Towards targeted therapies for phospholamban cardiomyopathy

Eijgenraam, Tim

DOI:
10.33612/diss.243703870

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2022

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Eijgenraam, T. (2022). Towards targeted therapies for phospholamban cardiomyopathy: data from a new preclinical model. University of Groningen. https://doi.org/10.33612/diss.243703870

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The phospholamban p.(Arg14del) pathogenic variant leads to cardiomyopathy with heart failure and is unresponsive to standard heart failure therapy

Tim R. Eijgenraam¹, Bastiaan J. Boukens²³, Cornelis J. Boogerd⁴, E. Marloes Schouten¹, Cees W. A. van de Kolk⁵⁶, Nienke M. Stege¹, Wouter P. te Rijdt⁷, Edgar T. Hoornije⁸⁹, Paul A. van der Zwaag², Eva van Rooij⁴, J. Peter van Tintelen⁹, Maarten P. van den Berg¹, Peter van der Meer¹, Jolanda van der Velden¹⁰, Herman H. W. Silljé¹ and Rudolf A. de Boer¹

¹Department of Cardiology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands. ²Department of Medical Biology, Amsterdam University Medical Center, University of Amsterdam, Amsterdam, the Netherlands. ³Department of Experimental Cardiology, Amsterdam University Medical Center, University of Amsterdam, Amsterdam, the Netherlands. ⁴Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences (KNAW), University Medical Center Utrecht, Utrecht, the Netherlands. ⁵Central Animal Facility, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands. ⁶Groningen Small Animal Imaging Facility, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands. ⁷Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands. ⁸Department of Genetics, University Medical Center Utrecht, University of Utrecht, Utrecht, the Netherlands. ⁹Department of Physiology, Amsterdam University Medical Center, University of Amsterdam, Amsterdam Cardiovascular Sciences, Amsterdam, the Netherlands.

Scientific Reports 2020 Jun;10:9819.
ABSTRACT

Phospholamban (PLN) plays a role in cardiomyocyte calcium handling as primary inhibitor of sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). The p.(Arg14del) pathogenic variant in the PLN gene results in a high risk of developing dilated or arrhythmogenic cardiomyopathy with heart failure (HF). There is no established treatment other than standard HF therapy or heart transplantation. In this study, we generated a novel mouse model with the PLN-R14del pathogenic variant, performed detailed phenotyping, and tested the efficacy of established HF therapies eplerenone or metoprolol. Heterozygous PLN-R14del mice demonstrated increased susceptibility to \textit{ex vivo} induced arrhythmias, and cardiomyopathy at 18 months of age, which was not accelerated by isoproterenol infusion. Homozygous PLN-R14del mice exhibited an accelerated phenotype including cardiac dilatation, contractile dysfunction, decreased ECG potentials, high susceptibility to \textit{ex vivo} induced arrhythmias, myocardial fibrosis, PLN protein aggregation, and early mortality. Neither eplerenone nor metoprolol administration improved cardiac function or survival. In conclusion, our novel PLN-R14del mouse model exhibits most features of human disease. Administration of standard HF therapy did not rescue the phenotype, underscoring the need for better understanding of the pathophysiology of PLN-R14del-associated cardiomyopathy. This model provides a great opportunity to study the pathophysiology, and to screen for potential therapeutic treatments.
INTRODUCTION

Phospholamban (encoded by the PLN gene) is a 52-amino acid protein that is present in the sarcoplasmic reticulum (SR) membrane. PLN plays a crucial role in cardiomyocyte calcium handling by acting as a primary regulator of the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), which transports calcium from the cytosol into the SR. In its dephosphorylated state, PLN lowers the affinity of SERCA for Ca\(^{2+}\), thereby inhibiting calcium uptake. Phosphorylation of PLN at serine 16 by protein kinase A (PKA) or threonine 17 by Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) relieves PLN-mediated inhibition of SERCA, thereby increasing SERCA activity and subsequent uptake of calcium. The PLN-SERCA interaction is essential for contraction and relaxation of the heart, and is under the regulation of the β-adrenergic receptor pathway to adapt cardiac output to physiological needs.

Several variants in the PLN gene have been described in heart failure (HF). The c.40_42delAGA pathogenic variant, a heterozygous deletion of arginine 14 (p.(Arg14del)) of the PLN protein, was originally described in a Greek family in 2006. Since then, this pathogenic variant has been identified in the USA, Canada, China, Germany, Spain and the Netherlands. Interestingly, this pathogenic variant was described as a founder mutation in the Netherlands, and was identified in ~14% of Dutch patients with dilated cardiomyopathy (DCM) or arrhythmogenic right ventricular cardiomyopathy (ARVC), which translates into thousands of carriers. PLN-R14del carriers have a high risk of developing malignant ventricular arrhythmias (VAs) and HF, and are often diagnosed with DCM or ARVC, which, given the presence of biventricular abnormalities, is better referred to as arrhythmogenic cardiomyopathy (ACM). The phenotype is typically characterized by ECG abnormalities, including low QRS potentials and inverted T waves in precordial leads, myocardial fibrosis and fibrofatty replacement, and, ultimately, severe biventricular dysfunction and HF. The severity of PLN-R14del-associated cardiomyopathy is evidenced by mutation carriers having higher incidences of malignant arrhythmias, premature sudden cardiac death (SCD) and cardiac transplantation, as compared to DCM and ARVC patients that do not carry this pathogenic variant.

To date, there is no specific therapeutic treatment for PLN-R14del-related cardiomyopathy, and thus the current guidelines for HF, VAs and SCD are applied, although cut-offs for recommendation of implantable cardioverter defibrillator (ICD) implantation are more lenient, given the malignant phenotype. Clearly, there is an urgent need to evaluate if treatment could slow down or even reverse the severe phenotype. In 2021 we expect the results of the PHOSpholamban RElated CArdiomyopathy STudy - Intervention (i-PHORECAST, www.clinicalTrials.gov identifier NCT01857856). As myocardial fibrosis is considered to be an early disease manifestation in this cardiomyopathy, the i-PHORECAST study aims to test the efficacy of the mineralocorticoid receptor antagonist (MRA) eplerenone, which has been shown to exert anti-fibrotic effects, in reducing disease progression or postponing onset of overt disease in asymptomatic mutation carriers.
Studies in human mutation carriers are laborious, expensive and take years before results of a single treatment may be evaluated. Therefore, we developed a novel mouse model of the PLN-R14del pathogenic variant. In this study, we demonstrate that this mouse model accurately resembles the phenotype of human patients, and is unresponsive to standard HF therapies eplerenone and metoprolol.

**METHODS**

Detailed descriptions of the methods are available in the Supplementary Information.

**Animals**

All animal experiments were approved by the animal ethical committee of the University of Groningen (permit numbers AVD10500201583, IVD1583-02-001, IVD1583-02-004, IVD1583-02-009 and IVD1583-05-002), and were performed conform the existing guidelines for the care and use of laboratory animals. To generate mice with the PLN-R14del pathogenic variant, a C57Bl6/N mouse line was generated, in which the third exon of the murine \( \text{Pln} \) gene, which contains the coding region for the PLN protein, was flanked by loxP sites (floxed) and followed by a third exon of the murine \( \text{Pln} \) gene with the c.40_42delAGA pathogenic variant (performed by PolyGene, Switzerland). To delete the floxed region, these mice were bred with mice expressing the Cre enzyme in the germline under the control of the hypoxanthine-guanine phosphoribosyltransferase (\( \text{Hprt} \)) promoter enhancer, replacing the murine wild-type (WT) \( \text{Pln} \) exon-3 with the murine R14del \( \text{Pln} \) exon-3 in the resulting offspring (Figure 1A).

**Sanger sequencing**

Total RNA was isolated from cardiac tissues using TRI Reagent (Sigma-Aldrich, MO, USA), and cDNA synthesis was performed using QuantiTect reverse transcription kit (Qiagen, Germany) as previously described.\(^{16}\) Total genomic DNA was isolated from cardiac tissues using the DNeasy Blood & Tissue kit (Qiagen). DNA fragments for Sanger sequencing were generated by PCR of cDNA and genomic DNA using Taq DNA Polymerase (Roche Diagnostics, Switzerland). PCR products were purified using agarose gel electrophoresis. Fragments of the correct length were excised and isolated using the QIAquick Gel Extraction kit (Qiagen). Sanger sequencing was performed by GATC-Biotech (Germany).

**RNA-seq analysis**

Total RNA was isolated from left ventricle (LV) tissues using TRI Reagent (Invitrogen, CA, USA). RNA quality was determined using RNA Pico Chips on a 2100 Bioanalyzer system (Agilent, CA, USA), and TruSeq Stranded mRNA libraries (Illumina, CA, USA) were generated from high quality (RIN > 8.0) total RNA. Samples were subjected to single-end sequencing using a NextSeq 500 sequencer (Illumina). Reads were aligned to mouse reference genome (mm10) using spliced transcript alignment to a reference (STAR, version 2.4.2a),\(^{17}\) and readcount analysis was performed using the HTSeq package (version 0.6.1) in Python.\(^{18}\) Differential expression analysis was performed using the DESeq2 package (version 1.24.0) in R.\(^{19}\) Gene
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set enrichment analysis was performed using fgsea package (version 1.10.0) in R\textsuperscript{20} with the MSigDB database (version 6.2).\textsuperscript{21}

**Cardiac magnetic resonance imaging**
Cardiac MRI measurements were performed using an AVANCE 400 MR system (Bruker BioSpin, Germany) as previously described.\textsuperscript{22} Volumetric analyses were performed using cvi42 software (version 5.6.6, Circle Cardiovascular Imaging, Canada).

**Echocardiography**
Echocardiographic measurements were performed using a Vevo 3100 preclinical imaging system, equipped with a 40-MHz MX550D linear array transducer (FUJIFILM VisualSonics, Canada). General techniques were reported before.\textsuperscript{23} Vevo LAB software (version 3.1.1, FUJIFILM VisualSonics) was used to assess cardiac morphology and function.

**Surface electrocardiography**
ECG recordings were acquired using two-lead subdermal needle electrodes, connected to a PowerLab 8/30 data acquisition device (model ML870, ADInstruments, Australia) and an animal Bio Amp biological potential amplifier (model ML136, ADInstruments) as previously reported.\textsuperscript{24} Analysis was performed using LabChart Pro software (version 8, ADInstruments).

**Implantable ECG telemetry**
Mice were subcutaneously implanted with telemeter transmitters (ETA-F10, Data Sciences International, MA, USA), and the electrode leads were subcutaneously secured in a lead II configuration as reported elsewhere.\textsuperscript{25} Continuous ECG recordings in conscious mice were acquired for 3 weeks, and analysed using Ponemah software (version 6.41, Data Sciences International).

**Isoproterenol infusion**
Following initial phenotyping, adult (10-weeks-old) PLN-R14\textsuperscript{Δ/Δ} mice and their WT littermates were randomly subjected to infusion of isoproterenol (30 mg/kg/day for 4 weeks, I6504, Sigma-Aldrich) using subcutaneously implanted ALZET osmotic mini pumps (model 2004, DURECT Corporation, CA, USA) or sham surgery as previously described.\textsuperscript{26}

**Eplerenone and metoprolol administration**
Following initial phenotyping, PLN-R14\textsuperscript{Δ/Δ} mice were randomly subjected to treatment with eplerenone or metoprolol or vehicle. Eplerenone (Inspra, 200 mg/kg/day, Pfizer, NY, USA) was mixed with the chow as described elsewhere.\textsuperscript{15} Metoprolol (350 mg/kg/day, M5391, Sigma-Aldrich) was dissolved in the drinking water as reported elsewhere.\textsuperscript{27} Drug administration was initiated at weaning (3 weeks of age) when cardiac abnormalities were still absent in PLN-R14\textsuperscript{Δ/Δ} mice (Supplementary Figure S1), and was continued until the endpoint was reached.
Chapter 3

**Sacrifice**

PLN-R14Δ/Δ mice and WT controls were sacrificed at the age of 20 months. PLN-R14Δ/Δ mice were sacrificed when they reached the endpoint. The endpoint is determined by presence of lethargy, dyspnoea and severe weight loss due to HF. During survival monitoring, it was observed that this occurred between 7 and 9 weeks of age. Euthanasia was performed as previously reported.28

**Ex vivo optical action potential recording and electrical stimulation**

Hearts were excised, cannulated, and perfused using a Langendorff setup as described elsewhere.29 *Ex vivo* ECGs were recorded (Biosemi, the Netherlands) and analysed using LabChart Pro software (version 8, ADInstruments). Optical action potentials were recorded with a CMOS camera (MiCAM05, SciMedia, CA, USA) using voltage-sensitive dye RH237 (Invitrogen). Conduction velocity was calculated at basic stimulation interval of 120 msec using dF/dt_{max} as local moment of activation. Arrhythmias were induced by decreasing basic stimulation interval with steps of 5 msec for a period of 20 sec until arrhythmias occurred or the ventricle failed to capture.

**Histological analysis**

Formalin-fixed cardiac transverse mid-slices were dehydrated, embedded in paraffin (Klinipath, the Netherlands), and cut into 4-μm thick sections. Masson’s trichrome stain was performed to detect collagen deposition as a measurement of fibrosis as previously described.30 Immunofluorescent staining was performed using an anti-PLN antibody (#MA3-922, Invitrogen) labelled with Alexa Fluor 555 (red) using an APEX antibody labelling kit (Invitrogen). Sections were co-stained with fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA, Sigma-Aldrich) to stain extracellular matrix green, and DAPI (Vector Laboratories, CA, USA) to stain nuclei blue as previously reported.31

**Quantitative PCR**

Total RNA was isolated from tissues using TRI Reagent (Sigma-Aldrich), and cDNA synthesis was performed using the QuantiTect RT kit (Qiagen). Gene expression levels were determined by qPCR analysis using iQ SYBR green supermix (Bio-Rad, CA, USA) as previously described.32 Gene expression was quantified by correcting for reference gene values of ribosomal protein lateral stalk subunit P0 (Rplp0, encoding 36B4) using CFX Manager software (version 3.0, Bio-Rad), and the calculated values were expressed relative to the control group per experiment. Primer sequences can be found in Supplementary Table S1.

**Western blot**

Total protein was isolated from LV tissue using RIPA lysis buffer as previously described.23 To obtain soluble and insoluble fractions, protein samples were centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was collected and was considered the soluble fraction. The remaining pellet was dissolved in urea solution (8 M) and was considered the insoluble fraction. Protein concentrations were determined using a Pierce bicinechonic acid (BCA) protein assay kit.
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(Thermo Scientific, MA, USA) according to the manufacturer’s protocol. Equal amounts of protein (5 μg) were denatured, separated by gel electrophoresis using Novex 10-20% Tricine Protein Gels (Invitrogen), and transferred onto Immun-Blot polyvinylidene fluoride (PVDF) membranes (Bio-Rad). After overnight incubation at 4°C with a primary antibody, membranes were incubated with an appropriate horseradish peroxide (HRP)-linked secondary antibody, and detection was performed using Western Lightning Ultra enhanced chemiluminescence (ECL, PerkinElmer, MA, USA) and an ImageQuant LAS 4000 digital imaging system (GE Healthcare, IL, USA). Antibodies that were used are described in Supplementary Tables S2 and S3.

Statistical analysis
All data are presented as means ± standard errors of the mean (S.E.M.). For statistical analysis of survival curves, a log-rank test was performed. Arrhythmia incidence was tested using a χ² test. For data with parametric distribution according to Shapiro-Wilk test for normality, and homogeneity of variance according to Levene’s test for homogeneity of variances, a two-tailed Student’s t-test was performed for comparisons between two groups, while for multi-group comparisons a one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was performed. For data without parametric distribution or homogeneity of variance, a Mann-Whitney U test was performed. We considered small sample sizes (n < 10) insufficient to test for parametric distribution or homogeneity of variance. A P-value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS Statistics software (version 23, IBM, NY, USA).

RESULTS
PLN-R14Δ/Δ mice exhibit heart failure and premature mortality
We generated mice carrying the PLN-R14del pathogenic variant by introducing an additional Pln exon-3 containing the R14del pathogenic variant, followed by Cre-loxP-mediated recombination to replace the WT Pln exon-3 with the mutant Pln exon-3 (Figure 1A), resulting in offspring carrying one PLN-R14del allele. The offspring of subsequent breeding of PLN-R14del mice was born in expected Mendelian ratios. Presence of the PLN-R14del pathogenic variant was confirmed by Sanger sequencing of LV genomic DNA. Furthermore, expression of the WT and/or mutant allele in the LV of WT, heterozygous (R14Δ/+), and homozygous (R14Δ/Δ) mutant mice was confirmed by Sanger sequencing of LV cDNA (Figure 1B). RNA-seq demonstrated that all groups had similar total levels of LV Pln expression (Supplementary Figure S1A). Expression of mutant Pln in PLN-R14Δ/Δ mice was similar to expression of the WT Pln gene in WT mice, and PLN-R14Δ/+ mice had equal expression levels of both WT and mutant Pln alleles (Supplementary Figure S1A), indicating that the mutant allele is not degraded by nonsense-mediated decay. Mice were monitored for up to 20 months of age (Figure 1C). Survival of PLN-R14Δ/+ mice was normal until this age. In contrast, PLN-R14Δ/Δ mice demonstrated significantly (p < 0.0001) decreased survival compared to WT littermates with a maximum life span of 2 months (n = 13, 54-61 days).
The early mortality of PLN-R14^{Δ/Δ} mice within 2 months, prompted us to determine cardiac function at the age of 6 weeks. LV end-diastolic and end-systolic volumes were increased, and stroke volume and ejection fraction were decreased in PLN-R14^{Δ/Δ} mice as compared to WT controls, indicating ventricular dilatation and contractile dysfunction (Figure 1D-H). At this age, no cardiac structural and functional abnormalities were observed upon MRI in PLN-R14^{Δ/+} mice.

**PLN-R14^{Δ/+} mice demonstrate severe cardiac remodelling**

To evaluate the effects of the PLN-R14del pathogenic variant on cardiac gene expression levels, RNA-seq was performed on LV tissue of 3- and 8-weeks-old WT, PLN-R14^{Δ/+} and PLN-R14^{Δ/Δ} mice. Principal component analysis revealed 3 groups with distinct gene expression profiles (Figure 2A). Most notably, 8-weeks-old PLN-R14^{Δ/Δ} mice clustered and segregated from other samples on the first principal component (Figure 2A, PC1). In contrast, at 3 weeks of age, PLN-R14^{Δ/+} mice clustered together with WT controls. This is in line with cardiac functional and histological analysis at this age, which showed no differences between PLN-R14 Δ/Δ mice and WT controls (Supplementary Figure S2A-E). Functional annotation of genes contributing to variance in PC1 (Figure 2A x-axis, accounting for 78% of variance across samples) indicated a strong increase in fibrosis-related gene expression in 8-weeks-old PLN-R14 Δ/Δ mice hearts (Supplementary Figure S1B). Furthermore, PLN-R14^{Δ/+} mice clustered together with WT mice at 8 weeks of age, which showed a more mature cardiac profile compared to 3-weeks-old hearts (Figure 2A, PC2) (e.g. elevated expression of genes encoding contractile (sarcomeric) proteins, and genes encoding enzymes for β-oxidation), whereas PLN-R14^{Δ/Δ} mice showed a less mature profile, including the upregulation of foetal genes in response to cardiac dysfunction (Figure 2A and Supplementary Figure S1B). Analysis of LV gene expression by qPCR confirmed elevated expression of atrial and brain natriuretic peptide genes, Nppa (ANP) and Nppb (BNP), respectively, and an increase in the ratio of beta (Myh7) to alpha (Myh6) myosin heavy chain (MHC) gene expression in 8-weeks-old PLN-R14^{Δ/Δ} mice, indicative for activation of the cardiac foetal gene program (Figure 2B).
Figure 1. Generation, validation, survival and cardiac MRI of PLN-R14del mutant mice. (A) Schematic overview of the genomic DNA encoding the murine Pln gene, and the modifications that were performed to replace the WT Pln exon-3 with the R14del Pln exon-3 (marked with 3*). (B) Fluorescent peak trace chromatograms of Sanger sequencing reactions including the coding region of the Pln gene in hearts of WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice. The codon for the 14th amino acid of the PLN protein is outlined by the grey rectangle. (C) Survival curve of male and female WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice (n = 6, 10 and 13, respectively). (D) Representative cardiac MRI images at the mid-papillary level in end-diastole and end-systole (scale bar: 1 mm) with quantification of LV end-diastolic volume (E), end-systolic volume (F), stroke volume (G) and ejection fraction (H) of 6-weeks-old WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice (n = 4, 5, and 5, respectively). Data are presented as means ± S.E.M. *p < 0.05 compared to WT (Mann-Whitney test).
Figure 2. Histological and molecular analysis of PLN-R14del mice hearts. (A) Principal component analysis plot of RNA-seq analysis of LVs of WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice of 3 or 8 weeks of age (n = 4 per group). 95% confidence ellipses of clusters are marked in grey. Information on genes contributing to variance in principal component 1 (PC1, x-axis) and principal component 2 (PC2, y-axis) is shown in Supplementary Figure 1B. (B) qPCR measurements of LV mRNA levels of Nppa (ANP), Nppb (BNP), and the ratio of Myh7 (β-MHC) to Myh6 (α-MHC) of 8-weeks-old WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice (n = 4, 5, and 5, respectively). (C) Representative images of LV sections stained with Masson’s trichrome (scale bar: 70 μm) with (D) quantification of myocardial fibrosis in WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice hearts (n = 4, 5, and 5, respectively). (E,F) qPCR measurements of LV mRNA levels of cardiac remodelling genes Col1a1, Col1a2, Col3a1, Lgals3 (Gal-3), Timp1, and Mmp2 of 8-weeks-old WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice (n = 4, 5, and 5, respectively). (G) Representative images of LV sections of 8-weeks-old WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice stained with an anti-PLN antibody (red), wheat-germ agglutinin (WGA) (green), and DAPI (blue) (scale bar: 35 μm). (H) Western blot analysis of monomeric PLN proteins in RIPA-soluble and RIPA-insoluble fractions of LVs of 8-weeks-old WT and PLN-R14Δ/Δ mice (n = 1 and 2, respectively). Images zoom in on the protein bands. Full blot images are presented in Supplementary Figure S3. Gene expression values are corrected for Rplp0 (36B4) gene expression, and shown as fold change compared to age-matched WT. Myocardial fibrosis is presented as fold change compared to age-matched WT. Data are presented as means ± S.E.M. *p < 0.05 compared to WT (Mann-Whitney test).
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Histological analysis of cardiac tissue at 8 weeks of age using Masson’s trichrome staining showed no difference in fibrosis between WT and PLN-R14Δ/Δ mice, whereas in PLN-R14Δ/Δ mice extensive myocardial fibrosis was present throughout the LV and right ventricle (RV) (Figure 2C,D). Elevated LV expression of fibrotic genes in PLN-R14Δ/Δ mice was confirmed by qPCR analysis of collagens (Col1a1, Col1a2, Col3a1), galectin-3 (Lgals3), tissue inhibitor of metalloproteinase-1 (Timp1), and matrix metalloproteinase-2 (Mmp2)33 (Figure 2E,F). Notably, at 3 weeks of age, no differences in myocardial fibrosis were identified between WT and PLN-R14Δ/Δ mice (Supplementary Figure S2E,F). Because histology of cardiac tissues of PLN-R14del patients not only revealed extensive scarring, but also aggregation of PLN protein, we also performed immunofluorescent staining of PLN in mouse cardiac sections. This revealed extensive aggregation of PLN proteins in all PLN-R14Δ/Δ mice at 8 weeks of age (Figure 2G). Quantification showed that 74.8 ± 2.7% (n = 4) of the cardiomyocytes of PLN-R14Δ/Δ hearts contained aggregates, whereas no such aggregation was present in WT (n = 3) and PLN-R14Δ/+ (n = 5) mice. Western blot of LV lysates confirmed the presence of RIPA-insoluble PLN proteins in PLN-R14Δ/Δ hearts (Figure 2H). As expected, the soluble housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was almost exclusively present in the RIPA-soluble fraction. Presence of low amounts of GAPDH in the RIPA-insoluble fraction is likely explained by contamination with the RIPA-soluble supernatant. At 3 weeks of age, PLN proteins were still mostly soluble in the PLN-R14Δ/Δ mice hearts (Supplementary Figure S2H).

**PLN-R14Δ/+ mice develop cardiomyopathy at a later age**

As indicated above, PLN-R14Δ/+ mice did not show any cardiac abnormalities at 8 weeks of age. However, long-term follow up demonstrated that in PLN-R14Δ/+ mice, although LV diameter was comparable to age-matched WT controls (Figure 3A,B), fractional shortening and global longitudinal strain were significantly (p < 0.05) impaired after 18 months of age (Figure 3C,D). At the age of 20 months, mice were sacrificed, and, in line with the echocardiographic findings, LVs of PLN-R14Δ/+ mice demonstrated higher gene expression levels of the cardiac foetal gene program (Figure 3E). In addition, cardiac sections of PLN-R14Δ/+ mice showed PLN protein aggregation in some of the cardiomyocytes (on average 7.3 ± 0.9 aggregates/mm² (n = 10)), whereas this was not observed in age-matched WT hearts (n = 6) (Figure 3F). Furthermore, there was a significant (p < 0.01) increase of myocardial fibrosis in cardiac sections of PLN-R14Δ/+ mice (Figure 3G,H). Consistently, fibrotic gene expression levels were elevated in the LVs of PLN-R14Δ/+ mice (Figure 3I,J).

Since PLN-R14Δ/+ mice developed a more subtle phenotype than PLN-R14Δ/Δ mice and at a later age, we investigated whether stimulation of the β-adrenergic pathway, in which PLN plays a role, using isoproterenol infusion, could accelerate disease onset in PLN-R14Δ/+ mice. Expectedly, isoproterenol infusion increased heart rate, and resulted in cardiac hypertrophy (20% increased heart weight) and cardiac fibrosis (4.5-fold) compared to non-stimulated mice, but there was no difference between PLN-R14Δ/+ and WT stimulated groups (Supplementary Figure S5).
Figure 3. Cardiac functional, histological and molecular analysis of 12- to 20-months-old PLN-R14Δ/Δ mice. Echocardiographic analysis of LV end-diastolic diameter (A), end-systolic diameter (B), fractional shortening (C) and global longitudinal strain (D) of 12-, 18- and 20-months-old WT and PLN-R14Δ/Δ mice (n = 9, 10, 6, 10, 5 and 9, respectively). (E) qPCR measurements of LV mRNA levels of Nppa (ANP), Nppb (BNP), and the ratio of Myh7 (β-MHC) to Myh6 (α-MHC) of 20-months-old WT and PLN-R14Δ/Δ mice (n = 6 and 10, respectively). (F) Representative images of LV sections of 20-months-old WT and PLN-R14Δ/Δ mice stained with an anti-PLN antibody (red), wheat-germ agglutinin (WGA) (green), and DAPI (blue) (scale bar: 35 μm) or (G) stained with Masson's trichrome (scale bar: 70 μm) with (H) quantification of myocardial fibrosis (fold change).
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fibrosis (n = 6 and 9, respectively). (I,J) qPCR measurements of LV mRNA levels of cardiac remodelling genes Col1α1, Col1α2, Col3α1, Lgals3 (Gal-3), Timp1, and Mmp2 of 20-months-old WT and PLN-R14Δ/+ mice (n = 6 and 10, respectively). Gene expression values are corrected for Rplp0 (36B4) gene expression, and shown as fold change compared to age-matched WT. Myocardial fibrosis is presented as fold change compared to age-matched WT. Data are presented as means ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001 compared to age-matched WT (Mann-Whitney test).

PLN-R14del mice are prone to develop ventricular arrhythmias ex vivo
Besides the DCM phenotype, the development of VAs is another key feature of PLN-R14del cardiomyopathy in human patients. ECG recordings of 6-weeks-old animals did not reveal any differences in heart rate between the different groups, nor in PR- and QT-interval and QRS-duration. However, QRS-amplitude was significantly (p < 0.05) diminished in PLN-R14Δ/Δ mice compared to WT controls (Figure 4A,B). This is also considered as one of the first signs of cardiac disease in human mutation carriers. We did not detect VAs in these mice during continuous in vivo monitoring with a telemetry device in the last month prior to the endpoint (presence of symptoms of severe HF). Since mice are in general very resilient to VAs, we tested whether arrhythmias could be induced using ex vivo programmed electrical stimulation during Langendorff perfusion. In isolated hearts, conduction velocity measured during central stimulation was lower in PLN-R14Δ/+ (3 months of age) and PLN-R14Δ/Δ (6 weeks of age) mice as compared to age-matched WT controls, which were combined into a single control group since there were no differences between 6-weeks-old and 3-months-old WT mice (Figure 4C,D). Action potential duration was not different. The incidence of induced VAs was higher in PLN-R14Δ/+ (2/4, p = 0.056) and PLN-R14Δ/Δ (5/6, p < 0.05) mice hearts than in WT controls (1/7) (Figure 4E-G). Thus, under conditions of ex vivo burst pacing both PLN-R14Δ/+ and PLN-R14Δ/Δ mice show a much higher propensity for VAs.

PLN-R14Δ/Δ mice are unresponsive to standard heart failure therapy
PLN-R14Δ/Δ mice showed similar cardiac characteristics as human patients, and in a timeframe that provides opportunities for therapeutic testing. We therefore investigated whether administration of the MRA eplerenone, which has been shown to inhibit cardiac fibrosis, or the β1-adrenergic receptor blocker metoprolol would attenuate disease progression. Drug administration was initiated at weaning when PLN-R14Δ/Δ mice were 3 weeks of age, and cardiac abnormalities were still absent (Supplementary Figure S2), and continued until the endpoint (presence of symptoms of severe HF) was reached. Similar to the initial phenotyping, in vivo cardiac analysis was performed at the age of 6 weeks. Efficacy of eplerenone administration was confirmed by increased kidney Ren (renin) gene expression compared to untreated PLN-R14Δ/Δ mice, reflecting a reported feedback mechanism (Supplementary Figure S6A). As expected, metoprolol significantly (p < 0.05) decreased heart rate (Supplementary Figure S6B). Neither eplerenone nor metoprolol administration increased the survival of PLN-R14Δ/Δ mice (Figure 5A). Furthermore, although eplerenone reduced ventricular dilatation, neither treatment prevented contractile dysfunction or cardiac remodelling (Figure 5B-F).
Figure 4. Electrophysiological characterization of PLN-R14del mice hearts. (A) Representative averaged views of 1-minute in vivo ECG measurements of 6-weeks-old WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice with (B) quantification of total QRS complex (peak-to-peak) amplitude (n = 4, 5, and 5, respectively). (C) Reconstructed activation maps during ex vivo LV stimulation (120 msec interval) with (D) quantification of longitudinal conduction velocity in WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice hearts (n = 6, 4, and 5, respectively). (E) Incidence of ex vivo induced VAs in WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice hearts (n = 7, 4, and 7, respectively). (F) Example of a pseudo-ECG (upper) with simultaneously recorded optical action potentials (OAPs) (middle), and reconstructed activation maps (lower) from a WT mouse, which indicates the order of activation from several beats that correspond to the traces above (indicated by the small letter). (G) Pseudo-ECG showing an induced arrhythmia in a PLN-R14Δ/Δ mouse heart following burst pacing. Data are presented as means ± S.E.M. *p < 0.05 compared to WT (Mann-Whitney test).
Figure 5. Effect of administration of eplerenone or metoprolol on survival and cardiac function of PLN-R14ΔΔ mice. (A) Survival curve of WT and PLN-R14ΔΔ mice without or with eplerenone (EPLE, 200 mg/kg/day) or metoprolol (METO, 350 mg/kg/day) administration (n = 11, 12, 12 and 10, respectively). (B-E) Echocardiographic analysis of LV end-diastolic and end-systolic diameter, fractional shortening and global longitudinal strain of 6-weeks-old WT and PLN-R14ΔΔ mice without or with eplerenone or metoprolol administration (n = 11, 12, 12 and 10, respectively). (F) Quantification of myocardial fibrosis in Masson's trichrome-stained LV sections of 16-weeks-old WT and 8-weeks-old PLN-R14ΔΔ mice without or with eplerenone or metoprolol administration (n = 11, 12, 12 and 10, respectively). Myocardial fibrosis is presented as fold change compared to age-matched WT. Data are presented as means ± S.E.M. *p < 0.05, ****p < 0.0001 compared to WT; *p < 0.05, **p < 0.01 compared to R14ΔΔ + VEH (one-way ANOVA followed by Tukey’s post hoc test).
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DISCUSSION

We demonstrated that the newly generated PLN-R14del mouse model closely mimics the human phenotype of PLN-R14del-related cardiomyopathy. We observed a delayed onset of cardiomyopathy in PLN-R14\(\Delta/\Delta\) mice, including impaired cardiac contractile function after 18 months of age, increased myocardial fibrosis, and the presence of PLN protein aggregation. This is consistent with the observations in human mutation carriers, which most often present with HF symptoms at middle age.\(^9\) Furthermore, the hearts of PLN-R14\(\Delta/\Delta\) mice were more susceptible to develop induced arrhythmias \textit{ex vivo}, even at an early age when other cardiac abnormalities were absent. In contrast to the DCM phenotype that is often found in human patients, PLN-R14\(\Delta/\Delta\) mice exhibited no ventricular dilatation. As dilatation may be secondary to contractile dysfunction, cardiac dilatation may occur at a later stage. However, longer follow up would be needed to confirm this.

Cohort screenings have reported to identify the PLN-R14del pathogenic variant in up to 1:200 of included patients, which translates to thousands of mutation carriers.\(^{35,36}\) However, so far only 1,000 carriers have been identified, and clinical heterogeneity has been observed, suggesting that additional factors may contribute to the disease onset and severity. In this study, we demonstrated that stimulation of the \(\beta\)-adrenergic pathway, in which PLN plays a role, could not accelerate disease onset in PLN-R14\(\Delta/\Delta\) mice, suggesting that \(\beta\)-adrenergic stimulation may not influence the disease. Investigating the effect of different disease modifiers in PLN-R14\(\Delta/\Delta\) mice could identify risk factors for this type of cardiomyopathy.

In addition to the findings in PLN-R14\(\Delta/\Delta\) mice, we observed that PLN-R14\(\Delta/\Delta\) mice exhibited the same cardiac phenotype as human cardiomyopathy patients that carry this pathogenic variant, but in an accelerated manner. Indeed, until now, mutations carriers have been found to be heterozygous for this pathogenic variant.\(^3,4\) We speculate that the presence of only the PLN-R14del protein, in the absence of the PLN-WT protein, accelerates the detrimental effects of the pathogenic variant (as seen in PLN-R14\(\Delta/\Delta\) mice), whereas in PLN-R14\(\Delta/\Delta\) mice, where equal amounts of PLN-WT and PLN-R14del are present, the disease onset is at a later age. It has been reported that cardiomyocytes independently transcribe both alleles of a gene, and that individual cells might favour expression of one allele over the other.\(^{37}\) Possibly, the expression level or accumulation of the PLN-R14del protein has to exceed a certain threshold to trigger cardiomyopathy. Thus, even in heterozygous individuals, not only SERCA superinhibition may occur but also loss of inhibition and PLN-R14del aggregation. This could contribute to the clinical heterogeneity that is observed in mutation carriers, and may also explain why in cardiac tissues of patients a mosaic pattern of cardiomyocytes with and without PLN aggregation is observed. The latter is also true for our PLN-R14\(\Delta/\Delta\) mice.

As there is no specific therapy for PLN-R14del-related cardiomyopathy, current HF guidelines, which recommend treatment with angiotensin-converting enzyme (ACE) inhibitors, \(\beta\)-blockers and MRAs or a combination, are applied for treatment.\(^{12}\) Since the accelerated phenotype of
PLN-R14^{Δ/Δ} mice allows for rapid therapeutic drug screening, we tested whether PLN-R14^{Δ/Δ} mice would benefit from standard HF therapy by administering eplerenone or metoprolol, which have been shown to have therapeutic effects in heart failure.\textsuperscript{15,38} Drug administration was initiated at an early age when cardiac abnormalities were still absent in PLN-R14^{Δ/Δ} mice to examine whether these drugs would attenuate disease development. Eplerenone administration resulted in decreased LV volume, most likely related to its diuretic effect and subsequent unloading of the heart. However, neither drug could prevent disease development or increase survival. The finding that inhibition of the β-adrenergic pathway does not prevent disease progression in PLN-R14^{Δ/Δ} mice is in line with the finding that stimulation of this pathway does not accelerate the phenotype of PLN-R14^{Δ/+} mice. These results show that common HF drugs could not attenuate the progression towards end-stage HF in this accelerated model. In humans, fortunately, such fast-forward presentations are rare, but in some patients, deterioration is very fast, which cannot be rescued by common guideline recommended drugs, in line with our findings. On the other hand, many patients present with more slowly progressive disease, and common HF drugs may attenuate progression. Our results do not exclude such a long-term beneficial effect.

Previously, Haghighi et al.\textsuperscript{3} have generated a heterozygous PLN-R14del mouse model with cardiac-specific overexpression of the PLN-R14del protein under control of the α-MHC promoter in the presence of endogenous PLN-WT. Overexpression of PLN-R14del resulted in superinhibition of SERCA, associated with cardiac hypertrophy, myocardial fibrosis, and premature death between 2 and 16 weeks of age. Later, the same group has also overexpressed PLN-R14del in a PLN-KO background (analogous to homozygous PLN-R14del expression).\textsuperscript{39} Although this model did not exhibit increased mortality, it exerted a similar cardiac phenotype at 3 months of age. However, PLN-R14del alone, in the absence of PLN-WT, was unable to inhibit SERCA, and was found to inhibit the Na⁺/K⁺-ATPase at the plasma membrane, implicating mechanistic differences. In contrast, our novel mouse model was generated by editing the murine genome to endogenously express PLN-R14del. We believe that this more accurately resembles the situation of human disease and provides normal endogenous levels of PLN-WT and PLN-R14del (which were present in equal amounts in PLN-R14^{Δ/+} mice hearts) rather than the classical method of (over)expressing PLN-R14del on top of endogenous PLN-WT. Accordingly, the cardiac phenotype of our model, which represents DCM with inducible arrhythmias, has specific aspects that are closer to the human disease phenotype than the models of Haghighi et al., which mostly revolve around cardiac hypertrophy, but lacked cardiac functional and electrophysiological data.\textsuperscript{3,39} Additionally, similar to human patients, we demonstrate PLN protein aggregates in the cardiomyocytes of this model, while this has not been demonstrated in other models. Finally, we have investigated the propensity towards VAs, which is an important part of the disease. Electrophysiological properties of the murine heart are different from the human, and spontaneous VAs are rare in mice, but we show that both PLN-R14^{Δ/+} and PLN-R14^{Δ/Δ} mice hearts were prone to develop VAs upon electrical stimulation. Although we did not study the relation between altered conduction and other electrophysiological differences in detail, the observed inducibility of VAs was...
also present at an early age and in the absence of extensive myocardial fibrosis, a known substrate for conduction irregularities,\(^4^0\) which is suggestive of aberrant calcium handling. As mentioned above, Haghighi et al. reported mechanistic differences between mice with heterozygous and homozygous PLN-R14del overexpression.\(^3^,^3^9\) However, we clearly observed a “dose-dependent” effect with regards to the amount of mutant PLN, i.e. the phenotype of PLN-R14\(^{\Delta/\Delta}\) mice was far more severe than in PLN-R14\(^{\Delta/+}\) mice, suggesting that the more mutant PLN is present, the more severe phenotype develops. Likely, this difference is the result of the different methods that were used to generate the mouse models.

te Rijdt et al.\(^1^0\) demonstrated that the PLN protein aggregates colocalized with autophagy markers p62 and microtubule-associated protein 1A/1B-light chain (LC) 3, suggesting that the protein quality control (PQC) system, the cell’s endogenous system to control correct protein folding, is unable to resolve the protein aggregates. Aggregation of PLN proteins in the cardiomyocytes and insufficiency of the PQC system to correct this, could play a causal role in the pathophysiology of PLN-R14del-related cardiomyopathy. Aggregation of PLN proteins likely affects the effect of PLN on SERCA. It is well known that protein aggregation is involved in several neurodegenerative diseases, such as Alzheimer’s, Parkinson’s, and Huntington’s disease,\(^4^1\) and it is becoming increasingly recognized that impairment in protein homeostasis and protein aggregation also play a role in HF, including hypertrophic,\(^4^2\) dilated,\(^4^3\) and desmin-related cardiomyopathy (desminopathy),\(^4^4\) ischemic\(^4^5\) and diabetic\(^4^6\) heart disease, and HF with preserved ejection fraction (HFpEF).\(^4^7\) Future experiments should define the contribution of protein aggregation and disturbed calcium handling to the development of PLN-R14del-related cardiomyopathy. Further, enhancement of the PQC system has been reported to be beneficial in multiple models of HF,\(^4^8^-^5^0\) and boosting the PQC system may be explored as a novel therapeutic approach.

In conclusion, we have generated a novel mouse model carrying the PLN-R14del pathogenic variant, and PLN-R14\(^{\Delta/\Delta}\) mice mimic human disease in a strikingly comparable but accelerated manner, whereas PLN-R14\(^{\Delta/+}\) mice exhibit cardiomyopathy at middle age similar to human carriers (Graphical abstract). Administration of standard HF therapy does not rescue the phenotype, underscoring the need for better understanding of the pathophysiology of PLN-R14del-related cardiomyopathy and PLN-targeted therapy. This model will be useful for a better understanding of cardiomyopathies that are primarily caused by PLN pathogenic variants or with secondary PLN abnormalities, and for screening of secondary disease modifiers and potential therapeutic treatments.
Graphical abstract. PLN-R14Δ/Δ mice (left) mimic human disease in a strikingly comparable but accelerated manner, evidenced by cardiac dilatation, contractile dysfunction, decreased ECG potentials, high susceptibility to ex vivo induced arrhythmias, cardiac fibrosis, PLN protein aggregation, and early mortality. Administration of standard HF therapy could not rescue the phenotype. PLN-R14Δ/+ mice (right) demonstrated increased susceptibility to ex vivo induced arrhythmias, and developed cardiomyopathy with comparable characteristics at middle age, similar to human carriers. The phenotype was not accelerated by β-adrenergic stimulation.

ACKNOWLEDGEMENTS

The authors thank Martin M. Dokter and Noa Keijzer (UMCG) for their excellent technical assistance. The authors thank the Utrecht Sequencing Facility for providing sequencing service and data.

FUNDING

This work was supported by the Netherlands CardioVascular Research Initiative, an initiative with support of the Dutch Heart Foundation (grants 2014-40 [CVON DOSIS], 2015-12 [CVON eDETECT], 2017-12 [CVON SHE-PREDICTS-HF] and 2017-11 [CVON RED-CVD]), the Cure PhosphoLambaN induced cardiomyopathy (CURE-PLaN) initiative of the Leducq Foundation, the de Boer Foundation, the Ubbo Emmius Fund and the PLN Foundation. Dr Boukens was supported by the Dutch Heart Foundation (grant 2016T047). Dr Boogerd was supported by the European Union’s Horizon 2020 research and innovation program (Marie Skłodowska-
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Curie grant 751988). Dr te Rijdt was supported by the Young Talent Program of the Dutch Heart Foundation (grant 2017T001, CVON PREDICT) and a Postdoctoral Fellowship of the Leducq Foundation (CURE-PLaN). Dr de Boer received support from the Innovational Research Incentives Scheme program of the Dutch Research Council (NWO VIDI grant 917.13.350) and a European Research Council Consolidator Grant (ERC CoG 818715, SECRETE-HF). The Utrecht Sequencing Facility is funded by the University Medical Center Utrecht, the Hubrecht Institute, the University of Utrecht, and the Netherlands X-omics Initiative (NWO project 184.034.019).

CONFLICTS OF INTEREST

The University Medical Center Groningen, which employs the majority of the authors, has received research grants and/or fees from Abbott, AstraZeneca, Bristol-Myers Squibb, Novartis, Novo Nordisk and Roche. Dr de Boer is a minority shareholder of scPharmaceuticals, and received personal fees from Abbott, AstraZeneca, MandalMed Inc. and Novartis. The other authors have nothing to disclose.
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Supplementary Figure S1. RNA-seq analysis of LVs of PLN-R14del mice. (A) Readcounts of the *Pln* R14 region in transcripts of LVs of WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice (n = 4 per group). Data are presented as means ± S.E.M. (B) Heatmap of examples of genes contributing to the principal components of the PCA of 3- and 8-weeks-old WT, PLN-R14Δ/+ and PLN-R14Δ/Δ mice shown in Figure 2A.
Supplementary Figure S2. Cardiac functional, histological and molecular analysis of 3-weeks-old PLN-R14Δ/Δ mice. Echocardiographic analysis of LV end-diastolic diameter (A), end-systolic diameter (B), fractional shortening (C) and global longitudinal strain (D) of 3-weeks-old WT and PLN-R14Δ/Δ mice (n = 10 and 11, respectively). (E) Representative images of LV sections of 3-weeks-old WT and PLN-R14Δ/Δ mice stained with Masson's trichrome (scale bar: 70 μm) with (F) quantification of myocardial fibrosis (n = 6 per genotype). (G) qPCR measurements of LV mRNA levels of Nppa (ANP) of 3-weeks-old WT and PLN-R14Δ/Δ mice (n = 6 per genotype). (H) Western blot analysis of monomeric PLN proteins in RIPA-soluble and RIPA-insoluble fractions of LVs of 3-weeks-old WT and PLN-R14Δ/Δ mice (n = 2 and 3, respectively). Images zoom in on the protein bands. Full blot images are presented in Supplementary Figure S4. Gene expression values are corrected for Rplp0 (36B4) gene expression, and shown as fold change compared to WT. Myocardial fibrosis is presented as fold change compared to age-matched WT. Data are presented as means ± S.E.M.
Supplementary Figure S3. Images of the full Western blot that is shown in Figure 2H. (A) White-light epi-illumination image of the full membrane to visualize the dye-stained molecular weight markers. (B) Chemiluminescence image of the full membrane to visualize PLN proteins. (C) White-light epi-illumination image of the membrane to visualize the dye-stained molecular weight markers after low-weight (<15 kDa) and high-weight (>50 kDa) proteins were cut off. (D) Chemiluminescence image of the cut-out part of the membrane to visualize housekeeping protein GAPDH.
Supplementary Figure S4. Images of the full Western blot that is shown in Supplementary Figure 2H. (A) White-light epi-illumination image to visualize the dye-stained molecular weight markers high-weight (>50 kDa) proteins were cut off. (B) Chemiluminescence image of the membrane to visualize PLN proteins. (C) White-light epi-illumination image of the membrane to visualize the dye-stained molecular weight markers after low-weight (<15 kDa) proteins were cut off. (D) Chemiluminescence image of the cut-out part of the membrane to visualize housekeeping protein GAPDH.
Supplementary Figure S5. The effect of isoproterenol infusion on hearts of PLN-R14 Δ/+ mice. Effect of 4 weeks of isoproterenol (ISO, 30 mg/kg/day) infusion on heart rate measured with surface ECG (A), and heart weight, corrected for tibia length (B) of WT and PLN-R14 Δ/+ mice (n = 10, 11, 12 and 11, respectively). (C, D) Echocardiographic analysis of fractional shortening and global longitudinal strain of WT and PLN-R14 Δ/+ mice without or with isoproterenol infusion (n = 10, 11, 12 and 11, respectively). (E) Quantification of myocardial fibrosis in Masson's trichrome-stained LV sections of WT and PLN-R14 Δ/+ mice without or with isoproterenol infusion (n = 10, 11, 12 and 11, respectively). Myocardial fibrosis is presented as fold change compared to WT. Data are presented as means ± S.E.M. ***p < 0.001 compared to Sham with the same genotype (one-way ANOVA followed by Tukey’s post hoc test).
Supplementary Figure S6. Effect of administration of eplerenone (EPL, 200 mg/kg/day) or metoprolol (METO, 350 mg/kg/day) on kidney mRNA levels of Ren (renin), a regulator of blood pressure, measured by qPCR (A) and heart rate measured with surface ECG (B) of 16-weeks-old WT and 8-weeks-old PLN-R14ΔΔ mice (n = 11, 12, 12 and 10, respectively). Gene expression values are corrected for Rplp0 (36B4) gene expression, and shown as fold change compared to age-matched WT. Data are presented as means ± S.E.M. *p < 0.05 compared to WT, *p < 0.05, ***p < 0.001 compared to R14ΔΔ + VEH (one-way ANOVA followed by Tukey’s post hoc test).
SUPPLEMENTARY METHODS

Animals
Mice were housed per nest, unless individual housing was required (e.g. in case of in vivo telemetry measurements) on a 12 h light / 12 h dark cycle with ad libitum access to chow and water. Surgery, cardiac analyses and euthanasia were performed under continuous anaesthesia of 2-3% isoflurane (TEVA Pharmachemie, the Netherlands) mixed with oxygen, administered via an aerial dispenser. Heart and respiration rates and body temperature were continuously monitored throughout the procedures. Surgical procedures were preceded by a subcutaneous injection of 5.0 mg/kg carprofen (Rimadyl) for analgesic purposes. Male mice have been used throughout the study unless stated otherwise.

Genotyping
For genotyping of PLN-R14del mice, DNA was isolated from ear cuts using the prepGEM Universal kit (ZyGEM, New-Zealand) following manufacturer’s instructions for DNA isolation from solid tissue. Briefly, ear cuts were incubated with ORANGE+ buffer (ZyGEM), Histosolv (ZyGEM) and prepGEM (ZyGEM) at 52°C for 5 min, 75°C for 10 min and 95°C for 3 min using a T100 thermal cycler (Bio-Rad, CA, USA).

To identify genotypes, qPCR analysis was performed using iQ SYBR green supermix (Bio-Rad) according to the manufacturer’s instructions. Briefly, DNA isolated from ear cuts was mixed 1:50 with 1.5 mM of forward (5’-ACCCAGGACAGTGAGAC-3’) and reverse (5’-GCTTTGCAGCAGCTCGTTC-3’) primers and iQ SYBR green supermix (Bio-Rad). The qPCR reaction was performed at 95°C for 5 min, 35 cycles of 95°C for 30 sec, 55°C for 15 sec and 69°C for 30 sec, followed by a melt curve from 81°C to 86°C with increments of 0.2°C every 5 sec using a CFX384 Touch real-time PCR detection system (Bio-Rad). Since after Cre-loxP recombination one loxP site (consisting of 117 base pairs, including the 34 base pairs of the loxP site) remains present in the R14del Pln allele (Figure 1A), presence of the WT and/or mutated allele is identified based on the size of the qPCR product (107 base pairs for the WT allele, 224 base pairs for the mutated allele) using CFX Manager software (version 3.0, Bio-Rad), which can be distinguished based on the temperature of the melt peak.

Sanger sequencing
Total RNA was isolated from cardiac tissue using TRI Reagent (Sigma-Aldrich, MO, USA) according to the manufacturer’s protocol. Briefly, snap-frozen LV tissues were mechanically disrupted, and approximately 25 mg of powdered LV tissue was homogenized in 1 ml of TRI Reagent (Sigma-Aldrich) using a TissueLyser LT (Qiagen, Germany) at 50 Hz for 5 min. After incubation for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes, phases were separated by thoroughly mixing with 0.2 ml chloroform (Merck Millipore, MA, USA), incubation for 2 min at room temperature, and centrifugation at 12,000 g for 15 min at 4°C. The RNA-containing colourless upper aqueous phase was isolated, and RNA was precipitated by mixing with 0.5 ml 2-propanol (Biosolve Chemicals, France) and
incubation for 10 min at room temperature, followed by centrifugation at 12,000 \( g \) for 10 min at 4°C. The supernatant was discarded, and the RNA pellet was washed twice by mixing with 1 ml 75% ethanol (Merck Millipore) and centrifugation at 12,000 \( g \) for 5 min at 4°C. The supernatant was removed, and the RNA pellet was air-dried before dissolving in RNase-free water. RNA concentrations were determined by spectrophotometry using a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA).

Next, cDNA synthesis was performed using QuantiTect reverse transcription (RT) kit (Qiagen) following manufacturer’s instructions. For every sample, 1 \( \mu g \) of isolated total RNA was incubated with gDNA wipe-out buffer (Qiagen) at 42°C for 2 min to remove any contaminating genomic DNA. After gDNA elimination, the purified RNA samples were converted to cDNA by reverse transcription by adding Quantiscript reverse transcriptase (Qiagen) and RT primer mix (Qiagen) in Quantiscript RT buffer (Qiagen). The RT reaction was performed at 42°C for 15 min and was subsequently inactivated at 95°C for 3 min using a T100 thermal cycler (Bio-Rad).

Total DNA was isolated from LV tissue using DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer’s instructions. Briefly, snap-frozen LV tissues were mechanically disrupted, and approximately 25 mg of powdered LV tissue was homogenized in buffer ATL (Qiagen) supplemented with proteinase K (Qiagen) by incubation at 56°C for 1 h while shaking and vortexing every 10 min. To remove residual RNA, 0.4 mg RNase A was added and incubated at room temperature for 2 min, followed by addition of buffer AL (Qiagen) and 100% ethanol (Merck Millipore) and homogenization by vortexing. The mixtures were then centrifuged at 6,000 \( g \) for 1 min through a DNeasy mini spin column (Qiagen) to selectively bind the DNA to the column membrane. To remove remaining contaminants, columns were washed with buffer AW1 (Qiagen) and AW2 (Qiagen) by centrifugation at 6,000 \( g \) for 1 min and 20,000 \( g \) for 3 min, respectively, to dry the membrane from residual ethanol. DNA was eluted from the column using water by centrifugation at 6,000 \( g \) for 1 min. DNA concentrations were determined by spectrophotometry using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

To generate DNA fragments for Sanger sequencing, PCR was performed using Taq DNA Polymerase (Roche Diagnostics, Switzerland) according to the manufacturer’s instructions. For every sample, 15 ng cDNA or 250 ng gDNA was mixed with 0.5 \( \mu M \) forward (5'-CATTTGGCTGCCTGTTGTCAAC-3') and reverse (5'-CGTTGTACGGTTGAGTCGAA-3') primers, 200 \( \mu M \) of dNTP solution (Roche Diagnostics), 1x PCR reaction buffer supplemented with 1.5 mM MgCl\(_2\) (Roche Diagnostics), and 1 U Taq DNA polymerase (Roche Diagnostics). The PCR reaction was performed at 94°C for 2 min followed by 35 or 30 cycles for cDNA and gDNA, respectively, of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and 72°C for 7 min using a T100 thermal cycler (Bio-Rad) to yield fragments of 286 base pairs including the \( Pln \) coding region (283 base pairs in case of c.40_42delAGA).

For DNA purification, PCR products were separated by agarose gel electrophoresis (2% agarose (Invitrogen, CA, USA) dissolved in Tris-acetate-EDTA (TAE) buffer (40 mM Tris (Sigma-
Aldrich), 20 mM glacial acetic acid (Merck Millipore) and 1 mM EDTA (Merck Millipore) in distilled water) with 0.5 μg/ml ethidium bromide (Sigma-Aldrich)), and fragments of the correct length (283 and 286 base pairs) were excised and isolated from the agarose gel using QIAquick Gel Extraction kit (Qiagen) following the manufacturer’s protocol. Briefly, the excised gel was dissolved in 3 gel volumes buffer QG (Qiagen) by incubation at 50°C for 10 min while shaking and vortexing every 2 min. After addition of 1 gel volume 2-propanol (Biosolve Chemicals), mixtures were centrifuged at 17,900 g for 1 min through a QIAquick mini spin column (Qiagen) to selectively bind the DNA to the column membrane. To remove residual agarose, buffer QG (Qiagen) was centrifuged twice through the column at 17,900 g for 1 min. Columns were washed with buffer PE (Qiagen) by centrifugation at 17,900 g for 1 min, and centrifuged at 17,900 g for 1 min to dry the membrane from residual ethanol. DNA was eluted from the column using water by centrifugation at 17,900 g for 1 min. DNA concentrations were determined by spectrophotometry using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Sanger sequencing was performed at GATC-Biotech (Germany) using a specific primer to include the Pln coding region: 5’-CTTCTCTTGACCACCTAG-3’.

**Cardiac magnetic resonance imaging**

Cardiac MRI was performed using an AVANCE 400 MR system (Bruker BioSpin, Germany). Mice were anesthetized (2-3% isoflurane (TEVA Pharmachemie) mixed with oxygen, administered via an aerial dispenser) and positioned in a quadrature-driven birdcage coil with an inner diameter of 30 mm. The coil was positioned in a 9.4-T vertical-bore superconducting magnet with a bore diameter of 89 mm, equipped with shielded gradients of 1.5 T/m. Heart and respiration rates were monitored using an ECG trigger unit (RAPID Biomedical, Germany). Heart rate was maintained at 400-600 beats per minute and respiration rate was maintained at 20-60 breaths per minute. After orthogonal scout imaging, short axis (oriented perpendicular to the septum) cardiac cine MR images were acquired and reconstructed using ParaVision 4.0 (Bruker BioSpin) and IntraGate software (Bruker BioSpin). Depending on the size of the heart, 7-9 slices with a slice thickness of 1 mm without gaps between slices were needed to cover the entire heart from base to apex. LV end-diastolic volume, end-systolic volume, stroke volume and ejection fraction were determined using cvi42 software (version 5.6.6; Circle Cardiovascular Imaging, Canada) by automatically delineating the end-diastolic and end-systolic epicardial and endocardial borders. Manual adjustments were made where necessary. Papillary muscles were included in the LV lumen.

**Echocardiography**

Echocardiography was performed using a Vevo 3100 preclinical imaging system (FUJIFILM VisualSonics, Canada), equipped with a 40-MHz MX550D linear array transducer (FUJIFILM VisualSonics). Prior to echocardiographic imaging, mice were anesthetized (2-3% isoflurane (TEVA Pharmachemie) mixed with oxygen, administered via an aerial dispenser) and the fur was removed from the chest area using a commercially available topical depilation agent with
potassium thioglycolate (Veet). Mice were placed on the temperature-maintained platform of the Vevo imaging station (FUJIFILM VisualSonics) in supine position with the paws taped over the electrode pads to monitor the heart and respiration rate. Vevo LAB software (version 3.1.1, FUJIFILM VisualSonics) was used for image analysis. LV parasternal long-axis B-mode images were used in circumferential strain (GLS) analysis. For cardiac dimension and function analysis, short-axis M-mode images were obtained at the mid-papillary level. The LV Trace tool was used to determine the LV end-diastolic internal diameter, LV end-systolic internal diameter and fractional shortening.

**Surface electrocardiography**

ECG recordings were acquired using two-lead subdermal needle electrodes, connected to a PowerLab 8/30 data acquisition device (model ML870; ADInstruments, Australia) and an animal Bio Amp biological potential amplifier (model ML136; ADInstruments). Mice were anesthetized (2-3% isoflurane (TEVA Pharmachemie) mixed with oxygen, administered via an aerial dispenser) and placed supine on a heating pad and recording needle electrodes were placed subcutaneously into the right axillary region and the left inguinal region (lead II configuration). After a stabilization period of two minutes, a recording of one minute was acquired, which was considered sufficient to provide a representative view of heart function (400-600 cardiac cycles). RR-, PR-, QRS- and QT-intervals, P-duration, P-, Q-, R-, S- and T-amplitudes, ST-height and heart rate were analysed using the ECG Analysis module in the LabChart Pro software (version 8, ADInstruments). One-minute recordings were averaged by overlaying all cycles using QRS-maximum for alignment. ECG parameters were detected and measured automatically and manually adjusted where appropriate. The QRS-interval was measured from the start of the Q wave to the end of the S wave on the isoelectric line. As in rodents the T wave directly succeeds the QRS complex, the end of the QRS complex marked the start of the T wave. The QT-interval was measured from the start of the Q wave to the end of the negative portion of the T wave. In contrast to humans, which show a pause between the QRS complex and T wave, ST segment is not clearly defined in mice. Therefore, ST height was measured according to the method provided by LabChart software, in which the ST height was determined 10 msec after R wave peak, which served as alignment point for cycle averaging.

**Implantable ECG telemetry**

Two 5-weeks-old PLN-R14Δ/Δ mice and WT littermates were subcutaneously implanted with telemeter transmitters (ETA-F10, Data Sciences International, MA, USA). Mice were anesthetized (2-3% isoflurane (TEVA Pharmachemie) mixed with oxygen, administered via an aerial dispenser) and placed supine on a heating pad. A single dose of 5.0 mg/kg carprofen (Rimadyl) was given via subcutaneous injection for analgesic purposes. A small incision was made in the skin of the abdomen, and a subcutaneous pocket was created on the right flank by spreading the subcutaneous connective tissues apart, in which the telemeter transmitter was inserted. The electrode leads were subcutaneously tunnelled, and the negative lead was secured to the right pectoral fascia, and the positive lead in the lower left abdominal
region (lead II configuration). Following surgery, mice were individually housed and allowed to recover for a week. Continuous signals were transmitted wirelessly to the receivers located below the cage. Conscious ECG recordings were monitored for 3 weeks, and analysed using Ponemah software (version 6.41, Data Sciences International) using the default settings for mice.

Isoproterenol infusion

Adult (10-weeks-old) PLN-R14Δ/Δ mice and their WT littermates were randomly subjected to infusion of 30 mg/kg/day isoproterenol (I6504, Sigma-Aldrich) for 4 weeks using subcutaneously implanted ALZET osmotic mini pumps (model 2004, DURECT Corporation, CA, USA) or sham surgery. Isoproterenol solution was prepared by dissolving isoproterenol hydrochloride (Sigma-Aldrich) in saline, and was injected into the osmotic mini pumps. Filled osmotic mini pumps were primed for at least 40 h in saline at 37°C prior to implantation in order for the infusion to start immediately upon implantation. Mice were anesthetized (2-3% isoflurane (TEVA Pharmachemie) mixed with oxygen, administered via an aerial dispenser) and placed prone on a heating pad. A single dose of 5.0 mg/kg carprofen (Rimadyl) was given via subcutaneous injection for analgesic purposes. A small incision was made in the skin between the scapulae, and a subcutaneous pocket was created on the right flank by spreading the subcutaneous connective tissues apart, in which the osmotic mini pump was inserted. Pumps were sufficient for drug delivery for 28 days and were present until the end of the experiment. After 4 weeks of isoproterenol infusion, in vivo cardiac analysis was performed, and mice were sacrificed.

Eplerenone and metoprolol treatment

PLN-R14Δ/Δ mice were randomly subjected to treatment with eplerenone or metoprolol or vehicle. Two-hundred mg/kg/day eplerenone (Inspra, Pfizer, NY, USA) was mixed with the chow. Three-hundred-fifty mg/kg/day metoprolol (M5391, Sigma-Aldrich) was administered orally via the drinking water, protected from light. Treatment was initiated at weaning when PLN-R14Δ/Δ mice were 3 weeks of age and cardiac abnormalities were still absent (Supplementary Figure S1). Monitoring of food and water intake during cardiac phenotyping established that for mice housed under the conditions of our animal facility, food and water intake increase from 2 mg or ml per day, respectively, at 3 weeks of age to 4 mg or ml day, respectively, at 6 weeks of age. Mice were weighed every week and the concentrations of eplerenone (0.8-2.0 mg/g) and metoprolol (1.2-2.5 mg/ml) were adjusted accordingly. Food and water intake were monitored throughout the study to ensure appropriate dosages were taken. Treatment was continued until the endpoint was reached. WT controls were sacrificed at the age of 16 weeks. In vivo cardiac analysis was performed at the age of 6 weeks as described earlier.

Sacrifice

Euthanasia was performed by anesthetizing (2-3% isoflurane (TEVA Pharmachemie) mixed with oxygen, administered via an aerial dispenser) the mice, after which the abdomen was opened, the abdominal part of the aorta was cut, and the circulation was perfused with
saline via injection into the heart to wash out the blood. The heart was quickly excised, rinsed in 1 M KCl (Merck Millipore) solution, weighed and dissected. A transverse mid-slice was fixed overnight in 4% buffered formaldehyde (10% formalin, Klinipath, the Netherlands) for histological analysis. Remaining LV tissue and kidneys were snap-frozen in liquid nitrogen, and stored at -80°C until further processing.

**Ex vivo optical action potential recording and electrical stimulation**

Mice were stunned by inhalation of CO₂ and euthanized by cervical dislocation, after which the heart was excised, cannulated, mounted on a Langendorff perfusion setup, and perfused at 37°C with Tyrode’s solution (128 mmol/L NaCl, 4.7 mmol/L KCl, 1.45 mmol/L CaCl₂, 0.6 mmol/L MgCl₂, 27 mmol/L NaHCO₃, 0.4 mmol/L NaH₂PO₄, and 11 mmol/L glucose (pH maintained at 7.4 by equilibration with a mixture of 95% O₂ and 5% CO₂)). Ex vivo ECGs were recorded (Biosemi, the Netherlands; sampling rate 2048 Hz, filtering DC 400 kHz (3 dB)) and analysed using LabChart Pro software (version 8, ADInstruments). Optical action potentials were recorded with a CMOS camera (MICAM05, SciMedia, CA, USA; 1 kHz sampling rate) using voltage-sensitive dye RH237 (Invitrogen). Blebbistatin was used to remove motion artefacts. Conduction velocity was calculated at basic stimulation interval of 120 msec using dF/dtₘₐₓ as local moment of activation. Arrhythmias were induced by decreasing basic stimulation interval with steps of 5 msec for a period of 20 sec until arrhythmias occurred or the ventricle failed to capture.

**Histological analysis**

After sacrifice, a transverse mid-slice of the heart was fixed overnight in 4% buffered formaldehyde (10% formalin, Klinipath), subjected to a dehydration series (70% ethanol (Klinipath) for 1 h, 80% ethanol for 1 h, 90% ethanol for 1 h, 99.5% ethanol for 3 h and xylene (Klinipath) for 3 h) using a Leica TP1020 tissue processor (Leica Microsystems, Germany), and embedded in paraffin (Klinipath) using a Leica EG1150 H paraffin embedding module (Leica Microsystems). Embedded tissue slices were cut into 4-µm thick transversal sections using a Leica RM2255 microtome (Leica Microsystems), mounted on StarFrost Adhesive silane-coated microscope slides (Knittel, Germany), and incubated overnight at 60°C for deparaffinization of the tissues, followed by histological analysis.

Masson’s trichrome stain was performed to detect collagen deposition as a measurement of fibrosis. For complete deparaffinization and rehydration of the tissues, sections were incubated in xylene for 20 min, 100% ethanol for 10 min, 96% ethanol for 5 min, 70% ethanol for 1 min and rinsed with distilled water. Nuclei are stained black by incubation in Weigert’s iron haematoxylin solution (0.05% haematoxylin solution (Gill I) (Sigma-Aldrich), 0.06% FeCl₃ (Sigma-Aldrich) and 0.5% HCl (Merck Millipore) in distilled water) for 10 min. Sections were washed in running tap water for 10 min and rinsed with distilled water. Next, cytoplasm was stained red by incubation in Biebrich scarlet-acid fuchsin solution (0.9% Biebrich scarlet (VWR Chemicals, PA, USA), 0.1% acid fuchsin (Sigma-Aldrich) and 0.5% glacial acetic acid (Merck Millipore) in distilled water) for 10 min. Sections were washed with distilled water.
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for 1 min before differentiation in phosphomolybdic-phosphotungstic (PP) acid solution (3% phosphomolybdic acid hydrate (Alfa Aesar, MA, USA) and 2.5% phosphotungstic acid (Sigma-Aldrich) in distilled water) for 15 min. Without rinsing, sections were transferred to aniline blue solution (1.26% aniline blue (Acros Organics, NJ, USA) and 2.0% glacial acetic acid in distilled water) for 5 min to stain collagen blue. Sections were washed with distilled water for 1 min before differentiation in 1% glacial acetic acid solution for 4 min. Subsequently, sections were rinsed with distilled water for 30 sec and dehydrated in 96% ethanol for 30 sec, 100% ethanol for 2 min and xylene for 10 min. Sections were covered with DPX (a mixture of distyrene, a plasticiser (tricresyl phosphate) and xylene) neutral mounting medium (Sigma-Aldrich) and a cover slip. To quantify the amount of fibrosis, whole stained sections were automatically imaged using a NanoZoomer 2.0-HT digital slide scanner (Hamamatsu, Japan), and fibrotic area was determined with Aperio’s ImageScope software (version 12.4, Leica Microsystems). Fibrosis fractions were quantified as a percentage of the total area of the entire stained section, and calculated as fold change compared to the control group per experiment.

Immunofluorescent staining for PLN was performed using a mouse monoclonal anti-PLN antibody (clone 2D12, #MA3-922, Invitrogen) labelled with Alexa Fluor 555 (red) using an APEX antibody labelling kit (Invitrogen) according to the manufacturer’s protocol. Briefly, anti-PLN antibody was loaded onto the prehydrated resin of the APEX antibody labelling tip together with the fluorescent label, and incubated for 2 h at room temperature, followed by elution of the labelled antibody. For complete deparaffinization and rehydration of the tissues, sections were incubated in xylene for 25 min, 100% ethanol for 10 min, 96% ethanol for 3 min, 70% ethanol for 3 min and rinsed with distilled water for 3 min. Sections were washed twice in PBS (1.76 mM KH₂PO₄ (Merck Millipore), 10 mM Na₂HPO₄ (Sigma-Aldrich), 0.14 mM NaCl (Merck Millipore) and 2.68 mM KCl (Merck Millipore) in distilled water) for 5 min. Antigen retrieval was done in a microwave at 400 W by incubating sections for 15 min in preheated antigen retrieval buffer (10 mM Tris (Sigma-Aldrich) and 1 mM EDTA (Merck Millipore) pH 9.0 in distilled water). After cooling down, excess antigen retrieval buffer was removed by washing three times in PBS for 5 min. Next, sections were incubated with labelled PLN antibody (1:200) to stain PLN red, and fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (1:100, WGA, Sigma-Aldrich, 2 mg/ml in PBS) for 1 h to stain extracellular matrix (ECM) green. After rinsing excess antibody three times with PBS for 10 min, sections were incubated in VECTASHIELD mounting medium with DAPI (Vector Laboratories, CA, USA) for 30 min to stain nuclei blue. Sections were sealed using blank nail polish and stored at 4°C protected from light until imaging. Fluorescent imaging was done using a Leica AF6000 fluorescence imaging system (Leica Microsystems). The number of PLN-aggregate-containing cardiomyocytes in PLN-R14Δ/Δ mice hearts was scored in 2 representative fields of 20x magnification (0.14 mm² per image) of PLN-immunostained sections, and shown as the percentage of the total number of cardiomyocytes in the fields. Since the prevalence was lower in PLN-R14Δ/+ mice hearts, the number of PLN-containing aggregates was assessed in the total area of PLN-immunostained sections, and shown as the number of aggregates per mm² of the images. These methods
are adapted from the method that was used by te Rijdt et al.\textsuperscript{10} to score PLN aggregates in human cardiac biopsies.

**Quantitative PCR**

Total RNA was isolated from tissues using TRI Reagent (Sigma-Aldrich), and cDNA synthesis was performed using the QuantiTect RT kit (Qiagen) as described earlier for Sanger sequencing. Gene expression levels were determined by qPCR analysis using iQ SYBR green supermix (Bio-Rad) according to the manufacturer’s instructions. Duplicates of 7.5 ng cDNA were mixed with 750 nM forward and reverse primers and iQ SYBR green supermix (Bio-Rad). The qPCR reaction was performed at 95°C for 3 min followed by 35 cycles of 95°C for 15 sec and 60°C for 30 sec using a CFX384 Touch real-time PCR detection system (Bio-Rad). Gene expression was quantified by correcting for reference gene values of ribosomal protein lateral stalk subunit P0 ($R_{plp0}$, encoding 36B4) using CFX Manager software (version 3.0, Bio-Rad), and the calculated values were expressed relative to the control group per experiment. Primer sequences can be found in Supplementary Table S1.

**Western blot**

For total protein isolation, snap-frozen LV tissue was mechanically disrupted, and approximately 25 mg of powdered tissue was homogenized in 0.2 ml ice-cold RIPA lysis buffer (50 mM Tris (Sigma-Aldrich) pH 8.0, 1.0% IGEPAL CA-630 (Sigma-Aldrich), 0.5% sodium deoxycholate (Sigma-Aldrich), 0.1% SDS (Sigma-Aldrich) and 150 mM NaCl (Merck Millipore) in distilled water) freshly supplemented with 4% cOmplete protease inhibitor (PI) cocktail (Roche Diagnostics), 1% phosphatase inhibitor cocktail 3 (Sigma-Aldrich), 15 mM sodium orthovanadate (Sigma-Aldrich), and 1 mM phenylmethylsulphonyl fluoride (PMSF) (Roche Diagnostics) using a TissueLyser LT (Qiagen) at 50 Hz for 5 min. After centrifugation at 12,000 g for 10 min at 4°C, the supernatant containing solubilized proteins was collected. The remaining pellet, which contains insoluble proteins, was dissolved in 50 μl urea solution (8 M urea (Sigma-Aldrich), 0.1 M NaH$_2$PO$_4$ (Merck Millipore) and 0.01 M Tris-HCl (Sigma-Aldrich) in distilled water).

Protein concentrations were determined using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) according to the manufacturer’s protocol. Duplicates of total protein samples were mixed 1:20 with working reagent (50 reagent A : 1 reagent B) in a flat-bottom 96-wells plate and incubated at 37°C for 30 min. Absorbance was measured at 562 nm using a Synergy H1 microplate reader (BioTek, VT, USA). Individual absorbance values were corrected for the absorbance value of the blank standard sample, after which total protein sample concentrations were determined according to a standard curve by plotting the absorbance of the bovine serum albumin (BSA) standard samples against their concentrations with a quadratic curve fit using Gen5 software (BioTek).

Protein expression levels were determined by Western blot analysis. Equal amounts of protein (5 μg) were denatured at 95°C for 5 min and separated by gel electrophoresis using Novex 10-20% Tricine Protein Gels (Invitrogen). Separated proteins were transferred onto
Immun-Blot polyvinylidene fluoride (PVDF) membranes (Bio-Rad) by semi-dry blotting. Next, membranes were blocked in block buffer (5% BSA (Serva, Germany) in Tris-buffered saline (TBS) (150 mM NaCl (Merck Millipore) and 10 mM Tris (Sigma-Aldrich) pH 8.0 in distilled water) with 0.1% TWEEN (polysorbate) 20 (Sigma-Aldrich) (TBST)) for 1 h at room temperature while shaking and incubated overnight at 4°C with an anti-PLN or anti-GAPDH primary antibody in block buffer while shaking. To remove unbound primary antibody, membranes were washed three times in TBST for 5 min while shaking, followed by 1-h incubation at room temperature with an appropriate horseradish peroxide (HRP)-linked secondary antibody in block buffer while shaking. After washing off unbound secondary antibody, detection was performed using Western Lightning Ultra enhanced chemiluminescence (ECL, PerkinElmer, MA, USA) and an ImageQuant LAS 4000 digital imaging system (GE Healthcare, IL, USA). Antibodies that were used are described in Supplementary Tables S2 and S3.
### SUPPLEMENTARY TABLES

#### Supplementary Table S1. List of primers used in this study for qPCR analysis.

| Transcript | Name   | Forward primer (5'-3') | Reverse primer (5'-3') | Product size |
|------------|--------|------------------------|------------------------|--------------|
| Col1a1     | COL1A1 | AGAGCATGACCGATGGATTC   | CGCTGTTCTTGCAATGATAG   | 138 bp       |
| Col1a2     | COL1A2 | CTACTGGATTGACCTAACC    | CGCCACATTGATATGCTC     | 177 bp       |
| Col3a1     | COL3A1 | ATATGCCACACGCTTCTAC    | CCACCAGTGGACATGATCC    | 185 bp       |
| Lgals3     | Gal-3  | CAGTGAAACCCAACGCAACAC  | AGGCAACATCATTCCCTCTC   | 60 bp        |
| Mmp2       | MMP2   | GGGAGCATGGAGATGGATAC   | CCACCTTTACGGGGACCAC    | 151 bp       |
| Myh6       | α-MHC  | AGCTCATGGCTACACTCTTC   | GTGGGTGCTTCACAGTTTG    | 158 bp       |
| Myh7       | β-MHC  | GAGCATTCTCTGCTGTCTTC   | GAGCCTTGATTCTCAAACG    | 136 bp       |
| Nppa       | ANP    | GCTTCCAGGGCCATATTGGAG  | GTGGTCTAGCGGCTTCTTG    | 86 bp        |
| Nppb       | BNP    | CTCTATCTCTCTGGGAAGTC   | CGGATCCGCTATCTACTTG    | 246 bp       |
| Ren        | Renin  | CGCACCGCTACCTTGAAC     | TCCAGGATTTTCCGGACAG    | 62 bp        |
| Riplp0     | 36B4   | AAGCGCGTCCTGGCATTTGC   | GCAGCCGCAAATGCAGATGG   | 98 bp        |
| Timp1      | TIMP1  | CAACGAGACCACCTTATAACC  | CATATCCACAGAGGCTTCTCC  | 131 bp       |

bp, base pairs; Col1a1/COL1A1, collagen type I alpha 1; Col1a2/COL1A2, collagen type I alpha 2; Col3a1/COL3A1, collagen type III alpha 1; Lgals3/Gal-3, galectin-3; Mmp2/MMP2, matrix metalloproteinase-2; Myh6/α-MHC, alpha myosin heavy chain; Myh7/β-MHC, beta myosin heavy chain; Nppa/ANP, atrial (A-type) natriuretic peptide; Nppb/BNP, brain (B-type) natriuretic peptide; Riplp0/36B4, ribosomal protein lateral stalk subunit P0; Timp1/TIMP1, tissue inhibitor of metalloproteinase-1.

#### Supplementary Table S2. List of primary antibodies used in this study for Western blot analysis.

| Antigen | Supplier | Product no. | Host | Dilution |
|---------|----------|-------------|------|----------|
| GAPDH   | Fitzgerald, MA, USA | 10R-G109a | Mouse | 1:30,000 |
| PLN     | Cell Signalling, MA, USA | 14562 | Rabbit | 1:1,000 |

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PLN, phospholamban.

#### Supplementary Table S3. List of secondary antibodies used in this study for Western blot analysis.

| Antigen | Supplier | Product no. | Host | Dilution | Label |
|---------|----------|-------------|------|----------|-------|
| Anti-mouse | Dako, CA, USA | P026002 | Rabbit | 1:2,000 | HRP   |
| Anti-rabbit | Dako | P044801 | Goat | 1:2,000 | HRP   |

HRP, horseradish peroxidase.
