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Phosphoinositide 3-Kinase γ Inhibits Cardiac GSK-3 Independently of Akt

Maradumane L. Mohan¹, Babal K. Jha², Manveen K. Gupta¹, Neelakantan T. Vasudevan¹, Elizabeth E. Martelli¹, John David Mosinski¹, and Sathyamangla V. Naga Prasad^{1,*}

¹Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, USA

²Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, USA

Abstract

Activation of cardiac phosphoinositide 3-kinase α (PI3K α) by growth factors, such as insulin, or activation of PI3K γ downstream of heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors stimulates the activity of the kinase Akt, which phosphorylates and inhibits glycogen synthase kinase-3 (GSK-3). We found that PI3K γ inhibited GSK-3 independently of the insulin-PI3K α -Akt axis. Although insulin treatment activated Akt in PI3K γ knockout mice, phosphorylation of GSK-3 was decreased compared to control mice. GSK-3 is activated when dephosphorylated by the protein phosphatase 2A (PP2A), which is activated when methylated by the PP2A methyltransferase PPMT-1. PI3K γ knockout mice showed increased activity of PPMT-1 and PP2A and enhanced nuclear export of the GSK-3 substrate NFATc3. GSK-3 inhibits cardiac hypertrophy, and the hearts of PI3K γ knockout mice were smaller compared to those of wild-type mice. Cardiac overexpression of a catalytically inactive PI3K γ (PI3K γ_{inact}) transgene in PI3K γ knockout mice reduced the activities of PPMT-1 and PP2A and increased phosphorylation of GSK-3. Furthermore, PI3K γ knockout mice expressing the PI3K γ_{inact} transgene had larger hearts than wild-type or PI3K γ knockout mice. Our studies show that a kinase-independent function of PI3K γ could directly inhibit GSK-3 function by preventing the PP2A-PPMT-1 interaction and that this inhibition of GSK-3 was independent of Akt.

INTRODUCTION

The phosphoinositide 3-kinase (PI3K) family of enzymes are involved in signal transduction that control various cellular events and play a pivotal role in the cardiovascular system (1). The PI3K family is organized into three classes, of which class 1 can be subdivided into class 1A and 1B (2, 3). PI3K α , β , and δ isoforms belong to class 1A and are primarily activated by receptor tyrosine kinases (1), whereas activation of PI3K γ belonging to class 1B is facilitated by heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) (1). PI3K α is ubiquitously distributed, whereas PI3K γ distribution is restricted to leukocytes and cardiovascular tissues (4). PI3K γ knockout (PI3K γ KO) mice show reduced immune response and enhanced cardiac contractility (5, 6). Both PI3K α and γ isoforms are found in the heart and play distinct roles in cardiac function. PI3K α promotes cell growth and physiological hypertrophy (7), whereas increased abundance of PI3K γ in

*To whom correspondence should be addressed. prasads2@ccf.org.

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pathological cardiac hypertrophy (8) promotes deleterious remodeling in response to cardiac stress through distinct kinase-dependent and kinase-independent functions leading to heart failure (9). In addition to playing a role in cardiac contractility (6), PI3K γ also inhibits β -adrenergic receptor (β AR) function (10, 11) and promotes endocytosis (12, 13) through its lipid or protein kinase activities (13, 14). Apart from these kinase-dependent functions, studies with knockout and knock-in mice have identified kinase-independent scaffolding functions for PI3K γ and β isoforms (15–17). However, the functional relevance of kinase independent scaffolding functions of PI3K γ is not well understood.

Signals from class 1A and B PI3Ks converge on phosphoinositide dependent kinase, which activates a downstream signaling pathway involving Akt and glycogen synthase kinase-3 (GSK-3) (1). GSK-3 is active in the dephosphorylated state, and its phosphorylation by activated Akt results in inhibition (18). The inhibitory phosphorylation on GSK-3 is released by dephosphorylation, which is primarily mediated by protein phosphatase 2A (PP2A) (19, 20). PP2A is the most abundant serine/threonine phosphatase, accounting for the majority of dephosphorylation in several different cell types (21). PP2A is a heterotrimeric enzyme, and its activity is regulated by phosphorylation and methylation (21). Reversible carboxy methylation of the PP2A catalytic subunit stimulates PP2A activity (22–26) and is catalyzed by a PP2A-specific leucine carboxyl methyltransferase (PPMT-1) (27, 28). Demethylation is mediated by the PP2A methylesterase (PME-1) (29).

We have shown that PI3K γ inhibits PP2A activity at the β AR complex, thereby blocking resensitization of the β AR. Because GSK-3 is dephosphorylated by PP2A (19, 20) and because PI3K γ inhibits PP2A activity (11), we tested whether PI3K γ directly inhibited GSK-3 downstream of the PI3K α –Akt–GSK-3 axis. PI3K γ can promote downstream signaling (30) including phosphorylation of GSK-3, but the underlying mechanisms are not well understood. Here, we showed that PI3K γ promoted phosphorylation of GSK-3 independently of Akt by inhibiting PP2A activity and thus reducing GSK-3 function downstream of the PI3K α –Akt pathway. We illustrated that PI3K γ suppressed PP2A activity by decreasing the methylation of the PP2A catalytic subunit through a kinase-independent mechanism. Furthermore, we also showed that the increased activity of GSK-3 in PI3K γ KO mice could potentially account for the reduced heart size observed with age, consistent with the antihypertrophic role of GSK-3, which, when activated, results in reduced heart size (31, 32).

RESULTS

PI3K γ independently promotes phosphorylation of GSK-3 downstream of the insulin-PI3K α –Akt axis

Because activation of the PI3K γ –Akt–GSK-3 signaling pathway occurs downstream of GPCRs, we used β ARs as a prototypical GPCR to test whether this signaling axis is preserved in vivo. PI3K γ KO mice and their wild-type littermate controls were administered a bolus of isoproterenol to acutely stimulate β ARs. Phosphorylation of Akt (as an indicator of activation) and phosphorylation of GSK-3 (as an indicator of inhibition) were assessed in cardiac lysates. Activation of Akt was significantly increased in wild-type mice after isoproterenol, a response that was attenuated in PI3K γ KO mice (Fig. 1A, upper and lower panels). Consistent with an absence of activation of Akt in PI3K γ KO mice, phosphorylation of cardiac GSK-3 α or β did not noticeably increase after isoproterenol treatment (Fig. 1B, upper and lower panels). Thus, the PI3K γ –Akt–GSK-3 signaling axis downstream of GPCRs is conserved in hearts as observed in previous studies (33, 34).

To determine the effect of PI3K γ on signaling downstream of PI3K α , we administered insulin to PI3K γ KO and wild-type mice to acutely stimulate signaling through PI3K α .

Activation of Akt was significantly increased in wild-type and PI3K γ KO mice after insulin administration (Fig. 1C, upper and lower panels), which in wild-type mice resulted in significantly increased phosphorylation of GSK-3 α and β (Fig. 1D, upper and lower panels). In contrast, PI3K γ KO mice showed marked reduction in phosphorylation of GSK-3 despite significant insulin-induced activation of Akt (Fig. 1D, left and right panels). The accelerated decrease in phosphorylation of GSK-3 in PI3K γ KO mice after insulin stimulation suggested that PI3K γ promotes GSK-3 phosphorylation independently of the PI3K α -Akt signaling axis. Consistent with this finding, steady-state GSK-3 phosphorylation was reduced in PI3K γ KO mice compared to their littermate controls. This loss of phosphorylation was specific to GSK-3 because phosphorylation of extracellular signal-regulated kinase 1 and 2 was not decreased in PI3K γ KO mice (fig. S1A).

Confocal microscopy of adult cardiac myocytes immunostained with anti-phospho-GSK-3 antibody showed that GSK-3 phosphorylation was lower in PI3K γ KO cardiomyocytes compared to wild-type controls (Fig. 1E). Together, these results establish that PI3K γ promotes phosphorylation of GSK-3 independently of the upstream PI3K α -Akt signaling axis. To investigate whether this PI3K γ pathway was specific to the heart, we assessed the phosphorylation status of GSK-3 in brain and spleen lysates from PI3K γ KO and wild-type mice. Steady-state phosphorylation of GSK-3 was lower in brain and spleen lysates from PI3K γ KO mice than in wild-type mice (fig. S1B), suggesting that PI3K γ mediated regulation of GSK-3 phosphorylation may not be restricted to the heart. Furthermore, these alterations are PI3K γ specific because no appreciable changes in PI3K α abundance were observed in PI3K γ KO or wild-type mice (fig. S1C).

PI3K promotes phosphorylation of GSK-3 by inhibiting PP2A activity

We have previously reported that PI3K γ inhibits PP2A activity (11), leading us to investigate whether PP2A activity was altered in PI3K γ KO mice. The activity of PP2A was significantly higher in the PP2Ac immunoprecipitates of cardiac lysates from PI3K γ KO mice than in those from wild-type mice (Fig. 2A). Furthermore, phosphatase activity associated with GSK-3 (both α and β) was higher in GSK-3 immunoprecipitates from PI3K γ KO mice than in those from wild-type mice (Fig. 2B), potentially providing an explanation for the reduced phosphorylation of GSK-3 in PI3K γ KO mice (Fig. 1). Immunoblotting of GSK-3 α and GSK-3 β immunoprecipitates from cardiac lysates indicated that the association of PP2A with GSK-3 α and β was increased in PI3K γ KO mice compared to wild-type mice (Fig. 2C), consistent with the increase in GSK-3-associated phosphatase activity (Fig. 2B). These results suggest that the absence of PI3K γ reduces phosphorylation of GSK-3 due to enhanced interaction of GSK-3 with PP2A, thereby manifesting in increased phosphatase activity.

PI3K γ inhibits PP2A activity by increasing methylation of PP2A

Our data indicated that the absence of PI3K γ led to increased GSK-3-associated PP2A activity, suggesting that PI3K γ inhibited the catalytic activity of PP2A. PP2A activity is inhibited through various mechanisms such as binding of PP2Ac by the endogenous peptide inhibitor I2-PP2A (35) and de-esterification of its carboxy-methylated terminal leucine residue Leu309 (29, 36). PP2A immunoprecipitates from cardiac lysates were immunoblotted for I2-PP2A, and in contrast to our study on the inhibition of PP2A by PI3K γ (11), PP2Ac interacted with I2-PP2A to a similar extent in both PI3K γ KO and wild-type mice (fig. S1D). Similarly, we did not observe a difference in the interaction of I2-PP2A with GSK-3 α or β (fig. S1E), suggesting that an alternative mechanism may be involved in regulating GSK-3-associated PP2A activity.

We next investigated whether PI3K γ regulated the methylation status of PP2Ac and thereby its activity. Methylation of PP2Ac was significantly higher in PI3K γ KO hearts than in wild-type mice (Fig. 2D, top and bottom panels), suggesting that PI3K γ may inhibit PP2A activity by decreasing its methylation. Immunoblotting of GSK-3 α and β immunoprecipitates indicated that the association of methylated PP2A with GSK-3 α and β was greater in PI3K γ KO hearts than in wild-type hearts (Fig. 2E, left and right panels), suggesting that methylation of PP2Ac may account for increased PP2A activity in PI3K γ KO mice.

PP2Ac is methylated by PPMT-1 and demethylated by PPME-1 (25, 26, 29). We immunoblotted GSK-3 α and β immunoprecipitates from cardiac lysates of PI3K γ KO and wild-type mice for PPMT-1 and PPME-1. We did not observe any differences in the interaction of PPMT-1 or PPME-1 with GSK-3 α and β (Fig. 2F, left and right panels). Because GSK-3-associated phosphatase activity was higher in PI3K γ KO mice, we assessed GSK-3 α - and GSK-3 β -associated methyltransferase activity. Methyltransferase activity assays using GSK-3 α and β immunoprecipitates and purified PP2Ac as substrate indicated that methyltransferase activity was higher in immunoprecipitates from PI3K γ KO mice than in those from wild-type mice (Fig. 2G) despite the absence of differences in the interaction between GSK-3 and PPMT-1 (Fig. 2F). Collectively, these data suggest that PI3K γ inhibits PP2A function by suppressing PPMT-1 activity, thereby inhibiting GSK-3-associated phosphatase activity. Furthermore, these data suggest that PI3K γ directly inhibits GSK-3 independently of Akt.

Knockdown of PPMT-1 rescues phosphorylation of GSK-3

To dissect the mechanism by which PI3K γ inhibits GSK-3, we assessed the phosphorylation status of Akt and GSK-3 in mouse embryonic fibroblasts (MEFs) from wild-type and PI3K γ KO mice stimulated with insulin. Although Akt was activated in both PI3K γ KO and wild-type MEFs (Fig. 3A), GSK-3 phosphorylation was reduced in PI3K γ KO MEFs (Fig. 3B), recapitulating our observation in hearts (Fig. 1). Because the PI3K α -Akt-GSK-3 signaling is present in MEFs from PI3K γ KO mice, we tested whether the PI3K γ -PP2A axis altered the phosphorylation state of GSK-3 independently of Akt activation. Okadaic acid treatment significantly increased basal and insulin-stimulated phosphorylation of GSK-3 (Fig. 3C), suggesting that the loss of GSK-3 phosphorylation in PI3K γ KO MEFs was mediated by PP2A. Similar to our observation in cardiac lysates (Fig. 2D), methylation of PP2Ac was significantly higher in PI3K γ KO MEFs compared to wild-type MEFs (Fig. 3D).

To demonstrate that PI3K γ inhibits PP2A through PPMT-1, we depleted PPMT-1 by small interfering RNA (siRNA) in PI3K γ KO MEFs. Phosphorylation of GSK-3 was increased in PI3K γ KO MEFs (Fig. 3E) after PPMT-1 knockdown and was accompanied by reduced PP2A methylation (Fig. 3E). Furthermore, the association of PP2A with GSK-3 was decreased after PPMT-1 knockdown (Fig. 3F). Together, these data suggest that PI3K γ alters the interaction of GSK-3 with PP2A by inhibiting PPMT-1, thereby decreasing PP2A methylation and increasing GSK-3 phosphorylation.

PI3K γ increases phosphorylation of GSK-3 through a kinase-independent manner

To elucidate mechanisms through which PI3K γ inhibits GSK-3 phosphorylation and associated phosphatase activity, we crossed transgenic mice with cardiac-specific overexpression of a catalytically inactive PI3K γ mutant (PI3K γ_{inact}) with PI3K γ KO mice to generate cardiac-specific overexpression of PI3K γ_{inact} in the PI3K γ KO background (PI3K γ_{inact} /PI3K γ KO). Similar to our previous results (Fig. 1), PI3K γ KO mice showed decreased phosphorylation of GSK-3 (Fig. 4A). Overexpression of PI3K γ_{inact} normalized the phosphorylation of GSK-3 in the hearts of PI3K γ KO mice (Fig. 4A) and in PI3K γ KO

MEFs (fig. S1F). Consistent with the increase in the phosphorylation of GSK-3, GSK-3 α - and GSK-3 β -associated phosphatase activity was reduced in PI3K γ_{inact} /PI3K γ KO mice compared to PI3K γ KO mice (Fig. 4B). GSK-3 α - and GSK-3 β -associated methyltransferase activity was reduced in PI3K γ_{inact} /PI3K γ KO mice to a similar extent to that in wild-type mice (Fig. 4C), showing that the presence of PI3K γ protein inhibited GSK-3-associated PPMT-1 activity. Furthermore, PI3K γ coimmunoprecipitated with GSK-3 α and β in cardiac lysates from PI3K γ_{inact} overexpressing hearts (fig. S1G), providing a potential explanation for the reduced methyltransferase activity in the GSK-3 complex. Together, these data suggest that a kinase-independent function of PI3K γ acts to inhibit GSK-3-associated PP2A by decreasing PPMT-1 activity.

PI3K γ inhibits the interaction between PPMT-1 and PP2Ac by sequestering PP2Ac

Because expression of PI3K γ_{inact} decreased PPMT-1 activity in the GSK-3 complex, we tested whether PI3K γ prevented binding of PPMT-1 to PP2A, thereby blocking methylation of PP2A. Immunoblotting of PP2Ac immunoprecipitates revealed that more PPMT-1 associated with PP2Ac in PI3K γ KO hearts than in wild-type hearts (Fig. 4D, left and right panels). These results suggest that in the absence of PI3K γ , more PPMT-1 binds to PP2Ac, leading to increased PP2Ac methylation, increased phosphatase activity (21, 28), and efficient dephosphorylation of GSK-3.

To directly explore whether the presence of PI3K γ inhibits the interaction between PP2Ac and PPMT-1, we generated human embryonic kidney (HEK) 293 cells stably expressing a short hairpin RNA (shRNA) that depleted PI3K γ without affecting the abundance of PI3K α (Fig. 4E). Immunoblotting of PP2Ac immunoprecipitates revealed that the association of PPMT-1 was increased in stable PI3K γ knockdown cells compared to control cells (Fig. 4F). Conversely, cardiac-specific overexpression of PI3K γ_{inact} in PI3K γ KO mice significantly reduced the PP2A–PPMT-1 interaction and PP2Ac methylation (Fig. 4G). These data suggest that PI3K γ inhibits binding of PPMT-1 to PP2Ac, which attenuates methylation and activity of PP2Ac in a manner dependent on the presence of PI3K γ protein, indicating that PI3K γ may shield PP2A from PPMT-1. Thus, the absence of PI3K γ (in vivo or in vitro) consistently increased the PP2A–PPMT-1 interaction, manifesting in reduced GSK-3 phosphorylation. In addition, overexpression of PI3K γ_{inact} disrupted this interaction, suggesting that kinase-independent function of PI3K γ could have an important role in cardiac hypertrophic response by inhibiting GSK-3 function.

To examine whether PI3K γ sequesters PP2Ac and shields it from PPMT-1 activity, we performed in vitro methyltransferase assays with purified PI3K γ (in conditions not conducive for PI3K γ activity due to the absence of adenosine 5'-triphosphate and lipids), PP2Ac, and PPMT-1 and found that PP2Ac methylation was significantly reduced in the presence of PI3K γ (Fig. 5A), suggesting that the presence of PI3K γ shields PP2Ac from PPMT-1 and that PI3K γ has a kinase-independent function in inhibiting PP2Ac activity. PPMT-1 activity was decreased in a concentration dependent manner, with increasing concentrations of PI3K γ in the reaction (Fig. 5A). Because our in vitro study showed that binding of PP2Ac by PI3K γ reduced its availability to PPMT-1, we tested whether PP2Ac directly interacted with PI3K γ . Surface plasmon resonance (SPR) assays (37) indicated that PI3K γ bound to PP2Ac with rapid kinetics in a concentration dependent manner with a dissociation constant (K_d) of 0.91 μ M (Fig. 5B). Immunoprecipitation studies using HEK293 cells confirmed the interaction between endogenous PI3K γ and PP2Ac (fig. S2A). Similarly, PI3K γ immunoprecipitated with PP2Ac from PI3K γ KO hearts overexpressing inactive PI3K γ (fig. S2B). Furthermore, purified PI3K γ bound to immobilized glutathione S-transferase (GST)–PP2Ac (Fig. 5C, left panel) and addition of purified PI3K γ to the PP2A–PPMT-1 complex displaced PPMT-1 from PP2Ac (Fig. 5C, right panel). Analysis of PP2Ac immunoprecipitates from PI3K γ KO MEFs expressing deletion mutants of PI3K γ_{inact} (Fig.

5D) indicated that the PI3K γ - Δ 144–1102 deletion mutant, but not the PI3K γ - Δ 1–143 deletion mutant, interacted with PP2Ac [Fig. 5E; IB: PI3K γ and hemagglutinin (HA)]. Consistent with these observations, the interaction of PPMT-1 with PP2Ac was increased with expression of PI3K γ - Δ 1–143 and expression of PI3K γ - Δ 144–1102 resulted in reduced interaction of PPMT-1 with PP2Ac (Fig. 5E). These results were confirmed by reciprocal immunoprecipitations of HA–PI3K γ - Δ 144–1100 and PI3K γ (fig. S2C).

Inhibition of GSK-3 by PI3K γ decreases nuclear export of NFAT

GSK-3 inhibits the cardiac hypertrophic response because it antagonizes the calcineurin-mediated nuclear localization of the transcription factor NFATc3 (31, 32). Whereas dephosphorylation of NFATc by calcineurin causes its nuclear import, phosphorylation by GSK-3 in the nucleus results in NFATc export. Immunoblotting of phosphorylated NFATc3 immunoprecipitates from nuclear and cytoplasmic fractions of cardiac lysates indicated that the amount of phosphorylated NFATc3 in the nuclear fraction and its association with GSK-3 α were increased in PI3K γ KO mice (Fig. 6A). In contrast, the cytosolic abundance of phosphorylated NFATc3 did not appreciably differ between PI3K γ KO and wild-type mice, although the association of GSK-3 α with phosphorylated NFATc3 was increased in PI3K γ KO mice (Fig. 6B). In addition, the nuclear localization of NFATc3 did not differ between PI3K γ KO and wild-type mice, although the association of GSK-3 α and β with NFATc3 was increased in PI3K γ KO mice (Fig. 6C). In contrast, NFATc3 abundance was increased in the cytosolic fraction of PI3K γ KO mice compared to wild-type mice, an effect that was associated with a concomitant increase in GSK-3 α interaction (Fig. 6D). The quality of cytosolic and nuclear fractions was assessed by using I κ B- β (inhibitor of nuclear factor κ B) as cytoplasmic marker and histone H1b as nuclear marker (fig. S2D). These data establish that the increased activity of GSK-3 in PI3K γ KO mice due to increased phosphatase activity results in phosphorylation and nuclear export of NFATc3, which may affect transcriptional regulation.

To inspect further whether differential distribution of NFATc affects transcriptional activity, we measured the expression of a set of NFATc controlled genes [interleukin 4 (IL4), IL2, myogenic differentiation 1 (MYOD1), forkhead box P3 (FOXP3), and interleukin receptor 2A (ILR2A)] by real-time reverse transcription polymerase chain reaction (RT-PCR). The abundance of ILR2A (Fig. 6E), but not that of other NFATc target genes (fig. S2E), was significantly decreased in PI3K γ KO hearts compared to wild-type hearts. The absence of altered abundance of other gene transcripts in our study suggests that transcriptional regulation of these genes by NFATc3 may require coordination with other transcriptional factors, which has been previously demonstrated (38, 39). To further test whether the kinase-independent inhibition of GSK-3 by PI3K γ affected cardiac hypertrophy, we analyzed the abundance of the cardiac hypertrophic marker atrial natriuretic peptide (ANP) in wild-type and PI3K γ KO cardiac lysates. ANP amounts were significantly lower in 6-month-old PI3K γ KO mice compared to wild-type mice (Fig. 6F). The abundance of PI3K γ increases with cardiac stress (8), and these results suggest that the kinase-independent function of PI3K γ that inhibits phosphorylation of GSK-3 may play a role in cardiac hypertrophy and failure.

PI3K γ promotes cardiac hypertrophy in a kinase-independent manner

To test whether PI3K γ promotes cardiac hypertrophy in a kinase independent manner, we compared the morphology of hearts from wild-type, PI3K γ KO, and PI3K γ_{inact} /PI3K γ KO mice at 24 weeks of age. Although the hearts of PI3K γ KO mice had increased wall thickness (Table 1), they were smaller than the hearts of wild-type and PI3K γ_{inact} /PI3K γ KO mice (Fig. 7A). Cardiac overexpression of PI3K γ_{inact} in the PI3K γ KO background further accentuated the size of hearts (Fig. 7A). Furthermore, the heart weight/body weight ratio of

PI3K γ_{inact} /PI3K γ KO mice was increased compared to wild-type or PI3K γ KO mice, whereas PI3K γ KO mice had a reduced heart weight/body weight ratio compared to wild-type or PI3K γ_{inact} /PI3K γ KO mice (Table 2), which was confirmed by whole heart section staining (Fig. 7B). To comprehensively assess the cardiac function, we performed M-mode echocardiography on 24-week-old mice. Echocardiographic and morphometric analysis showed that PI3K γ_{inact} /PI3K γ KO mice had significant cardiac dysfunction as measured by percentage of fractional shortening, which could be due to increased cardiac wall thickness (Fig. 7C and Table 1). Together, these *in vivo* studies show that a kinase-independent function of PI3K γ promotes cardiac growth potentially by inhibiting GSK-3.

DISCUSSION

Here, we show a mechanism that inhibits the activity of GSK-3 α and β in which PI3K γ promotes the phosphorylation of GSK-3 independently of Akt activation by inhibiting PP2A. Our study shows that the absence of PI3K γ protein increased PP2A activity, thereby resulting in increased dephosphorylation of GSK-3 in PI3K γ KO mice. We show that PI3K γ interacted with PP2A and shielded PP2A from methylation by PPMT-1, thereby resulting in inhibition of PP2A (27). Active GSK-3 in PI3K γ KO mice triggered nuclear export of the GSK-3 substrate NFATc3, leading to reduced transcription of the NFATc3 target gene ILR2A. Consistent with the role of GSK-3 in cardiac hypertrophy (31, 32), the increased GSK-3 activity in PI3K γ KO mice correlated with decreased heart size. Thus, PI3K γ suppresses GSK-3 activity by inhibiting its dephosphorylation by PP2A (Fig. 7E).

Our studies suggest the presence of cross-talk between PI3K α and PI3K γ signaling pathways in which PI3K γ inhibits GSK-3 activation downstream of the insulin-PI3K α -Akt pathway, thereby promoting PI3K α signaling. Increased dephosphorylation of GSK-3 downstream of insulin-PI3K α -Akt pathway in PI3K γ KO mice indicates a critical role for PI3K γ in inhibiting GSK-3 function independently of Akt. Reduced steady-state phosphorylation of GSK-3 (α and β) in the heart, brain, and spleen of PI3K γ KO mice suggests that this PI3K γ -mediated GSK-3 inhibition is not limited to a single tissue and could synergize with the insulin-PI3K α -Akt signaling axis to GSK-3.

The decreased steady-state phosphorylation of GSK-3 in PI3K γ KO mice, which has been observed by others (30), was associated with increased PP2A activity. Furthermore, inhibition of PP2A reverses phosphorylation of GSK-3 in PI3K γ MEFs (Fig. 3C). Although GSK-3 is phosphorylated by several kinases (40–45), the reduced phosphorylation of GSK-3 in PI3K γ KO mice suggests that increased PP2A activity may override kinase-dependent mechanisms. Because overexpression of PI3K γ_{inact} in PI3K γ KO mice rescued the effects of the absence of PI3K γ , our studies have unraveled a kinase-independent function of PI3K γ that results in inhibition of PP2A activity (Fig. 4B). Inhibition of PP2A activity by PI3K γ is due to loss in methylation of PP2A because GSK-3–associated methyltransferase activity and PP2A methylation were increased in PI3K γ KO mice. The increased methylation of PP2A in PI3K γ KO mice, which would be expected to increase its activity (21, 28), could account for increased dephosphorylation of GSK-3. Consistent with this kinase-independent function, expression of PI3K γ_{inact} in PI3K γ KO mice resets phosphatase and methyltransferase activity, thereby normalizing phosphorylation of GSK-3.

Although the molecular details of PP2A methylation by PPMT-1 are well understood (23), the mechanisms that control the activity of PPMT-1 are not. Loss of PI3K γ *in vivo* (in PI3K γ KO mice) or *in vitro* (by shRNA depletion) led to increased interaction of PPMT-1 with PP2A, resulting in higher PP2A activity. Conversely, overexpression of PI3K γ_{inact} in PI3K γ_{inact} /PI3K γ KO mice normalized GSK-3 phosphorylation and PP2A activity and

lowered PP2A–PPMT-1 interaction. These data support the idea that PI3K γ inhibits PP2A methylation by shielding PP2A from PPMT-1–mediated methylation.

Inhibition of GSK-3 by a kinase-independent function of PI3K γ has implications in cardiac hypertrophy because PI3K γ participates in deleterious remodeling in a kinase-independent manner (9, 17, 46, 47). Our data show that PI3K γ KO mice have reduced cardiac mass and increased GSK-3 activity. Normalizing GSK-3 phosphorylation by cardiac expression of PI3K γ_{inact} in PI3K γ KO mice was associated with increased cardiac mass and dysfunction by 6 months. The reciprocal correlative relationship between GSK-3 activity and cardiac hypertrophy suggests that GSK-3 may be one of the pathways through which the kinase-independent function of PI3K γ may mediate cardiac hypertrophic responses. In this context, GSK-3 antagonizes calcineurin-mediated nuclear export of the prohypertrophic transcription factor NFATc3 (31, 32), which contributes to cardiac remodeling (31, 32, 48, 49). Hearts of PI3K γ KO mice show substantial cytosolic localization of NFATc3 due to increased GSK-3 activity, decreased expression of the NFATc3-regulated gene ILR2A, and reduced abundance of the hypertrophic marker ANP. Therefore, age related or stress-induced changes in the abundance of PI3K γ may play a role in cardiac remodeling by affecting the activity of GSK-3.

In summary, our results establish that PI3K γ increases phosphorylation of GSK-3 by preventing the methylation of PP2A and decreasing its activity, thereby providing an explanation for enhanced GSK-3 phosphorylation in the presence of PI3K γ (1, 30). These findings may have implications in cardiac pathology because the abundance of PI3K γ is increased with cardiac stress (8). Therefore, increased abundance of PI3K γ may disrupt the PP2A–PPMT-1 interaction in a kinase independent manner, decreasing PP2A activity and resulting in the inhibition of GSK-3 function, which may be a mechanism through which PI3K γ mediates cardiac hypertrophic responses.

MATERIALS AND METHODS

Animals

C57/BL6 mice lacking PI3K γ have been described previously (10). Experiments involving live animals were performed in accordance with institutional and national guidelines and regulations, as approved by Cleveland Clinic Institutional Animal Care and Use Committee. PI3K γ KO and PI3K γ_{inact} (10) mice were bred to generate transgenic PI3K γ KO mice expressing PI3K γ_{inact} in the heart.

Antibodies, agonists, and inhibitors

Phospho-Akt (S473) (1:1000), Akt (1:1000), phospho-GSK-3 α (S21)/ β (S9) (1:1000), GSK-3 α (1:1000), (1:1000), and GSK-3 β (1:1000) antibodies were obtained from Cell Signaling Technology. Histone H1b antibody was from Millipore. GSK α/β (1:2000) antibody was also obtained from Assay Designs. Antibodies against I κ B- β (1:1000), PPME-1 (1:1000), PI3K α (1:500), PI3K γ (1:200), phospho-NFATc3 (1:1000), NFATc3 (1:1000), and I2-PP2A (1:5000) were from Santa Cruz Biotechnology. PP2Ac (1:2000), methyl-PP2Ac (1:500), and PPMT-1 (1:500) antibodies were from Upstate Biotechnology (Millipore). HA (1:1000) antibody was obtained from Roche Diagnostics GmbH. Anti-ANP (1:1000) antibody was from Abcam. Anti- β -actin (1:5000) antibody was from Sigma. Isoproterenol, insulin, and okadaic acid (phosphatase inhibitor) were obtained from Sigma. The mice were administered isoproterenol (1.5 mg/kg) or insulin (0.05 U), and hearts were excised after 0, 5, 10, and 20 min and snap-frozen for biochemical experiments.

Confocal microscopy

Adult cardiomyocytes from wild-type and PI3K γ KO mouse hearts were isolated as described previously (11) and plated onto coverslips treated with laminin (Sigma). The cells were fixed (4% paraformaldehyde), permeabilized (0.1% Triton X-100), and incubated with 10% horse serum. Anti-phospho-GSK-3 α/β (1:1000) and anti- α -actinin (1:1000) diluted in 5% horse serum were used as primary antibodies, and anti-rabbit Alexa Flour 488 (1:500) and anti-mouse Alexa Flour (1:500) were used as secondary antibodies (Molecular Probes). Samples were visualized using sequential line excitation at 488 and 568 nm for green and red, respectively.

Cell culture, immunoprecipitation, Western blotting, and DNA constructs

Standard procedures for cell culture, Western immunoblotting, and immunoprecipitations were followed. HEK293 cells and MEFs from wild-type (gift from R. Lefkowitz, Duke University, Durham, NC) and PI3K γ KO (10) were used. Human PP2Ac in pcDNA3.1 was a gift from D. Pallas (Emory University, Atlanta, GA), and human PPMT-1 complementary DNA (cDNA) was obtained from OriGene Technologies. PP2Ac and PPMT-1 were subcloned into pGEX4T1 to express GST-PP2Ac and GST-PPMT-1 fusion proteins in BL21 cells induced with 0.1 mM isopropyl- β -D-thiogalactopyranoside. The fusion proteins were purified using GST pull-down and the thrombin cleavage method. The PI3K γ - Δ 1-143 deletion mutant was generated from full-length PI3K γ_{inact} using QuikChange Lightning Site-Directed Mutagenesis Kit from Agilent Technologies (sense primer, CATACGATGTTCCAGATTACGCTTCCGAGGAGTCC; antisense primer, GGACTCCTCGGAAGCGTAATCTGGAACATCGTATG). PI3K γ - Δ 144-1102 deletion mutant was generated by PCR amplification using PI3K γ_{inact} as template (forward primer, GGATCCGCCACCATGTACCCATACGATGTTCCA; reverse primer, AAGCTTCTCGAGTTAGGGCGGGTGCCGCTGCACCAGGTGGAT). The PCR product was cloned into pcDNA3.1 vector. The recombinant deletion constructs were HA-tagged to determine expression. Hearts, MEFs, and HEK293 cells were homogenized in NP-40 lysis buffer for immunoblotting and 1% Triton X-100 buffer for immunoprecipitation. Both the buffers contained protease and phosphatase inhibitor cocktails (Sigma). Lysates were cleared by centrifugation at 13,000 rpm for 15 min at 4°C. Supernatants were used for immunoblotting analysis or for immunoprecipitation with the indicated antibodies. Nuclear and cytoplasmic fractions of mice hearts were prepared using NE-PER Nuclear and Cytoplasmic Extraction reagents from Pierce (Thermo Scientific).

Phosphatase assay

Phosphatase activity was measured using phosphatase assay kit (Upstate Biotechnology, Millipore). Immunoprecipitated samples were resuspended in phosphate-free assay buffer, and the assay was done according to the manufacturer's protocol (11).

Methyltransferase assay

GSK-3 α and β were immunoprecipitated from cardiac lysates. Immunoprecipitates were washed three times with 1x phosphate-buffered saline (PBS) and once with assay buffer containing 50 mM Mops (pH 7.2), 20 mM dithiothreitol, and 50 mM MnCl₂. The methyltransferase assay was carried out by adding purified PP2Ac (16 μ M) and [³H]-adenosylmethionine (SAM) (0.55 μ Ci) to the immunoprecipitates in assay buffer. The reaction was carried out for 30 min at 37°C. After spinning down the beads, 20- μ l aliquot of only supernatant (containing only PP2Ac) was transferred onto P81 phosphocellulose paper squares (Upstate, Millipore), and the squares were washed three times with 10% trichloroacetic acid for 15 min each and once with 95% ethanol for 5 min. The squares were allowed to dry, and the radioactivity was measured by liquid scintillation. In vitro

methylation assays were performed by mixing of purified PPMT-1 (1.6 μ M) and purified PP2Ac (16 μ M), and [3 H]SAM (0.55 μ Ci) either in the presence or absence of Sf9-purified PI3K γ (0.5, 1, and 2 μ M). The reaction mixture was transferred onto P81 phosphocellulose paper squares, and above-described procedure was followed.

siRNA knockdown of PPMT-1

Knockdown of endogenous PPMT-1 was mediated by transfection of a previously reported siRNA (GAAGGAGAUAAACCUAUUGA) that targets mouse PPMT-1 (23). All-Stars negative control siRNA and PPMT-1 siRNAs were made by Qiagen. The siRNA duplexes were resuspended and transfected using HiPerFect (Qiagen) into PI3K γ KO MEFs at 100 nM concentration. All assays were performed 48 hours after transfection.

siRNA knockdown and stable shRNA knockdown of PI3K γ

siRNA duplexes were generated from human PI3K γ cDNA sequence using Qiagen or Ambion siRNA design programs. The target 21-mer sequences for siRNAs were siRNA-1 [regions 2404 to 2424 base pairs (bp)] AAAGTAATGGCCTCCAAGAAA; siRNA-2 (regions 3287 to 3307 bp) AAGGAGAGAAACATTCAGCCT; and siRNA-3 (regions 777 to 795 bp) AAGGATATTCCCGAAAGCCAA, respectively. All-Stars negative control siRNA and siRNA (1, 2, and 3) were made by Qiagen. The siRNA duplexes were resuspended and transfected using HiPerFect (Qiagen) into HEK293 cells at 100 nM concentration. All assays were performed 48 hours after transfection. The shRNA sequence corresponding to siRNA-3 (GGATCCCGGATATTCCCGAAAGCCAATTGATATCCGTTGGCTTTTCGGAATA TCCTTTTTTCCAAAAGCTT) was cloned into pRNATU6.1/Hygro vector (GenScript).

SPR assay

SPR was performed with a Biacore 3000 (GE Healthcare Life Sciences). Response units (RU), a measure of binding, were monitored as a function of time. Purified anti-GST monoclonal antibodies (clone P1A12; BioLegend Inc.) were immobilized on Sensor Chip CM5 (GE Healthcare Life Sciences) to achieve a baseline gain of <1000 RU by amine group coupling as indicated by the manufacturer's instructions (Biacore Amine Coupling Kit, GE Healthcare Life Sciences). The purified GST-tagged PP2Ac (200 ng/ml) was captured on antibodies immobilized on a sensor chip at a flow rate of 10 μ l/min for 3 min at 25°C to achieve a response of 450 to 500 RU. An additional wash for 5 min at a flow rate of 20 μ l/min was performed with buffer alone. (His) $_6$ -PI3K γ was used as soluble analyte and injected at different concentrations over GST-PP2Ac. All analytes were in SPR buffer (HEPES-buffered saline with surfactant P-20, GE Healthcare Life Sciences, Biacore Inc.), and binding experiments were performed at 25°C in SPR buffer with a flow rate of 5 μ l/min. Data normalization was done against a reference channel immobilized with GST alone. Analysis and fitting were performed with BIAevaluation software, version 4.0.1 (Biacore Inc.), with the option for simultaneous association constant (K_a)/ K_d calculation. Fitting of sensogram data was carried out according to global fitting, and the K_a and K_d values were calculated with a 1:1 Langmuir model. The quality of the fit was determined by χ^2 values, as well as from the magnitude and distribution of the residuals (37).

In vitro interaction assay

Binding assays of immobilized 16 μ M GST-PP2Ac with 0.5 μ M free PI3K γ were performed in binding buffer [50 mM Tris-HCl (pH 7.4), 20 mM NaCl, 0.05% Triton X-100]. The reactions were incubated for 30 min at room temperature with shaking, and the beads were washed four times with binding buffer. Beads were heated at 95°C for 5 min in 2x Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. Competitive

binding assays of immobilized 16 μ M GST-PP2Ac with 1.6 μ M free PPMT-1 and 0.5, 1, and 2 μ M free PI3K γ were performed in binding buffer as described above.

Real-time RT-PCR

Two-step quantitative RT-PCR (qRT-PCR) was performed with standard procedures. Briefly, total RNA was extracted from cardiac samples using TRIzol Reagent (Invitrogen). First-strand cDNA was synthesized using 1 μ g of total RNA per sample with iScript cDNA synthesis kit (Bio-Rad). Subsequently, qRT-PCR was performed with iQ SYBR Green (Bio-Rad) on Bio-Rad iCycler. Primers and probes were obtained from Integrated DNA Technologies. Gene expression was analyzed with $2^{-\Delta\Delta C_t}$ relative quantification method, using glyceraldehyde-3-phosphate dehydrogenase as the endogenous control.

Immunohistochemistry

Freshly harvested cardiac samples were placed in sucrose/PBS solution at 4°C for 2 to 4 hours, placed in cross-section in OCT (Miles Pharmaceuticals), and snap-frozen in liquid nitrogen. Slides from frozen sections were subsequently fixed in 4% paraformaldehyde and stained with H&E. Photographs were taken using an Optometrics analog camera and Adobe Premier version 5.1, and were analyzed using a National Institutes of Health Image analysis system.

Echocardiography

Echocardiography was performed on lightly sedated 6-month-old mice using a Vevo 770 (Visualsonics) as previously described (50). The probe was gently lowered onto the mouse laid in a supine position, and M-mode views were recorded including left ventricular systolic and diastolic dimensions, septum, and posterior wall.

Statistical analysis

Results are expressed as means $\pm$ SE. Data were analyzed by unpaired t test for two-group comparison (for example, Akt or GSK-3 densitometric analysis). For comparison of more than two groups, we used one-way analysis of variance (ANOVA) if there was one independent variable (for example, the in vitro methyltransferase assay in Fig. 5A) and two-way ANOVA if there were two independent variables (for example, echocardiographic and morphometric analyses). To adjust for multiple comparisons, we performed a Newman-Keuls post hoc test. A probability value of <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Absence of PI3K γ leads to decreased phosphorylation of GSK-3. (A) Cardiac lysates from wild-type (WT) and PI3K γ KO mice were immunoblotted with anti-phospho-Akt (Ser473) antibody (p-Akt) after an isoproterenol bolus. The blots were stripped and reprobed for total Akt (lower panel). Amalgamated densitometry is shown in the bar graph. $n = 5$ to 6 mice per time point of treatment, and each lane represents one mice. $^*P < 0.001$ compared to the PI3K γ KO time points. (B) Cardiac lysates from WT and PI3K γ KO mice were immunoblotted using anti-phospho-GSK-3 (GSK-3 α and β) antibody after administration of an isoproterenol bolus. The blots were stripped and reprobed for total GSK-3 α or β (lower panel). Amalgamated densitometry is shown in the bar graphs. $n = 5$ to 6 mice per time point. $^*P < 0.0001$ compared to the PI3K γ KO time points. (C) Cardiac lysates from insulin-treated WT and PI3K γ KO mice were probed with anti-phospho-Akt antibody. The blots were stripped and reprobed for total Akt (lower panel). Bar graphs represent amalgamated densitometry (lower panel). $n = 5$ to 6 mice per time point. $^*P < 0.0001$ compared to the unstimulated time point (0 min) for WT and PI3K γ KO. (D) Cardiac lysates from WT and PI3K γ KO mice were immunoblotted with anti-phospho-GSK-3 (GSK-3 α and β) antibody after an insulin bolus. The blots were stripped and reprobed for total GSK-3 α or β (lower panel), and amalgamated densitometry is shown in the bar graphs. $n = 5$ to 6 mice per time point. $^*P < 0.0001$ compared to the PI3K γ KO time points. (E) Confocal microscopy of adult cardiac myocytes from WT or PI3K γ KO mice stained with anti-phospho-GSK-3 antibody (green), α -actinin (red; for myocyte staining), and 4',6-diamidino-2-phenylindole (blue; to stain nuclei). $n = 4$ mice per experiment and 20 to 30 cells per mice. Scale bar, 10 μ m.

Fig. 2.

GSK-3 is dephosphorylated in PI3K γ KO hearts due to increased PP2A activity. (A) In vitro phosphatase assays were carried out on PP2Ac immunoprecipitates from cardiac lysates of WT and PI3K γ KO mice. Data presented as fold change in PP2A activity in PI3K γ KO over WT. $n = 5$ to 6 mice per genotype. * $P < 0.0001$ compared to WT. (B) Phosphatase activity in GSK-3 α or β immunoprecipitates from cardiac lysates of PI3K γ KO or WT mice was measured and presented as fold change in GSK-3 α - or GSK-3 β -associated phosphatase activity in PI3K γ KO over WT. $n = 5$ to 6 mice per genotype. * $P < 0.005$ compared to WT. (C) GSK-3 α (upper panel) and β (lower panel) immunoprecipitates from cardiac lysates of PI3K γ KO or WT mice were immunoblotted for PP2Ac. Coimmunoprecipitating PP2Ac with GSK-3 α (upper panel) or β (lower panel) was normalized to total PP2Ac in the lysates. Amalgamated densitometry is presented in the bar graphs. $n = 5$ to 6 mice per genotype. * $P < 0.003$ compared to WT. (D) Cardiac lysates from PI3K γ KO or WT mice were immunoblotted with anti-methyl-PP2Ac. Amalgamated densitometry is shown in the bar graph. $n = 5$ to 6 mice per genotype. * $P < 0.0001$ compared to WT. (E) GSK-3 α and β immunoprecipitates from cardiac lysates of PI3K γ KO or WT mice were immunoblotted for coimmunoprecipitating methylated PP2Ac and total PP2Ac. Amalgamated densitometry is shown in the bar graphs. $n = 5$ to 6 mice per genotype. * $P < 0.005$ compared to WT normalized to total PP2Ac in the lysates. (F) GSK-3 α and β immunoprecipitates from cardiac lysates of PI3K γ KO or WT mice were immunoblotted with anti-PPMT-1 and anti-PPME-1 antibody, respectively. Amalgamated densitometry is shown in the bar graphs. $n = 5$ to 6 mice per genotype. (G) Methyltransferase activity in GSK-3 α and β immunoprecipitates from cardiac lysates of PI3K γ KO or WT mice was measured by in vitro methylation of purified PP2Ac substrate. Activity is represented as radioactive methyl groups (cpm) transferred to PP2Ac. $n = 5$ to 6 mice per genotype, and assay was carried out in duplicate for each sample. * $P < 0.002$ compared to WT.

Fig. 3.

Knockdown of PPMT-1 rescues phosphorylation of GSK-3. (A) Cell lysates from insulin-pretreated WT and PI3Kγ KO MEFs were immunoblotted with anti-phospho-Akt antibody. Amalgamated densitometry is shown as the bar graph. $n = 5$ independent experiments performed in duplicate. $*P < 0.001$ compared to the unstimulated time point (0 min). (B) Cell lysates from WT and PI3Kγ KO MEFs were immunoblotted with anti-phospho-GSK-3α or anti-phospho-GSK-3β antibody after insulin pretreatment. The blots were stripped and reprobed with anti-GSK-3α or anti-GSK-3β antibody. Amalgamated densitometry is shown in the bar graphs. $n = 5$ to 6 independent experiments performed in duplicate. $*P < 0.001$ compared to PI3Kγ KO. (C) Cell lysates from WT and PI3Kγ KO MEFs were immunoblotted with anti-phospho-GSK-3α or anti-phospho-GSK-3β antibody after okadaic acid pretreatment and insulin stimulation. Amalgamated densitometry is shown in the bar graphs. $n = 5$ to 6 independent experiments performed in duplicate. $*P < 0.01$ compared to vehicle (Veh). (D) Cell lysates from WT and PI3Kγ KO MEFs were immunoblotted with anti-methyl-PP2Ac. Amalgamated densitometry is shown in the bar graph. $n = 5$ to 6 independent experiments. $*P < 0.001$ compared to WT. (E) Cell lysates from PI3Kγ KO MEFs transfected with control and PPMT-1 siRNA were immunoblotted for anti-phospho-GSK-3α or β, anti-GSK-3α or β, anti-methyl-PP2Ac, or anti-PPMT-1

antibodies. Amalgamated densitometry is shown in the bar graphs. $n = 5$ to 6 independent experiments performed in triplicate. $*P < 0.001$ compared to Ctrl siRNA for pGSK-3 and $*P < 0.01$ compared to Ctrl siRNA for methyl-PP2Ac. (F) GSK-3 α and β immunoprecipitates from PI3K γ KO MEFs transfected with control (Ctrl) or PPMT-1 siRNA were immunoblotted for coimmunoprecipitating PP2Ac. Amalgamated densitometry for coimmunoprecipitating PP2Ac normalized to total PP2Ac in the lysates is shown in the bar graph. $n = 5$ to 6 independent experiments in triplicates. $*P < 0.05$ compared to Ctrl.

Fig. 4. PI3K γ inhibits PP2A by kinase independent mechanism. (A) Cardiac lysates from WT, PI3K γ KO, or PI3K γ inact/PI3K γ KO mice were immunoblotted with anti-phospho-GSK-3 α or β antibodies. The blots were stripped and reprobed for total GSK-3 α or β as loading control. Amalgamated densitometry is presented in the bar graphs. $n = 6$ mice per genotype. $*P < 0.0001$ compared to WT and PI3K γ inact/PI3K γ KO. (B) Phosphatase activity in GSK-3 α or β immunoprecipitates from cardiac lysates of WT, PI3K γ KO, or PI3K γ inact/PI3K γ KO mice was measured and presented as fold change over WT. $n = 6$ mice per genotype. $*P < 0.001$ compared to WT and PI3K γ inact/PI3K γ KO. $\#P < 0.01$ compared to PI3K γ KO. (C) Methyltransferase activity in GSK-3 α and β immunoprecipitates from cardiac lysates of WT, PI3K γ KO, or PI3K γ inact/PI3K γ KO mice was measured. $n = 6$ mice per genotype. $*P < 0.0001$ compared to WT and PI3K γ inact/PI3K γ KO. $\#P < 0.001$ compared to PI3K γ KO. (D) PP2Ac immunoprecipitates from cardiac lysates of WT and PI3K γ KO mice were immunoblotted with anti-PPMT-1 antibody. Cardiac lysates were immunoblotted with anti-PPMT-1 antibody. Amalgamated densitometry is shown in the bar graph. $n = 6$ mice per genotype. $*P < 0.001$ compared to WT. (E) Lysates of HEK293 cells transfected with three different siRNAs (1, 2, and 3) targeting PI3K γ were immunoblotted with anti-PI3K α antibody and anti-PI3K γ antibody. $n = 3$ independent experiments. (F) PP2Ac was immunoprecipitated from lysates of cells stably expressing either vector or shPI3K γ (which expressed siRNA-3) and stimulated with isoproterenol. Immunoprecipitates were

immunoblotted for co- immunoprecipitating PPMT-1, which was normalized to total PPMT-1 in the lysates. Amalgamated densitometry is shown in the bar graph. n = 5 to 6 experiments performed in triplicates. *P < 0.05 compared to Vector (Vec). (G) PP2Ac immunoprecipitates from cardiac lysates of WT, PI3K γ KO, and PI3K γ_{inact} /PI3K γ KO mice were immunoblotted for coimmunoprecipitating PPMT-1 and methyl-PP2Ac. Amalgamated densitometry is presented in the bar graph. n = 6 mice per genotype. *P < 0.001 compared to WT and PI3K γ_{inact} /PI3K γ KO. #P < 0.01 compared to PI3K γ KO and WT.

Fig. 5. PI3K γ inhibits methylation of PP2A by disrupting the PP2A–PPMT-1 interaction. (A) Purified PI3K γ was added to an in vitro PPMT-1 methylation reaction with PP2Ac as substrate, and loss in PPMT-1 activity was measured. Data are presented as the measurement of labeled PP2Ac (cpm). *P < 0.001 compared to 0 μ M PI3K γ . #P < 0.001 compared to 0.5 μ M PI3K γ . n = 4 independent experiments done in duplicate. (B) GST-PP2Ac was immobilized on the CM5 Biacore chip, and increasing concentrations of Sf9-purified PI3K γ were injected. The direct interaction was measured by SPR (K_d = 0.91 μ M). n = 3 independent experiments. (C) The binding of purified PI3K γ to GST-PP2Ac was assessed by immunoblotting with PI3K γ antibody. The blots were stripped and reprobed with GST antibody (left panel). The binding of PPMT-1 to GST-PP2Ac in the presence of purified PI3K γ was assessed by immunoblotting with anti-PPMT-1 antibody. The blots were stripped and reprobed with GST antibody (right panel). n = 4 independent experiments. (D) Cell lysates of PI3K γ KO MEFs transfected with deletion constructs of PI3K γ inact were immunoblotted for PI3K γ . The blots were stripped and reprobed with HA antibody. n = 3 independent experiments. (E) PP2Ac immunoprecipitates from cell lysates of PI3K γ KO MEFs transfected with deletion constructs of PI3K γ inact were immunoblotted for coimmunoprecipitating PI3K γ . The blots were stripped and reprobed for HA. n = 4 independent experiments.

Fig. 6.

Inhibition of PP2A-mediated dephosphorylation of GSK-3 by PI3K γ increases GSK-3-regulated transcription factor and target gene expression. (A and B) Phospho-NFATc3 (p-NFATc3) immunoprecipitates from cardiac nuclear (A) and cytosolic (B) fractions using anti-phospho-NFATc3 antibody were immunoblotted for phospho-NFATc3 and coimmunoprecipitating GSK-3 α and β , which was normalized to phospho-NFATc3. Nuclear and cytosolic lysates were immunoblotted for total GSK-3 α and β . Amalgamated densitometry is shown in bar graphs. $n = 6$ mice per genotype. * $P < 0.001$ compared to WT. (C and D) NFATc3 immunoprecipitates from cardiac nuclear (C) and cytosolic (D) fractions using anti-NFATc3 antibody and immunoblotted for NFATc3 and coimmunoprecipitating GSK-3 α and β , which was normalized to total NFATc3. Nuclear and cytosolic lysates were immunoblotted for total GSK-3 α and β . Amalgamated densitometry is shown as bar graphs. $n = 6$ mice per genotype. * $P < 0.005$ compared to WT. (E) Expression of the NFATc3-regulated gene ILR2A in WT and PI3K γ KO mice was measured using real-time RT-PCR and represented as fold change over WT. $n = 5$ mice per genotype. * $P < 0.0005$ compared to WT. (F) Cardiac lysates from WT and PI3K γ KO mice were immunoblotted with anti-ANP antibody. The blots were stripped and reprobed with anti- β -actin antibody. Amalgamated densitometry is shown in the bar graph. $n = 6$ per genotype. * $P < 0.001$ compared to WT.

Fig. 7.

Cardiac expression of inactive PI3Kγ (PI3Kγ_{inact}) in PI3Kγ KO increases heart size. (A) Hearts were excised from WT, PI3Kγ KO, or PI3Kγ_{inact}/PI3Kγ KO mice, and representative pictures depicting comparative sizes are shown. n=6 to 7 mice per genotype. Scale bar, 10 μm. (B) Hematoxylin and eosin (H&E)-stained whole heart sections of WT, PI3Kγ KO, or PI3Kγ_{inact}/PI3Kγ KO mice showing comparative sizes. n = 6 to 7 mice per genotype. Scale bar, 10 μm. (C) M-mode echocardiographic images of WT, PI3Kγ KO, or PI3Kγ_{inact}/PI3Kγ KO mice. n = 6 to 7 mice per genotype. (D) Illustration depicting Akt-independent inhibition of GSK-3 by PI3Kγ. Overview of the signaling pathway (left panel). Mechanistic view of PI3Kγ-mediated inhibition of GSK-3 that is independent of Akt (right panel). (E) Schematic representation of kinase-independent function of PI3Kγ in regulating PP2Ac methylation and phosphatase activity. C, catalytic subunit of PP2A; B, regulatory subunit of PP2A; A, scaffolding subunit of PP2A; Me, methylation on PP2A.

Echo parameters of wild-type, PI3K γ KO, and PI3K γ_{inact} /PI3K γ KO mice (at 24 weeks of age). LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; PW, posterior wall; IVS, interventricular septum; %FS, %fractional shortening; %EF, % ejection fraction. n = 6 to 7 mice per genotype for echocardiography analysis.

Table 1

	LVEDD	LVESD	PW	IVS	% FS	% EF
Wild-type	3.19 $\pm$ 0.05	1.69 $\pm$ 0.05	1.05 $\pm$ 0.20	1.32 $\pm$ 0.15	47.08 $\pm$ 3.18	79.64 $\pm$ 3.28
PI3K γ KO	3.04 $\pm$ 0.05 [*]	1.46 $\pm$ 0.05 [*]	1.20 $\pm$ 0.23	1.61 $\pm$ 0.16 [*]	51.95 $\pm$ 2.12	84.35 $\pm$ 1.90
PI3K γ_{inact} /PI3K γ KO	3.99 $\pm$ 0.06 [†]	2.94 $\pm$ 0.04 [†]	1.06 $\pm$ 0.18	0.93 $\pm$ 0.21 [†]	26.61 $\pm$ 3.28 [†]	52.55 $\pm$ 5.42 [†]

^{*} P < 0.01 compared to wild-type or PI3K γ_{inact} /PI3K γ KO.

[†] P < 0.005 compared to wild-type or PI3K γ KO.

Table 2

Age-dependent HW/BW ratios in wild-type, PI3K γ KO, and PI3K γ_{inact} /PI3K γ KO mice. HW/BW, heart weight/body weight ratio. n = 6 to 7 mice per genotype for morphometric analysis.

	HW/BW (10 weeks)	HW/BW (24 weeks)
Wild-type	4.09 $\pm$ 0.04	4.90 $\pm$ 0.09
PI3K γ KO	4.01 $\pm$ 0.08	4.32 $\pm$ 0.17 [*]
PI3K γ_{inact} /PI3K γ KO	4.17 $\pm$ 0.09	5.19 $\pm$ 0.03 [†]

^{*} P < 0.01 compared to wild-type or PI3K γ_{inact} /PI3K γ KO.

[†] P < 0.005 compared to wild-type or PI3K γ KO.