ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/10969596

The Cardiac Mechanical Stretch Sensor Machinery Involves a Z Disc Complex that Is Defective in a Subset of Human Dilated Cardiomyopathy

ARTICLE *in* CELL · JANUARY 2003

Impact Factor: 32.24 · DOI: 10.1016/S0092-8674(02)01226-6 · Source: PubMed

CITATIONS
506

READS
27

21 AUTHORS, INCLUDING:

Ralph Knöll
AstraZeneca and ICMC - Karolinska
58 PUBLICATIONS **2,043** CITATIONS
SEE PROFILE

Marie-Louise Bang
National Research Council/Humanitas Res…
36 PUBLICATIONS **3,528** CITATIONS
SEE PROFILE

Wolfgang Schaper
Max Planck Institute for Heart and Lung R…
592 PUBLICATIONS **19,939** CITATIONS
SEE PROFILE

Jeffrey H Omens
University of California, San Diego
127 PUBLICATIONS **4,201** CITATIONS
SEE PROFILE

Available from: Heinz-Peter Schultheiss
Retrieved on: 02 February 2016

Cell, Vol. 111, 943–955, December 27, 2002, Copyright ©2002 by Cell Press

The Cardiac Mechanical Stretch Sensor Machinery Involves a Z Disc Complex that Is Defective in a Subset of Human Dilated Cardiomyopathy

Ralph Knöll,[1,2,14] Masahiko Hoshijima,[1,2,14] Hal M. Hoffman,[2,3] Veronika Person,[6] Ilka Lorenzen-Schmidt,[4] Marie-Louise Bang,[1,2] Takeharu Hayashi,[7] Nobuyuki Shiga,[8] Hideo Yasukawa,[1,2] Wolfgang Schaper,[6] William McKenna,[9] Mitsuhiro Yokoyama,[8] Nicholas J. Schork,[5] Jeffrey H. Omens,[2,4] Andrew D. McCulloch,[4] Akinori Kimura,[7] Carol C. Gregorio,[10,11] Wolfgang Poller,[12] Jutta Schaper,[6] Heinz P. Schultheiss,[12] and Kenneth R. Chien[1,2,13]

[1]Institute of Molecular Medicine
[2]Department of Medicine
[3]Department of Pediatrics
[4]Department of Bioengineering
[5]Department of Psychiatry
University of California at San Diego
9500 Gilman Drive
La Jolla, California 92093
[6]Max-Planck Institute
61231 Bad Nauheim
Germany
[7]Department of Molecular Pathogenesis
Medical Research Institute
Tokyo Medical and Dental University
Tokyo 101-0062
Japan
[8]Division of Cardiovascular and Respiratory Medicine
Department of Internal Medicine
Kobe University Graduate School of Medicine
Kobe 650-0017
Japan
[9]Department of Cardiological Sciences
St. George's Hospital Medical School
Crammer Terrace
London SW170RE
England
[10]Department of Cell Biology and Anatomy
[11]Department of Molecular and Cellular Biology
University of Arizona
Tucson, Arizona 85724
[12]Universitätsklinikum Benjamin Franklin
Medizinische Klinik II
Freie Universität Berlin
12200 Berlin
Germany

Summary

Muscle cells respond to mechanical stretch stimuli by triggering downstream signals for myocyte growth and survival. The molecular components of the muscle stretch sensor are unknown, and their role in muscle disease is unclear. Here, we present biophysical/biochemical studies in muscle LIM protein (MLP) deficient cardiac muscle that support a selective role for this Z disc protein in mechanical stretch sensing. MLP interacts with and colocalizes with telethonin (T-cap), a titin interacting protein. Further, a human MLP mutation (W4R) associated with dilated cardiomyopathy (DCM) results in a marked defect in T-cap interaction/localization. We propose that a Z disc MLP/T-cap complex is a key component of the in vivo cardiomyocyte stretch sensor machinery, and that defects in the complex can lead to human DCM and associated heart failure.

Introduction

Dilated cardiomyopathy (DCM) and associated heart failure are major causes of human morbidity and mortality. Studies in gene-targeted animal models and familial forms of the human disease are now pointing to a previously unsuspected role of the cardiac myocyte cytoskeleton in the pathogenesis of DCM (Chien, 2000). The cytoskeleton of myocytes includes the Z disc, which defines the lateral boundaries of the sarcomere. Z discs constitute an anchoring site for actin filaments, titin, and nebulin filaments, and because of this anchoring property are the primary conduits of the force generated by contraction. One of the major components of the Z disc is α actinin, which crosslinks cardiac actin and titin molecules from neighboring sarcomeres. Interaction of titin with telethonin (T-cap) at the Z disc is required for sarcomeric function (Gregorio et al., 1998). Z discs of adjacent myofibrils are aligned, providing a means to coordinate contractions between individual myofibrils to a focal point where the Z disc is linked to the muscle membrane. The Z disc plays a pivotal role in diverse aspects of heart muscle structure and function (Clark et al., 2002), including sarcomeric assembly and organization, sarcolemmal membrane integrity, and muscle force generation and transmission.

Mice that harbor a deficiency in the muscle-specific LIM protein (MLP), another Z disc protein, exhibit chamber dilation and contractile dysfunction (Arber et al., 1997). This provides evidence that defects in the components of the Z disc can trigger disease pathways, and is supported by the phenotypes of mice deficient in other muscle-restricted Z disc LIM domain proteins (Pashmforoush et al., 2001; Zhou et al., 2001). Cardiac actin mutations have been identified in familial forms of human DCM (Olson et al., 1998) and a diverse group of cytoskeletal gene mutations have now been linked to DCM (Seidman and Seidman, 2001). The identification of the precise molecular mechanisms that lead from the cytoskeletal defects in the cardiac Z disc to DCM is critical for the design of therapeutic strategies to prevent disease progression.

Disease progression in cardiomyopathy and chronic heart failure can be driven by elevated mechanical stress on cardiomyocytes, which leads to cell injury and the

[13]Correspondence: kchien@ucsd.edu
[14]These authors contributed equally to this work.

loss of residual myocardial cells (Chien, 1999; Hoshijima and Chien, 2002). In the heart, this mechanical stress primarily arises from increases in cardiac chamber volume and pressure, secondary to myocardial injury or hypertension. In this regard, in vivo biomechanical stress has been documented to trigger the activation of multiple signaling pathways, which enhance myocardial cell survival and prevent the onset of dilated cardiomyopathy (Hirota et al., 1999; Yasukawa et al., 2001). This suggests a potential role for defective mechanical stress sensor pathways in heart failure progression. Accordingly, it is possible that the deficiency of MLP or other Z disc-linked proteins might lead to DCM as a result of an intrinsic role for the Z disc proteins in the pathways of cardiac muscle mechanical stress sensing. Although stretch activated receptors and channels have been suggested as potential mediators (Sadoshima and Izumo, 1997; Zeng et al., 2000), the molecular components of the muscle stretch sensor are completely unknown.

Here, we present a combination of biochemical and biophysical studies in MLP deficient cardiac muscle providing evidence suggesting that an MLP/T-cap complex is an essential component of the cardiac mechanical stretch sensor machinery. Screening of over 1400 patients identified a human MLP mutation (W4R) that results in the complete loss of T-cap interaction, the mislocalization of T-cap, and a strong association with DCM. Haplotype analysis of nine W4R patients from distinct families suggests a founder effect in a subset of the European population. We propose that defects in the MLP/T-cap/titin component of the cardiac muscle mechanical stretch sensor links cardiac Z disc proteins with human dilated cardiomyopathy and heart failure.

Results

A Selective Defect in the Mechanical Stretch Response of Intact Juvenile MLP$^{-/-}$ Cardiac Muscle

The echocardiographic measurement of multiple indexes in MLP$^{-/-}$ mice revealed normal cardiac structural and functional phenotypes at 2 weeks of age, with chamber dilation and contractile dysfunction appearing approximately at 4 weeks of age (Figure 1A). This age-dependent progression of heart failure offered an early time window to analyze the primary defects of MLP null myocardium in animals with normal cardiac phenotypes. To detect subtle intrinsic mechanical abnormalities in MLP$^{-/-}$ myocytes, we exposed papillary muscles to incremental passive stretch by increasing the length of the contracting cardiac muscle. Subsequently, we monitored the tension generated as a biophysical estimate of downstream stretch activation responses.

As shown in Figure 1C, the MLP$^{-/-}$ papillary muscles displayed a severe intrinsic defect in tension development for a given increase in muscle length. On the other hand, there was no difference in the recovery from this maximal passive stretch stimulus in MLP deficient papillary muscle as the muscle returned to its original length.

The time constant of the recovery from an active contraction was also not different between MLP$^{+/+}$ and MLP$^{-/-}$ papillary muscles (data not shown). Thus, the observed defects in the MLP$^{-/-}$ papillary muscle are reversible and restricted to the passive stretch properties. The generation of tension during passive stretch in cardiac muscle is mainly carried by two components: the intrinsic cytoskeletal protein titin and extracellular collagen (Granzier and Irving, 1995; Wu et al., 2000; for a review, Trombitas et al., 2000). Studies in rat trabeculae (Granzier and Irving, 1995) have identified titin as the major component that mediates the tension generated following increases in passive muscle stretch thereby suggesting that the MLP$^{-/-}$ papillary muscles may have an intrinsic defect in titin function.

A Selective Loss of Passive Stretch Sensing in MLP$^{-/-}$ Neonatal Cardiac Muscle Cells

The defective intrinsic stretch responses observed in juvenile MLP$^{-/-}$ papillary muscles prompted us to investigate in vitro mechanical stress-dependent signaling in MLP$^{-/-}$ cardiomyocytes from neonatal ventricles. Elastic silicon membranes were coated with collagen type I, and neonatal mouse myocardial cells were plated directly on the membranes. Then, cells derived from either wild-type or MLP$^{-/-}$ ventricles were exposed to either a biomechanical stimulus of a 10% passive stretch or hormonal hypertrophic agonists. We selected brain natriuretic peptide (BNP), a sensitive in vivo cardiac mechanical load indicator, and a clinically reliable index of heart failure (Maisel, 2002), as a molecular marker of stretch-regulated responses. As noted in Figure 1E, MLP$^{+/+}$ cells displayed marked induction of BNP mRNA, in response to exposure to a 10% passive stretch stimulus for 24 hr. In contrast, the MLP$^{-/-}$ cells displayed a complete loss of BNP induction following exposure to an equivalent stretch stimulus, while a bonafide hormonal Gq-coupling receptor agonist, endothelin, enhanced BNP expression. The response to an α-adrenergic agonist, phenylephrine, on the MLP$^{-/-}$ and MLP$^{+/+}$ cells was identical (Figure 1F), indicating that the primary defect in the MLP deficient cells was not located in the downstream pathway for gene induction of stretch responses, but in the initial sensing of the stretch stimulus. We also examined atrial natriuretic factor (ANF), another widely used marker of the cardiac embryonic gene program. The enhancement of ANF expression by stretch was similarly suppressed in MLP$^{-/-}$ cardiomyocytes (data not shown). Thus, there is a primary, selective defect in the cardiac muscle passive stretch sensing in the MLP$^{-/-}$ animals, which reflects an intrinsic abnormality of titin-related passive stretch response properties within cardiomyocytes.

The Z Disc Protein MLP Associates with T-Cap, a Titin Binding Protein

To identify the molecular components that underpin stretch sensor pathways, we characterized the protein partners, which interact directly with MLP in cardiomyocytes. Via a yeast two-hybrid screening, a total of over 10^6 clones were screened, and about 650 positive clones were sequenced. α-actinin was identified as a strongly interacting protein (Figure 2A), consistent with previous studies (Louis et al., 1997). In addition, approximately 30 positive clones encoded sequences of T-cap, a striated muscle specific protein bound to the NH2-terminus of titin at the Z disc. The interaction between MLP and

A

Age	2 weeks		4 weeks		8 weeks	
Genotype	MLP+/+	MLP-/-	MLP+/+	MLP-/-	MLP+/+	MLP-/-
LVEDd	2.3±0.1	2.1±0.1	3.1±0.1	3.6±0.5	3.6±0.3	5.3±0.4
LVEDs	1.1±0.1	0.9±0.1	1.7±0.8	2.4±0.8	2.5±0.3	4.4±0.3*
%FS	54±4	59±4	46±3	35±12	30±4	16±4*

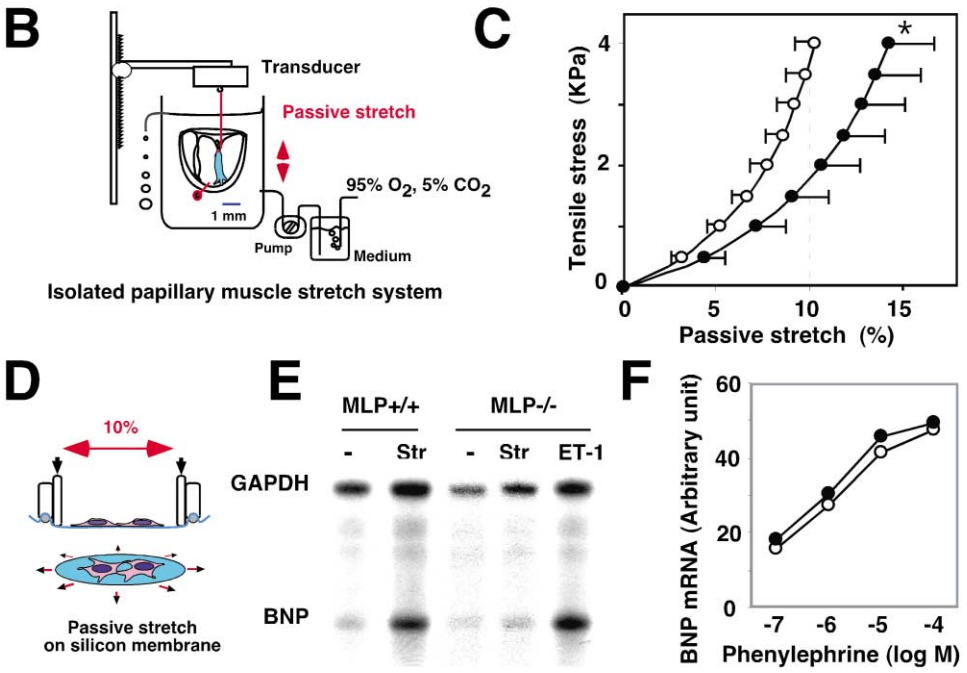


Figure 1. Selective Loss of Passive Stretch-Induced Responses in Neonatal/Perinatal MLP Null Cardiomyocytes

(A) Echocardiographic analysis of MLP$^{-/-}$ mice in the postnatal development. Data are mean ± SE from 2 week (MLP$^{+/+}$, n = 6; MLP$^{-/-}$, n = 8), 4 week (MLP$^{+/+}$, n = 6; MLP$^{-/-}$, n = 6) and 8 week (MLP$^{+/+}$, n = 5; MLP$^{-/-}$, n = 4) old animals. * $P < 0.01$, versus MLP$^{+/+}$ by Student's t test.

(B) The schematic view of papillary muscle stretch system used for the stress-strain relationship analysis.

(C) Passive stress-strain relationship of papillary muscle isolated from 2 week old MLP$^{-/-}$ mice (closed circles, n = 6) is strongly impaired compared to MLP$^{+/+}$ mice (open circles, n = 5) of the same age. * $P < 0.05$, versus MLP$^{+/+}$ by Repeated Measures ANOVA.

(D) The schematic view of the equibiaxial stretch system for cultured cardiomyocytes.

(E) Passive stretch (Str, 10%) activates BNP mRNA induction in neonatal MLP$^{+/+}$ cardiomyocytes, while MLP$^{-/-}$ neonatal cardiomyocytes do not respond to passive stretch. Note endothelin-1 (ET-1, 100 μM) upregulates BNP mRNA to the same level as mechanical stress activates in MLP$^{+/+}$ cells. The results are representative from three independent experiments.

(F) The dose-dependent induction of BNP mRNA by phenylephrine, a Gq-coupled receptor agonist, is similar between MLP$^{+/+}$ (open circles) and MLP$^{-/-}$ (closed circles) neonatal cardiomyocytes. The results are representative from three independent experiments.

T-cap was confirmed by in vitro GST-pull down assays (Figure 2B) and further supported in vivo by the colocalization via immunostaining of these molecules in neonatal rat cardiomyocytes (Figure 2C), consistent with previous observations that MLP and T-cap are both localized at the proximity of Z-disc, where titin anchors its most N-terminal end (Arber et al., 1997; Gregorio et al., 1998). Utilizing a series of truncated MLP constructs, we show that the short peptide sequence at the MLP NH2-terminal domain (T-cap interacting domain: TID), outside of the two LIM domains per se, is critical for the selective interaction with T-cap (Figure 2D). A series of T-cap truncated cDNAs were used to map the MLP interacting domain of T-cap (amino acids 53–81), which is adjacent to the titin binding domain (amino acids 78–125) (Gregorio et al., 1998) (Figure 2E). Taken together, these studies indicate that MLP binds to a muscle specific protein, T-cap, which is a docking protein of titin.

Z Disc Alignment Defects and Selective Loss of T-Cap Staining in MLP$^{-/-}$ Myocardium

Based on the interaction of MLP with T-cap at the Z disc, we critically examined Z disc structure in MLP$^{-/-}$ myocardium from 6–9 month old animals by electron microscopy. As shown in Figure 3, the MLP$^{-/-}$ Z discs, which were normally identified as tight electron dense bands in the middle of I-bands, displayed misalignment, appearing less dense, fluffy, and markedly more dispersed. The MLP$^{-/-}$ Z discs were about 1.4-fold wider than the controls (Figure 3). The Z disc defects in MLP$^{-/-}$ myocardium might reflect an abnormal conformation of the Z disc domain of titin or the lateral axis misalignment

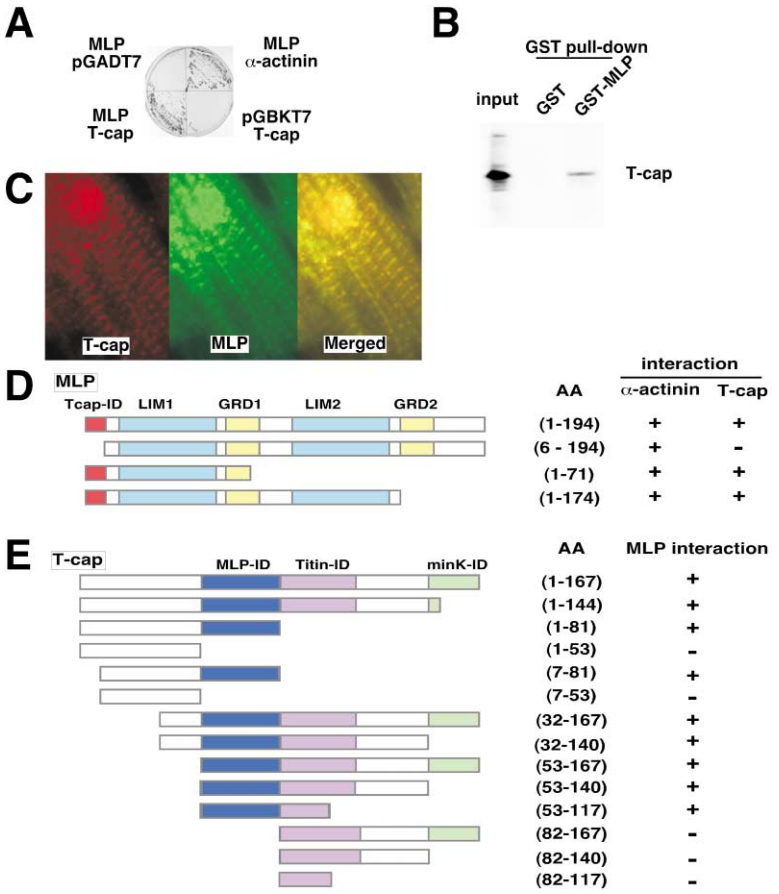


Figure 2. Direct Molecular Interaction of MLP and T-Cap

(A) Yeast two-hybrid analysis of MLP interaction with T-cap and α-actinin. pGADT7 and pGBKT7 are empty backbone plasmids.

(B) Interaction of T-cap with MLP in in vitro GST-pull-down analyses. Full-length T-cap translated in vitro (input) was incubated with a recombinant GST-MLP fusion protein (GST-MLP) or GST alone (GST) and pulled down with glutathione-Sepharose 4B beads (T-cap).

(C) Coimmunostaining analysis of MLP and T-cap in neonatal cultured rat cardiomyocytes.

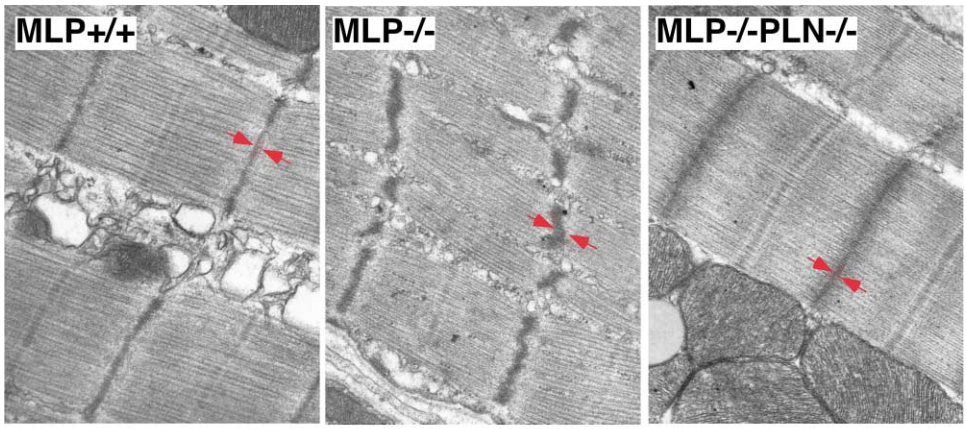
(D) MLP deletion mapping for the T-cap and α-actinin interactions by yeast two-hybrid analyses. Note the removal of the first 5 amino acids leads to the selective loss of MLP binding to T-cap. LIM1 and LIM2, LIM domain 1 and 2; GRD1 and GRD2, glycine-rich domain 1 and 2.

(E) T-cap mapping for the MLP interaction by yeast two-hybrid analysis. Amino acids 53 to 81 are responsible for MLP interaction. MLP-ID, MLP interacting domain; Titin-ID, titin interacting domain; mink-ID, minK interacting domain.

of Z disc components including the NH2-terminal region of titin that interacts with T-cap.

Previously, we reported that a deficiency in phospholamban (PLN), the physiologic inhibitor of the sarcoplasmic reticulum ATPase (SERCA 2A), can completely compensate for the cardiac phenotype due to loss of MLP (Minamisawa et al., 1999). We speculate that loss of PLN, leading to cardiac relaxation, which reduces wall stress and concomitantly passive myocardial stretch, prevents chamber dilation and the onset and progression of heart failure. Interestingly, as shown in Figure 3, the Z disc alignment in $MLP^{-/-}PLN^{-/-}$ mouse hearts was nearly completely preserved, suggesting that the Z disc abnormalities in $MLP^{-/-}$ myocardium are not primary, but represent an inducible phenotype that occurs in response to high wall stress.

The loss of a single component of the sarcoglycan/dystrophin complex can lead to the selective loss of other components of the complex in both clinical and experimental models of cardiomyopathy (Cohn and Campbell, 2000). To test whether the loss of MLP might secondarily result in the dislocation of T-cap in $MLP^{-/-}$ animals, hearts from $MLP^{-/-}$ mice were analyzed via immunohistological staining with polyclonal anti-T-cap antibodies or via biochemical subcellular fractionation. Microscopic examination of over 90,000 cells in three groups of specimens ($MLP^{+/+}$, $MLP^{+/-}$, and $MLP^{-/-}$) following immunostaining with anti-T-cap antibodies revealed a lacerated or patchy distribution of T-cap in a fraction (4%) of MLP deficient ventricular cells (Figures 4A–4E). The selectivity of the T-cap abnormality in $MLP^{-/-}$ cells was evidenced by double-staining of T-cap and f-actin (Figures 4A–4D), or the immunostaining of the adjacent specimen from continuous sectioning with an anti-α-actinin antibody (Figure 4E). Similarly, the T-cap abnormality was observed in a smaller population of cardiomyocytes (0.1%) from $MLP^{+/-}$ animals, indicating a gene dosage effect of MLP on T-cap stabilization (Figure 4E). Consistent with these immunological studies, the fractionation of myocardial homogenates by sequential centrifugation revealed that the T-cap protein can partially translocate from the particulate myofilament fraction to the soluble fraction following low-speed centrifugation in the $MLP^{-/-}$ mice (Figure 4F). Immunostaining studies documented that T-cap mislocalization did not occur in the $MLP^{-/-}PLN^{-/-}$ myocardium (data not shown), and electron microscopy revealed a completely normal Z-disc structure, as described above. The specificity of Z disc defects and T-cap mislocalization in the MLP deficient myocardium was confirmed by a similar analysis in two other heart failure models: actinin-associated LIM protein (ALP) knockout mice, which have a prominent right ventricular dominant dilated cardiomyopathy (Pashmforoush et al., 2001), did not show Z disc thickening at the electron microscopic level, or T-cap mislocalization following immunostaining. Similarly, a



Z-line thickness

Genotype	# counted	Thickness (nm)
MLP+/+	776	112±3
MLP-/-	567	160±4*
MLP-/-PLN-/-	1572	108±4**

Figure 3. Z Disc Misalignment in MLP Deficient Mice

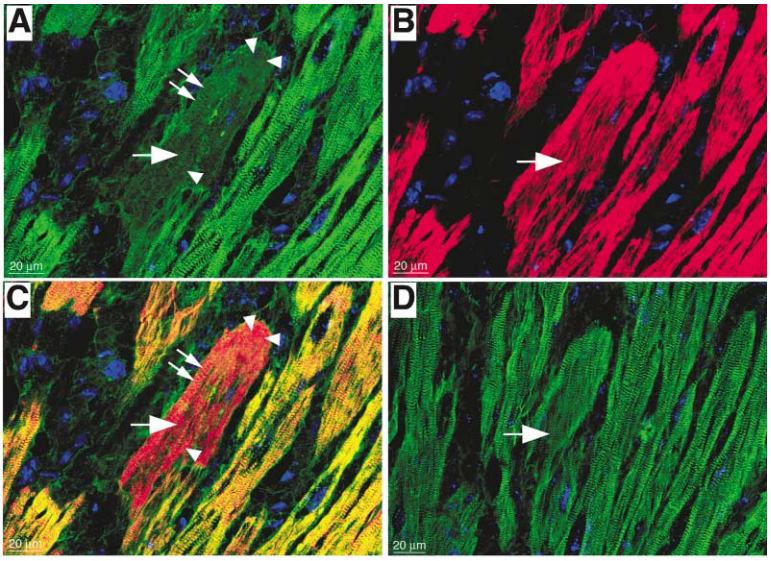
Note the tight dense Z discs in the wild-type hearts while there is Z disc widening, dispersion, and irregularities in MLP deficient myocardium. There is a completely preserved Z disc structure in the double MLP/PLN KO mice ($MLP^{-/-}PLN^{-/-}$), indicating that promotion of calcium cycling secondarily prevents the Z disc defects most likely via enhancing cardiac relaxation and reducing wall stress. Z disc thickness was randomly measured. * $P < 0.01$, versus $MLP^{+/+}$; ** $P < 0.01$, versus $MLP^{-/-}$ by ANOVA followed by post hoc test (Student-Newman-Keuls).

well-characterized rat model of severe postmyocardial infarction heart failure did not show any immunohistological abnormalities in T-cap (data not shown).

A Missense Mutation of a Highly Conserved Residue in the T-Cap Interacting Domain of MLP is Associated with Human Dilated Cardiomyopathy

Mutations in the T-cap gene lead to limb girdle muscular dystrophy (LGMD type G) in human populations (Moreira et al., 2000). The interaction of MLP with T-cap and the known association of MLP deficient animals with DCM in gene-targeted mice (Arber et al., 1997) suggested the value of screening human patients with idiopathic forms of DCM for sequence variations in the MLP coding region. As an initial step, the structure of human MLP was characterized. The human MLP gene is encoded by a 20 Kbp genomic region and organized in 6 exons (NCBI genome contig: NT_009307). Initially, 516 patients diagnosed with idiopathic DCM at the University Hospital Benjamin Franklin in Berlin (Germany) and 20 DCM patients from the St. Georges Hospital in London (UK), were enrolled for MLP mutation screening. All individuals were Caucasians, and all of the German patients underwent cardiac catheterization/angiography and cardiac biopsy at the University Hospital Benjamin Franklin in Berlin to document the presence of left ventricular dysfunction and to eliminate the involvement of coronary artery disease. In addition, cases with significant hypertension or other major causes of heart failure were excluded. An age-matched control Caucasian population ($n = 320$) was collected at the Berlin site, consisting of individuals diagnosed with coronary artery disease by cardiac catheterization at the same hospital and having normal cardiac function as judged by angiography. We examined three polymorphisms, G336AMLP (see below), the blood group duffy polymorphism (Reid et al., 2000) and Val92Met in the melanocortin 1 receptor gene (Rana et al., 1999) in both German DCM and control populations, confirming that these two groups are well matched with respect to their genetic background.

The entire MLP coding region was sequenced in more than 50 DCM patients, revealing one DCM patient with a heterozygous missense mutation with a unique base pair change (T to C). This single nucleotide mutation resulted in a severe charge change at position 4 in the MLP protein (W4R), that lies within the NH2-terminal T-cap interacting domain (TID) of MLP. As noted in Figure 5A, this single base pair change introduces a Nci I restriction site, allowing subsequent rapid screening of a total of 536 DCM patients by the PCR amplification followed by Nci I digestion, resulting in the identification of 9 additional patients with this identical heterozygous missense mutation in MLP. All the positive patients were confirmed by direct sequencing of this TID region. (See Table 1 for the clinical characterization of all the W4RMLP DCM patients). The genetic analysis of the kindred of three DCM patients suggested a dominant form of disease transmission (Figure 5C). Two-point linkage analysis was carried out for 7 German DCM families, resulting in a combined two-point lod score of 2.62. This



E

Genotype	Sampling size *cell number / mm2*	Affected cardiomyocytes *cell number (% of total cells)*
MLP+/+	36,239 / 24.1	0 (0.0%)
MLP+/-	34,230 / 24.1	34 (0.1 %)
MLP-/-	21,120 / 28.6	845 (4.0 %)

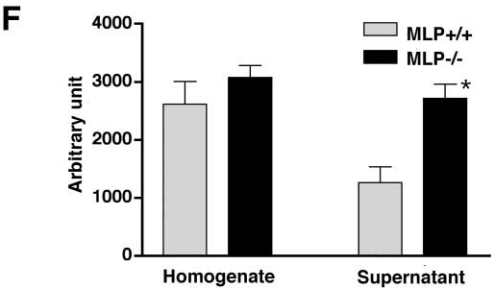


Figure 4. Selective Loss of T-Cap in a Subset of MLP Null Myocardium

(A–D) Adult (12–20 week old) MLP-KO mouse ventricles were triple-stained for T-cap (green), f-actin (red), and nuclei (blue) (A–C), or double-stained for α-actinin (green) and nuclei (blue) (D). The large arrows indicate representative defective cardiomyocytes with selective loss of T-cap signals (A and C) organized poorly for the cross-striated pattern (small arrows) or in diffused amorphously (arrowheads), while these cells have clearly organized myofibril depicted with f-actin staining with TRITC-Phalloidin (B and C).

(C) Merged images.

(D) α-actinin staining of the section serially to (A–C).

(E) Quantitative histological analysis. A blinded examiner analyzed two animals for each group.

(F) Biochemical fractionation of T-cap in $MLP^{+/+}$ ($n = 5$) and $MLP^{-/-}$ ($n = 5$) myocardium homogenates. Note higher level of extracted T-cap in low-speed supernatant in $MLP^{-/-}$ hearts. * $P < 0.01$, versus $MLP^{+/+}$ by Student's t test.

analysis was limited by the size of pedigrees and age dependent penetrance.

The W4R mutation is located outside of the LIM domains (see Figure 2D) and is highly conserved in avian through human MLP protein sequences, as well as across related members of the CRP gene family. The W4R mutation was not found in 320 control patients, indicating a highly significant association of the W4R mutation with DCM phenotype by exact Fisher test analysis ($P < 0.01$). During the analysis of the entire MLP coding sequence, another MLP variation was found in several individuals; however, this single nucleotide exchange is silent and appears also in normal human populations, its distribution is not significantly different between the control and DCM population (see GenBank human MLP coding sequence NM_003476, codon 112, position 3 G to A). The presence of W4RMLP mutation was examined in another distinct well-characterized DCM patient population with a large number of control individuals at the Kobe University Hospital in Japan (Figure 5B). These studies found no evidence of the W4R mutation in either the control or DCM Japanese populations, suggesting a founder effect of the W4RMLP mutation in the European population. In addition, 190 German DCM and 134 Japanese DCM patient populations were subjected to single-stranded conformation polymorphism (SSCP) analysis to search for additional MLP mutations, however, no additional MLP sequence variations were detected.

To confirm the founder effect of the W4RMLP mutation, we analyzed multiple microsatellite markers in the proximate region of the MLP gene. Genotyping revealed a shared haplotype of the mutation and 4 microsatellite markers (~2 Mb) in 4 of the families ($p = 0.081$) (shown in families 1 and 2 in Figure 5C) and 3 microsatellite markers (~74 Kb) in 8 of the families ($p = 0.079$). The allele (2) for D11S5039 was shared by all affected members of the 9 German DCM families (seen in family 3 in Figure 5C) ($p = 0.005$) and was not found in unaffected subjects from these families or in 120 control chromosomes from ethnically matched subjects.

Given the presence of a MLP-T-cap interaction, we also screened for mutations in T-cap via SSCP and direct gene sequencing in 380 DCM patients with 100 controls from the collected population at the University Hospital Benjamin Franklin in Berlin and found a T-cap missense mutation (R87Q) in one DCM patient who does not have the W4RMLP mutation, whereas this mutation

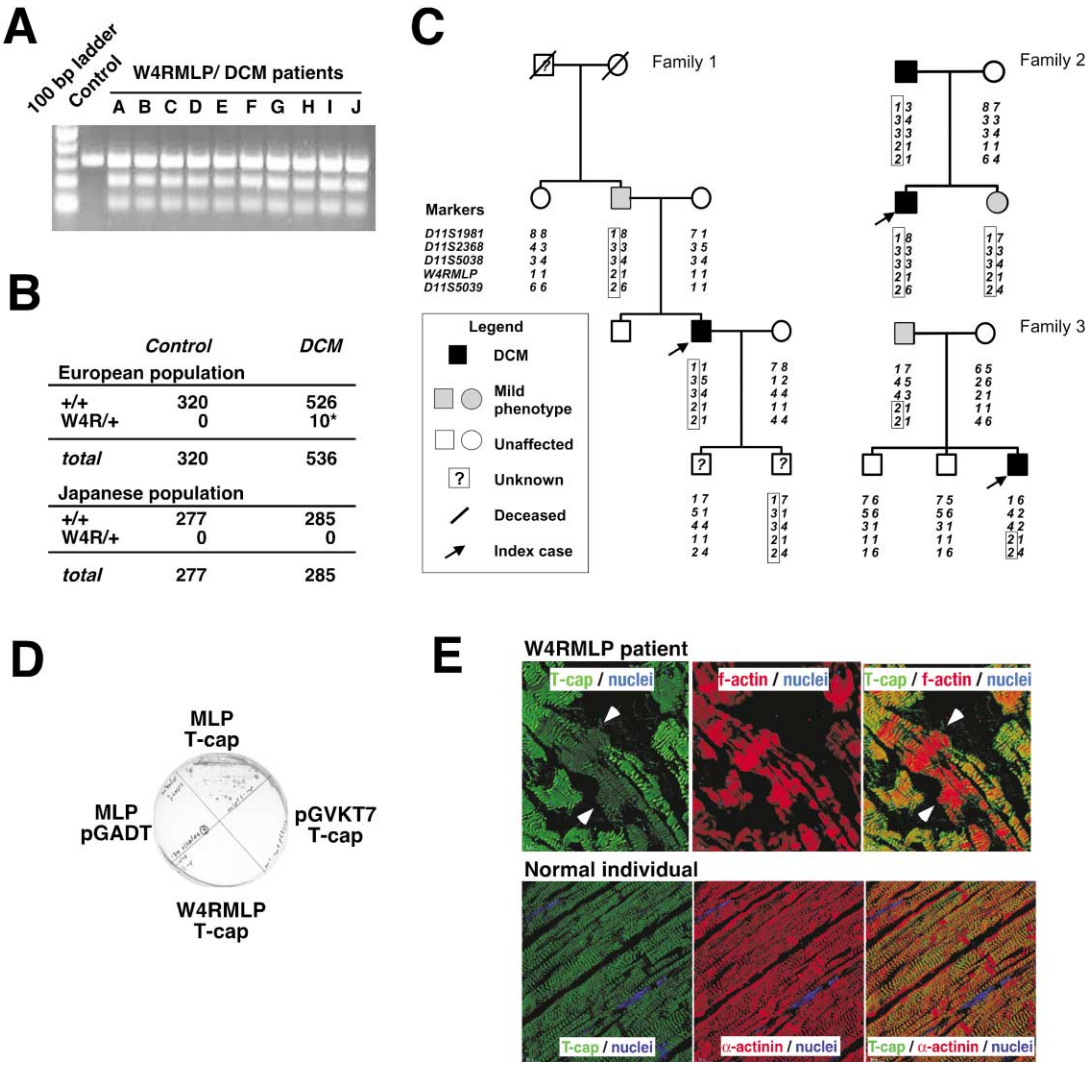


Figure 5. Identification of a W4R MLP Mutation in Human DCM Patients

(A) The result of the restriction analysis of PCR products from the genomic DNA from 10 DCM patients (patients A–J). The W4RMLP mutation introduces a Nci I restriction site, which yields additional 100 and 200 bp fragments after Nci I digestion.

(B) W4RMLP mutation in DCM patients versus control individuals analyzed in European and Japanese populations. $P < 0.01$ by exact Fisher test.

(C) Pedigrees of 3 representative DCM families demonstrating shared haplotype. Squares represent males and circles represent females. Microsatellite markers and W4RMLP mutation are listed in order on chromosome 11p15 to the left of Family 1. Below each subject are the allele numbers for each marker and (2) indicates mutated allele (W4RMLP). The shared haplotype is boxed.

(D) The W4R MLP does not interact with T-cap, shown by yeast two-hybrid analysis.

(E) The representative immunohistochemical analysis of biopsies for a W4RMLP DCM patient and a normal control individual. Note, arrowheads indicate cells with defective T-cap staining and the normal appearance of f-actin staining.

was not found in any individual of the control population and was not found in any of 400 control individuals in Japan.

The W4RMLP Protein Displays the Selective Loss of T-Cap Interaction and Is Associated with T-Cap Mislocalization

As noted, the W4R mutation lies within the NH2-terminal T-cap interacting domain of MLP. Accordingly, we directly examined whether the W4R mutation would affect the ability of MLP to interact with T-cap and found that the W4R mutation in MLP results in the complete loss of T-cap binding (Figure 5D). The inability of the mutant MLP protein to interact with T-cap is not simply due to the instability of W4RMLP peptide, nor to intracellular mislocalization of this mutant MLP protein, as NH2- and COOH-terminal GFP-tagged W4RMLP displayed a normal cardiac Z disc localization by adenoviral mediated forced expression in neonatal rat cardiomyocytes (data not shown). Similarly, a mutant MLP protein that lacks the first 5 amino acids displays normal localization (data not shown), suggesting that the NH2-terminal T-cap binding domain of MLP is not a critical determinant for basal MLP localization at the Z disc per se. In addition,

Table 1. Clinical Characteristics of 11 DCM Patients with MLP or T-Cap Mutations

Patient ID	Age	Sex	Biopsy	NYHA[a]	LV function[b]
W4RMLP					
A	35	m	Typical DCM[c]	II	ND[e]
B	41	m	Typical DCM	II-III	EF 44%, LVEDD 58mm
C	70	f	Typical DCM	III	EF 38%, LVEDV 204 ml
D	47	f	Typical DCM	III	EF 47%
E[d]	43	m	Typical DCM	II	LVEDD 58 mm
F[d]	38	m	NA	III	EF 46%, LVEDV 226 ml
G	32	m	NA	I	LVEDD 57 mm
H[d]	33	m	Typical DCM	III	LVEDD 61mmm; EF: 30%, EF 42%, LVEDV 279 ml
I	51	f	NA	II	ND[e]
J	52	f	NA	I	EF 46%
R87QT-cap					
K	46	f	NA	I	ND[e]

[a] New York Heart Association heart failure classification.
[b] Diagnosis by echocardiography or radionucleotide ventriculography.
[c] The diagnosis of typical DCM is based on histological observations.
[d] The pedigrees of these families are shown in figure 5C.
%FS, % fractional shortening; LVEDD, left ventricular end-diastolic diameter (normal 56 mm); EF, ejection fraction; LVEDV, left ventricular enddiastolic volume (normal: 180 ml); EF, ejection fraction (normal: 55%); NA, not available.
[e] ND, LV diameters not determined.

the R87Q mutant of T-cap, found in a single DCM patient, was also unable to form a stable complex with MLP via yeast two-hybrid molecular interaction analysis (data not shown), providing further independent evidence that the loss of MLP-T-cap interaction is associated with human DCM.

Taken together, these data support the concept that MLP/T-cap interaction is critical for maintaining normal cardiac function and that mutations of either MLP or T-cap, which are related to human DCM, disrupt this molecular interaction. Interestingly, the selective T-cap mislocalization, which was initially identified in MLP$^{-/-}$ ventricular myocardium (Figure 4), was also observed in cardiac biopsies from a single DCM patient harboring the W4RMLP (Figure 5E). Similar staining analysis of biopsies from normal human myocardium did not show any evidence of T-cap mislocalization.

Discussion

T-cap, a 19 kd muscle-specific protein was isolated as a titin binding protein at the Z disc (Gregorio et al., 1998). An expanding number of novel muscle-specific components of the cardiac Z disc have recently been uncovered by genomic databases (Clark et al., 2002), implying that the Z disc may have acquired a specialized function beyond serving in a purely structural role. The importance for understanding how the Z disc complex intersects with cardiac myocyte function has been also underscored by associations with dilated cardiomyopathy and heart failure both in experimental animal models and human disease (Chien, 2000; Hoshijima and Chien, 2002; Seidman and Seidman, 2001).

The present study provides evidence for the interaction of MLP and T-cap including yeast two-hybrid analysis, in vitro GST-pull down experiments, colocalization at the cardiac Z disc, and selective loss of T-cap staining in a subset of myocytes from MLP deficient hearts. In addition, T-cap is partially released into the cytosolic fraction in MLP deficient hearts. The latter finding provides direct evidence that MLP is required for the stabilization of T-cap with the titin complex. The mechanism by which MLP stabilizes T-cap at the Z disc is not clear from the current study; however, the independent association of MLP with α-actinin may consist of a T-cap/MLP/α-actinin complex at the Z disc with titin. The α-actinin docking domain of cysteine-rich protein (CRP) 1, a member of CRP protein family, which includes MLP, has been mapped (Harper et al., 2000) and our yeast two-hybrid screening of MLP interacting molecules supports the concept of MLP/α-actinin interaction. Taken together, MLP appears to be an intrinsic element of the Z disc/titin complex, acting to stabilize T-cap at the most proximal end of the titin complex. In this regard, the recent finding that MLP protein levels are decreased in human idiopathic and ischemic cardiomyopathy patients (Zolk et al., 2000) suggests the possibility that defects in stabilization of the macromolecular titin/Z disc complex including MLP could potentially underlie other genetic and acquired forms of heart failure.

The MLP/T-Cap Z Disc Complex Is a Key Component of the Intrinsic Cardiac Muscle Stretch Sensing

Stretch regulation is a highly conserved function in both cardiac and skeletal muscle cells. This stretch regulated response includes the triggering of a hypertrophic program that leads to an increase in sarcomeres, the activation of an embryonic gene program that leads to the upregulation of fetal genes (Hunter et al., 1999), the downregulation of adult-onset genes (Kuo et al., 2001), and the activation of counteracting cell survival and cell death pathways (Chien, 1999; Hoshijima and Chien, 2002). In the in vivo setting, this biomechanical stimulus is triggered by volume or pressure overload, and results in the activation of pathways for hypertrophy that are mediated by a diverse set of agonists, including adrenergic stimuli, angiotensin II, endothelin, and cardiotro-

phin-1. The trigger for the release of these hormonal stimuli appears to reside largely outside of myocytes and may reflect a paracrine pathway mediated by the stretch induced release of these peptides from neighboring cardiac fibroblast and endothelial cells. In this regard, the dominant effect of Gq-coupling receptor agonists in the murine model of pressure-overload induced cardiac hypertrophy has been documented (Wettschureck et al., 2001). In contrast, cardiac muscle cells possess intrinsic muscle stretch-regulated responses that are independent of these paracrine pathways.

To date, the molecular identity of the cardiac stretch sensor has remained very elusive. Following mechanical stretch of cultured cardiomyocytes, several transmembrane signaling pathways, including MAP kinase(s), JAK-STAT, and PI3K-AKT cascades, are activated, but the nature of the mechanical stretch sensor that triggers these kinase cascades is unknown. To our knowledge, no cardiac mutations have been shown to have a selective effect on stretch versus hormonal induced responses in either intact heart muscle preparations or cardiomyocytes. Mechanical stretch can lead to changes in the relative activity of ion channels and ion exchangers, but it has been difficult to determine whether these are serving as sensors to trigger the embryonic gene program or simply occur as a result of the hypertrophic process itself, with most of the data supporting the latter notion. Similarly, studies of the role of mechanical stretch activated channels have been negative thus far (Yamazaki et al., 1998). The current study now provides functional evidence that the titin/Z-disc structure is an essential part of a complex that triggers downstream effector pathways following mechanical stretch.

The titin protein contains intrinsic elastic elements that maintain the normal length of cardiac muscle cells during the continuous cycle of cardiac contraction and relaxation (Helmes et al., 1996). The possibility exists that the chamber dilation seen in the MLP deficient mice simply reflects an additional defect in the elastic recoil, rather than passive stretch-related responses caused by titin abnormality during DCM progression.

However, a detailed analysis of the recoil properties of MLP$^{-/-}$ papillary muscle reveals a completely normal response and supports the concept that the defect that leads to chamber dilation is not simply secondary to a structural defect that prevents recovery of the muscle to the initial sarcomeric length following the stretch stimulus, but rather is related to an inherent inability to sense the mechanical stretch stimulus and to generate a primary effect on muscle tension. In this regard, previous studies indicate that titin also contributes to the tension that is generated by increasing passive stretch of cardiac muscle (Granzier and Irving, 1995).

Defects in the Cardiac Muscle Stretch Sensing and Mechanistic Pathways for DCM and Heart Failure

In this study, by screening over 1400 well phenotyped control individuals and patients with DCM, we report a close association between a subset of human DCM and a mutation in a highly conserved amino acid residue in the N-terminal domain of MLP. The 10 patients harboring the heterozygous mutant MLP gene display a clinical phenotype that is consistent with the MLP deficient mouse heart, including chamber dilation, thin ventricular walls, decreased contractility and impaired relaxation, and no evidence of hypertrophic cardiomyopathy. Although the formal linkage analysis in these families was limited by several factors including the size of available pedigrees and age dependant penetrance, the haplotype analysis provides strong evidence of a founder effect in this population. The strong association of the W4RMLP mutation with allele 2 of D11S5039 that was not identified in normal ethnically matched controls suggests a distant common ancestor and supports vertical transmission of the DCM phenotype related to W4RMLP over many generations in the European population.

High fidelity left ventricular pressure measurements have revealed partial defects in cardiac contractility and β-adrenergic agonist sensitivity in MLP$^{+/-}$ animals (J. Ross, Jr., M.H., and K.R.C., unpublished data), thereby supporting haploinsufficiency as the most likely mechanistic basis for the appearance of the disease phenotype in the W4R patients. As noted earlier, the W4R mutation in MLP results in the complete loss of T-cap interaction, and myocardial biopsies from patients with this mutation show the same phenotype as the one found in the MLP$^{-/-}$ cardiac muscle. The low percentage of loss of T-cap in the MLP KO hearts indicates that MLP/T-cap interaction is not stoichiometrically essential for the initial correct localization of T-cap, presumably because the primary binding partner of T-cap is titin (Gregorio et al., 1998). In addition, our data supports the concept that T-cap binding to MLP is critical for maintenance of the cardiac Z disc, and that in the absence of MLP, additional mechanical stress facilitates the mislocalization of T-cap from the titin complex, which is linked to the onset of Z disc defects and progressive DCM. The studies of the PLN rescue of the MLP mutation support this concept, as PLN ablation rescues both the Z disc defects and the T-cap mislocalization concomitantly. Interestingly, we also found a T-cap mutation (R87QT-cap) in a DCM patient, leading to an impaired affinity of MLP interaction. In this study, the MLP interacting domain of T-cap was mapped at amino acids 53–81, thus, we speculate the R87Q mutation causes allosteric effects on the MLP-interacting domain. Previous studies have identified two titin mutations (Val54Met and Ala743Val) at the T-cap and α-actinin binding domain in Japanese DCM patients and these titin mutations caused a weaker interaction between titin and T-cap/α-actinin (Itoh-Satoh et al., 2002). These results suggest that the defect in MLP/T-cap interaction caused by genetic mutations could be causative for the development of a subset of human DCM through the conformational and/or functional modifications of titin as an essential component of the stretch sensor machinery.

Taken together, the current study suggests a molecular model for the onset of DCM via the loss of the MLP/T-cap/titin dependent mechanical stress sensor pathway (Figure 6) wherein the inherent elastic segments within the I domains (Granzier and Labeit, 2002) serve as the intrinsic cardiac mechanical stress sensor that requires the anchoring of T-cap to MLP to insure that the titin elastic elements are organized in proper alignment. The loss of MLP leads to a destabilization of the anchoring of the Z disc to the proximal end of the T-cap/

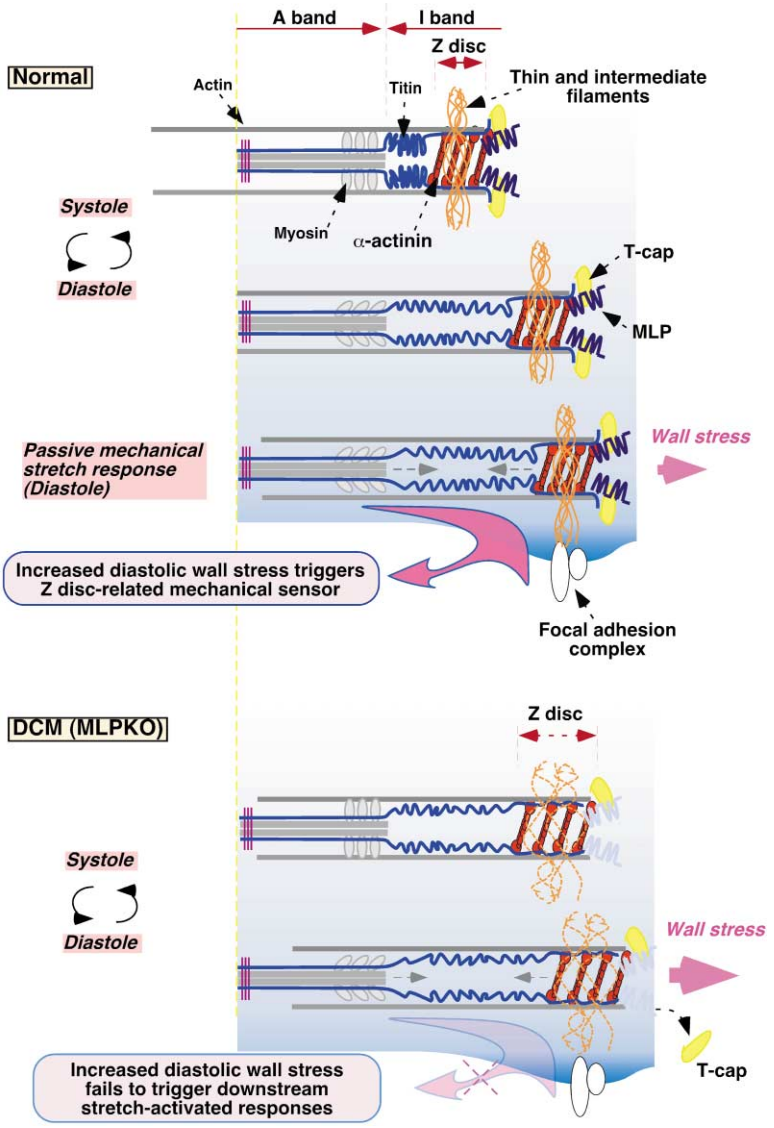


Figure 6. A Working Model for the Pathways that Link Defects in the Cardiac Stretch Sensor Machinery and Dilated Cardiomyopathy

Cardiac titin has multiple elastic sequences in the I-band and their elastic structures serve as molecular springs that generate tension in response to mechanical stretch following cardiac contraction (systole). At peak cardiac relaxation (diastole), as ventricular filling pressure rises, the titin elastic segments are reshaped and largely determine ventricular wall distensibility. With increases in diastolic wall stress, the titin/Z disc complex is stretched and the mechanical load is sensed and activates downstream signals for cardiomyocyte survival and hypertrophy. In the MLP$^{-/-}$ heart, the ventricular chamber dilation enhances diastolic wall stress leading to an increase in the mechanical stretch stimulus. T-cap, which is stabilized at the cardiac Z disc by MLP, gradually mislocalizes in the absence of MLP, and the loss of the MLP/T-cap complex causes Z-disc and related titin abnormalities resulting in a defect in mechanical stretch sensor function. The loss of downstream survival and hypertrophy cues leads to progressive DCM.

titin complex, thereby leading to a conformational alteration of the intrinsic titin molecular spring elements. In turn, this loss of elasticity results in the primary defect in the endogenous cardiac muscle stretch sensor machinery. The over-stretching of individual myocytes leads to the activation of cell death pathways, at a time when stretch-regulated survival cues are diminished due to the defect in the stretch sensing, ultimately leading to myocyte cell death and heart failure progression. Interestingly, this model is consistent with the rescue of the MLP$^{-/-}$ cardiomyopathy by PLN ablation, where the mechanical stretch stimulus is eliminated due to the inhibition of chamber dilation that accompanies the enhanced cardiac relaxation due to augmented SR calcium cycling.

Accordingly, genetic rescue of dilated cardiomyopathy in MLP mutant hearts by PLN most likely represent a secondary effect of a decrease in a mechanical stretch stimulus itself versus a primary effect on calcium signaling. It will become of interest to directly test the validity of this model via the generation of cardiac restricted germline mutations in critical regions of telethonin and titin itself. A recent report describes a human titin mutation in the cardiac specific N2B domain that is critical for its elastic properties and is associated with DCM (Gerull et al., 2002). In addition, in a zebrafish mutant (pickwick^{m171}), a missense mutation in an Ig-like element located at the Z disc/I-band segment of titin, is associated with a DCM like phenotype (Xu et al., 2002).

A more complete molecular understanding of the stretch sensor function will require identification of the signaling pathways that interact with the Z disc and titin complex. There are several attractive candidates, including calcineurin (interacts with Calsarcin/FATZ, a telethonin interacting protein) (Frey and Olson, 2002; Frey et al., 2000), protein kinase C (interacts with Cypher, another muscle restricted LIM domain protein associated with DCM in mutant mice) (Zhou et al., 2001), gp 130 (Hirota et al., 1999; Yasukawa et al., 2001), and minK and other related channels that have recently been shown to interact with telethonin (Furukawa et al., 2001). MLP itself has been proposed to translocate to the nu-

cleus and to serve as a transcriptional co-factor in in vitro transient assays in muscle cells. Finally, the endogenous titin kinase and/or the phosphorylation of T-cap itself (Mayans et al., 1998), which serves as a substrate of titin kinase, may play a role in the downstream signaling pathways.

Experimental Procedures

Stress-Strain Relationship in Papillary Muscles

Mouse hearts were arrested and isolated in modified Tyrode's solution, the papillary muscles were surgically exposed, and the base of the anterior papillary muscle was pinned to the bottom of a fluid-filled bath and a 7-0 silk suture was tied to cordae tendineae and connected to isometric force transducer (Harvard Apparatus). Titanium dioxide markers were arrayed on the surface of the muscle and a mirror was placed at 45° next to the muscle for the side view, allowing us to compute two-dimensional finite strain from video images. The muscle was continuously superfused with oxygenated modified Tyrode's solution and was field stimulated at 1 Hz, at a voltage of 10% above the threshold. The calcium concentration was gradually increased to 2.0 mM during a one hour equilibration period. Single directional (uniaxial) muscle forces were recorded during continuous loading up to a maximum diastolic stress of 5 kPa while videotaping deformations of the muscle. Diastolic stress data points were chosen immediately before the onset of contraction to ensure that the muscle was completely relaxed. Local muscle length was measured at the same time points with respect to the unloaded state using the markers. Yielding is the change in length (passive stretch) at 0 load (tensile stress) after a stretching protocol. Values are normalized to the slack length before stretch.

Passive Stretch-Induced Neonatal Mouse Cardiomyocytes BNP Expression

Mouse ventricular cardiomyocytes were isolated from day 0–2 mouse neonates as described previously (Wollert et al., 1996). The cells (2×10^5 cells/cm^2) were plated on silicon membranes coated with collagen type-1 and equipped on equibiaxial (uniform) stretcher devices (Lee et al., 1996). The cells were serum-depleted for 24 hr followed by passive equibiaxial 10% stretch for an additional 24 hr. Total RNA was extracted and subjected to RNA protection assay (RPA) using Direct RPA system (Ambion Inc.). Mouse BNP coding sequences were amplified by RT-PCR and used as the template for a BNP riboprobe synthesis with mouse GAPDH as a control.

Yeast Two-Hybrid Analyses, Immunoblotting, and Immunostaining

A Matchmaker Gal 4 two hybrid system 3 (Clontech) was used for interaction analyses and an adult mouse heart library was screened Proteins were homogenated in myofibrillar buffer (0.1 M KCl, 30 mM Tris HCl, and 5 mM EGTA) and subcellular fractionation performed by centrifugation for 10 min at 14,000 RPM, followed by separation on SDS PAGE gels and blotting with a MLP antibody. Immunohistochemistry was performed as described previously (Nguyen-Tran et al., 2000).

In Vitro Transcription/Translation and GST Pull-Down Experiments

In vitro transcription and translation of full-length T-cap cloned into pGADT7 (Clontech) was carried out in the presence of [^{35}S]Methionine using a TNT T7-coupled reticulocyte lysate system according to the manufacturer's instructions (Promega Corp.). MLP was fused to Glutathione S-transferase (GST) by cloning into the pGEX5x-2 vector (Amersham Pharmacia Biotech). Analysis of the interaction was essentially the same as described previously (Furukawa et al., 2001).

Screening for MLP Mutations in Control and DCM Patient Populations

Written informed consent for the study was obtained from all participants. Genomic DNA was isolated from whole blood using a Qiagen kit (Qiagen laboratories). The PCR and sequencing primers used for the W4R MLP mutation analyses were: TCTTAGAGATTGGTTCAC TCC and ACCACACTATGAGAACCACTGGC (PCR conditions: 95°C for 4 min; 35 cycles of: 94°C for 30 s, 62°C for 30 s, 72°C for 25 s followed by 72°C for 7 min). The exact Fisher test was used to estimate statistical significance.

Genotyping and Linkage Analysis

Four microsatellite markers within 2 cM of MLP were used to genotype all available subjects from the 9 German DCM families to evaluate the possibility of a founder effect. Two of these markers (D11S1981 and D11S2368) were available in public databases and 2 of the markers (D11S5038 and D11S5039) were designed by searching the public genome database sequence for short tandem nucleotide repeats in the MLP region (www.gdb.org). The marker order and approximate genomic distances are as follows: D11S1981 (distance to W4RMLP ~2 Mb), D11S2368 (distance to W4RMLP ~70 Kb), D11S5038 (distance to W4RMLP ~13 Kb), W4RMLP, (distance to W4RMLP ~4 Kb) D11S5039. Genotype assignments and haplotype analysis were performed as previously described (Hoffman et al., 2000). Family analysis was performed according to international guidelines (Mestroni et al., 1999) and two-point linkage analysis for the mutation was performed with the program MLINK of the LINKAGE 5.2 (Lathrop et al., 1984) package using 7 DCM families, because 2 of the families were uninformative for linkage. Family members with a mild phenotype were included. Disease status of subjects younger than 30 years was considered to be unknown. Combined lod scores were calculated with a disease penetrance of 90% and a phenocopy rate of 0.001. Shared haplotypes were evaluated by transmission dysequilibrium testing using the ETDT program (Sham and Curtis, 1995) for all 9 German families and all 4 microsatellite markers and the mutation using the same criteria as above.

Electron Microscopy

For electron microscopy, freshly isolated heart was quickly cut into 1–2 mm squares and submerged in 3% gluteraldehyde/0.1 M phosphate buffer, [pH 7.2–7.4]. Subsequently, the tissue was dehydrated in a series of ethanol washes and embedded in Spurr. Sections were viewed and photographed on a Philips CM12 electron microscope in the Arizona Research Laboratories, Division of Biotechnology Imaging Facility.

Acknowledgments

Drs. S. Labeit and G. Faulkner are gratefully acknowledged for providing their T-cap antibodies. The Resource Center is acknowledged for providing the MLP encoding genomic clones in support of R. Knöll. R. Knöll is supported by DFG Kn 448/2-1, DFG Kn 448/6-1. K. Jung is acknowledged for assistance with a portion of the PCR based patient analyses. N. Dalton is acknowledged for help in performing the echocardiography. Dr. H. Fechner is acknowledged for his support in genotyping and sequencing. G. Knöll, J. Q. Anderson, S. Woodward, and K. Weldy are acknowledged for their technical assistance. We thank the participating families and Prof. H.P. Schultheiss and Prof. M. Yokoyama for their support of this study. This work was entirely supported by the LeDucq Foundation. This study is dedicated to honor the memory and vision of Jean LeDucq.

Received: June 19, 2002
Revised: November 18, 2002

References

Arber, S., Hunter, J.J., Ross, J., Jr., Hongo, M., Sansig, G., Borg, J., Perriard, J.C., Chien, K.R., and Caroni, P. (1997). MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. Cell *88*, 393–403.

Chien, K.R. (1999). Stress pathways and heart failure. Cell *98*, 555–558.

Chien, K.R. (2000). Genomic circuits and the integrative biology of cardiac diseases. Nature *407*, 227–232.

Clark, K.A., McElhinny, A.S., Beckerle, M.C., and Gregorio, C.C.

(2002). Striated muscle cytoarchitecture: an intricate web of form and function. Annu. Rev. Cell Dev. Biol. *18*, 637–706.

Cohn, R.D., and Campbell, K.P. (2000). Molecular basis of muscular dystrophies. Muscle Nerve *23*, 1456–1471.

Frey, N., and Olson, E.N. (2002). Calsarcin-3, a novel skeletal muscle-specific member of the calsarcin family, interacts with multiple Z-disc proteins. J. Biol. Chem. *277*, 13998–14004.

Frey, N., Richardson, J.A., and Olson, E.N. (2000). Calsarcins, a novel family of sarcomeric calcineurin-binding proteins. Proc. Natl. Acad. Sci. USA *97*, 14632–14637.

Furukawa, T., Ono, Y., Tsuchiya, H., Katayama, Y., Bang, M., Labeit, D., Labeit, S., Inagaki, N., and Gregorio, C. (2001). Specific interaction of the potassium channel β-subunit minK with the sarcomeric protein T-cap suggests a T-tubule-myofibril linking system. J. Mol. Biol. *313*, 775–784.

Gerull, B., Gramlich, M., Atherton, J., McNabb, M., Trombitas, K., Sasse-Klaassen, S., Seidman, J.G., Seidman, C., Granzier, H., Labeit, S., et al. (2002). Mutations of TTN, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy. Nat. Genet. *30*, 201–204.

Granzier, H.L., and Irving, T.C. (1995). Passive tension in cardiac muscle: contribution of collagen, titin, microtubules, and intermediate filaments. Biophys. J. *68*, 1027–1044.

Granzier, H., and Labeit, D. (2002). Cardiac titin: an adjustable multifunctional spring. J. Physiol. *541*(Pt.2), 335–342.

Gregorio, C.C., Trombitas, K., Centner, T., Kolmerer, B., Stier, G., Kunke, K., Suzuki, K., Obermayr, F., Herrmann, B., Granzier, H., et al. (1998). The NH2 terminus of titin spans the Z-disc: its interaction with a novel 19-kD ligand (T-cap) is required for sarcomeric integrity. J. Cell Biol. *143*, 1013–1027.

Harper, B.D., Beckerle, M.C., and Pomies, P. (2000). Fine mapping of the α-actinin binding site within cysteine-rich protein. Biochem. J. *350*, 269–274.

Helmes, M., Trombitas, K., and Granzier, H. (1996). Titin develops restoring force in rat cardiac myocytes. Circ. Res. *79*, 619–626.

Hirota, H., Chen, J., Betz, U.A., Rajewsky, K., Gu, Y., Ross, J., Jr., Muller, W., and Chien, K.R. (1999). Loss of a gp130 cardiac muscle cell survival pathway is a critical event in the onset of heart failure during biomechanical stress. Cell *97*, 189–198.

Hoffman, H.M., Wright, F.A., Broide, D.H., Wanderer, A.A., and Kolodner, R.D. (2000). Identification of a locus on chromosome 1q44 for familial cold urticaria. Am. J. Hum. Genet. *66*, 1693–1698.

Hoshijima, M., and Chien, K.R. (2002). Mixed signals in heart failure: cancer rules. J. Clin. Invest. *109*, 849–855.

Hunter, J.J., Grace, A., and Chien, K.R. (1999). Mol. cell. biol. of cardiac hypertrophy and failure. In Molecular Basis of Cardiovascular Diseases (A Companion to Braunwald's Heart Disease), K.R. Chien, ed. (Philadelphia, London, Toronto, Montreal, Sydney, Tokyo: W.B.Saunders Company), pp. 211–250.

Itoh-Satoh, M., Hayashi, T., Nishi, H., Koga, Y., Arimura, T., Koyanagi, T., Takahashi, M., Hohda, S., Ueda, K., Nouchi, T., et al. (2002). Titin mutations as the molecular basis for dilated cardiomyopathy. Biochem. Biophys. Res. Commun. *291*, 385–393.

Kuo, H.C., Cheng, C.F., Clark, R.B., Lin, J.J., Lin, J.L., Hoshijima, M., Nguyen-Tran, V.T., Gu, Y., Ikeda, Y., Chu, P.H., et al. (2001). A defect in the Kv channel-interacting protein 2 (KChIP2) gene leads to a complete loss of I(to) and confers susceptibility to ventricular tachycardia. Cell *107*, 801–813.

Lathrop, G.M., Lalouel, J.M., Julier, C., and Ott, J. (1984). Strategies for multilocus linkage analysis in humans. Proc. Natl. Acad. Sci. USA *81*, 3443–3446.

Lee, A.A., Delhaas, T., Waldman, L.K., MacKenna, D.A., Villarreal, F.J., and McCulloch, A.D. (1996). An equibiaxial strain system for cultured cells. Am. J. Physiol. *271*, C1400–1408.

Louis, H.A., Pino, J.D., Schmeichel, K.L., Pomies, P., and Beckerle, M.C. (1997). Comparison of three members of the cysteine-rich protein family reveals functional conservation and divergent patterns of gene expression. J. Biol. Chem. *272*, 27484–27491.

Maisel, A. (2002). B-type natriuretic peptide levels: diagnostic and prognostic in congestive heart failure: what's next? Circulation *105*, 2328–2331.

Mayans, O., van der Ven, P.F., Wilm, M., Mues, A., Young, P., Furst, D.O., Wilmanns, M., and Gautel, M. (1998). Structural basis for activation of the titin kinase domain during myofibrillogenesis. Nature *395*, 863–869.

Mestroni, L., Maisch, B., McKenna, W.J., Schwartz, K., Charron, P., Rocco, C., Tesson, F., Richter, A., Wilke, A., and Komajda, M. (1999). Guidelines for the study of familial dilated cardiomyopathies. Collaborative research group of the European human and capital mobility project on familial dilated cardiomyopathy. Eur. Heart J. *20*, 93–102.

Minamisawa, S., Hoshijima, M., Chu, G., Ward, C.A., Frank, K., Gu, Y., Martone, M.E., Wang, Y., Ross, J., Jr., Kranias, E.G., et al. (1999). Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. Cell *99*, 313–322.

Moreira, E.S., Wiltshire, T.J., Faulkner, G., Nilforoushan, A., Vainzof, M., Suzuki, O.T., Valle, G., Reeves, R., Zatz, M., Passos-Bueno, M.R., and Jenne, D.E. (2000). Limb-girdle muscular dystrophy type 2G is caused by mutations in the gene encoding the sarcomeric protein telethonin. Nat. Genet. *24*, 163–166.

Nguyen-Tran, V.T., Kubalak, S.W., Minamisawa, S., Fiset, C., Wollert, K.C., Brown, A.B., Ruiz-Lozano, P., Barrere-Lemaire, S., Kondo, R., Norman, L.W., et al. (2000). A novel genetic pathway for sudden cardiac death via defects in the transition between ventricular and conduction system cell lineages. Cell *102*, 671–682.

Olson, T., Michels, V., Thibodeau, S., Tai, Y.-S., and Keating, M. (1998). Actin mutations in dilated cardiomyopathy, a heritable form of heart failure. Science *280*, 750–752.

Pashmforoush, M., Pomies, P., Peterson, K.L., Kubalak, S., Ross, J., Jr., Hefti, A., Aebi, U., Beckerle, M.C., and Chien, K.R. (2001). Adult mice deficient in actinin-associated LIM-domain protein reveal a developmental pathway for right ventricular cardiomyopathy. Nat. Med. *7*, 591–597.

Rana, B.K., Hewett-Emmett, D., Jin, L., Chang, B.H., Sambuughin, N., Lin, M., Watkins, S., Bamshad, M., Jorde, L.B., Ramsay, M., et al. (1999). High polymorphism at the human melanocortin 1 receptor locus. Genetics *151*, 1547–1557.

Reid, M.E., Rios, M., Powell, V.I., Charles-Pierre, D., and Malavade, V. (2000). DNA from blood samples can be used to genotype patients who have recently received a transfusion. Transfusion *40*, 48–53.

Sadoshima, J., and Izumo, S. (1997). The cellular and molecular response of cardiac myocytes to mechanical stress. Annu. Rev. Physiol. *59*, 551–571.

Seidman, J.G., and Seidman, C. (2001). The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms. Cell *104*, 557–567.

Sham, P.C., and Curtis, D. (1995). An extended transmission/disequilibrium test (TDT) for multi-allele marker loci. Ann. Hum. Genet. *59*, 323–336.

Trombitas, K., Freiburg, A., Greaser, M., Labeit, S., and Granzier, H. (2000). From connecting filaments to co-expression of titin isoforms. Adv. Exp. Med. Biol. *481*, 405–418.

Wettschureck, N., Rutten, H., Zywietz, A., Gehring, D., Wilkie, T.M., Chen, J., Chien, K.R., and Offermanns, S. (2001). Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of Galphaq/Galpha11 in cardiomyocytes. Nat. Med. *7*, 1236–1240.

Wollert, K.C., Taga, T., Saito, M., Narazaki, M., Kishimoto, T., Glembotski, C.C., Vernallis, A.B., Heath, J.K., Pennica, D., Wood, W.I., and Chien, K.R. (1996). Cardiotrophin-1 activates a distinct form of cardiac muscle cell hypertrophy. Assembly of sarcomeric units in series VIA gp130/leukemia inhibitory factor receptor-dependent pathways. J. Biol. Chem. *271*, 9535–9545.

Wu, Y., Cazorla, O., Labeit, D., Labeit, S., and Granzier, H. (2000). Changes in titin and collagen underlie diastolic stiffness diversity of cardiac muscle. J. Mol. Cell. Cardiol. *32*, 2151–2162.

Xu, X., Meiler, S.E., Zhong, T.P., Mohideen, M., Crossley, D.A., Burggren, W.W., and Fishman, M.C. (2002). Cardiomyopathy in zebrafish

due to mutation in an alternatively spliced exon of titin. Nat. Genet. *30*, 205–209.

Yamazaki, T., Komuro, I., Kudoh, S., Zou, Y., Nagai, R., Aikawa, R., Uozumi, H., and Yazaki, Y. (1998). Role of ion channels and exchangers in mechanical stretch-induced cardiomyocyte hypertrophy. Circ. Res. *82*, 430–437.

Yasukawa, H., Hoshijima, M., Gu, Y., Nakamura, T., Pradervand, S., Hanada, T., Hanakawa, Y., Yoshimura, A., Ross, J., Jr., and Chien, K.R. (2001). Suppressor of cytokine signaling-3 is a biomechanical stress-inducible gene that suppresses gp130-mediated cardiac myocyte hypertrophy and survival pathways. J. Clin. Invest. *108*, 1459–1467.

Zeng, T., Bett, G.C., and Sachs, F. (2000). Stretch-activated whole cell currents in adult rat cardiac myocytes. Am. J. Physiol. Heart Circ. Physiol. *278*, H548–557.

Zhou, Q., Chu, P.H., Huang, C., Cheng, C.F., Martone, M.E., Knoll, G., Shelton, G.D., Evans, S., and Chen, J. (2001). Ablation of Cypher, a PDZ-LIM domain Z-line protein, causes a severe form of congenital myopathy. J. Cell Biol. *155*, 605–612.

Zolk, O., Caroni, P., and Bohm, M. (2000). Decreased expression of the cardiac LIM domain protein MLP in chronic human heart failure. Circulation *101*, 2674–2677.