A Common MLP (Muscle LIM Protein) Variant Is Associated With Cardiomyopathy


Rationale: We previously discovered the human 10T→C (Trp4Arg) missense mutation in exon 2 of the muscle LIM protein (MLP, CSRP3) gene.

Objective: We sought to study the effects of this single-nucleotide polymorphism in the in vivo situation.

Methods and Results: We now report the generation and detailed analysis of the corresponding MlpW4R/+ and MlpW4R/W4R knock-in animals, which develop an age- and gene dosage–dependent hypertrophic cardiomyopathy and heart failure phenotype, characterized by almost complete loss of contractile reserve under catecholamine induced stress. In addition, evidence for skeletal muscle pathology, which might have implications for human mutation carriers, was observed. Importantly, we found significantly reduced MLP mRNA and MLP protein expression levels in hearts of heterozygous and homozygous W4R-MLP knock-in animals. We also detected a weaker in vitro interaction of telethonin with W4R-MLP than with wild-type MLP. These alterations may contribute to an increased nuclear localization of W4R-MLP, which was observed by immunohistochemistry.

Conclusions: Given the well-known high frequency of this mutation in Caucasians of up to 1%, our data suggest that W4R-MLP might contribute significantly to human cardiovascular disease. (Circ Res. 2010;106:695-704.)

Key Words: genetics ■ mechanosensation ■ mechanotransduction ■ cardiomyopathy ■ heart failure ■ circulation

Cardiomyopathies are primary disorders of cardiac muscle and represent major causes of morbidity and mortality at all ages.1 With a prevalence of 200 per 100,000 individuals, hypertrophic cardiomyopathy (HCM) is the most common cardiovascular disease inherited as an autosomal dominant trait and accounts for ≈36% of all sudden deaths in the United States in competitive athletes. Onset of disease is variable and can be late in adolescence.1 In the majority of cases, the disease is caused by mutations in sarcomeric and/or structural components.2 However, the underlying molecular mechanisms remain unclear.

We cloned and sequenced previously the human MLP gene (CLP, CRP3, or CSRP3), which is encoded by ≈20,000 bp and organized into 6 exons, giving rise to a 194-aa LIM only protein.3 Homozygous loss of Mlp in a genetically engineered mouse model results in cardiac hypertrophy followed by dilated cardiomyopathy (DCM),4 the first model for this condition in a genetically manipulatable organism. In addition, we discovered and characterized the 10T→C (Trp4Arg) missense mutation in exon 2 of the MLP gene, the first human MLP mutation (and together with a telethonin mutation, the first Z-disk mutation in general) linked to cardiomyopathy, and proposed a role for this gene in cardiac mechanosensation.3

Because of the well-known limitations of human studies, owing in part to differences in environmental and epigenetic factors, it is often impossible to perform an in depth functional analysis of mutations in the human setting. Therefore, we generated MlpW4R/+ and MlpW4R/W4R knock-in (KI) animals and compared the phenotype with Mlp+/+ littermates (Figure 1).

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.
Gene Targeting

Gene targeting was performed essentially as described in Arber et al.4

Echocardiography

All animal studies were performed in accordance with institutional, national, and international guidelines and regulations (local ethics committee #33.11.42502-04-075/08). For echocardiography, mice were anesthetized with isoflurane and transthoracic echocardiography was performed by a blinded investigator as described previously.5

Two-Dimensional Difference Gel Electrophoresis and Mass Spectrometry

MLP<sup>W4R/W4R</sup>(KI) or MLP<sup>++/++</sup> (wild-type [WT]) left ventricular (LV) tissue (12-month-old mice) was used to perform 2D difference gel electrophoresis (2D-DIGE)<sup>6,7</sup> as described in detail in the Online Data Supplement.

Glutathione S-Transferase Pull-Down Assays and MLP–Green Fluorescent Protein Overexpression in Embryonic Rat Cardiomyocytes

MLP, W4R-MLP, and telethonin (TCap) constructs were recombinantly expressed using pGEX4T2 vector and pull down assays were performed according to standard protocols.

Cardiomyocytes were isolated from embryonic day (E)18 hearts by enzymatic dissociation, plated at a density of 8×10<sup>5</sup> cells/mm<sup>2</sup>, and cultured at 37°C and 5% CO<sub>2</sub> in Iscove’s modified Dulbecco’s medium supplemented with 20% FCS. Transfections were performed with recombinantly expressed, enhanced green fluorescent protein (GFP)-coupled MLP or W4R-MLP constructs overexpressed in 24– to 48-hour cell cultures using standard lipofection methods (reagent, Escort IV; Sigma).

Statistics

For the comparison of different animal groups during echocardiography, the ANOVA F test, including sex adjustment, was used. For the parameters showing significance in the overall test, a pairwise comparison of the 3 different genotypes was performed by contrasts (closed testing procedure). Because of the high correlation of the different parameters to each other, no multiple testing or correction was used, and probability values of ≤0.05 were considered significant. For analysis of variations in fiber diameter the Wilcoxon signed-rank test for unpaired testing was applied. The Mann–Whitney U test was used for the analysis of hemodynamic parameters (data were not normally distributed), and the Student’s t test was applied for analysis of quantitative PCRs.

Results

MLP<sup>W4R/+</sup> and MLP<sup>W4R/W4R</sup> KI mice are born in the expected mendelian ratios, they are fertile and develop normal until adolescence. However, at an age between approximately 12 and 14 months (approximately half the life span of a mouse),
heterozygous, as well as homozygous, KI animals develop severe septal hypertrophy, a hallmark of HCM (Table). In contrast to the heterozygous animals, which develop a moderate but significant HCM phenotype, homozygous KI animals develop a severe septal hypertrophy, a hallmark of HCM (Table). In heterozygous, as well as homozygous, KI animals develop a severe form of HCM (Table): each parameter indicative of hypertrophy is significantly (P<0.05) or highly significantly increased (P<0.01) in homozygous KI animals. An important feature of the phenotype is the substantial gain in fractional shortening percentage, another hallmark of HCM, that is observed in all mutation carriers. An increase in fractional shortening percentage indicates, but is not necessarily associated with, a gain of function.9

We also performed in vivo hemodynamic measurements in this line and found, besides a significant increase in heart weight per body weight (BW), an increased lung weight per BW ratio, which might indicate pulmonary edema as an indirect sign of heart failure. However, we measured normal functional parameters under baseline conditions (n=6 for each group, Online Table I). WT animals responded to hemodynamic stress in form of adrenalin application for 2 and 3 minutes with the expected significant increase in myocardial performance as measured by decreased end-systolic volumes (Figure 2A), decreased end-diastolic volumes (Figure 2B), and increased LV contractility (Figure 2D). In contrast, W4R-MLP KI animals were unable to increase myocardial function under these conditions, thereby revealing a heart failure phenotype (Online Table I).

This striking observation led us to search for the underlying molecular mechanisms. In heterozygous, as well as homozygous, W4R-MLP KI animals, we found significantly reduced MLP mRNA (Figure 3A) and MLP protein expression levels (Figure 3B). Differential analysis of protein expression levels in 12-month-old Mlp+/+ and MlpW4R/W4R hearts using 2D difference gel electrophoresis (2D-DIGE) showed upregulation, among others, of myofibrillar proteins (eg, tropomyosin and α-actinin), consistent with a hypertrophy phenotype (Online Figures I and II; Online Table II).

Table. Parameters Indicating a Hypertrophic Phenotype in Animals Heterozygous or Homozygous for the 10T→C (Trp4Arg) Missense Mutation in MLP

<table>
<thead>
<tr>
<th></th>
<th>WT Mean Value (n=18)</th>
<th>Heterozygous Mean Value (n=18)</th>
<th>Homozygous Mean Value (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW (mm)</td>
<td>0.96±0.14</td>
<td>1.17±0.28††</td>
<td>1.11±0.18†</td>
</tr>
<tr>
<td>PW (mm)</td>
<td>0.88±0.11</td>
<td>0.97±0.19</td>
<td>1.03±0.24††</td>
</tr>
<tr>
<td>EDD (mm)</td>
<td>4.42±0.47</td>
<td>4.08±0.74</td>
<td>4.18±0.96</td>
</tr>
<tr>
<td>ESD (mm)</td>
<td>3.05±0.66</td>
<td>2.49±0.84†</td>
<td>2.65±1.19</td>
</tr>
<tr>
<td>h/r</td>
<td>0.43±0.08</td>
<td>0.55±0.19†</td>
<td>0.55±0.19††</td>
</tr>
<tr>
<td>FS (%)</td>
<td>31.7±9.3</td>
<td>40.4±10.8†</td>
<td>39.0±13.0†</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>521±82</td>
<td>569±82</td>
<td>547±69</td>
</tr>
<tr>
<td>Calc.LVM (mg)</td>
<td>181±33</td>
<td>188±48</td>
<td>196±66</td>
</tr>
<tr>
<td>BW (g)</td>
<td>39.0±6.0</td>
<td>41.7±9.3</td>
<td>34.8±5.7**</td>
</tr>
<tr>
<td>LVM/BW</td>
<td>4.65±0.59</td>
<td>4.56±0.83</td>
<td>5.71±2.07††</td>
</tr>
</tbody>
</table>

BW indicates body weight; Calc.LVM, calculated left ventricular mass; EDD, end-diastolic diameter; ESD, end-systolic diameter; FS, fractional shortening; h/r, wall thickness per radius (septum width/2 divided by diastolic radius); HR, heart rate in bpm; LVM/BW, LV mass per BW; PW, posterior wall width; SW, septum width. †P<0.05, ††P<0.01, heterozygous or homozygous KI animals vs WT littermates; *P<0.05, **P<0.01, heterozygous vs KI.

Figure 2. LV end-systolic volume (LVESV) (A), LV end-diastolic volume (LVEDV) (B), ejection fraction (EF) (C), and peak rate of LV pressure rise normalized for end-diastolic volume (dP/dtmax/Ved) as a marker for LV contractility (D), under basal as well as under stressed conditions (2 and 3 minutes after adrenalin injection). Under basal conditions, W4R-MLP KI animals showed a tendency for higher end-diastolic volumes (P=0.065) but no difference in ejection fraction, LV end-systolic volume, or LV contractility. However, KI animals developed severe heart failure under stressed conditions (as measured by end-systolic volumes, ejection fraction, and contractility), thus uncovering a cardiomyopathy phenotype. KI indicates homozygous W4R-MLP KI animals. *P<0.05 vs basal hemodynamic status of the same genotype; #P<0.05 vs WT animals under same stress conditions; §P<0.05 vs hemodynamic status 2 minutes after adrenalin injection of the same genotype (n=6 per group).
When we analyzed gene expression patterns, we found no change in brain natriuretic peptide (BNP) and atrial natriuretic factor mRNA expression levels but slightly increased myosin heavy chain protein expression levels in the W4R-MLP KI animals, indicating the presence of a hypertrophic gene program (Online Figure I). We also applied 10% biaxial stretch at 0.5 Hz to cultured cardiomyocytes, isolated from 8- to 12-week-old W4R-MLP KI hearts, as well as WT littermate controls, and analyzed BNP mRNA expression. Interestingly, similar to the situation in MLP-deficient cardiomyocytes,3 there was a clear trend suggesting the stretch-induced upregulation of BNP in cultured WT myocytes did not take place in W4R-MLP KI cardiomyocytes (Online Figure V).

MLP is known to interact with calcineurin,10 and increased calcineurin expression and/or activity levels are well known causes of myocardial hypertrophy.11 Therefore, we measured MCIP1 mRNA (modulatory calcineurin interacting protein, regulator of calcineurin 1), which has been used as an indicator of calcineurin activity previously12,13 but did not find any significant changes in the expression of this protein (Online Figure VI).

Moreover, in-depth analyses of the ultrastructure of KI hearts revealed myocardial disarray at the cellular, as well as subcellular, level (Figure 4A through 4G; Online Figure VII). Mutant animals also revealed significant fibrosis as detected by increased collagen I in the extracellular matrix (Figure 4H). Interestingly, in comparison to WT littermate control hearts, MLP in the KI animals was found to be less abundant at the sarcomeric Z-disk, whereas MLP in the nucleus was elevated (Figure 5A through 5E; Online Figure VIII).

Several lines of evidence indicate a critical role for MLP also in skeletal muscle.14 Histological analysis of the quadriceps femoris muscle of 1-year-old MlpW4R/W4R KI animals revealed normal fiber-type distribution and no increase in the proportion of centrally located nuclei but demonstrated mild myopathic features with a significantly increased variation in fiber diameter compared to controls (P<0.03) (Figure 6A and 6B). Measurements of passive elastic properties of fast muscle fibers from chemically permeabilized musculus vastus lateralis of WT and MlpW4R/W4R KI mice under relaxing conditions revealed a substantial decrease in fiber stiffness at low and high ionic strength in the homozygous KI mice (Figure 6C). Stiffness measurements under these conditions, performed with different speeds of stretch at low and high ionic strength, allowed the differentiation of contributions from passive structural components of the fibers and weak acto–myosin interactions.15 Therefore, the larger compliance found in MlpW4R/W4R muscle fibers is most likely attributable to changes in both passive structural components of the fibers and weak acto–myosin interactions. Confocal microscopy of skinned musculus vastus lateralis fibers immunofluorescently labeled with MLP-specific antibodies (Figure 6D and Online Figure IX) revealed specific staining at the Z-disk and M-band; the latter structure, however, was much more faintly stained. The observed changes in skeletal muscle compliance were not attributable to changes in titin isoform expression patterns, because we did not find any alterations in N2A-titin isoform size by 2% SDS-PAGE in W4R-MLP KI soleus skeletal muscle compared to WT muscle (Online Figure X).

In addition, the W4R-MLP mutation caused a significant decrease in the in vitro affinity of MLP to telethonin (TCap), a known MLP-interacting protein located at the periphery of the Z-disk (Figure 7A). However, W4R-MLP still localized to the...
Z-disk when the mutation was introduced into a MLP-GFP construct and overexpressed in late-embryonic rat cardiomyocyte cultures (Figure 7B and 7C). We note that WT and mutant MLP are not restricted to a Z-disk location but are found in the nucleus, the cytoplasm, and at gap junctions, as well.16,17

Discussion
Here, we report the generation and detailed analysis of Mlp\(^{W4R+}\) and Mlp\(^{W4R/W4R}\) KI mice, which develop an age- and gene dosage–dependent hypertrophic cardiomyopathy and heart failure phenotype. In addition, we provide evidence for the presence of skeletal muscle pathology in these animals, a fact that might have implications for human W4R-MLP mutation carriers in which, to the best of our knowledge, no skeletal muscle alteration has been described to date.

According to echocardiography measurements, W4R-MLP heterozygous, as well as homozygous, KI animals developed signs of hypertrophic cardiomyopathy, including increases in septum wall thickness, fractional shortening, and wall thickness per diameter (h/r). In addition, homozygous KI animals showed increased LV mass per BW and, interestingly, significantly reduced body weight. We do not have an explanation for the significantly decreased body weight in the homozygous KI group, but it is possible that the observed skeletal myopathic effects play a role. The increase in fractional shortening under baseline conditions in the genetically altered animals, although interesting, is unlikely to be attributable to enhanced myocardial function; rather, it may be explained by the smaller LV end-systolic and end-diastolic diameters found in these animals.9

Data obtained from animals undergoing hemodynamic analysis (Online Table I) support the findings obtained by echocardiography. Interestingly, the hemodynamic parameters under baseline conditions were normal (Online Table I). However, slightly but significantly increased lung weight per BW ratios might indicate the presence of pulmonary edema in the KI animals, an indirect sign of heart failure.

Importantly, hemodynamic stress in form of adrenalin application for 2 and 3 minutes revealed a blunted response in the KI animals, as judged by a lack of significant

Figure 4. Myofiber disarray, fibrosis, and MLP nuclear translocation in W4R-MLP KI mice. A and B, Myomesin (green) used as indicator of the M-band shows regular cross striation in control hearts (con) but myofiber disarray and loss of myocytes in KI. Nuclei are blue. C and D, Fibrosis: collagen I (green) is present in small amounts in control but significantly increased in KI hearts. Myocytes are red. E through G, Ultrastructural evidence of myofiber disarray. Compared to controls (E), the tissue from KI mice shows “star-like” arrangement of myofibers and myofilaments (F). G shows the framed area in F at higher magnification. Disarray of myofilaments is evident. H, Fibrosis (ie, collagen I) is significantly more present in the KI hearts.*P<0.01.
changes in end-systolic volumes (Figure 2A), end-diastolic volumes (Figure 2B), and LV contractility (Figure 2D). This finding is remarkable and suggests that the ventricular performance is maintained at the expense of limited myocardial contractile reserve, and that inotropic stimulation unmasks the heart failure phenotype present in these animals. In this context, we point out that two individuals previously identified as W4R-MLP mutation carriers developed heart failure after performing extreme sports (ie, marathon running and free climbing).

Although heterozygous MLP knock out animals, which express significantly less MLP protein than WT littermates, do not develop any spontaneous phenotype, heart failure can easily be induced in these animals by additional stressing, such as ischemia. Similarly, the significantly reduced MLP mRNA and protein expression levels observed in heterozygous and homozygous W4R-MLP KI animals (Figure 3A and 3B and Figure 7D) might ultimately lead to hypertrophy and heart failure.

Another potentially disease causing alteration is the reduced protein-protein interaction between the Z-disk protein telethonin and W4R-MLP compared to WT-MLP. Although we found that overexpressed W4R-MLP coupled to green fluorescent protein still bound to Z-disks in cultured cardiomyocytes, a reduced binding to telethonin might ultimately contribute to loss of MLP at the sarcomeric Z-disk and facilitate the observed translocation of W4R-MLP into the nucleus (Figure 5A through 5E and Figure 7D). In this context, we note that MLP translocates to the nucleus via its nuclear localization sequence located within the first glycine-rich domain; nuclear MLP is essential for proper sarcomeric organization. The function of MLP in the nucleus is poorly understood. However, this protein, which is unable to bind to DNA directly, can act as a cofactor of transcription by interacting with a variety of different transcription factors, such as MyoD, myogenin, and MRF. Therefore, increased W4R-MLP in the nucleus could readily affect transcription and may be one reason why W4R-MLP KI animals show blunted BNP gene expression (Online Figure V).

MLP was found to localize to the sarcomeric Z-disk in skeletal and cardiac muscle tissue, confirming results of several previous MLP localization studies in striated muscle. Notably, we used different anti-MLP antibodies, including a monoclonal described in a recent report, as well as one developed by Hoshijima and colleagues and another by Caroni and colleagues. Besides a Z-disk MLP localization in skeletal muscle tissue, we observed faint staining of MLP at the M lines (close to titin kinase domain of titin), the significance of which needs to be determined.

W4R-MLP KI skeletal muscles were significantly more compliant in comparison to WT littermate controls. This finding is consistent with the increased compliance of MLP deficient mouse heart samples reported earlier. The molecular mechanisms leading to reduced skeletal muscle passive stiffness remains to be determined, but we showed they do not include changes in titin isoform size. Whether post translational modifications of the elastic titin region (eg, phosphorylation or disulphide bridge formation) are involved in altering fiber stiffness is an issue worth studying in the future. Alternatively, the decrease in MLP fiber stiffness could be attributable to a direct structural role of MLP in the Z-disk and/or secondary effects via involve-
The mutation of MLP in signaling cascades that regulate myocyte remodeling. In any case, our finding that the W4R-MLP mutation affects the skeletal muscle confirms a previously reported role for MLP in skeletal muscle maintenance and regeneration, as well as a report on skeletal muscle pathology in patients carrying a MLP mutation. Our results might have implications for human 10T3(Trp4Arg)MLP carriers, which may show signs of both heart and skeletal muscle pathologies.

In W4R-MLP KI hearts, hemodynamic measurements showed no significant change in relaxation constant, Tau (Online Table I). Possibly, an increased compliance of the cardiomyocytes in W4R-MLP hearts had been cancelled out by the increased collagen I expression causing increased collagen-based stiffness.

Since our initial description of the 10T→C (Trp4Arg) MLP missense mutation, this mutation has been found in additional individuals affected by either DCM or HCM. Possibly, the individuals first described by us to have the 10T→C (Trp4Arg) MLP missense mutation had been at later stages of the disease, when the mutation is DCM-associated. However, at earlier stages, the mutation seems to be associated with HCM. In addition, confounding effects of incomplete penetrance, genetic modifiers, or different envi-

**Figure 6.** A and B, W4R-MLP KI animals are characterized by myopathic changes in skeletal muscle. A, In comparison to controls (CON), hematoxylin/eosin staining of transverse muscle sections (quadriceps femoris muscle) reveals an increased variation in fiber diameter in mutant animals. Scale bar=50 μm. B, Distribution of fiber diameters. Averages were formed from all individual means and from all individual variances for the animals of 1 group, yielding 2 different average Gaussian distribution curves. Interestingly, W4R-MLP KI animals display significantly larger variation in their myofiber diameters than WT animals (n=5 muscles from 12-month-old animals, with a total of 700 to 800 myofibers; P<0.03). C, Stiffness (passive elastic properties) of relaxed fibers isolated from chemically permeabilized musculus vastus lateralis of MlpW4R/W4R KI mice (open symbols) is significantly lower than in fibers from control mice (WT) (filled symbols). On average, stiffness recorded at different speeds of stretch at ionic strength 50 mmol/L (squares) and 220 mmol/L (circles) was reduced by 45% and 58%, respectively (n=5 to 9 fibers from 2 KI mice and 3 WT mice; error bars sometimes smaller than symbol). D, Longitudinal optical section of a single fiber derived from musculus vastus lateralis of a MlpW4R/W4R KI obtained by confocal microscopy shows colocalization (overlay, right image) of α-actinin (TRITC fluorescence, middle image; fiber not yet equilibrated with antibodies throughout cross-section) and MLP (FITC fluorescence, left image; antibody from P. Caroni) in the Z-disk (sarcomere length, 2.5 μm). Faint MLP labeling is also observed in the M-line of the sarcomeres.
environmental circumstances might be at play. Moreover, HCM and DCM phenotypes do not exclude one another and are sometimes difficult to distinguish (ie, “burnt out” phase HCM). This notion is supported by the spontaneous development of an HCM phenotype in the 10T\(\rightarrow\)C (Trp4Arg) MLP KI animals and by the fact that at least 10% to 20% of all HCM patients develop a DCM phenotype later in life\(^\text{30}\) (reviewed elsewhere\(^\text{31}\)). An alternative possibility is that this mutation is capable of causing either an HCM or a DCM phenotype, depending on modifier genes and/or epigenetic factors. This possibility is confirmed by the Syrian hamster model, where the same \(\delta\)-sarcoglycan mutation causes, in 2 different lines, either an HCM (BIO 14.6) or a DCM (TO-2) phenotype.\(^\text{32,33}\) Heterozygous, as well as homozygous, W4R-MLP KI animals develop the phenotype relatively late at an age of \(\approx12\) months. A late-onset phenotype, which has also been observed for myosin binding protein C mutations,\(^\text{34}\) might explain the presence of several healthy mutation carriers in younger human individuals and the fact that the fraction of healthy 10T\(\rightarrow\)C (Trp4Arg) MLP carriers drops in an older population.\(^\text{23}\) Similarly, late onset of disease might be a reason for the presence of this mutation in another control population, where the cardiovascular phenotype was not determined.\(^\text{29}\) In summary, our data support the notion that the 10T\(\rightarrow\)C (Trp4Arg) MLP missense mutation is a late-onset and gene dosage–dependent, disease-causing mutation.

Given the well-known association of cardiac hypertrophy with cardiovascular morbidity and mortality (reviewed by Hill and Olson\(^\text{35}\)), it is clear that the 10T\(\rightarrow\)C (Trp4Arg) MLP missense mutation conveys a significant risk for human individuals affected by this mutation (Figure 7D). To the best of our knowledge, the 10T\(\rightarrow\)C (Trp4Arg) MLP is the most frequent cardiovascular disease gene in European whites, comparable to a recently published myosin binding protein C variant common among Southern Asians.\(^\text{36,37}\) We speculate that the W4R-MLP mutation, because of its high frequency of up to 1%, may convey (partial) myocardial protection or at least offer an advantage under certain conditions (otherwise, evolution might not have allowed its widespread distribution) but, in the end, may exert detrimental effects. Gene dosage effects are well known to modify the progression of diseases like HCM and DCM considerably, both in human individuals\(^\text{38}\) and in animal models.\(^\text{39}\) In this context, our Mlp\(^{W4R/-}\) and Mlp\(^{W4R/W4R}\) KI animals may represent a novel “humanized” mouse model to recapitulate and study major features of cardiomyopathies. Future research should focus on elucidating the underlying age- and gene dosage–dependent, disease-causing mutation.

Moreover, innovative therapeutic strategies have to be developed to reverse the detrimental effects seen in HCM and DCM patients. Our novel model of Mlp\(^{W4R/W4R}\) mice could help facilitate progress in this field.

Figure 7. A through C, Effect of W4R-MLP mutation on MLP-TCap interaction and MLP localization in cultured embryonic (E18) rat cardiomyocytes. A, Results of glutathione S-transferase pull-down assays probing interaction between TCap and WT-MLP (WT) or W4R-MLP (W4R) recombinant constructs. Left, Western blots (12.5% SDS-PAGE) using MLP antibody (E1 to E4; 4 different experiments). Right, Average relative binding affinity (means\(\pm\)SD; \(N=4\)) between TCap and MLP constructs. \(*P<0.05\). B, Localization of WT-MLP-GFP in transfected E18 rat cardiomyocytes, in comparison to \(\alpha\)-actinin (primary antibody visualized using Cy3-conjugated IgG) and nuclear staining (DAPI). Scale bar=10 \(\mu\)m. C, Localization of W4R-MLP-GFP overexpressed in E18 rat cardiomyocytes. For comparison, a cardiomyocyte transfected with WT-MLP-GFP (upper right) or with GFP only (lower right) is shown. Scale bar=10 \(\mu\)m. D, Schematic diagram depicting molecular mechanisms underlying the observed cardiomyopathy phenotype in W4R-MLP animals.
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Disclosures

None.

References

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Novelty and Significance

What Is Known?

- MLP is expressed in muscle tissues, but Z-disk and/or nuclear localization remains unclear.
- 10T→C (Trp4Arg) MLP single-nucleotide polymorphism exists, but a disease causing role either for heart or skeletal muscle remains unclear.
- Loss of MLP in a mouse knockout model is associated with a severe form of cardiomyopathy and loss of MLP has been observed in human cardiomyopathic hearts.

What New Information Does This Article Contribute?

- Beside their presence in other cellular compartments, MLP as well as W4R-MLP are localized at the sarcomeric Z-disk and particularly W4R-MLP relocates into the nucleus.
- W4R-MLP mRNA, as well as W4R-MLP protein, levels are significantly decreased in the knock-in animals.
- 10T→C (Trp4Arg) MLP is a cardiomyopathy and heart failure and/or mild skeletal muscle myopathy causing mutation.

Although we continue to discover ever more novel cardiomyopathy causing mutations, the underlying molecular events that link the genetic defect to the complex disease phenotype remain elusive. We described previously a role for the muscle specific protein MLP (CSRP3) in mechanosensation and discovered the human 10T→C (Trp4Arg) missense mutation in this gene. However, recent publications provided evidence for the presence of this variant in different populations at high frequency and questioned some of the earlier findings on MLP such as the relevance of this mutation for human disease and/or the localization of MLP at the sarcomeric Z-disk. To study the effects of the above mentioned mutation, we generated W4R-MLP knock-in mice and found that these animals develop a cardiomyopathy and heart failure phenotype together with a mild skeletal myopathy. Moreover, we found relocation of the mutant MLP away from the sarcomeric Z-disk to the nucleus. This study provides unequivocal evidence for a disease causing role of the 10T→C (Trp4Arg) MLP missense mutation. Together with the unusually high frequency of up to 1% of the 10T→C (Trp4Arg) MLP missense mutation in different human populations, our findings may have relevance for human mutation carriers, who should be carefully examined for heart failure and skeletal muscle phenotypes.
A common MLP (Muscle LIM Protein) variant associated with cardiomyopathy

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Methods

Gene targeting
Gene targeting was performed essentially as described in Arber et al. Briefly, a mouse Mlp genomic sequence was subcloned and a 6 kb long arm and a 4 kb short arm were generated. After introduction of the 10T→C (Trp4Arg) MLP missense mutation by using the Quik Change site directed mutagenesis kit (Stratagene, La Jolla, California, USA), both arms were cloned into plasmid pflox, containing HSV-thymidine kinase and neomycin resistance genes (Fig. 1). After recombination and additional Cre transfection, positive stem cells were injected and chimeras were used to obtain germline transmission. Animals were analyzed in 2 strains: Black Swiss as well as C57 black 6 N; no differences between strains were found.

Echocardiography
All animal studies have been performed in accordance with institutional, national and international guidelines and regulations (local ethics committee # 33.11.42502-04-075/08). For echocardiography mice were anesthetized with avertin (125 mg/kg, IP) and transthoracic echocardiography was performed by a blinded investigator as described previously. Thickness at the interventricular septum (SW), posterior wall (PW), left ventricular end-diastolic diameter (EDD), and left ventricular systolic diameter (ESD) were measured. Heart rate (HR) was determined and fractional shortening (FS), as well as left ventricular mass/body weight ratio (LVM/BW), were calculated.

Hemodynamics
Hemodynamic measurements of the left ventricle (LV), under basal as well as stressed conditions, were performed using a conductance catheter as previously described. Six wildtype and homozygous knock-in animals at the age of 12-14 months were analyzed. The mice were anesthetized (0.8-1.2 g/kg urethane and 0.05 mg/kg buprenorphine intraperitoneally) and artificially ventilated. A 1.2F microconductance pressure-volume catheter (Scisense Inc., Ontario, Canada) was positioned in the left ventricle through the right carotid artery for continuous registration of LV pressure-volume loops in a closed chest model. The volumes were calibrated using the hypertonic saline technique. All measurements were performed three times while ventilation had been turned off momentarily. Indices of systolic and diastolic cardiac performance were derived from LV pressure-volume data obtained at steady state. Preload-independent measurements were obtained during short occlusion of the vena cava inferior. Systolic function and myocardial contractility were quantified by measurements of maximal LV-pressure, peak rate of rise of LV pressure (dP/dtmax), ejection fraction (EF), cardiac output (CO), end-systolic volume (ESV), stroke volume (SV) and maximal elastance (Ees). Diastolic
performance was measured by LV end-diastolic pressure (LVEDP), peak rate of LV pressure decrease dP/dtmin, end-diastolic volume (EDV), LV-stiffness and the time constant of LV relaxation Tau (τ).

Furthermore, a protocol was used to assess hemodynamic parameters of the left ventricle under stress conditions. Adrenaline (1mg/ml) was injected intraperitoneally at a dose of 5 μl and steady state measurements were obtained after 2 and 3 minutes. The animals were sacrificed after the experiment, the heart, the left ventricle and the lungs were weighted and snap frozen in liquid nitrogen for further analysis.

**Tissue sampling**

Tissue samples were taken by dissection from explanted hearts in control and knock-in mice. All tissues were immediately frozen in liquid nitrogen and stored at –80°C until further use. In addition, small samples were fixed in 3 % buffered glutaraldehyde for electron microscopy.

**Electron microscopy**

The tissue was embedded in Epon following routine procedures. Ultrathin sections were double stained with uranyl acetate and lead citrate and viewed in a Philips CM 10 or a CM 201 electron microscope.

**Immunolabeling**

Cryosections 5 μm thick were air dried and fixed with 3% paraformaldehyde. Primary antibodies were biotinconjugated rabbit anti-collagen type I (Rockland) and monoclonal mouse anti-myomesin (kind gift from J. Perriard, Zürich, Switzerland). Anti MLP antibodies were provided by Dr. Masahiko Hoshijima 5, Dr. P. Caroni 6 as well as by Dr. D. Fürst 7. The specificity of all antibodies was verified by omission of the first antibody. The secondary detection system was biotinylated anti-mouse IgG followed by streptavidin linked with either Cy 2 (myomesin) or Cy 3 (collagen) (Amersham). Myocyte identification for collagen labeled sections was done with TRITC labeled phalloidin (Sigma). Nuclei were stained with TOTO-3 (Molecular Probes). Sections were viewed and representative pictures taken in a confocal microscope (Leica).

**Quantitative immunofluorescent measurements**

Quantification of collagen I was performed as described previously 8,9 : In brief, cryosections from at least two different levels in each mouse were used. All samples were immunolabeled simultaneously with identical conditions of fixation and dilutions of primary and secondary antibodies. Sections exposed to PBS instead of primary antibodies served as negative controls. For each heart at least 10 random fields of vision were analyzed with a fluorescent microscope Leica (Leitz DMRB) using a x40 Planapo objective (Leica). Immunolabeled cryosections were studied using image analysis (Leica) and Image J software. Quantification of collagen I was performed blinded to the type of section having on the screen only one channel showing F-actin labeling. For each quantification a specific setting was established and kept constant in all measurements. Quantification of collagen I was performed by measurements of fluorescence intensity by using a range of 0 to 255 gray values. Arbitrary units of the fluorescence intensity were calculated per unit myocardial area (AU/μm2). The area of collagen I were calculated as percent of positive labeling per tissue area.

**Histology of skeletal muscle**

For morphometric analysis skeletal muscle of 12 month-old mice was flash-frozen in liquid nitrogen cooled isopentane. Subsequently, 10μm sections were stained with hematoxylin and eosin, and immunohistochemistry was undertaken to detect succinatehydrogenase and cytochrome C oxidase activity according to standard procedures. The diameter of all fibers in three different fields was measured at 20x magnification per animal (n=6). A total of 120 to 240 fibers per animal were measured. Within these fields the percentage of fibers with central location of nuclei were also measured. All images were taken using a Zeiss Axioplan microscope, and measurements were carried out applying Axiovision V4.6.3.0 software.
**GST-pulldown assays**
MLP, W4R-MLP and telethonin (TCap) constructs were recombinantly expressed using pGEX4T2 vector, according to standard protocols, and verified by sequencing. For GST-pulldown experiments, TCap was immobilized on beads, followed by incubation with MLP (WT or W4R-MLP) containing supernatant. Four washing steps were done with thrombin buffer. Samples were analyzed by SDS-PAGE, transferred to a PVDF membrane and probed by Western blot using MLP antibodies (anti-rabbit CSRP 3). For densitometric analysis, the signal intensity of the MLP-TCap interaction was considered to be 100%.

**MLP-GFP overexpression in embryonic rat cardiomyocytes**
Cardiomyocytes were isolated from embryonic day 18 (E18) hearts by enzymatic dissociation, plated at a density of 8×10⁵ cells/mm², and cultured at 37°C and 5% CO₂ in Iscove’s Modified Dulbecco’s medium supplemented with 20% fetal calf serum 10. Transfections were done with recombinantly expressed, EGFP-coupled, MLP or W4R-MLP constructs overexpressed in 24-48-h-old cell cultures using standard lipofection methods (reagent, Escort™ IV; Sigma). Controls were transfected with EGFP only. GFP signals were visualized under a Zeiss Axiovert 135 inverted microscope in epifluorescence mode, using a color-CCD camera (Sony). Paraformaldehyde-fixed cells were also stained against -actinin (primary antibody from Sigma; secondary antibody, Cy3-conjugated IgG), nuclei were visualized using DAPI (Hoechst) staining.

**Cell stretch experiments**
*Cardiac myocyte preparation:* Isolation of adult (8-12 week old) mouse ventricular myocytes was carried out as described previously. ¹¹ Briefly, hearts were excised from mice that were anesthetized in a gas chamber with isoflurane. Hearts were mounted on a Langendorff-perfusion apparatus driven by gravity and perfused with nominally Ca²⁺ - free Tyrode’s solution containing (in mM) NaCl 115, KCl 4.7, KH₂PO₄ 0.6, Na₂HPO₄, MgSO₄ 1.2, NaHCO₃ 12, KHCO₃ 10, HEPES 10, taurine 30, 2,3-butanedionemonoxime 10, glucose 5.5 (pH 7.46) for 2-4 min at 36°C. Perfusion was then switched to the same solution containing liberase blendzyme 1 (Roche) 0.25 mg/ml and Trypsin 0.14 mg/ml with perfusion continuing until the heart became flaccid (7-12 min). Ventricular tissue was removed, dispersed, filtered and suspensions were rinsed several times. After Ca²⁺ reintroduction (stepwise increase to 0.8 mM), isolated cardiomyocytes were then plated onto superfusion chambers, with the glass bottoms treated with laminin to allow cell adhesion. Isolated cardiomyocytes were then resuspended in MEM medium supplemented with 5% fetal bovine serum, 2 % BDM, 1 % penicillin/streptomycin and 0.01% linoleic & oleic acid and plated at 5 x 10⁵ cells/ml on 35-mm diameter laminin coated silicone membranes (Bioflex, Flexcell, McKeesport, PA, USA) for 1 h at 37°C in humidified air with 5% CO₂. Then the medium was changed to serum-free and BDM-free medium and replaced by fresh medium after 30 min and after 1 h.

*Application of Mechanical Stretch:* We applied biaxial cyclic stretch to attached cardiomyocytes after 1 h in complete serum-free medium by applying a computer-controlled vacuum suction under the flexible-bottomed bioflex laminin plates using a S-1 Cell- Stretching Apparatus (Cell Line Service, Eppelheim, Germany) ¹². The vacuum varied in two-second cycles (0.5 Hz) at a level to promote 10% elongation of the cardiomyocytes at the point of maximal distension of the culture surface. For controls (non-stretch) we applied quiescent cultures under the same conditions. Cardiomyocytes were either stretched for 2 hours or for 4 hours. After experiments, the cells were washed twice with phosphate-buffered saline and quickly harvested by scrapping in lysis buffer from the RNeasy Micro Kit (Qiagen GmbH, Hilden, Germany).

**Quantitative Real Time PCR**
*Isolation and Analysis of RNA:* RNA from left ventricles was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen GmbH, Hilden, Germany) whereas RNA from stretched isolated cardiomyocytes and controls was isolated using the RNeasy Micro Kit (Qiagen GmbH, Hilden, Germany). The integrity, purity and concentration of RNAs were determined using the RNA 6000 Nano Chip Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Reverse-
transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. qPCR was performed in duplicate using the Bio-Rad iCycler (Bio-Rad) and SYBR Green as fluorescent. Primer sequences are available upon request.

**Titin Isoform Analysis**

Skeletal muscle (soleus) tissue obtained from deep-frozen W4R-MLP or WT mice was homogenized in modified Laemmli buffer and proteins were separated on agarose-strengthened 2% SDS-PAGE as described previously. For comparison of titin molecular size, the mouse soleus tissue was mixed with rabbit psoas tissue, which has a known titin size of 3400 kDa. Protein bands were visualized by Imperial protein stain, scanned, and analyzed densitometrically.

**Stiffness measurements and confocal microscopy of permeabilized skeletal muscle fibers**

Skeletal muscle fiber stiffness of 8-9 month old wildtype (WT) and MlpW4R/W4R knock-in mice was determined according to a previously described method on single fibers isolated from Vastus lateralis muscle permeabilized with 0.5% Triton-X-100 (Boehringer, Mannheim). To obtain fiber stiffness under relaxed conditions fibers were immersed in solution without calcium at 50mM and 220mM ionic strength. To confirm that all fibers were of the same type (Iib), gel electrophoretic analysis of myosin heavy chain isoform was performed for all muscle fibers used for the stiffness measurements. To study MLP localization in Vastus lateralis muscle of WT and W4R-MLP knock-in mice, permeabilized unfixed fibers were incubated with MLP specific antibodies generously given to us by P. Caroni and C. Geier, respectively. As localization control a monoclonal anti-α-actinin antibody (Sigma-Aldrich, Munich) was used. Primary antibodies were visualized with TRITC and FITC conjugated secondary antibodies using confocal laser scanning microscopy (Leica Inverted MP) as described previously. Incubation occurred in relaxing solution for 1-2 hours with primary and secondary antibodies, respectively.

**Statistics**

For the comparison of different animal groups, the ANOVA F-test including sex adjustment was used. For the parameters showing significance in the overall test, a pairwise comparison of the three different genotypes was performed by contrasts (closed testing procedure). Because of the high correlation of the different parameters to each other, no multiple testing correction was used and p values of 0.05 or less were considered significant. For analysis of variations in fiber diameter the Wilcoxon signed-rank test for unpaired testing was applied.

**Two-dimensional difference gel electrophoresis (2D-DIGE)**

**Sample preparation**

Frozen MLPW4R/W4R (KI) or MLP+/+ (WT) LV tissue (12 months old mice) was homogenized on ice in 10 volumes of extraction buffer (8 M urea, 2 M thiourea, 4 % w/v CHAPS, 1 % w/v DTT [all Fisher Scientific], Plus One Amberlite IRN 150L [GE Healthcare]), as described previously. The protein concentration of each homogenate was estimated using the 2-D Quant Kit (GE Healthcare) according to the manufacturer’s protocol.

**2-dimensional gel electrophoresis**

For the 2D-DIGE experiments 5 left ventricles of 12 months old homozygous W4R-MLP mutant mice and 5 litter-matched wildtype hearts were used. Each IPG strip was loaded with 75 µg of CyDye (Ettan minimal CyDyes, Cy2, Cy3, Cy5; GE Healthcare) labeled sample (25 µg of protein per 400 pmol of each CyDye). For preparative silver stains the immobilized pH gradient gel strips (linear gradient IPG strips, pH 4-7; GE Healthcare) were loaded with 750 µg of protein.

Prior to the labeling with Ettan minimal CyDyes the samples were treated with the 2D Clean-Up Kit (GE Healthcare). As an internal standard 25 µg of pooled MLPW4R/W4R or MLP+/+ sample were labeled with Cy5. Sample clean-up and labeling were performed according to the manufacturers protocols. Supplementary 1% carrier ampholytes pH 4 – 7 (GE Healthcare), 1 % v/v hydroxy-ethyl-disulfide (HED, Acros), and a trace amount of bromophenol blue (Fisher Scientific) were added.

Active rehydration and isoelectric focusing (IEF) (18 cm IPG strips, linear gradient; GE Healthcare) were performed in a Protean IEF cell (Bio-Rad), while the following voltages in a rapid voltage ramp mode were applied: 50 V for 11 h, 150 V, 300 V, 600 V, 1,000 V, 2,000 V and 4,000 V for 1 h each
followed by 8,000 V for 2 h and 10,000 V for 10 h. Thereafter the IEF IPG strips were stored at -80 °C until the start of the second dimension separation.

Prior to the second dimension the focused proteins were reduced (DTT) and alkylated (iodoacetamide) in two 15 min steps. The equilibration buffer consisted of 50 mM Bis-Tris (pH 6.4), 30% glycerol, 1% SDS, a trace of bromophenol blue, and 100 mM DTT (Fisher Scientific) or 150 mM iodoacetamide (SIGMA), respectively. The equilibrated proteins were transferred from the IPG strip onto a 10% Bis-Tris gel as described previously.19

Preparative gels were silver stained according to.20 DIGE gels were washed twice with water and then scanned with a Typhoon 9410 Variable Mode Imager using ImageQuant Software (both GE Healthcare) according to.21 For further analysis the DIGE gels were submitted to Ludesi (Sweden) via the Ludesi software, Redfin. Spots showing a significant expression change were excised from silver stained gels (we thank Dr. Jennifer van Eyk for supporting the 2D-DIGE analysis).

**Liquid chromatography and mass spectrometry**

Protein identification for the 2D spots was performed by a combination of liquid chromatography (LC) (Easy nLC system; Bruker Daltonics, Bremen, Germany) and mass spectrometry (electrospray ion trap HCTultra; Bruker Daltonics, Bremen, Germany). The sample preparation was performed by the team of Carsten Bäßmann at Bruker Daltonics, Bremen, Germany, according to.20, 22

For the in-gel digestion porcine trypsin (Promega) was used. The dried peptides were resuspended in 20 µl of 0.1% trifluoroacetic acid (TFA). For LC the following columns (nanoseparations) were utilized: analytical column: particle size and material: 3 µm Biosphere C18, length (L)=10 cm, inner diameter (ID)=75 µm, outer diameter (OD)=360 µm (NS-AC-10-dp3); trapping column: particle size and material: 5 µm Biosphere C18, L=2 cm, ID=100 µm, OD=360 µm (NS-MP-10).

The injection volume was 5 µl. The LC pump settings were as follows: flow rate 30 µl/min, maximum pressure 300 bar, 30 min 95% of 0.1% formic acid in water (solvent A) and 5% of 0.1% formic acid in acetonitrile (solvent B), 2 min 65% solvent A and 35% solvent B, 8 min 100% solvent B. The parameter settings for the HCTultra were as follows: mass range mode: std/enhanced; ion polarity: positive; trap scan begin: 400 m/z; trap scan end: 1400 m/z.

For data acquisition, Compass 1.3 software (including HyStar 3.2 SR2, esquire ctrl 6.2, DataAnalysis 4.0 SR1, Bruker Daltonics) was used. Data processing was performed using ProteinScape 2 (Bruker Daltonics, Bremen, Germany). Proteins were identified by comparison to the SwissProtKB Mus musculus database (12/16/2008) utilizing Mascot (Matrix Science, London, UK). The search parameters were as follows: missed cleavage=1; peptide tolerance: 0.35 Da; MS/MS tolerance: 0.8 Da; number of 13C: 2; peptide charge 1+, 2+, 3+; cysteine treated with iodoacetamide. Identifications with a minimum Mascot score of 80 were considered to be high confidence.
Online Table I

<table>
<thead>
<tr>
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<th>Wildtype (n=6)</th>
<th>Homozygous (n=6)</th>
<th>p-value</th>
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</thead>
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<tr>
<td>BW (g)</td>
<td>29.17 +/- 3.4</td>
<td>26.17 +/- 2.48</td>
<td>0.145</td>
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<tr>
<td>HW (mg)</td>
<td>130 +/- 5</td>
<td>139 +/- 22</td>
<td>0.18</td>
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<tr>
<td>LVW (mg)</td>
<td>94 +/- 5</td>
<td>100 +/- 18</td>
<td>0.394</td>
</tr>
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<td>HW/BW (mg/g)</td>
<td>4.5 +/- 0.5</td>
<td>5.3 +/- 0.5</td>
<td>0.026</td>
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<td>LVW/BW (mg/g)</td>
<td>3.3 +/- 0.4</td>
<td>3.8 +/- 0.4</td>
<td>0.041</td>
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<td>LVW/tibia length (mg/mm)</td>
<td>4.7 +/- 0.4</td>
<td>4.7 +/- 0.4</td>
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<td>Tibia length (mm)</td>
<td>20 +/- 0.9</td>
<td>19.67 +/- 0.52</td>
<td>0.54</td>
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<tr>
<td>Lung weight (mg)</td>
<td>167 +/- 11</td>
<td>175 +/- 24</td>
<td>0.18</td>
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<tr>
<td>Lung weight/BW (mg/g)</td>
<td>5.8 +/- 0.5</td>
<td>6.6 +/- 0.6</td>
<td>0.026</td>
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<td>HR (beats/min)</td>
<td>480 +/- 77</td>
<td>471 +/- 60</td>
<td>0.94</td>
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<td>LVP_max (mmHg)</td>
<td>77.5 +/- 6.1</td>
<td>79.8 +/- 9.9</td>
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<td>LVP_end (mmHg)</td>
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<td>LVPdP/dt_max (mmHg x s^-1)</td>
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<td>LVPdP/dt_max/Ved (mmHg x s^-1 x μl^-1)</td>
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<td>LVPdP/dt_min (mmHg x s^-1)</td>
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<td>SV (μl)</td>
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<td>CO (μl x s^-1)</td>
<td>10978 +/- 2984</td>
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<td>Ees</td>
<td>3.9 +/- 2.6</td>
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<td>Stiffness (1/μl)</td>
<td>0.08 +/- 0.06</td>
<td>0.06 +/- 0.09</td>
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Online Table I: hemodynamic characteristics of wildtype and homozygous W4R-MLP knock-in animals at the age of 12-14 months under basal conditions. Homozygous animals develop significant left ventricular hypertrophy (HW/BW) and an increase in lung weight / body weight might indicate the presence of pulmonary edema, a sign of heart failure. But otherwise, under spontaneous conditions, there are no major differences between the groups. BW, body weight; HW, heart weight; LVW, left ventricular weight; HR, heart rate; LVPmax, maximal LV-pressure; LVPed, enddiastolic LV-pressure; LVPdP/dtmax, peak rate of LV-pressure rise; LVPdP/dtmin, peak rate of LV-pressure decrease; Ved, enddiastolic LV-volume; SV, stroke volume; CO, cardiac output; Ees, maximal elastance; PHT, pressure half time;
Two-dimensional difference gel electrophoresis / mass spectrometry: Additional Results

Supplementary Figures I and II and supplementary Table II show results of the 2D-DIGE analysis using left ventricular tissue from 12 months old homozygous W4R mutant mice and litter-matched wildtype control hearts (n=5 each). Supplementary Figure I (A) illustrates a greyscale image of a typical gel (first dimension: pH range, 4-7), on which we circled the protein spots for which significantly different expression levels were found by Ludesi software. Supplementary Figure II (B) and (C) show representative CyDye labelled images: in (B) MLP^{+/+} tissue was labeled red and MLP^{W4R/W4R} tissue green, in (C) MLP^{+/+} tissue was labeled green and MLP^{W4R/W4R} tissue red. Supplementary table II is a summary of the results of the 2D gel spot analysis. Note the upregulation of sarcomeric proteins, such as tropomyosin and alpha-actinin, in W4R-MLP mutant hearts. Supplementary Figure II shows the MS/MS spectra of the three 1-peptide-only hits.
Online Figure I: Comparison of 12 months old MLP\textsuperscript{WT/WT} and MLP\textsuperscript{W4R/W4R} mouse heart tissue on 2D DIGE gels. A) Gray scale image; circled are spots with significantly altered abundance (for numbers, see Table Z). B) and C) are typical 2D-DIGE gels on which MLP\textsuperscript{WT/WT} is labeled red and MLP\textsuperscript{W4R/W4R} green (B) or MLP\textsuperscript{WT/WT} green and MLP\textsuperscript{W4R/W4R} red (C). 1\textsuperscript{st} dimension: pH 4-7 IPG strips; 2\textsuperscript{nd} dimension: 10% Bis-Tris SDS-PAGE.
Online Figure II

Protein Name: Lumican, O5=Mus musculus GN=Lum, PE=1 D1=x2
Accession: LUM_MOUSE
Sequence: LPAGLPTSLTLTLDNK

Parent m/z: 972.0287, 2+

[Graph showing mass spectrometry data with peaks labeled with y and b ions, including y2, y3, y4, y5, y6, b4, b5, b6, etc.]
Online Figure II: MS/MS spectra of 1 peptide only hits.
**Online Table II:** Differentially regulated proteins in MLP+/+ versus MLP\(^{W4R/W4R}\) mouse hearts (12 months old) identified by 2D gel spot analysis.

<table>
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<th>Spot</th>
<th>Presence (n=5) [%]</th>
<th>p-Value</th>
<th>Ratio</th>
<th>Swiss-Prot Accession</th>
<th>Protein name</th>
<th>MW [kDa]</th>
<th>pI</th>
<th>Mascot Score</th>
<th># of peptides</th>
<th>SC [%]</th>
<th>p-Value</th>
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**Note:**
- The table lists the mass and mass-to-charge (m/z) values for various proteins and their corresponding accession numbers.
- The intensity values indicate the relative abundance of each mass.
- The table entries are sorted by the m/z values in ascending order.
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**Online Table II:** Ratio: abundance change according to 2D DIGE gel analysis by Ludesi Redfin software; positive value: up-regulation of protein abundance in MLP<sup>W4R/W4R</sup>; negative value: down-regulation of protein abundance in MLP<sup>W4R/W4R</sup>; pI: theoretical isoelectric point; SC [%]: sequence coverage of detected peptides in percent; RT [min]: run time in minutes.
Online Figure III

Online Figure III shows mRNA expression patterns in W4R-MLP (KI) as well as in corresponding wildtype littermate control animals at an age of about 50 weeks for Bnp and Anf (WT:n = 11; KI: n = 9).

Online Figure IV

Online Figure IV shows β-myosin heavy chain expression patterns in W4R-MLP (KI) as well as in corresponding wildtype littermate control animals at an age of about 50 weeks (n=3 per group).
Online Figure V shows the result of a combined analysis of cardiomyocyte cell stretch experiments. Wildtype control and W4R-MLP knock-in cardiomyocytes were isolated, stretched for 2 hours at 0.5 Hz (10% biaxial stretch) and BNP gene expression was analyzed. We combined 4 wildtype (WT, n=4) cardiomyocyte experiments without stretch and 2 wildtype cardiomyocyte experiments with stretch (n=2) as well as 3 W4R-MLP knock-in cardiomyocyte experiments without stretch (n=3) as well as 2 W4R-MLP knock-in cardiomyocyte experiments with stretch (n=2). P = 0.11 for WT without versus WT with stretch and p = 0.14 for W4R-MLP without versus W4R-MLP with stretch. There is a clear tendency for an increased BNP gene expression in wildtype cardiomyocytes, but no (or only a very marginal) increase in BNP gene expression in the W4R-MLP knock-in cardiomyocytes following stretch.
Online Figure VI shows the result of MCIP (modulatory calcineurin interacting protein or RCAN) gene expression (Wildtype = WT: n = 12; W4R-MLP knock-in = KI: n = 10). We did not find any significant changes in the expression of this gene.
Immunohistochemistry – additional results

Online Figure VII

Online Figure VII: Myofiber disarray at the cellular level. Toluidine stained semithin sections showing regular arrangement of myofibers in controls (A) and nonparallel arrangement of cardiac myocytes (myofiber disarray) in KI mice (B).
Online Figure VIII: W4R-MLP nuclear translocation. A, B, Control hearts derived from wildtype littermate control animals show regular MLP Z-disc staining, but W4R-MLP is less present at the sarcomeric Z-disc and translocates into the nuclei.
Skeletal muscle cell stretch experiments

Online Figure IX

Online Figure IX: Confocal images of two single muscle fibers isolated from M. vastus lateralis of a Mlp\textsuperscript{W4R/W4R} KI showing that the same MLP-labelling pattern is obtained at normal sarcomere length (SL 2,4\textmu m) and at long SL of 2,8\textmu m, where the sarcomeric I-band gets wider. This indicates that Z-disc labeling with the MLP antibody (here from P. Caroni) is not due to non-specific accumulation of the antibody in the I-band but rather to specific binding of MLP at the Z-disc. Both fibers were not double stained with anti-\textalpha-actinin to confirm the specificity of MLP localization and to exclude Z-disk labeling through fluorescence cross-talk between red and green channels.

Online Figure X

Online Figure X: Analysis of titin expression patterns in W4R-MLP knock-in and wildtype (WT) soleus muscles. We did not find any significant changes in titin isoform expression patterns. The average titin size of the soleus muscle was 3605±18 kDa in both WT and W4R-knockin muscle.
 References


