Altered Titin Expression, Myocardial Stiffness, and Left Ventricular Function in Patients With Dilated Cardiomyopathy

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Background—The role of the giant protein titin in patients with heart failure is not well established. We investigated titin expression in patients with end-stage heart failure resulting from nonischemic dilated cardiomyopathy, in particular as it relates to left ventricular (LV) myocardial stiffness and LV function.

Methods and Results—SDS-agarose gels revealed small N2B (stiff) and large N2BA (compliant) cardiac titin isoforms with a mean N2BA:N2B expression ratio that was significantly ($P<0.003$) increased in 20 heart failure patients versus 6 controls. However, total titin was unchanged. The coexpression ratio was highest in a subsample of patients with an impaired LV relaxation pattern (n=7), intermediate in those with pseudonormal filling (n=6), and lowest in the group with restrictive filling (n=7). Mechanical measurements on LV muscle strips dissected from these hearts (n=8) revealed that passive muscle stiffness was significantly reduced in patients with a high N2BA:N2B expression ratio. Clinical correlations support the relevance of these changes for LV function (assessed by invasive hemodynamics and Doppler echocardiography). A positive correlation between the N2BA:N2B titin isoform ratio and deceleration time of mitral $E$ velocity, $A$ wave transit time, and end diastolic volume/pressure ratio was found. These changes affect exercise tolerance, as indicated by the positive correlation between the N2BA:N2B isoform ratio and peak $O_2$ consumption (n=10). Upregulated N2BA expression was accompanied by increased expression levels of titin-binding proteins (cardiac ankyrin repeat protein, ankrd2, and diabetes ankyrin repeat protein) that bind to the N2A element of N2BA titin (studied in 13 patients).

Conclusions—Total titin content was unchanged in end-stage failing hearts and the more compliant N2BA isoform comprised a greater percentage of titin in these hearts. Changes in titin isoform expression in heart failure patients with dilated cardiomyopathy significantly impact filling by lowering myocardial stiffness. Upregulation of titin-binding proteins indicates that the importance of altered titin expression might extend to cell signaling and regulation of gene expression. *(Circulation. 2004;110:155-162.)*

**Key Words:** heart failure ◦ diastole ◦ echocardiography ◦ mechanics ◦ myocardium

Left ventricular (LV) diastolic dysfunction in patients with heart failure is associated with significant morbidity and mortality. Abnormalities in patients with diastolic dysfunction include impaired LV relaxation and increased chamber/myocardial stiffness and ventricular dilatation.1 Recently, molecular changes in sarcomeric and interstitial proteins as well as their relation to passive stiffness were evaluated in patients with systolic dysfunction. In particular, titin and collagen were reported to exhibit isoform changes that can adversely affect or compensate for increased chamber stiffness.2-5 Whereas the role of collagen is well investigated, there is controversy regarding changes in titin expression. Titin is a giant sarcomeric protein that extends from the Z disk to the M-line and is encoded by a single gene. Its differential splicing leads in the myocardium to the expression of N2B and N2BA isoforms that differ in size.6 Smaller mammals express predominantly N2B titin, whereas larger mammals, including humans, coexpress both N2B and N2BA titins.6 Because of its shorter extensible I-band region, expression of the N2B isoform results in a higher passive myocardial stiffness than that of the N2BA isoform.7 Recent studies in animal models of dilated cardiomyopathy and pressure overload have revealed adjustments in titin expression that increased myocardial passive stiffness. With

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respect to human studies, there has been a paucity of data.\(^3,8\) In one study, titin content was reported to be significantly reduced.\(^8\) However, this study was conducted prior to the development of tissue handling and solubilization techniques that limit titin degradation. In another study, titin-based stiffness of myofibrils isolated from patients with coronary artery disease who underwent cardiac transplantation was decreased.\(^7\) These previous studies did not determine whether titin-based changes impacted LV function. Furthermore, the recent findings from animal models may not all extrapolate to patients, where heart disease is usually more chronic in nature. We undertook this investigation to evaluate the changes in titin isoform expression in patients with end-stage heart failure, in particular as it relates to LV structure and function. To avoid the confounding effect of ischemia/infarction on the expression of titin, we selected patients with nonischemic dilated cardiomyopathy. We studied titin isoform expression at the transcript and protein levels and measured myocardial stiffness using skinned muscle strips and compared it to LV stiffness assessed by echocardiography. Finally, we evaluated the expression levels and cellular localization of several titin-binding proteins that might be involved in stretch-dependent regulation of gene expression.

### Methods

#### Patients

The patient sample included 20 end-stage heart failure patients with nonischemic dilated cardiomyopathy (DCM) who underwent cardiac transplantation over the previous 3 years. Clinical, echocardiographic, invasive hemodynamic evaluation by right heart catheterization and coronary angiography were performed (within 60 days of transplantation) when LV filling pressures and echocardiographic measurements were determined. Full-thickness specimens of the LV midanterior, midseptal, and midlateral walls were removed from explanted hearts as well as from normal subjects who died from noncardiac causes who were used as controls. Normal subjects (n = 6) were 40 to 51 years old (mean, 46 ± 2.4 years) and the cause of death was head trauma (n = 2), gunshot wound (n = 2), intracranial hemorrhage (n = 1), or drug overdose (n = 1). These controls had normal cardiac dimensions, wall thickness, and ejection fraction (EF: 60% to 70%). Samples were frozen in liquid nitrogen and stored at −80°C.

#### Echocardiographic Studies and Assessment of LV Stiffness

LV volumes (end diastolic [EDV], end systolic [ESV]) and ejection fraction (EF) were determined from 2D images per published criteria.\(^9\) Mitral inflow was recorded at the mitral valve tips. On the fraction (EF) were determined from 2D images per published criteria.\(^9\) Mitral inflow was recorded at the mitral valve tips. On the

#### Transcript Studies

The recently developed titin exon microarray that monitors all 363 titin exons of the human titin gene was used to study titin transcripts in myocardium from patients with DCM.\(^16\) As controls we used commercially available total RNA isolated from human fetal myocardium (9 donors: 7 male, 2 female, aged 18 to 21 weeks; Stratagene, La Jolla, Calif) and adult control LV myocardium (obtained from 6 males, aged 21 to 74 years, mean 37 ± 9 years; Ambion and Biochain Institute Inc). Expression levels in different muscle types were compared with a 2-tailed t test for samples of unequal variance. Exons identified as upregulated/downregulated had P < 0.05, and in fetal versus adult control, myocardium had ≥3-fold change of their mean values and ≥2-fold for DCM versus adult myocardium. (See also Lahmers et al\(^16\) and the Results section.)

#### Western Blot Analysis and Immunofluorescence

Western blots were probed with rabbit affinity–purified polyclonal antibodies, which were raised to unique regions of cardiac ankyrin repeat protein (CARP), ank2d, and diabetes ankyrin repeat protein (DARP), as described previously.\(^17\) Primary antibody incubations were followed by alkaline phosphatase–conjugated goat antirabbit IgG. Immunoreactive bands were visualized in detection buffer supplemented with nitro blue tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate substrate, according to the manufacturer’s instructions (Vector). Blots were scanned and their intensity quantified using Scanalytic’s 1DScan software, version 2.03. Results were normalized relative to actin. (For additional details, see Cazorla et al.\(^7\) For immunofluorescence, sections were cut from frozen tissue and labeled with anti-CARP as described previously,\(^8\) followed by either goat antirabbit Texas Red–conjugated IgG (1:800) or Alexafluor 488–conjugated IgG (1:800). Some sections were costained with avian anti-N2A,\(^18,19\) followed by goat anti-avian secondary antibody.

#### Muscle Mechanics

Frozen muscle samples were added to a solution consisting of 50% glycerol in relaxing solution (for composition of relaxing solution, see Wu et al\(^30\) at 4°C. We have previously shown that this results in excellent tissue preservation (Figure 3B in Freiburg et al.\(^11\) whereas others\(^12,11\) have shown that active and passive mechanical properties are well maintained in freeze-thawed samples. Muscle strips were dissected from LV specimens, skinned overnight at 4°C in relaxing solution containing 1% vol/vol Triton X-100, and then washed thoroughly with relaxing solution. All solutions contained protease inhibitors.\(^7,30\) Passive force was measured during a stretch from the slack length (L₀) with a velocity of 10% L₀/s and amplitude of 15% L₀ before and after muscle was extracted in relaxing solution.
containing first 0.6 mol/L KCl and then 1 mol/L KI. The KCl/KI-sensitive tension was assumed to be titin-based and KCl/KI-insensitive tension to be collagen-based. The cross-sectional area of muscle was measured and used to convert forces into tensions. Experiments were done at 20°C to 22°C.

Statistics
Analysis was performed with SigmaStat statistical software, version 2.0. The control and the heart failure groups were compared using a 2-tailed unpaired t test. Repeated measures ANOVA was used to compare the N2BA:N2B titin isoform ratios of the anterior, lateral, and septal walls and ANOVA for the titin isoform ratios among the 3 patient groups divided according to the mitral inflow pattern. Regression analysis (linear and nonlinear) was applied to relate the ratio of titin isoforms to echocardiographic indices of LV function and peak O₂ consumption (PVO₂). A P value ≤0.05 was considered significant.

Results
Patients
The heart failure group (20 patients; 5 women) had a mean age of 56±12 years. All patients had advanced heart failure with a New York Heart Association class III to IV with a mean EF of 21.5±7.4%. All underwent coronary angiography as part of the transplant evaluation, and none had coronary artery disease. The mean value for peak oxygen consumption for those able to exercise (n = 10) determined by respiratory gas analysis was 11.9±3 mL/kg/min. Findings are summarized in the Table.

<table>
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<th>Hemodynamic Findings in the Patient Sample</th>
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<td>Heart rate per minute</td>
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Data are presented as mean±SEM.

Titin Expression at Protein Level
High-resolution SDS-agarose gels revealed in both control and disease samples prominent N2B and N2BA bands and only limited levels of titin degradation (reflected by the minor T2 bands); examples are shown in Figure 1A. Densitometry showed that the N2BA band in patients was more diffuse with evidence for a second lower-mobility N2BA isoform that gives rise to a “shoulder” in the densitometry trace (see Figure 1B, arrows). To well represent the whole LV, for each DCM patient we used in the analysis shown below the mean value from the anterior, lateral, and septal walls. Because the N2BA:N2B ratios of the 3 walls were not significantly different (P=0.65), results were similar if comparisons were made between corresponding individual walls.

Densitometric gel analysis revealed that the total titin to MHC ratio and the ratio of T2 to MHC (reflecting titin degradation) in normal subjects and patients with dilated cardiomyopathy were similar (Figure 2, A and B). The mean ratio of N2BA to N2B titin was 0.56±0.06 in normal hearts (range, 0.4 to 0.8) and increased significantly to 0.97±0.07 in DCM hearts.
Thus, total titin was not different in patients, but the mean expression level of the more compliant N2BA isoform was increased at the expense of the stiffer N2B isoform. Segregating patients by Doppler-derived LV filling patterns into impaired relaxation, pseudonormal, and restrictive filling subgroups (see Methods section) revealed that the subgroups were significantly different with respect to titin expression (ANOVA: \( P < 0.004 \), using Kruskal-Wallis test, \( P < 0.05 \)). The mean N2BA:N2B expression ratio increased in the following order: restrictive filling, pseudonormal, and impaired relaxation subgroups, with significant differences in the mean expression ratio of the subgroups (Figure 2D).

**Titin Expression at Transcript Level**

Considering that reactivation of fetal gene expression occurs in many myocardial disease states\(^2\) and our recent finding of a fetal cardiac titin isoform that is larger than adult N2BA titin\(^1\), we explored whether expression of fetal cardiac titin occurs in DCM patients. We used the recently developed titin exon microarray to study titin at the transcript level and compared myocardium from DCM patients with that of fetal and adult controls (see Methods section). Hearts from patients with DCM were selected on the basis of their SDS-agarose electrophoresis patterns that showed the clearest evidence for a low-mobility N2BA band, i.e., that had the most pronounced shoulder in the densitometry profile (hearts 91, 95, and 100; mean N2BA:N2B expression ratio 0.99). Typical results are shown in Figure 3A. Comparing exon expression levels in human fetal myocardium with those of human adult control myocardium showed a large number of fetal specific exons (consistent with previous findings). However, comparing titin exon expression patterns in DCM myocardium with those in adult control myocardium revealed that the majority of these fetal titin specific exons were not elevated in DCM patients (Figure 3B). This analysis indicates that the examined DCM hearts do not express fetal cardiac titin. Upregulated N2BA expression is instead likely due to upregulation of adult N2BA-type isoforms.

**Correlations Between Titin Expression and LV Function and PVO\(_2\)**

To determine whether titin expression correlates with overall myocardial performance, we studied the correlation between the N2BA:N2B ratio and various clinical measures of LV function. Significant direct correlations existed between the ratio of N2BA to N2B titin isoforms and LV volumes: EDV and ESV were larger and EF was reduced in patients with a higher N2BA:N2B titin isoform ratio (Figure 4, A through C). The N2BA:N2B titin isoform ratio was also significantly related to mean wedge pressure \( (r = -0.61, P < 0.01) \), such that a higher ratio was associated with lower wedge pressure.

To assess LV stiffness, we focused on the mitral early diastolic velocity deceleration time (DT of E) and A velocity transit time from LV inflow to LV outflow (A wave transit time), both of which have been shown previously (see Methods section) to reflect LV stiffness (higher LV stiffness associated with shorter DT of E and shorter A wave transit times). Patients with higher N2BA:N2B titin isoform ratio had longer DT of E times and longer A wave transit times.

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**Figure 3.** A, Analysis of human cardiac muscle transcripts by a 50mer oligonucleotide array representing all 363 exons of the human titin gene. RNA isolated from human fetal myocardium and LV myocardium from adult control subjects and patients with DCM was reverse transcribed, biotinylated, and hybridized to the exon microarray. Hybridization quality was indistinguishable for biotinylated cDNA reverse transcribed from DCM myocardial RNA and control adult and fetal myocardial RNA. (Note large blocks of exons that are positive only in fetal myocardium.) B, Comparison of fetal and adult control transcripts reveals 21 exons located in the tandem Ig and PEVK region of titin that are upregulated in the fetal transcript of which 3 exons are 2-fold upregulated in DCM myocardium. Results were obtained with a t test. A Wilcoxon test gave identical results for the fetal versus adult control comparison [except that the \( P \) value was 0.002 for all exons identified in the Table]. As for the DCM versus adult control comparison, exon 54 was not significantly upregulated with the Wilcoxon test while exons 192 and 208 were still upregulated but had slightly different \( P \) values \( [0.04 \text{ and } 0.008, \text{ respectively}] \).
nificantly increased \( r=0.8; P<0.01 \) with the expression ratio. Overall, these data support the notion that an elevated N2BA:N2B expression ratio is associated with lower LV diastolic stiffness and filling pressures and, importantly, a better exercise tolerance (increased \( \text{PVO}_2 \)).

**Relation of Titin Expression to Myocardial Passive Stiffness**

Passive stiffness was measured in myocardial muscle strips dissected from the LV walls of hearts that had either a low N2BA:N2B expression ratio (0.54±0.06; \( n=4 \) hearts) or a high ratio (1.55±0.14; \( n=4 \) hearts), and the contributions of titin and collagen to passive myocardial tension and stiffness were determined (see Methods section). The hearts with the higher proportion of N2BA belonged to patients with impaired relaxation and longer DT, whereas DT was short (<160 ms) in the patients whose hearts had the lower N2BA:N2B expression ratio. Results disclosed that both titin-based tension and stiffness were significantly reduced in the high-expression-ratio hearts (Figure 5, A and B), with no significant changes in collagen-based tension and stiffness (Figure 5, C and D).

**Relation of Titin Expression to Titin-Binding Proteins**

We studied titin-binding proteins that bind to titin’s N2A element (found in N2BA titin and not in N2B titin) that have been suggested to be part of stress response pathways: CARP and the 2 closely related proteins, ankrd2 and DARP. Using Western blots, we identified an \( \approx 38 \)-kDa protein that interacts with anti-CARP and an \( \approx 40 \)-kDa protein that interacts with ankrd2, both of which are significantly upregulated in patients (Figure 6A, i and ii). (Andkrd2 cross-reacted with an \( \approx 70 \)-kDa protein, the identity of which has to be

Figure 4. LV function as assessed by echocardiographic methods in patients before heart transplantation versus N2BA:N2B expression ratio. Lines are second-order polynomial fits \( (R^2 \) ranged from 0.71 to 0.74 and \( P \) values were all <0.01). See text for additional details.

Figure 5. Myocardial passive tension–sarcomere length (SL) relationships derived from titin (A) and collagen (C) in patients with DCM. Inset of A shows the 2 groups of hearts that were studied: low and high N2BA:N2B expression ratio. Titin-based passive tension is significantly higher in the group with a low titin isoform ratio. The slopes of the passive tension–SL relationships were determined (changes in SL expressed as strain) and plotted against SL to derive titin-based (B) and collagen-based (D) stiffness. (*, significant differences.)

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Localization (Figure 6B, right), with limited evidence for nuclear localization in normal myocytes, which is in contrast to previous work with heart failure that reported a significant reduction in titin expression. This reduction might be attributable to the use of more severe disease states or to suboptimal techniques available at the time (such as absence of protease inhibitors). On the basis of the present study, we conclude that in hearts of DCM patients, total titin expression levels are not significantly different from those of control subjects.

In large mammals, total titin consists of the relatively large N2BA and smaller N2B isoforms. In the present study, we found that the N2BA:N2B expression ratio of human control myocardium is 0.56±0.06, a value similar to that reported by Neagoe et al for normal donor hearts (calculated from their Table 1 at 0.4). Together these studies establish that in human control myocardium, the expression of N2B titin dominates. As for heart failure patients with DCM, a novel finding of the present study is that the significant upregulation of N2BA titin (Figure 2C) and its correlation with diastolic function parameters of the patients (Figure 2D). Changes in titin expression have also been reported for animal models of DCM, but interestingly in an opposite direction of what was found here. Work on a rapid-pacing canine model of DCM heart failure revealed reduced N2BA expression. It is currently unclear why changes in titin expression in animal models are opposite to those in humans. It is possible that the canine rapid-pacing model is unique and that the titin response is different from that in human heart failure. Another explanation that warrants testing is the long-term nature of heart disease in humans with an initial adaptation similar to that seen in animal models (decrease in N2BA titin) and a subsequent change in the opposite direction (increase in N2BA titin) when hearts go into failure and become available for study.

Complex adaptations in titin isoform expression are also suggested by using diastolic filling patterns to segregate patients into impaired relaxation, pseudonormal, and restrictive filling subgroups (see Methods section for details) and the finding that these groups had the highest (1.19), intermediate (0.85), and lowest N2BA:N2B (0.63) expression ratios, respectively. Because the restrictive filling pattern is associated with poor prognosis, patients with a higher N2BA:N2B ratio may have a more favorable outlook.

**Titin Isoform Expression and Myocardial Stiffness**

The I-band region of titin is extensible and functions as a molecular spring that develops passive force when stretched, giving rise to passive myocardial stiffness. The differences in size of the N2BA (∼3.3 mega dalton) and N2B (∼3.0 mega dalton) isoforms are largely due to differential splicing of the tandem Ig and the PEVK segment of titin that comprise this molecular spring (longer segments in N2BA titin). As a result, a given change in sarcomere length gives rise to a titin-based force that is lower for N2BA titin (its longer extensible region results in lower fractional extension) than for the N2B isoform. Thus, as expression shifts toward the N2BA isoform, the contribution of titin to passive myocardial stiffness increases, which might be expected to result in a reduced myocardial stiffness. Indeed, patients with a higher N2BA:N2B ratio were found to have a lower LV fractional shortening, which is consistent with a decrease in myocardial stiffness. This finding is in contrast to previous work in patients with heart failure that reported a significant reduction in titin expression. This reduction might be attributable to the use of more severe disease states or to suboptimal techniques available at the time (such as absence of protease inhibitors). On the basis of the present study, we conclude that in hearts of DCM patients, total titin expression levels are not significantly different from those of control subjects.

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stiffness is expected to be less. Consistent with this notion, passive stiffness in muscle strips isolated from heart failure patients was significantly reduced in the group with high N2BA:N2B expression ratio (Figure 5, A and B). The average reduction in passive stiffness was 2.5-fold. This is slightly larger than can be explained by the 2-fold change that we calculated on the basis of the measured change in isoform expression ratio (for details concerning the calculations, see Lahmers et al).16 Although various explanations for this discrepancy exist, an interesting possibility is a difference in the phosphorylation state of the extensible region of titin (phosphorylation affects titin’s stiffness)25 between control subjects and patients that increases the difference in passive stiffness beyond what is expected from the change in isoform expression. Thus, minor discrepancies remain to be explained, but overall, our findings reveal that passive myocardial stiffness is significantly reduced in hearts with high N2BA:N2B expression ratio.

**Titin Isoform Expression and LV Function**

The clinical correlations that we found (Figure 4) support that the changes in titin expression are relevant for LV diastolic function. We noted a positive correlation between the N2BA:N2B titin isoform ratio and DT of mitral E velocity, a wave transit time, EDV/EDP ratio (all of which relate inversely to LV chamber stiffness), and EDV. These findings are consistent with the view that ventricles with a high N2BA:N2B ratio are more compliant (lower stiffness) than those in patients with lower N2BA:N2B ratios.

Correlations between titin expression and systolic function were also observed. (Figure 4B and C). Although the correlations may be serendipitous and not reflect a cause and effect, the recent finding that titin modulates the calcium sensitivity of active force25 is consistent with our observations. Fukuda et al reported that titin-based passive force enhances calcium sensitivity and this effect was reduced in myocardium that expresses a high level of N2BA titin (because it develops less passive force).26 Thus, the correlation between lower EF and higher N2BA:N2B ratio (Figure 4C) might be explained by a decreased calcium sensitivity of the contractile machinery in the presence of increased N2BA titin.

The finding that increased N2BA expression may negatively impact systolic function (reduced ESV and EF) but improve diastolic function (increased chamber compliance) leads to the question of whether the net effect is beneficial. The positive correlation between the N2BA:N2B isoform ratio and PVO2 that our work disclosed suggests that at least during exercise the positive effect on diastolic function dominates. Considering that exercise capacity strongly correlates with survival in patients with CHF,27 this finding may be clinically relevant and raises the possibility that differential splicing of titin may be an important determinant of outcome in patients with dilated cardiomyopathy.

**Titin-Binding Proteins**

Titin’s N2A element (found in the I-band region of the molecule) assembles a protein complex that contains CARP and the 2 closely related proteins, ankrd2 and DARP. A recent study of a muscular dystrophy mouse model revealed dysregulation of the N2A protein complex, with CARP upregulation as a possible primary event.17 The present finding of upregulation of CARP, ankrd2, and DARP in the myocardium of patients with DCM shows that changes in the N2A protein complex also occur in myocardium of patients with heart failure. Whether upregulation of these N2A-binding proteins is a result of N2BA upregulation (only N2BA titin contains the N2A element) or is differentially regulated remains to be established. Finally, in control cardiomyocytes, CARP is known not only to bind to titin’s N2A element but also to be present in the nucleus,23 where it exerts transcriptional control by interacting with the transcriptional regulator YB-1.6 Absence of nuclear labeling with anti-CARP antibodies in heart failure patients (Figure 6B, right) suggests possible changes in CARP-regulated gene expression in these patients. These are novel findings that warrant more extensive follow-up studies.

**Conclusion**

In heart failure patients with DCM, N2BA titin is upregulated at the expense of N2B titin, and these changes in titin expression correlate with parameters of LV functions. Various indices of LV chamber stiffness obtained by echocardiography indicate that diastolic stiffness is significantly lower in hearts with high N2BA:N2B expression ratio. This conclusion is supported by passive stiffness measurements on muscle strips. In contrast to the positive impact on diastolic function, upregulation of N2BA titin may negatively impact systolic function (reduced ESV and EF). Improvement of PVO2 suggests that during exercise, the positive effect on diastolic function is dominant. Finally, we recently reported that during normal postnatal heart development, titin expression switches from a predominance of compliant fetal N2BA titin to the more stiff N2B isoform, giving rise to increased passive stiffness.16 We now add that in DCM a reverse
process occurs with decreasing passive stiffness due to upregulation of adult N2BA isoforms (Figure 7).

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