unit, stained with Ponceau S, scanned, destained and labeled with antibodies to TCap, MLP, FHL1, FHL2, αβ-crystallin, CARP, ANKRD2, MARP3, MURF1 and MURF2 (available from www.myomedix.com) along with GAPDH.
or Tubulin (when necessary) for a loading control. Secondary antibodies conjugated with fluorescent dyes with infrared excitation spectra were used and membranes scanned using an Odyssey Infrared Imaging System. Images were analyzed using LiCOR infrared software and normalized either the loading control.

**QUANTITATIVE RT-PCR**

Total RNA was extracted using the RNeasy Fibrous Tissue Mini Kit with DNase treatment (Qiagen) from left ventricle tissue, which upon dissection had been immediately immersed into RNAlater (Ambion) and stored at -20°C. SuperScript III (Invitrogen) was used to reverse transcribe total RNA for expression studies. RNA quantification and quality assessment were performed using a NanoDrop 1000 Spectrophotometer and a 2100 Bioanalyzer (Agilent); control reactions with 25ng total RNA rather than cDNA (qPCR) were used to confirm that there was no DNA contamination in all samples. Quantitative RT-PCR was performed using a Rotor gene 6000 (Corbett Life Science) using Maxima SYBR Green qPCR Master Mix (Fermentas) with 60°C annealing temperature. Each reaction included the cDNA equivalent of 25ng total RNA per 20μl reaction. For each gene assayed, standard curves were used to quantify levels from averages of 3 technical replicates for 4 biological replicates from 3 month and 12 month (sedentary) samples. Samples were normalized using standard curves to the Polr2a, Ppia (Cyclophilin A, CypA), Hprt, and Rpl13a reference genes. The geNorm analysis method was used to select the best normalization (comparing results for all genes tested normalized with every single-gene, double-gene and triple-gene combination -- 14 normalizations total) to identify the Polr2a & Ppia & Rpl13a combination as having the most precise values, minimizing deviation within each group. Specificity of qPCR products was confirmed by melting point analysis; no outliers were removed. Oligonucleotides were synthesized by Integrated DNA Technologies or Invitrogen, sequences are found in Supplemental Table IV.

**IMMUNOELECTRON MICROSCOPY**

Immunoelectron microscopy methods were performed as previously described to estimated segment extension by measuring Z-disk to epitope distances. Briefly, 6 hearts from 6 month old male WT and IG KO mice were quickly excised from heparinized, anesthetized mice, and the LV opened along the septum and pinned to a Sylgard (Dow Corning, Midland MI)-coated petri dish in oxygenated HEPES buffer. Papillary muscles were dissected and placed in an Imidazole buffered relaxing solution ([in mmol/L] 40 Imidazole, 10 EGTA, 6.4 Mg-acetate, 5.9 NaATP, 1 DTT, 70 K-Proprionate, 10 creatine phosphate) with 1% Triton X-100 and protease inhibitors ([in mmol/L] 0.1 E64, 0.4 Leupeptin and 0.5 PMSF) for 24-48 hrs at 4°C with at least one solution exchange. The tissues were washed with Triton-free relaxing buffer for a minimum of 4 hrs (at 4°C). Fibers were microdissected (~150um in diameter, 2mm length), stretched to various lengths from slack up to ~150% of slack length and pinned to a Sylgard coated dish. Tissues were washed with PBS ([in mmol/L] 2.7 KCl, 1.5 KH2PO4, 137 NaCl, 8 NaH2PO4) and incubated with primary antibodies in PBS for 48 hours at 4°C. Primary antibodies used were: T12, a mouse monoclonal antibody, targeting Ig domains 2-3 (Roche, Indianapolis IN); Uc, targeting the C-terminal aspect of the N2B unique sequence; I84, Ig-domains 84-86 at the C-terminal side of the PEVK domain (Uc, Un and I84 are rabbit polyclonal antibodies courtesy of Dr. Siegfried Labeit); and I102 an affinity purified polyclonal antibody raised in a rabbit against nucleotide positions 18803-19600 of the Mus musculus titin N2-B cardiac isoform data library entry (GenBank Accession: NM_028004.2, KA Garvey and CC Gregorio, unpublished data). Primary antibodies were labeled in sets using T12, Uc and I102 or Un, I84 and I102 to minimize overlap of epitopes at lower sarcomere lengths. Excess primary antibody was washed from the preparations with fresh PBS for 24 hours, followed by incubation with secondary antibodies for 48
hours at 4°C. Goat anti-Mouse IgG (AP134, Millipore, Billeria MA) and Goat anti-Rabbit IgG (AP 132, Millipore) were co-incubated as secondary antibodies to provide added contrast during electron microscopy imaging. Samples were then washed again with PBS for 24 h and fixed for 20 min using a 3% glutaraldehyde solution, washed in PBS for 24 h to remove unconjugated glutaraldehyde, and incubated with 1%OsO₄ in PBS to post-fix the tissues for 30 minutes. Preparations were finalized after washing with ddH₂O then dehydrated in graded series of ethanol washes (25%, 50%, 75%, 95%, 100%, twice at each step for 15 minutes each). Tissues were then infiltrated with Araldite Epoxy Resin (Araldite 502 Kit, Ted Pella, Redding CA), incubated at 50°C for 2 hours, embedded in a size 3 BEEM capsule (Electron Microscopy Sciences, Hatford PA) and incubated at 60°C for 24-48 hrs. Samples were sliced in a cryotome and adhered to copper square mesh grids. Transmission electron microscopy was performed on a standard TEM system (CM-12 TEM, Phillips). Calibrated images were analyzed using a custom LabView VI (National instruments, Austin TX). Epitope locations were exported and fit using monoexponential curves; epitope separations were estimated via monoexponential fit to obtain sarcomere length dependent segment extensions and deviation estimated as the standard error between mean and epitope locations or distances.

HISTOLOGY
Six hearts each from 3 and 12 month old WT and IG KO mice were obtained and quickly cannulated from anesthetized animals. Hearts were perfused with a calcium free Tyrode solution with 2,3-Butanedione monoxide (BDM) and KCl to maximize relaxation ([in mmol/L] 25 NaHCO₃, 30 KCl, 118 NaCl, 1.2 MgCl₂, 1.2 Na₂HPO₄, 5 Glucose, 5 Na-Pyruvate, with 5 U/L Insulin and 80 mg/L Bovine Serum Albumin [BSA], 30 BDM). A 4-0 silk suture was advanced through the mitral valve and apex and left in place to eliminate fluid buildup in the ventricle. After 5 minutes, perfusion was rapidly exchanged with a 10% formalin solution (Sigma, St Louis MO) and allowed to perfuse for 10 minutes. These partially fixed hearts were then removed, sliced radially in 4 sections and post-fixed in 10% formalin for 24 hrs. Short-axis cross sections were embedded, sectioned, and stained using Picrosirius Red to quantify collagen content. Stained sections were then imaged on a Zeiss microscope (Imager.M1), and analyzed for collagen content and CSA using custom Axiovision scripts.

SKINNED CELL MECHANICS
Cardiomyocytes were isolated from old male WT and IG KO mice as previously described. Briefly, mice were injected with 100 units heparin IP 10 min prior to isolation. Mice were anesthetized via isoflurane inhalation, sacrificed via cervical dislocation, hearts were rapidly removed, and aorta rapidly cannulated. Langendorf perfusion was begun with a 3mL/min constant flow perfusion using a 37°C calcium free Perfusion Buffer solution ([in mmol/L] 10 HEPES; 113 NaCl, 4.7 KCl, 0.6 KH₂PO₄; 0.6 Na₂HPO₄; 1.2 MgSO₄, 12 NaHCO₃, 12 KHCO₃, 2.5 MgCl₂, 5.5 glucose, 10 BDM, and 10 Taurine, with 10 μg/mL Insulin, pH 7.46 at 37°C). After 5 minutes, this calcium free solution was replaced with a digestion solution (Perfusion Buffer with 240U/mg Type II Collagenase (Worthington Biochemical Company, Lakewood, NJ) and 25 μmol/L CaCl₂ for activation of the collagenase). Hearts were digested for approximately 10 minutes then washed for 10 minutes with a stop buffer (Perfusion Buffer buffered solution with 0.05 mg/mL bovine calf serum and 7.5 μmol/L CaCl₂). Hearts were then removed and placed in a small dish containing calcium free HEPES solution with 20mmol/L BDM and high concentration protease inhibitors ([in mmol/L] 0.1 E64, 0.4 Leupeptin and 0.5 PMSF). The right ventricles and atria were removed, and myocytes were then mechanically dissociated from the LV. Cells were placed in a 15mL tube and allowed to pellet. The supernatant solution was removed and cells resuspended in a BES buffered relaxing solution with 0.3% Triton X-100 (Thermo Scientific, Waltham MA) with a high concentration of protease inhibitors ([in mmol/L] 0.1 E64, 0.4 Leupeptin and 0.5 PMSF). Cells were incubated for 6 minutes then pelleted in a centrifuge at 4°C for 3
minutes at 400 rpm and again washed and resuspended in BES buffered relaxing solution buffer and stored on wet ice.

3-5 myocytes from each isolation were obtained and averaged from 5-6 isolations per genotype for mechanical measurements. For each cell, myocyte suspensions were added to a room-temperature flow-through chamber with relaxing solution, mounted on the stage of an inverted microscope. One end of a single cell was glued to a force transducer (Model 406A or 403A, Aurora Scientific). The free end was then bent with a pulled glass pipette attached to micromanipulator so that the myocyte axis aligned with the microscope optical axis and the cross-sectional area (CSA) was measured. The free end of the cell was released and glued to a high-resolution motor (308B, Aurora Scientific, Aurora, Ontario, Canada). Sarcomere length (SL) was measured using a CCD Camera and IonWizard Software (Ionoptix, Cambridge MA) and slack SL of skinned cells was not different (WT: 1.90 μm and IG KO: 1.89 μm, p=0.9 by Wilcoxon rank sum test). Cells were activated using a pCa 4.5 activating solution ([in mmol/L] 40 BES, 10 CaCO3 EGTA, 6.29 MgCl2, 6.12 Na-ATP, 1 DTT, 45.3 potassium-proprionate, 15 creatine phosphate) and protease inhibitors ([in mmol/L] 0.01 E64, 0.04 Leupeptin and 0.5 PMSF) at sarcomere length 2.0 μm to confirm cell viability and allowed to rest for 15 minutes. All maximal active tensions were >35mN/mm² with a statistically significant increase in the IG KO (WT: 47±2 mN/mm² (n=7) and IG KO: 58±5 mN/mm² (n=90), p=0.04 by Wilcoxon rank-sum test). All mechanical protocols were performed in BES buffered relaxing solution and protease inhibitors at room temperature (24°C).

To measure passive stiffness, cardiomyocytes were stretched from slack length to 2.3 μm, held for 20 seconds, and then released with a ramp speed 1.0 length/sec. All stretch protocols were carried out under sarcomere length control using constant strain rates. Recovery time of at least 7 minutes in between stretches was utilized to prevent memory-effects in subsequent measurements. To further quantify the viscoelastic behavior, sinusoidal oscillations were imposed at SLs of 2.05, 2.1, 2.2 and 2.3 μm using a frequency sweep from 0.1Hz to 100Hz with an amplitude of +/-5% of the cell length. Data was collected using a custom LabVIEW VI (National Instruments, Austin TX) at a sample rate of 1kHz and stored offline.

SKINNED FIBER MECHANICS
Skinned muscle fibers were obtained from 3 month old male WT, and IG KO mice as previously described. Briefly, hearts were quickly excised from heparinized, anesthetized mice, and the LV opened along the septum and pinned to a Sylgard (Dow Corning, Midland MI)-coated Petri dish in oxygenated HEPES buffer. Papillary muscles were dissected and placed in a BES buffered relaxing solution with 1% Triton X-100 and protease inhibitors ([in mmol/L] 0.1 E64, 0.4 Leupeptin and 0.5 PMSF) for 24-48 h at 4°C with at least one solution exchange. The tissues were washed with Triton-free relaxing buffer for a minimum of 4 h, infiltrated with a 50% (vol/vol) solution of relaxing solution with protease inhibitors and glycerol for a minimum of 8 h (both at 4°C), and then stored at -20°C. Small fibers (~0.02mm² CSA and ~1.0 mm length) were dissected from the papillary muscles and glued at their ends to aluminum clips. Fibers were attached to a force transducer (AE801, SensorOne, Sausalito CA) and length motor (308B Aurora Scientific). Cross sectional area (CSA) was calculated using the dimensions of two perpendicular measurements assuming an ellipsoid shape. To test fiber quality, all preparations were activated at sarcomere length 2.0 μm using a pCa 4.5 solution and achieved tensions of >35 mN/mm². The maximum active tensions were reduced in the IG KO, (WT: 47±2 mN/mm² (n=17) and IG KO: 42±2 mN/mm² (n=18), p=0.02 by Wilcoxon rank sum test). Fibers were washed with relaxing buffer, allowed to rest for 15 min below their slack length, and where then returned to their slack SL. Slack SL was not significantly different (WT: 1.86±0.02 μm and IG KO: 1.90±0.01 μm, p=0.10
by Wilcoxon rank sum test). Mechanical protocols were performed in BES buffered relaxing solution with protease inhibitors ([in mmol/L] 0.01 E64, 0.04 Leupeptin and 0.5 PMSF) at room temperature.

Cardiac muscle fibers were stretched using a stretch-hold-release protocol from their slack sarcomere length to a SL of 2.3 µm at a speed of 1.0 length/sec. The fibers were held at their stretched length for 90 seconds, followed by a release. Myofilaments were extracted using a high KCl concentration (1.0 mol/L) relaxing buffer to depolymerize the thick filaments and high KI concentration (0.6 mol/L) buffer to depolymerize the actin filaments. The stretch-hold-release was then repeated to determine ECM based stiffness. Data was collected from 6 animals per group and stored using a custom LabVIEW VI at a sample rate of 10 kHz and stored offline.

**ANALYSIS OF SKINNED MYOCYTE AND MUSCLE MECHANICS**

Data was analyzed off line in a custom LabVIEW VI. Stress was calculated by dividing measured force by cross sectional area. The force during the 1.0 length/s stretch was plotted against the sarcomere length and fitted with a monoexponential curve to derive stress-sarcomere length relationships. In cells, the total stiffness was attributed to titin; the slope of the monoexponential curve was calculated in ranges of ±0.5 µm to determine stiffness. In fibers, the stress post myofilament extraction was attributed to the ECM while the difference between the total (pre-extraction stress) and post-extraction stress was attributed to titin29, 31. The ECM based stiffness was calculated from 2.0-2.2 µm to overlap the physiologic range in the ex-vivo isolated heart studies.

Frequency sweep data from cells were analyzed to obtain viscous moduli32. Briefly, the stress was used to first calculate the complex stiffness as the stress divided by the percent length change. Phase delay was calculated as the phase difference between the stress and strain signals. (For myocyte mechanics, the internal timing/signal processing delay for the force transducer was determined as 12 ms using a glutaraldehyde fixed cell and measured phase was corrected for this delay; no delay was found for the fiber mechanics in the frequency range that was used.) The viscous modulus was then calculated as complex stiffness times the sine of the phase delay. To correct for preparation length, strain was normalized to cell or fiber length.

**ISOLATED HEART MECHANICS**

Isolated perfused mouse hearts were prepared as previously described16, 18. Briefly, WT and IG KO mice were heparinized and anesthetized. Mice were sacrificed (as above) and the hearts quickly cannulated for Langendorff perfusion. Constant pressure (90mmHg) perfusion was provided with an oxygenated 37°C Tyrode solution ([in mmol/L] 25 NaHCO3, 4.7 KCl, 118 NaCl, 1.2 MgCl2, 1.2 NaH2PO4, 1.75 CaCl2, 5 Glucose, 5 Na-Pyruvate, with 5 U/L Insulin and 80 mg/L Bovine Serum Albumin [BSA]). Left and right atria were opened for access and the AV-node was heat ablated. A small needle was used to guide a custom plastic balloon in the LV between the mitral valve orifice and LV apex. The balloon was connected to a chamber containing degassed water connected to a servomotor for volume control, and a micromanometric pressure catheter (SPR-471, Millar Instruments, Houston TX). The heart was submerged in a temperature-controlled chamber filled with Tyrode perfusate. Chamber temperature was adjusted to obtain right ventricular chamber (core) temperatures of 37°C. The balloon was inflated to a slightly preloaded volume achieving a diastolic pressure of 5-10 mmHg, and the ventricle was externally paced via field stimulation by platinum electrodes at 500 bpm, typically developing ~75 mmHg developed pressure. The heart was then allowed to equilibrate for approximately 10 minutes. A single beat Frank-Starling protocol was used to probe volumes below baseline (<0mmHg) up to
a volume where maximal developed pressure was resolvable. Frank-Starling protocols were repeated at perfusion pressures of 75, 60, and 45mmHg and returned to 90 mmHg to calculate the pure passive pressure-volume relationship and eliminate turgor effects\textsuperscript{16}. Once equilibrated, perfusion was changed to 2 µmol/L dobutamine to maximally stimulate the β-adrenergic pathway. After ~10 minutes of stabilization, the single beat Frank-Starling protocol was repeated. A 2 µmol/L dose of propranolol was also used to block the beta-adrenergic pathway, stabilized and studied via the Frank-Starling protocol. Hearts were then adjusted to one of 3 volume levels (baseline, near the volume of maximal developed pressure, or approximately half way between the two) then fixed in situ for 20 minutes using a 2% glutaraldehyde supplemented Tyrode solution. Hearts were then removed and post fixed for ~24 hrs in the same solution, then washed and stored in phosphate buffered solution (PBS, [in mmol/L] 2.7 KCl, 1.5 KH\textsubscript{2}PO\textsubscript{4}, 137 NaCl, 8 NaH\textsubscript{2}PO\textsubscript{4}). After heart removal the single beat Frank-Starling protocol was repeated to determine the elastic properties of the balloon. Volume control and data acquisition was performed using custom PC interface and stored for offline analysis. All data from isolated hearts were analyzed in a custom LabVIEW interface. Pressures were corrected with balloon-measurements at identical volumes with the heart removed.

Pressures were converted to wall stress in order to compare with myocyte and fiber experiments. Typically, spherical models of wall stress have been validated and utilized\textsuperscript{18, 33}. However, ventricular geometry is often more elliptical (with the apex to base major axis being longer than the minor axis). Therefore, we compared spherical and elliptical models of wall stress. The equation for a sphere has previously been derived and validated\textsuperscript{18, 33}:

$$\sigma_{sphere} = \frac{p}{\left(1+\frac{1.05W_{LV}}{V_{LV}}\right)^{2/3}-1}$$

Eq.S1

where \(W_{LV}\) is the ventricular weight calculated by M-mode echocardiography and \(V_{LV}\) is the ventricular volume\textsuperscript{18}. An idealized elliptical model can be derived in the same method as Eq.S1 by assuming that wall stress is approximately pressure divided by the area of the surrounding band of muscle\textsuperscript{33}. Thus, the surrounding muscle area can be calculated using \(\pi(r_{epi}^2 - r_{endo}^2)\).

Using \(V = \frac{4}{3} \pi r^2 l\), for a known volume and length we can calculate:

$$\sigma_{ellipse} = \frac{p}{\frac{3}{4}\left(V_{epi} - V_{endo}\right)}$$

Eq.S2

where \(l\) is length, \(V_{endo}\) is the chamber volume, and \(V_{epi}=V_{endo}+(1.05*W_{LV})\). In the isolated heart it is relatively simple to calculate spherical wall stress, but elliptical strain requires knowledge of the major (apex to base) length. We measured the major length in post-fixed hearts at known volumes and used this to calculate elliptical wall stress. Stress- volume relationships were significantly different between IGKO and WT for both elliptical and spherical wall stress models with the IG KO showing similar increases for both models (Fig. S3A-D). In the P-V analysis we attempted to get long-axis length from the ultrasound study that was carried out on each mouse prior to the P-V study. However, differences in cardiac parameters between echo and P-V (especially those that are sensitive to anesthesia such as LV volumes, heart rates, etc.) caused too much variability, i.e., the length obtained from echo was unlikely to accurately represent this value in the P-V study. Given the uncertainty in this data and the complexity of its analysis, we elected to use in the main article the validated spherical model to calculate wall stress.\textsuperscript{18, 33} In order to derive the passive pressure-volume and wall-stress volume relationship, the diastolic pressures were corrected for perfusion and turgor effects as previously described\textsuperscript{16}. Diastolic stiffness was calculated in the overlapping volume range (~22-28μL). Systolic stress-volume relationships were calculated using the 90mmHg perfusion pressure data at baseline and after dobutamine and propranolol treatment.
To determine stress-sarcomere length relationships, we determined sarcomere lengths as previously described\textsuperscript{16}. Briefly, we obtained a transmural section of the lateral free-wall of the heart. Sections were dissected in PBS until circumferential midwall fibers were obtained. Fibers were then placed into custom cut chambers in Sylgard (Dow Corning) coated Petri dishes; chambers were filled with PBS and sealed to prevent a meniscus and eliminate bubbles or evaporation. Sarcomere lengths were measured via laser diffraction and plotted against the volume during fixation to obtain a sarcomere length-volume relationship and a cylindrical model was applied via a square-root relationship. (Linear, quadratic and quartic (spherical model) relationships had minimal differences in this volume range. Since apex-base length was not significantly different (data not shown), a quadratic (square-root) relationship was used.) The relationship was fit for the IG KO and WT hearts separately and volumes converted to sarcomere lengths. This relationship was used to convert stress-volume relationships to stress-sarcomere length relationships. Stiffness was calculated in the physiologic sarcomere length range (2.0–2.3 μm).

**EXERCISE TOLERANCE**

Maximal and voluntary exercise tolerance testing was performed on 6 mice per genotype. Maximal exercise testing was performed using a 6-lane rodent treadmill system (Exer 3/6, Columbus Instruments, Columbus, OH). Exercise testing was performed at a 25% incline and having mice run at progressively increasing speeds (speed steps of 5 m/min). Mice ran for 160 s with a 50 s rest period between running periods, after which the protocol was repeated but at a 5 m/min greater speed. Maximal Tolerance was determined when the mouse left the treadmill and remained on a shock pad for 5 s. Tolerance was scored according to the speed of the last completed running period plus the fractional duration of the period before fatigue times 5 m/min (score=completed running period speed+5x pre-fatigue duration of final protocol /160s). Volunteer exercise tolerance was measured in 3 mo and 12 mo old animals\textsuperscript{34}. Briefly. Individual animals were housed in a large cage that contained a free wheel for 21 days. The exercise wheels have been previously described\textsuperscript{34}. Briefly, an 11.5cm-diameter wheel with a 5.0cm wide running surface (6208; PetSmart; Phoenix, AZ) was equipped with a digital magnetic counter (BC600, Sigma Sport, Olney II) that is activated by wheel rotation. All animals were given water and standard rodent feed ad libitum. Daily exercise values for time and distance run were recorded.

**IN-VIVO PRESSURE VOLUME RELATIONSHIPS**

In-vivo pressure volume analysis was performed in mice using a SciSense Advantage Admittance Derived Volume Measurement System and 1.2f catheters with 4.5 mm electrode spacing (SciSense, London, Ontario, Canada). Mice were anesthetized and ventilated with 1% isoflurane using an SAR-1000 Ventilator (CWE Inc) and body temperature maintained at 37°C using a TC-1000 Temperature Controller (CWE Inc). Three mo old anesthetized mice were secured and a mid-line incision was made down the neck. The muscles in the neck were separated and the right carotid artery was isolated from the vagus nerve. The right carotid artery was cannulated and the catheter guided past the aortic valve. The abdomen was opened below the sternum; the IVC was located and occluded during a sigh (pause) in ventilation to find load-independent indexes.

Data acquisition and preliminary analysis was performed in LabScribe2 (iWorx, Dover NH). End diastolic pressure-volume data was exported to MS Excel (Microsoft Corporation, Redmond WA) and a custom LabView VI where end diastolic and end systolic wall stress were calculated. Pressures were converted to wall stress using a spherical model as in Eq.S1. The spherical model provided a conservative estimate of wall stress without requiring values of ventricular lengths, which could not be measured simultaneously with PV measurements (the use of non-simultaneous measurements of major-axis length introduces a major source of error in the
calculation). PV data on 3 mo old mice, acquired via the carotid approach, was fit to linear PV and stress-volume curves with linear slopes reported as the stiffness (EDPVR). (Monoexponential fits did not converge on a unique parameter set.) The PV data on 12 mo old mice was acquired via apical stab (see above) and this type of data made it possible to fit stress-volume data using a monoexponential fit \(P = Ae^{\beta V}\) with the exponent (\(\beta\)) reported as the stiffness.

IN-VIVO ECHOCARDIOGRAPHY
Echocardiographic data was acquired on 12 mo WT and IG KO mice after 21 days of volunteer exercise tolerance using a Vevo 770 small animal echocardiography imaging system (VisualSonics, Toronto, Ontario). Briefly, chest hair was removed and mice were consciously echoed while scruffing the skin at the nape of the neck and a standard short axis (M-mode) cine loop was recorded at the level of the papillary muscles to assess chamber dimensions (LV systolic and diastolic dimensions (LVDs, LVDd)) posterior wall thickness (PWT) and cardiac function via fractional shortening (%FS). Left atrial dimensions were obtained from m-mode acquisition in parasternal long axis (PLA) view in conscious mice. Mice were subsequently anesthetized under a 1% isoflurane/oxygen mixture and secured to a temperature controlled scan table. Following anesthesia the 4-chamber view was used to obtain cardiac diastolic function (E-wave, A-wave, Tissue Doppler). Data was analyzed in Vevo770 3.0 software suite (VisualSonics).

SINGLE MOLECULE MODELING.
The force-SL relations were calculated using the wormlike chain (WLC) equation\(^{36}\) with three serially-linked WLCs, representing the combined tandem Ig segments, the PEVK, and N2B-Us spring elements\(^{37}\). We assumed a contour length (CL) of tandem Ig segments of 200 nm (40 Ig domains with an average spacing of 5 nm) in WT N2B cardiac titin and 155 nm in the IG KO (31 Ig domains with an average spacing of 5 nm). The PEVK contour length was assumed to be of 70 nm and the N2B Us CL 200 nm. The assumed persistence lengths (PL) were 12 nm, 1.0 nm and 0.65 nm, respectively. (For experimental evidence that underlies these values and additional details, see \(^{17,26,37,38}\).) We then calculated the force-SL relation of a single titin molecule and compared results for WT with IG KO, along the values obtained for N2B KO and PEVK KO molecules (calculated by setting the CL of N2B element/PEVK element to zero). From the obtained force values the stress ration was calculated between the IG KO and WT molecules.

STATISTICS
Non-parametric Mann-Whitney (Wilcoxon sign rank) tests were used to determine significant differences. Analysis was performed in SAS 9.3 (SAS Institute, Cary NC) or MS Excel 2010 (Microsoft Corporation, Redmond WA). Results are shown as mean ± SEM, with \(p<0.05\) taken as significant.
Supplemental Table I. Left ventricular properties derived from Isolated Mouse Heart (n=12) and In-vivo Pressure Volume (n=10) analysis at 3 mo. Data shown as mean±SEM. Abbreviations: dP/dtmax: maximum rate of pressure increase during contraction; dP/dtmin: minimum rate of pressure decrease during relaxation; tau: time constant of isovolumic relaxation; SSVR: systolic stress-volume relationship; DSVR: diastolic stress volume relationship; HR: heart rate; ESP: end systolic pressure; EDP: end diastolic pressure; ESV: end systolic volume; EDV: end diastolic volume; EF: ejection fraction; SW: stroke work; ESSVR: end systolic stress volume relationship; EDSVR: end diastolic stress volume relationship. * p<0.05; ** p<0.01.

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<tr>
<th>Isolated Heart Analysis</th>
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<th>IG KO</th>
</tr>
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<tr>
<td>Baseline LV Volume [μL]</td>
<td>23.5 ± 0.4</td>
<td>21.6 ± 0.4 **</td>
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<td>Developed LV Stress (max) [mmHg]</td>
<td>45.9 ± 3.1</td>
<td>54.1 ± 4.3</td>
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<tr>
<td>dP/dtmax [mmHg/ms]</td>
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<td>2.04 ± 0.10 *</td>
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<tr>
<td>dP/dtmin [mmHg/ms]</td>
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<td>-0.98 ± 0.05</td>
</tr>
<tr>
<td>Tau [ms]</td>
<td>-41.6 ± 3.0</td>
<td>-38.9 ± 1.9</td>
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<tr>
<td>SSVR [mmHg/μL]</td>
<td>2.68 ± 1.40</td>
<td>4.33 ± 0.94 **</td>
</tr>
<tr>
<td>DSVR [mmHg/μL]</td>
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<td>3.90 ± 0.66 *</td>
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<table>
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<td>HR [bpm]</td>
<td>572.3 ± 9.8</td>
<td>565.8 ± 22.2</td>
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<tr>
<td>ESP [mmHg]</td>
<td>100.9 ± 2.1</td>
<td>102.2 ± 3.4</td>
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<tr>
<td>EDP [mmHg]</td>
<td>4.2 ± 0.5</td>
<td>5.1 ± 1.0</td>
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<td>dP/dtmax [mmHg/s]</td>
<td>12,090 ± 440</td>
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<td>-10,260 ± 390</td>
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<tr>
<td>ESV [μL]</td>
<td>25.8 ± 1.8</td>
<td>25.0 ± 1.8</td>
</tr>
<tr>
<td>EDV [μL]</td>
<td>57.2 ± 1.9</td>
<td>55.0 ± 1.7</td>
</tr>
<tr>
<td>SV [μL]</td>
<td>31.4 ± 2.1</td>
<td>29.9 ± 2.2</td>
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<tr>
<td>EF [%]</td>
<td>54.8 ± 2.8</td>
<td>54.1 ± 3.5</td>
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<tr>
<td>SW [mmHg μL]</td>
<td>0.448 ± 0.025</td>
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<tr>
<td>Tau (Glantz) [ms]</td>
<td>7.03 ± 0.55</td>
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<td>ESSVR [mmHg/μL]</td>
<td>3.35 ± 0.10</td>
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<tr>
<td>EDSVR [mmHg/μL]</td>
<td>0.14 ± 0.01</td>
<td>0.19 ± 0.02 *</td>
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### Supplemental Table II. Comparison of body weight, cardiac mass, protein expression and gene expression at 3 and 12 months of age.

Data shown as mean±SEM. BW: body weight; HW: whole heart weight; LVW: left ventricular weight; RVW: right ventricular weight; LAW: left atrial weight; RAW: right atrial weight; tibial L: tibia length; N2B: short cardiac titin isoform; N2BA: long cardiac titin isoform; T2:T1: ratio of degraded to intact titin; TT:MHC: ratio of total titin protein to myosin heavy chain. Data based on the following n-numbers: for 3 mo: n>20 cardiac weights and tibia lengths, n>80 for BW; for 12 mo: n>8 cardiac weights and tibia lengths, n>15 for BW; n=6 for protein expression. * p<0.05; **p<0.01; ***p<0.001 for IG KO vs. WT per age group.

<table>
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<tr>
<th></th>
<th>3mo</th>
<th>12mo</th>
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<tr>
<td></td>
<td>WT</td>
<td>IG KO</td>
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<tr>
<td>HW [mg]</td>
<td>123.5 ± 2.3</td>
<td>112.8 ± 2.5</td>
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<tr>
<td>LVW [mg]</td>
<td>92.4 ± 1.5</td>
<td>83.3 ± 1.4 ***</td>
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<tr>
<td>RVW [mg]</td>
<td>24.5 ± 0.6</td>
<td>24.8 ± 1.4</td>
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<tr>
<td>LAW [mg]</td>
<td>3.90 ± 0.14</td>
<td>4.07 ± 0.18</td>
</tr>
<tr>
<td>RAW [mg]</td>
<td>3.80 ± 0.22</td>
<td>3.90 ± 0.29</td>
</tr>
<tr>
<td>Lung W [mg]</td>
<td>148 ± 3</td>
<td>138 ± 3 *</td>
</tr>
<tr>
<td>BW [g]</td>
<td>26.9 ± 0.3</td>
<td>23.8 ± 0.2 ***</td>
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<tr>
<td>HW:BW [mg/g]</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.1</td>
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<tr>
<td>LVV:BW [mg/g]</td>
<td>3.5 ± 0.1</td>
<td>3.5 ± 0.0</td>
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<tr>
<td>RVW:BW [mg/g]</td>
<td>0.92 ± 0.01</td>
<td>1.01 ± 0.03 **</td>
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<tr>
<td>LAW:BW [mg/g]</td>
<td>0.14 ± 0.01</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>RAW:BW [mg/g]</td>
<td>0.14 ± 0.01</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Lung W:BW [mg/g]</td>
<td>5.6 ± 0.1</td>
<td>5.9 ± 0.1 *</td>
</tr>
<tr>
<td>Tibial L [mm]</td>
<td>17.8 ± 0.1</td>
<td>17.7 ± 0.1</td>
</tr>
<tr>
<td>HW:Tibial L [mg/mm]</td>
<td>7.07 ± 0.18</td>
<td>6.83 ± 0.18</td>
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<td>LVV:Tibia L [mg/mm]</td>
<td>5.12 ± 0.13</td>
<td>4.85 ± 0.11</td>
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<td>RVV:Tibia L [mg/mm]</td>
<td>1.41 ± 0.04</td>
<td>1.54 ± 0.15</td>
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<tr>
<td>LAW:Tibia L [mg/mm]</td>
<td>0.23 ± 0.01</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>RAW:Tibia L [mg/mm]</td>
<td>0.22 ± 0.01</td>
<td>0.24 ± 0.02</td>
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<tr>
<td>Lung W:Tibial L [mg/mm]</td>
<td>8.5 ± 0.2</td>
<td>8.0 ± 0.2</td>
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<tr>
<td>Protein Expression</td>
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<tr>
<td></td>
<td>WT</td>
<td>IG KO</td>
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<tr>
<td>N2B:N2BA</td>
<td>0.27 ± 0.02</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>T2:T1</td>
<td>0.14 ± 0.02</td>
<td>0.16 ± 0.02</td>
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<tr>
<td>TT:MHC</td>
<td>0.19 ± 0.01</td>
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<tr>
<td>βMHC/αMHC ratio</td>
<td>0.0044 ± 0.0006</td>
<td>0.0044 ± 0.0006</td>
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**Supplemental Table III. Exercise tolerance and cardiac function in 12 mo mice after 21 days of volunteer exercise.** Abbreviations: BW: body weight; Tibia L: tibia length; LA W: left atrial weight; HR: heart rate; ESP: end systolic pressure; EDP: end diastolic pressure; dP/dtmax: maximum rate of pressure increase during contraction; dP/dtmin: minimum rate of pressure decrease during relaxation; ESV: end systolic volume; EDV: end diastolic volume; SV: stroke volume; EF: ejection fraction; SW: stroke work; tau: time constant of isovolumic relaxation; EDPVR (β): monoeXponential fit derived end diastolic pressure-volume relationship; EDSVR (β): monoeXponential fit derived end diastolic (spherical) wall stress-volume relationship; ESPVR: end systolic pressure-volume relationship; ESSVR: end systolic (spherical) wall stress-volume relationship; FS: fractional shortening; MV DT: mitral valve deceleration time; E/A: mitral valve peak E-wave to A-wave velocity ratio; LA: left atrium. Data shown as mean±SEM, n=6 * p<0.05; ** p<0.01. *** p<0.001.

<table>
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<tr>
<th>Measure</th>
<th>WT (mean±SEM)</th>
<th>IG KO (mean±SEM)</th>
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<tr>
<td>BW [g]</td>
<td>38.0 ± 1.6</td>
<td>34.5 ± 2.0</td>
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<tr>
<td>Tibia L [mm]</td>
<td>18.6 ± 0.1</td>
<td>18.4 ± 0.1</td>
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<tr>
<td>LA W [mg]</td>
<td>4.97 ± 0.41</td>
<td>6.87 ± 0.47**</td>
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<tr>
<td>LA W:Tibia L [mg:mm]</td>
<td>0.27 ± 0.02</td>
<td>0.37 ± 0.03**</td>
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<td><strong>Exercise Tolerance</strong></td>
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<tr>
<td>Distance [km/day]</td>
<td>4.1 ± 0.9</td>
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<td><strong>In vivo Pressure Volume Analysis</strong></td>
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<td>HR [bpm]</td>
<td>533 ± 23</td>
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<td>ESP [mmHg]</td>
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<td>EDV [µL]</td>
<td>58 ± 3</td>
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<td>SV [µL]</td>
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<td>EF [%]</td>
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<td>SW [mmHg µL]</td>
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<td>Tau (Giantz) [ms]</td>
<td>8.9 ± 0.9</td>
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<td>EDPVR (β) [mmHg/µL]</td>
<td>0.048 ± 0.002</td>
<td>0.068 ± 0.006**</td>
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<td>EDSVR (β) [mmHg/µL]</td>
<td>0.060 ± 0.001</td>
<td>0.081 ± 0.005**</td>
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<td>LA Dimension [mm]</td>
<td>2.2 ± 0.1</td>
<td>2.8 ± 0.1 ***</td>
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### Table IV. Primers used in qPCR

Ref: see Supplemental References, pg. 21; DIH: designed in house.
Supplemental Figure I. Generation and basic characterization of the IG KO mouse model.
A) targeting strategy for gene KO of titin exons 30-38 that encode Ig 3-11. Genotyping (P) and screening (F,P) primers are shown as described in the supplementary methods. Comparison of 3 mo old WT and IG KO cardiac sarcomeric protein expression (B) and phosphorylation (C) of sarcomeric protein relative to WT at 3 months of age. Horizontal lines denote mean±SEM, n=5. No significant differences were detected.
Supplemental Figure II. Expression of ECM-components in sedentary 3 and 12 mo old WT and IG KO mice. Panels A-H, qPCR data normalized to WT per age group and shown as mean±SEM (n=4). Panel I, Picrosirius Red staining (n=5). Horizontal lines denote mean±SEM, * p<0.05 for IG KO vs. WT per age group.
Supplemental Figure III. Wall Stress, sarcomere length and pressure-volume relationships. Elliptical (A,B) and spherical models (C,D) of wall stress of isolated hearts show in IG KO mice similar relative increases in diastolic stiffness of DSVR relationships. (n=12). After fixing hearts at specific preload, the sarcomere length volume relationship was determined (E) (n=4 per group per volume). Example in-vivo PV from a 3mo IG KO mouse (F) and stress-volume (G) relationships (top: full IVC occlusion, line shows end systolic P-V relationship; bottom: data for end diastolic relationships). Horizontal lines denote mean±SEM, * p<0.05.
Supplemental Figure IV. Markers for cardiac disease and failure. Atrial natriuretic peptide (A, ANP), b-type natriuretic peptide (B, BNP), MAPKAPK2 (C), and myosin heavy chain isoform expression and expression ratios (D-F) and skeletal actin (G) are shown. Data normalized to WT per age group and shown as mean±SEM, n=4. Horizontal lines denote mean±SEM, * p<0.05 for IG KO vs. WT per age group.
Supplemental Figure V. Expression of titin binding proteins (A) and calcineurin hypertrophic pathway markers (B-F). Expression of titin-binding proteins is unaltered except for the I-band binding proteins FHL1, FHL2, and CARP (A). Calcineurin (B) and NFAT (C-E) mRNA expression are also unchanged along with the primary downstream marker of the calcineurin hypertrophy pathway RCAN1,4 (F). Data normalized to WT per age group; horizontal lines denote mean±SEM, n=4. ** p<0.01.***p<0.001 for IG KO vs. WT per age group.
Supplemental Figure VI. Single molecule modeling. A) Predicted force-SL relationship of single titin molecule (N2B cardiac titin isoform). WT (gray) and IG KO molecules (thick black). Orange line is predicted force of PEVK KO molecule and blue line of N2B KO molecules. At SLs <~2.2 μm force is highest in the IG KO and only towards the upper limit of the physiological SL range the values in the N2B KO and PEVK KO models exceed those in the IG KO. The large increases in the IG KO model at short to intermediate SLs can be explained by the previous findings that tandem Ig segments dominate titin extension at short to intermediate SL (due to their long persistence length). Inset shows the fractional increase in stress of the IG KO vs. WT from model data (thick black line) and from the cardiomyocyte data (gray line, error bars denote SE; data from Fig.2A). For details, see Supplemental methods.
Supplemental Figure VII. Basic characterization of the IG KO mouse model (Same as Figure 1 with Box-and-whiskers plots). A) Location of Ig 3-11 (deleted in the IG KO) in the spring region of titin (Ig domains are indicated by the rectangular red structures). B) PCR products showing differential gene expression from WT, heterozygous and homozygous IG KO mice. C) Titin exon microarray analysis shows titin exon expression changes only in the 9 deleted exons. D) Titin protein analysis (1% agarose gel). Top: the shortened titin (IG KO) has a higher mobility when compared to the WT titin bands and a doublet can be seen in the HET mice. Bottom: Quantitative analysis shows that titin isoform expression is unchanged (n=6). E) Titin phosphorylation is unchanged in the IG KO. Top: PKA back-phosphorylation and phospho-specific pS26 and pS170 antibodies to PKC Western blotting examples. Bottom: quantification showing unchanged phosphorylation levels in the IG KO (n=4). CB: coomassie blue; AR: autoradiography; PonS: Ponceau S. Horizontal lines denote mean±SEM.
Supplemental Figure VIII. Cardiomyocyte mechanical properties in relaxing solution (Same as Figure 2 with Box-and-whiskers plots). A) Passive tension is higher in the IG KO cell (n=5 for WT and IG KO). Inset: Representative stretch-hold-release experiment. From these experiments, both peak stress (B) and steady state stress (C) is increased in the IG KO cells. D) Passive stiffness measured during the ramp stretch is also increased in the IG KO mouse at SLs>2.0 μm. Dynamic stiffness analysis using small amplitude sinusoidal length oscillations to determine elastic (E) and viscous (F) moduli of the myocytes. Elastic stiffness is increased at all SLs>2.0μm but viscous properties are increased only at SLs≥2.2 μm (n=6). Horizontal lines denote mean±SEM, * p<0.05; **p<0.01; ***p<0.001.
Supplemental Figure IX. Cardiac Trophicity (Same as Figure 6 with Box-and-whisker plots). LVW:Tibia Length (TL) ratio is increased in older mice (A, n=6 for 5d day, n=28 for 3 mo; n=8 for 12 mo). Representative Western blots from 3mo mice (B). Expression of titin binding proteins FHL1, FHL2, and CARP were normalized to the 3mo WT (C). (n=4 for 5 day, n=8 for 3, 12 mo) Box denotes 25th and 75th percentile and whiskers display min and max, * p<0.05; ** p<0.01; ***p<0.001.
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