Loss of mouse cardiomyocyte talin-1 and talin-2 leads to β-1 integrin reduction, costameric instability, and dilated cardiomyopathy

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Continuous contraction–relaxation cycles of the heart require strong and stable connections of cardiac myocytes (CMs) with the extracellular matrix (ECM) to preserve sarcolemmal integrity. CM attachment to the ECM is mediated by integrin complexes localized at the muscle adhesion sites termed costameres. The ubiquitously expressed cytoskeletal protein talin (Tln) is a component of muscle costameres that links integrins ultimately to the sarcomere. There are two talin genes, Tln1 and Tln2. Here, we tested the function of these two Tln forms in myocardium where Tln2 is the dominant isoform in postnatal CMs. Surprisingly, global deletion of Tln2 in mice caused no structural or functional changes in heart, presumably because CM Tln1 became up-regulated. Tln2 loss increased integrin activation, although levels of the muscle-specific β1D-integrin isoform were reduced by 50%. With this result, we produced mice that had simultaneous loss of both CM Tln1 and Tln2 and found that cardiac dysfunction occurred by 4 wk with 100% mortality by 6 mo. β1D integrin and other costameric proteins were lost from the CMs, and membrane integrity was compromised. Given that integrin protein reduction occurred with Tln loss, rescue of the phenotype was attempted through transgenic integrin overexpression, but this could not restore WT CM integrin levels nor improve heart function. Our results show that CM Tln2 is essential for proper β1D-integrin expression and that Tln1 can substitute for Tln2 in preserving heart function, but that loss of all Tln forms from the heart-muscle cell leads to myocyte instability and a dilated cardiomyopathy.

talin | costameres | cardiomyopathy | heart | integrins

The heart and all of its attendant cells, particularly the contracting cardiac myocytes (CMs), are constantly subjected to high forces. Proper linkage between the cellular cytoskeleton, the cell membrane (termed the sarcolemma in muscle cells), and the extracellular matrix (ECM) is necessary for synchronized force distribution among the cells of the myocardium (1–3). When these linkages are disturbed, forces may be distributed abnormally, cardiac muscle can be injured, and therefore whole-heart function can become abnormal. A number of intracellular proteins and membrane receptors have been found to be crucial for organizing this interactive arrangement between the intracellular CM environment and the surrounding ECM. The integrin family of adhesion receptors are key proteins operative in this response (4). Integrins are a large family of 18 α-subunits and 8 β-subunits, which heterodimerize noncovalently, forming over 24 unique transmembrane receptors on virtually all cells, including CMs. They bind directly to ECM proteins via their proportionally large extracellular domains, and their short cytoplasmic tails assist with organizing a large multiprotein complex that connects integrins to the actin cytoskeleton and may transduce signals to a range of downstream pathways. With these connections, integrins are recognized as key mechanotransducers, converting mechanical perturbations to biochemical signals (5, 6).

The complex of proteins organized by integrins has been most commonly termed focal adhesions (FA) by studies performed in cells such as fibroblasts in a 2D environment. It is recognized that this structure is important for organizing and regulating the mechanical and signaling events that occur upon cellular adhesion to ECM (7, 8). In addition, it is clear that there are other types of matrix adhesions in addition to the FA, and furthermore, that there can be substantial variation in these structures when studied in 2D cultured cells vs. 3D systems more akin to that found in vivo in tissues/organs such as the heart. In CMs, integrins organize complexes concentrated adjacent to the sarcolemma that are analogous to the FA, known as costameres (9). Costameres are like the hoops of a barrel, providing stability for the cell. They align circumferentially with the Z-disks of the sarcomere and ensure that the distortions of the sarcolemma that occur during myocyte contractions are small and periodic, protecting the membrane from excessive stress. In this manner, the costamere, with its connection from the ECM, through the sarcolemma and then through to the sarcomere, provides an essential structure for force transmission, mechanotransduction, and stability for the CM (2).

Talin (Tln) is a large (270-kDa) dimeric FA/costameric protein that binds the cytoplasmic domain of the integrin β-subunit

Significance

Heart failure is a significant health problem with a poorly understood molecular basis. Continuous contraction–relaxation cycles of the heart and its cardiomyocytes (CMs) require strong interactions between intracellular proteins and the surrounding extracellular matrix to maintain normal heart function. We exhaustively studied the function of the cytoskeletal protein talin (Tln), and its two isoforms, Tln1 and Tln2, in CMs and the intact heart. Both Tln isoforms can independently support costamere, CM, and whole-heart function. Yet, combined deletion of CM Tln1 and Tln2 destabilized the myocardium, leading to heart failure. This study significantly advances knowledge about the basic biology of talin, particularly given its study in vivo, and advances understanding of Tln's role in maintenance of normal heart muscle function.

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The authors declare no conflict of interest.

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and activates integrins to modulate ECM engagement. Talin links integrins to the cellular cytoskeleton by directly connecting to actin and also indirectly via its binding to vinculin (Vcl) (10). Studies in a variety of cells have demonstrated that Tln is necessary for proper integrin adhesion to ECM and that its deletion or down-regulation compromises FA formation (11). Recent studies in nonmuscle cells also show that Tln is a key regulator of force transmission and transduction, an especially important property in the myocardium, an organ that is subjected to mechanical forces continuously, in its basal state, and that must adapt to further mechanical changes with physiological stresses or pathological conditions (12, 13).

Vertebrates contain two *Tln* genes encoding closely related isoforms termed Tln1 and Tln2. In mammals, Tln1 is ubiquitously expressed, but Tln2 shows a more restricted distribution with high levels in the heart, brain, and skeletal muscle (14). *Tln1* knockout (KO) mice display an embryonic lethal phenotype by E8.5–E9.0 due to gastrulation defects (15), indicating that, although both *Tln* genes encode very similar proteins (~74% identical), Tln2 cannot replace Tln1 function in the entire embryo. *Tln2* KO mice develop a mild skeletal myopathy that is not evident until 3 mo of age due to defects in the myotendinous junction (16), again indicating that both isoforms cannot completely compensate for the loss of each other. In our previous work (17), we showed that, during embryogenesis, both Tln1 and Tln2 are highly expressed in CMs, but that, in normal resting adult CMs, Tln1 expression is very low, and Tln2 is the dominant isoform. In the mature CMs, Tln2 colocalizes with the muscle-specific β1D integrin isoform in costameres. Tln2 has a higher affinity for β1D integrin than Tln1 (18). Therefore, of the possible β1 integrin–Tln combinations in CMs, Tln2-β1D would appear to provide the strongest connection between β1 integrins and the actin cytoskeleton (19). This idea fits with the fact that Tln2 replaces Tln1 in the adult CMs.

To comprehensively investigate the role of Tln1 and Tln2 in the heart, we first studied cardiac structure and function in Tln2 KO mice. In the absence of Tln2, CMs Tln1 was up-regulated, and, remarkably, normal cardiac structure and function was maintained despite a 50% reduction in levels of β1D integrin. In contrast, deletion of Tln1 from CMs in mice lacking Tln2 led to improper assembly of integrin complexes on the myocyte surface, myocardial dysfunction, and early death, a phenotype that could not be mitigated by overexpression of integrin subunits.

Results

Mice with Global Deletion of Tln2 Display Normal Basal Cardiac Structure and Function. Tln2 is the major Tln isoform in adult CMs (17). To study its role in the heart, we used Tln2 knockout (KO) mice. The mice were born at the expected Mendelian ratios (*SI Appendix*, Table S1) and had normal life spans as shown previously (16). No morphological, morphometric, or histological changes were noted between Tln2KO and WT mice, up to 12 mo (Fig. 1*A* and *SI Appendix*, Table S2). Importantly, cardiac function evaluated by echocardiography (echo) at 6 mo and 12 mo also showed no differences between Tln2KO and WT mice (*SI Appendix*, Table S3). Molecular markers that change with even subtle cardiac stress, such as atrial natriuretic factor (ANF), B-type natriuretic peptide (BNP), and alpha skeletal actin (α–sk-actin) likewise showed no differences between the Tln2KO and control groups (Fig. 1*B*). Thus, it appears that the absence of Tln2 from CMs does not in itself predispose to a basal heart phenotype.

Tln1 Can Functionally Replace Tln2 in Cardiac Myocytes. Given these results, we asked whether Tln1 might be up-regulated in CMs from Tln2KO mice and compensate for loss of Tln2. However, no changes in Tln1 protein levels were found in whole-heart lysates from Tln2KO mice compared with WT controls (Fig. 1*C*). Tln1 is expressed at very low levels in normal CMs compared with other cell types (e.g., in endothelial cells or fibroblasts), so we wondered

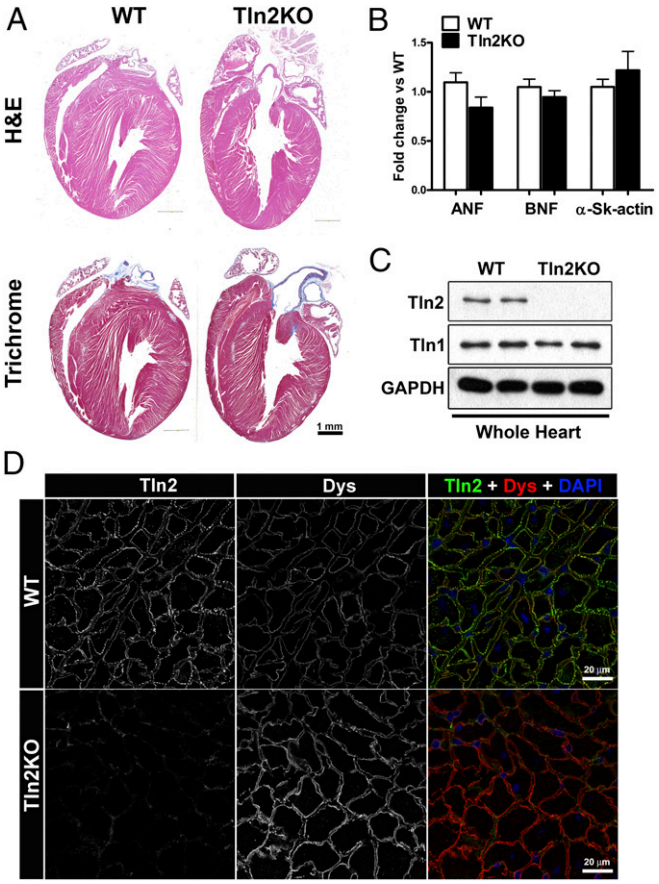

Fig. 1. Tln2KO shows no alterations in myocardial structure, hypertrophic markers, or whole-heart Tln1 expression. (*A*) Histological analyses showed no difference between WT and Tln2KO male mice at 1 y of age. [Hematoxylin/eosin staining (H&E) was used to display standard morphology; trichrome was used to detect fibrotic alterations.] (*B*) qRT-PCR was used to detect changes in the molecular markers of hypertrophy, ANF, BNP, and α-skeletal actin transcripts, using heart RNA from WT and Tln2KO male mice at 6 mo. GAPDH was used as loading control. Data are mean ± SEM; $n = 3$ in each group. (*C*) Western blotting of whole-heart protein lysates from 2-mo-old Tln2KO vs. WT shows absence of Tln2 protein but no changes in Tln1. GAPDH was used as loading control. WT: $n = 7$; Tln2KO: $n = 5$. (*D*) Dual staining was performed with anti-Tln2 (green) and anti-Dys (red), used as a myocyte membrane marker, on adult cardiac tissue from WT and Tln2KO male mice. A higher-quality version of this figure can be viewed in the *SI Appendix*.

whether the high Tln1 background in nonmyocyte cells might mask any up-regulation of Tln1 in CMs in the context of the intact heart. Therefore, we tested lysates derived from isolated CMs that clearly showed that Tln1 protein levels were increased about twofold in Tln2KO samples, compared with controls (Fig. 2 *A* and *B*). Despite the up-regulation of Tln1 protein, no change in Tln1 transcript levels was measured in the Tln2KO samples (Fig. 2*C*). Immunomicroscopy was then performed to localize Tln1 in hearts from the Tln2KO vs. controls. Because Tln1 is expressed in a variety of cell types, including nonmuscle cells, dystrophin (Dys) was used as a muscle marker to distinguish CMs from nonmyocytes. Tln1 expression was clearly increased in costameres of CMs from Tln2KO tissue (Fig. 2*D*). Therefore, when Tln2 is absent from CMs, Tln1 expression is increased, allowing the basal heart to maintain its normal structure and function.

Integrins and Proteins of the Integrin Complex Are Altered in Tln2KO CM. Tln2 has been reported to have a higher affinity for β1D integrin than Tln1 (18), and we therefore wondered whether loss

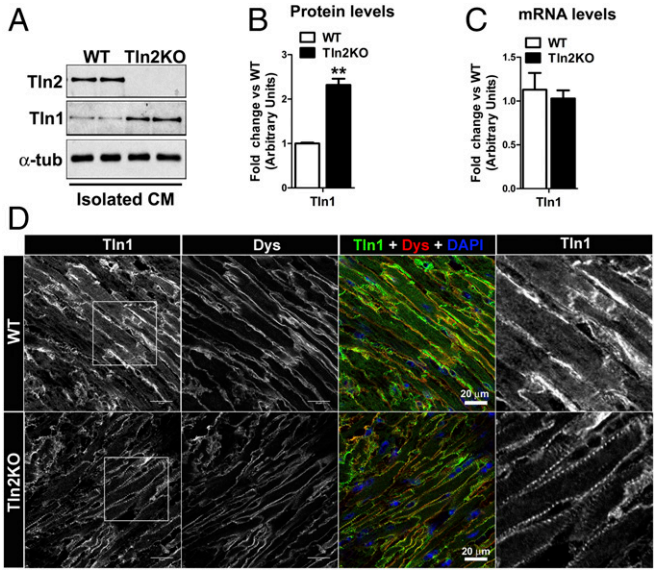

Fig. 2. Tln1 can functionally replace Tln2 in cardiac myocytes. (*A* and *B*) Western blotting (*A*) and associated densitometric analysis (*B*) of isolated cardiomyocyte lysate from 2-mo-old Tln2KO vs. WT. Data are mean ± SEM; $n = 7$ in each group. (**$P < 0.01$.) (*C*) qRT-PCR was used to quantify Tln1 in isolated CM from WT and Tln2KO male mice. Data are mean ± SEM; $n = 3$ in each group. Data were analyzed by *t* test vs. WT and were significant as indicated (**$P < 0.01$). (*D*) Immunofluorescent microscopy of WT and Tln2KO adult cardiac tissue shows strong localization of Tln1 at costameres of Tln2KO CM. Dys was used to mark and localize cardiac myocytes. A higher-quality version of this figure can be viewed in the *SI Appendix*.

of Tln2 might affect the levels of β1D integrin, the dominant β1-integrin subunit in striated muscle. Indeed, Western blots of whole-heart lysates showed a 50% reduction in β1D integrin in Tln2KO vs. controls (*SI Appendix*, Fig. S1). Surprisingly, no changes were seen in levels of the α7-integrin subunit, although it is the main partner of β1D in mature CMs (20). Furthermore, no changes were detected in Vcl, paxillin (Pax), or focal adhesion kinase (FAK) (*SI Appendix*, Fig. S1). Similarly, β1D-integrin protein levels were decreased by 50% in isolated Tln2KO CMs vs. control samples, whereas no changes in α7-integrin expression were detected (Fig. 3*A*). Importantly, no compensatory increase in either the ubiquitous β1A integrin or the β3-integrin protein was found in the Tln2KO CMs (*SI Appendix*, Fig. S2). In addition, Tln2KO CMs showed a significant decrease in integrin adapter protein integrin-linked kinase (ILK) a well-known regulator of cardiac function (21) and paxillin. Importantly, levels of kindlin-2 (KN2), a known coactivator of integrins along with Tln, and Vcl, the best-known binding partner of Tln1 and Tln2, were not altered in the Tln2-deficient CMs (Fig. 3 *A* and *B*).

Immunolocalization studies in heart from Tln2 KO mice using a pan anti–β1-integrin antibody showed a clear reduction in the Tln2KO CMs, compared with WT specimens (Fig. 3*D*) and consistent with the Western blot data showing a reduction in β1D integrin. However, the β1 integrin that remained was still properly localized at costameres. In agreement with the reduction of β1D at the Tln2KO CM membrane, α7 integrin was also reduced at this location, although it appeared to increase its localization in small reservoirs within the cytoplasm (*SI Appendix*, Fig. S3 *A* and *B*), which could explain why its protein levels did not change. Vcl and KN2 immunostaining remained unchanged (Fig. 3*E* and *SI Appendix*, Fig. S4) in the KO cells vs. controls.

To establish if changes in β1D-integrin expression were due to altered transcription or posttranscriptional mechanisms (such as protein degradation), we investigated β1D mRNA levels in isolated CMs. Integrin β1D mRNA levels were unchanged in the Tln2KO CM (Fig. 3*C*), suggesting that deletion of CM Tln2 more likely leads to decreased β1D integrin protein levels by increased degradation as opposed to reduced transcription. Thus, the compensatory increase of Tln1 protein in the Tln2KO CM does not completely protect against loss of β1D from the

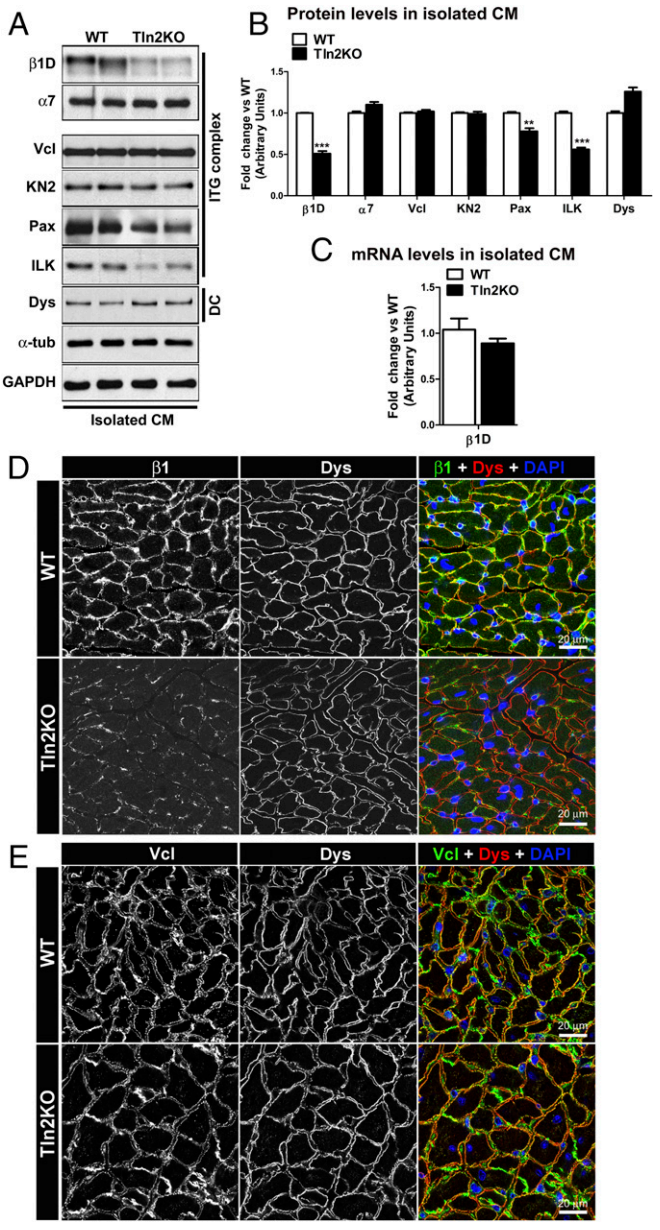

Fig. 3. Integrins and integrin complex proteins are altered in Tln2KO CM. (*A* and *B*) Western blotting (*A*) and densitometric analysis (*B*) of isolated cardiac myocytes from 2-mo-old WT and Tln2KO male mice show reduced β1D integrin, ILK, and Pax expression in the Tln2KO without changes in other proteins assessed—α7 integrin, Vcl, KN2, or dystrophin. GAPDH was used as a loading control. Data are mean ± SEM; $n = 7$ in each group. (*C*) β1D-integrin transcript expression was not changed in the Tln2KO examined by qRT-PCR analysis of RNA from WT and Tln2KO male mice isolated myocytes. Data are mean ± SEM; $n = 3$ in each group. (*D*) β1 integrin was visibly reduced, but normally localized in Tln2KO myocytes when compared with WT myocardium. (*E*) Vcl levels and localization remained unchanged when assessed by immunofluorescent microscopy of adult cardiac tissue from WT and Tln2KO male mice. (**$P < 0.01$ and ***$P < 0.001$: Tln2KO vs. WT.) ITG complex, integrin complex. A higher-quality version of this figure can be viewed in the *SI Appendix*.

sarcolemmal membrane. Despite this, the 50% of β1D-integrin protein remaining in the KO CM is clearly sufficient to maintain normal heart function, consistent with previously published work (22, 23).

β1 Activation in Cardiac Myocytes Is Increased in Tln2KO Myocardium. Although Tln serves as a structural protein linking integrins to the actin cytoskeleton, it also plays a key role in integrin activation in a variety of cell types (11, 24, 25). To investigate whether loss of CM Tln2 and the subsequent compensatory increase in Tln1 expression causes changes in integrin activation, we stained heart tissue with the 9EG7 antibody that specifically recognizes the activated form of the β1-integrin subunit (26, 27). Consistent with the reduction of total β1-integrin expression in CMs, the levels of activated β1 integrin detected by the 9EG7 antibody were also reduced in Tln2KO heart compared with WT. However, the remaining activated β1 integrin was properly localized at costameres (Fig. 4*A*). To quantify integrin activation, we isolated CMs and performed Western blot analysis. We compared levels of activated β1 integrin (marked by 9EG7) to total β1D integrin (the 9EG7/β1D ratio) in the Tln2KO compared with WT control and found significantly higher relative levels of β1-integrin activation in the Tln2KO CMs (Fig. 4*B*).

Tln2 Deletion Does Not Produce Changes in the Dystrophin–Glycoprotein Complex or Intercalated Disk Proteins. Costameres contain two main adhesive structures: the integrin complex and the dystrophin–glycoprotein complex (DGC). Previous studies, including ones from our laboratory, showed that these two complexes can compensate for one another to preserve CM integrity and heart function (28, 29). Given the changes in the β1D-integrin complex in Tln2KO heart, we therefore assessed if changes occur in DGC proteins. However, no changes in the key DGC component Dys was seen in Tln2KO hearts (*SI Appendix*, Fig. S1) or isolated CM (Fig. 3 *A* and *B*), compared with control samples.

Maintenance of cardiac integrity also depends on the link between adjacent CMs mediated by the intercalated disks (ICD) (30, 31). Although Tln is mainly localized at costameres, it has also been detected at low levels in ICDs (17). Therefore, we analyzed the expression levels of ICD proteins in adherens and gap junctions, as well as in desmosomes. No changes in protein levels of desmoplakin, plakophilin, *N*-cadherin, or connexin-43 were detected in Tln2KO hearts compared with controls (*SI Appendix*, Fig. S5).

Combined Loss of Tln1 and Tln2 from CMs Leads to Rapid Cardiac Dysfunction. Because up-regulation of Tln1 in Tln2KO CMs is able to functionally compensate for loss of Tln2, we developed a strategy to delete both Tln isoforms from heart. For this, we crossed the Tln1 CM-specific KO mice (Tln1flox/flox × α-MHC-Cre) that we generated previously (17) (Tln1cKO) with Tln2KO mice. In our prior studies, we showed that the α-MCH-Cre transgene is active beginning at E10.5 during heart development (17). Tln1cKO/Tln2KO mice were born at expected Mendelian ratios (*SI Appendix*, Table S4) and appeared morphologically normal at birth. However, beginning at 4 wk, the mice began to develop significant cardiac dilation, wall thinning, and dysfunction vs. Tln1flox/flox/Tln2KO controls, as determined by echo (*SI Appendix*, Fig. S6 *A* and *B*). Progressive heart failure ensued, and the Tln1cKO/Tln2KO mice showed significant mortality beginning at 8 wk with 100% mortality by 6 mo of age (Fig. 5*A*). In agreement with the functional and structural echo data, morphometric and histological examination of 8-wk hearts showed clear evidence of a dilated cardiomyopathy, with dilated chambers, increased total normalized muscle mass, thinned ventricular walls and significant fibrosis (Fig. 5 *B* and *C*).

Because previous studies have shown that CM Cre expression can potentially induce an independent cardiomyopathy (32), we evaluated the α-myosin heavy chain (MHC) Cre mice in the same mouse background used to generate the Tln1cKO/Tln2KO. No changes in morphometry were noted in these controls, indicating that the cardiomyopathic phenotype detected with loss of CM Tln1 and Tln2 is not simply due to CM Cre expression (*SI Appendix*, Fig. S6*C*). Furthermore, prior work by ourselves and others with this specific Cre mouse line has shown no evidence of heart failure (17, 32–34).

Loss of CM Tln1 and Tln2 Leads to Defects in Integrin Adhesion Complexes, Abnormal Costameres, and Subsequent Loss of Membrane Integrity. To determine the mechanism by which loss of CM Tln leads to cardiac dysfunction, we first evaluated the expression and cellular arrangement of other proteins in the cell-matrix adhesion complex. To be sure that we assessed only the primary effects of CM Tln loss, as opposed to those produced from general heart failure, we analyzed hearts from mice at 3–4 wk with normal cardiac function. Immunomicroscopic analyses were performed on cardiac tissue from Tln1cKO/Tln2KO and Tln1flox/flox/Tln2KO control littermates. In control hearts, Tln1 expression was detected in CM costameres as well as in cardiac interstitial cells, i.e., endothelial cells and fibroblasts. In contrast, in Tln1cKO/Tln2KO hearts, Tln1 expression was restricted to interstitial cells (indicated by arrows in Fig. 6*A*) although a few CMs still expressed Tln1 in line with the known incomplete activity of the α-MHC Cre transgene (17) (indicated by asterisks in Fig. 6*A*).

As for Tln2KO mice, we assessed the expression and localization of CM integrins and integrin-related proteins in Tln1cKO/Tln2KO hearts versus Tln1flox/flox/Tln2KO controls. In control hearts, which can express CM Tln1, β1 integrin was detected in CM costameres, as well as in interstitial cells. However, in Tln1cKO/Tln2KO hearts, β1-integrin levels were reduced in parallel with the

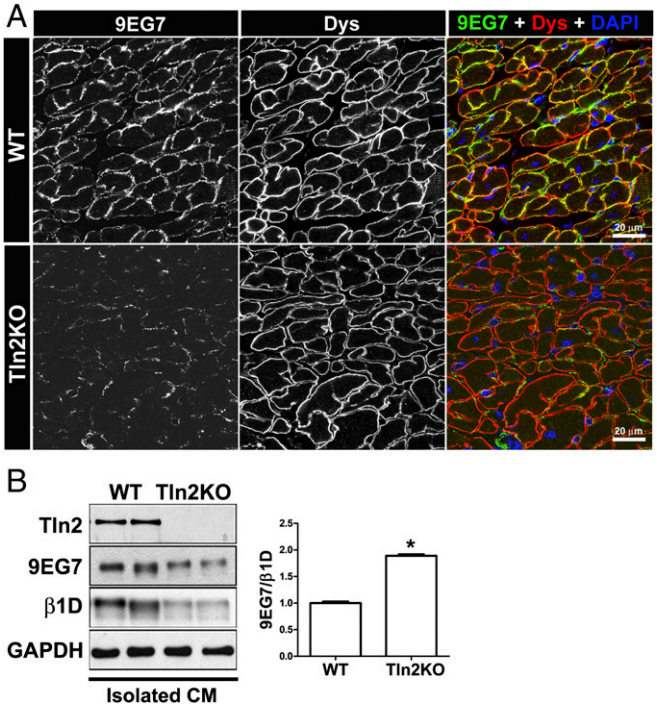

Fig. 4. β1 activation in cardiac myocytes is increased in Tln2KO myocardium. (*A*) Immunofluorescent microscopy using antibody 9EG7 to detect β1-integrin activation and Dys to detect myocytes in adult cardiac tissue from WT and Tln2KO male mice. (*B*) Western blotting and densitometric analysis for activated β1 integrin (9EG7) and total β1D integrin in isolated cardiac myocytes from 2-mo-old WT and Tln2KO male mice. GAPDH was used as loading control. Data are mean ± SEM; $n = 7$ in each group. Data were analyzed by *t* test vs. WT and were significant as indicated (*$P < 0.05$). A higher-quality version of this figure can be viewed in the *SI Appendix*.

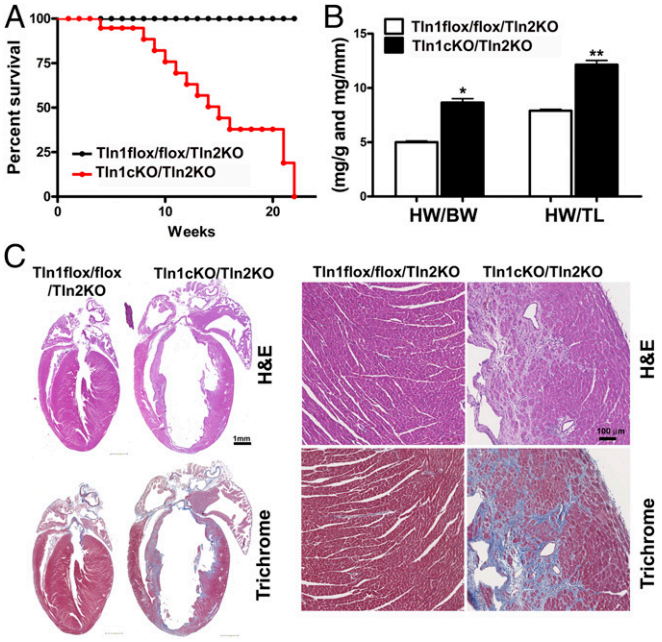

Fig. 5. Combined loss of Tln1 and Tln2 from CMs leads to a dilated cardiomyopathy and reduced survival. (*A*) Survival curve shows that Tln1cKO/Tln2KO male mice die between 2 and 6 mo of age [Tln1flox/flox/Tln2KO (control): $n = 23$; Tln1cKO/Tln2KO: $n = 16$]. (*B*) Morphometric data of male mice that survived to 8 wk showed increased HW/BW and HW/TL in the basal Tln1cKO/Tln2KO vs. Tln1flox/flox/Tln2KO used as controls. Data are mean ± SEM, Tln1flox/flox/Tln2KO $n = 7$ and Tln1cKO/Tln2KO $n = 10$. Data were analyzed by *t* test vs. Tln1flox/flox/Tln2KO and were significant as indicated (*$P < 0.05$; **$P < 0.01$). (*C*) Histological (H&E and trichrome staining) analyses showed cardiac dilation with fibrosis in 8-wk-old male Tln1cKO/Tln2KO mice vs. control Tln1flox/flox/Tln2KO. A higher-quality version of this figure can be viewed in the *SI Appendix*.

loss of Tln from CMs (Fig. 6*A*). Vinculin directly binds Tln and is localized at both costameres and ICDs in muscle cells. Loss of both Tln isoforms led to displacement of Vcl from its traditional costameric site, but it was still localized normally at ICDs that are not noted to be integrin/Tln-dependent structures (Fig. 6*B* and *SI Appendix*, Fig. S7). Together, these results suggest that loss of both Tln isoforms from CMs leads to altered expression and localization of costameric proteins.

Costameres link sarcomeres at the Z-disk to the sarcolemma, play important roles in mechanotransduction, and preserve muscle integrity during basal contraction and when muscle is subjected to increased workloads. Evans Blue Dye (EBD) is commonly used to detect early muscle damage with resultant increased membrane permeability (35) because it can be used as a vital stain administered to the intact animal. Since costameric protein changes were noted in the Tln-deficient mice, we used EBD staining to establish if early muscle damage was evident in Tln1cKO/Tln2KO mice before there was depressed whole-heart function that could be detected by echo. None of the CMs in control mice were stained by EBD. However, in multiple regions of Tln1cKO/Tln2KO hearts, there was clear indication that EBD–albumin complexes were present, indicating early muscle damage (Fig. 7). Thus, when both Tln1 and Tln2 are lost from the CM, altered expression and localization of costameric proteins occurs along with resultant muscle damage as evidenced by increased uptake of EBD.

Loss of Tln from Cultured CMs Recapitulates in Vivo Findings, Showing That Tln Is Required for Preservation of Normal CM Structure and Function. To further dissect the mechanism whereby loss of Tln from the CM leads to cellular and whole-heart dysfunction, we used an in vitro model created with neonatal mouse ventricular myocytes (NMVMs) derived from Tln1flox/flox/Tln2KO mice plated onto fibronectin. This allowed us to generate NMVMs deficient in both Tln1 and Tln2 by infection of cells with recombinant adenovirus (Ad) expressing Cre-recombinase (Ad/Cre). Tln1flox/flox/Tln2KO NMVMs infected with adenovirus expressing β-galactosidase (Ad/LacZ) were used as controls.

Initially, we performed a time course to test how Tln1 protein levels decreased over time. We observed that Ad/Cre-infected cells were visibly indistinguishable from Ad/LacZ control cells at 24 h post infection, at which point Tln1 protein expression did not differ between groups (*SI Appendix*, Fig. S8 *A* and *B*). By 48 h post infection, Ad/Cre-infected cells began to change morphology as Tln1 levels decreased. At 72 h, small numbers of Ad/Cre-infected cells began to detach from the substrate, and by 96 h, when Tln1 levels were substantially reduced, cell detachment was widespread. We found that β1D-integrin protein levels decreased in parallel with loss of Tln. In contrast, Vcl levels did not change (*SI Appendix*, Fig. S8*A*).

Given these results, we analyzed NMVMs 72 h post infection when Tln protein levels were significantly reduced. Immunomicroscopy showed that control cells displayed Tln1 in precostameric

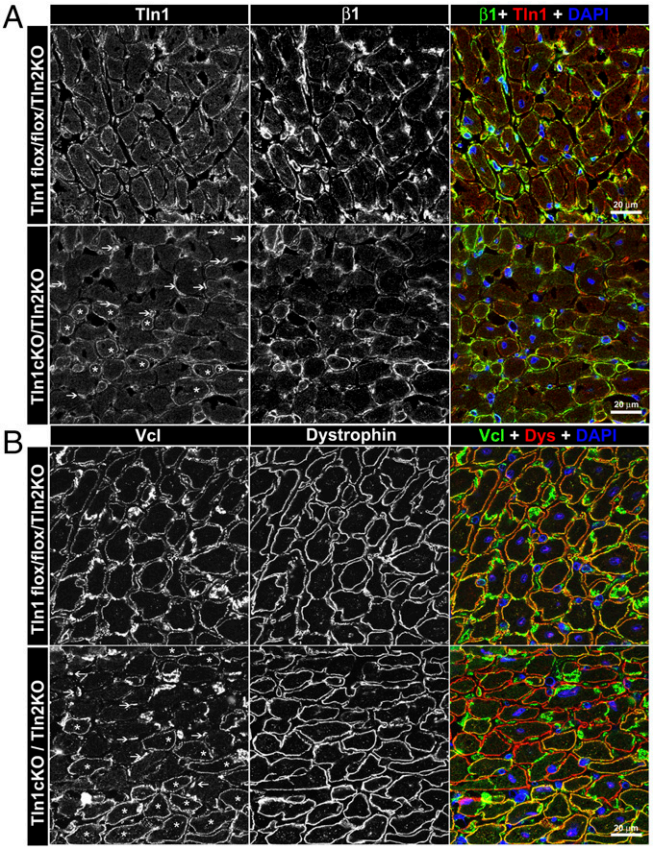

Fig. 6. Loss of all Tln forms from the CM leads to concomitant reduction of CM integrin and costameric proteins. Immunofluorescent microscopy of Tln1flox/flox/Tln2KO and Tln1cKO/Tln2KO cardiac tissue from 4-wk-old male mice for (*A*) Tln1 and β1 integrin (β1) and for (*B*) Vcl and Dys shows that, when all Tln isoforms in CMs are deleted, neither β1 integrin nor Vcl are highly localized at the CM membrane. DAPI (blue) was used to identify nuclei. Asterisks indicate CMs from Tln1cKO/Tln2KO hearts in which Tln1 expression remains, as is common given the chimeric expression of Cre transgenes. These Tln1 expressing CMs still show β1 integrin and Vcl expression at the membrane. Arrows indicate non-CMs in Tln1cKO/Tln2KO hearts. A higher-quality version of this figure can be viewed in the *SI Appendix*.

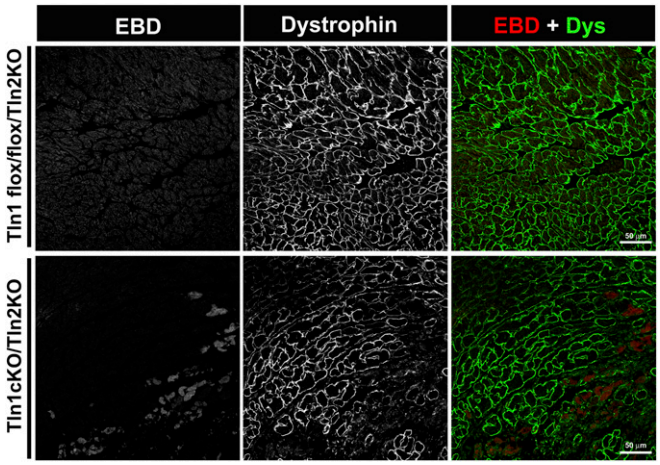

Fig. 7. CM membrane stability is lost in the Tln-deficient cell. EBD was used to assess for membrane permeability, indicative of early cell damage. Tln1flox/flox/Tln2KO and Tln1cKO/Tln2KO mice were injected i.p. with EBD at 4 wk, and heart tissue was analyzed for EBD localization, shown as red. Anti-Dys antibody was used to identify myocyte cell membranes. Significant EBD uptake was only detected in the Tln1cKO/Tln2KO hearts. A higher-quality version of this figure can be viewed in the *SI Appendix*.

structures. In contrast, Tln1 and β1D integrin were missing from these structures in Tln-deficient NMVMs, in agreement with the reduction of β1D observed by Western blotting (Fig. 8). Although expression of Vcl was maintained, it was displaced from costameres in the Tln-deficient NMVMs, even though it still localized at nascent cell–cell junctions (Fig. 8). FAK was also displaced from the costameric structures in the Tln-deficient cells (Fig. 8).

Sarcomeric α-actinin (s–α-actinin) is known to be located at the Z-lines of CMs, so we used it as a sarcomeric marker. In control NMVMs, s–α-actinin displayed a well-organized and compact Z-line pattern. In contrast, although the Z-line pattern was generally preserved in Tln-deficient cells, it appeared looser and less organized than in controls (Fig. 8). This result suggests that loss of Tln leads to disassembly of integrin-containing costameric complexes, likely resulting in a weaker membrane–sarcomere connection and sarcomeric disorganization. These data are consistent with the results presented above in the Tln1cKO/Tln2KO mouse heart.

Transgenic Overexpression of Integrins in the Tln-Deficient CM Cannot Improve Myocardial Dysfunction nor Restore the Normal CM Phenotype in Vitro. We reasoned that the phenotype in the Tln-deficient CMs and heart could result from (*i*) defects in the linkage between integrins and the cytoskeleton/sarcomere; (*ii*) the down-regulation of integrin protein levels; or (*iii*) a combination of both. To determine which of these two mechanisms is the main contributor to the phenotype, we sought to establish whether forced overexpression of integrin subunits could "rescue" the cellular defects.

First, we evaluated this using an in vivo model where we attempted rescue of the cardiomyopathic phenotype of the Tln1cKO/Tln2KO mice by overexpressing α7β1D integrins in CMs via transgenesis. For this, we took advantage of the α7 and β1D transgenic mice that we had generated previously (36). Crossing the Tln1cKO/Tln2KO mouse line with the α-myosin heavy chain–α7β1D transgenic mice allowed us to generate a model that lacks both Tln1 and Tln2 in CMs, but directs cardiomyocyte-specific expression of α7β1D transgenes. These mice were termed Tln1cKO/Tln2KO/α7β1D overexpression (OE) (Fig. 9*A*). The human β1D transgenic integrin was properly located at the costameres in the CM membrane of Tln1flox/flox/α7β1D OE and Tln2KO/Tln1flox/flox/α7β1D OE hearts (*SI Appendix*, Fig. S9). Interestingly, the transgenic human β1D isoform displaced the endogenous mouse β1 protein from its costameric location, probably due to the high amount of human β1D expressed by the transgene (*SI Appendix*, Fig. S9). Histological, morphometric, and echocardiographic analyses showed that integrin overexpression could not modify the phenotype in 8-wk-old Tln1cKO/Tln2KO mice (Fig. 9 *B–D*). The Tln1cKO/Tln2KO/α7β1D OE mice still showed cardiac fibrosis, chamber enlargement, and severe cardiac dysfunction compared with the two controls groups (Tln2KO/Tln1flox/flox and Tln2KO/Tln1flox/flox/α7β1D OE).

We then also tested if the CM-specific transgenic α7β1D integrin would allow β1D stabilization at the membrane in the mice lacking both Tln isoforms. Immunomicroscopy was used to detect β1-integrin protein localization. As shown in Fig. 9*E* and *SI Appendix*, Fig. S9, this approach was not successful in restoring β1D levels at the myocyte membrane.

Next, we moved from this in vivo model to a CM cell culture one to allow further evaluation of why this rescue attempt was not successful. To test this, we used the NMVM model outlined above and infected NMVMs with adenovirus constructs encoding human β1D integrin (Ad/hβ1D) and α5 integrin (Ad/α5). Adenoviral expression of the integrin subunits in Ad/LacZ control cells led to a marked up-regulation of α5 and β1D protein levels, but similar increases could not be achieved in the Tln-deficient cells plated on fibronectin, the α5β1 integrin ligand (Fig. 10*A*). Moreover, cell detachment and the cell death, measured by lactate dehydrogenase (LDH) levels in the cell media and observed in Ad/Cre/Tln-deficient cells, could not be rescued by infecting cells with the hβ1D and α5 viruses (Fig. 10 *B* and *D*). Because α7β1D, a laminin receptor, is the main integrin receptor in adult CMs (20, 37), we also infected cells with Ad/α7 and Ad/hβ1D and plated them on laminin. Similar to the experiments

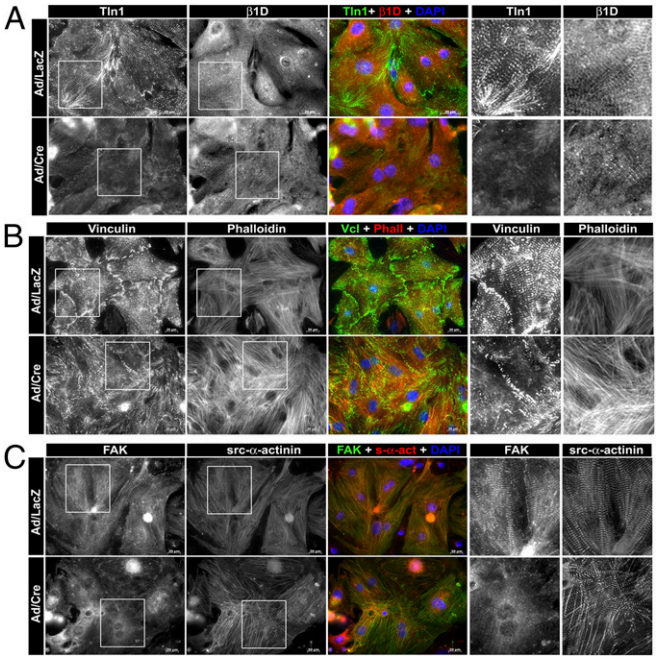

Fig. 8. Loss of Tln from cultured NMVMs recapitulates the in vivo findings, showing that talin protein is required for preservation of normal β1-integrin expression, costamere structure, and sarcomere organization. Immunofluorescent microscopy of Tln1flox/flox/Tln2KO NMVMs infected with Ad/LacZ (control) and Ad/Cre (to excise the floxed Tln1 gene) for 72 h for (*A*) Tln1 (green) and β1D (red), (*B*) vinculin (Vcl, green) and Phalloidin (Phall, red), and (*C*) focal adhesion kinase (FAK, red) and s–α-act (green). DAPI (blue) was used to localize nuclei. A higher-quality version of this figure can be viewed in the *SI Appendix*.

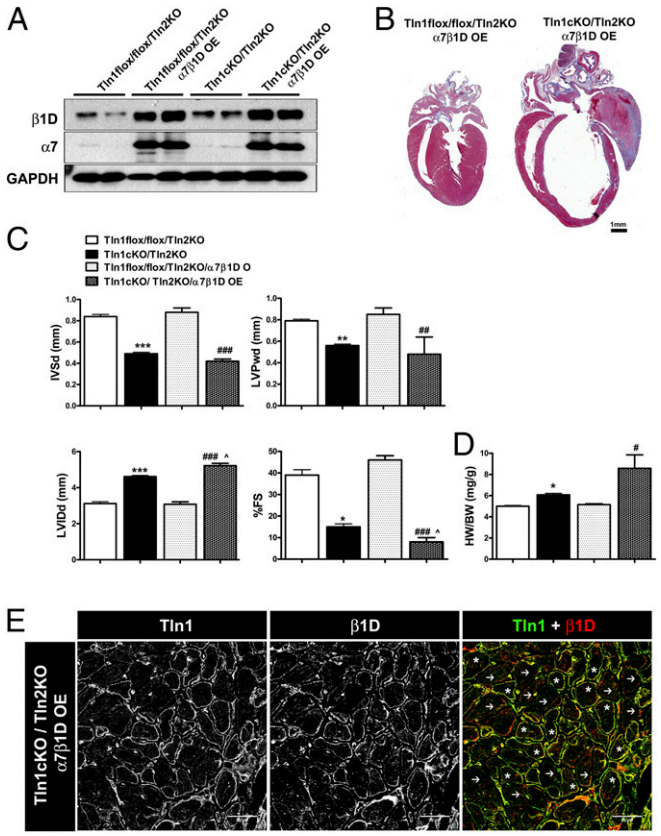

Fig. 9. α7β1D integrin overexpression in Tln-deficient mice does not rescue the cardiomyopathy or prevent premature death observed in Tln1cKO/Tln2KO. (*A*) Lysates from Tln1flox/flox/Tln2KO, Tln1cKO/Tln2KO, Tln1flox/flox/Tln2KO/α7β1D OE, and Tln1cKO/Tln2KO/α7β1D OE adult cardiac tissue (8 wk) were analyzed by Western blotting for β1D and α7 integrins. GAPDH was used as a loading control. (*B*) Histological (H&E and trichrome staining) analyses showed cardiac dilation with fibrosis in 8-wk-old male Tln1cKO/Tln2KO/α7β1D OE mice compared with Tln1flox/flox/Tln2KO/α7β1D OE. (*C*) Echocardiographic analysis. Cardiac function (%FS) and wall thickness (LVPwd and IVSd) were decreased, whereas left ventricle (LV) dilation occurred (LVID) in Tln1cKO/Tln2KO and Tln1cKO/Tln2KO/α7β1D OE mice compared with Tln1flox/flox/Tln2KO and Tln1flox/flox/Tln2KO /α7β1D OE mice (Tln1flox/flox/Tln2KO: $n = 4$; Tln1cKO/Tln2KO: $n = 8$; Tln1flox/flox/Tln2KO/α7β1D OE: $n = 4$; Tln1cKO/Tln2KO/α7β1D OE: $n = 4$, all at 8 wk). (*D*) Morphometry in 8-wk-old male mice (Tln1flox/flox/Tln2KO: $n = 10$; Tln1cKO/Tln2KO: $n = 7$; Tln1flox/flox/Tln2KO/α7β1D OE: $n = 7$; Tln1cKO/Tln2KO/α7β1D OE: $n = 6$). Data in *A* and *B* are mean ± SEM. Data were analyzed by *t* tests and were significant as indicated (Tln1flox/flox/Tln2KO vs. Tln1cKO/Tln2KO: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; Tln1flox/flox/Tln2KO/α7β1D OE vs. Tln1cKO/Tln2KO/α7β1D OE: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$; Tln1cKO/Tln2KO vs. Tln1cKO/Tln2KO/α7β1D OE: $\hat{}P < 0.05$). FS: fractional shortening; IVSd: interventricular septum in diastole; LVPwd: left ventricular posterior wall in diastole; LVIDd: left ventricular internal dimension in diastole. (*E*) Immunofluorescent microscopy of Tln1cKO/Tln2KO/α7β1D cardiac tissue from 8-wk-old male mice for Tln1 (green) and β1D integrin (β1D) (red) shows that β1D integrin could not be located at the cellular membrane in Tln1-deficient CMs, even with transgenic CM-specific β1D overexpression. Asterisks indicate CMs in which Tln1 expression remains, as is common given the chimeric expression of Cre transgenes. β1D integrin expression is still detected at the membrane in these cells. Arrows indicate CMs with total Tln (Tln1 and Tln2) deletion. A higher-quality version of this figure can be viewed in the *SI Appendix*.

with Ad/α5 and Ad/hβ1D, α7 and β1D were up-regulated in the Ad/LacZ control group, but this could not be achieved in the Cre-deleted Tln-deficient cells, and likewise, we could not alter the defect in cell adhesion to laminin (*SI Appendix*, Fig. S10).

With forced adenoviral overexpression of integrins in the above experiment, the integrin protein levels detected by Western blotting (Fig. 10*A*) would be the combined expression of both the endogenous mouse form and that produced by the viral expression of the human form. We reasoned that the inability to restore integrin protein levels toward normal with the viral overexpression could be due to transcriptional or posttranscriptional mechanisms. Therefore, we next investigated transcript levels of integrins in the cells because we could capitalize on species-specific sequences and discriminate endogenous mouse from the virally expressed human forms. We found that the endogenous mouse integrin β1D mRNA levels were significantly decreased in the Ad/Cre-infected, Tln-deficient cells, compared with the Ad/lacZ control group (Fig. 10*E*), indicating that reduced protein levels of β1D integrin in Tln-deficient cells is in part due to the down-regulation of its transcript levels.

Next, we investigated expression of the exogenous human β1D transcripts produced by the Ad/hβ1D viral vector. We found that the levels of the virally mediated human β1D transcript were similar in the cells expressing normal amounts of Tln protein, compared with the Tln-deficient cells (Fig. 10*E*). Therefore, the β1D transgene produced by the viral vector is able to generate integrin transcript, but is unable to increase integrin protein in the Tln-deficient CMs due to posttranscriptional changes, likely caused by protein degradation. Combined, these data suggest that, in the absence of both Tln isoforms, CM cannot maintain adequate integrin levels in the cell membrane due to two different mechanisms: (*i*) the down-regulation of β1D mRNA levels and (*ii*) the instability of integrin protein.

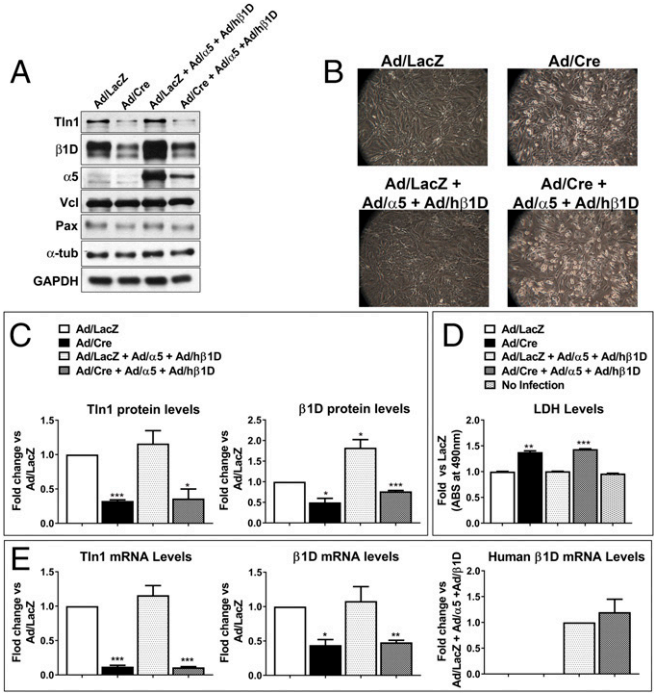

Fig. 10. Forced overexpression of β1 integrins cannot be achieved in Tln-deficient NMVMs, although β1D integrin mRNA levels are restored toward normal. Tln1flox/flox/Tln2KO NMVMs were infected with human Ad/β1D or Ad/α5 for 48 h and subsequently with Ad/LacZ or Ad/Cre for an additional 72 h. (*A*) Protein lysates were analyzed by Western blotting for Tln1, β1D, α5, Vcl, and Pax. GAPDH and α-tubulin were used as loading controls. (*B*) Phase-contrast images of cell groups. (*C*) Densitometric quantification of Western blot data from *A* analyzed for Tln1 and β1D. (*D*) Tln deletion from NMVM leads to increased LDH release, indicating cell injury, compared with Ad/LacZ-treated control cells. (*E*) qRT-PCR was used to quantify Tln1, endogenous β1D, and exogenous human β1D integrin. Data for all panels are mean ± SEM; $n = 3$. All data were analyzed by *t* tests vs. AdLacZ and were significant as indicated ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). A higher-quality version of this figure can be viewed in the *SI Appendix*.

Discussion

In this paper, we have provided a comprehensive study to examine, first, the role of Tln2 and, second, the function of Tln protein in general in the heart. Tln2 is the major Tln isoform in striated muscle, and here we provide a report on the role of Tln2 in mammalian cardiac myocytes. Initially, we used Tln2KO mice, which are viable and fertile. In the absence of Tln2, CMs showed a marked compensatory up-regulation of Tln1 protein but not Tln1 mRNA. Remarkably, we found no change in cardiac structure or function even in mice more than 1 y old, although we have previously shown that up-regulation of CM Tln1 occurs in WT mice provoked to develop a hypertrophic response (17). The one notable change in the CMs from Tln2KO mice was a 50% reduction in levels of the muscle-specific β1D-integrin protein, but not mRNA, although the remaining β1D integrin showed normal localization as well as increased activation. Next, we analyzed the effects of deleting both Tln1 and Tln2 on CM function using a Tln1flox/flox × α-MHC-Cre/Tln2KO mouse model. Loss of both Tln isoforms led to the onset of cardiac dysfunction with 100% mortality by 6 mo. Concomitant with this, β1 integrin, as well as other proteins (e.g., vinculin), were lost from costameres, and cell damage occurred likely due to loss of membrane integrity. CM-specific integrin transgenes could not rescue this phenotype in Tln1/Tln2-null cells or in the intact mouse heart. Thus, Tln protein is essential for proper β1D integrin expression at the CM membrane for preservation of costamere and cellular integrity and ultimately for normal myocardial function.

The finding that up-regulation of CM Tln1 compensates for loss of Tln2 indicates that the two Tln isoforms are interchangeable in CM. Indeed, both proteins have a very similar amino acid sequence and domain structure (10), and both can rescue cell spreading, integrin activation, and FAK phosphorylation in Tln-deficient fibroblasts (11). However, Tln1 was unable to maintain normal β1D-integrin protein levels in Tln2KO CMs. We suggest that substitution of Tln1 for Tln2 leads to increased β1D degradation or turnover because Tln1 binds β1D integrin with lower affinity than Tln2 (18) and is therefore less effective in stabilizing β1D integrin at the sarcolemmal membrane. Support for this hypothesis comes from our previous studies (17) where we showed that deletion of the Tln1 gene in CMs led to increased levels of β1D integrin. Likewise, work in other cell types has shown that Tln controls β1-integrin expression at the cell membrane (38, 39).

Integrin protein stability at the membrane is determined in part by its turnover rate. This is important because integrin-dependent cell adhesions are dynamic, and so they undergo constant renewal. This process involves disassembly of the integrin adhesion complexes, endocytosis, recycling back to the membrane, and degradation. Endocytosis occurs by two main routes: one that is clathrin-dependent and a second that is clathrin-independent (40). Two clathrin adaptors, disabled-2 (Dab-2) and Numb, bind the NPXY motif in the cytoplasmic tails of β-integrin subunits via their PTB domains (41), and Dab-2 colocalizes in FAs with clathrin, the primary clathrin adaptor AP-2, and dynamin-2 to facilitate β1-integrin internalization and FA disassembly (42). Numb also localizes at adhesions, and its binding to integrin is regulated by atypical protein kinase C (43). Tln binds the same NPXY motif in integrin β-subunits as these two clathrin adapters. Because the connection between Tln1 and β1D is weaker than with Tln2 (18), we hypothesize that loss of Tln2 makes the β1D NPXY motif more available to bind Dab-2/Numb, potentially generating an increase in integrin endocytosis and degradation and a faster turnover of β1D. Further studies will be necessary to test this hypothesis.

Many studies have previously shown that endocytosis and degradation of integrin α- and β-subunits occur simultaneously. In agreement with this, prior work by our laboratory and others in skeletal and cardiac muscle models (29, 44) showed that when β1-integrin expression was directly reduced, α7-integrin, the main partner of β1D in CMs, decreased in parallel. Thus, it was surprising to find here that the reduction in β1D protein levels in the Tln2KO CMs was not accompanied by any significant reduction in the α7-integrin subunit. The fact that there was no compensatory increase of other β-integrin subunits (i.e., β1A or β3) that might alternatively partner with α7, suggests that in CMs either α7 does not get degraded simultaneously with its β1 partner (β1D) or perhaps that α7 is degraded but then has augmented production, allowing it to accumulate out of proportion to β1D. In agreement with this second hypothesis, immunostaining of Tln2KO heart samples shows that α7 expression at the CM membrane is decreased in parallel with β1D. This study also detected increased α7 expression within small reservoirs in the cytoplasm. Thus, in the KO CMs vs. controls, the localization changed, but the total amount of α7 integrin did not. Previous studies in skeletal muscle have shown that the α7-integrin subunit can be retained in the endoplasmic reticulum when there is an excess of protein (45, 46). We suggest that this occurs in the Tln2KO CMs. Clarification will require further study.

Despite the 50% reduction of β1D protein in Tln2KO CMs, the expression and, more importantly, the localization of Vcl was unchanged compared with WT cells. Vcl cross-links Tln to actin. We hypothesize that the number of Vcl molecules binding the β1D-integrin/Tln1 complex in Tln2KO mice is higher (e.g., double) than the number of Vcl molecules bound to the β1D-integrin/Tln2 complex in WT mice. Tln directly links integrins to actin and is stretched as cells transmit forces to the ECM (13, 47). In so doing, it mediates cellular responses to force (12). The Tln rod domain contains up to 11 Vcl-binding sites, but structural studies show that the binding sites can be buried basally, making them unavailable for Vcl binding (10). However, in vitro experiments show that, when force causes Tln to unfold, these "cryptic" Vcl-binding sites (48–50) become exposed. Vcl then locks Tln into an active configuration and stabilizes the link between Tln and F-actin (51). Therefore, increased Vcl binding to Tln1 in the Tln2KO CMs may reinforce the connection of Tln1 to F-actin. In this scenario, despite the reduced levels of β1D integrin in Tln2KO CMs, the connection with the sarcomere would be as strong as in WT cells due to the higher number of Vcl molecules bound per integrin, reinforcing the linkage. This offers a plausible explanation as to why the Tln2KO CMs can still support strong forces, despite the 50% reduction in β1D-integrin levels. We hypothesize that the loading force per integrin could be increased in Tln2KO CMs vs. WT because the tension is distributed among fewer integrins. In turn, this increase in force could increase Tln1 unfolding, exposing the Vcl-binding sites (48, 52) and allowing more Vcl molecules to bind.

Although Tln has been shown to be a structural protein that links integrins and F-actin, it also plays an essential role in causing integrin to become activated and bind to ECM in a variety of cell types (11, 24, 25). In the Tln2-deficient CM, the fraction of activated β1 integrin is increased. This increase could be due to the reduction in the total number of integrin receptors present on the cell surface, as shown in the Tln2KO CMs. The remaining pool of integrins would then have increased activation, binding more avidly to ECM to handle the force imposed on the CMs. Alternatively, because Tln1 takes the place of Tln2 in these Tln2KO CMs, it may imply that Tln1 is more efficient than Tln2 at activating integrins. We would favor the first hypothesis because a recent paper using fibroblasts demonstrated that exchanging Tln1 by Tln2 does not change the activation state of β1 integrin (13). Additional studies are needed to precisely determine which of these two hypotheses is correct in the CM.

Tln1cKO/Tln2KO mice develop significant cardiac dilation at 4 wk that rapidly progresses to heart failure and premature death beginning at 8 wk with 100% mortality by 6 mo of age. In contrast, the Tln2KO mice have normal development, cardiac function, and life span. Although the Tln2KO CMs are seen to have a substantial

immunomicroscopic loss of β1D-integrin protein, the Tln1cKO/Tln2KO mice show almost complete absence of β1D in CMs devoid of both the Tln1 and Tln2 isoforms. Mislocalization of Vcl was detected only in the CMs of the double KO model. Likewise, in the NMVM culture model, when Tln forms are lost, both Vcl and FAK are mislocalized. Therefore, it is only when both Tln isoforms are lost from the CM, that assembly and maintenance of the integrin complex at costameres is compromised, presumably because of defects in integrin activation, stabilization of integrin-ECM binding, and likely loss of coupling of the elaborate ECM–integrin–costamere–sarcomere arrangement. These changes ultimately provoke loss of cellular integrity, suggesting that the integrin–costamere structure is required to maintain myocyte-membrane integrity.

In previous papers studying Tln deletion in nonmuscle cells such as fibroblasts (11) or platelets (53), localization of β1-integrin protein at the cell membrane was not affected, but integrin activation and assembly of FAs was disturbed. In skeletal myoblasts, Conti and colleagues (16) showed that deficiency of both Tln isoforms did not affect either β1 integrin membrane expression or its activation. Still, they described defects in the assembly of integrin adhesion complexes at the myotendinous junctions in these skeletal-muscle–specific Tln1cKO/Tln2KO mice. Conti's cellular work focused on quiescent skeletal myoblasts that express only the ubiquitous β1A isoform, but not the β1D integrin form that is present in differentiated skeletal muscle tissue. Together this suggests that although β1D protein expression is sensitive to Tln deficiency, β1A integrin expression may be less so, particularly in a cell that is subjected to continuous mechanical stress. Studies in *Drosophila melanogaster* support such a hypothesis, in that Tln has been shown to be required for FA assembly, as well as for stabilization of integrins at sites of adhesion in both skeletal muscle and the heart tube (39, 54). It is also important to note that studies with cardiac-specific deletion of other integrin-associated proteins, including several by our group, such as Vcl (31), KN2 (55), PINCH (56) and ILK (21), showed β1-integrin down-regulation and development of a dilated cardiomyopathy. This indicates that preservation of an intact integrin complex at the cardiac costamere requires proteins, apart from Tln, to maintain proper integrin expression at this cellular location.

Furthermore, the Tln1cKO/Tln2KO mouse model that we have analyzed in this study has a much more severe postnatal phenotype than that which occurs with loss of other integrin-related proteins such as KN2. Comparison of our mice and the KN2cKO model that we reported recently (55) is particularly pertinent because in both cases we used the identical α-MHC Cre transgene to produce gene excision, and because in both models a reduction in β1D integrin was noted. We hypothesize that, in the Kn2cKO model, although there is a significant reduction in β1D-integrin protein expression due to increased turnover/degradation, the remaining β1D-integrin protein is sufficient to support proper costameric structure and cardiac function. In contrast, we suggest that the phenotype detected in the Tln1cKO/Tln2KO model is provoked by a more significant loss of β1D-integrin expression at the sarcolemmal membrane, than in the Kn2cKO, leading to disassembly of the costamere in the Tln-deficient CMs.

Attempts to restore integrin expression levels to rescue the phenotype of the Tln1cKO/Tln2KO mice and Tln-deficient NMVMs were not successful. One might question whether species (human vs. mouse) differences in integrin subunits could have led to this failure. Human and mouse β1D-integrin proteins are 92% similar whereas α7 integrins are 89% similar in these species. More importantly, the β1-integrin immunostaining studies (*SI Appendix*, Fig. S10) show that human β1 is properly localized at the CM membrane in the transgenic OE hearts. Therefore, it seems that the difficulty in rescuing the Tln1cKO/Tln2KO phenotype is unlikely due to integrin species variation, but occurs because β1 integrin cannot be stabilized at the sarcolemmal membrane in the absence of Tln.

Attempts to overexpress β1D in cultured NMVMs showed that Tln regulates β1D-integrin expression, likely through combined transcriptional and posttranscriptional means. Therefore, we hypothesize that Tln deletion generates two different effects in the CM. First, with loss of Tln, decreased costameric tension occurs because integrins are disconnected from sarcomeres, resulting in a decrease in β1D-integrin mRNA. Second, with loss of CM Tln, Dab-2 or Numb could increase their integrin binding, leading to increased integrin protein degradation/turnover. These two effects would generate a rapid and complete β1D down-regulation resulting in costamere disassembly.

In conclusion, our studies using both mouse models and Tln-deficient CMs show that Tln is necessary for stabilizing β1D-integrin expression at the CM membrane and for maintaining proper CM integrity. Because attempts to rescue this phenotype by restoration of CM integrin expression were not successful, it appears that proper expression of Tln protein is necessary for maintenance of the integrin adhesion complex and its attendant proteins in the CM and, ultimately, for normal CM and whole-heart function. Future work will be necessary to determine precisely how Tln controls integrin complexes in the mature cardiac myocyte and to establish the significance of the switch from expression of Tln1 to Tln2 during cardiac development.

Materials and Methods

Mouse Lines. The generation of *Tln1-flox*, *Tln2KO*, MHC nuclear Cre, and the CM-specific overexpressing α7 and β1D transgenic mice has been described (16, 36, 57). The mice were analyzed on a mixed 129/C57BL/6 genetic background, and, unless otherwise stated, male mice that were littermate controls or from an identical genetic background were analyzed. All animal use protocols were approved by the University of California at San Diego and Veterans Administration San Diego Institutional Animal Care and Use Committees. These investigations conformed to the *Guide for the Care and Use of Laboratory Animals* adopted by the NIH (58).

Cardiac Myocyte Isolation, Infection, and Culture. Neonatal and adult mouse CMs were isolated as has been outlined previously (17), and information about culture conditions and viral treatments used are outlined in *SI Appendix, Supplemental Materials and Methods*.

Cytotoxicity Assay. LDH release was measured in NMVM cell culture supernatants using a colorimetric assay kit according to the manufacturer's instructions (catalog no. 04744926001, Roche Applied Science).

Western Blotting and Immunofluorescent Microscopic Analyses. At study termination, cardiac tissue or cell protein was prepared as outlined previously (17) and then used for Western blotting or immunomicroscopic procedures. Antibodies and concentrations used are outlined in *SI Appendix, Supplemental Materials and Methods*. For immunomicroscopic analyses, heart tissues or NMVMs were stained with primary and secondary antibodies using methods as outlined previously (17). Antibodies and concentrations used are outlined in *SI Appendix, Supplemental Materials and Methods*.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR. RNA was extracted and purified from heart tissue, NMVM, or isolated adult CMs using RNA-Bee (Tel-Test, Inc.) according to the manufacturer's protocol. Primer sequences and reaction protocols used are outlined in *SI Appendix, Supplemental Materials and Methods*.

EBD Uptake. EBD (Sigma) was dissolved in PBS (10 mg/mL) and 100 μL/10 g body weight was injected i.p. Twelve hours after injection, mice were killed, and hearts were dissected and cryoprotected in 30% sucrose/PBS overnight at 4 °C, embedded in optimal cutting temperature mounting media, and then cut to a 10-μm thickness on a standard cryotome. Sections were analyzed using a Zeiss AxioObserver microscope.

Echocardiography Analyses. The echo was performed on animals under isoflurane anesthesia and measured by investigators blinded to the genotype of the animal, as previously described (17), using a Vevo 2100 machine (Visualsonics).

Statistical Methods. Data are shown as means ± SEs of the mean (SEM). Data were evaluated using unpaired, two-tailed *t* tests (95% confidence interval)

and GraphPad Prism4 software (GraphPad Inc.). A P value < 0.05 was considered significant.

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