MLP and CARP are linked to chronic PKCα signalling in dilated cardiomyopathy

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MLP (muscle LIM protein)-deficient mice count among the first mouse models for dilated cardiomyopathy (DCM), yet the exact role of MLP in cardiac signalling processes is still enigmatic. Elevated PKCα signalling activity is known to be an important contributor to heart failure. Here we show that MLP directly inhibits the activity of PKCα. In end-stage DCM, PKCα is concentrated at the intercalated disc of cardiomyocytes, where it is sequestered by the adaptor protein CARP in a multiprotein complex together with PLCβ1. In mice deficient for both MLP and CARP the chronic PKCα signalling chain at the intercalated disc is broken and they remain healthy. Our results suggest that the main role of MLP in heart lies in the direct inhibition of PKCα and that chronic uninhibited PKCα activity at the intercalated disc in the absence of functional MLP leads to heart failure.
MLP (Muscle LIM protein, encoded by the Csrp3 gene) was initially discovered as a protein up-regulated in skeletal muscle following denervation. It was subsequently shown to be expressed only in the heart and in adult slow-twitch skeletal muscle, and suggested to play a role during muscle differentiation. MLP consists of two LIM domains, structural domains composed of two zinc fingers, which are well known for their role in protein–protein interactions. Among the many binding partners that were described for MLP are the cytoskeletal proteins actin, α-actinin, N-RAP, telethonin (T-cap) and spectrin as well as the skeletal muscle transcription factors MyoD, MRF4 and myogenin. Based on these interactions and the presence of a nuclear localization signal it was proposed that MLP acts as a signalling protein between the myofilaments or the cytoplasm, and the nucleus in myocytes, which is responsive to pharmacological or mechanical stimuli. Pathological mutations in MLP can lead to familial hypertrophic cardiomyopathy (HCM) or dilated cardiomyopathy (DCM). MLP knockout mice count among the first published models for DCM in a genetically manipulated animal. They show all the anatomical and physiological hallmarks of DCM, and present with up-regulated expression levels of classical biomarkers for hypertrophy such as ANF (atrial natriuretic factor), BNP (brain natriuretic peptide) and β-myosin heavy chain as well as stress markers such as CARP (Cardiac-specific ankyrin repeat protein, CARP1/Ankrd1). While MLP knockout mice have been used by many laboratories as a mouse model to investigate DCM, the exact role that MLP plays in myocytes remains unclear. It was proposed that MLP could act as a mechanosensor at the Z-disc transmitting stress signals to the nucleus. Signalling roles of MLP in the heart are well characterized, its function as a mechanosensor is less clear. Considering its molecular structure and subcellular localization, it is not obvious how a 20 kDa protein that exclusively consists of LIM domains can sense changes in mechanical force. Given that LIM domains have well-known protein–protein interaction interfaces, it seems more likely that MLP functions in signal transmission rather than as a direct mechanosensor. Additionally, the exclusive Z-disc localization has been challenged, since several groups have reported a more widespread distribution throughout several subcellular compartments in myocytes both for endogenous and transfected MLP, including the nucleus, plasma membrane, cytoplasm, cytoskeleton and myofibrillar localizations other than the Z-disc.

Over the years numerous rescue models were published using MLP knockout mice including double knockout mice with the SERCA-2A (Sarcoplasmic reticulum Ca²⁺ ATPase) regulator phospholamban and overexpression of calcineurin. Other MLP knockout rescue reports involve inhibition of adrenergic signalling and interference with PKCα (Protein Kinase C) signalling.

Increased PKCα expression and activity are well established in end stage heart failure models in rodents. The phosphorylation substrates for PKCα range from phospholamban to sarcomeric proteins such as troponin and titin, and phosphatases such as PP2A with ensuing effects on calcium handling and contractility. Intrigued by recent propositions that MLP may interact directly with PKCα, and reports that MLP expression is down-regulated in failing mouse and human hearts, we speculated that MLP may directly affect PKCα activity. Our assays reveal that the presence of MLP inhibits autophosphorylation of PKCα as well as phosphorylation of downstream targets such as phospholamban. We demonstrate that in failing hearts PKCα is concentrated at the intercalated discs (ID) (specialized cell–cell contacts in cardiomyocytes) in a complex with the adaptor protein CARP1 and PLCβ1. In double knockout mice for CARP1 and MLP (CMP1), PKCα is no longer detected at the ID and the DCM phenotype does not develop. This is accompanied by normalized PKCα phosphorylation and expression levels. We propose a signalosome complex consisting of muscle ankyrin repeat proteins (MARPs) and PKCα that is regulated by MLP and whose persistent activation may play a role in chronic stress signalling in the failing heart.

**Results**

**PKCα concentrates at the ID in failing hearts.** While an increase in PKCα expression and activity is well established in the failing rodent heart, the situation in humans is less clear. Analysis of PKCα/β expression in heart samples from patients with idiopathic dilated cardiomyopathy (IDCM) by immunoblotting revealed a marked up-regulation of PKCα phosphorylation at Thr638/641 in most patient samples (Fig. 2a). Confocal microscopy on immunostained heart sections from DCM patients showed a remarkable concentration of PKCα at the ID (Fig. 2b, quantification in 2c), which was not as evident in heart samples from NF controls. This supports published data that reported an increased signal for PKCα at the ID in human heart failure. How does PKCα specifically target the ID? CARP1 is a stress marker that is consistently up-regulated in heart failure, and a well-known scaffold protein (for reviews see refs 32,33). Immunoblot data (Fig. 2a) showed increased expression of CARP1, but not the closely related CARP2/Ankrd2 in failing human heart samples. At the cellular level CARP1 is known to localize to the sarcomere and the nucleus, with increased nuclear presence following mechanical strain. We observed no differences in nuclear versus cytoplasmic localization of CARP1 in either normal or DCM hearts (Supplementary Fig. 1c). However, in all functionally compromised hearts that we studied (MLP knockout and human DCM samples), CARP1 translocated from the sarcomere to the ID (Fig. 2d, for quantification see Supplementary Fig. 1d,e), whereas the localization of CARP2 was less affected (Fig. 2d, for quantification see Supplementary Fig. 1e). ID association for both proteins is consistent with a previously reported interaction between CARP1 and phospholipase-C (PLC) in chronic stress signalling in the failing heart.
pull-down assays that the coiled coil region of PLCβ1, the main PLC isoform in hearts, binds to both CARP1 and CARP2 (Fig. 2e). Protein complementation assays revealed that all CARP family members can interact with the coiled coil region of PLCβ1 and that this interaction happens in an antiparallel fashion (Fig. 2f). To investigate whether this complex also occurs in cardiac tissue in situ, we carried out immunoprecipitation experiments on cardiac extracts from MLP knockout hearts. CARP1 is exclusively expressed in cardiomyocytes and cannot be detected in fibroblasts (Supplementary Fig. 1f), therefore confirming that we specifically assayed the cardiomyocyte signalosome. Using CARP1 antibodies, we identified the presence of a signalosome complex in MLP knockout hearts containing CARP1, CARP2, PKCα and PLCβ1 (Fig. 2g). In contrast, these proteins were not pulled down from WT or CARP1 knockout hearts, adding support to our hypothesis that this complex only assembles in DCM/failing hearts. Using recombinant CARP1 or CARP2, we demonstrated that it is also possible to pull-down PLCβ1 and PKCα from MLP knockout hearts (Supplementary Fig. 1g). In left ventricle extracts from a subset of end-stage DCM patients the entire complex could be pulled down (Supplementary Fig. 1h).

### Figure 1: MLP is a substrate and inhibitor of PKCα kinase activity in vitro.

(a) In vitro kinase assay using PKCα and GST tagged phospholamban cytoplasmic N-terminus (GST-PLN(N)) as substrate with increasing GST-MLP or GST amounts. Shown are representative autoradiography images for phosphorylated proteins (upper panel) or Coomassie staining for total proteins (lower panel). Dashed line indicates that proteins were run on the same gel, but imaged using different exposure times. (b) Quantification of GST-PLN(N) phosphorylation or PKCα auto-phosphorylation with increasing GST-MLP or GST concentrations. Mean values with s.e., sample size (n) and P values are shown. (c) In vitro kinase assay using PKCα and GST tagged WT and mutant MLP as substrate. Representative autoradiography images for either Leu44Pro (L44P), Lys69Arg (K69R), Gly72Arg (G72R) mutant MLP (top panel), or Ser54Arg/Glu55Gly (S54R/E55G) or Cys58Gly (C58G; bottom panel) mutant MLP (bottom panel) in comparison with WT MLP are shown. Dashed line indicates proteins were run on the same gel, but non-consecutive. (d) Quantification of MLP phosphorylation by PKCα. Phosphorylation efficiency of WT MLP was arbitrarily set to 1. Mutants identified in human HCM patients show lower MLP phosphorylation, while MLP mutants associated with DCM show increased phosphorylation values. A representative Coomassie stained gel showing total GST-tagged MLP used for kinase assays is depicted (bottom panel; dashed line indicates that proteins were run on the same gel, but non-consecutive.). Shown are mean values and s.e., as well as P values and sample sizes (n, in brackets below bar graphs). (e,f) Analysis of MLP phosphorylation in IDCM. (e) Quantification of MLP phosphorylation indicates significantly elevated phosphorylation levels in IDCM patients, compared to NF controls. Shown are mean values and s.e., as well as sample size (n, in brackets below bar graphs), and P values. (f) MLP phosphorylation levels for quantification in (e) were determined by Phostag analysis. SDS samples of protein extracts of NF and IDCM patient heart samples were run on conventional SDS PAGE (middle) and on 12% polyacrylamide gels with 50 μM Phostag reagent (top) and immunoblotted for MLP. A human control sample with uncharacterized disease status (ctl) showing non-phosphorylated (P0) and phosphorylated (P1) MLP was used to indicate Phostag gel migration pattern. Phosphorylated proteins (P1) migrate slower due to their interaction with the Phostag reagent compared to unphosphorylated protein (P0). GAPDH (bottom) was used to show equal loading.

| Condition   | GST-MLP (μg) | GST (μg) | PKCα (mM) | MLP | GAPDH |
|-------------|--------------|----------|------------|-----|-------|
| Control     | 0            | 0        | 0.5        | 0   | 0     |
| MLP         | 0            | 0.5      | 0.5        | 1   | 0     |
| Phosphorylated | 0      | 0        | 0.5        | 0   | 0     |
These results indicate that CARP1 and CARP2 can sequester PKCz into a complex with PLCβ1. In the failing heart CARP1 is up-regulated and relocates from the sarcomere to the ID. This relocation and the ensuing retention of PKCz at the ID appear to be the crucial step in maladaptive signalling in failing hearts.

**PKC activity affects CARP and MLP expression.** CARP1 is reportedly a target for many protein kinases including PKCz, which we recently demonstrated through *in vitro* kinase assays. Since CARP1 up-regulation and subcellular relocation appear to be crucial in setting up the pathological signalosome complex together with PKCz and PLCβ at the ID, we considered the possibility of an initial crosstalk between PKCz and CARP. Addition of kinase inhibitors to cultures of neonatal mouse cardiomyocytes (NMC) revealed that exposure to PKC inhibitors (bisindolylmaleimide (BI) or calphostin C (Ca)) not only reduced PKCz phosphorylation levels at Thr638/641, but also downregulated CARP1 expression levels (Fig. 3a). No effect was seen with a Map kinase kinase inhibitor (U0126; U0), again supporting the idea that the effect on CARP1 expression levels may be due to PKCz activity (Fig. 3a). The reduction in CARP1 expression following PKC inhibition could also be observed in cardiomyocytes cultured over several days as well as *in vivo*, when MLP knockout mice were peritoneally injected with BI (Fig. 3b,c, Supplementary Fig. 1i). The effect was not limited to CARP1, but also extended to CARP2 expression (Fig. 3b,c), which was significantly down-regulated in hearts of BI treated mice. More intriguingly, however, was the effect we observed on MLP phosphorylation and expression levels. PKC inhibition by BI decreased MLP expression levels, and more importantly MLP phosphorylation in cardiomyocytes (Fig. 3d). These data indicate that MLP is a direct phosphorylation target of PKC, and that this phosphorylation may affect MLP activity and protein levels (Supplementary Fig. 1k). In immunofluorescence-stained heart sections, PKC inhibition shifted CARP1 but not CARP2 localization from a more pronounced ID association to an increased sarcomeric signal (Fig. 3e, for quantification see 3f). Label-free measurements of NMC contractility demonstrated that inhibition of PKCz by BI normalized the higher beating frequencies usually observed in neonatal MLP knockout cardiomyocytes compared to WT controls (Fig. 3g,h).

These results show that elevated PKCz phosphorylation increases CARP expression levels. This is accompanied by the subcellular relocation of both PKCz and CARP1 to the ID. The assembly of a signalosome consisting of CARP, PKCz and PLCβ1 at the ID leads to a stabilization of PKCz signalling activity. Inhibition of PKCz results in down-regulation of CARP expression, changes its subcellular targeting and probably leads to the disassembly of the signalosome. In addition, PKCz inhibition
in WT cardiomyocytes leads to a decrease in MLP phosphorylation and expression.

We propose that acute signalling of this complex may be beneficial for the adaptation of the heart to stress⁴⁰, while its chronic or pathological activation is maladaptive.

**Absent DCM phenotype in MLP CARP1/CARP2 double knockout mice.** If CARP1 is indeed a crucial adaptor required to recruit PKCα stably to the ID, and to trigger pathological signalling in failing hearts, then its removal should prevent the development of heart failure in MLP knockout mice. Genetic ablation of any of the MARP family members in mice (CARP1/Ankrd1/MARP—encoded by the Ankrd1 gene; CARP2/Ankrd2/ARPP encoded by the Ankrd2 gene; CARP3/Ankrd23/DARP—encoded by the Ankrd23 gene) had no effect on heart function at baseline, even in the case of a triple knockout⁴¹. To test our hypothesis, we crossed MLP knockout mice with the three MARP knockout lines and examined their cardiac phenotype. Immunoblot analysis of MLP and MARP expression revealed basal expression of CARP1, but no CARP2 in control hearts (Fig. 4a and Supplementary Fig. 2a,b). Hearts of MLP knockout mice displayed increased expression as well as altered posttranslational modification of CARP1 and induced CARP2 expression (Fig. 4a, Supplementary Fig. 2a–c). CARP3 was not expressed in control hearts and was not induced in MLP knockout hearts (Supplementary Fig. 2a). Histological and functional analyses revealed the previously reported phenotype of MLP knockout hearts (that is, dilation and heart failure⁴²); in contrast double knockout mice for MLP and CARP1 (CMP1), and MLP and CARP2 (CMP2) had normal healthy hearts. Thus, the DCM phenotype does not develop in the absence of CARP1 or CARP2 (Fig. 4b,c, Supplementary Fig. 2d,e, Supplementary Tables 1 and 2). The hearts of double knockout mice for MLP and CARP3 (CMP3) were dilated and exhibited heart failure similar to the single MLP knockout mice (Fig. 4b,c, Supplementary Fig. 2d,e, Supplementary Table 2).
Discussion

We demonstrate here the existence of a multiprotein complex composed of PKC\(\alpha\), PLC\(\beta\), CARP1 and CARP2 at the ID specifically in failing hearts. The retention of PKC\(\alpha\) to this signalsome at the ID may lead to an amplification of signalling. The persistent shift in subcellular localization makes the difference between acute activation of PKC\(\alpha\) that is beneficial for the heart to cope with transient stress, and chronic activation, which leads to detrimental downstream effects on calcium handling and contractile parameters. We propose that the up-regulation in expression and relocation of CARP1 or CARP2 from a sarcomeric localization to the ID is the crucial switch between acute and chronic PKC\(\alpha\) activation (Fig. 5). We further show that MLP can directly inhibit PKC\(\alpha\) activity in vitro, and demonstrate that removal of CARP by genetic means prevents the formation of this maladaptive signalling complex, thereby preventing the morphological, functional and molecular phenotype of DCM in MLP knockout mice.

These results explain at a molecular level, why previous strategies that prevented the development of a DCM phenotype in the MLP knockout mouse were successful\(^{15,19,20}\). Under normal circumstances MLP reduces excessive PKC\(\alpha\) signalling in the heart. In MLP knockout mice this does no longer happen and DCM develops. Direct interference with the maladaptive PKC\(\alpha\) signalling pathway by genetic or pharmacological strategies thus rescues the effect of the lack of MLP. The novel role of MLP as a PKC\(\alpha\) inhibitor could also provide an explanation why overexpression of MLP does not have drastic effects on heart function under basal conditions\(^{44}\). Removal of phospholamban, which was found to rescue the MLP knockout phenotype\(^{15}\), leads to a reduction of free cytosolic calcium known to be required for PKC\(\alpha\) activation.

We also noted that MLP itself is a substrate for PKC\(\alpha\) activity. Indeed, DCM patient samples that show higher PKC\(\alpha\) phosphorylation levels correlated with increased MLP phosphorylation levels compared to NF controls. Moreover we observed that DCM-causing MLP mutants were hyper-phosphorylated by PKC\(\alpha\) in vitro, while MLP mutants that were associated with Supplementary Table 3). CMP3 hearts had elevated expression levels of CARP1 and CARP2 as well as increased PKC\(\alpha\) phosphorylation, suggesting activity of the same maladaptive signalling complex that we propose for MLP knockout mice (Supplementary Fig. 2a,f). The absence of a failing heart phenotype in the CMP1 animals was accompanied by redistribution of CARP2 to the sarcomeres (Fig. 4d, for quantification see Supplementary Fig. 2g). Most importantly PKC\(\alpha\) no longer concentrated at the ID, while the PLC\(\beta\) localization was indistinguishable between genotypes (Fig. 4e, Supplementary Fig. 2h,i). Analysis of PKC\(\alpha\) phosphorylation levels revealed their normalization to control levels in the CMP1 heart compared to the MLP knockout heart (Fig. 4f). This suggests that it is the concentration of PKC\(\alpha\) at the ID that triggers its pathological activity. Previously we characterized pathological changes at the cellular level in murine and human DCM samples such as elevated expression levels of N-RAP at the ID and re-expression of the foetal M-band marker EH-myomesin\(^{3,42,43}\). These changes were no longer seen in CMP1 or CMP2, but were still evident in CMP3 hearts (Supplementary Fig. 3a,b). However, while the lack of CARP1 or CARP2 expression prevents the development of a DCM phenotype, biomarkers for hypertrophy such as ANF, BNP and skeletal actin continued to be elevated at the mRNA level in CMP1 and CMP2 mice (Supplementary Fig. 4a–c). Also, double knockout hearts still showed some evidence of fibrosis (Supplementary Fig. 4d–f), although the amount was markedly reduced compared to MLP knockout mice.

In conclusion, we propose that the existence of a multiprotein complex consisting of CARP, PKC\(\alpha\), and PLC\(\beta\)1 at the ID of failing hearts is crucial for the maintenance of pathological signalling activity of PKC\(\alpha\). MLP can directly inhibit PKC\(\alpha\) activity, and its absence is sufficient to lead to a DCM phenotype over time. Removal of CARP leads to the dissolution of the complex, where PKC\(\alpha\) no longer concentrates at the ID, preventing the DCM phenotype in MLP knockout mice.
the development of HCM were hypo-phosphorylated. Inhibition of PKCζ activity in vitro leads to decreased MLP phosphorylation and expression in cardiomyocytes. The exciting finding that the MLP phosphorylation state can be modulated by PKCζ, correlates with different forms of cardiomyopathy and may affect its protein levels and/or activity again underlines the important role of this protein in cardiac stress signalling. At present it is unknown whether mutations in MLP or its various posttranslational modifications have an effect on oligomerization, which determines the subcellular localization and actin bundling activity of MLP. Phastag blots of neonatal rat cardiomyocytes (NRC) indicate the presence of several distinct MLP phosphorylation sites, one of which was significantly changed upon PKC inhibition. It will be intriguing to investigate which posttranslational modifications of MLP indeed change its cell-biological properties. We propose that these posttranslational modifications of MLP are key to its biological function, and may ultimately determine where, when and how effectively it interferes with PKCζ signalling.

CARP1 has for a long time been associated with mechanosignalling and was shown to be up-regulated in heart failure by several studies. Recently its upregulated expression was shown to correlate directly with heart failure progression in IDCM. So far most studies have proposed that nuclear signalling of CARP via ERK1/2 to molecules such as p53 and GATA4 is crucial for a hypertrophic response. While nuclear signalling of CARP via ERK1/2 to molecules such as p53 and GATA4 is crucial for a hypertrophic response, the mechanism underlying the importance of this protein in cardiac stress signalling is not fully understood. At present it is unknown whether mutations in MLP or its various posttranslational modifications have an effect on oligomerization, which determines the subcellular localization and actin bundling activity of MLP. Phastag blots of neonatal rat cardiomyocytes (NRC) indicate the presence of several distinct MLP phosphorylation sites, one of which was significantly changed upon PKC inhibition. It will be intriguing to investigate which posttranslational modifications of MLP indeed change its cell-biological properties. We propose that these posttranslational modifications of MLP are key to its biological function, and may ultimately determine where, when and how effectively it interferes with PKCζ signalling.

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Our results demonstrate that one of the main differences between a healthy and a failing heart is the presence of PKCζ at the ID. The analysis of PKCζ/β expression levels in human hearts was inconclusive and it seems to be the change in subcellular targeting rather than mere expression levels that makes the crucial difference between a healthy and a failing heart. Why would active PKCζ/β at the ID be so detrimental for the heart? Cell membranes are known to be the major site of PKC function (for review see ref. 52) and the signalosome complex that we describe here appears to be similar to previously described scaffold interactions that were shown to be crucial for PKC regulation. Increased translocation of PKCζ from the cytosolic to the membranous fraction marked the transition from pressure overload induced hypertrophy to congestive heart failure in guinea pigs. In addition, constitutively active PKCζ at the ID may interfere with another level of regulation in the challenged heart. β-adrenergic receptors are known to become insensitive following PKCζ activation and adrenergic signalling is well known to play a role in heart disease. Adrenergic receptors were previously shown to be concentrated at the ID in cardiomyocytes. A failure to respond to fine-tuning by adrenergic signalling as shown by the altered contractile behaviour that we observed in cultured cardiomyocytes from MLP knockout mice, may over time lead to a failing heart phenotype.

Taken together, our results highlight novel roles for MLP and CARP in pathological signalling via their interactions with PKCζ.
This signalosome seems to be crucial for eliciting the maximal maladaptive response in the stressed heart, and CARP may be the proposed missing link.27 Modulation of this complex may offer new therapeutic options to prevent heart failure.

Methods

Bacterial protein expression and in vitro kinase assay. Expression of GST-fusion proteins was done using BL21 cells (C601003; Invitrogen; Life Technologies). Culture (2 L, 30 °C, 150 rpm) of the bacterial strain BL21 (DE3) transformed with the vector (pGEX-2T bacterial expression vector; Amersham Pharmacia39). Generation of constructs with the HA-tag), or pGST-C1 vector (a modified version of the human phospholipase C (human CARP2/Ankrd2 (NM_020349), human CARP3/Ankrd23 (NM_144994), or pGST-PLN(N)) and grown in 400 mL LB-medium supplemented with 50 μg/mL carbenicillin on an orbital shaker at 37 °C to an OD600 of >1. After incubation on ice for 1 min, expression of GST or GST-fusion proteins was induced by addition of 0.2 mM IPTG (isopropyl β-D-thiogalactopyranoside) and production of proteins was allowed to proceed over night at 18 °C. Cells were collected by centrifugation and lysed by 40 mL ice cold lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 8, 1% Triton X100), followed by sonication for 1 min at 4 °C at 70% output (Vibracell, Sonics & Materials Inc., Newtown, CT). Removal of insoluble cell debris was done by centrifugation (45 min at 11,000 r.p.m., 4 °C; Sorvall) and the supernatant was incubated with 400 μl glutathionese-phore proteins (Pharmacia) for 2 h at 4 °C with agitation. After washing of beads with ice cold PBS for four times, bound protein was eluted with GST-elution buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 150 mM reduced glutathione) and dialyzed overnight at 4 °C against 500 mL HEPES pH 7.4, 10 mM MgO4, 0.1 mM CaCl2. Measurement of protein concentration was done using the Bradford assay (BioRad) or through densitometry of Coomassie stained SDS-PAGE gels. Proteins were frozen in liquid nitrogen and stored at −80 °C until further use in in vitro kinase assays or for GST pull-down assays.

For in vitro kinase assays 2 μg of GST-PLN(N) or GST-MLP (WT or mutant) were combined with purified PKCζ (approximately 10 ng/reaction, P0329, Sigma-Aldrich) in kinase buffer (20 mM HEPES KOH pH7.4, 10 mM MgSO4, 0.1 mM CaCl2) supplemented with either radioactively labelled [gamma-32P]ATP (Amersham Biosciences) or unlabelled ATP (20 mM) and increasing amounts (0.25 μg to 1 μg) of either GST-MLP or GST. The reaction mixture was supplemented with a combination of 1 μg/ml phosphatidylserine (1,2-diacyl-sn-glycerol-3-phospho-s-erine) and 2 μg/ml diacylglycerol (DAG; 1,2-Dioleoyl-sn-glycerol) dissolved in resuspension buffer (10 mM HEPES pH 7.4, 0.3% Triton X-100). Following incubation at 30 °C for 30 min, samples were mixed with SDS-sample buffer (2X) and 2 μg phosphatidylserine (1,2-diacyl-sn-glycerol-3-phospho-s-erine) were used for further analysis via Phostag gel analysis58 after determination of protein concentrations. Protein samples with normalized concentrations were supplemented with SDS sample buffer, boiled for 5 min, and either run on a 12% polyacrylamide SDS-PAGE, or a 12% polyacrylamide SDS-PAGE supplemented with 50 μM Manganese Phostag (WAKO Chemicals). After end of electrophoresis, gels were washed once in immunoblot transfer buffer supplemented with 10 mM EDTA for 10 min, followed by a wash in immunoblot transfer buffer for another 10 min and prepared for immunoblot transfer and subsequent detection and analysis following standard procedures.

Human tissues. Human specimens were obtained from the Sydney Heart Bank at the University of Sydney and signed patient consent was obtained for all samples in this tissue bank. All failing samples were collected in the heart transplant theatres at St Vincent’s Hospital in patients in end-stage heart failure and were frozen in liquid nitrogen within 15–20 min of the loss of coronary artery flow (cross-clamp). Human Research Ethics Committee approval was obtained by St Vincent’s Hospital (#H03/118), and by the University of Sydney Human Research Ethics Committee (#12146, #159401). Human tissue was used in accordance with the ethical guidelines of King’s College London (College Research Ethical Committee 04/05–74; REC reference 12/EM/0106), under current UK law. For protein, histological and immunofluorescence analysis, samples from the left ventricular free walls of patients in end-stage heart failure with familial (FDCM) or IDCM were used. NF donor hearts, which were cardioplegic, but not required for heart transplantation, were provided by the Australian Red Cross Blood Transfusion Service. Typically these hearts were not transplanted because of tissue incompatibility.

Generation of constructs. Bacterial and eukaryotic expression constructs for human MLP (HSU49837), the cytoplasmic domain of mouse phospholamban (PLN(N), amino acids 1–34; NM_023129), human CARP1/Ankrd1 (NM_014391), human CARP2/Ankrd2 (NM_020349), human CARP3/Ankrd23 (NM_144994), human phospholamban Cβ coiled-coil domains (NM_015192, amino acids 980–1140) into the protein complementation vectors (split-YFP) YN-C1, YC-C1 or YC-N1 (ref. 39). This effectively generates either N-terminally tagged fusion proteins with the N-terminal (YN) or C-terminal (YC) half of YFP, or C-terminally tagged fusion proteins with the C-terminal half of YFP. All constructs were verifie for in-frame integration and correct sequence by sequencing.

Protein expression analysis. Total protein extracts of hearts for immunoblot or biochemical analysis were generated by homogenizing ventricular samples either directly into SDS-sample buffer (BioRad) or ice-cold IP-buffer (150 mM NaCl, Tris-HCl pH 8, 1 mM DTT, 1 × Complete Protease Inhibitor EDTA-free (Roche), 1 × PhosSTOP (Roche), 0.2% NP-40, 0.2% SDS) by using a polytron blade homogenizer (Pro Scientific Inc). Total proteins from NMC, neonatal cardiac fibroblasts, transfected COS cells or extracts were used with PBS at room temperature and solubilization of cells directly into SDS-sample buffer or ice-cold IP-buffer using a cell scraper. Protein extracts were immediately transferred to Eppendorf tubes and stored on ice for immediate use or snap-frozen into liquid nitrogen and stored at −20 °C or −80 °C. Protein samples were normalized by densitometry of Coomassie stained SDS-PAGE.

Normalised total protein extracts were run on uniform 15% acrylamide, 10% acrylamide, or 4–20% acrylamide gradient SDS-PAGE gels (BioRad, Invitrogen), followed by immunoblotting on nitrocellulose membranes (BioRad) using wet blot technology. The nitrocellulose membranes were stained with Ponceau Red, blocked with 5% non fat dry milk or 0.4% Bovine serum (Sigma-Aldrich) in Low Salt Buffer (0.9% NaCl, 9 mM Tris pH 7.4, 0.1% Tween-20) and sequential incubation with the appropriate primary and secondary antibodies with intermittent washing in Low Salt Buffer was performed. Results from the chemiluminescence reaction were visualized on Fuji medical X-ray films. For gel analysis, protein samples were diluted 1:1 into IEF sample buffer (Life Technologies), and first dimension was run on IEF pH3–10 gels (Life Technologies), followed by 12% acrylamide SDS-PAGE according to the manufacturer’s instructions.

Phostag gels. For analysis of MLP phosphorylation levels, 20 mg of frozen cardiac tissue samples were homogenized into 200 μl freshly prepared ice-cold TBL lysis buffer (20 mM Tris HCL pH 7.6, 138 mM NaCl, 5% glycerol, 1% Triton, 5 mM DTT, 0.2% sodium ortho-oxalate, Mini Protease inhibitor tablet (no EDTA; Roche), Phos-stop tablet (Roche)). Samples were briefly sonicated, incubated on ice for 20 min, centrifuged at 4 °C at 14,000 r.p.m. for 15 min, and supernatants were used for further analysis via Phostag gel analysis58 after determination of protein concentrations. Protein samples with normalized concentrations were supplemented with SDS sample buffer, boiled for 5 min, and either run on a 12% polyacrylamide SDS-PAGE, or a 12% polyacrylamide SDS-PAGE supplemented with 50 μM Manganese Phostag (WAKO Chemicals). After end of electrophoresis, gels were washed once in immunoblot transfer buffer supplemented with 10 mM EDTA for 10 min, followed by a wash in immunoblot transfer buffer for another 10 min and prepared for immunoblot transfer and subsequent detection and analysis following standard procedures.

Co-immunoprecipitation and GST pull-down assays. For biochemical protein–protein interaction assays, protein extracts in IP-buffer were sonicated for 1 min at 30% output on ice (Vibracell, Sonics & Materials Inc.), followed by centrifugation at 14,000 r.p.m. (4 °C) for 10 min to separate insoluble proteins. Soluble proteins were either incubated with 1 μg of primary antibody or control serum for 2 h, or incubated with 2 μg of GST or GST-fusion protein at 4 °C. Following immuno-complex formation in co-immunoprecipitation assays, protein extracts were incubated with protein G-linked magnetic beads (Dynabeads; Invitrogen) for an additional hour at 4 °C with agitation. For GST pull-down assays, protein extracts were incubated with glutathione-sepharose 4B resin (Pharmacia) for an additional 2 h at 4 °C with agitation. After incubation, beads were washed three times with ice-cold PBS, resuspended in sample buffer (BioRad), and analysed by SDS PAGE, followed by immunoblotting on nitrocellulose membranes24.

Animals. All procedures were reviewed and approved by the Animal Care and Use Committee at the University of California San Diego. MLP and MRP knockout mice as well as Gz (q) overexpression mice were generated.21 All animals were in a mixed sv129/black swiss background. For physiological experiments only male mice were used, samples used for immunohistology and immunoblotting were from both genders. Unless stated otherwise, animals in the age range between 4 and 8 months were used for the experiments. Oligonucleotides used for geneticotyping via PCR analysis of tail DNA can be found in Supplementary Table 4. The mice were fed ad libitum with a standard diet and maintained in a temperature and light-controlled room (22 °C, 14 h light/10 h dark). Treatment of MLP knockout animals with PKC inhibitor was done similar to a previously described method.26 In short, 4-months old male MLP mice were injected subcutaneous with a single dose of either PKC inhibitor BI I-HCl (BI; sc-24004, Santa Cruz Biotechnology, 1 μg −1 body weight) or DMSO (vehicle). Ventricular This page is a continuation of the previous page.
heart tissue of mice was dissected after 24 h following the injection and snap frozen in liquid nitrogen for further analysis, or processed for immunofluorescence analysis.

The protocol for animal handling and treatment procedures was in accordance with the guidelines of the Laboratory Animal Services at the University of California in Davis and guidelines presented in the National Research Council's (NRC) 'Guide for Care and Use of Laboratory Animals' published by the Institute for Laboratory Animal Research of the National Academy of Science, Bethesda, MD, 2011.

For echocardiography analysis 2-, 4-, 6- and 12-month-old male control, MLP, MARP and MLP-MARP double knockout mice were analysed. For protein, mRNA, histological, immunofluorescence and morphological analysis, the left ventricular free wall of hearts from 3–9-month-old adult male and female mice were used. Investigation of gross cardiac morphology by histology used whole hearts that included ventricles and atria.

**Transthoracic echocardiography.** Adult male mice at 2, 4, 6 and 12 months of age were analysed as previously described. Briefly, mice were anesthetized with 1% isoflurane and cardiac function was measured with a Philips Sonos 5500 machine (Philips Medical Systems, Andover, MA) equipped with a 15-MHz transducer. M-mode tracings of semi-conscious mice were recorded and analysed for left ventricular posterior wall and inter-ventricular septal thickness, as well as left ventricular chamber dimensions (LVID) at both end systole and end diastole. Heart contractility, shown as fractional shortening (%) was calculated as previously described.

**Antibodies.** The following primary antibodies were used for biochemical analysis of protein and phosphorylation levels (dilutions given in brackets are for immunoblotting, the antibodies were used 10 times more concentrated for immunohistology): GAPDH (clone 6C5; Santa Cruz Biotechnology; 1:1000), cardiac Actin (clone A1C-20-4.2; Progen Biotechnik; 1:2000), collagen-1 (ab34710, Abcam; 1:1000), smooth-muscle actin (clone 1A4, DAKO: 1:1000); CD-5 (550274, BD Pharmingen; 1:5000), α-smooth-muscle actin (clone EA53-A5E3, A7811, Sigma-Aldrich; 1:5000; PLCβ (sc-205, Santa Cruz Biotechnology; 1:1000), PKCz (sc-208, Santa Cruz Biotechnology; #2056, Cell Signalling; 1:1000), m-PPCK (T638/641 9375, Cell Signalling; 1:1000), plakoglobin/α-catenin (XAB20802, Sigma; 1:1000), sarcomytic myosin-heavy chain (A41025, Developmental Studies Hybridoma Bank, University of Iowa; 1:1000), PDGF-Receptor α (AF1062, R&D; 1:1000), GFAP-tag (1181446001 Roche; 1:1000).

Normal rabbit IgG was from Santa Cruz Biotechnology (sc-2027), while normal mouse IgG was from development studies hybridoma bank, University of Iowa; 1:100), PDGF-Receptor α (AF1062, R&D; 1:1000), GFAP-tag (1181446001 Roche; 1:1000).

Normal rabbit IgG was from Santa Cruz Biotechnology (sc-2027), while normal mouse IgG was from development studies hybridoma bank, University of Iowa; 1:100), PDGF-Receptor α (AF1062, R&D; 1:1000), GFAP-tag (1181446001 Roche; 1:1000).

Quantitative real-time PCR analysis (qPCR).** Ventricular samples were homogenized directly into Trizol reagent (Invitrogen) to extract total RNA according to the manufacturer's instructions. First strand cDNA was generated from 2 μg of total mRNA using random hexamers and Superscript II reverse transcriptase. Oligonucleotides optimized for qPCR (Supplementary Table 5) of murine ANF, BNP, skeletal actin, CARP1 and GAPDH were used in reactions employing the PerfeCTa SYBR green real-time PCR mix (Quanta Biosciences) and a CFX96 thermocycler (BioRad). Samples were normalized to GAPDH. If not stated otherwise, three biological replicates were analysed per sample group.

**Cell culture.** The isolation of NMC and NRC was performed using collagenase/ dispase digestion, respectively. Hearts from neonatal rats and mice were initially dissected in PBS containing 20 mM BDM (2,3-butanedione monoxime; Sigma) and minced. Subsequently they were subjected to digestion with collagenase/dispase (Roche; 150U in 10 ml medium with 20 mM BDM) for 30–20 min at 37 °C with gentle agitation. Alternatively, cardiomyocytes were isolated using the Neonatal Cardiomyocyte isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. Individual cells were released by trituration with a 10 ml tissue culture pipette and undigested tissue pieces were removed using a cell strainer (Falcon). After centrifugation at 80 g, the pellet of isolated cells was resuspended in Plating Medium (68% DMEM, 17% Medium M199, 10% horse serum, 5% foetal calf serum, 4 mM glutamine, 1% penicillin/streptomycin, 0.1 mM phenylthiourea, 1.4 mM NaHCO₃, 2 mM HEPES). The next day, the cells were switched to Maintenance Medium (20% Medium M199, 75% DBSS-K, 4% horse serum, 4 mM glutamine, 1% penicillin/streptomycin, 0.1 mM phenylthiourea, 1.4 mM NaHCO₃, 2 mM HEPES). 1 μM DMSO was then added in the absence of DBSS-K to allow for the propagation of cells.

**Conventional microscopy.** Fluorescently stained samples were imaged using an Olympus Fluoview 1000 confocal microscope equipped with 40 × oil immersion lens, or a LEICA TCS-SP5 confocal microscope equipped with a 63 × glycerol immersion objective in sequential scanning mode and zoom rates between one and three. Histology samples were processed by a SPOT camera and imaging software, using a Nikon epifluorescence microscope equipped with a 5× objective in bright-field mode.

**Image processing and statistical analysis.** Images were processed using ImageJ (NIH) equipped with the LOCI bio-formats plugin and Photoshop (Adobe). Statistical analysis was used using Excel (Microsoft). Data presented are mean ± s.e. Significance was evaluated by the two-tailed student’s t-test. Sample size (n-values) and P values are indicated in the figures and/or figure legends. For analysis of PKC localization over ID (Fig. 2e, Supplementary Fig. 2h), profile plots were measured over aligned ID using the ImageJ plugin ‘RGB profile plot’. Plot values (PKC fluorescence intensity and plakoglobin fluorescence intensity) were exported into Excel. The plakoglobin profile was used to establish the precise localization of the ID. The corresponding PKC intensity (F) at the ID (for example) and the background PKC intensity (Fo) was used to calculate the background corrected profile PKCz plots (F–Fo). Profile plots were centred at the ID, and the average profile plot and s.e. were calculated for each group, and used to generate the figure. Data presented in Fig. 2e and Supplementary Fig. 2h are mean values ± s.e. Significance was evaluated by the two-tailed student’s t-test over fluorescence intensity values (F) at the ID. Sample size (n-values) and P values are indicated in the figures and/or figure legends.

For Phostag profile plot analysis (Fig. 3d), MLP bands were analysed using ImageJ and the ‘Plot Profile’ tool. Data were exported to Excel, aligned and normalized, and the resulting average plot profile for each group, including s.e. were used to generate the figure. Significance was evaluated by student’s t-test analysis.

For analysis of CARP1, CARP2 and α-Actinin fluorescence intensity ratios (I1/I2) between the ID and the sarcomere (S), identical-sized boxes enclosing a portion of the ID, and an adjacent sarcomere were selected, and RGB values were measured in ImageJ using ‘image’ as described previously. Briefly, images were used to determine the background corrected fluorescence intensities, individual and average intensity ratios as well as s.e. and significance was calculated in Excel. Data presented in Fig. 3f, Supplementary...
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Author contributions
S.L. guided the project and carried out most experiments; K.G. supplied MLP expression constructs and antibodies and contributed to the Phostag experiments; A.S.L. and C.H. helped with experiments; J.C., N.D.D., E.A.A., X.Z. and M.-L.B. generated and characterized the CARP knockout mice; Y.A. helped with analysis of contractile behaviour; C.G.d.R. processed and supplied the human heart tissue; K.L.P. and J.C. supervised experiments; S.L., K.G. and C.G.d.R. revised the manuscript; E.E. conceived the project, carried out some experiments and wrote the manuscript.

Additional information
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