Supplemental Data

Tyrosyl phosphorylation of PZR promotes hypertrophic cardiomyopathy in *PTPN11*-associated Noonan syndrome with Multiple Lentigines

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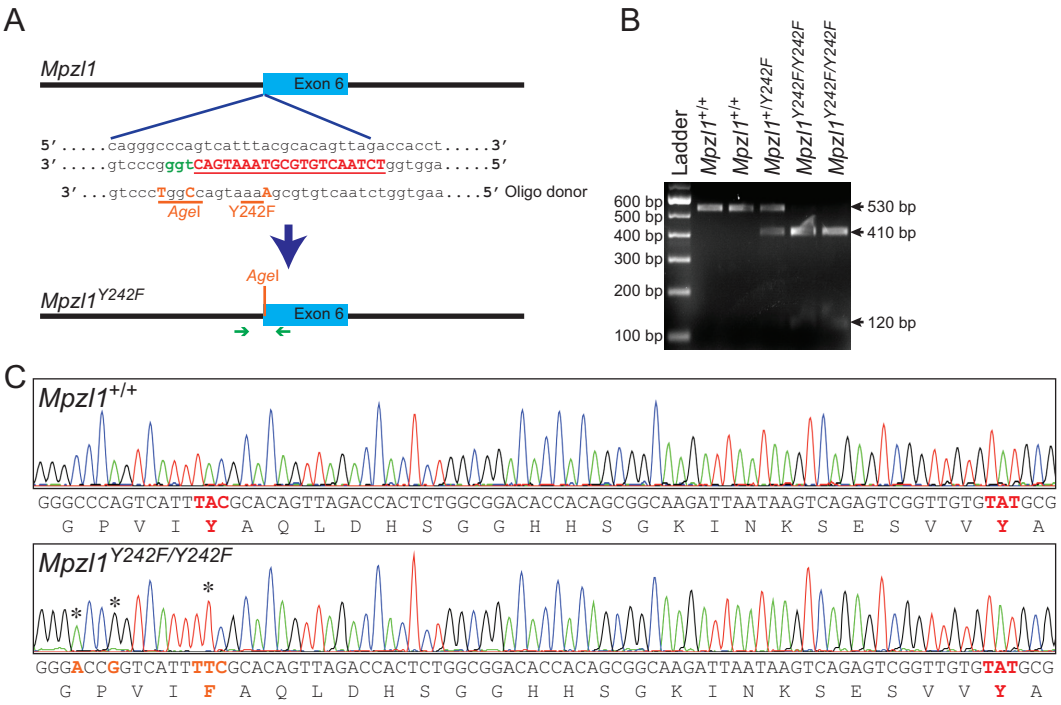
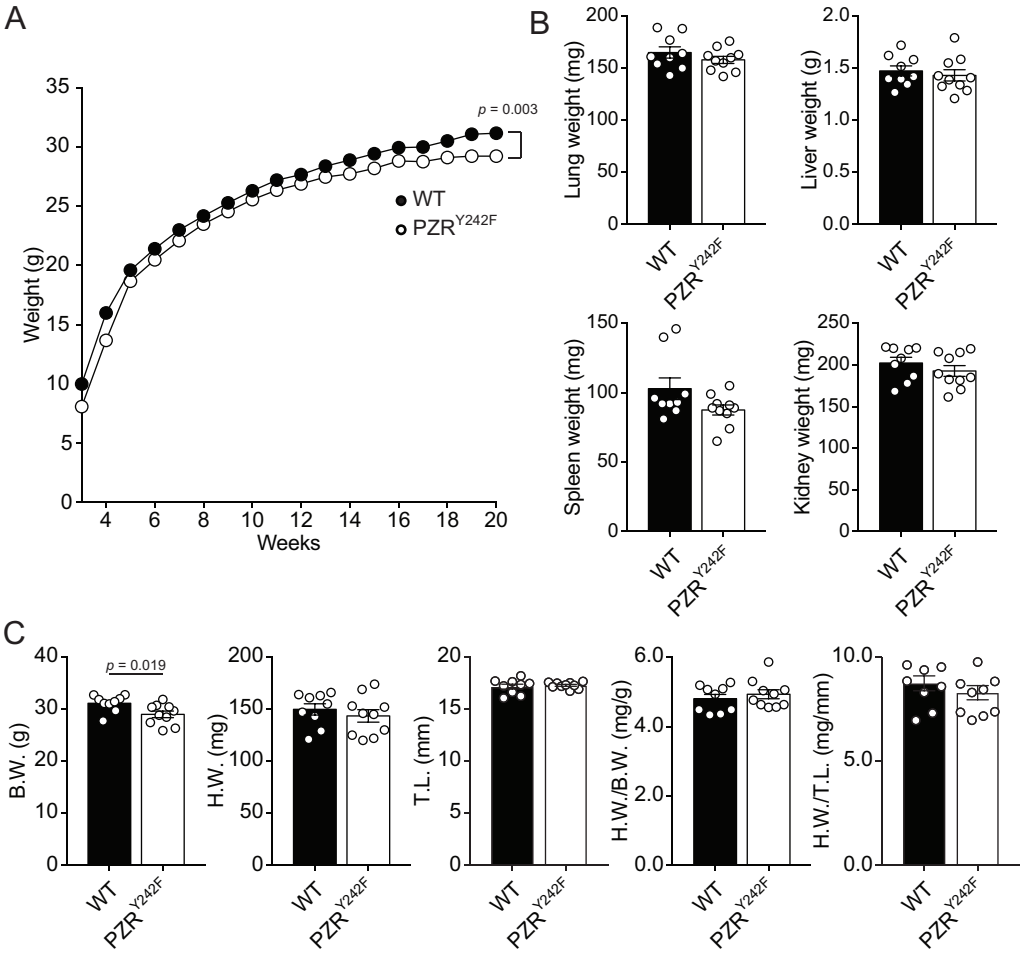
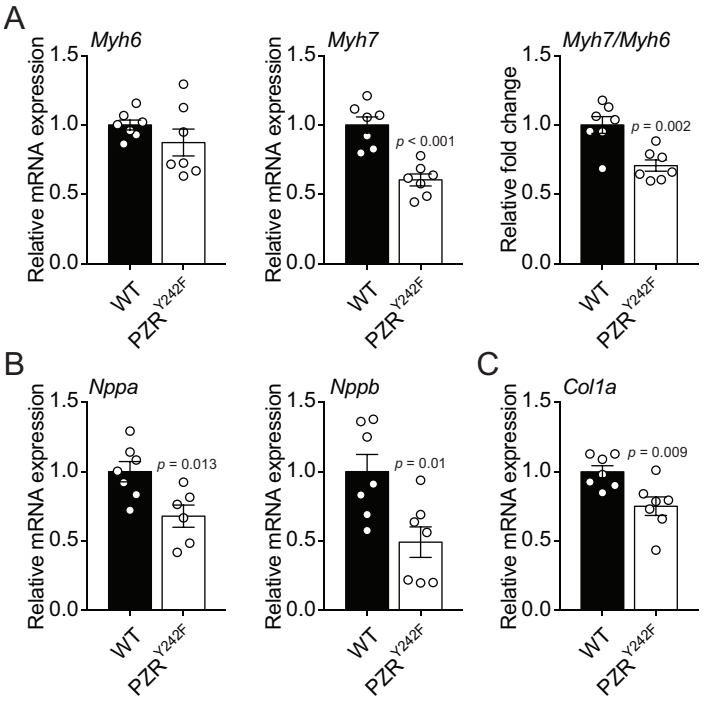


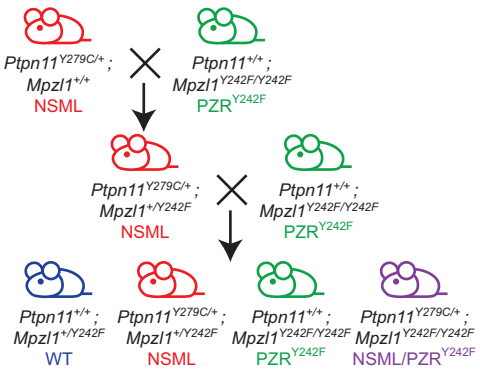
Supplemental Figure 1. Generation of *Mpzl1*^{Y242F} allele. (**A**) Schematic of the Cas9/sgRNA/oligo-targeting site at *Mpzl1* exon 6. The sgRNA coding sequence is underlined, capitalized and labeled in red. The protospacer adjacent motif (PAM) sequence is labeled in green. The mutations of *AgeI* and Y242F are labeled in orange. The oligo donor has a 60 bp homology on both sides of the mutated sequence. The location of PCR primers used for genotyping are shown as green arrows. (**B**) Genotyping PCR and subsequent *AgeI* digestion produced bands with the correct size in *Mpzl1*^{Y242F} heterozygotes (*Mpzl1*$^{+/Y242F}$) and homozygotes (*Mpzl1*$^{Y242F/Y242F}$), but not in WT (*Mpzl1*$^{+/+}$). (**C**) PCR products generated from genotyping in (**B**) were sequenced. Sequence across the targeting region confirmed correct mutations (*) in exon 6 of *Mpzl1* gene.



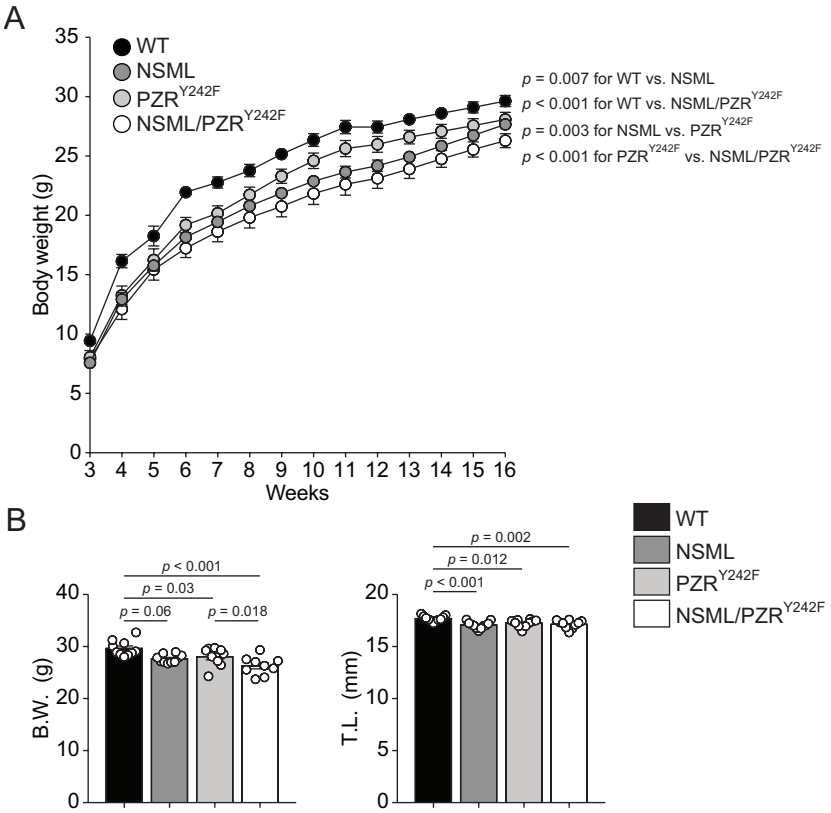
Supplemental Figure 2. Post-developmental characterization of PZR^{Y242F} mice. (**A**) Post-developmental body weight of WT (*Mpzl1*$^{+/+}$) and PZR^{Y242F} (*Mpzl1*$^{Y242F/Y242F}$) mice. (**B** and **C**) Lung, liver, spleen, kidney weight (**B**), body weight (B.W.), heart weight (H.W.), tibia length (T.L.), the ratio of heart weight to body weight (H.W./B.W.) and heart weight to tibia length (H.W./T.L.) were measured from 20-weeks-old PZR^{Y242F} mice (**C**) (n = 9 for WT and n = 10 for PZR^{Y242F}). All data represent mean ± SEM. Statistical significance was analyzed with Two-way ANOVA (**A**) or two-tailed Student's t-test (**B** and **C**).



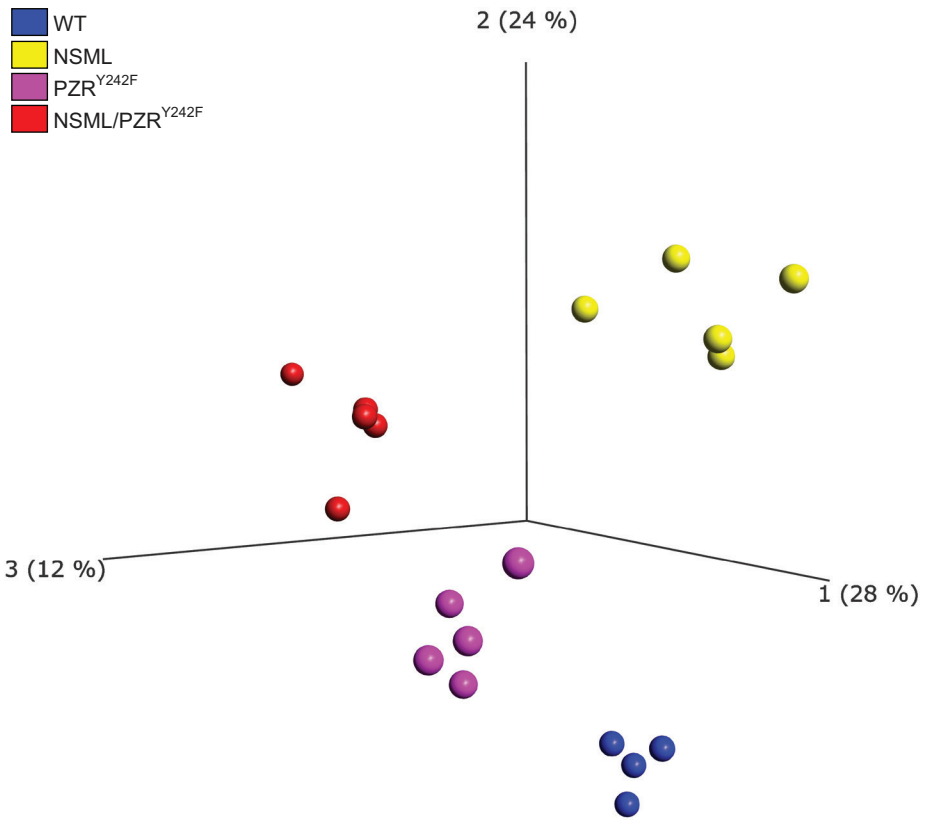
Supplemental Figure 3. Expression of cardiomyopathy-related genes in PZR^{Y242F} mice. Relative mRNA expression levels of *Myh6*, *Myh7* and ratio of *Myh7*/*Myh6* (**A**), *Nppa*, *Nppb* (**B**) and *Col1a* (**C**) in the heart of 20-week-old WT (*Mpzl1*$^{+/+}$) and PZR^{Y242F} (*Mpzl1*$^{Y242F/Y242F}$) mice were measured by quantitative RT-PCR (n = 7 for each group). All data represent mean ± SEM. Statistical significance was analyzed by two-tailed Student's t-test.



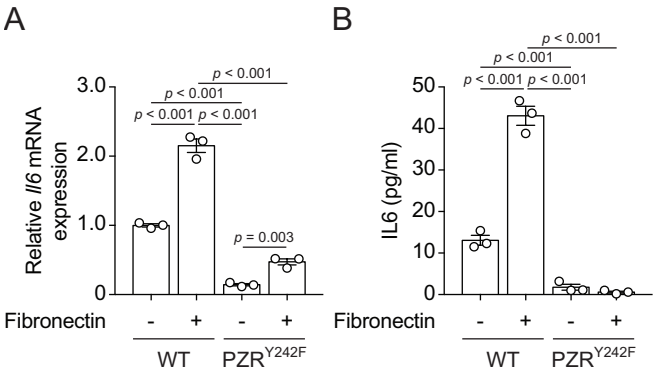
Supplemental Figure 4. Schematic of NSML/PZR^{Y242F} mice generation. NSML mice (*Ptpn11*$^{Y279C/+}$) were crossed with PZR^{Y242F} mice (*Mpzl1*$^{Y242F/Y242F}$). Heterozygotes (*Ptpn11*$^{Y279C/+}$;*Mpzl1*$^{+/Y242F}$) in the first generation were back-crossed with PZR^{Y242F} mice. The resultant four genotypes are shown WT (*Ptpn11*$^{+/+}$; *Mpzl1*$^{+/Y242F}$), NSML (*Ptpn11*$^{Y279C/+}$; *Mpzl1*$^{+/Y242F}$), PZR^{Y242F} (*Ptpn11*$^{+/+}$; *Mpzl1*$^{Y242F/Y242F}$) and NSML/PZR^{Y242F} (*Ptpn11*$^{Y279C/+}$; *Mpzl1*$^{Y242F/Y242F}$).



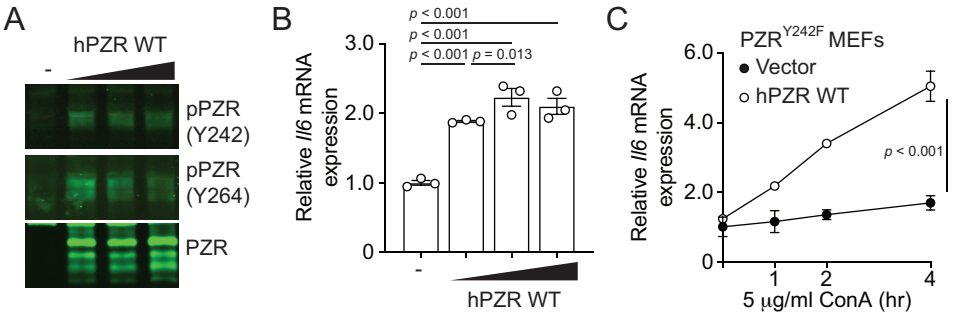
Supplemental Figure 5. Body weight and tibia length of NSML/PZR^{Y242F} mice. (**A**) Body weight of WT (*Ptpn11*$^{+/+}$*; Mpzl1*$^{+/Y242F}$), NSML (*Ptpn11*$^{Y279C/+}$*; Mpzl1*$^{+/Y242F}$), PZR^{Y242F} (*Ptpn11*$^{+/+}$*;Mpzl1*$^{Y242F/Y242F}$) and NSML/PZR^{Y242F} (*Ptpn11*$^{Y279C/+}$*;Mpzl1*$^{Y242F/Y242F}$) mice were measured weekly from the age of 3 weeks to 16 weeks. (**B**) Body weight (B.W.) and tibia length (T.L.) were measured from 16-week-old mice (n = 10 for WT and NSML, n = 9 for PZR^{Y242F} and NSML/PZR^{Y242F}). All data represent mean ± SEM. Statistical significance was analyzed by Two-way ANOVA (**A**) or One-way ANOVA with multiple comparisons, Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (**B**).



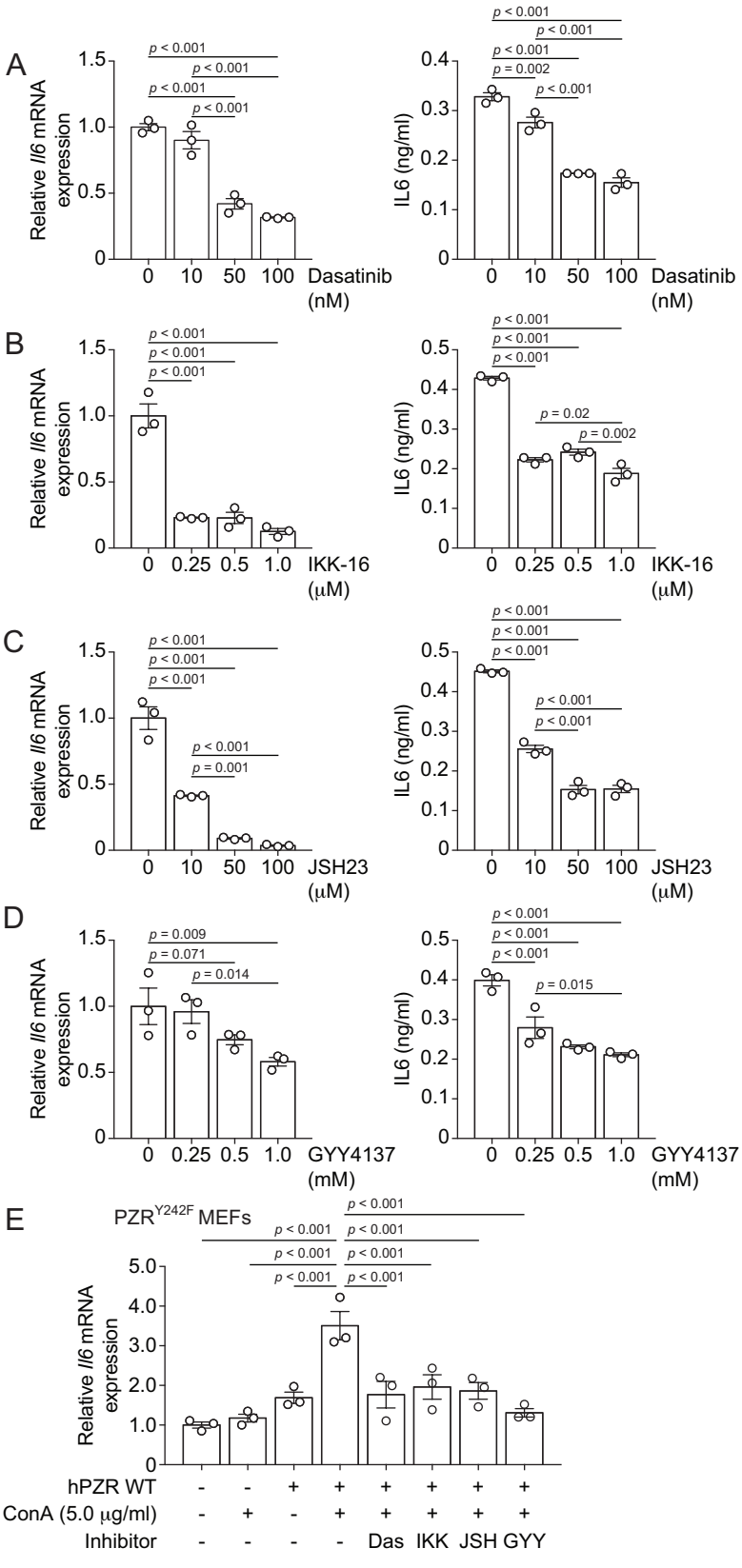
Supplemental Figure 6. PCA plot of RNA-seq data. Three dimensional principal component analysis (PCA) was carried out on the different genotypes (WT (*Ptpn11*$^{+/+}$;*Mpzl1*$^{+/Y242F}$), NSML (*Ptpn11*$^{Y279C/+}$;*Mpzl1*$^{+/Y242F}$), PZR^{Y242F} (*Ptpn11*$^{+/+}$;*Mpzl1*$^{Y242F/Y242F}$) and NSML/PZR^{Y242F} (*Ptpn11*$^{Y279C/+}$;*Mpzl1*$^{Y242F/Y242F}$)) based on 185 genes ($p < 0.01$). Each spot represents an individual mouse and colored according to the corresponding genotypes. The percentage of the total variance (64%) described by each of the three principal components is given in the parentheses near each axis.



Supplemental Figure 7. PZR tyrosyl phosphorylation is required for IL6 expression and secretion upon fibronectin engagement. Mouse embryonic fibroblasts (MEFs) from WT (*Mpzl1*$^{+/+}$) and PZR^{Y242F} (*Mpzl1*$^{Y242F/Y242F}$) mice were serum-starved, trypsinized, suspended and then plated onto fibronectin-coated petri dish for 1 hr. (**A**) Total RNA was isolated and the relative expression of *Il6* was measured by quantitative RT-PCR. (**A**) Medium was collected and secreted IL6 levels measured by ELISA (n = 3 for each group). All data represent mean ± SEM. Statistical significance was analyzed with Two-way ANOVA with multiple comparisons, Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.



Supplemental Figure 8. PZR tyrosyl phosphorylation is required for IL6 expression. (**A-C**) Human PZR WT cDNA was overexpressed into PZR^{Y242F} (*Mpzl1*$^{Y242F/Y242F}$) MEFs. Whole cell lysates were immunoblotted with anti-pPZR (Y242), pPZR (Y264) and PZR antibodies (**A**). Total RNA was isolated and the relative expression of *Il6* was measured by quantitative RT-PCR (**B**) (n = 3). After serum starvation, cells were stimulated with 5 μg/ml of Concanavalin A (ConA) for 1, 2 and 4 hr. Total RNA was isolated and the relative expression of *Il6* was measured by quantitative RT-PCR (**C**) (n = 3). 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 (**B**) or Two-way ANOVA (**C**).


A
Relative Il6 mRNA expression
IL6 (ng/ml)
Dasatinib (nM)
B
IKK-16 (μM)
C
JSH23 (μM)
D
GYY4137 (mM)
E
PZR^Y242F MEFs
hPZR WT
ConA (5.0 μg/ml)
Inhibitor
Das IKK JSH GYY

Supplemental Figure 9. NFκB signaling is required for Concanavalin A-induced IL6 expression. (**A-D**) WT (*Mpzl1*$^{+/+}$) MEFs were treated with dasatinib (**A**), IKK-16 (**B**), JSH23 (**C**) and GYY4137 (**D**) for 16 hr with the indicated concentrations, and then stimulated with 5 μg/ml of Concanavalin A (ConA) for 1 hr. Total RNA was isolated and the relative expression of *Il6* was measured by quantitative RT-PCR (n = 3). Medium was collected and secreted IL6 levels measured by ELISA (n = 3). (**E**) Human PZR WT cDNA was overexpressed into PZR^{Y242F} MEFs. Cells were treated with 50 nM dasatinib (Das), 0.5 μM IKK-16 (IKK), 50 μM JSH23 (JSH) and 1 mM GYY4137 (GYY) for 16 hr, and then were stimulated with 5 μg/ml of Concanavalin A for 1 hr. Total RNA was isolated and the relative expression of *Il6* was measured by quantitative RT-PCR (n = 3). All data represent mean ± SEM. Statistical significance was analyzed with One-way ANOVA with multiple comparisons.

		WT	*Mpzl1*$^{+/Y242F}$	*Mpzl1*$^{Y242F/Y242F}$	c^2	*p*	*n*
P10	All	88	183	93	0.1484	0.9285	364
	Male	46	96	42	0.5217	0.7704	184
	Female	42	87	51	1.1000	0.5769	180

Supplemental Table 1. Mendelian inheritance in the offspring of PZR^{Y242F} mice.

	WT (n=6)	PZR^{Y242F} (n=7)
IVS,d (mm)	0.66±0.02	0.69±0.03
IVS,s (mm)	1.10±0.03	1.12±0.06
LVID,d (mm)	3.94±0.15	3.65±0.11
LVID,s (mm)	2.70±0.13	2.39±0.09
LVPW,d (mm)	0.75±0.03	0.85±0.04
LVPW,s (mm)	1.10±0.06	1.23±0.05
LV vol,d (mm^3)	68.21±6.08	56.48±4.07
LV vol,s (mm^3)	27.47±3.20	20.09±1.90
%EF	60.10±1.70	64.57±1.51
%FS	31.59±1.12	34.60±1.11

Supplemental Table 2. Echocardiography parameters of 16-week-old WT (*Mpzl1*$^{+/+}$) and PZR^{Y242F} (*Mpzl1*$^{Y242F/Y242F+}$) mice. Data represents the mean ± SEM for WT (n=6) and PZR^{Y242F} (n=7). IVS, Intraventricular septum wall thickness; LVID, left ventricular internal dimension; LVPW, left ventricular posterior wall thickness; LV vol, left ventricle volume; EF, ejection fraction; FS, fractional shortening; d, diatole; s, systole.

	WT (n=7)	NSML (n=7)	PZR^{Y242F} (n=7)	NSML/PZR^{Y242F} (n=7)
IVS,d (mm)	0.81±0.03	0.91±0.03*	0.77±0.03†††	0.81±0.02†
IVS,s (mm)	1.38±0.05	1.33±0.05	1.17±0.03**,†	1.18±0.06**,†
LVID,d (mm)	4.01±0.08	4.16±0.09	4.20±0.06	4.24±0.12
LVID,s (mm)	2.58±0.09	2.73±0.12	2.74±0.11	2.78±0.09
LVPW,d (mm)	0.81±0.01	0.91±0.05*	0.81±0.01†	0.82±0.01†
LVPW,s (mm)	1.12±0.05	1.22±0.05	1.09±0.04	1.17±0.03
LV vol,d (mm^3)	71.35±3.48	79.02±3.69	78.80±3.26	83.09±5.18
LV vol,s (mm^3)	24.5±2.17	28.35±3.01	28.56±2.75	29.38±2.22
%EF	65.94±1.75	64.35±3.17	63.97±2.84	64.62±1.66
%FS	35.97±1.33	35.12±2.33	34.79±2.17	35.14±1.23

Supplemental Table 3. Echocardiography parameters of 20-week-old 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. Data represents the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$ denotes significance compared with WT mice. †, $p < 0.05$; ††, $p < 0.01$ denotes significance compared with NSML mice. All p values were derived using One-way ANOVA with multiple comparisons. IVS, Intraventricular septum wall thickness; LVID, left ventricular internal dimension; LVPW, left ventricular posterior wall thickness; LV vol, left ventricle volume; EF, ejection fraction; FS, fractional shortening; d, diastole; s, systole.

Name	Sequences
Guide Mpzl1-2 primer	5'-TGTAATACGACTCACTATAGGTCTAACTGTGCGTA AATGACGTTTTAGAGCTAGAAA-3'
sgRNA reverse primer	5'-AAAAGCACCGACTCGGTGCC-3'
Cas9FWpX330 primer	5'-TGTAATACGACTCACTATAGGGAGAATGGACTATA AGGACCACGAC-3'
Cas9revpX330 primer	5'-GCGAGCTCTAGGAATTCTTAC-3'
Template ssODN	5'-TCCTGTGGCTCAGGGACCATCAGTTCTTCCAAACCT CTAATTGGTTTCTCTTCTCCAGGGACCGGTCATTTTCG CACAGTTAGACCACTCTGGCGGACACCACAGCGGCAA GATTAATAAGTCAGAGTCGGTTGTGTTTGCGGACATCC GGAAAGACTAAGAGAACACCCAAACATTTCCAAACTG GACGCTTGTGCAGA-3'

Supplemental Table 4. Oligo nucleotide sequences for *Mpzl1*^{Y242F} mutant mice generation.

Primer Name	Sequences
18S rRNA	5’-ACCGCAGCTAGGAATAATGGA-3’ 5’-ACCAAAAGCCTTGACTCCG-3’
Myh6	5’-GTCCCGGACACTGGACCAGGCC-3’ 5’-CTCCTTTTCTTCCAGTTGCCTAGCCAA-3’
Myh7	5’-GAGCAAGGCCGAGGAGACGCAGCGT-3’ 5’-GAGCCTCCTTCTCGTCCAGCTGCCGG-3’
Nppa	5’- CCTGGAGGAGAAGATGCCGGTAGAA-3’ 5’-CCCCAGTCCAGGGAGGCACCTCGG-3’
Nppb	5’-CACTTCAAAGGTGGTCCCAGAGCTGC-3’ 5’-GACCGGATCGGATCCGTCAGTCG-3’
Col1a	5’-AGGTCTTCCTGGAGCTGATG-3’ 5’-ACCCACAGGGCCTTCTTTAC-3’
Col3a	5’-ACAGCAAATTCACTTACACAGTTC-3’ 5’-CTCATTGCCTTGCGTGTTT-3’
Il1b	5’-CCAAGCAACGACAAAATACC-3’ 5’-GTTGAAGACAAACCGTTTTTCC-3’
Il4	5’-GGTCTCAACCCCCAGCTAGT-3’ 5’-GCCGATGATCTCTCTCAAGTGAT-3’
Il6	5’-CCACGGCCTTCCCTACTTC -3’ 5’-TGGGAGTGGTATCCTCTGTGAA -3’
Il6r	5’-AAGCAGCAGGCAATGTTACC-3’ 5’-CATAAATAGTCCCCAGTGTCG-3’
Il10	5’-CTTACTGACTGGCATGAGGATCA-3’ 5’-GCAGCTCTAGGAGCATGTGG-3’
Il13	5’-CCTGGCTCTTGCTTGCCTT-3’ 5’-GGTCTTGTGTGATGTTGCTCA-3’
Tnf	5’-CATCTTCTCAAAATTCGAGTGACAA-3’ 5’-TGGGAGTAGACAAGGTACAACCC-3’
Ifng	5’-TCAAGTGGCATAGATGTGGAAGAA-3’ 5’-TGGCTCTGCAGGATTTTCATG-3’
Crp	5’-CAGACTTTTCCGCACCTTGGCTTT-3’ 5’-AGTGGGTGGTGCTGAAGTACGATT-3’

Supplemental Table 5. The list of primers for quantitative real-time PCR analysis.