

## Research Article

## Subcellular Localizations of PP1 Isoforms Determine their Specific Functions

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### Abstract

Although highly homologous in the catalytic domains, the three protein phosphatase 1 isoforms  $\alpha$ ,  $\beta$ , and  $\gamma$  (PP1 $\alpha$ , PP1 $\beta$  and PP1 $\gamma$ ) are hypothesized to have different subcellular localizations. In the heart, PP1 $\beta$  has been shown to localize to the myofilament proteins, but little is known whether it is also present in the nucleus. In this study, we first compared the subcellular localizations of PP1 isoforms and their specific substrates, and then determined their effects on cardiac growth. In neonatal cardiac myocytes, PP1 $\beta$  was mainly localized to the nucleus, compared to the predominant presence of PP1 $\alpha$  and PP1 $\gamma$  in the cytoplasm. Adenovirus-mediated overexpression of PP1 $\alpha$  led to reduced phosphorylation of phospholamban, which was not found upon overexpression of PP1 $\beta$ . Genetic deletion of PP1 $\beta$  from the adult mouse hearts resulted in increased HDAC7 phosphorylation, consistent with the nuclear localization of PP1 $\beta$ . All three isoforms were expressed differently in neonatal and adult cardiac myocytes and fibroblasts, and had increased levels during post-natal growth. However, deletion of neither PP1 $\alpha$  nor PP1 $\beta$  altered the cardiac function of the mice following pressure overload stimulation, indicating that PP1s are not involved in the stress responses. Together, our data suggest that PP1 isoforms reside in distinct cellular compartments to regulate the phosphorylation of specific substrates for the physiological cardiac function.

**Keywords:** Cardiac function, Histone deacetylase, Phospholamban, Protein phosphatase 1

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### Introduction

The balance between protein phosphorylation and dephosphorylation regulates almost every aspect of biological processes [1]. In contrast to protein phosphorylation mediated by a vast number of protein kinases, only a small number of protein phosphatases have been reported [2]. Protein phosphatases are classified into three subgroups, namely serine/threonine, tyrosine, or dual specific phosphatases [3]. The serine/threonine protein phosphatases, which include Protein Phosphatase 1 (PP1), Protein Phosphatase 2A (PP2A), and Protein Phosphatase 2B (PP2B, also called calcineurin), account for ~98% of the phosphatase activities in the mammalian heart [4]. A common feature for serine/threonine phosphatases is that they are holoenzymes composed of both catalytic subunits for the enzymatic activity and regulatory subunits that are either targeting proteins or substrates [4]. The three catalytic subunits of PP1s (PP1 $\alpha$ , PP1 $\beta$ , and PP1 $\gamma$ ), encoded by three distinct genes, are highly homologous in their catalytic domains. Their divergent N- and C-termini are hypothesized to underlie the target selectivity [5-7].

Sympathetic stimulation upon catecholamine binding generates cAMP in the heart that activates Protein Kinase A (PKA). PKA phosphorylates many Ca<sup>2+</sup> handling and myofilament proteins, such as Ryanodine Receptor 2 (RyR2), Phospholamban (PLB), Myosin Light Chain 2 V (MLC2V), and myosin binding protein C [8-11]. Phosphorylation of SR protein PLB at serine 16 by PKA or threonine 17 by Calcium-Calmodulin-Dependent Protein Kinases (CAMKs) increases the ATPase activity of SR Ca<sup>2+</sup> transport ATPase (SERCA) to enhance Ca<sup>2+</sup> recycling into the SR [12]. In heart failure patients, PP1 activities were found to be increased, suggesting PP1s might be the therapeutic targets to treat heart failure patients. Consistent with this notion, Adeno-Associated Virus (AAV) 9 mediated-overexpression of phosphatase 1 inhibitor-1 (I-1) improved cardiac function, while overexpression of a catalytic PP1 $\alpha$  isoform in mice led to reduced cardiac function and heart failure [13,14]. However, cardiac specific deletion of PP1 $\beta$  led to heart failure and cardiac remodeling in mice [15]. These findings extended the functional complexity of PP1 isoforms in the heart, possibly due to their subcellular locations such as SR and nucleus [15-17].

Histone Deacetylases (HDACs) are a group of enzymes that remove the acetyl groups from lysine residues of a variety of target proteins including histone and non-histone substrates [18,19]. In mammalian cells, 18 HDACs have been identified and grouped into 5 different subgroups I, IIa, IIb, III, IV. In the heart, group IIa HDACs (HDAC4, HDAC5, HDAC7, and HDAC9) have been extensively studied and shown to be the endogenous inhibitors for cardiac hypertrophy [18]. For example, overexpression of HDAC4, HDAC5, and HDAC9 suppressed Myocyte Enhancer Factor 2 (MEF2) mediated gene expression to prevent pathological stimuli-induced cardiac hypertrophy [20-22]. Loss of either HDAC5 or HDAC9 led to cardiac hypertrophy in mice [21,23]. Moreover, class II HDACs are transcriptional repressors at resting state. Upon stimulation, phosphorylation of class II HDACs led to their nuclear export, resulting in the de-repression of their targeting genes [17,24-26]. However, the exact

protein phosphatases that regulate the phosphorylation status of HDACs have yet to be explored.

In the present study, we hypothesized that PP1s are localized differently in the cells to regulate the phosphorylation of their substrates. In addition, we determined whether PP1s play a role in pressure-overload induced cardiac hypertrophy, and their levels during postnatal growth of the heart.

## Materials and Methods

### Mice husbandry and transverse aortic constriction surgery

All animal experimentation was approved by the Institutional Animal Care and Use Committee at Grand Valley State University (Protocol Number: 17-02-A). Mice were housed in standard rodent cages, and observed daily for their wellbeing such as physical activity and food/water intake. The procedures for pressure overload by Transverse Aortic Constriction (TAC) to induce cardiac hypertrophy have been previously described [15]. In brief, 8-week-old sex-matched mice were anesthetized by 1.5% isoflurane to expose the transverse aortas through a thoracotomy procedure. Constriction was performed with 7-0 silk (Ethicon, Somerville, NJ) around a 27-gauge wire which was slowly removed after the constriction. After the surgery, mice were given pain medicine buprenex (0.1 mg/kg), and transferred to 30°C chambers for overnight recovery before being transferred back to the standard housing. After 2 weeks of TAC, Doppler echocardiography was first applied to the mice to determine pressure gradients across the constrictions to ensure similar stimulation for all the mice. Cardiac function of the mice was analyzed by echocardiography using a SONOS 5500 instrument (Hewlett-Packard, Palo Alto, CA) and a 15-MHz transducer. Left ventricular Fractional Shortening percentage (FS%) was calculated using Left Ventricle Internal Diameters at the end of systole (LVIDs) and diastole (LVIDd) based on the formula:  $([LVIDd-LVIDs]/LVIDd) \times 100$  (%). At the end of the echocardiographic analysis, mice were euthanized by CO<sub>2</sub> inhalation for 5 minutes followed by cervical dislocation before the hearts were removed for the weight measurement. Hearts were then fixed overnight in 10% formalin-containing phosphate buffered saline and dehydrated for embedding with paraffin. Serial 5- $\mu$ m heart sections were stained with Masson's trichrome to detect interstitial fibrosis shown as the blue staining. Fibrosis was quantified as percentage of blue staining of the tissue section using ImageJ.

### Isolation of cardiomyocytes and fibroblasts from neonatal and adult hearts

Neonatal rat hearts were digested with 0.01% trypsin (Worthington, code TRLS, Lakewood, NJ) in sterile calcium and magnesium-free Hank's Balanced Salt Solution (HBSS) for 16–20 hours at 4°C with gentle rotation. The next day, trypsin inhibitors (Worthington, code SIC, Lakewood, NJ) were added to the digestion tube and incubated for 15 minutes at 37°C to stop the trypsin activity. Collagenases of 1,500 units (Worthington, code CLSPA, Lakewood, NJ) were then added to the digestion tube for an additional digestion for 60 minutes. Cells were collected through a cell strainer, and plated into 0.01% gelatin-coated dishes with 5% fetal bovine serum containing medium M199 (Corning, 10-060-CV, Corning, NY). After 60 minutes of culture, myocytes were washed off the plates and cultured into new gelatin-coated dishes, while the cells attached to the plates were considered as non-myocytes/fibroblasts.

Isolation of adult ventricular myocytes and fibroblasts has been described previously [27]. Hearts were surgically removed from 2-month-old mice after treatment with heparin (0.35 units) under anesthesia (Nembutal, 100mg/kg), and cannulated for retrograde perfusion with a solution containing liberase blendzyme (Roche, 05401151001, Indianapolis, IN). Heart tissues were dissociated by gentle pipetting and transferred to a new tube. Myocytes were allowed to settle by gravity followed by CaCl<sub>2</sub> re-introduction. The cells in the pellet were considered as myocytes, while those in the supernatant were considered as non-myocytes/fibroblasts. After examination under the light microscope for their shape and quantity, cells were harvested immediately for RNA analysis.

### RNA isolation and real-time PCR

Total RNA was purified from myocytes or fibroblasts using the RNeasy Fibrous Tissue Kit (Qiagen, 74704, Hilden, Germany) and quantified with a NANODROP 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). cDNA synthesis was performed using the SuperScript III First-Strand Synthesis Kit (Invitrogen, 18080-051, Carlsbad, CA) on a regular PCR machine. Real-time PCR analysis was performed using SYBR green dye (Bio-Rad, 172–5274, Hercules, CA) on a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). The primers to determine the mRNA level of PP1s were as follows: PP1 $\alpha$ , forward: 5'-cctccagagagcaactacctctc-3', reverse: 5'-acgtctccacagttgatgtgt-3'; PP1 $\beta$ , forward: 5'-aatatggaggtttccacagaag-3', reverse: 5'-attgatgctagcacactcatggtt-3'; PP1 $\gamma$ , forward: 5'-tctcctcagtcagcctatcctt-3', reverse: 5'-ctccggatactgattttgtaggc-3'; ribosomal protein 27 as the internal control, forward: 5'-ggacgctactcggacgcaag-3', reverse: 5'-ctcttgccatggcagctgtcac-3'.

### Nuclear fractionation and Western blot

Procedures for nuclear fractionation of cell culture has been previously described [27]. In brief, neonatal rat cardiac myocytes or HEK293 cells were collected into hypotonic buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.4% IGEPAL and protease inhibitors) and were then lysed on ice for 10 minutes. After being centrifuged for 5 minutes at 15,000 x g, the supernatant was collected as cytosolic fraction and the pellet was further re-suspended into buffer B (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol and protease inhibitors). After 2 hours of incubation on ice, proteins eluted from the pellet were collected by centrifugation and considered as nuclear proteins. Equal amount of cytosolic and nuclear proteins were separated by 10% SDS-PAGE, transferred onto PVDF membrane, blocked with 5% non-fat milk, and incubated with the following primary antibodies: Flag (Cell signaling technology, 2368, Danvers, MA), PP1 $\alpha$  (Santa Cruz Biotechnology, sc-6104, Dallas, TX), PP1 $\beta$  (Millipore, 07-1217, Burlington, MA), PP1 $\gamma$  (Santa Cruz Biotechnology, sc-6108, Dallas, TX), Gapdh (Fitzgerald, 10-1500, Acton, MA), Lamin A/C (Cell signaling technology, 2032, Danvers, MA). Additional primary antibodies that were also used for the Western blot: HDAC7 (Santa Cruz 7 Biotechnology, sc-74563, Dallas, TX), phospho-HDAC7 (Cell signaling technology, 3424, Danvers, MA), p<sup>Ser16</sup>-phospholamban (Badrilla, A010-12AP, Leeds, UK), p<sup>Thr17</sup>-phospholamban (Badrilla, A010-13AP, Leeds, UK), phospholamban (Thermo Fisher, MA3-922, Waltham, MA). Goat-anti-rabbit secondary antibody (Li-COR, P/N 925-32211, Lincoln, NE) and goat-anti-mouse secondary antibody (Li-COR, P/N 925-32211, Lincoln, NE) were utilized to visualize the results.

## Immunocytochemistry

Neonatal rat cardiac myocytes grown in gelatin-coated dishes were fixed for 20 minutes with 4% paraformaldehyde followed by rinses with Phosphate-Buffered Saline (PBS) for three times. Cells were permeabilized with 0.3% triton X-100 for 15 minutes, then blocked with 3% Bovine Serum Albumin (BSA) for 20 minutes. Isoform specific PP1 antibodies were added to the cells for overnight staining of endogenous PP1 proteins, which were visualized by Alexa Fluor 488 conjugated goat-anti-rabbit secondary antibody (Thermo Fisher, A11034, Waltham, MA).

## Statistics

All the results were presented as mean  $\pm$  SEM from at least three independent experiments. Statistical analysis was performed using Microsoft Excel using Student's t-test for 2 group analysis or ANOVA followed by a Bonferroni post hoc test for comparison of differences across multiple groups.  $P < 0.05$  was considered statistically significant.

## Result

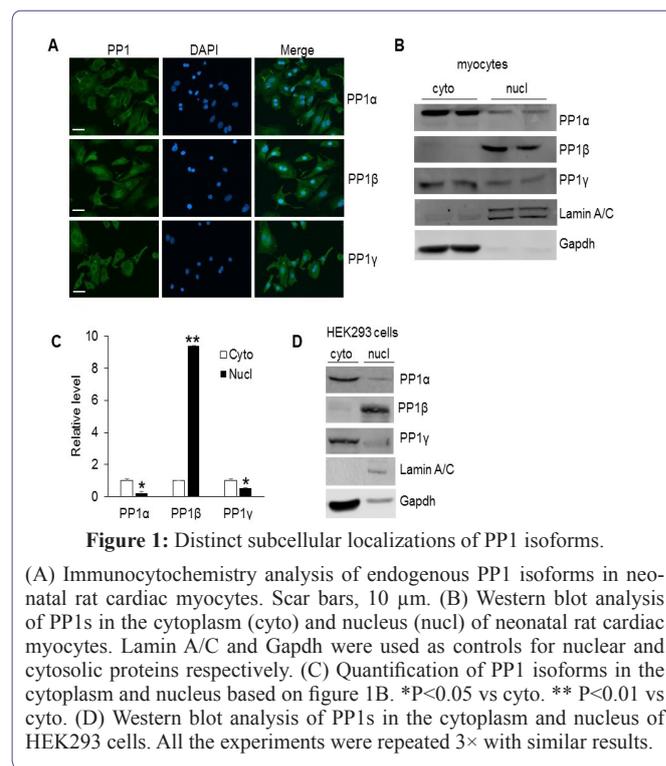
### Distinct subcellular localizations of PP1 isoforms

PP1 isoforms are highly homologous in the catalytic domains, but vary in the unique N and C termini, a mechanism underlying the subcellular localizations of PP1s. To further investigate the biology of PP1s in the mammalian hearts, we first compared the subcellular localizations of endogenous PP1s in neonatal cardiac myocytes using isoform specific antibodies. The majority of the PP1 $\alpha$  and PP1 $\gamma$  proteins were localized in the cytoplasm, whereas PP1 $\beta$  was present in both the cytoplasm and the nucleus (Figure 1A). To further verify this result, we performed a nuclear fractionation experiment to compare the subcellular distribution of PP1s in neonatal cardiomyocytes. PP1 $\beta$  was predominantly present in the nucleus compared to PP1 $\alpha$  and PP1 $\gamma$ , which also had a higher expression in the cytoplasm (Figure 1B and 1C). This result was further confirmed by biochemical fractionation of PP1 isoforms from Human Embryonic Kidney (HEK) 293 cells (Figure 1D). In both Western blots, Gapdh and Lamin A/C proteins were used as markers for cytoplasmic and nuclear proteins respectively (Figure 1B and 1D).

### Distinct substrates of PP1 $\alpha$ and PP1 $\beta$ in the heart

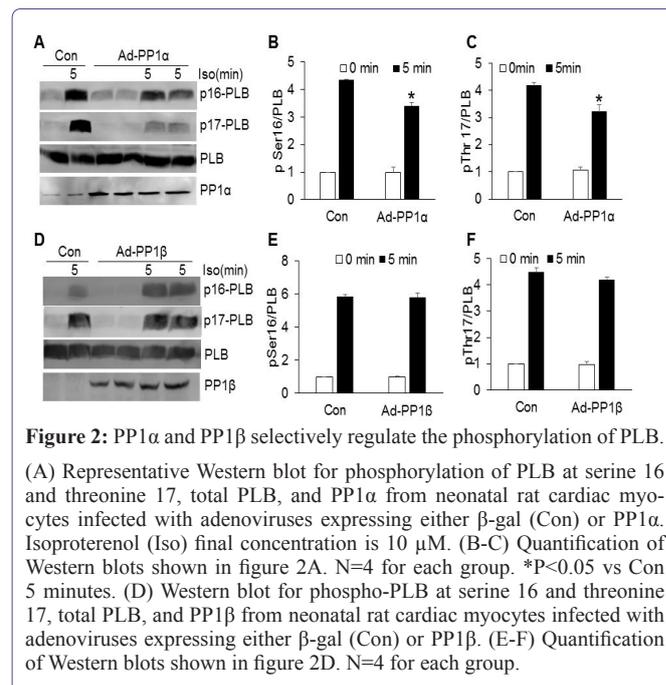
Based on the subcellular localizations of PP1 isoforms (Figure 1), we sought to investigate whether PP1s dephosphorylate different substrates in the cells. The functional role for PP1 $\gamma$  in the heart still remains elusive; hence PP1 $\alpha$  and PP1 $\beta$  were focused for the rest of the study. We first determined whether PP1s regulate the Phosphorylation of Phospholamban (PLB), a critical protein on the SR for Ca<sup>2+</sup> recycling. Neonatal rat cardiac myocytes were infected with adenoviruses expressing either  $\beta$ -gal (Con) or PP1 for 36 hours, and the PLB phosphorylation was assessed at baseline and upon isoproterenol (Iso) stimulation for 10 minutes to induce PLB phosphorylation. Overexpression of PP1 $\alpha$  significantly reduced the phosphorylation of PLB in cells challenged with isoproterenol, an agonist for adrenergic receptors to elicit cAMP-PKA pathway (Figure 2A-2C). However, overexpression of PP1 $\beta$  did not alter the phosphorylation status of PLB (Figure 2D-2F). These data suggest that PP1 $\alpha$ , the PP1 isoform localized in the cytoplasm, regulates the phosphorylation of PLB in the cytoplasm. Because PP1 $\beta$  was enriched in the nucleus (Figure 1B),

we sought to identify the nuclear substrate for PP1 $\beta$ . We first assessed HDAC7, a potential target of PP1 in the nucleus [17], using PP1 $\alpha$  (PP1 $\alpha$  fl/fl<sup>NKX-Cre</sup>) and PP1 $\beta$  (PP1 $\beta$  fl/fl<sup>NKX-Cre</sup>) cardiac specific PP1 deletion mice [15]. Compared to the NKX-Cre control mice, deletion of PP1 $\beta$ , but not PP1 $\alpha$ , significantly enhanced the phosphorylation of HDAC7, suggesting that PP1 $\beta$  is the major isoform localized in the nucleus to regulate the phosphorylation level of HDAC7 (Figure 3A-3C).



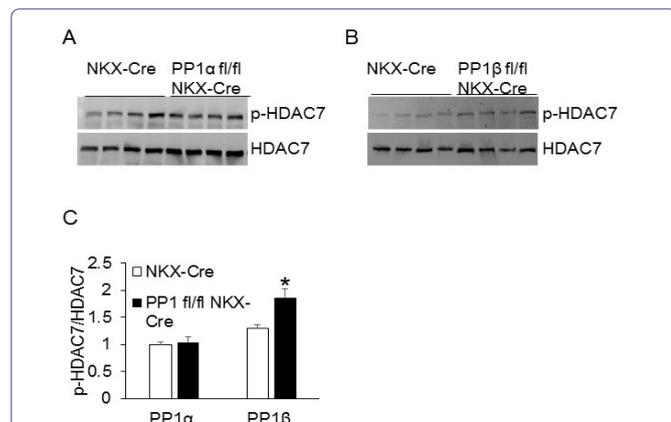
**Figure 1:** Distinct subcellular localizations of PP1 isoforms.

(A) Immunocytochemistry analysis of endogenous PP1 isoforms in neonatal rat cardiac myocytes. Scar bars, 10  $\mu$ m. (B) Western blot analysis of PP1s in the cytoplasm (cyto) and nucleus (nucl) of neonatal rat cardiac myocytes. Lamin A/C and Gapdh were used as controls for nuclear and cytosolic proteins respectively. (C) Quantification of PP1 isoforms in the cytoplasm and nucleus based on figure 1B. \* $P < 0.05$  vs cyto. \*\* $P < 0.01$  vs cyto. (D) Western blot analysis of PP1s in the cytoplasm and nucleus of HEK293 cells. All the experiments were repeated 3 $\times$  with similar results.



**Figure 2:** PP1 $\alpha$  and PP1 $\beta$  selectively regulate the phosphorylation of PLB.

(A) Representative Western blot for phosphorylation of PLB at serine 16 and threonine 17, total PLB, and PP1 $\alpha$  from neonatal rat cardiac myocytes infected with adenoviruses expressing either  $\beta$ -gal (Con) or PP1 $\alpha$ . Isoproterenol (Iso) final concentration is 10  $\mu$ M. (B-C) Quantification of Western blots shown in figure 2A. N=4 for each group. \* $P < 0.05$  vs Con 5 minutes. (D) Western blot for phospho-PLB at serine 16 and threonine 17, total PLB, and PP1 $\beta$  from neonatal rat cardiac myocytes infected with adenoviruses expressing either  $\beta$ -gal (Con) or PP1 $\beta$ . (E-F) Quantification of Western blots shown in figure 2D. N=4 for each group.



**Figure 3:** Cardiac-specific deletion of PP1 $\beta$  leads to increased HDAC7 phosphorylation.

(A-B) Western blot for phospho-HDAC7 and total HDAC7 from the adult hearts of the indicated mice at 2 months of age. This result was representative of at least two independent experiments with multiple mouse hearts. (C) Quantification of Western blots shown in figure 3A-B. N=4 for each group. \*P<0.05 vs NKX-Cre.

### PP1 isoforms are not involved in cardiac stress responses

A previous study on the role of PP1 isoforms has demonstrated that cardiac specific deletion of PP1 $\beta$  from adult mouse hearts led to disease such as cardiac dysfunction and interstitial fibrosis [15]. However, it is still unknown whether PP1s play a role in cardiac stress responses. To address this question, we subjected 8-week-old NKX-Cre control mice or mice with cardiac specific deletion of PP1 $\alpha$  (PP1 $\alpha$  fl/fl<sup>NKX-Cre</sup>) or PP1 $\beta$  (PP1 $\beta$  fl/fl<sup>NKX-Cre</sup>) to either a sham procedure or pressure overload stimulation by Transverse Aortic Constriction (TAC). Cardiac function analysis by echocardiography demonstrated that after 2 weeks of pressure overload, there was no significant difference among all three groups of mice (Figure 4A). Similar cardiac hypertrophic growth after TAC was achieved among all three groups (Figure 4B). Moreover, histological analysis by Mason's trichrome staining also demonstrated that deletion of individual PP1 isoforms led to similar interstitial fibrosis (blue staining) compared to the NKX-Cre control mice (Figure 4C and 4D).

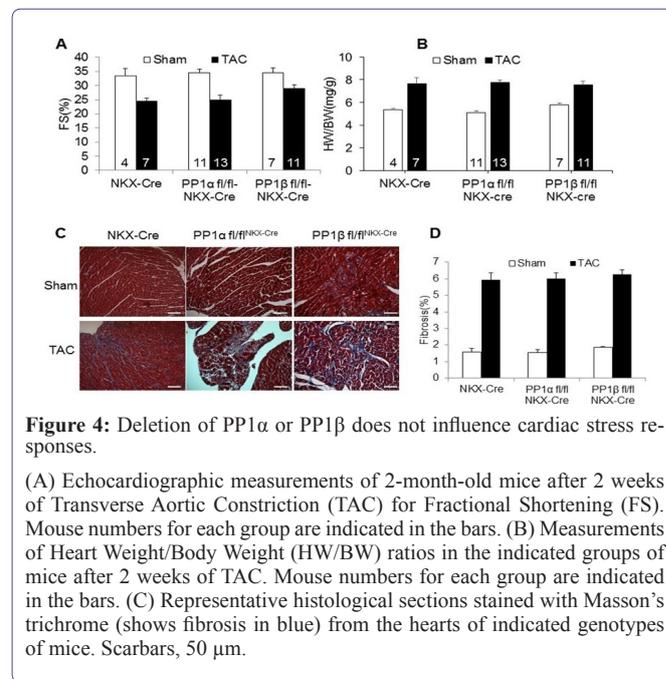
### PP1 levels are different in neonatal and adult hearts

Next, we compared the expression levels of PP1s in neonatal and adult hearts to understand whether PP1s have any role in post-natal growth of the heart. The expression of all three PP1 isoforms increased in adult hearts compared to the neonatal hearts (Figure 5A and 5B). Mammalian hearts are composed of two major cell types: Myocytes, for cardiac contraction and fibroblasts that are involved in secretion of extracellular matrix proteins for tissue remodeling, such as scar formation [28]. To further determine which cell population expresses PP1s, we isolated primary cardiac myocytes and fibroblasts from neonatal and adult hearts and assessed their expression levels by real-time PCR. PP1 $\alpha$  had a higher expression than PP1 $\beta$  and PP1 $\gamma$  in both cell types in neonatal hearts. In both cell types of adult hearts, PP1 $\beta$  expression was slightly higher than PP1 $\alpha$  and PP1 $\gamma$  (Figure 5C).

### Discussion

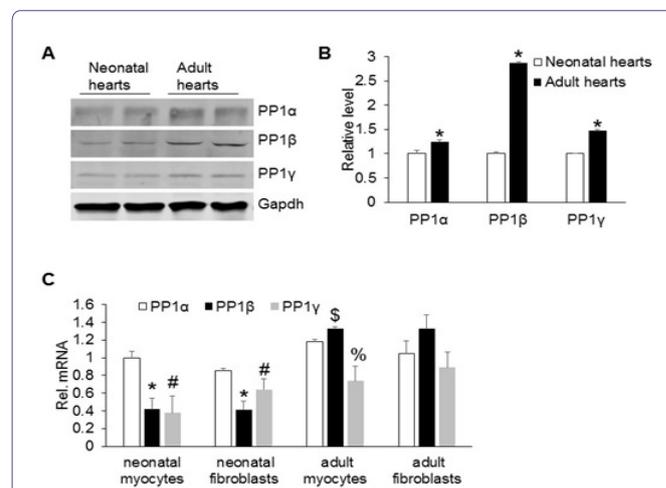
In this study we demonstrated that PP1 isoforms had distinct subcellular localizations to regulate the phosphorylation of specific substrates. PP1 $\beta$  was localized in the nucleus to regulate HDAC7

phosphorylation and PP1 $\alpha$  dephosphorylated PLB in the cytoplasm (Figure 1-3). Our data also suggested that PP1s were not involved in the pathological cardiac hypertrophy, but might play a role in postnatal growth of the heart (Figure 4 and 5).



**Figure 4:** Deletion of PP1 $\alpha$  or PP1 $\beta$  does not influence cardiac stress responses.

(A) Echocardiographic measurements of 2-month-old mice after 2 weeks of Transverse Aortic Constriction (TAC) for Fractional Shortening (FS). Mouse numbers for each group are indicated in the bars. (B) Measurements of Heart Weight/Body Weight (HW/BW) ratios in the indicated groups of mice after 2 weeks of TAC. Mouse numbers for each group are indicated in the bars. (C) Representative histological sections stained with Masson's trichrome (shows fibrosis in blue) from the hearts of indicated genotypes of mice. Scale bars, 50  $\mu$ m.



**Figure 5:** Relative expression levels of PP1 isoforms in the cardiac cells.

(A) Western blot analysis for each PP1 isoform in the hearts of neonatal rats and 2-month-old mice. (B) Quantification of protein levels of PP1 isoforms in figure 5A. N=4 for each group. \*P<0.05 vs neonatal hearts. (C) Real-time PCR analysis of PP1 isoform expression in cardiac myocytes and fibroblasts isolated from neonatal and adult mouse hearts. N=4 for each group. \*P<0.05 vs PP1 $\alpha$  from neonatal cells; # P<0.05 vs PP1 $\alpha$  from neonatal cells. \$ P<0.05 vs PP1 $\alpha$  from adult myocytes; %P<0.05 vs PP1 $\alpha$  from adult myocytes. All the data were representative of three independent experiments.

Numerous *in vitro* studies have suggested that the substrate specificity of PP1s is achieved by binding of PP1s to a large of number of

structurally unrelated proteins that target them to distinct subcellular localizations [29,30]. Nearly 200 of PP1-Interacting Proteins (PIPs) have been identified in vertebrates, many of which bind to all three PP1 isoforms without isoform specificity [31]. Binding of targeting proteins to PP1s is mediated by single or combinational docking motifs on the surface of PP1s such as RVxF, RNYF, SILK [30,31]. In addition, all three PP1 isoforms demonstrate an overlapping but also distinct and dynamic localizations as well as tissue-specific expression patterns [31,32]. Further study using *in vitro* methods such as co-immunoprecipitation and GST-pulldown followed by mass spectrometry will help identify isoform-specific binding proteins of PP1 in the heart.

Previous studies have demonstrated that PP1 and PP2A are also targeted to the mitochondria, but their specific function remains unclear [33,34]. Although we confirmed the presence of lamin A/C proteins in the nuclear fraction, we cannot rule out the possibility that proteins from other organelles such as mitochondria might be present in this crude fraction. However, we did observe a significant amount of PP1 $\beta$  in the nuclear fraction, deletion of which led to a moderate increase of HDAC7 phosphorylation (Figure 3), indicating that PP1 $\beta$  might play a role in regulating the transcriptional activity of HDAC7. Interestingly, arginine 19 in the N terminal of PP1 $\beta$  has been suggested for the nuclear targeting of PP1 $\beta$  [32].

PP1s are serine/threonine protein phosphatases that dephosphorylate a variety of substrates. Our study here showed that PP1 $\alpha$  was localized in the cytoplasm to regulate the PLB phosphorylation, consistent with a previous study of PP1 $\alpha$  in the heart using transgenic mouse approach [14]. PP1 $\beta$  seems to have functional roles in both the cytoplasm and nucleus. However, literatures regarding the role of PP1 $\beta$  in the cytoplasm are not consistent, possibly due to the different approaches being used. For example, using a siRNA-based approach to knock down individual PP1s from the adult cardiac myocytes, PP1 $\beta$  was demonstrated to be the major isoform regulating the phosphorylation of PLB [16]. Using a LoxP-Cre approach to specifically delete the PP1 isoforms from the heart, another research group demonstrated that deletion of neither PP1 isoforms influenced the phosphorylation of PLB [15]. In this study, we further demonstrated that PP1 $\beta$  had no effect on PLB phosphorylation (Figure 2D-2F). Instead, PP1 $\beta$  was localized to the nucleus to regulate HDAC7 phosphorylation. However, our study didn't provide any mechanism on how PP1 $\beta$  regulates HDAC7. It is possible that dephosphorylation of HDAC7 by PP1 $\beta$  promotes its nuclear localization to suppress target gene expression as shown in a thymocytes study [17]. In addition, the functional role of PP1 $\beta$  involving gene expression through HDAC7 may also help explain the fibrotic phenotype observed in PP1 $\beta$  fl/flNKX-Cre mice [15].

Congenital heart disease is one of the most common development disorders that affects ~8:1000 live births worldwide [35,36]. Mutation of multiple proteins have been linked to this disease, including transcription factors NKX2-5, protein tyrosine phosphatase SHP2 [35,37]. Missense variants in PPP1C $\beta$  gene were also implicated in congenital heart disease [38]. Moreover, a recent study demonstrated greater abundance of PP1 $\alpha$ , but unaltered levels of PP1 $\beta$  in heart failure patients [39]. Based on these studies, we initially hypothesized that PP1 $\beta$  regulates baseline cardiac function, whereas PP1 $\alpha$  is involved in pathological responses. However, upon 2 weeks of pathological stimulation by TAC, a well-established approach in the field to induce

cardiac hypertrophy, mice with loss of either PP1 $\alpha$  or PP1 $\beta$  developed similar hypertrophy and dysfunction compared to the controls, suggesting PP1s are not involved in this process. It will be interesting to extend the TAC stimulation to longer period such as 14 weeks to assess whether PP1s are involved in the decompensation of the heart to failure. It is also possible that deletion of one isoform is compensated by increased level of the other two isoforms, so the overall PP1 activity is not influenced. However, even deletion of PP1 $\beta$  led to upregulation of both PP1 $\alpha$  and PP1 $\gamma$ , mice still developed cardiac dysfunction, suggesting each PP1 isoform plays a unique function possibly due to their distinct subcellular localizations [15]. Although not involved in the cardiac stress responses, we found out that PP1s were increased during postnatal development, suggesting PP1s might play a role in postnatal growth of the heart (Figure 5A). Indeed, cardiac deletion of PP1 $\beta$  from fetal stage using NKX2.5-Cre led to cardiac dysfunction in mice as early as 4 weeks of age [15].

## Conclusion

PP1 isoforms have distinct subcellular localizations to regulate the phosphorylation of specific substrates. PP1s are not found to be involved in cardiac stress responses, but have increased expression in postnatal growth of the heart.

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