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Phosphoproteomics distinguishes disease-specific mechanisms for human phospholamban cardiomyopathy reversible by RNA therapy

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Phospholamban (PLN) p.Arg14del (R14^{Δ/+}, also known as R14del) is a pathogenic variant that causes inherited cardiomyopathy. RNA therapy improves cardiac function and survival in murine PLN R14^{Δ/+}. However, the molecular disease mechanisms and potential therapeutic effects of RNA therapy in the human setting remain poorly defined. Proteomic and phosphoproteomic profiling was performed on cardiac tissue from R14^{Δ/+} patients ($N=6$) and compared to other causes of dilated cardiomyopathy (DCM; $N=10$). Findings were validated in CRISPR-Cas9-engineered R14^{Δ/+} induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) and isogenic controls. To assess reversibility, PLN-targeted RNA therapy using antisense oligonucleotides was applied to iPSC-CMs. Proteomics revealed enrichment of fibrotic pathways, while phosphoproteomics highlighted altered actomyosin structural organization uniquely distinguishing R14^{Δ/+} from other DCM. This phosphoproteomic profile was recapitulated in R14^{Δ/+} iPSC-CMs. RNA therapy concentration-dependently reduced PLN expression and modified the disease-specific phosphorylation profile. Twenty-eight phosphorylation sites were consistently altered across patient tissue and iPSC-CMs; twenty-two were reversed by RNA therapy and were enriched for cadherin- and actin-binding functions, implicating cytoskeletal remodeling. PLN/LC3 protein aggregates, a hallmark of PLN cardiomyopathy, were reduced after RNA therapy. Functionally, R14^{Δ/+} cardiomyocytes exhibited accelerated calcium handling and contractile kinetics, which increased further upon RNA therapy. Human PLN R14^{Δ/+} cardiomyopathy is characterized by a distinct phosphoproteomic signature involving cytoskeletal and contractile machinery. PLN-targeted RNA therapy reduced PLN expression, partially normalized these alterations, diminished protein aggregation, and enhanced calcium handling and contractile performance. These findings clarify the molecular mechanisms underlying R14^{Δ/+} pathogenesis and support RNA therapy as a promising therapeutic strategy for PLN cardiomyopathy.

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INTRODUCTION

Phospholamban (PLN) is a key regulator of cardiac contractility through its inhibition of sarco/endoplasmic reticulum Ca²⁺-ATPase 2a (SERCA2a)-mediated calcium reuptake.¹ The most prevalent pathogenic PLN variant, p.Arg14del (R14^{Δ/+}, also known as R14del), causes dilated and arrhythmogenic cardiomyopathy, characterized by substantial variability in disease presentation and progression, and associated with a high risk of heart failure and sudden cardiac death.^{2–4} Despite its clinical severity, standard heart failure therapy offers limited benefit.^{5,6} At the cellular level, PLN R14^{Δ/+} cardiomyopathy is characterized by impaired contractility, metabolic dysregulation, and calcium-handling defects,^{7–10} accompanied by sarcoplasmic reticulum (SR)-associated PLN aggregation.^{11–13} Although these features are well described, the molecular mechanisms that link the PLN R14^{Δ/+} variant to downstream cellular dysfunction remain incompletely understood. Notably, disease phenotypes appear highly species dependent, underscoring the

importance of studying human patient-derived material to resolve clinically relevant mechanisms.¹⁴

RNA sequencing and global proteomics are often the starting point for defining molecular disease signatures, as they provide comprehensive insight into transcriptional programmes and protein abundance.^{4,10,15} However, neither approach captures posttranslational regulation, which frequently represents the immediate and functionally decisive layer of cellular control. Posttranslational modifications, particularly phosphorylation, enable rapid and reversible modulation of protein function in response to changing cellular conditions. In cardiomyocytes, where excitation-contraction coupling operates on a beat-to-beat basis, such dynamic regulation is essential for maintaining contractile performance and adapting to physiological stress.^{1,16} This limitation is particularly relevant in PLN R14^{Δ/+} cardiomyopathy, where disturbed intracellular calcium handling is a hallmark feature and many cardiac kinases and phosphatases are directly

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calcium-dependent.^{5,17} Given that calcium signaling tightly regulates excitation-contraction coupling, even subtle alterations in kinase and phosphatase activity can have profound effects on cardiomyocyte function.^{5,17} Consequently, pathogenic signaling alterations may arise without detectable changes at the RNA or total protein level, potentially obscuring key regulatory mechanisms that drive disease progression. This limitation necessitates approaches that directly assess posttranslational regulatory mechanisms.

Phosphoproteomics provides a complementary approach by enabling direct quantification of phosphorylation events that regulate cardiomyocyte contraction, metabolism, and cytoskeletal dynamics.^{18,19} By capturing dynamic, calcium-responsive signaling networks, phosphoproteomic profiling can reveal how perturbations in calcium homeostasis propagate to downstream functional pathways. Phosphorylation of key sarcomeric proteins, including components of the actomyosin complex, directly influences contractile force generation and relaxation kinetics. In addition, cytoskeletal organization is tightly regulated by phosphorylation-dependent processes that govern cellular structure and mechanical stability.^{16,20–22} Integrating phosphoproteomics with global proteomics therefore offers a powerful strategy to uncover disease-specific signaling alterations that remain undetected by transcriptomics or proteomics alone. In the context of PLN R14^{Δ/+} cardiomyopathy, such an approach is particularly suited to identify mechanistic links between altered calcium handling, cytoskeletal remodeling, and contractile dysfunction, thereby providing a more comprehensive understanding of disease pathogenesis.

RNA therapeutics have recently emerged as a therapeutic platform for cardiovascular disease, with clinically validated success in hypercholesterolemia, muscular dystrophy and trans-thyretin amyloidosis.²³ These therapies enable selective suppression of pathogenic mRNAs and represent a promising strategy for pathogenic variant-targeted treatment of cardiomyopathy. Currently, RNA therapy is not yet applied to treat inherited cardiomyopathies. Previous studies demonstrated that PLN-targeted RNA therapy using PLN antisense oligonucleotides (PLN-ASOs) improved cardiac function and survival in PLN R14^{Δ/+} murine models.²⁴ However, RNA therapeutics are inherently species specific, and the effects of the PLN R14^{Δ/+} variant are highly model specific,¹⁴ necessitating validation in human models. To address this, we investigated the effects of human-specific PLN-ASOs in induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) carrying the PLN R14^{Δ/+} variant. To date, the integration of phosphoproteomic profiling with human patient-derived cardiac tissue and RNA therapeutic intervention has not been systematically explored in PLN cardiomyopathy. In this study, we combined global proteomic and phosphoproteomic profiling of PLN R14^{Δ/+} patient-derived cardiac tissue with analyses in CRISPR-Cas9-engineered induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) to define disease-associated signaling alterations. Furthermore, we assessed whether these molecular signatures are reversible upon treatment with human-specific PLN-targeted RNA therapy. By integrating patient-derived tissue, human cellular models, and phosphoproteomic analysis, this work provides mechanistic insight into PLN R14^{Δ/+} pathogenesis and evaluates the therapeutic potential of RNA therapy in PLN cardiomyopathy.

RESULTS

PLN R14^{Δ/+} patient heart tissue displays a distinct, phosphorylation-driven disease signature
To define the molecular consequences of PLN R14^{Δ/+} cardiomyopathy in human tissue, we first performed global proteomics on left ventricular (LV) samples (Table 1). Mean age did not differ between groups (R14^{Δ/+} vs. DCM: 53.2 years [95% CI 42.2–64.1] vs. 48.1 years [95% CI 38.4–57.8]; Welch's *t* test *p* = 0.42). Left

ventricular ejection fraction did not differ between groups (R14^{Δ/+} vs. DCM: 16.8% [95% CI 10.5–23.2] vs. 18.6% [95% CI 16.1–21.1]; Welch's *t* test *p* = 0.53), indicating comparable end-stage disease severity. The sex distribution differed between groups (R14^{Δ/+} vs. DCM: 17% vs. 80% male; Fisher's exact test *p* = 0.035). Across 4775 quantified proteins, 246 were differentially expressed between PLN R14^{Δ/+} patients (*N* = 6) and other genetic forms of DCM (*N* = 10; Fig. 1a, b). Enrichment analysis highlighted extracellular matrix organization and collagen fibril formation (GO:0030198, *P* = 4.63E-10; GO:0030199, *P* = 3.39E-08; Fig. 1c), consistent with a fibrosis-associated remodeling phenotype. These findings reflect structural consequences of the disease but do not fully explain the contractile and calcium-handling defects characteristic of PLN R14^{Δ/+} cardiomyopathy.

To capture regulatory mechanisms not resolved by protein abundance alone, we next profiled the phosphoproteome. Phosphoproteomics revealed a markedly broader molecular footprint, with 1507 differentially expressed phosphorylated sites (DEPSs) across 740 proteins (Fig. 1d). The most enriched pathways, actomyosin structure organization (GO:0031032, *P* = 3.09E-15) and regulation of cardiac muscle contraction (GO:0086004, *P* = 3.61E-11; Fig. 1e), directly mapped to cytoskeletal integrity, contractile function, and calcium-responsive signaling. Several key contractile and Ca²⁺-handling proteins showed differential phosphorylation, including hypophosphorylation of PLN, HRC, RYR2, and SYNPO2L and hyperphosphorylation of PKP2, DES, and JPH2 (Fig. 1f). These changes suggest impaired excitation-contraction coupling and cytoskeletal remodeling, core processes predicted to be sensitive to altered calcium dynamics in PLN R14^{Δ/+} cardiomyopathy.

The phosphoproteomic disease signature is recapitulated in R14^{Δ/+} iPSC-CMs

To confirm that these phosphorylation-dependent alterations reflect pathogenic variant-driven biology rather than end-stage remodeling alone, we performed matched proteomic and phosphoproteomic profiling in CRISPR-Cas9 engineered R14^{Δ/+} iPSC-derived cardiomyocytes (iPSC-CMs) and isogenic controls (Fig. 2a). R14^{Δ/+} iPSC-CMs exhibited 445 differentially expressed proteins (DEPs; Fig. 2b) enriched for glycolysis (GO:0006096, *P* = 3.60E-05) and GTPase signaling (Fig. 2c), consistent with metabolic adaptations observed in patient tissue.

Strikingly, phosphoproteomics again revealed more extensive alterations, with 1757 DEPSs (Fig. 2d). Enriched pathways included chromatin organization (GO:0006325, *P* = 5.94E-08), actomyosin structure organization (GO:0031032, *P* = 1.07E-07), and regulation of cardiac conduction (GO:0086091, *P* = 1.72E-07; Fig. 2e). Differential phosphorylation of key contractile and calcium-handling proteins, such as hyperphosphorylation of SCN5A, SORBS1, LMOD2, and EPB41L3 and hypophosphorylation of RYR2, PKP2, ANK2, TTN, OBSCN, and SYNPO2L, mirrored the cytoskeletal and contractile abnormalities detected in patient tissue (Fig. 2f).

Together, these findings show that phosphoproteomics identifies a robust, variant-specific signaling signature shared across patient tissue and iPSC-CMs, linking PLN dysfunction to disrupted calcium-dependent cytoskeletal and contractile pathways.

RNA therapy reverses disease-associated phosphorylation changes linked to cytoskeletal organization

To assess whether targeting PLN can reverse the signaling abnormalities underlying these phenotypes, R14^{Δ/+} iPSC-CMs were treated with PLN-targeted RNA therapy (Fig. 3a). RNA therapy reduced PLN mRNA (Fig. 3b; PLN-ASO specificity is reported in Supplementary Table S1) and protein in a dose-dependent manner (4.7-fold at 1 μM; 9.6-fold at 10 μM; Fig. 3c). Proteomics identified 60 DEPs (Fig. 3d) enriched for metabolic and mitochondrial pathways (Fig. 3e). In contrast, phosphoproteomics revealed a broad therapeutic impact, with 614 DEPSs (Fig. 3f)

Table 1. (Phospho-)proteomics R14^{Δ/+} and other etiologies of HF; demographics, clinical characteristics, and concomitant medication of the patients

Patient	Age	Sex	Diagnosis	LVEF	Medications
1	50	Male	PLN R14del	23%	Bisoprolol, Bumetanide, Eplerenon, Movicolon, Milrinon, Fraxiparine, Noradrenaline, Paracetamol, Zolpidem
2	60	Female	PLN R14del	20%	Acenocoumarol, Bumetanide, Colchicine, Dobutamine, Kaliumchloride, Metoclopramide, Milrinon, Nebivolol, Oxazepam, Paracetamol, Perindopril, Spironolacton, Temazepam
3	43	Female	PLN R14del	10%	Bumetanide, Carvedilol, Kaliumchloride, Milrinon, Fraxiparine, Oxazepam, Pantoprazol, Spironolacton, Zopiclon
4	41	Female	PLN R14del	15%	Amiodaron, Bumetanide, Carvedilol, Dapagliflozine, Dobutamine, SlowK, Macrogl, Metoclopramide, Milrinon, Noradrenaline, Omeprazol, Oxazepam, Oxyodon, Paracetamol, Sertraline, Spironolacton, Valsartan
5	68	Female	PLN R14del	23%	Amiodaron, Bisoprolol, Bumetanide, Eplerenon, Esomeprazol, Milrinon, Oxazepam, Paracetamol, Sacubitril/Valsartan
6	57	Female	PLN R14del	10%	Bisoprolol, Bumetanide, Dapagliflozine, Eplerenon, Macrogl, Milrinon, Fraxiparine, Noradrenaline
7	41	Male	DCM	21%	Bisoprolol, Bumetanide, Dapagliflozine, Dobutamine, Movicolon, Fraxiparine, Noradrenaline, Paracetamol, Perindopril, Spironolacton, Thiamine, Tramadol, Vitamin B Complex
8	54	Male	DCM	15%	Ascal, Ascorbinezuur, Atorvastatine, Bisoprolol, Bumetanide, Cefuroxim, Milrinon, Fraxiparine, Noradrenaline, Pantoprazol, Paracetamol, Spironolacton, Thiamine, Tiotropium, Vitamin B complex forte
9	59	Male	DCM, TTN mutation	15%	Allopurinol, Bumetanide, Carvedilol, Dapagliflozine, Eplerenon, Kaliumchloride, Movicolon, Magnesiumgluconaat, Milrinon, Noradrenaline, Paracetamol, Sacubitril/Valsartan
10	49	Male	DCM, TTN and MYPN mutation	15%	Bumetanide, Dobutamine, Macrogl, Milrinon, Fraxiparine, Noradrenaline, Omeprazol, Spironolacton
11	27	Male	DCM	23%	Bumetanide, Dobutamine, Eplerenon, Kaliumchloride, Movicolon, Metoclopramide, Milrinon, Noradrenaline, Oxybutynine, Paracetamol
12	63	Male	DCM	22%	Amiodaron, Bumetanide, Capsaicine, Eplerenon, Gabapentine, Milrinon, Noradrenaline, Pravastatine, Prednisolon, Sacubiril/Valsartan
13	64	Male	DCM, TTN mutation	15%	Allopurinol, Amiodaron, Amoxicilline, Bumetanide, Clonazepam, Dobutamine, Insuline/Glargine, Levetivucetam, Macrogl, Metoprolol, Midazolamizon, Paracetamol, Temazepam, Xylometazoline
14	26	Male	DCM	20%	Allopurinol, Bumetanide, Cefuroxim, Dapagliflozine, Digoxine, Kaliumchloride, Macrogl, Magnesiumgluconaat, Metoprolol, Milrinon, Mupirocine, Fraxiparine, Noradrenaline, Oxyodon, Paracetamol, ramipril, Sevelameer hydrochloride, Spironolacton, Temazepam
15	45	Female	DCM, doxorubicin induced HF	17%	Bumetanide, Dapagliflozine, Digoxine, Eplerenon, Kaliumchloride, Movicolon, Milrinon, Fraxiparine, Oxazepam, Paracetamol, Temazepam, Venlafaxine
16	53	Female	DCM, TPM1 mutation	23%	Amiodaron, Bumetanide, Dobutamine, Eplerenon, Fluticason, Ipratropium, Kaliumchloride, Levothyroxine, Macrogl, Magnesiumgluconaat, Metoclopramide, Metoprolol, Milrinon, Nadroparine, Noradrenaline, Oxazepam, Pantoprazol, Paracetamol, Temazepam
DCM dilated cardiomyopathy, HF heart failure, LVEF left ventricular ejection fraction, MYPN myopalladin, PLN R14del phospholamban p.Arg14del, TPM1 tropomyosin 1, TTN titin					

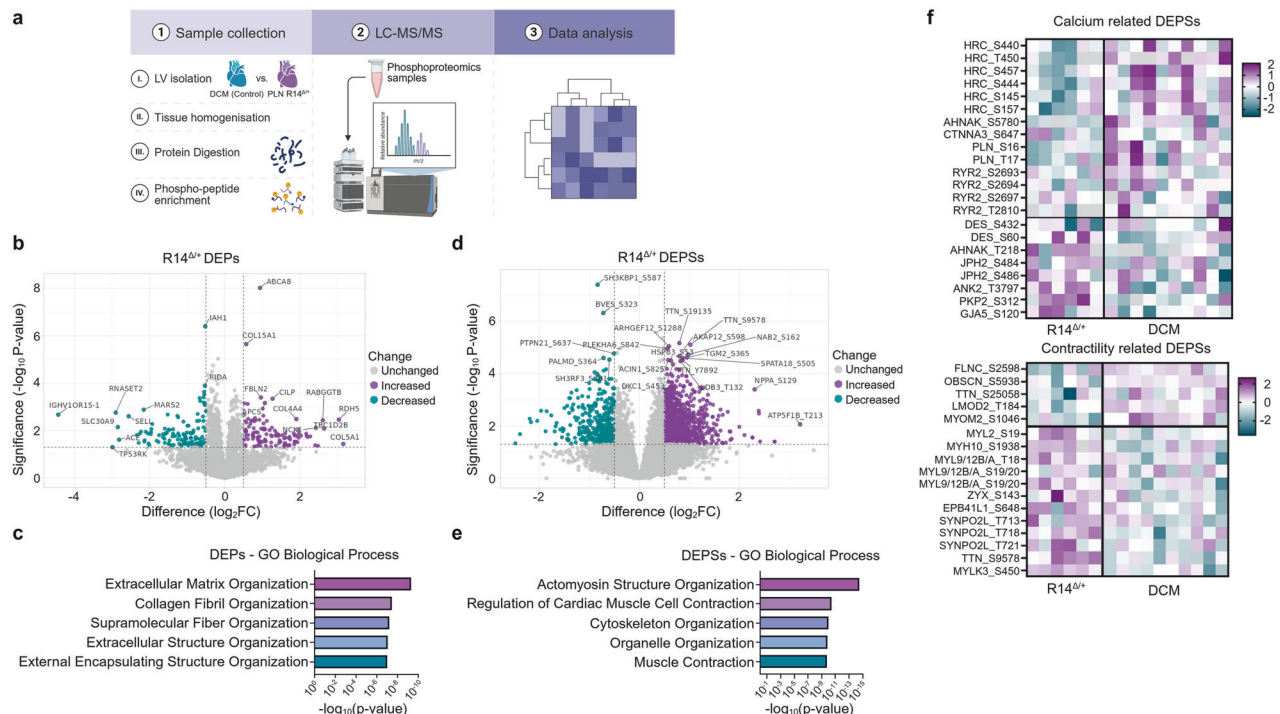


Fig. 1 Phosphoproteomics analysis of R14 Δ / $+$ heart tissue reveals an impact on calcium handling and contractility. **a** Workflow for (phospho-) proteomic examination of LV heart tissue from R14 Δ / $+$ ($N = 6$) and other genetic forms of dilated cardiomyopathy (DCM; $N = 10$). **b** Volcano plot of the identified DEPs in R14 Δ / $+$ heart tissue vs. DCM. **c** Gene ontology enrichment analysis of biological processes related to the identified DEPs. **d** Volcano plot of the identified DEPs in R14 Δ / $+$ heart tissue vs. DCM. **e** Gene ontology enrichment analysis of biological processes related to the identified DEPs. **f** Calcium-related DEPs associated with the GO term regulation of cardiac muscle cell contraction and contractility-related DEPs associated with the gene enrichment term actomyosin structure organization

enriched for transcriptional regulation, calcium handling, and contractile function (Fig. 3g), consistent with modulation of the pathways disrupted in PLN R14 Δ / $+$ cardiomyocytes.

Integrative analysis across PLN R14 Δ / $+$ patient myocardium, isogenic R14 Δ / $+$ iPSC-CMs, and RNA therapy-treated iPSC-CMs identified 28 phosphosites consistently altered across datasets (Table 2). This overlap was not driven by TTN variant carriers in the DCM control group (sensitivity analysis; Supplementary Table S2). Twenty-two of these were oppositely regulated between disease and RNA therapy and mapped to cadherin- and actin-binding proteins, including PI4KA, PLEC, SLK, and SPTBN1 (Fig. 3h), highlighting restoration of cytoskeletal signaling as a central effect of RNA therapy. Exploratory kinase enrichment analysis of these sites highlighted CK1 isoforms (CK1A, CK1G1), PKG2, BIKE, and IKK β as top-ranked kinases based on mean rank (Supplementary Table S3). The integrative workflow and overlap across datasets are summarized in Supplementary Fig. S1 (with kinase enrichment analysis of the R14 Δ / $+$ specific signature in myocardium and iPSC-CMs reported in Supplementary Table S4). The protein abundance of the identified phosphorylation sites was unaltered (Supplementary Fig. S1c).

Cellular validation and therapeutic reversal of phenotypes predicted by their phosphoproteomic profile
Consistent with the signaling disruptions identified by phosphoproteomics, we examined calcium and contractile behavior in R14 Δ / $+$ iPSC-CMs (Fig. 4a). Compared to isogenic iPSC-CMs, R14 Δ / $+$ iPSC-CMs showed consistently accelerated calcium transients and shortened contraction-relaxation kinetics (Fig. 4b–e), matching the predicted impairment in excitation-contraction coupling and formation of perinuclear PLN/LC3 aggregation clusters, a hallmark of the disease. These phenotypes provided a functional and structural reflection of the signaling defects identified in both patient tissue and engineered cells.

Treatment with PLN-targeted RNA therapy attenuated these abnormalities. RNA therapy treatment enhanced calcium kinetics (Fig. 4b, c, and Supplementary Fig. S2) and restored contractile amplitude (Fig. 4d, e, and Supplementary Fig. S3). Notably, RNA therapy reduced PLN/LC3 clusters (Fig. 4f–h), demonstrating rescue of the aggregation phenotype.

DISCUSSION

Inherited cardiomyopathies, such as PLN R14 Δ / $+$, lack targeted therapies, despite being major causes of heart failure and sudden cardiac death. Here, we applied an integrated multilayer strategy, combining patient myocardial tissue, phosphoproteomics, and human iPSC-CM modeling, to resolve how the R14 Δ / $+$ variant alters cardiomyocyte signaling and to evaluate the therapeutic potential of PLN-targeted RNA therapy (Fig. 5). Patient myocardial tissue was used as the primary discovery platform, while iPSC-CMs served as a controlled system to interrogate whether patient-defined signaling alterations could be recapitulated and modulated at the cardiomyocyte level. Our results demonstrate that phosphoproteomics, rather than protein abundance alone, is necessary to uncover the regulatory pathways driving R14 Δ / $+$ disease.

This study was designed to define disease-associated signaling alterations in PLN R14 Δ / $+$ cardiomyopathy and to assess whether these alterations are reversible upon RNA-based modulation. Accordingly, the phosphoproteomic findings should be interpreted as mechanistically informative rather than causal. The observed phosphorylation changes are therefore interpreted as consistent with downstream consequences of altered calcium handling and associated remodeling of calcium-responsive kinase and phosphatase signaling, rather than primary disease drivers, although direct causal relationships cannot be established in the present study. Exploratory kinase enrichment analysis further

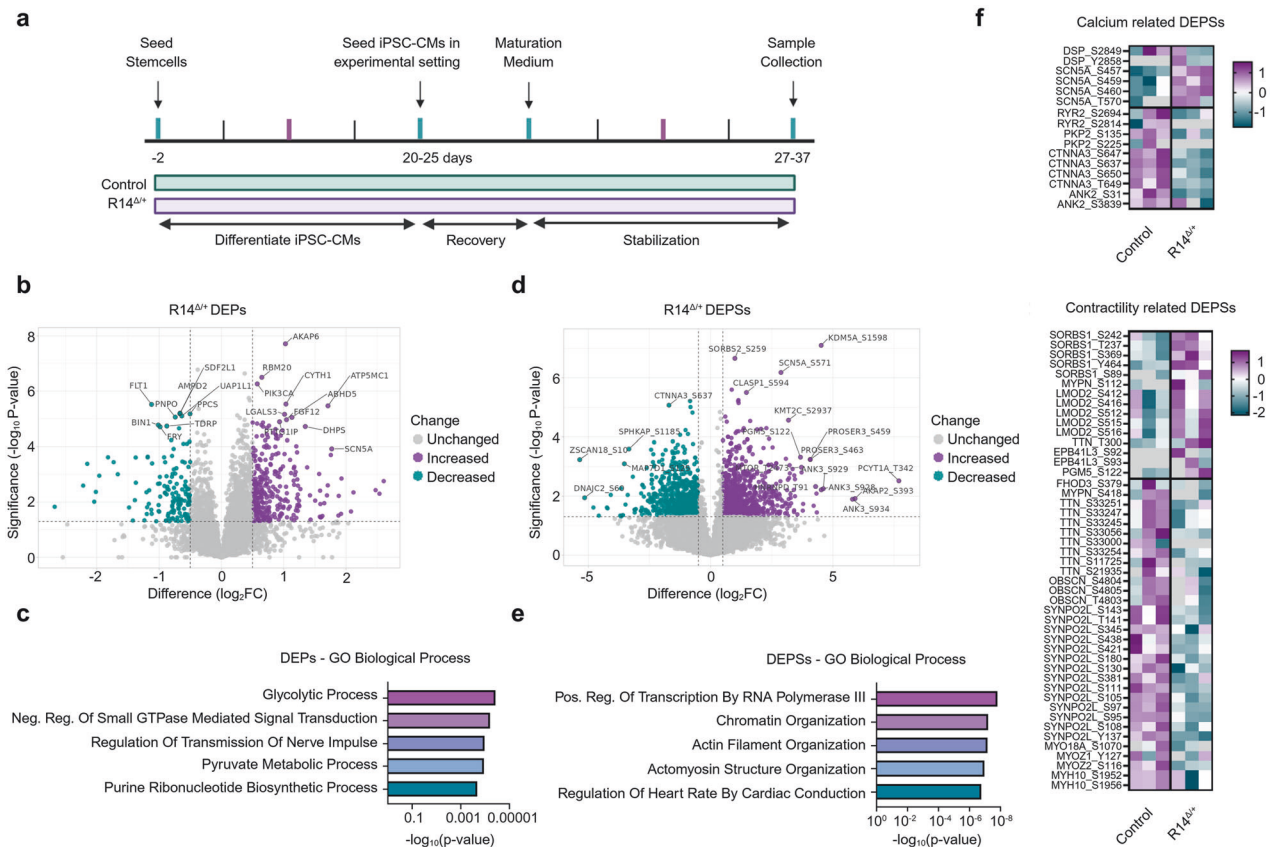


Fig. 2 Phosphoproteomics analysis of R14 Δ / $+$ iPSC-CM confirms the impact of R14 Δ / $+$ on calcium handling and contractility. **a** Workflow for (phospho-)proteomic examination of R14 Δ / $+$ iPSC-CM vs. isogenic control (3 biological replicates, 2 technical replicates). **b** Volcano plot of the identified DEPs in R14 Δ / $+$. **c** Gene ontology enrichment analysis of biological processes related to the identified DEPs. **d** Volcano plot of the identified DEPs in R14 Δ / $+$. **e** Gene ontology enrichment analysis of biological processes related to the identified DEPs. **f** Calcium-related DEPs associated with the gene ontology enrichment term regulation of heart rate by cardiac conduction and contractility-related DEPs associated with the gene ontology enrichment term actomyosin structure organization

prioritized candidate upstream regulators of the shared phosphorylation signature, including CK1 isoforms, PKG2, BIKE, and IKK β , which should be interpreted as hypothesis-generating rather than evidence of direct kinase activity. Importantly, these phosphorylation patterns are not proposed as diagnostic or prognostic biomarkers but rather as molecular readouts of disease-associated signaling states. While RNA-based modulation normalized several disease-associated signaling signatures, this does not establish direct causal links between individual phosphosites and functional outcomes, nor does it define therapeutic efficacy or dosing parameters. However, upon appropriate clinical validation, such signaling signatures may enable early identification of therapeutic responders and support treatment stratification and longitudinal monitoring. Prior studies have demonstrated that PLN-targeted RNA therapy improves cardiac function and survival in rodent models, including R14 Δ / $+$ cardiomyopathy.^{24,25} In contrast to these functional rescue studies, the present human phosphoproteomic analysis provides site-resolved mapping of disease-associated signaling in native myocardium, revealing prominent cytoskeletal phosphorylation remodeling. By integrating patient tissue and iPSC-CMs, this work extends preclinical observations into human disease biology. Notably, a first-in-human phase 1 clinical trial of PLN-targeting RNA therapy is currently underway (ClinicalTrials.gov ID: NCT07241104), underscoring the immediate translational relevance of these insights.

Global proteomics revealed a primarily fibrosis-related signature in patient hearts, reflecting end-stage structural remodeling but

providing limited insight into the contractile and calcium-handling abnormalities characteristic of PLN cardiomyopathy.²⁶ In contrast, phosphoproteomics uncovered a robust, disease-specific signaling signature enriched for actomyosin organization, contractility, and calcium-responsive pathways, directly implicating dysregulated posttranslational signaling as a primary driver of R14 Δ / $+$ pathophysiology. This phosphorylation-based signature was recapitulated in CRISPR-Cas9-engineered R14 Δ / $+$ iPSC-CMs, demonstrating that variant-driven signaling abnormalities are preserved outside the fibrotic environment and can be captured in human cell models. Importantly, RNA therapy reversed key components of this phosphoproteomic signature in cytoskeletal organization and excitation-contraction coupling. These molecular improvements translated into enhanced calcium and contractile kinetics and reduced PLN/LC3 aggregation, hallmark phenotypes of the R14 Δ / $+$ variant. Together, these results position phosphoproteomics as an essential mechanistic layer for understanding PLN cardiomyopathy and evaluating targeted interventions.

The observed normalization of phosphorylation patterns following RNA therapy likely reflects downstream consequences of altered calcium handling and associated kinase-phosphatase activity or reduced proteotoxicity, rather than direct correction of specific signaling nodes. Establishing causality would require targeted perturbation of individual kinases or phosphatases or site-directed mutagenesis of specific phosphorylation sites, which was beyond the scope of the present study. These approaches represent important future directions to further dissect the mechanistic hierarchy linking PLN dysfunction, calcium signaling,

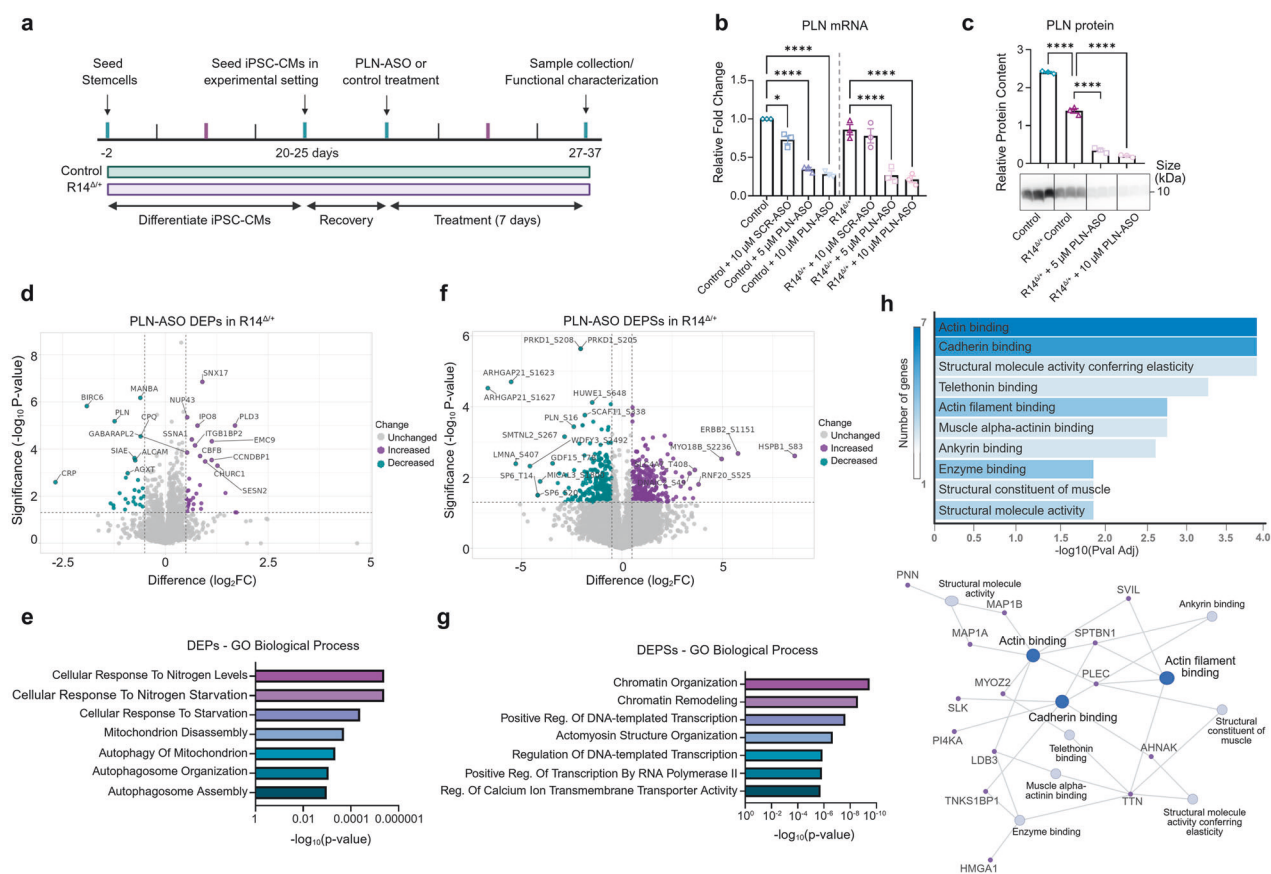


Fig. 3 PLN-ASO efficiency and phosphoproteomic examination. **a** Workflow for (phospho-) proteomics examination of PLN-ASO therapy in R14 $\Delta^{+/+}$ ($N = 3$) and isogenic control iPSC-CMs ($N = 3$). **b** PLN mRNA downregulation. **c** PLN protein content. **d** Volcano plot of DEP in R14 $\Delta^{+/+}$, including downregulation of PLN. **e** Gene Ontology Enrichment DEP in R14 $\Delta^{+/+}$. **f** Volcano plot of DEPS in R14 $\Delta^{+/+}$. **g** Gene Ontology enrichment of DEPSs in R14 $\Delta^{+/+}$. **h** Integrative analysis of DEPSs of R14 $\Delta^{+/+}$ cardiac tissues, iPSC-CMs and RNA therapy. Bar graphs represent the mean $\pm$ SEM. Significance was examined by one-way ANOVA; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$

and cytoskeletal remodeling. In addition, altered calcium transient kinetics should be interpreted cautiously. In PLN R14 $\Delta^{+/+}$ iPSC-CMs, accelerated calcium reuptake likely reflects an impaired SERCA/PLN axis, while RNA-based PLN reduction further enhances reuptake through partial SERCA disinhibition. Importantly, this effect was accompanied by improved contractile performance and reduced protein aggregation, indicating altered calcium handling rather than complete functional normalization. The long-term electrophysiological consequences of this acceleration remain to be determined.

Differences between patient myocardium and iPSC-derived cardiomyocytes should be interpreted in the context of model-specific biology. To explicitly assess cross-model consistency, phosphorylation sites were first identified as disease-associated in human PLN R14 $\Delta^{+/+}$ myocardium (R14 $\Delta^{+/+}$ vs non-PLN DCM), subsequently evaluated for concordant regulation in isogenic iPSC-derived cardiomyocytes (R14 $\Delta^{+/+}$ vs isogenic control), and finally examined for reversal following RNA therapy. Using this stepwise integrative approach, the phosphosites summarized in Table 2 therefore represent phosphorylation events that are shared between patient tissue and iPSC-derived cardiomyocytes and are reversed after PLN-targeted RNA modulation, defining a cardiomyocyte-intrinsic phosphorylation subset detectable across experimental systems.

In contrast, incomplete global phosphoproteomic concordance reflects established biological differences between end-stage human myocardium and iPSC-derived cardiomyocytes cultured in vitro, including differences in cellular maturity, tissue composition, and chronic neurohumoral remodeling. Thus, lack of full

phosphosite overlap does not indicate model inconsistency but rather underscores the complementary nature of patient tissue and iPSC-based systems. Importantly, the presence of a reproducible phosphorylation subset observable across both models and reversible following RNA therapy supports the relevance of the iPSC-CM platform for interrogating variant-driven signaling in PLN R14 $\Delta^{+/+}$ cardiomyopathy.

Across patient tissue, iPSC-CMs, and RNA therapy-treated cells, cytoskeletal signaling emerged as a consistent and disease-defining node. Although cytoskeletal remodeling is well established in heart failure,^{27–29} our phosphoproteomic analyses highlighted posttranslational dysregulation of cytoskeletal proteins, rather than merely changes in protein abundance, as a central feature of R14 $\Delta^{+/+}$ cardiomyopathy. Key regulators of structural integrity and force transmission, including TTN, PLEC and SPTBN1, displayed disease-associated phosphorylation patterns that were reversed by RNA therapy. These findings shift the mechanistic focus beyond isolated calcium or metabolic abnormalities toward broader disruption of cytoskeletal organization and mechanotransductive signaling.

Given the known sensitivity of cytoskeletal dynamics to intracellular calcium, the observed phosphorylation changes likely arise from the altered calcium environment imposed by the R14 $\Delta^{+/+}$ variant. Thus, PLN may influence cytoskeletal remodeling not only through SERCA inhibition but also by reshaping the calcium-dependent kinase and phosphatase landscape.³⁰ This provides a unifying model in which calcium dysregulation and cytoskeletal impairment are mechanistically linked through aberrant phosphorylation signaling.

Table 2. Integrative analysis of phosphorylation in R14^{Δ/+} heart tissues, R14^{Δ/+} iPSC-CMs and RNA therapy in R14^{Δ/+} iPSC-CMs

Condition	DEPSs	Protein	log ₂ FC (vs. comparator)	-log(P-value)
ASO	AHNAK_S210	AHNAK	2.273	1.462
R14 ^{Δ/+}	AHNAK_S210	AHNAK	0.920	1.638
ASO	ARHGAP23_S361	ARHGAP23	-0.444	2.314
R14 ^{Δ/+}	ARHGAP23_S361	ARHGAP23	0.898	3.580
ASO	CCDC61_T285	CCDC61	-0.477	2.070
R14 ^{Δ/+}	CCDC61_T285	CCDC61	0.362	1.759
ASO	DENND5B_S1076	DENND5B	-1.119	1.705
R14 ^{Δ/+}	DENND5B_S1076	DENND5B	1.633	3.665
ASO	DNAJC2_S47	DNAJC2	3.071	1.873
R14 ^{Δ/+}	DNAJC2_S47	DNAJC2	-4.571	1.339
ASO	HMGA1_S102	HMGA1	-1.591	1.990
R14 ^{Δ/+}	HMGA1_S102	HMGA1	-0.560	1.454
ASO	INTS1_S307	INTS1	1.057	1.332
R14 ^{Δ/+}	INTS1_S307	INTS1	-1.948	2.232
ASO	LDB3_T135	LDB3	-0.449	2.190
R14 ^{Δ/+}	LDB3_T135	LDB3	0.668	2.103
ASO	MAP1A_S1797	MAP1A	0.862	1.865
R14 ^{Δ/+}	MAP1A_S1797	MAP1A	-1.558	3.193
ASO	MAP1B_S831	MAP1B	1.391	1.591
R14 ^{Δ/+}	MAP1B_S831	MAP1B	-1.180	1.966
ASO	MAP1B_S832	MAP1B	1.401	1.583
R14 ^{Δ/+}	MAP1B_S832	MAP1B	-1.165	1.960
ASO	MYOZ2_T107	MYOZ2	-0.375	1.877
R14 ^{Δ/+}	MYOZ2_T107	MYOZ2	0.453	1.764
ASO	MYOZ2_T111	MYOZ2	-0.374	1.871
R14 ^{Δ/+}	MYOZ2_T111	MYOZ2	0.451	1.762
ASO	OSBP_S379	OSBP	0.855	2.232
R14 ^{Δ/+}	OSBP_S379	OSBP	0.999	1.531
ASO	PI4KA_S257	PI4KA	-1.099	2.031
R14 ^{Δ/+}	PI4KA_S257	PI4KA	1.363	1.507
ASO	PLEC_S4622	PLEC	-0.494	1.520
R14 ^{Δ/+}	PLEC_S4622	PLEC	1.682	1.657
ASO	PNN_S66	PNN	0.522	3.768
R14 ^{Δ/+}	PNN_S66	PNN	0.530	1.739
ASO	RAP1GAP2_S609	RAP1GAP2	-1.066	1.848
R14 ^{Δ/+}	RAP1GAP2_S609	RAP1GAP2	0.857	1.344
ASO	SLK_S565	SLK	0.742	1.406
R14 ^{Δ/+}	SLK_S565	SLK	-0.583	1.301
ASO	SLK_S571	SLK	0.475	1.556
R14 ^{Δ/+}	SLK_S571	SLK	-0.463	1.517
ASO	SPTBN1_S2138	SPTBN1	-0.444	1.723
R14 ^{Δ/+}	SPTBN1_S2138	SPTBN1	0.426	1.947
ASO	SVIL_S319	SVIL	-0.431	1.300
R14 ^{Δ/+}	SVIL_S319	SVIL	1.750	1.533
ASO	TFIP11_S98	TFIP11	1.058	1.590
R14 ^{Δ/+}	TFIP11_S98	TFIP11	-0.954	1.452
ASO	TNKS1BP1_S1620	TNKS1BP1	-0.482	2.722
R14 ^{Δ/+}	TNKS1BP1_S1620	TNKS1BP1	-0.353	1.574
ASO	TNKS1BP1_S1621	TNKS1BP1	-0.377	2.620
R14 ^{Δ/+}	TNKS1BP1_S1621	TNKS1BP1	-0.426	1.792
ASO	TTN_S12009	TTN	-0.421	2.088

Table 2. continued

Condition	DEPSs	Protein	log ₂ FC (vs. comparator)	-log(P-value)
R14 ^{Δ/+}	TTN_S12009	TTN	0.417	1.474
ASO	TTN_T12007	TTN	-0.421	2.088
R14 ^{Δ/+}	TTN_T12007	TTN	0.417	1.474
ASO	ZNF318_S136	ZNF318	-0.345	2.599
R14 ^{Δ/+}	ZNF318_S136	ZNF318	0.376	2.831

DEPSs identified in PLN R14^{Δ/+} myocardium and evaluated in R14^{Δ/+} iPSC-CMs following PLN-targeted RNA therapy. The table lists the phosphosite, corresponding protein, direction and log₂FC, and associated -log(p value) for comparisons between conditions. Bold entries indicate phosphosites that were PLN R14^{Δ/+}-specific and modulated after RNA therapy, representing disease-associated signaling alterations reversible by PLN reduction

Previous therapeutic strategies for PLN R14^{Δ/+} have targeted isolated aspects of disease, such as reducing SR clustering,¹³ activating the unfolded protein response,¹⁰ or buffering intracellular calcium,⁷ but none have produced broad correction of pathogenic signaling. In contrast, RNA therapy provides a broader and more integrated effect. By reducing PLN expression, RNA therapy not only reduces PLN aggregation but also reverses disease-specific phosphorylation of cytoskeletal and contractile proteins and enhances excitation-contraction coupling. This ability to correct upstream signaling distinguishes RNA therapy from earlier approaches and positions it as a promising targeted therapy for PLN R14^{Δ/+} cardiomyopathy. Moreover, demonstrating RNA therapy efficacy in human iPSC-CMs supports its translational potential and suggests that PLN suppression may be a generalizable strategy for additional pathogenic PLN variants.

The phosphoproteomic changes identified in this study reflect disease-associated phosphorylation signatures that provide mechanistic insight into signaling pathways altered in PLN R14^{Δ/+} cardiomyopathy. These signatures were used to interrogate molecular disease mechanisms and to assess whether such alterations are reversible upon RNA-based modulation. The DEPSs are not intended to represent clinical biomarkers, as their potential utility as diagnostic or prognostic biomarkers would require independent validation in larger, clinically annotated cohorts, which was beyond the scope of the present study. Our findings extend prior murine studies by providing the first demonstration that PLN-targeted RNA therapy improves molecular, structural, and functional phenotypes in human models of inherited cardiomyopathy. Despite concerns about complete PLN loss causing dilated cardiomyopathy,³¹ our data reveal biological responsiveness in which partial PLN reduction enhances calcium and contractile performance and reduces protein aggregation without overt toxicity. These results support the advancement of PLN-targeted RNA therapy toward clinical testing (ClinicalTrials.gov ID: NCT07241104) and establish a framework for applying RNA therapy-based approaches to other genetic forms of cardiomyopathy.

A major strength of this study is the integration of patient-derived myocardial tissue with human iPSC-derived cardiomyocytes, allowing variant-specific disease mechanisms in PLN R14^{Δ/+} cardiomyopathy to be interrogated while partially disentangling these effects from end-stage remodeling. All patient samples were obtained surgically during LVAD implantation, ensuring high tissue quality. In combination with prior in vivo work, this multilayered approach provides a robust human disease-anchored framework for mechanistic investigation and translational development of PLN-targeted RNA therapy.

Several limitations should be considered. Inclusion of nonfailing human myocardium would provide additional context; however,

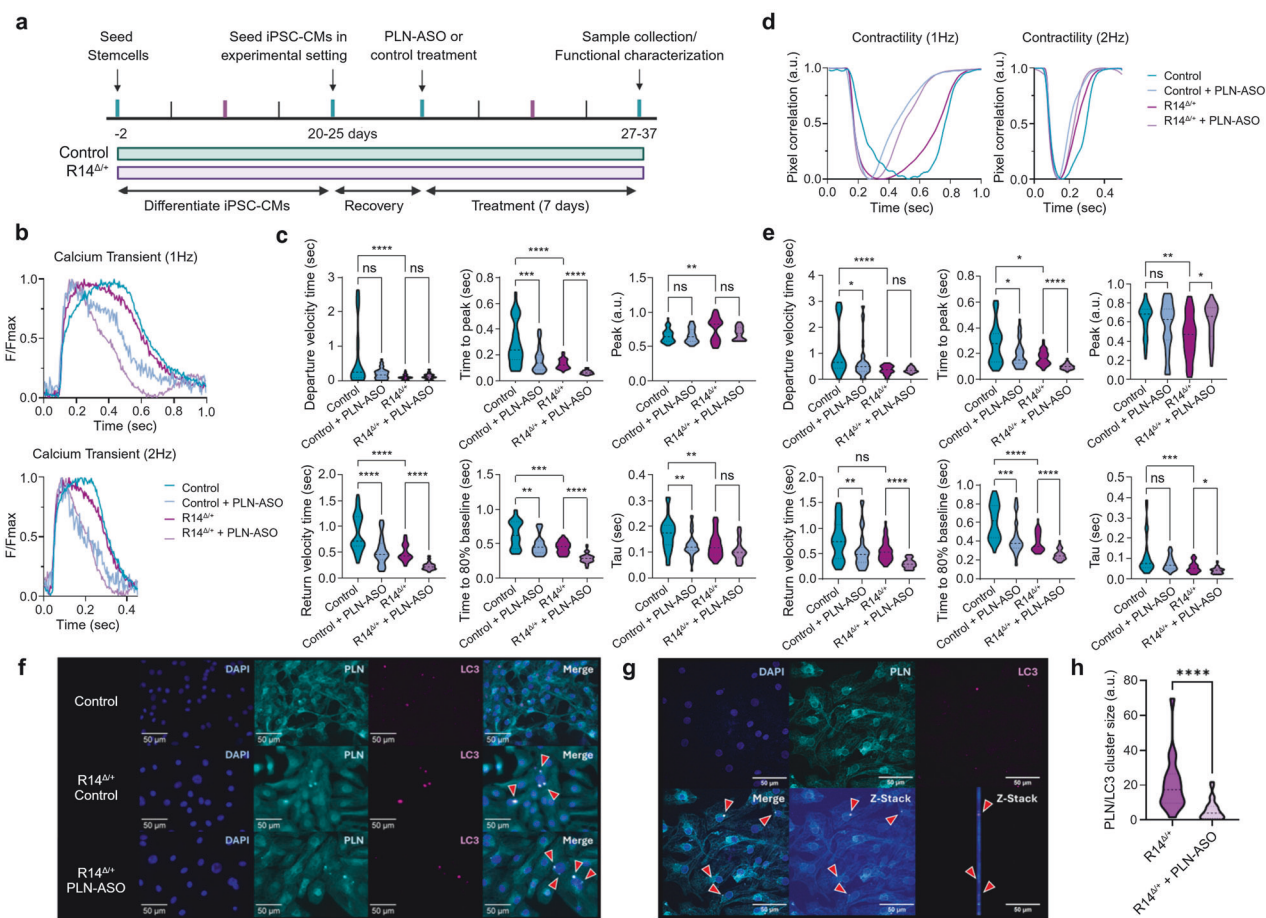


Fig. 4 Functional characterization of PLN R14 $\Delta/+$ iPSC-CM and PLN-ASO therapy. **a** Workflow for in vitro characterization of CRISPR-Cas9 engineered R14 $\Delta/+$ iPSC-CM vs. isogenic after PLN-ASO therapy or vehicle treatment. **b** Representative calcium transients under pacing conditions. **c** Calcium parameters derived from calcium transient at 1 Hz (with $N = 7$ control, and $N = 6$ R14 $\Delta/+$ iPSC-CM), including departure velocity time (time from the beginning of the transient to reach maximal slope), time to peak (time from beginning of the transient till the maximal deflection), peak (maximal deflection from baseline), return velocity time (duration between reaching 95% of the peak value and achieving the maximum return velocity during the recovery phase), time to 80% baseline (time to reduce systolic calcium level by 80%) and tau (decay time constant). **d** Representative contractility traces under pacing conditions. **e** Contractility parameters derived from contractility traces at 1 Hz, including departure velocity time, time to peak, peak, return velocity time, time to 80% baseline and tau. **f** Immunofluorescence labeling of DAPI, PLN and LC3. Red arrows indicate PLN/LC3 clustering. **g** Confocal microscopy assessment of PLN/LC3 clusters. PLN and LC3 can show colocalization in R14 $\Delta/+$ iPSC-CMs. Subsequent Z-stacking revealed that these PLN/LC3 clusters are intracellular PLN/LC3 clusters. **h** Quantification of immunofluorescent PLN/LC3 clusters in a violin plot indicating the mean and quartiles. Scale bars indicate 50 μ m. For the kinetic experiments, significance was examined by Kruskal–Wallis with Dunn's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. For the PLN/LC3 cluster sizes, the Mann–Whitney U test was used **** $P < 0.0001$.

such tissue is rarely available in comparable quality, as control samples are typically obtained postmortem. Consequently, patient-based comparisons were performed against non-R14 $\Delta/+$ DCM, and identified signaling differences should be interpreted as PLN-specific features within advanced cardiomyopathy rather than deviations from healthy myocardium. Moreover, the limited number of patients from whom myocardial tissue could be surgically obtained represents a fundamental constraint in studies of inherited cardiomyopathies, particularly for variants such as R14 $\Delta/+$, and restricts the ability to control for potential confounders, including sex or comorbidities. Because myocardial samples were obtained from patients with end-stage HF, differences in cellular composition, including increased fibrosis and a higher proportion of noncardiomyocytes, may contribute to the observed proteomic and phosphoproteomic profiles. While iPSC-derived cardiomyocytes enable controlled interrogation of cardiomyocyte-intrinsic mechanisms, they remain limited by structural immaturity, lack of physiological loading, and absence of the multicellular and neurohumoral environment present in the human heart, which likely contributes to differences observed between patient

tissue and iPSC-CMs. In this context, incomplete overlap between models should be interpreted as reflecting biological differences rather than inconsistency. In addition, variations in cell density and morphology may influence experimental readouts.

Phosphoproteomic analyses provide sensitive insight into signaling alterations but are inherently associative. Although statistical thresholds were applied, the identified phosphorylation changes should be interpreted as mechanistically informative rather than causal, and validation of individual phosphosites or kinase-substrate relationships was beyond the scope of this study. Although scrambled ASO controls were included for transcript and protein-level analyses, comprehensive phosphoproteomic profiling of SCR-ASO-treated R14 $\Delta/+$ iPSC-CMs was not performed. As such, subtle phosphorylation changes related to ASO chemistry rather than sequence specificity cannot be fully excluded. Future studies incorporating phosphoproteomic and functional analyses of scrambled ASO controls will be required to definitively distinguish sequence-specific effects from nonspecific ASO-related signaling changes. Finally, although RNA-based modulation reversed multiple disease-associated molecular and

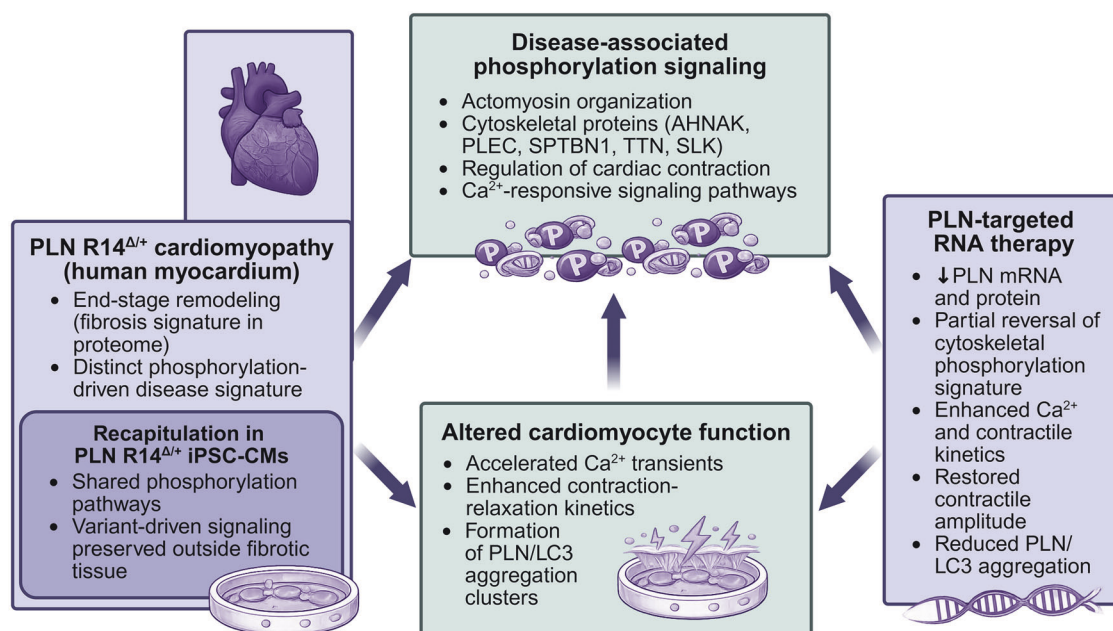


Fig. 5 Integrated schematic of PLN R14 $\Delta/+$ cardiomyopathy and RNA therapy-mediated rescue. The R14 $\Delta/+$ variant disrupts cardiomyocyte function and induces a disease-associated phosphorylation signature enriched for cytoskeletal and actomyosin-related proteins, contributing to cytoskeletal remodeling. Phosphoproteomic profiling of patient myocardium identified these signaling alterations, which were partially recapitulated in iPSC-derived cardiomyocytes. PLN-targeted RNA therapy reduced PLN aggregation and reversed key phosphorylation signatures, accompanied by enhanced calcium and contractile dynamics in vitro. The schematic summarizes the proposed relationship between PLN dysfunction, altered calcium-dependent signaling, cytoskeletal remodeling, and RNA-based modulation

functional phenotypes in vitro, this study was not designed to define therapeutic windows, long-term safety, or clinical efficacy. Accordingly, the phosphoproteomic signatures identified here should not be interpreted as clinical biomarkers but rather as molecular readouts of disease-associated signaling states that may guide future mechanistic and translational investigations.

In summary, PLN R14 $\Delta/+$ cardiomyopathy is defined by a distinct phosphoproteomic signature involving cytoskeletal remodeling, contractility, calcium handling and signaling, which are changes not detectable by proteomics alone. Phosphoproteomics thus functions as the crucial layer linking the pathogenic variant to altered calcium handling, structural remodeling and cardiomyocyte dysfunction. RNA therapy partially reverses this molecular profile, restores cytoskeletal phosphorylation patterns, enhances calcium and contractile kinetics, and reduces PLN aggregation. Compared to alternative therapeutic approaches, RNA therapy exerts broader and more integrated effects, establishing it as the most promising strategy to date for the treatment of R14 $\Delta/+$ cardiomyopathy. This integrative approach demonstrates how phosphoproteomics can accelerate mechanistic insight and therapeutic development in inherited cardiomyopathies.

MATERIALS AND METHODS

Collection of cardiac tissues

Heart tissue of the left ventricle (LV) from 6 patients with R14 $\Delta/+$ and 10 patients with other etiologies of HF (Genetic TTN or TPM1, Doxorubicin-induced HF, DCM with unknown etiology; Table 1) was obtained during left ventricular assist device implantation at the apex of the left ventricle in patients with advanced heart failure (NYHA class III-IV). All participants provided written informed consent prior to inclusion in the study.³² The scientific advisory board of the University Medical Centre Groningen provided ethical approval for the collection of human heart tissue. This study complies with the Declaration of Helsinki (Protocol number 2020.327, UMC Research Register no: 202000351, ABR-number NL73976.042.20). Due to the limited

number of patients from whom myocardial tissue could be surgically obtained, the study was not powered to control for all demographic variables beyond age. Consequently, phosphoproteomic differences should be interpreted as disease-associated rather than causal, and sex- and/or comorbidity-related effects cannot be fully excluded.

CRISPR-Cas9 engineered R14 $\Delta/+$ iPSC line generation

The human iPSC line 22 (ChiPSC22) (Takara Bio, #Y00320) was cultured following the manufacturer's guidelines using the Cellartis DEF-CS 100 Culture System (Takara Bio #Y30020). To create the R14 $\Delta/+$ iPSC line, the CRISPR-Cas9 technique was employed to introduce the R14 $\Delta/+$ variant into the ChiPSC22 wild-type cell line. iPSC cells were transfected with plasmids carrying spCas9-T2A-GFP and guide RNA targeting the PLN gene (TTCTATAGCTGAGCGAGTG), along with a ssDNA donor (Merck) that contained 50-base homology arms flanking the 3-nucleotide deletion (AGA) necessary for the pathogenic variant. To enhance knock-in efficiency, 1 μ M of the DNA-PK inhibitor (MedChemExpress, #HY-111783) was added to the cell cultures after transfection. Transfection was conducted during the log phase of cell growth using FuGene (Promega, #E2311). After 48 h, cells were sorted based on GFP expression, and single-cell clones were generated through limited dilution from GFP-enriched cell pools. Clones were screened for deletion via ddPCR (Bio-Rad) and further analyzed using next-generation sequencing to confirm the variant. Clones with the desired variant were then expanded and characterized (Supplementary Fig. S4).

Cardiomyocyte differentiation and maintenance

To induce differentiation into cardiomyocytes, we adhered to a previously established protocol. iPSCs were dissociated with TrypLE (Thermo Fisher, #12604-021) and plated as single cells in Essential 8 medium with 5 μ M Y27632 (Selleck Chemicals, #S1049). The medium was refreshed daily. Upon reaching 80% confluency, differentiation was initiated on day 0 using CDM3K5 medium, which included RPMI with 5% knockout serum replacement

(Thermo Fisher, #10828028), 500 µg/ml recombinant human albumin (RHA; Sigma-Aldrich, #A0237), 213 µg/ml ascorbic acid 2-phosphate (AA2P; Sigma-Aldrich, #A8960), and 1% Pen/Strep supplemented with 6 µmol/L CHIR99021 (Cayman Chemical, #13122) and 50 ng/ml activin A (PeproTech, #AF-120-14E). After 48 h, this medium was replaced with CDM3K5 medium containing 2 µmol/L Wnt-C59 (Tocris, #5148) and 5 ng/ml BMP4 (PeproTech, #AF-120-05ET). The medium was refreshed every other day and switched to CDM3 medium on day 8, leading to spontaneously contracting cardiomyocytes between days 8 and 12. Cardiomyocytes were then purified by enrichment in glucose-free RPMI1640-based CDM3 medium with 5 mM sodium DL-lactate (Sigma-Aldrich, #L4263) on day 16, resulting in pure spontaneously beating cardiomyocytes. Experiments began on days 20–25. For the experiments, iPSC-CMs were passaged as single cells using 1× TrypLE and then seeded onto experimental plastic in CDM3K5. Following recovery, the cells underwent a brief 24-h starvation in glucose-free RPMI medium (Thermo Fisher, #11879020) supplemented with 500 µg/ml RHA (Sigma-Aldrich, #A9731), 213 µg/ml AA2P (Sigma-Aldrich, #A8960), and 0.1% KnockOut Serum Replacement (Thermo Fisher, #10828028) to deplete glucose stores. The iPSC-CMs were then treated with PLN-ASO. The PLN-ASO has a gapmer design with the sequence motif kkk-10-kkk, consisting of a central DNA gap flanked by chemically modified nucleotides. The wings are composed of constrained ethyl (cEt)-modified nucleotides for enhanced stability and affinity, while the central 10-mer DNA gap supports RNase H activity (10 µM concentration was used for (phospho-)proteomics and functional characterization unless otherwise indicated; ASO concentrations were selected based on prior validation studies to assess biological responsiveness rather than to define a therapeutic dose range²⁴; ASO chemistry is listed in Supplementary Fig. S5), SCR-ASO (10 µM; To assess potential nonspecific transcriptional effects of ASO exposure, RNA-seq was performed following SCR-ASO treatment in two independent R14^{Δ/+} iPSC-CM lines; supplementary materials; Supplementary Fig. S6) or vehicle treatment, and stabilized for a week in a fatty acid-dependent maturation medium, consisting of a 1:1 mix of RPMI 1640 (Thermo Fisher, #21875034) and glucose-free RPMI 1640 (Thermo Fisher, #11879020). The medium was further supplemented with 1× linoleic acid-oleic acid-albumin (Sigma-Aldrich #L9655), 500 µg/ml RHA, 213 µg/ml AA2P, 1% Pen/Strep (Thermo Fisher, #15070063), 10 nM insulin-transferrin-selenium (Thermo Fisher, #41400045), 68.75 nM transferrin (Sigma-Aldrich, #T8158), 60 nM selenium (Sigma-Aldrich, #S5261), 200 µM carnitine (Sigma-Aldrich, #C0283), 200 µM 3-hydroxybutyrate (Sigma-Aldrich, #54965), 14.8 nM triiodothyronine (Sigma-Aldrich, #T6397), 30 nM CP775146 (Tocris, #4190/10), 250 nM cis-retinoic acid (Sigma-Aldrich, #R4643), and 250 nM dexamethasone (Sigma-Aldrich, #D4902). All functional experiments were performed on matched differentiation batches to minimize variability related to cell composition.

Sample preparation prior to phosphoproteomics and proteomics
For cardiac tissues, 100 mg of cardiac tissue, and for iPSC-CMs, approximately 5 million iPSC-CMs were lysed in SDS lysis buffer (5% sodium dodecyl sulfate (SDS), 50 mM TEAB, and 12% phosphoric acid), and lysates were clarified at 15,000 rpm for 10 min. Protein concentration was determined using the Pierce™ Rapid Gold BCA Protein Assay Kit according to the manufacturer's instructions. Proteins from each sample (300 µg) were reduced with 10 mM tris(2-carboxyethyl)phosphine (TCEP) and alkylated with 40 mM chloroacetamide in a thermomixer for 15 min at 90 °C and 1200 rpm. Sample digestion was performed on a S-Trap™ (Protifi) 96-well plate according to the manufacturer's instructions with slight modifications. Trypsin/LysC (Promega) in 50 mM ammonium bicarbonate solution was added to S-Trap columns at a ratio of 1:25 (enzyme:protein) and spun down prior to

digestion. The flow-through was reloaded, and samples were allowed to digest overnight at 37 °C. Digests were eluted, acidified and desalted using Oasis HBL plates (5 mg sorbent weight).

An aliquot of 5% was used for global proteomics, dried in vacuo and then reconstituted in 0.15% FA in water. The peptide concentration was measured using a nanodrop at A220 nm. Enrichment of phosphopeptides using IMAC was performed on the remaining desalted peptides using the King Fisher Flex platform along with PureCube Fe-NTA MagBeads (Cube Biotech, 31501-Fe) as described previously.³³

LC-MS/MS analysis

LC-MS/MS analysis was conducted on a timsTOF Pro mass spectrometer (Bruker) coupled with an Evosep-One LC-system and nanoelectrospray ion source (CaptiveSpray Source, Bruker). The mobile phases comprised 0.1% FA for solution A and 0.1% FA/100% ACN as solution B.

Two hundred nanograms of digested peptides were loaded onto an Evotip. Peptides were separated on a reversed-phase C18 column (8 cm × 150 µm ID, 1.5 µm, Bruker) maintained at 50 °C using 60 samples per day gradients. Data-dependent acquisition (DDA) was performed in PASEF mode with six PASEF scans at a duty cycle close to 100%. MS was acquired from 100 to 1700 *m/z*, and ion mobility was scanned from 0.85 to 1.30 Vs/cm² over a ramp time of 100 ms. The total cycle time was 0.74 s for six PASEF. The collision energy was linearly increased from 27 to 45 eV as a function of ion mobility. Singly charged precursors were filtered out by their position in the *m/z*-IM plane, and only precursor signals over an intensity threshold of 2000 arbitrary units were picked for fragmentation. An active exclusion of 0.4 min was applied to precursors that reached a target intensity of 20,000 arbitrary units. Data-independent acquisition (DIA)-PASEF mode was performed with a scheme that consisted of 24 windows with a 26 *m/z* isolation width. The mass scan range was from 100 to 1700 *m/z*, and ion mobility was scanned from 0.70 to 1.30 Vs/cm² over a ramp time of 100 ms. The collision energy was ramped linearly from 20 to 52 eV as a function of mobility. The total cycle time was 1.38 s.

For phosphoproteomics experiments, the collision energy was decreased from 60 eV at 1.3 Vs/cm² to 55 eV at 1.2 Vs/cm² to 50 eV at 1.1 Vs/cm² to 45 eV at 1.0 Vs/cm² to 40 eV at 0.9 Vs/cm² to 30 eV at 0.8 Vs/cm². (DIA)-PASEF mode was performed with a scheme that consists of 32 windows with a 26 *m/z* isolation width. The total cycle time was 1.8 s.

To generate a comprehensive spectral library for the DIA analysis, a hybrid library that contained MS data analyzed in both DDA and DIA modes for all individual samples was created. The combined DDA and DIA raw files were analyzed in Spectronaut version 16.1 (Biognosys AG) software with the Pulsar search engine using the UniProt Human (UP000005640, 82,493 entries) database. The search parameters were set as default with an additional deamidation (NQ) and phosphorylation (STY) in variable modifications. The dataset was analyzed using the default settings with precursor and protein FDR cutoff set to 0.01, quantification data/precursor filtering set to Q-value, no imputation, MaxLFQ, and cross-run normalization strategy set to automatic on precursors identified in all runs for global proteomics. Statistical analysis and plotting were performed using Perseus v2.0.7.0. Kinase enrichment analysis was performed using Kinase Enrichment Analysis 3 (KEA3; Maayan lab).

RNA isolation and qPCR

To analyze PLN and SERCA2a gene expression, total RNA was isolated using TRIzol reagent (Sigma-Aldrich, #T9424) according to the manufacturer's protocol. RNA concentrations were determined with a Nanodrop 2000 (Thermo Fisher), and cDNA was synthesized using the QuantiTect Reverse Transcription kit (Qiagen, #205313). Gene expression analysis was performed by qRT-PCR using IQ SYBR

Green (Bio-Rad, #170-8885). The protocol used was 95 °C for 3 min, followed by 35 cycles of 15 s at 95 °C and 1 min at 60 °C. The reference gene used was PPIA. The primers used are as follows: for PPIA, forward primer: GCTGTTTGACACAAGGTCC and reverse primer: GAAGTACCACCTGACACA. The PLN forward primer was ACAGTGCCAAGGTACCTA, and the reverse primer was TCCATGATACCAGCAGGACA. For SERCA2a forward primer, CGAACCTTGCACTCATCT and reverse primer, CCACTATTGCAGGTTCCAGGT.

Western blot

PLN protein levels were measured using a Western blot. Proteins were extracted using radioimmunoprecipitation (RIPA) buffer containing 1% phosphatase inhibitor cocktail 3 (Sigma-Aldrich, #P0044), 1× complete protease inhibitor cocktail (Roche, #11873580001), and 15 mM sodium orthovanadate (Sigma-Aldrich, #S6508). The protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher, #23225). Proteins were separated on a 15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and transferred to a PVDF membrane through semidry western blotting. Detection of PLN involved incubation with rabbit anti-PLN (1:1000 Cell Signaling, #145625) for 1 h at room temperature and monoclonal mouse anti-GAPDH IgG (1:10,000; Sigma-Aldrich, #T5168) for 30 min at room temperature. Following washing, the blots were incubated with polyclonal goat anti-rabbit IgG-HRP (1:2000; DAKO, #P0448) for 1.5 h at room temperature and polyclonal rabbit anti-mouse IgG-HRP (1:2000; DAKO, #P0260) for 1 h at room temperature. Signal detection was accomplished using enhanced chemiluminescence (ECL; PerkinElmer, #NEL120001EA), and densitometry analysis was performed with an ImageQuant LAS 4000 (GE Healthcare). All Western blot quantifications used target and loading control bands from the same membrane. Full, uncropped membranes are provided in Supplementary Fig. S7.

Calcium and contractility measurements

Cardiomyocyte contractility and calcium handling were simultaneously assessed using FURA-2 AM for calcium transients and pixel correlation for contractility in iPSC-CM monolayers. R14^{Δ/+} and isogenic controls were seeded in fluorodishes (World Precision Instruments, #FD35-100). The culture medium was replaced with a staining medium comprising RPMI 1640 without phenol red (Life Technologies, #11835-030), 1% HEPES (Gibco, #15630), and B27+ supplement (Gibco, #17504-44). Fura-2 AM (Thermo Fisher, #F1221) was prepared in 20% Pluronic F-127 (Sigma-Aldrich, #P2443) in DMSO (Sigma-Aldrich, #276855). Cardiomyocytes were treated with 5 μM FURA-2 AM in the staining medium for 45 min and then washed and stabilized in the staining medium at 37 °C. For imaging, iPSC-CMs were paced at 1 and 2 Hz using MyoPacer (IonOptix) at 10 volts and imaged with the CytoCypher MultiCell High Throughput System (CytoCypher BV). Measurements were taken within 30 min after FURA-2 AM staining, with 7 to 15 regions selected per fluorodish, and each monolayer was measured for 15 s. Calcium and contractility data were extracted using CytoSolver software (IonOptix), outliers were excluded using the Grubbs test, and the results were visualized in violin plots using GraphPad (GraphPad Software).

Immunofluorescence

To study PLN protein aggregation, PLN/LC3 clusters were measured as an in vitro proxy. iPSC-CMs seeded on coverslips were washed twice with cold PBS (1×; VWR, #392-0440) and fixed with PFA (4%; VWR, #VWRK4078.9010) on ice for 10 min. iPSC-CMs were washed 3 times with PBS and permeabilized with PBS + Triton-X100 (0.3%; Sigma-Aldrich, #T9284) on ice for 5 min. iPSC-CMs were blocked for 1 h at room temperature with PBS with Tween (0.1%; Sigma-Aldrich, #P1379) containing BSA (3%; Serva, #11930) and donkey serum (2%; Sigma-Aldrich, #D993). The iPSC-CMs were subsequently incubated with mouse anti-PLN (1:200;

Thermo Fisher, #MA3-922) and rabbit anti-LC3 (1:250; Cell Signaling, #127415) in blocking solution for 1 h. After washing, the cells were incubated with ALEXA Fluor 488 donkey-anti-mouse IgG (1:1000; Thermo Fisher, #A21202) and Alexa Fluor 555 donkey-anti-rabbit IgG (1:1000; Thermo Fisher, #A31572). iPSC-CMs were mounted with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, #H150010), and images were obtained with an AF6000 microscope (Leica) or SP8 confocal microscope (Leica). On the SP8, Z-stacks were obtained from three independent channels and analyzed using ImageJ (National Institute of Health). For PLN/LC3 cluster quantification, images were converted into grayscale and threshold to differentiate the aggregates from the background. After thresholding, PLN/LC3 clusters were measured by pixel size. PLN/LC3 cluster quantification was performed in a blinded fashion across three differentiations. Assessment of PLN aggregation was performed under low-density culture conditions to allow reliable discrimination of intracellular PLN/LC3-positive aggregates. At higher cell densities, extensive membrane contacts and increased PLN signals preclude accurate distinction between physiological membrane localization and aggregation. To visualize cardiac markers, iPSC-CMs were stained using anti-cTNT (Abcam, #ab45932) and anti-α-actinin (Sigma, #A7732), ALEXA 488 donkey-anti-mouse (Invitrogen, #A21202) and ALEXA 488 donkey-anti-rabbit (Invitrogen, #A31572) antibodies.

Statistical analyses

For the phosphoproteomic analysis, a protein FDR cutoff of 0.01 was used, and only DEPs and DEPSs with a $-\log(p)$ value of at least 1.3 and a $|\log_2FC|$ of at least 0.5 were included (with 0.3 for the integrative analysis). The identified DEPs and DEPSs are displayed as volcano plots created using VolcanoR (University of Amsterdam). Gene Ontology enrichment analysis was conducted with the Enrichr tool utilizing the 2023 Biological Processes Gene Ontology knowledgebase (Maayan lab). Heatmaps of DEPSs related to contractility and calcium handling were generated using DEPSs with a $|\log_2FC|$ of at least 1.0 in GraphPad Prism (GraphPad Software), with LC-MS-derived values transformed into Z values for plotting. In the in vitro experiments, calcium transient and contractility data are shown as truncated violin plots, with medians and quartiles indicated by lines. For the in vitro experiments, quantitative PCR and western blot data are presented as bar graphs, with visible data points. Statistical significance was determined using ANOVA followed by a multiple comparison test between relevant groups (cell line (control or R14^{Δ/+}) versus ASO (10 μM SCR-ASO, 5 μM PLN-ASO, 10 μM PLN-ASO treatments). In addition, calcium transient and contractility data are presented as truncated violin plots (median and quartiles are indicated by lines). To determine significance, the Kruskal–Wallis test followed by Dunn's post hoc test was used for multigroup comparisons between relevant groups (Control vs. Control + PLN-ASO, Control vs. R14^{Δ/+} and R14^{Δ/+} vs. R14^{Δ/+} + PLN-ASO). Statistical significance for the quantification of PLN/LC3 clusters was determined using a Mann–Whitney U test. All statistical analyses were performed using GraphPad Prism (version 9.1.0; GraphPad Software), with p values below 0.05 considered statistically significant.

DATA AVAILABILITY

The datasets generated in this study have been deposited in the Zenodo public repository and are available at <https://doi.org/10.5281/zenodo.18602605>. All data supporting the findings of this study are included within the article and its supplementary information files. Source clinical data are subject to institutional privacy regulations but may be made available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

F.E.D., P.D., D.S., C.A., K.M.H., A.M., N.B., N.G.B., and P.M. conceived the study and designed the experiments. F.E.D., P.D., A.N.L., I.B.D., K.F.A.G., A.E.G., J.Z., A.W., and D.E. performed the experiments, collected the data, and analyzed the results and performed statistical analyses. D.S., A.M., S.E., N.H., and H.S. provided critical resources. N.B., N.G.B., and P.M. contributed to data interpretation and provided critical feedback. F.E.D. drafted the manuscript, with writing review and editing by P.D., N.B., N.G.B., and P.M. All authors have read and approved the article.

ADDITIONAL INFORMATION

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