Inpp5f Is a Polyphosphoinositide Phosphatase That Regulates Cardiac Hypertrophic Responsiveness

Wenting Zhu, Chinmay M. Trivedi, Diane Zhou, Lijun Yuan, Min Min Lu, Jonathan A. Epstein

Rationale: Cardiac hypertrophy occurs in response to a variety of extrinsic and intrinsic stimuli that impose increased biomechanical stress. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway has previously been shown to be strongly associated with hypertrophic signaling in the heart, and with the control of cell size in multiple contexts. This pathway is tightly regulated by many factors, including a host of kinases and phosphatases that function at multiple steps in the signaling cascade. For example, the PTEN (phosphatase and tensin homolog) tumor suppressor protein is a phosphoinositide 3-phosphatase that, by metabolizing phosphatidylinositol 3,4,5-trisphosphate (PtdIns[3,4,5]P3,PIP3), acts in direct antagonism to growth factor–stimulated PI3K. Inhibition of PTEN leads to cardiomyocyte hypertrophy. Another polyphosphoinositide phosphatase, inositol polyphosphate-5-phosphatase F (Inpp5f) has recently been implicated in regulation of cardiac hypertrophy. Like PTEN, this phosphatase can degrade PtdIns(3,4,5)P3 and thus modulates the PI3K/Akt pathway.

Objective: To characterize the role of Inpp5f in regulating cardiac hypertrophy.

Methods and Results: We generated homozygous Inpp5f knockout mice and cardiac specific Inpp5f overexpression transgenic mice. We evaluated their hearts for biochemical, structural and functional changes. Inpp5f knockout mice have augmented hypertrophy and reactivation of the fetal gene program in response to stress when compared to wild-type littermates. Furthermore, cardiac overexpression of Inpp5f in transgenic mice reduces hypertrophic responsiveness.

Conclusions: Our results suggest that Inpp5f is a functionally important endogenous modulator of cardiac myocyte size and of the cardiac response to stress. (Circ Res. 2009;105:1240-1247.)

Key Words: Inpp5f ■ hypertrophy ■ Akt ■ GSK3β ■ PIP3 ■ Hdac2

Cardiovascular disease remains the number one cause of mortality in the Western world, with heart failure representing the fastest growing subclass over the past decade. In many cases, cardiac hypertrophy precedes cardiac dilation and heart failure. Cardiac hypertrophy can occur in response to normal physiological stimuli or may be maladaptive leading to cardiac dilation and congestive heart failure. However, the cellular mechanisms that regulate the hypertrophic response to agonists or to stretch remain poorly understood, and the transition from hypertrophy to failure is also ill defined. On the other hand, evidence accumulating over the last few decades confirms that intercellular signaling pathways and gene expression are fundamentally altered in states of hypertrophy and failure and a thorough understanding of these changes will provide therapeutic targets.

Recently, we showed that Hdac2 deficient mice are resistant to cardiac hypertrophy when exposed to hypertrophic stimuli. Resistance to hypertrophy in Hdac2−/− mice is associated with increased expression of the gene encoding inositol polyphosphate-5-phosphatase (Inpp5f) and constitutive activation of glycogen synthase kinase (Gsk)3β via inactivation of Akt and Pdk1. Conversely, transgenic overexpression of Hdac2 in the heart reactivates fetal genes and induces cardiac hypertrophy. In these transgenic hearts, Inpp5f is significantly downregulated, Akt is activated, and Gsk3β is inactive. Further studies suggest that Hdac2 is a direct regulator of Inpp5f. Thus, we hypothesized that Inpp5f might functionally contribute to cardiac regulation of the Akt/Gsk3β pathway in response to stress.

Inpp5f is one of several polyphosphoinositide phosphatases that have been identified and partially characterized. Prior work demonstrates that Inpp5f can degrade both phosphatidylinositol 4,5-bisphosphate (PtdIns[4,5]P2 [PIP2]) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns[3,4,5]P3 [PIP3]) by removing the 5’ phosphate from the inositol ring. Although the PIP3 3’ phosphatase PTEN (phosphatase and tensin homolog) has previously been shown to regulate cardiac myocyte hypertrophy and Akt signaling in the heart, a functional role for a PIP3 3’ phosphatase in the heart has been less clear. However, in various noncardiac
model systems, both 3' and 5' inositol phosphatases are important regulators of PIP3 activity and downstream signaling. For example, the SH2 domain-containing inositol 5'-phosphatase (SHIP) and PTEN both play important roles to regulate PIP3 and Akt in immune cells.8

To determine the role of Inpp5f in the adult heart, we have created both gain- and loss-of-function models. Our results indicate that Inpp5f transgenic mice are unable to reactivate fetal genes or to exhibit normal hypertrophic responses to adrenergic agonists, whereas Inpp5f knockout mice exhibit augmented hypertrophy and exaggerated reactivation of the fetal gene program under stress.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Inpp5f−/− Knockout Mice

An Inpp5f gene-trap ES clone was obtained from International Gene Trap Consortium (ES clone no. XL0571). The Inpp5f genomic locus is interrupted by insertion of the pGTO1xf vector, which integrated into intron 6. Chimeric mice were produced by blastocyst injection according to standard protocols. Mice were genotyped by PCR analysis of genomic DNA. Cardiac specific expression of Inpp5f was revealed by quantitative RT-PCR (see the Online Data Supplement) and Western blotting analysis using antibodies to Flag (Sigma, F3165) or Inpp5f (generated by our laboratory; see Online Data Supplement). The loss of Inpp5f was confirmed by PCR analysis of genomic DNA. Cardiac specific expression of Inpp5f was revealed by quantitative RT-PCR (see the Online Data Supplement) and Western blotting analysis using antibodies to Flag (Sigma, F3165) or Inpp5f (generated by our laboratory; see Online Data Supplement).

Inpp5f Transgenic Mice

A cDNA encoding human Flag-tagged Inpp5f was cloned into an expression plasmid containing the myosin heavy polypeptide (Myh)6 (encoding α-myosin heavy chain) promoter,9 and transgenic mice were generated by standard techniques. Genotyping was performed by PCR analysis of genomic DNA. Cardiac specific expression of Inpp5f was revealed by quantitative RT-PCR (see the Online Data Supplement) and Western blot analysis using antibodies to Flag (Sigma, F3165) or Inpp5f (generated by our laboratory; see Online Data Supplement).

Inpp5f Antibody Generation

GST-Inpp5f fusion protein was expressed in bacteria, purified by standard techniques and used to immunize rabbits (Cocalico Biologicals Inc). A region of mouse Inpp5f cDNA encoding 61 C-terminal amino acids was amplified by PCR and cloned into the pGEX-2T vector (see the Online Data Supplement). The GST fusion protein was expressed in BL21 bacteria, extracted and purified using glutathione Sepharose 4B (GE Healthcare) according to the instructions of the manufacturer. Purified protein was used to immunize rabbits (Cocalico Biologicals Inc).

Western Blotting

Tissue lysates were prepared in lysis buffer consisting of 20 mmol/L Tris HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1% Triton X-100, 1 μg/ml leupeptin, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L Na3VO4, 1 mmol/L β-glycerophosphate, 1 mmol/L phenylmethylsulfonyl fluoride was added before use. Samples were separated by SDS-PAGE and transferred to poly(vinylidene difluoride) membranes. We used antibodies to Gapdh (1:5000 dilution, Chemicon, MAB374), phospho-Gsk3β (Ser 9), total Gsk3β, phospho-Akt (Ser 473), total Akt (1:1000 dilution, Cell Signaling, #9336, #9315, #4058, #9272).

Primary antibody binding was visualized by using the Western Breeze Kit (Invitrogen) according to the instructions of the manufacturer. Inpp5f antibody was purified by Melon Gel IgG Spin Purification kit (Thermo Scientific) and then diluted 1:100 in 5% milk.

Treatment With Isoproterenol

Isoproterenol (ISO) (Sigma, I5627) was delivered by implanting a microosmotic pump (Alzet, Durect; model 1002) subcutaneously under pentobarbital anesthesia. ISO (30 mg/kg per day) or vehicle (Dulbecco's PBS, Gibco) was infused subcutaneously for 14 days as described previously.10

Histology

Adult mouse hearts were collected in ice-cold PBS, fixed overnight in 4% paraformaldehyde at 4°C, washed with PBS, and dehydrated through an ethanol series before paraffin embedding. Masson's trichrome (to reveal fibrosis) and hematoxylin/eosin (H&E) stains were performed according to standard protocols.

Apoptosis Analysis

Apoptosis was measured by TUNEL assay (Roche, 1684795). The total number of cells was quantified using ImageJ software.

Quantitative Real-Time PCR

Total RNA was isolated from dissected mouse hearts using TRizol (Invitrogen). RNA was reverse-transcribed using random hexamers and the Superscript First Strand Synthesis Kit (Invitrogen). Gene expression was then evaluated by quantitative RT-PCR using ABI PRISM 7900) using the SYBR Green (Applied Biosystems). Signals were normalized to their corresponding Gapdh controls and the ratios expressed as fold changes compared to wild type. PCR conditions and primer set sequences are available on request.

Insulin-Like Growth Factor-1 Treatment

Hearts from 1-month old mice were minced and incubated with 100 nmol/L insulin-like growth factor (IGF)-1 (Sigma, I8779) for 30 minutes at 37°C followed by lipid extraction.

PIP3 ELISA

Mouse heart tissue was pulverized under liquid nitrogen using a mortar and pestle, followed by lipid extraction and ELISA assays as per manufacture's instruction (Echelon Biosciences Inc, K-2500). ELISA measurements were performed in triplicate after combining tissue from 4 hearts.

Statistical Analysis

All data are expressed as the means ± SD. Student’s t test was used to compare heart to body weight ratios and heart weight/tibia length ratios. Probability values of <0.05 were considered statistically significant.
Inpp5f Regulates Stress-Induced Hypertrophy

Next, we sought to determine whether adult mice lacking Inpp5f would be more susceptible to agonist-induced cardiac hypertrophy. Wild-type and Inpp5f−/− littermates at 2 months of age were treated with a constant infusion of saline or ISO delivered by osmotic mini-pump for 14 days. Animals lacking Inpp5f showed an increase in ISO-induced cardiac hypertrophy as measured by either the heart to body weight ratio or the heart weight/tibia length ratio (Figure 3A). In response to ISO, Inpp5f−/− hearts also showed more potent reactivation of the fetal program of gene expression than wild-type littermates; transcripts for natriuretic peptide precursor type A (NPPA) and B (NPPB) were increased more in Inpp5f−/− hearts than in wild-type hearts (Figure 3B). These data suggest that Inpp5f may have a role in regulating the fetal program of gene expression in response to ISO stimulation.

Table 1. Loss of Inpp5f Does Not Lead to Embryonic or Perinatal Lethality

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>Expected</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inpp5f+/+</td>
<td>105</td>
<td>123</td>
</tr>
<tr>
<td>Inpp5f+/−</td>
<td>210</td>
<td>192</td>
</tr>
<tr>
<td>Inpp5f−/−</td>
<td>105</td>
<td>105</td>
</tr>
<tr>
<td>Total</td>
<td>420</td>
<td>420</td>
</tr>
</tbody>
</table>

*Offspring of heterozygous crosses. χ²=4.629, 2 degrees of freedom, P=0.10.

Further in depth analysis of the Inpp5f genomic locus affected by the gene-trap revealed that the insertion event was accompanied by a loss of endogenous genomic sequence spanning exons 7 to 13 (Figure 1A and Online Figure II). Thus, the affected allele cannot produce a full-length protein or one with functional phosphatase activity. We confirmed the lack of full length Inpp5f transcripts by real-time PCR using mRNA from adult Inpp5f−/− and wild-type hearts (Figure 1D). Western blot using antibody recognizing the C-terminus of Inpp5f demonstrated a lack of protein expression in the knockout (Figure 1E). Thus, we conclude that we have generated a null allele for Inpp5f.

Inpp5f-Null Hearts Appear Normal

We examined cardiac histology, size and function in Inpp5f−/− and littermate control mice. H&E staining of hearts was performed at E14.5, P0 and 2 months of age. At these time points, we could not identify any differences and Inpp5f-null mice appear normal by gross and histological assessment (Figure 2A). We measured cardiac weight in relation to total body weight to determine relative cardiac size. At both 2 and 9 months of age, there was no difference in heart to body weight ratio between wild-type and Inpp5f−/− littermates (Figure 2B and 2C).

To evaluate the functional impact of the loss of Inpp5f, we performed echocardiography on Inpp5f−/− and wild-type littermates at 2 and 9 months (Table 2). We did not observe any significant difference in the interventricular septum or the left ventricular internal diameter measurements at either end-diastole or end-systole. Left ventricular ejection fraction and fractional shortening also were not different between groups. Thus, under basal conditions, Inpp5f−/− mice appear to have normal cardiac form and function.
A (Nppa) (encoding atrial natriuretic factor, ANF), Myh7 (encoding β-myosin heavy chain), and Nppb (encoding BNP) increased more dramatically in the knockout hearts (Figure 3B). Cellular hypertrophy, as revealed by wheat germ agglutinin staining, followed by quantification, was more pronounced in the Inpp5f<sup>−/−</sup> hearts compared to wild-type hearts after ISO (Figure 3C) (256.5±21.8 μm<sup>2</sup> for Inpp5f<sup>−/−</sup> mice treated with ISO, n=446 cells from 3 hearts; 194.8±6.8 μm<sup>2</sup> for wild-type mice treated with ISO, n=514 from 3 hearts). Patchy areas of fibrosis were more evident in ISO-treated Inpp5f<sup>−/−</sup> hearts when compared to controls (Figure 3D) (quantification of fibrotic area was 7.3% for Inpp5f<sup>−/−</sup> hearts, 25 sections, 3 hearts; 1.8% for wild-type mice, 27 sections, 3 hearts). We also observed more apoptotic cells in the Inpp5f<sup>−/−</sup> hearts after ISO treatment compared to wild-type (0.37% of 16,804 cells from 4 Inpp5f<sup>−/−</sup> hearts, 0.24% of 21,558 cells from 5 wild-type hearts, P<0.01). Functional assessment by echocardiography after 2 weeks of ISO treatment revealed relative preservation of fractional shortening and ejection fraction, with thickening of the posterior wall in Inpp5f<sup>−/−</sup> animals at this time point (Online Table I).

Neonatal myocytes isolated from Inpp5f<sup>−/−</sup> and wild-type littermates revealed similar levels of Akt and Gsk3β phosphorylation under basal conditions (Figure 3E). Treatment with ISO resulted in increased phosphorylation of Akt and Gsk3β in knockout cells when compared to controls (Figure 3E), whereas no significant differences in Erk activation were noted (data not shown). IGF-1 treatment of myocytes revealed similar results (Online Figure III). Taken together, our data suggest that cardiac myocytes lacking Inpp5f are sensi-

### Table 2. Inpp5f<sup>−/−</sup> Mice Have Normal Cardiac Function by Echocardiography

<table>
<thead>
<tr>
<th></th>
<th>2 Months</th>
<th></th>
<th></th>
<th>9 Months</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type (n=3)</td>
<td>Inpp5f KO (n=3)</td>
<td>p Value</td>
<td>Wild Type (n=4)</td>
<td>Inpp5f KO (n=4)</td>
<td>p Value</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.69±0.06</td>
<td>0.66±0.03</td>
<td>NS</td>
<td>0.66±0.06</td>
<td>0.67±0.07</td>
<td>NS</td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>1.13±0.12</td>
<td>1.00±0.04</td>
<td>NS</td>
<td>0.96±0.10</td>
<td>1.02±0.09</td>
<td>NS</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.69±0.06</td>
<td>0.64±0.07</td>
<td>NS</td>
<td>0.65±0.03</td>
<td>0.70±0.06</td>
<td>NS</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>1.02±0.13</td>
<td>0.90±0.07</td>
<td>NS</td>
<td>0.95±0.14</td>
<td>1.04±0.07</td>
<td>NS</td>
</tr>
<tr>
<td>LVIdd (mm)</td>
<td>3.92±0.16</td>
<td>3.76±0.13</td>
<td>NS</td>
<td>4.13±0.22</td>
<td>4.13±0.48</td>
<td>NS</td>
</tr>
<tr>
<td>LVIds (mm)</td>
<td>2.57±0.10</td>
<td>2.54±0.17</td>
<td>NS</td>
<td>2.98±0.12</td>
<td>2.92±0.40</td>
<td>NS</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>64±2</td>
<td>60±4</td>
<td>NS</td>
<td>56±4</td>
<td>57±4</td>
<td>NS</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>34±1</td>
<td>31±3</td>
<td>NS</td>
<td>29±3</td>
<td>29±2</td>
<td>NS</td>
</tr>
</tbody>
</table>

IVSd indicates interventricular septum thickness in diastole; IVSs, interventricular septum thickness in systole; LVPWd, left ventricular (LV) posterior wall thickness in diastole; LVPWs, LV posterior wall thickness in systole; LVIdd, LV internal dimension in diastole; LVIds, LV internal dimension in systole; LVEF, LV ejection fraction; LVFS, LV fractional shortening.
tive to stress-induced cardiac hypertrophy and activation of the Akt pathway.

**PIP3 Levels Are Altered in Inpp5f-Null Mice**

Although a prior report has indicated that Inpp5f exhibits 5-phosphatase activity when PIP3 and PIP2 are used as substrates in vitro, we sought to determine directly if loss of Inpp5f alters endogenous cardiac PIP3 levels in vivo. Endogenous PIP3 levels are low, and we are unaware of prior studies that directly measure levels of this important signaling molecule in vivo in genetically engineered animals. Therefore, we adapted an ELISA-based sensitive assay that has been used for cultured cells and compared PIP3 levels in control and Inpp5f/−/− hearts before and after ligand stimulation intended to augment PIP3 levels. Heart tissue from 4 mice age 3 to 5 weeks was combined for each condition, and some samples were treated ex vivo with IGF-1, a potent activator of PI3K (phosphatidylinositol 3-kinase) and AKT in the heart. Our data shows that PIP3 levels in Inpp5f/−/− mice are 1.6 fold greater than wild-type littermates. However, on IGF-1 stimulation, PIP3 levels in the knockout hearts increased to 4.9 fold those of stimulated wild-type litters (Figure 3F). Thus, loss of Inpp5f sensitizes the heart to hypertrophic stimulation.

**Cardiac-Specific Inpp5f Transgenic Mice**

We used the well-characterized α-myosin heavy chain promoter to direct cardiac-restricted expression of Inpp5f in transgenic mice (Figure 4A). We evaluated 2 independent transgenic lines of mice to control for effects mediated by sites of insertion. Germline transmission of the transgene was verified by PCR (data not shown) and the transgene mRNA level was determined by real-time PCR (data not shown). Transgenic expression of Inpp5f was verified by Western blotting (Figure 4B) which revealed similar levels of protein expression in the 2 independent lines (Figure 4B).

**Inpp5f Transgenic Mice Are Resistant to Stress-Induced Hypertrophy**

Adult Inpp5f transgenic mice appeared healthy. Heart to body weight ratios of transgenic and wild-type littermates were not significantly different at P60 (Figure 4C). Control and Inpp5f transgenic littersmates at 2 months of age were treated with a constant infusion of saline or ISO for 14 days. As predicted, wild-type mice exhibited cardiac hypertrophy, as revealed by an increase in both the heart to body weight ratio and the heart weight/tibia length ratio (Figure 4C). Hypertrophy of Inpp5f transgenic mice in response to ISO was significantly blunted (Figure 4C). Likewise, reactivation of the fetal gene program that accompanied hypertrophy in control animals was mark-
edly attenuated in transgenic animals (Figure 4D). After treatment with ISO, we noted activation of the Akt pathway with enhanced phosphorylation of Akt and Gsk3β in the control hearts (Figure 4E). However, ISO-induced increases in phospho-Akt and phospho-Gsk3β were blunted in transgenic hearts.

Discussion

In this study, we investigated the effects of gain- and loss-of-function of Inpp5f. Our results suggest that Inpp5f modulates stress-induced hypertrophic responsiveness in the heart. Under basal conditions, Inpp5f knockout and transgenic mice appear normal, with preserved cardiac structure and function. However, in the setting of adrenergic stimulation produced by infusion of ISO, Inpp5f−/− animals had elevated level of PIP3 and showed accentuated hypertrophy as measured by heart size, myocyte size and gene expression. Isolated myocytes lacking Inpp5f were hypersensitive to ISO and IGF-1 as reflected by accentuated activation of Akt compared to control myocytes. Conversely, Inpp5f transgenic mice were relatively resistant to hypertrophic stimulation.

Inpp5f encodes a 5’ PIP3 phosphatase that is predicted to reduce PIP3 levels and subsequent activation of Akt and downstream signals. Our biochemical findings in Inpp5f transgenic and knockout hearts are consistent with this mode of action. The Akt signaling network in the adult heart has been extensively examined and has been shown to contribute to both adaptive (physiological) and maladaptive (pathological) hypertrophy.7 Akt1−/− mice have a 20% reduction in body size, with a concomitant reduction in heart size11 and they are defective in exercise-induced cardiac hypertrophy.12 Cardiac-specific overexpression of constitutively active or dominant negative forms of Akt lead to larger or smaller hearts respectively.13–15 Enhanced Akt activity is associated with increased p70S6 kinase activity and increased phospho-Gsk3β.13,14 Cardiac-specific overexpression of constitutively active Gsk3β is associated with reduced agonist and pressure-overload hypertrophy confirming that Gsk3β functions as a negative regulator of hypertrophy in vivo.16 Thus, the alterations in Akt and Gsk3β phosphorylation that we observed in Inpp5f knockout and transgenic hearts are consistent with a model in which Inpp5f regulates PIP3 levels, Akt and Gsk3β activity and subsequent hypertrophic responsiveness.

The inositol polyphosphate 5-phosphatases are a large family of enzymes comprising at least 10 mammalian and 4 yeast members.17 Inpp5f has a SAC phosphatase domain which exhibits phosphatidylinositol polyphosphate phosphatase activity.5 The SAC domain is approximately 400 amino acids in length and is defined by seven conserved motifs encompassing the catalytic and regulatory regions of the phosphatase.18 The highly conserved sequence RXNCXDCLDRTN in the sixth motif is proposed to be the catalytic core of the SAC domain phosphatases. The CX3R(T/S) motif within this sequence is also found in a number of metal-independent protein phos-
phosphatases and other inositol polyphosphate phosphatases.\textsuperscript{18,19} Interestingly, the SAC domain of the yeast synaptojanin-like protein Inp51p does not exhibit phosphatase activity, and the cysteine, arginine and threonine/serine residue are absent from CX\textsubscript{3}R(T/S) motif of this protein, being replaced by alanine, lysine, and proline respectively. Mutation of the first conserved aspartate residue in the RXNCRX-DCLDRGN sequence as seen in the yeast sac1-8 and sac1-22 mutant alleles were demonstrated to inactivate the Sac1p functions.\textsuperscript{18,20,21}

Some 5-phosphatases, such as the Src homology 2 (SH2) domain-containing inositol polyphosphate 5-phosphatases 1 and 2 (Ship and Ship2) have been extensively characterized.\textsuperscript{22–26} Ship is expressed predominantly in hematopoietic cells where it is an important negative regulator of cytokine signaling. \textit{Ship}\textsuperscript{−/−} mice have a short life span associated with massive myeloid cell infiltration of the lungs and numerous hematopoietic abnormalities.\textsuperscript{27,28} Ship2 is more widely expressed, with high expression in brain, skeletal muscle and heart.\textsuperscript{17} It plays an important role in insulin signaling and obesity regulation.\textsuperscript{29,30} Ship2 has been reported to be a negative regulator of Akt activation. Although loss of Ship2 is not sufficient to activate Akt, the absence of Ship2 allows for greater activity on Akt stimulation.\textsuperscript{31,32} These findings are reminiscent of our results with regard to Inpp5f-mediated regulation of basal and agonist-induced activation of Akt in the heart.

Although PTEN and the 5-phosphatases can all degrade PIP\textsubscript{3}, the degradation products are distinct. Whereas PTEN converts PIP\textsubscript{3} to PI(4,5)P\textsubscript{2}, the 5-phosphatases convert PIP\textsubscript{3} to PI(3,4)P\textsubscript{2}, which can function as a second messenger.\textsuperscript{33} Therefore, the production of PI(3,4)P\textsubscript{2} from PIP\textsubscript{3} by Inpp5f and Ship phosphatases may function in part via active signaling, whereas PTEN action appears to be mediated through loss of active PI(3,4)P\textsubscript{2} signaling.\textsuperscript{34} For example, PIP(3,4)P\textsubscript{2} has been shown to activate reactive oxygen species\textsuperscript{35} and the generation of reactive oxygen species is a process that is increasingly recognized as an important contributor to depressed cardiac function and maladaptive remodeling.\textsuperscript{36} Thus, this process could also contribute to the phenotype we observed. Moreover, PI(3,4)P\textsubscript{2} activity has been shown to correlate with Akt phosphorylation and activity.\textsuperscript{37} Inpp5f can also dephosphorylate PI(4,5)P\textsubscript{2},\textsuperscript{3} and depletes of PI(4,5)P\textsubscript{2} may contribute to cardiomyocyte apoptosis and subsequent heart failure.\textsuperscript{38} Thus, regulation of PI(4,5)P\textsubscript{2} levels may contribute to the mechanism by which Inpp5f regulates hypertrophy.

Under sedentary conditions, we found that Inpp5f-null mice have slightly higher level of cardiac PI(3,4)P\textsubscript{2}, though phospho-Akt and phospho-Gsk3\textbeta are unaltered and the mice do not show abnormal hypertrophy. However, on IGF-1 stimulation, PI(3,4)P\textsubscript{2} levels in the knockout mice increase dramatically and they are more sensitive to hypertrophic stimuli. Hence, unlike PTEN, which alters the basal level of PI(3,4)P\textsubscript{2}, Inpp5f appears to modulate PI(3,4)P\textsubscript{2} levels primarily on agonist-induced stimulation.

Our prior studies have suggested that Inpp5f is repressed at the transcriptional level by Hdac2. Inpp5f transcripts are elevated in Hdac2 knockout hearts and are diminished by overexpression of Hdac2 in the heart.\textsuperscript{3} siRNA-mediated knockdown of Hdac2 or chemical Hdac inhibition in H9C2 myocytes results in increased levels of Inpp5f and decreased phosphorylation of Akt and Gsk3\textbeta. Although Hdac2 may modulate cardiac myocyte size and hypertrophic signaling via multiple pathways, our analysis of Inpp5f knockout and transgenic mice are consistent with a functional relationship between Hdac2 and Inpp5f in the setting of hypertrophic stimulation.

Taken together, the work presented here suggests that Inpp5f functions as a negative regulator of cardiac hypertrophy and Akt signaling. Loss of Inpp5f sensitizes the heart’s response to hypertrophic stimuli by modulating PI(3,4,5)P\textsubscript{3} levels.

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Disclosures
None.

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Inpp5f knockout mice. Inpp5f+/− and Inpp5f−/− mice were genotyped by PCR using:

Wt F: 5' -AAAGCAGGTGTAAGTGAGCTG- 3',
Wt R: 5' -TGCTCTTTGTCATCATCCGAGGACT- 3',
Mut F: 5' -ATATTGAAACCCACCGCATTGGTGC- 3' and
Mut R: 5' -TTTGATGGACCATTTCGCGACAGC- 3' primers.

The mutant mice have one band at 323 bp, and the wild type mice have one band at 1039 bp.

The loss of Inpp5f was confirmed by qRT-PCR using
5' -ACAGGAGAAAGGAAGTTGGCAGGA -3' and
5' -AGCTTCATGCTCTTCTTCTTTTGT -3' primers.

Inpp5f-transgenic mice. Genotyping was performed by PCR analysis of genomic DNA using 5'-TCTTCCAAGCCAAGGCCACTACA- 3' and 5' -TCTGCACCGAATTGGTCAGGTCAT- 3', and cardiac specific expression of Inpp5f was revealed by qRT-PCR using 5' -ACAGGAGAAAGGAAGTTAGCAGGA- 3' and 5' -AGCTTCATGTTCTTCTCCTTGTG- 3' primers.

Inpp5f antibody generation. The primers for amplifying mouse Inpp5f cDNA are 5'-CGGACTGGCTTCACAAGCCCA-3', 5'-AGGAGGCGTCTGGTCACATTTG-3'.
Online Table I: Echocardiography of wild type and *Inpp5f*<sup>−/−</sup> mice after 2 weeks of ISO infusion.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type (n=5)</th>
<th>Inpp5f&lt;sup&gt;−/−&lt;/sup&gt; (n=5)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSd (mm)</td>
<td>0.69±0.10</td>
<td>0.78±0.15</td>
<td>ns</td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>1.01±0.17</td>
<td>1.16±0.25</td>
<td>ns</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.65±0.06</td>
<td>0.74±0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>0.98±0.13</td>
<td>0.98±0.16</td>
<td>ns</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.97±0.36</td>
<td>3.96±0.45</td>
<td>ns</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.80±0.45</td>
<td>2.76±0.60</td>
<td>ns</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>57±12</td>
<td>59±12</td>
<td>ns</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>30±8</td>
<td>31±8</td>
<td>ns</td>
</tr>
</tbody>
</table>
Existence of the fusion mRNA transcript. (A) Schematic representation of the Inpp5f fusion mRNA transcript. The primers for the Real-time PCR are shown. (B) Real-time PCR of mRNA from four individual knockout mice and their wild type littermate controls.
Examination of the endogenous Inpp5f genomic sequence. PCR results show that exons 7 to 13 are deleted in the Inpp5f locus.
Inpp5f null myocytes are more sensitive to IGF-1 treatment. Western blots from neonatal cardiomyocytes treated with or without 100 nM IGF-1 for 10 min are shown.