Phospholamban antisense oligonucleotides improve cardiac function in murine cardiomyopathy

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Heart failure (HF) is a major cause of morbidity and mortality worldwide, highlighting an urgent need for novel treatment options, despite recent improvements. Aberrant Ca2+ handling is a key feature of HF pathophysiology. Restoring the Ca2+ regulating machinery is an attractive therapeutic strategy supported by genetic and pharmacological proof of concept studies. Here, we study antisense oligonucleotides (ASOs) as a therapeutic modality, interfering with the PLN/SERCA2a interaction by targeting Pln mRNA for downregulation in the heart of murine HF models. Mice harboring the PLN R14del pathogenic variant recapitulate the human dilated cardiomyopathy (DCM) phenotype; subcutaneous administration of PLN-ASO prevents PLN protein aggregation, cardiac dysfunction, and leads to a 3-fold increase in survival rate. In another genetic DCM mouse model, unrelated to PLN (Cspr3/Mlp −/−), PLN-ASO also reverses the HF phenotype. Finally, in rats with myocardial infarction, PLN-ASO treatment prevents progression of left ventricular dilatation and improves left ventricular contractility. Thus, our data establish that antisense inhibition of PLN is an effective strategy in preclinical models of genetic cardiomyopathy as well as ischemia driven HF.
Despite advances in treatment, HF patients have poor long-term outcomes with 5-year mortality rates of 50%, exceeding common forms of cancer such as prostate and breast cancer\(^1\). The need for a therapy capable of addressing the root cause of cardiac dysfunction in HF remains high\(^2\). One of the key features of HF is aberrant Ca\(^{2+}\) cycling, which impairs cardiac muscle contraction, and is associated with cardiac arrhythmias and adverse remodeling\(^3\). A critical contributor is reduced activity of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a), accompanied by a relative increase of unphosphorylated phospholamban (PLN), a SERCA2a inhibitor\(^4\). PLN is a principal regulator of SERCA2a activity, maintaining appropriate sarcoplasmic reticulum Ca\(^{2+}\) cycling, which has been shown critical for cardiac relaxation and contraction\(^5\). Evidence based HF treatments primarily target neurohormonal systems, while the assumed crucial calcium handling proteins are left untouched.

There have been attempts to target SERCA2a and its partners. To date, most efforts have focussed on increasing SERCA2a expression using adeno-associated virus based gene therapy in multiple date, most efforts have focused on increasing SERCA2a expression using adeno-associated virus based gene therapy in multiple

Antisense oligonucleotides (ASOs) provide the possibility to treat PLN-ASO treatment prevents cardiac dysfunction and mortality in a PLN R14del mouse model. To assess in vivo functionality of the identified PLN-ASO leads, we turned to a model of genetic cardiomyopathy caused by a pathogenic variant of PLN. Several PLN mutations are known to cause cardiomyopathy in humans, with the deletion of arginine at position 14 of the PLN protein (R14del) being one of the most prevalent variants which leads to severe HF and malignant ventricular arrhythmias, and has recently been increasingly studied\(^16\). Although the direct effects on Ca\(^{2+}\) cycling are part of ongoing discussion, the PLN R14del pathogenic variant is reported to result in PLN protein aggregates, myocardial fibrosis and cardiac dysfunction\(^17\). We reasoned that ASO induced reductions of PLN protein expression has the potential to prevent disease development and progression, thereby representing a possible precision medicine approach to treat PLN R14del induced cardiomyopathy. A mouse model carrying the PLN R14del pathogenic variant in both alleles (PLN R14\(^{Δ/Δ}\)) recapitulated all common features of the human
phenotype observed in heterozygous carriers, yet in an accelerated fashion, with rapid development of DCM, myocardial fibrosis, and PLN protein aggregates resulting in premature death at the age of 8–9 weeks. This phenotype is observed despite endogenous downregulation of PLN RNA and protein expression in untreated PLN R14Δ/Δ mice compared to wild-type mice. We hypothesize this phenotype to be caused by the residual expression of mutant PLN, we aim to further reduce this expression utilizing the PLN-ASO.

As the PLN R14Δ/Δ mice previously showed increased cardiac dimensions and a reduced left ventricular ejection fraction (LVEF) at 6 weeks of age, we started our 4-week dosing regimen of PLN-ASO at 3 weeks of age (Fig. 2a). This resulted in 42% reduction of cardiac Pln mRNA (±6%, P value = 0.0153, Fig. 2b) and 50% reduction of total (both soluble and insoluble/aggregated) PLN protein levels (±18%, P value = 0.0142) at treatment week 4 (T4) and 7 weeks of age (Fig. 2c and Supplementary Fig. 2).
remained unchanged (Supplementary Figs. 3–6). Combined, this resulted in a change of the SERCA2/PLN ratio in favor of SERCA2 (Supplementary Figs. 5, 6). Immunofluorescence showed a lower abundance of PLN protein aggregates in PLN-ASO treated mice (Fig. 2d). Additionally, RIPA-insoluble protein fractions of PLN R14Δ/Δ mice hearts contained more PLN protein as compared to wild-type mice, indicating aggregated PLN protein complexes (Supplementary Figs. 3–5). In PLN-ASO treated mice hearts, RIPA-insoluble PLN protein levels were 89% lower than values in vehicle control (±12%, Supplementary Figs. 4, 5, P value < 0.0001). Strikingly, magnetic resonance imaging (MRI) of the heart at 7 weeks of age revealed almost completely normal cardiac dimensions and function in PLN-ASO treated mice, whereas severe DCM was observed in vehicle-treated mice (Fig. 2e, f, LVEF 53 ± 2 vs. 27 ± 2%, P value < 0.0001, left ventricular end-diastolic volume [LVEDV] 41 ± 2 vs. 54 ± 2.0 mL, P value < 0.0001 and left ventricular end-systolic volume [LVESV] 20 ± 1 vs. 39 ± 2 mL, P value < 0.0001). No changes in heart rate were observed on ECG (Supplementary Fig. 7). Also, no signs of treatment toxicity were observed based on plasma AST and ALT, haematoxylin and eosin (H&E) staining of kidney, liver or spleen sections (Supplementary Fig. 7).

Genome wide transcriptome profiling by RNA-Seq revealed that PLN R14Δ/Δ mutant hearts displayed marked differences in global gene expression compared to wild-type control hearts (Fig. 2g). Moreover, PLN-ASO treatment attenuated gene expression changes in mutant hearts, thereby indicating that PLN-ASO treated hearts more closely resemble wild-type hearts than the PBS treated mutant counterparts. When assessing the functions of genes mostly contributing to the difference between PLN R14Δ/Δ mice and controls (principle component 1; PC1, and genes differentially expressed in PLN R14Δ/Δ vs. wild-type and subsequently reversed by PLN-ASO vs. PBS), we observed increased expression of genes involved in fatty acid metabolism, and decreased expression of genes involved in protein processing in the endoplasmic reticulum and extracellular matrix, providing further confirmation that key determinants of cardiomyopathy such as energy metabolism and fibrosis are attenuated by the PLN-ASO treatment (Fig. 2g and Supplementary Fig. 8a–c).

Another typical hallmark of PLN R14del cardiomyopathy in humans is a low voltage ECG, which was also observed in PLN R14Δ/Δ mice (Fig. 2h, i, expressed by low R-amplitude), and was partially restored by PLN-ASO treatment (0.84 ± 0.05 vs. 0.55 ± 0.05 mV, P value < 0.0001). We excluded known confounders of a low voltage ECG including obesity (body weight to tibia length ratio 1.14 ± 0.05 vs. 1.46 ± 0.02, P value = 0.0012, of PLN R14Δ/Δ vs. wild-type, Supplementary Fig. 9), and cardiac effusion which was not observed at sacrifice in PLN R14Δ/Δ mice. Masson's trichrome staining revealed a significant prevention of myocardial fibrosis in PLN-ASO treated mice (Fig. 2j, k, fold change compared to WT 2.4 ± 0.4 vs. 17.17 ± 4.1%, P value = 0.0032). The significantly higher ECG voltages seen after 4 weeks of PLN-ASO treatment, are therefore arguably explained by the prevention of fibrosis and PLN protein aggregation.

Since cardiac function was almost entirely preserved, we investigated the effects on lifespan. After an initial loading phase with a high-dose (100 mg/kg from 3 to 6 weeks of age), mice were treated with a lower maintenance dose (50 mg/kg every 4 weeks, Fig. 2a). This increased lifespan by over 3-fold from the average of 7.5 weeks to at least 24 weeks (Fig. 2b, log-rank P value < 0.0001), with only 1 mouse dying from HF before study termination at the age of 26 weeks. To gain insight into the development and progression of the disease-phenotype and to identify disease contributors, a set of mice were sacrificed every 4 weeks during the late study phase (follow-up). Age-matched vehicle-treated control PLN R14Δ/Δ mice were not available for these analyses as they all died before the age of 9 weeks, which hampers accurate interpretation of follow-up PLN mRNA and protein analyses in PLN-ASO treated mice. Based on known PLN ASO pharmacokinetics, this dosing regime was chosen not to keep PLN mRNA and protein levels knocked down at steady state, but to allow a slow incline of PLN levels over time and therefore being insufficient to prevent disease progression. PLN-ASO treated PLN R14Δ/Δ mice slowly progressed to HF, with first signs of cardiac disease at T19, which corresponds to the age of 22 weeks (Supplementary Fig. 10).

We correlated global transcriptome data with cardiac function (LVEF) acquired just before sacrifice at different time points during the study to identify possible pathways associated with a positive treatment effect. Gene ontology analysis revealed oxidative phosphorylation and other metabolic pathways and protein processing in the endoplasmic reticulum as the top affected pathways (Supplementary Fig. 8d, e). Additionally, we observed a durable downregulation of genes involved in the unfolded protein response in PLN-ASO treated PLN R14Δ/Δ mice (Supplementary Fig. 8f). These findings provide further support to the idea that a reduction of toxic PLN protein aggregates by PLN-ASO might contribute to the significant benefits observed after treatment. Sarcoplasmic reticulum protein processing is dependent on local Ca2+ levels, but no further indication of altered Ca2+ related pathways was observed. In summary, PLN-ASO treatment slowed down the development of cardiac fibrosis, protein aggregation and DCM in PLN R14Δ/Δ mice and extended survival over threefold.
PLN-ASO treatment reverses signs and symptoms of heart failure in \textit{Cspr3/Mlp} \textsuperscript{−/−} mice. In PLN \textsuperscript{R14\textDelta}/\textDelta mice, PLN-ASO directly addressed the root cause of the disease, resulting in a dramatic treatment benefit. Therefore, we were also interested in assessing PLN-ASO effects in a cardiac disease model where calcium handling is impaired, but where PLN is not believed to be the direct instigator of disease. To this end, we evaluated mice lacking muscle LIM protein (\textit{Cspr3/Mlp} \textsuperscript{−/−}), an established model for DCM\textsuperscript{29}. \textit{CSPR3/MLP} is a structural protein located at the Z-line in cardiac and slow-twitch skeletal muscle cells, but also has a nuclear translocation signal. It has been implicated to play a role in muscle differentiation and as a mechanical stress sensor converting upstream signals into both intra- and intercellular functional responses\textsuperscript{30}. Mutations in this gene can lead to both DCM and familial hypertrophic cardiomyopathy in humans\textsuperscript{31}. \textit{Cspr3/Mlp} \textsuperscript{−/−} mice develop a DCM phenotype, typically at the age of 8–12 weeks; however, disease onset variability was observed\textsuperscript{29}. Previous work has established this model to be amenable to improvements in cardiac function by additional genetic ablation of \textit{Pln}\textsuperscript{8}. Recently, this has been confirmed in a
study showing that DWORF overexpression, a micropeptide enhancing SERCA2a activity by displacing PLN, prevents cardiac dysfunction in Csp3/Mlp−/− mice.

To test whether therapeutic reductions of PLN protein levels would improve cardiac function in already diseased mice, PLN-ASO (ASO#26_C or ASO#27) or vehicle control (PBS or control ASO) were subcutaneously administered to Csp3/Mlp−/− mice after onset of disease (Fig. 3a, Supplementary Data 1). Mice were followed for 28 days before terminal analyses. PLN-ASO treatment resulted in a 70–90% reduction in Pln mRNA (Fig. 3f) and total PLN protein levels (both pentamer and monomer) in the heart (Fig. 3b and Supplementary Fig. 11). Expression of SERCA2a was not significantly affected by the disease condition or PLN-ASO treatment at the mRNA or protein level, while such treatment increased the ratio of SERCA2a to its inhibitor PLN (Fig. 3b, f and Supplementary Fig. 11). PLN protein downregulation appeared uniformly across cardiomyocytes (Supplementary Fig. 12). A PLN ASO pharmacokinetics and dynamics study showed an almost immediate onset of PLN downregulation on the mRNA level, followed by protein levels around 7 days later, and a sustained PLN knockdown effect post treatment (Supplementary Fig. 13).

Echocardiography was performed at baseline and after 4 weeks of treatment to compare responses to treatment, and mice with signs of HF at study initiation (LVEF ≤ 45%; median, 37.6%; interquartile range, 32.2–42.0%) were analyzed. Improvements of LVEF (60 ± 8 vs. 46 ± 12%), LVESV (31 ± 11 vs. 56 ± 21 mL) and LVEDV (79 ± 22 vs. 100 ± 25 mL) were observed in PLN-ASO treated versus control mice respectively (all P < 0.001, Fig. 3c, d, Supplementary Data 1). Hemodynamic baseline measurements of WT (Csp3/Mlp+/+) and Csp3/Mlp−/− mice, either PBS or PLN-ASO treated (Supplementary Data 1), showed that left ventricular contractility (assessed by LV dp/dtmax) was significantly enhanced by PLN-ASO treatment compared to Csp3/Mlp−/− control mice. In addition, an impaired diastolic function was detected in Csp3/Mlp−/− mice (assessed by lower LV dp/dtmin and increased Tau) which was rescued by PLN-ASO treatment to functional levels comparable to wild-type mice (Fig. 3e and Supplementary Fig. 14). In response to increasing doses of the beta-adrenergic agonist dobutamine, Csp3/Mlp−/− mice showed a blunted response in contractility and relaxation compared to littermate wild-type controls, which suggested a reduced cardiac reserve in Csp3/Mlp−/− mice. The contractility response was mostly normalized by PLN-ASO treatment, and relaxation was enhanced but not normalized upon increasing dobutamine doses (Fig. 3e).

Heart rate was not affected by PLN-ASO treatment (Supplementary Fig. 14). Furthermore, there was no effect on peak systolic and end-diastolic LV pressure (Supplementary Fig. 14) and no effect on heart and body weight (Supplementary Fig. 15). Whole cell Ca2+ flux recordings of adult cardiomyocytes isolated from Csp3/Mlp−/− mice after 4 weeks of treatment with PLN-ASO versus control in vivo (Fig. 3a), showed significantly enhanced amplitude and uptake- and decay velocity, without changes in Ca2+ flux durations due to pacing at 1 Hz (Supplementary Fig. 16). Finally, RT-PCR analysis of cardiac transcripts demonstrated reversal of HF associated gene expression, such as Myh7, Acta1, Nppa, Nppb in Csp3/Mlp−/− mice upon PLN-ASO treatment (Fig. 3f). A reduction of ANP levels was also observed in plasma samples (Fig. 3f). A validation study was performed comparing PLN-ASO (ASO#26_C) to a control ASO, using a similar design as the other studies (Supplementary Fig. 17). PLN-ASO significantly improved LVVEF and LVESV over the control ASO treated mice. In summary, PLN-ASO treatment substantially reversed the DCM phenotype of Csp3/Mlp−/− mice towards that of healthy mice.

PLN-ASO treatment improves contractility and reduces cardiac dimensions after myocardial infarction. Acquired HF is the most prevalent form of cardiomyopathy in humans. Therefore, we aimed to test the PLN-ASO treatment effects in an acquired HF model. For this purpose, we employed the rat post myocardial infarction (MI) model, which is widely used to test potential therapeutic interventions to treat human disease. In this model, the ischemic event triggers massive cell loss, while cardiac function progressively declines, accompanied by pathological remodeling and impaired intracellular Ca2+ handling. This has been extensively evaluated in preclinical MI models and isolated cardiomyocytes from patients with post-MI HF. A previous study showed that reduction of inhibitory PLN, and thereby enhanced SERCA2a activity, in post-MI rats could prevent progressive cardiac dysfunction and pathological remodeling. An additional ASO screen for a rat-specific PLN-ASO was
performed, similar to that in mouse (Supplementary Fig. 18). Rats underwent permanent left anterior descending artery ligation to induce MI, and then a 6-week interim period to allow disease progression. At this time point, rats underwent an MRI to obtain baseline measurements (average LVEF = 46%) and were randomized into the following treatment groups: PBS, scrambled Control-ASO, PLN-ASO low (25 mg/kg) and high (50 mg/kg) dose, and a repeated dosing regimen was initiated (Fig. 4a). After 5 weeks of treatment, PLN-ASO resulted in a 29% (±12%) or 66% (±12%) reduction of Pln mRNA levels (P = 0.07 and P = 0.0001, respectively).
Supplementary Fig. 19), and 30% (±4%) or 57% (±4%) reduction in PLN protein levels (both \( P < 0.0001 \), Fig. 4b and Supplementary Fig. 20) in the low and high-dose group respectively. SERCA2 protein expression levels were not significantly affected by MI or PLN-ASO treatment, and thus the ratio of SERCA2 to PLN expression was significantly increased in rats treated with 50 mg/kg PLN-ASO (Fig. 4b and Supplementary Figs. 19, 20). MRI functional analyses showed no differences between the two control groups (PBS and scrambled ASO treated rats). The average LVEF at the start of treatment was reduced in MI compared to sham (46% (±1%) versus 62% (±1%), \( P \) value < 0.001), suggesting a moderate HF state, but no further deterioration in LVEF was observed in the following 5-week period in any of the treatment groups (Fig. 4c). However, both LVESV and LVEDV tended to increase with time in the control post-MI groups, and this effect was significantly reversed by high-dose PLN-ASO treatment (change from baseline: LVESV, +51 µl (±8 µl) versus −27 µl (±12 µl), \( P \) value < 0.0001, LVEDV, +68 µl (±16 µl) versus...
of PLN ASO in rats with a myocardial infarction improves contractility and reduces cardiac dimensions. a Experimental design of the rat post myocardial infarction intervention study. Six weeks after myocardial infarction, induced by permanent left anterior descending (LAD) artery ligation, an MRI was performed, and rats were randomized into groups. Treatment of ASO#136 was initiated at a 2×
daily dose for the first 2 weeks followed by 1×
daily dose (sham = 5, PBS = 7, Control-ASO = 5, ASO = 6, 25 mg/kg). MRI, invasive hemodynamics and sacrifice was performed 5 weeks after treatment initiation, 11 weeks after myocardial infarction. b Western blot results of LV protein lysates stained for PLN and SERCA2 protein and semi-quantified relative to PBS treated control samples. Intensities were normalized to GAPDH (n = 5 for sham, n = 7 for PBS and PLN-ASO 50 mg/kg, and n = 6 for control ASO and PLN-ASO 25 mg/kg). Representative image of 1 out of 4 membrane stains (Supplementary Fig. 20). Ratio of SERCA2 to PLN is shown as fold change relative to PBS. c Individual MRI assessment and quantification of the change in left ventricular ejection fraction (LVEF), left ventricular end-diastolic volume (LVEDV), and left ventricular end-systolic volume (LVESV), and left ventricular end-diastolic volume (LVEDV) between treatment initiation (6 weeks post MI) and 5 weeks after start of treatment showing (n = 5 for sham, n = 7 for PBS, n = 8 for control ASO, n = 8 for PLN-ASO 25 mg/kg, and n = 6 for PLN-ASO 50 mg/kg). d Hemodynamic assessment of the maximum/minimum first derivative of LV pressure (Max dP/dt and Min dP/dt) performed at study end, 5 weeks after treatment start, at baseline and upon increasing dobutamine doses of 0.1, 2, and 6 μg/kg/min (n = 5 for sham, n = 7 for PBS and PLN-ASO 50 mg/kg and n = 8 for control ASO and PLN-ASO 25 mg/kg). e Left ventricular sections from all treatment groups were stained for Caveolin-3 to visualize t-tubules. The density and proportions of transverse and longitudinally-oriented elements were determined using custom-made software in MatLab (see methods for details). Number of cells/animals analyzed: sham: n = 83 (rats n = 5), PBS: n = 82 (rats n = 5), control ASO n = 76 (rats n = 4), PLN-ASO 25 mg/kg n = 35 (rats n = 3), and PLN-ASO 50 mg/kg n = 54 (rats n = 5). A repeated measures model with one-sided paired contrasts was used to compare the mean differences between treatments and a control group (PBS). Dunnett’s test was used to adjust p values for multiple contrasts with the vehicle/reference group. For other data, one or two-way analysis of variance was used for analyses, with PBS treated animals as the reference group in multiple comparison analyses. Asterisk denotes significance level based on two-sided P values compared to PBS with: * P value < 0.05; ** P value < 0.01; *** P value < 0.001; **** P value < 0.0001. Single values are depicted, and error bars represent standard error of the mean (SEM). ASO antisense oligonucleotide, LAD left anterior descending artery, MRI magnetic resonance imaging. Source data are provided as a Source Data file.

Discussion
The PLN/SERCA2a interaction is a well-accepted therapeutic HF target, but to date no successful pharmacological strategy has been identified. Previous efforts have used different approaches, mostly focusing on gene therapy, since small-molecule approaches to specifically target the PLN-SERCA2a interaction have been challenging. In this study, we present a strategy using ASOs to decrease Pln mRNA. The resulting reductions in PLN protein expression prevented or reversed cardiac dysfunction in three different rodent models of HF and cardiomyopathy, and significantly increased lifespan in a model of hereditary PLN R14del cardiomyopathy.

Our limited understanding of the mechanisms involved in the complex spectrum of HF syndromes and specifically DCM is evident by the scarcity of effective treatments. A common pathophysiological hallmark however is aberrant Ca2+ handling in cardiomyocytes, which can include dysfunctional PLN and/or SERCA2a to a variable extent39. The contribution of aberrant Ca2+ cycling and a dysfunctional PLN/SERCA2a interaction differs between diseases. Whereas PLN is likely a key disease driver in carriers of pathological PLN variants, abnormal Ca2+ cycling is rather a consequential aggravating factor in common acquired heart disease, such as MI. In a murine transverse aortic constriction model, simulating cardiac pressure overload induced HF, Morihara et al. showed the short-term effects of a locked nucleic acid ASO targeting PLN administered via a single intravenous dose using a proprietary hydrodynamic delivery system23. A 6.5% improvement in FS after 1 week was observed in treated mice23. This minor and isolated improvement in FS was observed in a total of five mice, with no safety or efficacy data on repeated administrations, long-term follow-up or other clinically relevant models. The clinical tolerability and safety of this delivery solution and methodology is untested, as it is only used as a research tool. Conversely, we set out to evaluate a therapeutically relevant means to reduce PLN expression using eET-modified gapmer ASOs administered subcutaneously. Such ASOs, commonly referred to as generation 2.5, are currently under clinical evaluation across a number of different indications40. To robustly
test the efficiency and potential clinical translatability of PLN-ASO treatment in a variety of cardiac disease, covering the aforementioned aspects, we selected three different rodent HF models: two mouse DCM models, one driven by mutant PLN R14del, and the other driven by a Cspr3/Mlp\textsuperscript{−/−} model. PLN functional defects are causative to the pathology observed in PLN R14\textsuperscript{Δ/Δ}, early contributing to the pathology in Cspr3/Mlp\textsuperscript{−/−}, and consequential and aggravating in myocardial infarction. In line with previous studies, the beneficial effects of the PLN-ASO were most abundant in the PLN R14\textsuperscript{Δ/Δ} and Cspr3/Mlp\textsuperscript{−/−} models, showing almost complete prevention or reversal of disease phenotype. In our myocardial infarction model, the PLN-ASO restored cardiac dimensions and contractility, but not relaxation or left ventricular ejection fraction. ASO antisense oligonucleotide, ANP atrial natriuretic peptide.

The exact mechanism by which Cspr3/Mlp\textsuperscript{−/−} results in cardiac disease is still under investigation. However, Ca\textsuperscript{2+} handling abnormalities were shown to be present, and to contribute to the phenotype in Cspr3/Mlp\textsuperscript{−/−} mice in previous in vivo studies\textsuperscript{32,42}. Recently, engineered Cspr3/Mlp\textsuperscript{−/−} induced pluripotent stem cell-derived cardiomyocytes were shown to develop a hypertrophic and HF phenotype due to abnormal Ca\textsuperscript{2+} handling which could be improved by exposure to a L-type Ca\textsuperscript{2+} channel blocker\textsuperscript{43}. Although we did not observe impaired Ca\textsuperscript{2+} fluxes in the Cspr3/Mlp\textsuperscript{−/−} cardiomyocytes, the PLN-ASO improved Ca\textsuperscript{2+} handling as hypothesized. Correspondingly, the PLN-ASO normalized cardiac function and dimensions in vivo, comparable to earlier results describing the prevention of the Cspr3/Mlp\textsuperscript{−/−} cardiac phenotype when combined with the genetic deletion of Pln or DWORF, a SERCA2a activator, overexpression\textsuperscript{39,42}.

Ablation Ca\textsuperscript{2+} signaling and downregulation of SERCA2a has been established by many groups in the well-studied rat MI model. Interference with PLN/SERCA2a in MI models was reported to have some beneficial effects, mostly when used as a protective strategy and not reducing arrhythmias\textsuperscript{35,44,45}. Here, we aimed to intervene with the progression of LV remodeling, both at the whole-heart and subcellular level, after this process was already set in motion. Thus, we initiated PLN-ASO treatment 6 weeks post-MI, and tracked cardiac structure and function over the ensuing weeks. We observed that cardiac dimensions progressively increased over the course of the study in placebo-treated rats, and these changes were halted or even improved by PLN-ASO. In addition, impairment of cardiac contractility in post-MI hearts was significantly reversed by PLN-ASO. Cardiac relaxation was not improved, potentially highlighting the complexity of diastolic relaxation and its dependence on cardiomyocyte morphology and cardiac tissue composition alongside diastolic Ca\textsuperscript{2+} uptake\textsuperscript{46,47}. Interestingly, in agreement with a moderate stage of HF induced in placebo-treated rats, overall SERCA levels did not decline compared to sham control rats. These findings suggest that a supranormal SERCA/PLN ratio can be therapeutic in treating HF, even in the absence of SERCA depression and an advanced stage of the disease. Interestingly, improvement of cardiac contractility was linked to preservation of cardiomyocyte t-tubule structure. Indeed, t-tubule disruption is a well-established mechanism for impaired contractile function in HF, and pharmacological treatment of heart failure is frequently associated with t-tubule repair\textsuperscript{36}. Our current observations appear to be in agreement with previous work that has indicated that SERCA overexpression can directly repair t-tubule derangement in this disease\textsuperscript{47}. While the underlying mechanisms are unclear, recent work has indicated that declining SERCA expression and t-tubule structure are closely linked to the dilation of ventricular dimensions during HF progression\textsuperscript{38,48}. The current study supports this link, and indicates that increasing SERCA activity can reverse detrimental remodeling at both the whole-heart and subcellular level. We speculate these effects could be more pronounced with an earlier timed intervention, prior to the onset of extensive scarring remodeling. Compared to gene therapy utilized in earlier studies, these promising results in rodent models of MI, in PLN R14del related cardiomyopathy and DCM (Cspr3/Mlp\textsuperscript{−/−}), highlight the therapeutic potential of targeting cardiac PLN\textsuperscript{34,44,45}.

Improvements in cardiac function were achieved with a range of PLN reductions spanning 30–80% in Cspr3/Mlp\textsuperscript{−/−}, PLN R14\textsuperscript{Δ/Δ}, and post-MI rats (Figs. 2, 3, 4). This would be expected given previous observations of the gene dosage effects of decreased PLN expression and increased contractility in PLN intact, heterozygous and null mice\textsuperscript{49}. Understanding the therapeutic window of PLN inhibition remains speculative and will ultimately require careful dose titration studies in patients.
data demonstrate that mice with 50% PLN reductions maintain contractile reserve to β-adrenergic stimulation, and similar observations were made in post-MI rats. Due to the physiological differences in cardiac SERCA2a activity, Ca2+ cycling and cardiac reserve in rodents vs. humans, future studies in large animal models to define the therapeutic index of PLN inhibition will be necessary before proceeding to clinical studies.

There are several limitations to our approach. As ASOs do not permanently affect gene expression, as might be achieved with gene therapy, repeated subcutaneous administrations will be required for chronic treatment. We feel this is a beneficial attribute, as repeated dosing will allow careful titration of PLN suppression, as repeated dosing will allow careful titration of PLN sup-

Methods

Overall, our PLN-ASO experiments suggest potential to further develop such a therapeutic strategy as both a personalized medicine for PLN mutation carriers and as a more generalized treatment for patients with DCM or HF with reduced ejection fraction (HFrEF).

Antisense oligonucleotides. ASOs used in this report were 16mer (S)-constrained ethyl (cEt) modified 3′-3′ gapmers containing three cEt-modified ribonucleotides in each end and 2′-deoxyribonucleotides in the middle portion of the molecule with a phosphorothioate backbone as previously reviewed11. For palmitic acid conjugated ASOs, palmitate was conjugated at the 5′-terminus of the ASO with a phospho-

In vivo studies

Treatment of PLN R14Δ/Δ. Generation of PLN-R14Δ/Δ mice was previously described12. In short, the third, and coding, exon of the Pln gene was flanked by loxP sites (replacement, deletion, and reinsertion) and followed by a third exon of the Pln gene with the c.40-42del AGA mutation in a C57BL6/N mouse line. Mice were crossed with germline Cre-expressing mice, replacing the wild-type PLN exon-3 with the PLN-R14Δdel AGA mutation in a C57BL6/N mouse line. Mice were crossed with germline Cre-expressing mice, replacing the wild-type PLN exon-3 with the PLN-R14Δdel exon-3. The offspring were backcrossed into a C57BL6/J background for at least five generations. ASOs were formulated in PBS and subcutaneously injected into the neck compartment at a dose of 100 mg/kg on days 0, 7, 14, and 21 followed by 50 mg/kg on days 49, 77, 105, and 133 (see Fig.3a for dosing scheme). Mice were housed on a 12 h light/12 h dark cycle with free access to food and water, ambient temperature at 24 °C, and 45–65% humidity. Imaging and euthanasia were performed under 2–3% isoflurane (TEVA Pharmachemie, The Netherlands) anesthesia mixed with oxygen via an aerial dispenser. Heart and respiratory rate and body temperature were continuously monitored.

Cardiac MRI of PLN R14Δ/Δ mice. Mice were anesthetized with isoflurane (2%) and imaged in a vertical 9.4 T, 89-mm bore size magnet equipped with 1500 mT/m gradients and connected to an advanced 400 MR system (Bruker Biospin) using a quadrature-driven birdcage coil with an inner diameter of 3 cm. Respiration and ECG were continuously monitored and maintained at 20–60 breaths per minute and 400–600 bpm, respectively. Paravision 4.0 and IntraGate software (Bruker Biospin GmbH) were used for MRI acquisition and analysis. After MR acquisition and reconstruction, new experiments were performed on previously acquired material. The investigators were blinded to experimental settings during in vivo experiments, data acquisition and analysis. Equal ratios of male and female mice were used for all experiments. Mice were housed on a 12 h light/12 h dark cycle with ad libitum access to chow and water, ambient temperature at 20–24 °C and 45–65% humidity. Imaging and euthanasia were performed under 2–3% isoflurane (TEVA Pharmachemie, The Netherlands) anesthesia mixed with oxygen via an aerial dispenser. Heart and respiration rate and body temperature were continuously monitored.

Neonatal mouse cardiomyocyte isolation and culture. Neonatal mouse cardiomyo-

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Table 1 Antisense oligonucleotide sequences.

| ASO  | Sequence (5′-3′)                      |
|------|--------------------------------------|
| ASO26 | GATAATGGTACCTCCTG                  |
| ASO27 | GCATATCAATTCTCCTG                  |
| ASO136 | GTAACTTATATCTTGG                  |
| Control-ASO | GGCACCATGCCGCTCA        |

The sequences of the antisense oligonucleotides used for the in vivo experiments.
Echocardiography of PLN R141A mice. ECG recordings were acquired using two lead subdural needle electrodes, connected to a PowerLab 8/30 data acquisition device (model ML670, ADInstruments, Australia) and an animal Bio Amp biological potential amplifier (model ML136, ADInstruments) as previously reported \(^5\), RR, RR, QRS- and QT-intervals, 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).

Cap3/MlpKO mice mice maintenance, and study details. C57BL/6N Cap3/MlpKO/+/− males and females were used for several in vivo studies, see Supplementary Data 1 for experimental details. Animals were housed on a 12 h light/12 h dark cycle, ad libitum access to chow and water, ambient temperature at 21±22°C and 50% humidity. ASOs were formulated in PBS and subcutaneously injected into the flank or neck compartment at a dose of 100 µg/kg on Day 0 and 50 µg/kg on Days 7 and 14 for palmitate conjugated PLN-ASO, and at a dose of 100 µg/kg on Days 0, 7, 14, and 21 for parent PLN-ASO (see Fig. 3a for dosing scheme). The mice were randomly allocated to groups based on body weight, gender, age, and LVEF. For echocardiography measurements, animals with LVEF <45% were included in analysis. For hemodynamic and RT-PCR analysis, the entry criteria for mice were high plasma ANP levels (>119 ng/ml), followed by randomization into two treatment groups based on EF% baseline levels (Supplementary Table 1).

Echocardiography of Cap3/MlpKO mice. Mice were anesthetized using 2.5% isoflurane (Forene®) mixed to air and then maintained on 1.5–2% isoflurane during the assessment. During the examination the mice were kept on a Physo Plate (Vevo 2100 System, Visualsonics, Canada). An LV long-axis parasternal B-mode in long-axis view was captured, followed by a 90° clockwise rotation of the ultrasound probe to adjust the level just caudal to the mitral leaflet to obtain short-axis B-MODE sequences and motion-mode (M-MODE). Anterior and posterior wall thickness as well as end-diastolic LV dimension was measured using the American Society of Echocardiography leading edge. End diastolic was defined as the R-tag in the ECG signal. LV wall thickness was calculated as the average of the anterior and the posterior wall thickness. LVM was calculated based on the uncorrected cubic formula: LVM = 1.05 × [(Pp + AED) × (EDD)] − EW; where Pp and A indicate Posterior and anterior wall thickness, respectively, and EDD is the end-diastolic LV diameter. FS and EF were calculated from short-axis MMODE measurements as follows: FS (%) = 100 × ((LVIDd − LVIDs)/LVIDd; EF(%)) = 100 × ((LVIDd − LVIDs)/LVIDd); CO (ml/min) = EF × LVIDd × heart rate/100. LVIDd and LVIDs are LV internal dimensions (LVIDd during diastole and systole, respectively. All measurements were calculated from three consecutive cardiac cycles with animal’s treatment group blinded to the sonographer. The animals were euthanized following echocardiographic examination. The hearts were punctured for a terminal blood sample using 1 mL syringes. Mice were sacrificed by an infusion pump, at a rate at 0, 0.75, 2, 4, 6, and 8 μg/min. When a stable and reproducible pressure reading was obtained, LV pressure and heart rate became stable, dobutamine was given intravenously by an infusion pump, at a rate of 80 μg/kg/min. Heart rate and blood pressure were recorded and averaged from 12 beats. The body temperature was maintained at 37°C with external heating both from the table and from a heating lamp during the experiment.

T-tubule imaging and analysis. Myocardial rat sections were first de-paraffinized (heating at 60°C for 60 min) and then submerged in Histo Clear Tissue Clearing Agent (National Diagnostics) for 2 x 3 min. Thereafter, sections were sequentially deparaffinized in xylene (100%) and xylene (50%), followed by a 20 min incubation in 100% ethanol. The sections were then incubated in 95% ethanol and 70% ethanol and then submerged in Histo Clear Tissue Clearing Agent (National Diagnostics). Antigen retrieval was performed by submerging sections in boiling sodium citrate buffer (pH 6.0), followed by incubation with 0.3% hydrogen peroxide in methanol for 10 min, and then washed with DP/dt max/min, ESP, EDP and Tau were measured using ImageJ (version 1.48) and ImageJ (version 1.48).

Hemodynamic measurements of rats. At study week 10–11, the animals were anesthetized with isoflurane (Attane vet, VM Pharma AB, Stockholm, Sweden) and the rats were subjected to left thoracotomy at the fifth intercostal space –2 to 3 mm to the left of the sternum. A rib spreader was used to keep the incision open. The pericardium was then opened and a ligature with a 7–0 suture was placed around the left coronary artery and the artery was occluded by tying the ligature. Induction of ischemia was confirmed by observed paleness of the heart distal from the ligature and ST-elevation of the ECG signal. After the ischemia the rat was monitored during continued maintenance of body temperature and ventilation until it regained consciousness and could be disconnected. Six weeks post LAD occlusion rats were randomized into four treatment groups (PBS, Control, Acetylcholine 0.25 mg/kg, PLN ASO 50 mg/kg) and treatment was initiated. Randomization was based on plasma cTnI levels one day post-infarction (LVF% to cTnI correlation was used to estimate LVF%), as well as visual infarct size score55, BW, and HR at baseline (6 weeks post MI). Parent rat PLN-ASO and Control ASO were formulated in PBS, and were subcutaneously injected into the neck compartment at a dose of 30 mg/kg (PLN-ASO, Control-ASO) or 25 mg/kg (PLN-ASO) twice weekly at week 6 and 7, and once weekly at week 8–11 of study (see Fig. 3a for dosing scheme).

Magnetic resonance imaging of rats. All MRI scanning was performed on a 4.7-T Bruker Biospec (Bruker BioSpin GmbH, Germany) using a 72-mm quadrature coil (Rapid Biomedical GmbH, Germany). Animals were placed in the supine position and exposed to inhalation anesthesia (Isoba® isoflurane, Schering-Plough Ltd, England). Physiological monitoring included temperature assessments with a rectal probe, respiration rate and ECG evaluation via the SA instruments platform (SA Instruments, Inc., USA). Animals temperature was maintained at 37.0 ± 0.2 °C via a near infrared laser and 1% isoflurane and respiration rate was kept at 20 ± 2 breaths per minute. Twelve short-axes CINE time series (temporal resolution 8 ms) covering the left ventricle and two and perpendicularly-long-axis slices were acquired for the assessment of systolic function using a gated gradient-echo imaging sequence. The other sequence parameters were a 2.1-ms echo time, 25° flip angle, 128 × 192 image matrix, 200-KHz/pixel bandwidth, 1.5 mm slice thickness, and 40 × 60 mm 2 field-of-view. The left ventricles were manually delineated by an expert, and LVEF, LVESV, and LVEDV were derived via the post-processing software package Segment (Medviso AB, Sweden).

Hemodynamic measurements of rats. At study week 10–11, the animals were anesthetized with isoflurane (Attane vet, VM Pharma AB, Stockholm, Sweden) gas (5%) in a gas chamber and kept anesthetized by breathing isoflurane gas through a mask. A catheter (plastic tubing PE 10 connected to a PE 50, Intramedic® polyethylene tubing, Becton Dickinson, Sparks, MD, USA) was inserted in the right femoral artery for registration of mean arterial blood pressure (MAP) and heart rate (HR). The arterial catheter was flushed with 10 µL/min of saline (9 mg/ml, Fresenius Kabi AG, Bad Homburg, Germany) throughout the experiment to maintain patency. An electrocardiograph (ECG) was recorded from skin electrodes. Signals from ECG, MAP, and HR were recorded and sampled by using a computer and software (PharmLab V6.6, AstraZeneca R&D Mölndal, Sweden. Ventricular function was measured via a pressure catheter (1.4F, SPR-847, Millar Instruments, Houston, TX, USA), inserted via the right carotid artery and fixed by a miniaturized Doppler (Fresenius Kabi AG, Bad Homburg, Germany) or neck compartment at a dose of 30 mg/kg (PLN-ASO, Control-ASO) or 25 mg/kg (PLN-ASO) twice weekly at week 6 and 7, and once weekly at week 8–11 of study.

Heart weight density was then calculated for the cell interior, de

(CV125, version 5.6.6, Circle Cardiovascular Imaging, Canada) was used for the determination of the LV end-diastolic volume, LV end-systolic volume, stroke volume, and ejection fraction (EF), as described53.
qPCR. Total RNA from cells and tissues were isolated using TRIzol Reagent (Thermo Fisher Scientific). cDNA was constructed using the Quantitect RT kit (Qiagen). Relative gene expression was determined by quantitative real-time PCR (qRT-PCR) on a BioRad CFX384 real-time system using Absolute qPCR SYBR Green mix (Thermo Fisher Scientific) or Taqman assay. Gene expression levels were corrected for reference gene expression (36B4), and relative ratios compared to the experimental control group are expressed. The primers used are listed in Supplementary Data 2.

Western blot. Cells and tissues were homogenized in ice-cold RIPA (50 mM Tris pH 8.0, 1% nonidet P40 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl or ThermoFisher, 89900) or urea containing phosphatase inhibitor cocktail 3 (Sigma-Aldrich) and protease inhibitor (Roche Diagnostics or ThermoFisher, 78440) and 15 mM Na3Vodate in selected experiments. Protein concentrations were determined with a DC protein assay kit (BioRad) or BCA protein Assay Kit (Pierce, 23225). Equal amounts of protein were loaded on 10% polyacrylamide gels or a Mini Protein precast gel (Biorad, 4561085). After electrophoresis, the gels were blotted onto PVDF or nitrocellulose membranes. Membranes were then blocked and incubated (night at 4 °C with the primary antibody, followed by 1 h incubation at room temperature with an HRP secondary antibody. Detection was performed by ECL and analyzed using ImageJ version 2.0.0. Protein quantifications were normalized to total protein staining (Revert 700) or GAPDH protein expression levels. Primary antibodies used: anti-PLN monoclonal (clone 2D12, #MA3-922, Invitrogen) (1:200) labeled with Alexa Fluor 555 (red) using an APEX antibody labeling kit (Invitrogen) according to the manufacturer’s protocol. Sections were co-stained for cardiac troponin I (Abcam 47003 1:100) and DAPI (Vector Laboratories, CA, USA) to stain nuclei blue.

Plasma measurements. For the Pln R414del studies, Troponin I was quantified using a Sandwich ELISA (LSBio, LS-F24180) in 10× diluted plasma according to manufacturer protocol. AST and ALT were quantified in plasma diluted 4× with NaCl 0.9% according to IFCC with pyridoxal phosphate activation, on a Cobas 6800 (Roche, Mannheim, Germany). For the other studies, AST and ALT were measured on a clinical analyzer (Olympus, Center Valley, PA). ANP was measured following plasma extraction via a competitive ELISA, as described in Catalog # ELAANP (ThermoFisher, CA).

RNA sequencing. RNA was extracted from pulverized LV tissue using Lysis matrix D beads (MP Biomedicals) and standard TRIzol extraction (ThermoFisher scientific), RNA quality was determined using RNA Pico Chips on Bioanalyzer 2100 (Agilent) and Truseq stranded mRNA libraries (Illumina) were generated from high quality total RNA (RNA integrity number >8.0). Samples were subjected to single end sequencing on Next Seq 500 platform (Illumina). Reads were aligned to mouse reference genome (mm10) using STAR 2.4.2a58 and read count analysis to single end sequencing on Next Seq 500 platform (Illumina). Reads were aligned to mouse reference genome (mm10) using STAR 2.4.2a58 and read count analysis

Data availability
The codes for the RNA-sequencing analyses are available as Supplementary Data 4, 5 and the codes for the T-tubule analysis in Matlab are available at: https://gitlab.com/louch-group/t-tubules-script.

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References
1. Chang, P. P. et al. Trends in hospitalizations and survival of acute decompensated heart failure in four US communities (2005–2014). Circulation 138, 12–24 (2018).
2. Mamas, M. A. et al. Do patients have worse outcomes in heart failure than in cancer? A primary care-based cohort study with 10-year follow-up in Scotland. Eur. J. Heart Fail. 19, 1095–1104 (2017).
3. Dagenais, G. R. et al. Variations in common diseases, hospital admissions, and deaths in middle-aged adults in 21 countries from five continents (PUIF): a prospective cohort study. Lancet 320, 32007-0 (2019).
4. Gheorgheida, M. et al. Developing new treatments for heart failure: focus on the heart. Circ. Heart Fail. 9, 1–8 (2016).
5. Bers, D. M. Calcium cycling and signaling in cardiac myocytes. Annu. Rev. Physiol. 70, 23–49 (2008).
6. Eisner, D. A., Caldwell, J. L., Kistamás, K. & Trafford, A. W. Calcium and excitation-contraction coupling in the heart. Circ. Res. 121, 181–195 (2017).
7. Kranias, E. G. & Hajjar, R. J. Modulation of cardiac contractility by the phospholamban/SERCA2a regulatome. Circ. Res. 110, 1646–1660 (2012).
8. Minamisawa, S. et al. Chronic phospholamban-sarcoplasmic reticulum calcium atpase interaction is the critical calcium cycling defect in dilated cardiomyopathy. Cell 99, 313–322 (1999).
9. Meyer, M. et al. Alterations of sarcoplasmic reticulum proteins in failing human dilated cardiomyopathy. Circulation 92, 778–784 (1995).
10. Helms, A. S. et al. Genotype-dependent and -independent calcium signaling dysregulation in human hypertrophic cardiomyopathy. Circulation 134, 1738–1748 (2016).
11. MacLennan, D. H. & Kranias, E. G. Phospholamban: a crucial regulator of cardiomyopathy. Nat. Rev. Mol. Cell Biol. 4, 566–577 (2003).
12. Miyamoto, M. I. et al. Adenoviral gene transfer of SERCA2A improves left-ventricular function in aortic-banded rats in transition to heart failure. Proc. Natl Acad. Sci. U.S.A. 97, 793–798 (2000).
13. Kawase, Y. et al. Reversal of cardiac dysfunction after long-term expression of SERCA2a by gene transfer in a pre-clinical model of heart failure. J. Am. Coll. Cardiol. 51, 1112–1119 (2008).
14. del MonteF et al. Restoration of contractile function in isolated cardiomyocytes from failing human hearts by gene transfer of SERCA2a. Circulation 100, 2308–2311 (1999).
15. Greenberg, B. et al. Calcium upregulation by percutaneous administration of gene therapy in patients with cardiac disease (CUPID 2): a randomised, multinational, double-blind, placebo-controlled, phase 2b trial. Lancet 387, 1178–1186 (2016).
16. Hoshijima, M. et al. Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via in vivo cardiac rAAV gene delivery. Nat. Med. 8, 864–871 (2002).
17. Haghighi, K. et al. A mutation in the human phospholamban gene, deleting arginine 14, results in lethal, hereditary cardiomyopathy. Proc. Natl Acad. Sci. U.S.A. 103, 1388–1393 (2006).
18. von der Zwaag, P. A. et al. Phospholamban R14del mutation in patients diagnosed with dilated cardiomyopathy or arrhythmogenic right ventricular cardiomyopathy: evidence supporting the concept of arrhythmogenic cardiomyopathy. Eur. J. Heart Fail. 14, 1199–1207 (2012).
19. Fry, D. C. Targeting Protein-Protein Interactions for Drug Discovery. In Protein-Protein Interactions: Methods and Applications (eds Meyerkord, C. L. & Fu, E.) 93–106 (Springer New York). https://doi.org/10.1007/978-1-4939-2425-7_6 (2015).

Statistics. Distinct samples were used for all analyses. All data are represented as means ± standard error of the mean (SEM). Two sample $t$ test were used to compare two groups with normal distributions, and Wilcoxon rank-sum for 2 group comparisons when non-normally distributed. In case of more than two group comparisons, one-way or two-way ANOVA with post hoc Bonferroni tests were utilized, unless mentioned otherwise. All analyses were carried out using GraphPad Prism software version 8.02 (GraphPad Software Inc.) or STATA version SE 16.0 (StataCorp). All reported $P$ values are two-sided unless otherwise noted, a two-sided $P$ value of $<0.05$ was considered statistically significant. All exact $P$ values are listed in Supplementary Data 3.
20. Levin, A. A. Treating Disease at the RNA Level with. Oligonucleotides. N. Engl. J. Med. 380, 57–59 (2019).

21. Prakash, T. P. et al. Glycine acid conjugation enhances potency of antisense oligonucleotides in muscle. Nucleic Acids Res. 47, 6029–6044 (2019).

22. Østergaard, M. E. et al. Conjugation of hydrophobic moieties enhances potency of antisense oligonucleotides in the muscle of rodents and non-human primates. Nucleic Acids Res. 47, 6045–6058 (2019).

23. Mehta, H. R. et al. Phospholamban Inhibition by a single dose of locked nucleic acid antisense oligonucleotide improves cardiac contractility in pressure overload-induced systolic dysfunction in mice. J. Cardiovasc. Pharmacol. Ther. 22, 273–282 (2017).

24. van Rijssing, I. A. W. et al. Outcome in phospholamban R14del carriers: results of a large multicentre cohort study. Circ. Cardiovasc. Genet. 7, 455–465 (2014).

25. Kawai, H. et al. Correction of human phospholamban R14del mutation associated with cardiomyopathy using targeted nucleases and combination therapy. Nat. Commun. 6, 6955 (2015).

26. te Rijdt, W. P. et al. Phospholamban p.Arg14del cardiomyopathy is characterized by phospholamban aggregates, aggresomes, and autophagic degradation. Histopathology 69, 542–550 (2016).

27. Eiggenaarm, T. et al. A novel mouse model of phospholamban p(Arg14del)-associated cardiomyopathy develops heart failure and is unresponsive to standard heart failure therapy. Sci. Rep. (2020) (in the press).

28. Mekahli, D., Bulletyn, G., Parys, J. B., De Smedt, H. & Missiaen, L. Endoplasmic-reticulum calcium depletion and disease. Cold Spring Harb. Perspect. Biol. 3, 1–9 (2011).

29. Arber, S. et al. MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. Cell 88, 393–403 (1997).

30. Knoll, R. et al. The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy. Circ. Res. 111, 943–955 (2012).

31. Knoll, R. et al. A common MLP (muscle LIM protein) variant is associated with cardiomyopathy. Circ. Res. 106, 695–704 (2010).

32. Makarewich, C. A. et al. The DWRGF micropeptide enhances contractility and prevents heart failure in a mouse model of dilated cardiomyopathy. Elife 7, 1–23 (2018).

33. Haydald, M. A. et al. Human cardiomyocyte calcium handling and transverse tubules in mid-stage of post-myocardial-infarction heart failure. ESC Heart Fail. 5, 332–342 (2018).

34. Heinzel, F. R. et al. Remodeling of T-tubules and reduced synthetic of Ca2+-release in myocytes from chronically ischemic myocardium. Circ. Res. 102, 338–346 (2008).

35. Iwanaga, Y. et al. Chronic phospholamban inhibition prevents progressive cardiac dysfunction and pathological remodeling after infarction in rats. J. Clin. Investig. 113, 727–736 (2004).

36. Jones, P. P., MacQuaide, N. & Louch, W. E. Dyadic plasticity in cardiomyocytes. Front. Physiol. 6, 1773 (2018).

37. Lyon, A. R. M. Architecture of myocardial structures and β2-adrenergic receptor localization in failing ventricular cardiomyocytes during recovery from heart failure. Circ. Heart Fail. 5, 357–365 (2012).

38. Frisk, M. et al. Elevated ventricular wall stress disrupts cardiomyocyte t-tubule structure and calcium homeostasis. Cardiovasc. Res. 112, 443–451 (2015).

39. Luo, M. & Anderson, M. E. Mechanisms of altered Ca2+ handling in heart failure. Circ. Res. 113, 690–708 (2013).

40. Crooke, S. T., Liang, X.-H., Crooke, R. M., Baker, B. F. & Geary, R. S. Anti-sense drug discovery and development technology considered in a pharmacological context. Biochem. Pharmacol. 114196 https://doi.org/10.1016/j.cbpc.2020.114196 (2020).

41. Hof, I. E. et al. Prevalence and cardiac phenotype of patients with a phospholamban mutation. Neth. Heart J. 27, 64–69 (2019).

42. Hoshijima, M., Knoll, R., Pashmforoush, M. & Chien, K. R. Reversal of cardiomyopathy in failing ventricular cardiomyocytes during recovery from heart failure with a preserved ejection fraction: contributions of collagen and titin. Circulation 131, 1247–1259 (2015).

43. Zile, M. R. et al. Myocardial stiffness in patients with heart failure and a preserved ejection fraction: contributions of collagen and titin. Circulation 131, 1247–1259 (2015).

44. Roe, A. T. et al. Regional diastolic dysfunction in post-infarction heart failure: role of local mechanical load and SERCA expression. Cardiovasc. Res. 115, 752–764 (2019).

45. Løken, R. N. et al. Comprising the SEBTF Southern European Biogeriatric Research Platform. Scand. J. Respir. 30, 1–8 (2016).

46. Crooke, S. T., Witztum, J. L., Bennett, C. F. & Baker, B. F. RNA-Targeted Therapeutics. Cell Metab. 27, 714–739 (2018).

47. Klijstra, J. D. et al. Integrated analysis of contractile kinetics, force generation, and electrical activity in single human stem cell-derived cardiomyocytes. Stem Cell Rep. 5, 1226–1238 (2015).

48. Boujel, M. G. & Riascos, E. Optimization of A kinase interacting protein 1 attenuates myocardial ischaemia/reperfusion injury but does not influence heart failure development. Cardiovasc. Res. 111, 217–226 (2016).

49. van der Pol, A. et al. OPLAH ablation leads to accumulation of 5-oxoproline, oxidative stress, fibrosis, and elevated filling pressures: a murine model for heart failure with preserved ejection fraction. Cardiovasc. Res. 114, 1–18 (2018).

50. Colucci, W. & Braunwald, E. Atlas of Heart Failure. (Current Medicine Group, 2002).

51. Frisk, M. et al. Variable t-tubule organization and Ca2+-homeostasis across the atria. Am. J. Physiol. Heart Circ. Physiol. 307, H609–H620 (2014).

52. Van der Pol, A. et al. Accumulation of 5-oxoproline in myocardial dysfunction and the protective effects of OPLAH. Sci. Transl. Med. 9, eaam8574 (2017).

53. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–23 (2013).

54. Anders, S., Pyl, P. T. & Huber, W. HTSeq-a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169 (2015).

55. Liu, M. I., Huber, W. & Irizarry, R. A. Estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550 (2014).

56. Sergusheicz, A. A. An algorithm for fast prernanked gene set enrichment analysis using cumulative statistic calculation. bioRxiv 060012. https://doi.org/10.1101/060012 (2016).

57. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl Acad. Sci. U.S.A. 102, 15545–15550 (2005).

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Author contributions

N.G.B.: designed and conducted the PLN R14del studies, acquired funding, analysed and interpreted the data and wrote the paper. D.S.: designed and supervised Csp3/Mlp−/− mice studies 1 and 2, and the adult cardiomyocyte calcium imaging, as well as the rat MI study. Additionally, Kirk Peterson, Nancy Dalton, and Yusu Gu of the Sea—World Park contributed a great deal to the cardiac imaging. Funding for this work was provided by the National Institutes of Health (NIH), the National Heart, Lung, and Blood Institute (NHLBI), the American Heart Association (AHA), the Diabetes Foundation (DFG), and the Swedish Research Council.

Conflicts of interest

The authors declare no competing interests.
performed and analyzed echocardiography of Cspr3/Mlp−/− study #1, and performed rat MI surgery. T.A.: designed, and performed rat MI surgery. M.Z.: designed, performed, and analyzed MRI measurements for rat MI study. S.P.: designed, performed, and analyzed hemodynamic measurements for rat MI study. T.R.E.: assisted in part of the PLN R14del studies and analysed and interpreted data. N.B.: assisted in part of the PLN R14del studies, interpreted data. M.F.H.: performed experiments and interpreted data. C.J.B.: performed RNA-sequencing experiments, analyzed, and interpreted data. M.I.F.: performed T-tubule analysis, analyzed, and interpreted data. E.M.R.: contributed to the experimental design of the RNA-sequencing experiments and interpreted the data. S.D.: performed RNA-sequencing experiments, analyzed, and interpreted data. W.E.L.: contributed to the experimental design of the T-tubule analysis and post-MI rat study, interpreted data. Q.D.W.: contributed to experimental design and data interpretation of Cspr3/Mlp−/− in vivo studies and rat MI study. K.R.C.: contributed to experimental design and data interpretation of all in vivo studies. R.F.D.: contributed to experimental design and data interpretation of Cspr3/Mlp−/− in vivo studies and rat MI study, secured funding. K.H.: designed PLN R14del, Cspr3/Mlp−/−, and rat MI study, acquired funding, and interpreted data. R.K.: designed and supervised Cspr3/Mlp−/− studies and interpreted data. A.E.M.: designed and supervised PLN ASO lead identification, Cspr3/Mlp−/− studies, designed PLN R14del study, acquired funding, interpreted data, and wrote the paper. R.B.: designed the PLN R14del mouse model, acquired funding, and interpreted data. P.v.d.M.: designed and supervised the PLN R14del studies, acquired funding, interpreted the data, and wrote the paper. All authors discussed the results and critically revised the paper.

**Competing interests**

A.H., Z.E., H.S., C.J.B., M.F., E.R. and W.E.L. declare no competing interest. D.S., K.R., A.H., M.Z., M.P., S.P., T.A., Q.W., R.F. and K.H. are employees of AstraZeneca. S.Y., S.D. and A.E.M. are employees of Ionis Pharmaceuticals. N.G.B., T.R.E., H.S., N.B., M.F.H., R.A.B. and P.v.d.M. are employees of the UMCG which received research grants and/or fees from AstraZeneca, Abbott, Bristol-Myers Squibb, Novartis, Novo Nordisk, and Roche. K.R.C. is a member of the Scientific Advisory Board and receives research support from AstraZeneca, and is a Co-Founder and Equity holder in Moderna Therapeutics. R.d.B. received speaker fees from Abbott, AstraZeneca, Novartis, and Roche.

**Additional information**

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