

Talin1 Has Unique Expression *versus* Talin 2 in the Heart and Modifies the Hypertrophic Response to Pressure Overload^{*[5]}

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Background: Talin is an integrin-actin linker essential for integrin activation.

Results: Talin1 has distinct developmental and postnatal expression in heart *versus* Talin2. Cardiac-myocyte specific Talin1 deletion alters physiological and molecular responses of the myocardium to stress.

Conclusion: Talin1 has a unique mechanotransductive role in the cardiomyocyte.

Significance: Reduction of talin1 in cardiomyocytes may have beneficial effects in the stressed myocardium.

Integrins are adhesive, signaling, and mechanotransduction proteins. Talin (Tln) activates integrins and links it to the actin cytoskeleton. Vertebrates contain two talin genes, *tln1* and *tln2*. How Tln1 and Tln2 function in cardiac myocytes (CMs) is unknown. Tln1 and Tln2 expression were evaluated in the normal embryonic and adult mouse heart as well as in control and failing human adult myocardium. Tln1 function was then tested in the basal and mechanically stressed myocardium after cardiomyocyte-specific excision of the Tln1 gene. During embryogenesis, both Tln forms are highly expressed in CMs, but in the mature heart Tln2 becomes the main Tln isoform, localizing to the costameres. Tln1 expression is minimal in the adult CM. With pharmacological and mechanical stress causing hypertrophy, Tln1 is up-regulated in CMs and is specifically detected at costameres, suggesting its importance in the compensatory response to CM stress. In human failing heart, CM Tln1 also increases compared with control samples from normal functioning myocardium. To directly test Tln1 function in CMs, we generated CM-specific Tln1 knock-out mice (Tln1cKO). Tln1cKO mice showed normal basal cardiac structure and function but when subjected to pressure overload showed blunted hypertrophy, less fibrosis, and improved cardiac function *versus* controls. Acute responses of ERK1/2, p38, Akt, and glycogen synthase kinase 3 after mechanical stress were strongly blunted in Tln1cKO mice. Given these results, we conclude that Tln1 and Tln2 have distinct functions in the myocardium. Our data show that reduction of CM Tln1 expression can lead to improved cardiac remodeling following pressure overload.

The molecular basis of cardiomyopathies remains poorly understood, but recent studies have shown that they can be caused by defects in proteins coordinating cell matrix as well as cell-cell adhesion (1, 2). Cell-matrix adhesion is mainly mediated by integrins that connect the extracellular matrix with the actin cytoskeleton of the cell. Integrins are heterodimeric surface receptors composed of α and β subunits that function by assembling a complex of proteins in structures termed focal adhesions (3). Muscle cells have similar structures termed costameres that share many of the protein components of focal adhesions. Costameres circumferentially align with the Z-disk of the myofibrils and are critical for muscle attachment, sarcomeric integrity, and cellular mechanotransduction (4).

The connection between integrins and the actin cytoskeleton is indirect and occurs through a series of structural proteins including vinculin (Vcl),² α -actinin, integrin-linked kinase (ILK), PINCH, parvin, kindlin, filamin, tensin, and the subject of this manuscript, talin (Tln) (5).

Tln is a large dimeric cytoskeletal protein, 270 kDa, 2540 amino acids in size. It links integrins to the actin cytoskeleton via its connections to the cytoplasmic domain of the integrin β subunit. Importantly, in addition to its role as a structural linker protein, Tln has been shown to be essential for integrin activation (6). Studies in *Caenorhabditis elegans* (7), *Drosophila* (8), and mice (9) have demonstrated that Tln is an essential protein for integrin adhesion. Tln orthologs have been identified in all multicellular eukaryotes. Recent work has shown that vertebrates contain two *Tln* genes encoding closely related isoforms, Tln1 and Tln2 (10), whereas slower eukaryotes possess only a single *Tln* gene (11). In mammals Tln1 is ubiquitously

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[5] This article contains supplemental Tables 1–4 and M1–M4 and Figs. 1–7.

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² The abbreviations used are: Vcl, vinculin; ILK, integrin-linked kinase; Tln, talin; CM, cardiac myocytes; NRVM, neonatal rat ventricular myocyte; POL, pressure overload; TAC, transverse aortic constriction; EC, endothelial cell; CF, cardiac fibroblast; PE, phenylephrine; ANF, atrial natriuretic; DCM, dilated cardiomyopathy; HW, heart weight; TL, tibia length; BW, body weight; CARP, cardiac ankyrin repeat protein; NF, non-failing; PINCH, Particularly Interesting Cys-His-rich Protein.

expressed but Tln2 shows a more restricted distribution with high levels in the heart, brain, and skeletal muscle (10). Global *Tln1* knock-out (KO) mice display an embryonic lethal phenotype by E8.5–9.0 due to gastrulation defects (9), indicating that although both *Tln* genes encode very similar proteins (74% identical), Tln2 cannot replace Tln1 function in the entire embryo. *Tln2* KO mice have also been generated and develop a mild skeletal myopathy due to defects in myotendinous junction integrity, again indicating that alternative isoforms cannot completely compensate for loss of another (12).

Integrins are key mechanotransducers in many cells, including cardiac myocytes (CM) and, therefore, are intimately involved in the process of cardiac hypertrophy. The role of Tln in the CM has not been previously explored and is the focus of this study.

MATERIALS AND METHODS

Detailed methods and reagents used are provided in the [supplemental material](#).

Generation of *Tln1* Conditional KO—Tln1-flox mice and α -MHC nuclear Cre mice have been described (13, 14). The mice were maintained on a mixed 129/C57Bl/FVB strain genetic background. All mice used in the study were males. Control and Tln1cKO mice were matched for age in each experiment, and littermates were used as controls for cKO. Methods for genotyping and analysis of recombination are shown in the [supplemental data](#) (primers used are shown in [Supplemental Table M1](#).)

Cardiac Myocyte Culture—Adult mouse CMs and neonatal rat ventricular myocytes (NRVMs) were isolated as previously (15, 16). Adult CMs were plated onto laminin-coated plates, whereas NRVMs were plated onto fibronectin-coated plates, both at 10 μ g/ml.

RNA and Protein Analyses—RNA was extracted and purified from heart tissue or isolated adult CMs and then analyzed using real-time (RT) quantitative PCR with an Applied Biosystems 7300 machine (Foster City, CA). For quantification of Tln transcripts in CM, standard curves for Tln1 and Tln2 were generated using known Tln fragments, directly measured, and used as input templates. Detail methodology (including primers used for this study ([Supplemental Tables M2–M4](#))) is provided in the [supplemental material](#).

Western Blotting and Immunoprecipitation Analyses—At study termination, protein was prepared from cardiac tissue or cells and analyzed by immunoblotting. For immunoprecipitation experiments 500 μ g of NRVM protein lysate was incubated with 3 μ g of Tln1 (97H6) or Tln2 (68E7) primary antibody and rotated overnight at 4 °C. The following day the solution was incubated with 50 μ l of protein G-agarose beads (Roche Applied Science) at 4 °C for 1 h. Beads were washed 3 times with lysis buffer (10 mM Tris-HCl, pH 8, 100 mM NaCl, 1% Nonidet P-40, 2 mM sodium orthovanadate, protease inhibitor mixture (Roche Applied Science)) and resuspended in 50 μ l of 2 \times Laemmli sample buffer. Samples were analyzed by Western blotting using the appropriate antibodies. Densitometric quantitation of protein bands was with a ChemImagerTM 4400 imaging system and Alphaes software (Alpha Innotech Corp., San Leandro, CA). Further information is provided in the [supplemental material](#).

Microscopy—Immunomicroscopic analyses of embryos or adult hearts were performed on 5–8- μ m (embryos) or 10- μ m (adult heart) cryosections using previously published techniques (17). Primary antibodies were diluted as follows: anti- β 1D integrin (1:1500), vinculin (1:100), Tln1 (1:10), Tln2 (1:50), PECAM (1:100), dystrophin (1:100), titin (1:10), and Cre (1:1000). Alexa Fluor secondary antibodies were diluted (1:800), Cy secondaries (1:100), and DAPI (1:2000). Results were visualized via deconvolution optics using a Deltavision deconvolution microscope (Applied Precision, Inc., Seattle, WA) or with a Zeiss AxioObserver microscope and Axiovision software (Zeiss, Thornwood, NY). In addition some data were obtained with an Olympus Fluoview confocal microscope. Cross-sectional measurements were performed and calculations made using NIH ImageJ.

Surgeries and Physiological Analyses—Chronic pressure overload (POL) was imposed via transverse aortic constriction (TAC) on 10–12-week-old male mice, as previously described (15). At study termination, systolic pressure gradients were measured by selective cannulation of the left and right carotid arteries; morphometry was recorded, and heart tissue was utilized for histological, protein, and RNA analyses. Echocardiography was performed on animals under isoflurane anesthesia and measured by investigators blinded to the genotype of the animal, as previously described, using a Vevo 770 machine (Visualsonics, Toronto, Canada) (15). For acute TAC, mice were subjected to pressure overload or SHAM surgery for 10 min, after which hearts were removed and frozen immediately in liquid nitrogen for subsequent analysis.

Statistical Methods—Data were compiled and shown as the means \pm S.E. Data were evaluated using unpaired, two-tailed t-tests (95% confidence interval) using GraphPad Prism software (GraphPad Inc., San Diego, CA). A *p* value < 0.05 was considered significant.

RESULTS

***Tln1* and *Tln2* Are Both Highly Expressed in Embryonic CMs, but *Tln2* Becomes the Dominant Isoform in Adult CMs**—To begin to determine the role of the Tln in the heart and, more specifically, in CMs, we analyzed the expression level and pattern of both Tln isoforms during embryogenesis as well as in adult mouse heart using isoform-specific antibodies (18). Western blot analyses were performed on cardiac tissue from embryonic day (E) 16, postnatal day (P) 1, and adult (1- and 3-month) samples. This showed that both Tln isoforms were highly expressed in the embryonic heart and that Tln1 expression becomes significantly reduced in the adult heart as compared with the level of Tln2 (Fig. 1A).

To precisely localize the expression of these isoforms, immunomicroscopic analyses were performed. Tln2 was detected from E9 onward. Co-staining of Tln2 with titin (used as a muscle marker) and PECAM (an endothelial cell marker) showed that Tln2 expression was restricted to muscle cells in the developing myocardium, with no expression in endocardium/endothelium. Tln1 was also detected at E9 but in contrast was found to be expressed in both myocardial and endocardial cells (Fig. 1, B and C).

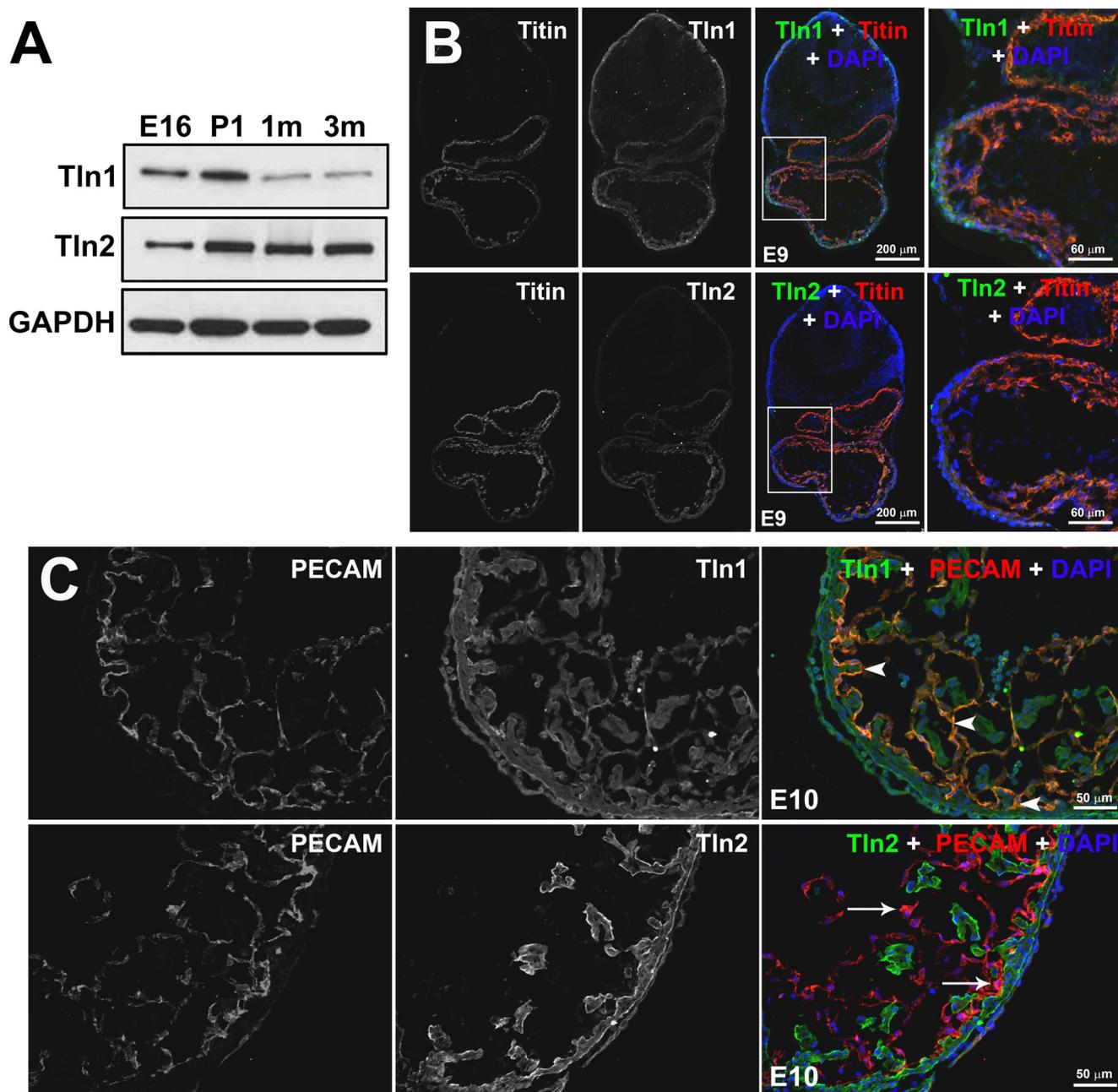


FIGURE 1. Tln1 was detected in both embryonic myocytes and endothelial cells, whereas Tln2 showed muscle-specific expression in the embryonic heart. *A*, Western blot analyses from embryonic (*E16*), neonatal (*P1*), and adult hearts (*1m* and *3m*) show that Tln1 and Tln2 are both expressed in the embryonic heart but that only Tln1 expression becomes reduced with aging, whereas Tln2 expression remains high. *B* and *C*, cardiac tissue from mouse embryos was stained with isoform-specific antibodies to microscopically evaluate the expression pattern of Tln1 and Tln2 in combination with a muscle-specific marker (Titin) (*B*) and an EC-specific marker (PECAM) (*C*). Tln1 was found in both CM (*B*, co-localized with titin) and also in ECs (arrowheads in *C*). Tln2 was found in CM (*B*) but not in ECs (arrows, *C*). *B*: green = Tln1 or Tln2, red = titin; blue = DAPI. *C*: green = Tln1 or Tln2; red = PECAM; blue = DAPI.

Next we determined how the Tln forms were expressed in adult myocardium. Microscopic studies of adult heart tissue showed that Tln1 was easily visualized in endothelial cells (ECs), with only minimal expression detected in adult CMs (Fig. 2*A*). In contrast, Tln2 was strongly expressed at the CM membrane where it colocalized with dystrophin that was used as a myocyte marker, and at intercalated disks (Fig. 2*B*). In addition we proved that Tln 2 localized in myocyte costameres, where it co-localized with β 1D integrin but not in the sarcomere itself, which was marked by anti-titin antibodies (Fig. 2*C*). Correlative staining of isolated adult CMs also showed that

Tln1 was detected minimally and only in a diffuse pattern, whereas Tln2 localized strongly at costameres. (Fig. 2, *D* and *E*).

To analyze the relative protein expression of the Tln forms in the various cell types within the myocardium, we analyzed expression in isolated adult CMs, ECs, and cardiac fibroblasts (CF). Tln1 was expressed in all three cell types, but its expression in adult CMs was significantly less than the robust expression detected in adult ECs and CFs. In contrast Tln2 was expressed only in CMs and CFs and was not detected in ECs (Fig. 2*F*). We also determined the amount of Tln1 versus Tln2 transcripts in CM using quantitative reverse transcriptase PCR.

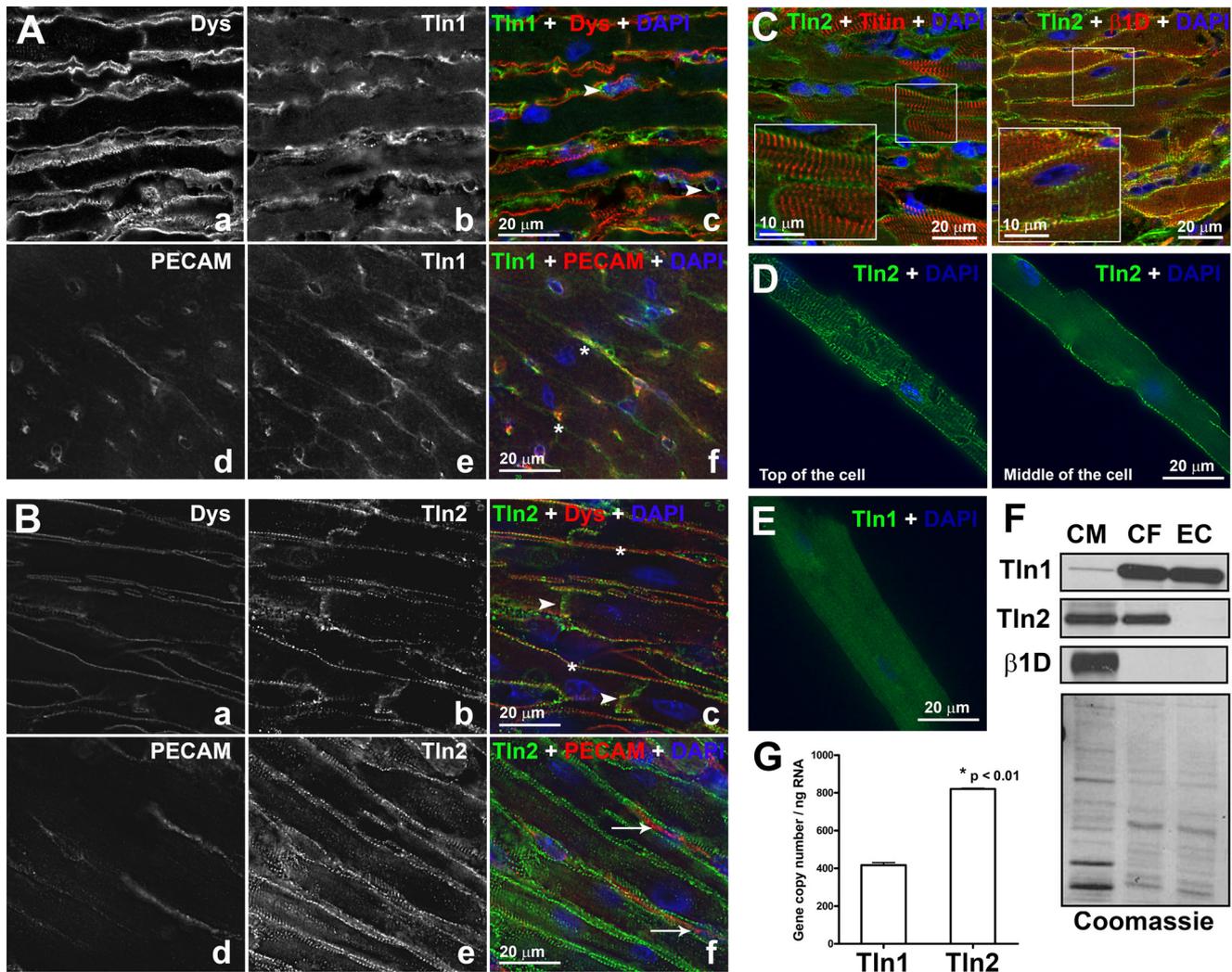


FIGURE 2. Tln1 was dominantly localized to endothelial cells, whereas Tln2 expression was restricted to cardiac myocytes in the adult heart. Adult mouse cardiac tissue (>2 months of age) was evaluated for expression of Tln1 or Tln2. *A–C*, Tln1 and Tln2 were localized with isoform-specific antibodies. Dystrophin (*Dys*) was used as a muscle marker, and PECAM was used to mark ECs. *A*, Tln1 was not detected in the CM membrane (*arrowheads*, *Ac*) but only in ECs (***, *Af*). *B*, Tln2 was detected in CMs (***, *Bc*) but not in ECs (*arrow*, *Bf*). Tln2 was also detected in intercalated disks in CMs (*arrowhead*, *Bc*). *C*, Tln2 was localized at the cardiomyocyte costamere. Microscopic analyses were performed to evaluate the subcellular localization of Tln2 using titin as a sarcomeric marker and β 1D integrin as a costameric marker. Tln2 did not colocalize with titin but was clearly detected at the myocyte costamere where it colocalized with β 1D integrin. *D* and *E*, microscopic analyses were also performed in isolated adult cardiac myocytes to evaluate the subcellular localization of Tln1 and Tln2. *D*, Tln2 was localized clearly in the costameres at the sarcolemmal membrane. This is most easily seen in the image labeled *Top of the cell*, which was acquired via a three-dimensional Z-stack analysis. *E*, Tln1 was only visible as diffuse staining through the CM. *F*, Western blotting of protein from isolated adult CMs, cardiac ECs, and CFs showed that Tln1 was detected minimally in CMs but highly expressed in ECs and CFs. Tln2 was expressed highly in CMs and CFs but not in ECs. β 1D integrin was used as a myocyte marker. Coomassie staining shows equally protein loading. *G*, the copy number of Tln1 and Tln2 gene transcripts in isolated CMs was calculated from Ct values derived from standard curve amplification plots using quantitative RT-PCR. Tln2 copy number were significantly higher compared with Tln1 (***, $p < 0.01$ versus Tln1, Student's *t* test, $n = 3$). Data are expressed as the mean \pm S.E. ($n = 3$).

Transcript copy number per ng of CM RNA was calculated using calibrated curves with known amounts of Tln input template. CM Tln2 transcripts were expressed at twice the copy number of Tln1 transcripts, supporting the hypothesis that Tln2 is the main Tln isoform in CM (Fig. 2*G*).

To extend our data from mouse to man, we next performed immunomicroscopic analyses of human adult cardiac tissue. Like the mouse, we found that Tln2 was strongly expressed in human CMs and also that it was detected in a costameric pattern. In contrast, Tln1 was expressed in non-myocytes as well as in CMs, with only weak staining in the CM membrane as shown by colocalization with dystrophin (supplemental Fig. 1, *A* and *B*).

Together, these results indicate that during embryogenesis, Tln1 and Tln2 are both highly expressed in CMs, yet with maturation of the myocardium, Tln1 becomes regionalized to ECs with only minimal expression in CMs. In contrast, Tln2 is expressed in developing CMs and postnatally becomes the main Tln isoform in cardiac muscle. Data in human heart were similar to mouse but with higher Tln1 expression noted in human cardiac muscle compared with mouse. Given the varied expression of Tln1 and Tln2, we hypothesized that the two Tln isoforms would have distinct functions in the myocardium.

Cardiac Stress Regulates the Expression and Localization of Tln1 in CMs—Our group and others have shown that integrins play important roles in the response to varied types of cardiac

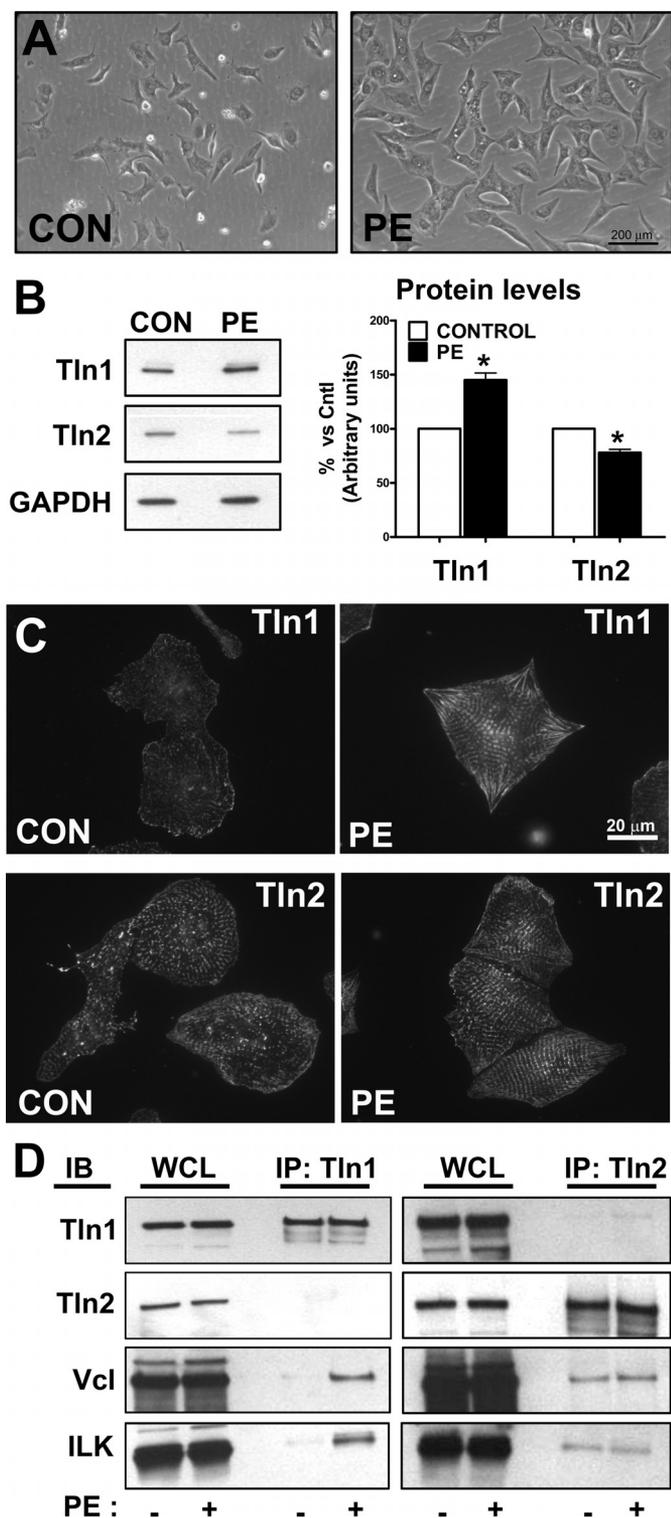


FIGURE 3. Hypertrophic induction of NRVMs with adrenergic stimulation increased Tln1 expression, its association with Vcl and ILK, and its localization to pre-costameric structures; Tln2 localization and association were not changed, and expression became reduced. NRVMs stimulated with PE for 48 h were used as a model of cardiac hypertrophy. *A*, phase-contrast images of NRVMs show PE causes cellular hypertrophy as compared with control (CON) cells. Scale bar = 200 μ m. *B*, Western blotting shows that PE causes an increase in Tln1 but a decrease in Tln2. Densitometry of each protein band was obtained, and values of Tln1 or Tln2 expression were normalized to GAPDH for each sample. Control expression (Tln/GAPDH) was considered 100% in all experiments, and changes in Tln/GAPDH after PE stimulation were then compared with normalized control values as shown. Graphics data are the mean \pm S.E., $n = 4$ independent experiments. *, $p < 0.05$ versus

stress such as that caused by adrenergic stimulation and POL, both of which can lead to a hypertrophic response of the myocyte (15, 19). Because the expression and localization of integrins and integrin-related proteins in the CM have been shown to be regulated by these events, we next evaluated the responses of Tln1 and Tln2 in two types of hypertrophic models; that is, a well characterized *in vitro* model in which NRVMs are treated with phenylephrine (PE) as an adrenergic stressor and an *in vivo* model in which the mouse is subjected to POL produced via TAC. The first model required use of rat as opposed to mouse cells as it has been recognized that mouse neonatal myocytes develop autonomous hypertrophy and that they are unresponsive to many stimuli that provoke hypertrophic growth of NRVMs (20) (Fig. 3A). A Western blot and microscopic analyses showed that PE stimulation of NRVMs resulted in atrial natriuretic (ANF) stimulation as expected (supplemental Fig. 2) (16) and also increased expression of Tln1 but not Tln2. With stimulation, Tln1 became localized to precostameric structures (Fig. 3, B and C). Confirmation of the costameric localization was shown as Tln1 was co-localized with β 1D integrin (supplemental Fig. 3). PE did not change Tln2 localization (Fig. 3C).

Given these changes in localization, we hypothesized that adrenergic induction of CM hypertrophy might alter complex formation of Tln forms with integrin-associated proteins. Thus we performed immunoprecipitation of CM lysates using Tln antibodies. PE stimulation caused Tln1 to increase its association with Vcl and ILK but did not alter Tln2 complex formation with these proteins (Fig. 3D). The increased CM expression of Tln1 after adrenergic stimulation supports the hypothesis that Tln1 has unique functions in the CM and suggests a potential role in the CM stress response.

Our previous work (19) combined with studies from others groups (21) has shown that integrin expression levels are regulated by POL and that integrin signaling constitutes an important component of the mechanically mediated hypertrophic response. Thus we next evaluated how Tln1 and Tln2 expression and localization might be changed during POL. TAC performed for 1 week caused induction of significant morphometric and molecular hypertrophy (Fig. 4A). Echocardiographic analysis showed preserved cardiac function in the post-TAC hearts (data not shown). Thus the TAC mice examined here were in a state of compensated hypertrophy.

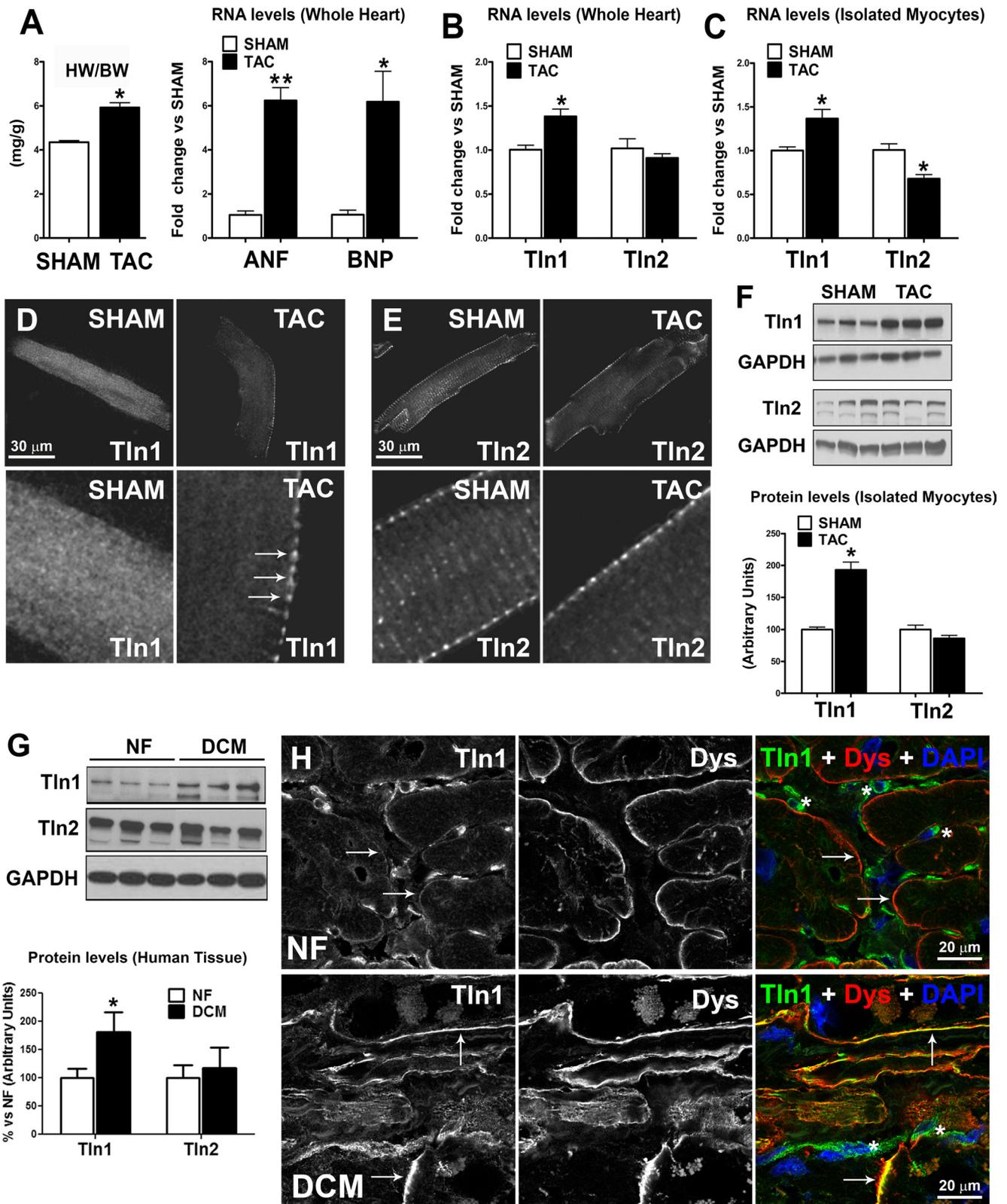
Tln1 transcript expression was increased after TAC in both whole heart and isolated CM samples, whereas Tln2 was not (Fig. 4, B and C). Microscopically, isolated CMs from SHAM-operated hearts showed only a small amount of diffuse Tln1 protein in CMs, yet after TAC, increased expression of Tln1 was detected specifically in costameres (Fig. 4D). The base-line costameric pattern of Tln2 protein detected in SHAM-operated controls was not altered by POL (Fig. 4E). Protein levels of Tln1 in isolated CMs were increased only modestly at 1 week post-TAC (data not shown), but by 4 weeks post-TAC was sig-

control. *C*, immunofluorescence analysis shows that PE causes increased NRVM organization and induces Tln1 localization in pre-costameric structures. Tln2 localization was not changed. Scale bar = 20 μ m. *D*, immunoprecipitation (IP) shows that PE stimulation caused increased association of Tln1 with Vcl and ILK but did not change Tln2 associations with these proteins. *IB*, immunoblotting; *WCL*, = whole cell lysis; *Vcl*, vinculin.

nificantly increased (Fig. 4F). In contrast, Tln2 levels were not changed at either time point.

We also examined the protein expression of Tln1 and Tln2 in non-failing human LV tissue and compared it to that found in LV tissue obtained from patients with failing hearts that were

explanted at the time of cardiac transplantation. The heart failure samples were all from patients with end-stage, non-ischemic dilated cardiomyopathy (DCM). The dilated ventricle typically has increased wall stress and in many cases occurs after a period of compensated cardiac hypertrophy. As shown in Fig.



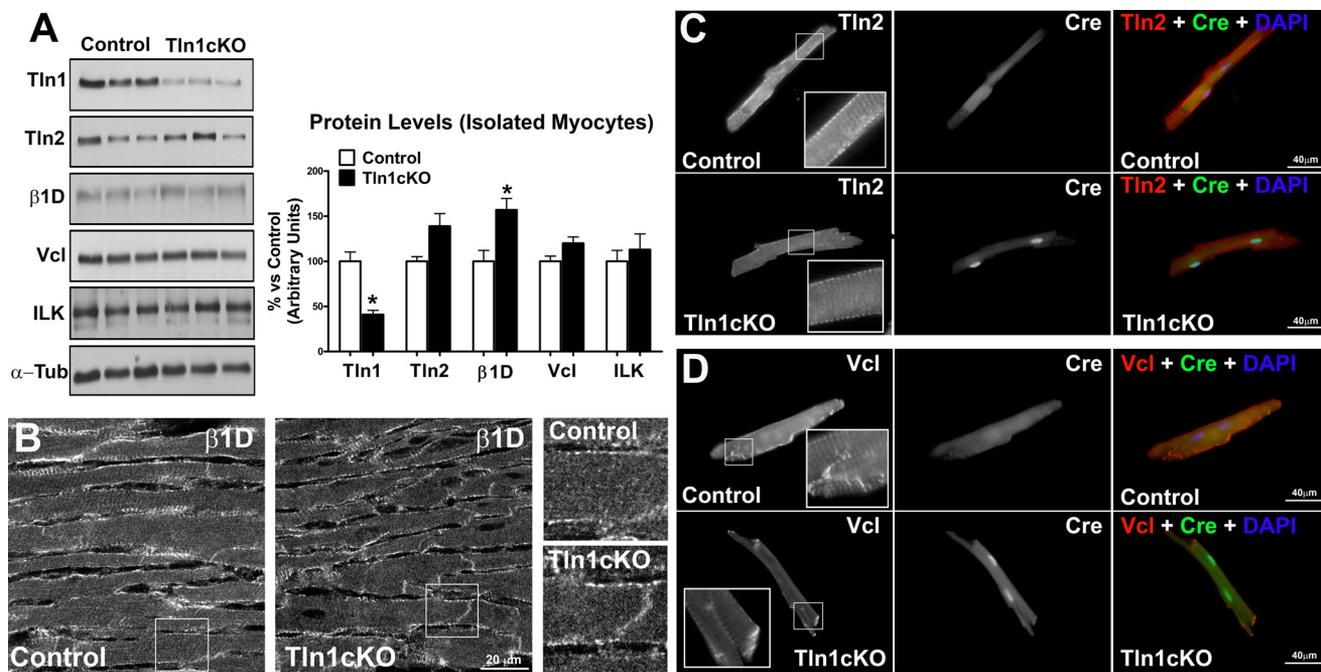


FIGURE 5. Mice with CM-specific excision of the *Tln1* gene displayed normal basal cardiac costameric structure. A, *Tln1* protein levels were decreased ~60% in CMs isolated from 2-month-old *Tln1*cKO mice versus controls, as analyzed by Western blotting. *, $p < 0.05$ versus control; $n = 3$ each group. Western blot analyses with *Tln2*, *Vcl*, *ILK*, and β 1D integrin antibodies in control and *Tln1*cKO CMs showed that deletion of the *Tln1* gene in heart did not alter the protein levels of *Tln2*, *Vcl*, or *ILK* but increased the levels of β 1D integrin. α -*Tub*, tubulin. Cardiac tissue (B) isolated CMs from *Tln1*cKO and control mice (C and D) were immunostained for β 1D, *Tln2*, and *Vcl* protein to microscopically evaluate the structural integrity of costameres. β 1D (B), *Tln2* (C), and *Vcl* (D) show similar microscopic localization in *Tln1*cKO and control specimens. Anti-Cre recombinase immunostaining was used to mark *Tln1*cKO CMs in the isolated cells.

4G, *Tln1* protein levels were increased in failing myocardium when compared with non-failing, control hearts. In contrast, *Tln2* expression was unchanged. To determine whether the site of *Tln1* protein increase in failing heart was due to augmented expression in CMs or non-myocytes, we also performed immunomicroscopic analyses. As shown in Fig. 4H, control specimens expressed *Tln1* mainly localized in interstitial cells, with only weak staining in CMs, where it co-localized with dystrophin, a myocyte-specific marker protein. In the DCM samples, *Tln1* was highly expressed in CMs, although it was still also detected in non-myocytes. *Tln2* expression and localization did not show any differences between the non-failing (control) and the DCM samples (Fig. 4G and supplemental Fig. 4). Taken together these data led us to hypothesize that *Tln1* would be involved in the integrin-dependent hypertrophic response of the myocardium.

Mice with CM-specific Excision of the *Tln1* Gene Display Normal Basal Cardiac Structure and Function— To directly test the role of *Tln1* in the basal and stressed CM, we generated *Tln1* CM-specific KO mice using the Cre/Lox system (supple-

mental Fig. 5A). Mice with a *Tln1* floxed allele (*Tln1*^{fllox/fllox}) have been described previously (14). To excise the *Tln1* gene and reduce *Tln1* expression only in CMs, we crossed *Tln1*^{fllox/fllox} mice with α -MHC nuclear Cre transgenic mice (α -MHC *nCre*) that express Cre recombinase selectively in CMs (supplemental Fig. 5, B and C) (13). Homozygous floxed *Tln1* mice (*Tln1*^{fllox/fllox}) that also expressed α -MHC nuclear Cre (*Tln1*^{fllox/fllox} α -MHC *nCre*) were termed *Tln1*cKO. Cre-negative littermates (*Tln1*^{fllox/fllox}) were used as control mice. These Cre recombinase-expressing mice were specifically shown not to have any abnormalities in cardiac function (22). *Tln1*cKO mice were born at the expected Mendelian ratios and had normal life spans (supplemental Table S1). Isolated CMs from 2-month-old *Tln1*cKO mice demonstrated a 60% reduction of *Tln1* protein levels versus control mice (Fig. 5A). We would suggest that this quantification is an overestimation of the *Tln1* that remained in the CM after Cre-mediated excision of the *Tln1* floxed alleles. This is because even under stringent conditions, we detected rare non-myocytes in our isolates of myocytes and also because, as shown in Fig. 2F earlier and in supplemental Fig. 6, these non-myocytes have a 10-fold higher expres-

FIGURE 4. Hemodynamic loading of the intact mouse heart caused increased CM expression and costameric localization of *Tln1* but did not change *Tln2*. *Tln1* was also increased in human cardiac samples from DCM patients compared with non-failing (NF) samples. A–F, 10-week-old male mice were subjected to SHAM surgery or TAC as a means to produce POL. A, 1-week post-TAC mice developed cardiac hypertrophy as indicated by morphometric changes (HW/BW) and increased transcript levels of the hypertrophic markers ANF and BNP. *, $p < 0.05$ versus SHAM; **, $p < 0.005$ versus SHAM, as indicated for HW/BW, ANF, and BNP; $n = 4$ each group. B and C, TAC increases expression of *Tln1* transcript in whole heart (B) and isolated CMs (C), whereas *Tln2* was not changed. *, $p < 0.05$ TAC versus SHAM; $n = 4$ for both groups (SHAM and TAC) whole heart; $n = 3$ (TAC) and $n = 4$ (SHAM) for isolated CM samples; GAPDH was used as a loading control for all samples. F, Western blotting of protein from isolated CMs shows *Tln1* but not *Tln2* protein levels increase 4w post-TAC. *, $p < 0.05$ TAC versus SHAM; $n = 6$ each group. D and E, immunomicroscopy of isolated CMs 1-week post-TAC or SHAM surgery shows TAC leads to visibly increased CM *Tln1*, particularly in costameres (arrows) (D), whereas *Tln2* localization in costameres is similar in SHAM and TAC samples (E). Scale bar = 30 μ m. G and H, human LV tissue from NF and dilated DCM hearts were analyzed. G, Western blotting and densitometric quantification shows increased *Tln1*, but not *Tln2*, in DCM compared with NF samples. *, $p < 0.05$ versus NF; $n = 6$ all groups. GAPDH was used as a loading control. H, immunomicroscopy shows that in NF samples *Tln1* is detected in non-myocytes (*) with only a weak signal in the CM membrane (arrow). In DCM samples *Tln1* remained in non-myocytes (*) but also showed an increased signal in CMs (arrow) versus NF. Dystrophin (*Dys*) was used as a muscle marker. Scale bar = 50 μ m.

sion of Tln1 protein *versus* that detected in CMs. Furthermore, we show that the efficiency of α -MHC nCre was high in our mice (supplemental Fig. 5, D and E), in agreement with prior studies that also used this Cre model (13, 23). No changes in Tln2 protein levels or cellular localization were detected in Tln1cKO CMs (Fig. 5A). In addition, the Tln1cKO CMs did not show changes in protein expression of a range of other costameric proteins such as Vcl or ILK, although increased expression of β 1D integrin was detected. Furthermore, localization of Vcl as a representative costameric marker was not altered in the Tln1cKO CMs (Fig. 5, A, B, and C, and supplemental Fig. 7). The Tln1cKO hearts exhibited a small, but significant increase in heart weight/tibia length (HW/TL) at 6 months of age (supplemental Table S2) but no differences in chamber dimensions, wall thicknesses, or function at 3 months (supplemental Table S3) or at 1 year of age (data not shown). Histological analysis of 6-month-old Tln1cKO did not reveal any changes compared with controls (data not shown). Together, these data suggest that normal expression of CM Tln1 is not necessary to maintain basal cardiac structure and function in the adult mouse myocardium, although a minimal stress response occurred, suggested by the mild increase in HW/TL and β 1D integrin expression as well as an increase in the molecular marker of hypertrophy, ANF, as discussed below.

Tln1cKO Mice Exhibit a Blunted Response to Pressure Overload—Given our data showing how Tln1 becomes up-regulated in the CM subjected to stress, we next tested the consequences of TAC-induced hemodynamic stress in the Tln1cKO. The Tln1cKO hearts developed a blunted response to TAC compared with controls, although transaortic pressure gradients were similar in control and Tln1cKO mice (supplemental Table S4). This was reflected in the reduced HW/body weight (BW) and HW/TL ratios (Fig. 6A) and in the reduced CM cross-sectional area (Fig. 6B) detected in the cKO mice after TAC. An inhibition of the hypertrophic response in Tln1cKO was further indicated by the significantly smaller increase in expression of the molecular markers of hypertrophy, ANF and BNP, in the cKO mice, *versus* controls (Fig. 6C). In addition, Fig. 6C highlights, as mentioned above, that the Tln1cKO mice show a small increase in ANF before TAC, consistent with a mild basal stress response in these mice. Remarkably, the Tln1cKO mice were able to maintain normal cardiac function in the face of POL, as compared with control mice that showed a significant decrease in % fractional shortening and left ventricular ejection fraction (LVEF) and also displayed more rapid chamber dilatation (Fig. 6D and Table 4). In addition to these physiological differences, after 8 weeks of TAC, the Tln1cKO mice showed reduced fibrosis and collagen I transcript levels compared with controls (Fig. 6, E and F). These data indicate that reduction of CM Tln1 limits adverse LV remodeling after POL.

Acute Mechanotransductive Signaling Is Altered in Tln1cKO Myocardium—Because we detected a beneficial response after TAC when Tln1 was reduced in the Tln1cKO mice, we investigated the effect that loss of Tln1 would have on CM mechanical signaling. For this we analyzed the acute activation of ERK1/2, p38, Akt, and glycogen synthase kinase 3 β as representative proteins that have been shown to be involved in cardiac hypertrophy and the transition of the heart to failure (24). Acute POL was used to study mechanical stress in the intact

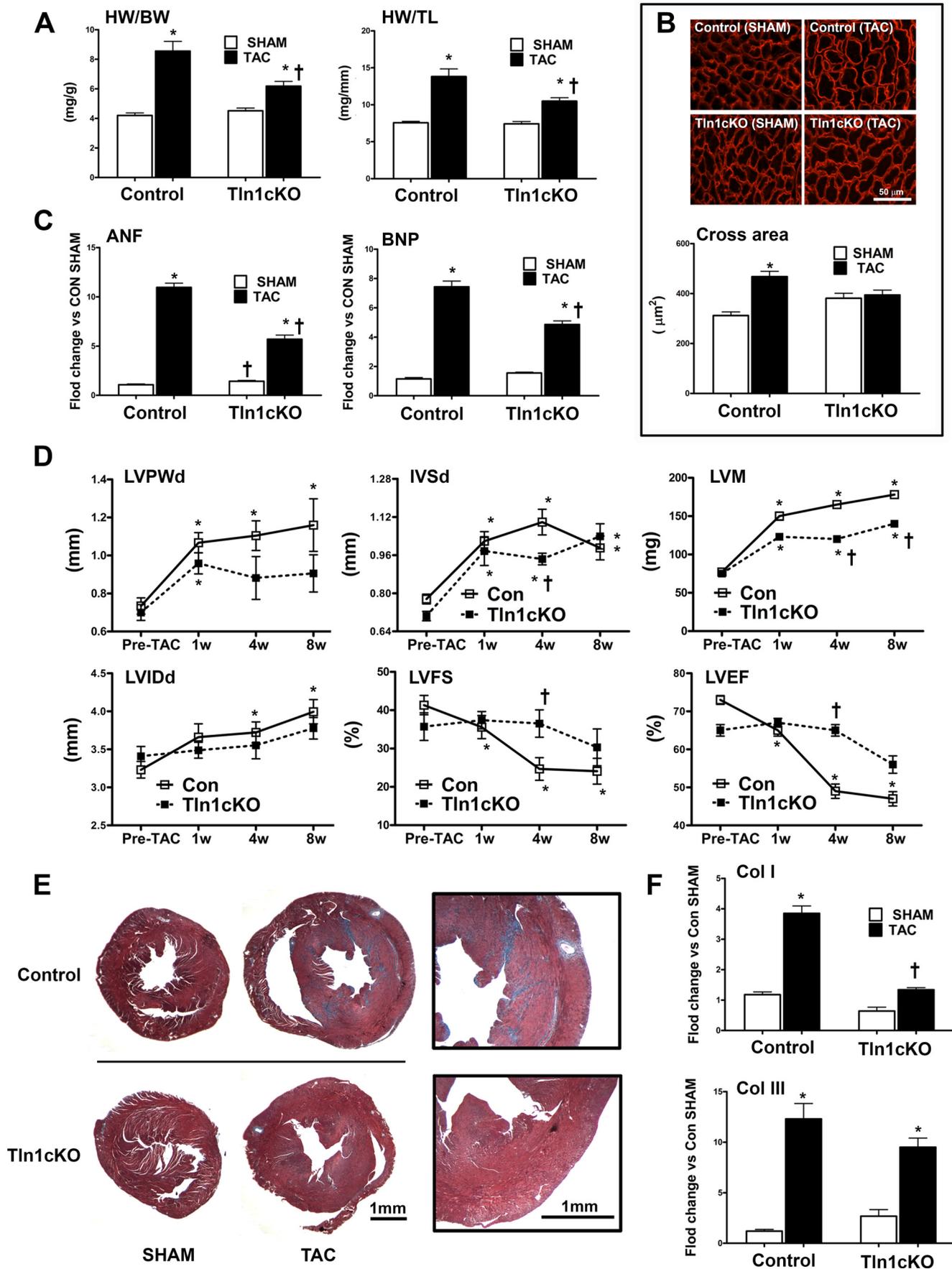
heart, as longer term POL can also evoke changes in humoral factors. Our prior studies showed that kinase activation was robustly increased in whole heart by 10 min (min) post-TAC; therefore, we chose this time point for analysis. Control mice showed phosphorylation of all proteins analyzed at this time (Fig. 7). In contrast, Tln1cKO mice showed complete loss of ERK1/2, Akt, and glycogen synthase kinase 3 β activation and reduced phosphorylation of p38. These data indicate that the reduction of Tln1 in CMs alters acute biomechanical signaling and offers an apparent beneficial response to cardiac remodeling after hemodynamic loading.

DISCUSSION

Tln serves as a critical linker between cell surface integrins and the actin cytoskeleton. It binds and activates integrins. Previous studies have shown the essential role of multiple integrins (*e.g.* α 5, α 7, β 1, and β 3) and integrin-related proteins (melusin, focal adhesion kinase, ILK, and PINCH) in heart development (25), in the preservation of normal cardiac function (19, 26, 27), and in the response of the heart to stress (21, 28). The contribution of Tln to these processes has not been previously studied.

Our results show that during embryogenesis, Tln1 and Tln2 are both highly expressed in CMs. Yet in fully mature CMs, Tln1 protein expression is reduced, and Tln2 becomes the main Tln isoform. With induction of hypertrophy, Tln1 protein expression increases in CMs and becomes localized to costameres, which suggests that it is involved in the adaptive mechanisms triggered in the stressed heart. In addition we also showed a similar Tln1 protein increase in CMs of human failing heart specimens compared with non-failing, control hearts. Our data along with prior work (29) support the important role of Tln1 and the integrin complex in the stress response of the myocardium in both mouse and man. To directly test this possibility we produced and characterized Tln1cKO mice. These mice showed normal basal cardiac structure and function, but after chronic POL, they had blunted hypertrophy, less fibrosis, and preserved cardiac function *versus* littermate controls. In addition, Tln1cKO showed attenuated Akt, ERK1/2, and p38 activation after acute POL. These data show that normal expression of Tln1 is not necessary to maintain basal cardiac structure and function and that reducing CM Tln1 leads to a beneficial response in the face of POL.

Tln1 and Tln2 Are Both Highly Expressed during Cardiogenesis, but Tln2 Is the Predominant Tln Isoform in Adult Cardiac Muscle—Our findings in the adult CM are concordant with previous studies which showed that Tln2 is the dominant Tln isoform in adult skeletal muscle (10, 30, 31). Tln2 has a higher affinity than Tln1 for the muscle-enriched β 1 integrin isoform β 1D (32) and, therefore, as appropriate for contracting muscle, provides the strongest connection possible between integrins and any Tln form. Biochemical studies also showed that Tln2 had a higher affinity for F-actin than Tln1 (33), which again would provide for a robust connection between Tln2 in the CM cytoskeleton and sarcomere. Therefore, it is intuitively clear that having Tln2 as the dominant CM Tln form allows CMs to support the high and constant forces that they must endure. In contrast, the co-expression of both Tln isoforms in embryonic



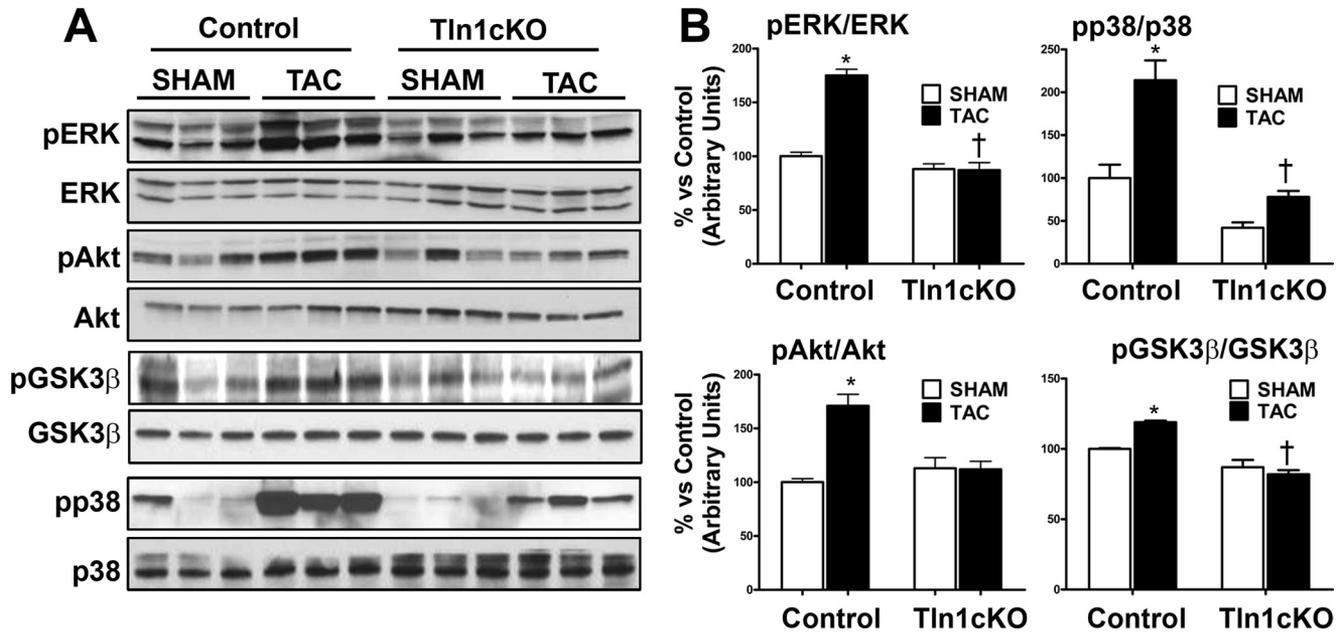


FIGURE 7. Acute mechanotransductive signals were blunted in Tln1cKO mice. Control and Tln1cKO mice were subjected to SHAM or TAC surgery, and cardiac tissue was harvested 10 min post-operatively. *A*, Western blot analysis shows that ERK, Akt, glycogen synthase kinase 3 (GSK3 β), and p38 were rapidly phosphorylated in response to TAC in control mice, but activation of ERK, p38, Akt, and glycogen synthase kinase 3 β was strongly impaired in Tln1cKO mice. *B*, shown are normalized densitometric analyses for each kinase (phospho/total) ($n = 6$ for all groups; *, $p < 0.05$ versus SHAM; †, $p < 0.05$ versus control TAC).

CMs would satisfy a requirement for weaker connections between integrins and the cytoskeleton necessary to facilitate processes such as migration and proliferation of CMs as the heart forms. In agreement with this hypothesis, during early heart development, cardiac muscle expresses the β 1A integrin subunit instead of β 1D (34), forming weaker connections with the cytoskeleton (35). We speculate that the two Tln isoforms may have unique binding partners other than those already characterized, which would allow for distinct roles of these Tln isoforms during development and postnatal function of the heart. Our preliminary data suggest that one of these partners could be cardiac ankyrin repeat protein (CARP) as CARP is up-regulated during cardiac development and hypertrophy (36) and binds to Tln1 but not Tln2 when NRVM are stimulated with PE (data not shown).

Cardiac Stress Regulates the Expression and Localization of Tln1 in CM— In line with previous studies that demonstrated increased Z-line distribution of extracellular matrix proteins (fibronectin, collagen) and integrins (α 1, α 3A, α 5, and β 1) as well as important integrin binding proteins (ILK, PINCH, parvin, and hic-5) in NRVMs exposed to PE (37–39), we found Tln1 was also induced and localized to the costameres by this adrenergic agent. These data highlight the importance of costameric proteins in forming a signaling complex that regulates

myocyte growth. Our data also show similarities with studies of the integrin-associated proteins paxillin and hic-5. Like Tln1 and -2, paxillin and hic-5 are highly homologous and share similar binding partners. In parallel with our Tln data, hic-5, but not paxillin, is up-regulated and localized to the costameres after PE treatment of NRVMs (39). This study also showed that only hic-5 but not paxillin was required for PE-stimulated increases in ANF and α -skeletal actin expression as well as for the increased organization of the cytoskeleton.

Similar to the NRVM results, Tln1 protein was increased in CM costameres after POL. In contrast, Tln2 expression decreased and did not show any change in its location. Similar data have been reported in human heart samples for another group of integrin-associated proteins, the ILK-PINCH-Parvin complex (29). Like Tln, PINCH and parvin have multiple isoforms (PINCH1 and -2, and α , β , and γ parvin, respectively), whereas ILK does not. Although PINCH1 and PINCH2 are co-expressed in adult cardiac muscle (27), PINCH2 has been demonstrated to show a higher affinity for ILK (40, 41). Similar to our data on Tln1 and Tln2 expression in adult cardiac muscle, α -parvin is expressed at only low levels in the adult CMs, whereas β -parvin is the dominant muscle isoform. Likewise, in failing human hearts as well as mouse hearts stressed with POL, the non-muscle isoform, α -parvin, is the isoform that is

FIGURE 6. Tln1 regulated cardiac remodeling after pressure overload. *A–C*, reduction of CM Tln1 in Tln1cKO caused a blunted response 8 weeks post-TAC as demonstrated by morphometry (HW/BW and HW/TL) ($n = 7$ each group) (*A*), a CM cross-sectional area ($n = 5$ mice each group with >500 cells analyzed/mouse; top, immunomicroscopy of representative myocardial sections stained with the CM membrane marker dystrophin; bottom, a graphical display of CM cross-sectional area) (*B*), and molecular markers of hypertrophy (ANF and BNP with GAPDH use as loading control; $n = 5–7$ each group; *, $p < 0.05$ versus SHAM of each group; †, $p < 0.05$ versus control TAC, $n = 7$ each group) (*C*). *D*, serial echocardiography revealed a blunted hypertrophic response in Tln1cKO mice after TAC. Analyses demonstrated that intraventricular septal thickness in diastole (IVSd) and LV mass had varied responses in the Tln1cKO versus control mice. *, $p < 0.05$ versus Pre-TAC; †, $p < 0.05$ versus control at same point, $n = 7$ each. LV fractional shortening as well as LV ejection fraction were improved at 4 weeks post-TAC in Tln1cKO versus control mice. *E* and *F*, cardiac fibrosis evaluated by Mallory's trichrome stain (*E*) as well as collagen I (Col1) and collagen III transcript levels (*F*) were reduced in Tln1cKO versus control 8-week post-TAC. *, $p < 0.05$ versus SHAM; †, $p < 0.05$ versus control TAC, $n = 4–7$ each group. All mice were 12 weeks old at the time of surgery. LVPWd = LV posterior wall in diastole; IVSd = interventricular septum in diastole; LVM = LV mass; LVIdd = LV internal dimension in diastole; LVFS = LV fractional shortening; LVEF, LV ejection fraction.

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induced in concert with PINCH1, whereas expression of β -parvin and PINCH2 do not change (29).

These data together with our results suggest the possibility of two unique and parallel systems associated with integrins at the myocyte costamere; one that is more permanent and has an essential structural role connecting integrins with the cytoskeleton/sarcomeres (Tln2, paxillin, β -parvin, and PINCH2 related) and another one that is activated under stress conditions (Tln1, hic-5, α -parvin, and PINCH1) to trigger signaling required for the integrin-dependent hypertrophic response. Prior studies are limited by the fact that observations were made in whole heart samples, so it is not clear if these findings relate to changes in CMs or non-myocytes. Our data using cultured CMs and *in vivo* models show for the first time the specific role of Tln1 in myocytes of the hemodynamically stressed heart.

Tln1 Regulates Cardiac Remodeling upon Pressure Overload—To directly test the role of Tln1 in the basal and hemodynamically stressed heart, we generated Tln1cKO mice. Tln1 was shown to be dispensable for normal cardiac development as the α -MHC nCre transgene we used to excise the Tln1 “floxed” allele is effective at causing gene excision by E11 during cardiogenesis (23). Still, we cannot rule out the possibility that the residual CM Tln1 which remained during heart development enabled normal cardiogenesis. Furthermore, although our Cre-loxP-mediated Tln1cKO hearts appeared to have residual expression of the protein at the whole heart level, it is generally accepted that this is due to chimeric expression of the Cre transgene. Thus at the single cell level, when Cre expression is detected, the Tln1 gene would be excised, and these cells would be null for Tln1. In addition, as outlined above, given the small amount of Tln1 basally expressed in CMs compared with the large amount in the non-myocyte pool, we suggest that our Western blot data overestimates the amount of residual Tln1 remaining in cKO hearts due to a small amount of non-myocytes contaminating our myocyte sample. The Tln1cKO survived to adulthood and basally showed normal cardiac structure and function with a minimal but significant increase in heart size (HW/TL), β 1D integrin protein levels, and ANF transcript levels. Taken together, this data suggest that Tln1 is not essential in the adult myocardium and that its loss from CMs only leads to a mild stress response.

Previous studies have shown that Tln1 is required for the assembly and support of focal adhesion complexes in non-muscle cells (42). Tln1cKO CMs show only a minimal increase in β 1D integrin protein expression, with no change in protein expression of other costameric proteins such as Vcl or ILK. In addition, the location of all of these costameric proteins was similar in Tln1cKO and control mice. These results indicate that Tln1 deletion from the CM causes little alteration in the structural integrity of CM costameres. This result is in agreement with data in skeletal muscle, where costameric structure was also preserved in the absence of Tln1 (30). Furthermore, CMs from Tln1cKO mice did not show any compensatory changes in Tln2 expression or localization compared with control cells. Therefore, Tln2 appears sufficient for normal CM function and particularly for the maintenance of costameric structure in adult cardiac myocytes.

The vertebrate heart is able to sense and adapt to changing stretch. For example mechanical stimuli triggered by pressure loading can result in hypertrophic growth. The role of integrins in response to POL as well as in mechanical stress has been previously described by our group and others and has been linked to hypertrophic growth and the activation of signaling pathways including PI3K-Akt and mitogen-activated protein kinases (15, 21). In our study Tln1cKO mice subjected to POL showed a beneficial remodeling response with less hypertrophy, less fibrosis, and an improvement in cardiac function. The fetal gene program is known to be reactivated in adult CMs in response to a pathological hypertrophic stimuli and functions as an adaptive response to protect the heart (43). The Tln1cKO mice had blunted induction of ANF and BNP after TAC, concordant with this beneficial adaptive response to hemodynamic loading.

Cardiomyocyte enlargement is a feature of physiological and pathological hypertrophy, yet the molecular mechanisms that lead to a beneficial *versus* deleterious hypertrophic response remain poorly understood. Our data suggest that the pressure-mediated induction of Tln1 in the normal CM may in fact be deleterious to preservation of myocardial function in the face of this abnormal stress. Tln1 may be an important factor in the evolution of pathological hypertrophy, as its deletion allows for better preservation of cardiac function and delay in the onset of cardiac failure in the face of POL. Still, it is important to point out that this improvement is transient, and Tln1 deletion only delays the onset of the cardiac failure with the extreme stress of TAC.

The Tln1cKO mice subjected to POL also showed decreased fibrosis *versus* controls. Accumulation of fibrotic tissue is a deleterious feature of pathological cardiac hypertrophy that affects the viscoelastic properties of the myocardium, impairs diastolic function, and may propagate the transition to heart failure. Therefore, the reduced fibrosis seen in Tln1cKO mice is another factor that assists to preserve the cardiac function of these mutant mice after POL.

Mechanical stress on the CM induces the activation of a range of signaling pathways, including ERK, p38, and Akt. These events have been associated with various aspects of hypertrophic growth in isolated CMs and the intact heart. β 1 integrin is an important mechanosensor and has been shown to be implicated in the hypertrophic growth of CMs (16). In a previous study we demonstrated that β 1 integrin is essential for the proper activation of ERK, p38, and Akt after acute POL (15), a role that has been also demonstrated in isolated CM under stretch (44). Tln1cKO and β 1 integrin cKO mice show similar responses to acute POL. This result is concordant with the key role that Tln1 has as an essential mediator in the β 1 integrin-dependent response to mechanical stress.

Our data showing that Tln1 plays a key role in acute mechanical responses of the myocardium despite its low expression level in adult CMs highlights the fact that the functional effects of proteins in the CM does not always depend on how much total protein is detected in the cell. Rather, protein location, for example in discrete microdomains, might greatly influence its function. Several studies using cell types other than CMs have shown that various stimuli can cause Tln1 to change its location

from the cytosol to the membrane, where it associates with integrins (45, 46). We hypothesize that acute mechanical stimulation of the CM could similarly change Tln1 localization from the cytosol to the CM costamere, where it plays an essential role with other costameric proteins to trigger key intracellular signaling events.

A critical feature of our results is that the function of Tln1 cannot be compensated by Tln2 protein when the CM undergoes acute mechanical loading. In agreement with this, Tln1 has been shown to be essential in the force-dependent reinforcement of integrin-cytoskeleton connections in non-muscle cells, where it was shown to be essential for stretch-dependent adhesion site formation at early, but not late, times (47). Like our results, Tln2 expression could not compensate for the loss of Tln1 in that study (47). Therefore, Tln1 appears to be critical in acute costameric force-sensing and mechanical signaling.

Our expression data in human control and failing myocardium was in agreement with that found in mouse. This suggests that the role of Tln1 in the cardiac stress response may also be applicable to man. In support of this is a recent study that linked mutation of the mechanosensory protein CARP to human familial cardiomyopathy (48). Interestingly, these CARP mutants lost binding to Tln1.

In conclusion these are the first data to show the differential expression and regulation of the two Tln isoforms in the myocardium and point to unique functions of Tln1 as compared with Tln2. Further work is necessary to fully understand the function of Tln1 and Tln2 in the cardiac myocyte and how normal expression of Tln1 might actually predispose the heart to failure in the face of a "second hit" such as pressure loading (e.g. hypertension in man), specifically how reduction of Tln1 in the CM might protect the myocardium from stress-induced myopathies needs additional study.

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