Published in final edited form as:

J Mol Cell Cardiol. 2015 February ; 79: 104–114. doi:10.1016/j.yjmcc.2014.10.020.

INCREASED MYOCARDIAL STIFFNESS DUE TO CARDIAC TITIN ISOFORM SWITCHING IN A MOUSE MODEL OF VOLUME OVERLOAD LIMITS ECCENTRIC REMODELING

Kirk R Hutchinson, PhD, Chandra Saripalli, MS, Charles S. Chung, PhD, and Henk Granzier, PhD

Department of Cellular and Molecular Medicine and Sarver Molecular Cardiovascular Research Program, University of Arizona, Tucson, AZ, USA 85724.

Abstract

We investigated the cellular and molecular mechanisms of diastolic dysfunction in pure volume overload induced by aortocaval fistula (ACF) surgery in the mouse. Four weeks of volume overload resulted in significant biventricular hypertrophy; protein expression analysis in left ventricular (LV) tissue showed a marked decrease in titin's N2BA/N2B ratio with no change in phosphorylation of titin's spring region. Titin-based passive tensions were significantly increased; a result of the decreased N2BA/N2B ratio. Conscious echocardiography in ACF mice revealed eccentric remodeling and pressure volume analysis revealed systolic dysfunction: reductions in ejection fraction (EF), +dP/dt, and the slope of the endsystolic pressure volume relationships (ESPVR). ACF mice also had diastolic dysfunction: increased LV end-diastolic pressure and reduced relaxation rates. Additionally, a decrease in the slope of the end diastolic pressure volume relationship (EDPVR) was found. However, correcting for altered geometry of the LV normalized the change in EDPVR and revealed, in line with our skinned muscle data, increased myocardial stiffness in vivo. ACF mice also had increased expression of the signaling proteins FHL-1, FHL-2, and CARP that bind to titin's spring region suggesting that titin stiffening is important to the volume overload phenotype. To test this we investigated the effect of volume overload in the RBM20 heterozygous (HET) mouse model, which exhibits reduced titin stiffness. It was found that LV hypertrophy was attenuated and that LV eccentricity was exacerbated. We propose that pure volume overload induces an increase in titin stiffness that is beneficial and limits eccentric remodeling.

© 2014 Elsevier Ltd. All rights reserved.

Corresponding Author: Henk Granzier, Department of Cellular and Molecular Medicine, MRB 325, 1656 E Mabel Street, University of Arizona, PO Box 245051, Tucson, AZ 85724-5217, Voice: (520)626-3641; granzier@email.arizona.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosures:
None.

Keywords

diastole; titin; cardiomyocyte; contractility; physiology

1. Introduction

A greater understanding of the mechanisms that govern diastolic function and cardiac dilation is important for understanding and developing therapeutics to treat pure volume overload, a disease resistant to medical therapy[1-4]. The aortocaval fistula (ACF) model has been used in multiple animal species to induce pure volume overload, such as that observed in isolated mitral or aortic valve regurgitation; conditions that do not involve concomitant increases in aortic pressure. Consistent with pure volume overload in humans, ACF leads to eccentric hypertrophy characterized by an increase in the left ventricular (LV) diameter to wall thickness ratio[2, 4-8]. Numerous studies have characterized the molecular mechanisms underlying systolic dysfunction[6, 7, 9-11]. Conversely, the molecular basis of diastolic dysfunction in pure volume overload is less well-understood. Diastolic function is comprised of active and passive properties. Active relaxation is thought to be regulated predominately by Ca^{2+} reuptake into the sarcoplasmic reticulum and extrusion from the cell. The passive properties are regulated by titin and the ECM, with the former dominating at physiological sarcomere lengths[12-16].

Titin is the largest protein known and spans the entire half-sarcomere from the Z-disk to the M-band. It functions as a molecular spring that develops passive tension during diastole when sarcomeres elongate. Titin is encoded by a single gene that is differentially spliced in the heart to express one of two isoforms; the larger and more compliant N2BA (~3.4MDa) and the smaller and stiffer N2B isoform (~3.0MDa). Both isoforms are co-expressed in the adult heart and the expression ratio largely determines titin's passive tension[17, 18]. Furthermore, kinases have been found to phosphorylate titin's spring region and tune passive stiffness. Specifically, phosphorylation by protein kinase A (PKA)[19] and protein kinase G (PKG)[20] has been found to decrease titin's passive stiffness. Phosphorylation of conserved regions in titin's proline, glutamate, valine, and lysine (PEVK) element (S26 and S170) by protein kinase C (PKC α) increase titin's passive stiffness[21-23]. More recently titin phosphorylation of S26 by PKC α was found to increase passive tension in a model of pressure overload induced heart failure despite an increase in N2BA/N2B ratio[12]. In addition to its role in regulating passive tension, titin is also thought to act as a mechanosensor that triggers hypertrophy in response to increased strain[13, 24-26].

Here, we studied titin's role in modulating diastolic function in mice with pure volume overload using the ACF model and focused on tissue and chamber mechanics. We found that volume overload increases titin-based tissue stiffness but that nevertheless left ventricular (LV) chamber stiffness is reduced due to altered chamber geometry. Correcting the pressure-volume relationship for geometric factors produced an increased wall-stress-sarcomere length relationship that mimics the increase in titin-based stiffness. We also tested the role of titin's stiffness in volume overload by performing the ACF surgery in RBM20 heterozygous (HET) mice, which express giant and compliant titins and have low diastolic

stiffness[14]. Obtained findings support that the increased titin stiffness in WT mice might be a beneficial adaptation that limits remodeling.

2. Methods

2.1. ACF Surgery and Tissue Collection

Mice were anesthetized with 2% isoflurane, and cardiac volume overload was induced[27] in 3 month old male C57BL/6J mice. Briefly, a midline abdominal incision was made and the abdominal aorta and inferior vena cava were exposed. Vascular clips were placed above the iliac bifurcation and below the iliolumbar vessels. A 23-g needle was inserted into the aorta and through the common midwall, creating an ACF. The needle was removed, and cyanoacrylate glue (Vetbond) was used to seal the aortic puncture. Shunt patency was visually confirmed by mixture of bright red arterial blood in the vena cava. The abdominal muscle and skin were closed using 6-0 vicryl absorbable suture and 7-mm surgical staples, respectively. Sham animals were treated identically except no aortic puncture was made. Mice were sacrificed 1 or 4 wks following surgery. All experiments were performed in accordance with the Principles of Laboratory Animal Care from the National Institutes of Health (8th Revision, 2011) and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arizona.

2.2. Tissue Collection and Quantification of Protein Expression

Mice were weighed, anesthetized with isoflurane and sacrificed by cervical dislocation. The hearts were rapidly excised and placed into a dish containing HEPES buffer ([in mmol/L] 133.5 NaCl, 5 KCl, 1.2 NaH₂PO₄, 1.2 MgSO₄, 30 BDM, 10 HEPES). All four chambers were removed, blotted, and weighed separately. The left ventricle (LV) was further separated into two sections, one of which was snap frozen in liquid nitrogen and the other placed into formalin for collagen volume fraction analysis using picosirius red staining. Tibias were removed and tibia length was measured using a caliper.

Protein expression analysis was performed using flash-frozen LV tissues as previously described[28]. Blots were stained with Ponceau S (Sigma) to visualize the total protein transferred. Blots were then probed with primary antibodies followed with secondary antibodies conjugated with fluorescent dyes with infrared excitation spectra (Ponceau S scans were analyzed to normalize WB signal to protein loading).

The collagen volume fraction (CVF) was measured on tissues fixed in formalin. These fixed hearts were sliced radially into sections, 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 using custom Axiovision scripts[26].

2.3. Skinned Fiber Mechanics

We studied 3 month old male WT, sham and ACF mice and small fibers (~0.02mm² CSA and ~1.0 mm length) were dissected from the LV muscle 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)[29]. Cross sectional area (CSA) was

calculated using the dimensions of two perpendicular measurements assuming an ellipsoid geometry. To test fiber quality, all preparations were activated at sarcomere length 2.0 μm using a pCa 4.5 solution and achieved tensions of $\sim 40 \text{ mN/mm}^2$. Fibers were washed with relaxing buffer, allowed to rest for 15 min below their slack length, and were then returned to their slack SL. 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/s. 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 the remaining stiffness. 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. The post-extraction stress was attributed to the ECM and the difference between the total (pre-extraction stress) and post-extraction stress was attributed to titin. Data was collected from 7 animals per group and stored using a custom LabVIEW VI at a sample rate of 10 kHz and stored offline. Data was analyzed off line in a custom LabVIEW VI. Stress was calculated by dividing measured force by cross sectional area.

2.4. Tail Cuff Blood Pressure Measurements

Mice underwent blood pressure analysis utilizing Hatteras Instruments Blood Pressure Analysis Systems (Model MC4000) placing individual tails in small cuffs after taping the tail and placing a magnetic cover over each mouse. Mice were conditioned three consecutive days prior to final analysis by taking the measurements at the same time of the day. Analysis included five preliminary and ten measurement cycles holding a constant temperature of 90°F. Conditioning the mice allowed for stable blood pressure measurements to be taken with low errors (at least 7/10 measurement cycles completed successfully).

2.5. Transthoracic Echocardiography

Echo images were obtained with a Vevo 2100 High Resolution Imaging System (Visual-Sonics, Toronto, Canada) using the model MS250 scan head designed for murine cardiac imaging. Imaging was performed at a depth setting of 1 cm. Images were collected and stored as a digital cine loops for off-line calculations. Standard imaging planes, M-mode, Doppler, and functional calculations were obtained according to American Society of Echocardiography guidelines. Conscious echo was chosen to evaluate geometry and functional parameters in order to reduce the impact of decreased heart rate associated with anesthesia. Conscious echo was performed while scruffing the skin at the nape of the neck and a standard mid-wall cross-sectional view of the left ventricle (LV) was obtained. A 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). Subsequently, mice were placed under isoflurane anesthesia for analysis of atrial diameter and LV filling patterns, which are necessary to obtain appropriate views and lower heart rates that allow assessment of filling patterns. Following anesthetic induction in 3% isoflurane, mice were placed in a supine position on a heated platform for echocardiography. Body temperature was maintained at 37°C and anesthesia was maintained with 0.5-1.0% isoflurane (USP, Phoenix)

in 100% oxygen. The left atrial dimension was measured in the long-axis view directly below the aortic valve leaflets. Doppler imaging was obtained from an apical 4-chamber view. Passive LV filling peak velocity, E (cm/sec), and atrial contraction flow peak velocity, A (cm/sec). Tissue velocity of the septal mitral valve annulus was measured and the e' and a' velocities (cm/sec) were determined. A sweep speed of 100 mm/sec was used for M-mode and Doppler studies. Considering that heart rate positively correlates with systolic performance, the heart rate of anesthetized animals during echocardiographic study was maintained in the range of 400 to 450 beats/min for Doppler studies.

2.6. In Vivo Pressure Volume Relations

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). Anesthetized animals were secured and the skin and muscle were cut below the ribcage. The diaphragm was then cut to expose the apex of the heart. A small puncture was made into the LV apex with a 28G needle and the catheter was then advanced into the LV. Baseline function was evaluated during a sigh (pause) in ventilation to prevent respiratory influences. Data acquisition and preliminary analysis was performed in LabScribe2 (iWorx, Dover NH). The IVC was also occluded during a sigh in ventilation to obtain load-independent indices and the end-systolic and end-diastolic pressure (P) volume (V) data was exported to MS Excel (Microsoft Corporation, Redmond WA). PV data were fit using linear regression analysis.

To investigate the effects of altered geometry on active and passive LV function and to compare PV data to fiber skinned fiber experiments, PV data were converted to spherical wall stress (σ) – strain (S) relations. P was converted to σ using a thick-walled spherical model[15]:

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

where W_{LV} is the ventricular weight calculated by M-mode echocardiography and V_{LV} is the ventricular volume. Volumes were converted to spherical strain (S) using the following formula:

$$S = 2\pi \left(\frac{3 \left(V_{blood} + 1.05 * \frac{M}{2} \right)}{4\pi} \right)^{1/3}$$

where V_{blood} is the blood volume and M is the LV mass, which is divided by 2 to calculate mid-wall strain.

2.7. Measurement of SL

To investigate the cellular mechanisms underlying LV stiffness in sham and ACF we measured the diastatic sarcomere length (SL) in 6 (per group) hearts from which PV relationships had been determined. Following the PV loop study, we infused HEPES with the addition of 30mM KCl and 30mM 2,3-Butanedione monoxide (BDM) into the left atrium to hyperpolarize the cells and inhibit cross-bridge formation. A 30G needle was advanced into the apex of the LV to prevent fluid buildup and maintain pressure in the ventricle at zero. After 3 min the perfusion was rapidly exchanged with a 2% glutaraldehyde fixation and allowed to fix for 5 min. The hearts were then post-fixed in glutaraldehyde and subsequently stored in PBS solution at 4C. Pressure was monitored during fixation and no significant changes occurred during perfusions. Hearts were cut into 2mm equatorial rings and circumferential sections of the myocardial wall were dissected. Thin strips of circumferential fibers were dissected from the midwall region and placed in a small chamber and SL was measured using laser diffraction with at least 20 fiber measurements averaged per heart. LV midwall strain at the volume at which the LV was fixed and the mean SL measured at that volume was used to convert strain at different LV volumes into SL and subsequently determine the diastolic LV wall stress-SL relationships.

2.8. Statistics

A student's t-test was to determine significant differences when two groups were present. A one-way ANOVA with a Bonferroni post-hoc analysis was performed to assess differences with four groups. F- tests of the non-linear regression analysis of passive tension curves were used to determine if passive tension curves were different. Analysis was performed with Graphpad Prism (La Jolla, CA). Results are shown as mean $\pm$ SEM. $p < 0.05$ was considered significant.

3. Results

3.1. ACF-Induced Cardiac Volume Overload

To address titin's role in diastolic function and hypertrophy in the context of pure volume overload we employed the ACF model and studied changes in titin from the molecular to the in vivo functional levels. Consistent with biventricular overload, morphometric analysis revealed significant LV hypertrophy (Table 1). ACF mice also had increased lung weight/tibia length (Table 1) indicating mild pulmonary edema. Since increases in arterial pressure resulting from increased afterload can also lead to hypertrophy, we analyzed arterial pressures in conscious mice. We found that arterial blood pressures were not different between ACF and sham (Table 2, top), confirming that the hypertrophy was not a result of increased afterload. Thus, using the ACF surgical model we successfully induced pure cardiac volume overload in mice. We studied titin size in left ventricular tissue from ACF and sham mice. Using high-resolution gel-electrophoresis (representative image Fig. 1A), we found that there was a decrease in the N2BA/N2B ratio (Fig. 1B). The total titin (TT) to myosin heavy chain (MHC) expression ratios were not different between groups indicating that there was no change in the number of titin molecules per half thick filament (Fig. 1B). Additionally, we found no change in titin degradation (T2/ TT, Fig. 1B). The ACF mice showed a decrease in the N2BA/N2B ratio that correlated with LV/BW ratio (Fig. 1C), a

result opposite that previously observed in pressure overload hypertrophy[12]. Phosphorylation of titin's spring elements has been found to fine-tune passive stiffness, with PKC phosphorylation increasing stiffness and PKA and PKG phosphorylation decreasing stiffness. Western blotting indicated no change in PKC α phosphorylation of S26 and S170 in the PEVK region of the N2B cardiac isoform (Fig. 1D) and no change in PKA phosphorylation (Fig. 1E). The decreased N2BA/N2B expression ratio with no change in titin phosphorylation is expected to increase titin-based stiffness.

3.3. Extracellular Matrix (ECM)

Changes in ECM composition often accompany alterations in hemodynamic load. We characterized the ECM in sham and ACF mice using Western blotting and found a significant decrease in collagen I and a trend toward a decrease in collagen III protein expression (Fig. 2A and 2B). Accordingly, analysis using picrosirius red, which stains mature collagen fibers, indicated that myocardial collagen volume fraction (CVF) was significantly decreased (Fig. 2C). Lysyl oxidase is associated with collagen crosslinking, which can increase the stiffness of collagen fibers. Therefore, we also measured the expression of LOX1 using Western blotting, but found no change in its expression (Fig. 2D). The decreased in collagen content observed in ACF mice is consistent with that found by others[3-5, 7, 8] and is thought to be a result of increased expression and activation of matrix metalloproteinases (MMPs)[8, 10, 30-32].

3.4. Passive tension in ACF and sham mice

The altered differential splicing of titin in ACF mice is expected to increase passive tension. Therefore, we performed mechanics on skinned passive muscle tissue to determine if LV titin tension was increased in ACF mice. Slack sarcomere length (SL) measured in relaxing solution using laser-diffraction was not different between groups (Fig. 3A). Maximal active tension at a SL of 2.0 μm was also not different (Fig. 3B). We next measured total passive tension, ECM-based passive tension and titin-based passive tension at SLs ranging from 1.9-2.3 μm . Comparisons of the obtained results indicated that ECM tension was unaltered but that in ACF mice total passive tension was increased due to an increase in titin-based passive tension (Fig. 3C). This finding is consistent with the decreased N2BA/N2B ratio from our titin protein analysis.

3.5. Integrative Physiology

3.5.1. LV Chamber Dimensions—To assess the effects of volume overload on LV dimension and function under physiological conditions we performed conscious echocardiography and pressure-volume (PV) analysis. Representative m-mode echocardiography images demonstrate the increased chamber dimensions observed in ACF mice compared to sham (Fig. 4A). Quantitative analysis revealed an increased LV internal diastolic diameter in ACF mice (Fig. 4B and Table 2) and an increased eccentricity (eccentricity index; LV internal diameter diastole/wall thickness in diastole) (Fig. 4C and Table 2). These results were consistent with increases in both LV end-diastolic volume (EDV) and LV end-systolic volume (ESV) determined from PV analysis (Table 3). LV systolic dimensions were also increased in ACF mice (Fig. 4D and Table 2).

3.5.2. Systolic Function—Systolic function was measured to determine if four weeks of cardiac volume overload led to a decrease in contractile function. Echocardiography indicated a decrease in FS (Fig. 4E and Table 2) and PV analysis indicated that ejection fraction (EF) and $+dP/dt$ were decreased in ACF mice (Table 3), consistent with a decrease in load dependent function. Despite having a decreased EF, there was an increase in stroke volume (SV) and pressure volume loop area (PVA) (Table 3); a result of the increased preload and increased chamber dimensions. The load-independent contractility can be assessed by performing a transient inferior vena cava occlusion to generate PV loops at a range of filling volumes, with a decrease in the slope of the end-systolic pressure volume relationship (ESPVR) indicating a decrease in contractility. ACF mice exhibited a decrease in the slope of the ESPVR indicating a decreased contractility (Table 3). However, increases in muscle mass and LV geometry, such as that observed in the ACF model confound, ESPVR results[33]. Therefore, to evaluate myocardial contractility independent of muscle mass and changing geometry we converted the LV pressure to stress (σ) and volumes to strain (S) yielding the end-systolic stress strain relationship (ESSSR)[15, 26]. No statistically significant change was detected in ESSSR slope (Fig. 5A and Table 3). The unchanging ESSSR is consistent with the unchanged active tension that was found in skinned muscle experiments (Fig. 3B). These data suggest that the decrease in EF and FS measured using echocardiography is likely due to alterations in chamber geometry, rather than reductions in myofilament function.

3.5.3. Diastolic Function—PV analysis and echo were also used to evaluate load-dependent and -independent diastolic function. Using PV analysis we found a decrease in $-dP/dt$ and an increase in tau in ACF mice indicating impaired relaxation of the LV (Table 3). Western blotting analysis of proteins involved in diastolic calcium handling indicated that the protein expression of NCX-1, SERCA2a, PLB and phosphorylated PLB was unaltered (Fig. S1); suggesting that they were not the source of the diastolic dysfunction. Increases in end diastolic pressure (EDP) occur in response to increased stiffness during LV filling. Using anesthetized echo to lower heart rate and separate E and A waves, we first evaluated mitral valve inflow and found an increase in transmitral Doppler E-wave velocity (MV E) in ACF mice relative to the tissue Doppler movement of the mitral valve (MV e') (Table 2). This resulted in an increase in the (E/e') of (Table 2), indicating an increase in LV filing pressures, which was confirmed by PV analysis that found a doubling of EDP in ACF mice (Table 3). Left atria (LA) are highly sensitive and increases in weight and diameter occur when EDP is increased. We measured the LA dimensions using echocardiography and representative images are depicted in Fig. 4F. Both the LAweight/TL ratio (Table 1) and LA diameters (LAD) (Fig. 4G and Table 2) were increased in ACF, which is suggestive of an increase in LV stiffness. Indeed, the inferred increase in EDP was confirmed using PV analysis (Table 3).

3.5.4. Diastolic Function-Passive LV Stiffness—The slope of the end-diastolic pressure volume relation (EDPVR) obtained from inferior vena cava occlusion is widely used as a measure of diastolic stiffness. Compared to sham mice, ACF mice had a significant decrease in the slope of the EDPVR indicating a more compliant LV chamber (Table 3). However, the EDPVR is sensitive to changes in mass and geometry[33];

therefore, we calculated the end-diastolic stress strain relationship (EDSSR) to examine the intrinsic myocardial stiffness. We converted the LV pressure to stress (σ) and volumes to strain (S) using methods identical to those used for calculating the ESSSR in the preceding section 3.5.2. The conversion to EDSSR eliminated the differences in slope between sham and ACF mice (Fig. 5B and Table 3) indicating that the diastolic wall stress strain relationship was maintained in volume overload. Severe alterations in LV geometry might induce sarcomere remodeling and changes in SL, which could affect our comparison of passive LV tissue properties from skinned muscle experiments and PV analysis. Accordingly, we measured SL in sham and ACF mice after in vivo fixation. The diastatic (0 mmHg) SL was not different between sham and ACF mice (Fig. 5C). We next plotted the diastolic LV wall stress obtained from PV analysis vs. SL and determined the slope as an in-vivo measure of diastolic myocardial stiffness. Volume overload increased the in vivo stiffness determined by End Diastolic wall Stress-SL Relationship (EDSSLR), in line with measurements in vitro.

3.5.6. Hypertrophy—Titin has been proposed to be a mechanosensor that in response to stretch induces hypertrophy, the role of which has never been tested in pure volume overload. As such, we performed a Western blot study to evaluate expression of titin-binding proteins that have been linked to stress-induced cardiac hypertrophy. We found no change in MARP3, MLP, ANKRD2, α -crystallin, MURF2, or T-cap protein (data not shown). However, four-and-half LIM protein (FHL) 1, FHL2 and cardiac ankyrin repeat protein (CARP), which bind to the extensible region of titin, were all significantly increased (Fig. 6).

3.6. Volume overload in the RBM20 mouse model

We hypothesized that the increased titin stiffness that we found in ACF mice is a beneficial adaptation that limits the increase in chamber size. We tested this hypothesis by experimentally increasing titin compliance and studying whether this leads to an exacerbation of LV chamber remodeling under conditions of volume overload. We induced volume overload in the RBM20 heterozygous (RBM HET) mouse model, which has an upregulation of compliant titin's through a deletion of the RNA Recognition Motif of the splicing factor RBM20 and a large reduction in LV chamber stiffness[14]. One week post ACF was chosen as an end-point due to high post-surgical mortality in the RBM HET ACF group when using the 4-week end points used above. RBM HET ACF mice exhibited significantly less LV and LA hypertrophy (Fig. 7A and Table 4). Echo analysis indicated that LV internal diameter in diastole was increased in WT and HET groups (Fig. 7B and Table 5). Compared to RBM WT mice, RBM HET mice exposed to ACF had a significant decrease in wall thickness (Fig. 7C and Table 5) and a profound increase in eccentricity (Fig. 7D and Table 5).

4. Discussion

We investigated the role of titin in compensated pure volume overload in mice and used the ACF model because of its similarities to volume overload in patients. Key findings include decreased N2BA:N2B expression ratio with no change in titin phosphorylation.

Accordingly, titin-based stiffness and thus titin's contribution to muscle stiffness is increased. At the organ level, LV geometry causes an apparent reduction in diastolic stiffness due to extensive eccentric chamber remodeling with a doubling in LV volumes. Correcting for the LV geometry recapitulates in vivo the titin-based increase in myocardial stiffness. Eccentric remodeling was amplified in a genetic mouse model with reduced titin-based stiffness, suggesting that the elevated titin stiffness in WT mice is a beneficial adaptation that limits remodeling. Below we discuss our findings in detail.

4.1. Mechanisms of increased titin-based stiffness

In the myocardium two titin isoforms are coexpressed: N2BA titin contains a longer extensible I-band region and has a lower passive stiffness than N2B titin. Small shifts in the expression ratio that occur through altered splicing have been observed in numerous diseases and can have large effects on myocardial passive stiffness[13]. Titin-based passive tensions were significantly increased in ACF mice and our molecular analysis uncovered a decrease in the N2BA:N2B ratio. This isoform shift and corresponding increase in passive tension is consistent with results in dog models of DCM induced by tachypacing [34-36]. It also mimics a decreased N2BA:N2B ratio in human patients with non-ischemic DCM in early stage HF[37], patients with aortic stenosis[38], and in dogs with experimentally induced HF with preserved ejection fraction (HFpEF)[39]. In contrast an increase in N2BA:N2B ratio and a reduction in titin-based passive tension has been documented in explanted hearts from human patients with decompensated DCM[40-42], NYHA grade IV HF patients with aortic stenosis and HFpEF[43], and in mice with end stage HF resulting from surgically induced pressure overload[12]. A possible explanation for the discrepancy in results is that temporal variations occur within the disease process, with compensated states exhibiting a *decreased* N2BA:N2B ratio and decompensated failing hearts having an *increased* N2BA:N2B ratio. Accordingly, the preceding human studies that demonstrated a *decreased* N2BA:N2B ratio were obtained from endocardial biopsies[37, 38], whilst the studies finding an *increase* were obtained from failing explanted hearts[40-43]. The results from the present study are similar to the preceding studies with compensated hearts and are more likely to reflect causative and/or adaptive mechanisms rather than responses to end-stage decompensated HF.

Altered phosphorylation of titin's spring elements can also tune titin's passive stiffness. PKA and PKG phosphorylation of the cardiac-specific N2B element have been found to lower passive stiffness[19, 20]; however, PKC α phosphorylation of the PEVK element increases passive stiffness[21, 22]. Accordingly, we probed titin's phosphorylation status in cardiac disease. Early investigations revealed that increased passive tension found in HFpEF and HF with reduced ejection fraction (HFrEF) was attenuated by phosphorylation with PKA/PKG, suggesting that hypophosphorylation of these sites increased passive stiffness[43]. The finding that hypophosphorylation of PKA/PKG increased cellular stiffness has since been substantiated in failing human hearts[37, 44] and in a rodent model of HFpEF[45]. Using tissues from pressure overload induced HF in mice we demonstrated that phosphorylation of PKC α sites on the PEVK element can increase passive tension. Specifically in pressure overload myocardium, we found increased phosphorylation of S26 and decreased phosphorylation of S170 that lead to an increased passive stiffness, an effect that was

normalized with phosphatase treatment[12]. Given the plethora of evidence supporting alterations in phosphorylation in HF, it was surprising that S26 and S170 in the PEVK region were not affected and PKA phosphorylation was unaltered in volume overload. Altered neurohormal stimuli, including β -adrenergic receptor activity, provide a mechanism for altered kinase activity that causes altered titin phosphorylation[13, 46], however most of the aforementioned studies documented deranged phosphorylation in end-stage HF. Thus, it is possible that absence of alterations in titin phosphorylation in our model may be explained by the fact that the mice in this study were not in end-stage heart failure.

4.2. Chamber geometry and LV systolic and diastolic function

In-depth analysis of myocardial function in the ACF mouse model uncovered the presence of load-dependent (decreased FS, EF, $+dP/dt$) and load-independent systolic dysfunction (decreased ESPVR). Although in line with results found by others[11, 47], it was surprising given that we found maximally activated active tensions in ACF wall muscle were not reduced in skinned muscle preparations. Similarly, Toisher, et al found that cells from 4wk ACF mice had identical fractional shortening despite reduced systolic function in vivo[11]. We hypothesized that the changing geometry of the LV might obscure the true myocardial properties and thus we converted P-V relations to stress-strain relations. This normalized the reduction in slope between ACF and sham mice (Fig. 5A), which suggests that much of the systolic dysfunction observed in this model is a result of altered chamber geometry.

LV diastolic function is determined by myocardial relaxation, passive properties, and chamber geometry[33, 48]. As such, we used experimental conditions that allowed us to determine the attributes of each and importantly compare isolated myocardial and ventricular chamber mechanics. Myocardial relaxation ($-dP/dt$ and τ), which are independent of chamber geometry, was slowed. We detected no changes in the expression or phosphorylation of proteins responsible for reuptake of Ca^{2+} into the SR, which supports prior studies indicate that Ca^{2+} transients were normal at this time point in ACF mice[11]. LV passive stiffness (dP/dV), which is dependent on chamber geometry, revealed other changes in cardiac function. As others have reported[5, 7, 8, 48] we found a decrease in the in-vivo EDPVR, indicating an increase in chamber compliance (decrease in stiffness). The results are also in accordance with increased chamber compliance in patients with mitral valve regurgitation[49, 50]. However, this contrasts with the significant increase in myocardial stiffness of skinned muscle that was driven by titin isoform switching. Because the pressure-volume relationships do not purely reflect the myocardial wall stress and strain the difference between the tissue-stiffness and in vivo stiffness is likely based on the chamber geometry. Eccentric dilation was present as the LV chamber was enlarged, while the wall thickness was unchanged. Comparison of the End Diastolic Stress Strain Relationships (EDSSR) normalized the stiffness ($d\sigma/dS$) between groups indicating preserved myocardial stiffness, similar to findings in rat[48]. This normalization in the EDSSR may reflect a natural homeostasis in the in vivo stress-strain relationship in the heart. While the in vivo resting (diastatic) sarcomere length was unchanged, the SL traversed for the same change in wall strain will be different in the volume overloaded hearts. Accounting for these differences we obtained an increase in End Diastolic wall Stress-SL Relationships (EDSSLR, $d\sigma/dSL$) in volume overloaded hearts. Without this titin-

based increase in stiffness to compensate, the EDSSR would likely fall due to the dilation-dependent change in ventricular geometry. Thus the isoform switch that we found in volume overload is likely a beneficial adaption.

4.3. Titin's role as a mechanosensor in volume overload

Mice exposed to volume overload developed hypertrophy with increased expression levels of the titin-binding proteins CARP, FHL-1, and FHL-2. These findings are similar to those found in the IG KO mouse model, which also develops hypertrophy and that also has increased CARP, FHL-1, and FHL-2 expression, changes that have been attributed to increased strain of the N2B element (a cardiac-specific subregion in titin's spring) [26]. That hypertrophy in mice with volume overload might have an origin in altered titin strain is supported by our findings the RBM20 heterozygous (RBM HET) mouse model, which has upregulation of compliant titin and thus a reduced N2B element strain[14] and which when exposed to volume overload developed less hypertrophy (but much more severe eccentricity) than WT mice. The mechanism by which increased titin strain causes hypertrophy in volume overload is unclear but it is worth considering that it involves the upregulated titin-binding proteins CARP, FHL-1 or FHL-2. CARPs role in responding to titin strain is unclear given its elevation in IG KO mice with hypertrophy and elevation in N2B KO mice with atrophy[26, 51]. As for FHL-1, recent evidence indicates that deficiency in the titin-binding protein FHL-1 confers resistance to pressure overload hypertrophy in response to stretch of titin's extensible N2B element[52]. Additionally, atrophy is present in the N2B KO mouse, which is devoid of the N2B region of titin where FHL1 binding occurs[51]. We previously studied FHL-1 expression in the PEVK KO and the IG KO models that have increased N2B strain and found that both titin models had increased FHL-1 expression and hypertrophy [26, 53]. Thus FHL-1 is an attractive candidate for being involved in hypertrophy. As for FHL-2, the first study to investigate its role in hypertrophy employed an FHL-2 KO mouse exposed to isoproterenol induced HF and found that hypertrophy was exaggerated, suggesting that FHL-2 *suppresses* hypertrophy[54]. Similarly, a recent study found that increased FHL-2 expression suppressed hypertrophy in a calcineurin dependent manner[55]. Thus it seems unlikely that the upregulated FHL-2 in ACF mice is part of the titin-based signaling pathways that leads to hypertrophy. In summary, we propose that titin plays an intimate role in the LV hypertrophy seen in volume overloaded mice and that of the titin-binding protein with altered expression, FHL-1 is the best candidate for linking altered titin strain to downstream hypertrophy signaling.

5. Conclusion

We characterized the LV diastolic properties in a surgery model of volume overload in the mouse. We obtained novel insights in the role of titin and found titin isoform switching that leads to increased titin-based passive stiffness. We also demonstrate that ventricular chamber stiffness (EDSSR) is unaltered in volume overload, and that titin-based tension normalizes muscle stiffness in the presence of decreased collagen. Our data on both WT mice and RBM20 HET mice also support that titin acts as a mechanosensor that regulates hypertrophy and plays a pivotal role in the adaptive and compensatory response to volume overload.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are very grateful to our current and former lab members (particularly Dr. Carlos Hidalgo and Mr. Javier Saldana Perez). Support was through the LeDucq Foundation 13CVD04 (to H.L.G.), T32HL 07249 (KH), NIH HL062881 (HG), and HL115988 (HG).

References

1. Wei CC, Ferrario CM, Brosnihan KB, Farrell DM, Bradley WE, Jaffa AA, et al. Angiotensin peptides modulate bradykinin levels in the interstitium of the dog heart in vivo. *J Pharmacol Exp Ther.* 2002; 300:324–9. [PubMed: 11752132]
2. Wei CC, Lucchesi PA, Tallaj J, Bradley WE, Powell PC, Dell'Italia LJ. Cardiac interstitial bradykinin and mast cells modulate pattern of LV remodeling in volume overload in rats. *American journal of physiology Heart and circulatory physiology.* 2003; 285:H784–92. [PubMed: 12663259]
3. Ryan TD, Rothstein EC, Aban I, Tallaj JA, Husain A, Lucchesi PA, et al. Left ventricular eccentric remodeling and matrix loss are mediated by bradykinin and precede cardiomyocyte elongation in rats with volume overload. *J Am Coll Cardiol.* 2007; 49:811–21. [PubMed: 17306712]
4. Wei CC, Chen Y, Powell LC, Zheng J, Shi K, Bradley WE, et al. Cardiac kallikrein-kinin system is upregulated in chronic volume overload and mediates an inflammatory induced collagen loss. *PLoS One.* 2012; 7:e40110. [PubMed: 22768235]
5. Gardner JD, Murray DB, Voloshenyuk TG, Brower GL, Bradley JM, Janicki JS. Estrogen attenuates chronic volume overload induced structural and functional remodeling in male rat hearts. *American journal of physiology Heart and circulatory physiology.* 2010; 298:H497–504. [PubMed: 19933421]
6. Guggilam A, Hutchinson KR, West TA, Kelly AP, Galantowicz ML, Davidoff AJ, et al. In vivo and in vitro cardiac responses to beta-adrenergic stimulation in volume-overload heart failure. *Journal of molecular and cellular cardiology.* 2013; 57:47–58. [PubMed: 23220155]
7. Hutchinson KR, Guggilam A, Cismowski MJ, Galantowicz ML, West TA, Stewart JA Jr. et al. Temporal pattern of left ventricular structural and functional remodeling following reversal of volume overload heart failure. *J Appl Physiol (1985).* 2011; 111:1778–88. [PubMed: 21885799]
8. Hutchinson KR, Stewart JA Jr. Lucchesi PA. Extracellular matrix remodeling during the progression of volume overload-induced heart failure. *Journal of molecular and cellular cardiology.* 2010; 48:564–9. [PubMed: 19524591]
9. Gladden JD, Zelickson BR, Guichard JL, Ahmed MI, Yancey DM, Ballinger S, et al. Xanthine oxidase inhibition preserves left ventricular systolic but not diastolic function in cardiac volume overload. *American journal of physiology Heart and circulatory physiology.* 2013; 305:H1440–50. [PubMed: 24014679]
10. Mazzo FR, de Carvalho Frimm C, Moretti AI, Guido MC, Koike MK. Acute aortocaval fistula: role of low perfusion pressure and subendocardial remodeling on left ventricular function. *Int J Exp Pathol.* 2013; 94:178–87. [PubMed: 23593971]
11. Toischer K, Rokita AG, Unsold B, Zhu W, Kararigas G, Sossalla S, et al. Differential cardiac remodeling in preload versus afterload. *Circulation.* 2010; 122:993–1003. [PubMed: 20733099]
12. Hudson B, Hidalgo C, Saripalli C, Granzier H. Hyperphosphorylation of mouse cardiac titin contributes to transverse aortic constriction-induced diastolic dysfunction. *Circ Res.* 2011; 109:858–66. [PubMed: 21835910]
13. LeWinter MM, Granzier H. Cardiac titin: a multifunctional giant. *Circulation.* 2010; 121:2137–45. [PubMed: 20479164]
14. Methawasin M, Hutchinson KR, Lee EJ, Smith JE 3rd, Saripalli C, Hidalgo CG, et al. Experimentally increasing titin compliance in a novel mouse model attenuates the frank-starling mechanism but has a beneficial effect on diastole. *Circulation.* 2014; 129:1924–36. [PubMed: 24599837]

15. Wu Y, Tobias AH, Bell K, Barry W, Helmes M, Trombitas K, et al. Cellular and molecular mechanisms of systolic and diastolic dysfunction in an avian model of dilated cardiomyopathy. *Journal of molecular and cellular cardiology*. 2004; 37:111–9. [PubMed: 15242741]
16. Chung CS, Granzier HL. Contribution of titin and extracellular matrix to passive pressure and measurement of sarcomere length in the mouse left ventricle. *Journal of molecular and cellular cardiology*. 2011; 50:731–9. [PubMed: 21255582]
17. Cazorla O, Freiburg A, Helmes M, Centner T, McNabb M, Wu Y, et al. Differential expression of cardiac titin isoforms and modulation of cellular stiffness. *Circ Res*. 2000; 86:59–67. [PubMed: 10625306]
18. Fukuda N, Granzier HL, Ishiwata S, Kurihara S. Physiological functions of the giant elastic protein titin in mammalian striated muscle. *The journal of physiological sciences : JPS*. 2008; 58:151–9. [PubMed: 18477421]
19. Yamasaki R, Wu Y, McNabb M, Greaser M, Labeit S, Granzier H. Protein kinase A phosphorylates titin's cardiac-specific N2B domain and reduces passive tension in rat cardiac myocytes. *Circ Res*. 2002; 90:1181–8. [PubMed: 12065321]
20. Kruger M, Kotter S, Grutzner A, Lang P, Andresen C, Redfield MM, et al. Protein kinase G modulates human myocardial passive stiffness by phosphorylation of the titin springs. *Circ Res*. 2009; 104:87–94. [PubMed: 19023132]
21. Anderson BR, Bogomolovas J, Labeit S, Granzier H. The effects of PKC α phosphorylation on the extensibility of titin's PEVK element. *Journal of structural biology*. 2010; 170:270–7. [PubMed: 20149875]
22. Hidalgo C, Hudson B, Bogomolovas J, Zhu Y, Anderson B, Greaser M, et al. PKC phosphorylation of titin's PEVK element: a novel and conserved pathway for modulating myocardial stiffness. *Circ Res*. 2009; 105:631–8. 17 p following 8. [PubMed: 19679839]
23. Hudson BD, Hidalgo CG, Gotthardt M, Granzier HL. Excision of titin's cardiac PEVK spring element abolishes PKC α -induced increases in myocardial stiffness. *Journal of molecular and cellular cardiology*. 2010; 48:972–8. [PubMed: 20026128]
24. Linke WA. Sense and stretchability: the role of titin and titin-associated proteins in myocardial stress-sensing and mechanical dysfunction. *Cardiovasc Res*. 2008; 77:637–48. [PubMed: 17475230]
25. Miller MK, Granzier H, Ehler E, Gregorio CC. The sensitive giant: the role of titin-based stretch sensing complexes in the heart. *Trends Cell Biol*. 2004; 14:119–26. [PubMed: 15003620]
26. Chung CS, Hutchinson KR, Methawasin M, Saripalli C, Smith JE 3rd, Hidalgo CG, et al. Shortening of the elastic tandem immunoglobulin segment of titin leads to diastolic dysfunction. *Circulation*. 2013; 128:19–28. [PubMed: 23709671]
27. Scheuermann-Freestone M, Freestone NS, Langenickel T, Hohnel K, Dietz R, Willenbrock R. A new model of congestive heart failure in the mouse due to chronic volume overload. *Eur J Heart Fail*. 2001; 3:535–43. [PubMed: 11595601]
28. Lahmers S, Wu Y, Call DR, Labeit S, Granzier H. Developmental control of titin isoform expression and passive stiffness in fetal and neonatal myocardium. *Circ Res*. 2004; 94:505–13. [PubMed: 14707027]
29. Watanabe K, Muhle-Goll C, Kellermayer MS, Labeit S, Granzier H. Different molecular mechanics displayed by titin's constitutively and differentially expressed tandem Ig segments. *Journal of structural biology*. 2002; 137:248–58. [PubMed: 12064950]
30. Levick SP, Gardner JD, Holland M, Hauer-Jensen M, Janicki JS, Brower GL. Protection from adverse myocardial remodeling secondary to chronic volume overload in mast cell deficient rats. *Journal of molecular and cellular cardiology*. 2008; 45:56–61. [PubMed: 18538342]
31. Melendez GC, Li J, Law BA, Janicki JS, Supowit SC, Levick SP. Substance P induces adverse myocardial remodelling via a mechanism involving cardiac mast cells. *Cardiovasc Res*. 2011; 92:420–9. [PubMed: 21908647]
32. Murray DB, Levick SP, Brower GL, Janicki JS. Inhibition of matrix metalloproteinase activity prevents increases in myocardial tumor necrosis factor- α . *Journal of molecular and cellular cardiology*. 2010; 49:245–50. [PubMed: 20403361]

33. Burkhoff D, Mirsky I, Suga H. Assessment of systolic and diastolic ventricular properties via pressure-volume analysis: a guide for clinical, translational, and basic researchers. *American journal of physiology Heart and circulatory physiology*. 2005; 289:H501–12. [PubMed: 16014610]
34. Bell SP, Nyland L, Tischler MD, McNabb M, Granzier H, LeWinter MM. Alterations in the determinants of diastolic suction during pacing tachycardia. *Circ Res*. 2000; 87:235–40. [PubMed: 10926875]
35. Wu Y, Bell SP, Trombitas K, Witt CC, Labeit S, LeWinter MM, et al. Changes in titin isoform expression in pacing-induced cardiac failure give rise to increased passive muscle stiffness. *Circulation*. 2002; 106:1384–9. [PubMed: 12221057]
36. Jaber WA, Maniu C, Krysiak J, Shapiro BP, Meyer DM, Linke WA, et al. Titin isoforms, extracellular matrix, and global chamber remodeling in experimental dilated cardiomyopathy: functional implications and mechanistic insight. *Circ Heart Fail*. 2008; 1:192–9. [PubMed: 19808289]
37. van Heerebeek L, Borbely A, Niessen HW, Bronzwaer JG, van der Velden J, Stienen GJ, et al. Myocardial structure and function differ in systolic and diastolic heart failure. *Circulation*. 2006; 113:1966–73. [PubMed: 16618817]
38. Williams L, Howell N, Pagano D, Andreka P, Vertesaljai M, Pecor T, et al. Titin isoform expression in aortic stenosis. *Clin Sci (Lond)*. 2009; 117:237–42. [PubMed: 19154184]
39. Hamdani N, Bishu KG, von Frieling-Salewsky M, Redfield MM, Linke WA. Deranged myofilament phosphorylation and function in experimental heart failure with preserved ejection fraction. *Cardiovasc Res*. 2013; 97:464–71. [PubMed: 23213108]
40. Makarenko I, Opitz CA, Leake MC, Neagoe C, Kulke M, Gwathmey JK, et al. Passive stiffness changes caused by upregulation of compliant titin isoforms in human dilated cardiomyopathy hearts. *Circ Res*. 2004; 95:708–16. [PubMed: 15345656]
41. Nagueh SF, Shah G, Wu Y, Torre-Amione G, King NM, Lahmers S, et al. Altered titin expression, myocardial stiffness, and left ventricular function in patients with dilated cardiomyopathy. *Circulation*. 2004; 110:155–62. [PubMed: 15238456]
42. Neagoe C, Kulke M, del Monte F, Gwathmey JK, de Tombe PP, Hajjar RJ, et al. Titin isoform switch in ischemic human heart disease. *Circulation*. 2002; 106:1333–41. [PubMed: 12221049]
43. Borbely A, Falcao-Pires I, van Heerebeek L, Hamdani N, Edes I, Gavina C, et al. Hypophosphorylation of the Stiff N2B titin isoform raises cardiomyocyte resting tension in failing human myocardium. *Circ Res*. 2009; 104:780–6. [PubMed: 19179657]
44. Kotter S, Gout L, Von Frieling-Salewsky M, Muller AE, Helling S, Marcus K, et al. Differential changes in titin domain phosphorylation increase myofilament stiffness in failing human hearts. *Cardiovasc Res*. 2013; 99:648–56. [PubMed: 23764881]
45. Hamdani N, Franssen C, Lourenco A, Falcao-Pires I, Fontoura D, Leite S, et al. Myocardial titin hypophosphorylation importantly contributes to heart failure with preserved ejection fraction in a rat metabolic risk model. *Circ Heart Fail*. 2013; 6:1239–49. [PubMed: 24014826]
46. Ahmed SH, Lindsey ML. Titin phosphorylation: myocardial passive stiffness regulated by the intracellular giant. *Circ Res*. 2009; 105:611–3. [PubMed: 19797191]
47. Perry GJ, Mori T, Wei CC, Xu XY, Chen YF, Oparil S, et al. Genetic variation in angiotensin-converting enzyme does not prevent development of cardiac hypertrophy or upregulation of angiotensin II in response to aortocaval fistula. *Circulation*. 2001; 103:1012–6. [PubMed: 11181478]
48. De Stefano LM, Matsubara LS, Matsubara BB. Myocardial dysfunction with increased ventricular compliance in volume overload hypertrophy. *Eur J Heart Fail*. 2006; 8:784–9. [PubMed: 16920023]
49. Corin WJ, Murakami T, Monrad ES, Hess OM, Kräyenbühl HP. Left ventricular passive diastolic properties in chronic mitral regurgitation. *Circulation*. 1991; 83:797–807. [PubMed: 1825625]
50. Wong CY, Spotnitz HM. Systolic and diastolic properties of the human left ventricle during valve replacement for chronic mitral regurgitation. *Am J Cardiol*. 1981; 47:40–50. [PubMed: 7457406]
51. Radke MH, Peng J, Wu Y, McNabb M, Nelson OL, Granzier H, et al. Targeted deletion of titin N2B region leads to diastolic dysfunction and cardiac atrophy. *Proc Natl Acad Sci U S A*. 2007; 104:3444–9. [PubMed: 17360664]

52. Sheikh F, Raskin A, Chu PH, Lange S, Domenighetti AA, Zheng M, et al. An FHL1-containing complex within the cardiomyocyte sarcomere mediates hypertrophic biomechanical stress responses in mice. *J Clin Invest.* 2008; 118:3870–80. [PubMed: 19033658]
53. Granzier HL, Radke MH, Peng J, Westermann D, Nelson OL, Rost K, et al. Truncation of titin's elastic PEVK region leads to cardiomyopathy with diastolic dysfunction. *Circ Res.* 2009; 105:557–64. [PubMed: 19679835]
54. Kong Y, Shelton JM, Rothermel B, Li X, Richardson JA, Bassel-Duby R, et al. Cardiac-specific LIM protein FHL2 modifies the hypertrophic response to beta-adrenergic stimulation. *Circulation.* 2001; 103:2731–8. [PubMed: 11390345]
55. Hojaye B, Rothermel BA, Gillette TG, Hill JA. FHL2 binds calcineurin and represses pathological cardiac growth. *Mol Cell Biol.* 2012; 32:4025–34. [PubMed: 22851699]

Highlights

- Volume overload (VO) studies were conducted in WT and RBM20 deficient mice.
- VO resulted in hypertrophy and an increase in titin's N2BA/N2B ratio.
- VO increased LV end-diastolic pressure and reduced relaxation rates.
- The LV had increased myocardial stiffness in vitro and in vivo.
- Conclusion: Titin's increased stiffness in VO limits eccentric remodeling.

Figure 1.

(A) Representative image of 1% agarose gel for titin analysis. (B) Quantitative analysis indicates a decrease in titin N2BA/N2B ratio in LV myocardial tissue with no change in total titin (TT) to myosin heavy chain (MHC) or titin degradation product (T2) to TT ratios (n=8). (C) N2BA/N2B ratio decreased with increasing LV/BW ratios. (D) Quantitative analysis and representative Western blots indicating no change in PEVK phosphorylation (n=8). (E) Back phosphorylation assay reveals no change in PKA phosphorylation. MHC: myosin heavy chain; TT: total titin; OD: optical density; CB: coomassie blue; AR: autoradiography; LV: left ventricle; BW: body weight. *** p < 0.001.

Figure 2.

(A) Western blot analysis revealed a decrease in collagen I expression after four weeks of ACF induced volume overload (n=6). (B) Collagen III expression was not different (n=6). (C) Collagen volume fraction (CVF) indicated a significant decrease in myocardial collagen content (n=7). (D) LOX1 expression was not different between sham and ACF mice (n=6). * $p < 0.05$.

Figure 3.

Resting sarcomere length in relaxing solution (A) and maximal activated tension (B) measured at SL 2.0 were not different between sham and ACF mice. Total, ECM, and titin-based passive tension (C) obtained from skinned muscle experiments (n=7). Total- and titin-based passive tension were increased in ACF mice

Figure 4.

Echo characterization. (A) Representative example of m-mode echo's demonstrating an increase in LV chamber dimensions. LVID;d (B), LVID;d/PWT;d (C), and LVID;s (D) were all significantly increased in ACF mice. Fractional shortening was decreased in ACF mice. Representative LA images obtained in B-mode (F, top) and m-mode (F, bottom). (G) Quantitative analysis indicates a significant increase in LA diameter. LV; left ventricle, ID; internal diameter, P; posterior, d; diastole, s; systole. n=16 for sham and n=20 for ACF. *** p< 0.001.

Figure 5.

PV analysis indicates a decrease in ESSSR (A) and no change in EDSSR (B) (n=13 for sham and n=14 for ACF). (C) SL in diastasis (0mmHg) was not different. (D) Slope of the diastolic stress vs. SL. ESSSR; end-systolic stress-strain relationship, EDSSR; end-diastolic stress-strain relationship, SL; sarcomere length. ** p< 0.01.

Figure 6.

Western blot analysis of titin binding proteins indicated that FHL-1, FHL-2, and CARP2 were increased after four weeks of ACF induced volume overload (n=6). *p<0.05 and ** p<0.01.

Figure 7.

Morphometry and echo of WT and RBM20 HET mice on week after they underwent sham or ACF surgery. (A) LV/TL ratio indicated a significant increase in WT mice exposed to ACF and no change in RBM HET mice. (B) LVID;d was increased in both genotypes after ACF. (C) PWT;d was unchanged in WT mice, but significantly decreased in RBM HET mice after ACF. (D) Eccentricity was significantly increased in RBM HET mice after one week of ACF. $n=7,9,10$, and 10 for RBM WT sham, RBM WT ACF, RBM HET sham, and RBM HET ACF, respectively. PWT; posterior wall thickness, d; diastole. RBM WT ACF vs. RBM WT Sham at ** $p < 0.01$; RBM HET ACF vs. RBM HET Sham at ### $P < 0.001$; RBM HET ACF vs. RBM WT ACF † $p < 0.05$ and ††† $p < 0.01$.

Table 1

Tissue morphometric analysis of Sham and ACF mice.

Morphometry		
	Sham	ACF
BW [g]	28.5 ± 1.1	29.1 ± 0.6
n=	16	20
LV [mg]	92.6 ± 2.5	142.3 ± 3.4 ***
RV [mg]	27.1 ± 0.9	41.6 ± 1.2 ***
LA [mg]	3.8 ± 0.3	8.3 ± 0.4 ***
RA [mg]	4 ± 0.3	7.9 ± 0.3 ***
Lung [mg]	139 ± 2.5	152.1 ± 8.2 ***
LV/TL [mg/mm]	5.16 ± 0.13	7.88 ± 0.18 ***
RV/TL [mg/mm]	1.51 ± 0.05	2.3 ± 0.07 ***
LA/TL [mg/mm]	0.21 ± 0.02	0.46 ± 0.02 ***
RA/TW [mg/mm]	0.22 ± 0.02	0.44 ± 0.02 ***
Lung/TL [mg/mm]	7.75 ± 0.13	8.84 ± 0.17 ***

BW: body weight; LV: left ventricle; RV: right ventricle; LA: left atria; RA: right atria; TL: Tibia length.

*** Significant vs. Sham (p< 0.001)

Table 2

Echocardiography analysis of Sham and ACF mice.

Tail Cuff Blood Pressure and Echocardiographic Analysis		
	Sham	ACF
Tail-Cuff Blood Pressure (conscious)		
n=	5	8
HR [BPM]	635 ± 14	575 ± 23
Systolic [mmHg]	121 ± 6	127 ± 6
Diastolic [mmHg]	100 ± 4	105 ± 5
MAP [mmHg]	107 ± 4	112 ± 5
Pulse Pressure [mmHg]	21 ± 3	22 ± 2
M-mode (conscious)		
n=	16	20
LVID;d [mm]	3.35 ± 0.06	4.6 ± 0.09 ***
LVPWT;d [mm]	0.86 ± 0.02	0.85 ± 0.02
LVID;s [mm]	1.79 ± 0.06	2.73 ± 0.1 ***
LVPWT;s [mm]	1.41 ± 0.03	1.46 ± 0.04
FS [%]	46.6 ± 1.3	41 ± 1.4 **
LVID;d/PWT;d	3.95 ± 0.13	5.48 ± 0.19 ***
LAD [mm]	2.42 ± .07	3.30 ± 0.10 ***
Pulsed Wave Doppler		
Ao Vel [mm/s]	770 ± 20	1296 ± 58 ***
MV E [mm/s]	690 ± 23	922 ± 22 ***
MV A [mm/s]	496 ± 26	700 ± 27 ***
MV Decel Time [ms]	25 ± 0.6	29.7 ± 0.7 ***
MV E/A	1.42 ± 0.04	1.33 ± 0.03
MV e' [mm/s]	16 ± 1.1	15.7 ± 0.7
MV a' [mm/s]	20.9 ± 1.2	23.4 ± 1.3
e'/a'	0.77 ± 0.04	0.69 ± 0.03
E/e'	45.3 ± 2.5	60.5 ± 2.5 ***

Significant vs. Sham at

Abbreviations: HR, heart rate; BPM, beats per minute; LV, left ventricle; LVIDd, LV internal diameter diastole; LVPWTd, LV posterior wall thickness diastole ; LVIDs, LV internal diameter systole; LVPWTs, LV posterior wall thickness systole; FS, fractional shortening percent; Eccentricity: LVIDd/PWTd; LAD, left atrial diameter; Ao Vel, aortic velocity; MV E, mitral valve E-wave velocity; MV A, mitral valve A-wave velocity; MV e', septal mitral valve annular tissue velocity; MV a', septal mitral valve annular tissue velocity

**
p< 0.01

***
P<0.001

Table 3

Pressure-Volume Loop analysis of Sham and ACF mice.

Pressure-volume analysis		
	SHAM	ACF
n=	13	14
HR [bpm]	564 ± 9	566 ± 6
Systolic Properties		
ESP [mmHg]	95.2 ± 1.2	99.2 ± 2.8
ESV [mmHg]	20.5 ± 1.2	51.9 ± 2.9 ***
SV [μL]	34.4 ± 1.7	61 ± 2.6 ***
CO [μL/min]	19379 ± 956	34454 ± 1490 ***
EF [%]	63 ± 2	54 ± 2 **
SW [mJoules]	0.45 ± 0.02	0.82 ± 0.03 ***
Ea [mmHg/μL]	2.9 ± 0.1	1.7 ± 0.1 ***
+dP/dt [mmHg/s]	12122 ± 329	10634 ± 508 *
PVA [mJoules]	0.6 ± 0.03	1.07 ± 0.08 ***
ESPVR linear [mmHg/μL]	7.5 ± 0.6	2.9 ± 0.2 ***
ESSSR [mmHg/μL]	57.3 ± 3.4	48.4 ± 3.6
Diastolic Properties		
EDP [mmHg]	2.1 ± 0.3	4.5 ± 0.7 **
EDV [μL]	54.8 ± 1.6	112.8 ± 3.6 ***
-dP/dt [mmHg/s]	-11481 ± 343	-9812 ± 606 *
Tau [ms]	5.0 ± 0.1	5.9 ± 0.2 **
EDPVR linear [mmHg/μL]	0.121 ± 0.008	0.071 ± 0.007 ***
EDSSR [mmHg/μL]	2.49 ± 0.23	2.68 ± 0.28

Significant vs. Sham at

Abbreviations: HR, heart rate; BPM, beats per minute; ESP, end-systolic pressure; ESV, end-systolic volume; SV, stroke volume; CO, cardiac output; EF, ejection fraction percent; SW, stroke work; Ea, arterial afterload; PVA, pressure-volume loop area; ESPVR, end-systolic pressure volume relationship; ESSSR, end-systolic stress-strain relation; EDP, end-diastolic pressure; EDV, end-diastolic volume; EDPVR, end-diastolic pressure volume relationship; EDPVR_{WV}, unitless chamber stiffness; EDSSR, end-diastolic stress-strain relationship.

*
p<0.05**
p< 0.01***
P<0.001

Table 4

Tissue morphometric analysis of RBM WT and RBM HET, Sham and ACF mice.

Morphometric Analysis				
	RBM WT		RBM HET	
	Sham	ACF	Sham	ACF
n=	7	9	10	10
BW	28.1 ± 1.1	25.8 ± 0.9	26.4 ± 1	25.4 ± 0.5
LV mg	93.7 ± 2.4	112.7 ± 5.4 ^{**}	87.1 ± 2.9	98.3 ± 2.1 [†]
RV mg	25.2 ± 1.4	34.1 ± 1.7 ^{**}	27.2 ± 1.3	33.3 ± 1.7 ^{##}
LA mg	3.8 ± 0.3	8.2 ± 0.3 ^{***}	3.7 ± 0.2	6.2 ± 0.6 ^{###,†}
RA mg	4.5 ± 0.3	7.6 ± 0.4 ^{***}	3.5 ± 0.3	6.4 ± 0.5 ^{###}
Lung g	149.1 ± 2.8	152.3 ± 5.5	142.5 ± 4.7	147.4 ± 4.1
LV/TL	5.17 ± 0.12	6.19 ± 0.29 ^{**}	4.83 ± 0.17	5.45 ± 0.11 [†]
RV/TL	1.39 ± 0.08	1.87 ± 0.09 ^{**}	1.51 ± 0.08	1.84 ± 0.09 [#]
LA/TL	0.21 ± 0.01	0.45 ± 0.02 ^{***}	0.21 ± 0.01	0.35 ± 0.03 ^{###,††}
RA/TL	0.25 ± 0.02	0.42 ± 0.02 ^{***}	0.19 ± 0.01	0.35 ± 0.03 ^{###}
Lung/TL	8.23 ± 0.16	8.35 ± 0.24	7.91 ± 0.27	8.17 ± 0.22

Significant RBM WT ACF vs. RBM WT Sham at

RBM HET ACF vs. RBM HET Sham at

RBM HET ACF vs. RBM WT ACF

BW: body weight; LV: left ventricle; RV: right ventricle; LA: left atria; RA: right atria.

^{**}
p<0.01^{***}
P<0.001[#]
p<0.05^{##}
p<0.01^{###}
P<0.001[†]
p<0.05^{††}
p<0.01.

Table 5

Conscious echocardiographic analysis of RBM WT and RBM HET, Sham and ACF mice.

Echocardiography Analysis				
	RBM WT		RBM HET	
	Sham	ACF	Sham	ACF
n=	7	9	10	10
LVID;d [mm]	3.72 ± 0.13	4.47 ± 0.17 ^{**}	3.58 ± 0.06	4.73 ± 0.12 ^{###}
LVPWT;d [mm]	0.86 ± 0.02	0.84 ± 0.02	0.92 ± 0.02	0.67 ± 0.03 ^{###,†††}
LVID;s [mm]	2.05 ± 0.15	2.65 ± 0.19 ^{**}	1.9 ± 0.07	1.15 ± 0.06 ^{###}
LVPWT;s [mm]	1.39 ± 0.06	1.41 ± 0.03	1.4 ± 0.04	1.27 ± 0.06
FS [%]	45.13 ± 2.88	41.17 ± 2.63	47.02 ± 1.27	41.94 ± 1.55
LVID;d/PWT;d	4.38 ± 0.22	5.36 ± 0.22	3.92 ± 0.12	7.27 ± 0.45 ^{###,†††}
Ao Velocity [mm/s]	838.5 ± 52.5	1319.6 ± 50.6 ^{***}	849.5 ± 31.1	1262.6 ± 50.7 ^{###}

Significant RBM WT ACF vs. RBM WT Sham at

RBM HET ACF vs. RBM HET Sham at

# p<0.05

p<0.01

RBM HET ACF vs. RBM WT ACF

† p<0.05

†† p<0.01

Abbreviations: HR, heart rate; BPM, beats per minute; LV, left ventricle; LVIDd, LV internal diameter diastole; LVPWTd, LV posterior wall thickness diastole; LVIDs, LV internal diameter systole; LVPWTs, LV posterior wall thickness systole; FS, fractional shortening percent; Eccentricity: LVIDd/WTd.

** p<0.01

*** P<0.001

P<0.001

††† p<0.001.