Myocardial Titin Hypophosphorylation Importantly Contributes to Heart Failure with Preserved Ejection Fraction in a Rat Metabolic Risk Model

Hamdani et al: Titin Hypophosphorylation and Metabolic Risk

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DOI: 10.1161/CIRCHEARTFAILURE.113.000539

Journal Subject Codes: (110) Congestive heart failure; (148) Heart failure – basic studies; (105) Contractile function
Abstract

**Background**—Obesity and diabetes are important metabolic risk factors and frequent comorbidities in heart failure with preserved ejection fraction (HFPEF). They contribute to myocardial diastolic dysfunction (DD) through collagen deposition or titin modification. The relative importance for myocardial DD of collagen deposition and titin modification was investigated in obese, diabetic ZSF1 rats after HFPEF development at 20 weeks of age.

**Methods and Results**—Four groups or rats (Wystar Kyoto, n=11; lean ZSF1, n=11; obese ZSF1, n=11 and obese ZSF1 with high fat diet, n=11) were followed over 20 weeks with repeat metabolic, renal and echocardiographic evaluations and hemodynamically assessed at sacrifice. Myocardial collagen, collagen crosslinking, titin isoforms and phosphorylation were also determined. Resting tension (F_{passive})-sarcomere length relations were obtained in small muscle strips before and after KCl-KI treatment, which unanchors titin and allows contributions of titin and extracellular-matrix to F_{passive} to be discerned. At 20 weeks of age, the lean ZSF1 group was hypertensive whereas both obese ZSF1 groups were hypertensive and diabetic. Only the obese ZSF1 groups had developed HFPEF, which was evident from increased lung weight, preserved LVEF and LV DD. The underlying myocardial DD was obvious from high muscle strip stiffness, which was largely (±80%) attributable to titin hypophosphorylation. The latter occurred specifically at the S3991 site of the elastic N2Bus segment and at the S12884 site of the PEVK segment.

**Conclusions**—Obese ZSF1 rats developed HFPEF over a 20 weeks time span. Titin hypophosphorylation importantly contributed to the underlying myocardial DD.

**Key Words:** diastole; heart failure; diabetes mellitus; obesity; myocardium
Heart failure with preserved ejection fraction (HFPEF) is currently observed in 50% of all heart failure patients. The incidence of HFPEF relative to heart failure with reduced ejection fraction (HFREF) continues to rise and its prognosis fails to improve partly because of lack of a specific HFPEF therapy.

Prevalence of comorbidities is higher in HFPEF than in HFREF. Comorbidities such as obesity and diabetes mellitus (DM) are key constituents of metabolic risk and known to be associated with the progressive left ventricular (LV) remodeling and dysfunction characteristically observed in HFPEF. In HFPEF, body mass index has a U-shaped relation to mortality in contrast to HFREF where it displays an inverse relation with mortality. DM has long time been recognized to be associated with LV diastolic dysfunction. In HFPEF, HFREF and aortic stenosis (AS), DM worsens diastolic LV stiffness through a variety of mechanisms such as myocardial fibrosis, advanced glycation endproducts (AGEs) deposition and high cardiomyocyte stiffness. High cardiomyocyte stiffness was especially evident in HFPEF and AS patients with DM, was associated with hypophosphorylation of the giant cytoskeletal protein titin and corrected in-vitro by administration of protein kinase A (PKA) or G (PKG). Furthermore, patients with high metabolic risk frequently suffer from salt-sensitive hypertension, which is like obesity associated with systemic oxidative stress.

To elucidate the mechanisms underlying myocardial dysfunction in metabolic risk-related HFPEF, the present study investigated: 1) LV hemodynamics; 2) myocardial histology; 3) in-vitro stiffness of small muscle strips; 4) cardiomyocyte stiffness and 5) myocardial titin phosphorylation in hypertensive ZSF1 rats, which became over a 20 weeks period morbidly obese and diabetic because of absence of satiation and unlimited access to a regular (ZSF1-obese) or high-fat diet (ZSF1-obese+HFD).
Methods

An expanded Methods section is available in the Online Data Supplement.

Animal model

Nine-week old male ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11) were obtained from Charles River (Barcelona, Spain) and fed with Purina Diet (#5008). After a 1 week laboratory adaptation period, animals underwent phenotypic evaluation consisting of metabolic cage studies, blood sample collection and echocardiographic evaluation. To assess diastolic function, peak velocity of early (E) and late (A) mitral inflow signals and the ratio of E over E’ (peak velocity of early diastolic lateral mitral annular motion) were measured as an indication of LV filling pressure. From this point onward, a subgroup of ZSF1 obese rats (ZSF1-Obese+11W, n=11) was randomly allocated to receive HFD (Research Diet Inc. #D12492). Weight gain and energy intake were recorded every third day. Phenotypic evaluation was repeated at the 14th and 18th week of life. At 20 weeks of age, animals underwent hemodynamic evaluation under anesthesia and were subsequently sacrificed with procurement of myocardial tissue samples for histological, biochemical and biomechanical studies. Animals were kept in individually ventilated chambers in a controlled environment with a 12-h-light/12-h-dark cycle at 22°C room temperature and had unlimited access to food. Investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH (NIH Publication no. 85–23, revised 1996) and was approved by the ethics committee of the Faculty of Medicine of Porto.

Histology and Collagen Gene Expression

Collagen volume fraction (CVF) was determined by quantitative morphometry with an automated image analysis system in sections stained with collagen-specific picrosirius red. A sircol-based assay was performed to obtain and quantify total, soluble and insoluble collagen, which was calculated by subtracting the amount of soluble collagen from the
amount of total collagen. The degree of cross-linking was calculated as the ratio between soluble and insoluble collagen. Gene expression of Collagen1A1 and Collagen3A1 was performed using real-time PCR.

**Force measurements on small muscle strips and cardiomyocytes**

Cardiomyocytes and muscle strips were incubated respectively for 5 and 30 minutes in relaxing solution supplemented with 0.2 % TritonX-100 to remove all membrane structures and subsequently attached between a force transducer and length motor. Resting tension ($F_{\text{passive}}$) was recorded between 1.9 and 2.3 μm sarcomere length (SL). $F_{\text{passive}}$ of cardiomyocytes was measured before and after PKG incubation. In muscle strips, thick and thin filaments were extracted by immersing the preparation in relaxing solution containing 0.6 M KCl (45 min at 20°C) followed by relaxing solution containing 1.0 M KI (45 min at 20°C). Following the extraction procedure, the muscle bundles were stretched again and the passive force remaining after KCl/KI treatment was assumed to be extracellular matrix-based. Titin-based passive force was determined as total passive force minus extracellular matrix-based passive force.¹⁴

**Titin analysis**

*Titin isoform separation:* Homogenized myocardial samples were analyzed by 1.8% sodium dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE). Protein bands were visualized by Coomassie blue or SYPRO Ruby, scanned, and analyzed densitometrically.¹⁵;¹⁶

*Titin phosphorylation assays:* Following 1.8% SDS-PAGE, gels were stained with Pro-Q Diamond for 1 hour and subsequently with Sypro Ruby overnight. Phosphorylation signals on Pro-Q Diamond-stained gels were indexed to Sypro Ruby-stained titin signals.⁹;¹⁰;¹⁵;¹⁶

*Titin/Phosphotitin immunoblots:* 1.8% SDS-PAGE and Western blot were performed to measure site-specific phosphorylation and expression of titin using custom-made, affinity-
purified, phosphosite-specific antibodies against phospho-S3991 (N2Bus), phospho-S12742 (PEVK) and phospho-S12884 (PEVK) (positions in mouse (*Mus musculus*) titin according to UniProtKB identifier A2ASS6), and antibodies recognizing the corresponding non-phosphorylated sequence around these sites.16

**Statistical analysis**

Groups were compared by two-way repeated measures ANOVA whenever serial acquisitions were obtained for the same animal, and by one-way ANOVA for single acquisitions. Pressure-volume loop analysis was analyzed using LabChart 7 Pro v7.3.1. Values are given as mean±SEM. A 2-tailed test with a probability of value <0.05 was considered significant. Single comparisons were assessed by an unpaired Student *t* test. Bonferroni-adjusted *t* tests were used subsequent for multiple comparisons after repeated measure ANOVA. Statistical analysis was performed with SPSS (Version 15.0; SPSS Inc, Chicago, Ill).

**Results**

**Cardiometabolic risk in obese ZSF1 rats**

ZSF1-Obese and ZSF1-Obese+HFD rats had higher weight gain at 20 weeks of age (Figure 1A). Energy intake was initially also higher in these animals, but leveled off at 20 weeks of age (Figure 1A). In both obese groups, glycemia levels, glucose tolerance and insulin resistance were higher (Figure 1B, Table 1). Hyperglycemia caused glycosuria, increased urine output and compensatory water intake. Proteinuria suggested presence of diabetic nephropathy despite preserved creatinine clearance and plasma protein levels (Table 1).

**Echocardiography, hemodynamics and morphometrics**

Serial echocardiographic studies at 10, 14 and 18 weeks of age demonstrated normal systolic function in all groups (Figure 2A, 2B and Table 2). Concentric LV remodeling was present throughout the entire study in both obese groups (Figure 2A, 2B and Table 2), which
progressively developed diastolic LV dysfunction, evident from a restrictive LV-inflow signal, higher E/E’ and increased left atrial area (LAA) at 14 and 18 weeks (Figure 2B and Table 2).

At 20 weeks of age, hemodynamic evaluation confirmed normal LV systolic performance, evident from LVEF, LVdP/dtmax and ESPVR EESI (Figure 2C and Table 3). Diastolic LV dysfunction was again evident from a prolonged τ, elevated LVEDP, an upward shift of the LV diastolic pressure-volume relationship and a higher LV diastolic chamber stiffness constant (β) (Figure 2C and Table 3).

At sacrifice, lung and heart weights were increased in both obese groups (Figure 3A and Table 4). There was evidence of visceral adiposity with more perirenal and perigonadal fat (Table 4).

**Histology**

Cardiomyocyte hypertrophy was confirmed histologically in both ZSF1-Obese groups (Figure 3B). Collagen volume fraction, collagen cross-linking, procollagen carboxyl-terminal proteinase type I (PCP) and PCP enhancer (PCPE) were similar in all groups (Figure 4). In line with these findings, the relative mRNA expression of collagen 1A1 and collagen 3A3 were also similar among all groups (Figure 4). No significant differences of Lysyl oxidase (LOX) expression were observed between all groups (WKY (1.75±0.44), ZSF1-Lean (1.76±0.19), ZSF1-Obese (1.50±0.35) and ZSF1-Obese+HFD (1.70±0.12)).

**F<sub>passive</sub> in small muscle strips and cardiomyocytes**

The relative contributions of collagen and titin were determined in small muscle strips (Figure 5). F<sub>passive</sub>-SL relations were constructed for SL ranging from 1.9 to 2.3 μm. F<sub>passive</sub> was higher in both obese groups from a SL of 2.075 μm onwards (Figure 5A). To discern the contribution of extracellular matrix (E-matrix), F<sub>passive</sub>-SL relations were also constructed following extraction with KCl/KI (Figure 5B). The contribution of titin was calculated by subtracting at
each SL E-matrix based $F_{\text{passive}}$ from total $F_{\text{passive}}$ (Figure 5C). $F_{\text{passive}}$ attributable to E-matrix and titin were higher in both obese groups respectively from a SL of 2.175 and 2.025 μm onwards. At the upper limit of the physiological SL-range (2.2 μm) titin accounted for 82 and 78% of $F_{\text{passive}}$ in respectively ZSF1-Obese and ZSF1-Obese+HFD groups.

$F_{\text{passive}}$-SL relations of isolated skinned cardiomyocytes were steeper in ZSF1-Obese and ZSF1-Obese+HFD (Figure 5D). Incubation with PKGα returned the $F_{\text{passive}}$-SL relations to control levels (Figure 5D). No significant differences of active tension were observed between groups in single skinned small strips as well as skinned cardiomyocytes.

**Titin hypophosphorylation**

N2B titin isoform expression relative to WKY was similar in all groups (WKY 100±9.4%; ZSF1-Lean 100.5±9.1%; ZSF1-Obese 95.03±9.6%; ZSF1-Obese+HFD 71.58±11.4%), but titin phosphorylation decreased by 67 and 82% in ZSF1-Obese and ZSF1-Obese+HFD rats respectively (Figure 6A). *Ex vivo* phosphorylation by PKG significantly increased all-titin phosphorylation in ZSF1-Obese and ZSF1-Obese+HFD, up to the level measured in WKY and ZSF1-Lean (Figure 6B). Using affinity-purified phosphospecific antibodies phosphorylation status was assessed by Western blot at a conserved serine within the N2Bus segment (S3991 of full-length mouse titin) and at two conserved serines within the PEVK segment (S12742 and S12884) (Figure 6C, D and E). In the obese groups, significant hypophosphorylation was observed at the phospho-N2Bus S3991 site and at the phospho-PEVK S12884 site but comparable phosphorylation at the phospho-PEVK S12742 site. Protein loading was checked by a sequence-specific antibody that corresponded to the phosphospecific antibody.
Discussion

The present study identified cardiac titin hypophosphorylation to be associated with high myocardial stiffness and HFPEF in an obese ZSF1 rat model with high metabolic risk.

**Metabolic risk-related HFPEF model**

At the time of sacrifice at 20 weeks of age, high metabolic risk was clearly evident in the obese ZSF1 rats fed either regular diet or high fat diet. Compared to lean ZSF1 rats or WKY rats, obese ZSF1 rats showed many features of high metabolic risk such as visceral obesity evident from elevated perirenal and perigonadal fat, insulin resistance, hyperglycemia and physical inactivity evident from striated muscle wasting. Arterial blood pressure was elevated in both obese and lean ZSF1 rats. At the time of sacrifice, HFPEF was however only present in the obese ZSF1 rats and high metabolic risk therefore seemed to be a prerequisite in this model for HFPEF development. As such, the current model differs from previous experimental HFPEF models, which largely disregarded metabolic risk as they were carried out in old, hypertensive dogs or in Dahl salt sensitive hypertensive rats. The current model however closely resembles clinical HFPEF where metabolic risk is highly prevalent as evident from numerous HFPEF registries or large outcome trials.

The HFPEF presentation observed in this metabolic risk model also shares characteristic features with clinical HFPEF presentation. After 18 weeks, during closed chest echocardiographic evaluation, the E/E’ ratio was diagnostic of diastolic LV dysfunction (ZSF1-Obese: 17.2±0.8; ZSF1-Obese+HFD: 15.8±1.1). At sacrifice, lung weight was 60% higher in obese animals. The latter probably resulted from episodes of pulmonary edema occurring during physical activity. A similar situation occurs in HFPEF patients who frequently have moderate abnormalities in diastolic LV function at rest but striking elevations of left ventricular filling pressure during exercise because of a steep diastolic LV pressure-volume relation. Steep diastolic LV pressure-volume and myocardial F_{passive-length}
relations were also present in the ZSF1 obese rats. The limited elevation of LVEDP in ZSF1 obese rats during open chest hemodynamic evaluation probably resulted from a reverse effect: thoracotomy and anaesthesia reduced venous return to the heart, which led to a prompt fall in LV filling pressures because of steep diastolic LV pressure-volume and myocardial $F_{\text{passive}}$-length relations. Apart from elevated E/E’, high diastolic LV chamber stiffness and high myocardial stiffness, obese ZSF1 rats also had other evidence of diastolic LV dysfunction such as progressive LA enlargement and a significant increase in $\tau$. The latter could however also be partially accounted for by the higher SAP in ZSF1-obese rats.

Systolic LV function in the ZSF1 obese rats closely resembled systolic LV function of HFPEF patients$^{24}$ as global indices of LV systolic performance (LV $dP/dt_{\text{max}}$, LVEF and Ees) were all preserved. In ZSF1 obese rats, Ees was even higher than in control WKY rats because of a steep end-systolic LV pressure-volume relation. The simultaneous presence of steep end-systolic and end-diastolic LV pressure-volume relations forces the left ventricle to function as a fixed stroke volume pump and explains the swings from pulmonary edema to low output frequently observed in HFPEF patients.$^{25}$

**Titin versus extracellular-matrix**

ZSF1-Obese rats had a steeper myocardial $F_{\text{passive}}$-sarcomere length relation (Figure 5A). After extraction of the cardiac muscle strips with KCl/KI, which depolymerised thick and thin filaments thereby leaving titin unanchored, the contribution of the E-matrix to myocardial $F_{\text{passive}}$ became evident (Figure 5B). Subsequently, the contribution of titin could be calculated by subtracting at each SL the contribution of the E-matrix from the measured $F_{\text{passive}}$ (Figure 5C). For SLs ranging from 2.0 to 2.2 $\mu$m, the contribution of titin greatly exceeded the contribution of the E-matrix. At 2.0 $\mu$m, the contribution of titin was 6.8 and 9.3 times larger than of E-matrix for ZSF1-obese and ZSF1-obese+HFD rats. At 2.2 $\mu$m, the contribution of titin was still 4.6 and 3.6 times larger. SLs ranging from 2.0 to 2.2 $\mu$m covered the
physiological range of LV filling pressures (from 5 to 40 mmHg). Using a thick wall ellipsoid model of the LV and the measured LVEDP of 5 mmHg (Table 3; WKY rats), the calculated LV end-diastolic wall stress (=1.25 kN/m²) corresponded with measured F
\textsubscript{passive} (1.26 kN/m²) at a 2.0μm SL. Similarly, after adjusting the values of LVEDVI and dLVPW-thickness for a 2.2μm SL and substituting LV end-diastolic wall stress by the measured F
\textsubscript{passive} at 2.2μm SL (13.70 kN/m² in ZSF1-obese+HFD), the same thick wall ellipsoid model yielded a LVEDP of 41 mmHg. Hence, up to filling pressures exceeding 40 mmHg, titin accounted for 82 and 78% of F
\textsubscript{passive} in ZSF1-obese and ZSF1-obese+HFD rats respectively. High titin-based stiffness is therefore the main contributor to high myocardial stiffness and likely also to HFPEF development in this metabolic risk-related rat HFPEF model. The importance of intrinsic cardiomyocyte stiffness was also evident from the F
\textsubscript{passive}-SL relation of isolated skinned cardiomyocytes (Figure 5D), which was steeper and shifted upward in both ZSF1-obese and ZSF1-obese+HFD rats. Furthermore, in-vitro administration of PKG to the isolated cardiomyocytes corrected the F
\textsubscript{passive}-SL relations. This in-vitro reversibility suggests the high F
\textsubscript{passive} of cardiomyocytes of obese ZSF1 rats to result more from altered phosphorylation status, than from structural changes of titin, such as isoform shifts or oxidative damage. In-vitro reversibility of high F
\textsubscript{passive} was also observed in the ZSF1-obese+HFD rats, which were exposed to the highest systemic oxidative stress.

Limited involvement of the E-matrix in the high F
\textsubscript{passive} of the obese ZSF1 rats was evident also from histological/biochemical analyses of myocardial tissue. Global myocardial collagen volume fraction, collagen cross linking, collagen 1A1 or collagen 3A3 gene expression and PCP or PCPE activity were unaltered in the obese ZSF1 rats (Figure 4). Despite these findings, there was a small increase in myocardial F
\textsubscript{passive} attributable to the E-matrix at SL > 2.175μm in ZSF1-obese and ZSF1-obese+HFD rats (Figure 5B). This increase
could have resulted from subtle alterations in endomysial collagen which remained undetected by histological analysis or by components of the E-matrix other than collagen.

**Titin hypophosphorylation**

Titin stiffness can be modulated mainly through isoform shifts or alterations of the phosphorylation status. In patients presenting with eccentric LV remodeling after myocardial infarction or with dilated cardiomyopathy, a titin isoform shift from the stiff N2B to the compliant N2BA isoform has been reported.\(^{26,27}\) In patients with concentric LV remodeling related to HFPEF or AS, most studies failed to observe a major shift in titin isoform expression.\(^{9,28}\) Rat hearts predominantly express N2B titin isoform, the proportion of which remained unaffected in the present study by the concentric LV remodeling observed in lean and obese ZSF1 rats.

As previously observed in HFPEF patients, AS patients with type 2 diabetes and old hypertensive dogs with HFPEF,\(^{9,10,16}\) overall N2B titin isoform phosphorylation was greatly reduced in the obese ZSF1 rats, especially when exposed to HFD (Figure 6A). Using site-specific antibodies, the S3991 site and the S12884 of the N2Bus and PEVK segments of titin were identified as being hypophosphorylated (Figure 6C and E). The S3991 site can be phosphorylated by both PKA and ERK2 (extracellular regulated kinase 2)\(^{29,30}\) and was recently also shown to be hypophosphorylated in old hypertensive HFPEF dogs.\(^{16}\) Phosphorylation of the S12742 site of the PEVK segment was unaltered in contrast to old hypertensive HFPEF dogs where it was hyperphosphorylated.\(^{16}\) Increased phosphorylation of the N2Bus segment is reported after PKA or PKG administration\(^ {31-35}\) and shown to lower \(F_{\text{passive}}\) whereas increased phosphorylation of the S12742-PEVK occurs after PKC\(\alpha\) administration and raises \(F_{\text{passive}}\).\(^{36}\) Both PKC\(\alpha\) and CaMKII can phosphorylate the S12884-PEVK site. Phosphorylation of the S12884-PEVK site by CaMKII leads to a reduction of \(F_{\text{passive}}\).\(^{37}\) The elevated \(F_{\text{passive}}\) observed in the present study in the obese ZSF1 rats is
consistent with the observed hypophosphorylation of the S3991 site within the N2Bus segment and the observed hypophosphorylation of the S12884 site within the PEVK segment.

**Conclusions**

Obese ZSF1 rats with a high metabolic risk profile developed HFPEF at 20 weeks of age. The diagnosis of HFPEF was based on lung congestion, preserved global LV systolic function and diastolic LV dysfunction. The latter was evident from elevated E/E’, LA enlargement, high LV diastolic chamber stiffness and high myocardial stiffness. High myocardial stiffness was largely (±80%) attributable to high cardiomyocyte stiffness, which resulted from hypophosphorylation of titin.

**Sources of Funding**

Supported by a grant from the Dutch Heart Foundation (2006B035)(W.J.P.) and from the European Commission (FP7-Health-2010; MEDIA-261409)(W.J.P.)

**Disclosures**

None.

**References**


Table 1. Metabolism and renal function

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<td>Plasma creatinine (mg.dL⁻¹)</td>
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<td>98 ± 17†</td>
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</tr>
<tr>
<td>CCr (mL.min⁻¹.Kg⁻¹)</td>
<td>10.1 ± 0.4</td>
<td>9.2 ± 0.8</td>
<td>7.4 ± 0.8*</td>
<td>9.3 ± 0.5</td>
<td>5.6 ± 0.2†‡</td>
<td>4.4 ± 0.2†‡</td>
</tr>
<tr>
<td>Water intake (mL.Kg⁻¹.d⁻¹)</td>
<td>203 ± 23†</td>
<td>110 ± 16†</td>
<td>91 ± 16†</td>
<td>208 ± 24†</td>
<td>179 ± 20†‡</td>
<td>182 ± 17†‡</td>
</tr>
</tbody>
</table>

OGT, oral glucose tolerance; AUC, area under curve; IR, insulin resistance; CCr, creatinine clearance.

Values are mean ± SEM, n=11, each group. *P<0.05 vs 10th wk; †P<0.05 vs WKY; ‡P<0.05 vs ZSF1-Lean; §vs ZSF1-Obese.
Table 2. Echocardiography

<table>
<thead>
<tr>
<th></th>
<th>10th wk</th>
<th>14th wk</th>
<th>18th wk</th>
<th>10th wk</th>
<th>14th wk</th>
<th>18th wk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WKY</td>
<td>ZSF1-Lean</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>281 ± 16</td>
<td>301 ± 10</td>
<td>290 ± 9</td>
<td>383 ± 11†</td>
<td>372 ± 11†</td>
<td>336 ± 9†</td>
</tr>
<tr>
<td>dLVPW (mm)</td>
<td>1.28 ± 0.07</td>
<td>1.28 ± 0.05†</td>
<td>1.26 ± 0.06†</td>
<td>1.45 ± 0.03†</td>
<td>1.39 ± 0.10‡</td>
<td>1.35 ± 0.09‡</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>447 ± 24</td>
<td>423 ± 16</td>
<td>412 ± 18</td>
<td>490 ± 32</td>
<td>485 ± 16</td>
<td>517 ± 29†</td>
</tr>
<tr>
<td>CI (µL.min⁻¹.cm⁻²)</td>
<td>99 ± 16</td>
<td>131 ± 20†</td>
<td>214 ± 18†</td>
<td>133 ± 20</td>
<td>223 ± 20†</td>
<td>212 ± 16†</td>
</tr>
<tr>
<td>FS (%)</td>
<td>37 ± 2</td>
<td>36 ± 1</td>
<td>38 ± 2</td>
<td>36 ± 1</td>
<td>36 ± 3</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>EF (%)</td>
<td>72 ± 3</td>
<td>72 ± 1</td>
<td>72 ± 4</td>
<td>71 ± 1</td>
<td>71 ± 3</td>
<td>74 ± 2</td>
</tr>
<tr>
<td>S (mm.s⁻¹)</td>
<td>66 ± 7</td>
<td>61 ± 3</td>
<td>54 ± 2</td>
<td>56 ± 4</td>
<td>58 ± 1</td>
<td>64 ± 5</td>
</tr>
<tr>
<td>E/A</td>
<td>1.64 ± 0.09</td>
<td>1.75 ± 0.08</td>
<td>1.88 ± 0.09</td>
<td>1.38 ± 0.04</td>
<td>1.53 ± 0.11</td>
<td>1.61 ± 0.12</td>
</tr>
<tr>
<td>E/E’</td>
<td>11.5 ± 1.0</td>
<td>11.4 ± 0.4</td>
<td>11.2 ± 0.8</td>
<td>11.0 ± 0.4</td>
<td>11.7 ± 0.9</td>
<td>12.0 ± 0.6</td>
</tr>
<tr>
<td>MPI (Tei)</td>
<td>0.70 ± 0.03</td>
<td>0.78 ± 0.02</td>
<td>0.78 ± 0.03</td>
<td>0.77 ± 0.02</td>
<td>0.76 ± 0.04</td>
<td>0.76 ± 0.05</td>
</tr>
<tr>
<td>EDVI (µL.cm⁻³)</td>
<td>1.51 ± 0.05</td>
<td>1.62 ± 0.10</td>
<td>1.90 ± 0.08</td>
<td>1.60 ± 0.05</td>
<td>1.64 ± 0.11</td>
<td>1.87 ± 0.11†</td>
</tr>
<tr>
<td>LAA (mm³)</td>
<td>2.0 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>2.2 ± 0.3</td>
<td>2.1 ± 0.2</td>
<td>2.6 ± 0.2</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>ZSF1-Obese</td>
<td>ZSF1-Obese+HFD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>344 ± 11†</td>
<td>347 ± 11†</td>
<td>328 ± 16</td>
<td>335 ± 12†</td>
<td>329 ± 10</td>
<td>321 ± 11</td>
</tr>
<tr>
<td>dLVPW (mm)</td>
<td>1.56 ± 0.04†</td>
<td>1.50 ± 0.05†</td>
<td>1.49 ± 0.06†</td>
<td>1.53 ± 0.12</td>
<td>1.56 ± 0.11</td>
<td>1.44 ± 0.07</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>698 ± 45</td>
<td>686 ± 42‡</td>
<td>714 ± 33†</td>
<td>642 ± 24</td>
<td>654 ± 48†</td>
<td>671 ± 24†</td>
</tr>
<tr>
<td>CI (µL.min⁻¹.cm⁻²)</td>
<td>136 ± 23</td>
<td>215 ± 20†</td>
<td>255 ± 22†</td>
<td>138 ± 16</td>
<td>224 ± 12†</td>
<td>268 ± 19†</td>
</tr>
<tr>
<td>FS (%)</td>
<td>36 ± 2</td>
<td>37 ± 2</td>
<td>39 ± 2</td>
<td>38 ± 2</td>
<td>40 ± 2</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>EF (%)</td>
<td>71 ± 3</td>
<td>73 ± 3</td>
<td>75 ± 2</td>
<td>73 ± 2</td>
<td>72 ± 3</td>
<td>74 ± 1</td>
</tr>
<tr>
<td>S (mm.s⁻¹)</td>
<td>66 ± 4</td>
<td>67 ± 4</td>
<td>63 ± 6</td>
<td>69 ± 5</td>
<td>66 ± 4</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>E/A</td>
<td>1.33 ± 0.03</td>
<td>1.34 ± 0.09†</td>
<td>1.32 ± 0.08†</td>
<td>1.44 ± 0.04</td>
<td>1.36 ± 0.15†</td>
<td>1.40 ± 0.10‡</td>
</tr>
<tr>
<td>E/E’</td>
<td>12.4 ± 0.4</td>
<td>14.2 ± 0.5†‡</td>
<td>17.2 ± 0.8†‡</td>
<td>12.3 ± 0.2</td>
<td>15.4 ± 0.4†‡</td>
<td>15.8 ± 1.1†‡</td>
</tr>
<tr>
<td>MPI (Tei)</td>
<td>0.78 ± 0.05</td>
<td>0.77 ± 0.06</td>
<td>0.78 ± 0.02</td>
<td>0.77 ± 0.03</td>
<td>0.76 ± 0.04</td>
<td>0.79 ± 0.04</td>
</tr>
<tr>
<td>EDVI (µL.cm⁻³)</td>
<td>1.58 ± 0.13</td>
<td>1.59 ± 0.07</td>
<td>1.86 ± 0.07†</td>
<td>1.59 ± 0.17</td>
<td>1.60 ± 0.12</td>
<td>1.91 ± 0.03†</td>
</tr>
<tr>
<td>LAA (mm³)</td>
<td>2.5 ± 0.2</td>
<td>3.0 ± 0.2†</td>
<td>3.6 ± 0.2†‡</td>
<td>2.6 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>3.4 ± 0.1†‡</td>
</tr>
</tbody>
</table>
HR, heart rate; dLVPW, left ventricular posterior wall measured in diastole; LV, left ventricular; CI, cardiac index; FS, fractional shortening; EF, ejection fraction; S, peak systolic tissue Doppler velocity; E/A, ratio between peak E and A waves of pulsed-wave Doppler mitral flow velocity; E/E’, ratio between peak E wave velocity of pulsed-wave Doppler mitral flow and peak E’ wave velocity of tissue Doppler at the lateral mitral annulus; MPI, myocardial performance or Tei index; EDVI, end-diastolic volume indexed for body surface area; LAA, left atrial area; Values are mean ± SEM, n=6, each group. *P<0.05 vs 10th wk; †P<0.05 vs WKY; ‡P<0.05 vs ZSF1-Lean; §vs ZSF1-Obese.
Table 3. Hemodynamics at age 20 weeks

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>ZSF1-Lean</th>
<th>ZSF1-Obese</th>
<th>ZSF1-Obese+HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA (cm²)</td>
<td>468 ± 5</td>
<td>508 ± 7</td>
<td>651 ± 5*</td>
<td>616 ± 4†</td>
</tr>
<tr>
<td>SAP (mmHg)</td>
<td>117 ± 4</td>
<td>146 ± 6*</td>
<td>181 ± 6*</td>
<td>170 ± 6*</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>94 ± 6</td>
<td>127 ± 6*</td>
<td>149 ± 5*</td>
<td>140 ± 5</td>
</tr>
<tr>
<td>DAP (mmHg)</td>
<td>75 ± 7</td>
<td>106 ± 7*</td>
<td>125 ± 6*</td>
<td>113 ± 5</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>346 ± 10</td>
<td>391 ± 9*</td>
<td>354 ± 12†</td>
<td>336 ± 8</td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>5 ± 0</td>
<td>4 ± 1</td>
<td>9 ± 1*</td>
<td>7 ± 1*</td>
</tr>
<tr>
<td>dP/dt max (mmHg.s⁻¹)</td>
<td>9430 ± 770</td>
<td>11700 ± 964</td>
<td>13000 ± 680*</td>
<td>11000 ± 497</td>
</tr>
<tr>
<td>dP/dt min (mmHg.s⁻¹)</td>
<td>-7880 ± 838</td>
<td>-12600 ± 686*</td>
<td>-11700 ± 541*</td>
<td>-11700 ± 429*</td>
</tr>
<tr>
<td>τ (ms)</td>
<td>8.3 ± 0.3</td>
<td>7.6 ± 0.4</td>
<td>10.5 ± 0.6†</td>
<td>9.5 ± 0.4†</td>
</tr>
<tr>
<td>EF (%)</td>
<td>60 ± 4</td>
<td>55 ± 3</td>
<td>59 ± 4</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>ESPVR EESI (mmHg.µL⁻¹.cm²⁻¹)</td>
<td>0.64 ± 0.15</td>
<td>2.49 ± 0.71*</td>
<td>2.00 ± 0.39*</td>
<td>2.04 ± 0.39*</td>
</tr>
<tr>
<td>EDPVR βI (µL⁻¹.cm²⁻¹)</td>
<td>0.0016 ± 0.002</td>
<td>0.0023 ± 0.002</td>
<td>0.0028 ± 0.002*</td>
<td>0.029 ± 0.004*</td>
</tr>
</tbody>
</table>

BSA, body surface area; SAP, systolic arterial pressure; MAP, mean blood pressure; DAP, diastolic arterial pressure; HR, heart rate; EDP, end-diastolic pressure; dP/dt max, maximum rate of pressure rise; dP/dt min, maximum rate of pressure fall; τ, time constant of isovolumetric relaxation; EF, ejection fraction; EESI, slope of linear ESPVR for indexed volumes; EDPVR, end-diastolic pressure-volume relationship; β I, chamber stiffness constant for indexed volumes, derived from exponential EDPVR.

For the purpose of volume indexation, BSA was estimated as 9.1*(body weight in g)²/³. Values are mean ± SEM, n=11, each group. *P<0.05 vs WKY; †P<0.05 vs ZSF1-Lean; ‡P<0.05 vs ZSF1-Obese.
<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>ZSF1-Lean</th>
<th>ZSF1-Obese</th>
<th>ZSF1-Obese+HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TL (mm)</strong></td>
<td>41.5 ± 0.1</td>
<td>42.1 ± 0.4</td>
<td>40.9 ± 0.4 †</td>
<td>39.6 ± 0.3 *†‡</td>
</tr>
<tr>
<td><strong>LV+IVS weight/ TL (mg.mm⁻¹)</strong></td>
<td>15.4 ± 0.6</td>
<td>16.6 ± 0.8</td>
<td>19.7 ± 1.1 *</td>
<td>20.2 ± 1.3 *†</td>
</tr>
<tr>
<td><strong>RV weight/ TL (mg.mm⁻¹)</strong></td>
<td>4.8 ± 0.4</td>
<td>4.5 ± 0.3</td>
<td>5.5 ± 0.3</td>
<td>5.6 ± 0.2 †</td>
</tr>
<tr>
<td><strong>Lung weight/ TL (mg.mm⁻¹)</strong></td>
<td>51 ± 2</td>
<td>53 ± 2</td>
<td>75 ± 3 †</td>
<td>75 ± 2 †</td>
</tr>
<tr>
<td><strong>Liver weight/ TL (mg.mm⁻¹)</strong></td>
<td>252 ± 9</td>
<td>314 ± 12</td>
<td>906 ± 55 ††</td>
<td>769 ± 29 ††</td>
</tr>
<tr>
<td><strong>Kidney weight/ TL (mg.mm⁻¹)</strong></td>
<td>23.3 ± 0.3</td>
<td>26.0 ± 0.8</td>
<td>37.1 ± 0.9 ††</td>
<td>33.9 ± 1.5 ††</td>
</tr>
<tr>
<td><strong>Perirenal fat weight/ TL (mg.mm⁻¹)</strong></td>
<td>64 ± 4</td>
<td>58 ± 6</td>
<td>362 ± 12 ††</td>
<td>415 ± 14 ††</td>
</tr>
<tr>
<td><strong>Perigonadal fat weight/ TL (mg.mm⁻¹)</strong></td>
<td>62 ± 3</td>
<td>56 ± 5</td>
<td>161 ± 6 †</td>
<td>145 ± 5 ††</td>
</tr>
<tr>
<td><strong>Gastrocnemius weight/ TL (mg.mm⁻¹)</strong></td>
<td>56 ± 1</td>
<td>63 ± 2</td>
<td>53 ± 1 †</td>
<td>48 ± 1 †</td>
</tr>
</tbody>
</table>

TL, tibial length; LV, left ventricle; IVS, interventricular septum; RV, right ventricle. Values are mean ± SEM, n=11, each group. *P<0.05 vs WKY; †P<0.05 vs ZSF1-Lean; ‡P<0.05 vs ZSF1-Obese.
Figure Legends

Figure 1. Body weight, energy intake and metabolism. A. Body weight and energy intake in all groups. B. Glycemia, oral glucose tolerance (OGT) and insulin resistance (IR) in all groups at 10, 14 and 18 weeks of age. *P<0.05 vs. WKY, †P<0.05 vs. ZSF1-Lean, ‡P<0.05 vs ZSF1-Obese. ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11).

Figure 2. Echocardiography. A. Representative examples of sequential Dopplerechocardiographic imaging in all groups. B. LV Mass, Cardiac Index and LV EF in all groups at 10, 14 and 18 weeks of age. C. Representative LV pressure-volume from all groups at 20 weeks of age. *P<0.05 vs. WKY, †P<0.05 vs. ZSF1-Lean, ‡P<0.05 vs. ZSF1-Obese. ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11).

Figure 3. Lung weight, heart weight and cardiomyocyte hypertrophy. A. Lung and heart weights in all groups at sacrifice (20 weeks of age). B. Representative histological images of LV myocardium in all groups showing progressive cardiomyocyte hypertrophy. *P<0.05. ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11).

Figure 4. Collagen-volume fraction and cross-linking. A. Representative images of myocardial fibrosis (Picosirius red; 200x magnification) in all groups. B-G. Collagen volume fraction, collagen cross-linking, procollagen carboxyl-terminal proteinase type I (PCP), PCP enhancer (PCPE), relative mRNA expressions of Collagen1A1 and Collagen3A1
in all groups. Nine-week old male ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11).

**Figure 5.** F\textsubscript{passive} of small muscle strips and cardiomyocytes. A. F\textsubscript{passive}-Total vs. SL curves for all groups. B. F\textsubscript{passive}-E matrix vs. SL curves for all groups. C. F\textsubscript{passive}-Titin vs. SL curves for all groups. D. F\textsubscript{passive}-cardiomyocyte vs. SL curves for all groups before (solid curves) and after PKG treatment (dashed curves). Curves are second-order polynomial fits to the means (n=16/4 muscle strips/group and n=15/4 cardiomyocytes/group). In A-C: *P<0.05 ZSF1-Obese+HFD vs. WKY, #P<0.05 ZSF1-Obese vs. WKY, †P<0.05 ZSF1-Obese+HFD vs. ZSF1-lean; In D: *P<0.05 ZSF1-Obese+HFD vs. WKY and ZSF1-Lean, #P<0.05 ZSF1-Obese vs. WKY and ZSF1-Lean and †P<0.05 effect of PKG.

**Figure 6.** Total and site-specific phosphorylation of titin N2Bus/PEVK segments. A. Total titin phosphorylation in all groups. B. Effect of Ex-\textit{vivo} phosphorylation by PKG on all-titin phosphorylation in all groups. C. Titin phosphorylation at S3991 site in all groups. D. Titin phosphorylation at S12742 site in all groups. E. Titin phosphorylation at S12884 in all groups. *P<0.05. ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=10) and Wistar-Kyoto rats (WKY, n=5).
Figure 1

Body weight and energy intake and metabolism
Figure 2

Echocardiography

A

2D Parasternal Short axis view

Transmitral flow Pulsed Doppler

Myocardial velocity Tissue Doppler

B

<table>
<thead>
<tr>
<th>Time (wks)</th>
<th>WKY</th>
<th>ZSF1-Lean</th>
<th>ZSF1-Obese</th>
<th>ZSF1-Obese+HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td></td>
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<td>14</td>
<td></td>
<td></td>
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<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

LV mass (mg)
Cardiac Index (L/min/m²)
Ejection Fraction (%)

LV Pressure (mmHg)
LV Volume Indexed (μL/cm²)
Figure 3

Lung weight, heart weight and cardiomyocyte hypertrophy

A

Lung weight

Heart weight

B

Cardiomyocyte area (μm²)

WKY, ZSF1-Lean, ZSF1-Obese, ZSF1-Obese+HFD

* indicates significant difference compared to WKY
**Figure 4**

**Collagen volume fraction and cross-linking**

A

![Collagen images](http://circheartfailure.ahajournals.org/Downloaded from)

B

Collagen volume fraction (%)

C

Collagen cross-linking

D

Carboxy-termini proteinase of procollagen type I (a.u.)

E

Carboxy-termini proteinase of procollagen type III (a.u.)

F

PCR: Collagen 1α1

G

PCR: Collagen 3α1

- WKY
- ZSF1-Lean
- ZSF1-Obese
- ZSF1-Obese+HFD
Figure 5

$F_{\text{passive}}$ of small muscle strips and cardiomyocytes

A

B

C

D
Figure 6

Total and site-specific phosphorylation of titin-N2Bus/PEVK titin segments
Myocardial Titin Hypophosphorylation Importantly Contributes to Heart Failure with Preserved Ejection Fraction in a Rat Metabolic Risk Model


Circ Heart Fail. published online September 6, 2013;
Circulation: Heart Failure is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-3289. Online ISSN: 1941-3297

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Supplemental Material

Myocardial Titin Hypophosphorylation Importantly Contributes to Heart Failure with Preserved Ejection Fraction in a Rat Metabolic Risk Model
METHODS AND MATERIALS

Animal model

Nine-week old male ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11) were obtained from Charles River (Barcelona, Spain) and fed with Purina Diet (#5008). After a 1 week laboratory adaptation period, animals underwent phenotypic evaluation consisting of metabolic cage studies, blood sample collection and echocardiographic evaluation. From this point onward, a subgroup of ZSF1 obese rats (ZSF1-Obese+HFD, n=11) was randomly allocated to receive HFD (Research Diet Inc. #D12468). Weight gain and energy intake were recorded every third day. Phenotypic evaluation was repeated at the 14th and 18th week of life. At 20 weeks of age, animals underwent hemodynamic evaluation under anaesthesia and were subsequently sacrificed with procurement of myocardial tissue samples for histological, biochemical and biomechanical studies. Animals were kept in individually ventilated chambers in a controlled environment with a 12-h-light/-dark cycle at 22°C room temperature and had unlimited access to food. Investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH (NIH Publication no. 85–23, revised 1996) and was approved by the ethics committee of the Faculty of Medicine of Porto.

ZSF1 rats (Charles River, Barcelona, Spain) are first generation hybrids between the ZDF (Zucker diabetic fatty) and SHHF (spontaneously hypertensive heart failure) rats, which carry two different leptin receptor mutations (fa and fa^p). A female ZDF (+/fa) is crossbred with a male SHHF (+/fa^p) rat, leading to either lean ZSF1 or obese ZSF1 rats (fa/fa^p). Both ZSF1 rats are hypertensive, since they inherit the hypertension gene from the SHHF rats but only obese ZSF1 rats develop diabetes and dyslipidemia.

Metabolic studies and renal function

After a 24h acclimatization period, water and energy intake, weight gain and urine output were recorded, and a 24h urine sample was collected in metabolic cages (Techniplast, Buguggiate). After successive 24h rest intervals, all rats underwent oral glucose and insulin resistance testing, at the end of 12h feed-deprivation periods. Glycemia was recorded at baseline and 15, 30, 60, 90 and 120 min
(Freestyle-Mini) after a 1 g.Kg\(^{-1}\) glucose gavage or a 0.5 U.Kg\(^{-1}\) intraperitoneal insulin injection, respectively. Echocardiographic studies were conducted, after another 24h rest period, and a blood sample (1.5 mL) was collected from the subclavian vein under anaesthesia, at the end of the procedure.

**Echocardiography**

Rats (n=11 per group) were anaesthetized by inhalation of 8% sevoflurane in vented containers, orotracheally intubated and mechanically ventilated. Anaesthesia was maintained with sevoflurane (1-2.5%) titrated to avoid the toe pinch reflex. Rats were placed in left-lateral decubitus on a heating pad, the ECG was monitored (lead II) and their temperature was kept at 38ºC. The skin was shaved and depilated. After applying prewarmed echocardiography gel a linear 15MHz probe (Sequoia 15L8W) was gently positioned on the thorax. Systolic and diastolic wall thickness and cavity dimensions were recorded, in M-mode and 2D echocardiography, at the level just above the papillary muscles in the parasternal short axis view. The long axis diastolic dimensions of the left ventricle and transverse aortic root diameter were recorded by 2D and M-mode echocardiography, respectively, in the parasternal long axis view. Aortic flow velocity was recorded by pulsed-wave Doppler just above the aortic valve. Mitral flow velocity tracings were obtained with pulsed-wave Doppler just above the mitral leaflets, peak systolic tissue velocity and E’ were measured with tissue Doppler at the medial mitral annulus and lateral mitral annulus, respectively, and left atrial dimensions were measured, at their maximum, by 2D echocardiography in the four chamber view. Acquisitions were done while transiently suspending mechanical ventilation and recordings were averaged from three consecutive heartbeats (Siemens Acuson Sequoia C512). Left ventricular (LV) mass and volumes were calculated by the 2D area-length method. Myocardial performance index was retrieved from the mitral flow pattern.

**Haemodynamic evaluation**

After sedation (100 µg.kg\(^{-1}\) and 5 mg.kg\(^{-1}\) intraperitoneal fentanyl and midazolam, respectively), anaesthesia (8 and 2.5–3% sevoflurane for induction and maintenance, respectively; Penlon Sigma Delta), endotracheal intubation, mechanical ventilation (TOPO, Kent scientific), 8 mL.kg\(^{-1}\).h\(^{-1}\)
intravenous warm Ringer’s solution infusion (NE-1000, New Era Pump Systems), temperature maintenance at 38ºC on a heating pad, left thoracotomy, LV and right ventricular (RV) pressure-volume catheter insertion through the apex (SPR-838 and PVR-1045 Millar Instruments, respectively), and ascending aorta probe placement (Transonics) that allowed CO measurement (Active Redirection Transit Time Flowmeter, Triton Technology), signals were continuously acquired (MPVS 300, Millar Instruments), recorded at 1000 Hz (ML880 PowerLab 16/30, ADinstruments), and analyzed (PVAN 3.5, Millar Instruments). Recordings were obtained at suspended end-expiration. The LV catheter was advanced to record systemic arterial pressure. Parallel conductance was assessed with hypertonic saline. After euthanasia (100 mg.kg\(^{-1}\) intravenous pentobarbital), blood (4mL) was collected for storage (-80ºC) and for volume calibration (910–1048, Millar instruments). LV volumes were varied using transient inferior vena cava constrictions by adjusting a sling around the inferior vena cava. Organs were weighed, RV and LV + interventricular septum (IVS) were weighed after dissection, and tibia length (TL) was measured. Samples were either snap-frozen and stored at -80ºC or processed for histology. Weights were normalized to TL due to the large body weight differences between groups.

Histomorphological analysis was performed on elastica-von-Giesson and hematoxylin-eosin stained, 4 \(\mu\)m thick, sections of tissue placed in 4% buffered formaldehyde solution. As previously validated,\(^3\) MyD was determined perpendicularly to the outer contour of the cell membrane at nucleus level.

**Collagen- volume fraction and cross-linking**

The collagen volume fraction (CVF) was determined by quantitative morphometry with an automated image analysis system in sections stained with collagen-specific picro-sirius red, as previously reported in ZSF1 lean (ZSF1-Lean, \(n=11\)), ZSF1 obese (ZSF1-Obese, \(n=22\)) and Wistar-Kyoto rats (WKY, \(n=11\)).\(^3\)\(^4\) All measurements were performed in duplicate by 2 independent observers. The inter- and intra-observer coefficients of variation were <4%. To distinguish between cross-linked (insoluble) and non-cross-linked (soluble) collagen a colorimetric procedure was employed. First, a fast green-sirius red assay was performed to identify and quantify total collagen. In a second step, a sircol-based assay was performed to obtain and quantify soluble collagen. The amount of insoluble
collagen was calculated by subtracting the amount of soluble collagen from the amount of total collagen. The degree of cross-linking was calculated as the ratio between the insoluble and the soluble forms of collagen. All measurements were performed in duplicate. The inter- and intra-assay coefficients of variation were 5 and 3%, respectively.\textsuperscript{3-5}

**RNA isolation and gene expression analysis**

Frozen tissue sections ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=11) and Wistar-Kyoto rats (WKY, n=5) were minced in Trizol and further disrupted during 10 minutes of vigorous shaking. To extract the RNA, chlorophorm was added, mixed, and centrifuged. The aqueous phase containing the RNA was collected in a separate tube, and isopropanol was added. For precipitation, the RNA solution was centrifuged 15 minutes at 4°C at high speed. The RNA pellet was then further purified using the RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. One $\mu$g of RNA was reverse transcribed into cDNA using the High Capacity Kit (Applied Biosystems) and then further diluted to a final concentration of 5 ng/$\mu$L cDNA.

The relative quantification of mRNA levels was carried out on a 7900 HT (Applied Biosystem). To assess the mRNA expression of the target genes, real-time PCR was performed using 5 $\mu$L of the gene expression master mix (Applied Biosystems) and 0.5 $\mu$L of the gene expression assay for Col1A1 (Rn01463848_m1) and Col3A1 (Rn01437683_m1) (each includes forward and reverse primers as well the fluorescently FAM-labelled probe) from Applied Biosystems, and 1 $\mu$L of cDNA in a final volume of 10 $\mu$L. Quantification of the house keeping gene GAPDH (Rn99999916_s1) as an internal control was performed for each sample. Data were normalized to 18S RNA level as an endogenous control and are expressed using the formula $2^{-\Delta\Delta C_T}$ in comparison to the corresponding untreated controls.

**Force measurements on skinned cardiomyocytes and strips**

**Single skinned cardiomyocytes**

Force measurements were performed on single skinned cardiomyocytes as described.\textsuperscript{3;4;6} Cardiomyocytes were isolated from WKY, ZSF1-Lean, ZSF1-Obese and ZSF1-Obese+HFD rat hearts
Briefly, samples were defrozen in relaxing solution (in mmol/L: free Mg, 1; KCl, 100; EGTA, 2; Mg-ATP, 4; imidazole, 10; pH 7.0), mechanically disrupted and incubated for 5 min in relaxing solution supplemented with 0.5% Triton X-100. The cell suspension was washed 5 times in relaxing solution. Single cardiomyocytes were selected under an inverted microscope (Zeiss Axiovert 135, 40x objective) and attached with silicone adhesive between a force transducer and a piezoelectric motor as part of a "Permeabilized Myocyte Test System" (1600A; with force transducer 403A; Aurora Scientific, Aurora, Ontario, Canada).

Cardiomyocyte $F_{\text{passive}}$ was measured in relaxing buffer at room temperature within a sarcomere-length range between 1.9 and 2.3 µm. Force values were normalized to myocyte cross-sectional area calculated from the diameter of the cells, assuming a circular shape. As a test of cell viability, each cardiomyocyte was also transferred from relaxing to maximally activating solution (pCa4.5), at which isometric force developed. Once a steady state force was reached, the cell was shortened within 1 ms to 80% of its original length to determine baseline force. Only cells developing active forces >20 kN/m² were included in the analysis. The passive tension was measured under steady state shear (viscous and elastic properties). Subsequently cardiomyocytes were incubated in relaxing solution supplemented with the PKG1α (0.1 U/mL; Sigma, batch 034K1336), guanosine cGMP (10 µmol/L, Sigma) and dithiothreitol (6 mmol/L; Sigma). After 40-min-long incubation with PKG1α, $F_{\text{passive}}$ measurements were again performed in relaxing solution at SL 1.9-2.3 µm.

**Skinned muscle strips**

Left papillary muscles were dissected after sacrificing the animals (n=16/4 muscle strips/group). Small muscle strips were created and chemically permeabilized in a 1% Triton X-100 solution for 30 minutes. After clipping both ends, the strips were mounted between a length motor and a force transducer on top of an inverted microscope. Strips were activated at 20°C with solutions containing a saturating Ca²⁺ concentration to determine maximal active tension (force/cross sectional area) at a sarcomere length (SL) of 2.0 µm. Subsequently, strips were stretched from SL 1.9 µm to 2.3 µm, with a velocity of 10% muscle length per second and while in a solution with low Ca²⁺ (pCa9), to determine passive tension generation. Afterwards, the strips underwent an extraction protocol of 45 minutes in
0.6M KCl followed by 45 minutes in 1.0M KI to depolymerize thick and thin filaments respectively, leaving titin unanchored, and the strips were again passively stretched as described above. The remaining tension after this procedure is extraction-insensitive and caused by extracellular matrix, i.e. collagen. The passive tension was measured under steady state shear (viscous and elastic properties).

**Titin-isofrom separation and titin phosphorylation by Pro-Q Diamond/Sypro Ruby**

Titin isoforms were separated as described. Briefly, tissue samples from ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=10) and Wistar-Kyoto rats (WKY, n=5) were solubilized in 50 mM Tris-sodium dodecyl sulfate (SDS) buffer (pH 6.8) containing 8 µg/mL leupeptin (Peptin Institute, Japan) and phosphatase inhibitor cocktail (PIC [P2880], 10 µL/mL; Sigma). Samples were heated for 3 minutes at 96°C and centrifuged. Then, samples (20 µg; equal concentration checked by spectroscopic methods) were separated by agarose-strengthened 1.8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run at 5 mA constant current for 16 hours. The phosphorylation state of cardiac titin was determined using Pro-Q Diamond phosphoprotein. Titin gels were stained for one hour with Pro-Q Diamond, and then overnight with Sypro Ruby (Molecular Probes). Staining was visualized using the LAS-4000 Image Reader (Fuji Science Imaging Systems) and signals were analyzed using Multi Gauge V3.2 or AIDA software. Finally signals obtained from Pro-Q Diamond staining were normalized to signals obtained from Sypro Ruby staining.

**Titin and phospho-titin analysis by Western blot**

1.8% SDS-PAGE followed by Western blot was performed to measure expression and site-specific phosphorylation of titin. Samples from ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=10) and Wistar-Kyoto rats (WKY, n=5) were applied at a concentration that was within the linear range of the detection system (30 µg dry weight; checked by spectroscopic methods). Sequence-specific or phosphosite-specific anti-titin antibodies were custom-made by Eurogentec, Belgium (positions in mouse (Mus musculus) titin according to UniProtKB identifier A2ASS6)). The following affinity-purified antibodies were used:

9;10:
- Anti-titin- mouse N2Bus against EEGKSLSFPLA (rabbit polyclonal; 1:1000).
- Anti-phospho-N2Bus (S3991 in mouse titin and S4010 in human titin) against EEGK(PO3H2)LSFPLA (rabbit polyclonal; 1:500).
- Anti-PEVK-domain against (cross-species conserved) sequence EVVLKSVLRK (1:1000)
- Anti-phospho-PEVK-domain (S12742 in mouse titin and S11878 in human titin) against EVVLK(PO3H2)VLRK (1:500)
- Anti-PEVK-domain against (cross-species conserved) sequence KLRPGSGGEKPP (1:100)
- Anti-phospho-PEVK-domain (S12884 in mouse titin and S12022 in human titin) against KLRPG(PO3H2)GGEKPP (1:500)

The amino acid sequences of rat are identical to the amino acid sequences of mouse.9;10

Titin antibodies gave specific signals on Western blots with cardiac tissue. Following SDS-PAGE, proteins were transferred to Hybond ECL nitrocellulose membranes. Blots were pre-incubated with 3% bovine serum albumin in Tween Tris-buffered saline (TTBS); 10 mmol/L Tris-HCl; pH 7.6; 75 mmol/L NaCl; 0.1% Tween) for 1 hour at room temperature. Then, blots were incubated overnight at 4°C with the primary antibodies against the respective (phospho) protein. After washing with TTBS, primary antibody binding was visualized using secondary horseradish peroxidase-labeled, goat-anti-rabbit/mouse antibodies (dilution 1:1000; DakoCytomation) and enhanced chemiluminescence (ECL Western blotting detection; Amersham Biosciences). Staining was visualized using the LAS-4000 Image Reader and analyzed with Multi Gauge V3.2 or AIDA software. PVDF stains were saved for comparison of protein load. Loading was also controlled by comparing signals of the phospho-specific antibodies with those of the respective sequence-specific antibodies. Finally signals obtained from phospho-specific antibodies were normalized to signals obtained from sequence-specific antibodies.

**Data analysis**

Circumferential LV end-diastolic wall stress ($\sigma$) was computed using a thick wall ellipsoid model of the LV:
\[ \sigma = \frac{PD}{2h} \times \left[ 1 - \left( \frac{h}{D} \right) - \frac{D^2}{2L^2} \right] \]

where \( P \) is LV end-diastolic pressure, \( h \) is LV wall thickness, and \( D \) and \( L \) are LV short axis diameter and long axis length at the midwall.

Groups were compared by two-way repeated measures ANOVA whenever serial acquisitions were obtained for the same animal, and by one-way ANOVA for single acquisitions. Pressure-volume loop analysis was analyzed using LabChart 7 Pro v7.3.1. Values are given as mean±SEM. A 2-tailed test with a probability of value <0.05 was considered significant. Single comparisons were assessed by an unpaired Student \( t \) test. Bonferroni-adjusted \( t \) tests were used subsequent for multiple comparisons after repeated measure ANOVA. Statistical analysis was performed with SPSS (Version 15.0; SPSS Inc,Chicago,III).

Supplemental References