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Long-term Rescue of a Familial Hypertrophic Cardiomyopathy Caused by a Mutation in the Thin Filament Protein, Tropomyosin, via Modulation of a Calcium Cycling Protein

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Abstract

We have recently shown that a temporary increase in sarcoplasmic reticulum (SR) cycling via adenovirus-mediated overexpression of sarcoplasmic-endoplasmic reticulum ATPase (SERCA2) transiently improves relaxation and delays hypertrophic remodeling in a familial hypertrophic cardiomyopathy (FHC) caused by a mutation in the thin filament protein, tropomyosin (i.e., α -TmE180G or Tm180). In this study, we sought to permanently alter calcium fluxes via phospholamban (PLN) gene deletion in Tm180 mice in order to sustain long-term improvements in cardiac function and adverse cardiac remodeling/hypertrophy. While similar work has been done in FHCs resulting from mutations in thick myofilament proteins, no one has studied these effects in an FHC resulting from a thin myofilament protein mutation.

Tm180 transgenic (TG) mice were crossbred with PLN knockout (KO) mice and four groups were studied in parallel: 1) non-TG (NTG), 2) Tm180, 3) PLNKO/NTG and 4) PLNKO/Tm180. Tm180 mice exhibit increased heart weight/body weight and hypertrophic gene markers compared to NTG mice, but levels in PLNKO/Tm180 mice were similar to NTG. Tm180 mice also displayed altered function as assessed via *in situ* pressure-volume analysis and echocardiography at 3–6 months and one year; however, altered function in Tm180 mice was rescued back to NTG levels in PLNKO/Tm180 mice. Collagen deposition, as assessed by Picrosirius Red staining, was increased in Tm180 mice but was similar in NTG and in PLNKO/Tm180 mice. Extracellular signal-regulated kinase (ERK1/2) phosphorylation increased in Tm180 mice while levels in PLNKO/Tm180 mice were similar to NTGs.

The present study shows that by modulating SR calcium cycling, we were able to rescue many of the deleterious aspects of FHC caused by a mutation in the thin myofilament protein, Tm.

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CONFLICTS OF INTEREST. None

Keywords

Familial Hypertrophic Cardiomyopathy; Phospholamban; cardiac function; diastolic dysfunction; interstitial fibrosis; Extracellular signal-regulated kinase

1. Introduction

Hypertrophic cardiomyopathy affects one in 500 people and is characterized by unexplained left ventricular hypertrophy [1]. The genetic form of the disease, referred to as Familial Hypertrophic Cardiomyopathy (FHC), is the number one cause of sudden death in young people, especially athletes [2]. Typical phenotypes of FHCs include predominantly septal hypertrophy, diastolic dysfunction, myocyte disarray and interstitial fibrosis [3]. Historically, FHCs have been ascribed to mutations in myofilament A and I band proteins [4, 5] but recent findings have shown them to be additionally linked to Z-disc proteins [6]. Although FHCs were first linked to mutations in the thick filament protein, i.e. myosin heavy chain [7], they were soon afterwards also linked to thin filament proteins, such as tropomyosin [8]. To date 11 FHC-causing and 2 DCM-causing mutations in α -tropomyosin (Tm) have been reported [9]. A mutation at residue 180 of α -Tm (i.e., α -Tm E180G) is an example of FHC [4] that has recently been generated in transgenic (TG) mouse models by different laboratories [10, 11]. In FVB/N mice, the phenotype of α -Tm E180G TG (or Tm180) includes left ventricular hypertrophy, a grossly enlarged left atrium, extensive fibrosis, increased myofilament Ca^{2+} sensitivity and diastolic dysfunction [10].

Since diastolic dysfunction is a common symptom of FHC, interventions to correct diastolic dysfunction in FHC are of great interest. We [12] have previously reported that the hypertrophic phenotype of Tm180 can be reduced by desensitizing the myofilament to Ca^{2+} and improving relaxation. Coutu *et al.* [13] have previously reported that increased expression of parvalbumin, a cytoplasmic Ca^{2+} -binding protein, corrects the slower relaxation in adult cardiac myocytes expressing Tm180. We have also recently shown that injection of SERCA2 adenovirus into the hearts of one day-old mice was sufficient to delay the development of hypertrophy and temporarily improve whole heart function in Tm180 mice [14]. However, the protective effects were lost after three months of age suggesting that permanent and/or stronger intervention in sarcoplasmic reticulum (SR) Ca^{2+} uptake is required to sustain the improved morphology and cardiac function of this FHC phenotype.

One of the possibilities to permanently intervene and improve cardiac relaxation is to ablate the PLN gene. PLN is a Ca^{2+} -handling protein that normally inhibits the SERCA2-mediated Ca^{2+} uptake into the SR. Phosphorylation of PLN removes its inhibition of SERCA2 allowing faster resequestration of Ca^{2+} and thus a shorter diastole duration and stronger enhanced contraction (for a review see [15]). Mice in which the PLN gene was ablated (i.e., PLNKO) exhibit hypercontractility with no change in morphology or heart rate, an attenuated response to β -adrenergic agonists [16-19], no alterations in myofilament Ca^{2+} sensitivity and myosin ATPase activity [20] and normal cardiac performance during exercise [21]. PLNKO mice have been used to restore depressed contractility and adverse cardiac remodeling in several mouse models of cardiomyopathies [22-26]; however, these have been in either dilated cardiomyopathies or FHCs resulting from thick myofilament protein mutations.

Knowing that PLN influences SR Ca^{2+} uptake and thus relaxation and based on our previous experiments using temporary, adenoviral overexpression of SERCA2 in young Tm180 mice [14], we hypothesized that a permanent increase in the rate of Ca^{2+} removal by the SR via PLNKO will yield sustained improvements in relaxation, cardiac function and cardiac

remodeling in Tm180 mice. Our results show that PLN ablation in Tm180 mice rescues cardiac function and morphological abnormalities for up to one year. To the best of our knowledge, this is the first study showing both prolonged rescue of cardiac performance and reversal of cardiac remodeling in a model of FHC linked to a mutation in a thin filament protein.

2. Methods

2.1 Generation of TG mice

New TG mouse lines were generated by crossbreeding existing lines of mice, Tm180 TG [10] and PLNKO [16]. Both sets of breeders were FVB/N strain. Four groups of mice were then selected for experiments: 1) NTG, which express normal levels of PLN protein and wild-type Tm; 2) Tm180, which express normal levels of PLN protein and mutated Tm (E180G); 3) PLNKO/NTG, which lack the PLN gene and express wild-type Tm; 4) PLNKO/Tm180, which lack the PLN gene and express mutated Tm.

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Real-time PCR

Expression of the hypertrophic gene markers, atrial natriuretic factor (ANF) and β -myosin heavy chain (MHC), was determined using quantitative RT-PCR [24] as described earlier [14] in 3-6 months-old and one year-old mice.

2.3 Western blots

Apexes from the left ventricle 3–4 month-old mice were obtained after each experiment and immediately frozen in liquid nitrogen. The ventricular homogenates were analyzed by standard Western blotting to compare phospholamban (PLN), SERCA2 and phosphorylation of ERK 1/2 at Thr202/Tyr204.

2.4 *In situ* pressure-volume measurements

Pressure-volume analyses were performed as recently described [27, 28]. To gain access to the femoral vein for infusion of isoproterenol (ISO), the right femoral vein was isolated, the distal end tied off and the proximal end catheterized with stretched PE-10 tubing. This tubing was connected to a 250 μ L glass syringe mounted on a Model PHD 2000 micro infusion pump (Harvard Instruments, Cambridge, MA), which allowed for precise delivery of ISO at 0.08 ng/g*min delivered 1.5 μ L per minute.

2.5 Histology

Excised hearts from one-year old mice were cannulated via the aorta and the coronary arteries retrogradely perfused with ice-cold saline followed by several mL of Methacarn. The heart was then transversely sliced into four pieces and each piece placed into a cassette. Cassettes were kept in Methacarn overnight at 4° C, placed in 100% ethanol for one hour (with two changes), xylene for one hour (with two changes) and finally paraffin at 60° C (with one change). Hematoxylin & Eosin (H & E) and Picrosirius Red staining were conducted on 5 μ m sections.

2.6 Transthoracic echocardiography

Echocardiography was conducted using a 30 MHz high resolution transducer (Vevo 770 High-Resolution Imaging System, Visualsonics, Toronto, ON, Canada) as previously

described [29]. Mice (four months and one year-old) were anesthetized via 0.5-1.5% isoflurane mixed with 100% oxygen delivered via a face mask. Proper anesthesia levels were checked via absence of the pedal reflex. Body temperature was monitored with a rectal thermometer and maintained at 37°C using the system's heated stage.

Briefly, left atrium (LA) measurements were taken from the parasternal long axis view. LV internal dimension (LVIDd) and septal wall thickness (SWT) were taken from the parasternal short axis view at the level of the papillary muscles. E/A ratio (E = peak velocity of mitral flow in the early phase of diastole; A = peak velocity of mitral flow during atrial contraction) was measured via Pulsed Doppler in the apical, four-chamber view. Peak myocardial velocity in the early phase of diastole (E_m) and peak myocardial velocity during systole (S_m) were obtained with the sample volume at the septal side of the mitral annulus in the four chamber view using tissue Doppler imaging. All measurements and calculations were averaged from 3 consecutive cycles as previously described [29].

2.7 Statistical analyses

All results are presented as means $\pm$ SE. Statistical analyses were conducted using an ANOVA with Newman-Keul's post-hoc test for RT-PCR, heart weight/body weight (HW/BW) ratios and echocardiography results and two way repeated measures ANOVA with Bonferroni's post-hoc test for *in vivo* results. $P \leq 0.05$ was regarded as statistically significant.

3. Results

3.1 Heart weight/body weight and hypertrophic marker gene expression

Figure 1 shows Tm180 HW/BW was significantly higher than NTGs at both 3 - 6 months (A) one year of age (B). This was largely due to the grossly enlarged left atria (C and D) that typifies this particular FHC mutation [10, 30]. Atrial mass in Tm180 was reduced to NTG levels after PLN ablation (i.e., in PLNKO/Tm180 mice).

PLN ablation also reversed the increased expression of two hypertrophic marker genes, β -MHC and ANF, in Tm180 mice. At both 3 - 6 months (E) and one year of age (F), β -MHC expression in Tm180 mice was much higher than NTG levels. Following PLN ablation, these levels were reversed back to NTG levels at both time points. Similar results were seen with ANF although it did not return to NTG levels. However, they were still significantly lower than those of Tm180 mice at both 3 - 6 months (G) and one year of age (H).

3.2 *In situ* pressure-volume measurements

At 3 - 6 months of age, PLN ablation in Tm180 mice increased dP/dt_{max} (Fig. 2A), the maximum rate of LV pressure development, to a value higher than either NTG or Tm180 mice. Following intravenous injection of 0.08 ng/g*min ISO, dP/dt_{max} in Tm180 mice was reduced compared to NTG mice. This is in agreement with previous studies that showed a blunted response to ISO in this model of FHC [10, 30]. However, dP/dt_{max} in PLNKO/Tm180 mice was similar to NTGs following ISO. The load-independent parameter of systolic function, E_{max} (Fig. 2B), was lower in Tm180 compared to NTG mice at baseline and following ISO. Ablation of PLN in Tm180 mice increased E_{max} back to NTG levels in both conditions. Another systolic parameter, E_{es} (i.e., end systolic elastance, which represents the slope of the load-independent end systolic pressure-volume relationship) was not significantly different from NTG values in any of groups at baseline (NTG=12.57 $\pm$ 2.22, Tm180=5.56 $\pm$ 0.65, PLNKO/NTG=17.62 $\pm$ 4.17, PLNKO/Tm180=10.67 $\pm$ 1.92). However, Tm180 mice showed a trend towards lower values compared to NTG at baseline, and this negative trend appears to improve in PLNKO/Tm180 mice. Following ISO, NTG was the

only group to exhibit a significant increase in E_{es} compared to baseline (data not shown). V_o (i.e., the volume intercept of the end systolic pressure-volume relationship) did not vary significantly from NTG values in any of the groups at baseline (NTG=-0.37±0.92, Tm180=-1.78±3.37, PLNKO/NTG=-0.48±1.78, PLNKO/Tm180=-2.67±2.28) or after ISO (data not shown).

PLNKO/Tm180 mice also improved the diastolic parameters, dP/dt_{min} (Fig. 2D) and τ (Fig. 2E). As expected, dP/dt_{min} , the maximum rate of LV pressure decline, was lower in Tm180 mice compared to NTGs at both baseline and following ISO [10, 30]. Ablation of PLN significantly improved this parameter (compare PLNKO/Tm180 to Tm180 mice) returning the speed of relaxation back to NTG values in both instances. τ , an index of the time needed for cardiac relaxation, was increased (i.e., lengthened) in Tm180 mice at baseline and following ISO. PLNKO/Tm180 mice again reversed this parameter back to NTG levels at baseline while showing a similar, although nonsignificant, trend following ISO.

3.3 Echocardiography

High-resolution echocardiography was utilized in four months and one-year old mice to assess cardiac morphology and function. Results were very consistent at both time points (Fig. 3A-I). Morphological analysis revealed an increased left atrium size in Tm180 mice that was reduced in PLNKO/Tm180 mice (Fig. 3A) to a size similar to NTG atria. Left ventricular internal dimension during diastole (LVIDd; Fig. 3B) was similar for all four groups while septal wall thickness (SWT; Fig. 3C) increased in Tm180 mice but was reduced in PLNKO/Tm180 mice.

Diastolic function was assessed using both pulsed (i.e., E/A ratio) and tissue Doppler (i.e., Em; see Fig 3 for explanation of this parameter). The E/A ratio (Fig. 3D) was increased to a very high value in Tm180 mice suggesting restrictive filling, yet it decreased back to NTG levels in PLNKO/Tm180 mice. Em (Fig. 3E) was decreased in Tm180 compared to NTG mice, indicating decreased velocity of myocardial relaxation, yet this was reversed in PLNKO/Tm180 mice. E/Em ratio (Fig. 3F), an indicator of LV filling pressure [31], was also increased in Tm180 but returned to NTG levels in PLNKO/Tm180 mice.

For systolic performance, both ejection fraction (Fig. 3G) and cardiac output (Fig. 3H) were similar in Tm180 and NTG mice. As expected, PLNKO/NTG mice exhibited an increase in cardiac output compared to NTGs. The peak systolic myocardial velocity, S_m (Fig. 3I), an indicator of myocardial contractility, was decreased in Tm180 mice but returned to NTG levels in PLNKO/Tm180 mice. Thus, S_m is congruous with our E_{es} data discussed in the pressure-volume measurements section above, although the latter shows only trends and not significance. Therefore, it appears that the ability of the myocyte to contract in Tm180 is hindered, possibly due to increased fibrosis (see the next section, histology). However, Tm180 mice are in the compensated stage since they show normal ejection fraction, cardiac output and LV chamber diastolic dimensions.

3.4 Histology

In order to elucidate the effects of PLN ablation on myocyte disarray and interstitial fibrosis, two hallmarks of FHC, histological analysis was performed via hematoxylin/eosin (H & E; Fig. 4, left) and Picrosirius Red staining (Fig. 4, right) in one year-old mice. H & E results show a disorganized pattern of myocyte arrangement in Tm180 mice as compared to the structured pattern of myocytes in NTG mice. However, PLNKO/Tm180 exhibited a restoration of the orderly arrangement of myocytes. Picrosirius Red staining shows a large degree of fibrosis (red color) in Tm180 mice compared to NTGs, yet PLN ablation reduces

fibrosis as seen in PLNKO/Tm180. Also, PLNKO/NTG has a higher degree of fibrosis compared to NTG, but it is considerably less than that seen in Tm180.

3.5 Western blots

Since it has been previously shown that phosphorylation of ERK1/2, one of the Mitogen Activated Protein (MAP) kinases, at residues Thr202 and Tyr204 is increased in a rabbit model of FHC [32], we performed Western blots against phospho-ERK1/2 in all four groups of mice at 3-4 months of age. As expected, Tm180 exhibited increased phosphorylation at residues Thr202 and Tyr204 compared to NTG (Fig. 5). However, in PLNKO/Tm180 mice, ERK1/2 phosphorylation levels decreased back to NTG levels.

PLN levels were not significantly different between Tm180 and NTG mice while PLNKO/NTG and PLNKO/Tm180 mice did not have any detectable levels of PLN (data not shown). In addition, SERCA2a levels were not different between any of the four groups (data not shown).

4. Discussion

We have found that permanent increases in SR Ca^{2+} uptake by PLNKO normalizes relaxation, prevents cardiac hypertrophy, reduces myocyte disarray and fibrosis and improves both systolic and diastolic function up to one year in a mouse model of FHC. These improvements are associated with restoration of phosphorylation of ERK1/2 to control levels.

4.1. Morphology and function of mice expressing mutated Tm are rescued by interventions in SR Ca^{2+} uptake

We have previously reported that the phenotype of Tm180 mice is characterized by severe left atrium enlargement, interstitial fibrosis, myocyte disarray, hypertrophy, increased myofilament sensitivity to Ca^{2+} and diastolic dysfunction [10]. Diastolic dysfunction in Tm180 mice (as evidenced by decreased $\text{dP/dt}_{\text{min}}$ and increased E/A ratio) causes prolonged LV relaxation/distension (increased tau and decreased E_{m}). Fibrosis, as seen in the histological sections, also creates a less compliant heart that takes longer to distend during diastole. Inevitably, LV diastolic filling pressure increases, as evidenced by the increased E/ E_{m} ratio, which decreases the pressure gradient between the LV and left atrium. Left atrial pressure must therefore increase to maintain adequate LV filling and the resulting increased wall tension causes the left atrium to expand [33]. In fact, it has been postulated that the volume of the left atrium is indicative of the severity of diastolic dysfunction [34].

We have shown that permanent increases in SR Ca^{2+} uptake through PLN ablation not only prevents left atrial enlargement but also reduces interstitial fibrosis, myocyte disarray and cardiac hypertrophy (Figures 3 and 4). The signaling mechanisms regarding how PLNKO rescues this FHC phenotype are not completely understood, but they most likely involve an improvement in diastolic function in Tm180 mice following PLN ablation. Ablation of PLN relieves the inhibitory action of PLN on Serca2 which allows Ca^{2+} to be pumped back into the SR more quickly [35]. The heart then relaxes faster thus improving diastolic function in PLNKO/Tm180 mice. Improved diastolic function may then somehow negate the maladaptive cardiac remodeling that causes fibrosis, hypertrophy and even left atrium enlargement in Tm180. From our data, it appears that one of the mechanisms involved in this phenomenon is inactivation of ERK1/2.

4.2. Role of ERK1/2 pathway

ERK1/2, along with the c-Jun N-terminal kinases (JNKs) and p38 kinases, comprise the MAPK signaling pathways involved in cardiac hypertrophy [36]. All three subfamilies of kinases phosphorylate a wide array of transcription factors involved in cardiac hypertrophy [37]. However, activation of the ERK1/2 pathway occurs primarily by hypertrophic stimuli whereas the JNKs and p38 kinases are primarily activated by cellular stresses (e.g., oxidative stress, ischemia/reperfusion or hypoxia) [38]. MAPK kinase (also known as MEK1/2) activates ERK1/2 by phosphorylating ERK1 at Thr202 and Tyr204 and ERK2 at Thr185 and Tyr187. A recent study in which MEK1 was over expressed in the mouse yielded increased levels of phosphorylated ERK1/2 and a compensated hypertrophy phenotype [39]. Since the latter is the condition found in Tm180 mice, as evidenced by increased SWT with normal LVIDd and ejection fraction, we sought to determine if the phosphorylation status of ERK1/2 changes in Tm180 mice following PLNKO.

Our data indicate that ERK1/2 phosphorylation decreases in Tm180 mice following PLN ablation when compared to Tm180 mice alone. Since hypertrophy also decreases in PLNKO/Tm180 hearts compared to Tm180, we can assume that the transcription of genes involved in the hypertrophic response are down regulated in PLNKO/Tm180 mice compared to Tm180. But how does ablation of PLN lead to decreased phosphorylation of ERK1/2? We can only speculate that the decreased duration of the Ca^{2+} transients in PLNKO mice [40] downregulates signals that activate of the fetal gene program [41]. Calcium/calmodulin kinase II may be an intermediate in this pathway since it is able to phosphorylate ERK1/2 in vascular smooth muscle cells [42], and its activity decreases as calcium pulse duration decreases [43]. Future studies are needed to see if these conjectures prove to be true or not.

4.3. Use of PLNKO mice in rescuing other models of cardiomyopathies

Recent work has shown varying success rescuing different models of cardiomyopathies via PLN gene ablation. In the calsequestrin TG mouse model of dilated cardiomyopathy (DCM), crossbreeding with PLNKO mice suprarescued depressed contractile parameters and reverted the prolonged action potential back to control levels [25]. In the muscle-specific LIM protein KO mouse model of DCM, crossbreeding with PLNKO mice also resulted in a suprarescue of systolic parameters and reversal of depressed Ca^{2+} transients, chamber dilation, myofibrillar disarray and large scale fibrosis [24]. However, in the tropomodulin TG mouse model of DCM, chamber dimensions and heart weight/body weight ratios remained increased even after PLN ablation [22]. In TNF- α TG mice, PLN ablation increased contractile function and Ca^{2+} transients (amplitude and kinetics) in isolated cardiomyocytes, but it did not improve survival, cardiac remodeling or *in vivo* cardiac function [44]. In CaMKII δ_c TG mice, PLNKO exacerbated DCM due to a persistent leak of Ca from the RyR [45]. Gene transfer of a PLN-targeted antibody also improved myocyte contractility and Ca^{2+} handling in a hamster model of heart failure [46]. In human cardiomyocytes from failing hearts, PLNKO restored cell shortening and calcium transient amplitude back to normal levels [47].

Rescue of FHCs via PLN ablation has also witnessed mixed success. α -MHC R403Q TG mice are an example of an FHC resulting from a mutation in the thick myofilament. Consequently, these mice have reduced crossbridge rate constants, which indicate decreased affinity for strong binding of myosin to actin [48]. Upon ablation of PLN, systolic function was restored at 12 months, most likely due to the increase in Ca^{2+} resequestration and the subsequent release at each cardiac cycle. However, hypertrophy persisted at 10.5 months, which could be an adaptation to compensate for R403Q's decreased actin-myosin affinity. In a mouse model of FHC resulting from a truncation of myosin binding protein C, PLN ablation failed to rescue the hypertrophy, fibrosis and *in vivo* function [26]. Once again, this

involved a thick filament protein, but here the truncated protein may lack the ability to bind either titin or the myosin heavy chain resulting in decreased force transmission. As a result, PLN ablation was not able to overcome this defect at the whole heart level, although it did improve cell shortening in isolated cardiomyocytes.

In contrast to these previous reports employing two models of FHC resulting from thick filament mutations, our model of FHC from a thin filament mutation, TmE180G, showed successful rescue of both phenotype and whole heart function. The difference could be due to both the location of the mutation, thin versus thick filament, and the degree to which the TmE180G mutation alters near neighbor protein interactions. Similar to myosin R403Q and truncated myosin binding protein C, TmE180G has altered interactions with nearby myofilament proteins for it has decreased affinity for actin [49] and altered interactions with troponin T [50]. Nonetheless, our study shows that many of the maladaptive consequences of these altered interactions in Tm180 mice can be overcome via targeting calcium fluxes and improving relaxation.

4.4. Modulation of Ca^{2+} regulatory proteins as a potential target in human FHCs

Although our studies clearly show that PLN ablation can rescue a mouse model of FHC linked to a mutation in the thin filament protein, Tm, this result may not be directly translatable to treating human FHCs. Specifically, it has been shown in a case study involving two individuals expressing a truncated form of PLN (PLN L39stop, which essentially mimics PLNKO), both patients exhibited DCM and required a heart transplant early in life [51]. Thus, it appears that PLN ablation in humans has negative repercussions. However, in humans with heart failure in which the PLN and SERCA interaction is altered [52-54], gene therapy trials in heart failure patients are currently utilizing an adeno-associated virus to increase SERCA expression [55]. Thus, successful modulation of calcium cycling proteins to rescue human cardiomyopathies may simply be a matter of fine-tuning PLN to SERCA ratios or the phosphorylation status of PLN. Our data gives credence to the promise of modulating calcium cycling proteins in FHCs.

4.5. Conclusions

In conclusion, we have demonstrated that PLN ablation in a model of severe FHC caused by a mutation in a thin filament protein, Tm, reverses both adverse cardiac remodeling and depressed cardiac function. Previous studies from other FHC models involving thick filament mutations have shown improvement in one parameter but not in both [23, 26]. Finally, the full range of molecular mechanisms involved in rescuing Tm180 remains to be fully elucidated, yet it is clear that ERK1/2 signaling is a component of the process.

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Figure 1. Ablation of PLN reduces hypertrophy and expression of hypertrophic marker genes in Tm180 mice

Heart weight/body weight ratios were increased in Tm180 mice but returned to NTG levels in PLNKO/Tm180 mice at both 3 - 6 months (A) and one year of age (B). Atrial weights were also increased in Tm180 hearts at both 3 - 6 months (C) and one year (D) but returned to NTG levels in PLNKO/Tm180 hearts. $n=10-25$ for all heart weight values. The hypertrophic gene makers, β -MHC (E, F) and ANF (G, H), were also increased in Tm180 mice but decreased in PLNKO/Tm180 mice ($n=4-10$ for hypertrophic gene marker data). * denotes $P \leq 0.05$ vs. NTG, + denotes $P \leq 0.05$ vs. Tm180, # denotes $P \leq 0.05$ vs. PLNKO/NTG.

Figure 2. Ablation of PLN improves systolic and diastolic hemodynamic parameters in Tm180 mice at 3 – 6 months of age (*in situ* pressure-volume recordings)

(A) dP/dt_{max} , maximum rate of LV pressure development (an index of contractility), increases in PLNKO/Tm180 mice compared to Tm180 mice at baseline. Following intravenous infusion of the β -adrenergic agonist (ISO, 0.08 ng/g*min), dP/dt_{max} decreases in Tm180 mice compared to NTGs, yet PLNKO/Tm180 mice are similar to NTG values. (B) E_{max} , peak value of time-varying elastance (a load-independent parameter of contractility) during inferior vena cava occlusion. E_{max} decreases in Tm180 mice but is restored to NTG values in PLNKO/Tm180 mice. (C) dP/dt_{min} , maximum rate of LV pressure decline (an index of relaxation), decreases in Tm180 mice compared to NTGs but is restored to NTG values in PLNKO/Tm180 mice both at baseline and following ISO. (D) Tau, an index of relaxation time, is prolonged in Tm180 mice compared to NTGs, but this is restored to NTG values in PLNKO/Tm180 mice (baseline only). * denotes $P \leq 0.05$ vs. NTG, + denotes $P \leq 0.05$ vs. Tm180, # denotes $P \leq 0.05$ vs. PLNKO/NTG, $\blacklozenge$ denotes $P \leq 0.05$ vs. same group at baseline (NTG, Tm180, PLNKO/NTG or PLNKO/Tm180), $n=8-12$ animals for each group.

Figure 4. Ablation of PLN reduces myocyte disarray and fibrosis in Tm180 mice
 Histological sections (40x amplification) from one year-old mice stained with H&E (left panels) and Picrosirius Red (right panels). Note the myocyte disarray (H&E) and fibrosis (Picrosirius Red) in Tm180 compared to NTG and its reduction in PLNKO/Tm180.

Figure 5. Ablation of PLN reduces phosphorylation of ERK1/2

Left. Densitometry of Phospho-ERK1/2 T202,Y204/ ERK1/2 in 3-4 month-old mice. Right. Western blots of Phospho-ERK1/2 T202,Y204 and ERK1/2. * denotes $P \leq 0.05$ vs. NTG, + denotes $P \leq 0.05$ vs. Tm180, n=6 per group.