Tyrosyl phosphorylation of PZR promotes hypertrophic cardiomyopathy in PTPN11-associated Noonan syndrome with multiple lentigines

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Conflict of Interest

The authors have declared that no conflict of interest exists.
Abstract

Noonan syndrome with multiple lentigines (NSML) is a rare autosomal dominant disorder that presents with cardio-cutaneous-craniofacial defects. Hypertrophic cardiomyopathy (HCM) represents the major life-threatening presentation in NSML. Mutations in the \textit{PTPN11} gene that encodes for the protein tyrosine phosphatase (PTP), SHP2, represents the predominant cause of HCM in NSML. NSML-associated \textit{PTPN11} mutations renders SHP2 catalytically inactive with an “open” conformation. NSML-associated \textit{PTPN11} mutations cause hypertyrosyl phosphorylation of the transmembrane glycoprotein, protein zero-related (PZR) resulting in increased SHP2 binding. Here we show that NSML mice harboring a tyrosyl phosphorylation-defective mutant of PZR (NSML/PZR\textsuperscript{242F}) that is defective for SHP2 binding fail to develop HCM. Enhanced AKT/S6K signaling in heart lysates of NSML mice was reversed in NSML/PZR\textsuperscript{242F} mice demonstrating that PZR/SHP2 interactions promote aberrant AKT/S6K activity in NSML. Enhanced PZR tyrosyl phosphorylation in the hearts of NSML mice was found to drive myocardial fibrosis by engaging a Src/NFkB pathway resulting in increased activation of interleukin-6 (IL6). Increased expression of IL6 in the hearts of NSML mice was reversed in NSML/PZR\textsuperscript{242F} mice and PZR\textsuperscript{242F} mutant fibroblasts were defective for IL6 secretion and STAT3-mediated fibrogenesis. These results demonstrate that NSML-associated \textit{PTPN11} mutations that induce PZR hypertyrosyl phosphorylation trigger pathophysiological signaling that promotes HCM and cardiac fibrosis.
Introduction

Hypertrophic cardiomyopathy (HCM) is a genetic myocardial disorder that is characterized by left ventricular hypertrophy, cardiomyocyte disarray and myocardial fibrosis (1-3). Although most familial HCM is caused by mutations in genes that encode for sarcomeric proteins, such as myosin subunits, myosin binding proteins and filament proteins, a variety of disorders also exhibit apparent left ventricular hypertrophy in nonsarcomeric proteins (4). Noonan syndrome with multiple lentigines (NSML, OMIM 151100) is one of the nonsarcomeric forms of HCM that shows multiple clinical characteristics, such as growth retardation, dysmorphic facial features and post-developmental abnormalities (5, 6). Approximately 85% of NSML cases exhibit cardiac defects that includes HCM and pulmonary stenosis. Up to 85% of NSML patients have mutations in the \textit{PTPN11} gene, which encodes for the SH2 domain-containing protein tyrosine phosphatase 2 (SHP2) (7-9).

SHP2 is a non-receptor protein tyrosine phosphatase (PTP) that is ubiquitously expressed (7-9). The phosphatase activity of SHP2 is required for the propagation of multiple signaling pathways, including the Ras-MAPK and phosphatidylinositol 3’-kinase (PI3K)/AKT pathways, which are involved in proliferation, differentiation, migration and development (10-12). The catalytic activity of SHP2 is tightly regulated by intramolecular conformational constraints (13). The ‘closed’ conformation, which is mediated by the interaction between the SH2 and phosphatase domains, is destabilized by the engagement of the N-terminal SH2 domain to phosphotyrosine peptides, resulting in an ‘open’ conformation that renders the catalytic domain substrate accessible (14, 15). NSML-associated SHP2 mutations occur in the PTP domain that result in reduced phosphatase activity and an open conformation (16, 17). The open conformation of NSML-associated SHP2 mutations enhances SH2 domain protein-protein interactions that likely contribute to propagating aberrant signaling that promotes the pathogenesis of NSML (18, 19). However, definitive evidence of such SHP2-mediated SH2 domain engagement in NSML has yet to be established.
We previously reported that PZR is hypertyrosyl phosphorylated in the heart of NSML mice (20). PZR is a transmembrane glycoprotein comprised of an extracellular immunoglobulin like domain and two intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (21-23). PZR was identified as a SHP2 binding partner, with the SH2 domains of SHP2 binding phosphorylated tyrosine residues (Y241 and Y263) within the ITIM of human PZR (hPZR) (20, 23). Much of the information known about PZR relates to its role in adhesion-mediated cell signaling and migration (24-26). A zebrafish model study revealed that PZR tyrosyl phosphorylation is necessary for convergence and extension cell movements during zebrafish gastrulation (20). We demonstrated NSML-associated SHP2 mutations (SHP2<sup>Y279C</sup>, NSML-SHP2), which have an “open” conformation, have increased PZR association (20). In order to understand the signaling mechanisms of NSML-associated congenital heart disease, Marin et al generated a mouse model that expresses a knock-in of <i>Ptpn11<sup>Y279C/+</sup></i> NSML mutation (referred to herein as NSML mice) (27). These NSML mice exhibit features of the human disease including that of HCM (27, 28). We found that a low dose of the Src family kinase inhibitor, dasatinib, ameliorated PZR hyper tyrosyl phosphorylation and normalized the expression of molecular markers of HCM in the hearts of NSML mice (29). Although our findings suggested the involvement of aberrant PZR/NSML-SHP2 interactions in NSML-associated HCM, it was not definitively addressed as to whether PZR hypertyrosyl phosphorylation and enhanced PZR/SHP2 binding is essential to the development and/or progression of congenital heart disease in general and HCM specifically, in NSML mice.

In this study, we generated a tyrosyl phosphorylation defective PZR knock-in mutant mouse. Although PZR tyrosyl phosphorylation-defective mice did not exhibit a cardiac phenotype, when intercrossed with NSML mice, PZR hypertyrosyl phosphorylation and enhanced PZR/SHP2 binding were inhibited. Remarkably, NSML mice lacking the ability to generate hypertyrosyl phosphorylated PZR failed to promote enhanced AKT activity in the heart which correlated with a complete abrogation in the development of HCM. Further, we show that PZR/SHP2 engages nuclear factor kappa B (NFκB) signaling that stimulates secretion of interleukin 6 (IL6) that drives the fibrotic sequelae of HCM. These results demonstrate an essential function for PZR in the development of NSML-associated HCM. Our results
provide genetic evidence for targeting PZR tyrosyl phosphorylation as a therapeutic strategy to treat certain forms of RASopathy-associated HCM where PZR is hypertyrosyl phosphorylated.
Results

Generation of NSML mice defective for PZR tyrosyl phosphorylation. To investigate the role of PZR tyrosyl phosphorylation and hence PZR/SHP2 binding in NSML pathogenesis, we generated a tyrosyl phosphorylation defective PZR knock-in mutant mouse. Using CRISPR-Cas9 we introduced a tyrosine 242 to phenylalanine mutation in exon 6 of the *MpzIl* gene that encodes for PZR (Figure 1A and Supplemental Figure 1). Homozygous PZR knock-in mice (*MpzIl*Y242F/Y242F, referred to herein as the PZR<sup>Y242F</sup> mice) were born from heterozygous (*MpzIl*<sup>+</sup>/Y242F) intercrosses. PZR<sup>Y242F</sup> mice were fertile and showed the anticipated ratio of Mendelian offspring (Supplemental Table 1). We confirmed PZR tyrosyl phosphorylation deficiency by immunoblotting with PZR phospho-specific antibodies and found that phosphorylation of both tyrosine resides (Y242 and Y264) were abolished in heart lysates derived from PZR<sup>Y242F</sup> mice (Figure 1B). These results were consistent with previous reports indicating that mutation of one of the ITIM motifs was sufficient to inhibit the phosphorylation of the other (20, 23). PZR<sup>Y242F</sup> mice lack any major developmental defects and gained weight at a slightly reduced, but significant, rate as compared with wild type (WT) mice (Supplemental Figure 2A) and there were no differences in the weight of organs, such as lung, liver, spleen and kidney (Supplemental Figure 2B). Notably, at the age of 20-weeks PZR<sup>Y242F</sup> mice showed similar heart weight (H.W.) to body weight (B.W.) or tibia length (T.L.) ratios (Supplemental Figure 2C). We also performed echocardiography at 16-weeks of age, and no differences in interventricular septum wall thickness (IVS), left ventricular posterior wall thickness (LVPW) and ejection fraction (EF) in PZR<sup>Y242F</sup> mice as compared with WT mice were observed (Supplemental Table 2). However, analysis of the cardiac fetal gene, *Myh7* (myosin heavy chain β), *Nppa* (atrial natriuretic peptide) and *Nppb* (brain natriuretic peptide) and fibrotic gene (*Col1a*) were all reduced in PZR<sup>Y242F</sup> mice as compared with WT mice (Supplemental Figure 3). These data demonstrate that in context of PZR tyrosyl phosphorylation deficiency alone mice develop normally without impairment to cardiac functionality.

To test the hypothesis that hypertyrosyl phosphorylation of PZR and increased PZR/SHP2 interactions promotes HCM in NSML, we introduced the *MpzIl*Y242F mutation into NSML (*Ptpn11<sup>Y279C/+</sup>) mice
We back-crossed heterozygous $Ptnl1^{Y279C/+}$; $Mpzl1^{+/Y242F}$ mice in the first founder with $Pzr^{Y242F}$ mice to generate four genotypes; WT ($Ptnl1^{+/+}$; $Mpzl1^{+/Y242F}$), NSML ($Ptnl1^{Y279C/+}$; $Mpzl1^{+/Y242F}$), PZR ($Ptnl1^{+/+}$; $Mpzl1^{Y242F/Y242F}$) and NSML/PZR ($Ptnl1^{Y279C/+}$; $Mpzl1^{Y242F/Y242F}$). Analysis of PZR tyrosyl phosphorylation in lysates derived from the hearts of these mice demonstrated that NSML mice exhibited increased PZR tyrosyl phosphorylation as compared with WT mice as demonstrated previously (20). Whereas, in NSML/PZR $Y242F$ mice PZR tyrosyl phosphorylation at both Y242 and Y264 was undetectable (Figure 1C). We evaluated whether PZR tyrosyl phosphorylation deficiency in NSML mice disrupted PZR/SHP2 binding. Heart lysates from NSML hearts incubated with GST-conjugated SH2 domains of SHP2 (GST-SHP2 N+C) showed increased interaction to PZR as compared with WT and this binding was abrogated in heart lysates prepared from PZR $Y242F$ mice. Similarly, heart lysates prepared from NSML/PZR $Y242F$ mice also failed to interact with the SHP2 N+C SH2 domains (Figure 1D). These results demonstrate that PZR $Y242F$ is phosphorylation defective and fails to bind the SH2 domains of SHP2. Thus, PZR $Y242F$ and NSML/PZR $Y242F$ mice represent a loss-of-function for PZR tyrosyl phosphorylation and SHP2 binding.

**PZR tyrosyl phosphorylation deficiency rescues hypertrophic cardiomyopathy in NSML mice.** We investigated whether the PZR $Y242F$ mutation in NSML mice interferes with the progression of HCM. As previously reported, NSML mice develop HCM by 16-weeks of age (Figure 2, A-C) (27, 28). Although NSML/PZR $Y242F$ mice exhibit retarded post-developmental growth (Supplemental Figure 5), their cardiac mass and cardiac mass normalized to either body mass or tibia length was reduced to levels comparable with WT mice (Figure 2, A-C). Morphometric analysis of individual cardiomyocytes from 16-week old mice demonstrated that the cross-sectional area of cardiomyocytes in NSML mice were significantly increased as compared with WT cardiomyocytes (Figure 2, D-F). In contrast, PZR $Y242F$ mutation rescued the NSML-associated cardiomyocyte enlargement (Figure 2, D-F). Additional cardiac parameters were assessed by echocardiography and these results showed that diastolic left ventricular posterior wall thickness (LVPW, d) and interventricular septum wall thickness (IVS, d) were significantly reduced in
NSML/PZR<sup>Y242F</sup> mice compared with NSML mice (Figure 2, G and H, and Supplemental Table 3). The transition of sarcomeric structural gene expression from myosin heavy chain α (<i>Myh6</i>) to myosin heavy chain β (<i>Myh7</i>) is indicative of cardiomyopathy (30). Whereas, <i>Myh7</i> and the ratio of <i>Myh7</i> to <i>Myh6</i> were prominently re-expressed in NSML mice compared with WT mice, this ratio was normalized in NSML/PZR<sup>Y242F</sup> mice (Figure 2I). We also assessed the mRNA expression of clinical markers of cardiomyopathy. Both <i>Nppa</i> and <i>Nppb</i> mRNA expression levels were significantly upregulated in NSML mice as compared with WT mice, while NSML/PZR<sup>Y242F</sup> mice had their levels restored to normal (Figure 2J). Together, these data demonstrate that PZR tyrosyl phosphorylation and thus, PZR/SHP2 complex formation contributes to the development of HCM in NSML mice.

**Effect of PZR tyrosyl phosphorylation on NSML signaling.** It has been shown that the hearts of NSML mice exhibit HCM as a result of increased levels of AKT phosphorylation suggesting that the PI3K/AKT pathway plays a central role in promoting NSML-associated HCM (27, 28, 31). We therefore investigated whether AKT was affected in the hearts of NSML/PZR<sup>Y242F</sup> mice. As previously reported (27), lysates derived from the hearts of NSML mice showed increased levels of AKT and S6 kinase (S6K) phosphorylation as compared with WT mice (Figure 3). Whereas, heart lysates from NSML/PZR<sup>Y242F</sup> mice failed to show increased AKT and S6K phosphorylation levels as compared with NSML mice (Figure 3). Notably, ERK1/2 phosphorylation levels in heart lysates were comparable across all four genotypes (Figure 3). These results demonstrate that PZR tyrosyl phosphorylation, and hence PZR/SHP2 interactions are required for the hyperactivation of the PI3K/AKT pathway correlating with the progression of HCM in NSML mice.

**Transcriptome analysis in the heart of NSML/PZR<sup>Y242F</sup> mice.** To provide insight into the effects of PZR<sup>Y242F</sup> mutation in NSML-associated gene expression in the heart, whole transcriptome RNA sequencing (RNA-seq) was performed in tissue derived from the hearts of these mice. We generated 22 ~ 49 million
reads per sample, filtered reads to have high quality scores and mapped 84.1 - 86.3% of those reads to the mouse genome. Principal component analysis shows that samples from the same genotype cluster, suggesting the transcriptome profiles were different in the 4 genotypes (Supplemental Figure 6). In order to identify candidate genes that are regulated by either NSML-SHP2 mutation and/or PZR tyrosyl phosphorylation in the heart, we identified differentially expressed genes ($p$ value < 0.05, [fold change]$ \geq 2.0$). 180 genes were found to be differentially expressed between NSML and WT, with 63 down-regulated and 117 up-regulated in the NSML group, as shown in the volcano plot (Figure 4A). When NSML mice were compared with PZR$^{Y242F}$ mice, 142 differentially expressed genes were identified, with 54 down-regulated and 88 up-regulated (Figure 4B). 129 genes were differentially expressed in NSML compared with NSML/PZR$^{Y242F}$ group, in which 55 genes were down-regulated and 74 up-regulated (Figure 4C). A Venn diagram shows the number of differentially expressed genes among the three pairwise comparisons (Figure 4D). Interestingly, we found 8 genes (Acaa1b, Actg2, Gm10401, Gm45909, Gm8206, Itih4, Prss5 and Rpl27-ps1) that were commonly downregulated and 1 gene (Fos) was commonly upregulated in NSML compared to the other three groups (Figure 4D). Ingenuity Pathway Analysis identified cardiac hypertrophy-related genes ($p = 0.0187$ for NSML vs. WT and $p = 0.0124$ for NSML vs. NSML/PZR$^{Y242F}$) such as Myh7 and Igf1 were differentially expressed in the heart of NSML mice (Figure 4E). The hierarchical clustering of log ratio transformed gene expression was represented in a heatmap and showed 185 genes that were differentially expressed between 4 genotypes ($p < 0.01$) (Figure 5A). Further, we identified 4 groups (groups #1, #2, #3 and #4) of genes in the hearts of NSML/PZR$^{Y242F}$ mice that were apparently reverted in their expression to WT levels (Figure 5A). Genes identified in groups #1 - #4 exhibiting revertant behavior (Figure 5B) demonstrate that they are dependent upon PZR tyrosyl phosphorylation and hence represent a transcriptomic “fingerprint” of NSML/PZR target genes that are potentially involved in the progression of HCM.

**PZR tyrosyl phosphorylation in NSML drives cardiac fibrosis and dysregulated IL6 secretion.**

Myocardial fibrosis is one of the clinical sequelae of HCM that contributes to left ventricular dysfunction,
heart failure and sudden cardiac death (1). As reported previously (27, 29), the hearts of NSML mice exhibited myocardial fibrosis as detected by Sirius red staining and increased expression of fibrotic genes (Col1a and Col3a) (Figure 6, A, B and C). However, hearts of NSML/PZR$^{Y242F}$ mice were devoid of myocardial fibrosis and the expression of fibrotic genes were equivalent to WT mice (Figure 6, A, B and C). These observations suggested that PZR tyrosyl phosphorylation in NSML contributes to myocardial fibrosis. To explore a potential mechanism through which PZR tyrosyl phosphorylation contributes to myocardial fibrosis in NSML mice, we examined the mRNA expression of cytokines in the heart of NSML mice (Figure 6D). We found that Il1b, Il6 and Tnf mRNA expression levels were significantly upregulated in the heart of NSML mice as compared with WT mice (Figure 6D). To determine whether the expression of these cytokines were dependent upon PZR tyrosyl phosphorylation we utilized mouse embryonic fibroblasts (MEFs) derived from WT and PZR$^{Y242F}$ mice. PZR tyrosyl phosphorylation was induced by treating cells with concanavalin A (ConA) (32) (Figure 6E). We found that Il6 and Tnf mRNA expression were significantly increased in WT MEFs by ConA (Figure 6F) but Il1b expression was undetectable. Treatment of PZR$^{Y242F}$ MEFs with ConA which did not induce PZR tyrosyl phosphorylation (Figure 6E) resulted in the diminution of Il6 but not Tnf mRNA expression (Figure 6F) indicating that the induction of Il6 rather than Tnf mRNA was dependent upon PZR tyrosyl phosphorylation. Furthermore, ConA was capable of inducing IL6 secretion in WT MEFs however, in PZR$^{Y242F}$ MEFs IL6 secretion was undetectable (Figure 6G). Similar results were obtained when PZR tyrosyl phosphorylation was induced by plating cells on to fibronectin (Supplemental Figure 7), further substantiating the interpretation that IL6 secretion is dependent upon PZR tyrosyl phosphorylation. We also re-introduced PZR WT into PZR$^{Y242F}$ MEFs and demonstrated that expression of PZR WT rescues Il6 expression in both basal and upon ConA induction (Supplemental Figure 8). Notably, low-dose dasatinib (0.1 mg/kg/day) treatment from the age of 12-weeks for 4 weeks which, prevents the progression of fibrosis in NSML mice (29), also reduced Il6 mRNA expression in the heart of NSML mice (Figure 6H). Importantly, the increased levels of Il6 mRNA expression in the heart and increased circulating IL6 levels in NSML mice were completely restored to WT levels in NSML/PZR$^{Y242F}$ mice (Figure 6I). Given the established role of IL6 in promoting cardiac fibrosis
These data demonstrate that PZR tyrosyl phosphorylation in NSML stimulates IL6 production which likely contributes to the later development of myocardial fibrosis in NSML mice.

**PZR tyrosyl phosphorylation activates IL6-mediated fibrogenesis by coupling to the NFκB pathway.**

The inhibitor of NFκB (IκB) kinase (IKK) and its downstream NFκB signaling pathway is an important regulator of inflammation and is implicated in HCM and heart failure (35-39). *Il6* is a major NFκB target gene and ablation of NFκB activity ameliorates HCM in a variety of mouse models (35, 36, 39-47). IKK is an enzyme complex that is composed of IKKα, IKKβ and IKKγ, that facilitates NFκB activation through phosphorylation-mediated protein degradation of IκB (48, 49). c-Src kinase has been shown to phosphorylate and activate IKKβ (50). We previously suggested that PZR hypertyrosyl phosphorylation results in enhanced Src signaling through increased recruitment of SHP2/Src to PZR thereby driving aberrant signaling in NSML (20, 29). We hypothesized that enhanced PZR/SHP2/Src signaling promotes IKKβ phosphorylation leading to NFκB-mediated *Il6* expression. To test this, we determined whether NFκB signaling is dependent on PZR tyrosyl phosphorylation. When WT MEFs were treated with ConA, PZR tyrosyl phosphorylation was induced (Figure 7A). In contrast, ConA-treated PZR<sup>Y242F</sup> MEFs failed to promote tyrosyl phosphorylation of IKKβ (Figure 7A). Consistent with defective IKKβ activation in PZR<sup>Y242F</sup> MEFs, IκB protein levels were increased in these cells as compared with WT MEFs (Figure 7A). These data suggest that increased activation of *Il6* gene expression in the hearts of NSML mice is driven by PZR tyrosyl phosphorylation which activates IKK-NFκB signaling. To further substantiate this interpretation, we examined the effects of an array of small molecule inhibitors of the Src and IKK-NFκB pathway on ConA-induced *Il6* mRNA expression in WT MEFs. Consistent with the notion that PZR/SHP2 couples to the NFκB pathway through c-Src, the Src kinase inhibitor (dasatinib), IKKβ inhibitor (IKK-16), NFκB nuclear transportation inhibitor (JSH23) and NFκB DNA binding inhibitor (GYY4137) all significantly reduced *Il6* mRNA expression and IL6 secretion in ConA-treated WT MEFs (Supplemental Figure 9, A-D). We also confirmed that these inhibitors ameliorated *Il6* mRNA expression in PZR<sup>Y242F</sup>
MEFs re-expressing WT PZR (Supplemental Figure 9E). Collectively, these data demonstrate that PZR hyper tyrosyl phosphorylation promotes NFκB signaling resulting in enhanced IL6 secretion.

IL6 activates the signal transducer and activator of transcription 3 (STAT3) which upregulates fibrotic genes, such as Col1a (51, 52). Since IL6 secretion was dependent upon PZR tyrosyl phosphorylation (Figure 6, F and G), we hypothesized that STAT3 activity in PZR$^{Y242F}$ MEFs would be reduced. Indeed, we found that ConA-induced STAT3 tyrosyl phosphorylation and Colla expression were dramatically reduced in PZR$^{Y242F}$ MEFs as compared with WT MEFs (Figure 7, B and C). These data demonstrate that PZR tyrosyl phosphorylation is responsible for the upregulation of IL6 which acts in an autocrine manner to induce STAT3 phosphorylation. Furthermore, conditioned medium from ConA-treated WT MEFs (CM-WT) induced STAT3 phosphorylation and Colla expression in PZR$^{Y242F}$ MEFs, which are defective for IL6 secretion (Figure 8A), but not by conditioned media from ConA-treated PZR$^{Y242F}$ MEFs (CM-PZR$^{Y242F}$) (Figure 8A). We confirmed that the effects of the CM-WT on Colla expression, was specifically due to IL6 by treating the CM-WT with an IL6 neutralizing antibody followed by assessment of Colla gene expression in PZR$^{Y242F}$ MEFs (Figure 8B). These experiments showed that STAT3 phosphorylation and Colla gene expression were reduced by IL6 neutralizing antibody treatment in a dose-dependent manner (Figure 8B). These data suggest that PZR hyper tyrosyl phosphorylation in the heart of NSML mice promotes cardiac fibrosis through increased secretion of IL6 via NFκB followed by autocrine activation of IL6/STAT3-mediated fibrotic gene expression.
Discussion

Previously, we reported that in the heart of NSML mice the ITIM-containing transmembrane glycoprotein PZR is hyper tyrosyl phosphorylated resulting in enhanced plasma membrane recruitment of SHP2 (20, 29). Although proposed to be involved in congenital heart disease, and possibly HCM in NSML, a causal role of PZR tyrosyl phosphorylation in HCM remained to be formally established. To address the question of whether PZR tyrosyl phosphorylation, and hence SHP2 binding, plays a role in NSML-associated HCM we generated a knock-in mouse harboring a mutant form of PZR (PZR$^{Y242F}$ mouse) that is defective for tyrosyl phosphorylation and thus, SHP2 binding. PZR$^{Y242F}$ mice show no developmental or post-developmental defects and exhibit normal postnatal growth. Therefore, tyrosyl phosphorylation of PZR in this context does not appear essential for cardiac development under normal physiological conditions. Whether there are defects in these PZR$^{Y242F}$ mice in other tissues cannot be excluded. However, when PZR$^{Y242F}$ mice were intercrossed with NSML mice the development of HCM seen in NSML mice was completely abrogated. These results demonstrate that in context of a NSML mutation PZR tyrosyl phosphorylation and SHP2 association plays a direct role in promoting HCM. Mechanistically, PZR/SHP2 binding in NSML promotes AKT activity in the hearts of NSML mice (Figure 8C). While, PZR/SHP2 binding induces the activation of NFκB resulting in the paracrine/autocrine secretion of IL6 that promotes myocardial interstitial fibrosis (Figure 8C). Thus, NSML-SHP2 mutants engage PZR to activate pathways distinct from, but parallel to, the canonical “RASopathy” pathway assigned to these mutations.

Multiple signaling pathways have been attributed to the development of HCM and the interplay of these mechanisms further complicates a clear understanding of how non-sarcomeric-driven HCM manifests. Nevertheless, the molecular basis, at least in part, for the development of HCM in NSML mice has been attributed largely to enhanced activation of AKT/mTOR signaling (27, 28, 31, 53). Remarkably, we observed that NSML/PZR$^{Y242F}$ mice showed completely normal heart weight/body weight as well as heart weight/tibia length as compared with wild type mice. Consistent with this, cardiomyocyte size in NSML/PZR$^{Y242F}$ mice was completely reverted to wild type cardiomyocyte size. These results correlated with the observed increased AKT activity in heart lysates derived from NSML mice as expected, but in
NSML/PZR<sup>Y242F</sup> mice, AKT and S6K activity were equivalent to that observed in wild type animals. Collectively, these results demonstrate that the observed hypertyrosyl phosphorylation of PZR in the hearts of NSML mice contributes to the hyperactivation of the AKT/mTOR pathway to promote HCM (Figure 8C). The hyperactivation of AKT seen in the hearts of NSML mice that is rescued upon introduction of the PZR<sup>Y242F</sup> allele occurs without appreciable changes in ERK1/2 phosphorylation. This raises the question of the role of ERK1/2 downstream of the NSML-SHP2 mutation in the development of HCM. Our data imply that despite the NSML-SHP2 mutation being inactivating for SHP2 phosphatase activity there is an evident dispensability for ERK1/2 for the development of NSML-associated HCM. It is important to note that our studies do not define the extent to which PZR hyper tyrosyl phosphorylation, AKT and ERK1/2 act in a particular cell type(s) <em>in vivo</em> to promote NSML-mediated HCM. Studies employing cell type-specific PZR tyrosyl phosphorylation knockin mice will be required to uncover these <em>in vivo</em> details as well as extending these studies to assess potential sex differences by examining female mice. Despite the aforementioned limitations, from a molecular standpoint NSML-SHP2 mutations adopt an open conformation which is critical for PZR hypertyrosyl phosphorylation and enhanced PZR/SHP2 binding these results indicate that NSML-SHP2 mutants behave as gain-of-function mutants. We suggest that acquisition of the open SHP2 conformation rendered by the NSML-associated mutations largely defines the molecular basis for NSML, at least for the progression of HCM. However, this interpretation is not mutually exclusive of the possibility that the lower levels of catalytic activity of NSML-associated SHP2 mutations also contribute to the development of HCM through other pathways.

We have previously suggested that low-dose dasatinib could serve as a potential therapeutic for the treatment of HCM in NSML (29). Our results suggest that low-dose dasatinib which inhibits PZR tyrosyl phosphorylation and prevents the development of HCM in NSML mice could act by blocking PZR tyrosyl phosphorylation and association with SHP2, and subsequently AKT activity. Additionally, low-dose dasatinib treatment in NSML mice also reduced IL6 expression levels. Given that other therapeutic strategies against NSML have targeted the AKT/mTOR pathway, although effective in animal models and
in a patient (27, 31, 53), toxicity at the effective doses makes this line of therapy prohibitive. If as proposed, enhanced AKT activation can be curtailed through modulating PZR tyrosyl phosphorylation using a low-dose tyrosine kinase inhibitor, such as dasatinib, this strategy might offer an attractive therapeutic path to treat certain NSML cases with HCM with minimum toxicity. PZR is also hypertyrosyl phosphorylated in the hearts of Noonan syndrome mice that also exhibit congenital heart disease phenotypes that are rescuable by low-dose dasatinib treatment (29). Whether PZR hypertyrosyl phosphorylation and increased PZR/SHP2 interactions are also similarly causal in Noonan syndrome-related HCM as well as other forms of HCM such as that induced by pressure overload remains to be determined.

Initiation of promiscuous signaling as a result of a SHP2 mutation in NSML that inactivates the phosphatase activity whilst simultaneously generating an “open” SHP2 conformation creates the formal possibility for both a loss-of- and gain-of-function attributes for SHP2 signaling (18). This complexity is further amplified as a result of enhanced upstream SHP2 target binding as observed through an upregulation of PZR/SHP2 interactions in the hearts of NSML mice which sustains the steady-state lifetime of this and other SHP2 SH2 domain-mediated complexes. Conceivably, these mechanisms are concurrently operable and potentially inter-operable culminating in the dysregulation of multiple downstream pathways that regulate critical gene transcription events that drive congenital heart disease and in particular HCM. Here we define a novel link between PZR hypertyrosyl phosphorylation and downstream signaling to AKT. However, multiple pathways likely contribute to the development of HCM in NSML and as such PZR represents one such signaling component in the manifestation of this disease. Given that PZR tyrosyl phosphorylation rescues NSML-mediated HCM we assessed in an unbiased manner the potential target genes dependent on PZR tyrosyl phosphorylation/SHP2 binding by performing RNA-seq transcriptome analysis in NSML, PZR<sup>Y242F</sup> and NSML/PZR<sup>Y242F</sup> mice. A global analysis between these genotypes revealed qualitatively 4 major cohorts of gene sets that were differentially expressed in NSML mice that were reverted back to “normal” in NSML mice expressing the PZR tyrosyl phosphorylation site mutant. These apparent “revertant” groups of genes suggest the existence of an identifiable “fingerprint” of PZR
tyrosyl phosphorylation-dependent genes that are dysregulated in context of the pathophysiological signals driven by the expression of SHP2-associated NSML mutants. Identifying the function of such genes may provide insight into the molecular program governing either the initiation and/or maintenance of HCM and possibly other NSML-associated phenotypes. We identified Fos as upregulated by 3-7 fold in NSML mouse hearts and the upregulation of Fos in the hearts of NSML mice was dependent upon PZR tyrosyl phosphorylation. Provocatively, Fos is an immediate early gene that has been shown to be upregulated in the hearts of patients with HCM (54, 55). Additionally, we identified Grin2c (GluN2C/NR2C) which encodes for the N-methyl-D-aspartate (NMDA) receptor subunit C (19), as a NSML target gene that is reverted to wild type levels in NSML/PZR\(^{Y242F}\) mice. Interestingly, chronic NMDA receptor activation has been associated with cardiac remodeling, cardiac electrical dysfunction and myocardial fibrosis (19). Given that NSML patients exhibit electrical conduction disorders of unknown etiology the identification of potential targets affecting this symptom may be of clinical significance.

A precipitating consequence of HCM is the development of myocardial fibrosis which underlies the deterioration of cardiac contractility that accompanies heart failure (3, 56, 57). The basis for myocardial fibrosis has been attributed, at least in part, to both local and systemic inflammation caused by increased circulation of pro-inflammatory cytokines (58). NSML/PZR\(^{Y242F}\) mice were resistant to the development of myocardial fibrosis consistent with the inhibition of HCM as compared with NSML mice. Although we identified a set of pro-inflammatory cytokines that were significantly upregulated in NSML mice, only IL6 expression levels were restored to wild type levels in NSML/PZR\(^{Y242F}\) mice. These observations demonstrate that in NSML mice the PZR/SHP2 interaction is sufficient to promote the upregulation of IL6 secretion (Figure 6F). The involvement of IL6 in the development of myocardial fibrosis in HCM is well established (19, 33, 34, 59). IL6 infusion results in concentric hypertrophy in rats and IL6 deletion attenuates left ventricular hypertrophy, myocardial fibrosis and left ventricular dysfunction in response to pressure overload in mice (34). IL6 also acts on fibroblasts to promote fibroblast proliferation and collagen production, as such the actions of IL6 on fibroblasts is a recognized contributing factor of myocardial
fibrosis (34, 60, 61). Our results demonstrating that fibroblasts expressing PZR<sup>Y242F</sup> fail to promote IL6 expression supports the interpretation that PZR/SHP2 interactions regulate IL6 expression and secretion. Mechanistically, we demonstrated that PZR/SHP2 interactions activate IL6 via a Src-dependent pathway that stimulates the IKK enzyme complex, phosphorylation of IκB and NfκB nuclear translocation (Figure 8C). Consistent with the notion that this pathway proceeds through Src-dependent mechanism, low-dose dasatinib treatment of NSML mice restores the elevated Il6 levels to those comparable to wild type mice. We further uncovered that the actions of PZR to promote IL6 secretion in fibroblasts correlated with the upregulation of collagen expression. This was likely a result of IL6 acting in an autocrine manner on fibroblasts to stimulate STAT3-mediated expression of Coll1a1. Although our data showed that at least fibroblasts are a target of IL6, we cannot exclude other cell types, such as cardiomyocytes, endothelial cells or hematopoietic cells that could also be receptive to the supporting auto- or paracrine actions of IL6 in NSML pathogenesis. Notably, STAT3 has been implicated in HCM (19), at this juncture our data does not exclude a role for STAT3 in HCM of NSML mice.

In summary, our data demonstrates using a PZR tyrosyl phosphorylation-defective mouse model that PZR/SHP2 interactions are critical for the development of NSML-associated HCM. These data identify the NFκB signaling pathway as a novel PZR/SHP2 downstream target in NSML and suggest that this pathway contributes to the cardiac pathogenesis associated with NSML.
Methods

Generation of *Mpzl1*<sup>Y242F</sup> mouse strain – The *Mpzl1*<sup>Y242F</sup> mutation was introduced via CRISPR-Cas9 genome editing essentially as described (62). Potential Cas9 target guide (protospacer) sequences were screened using the MIT CRISPR tool (crispr.mit.edu) over a genomic sequence spanning two potential Tyr codons: 5’-CTCTTCTCCAGGGCCAGTCATTTACGCACAGTTAGACCACCCTGCGGACACCACA GCGGCAAGATTAATAAGTCAGAGTCGGTTGTGTATGCGGACATCCGG-3’. The protospacer sequence TCTAACTGTGCGTAAATGAC(PAM TGG) was selected based on its overlap with the Y242 codon, its high specificity score 81, and the tool’s prediction of no likely off-target sites on chromosome 1. A PCR primer was synthesized containing a T7 promoter, the protospacer and the 5’ end of the sgRNA scaffold sequence (Supplemental Table 4). The template for sgRNA *in vitro* transcription was generated by PCR amplification using this primer and the “universal” reverse primer from the pX330 sgRNA expression template (Addgene) (63). sgRNA was transcribed from the template using the MEGAShortscript kit (Invitrogen). Cas9 mRNA was similarly transcribed *in vitro* from the pX330 plasmid using the primers listed. Transcribed RNAs were purified using MEGAclear kit (Invitrogen) and eluted using microinjection buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl and 0.25 mM EDTA). Template oligonucleotide (ssODN) containing the specified base pair changes was synthesized by IDT. Components were mixed in injection buffer at a concentration of 30 ng/µl Cas9 mRNA, 15 ng/µl each sgRNA, and 35 ng/µl ssODN, centrifuged, and microinjected into the cytoplasm of C57BL/6J zygotes. Embryos were transferred to the oviducts of pseudopregnant CD-1 foster females using standard techniques (64). 21 pups were screened for the *Mpzl1*<sup>Y242F</sup> mutation and 4 pups contained the correct base pair substitutions.
Animal handling – *Mpzll^{+/Y242F}* heterozygotes were crossed with each other, and offspring were genotyped by PCR and digestion with *Aeg1* for the Y242F allele. *Ptpn11^{Y279C/+}* male mice were crossed with wild type C57BL/6J female, and offspring were genotyped by PCR for the Y279C allele. All mice were maintained on C57BL6/J backgrounds, backcrossed for more than 10 generations and genotyped by PCR using tail DNA. We used 16-20 weeks old male mice in this study. Dasatinib was intraperitoneally injected (0.1 mg/kg/day) into male 12-weeks-old mice for 4 weeks.

Antibodies, chemicals, cell lines and plasmids – The following antibodies were used for immunoblotting. Rabbit polyclonal SHP2 (C-18) (sc-280, IB-1:1,000) and mouse monoclonal GST (B-14) (sc-138, IB-1:1,000) antibodies were from Santa Cruz Biotechnology. Rabbit monoclonal phospho-PZR (Y241; D6F9) (#8131, IB-1:1,000), rabbit monoclonal phospho-PZR (Y263; D6A5) (#8088, IB-1:1,000), rabbit polyclonal phospho-AKT (S473; #9271, IB-1:1,000), rabbit polyclonal phospho-AKT (T308; #9275, IB-1:1,000), rabbit polyclonal phospho-AKT (T389; #2708, IB-1:1,000), mouse monoclonal AKT1 (C73H10) (#2938; IB-1:1,000), rabbit monoclonal phospho-S6K (T389; 108D2) #9234; IB-1:1,000), rabbit monoclonal S6K (49D7) (#2708; IB-1:1,000), rabbit polyclonal phospho-ERK1/2 (T202/Y204; #9101, IB-1:1,000), mouse monoclonal ERK (3A7) (#9107, IB-1:1,000), rabbit monoclonal PZR (D17B10) (#9893s, IB-1:1,000), rabbit monoclonal IKKβ (D30C6) (#8943; IB-1:1,000), rabbit monoclonal IκBα (L35A5) (#4814; IB-1:1,000), rabbit monoclonal phospho-STAT3 (Y705; D3A7) (#9145; IB-1:1,000) and rabbit monoclonal STAT3 (79D7) (#4904; IB-1:1,000) antibodies were purchased from Cell Signaling Technology. Rabbit polyclonal phospho-IKKβ (Y188; ab192820, IB-1:1,000) was obtained from Abcam. Goat polyclonal IL6 neutralizing antibody (#AB-406-NA) and normal goat IgG control antibody (#AB-108-C) were purchased from R&D
system. Rabbit polyclonal PZR antibody (105-6, IB-1:1,000) was generously provided by Z. J. Zhao(21). IL6 levels in serum and medium were measured using an enzyme-linked immunosorbent assay (ELISA) kit (mouse IL6 Quantikine ELISA, R&D system, #M6000B). Dasatinib (#1586-100) was purchased from Biovision. IKK16 (#13313), JSH-23 (#10536), GYY4137 (#13345) were from Cayman Chemical. Mouse embryonic fibroblasts (MEFs) were isolated from wild type and PZR<sup>Y242F</sup> mice. Cells were grown in growth medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum) in a 5% CO<sub>2</sub> incubator at 37°C. The mammalian plasmid expressing human PZR (pCDNA3-hPZR-WT) were described previously(24) and was transfected into MEFs using the Lipofectamine 2000 (Invitrogen).

**Immunoblotting** – Cells or heart tissue were lysed on ice in lysis buffer (25 mM Tris-HCl, pH 7.4, 136 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1% Nonidet P-40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 1 mM benzamidine, 1 mM PMSF, 1 µg/ml pepstatin A, 5 µg/ml aprotinin, and 5 µg/ml leupeptin). Cell or tissue lysates were incubated at 4 °C for 30 min and clarified by centrifugation at 14,000 rpm at 4 °C for 10 min. Protein concentration was determined using the BCA reagent according to the manufacturer’s instructions (#23225; Thermo Fisher Scientific). Total lysates were subjected to SDS-PAGE and immunoblotting. The sites of antibody binding were visualized and were quantified using the Odyssey CLx Imaging System (LI-COR Bioscience).

**In vitro GST-affinity precipitation assay** – Recombinant GST-SH2 domains of human SHP2 protein was purified from bacterial cells (pGEX-2TK SHP2 N+C, BL21(DE3)). Affinity precipitation assays were carried out in 1 mg of heart lysates with GST-SH2 of SHP2 protein.
overnight at 4 °C. SH2 domain-bound PZR proteins were affinity purified by BSA-coated GST-Sepharose 4B (#17-0756-01; GE Healthcare) for 1 hr at 4 °C. The interaction between SH2 domains of SHP2 and PZR was examined by immunoblotting with anti-PZR antibodies.

**Histology** – Hearts were isolated, fixed in 4% paraformaldehyde in phosphate-buffer saline (PBS), processed for paraffin sections and stained with hematoxylin and eosin (H&E), Sirius Red or Alexa-488 conjugated wheat germ agglutinin (WGA; Molecular probes; #W11261). Tissue images were obtained under bright field microscopy or fluorescence microscopy (Axiovert 200M; Carl Zeiss).

**Echocardiography** – Transthoracic echocardiography was performed by the Mouse UltraSound Imaging Core (MUSIC) at Yale Cardiovascular Research Center. Mice were kept anesthetized with 1.5% isoflurane supplied by a nose cone connected to the isoflurane vaporizer while maintaining physiological temperature. Standardized cardiac views were obtained with a high-resolution ultrasound system (Vevo 2100, VisualSonics) equipped with an ultra-high frequency (40 MHz) linear array transducer. Left Ventricular (LV) ejection fraction, LV end-diastolic and end-systolic volumes, and LV wall thickness were measured offline using Vevo Lab software (version 3.1.0, VisualSonics).

**RNA extraction and quantitative real-time PCR analysis** – RNA was isolated from mice heart using an RNeasy kit (#74104; Qiagen) according to the manufacturer's instructions. A total of 1 μg RNA was reverse transcribed to generate cDNA using a reverse transcriptase PCR kit (#4368814; Applied Biosystems). Real-time quantitative PCR was carried out in triplicate using
the Applied Biosystems 7500 Fast real-time PCR system and PowerUp SYBR green master mix (#A25742; Applied Biosystems) with primer pairs listed in Supplemental Table 5. All relative gene expression levels were analyzed using the ΔΔC_{T} method and normalized to 18S rRNA expression.

**RNAseq analysis** – Total RNA was isolated from the heart of mice using an RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA quality was determined by estimating the A260/A280 and A260/A230 ratios by nanodrop. RNA integrity was determined by running an Agilent Bioanalyzer gel, which measures the ratio of the ribosomal peaks. mRNA was purified from approximately 50 ng of total RNA with oligo-dT beads and sheared by incubation at 94 °C in the presence of Mg^{2+} (Kapa mRNA Hyper Prep). Following first-strand synthesis with random primers, second strand synthesis and A-tailing were performed with dUTP to generate strand-specific sequencing libraries. Adapter ligation with 3’-dTMP overhangs were ligated to library insert fragments. Library amplification amplified fragments carrying the appropriate adapter sequences at both ends. Strands marked with dUTP were not amplified. Indexed libraries that meet appropriate cut-offs for both were quantified by qRT-PCR using a commercially available kit (KAPA Biosystems) and insert size distribution determined with the LabChip GX or Agilent Bioanalyzer. Samples with a yield of ≥0.5 ng/µl were used for sequencing. Sample concentrations were normalized to 1.2 nM and loaded onto an Illumina NovaSeq flow cell at a concentration that yields 25 million passing filter clusters per sample. Samples were sequenced using 100 bp paired-end sequencing on an Illumina NovaSeq according to Illumina protocols. The 10 bp dual index was read during additional sequencing reads that automatically follows the completion of read 1. Data generated during sequencing runs were simultaneously transferred to the YCGA high-
performance computing cluster. A positive control (prepared bacteriophage Phi X library) provided by Illumina is spiked into every lane at a concentration of 0.3% to monitor sequencing quality in real time. Signal intensities were converted to individual base calls during a run using the system's Real Time Analysis (RTA) software. Base calls were transferred from the machine's dedicated personal computer to the Yale High Performance Computing cluster via a 1 Gigabit network mount for downstream analysis.

Analysis of RNA-seq data and pathway analysis – The RNAseq and statistical analysis was performed using Partek Flow Genomic Analysis software build version 8.0.19.1125 (Partek Inc.). Paired-end reads were trimmed and aligned to the Genome Reference Consortium Mouse Build 38 (mm10) with the STAR alignment tool (ver. 2.6.1d). Total counts per gene were quantified and normalized to identify differentially expressed genes (DEGs). List of differentially expressed genes or transcripts were generated by DESeq2. Qlucore Omics Explorer 3.5 (Qlucore AB) was used for PCA of log₂ transformed of global expression values, heatmap generation and hierarchical clustering. DEGs used in pathway analysis were determined by using a filtering criteria fold change $\leq -1.2$ or $\geq 1.2$ ($p \leq 0.05$), Benjamini-Hochberg multiple testing Correction $p$-Value. Ingenuity Pathway Analysis (QIAGEN), IPA software (ver. 10-14) was used to identify top upstream regulators, top canonical pathways, diseases and functions overrepresented in the DEG.

Statistical analysis – No statistical methods were used to predetermine sample size. The number of samples ($n$) used in each experiment is shown. All in vitro experiments were performed at least three times independently. Sample size for animal studies was not estimated and randomization was not applied. The investigators were blinded during echocardiography experiments,
histological analyses and outcome assessment. Statistical analysis and graphing were performed using GraphPad Prism 8 software. We did not estimate variations in the data. The variances are similar between the groups that are being statistically compared. All data represent the means ± standard errors of the means (SEM). For $p$ value determinations, we used two tailed student’s $t$-test or ANOVA with multiple comparison, Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.

**Study Approval**

Animals were housed and cared for in facilities run by the Division of Animal Care and were routinely monitored by the veterinary staffs. Animal handling was approved by Yale University Institutional Animal Care and Use Committee.
Author Contributions

J.-S.Y. and A.M.B. conceptualized the research, designed the experiments, and wrote the manuscript. J.-S.Y. performed the majority of the experiments and analyzed data. J.-S.Y. and S.P. conducted RNA-seq data analysis. J.-S.Y. and L.E. conducted histological experiments and analyzed data.
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**Figures and Figure Legends**

**Figure 1. Generation of PZR tyrosyl phosphorylation deficient knockin mice.** (A) Schematic of mouse PZR\(^{Y242F}\) mutation. (B) Heart lysates from WT (\(Mpz1^{+/}\)) and PZR\(^{Y242F}\) (\(Mpz1^{Y242F/Y242F}\)) mice were immunoblotted with anti-SHP2, pPZR (Y242), pPZR (Y264) and PZR antibodies. (C and D) Heart lysates from WT (\(Ptpn11^{+/+};Mpz1^{+/Y242F}\)), NSML (\(Ptpn11^{Y279C/+};Mpz1^{+/Y242F}\)), PZR\(^{Y242F}\) (\(Ptpn11^{+/+};Mpz1^{Y242F/Y242F}\)) and NSML/PZR\(^{Y242F}\) (\(Ptpn11^{Y279C/+};Mpz1^{Y242F/Y242F}\)) mice were immunoblotted with anti-SHP2, pPZR (Y242), pPZR (Y264) and PZR antibodies. The phosphorylation level of PZR was quantitated (n = 6) (C). Heart lysates were incubated with GST-conjugated human SHP2 SH2 domains (GST-N+C SH2). WT lysate was incubated with GST as a negative control. Proteins were affinity purified with glutathione agarose beads and immunoblotted with anti-PZR and GST antibodies (D). pPZR and PZR bands are marked with brackets. Data represent mean ± SEM. Statistical significance was analyzed by One-way ANOVA with multiple comparisons, Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.
A. Graph showing HW/BW (mg) with comparisons between WT, NSML, PZR^242F, and NSML/PZR^242F.

B. Images of hearts from WT, NSML, PZR^242F, and NSML/PZR^242F.

C. Graph showing HW/TL (mg/mm) with comparisons.

D. Images showing WT, NSML, PZR^242F, and NSML/PZR^242F.

E. Images showing cross-sectional area (μm²) with comparisons.

F. Graph showing cross-sectional area (μm²) with comparisons.

G. Images showing IVS,d (mm) with comparisons.

H. Images showing LVPW,d (mm) with comparisons.

I. Graph showing relative mRNA expression of Myh6 and Myh7 with comparisons.

J. Graph showing relative mRNA expression of Nppa and Nppb with comparisons.
Figure 2. PZR$^{Y242F}$ mutation rescues NSML-associated cardiomyopathies. (A) Heart weight (H.W.), heart weight to body weight (H.W./B.W.) and heart weight to tibia length (H.W./T.L.) ratios were measured from 16-week-old WT ($Ptpn11^{+/-};Mpzl^{+/-Y242F}$), NSML ($Ptpn11^{Y279C/+};Mpzl^{+/-Y242F}$), PZR$^{Y242F}$ ($Ptpn11^{+/-};Mpzl^{Y242F/Y242F}$) and NSML/PZR$^{Y242F}$ ($Ptpn11^{Y279C/+};Mpzl^{Y242F/Y242F}$) mice (n = 10 for WT and NSML, n = 9 for PZR$^{Y242F}$ and NSML/PZR$^{Y242F}$). (B) Gross morphology of heart from 16-week-old mice. (C) Hematoxylin and eosin (H&E) stained longitudinal sections of heart from 16-week-old mice (bar = 1 mm). (D-F) H&E stain (D) and Alexa488-conjugated wheat germ agglutinin (WGA) stain (E) of left ventricles from 16-week-old mice (scale bar = 50 µm). The cross-sectional area of cardiomyocytes of each genotype was quantitated (F). Quantified data are represented as a box-and-whisker plot, with bonds from 25th to 75th percentile, median line, and whiskers ranging from minimum to maximum values (n = 1067 for WT, n = 698 for NSML, n = 1023 for PZR$^{Y242F}$ and n = 1241 for NSML/PZR$^{Y242F}$). (G and H), Representative echocardiographic images of 16-week-old mice (G). Left ventricular posterior wall thickness in diastole (LVPW, d) and interventricular septum wall thickness in diastole (IVS, d) were measured from echocardiograms (H) (n = 7 for each group). (I and J) The relative mRNA expression levels of $Myh6$, $Myh7$ and the ratio of $Myh7/Myh6$ (I) and $Nppa$ and $Nppb$ (J) in the heart of 16-week-old mice were measured by quantitative RT-PCR (n = 7 for each group). All data represent mean ± SEM. Statistical significance was analyzed with One-way ANOVA with multiple comparisons, Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.
Figure 3. **PZR<sup>Y242F</sup> mutation reverses NSML signaling in the heart.** Heart lysates from 16-week-old WT (Ptpn11<sup>+/+</sup>;Mpz1<sup>+/Y242F</sup>), NSML (Ptpn11<sup>Y279C/+</sup>;Mpz1<sup>+/Y242F</sup>), PZR<sup>Y242F</sup> (Ptpn11<sup>+/+</sup>;Mpz1<sup>Y242F/Y242F</sup>) and NSML/PZR<sup>Y242F</sup> (Ptpn11<sup>Y279C/+</sup>;Mpz1<sup>Y242F/Y242F</sup>) mice were immunoblotted with anti-pAKT (T308), pAKT (S473), AKT, pS6K (T389), S6K, pERK1/2 and ERK1/2 antibodies. The phosphorylation level of AKT, S6K and ERK1/2 were quantitated (n = 6). Data represent mean ± SEM, One-way ANOVA with multiple comparisons, Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.
Figure 4. RNAseq transcriptome analysis of NSML and NSML/PZR<sup>Y242F</sup> mice. Total RNA was isolated from the heart of 16-week-old mice and RNAseq analysis performed. (A-C) Differentially expressed genes of NSML (Ptpn11<sup>Y279C/+;Mpzl1<sup>+/Y242F</sup>) compared to WT (Ptpn11<sup>+/+</sup>;Mpzl1<sup>+/Y242F</sup>), PZR<sup>Y242F</sup> (Ptpn11<sup>+/+</sup>;Mpzl1<sup>Y242F/Y242F</sup>) or NSML/PZR<sup>Y242F</sup> (Ptpn11<sup>Y279C/+;Mpzl1<sup>Y242F/Y242F</sup>) mice were displayed as volcano plots. A cut-off of p-value ≤ 0.05 and fold-change ≤ -2.0 (green) or ≥ 2 (red) was used to determine significance. (D) Venn diagram representation showing the number of significantly differentially expressed genes in NSML compared to WT, PZR<sup>Y242F</sup> or NSML/PZR<sup>Y242F</sup> group. Negatively (left) and positively (right) regulated genes in NSML are shown. (E) Ingenuity pathway analysis ( IPA) performed on statistically significant genes (p < 0.05) for cardiac hypertrophy. Fold changes are shown in the heat map with values indicated.
Figure 5. Hierarchical clustering analysis of the relative gene expression of NSML and NSML/PZR Y242F mice. (A) Hierarchical clustered heatmap of log2 transformed gene expression in the heart of WT (Ptpn11+/+;Mpzl1+/Y242F), NSML (Ptpn11 Y279C/++;Mpzl1+/Y242F), PZR Y242F (Ptpn11+/+;Mpzl1/Y242F/Y242F) and NSML/PZR Y242F (Ptpn11 Y279C/++;Mpzl1/Y242F/Y242F) mice (p < 0.01). Each column represents an individual mouse and each row represents a gene. Differential gene expression was shown in the heat map. (B) Relatively up-regulated (group #2 and #3) or down-regulated (group #1 and #4) genes in NSML compared to WT, PZR Y242F or NSML/PZR Y242F were clustered.
Figure 6. PZR tyrosyl phosphorylation promotes myocardial fibrosis and positively regulates IL6 expression. (A-C) Picrosirius red stains of left ventricles from 16-week-old WT (Ptpn11+/++;Mpzl1+/Y242F), NSML (Ptpn11Y279C+;Mpzl1+/Y242F), PZR (Ptpn11+/++;Mpzl1Y242F) and NSML/PZR (Ptpn11Y279C+;Mpzl1Y242F/Y242F) mice (A) (scale bar = 50 µm). Sirius Red positive area were quantified (n = 9 for each group; 3 images per mouse, 3 mice per group) (B). The relative mRNA expression levels of Col1a and Col3a in the heart were measured by quantitative RT-PCR (C) (n = 7 for each group). (D) The relative mRNA expression levels of Il1b, Il4, Il6, Il6r, Il10, Il13, Tnf, Ifng and Crp in the heart of 16-week-old WT (Ptpn11+/+) and NSML (Ptpn11Y279C+) mice were measured by quantitative RT-PCR (n = 8 for each group). (E-G) Mouse embryonic fibroblasts (MEFs) from WT (Mpzl1+/+) and PZR (Mpzl1Y242F)
mice were serum-starved and stimulated with 5 µg/ml of Concanavalin A (ConA) for 2 hr. Whole cell lysates were immunoblotted with anti-pPZR (Y242), pPZR (Y264) and PZR antibodies (E). The relative mRNA expression of Il6 and Tnf were measured by quantitative RT-PCR (F) (n = 3). (G) MEFs were serum-starved and stimulated with 5 µg/ml of Con A for 1, 2 and 4 hr. Secreted IL6 protein levels were measured by enzyme-linked immunosorbent assay (ELISA) (n = 3 for each group). (H) Low-dose dasatinib (0.1 mg/kg/day, i.p.) was administered to 12-week-old WT (Ptpn11+/+) and NSML (Ptpn11Y279C/+) mice for 4 weeks. The relative mRNA expression of Il6 in the heart was measured by quantitative RT-PCR (n = 5 for vehicle-treated WT, n = 7 for vehicle-treated NSML, n = 6 for dasatinib-treated WT and n = 9 for dasatinib-treated NSML). (I) The relative mRNA expression level of Il6 in the heart of 16-weeks old WT (Ptpn11+/+;Mpzl1+/Y242F), NSML (Ptpn11Y279C/+,Mpzl1+/+Y242F), PZRY242F (Ptpn11+/+;Mpzl1Y242F/Y242F) and NSML/PZRY242F (Ptpn11Y279C/+,Mpzl1Y242F/Y242F) mice were measured by quantitative RT-PCR (n = 7 for WT, NSML and PZRY242F and n = 8 for NSML/PZRY242F). Serum was collected from 16-week-old mice and IL6 protein levels were measured by ELISA (n = 8 for WT and PZRY242F, n = 7 for NSML and NSML/PZRY242F). All data represent mean ± SEM. Statistical significance was analyzed with either two-tailed Student’s t-test (D), Two-way ANOVA (F-H) or One-way ANOVA (B, C and I) with multiple comparisons, Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.
Figure 7. PZR tyrosyl phosphorylation activates the NFκB pathway and Col1a expression. Mouse embryonic fibroblasts (MEFs) from WT (Mpzl1+/+) and PZR<sup>Y242F</sup> (Mpzl1<sup>Y242F/Y242F</sup>+) mice were serum-starved and stimulated with 5 µg/ml of Concanavalin A (ConA) for 1, 2 and 4 hr. (A and B) Whole cell lysates were immunoblotted with anti-pPZR (Y242), pPZR (Y264), PZR, pIKKβ (Y188), IKKβ, IκBα and ERK1/2 antibodies (A) and pSTAT3 (Y702) and STAT3 antibodies (B). The phosphorylation level of PZR, IKKβ, IκBα protein level (A) and phosphorylation of STAT3 (B) were quantitated (n = 3). (C) Total RNA was isolated and the relative expression of Col1a was measured by quantitative RT-PCR (n = 3). All data represent mean ± SEM. Statistical significance was analyzed with either Two-way ANOVA (A-C) with multiple comparisons, Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.
Figure 8. PZR tyrosyl phosphorylation induces autocrine/paracrine mediated IL6 secretion to activate STAT3 phosphorylation and Col1a expression. (A) WT (Mpzl1+/+) and PZR\textsuperscript{Y242F} (Mpzl1\textsuperscript{Y242F/+Y242F+}) MEFs were serum starved and stimulated with 5 µg/ml of Concanavalin A for 4 hr and medium was collected (conditioned medium, CM). CM from WT MEFs (CM-WT) or PZR\textsuperscript{Y242F} MEFs (CM-PZR\textsuperscript{Y242F}) was treated to PZR\textsuperscript{Y242F} MEFs for 2 hr. Whole cell lysates were immunoblotted with anti-pSTAT3 (Y702) and STAT3 antibodies. Total RNA was isolated and the relative expression of Col1a was measured by quantitative RT-PCR (n = 3). (B) Conditioned
medium from WT MEFs (CM-WT) was incubated with either mock IgG or anti-IL6 neutralizing antibody and then incubated on to PZR<sup>Y242F</sup> MEFs for 2 hr. Whole cell lysates were immunoblotted with anti-pSTAT3 (Y702) and STAT3 antibodies. Total RNA was isolated and the relative expression of *Col1a* was measured by quantitative RT-PCR (*n* = 3). (C) Schematic representation of PZR-SHP2 signaling in NSML. All data represent mean ± SEM. Statistical significance was analyzed with One-way ANOVA with multiple comparisons, Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.