PKA Phosphorylation of Shp2 Inhibits Its Phosphatase Activity and Modulates Substrate Specificity

BY

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THESIS

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This thesis is dedicated to the memory of Graeme Carnegie, who will always be an inspiration.

And to my parents. Even though they may not understand most of this dissertation, it would not have been possible without them.

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LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
AKAP	A-kinase anchoring protein
AML	Acute myelogenous leukemia
AR	Adrenergic receptor
B-ALL	B-cell acute lymphoblastic leukemia
BiFC	Bimolecular fluorescence complementation
cAMP	3′-5′-cyclic adenosine monophosphate
CFP	Cyan fluorescent protein
cMyBP-C	Cardiac myosin binding protein C
cTnI	Cardiac troponin I
DAG	Diacylglycerol
DH	Dbl-homology
DMSO	Dimethyl sulfoxide
EGF	Epidermal growth factor
ERK	Extracellular-signal-regulated kinase
FAK	Focal adhesion kinase
GAB1	Grb2-associtated-binding protein 1
GAB2	Grb2-associtated-binding protein 2
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GPCR	G-protein-coupled receptor
GST	Glutathione S-transferase
HCM	Hypertrophic cardiomyopathy
HDAC5	Histone deacetylase 5
IBMX	3-isobutyl-1-methylxanthine
IGF	Insulin-like growth factor

IL-6	Interleukin-6
IRS-1	Insulin receptor substrate 1
ISO	Isoproterenol
JMML	Juvenile myelomonocytic leukemia
JNK	c-Jun N-terminal kinase
KSR-1	Kinase suppressor of Ras 1
LPA	Lysophosphatidic acid
LS	LEOPARD Syndrome
mAKAP	Muscle-selective AKAP
MAPK	Mitogen-activated protein kinase
Mef2	Myocyte enhancer factor 2
MKK3	Mitogen-activated protein kinase kinase 3
MLTK	MLK-like mitogen-activated protein triple kinase
MP1	MEK partner-1
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NRVM	Neonatal rat ventricular myocyte
NS	Noonan Syndrome
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PH	Pleckstrin-homology
PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D
PKNα	Protein kinase Nα
PLB	Phospholamban
PTP	Protein tyrosine phosphatase
pY	Phosphotyrosyl

RFP	Red fluorescent protein
RTK	Receptor tyrosine kinase
RyR	Ryanodine receptor
SERCA	Sarcoplasmic reticulum Ca^{2+} ATPase
SH2	Src homology 2
Shp2	SH2 domain-containing phosphatase 2
TAC	Transverse aortic constriction
YFP	Yellow fluorescent protein

SUMMARY

Pathological cardiac hypertrophy (an increase in cardiac mass resulting from stress-induced cardiac myocyte growth) is a major factor underlying heart failure. Src homology 2-domain containing phosphatase 2 (Shp2) is critical for cardiac function and mutations resulting in loss of Shp2 catalytic activity are associated with congenital cardiac defects and hypertrophy.

I have identified a novel mechanism of Shp2 inhibition that may promote cardiac hypertrophy. I demonstrate that Shp2 is a component of the A-kinase anchoring protein (AKAP)-Lbc complex. AKAP-Lbc facilitates protein kinase A (PKA) phosphorylation of Shp2, which inhibits Shp2 phosphatase activity. I have identified two key amino acids in Shp2 that are phosphorylated by PKA. Utilizing double mutant PKA phospho-deficient (T73A/S189A) and phospho-mimetic (T73D/S189D) constructs, *in vitro* binding assays and phosphatase activity assays indicate that phosphorylation of these residues disrupts Shp2 interaction with tyrosine-phosphorylated substrates and inhibits its protein tyrosine phosphatase activity.

Overall, my data indicate that AKAP-Lbc integrates PKA and Shp2 signaling in the heart and that AKAP-Lbc-associated Shp2 activity is reduced in hypertrophic hearts in response to chronic β-adrenergic stimulation and PKA activation. Thus, while induction of cardiac hypertrophy is a multifaceted process, inhibition of Shp2 activity through AKAP-Lbc-anchored PKA is a previously unrecognized mechanism that may promote cardiac hypertrophy.

I. INTRODUCTION

(Portions of this chapter were originally published in The Journal of Cardiovascular Pharmacology and used by permission. Carnegie G. K. & Burmeister, B. T. (2011) A-kinase anchoring proteins that regulate cardiac remodeling. J Cardiovasc Pharmacol. 58(5), 451-8.)

A. Cardiac Remodeling and Hypertrophy

The adult heart responds to injury or stress by activating a variety of intracellular signaling pathways that promote re-expression of an embryonic gene program, myocyte hypertrophy, and remodeling of the extracellular matrix (Chien et al., 1999; Frey et al. 2003; Heineke et al., 2006). Collectively these changes are defined as cardiac remodeling and are associated with the progression of heart failure. This dysregulation of cardiac signaling leads to a decline in ejection fraction and the onset of dilated cardiomyopathy. Many diseases including hypertension, diabetes, valvular disorders, and coronary artery disease promote cardiac remodeling (Hill et al., 2008; Barry et al., 2010). Dilated cardiomyopathy can be viewed as the final phenotype for each of these disorders.

Typically, cardiac stress occurs through injury to the ventricles due to acute myocardial infarction, which promotes left ventricular remodeling and myocardial necrosis. After a myocardial infarction, remodeling occurs in order to repair the area of necrotic tissue, resulting in myocardial scarring. This remodeling may be considered beneficial to some extent, because there is an improvement in or stabilization of left ventricle function and cardiac output.

However, over time ventricular remodeling is a maladaptive process that can lead to a progressive decline in left ventricle performance (Chien et al., 1999; Frey et al. 2003; Heineke et al., 2006; Hill et al., 2008; Barry et al., 2010).

Cardiac remodeling describes changes in the heart that are caused by myocardial infarction, as well as nonischemic cardiomyopathies, such as chronic myocarditis and idiopathic dilated cardiomyopathy, suggesting that these distinct disorders may share common mechanisms that promote cardiac dysfunction. Factors that increase pressure or volume overload, which include chronic hypertension, congenital heart disease, and valvular heart disease, will induce cardiac remodeling.

Heart failure is a deficiency in the capability of the heart to adequately pump enough blood to meet systemic demands. It is typically caused by a number of common disease triggers, including: hypertension, myocardial infarction, chronic ischemia, inflammation, prolonged tachycardia or bradycardia, valvular heart disease, and congenital malformations (Burchfield et al., 2013). Before progression to heart failure, most of these stimuli first induce a phase of cardiac hypertrophy where adult myocytes respond to an increase in workload through an increase in cell size (hypertrophy), rather than proliferation. Hypertrophy is initially a beneficial compensatory process as it increases cardiac pump function and decreases ventricular wall tension (Haider et al., 1998; Berenji et al., 2005). However, prolonged cardiac hypertrophy is maladaptive, predisposing individuals to arrhythmia, cardiac failure, and sudden death (Berenji et al., 2005).

Initiating hypertrophic stimuli can be classified into biomechanical/stretch-sensitive mechanisms or neurohumoral mechanisms that result in the release of hormones, peptide growth factors, cytokines, and chemokines. In myocytes, these ligands activate various G-protein-coupled receptors (GPCRs), receptor tyrosine kinases, and gp130-linked receptors. Biomechanical signals are mediated through internal stretch-sensitive receptors, although the identities of these receptors and their signaling mechanisms remain unclear. These receptors converge on intracellular signaling pathways that induce myocyte growth. These signal transduction pathways coordinate hypertrophic growth by modifying gene expression in the nucleus and inducing protein synthesis.

The type of hypertrophic remodeling depends on the stimuli and accompanying progression of cardiac dysfunction. Cardiac growth can be classified as either physiological or pathological. Physiological hypertrophy includes normal postnatal growth as well as pregnancy and exercise-induced hypertrophy. In contrast, pathological hypertrophy manifests in a diseased state. Stimuli include myocardial infarction or ischemia, cardiomyopathy, or conditions of prolonged pressure or volume overload. While the onset of both physiological and pathological hypertrophy causes the overall size of the heart to increase, physiological hypertrophy is characterized by normal cardiac structure and normal or enhanced cardiac function. Importantly, physiological hypertrophy is reversible (Ferrans et al., 1976; Schaible et al., 1984; Fagard 1997). Conversely, pathological hypertrophy is associated with cardiac dysfunction, fibrosis, myocyte apoptosis, and increased risked of heart failure (Levy et al., 1990; Weber et al.,

1993; Cohn et al., 1997).

Physiological and pathological hypertrophy can be further classified as concentric or eccentric. Concentric hypertrophy occurs in response to an overload in pressure and triggers an increase myocyte thickness. Sarcomeres are added in a parallel pattern causing an increase in myocyte width. Eccentric hypertrophy is caused by volume overload, promoting myocyte elongation. In this manner of hypertrophy, sarcormeres are added in series, causing increased myocyte length (Sawada et al., 1991; Gerdes et al., 1992; Heineke et al., 2006).

Prolonged cardiac hypertrophy transitions to decompensation and cardiac failure, where there is downregulation of β-adrenergic receptors (ARs) and dysregulation of protein kinase A (PKA) signaling events (Movsesian et al., 2005). A change in PKA localization is observed under these conditions, which is caused by decreased phosphorylation of the type II regulatory subunit of PKA (Zakhary et al., 2000). This change in RII phosphorylation results in altered binding to A-kinase anchoring proteins (AKAPs), which have different affinities for phosphorylated versus unphosphorylated PKA (Zakhary et al., 2000). AKAPs are a diverse family of proteins that share the common function of binding and targeting PKA to discrete locations within the cell. Importantly, our lab has shown that AKAP-Lbc promotes the induction of hypertrophy through the modulation of myocyte enhancer factor 2 (MEF2)-mediated gene remodeling (Carnegie et al., 2008).

B. cAMP and Protein Kinase A (PKA) Signaling

3′-5′-cyclic adenosine monophosphate (cAMP) is a diffusible second messenger that is essential for intracellular signal transduction. The production of cAMP is triggered by the binding of a wide array of extracellular ligands to GPCRs leading to subsequent activation of adenylyl cyclases (ACs), which produce cAMP from adenosine triphosphate (Berthet et al., 1957; Sutherland et al., 1958; Ross et al., 1978). Along with GPCRs and ACs, cAMP-specific phosphodiesterases (PDEs) shape and maintain a cAMP gradient throughout the cell (Sutherland et al., 1958; Cheung et al., 1970). PDEs degrade the phosphodiester bond in cAMP and thus regulate the localization, duration, and amplitude of cAMP signaling within subcellular domains. cAMP regulates critical physiological processes including metabolism, muscle contraction, calcium homeostasis, gene transcription, and neurotransmitter release.

Importantly, cAMP activates the serine/threonine kinase Protein Kinase A (PKA). PKA phosphorylates a diverse group of proteins, and has been shown to play a pivotal role in regulating many physiological processes including exocytosis (Szaszak et al., 2008) metabolism (McKnight et al., 1998), and learning and memory (Abel & Nguyen, 2008). PKA is involved in many signaling pathways that contribute to various disease phenotypes, and may represent a novel drug target for treatment of disease. For example, because of its critical role in regulating pancreatic B-cell function, PKA may provide a potential drug target for the treatment of diabetes (Nesher et al., 2002). PKA also functions in cognitive processes including learning and memory. Therefore, targeting PKA

signaling may represent a potential therapeutic approach to those attempting to slow the symptoms of neurogenerative diseases (Bauman et al., 2004).

PKA is a tetrameric holoenzyme, composed of two regulatory subunits that form a dimer that binds two catalytic subunits. Basally inactive, activation of PKA occurs through the binding of cAMP to the regulatory dimer. The binding of cAMP triggers dissociation of the catalytic subunits (Corbin et al., 1973; Kim et al., 2005; Kim et al., 2007; Carnegie et al., 2009), allowing them to phosphorylate substrate proteins. PKA substrate specificity is partially conferred by different regulatory subunits (RIα, RIβ, RIIα, RIIβ). Both RIα and RIIα are ubiquitously expressed. RIβ is expressed only in the brain, spinal cord, and testes, while RIIβ is specifically expressed in the brain, testes, heart, and adipose tissue. While both RII isoforms are targeted to subcellular compartments, the RI isoforms are more diffusely dispersed throughout the cytoplasm (Diskar et al., 2007; Martin et al., 2007).

In the heart, PKA is crucial in mediating the effects of adrenergic stimulation. Catecholamines activate PKA, leading to phosphorylation of substrates that include the L-type Ca^{2+}-channel and the ryanodine receptor (RyR). PKA-mediated phosphorylation of these substrates increases the amount of Ca^{2+} available for contraction and strengthens the heart's contractions (Olson, 2004). Thus, PKA plays a key role in the regulation of contractility. Additionally, β-adrenergic stimulation leads to PKA phosphorylation of cardiac troponin I (cTnI) and results in reduced Ca^{2+} sensitivity, increased contractile force, and accelerated rate of relaxation (Bers, 2008). Catecholamines stimulate PKA-

mediated phosphorylation of phospholamban (PLB), which negatively regulates the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA). PKA-mediated phosphorylation of PLB results in increased Ca^{2+} re-uptake in the sarcoplasmic reticulum and relaxation of myofilaments (Zaccolo, 2009).

Although acute stimulation of PKA has beneficial effects on heart function, chronic heart failure is associated with elevated catecholamines and is characterized by dysregulation in β-adrenergic receptor function and downstream PKA signaling (Bristow et al., 1984; Movsesian et al., 2005). Overexpression of β_1-ARs in transgenic mice initially increases contractile function and responsiveness to the β-AR agonist isoproterenol. However, chronic over-stimulation of β-ARs leads to progressive deterioration of cardiac performance, cardiac hypertrophy, and heart failure (Bisognano et al., 2000). Transgenic overexpression of the catalytic subunit of PKA results in hypertrophy and fibrosis, suggesting that prolonged signaling through this protein kinase causes some of the detrimental consequences associated with chronically elevated β-adrenergic signaling (Antos et al., 2001). Despite such evidence, how the activation of different GPCRs can trigger distinct signaling pathways through the production of cAMP remains largely undefined.

C. A-Kinase Anchoring Proteins (AKAPs)

One manner of regulating the specificity of cAMP signaling is through spatiotemporal control of its primary effector PKA. AKAPs are a diverse family of

scaffold proteins that bind PKA and target it to discrete subcellular locations. AKAPs regulate spatiotemporal intracellular signaling by forming multienzyme complexes with other enzymes in order to integrate signaling pathways (Fig. 1). Dysregulation of these scaffold complexes may disrupt proper signal transduction, causing pathophysiological conditions and leading to the development of a disease state (Carnegie et al., 2009; Scott et al., 2009).

During the early 1980s, little was known about the compartmentalization of cAMP signaling, and there was much speculation regarding the molecular mechanism behind this regulation. The concept of random diffusion could not explain how the cAMP-dependent cascade could transduce signal between receptors and effectors. In 1982, Vallee et al. demonstrated that the type II regulatory subunit of PKA co-purifies with the microtubule-associated protein MAP2 (Theurkauf and Vallee, 1982). This observation would later prove to be the first example of an AKAP. Shortly thereafter in 1984, a several RII-binding proteins were identified using an RII overlay technique (Lohmann et al., 1984). This protocol is still widely used today as the primary method of identifying AKAPs. This technique of identifying bound RII entails probing protein-containing nitrocellulose membranes with purified RII subunit and an antibody-^{125}I-protein A complex. This procedure was later adapted to screen phage cDNA libraries to indentify proteins that bind RII (Smith et al., 2006). This screening identified several novel AKAPs, including Ht31, which is now known as AKAP-Lbc (Carr et al., 1992). Interestingly, the 18-amino-acid RII-binding region of Ht31 can be used as a disrupting peptide that blocks RII interaction with all currently identified

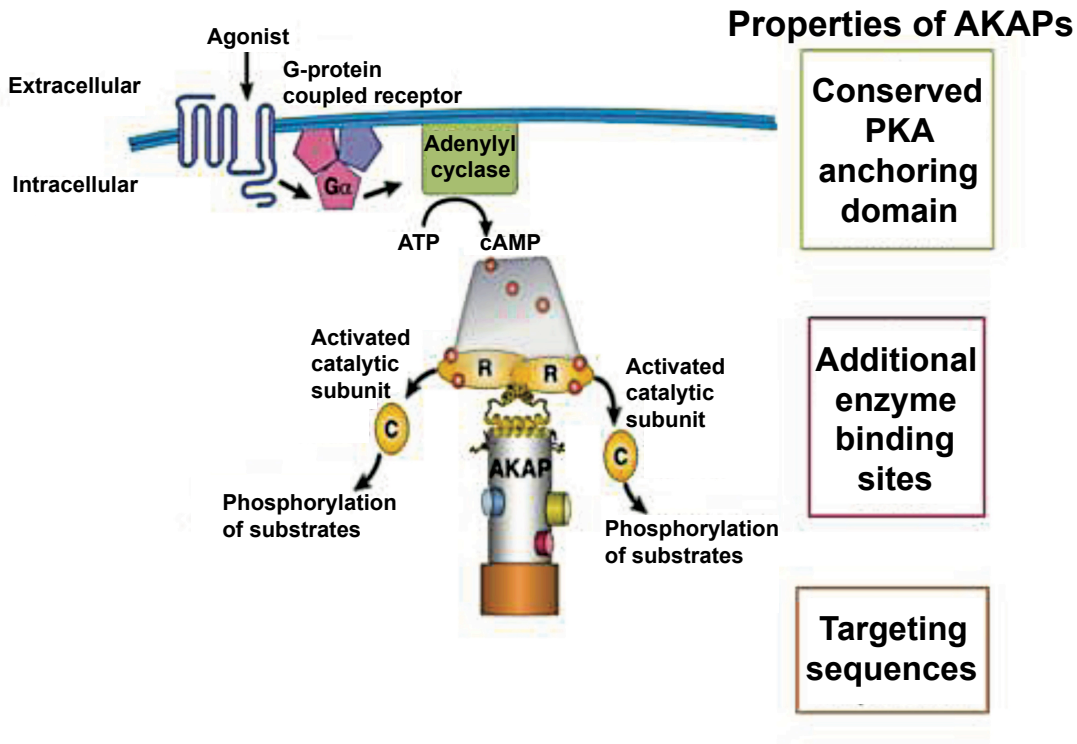

Figure 1. Properties of A-kinase anchoring proteins (AKAPs). AKAPs are a diverse family of scaffolding proteins that are defined solely by their ability to tether protein kinase A (PKA). AKAPs nucleate signaling complexes composed of PKA and additional signaling enzymes. AKAPs possess targeting sequences, directing signaling complexes to discrete subcellular locations. Agonist stimulation of $G\alpha_s$-containing G-protein-coupled receptors activates adenylyl cyclase, which catalyzes the synthesis of cyclic AMP from ATP. cAMP binds to the regulatory (R) subunits of PKA, which causes a conformational change that releases the active catalytic (C) subunit. The catalytic subunit of PKA can then phosphorylate nearby substrates.

AKAPs.

The cloning of several members of the AKAP family allowed for comparison of their primary sequences. These studies revealed that all members of the AKAP family possess a conserved amphipathic α-helical domain that anchors PKA. The only common feature of AKAPs is their ability to bind the regulatory subunit of PKA. However, each AKAP associates with a particular subset of signaling enzymes. Signaling components of AKAP complexes include protein kinases, protein phosphatases, small GTPases, phosphodiesterases, transmembrane receptors, and ion channels (Esseltine et al., 2013). Moreover, these anchoring proteins target signaling complexes to discrete subcellular locations, thereby providing compartmentalization of signaling. The ability of AKAPs to anchor PKA localizes PKA to a subset of potential substrates, thereby generating substrate specificity (Wong et al., 2004). PKA is further classified by the identity of its regulatory subunits. The primary subtypes of PKA are termed RI and RII (Scott, 1991). Most of the currently characterized AKAPs bind specifically to the RII subunit of PKA. However, RI-specific as well as dual specific AKAPs that bind to both PKA regulatory subtypes have been identified (Jarnaess et al., 2008). While the regulation of PKA signal transducation by AKAPs has been well studied, specific physiological roles for AKAPs have been more difficult to determine. Up until recently, the central research focus has been on identifying AKAPs and characterizing the components of their signaling complexes (Smith & Scott, 2006). It is only now that we are beginning to understand the specific biological roles of AKAPs and how their regulation of PKA affects cellular

physiology.

Many AKAPs have been indentifed in the heart. Characterization of their cellular function has demonstrated that AKAPs are essential in modulating phosphorylation of diverse PKA-dependent substrates that are important in regulating cardiac function (Diviani et al., 2008; Pidoux et al., 2010; Welch et al., 2010). AKAP-Lbc, Ezrin, AKAP15/18, muscle-selective AKAP (mAKAP), Yotiao, and AKAP220 have all been identified in adult cardiac myocytes (Diviani et al., 2001; Fraser et al., 1998; Gray et al., 1997; Fan et al., 2001; Potet et al., 2001; Schillace et al., 1999; Bretscher, 1999). The aim of my dissertation work has been to characterize AKAP-Lbc's role in cardiac signaling.

D. AKAP-Lbc

The four members of the Lbc family of Rho guanine nucleotide exchange factor (GEF) proteins were first identified in a screen for transforming genes from human chronic myeloid leukemias (Toksoz et al., 1994). Onco-Lbc contains 424 amino acids residues and displays unregulated exchange factor activity. Onco-Lbc has been shown to transform NIH-3T3 cells in a Rho-dependent manner (Zheng et al., 1995). A proto-oncogenic form containing a COOH-terminal region that attenuates its transforming ability was subsequently discovered (Sterpetti et al., 1999), along with the identification of a splice variant termed Brx (Rubino et al., 1998). Brx is expressed only in testis and estrogen-sensitive tissues (Rubino et al., 1998). AKAP-Lbc is the largest known splice variant of the Lbc oncogene

members and is named as such because of its ability to anchor PKA. The full-length cDNA of AKAP-Lbc was originally ampilified form human heart mRNA by reverse transcriptase-PCR (Diviani et al. 2001).

AKAP-Lbc is a large protein containing 2813 amino acids that plays a pivotal role in coordinating multiple signaling pathways involved in the induction of pathological cardiac hypertrophy (Fig. 2). Expression of AKAP-Lbc is upregulated in hypertrophic cardiac myocytes, and silencing of AKAP-Lbc expression in primary rat neonatal ventricular myocytes by siRNA leads to a reduction in phenylephrine-stimulated hypertrophy (Appert-Collin et al., 2007; Carnegie et al., 2008). AKAP-Lbc serves as a scaffold for PKA, PKC, protein kinase D (PKD) (Liu et al., 2004), and also contains a DH (Dbl-homology) domain and a PH (pleckstrin-homology) domain that functions as a GEF for the GTPase Rho (Diviani et al., 2001), which is a known to mediate the induction of cardiac hypertrophy. Interestingly, AKAP-Lbc-associated Rho-GEF activity is critical for activation of Rho following α1-AR stimulation in the heart. α1-ARs promote AKAP-Lbc activation through a $G\alpha_{12}$-coupled receptor signaling pathway (Diviani et al., 2001; Appert-Collin et al., 2007).

Additionally, the kinase IKKβ is a component of the AKAP-Lbc complex. IKKβ mediates hypertrophic signaling from α1-ARs to NF-κB (del Vescovo et al., 2013). In response to α1-AR stimulation, AKAP-Lbc-associated Rho-GEF activity is increased (Appert-Collin et al., 2007), which promotes the downstream activation of Rho kinase. Activated Rho kinase increases the activity of AKAP-Lbc-anchored IKKβ. Enhanced kinase activity of IKKβ promotes the prouction of

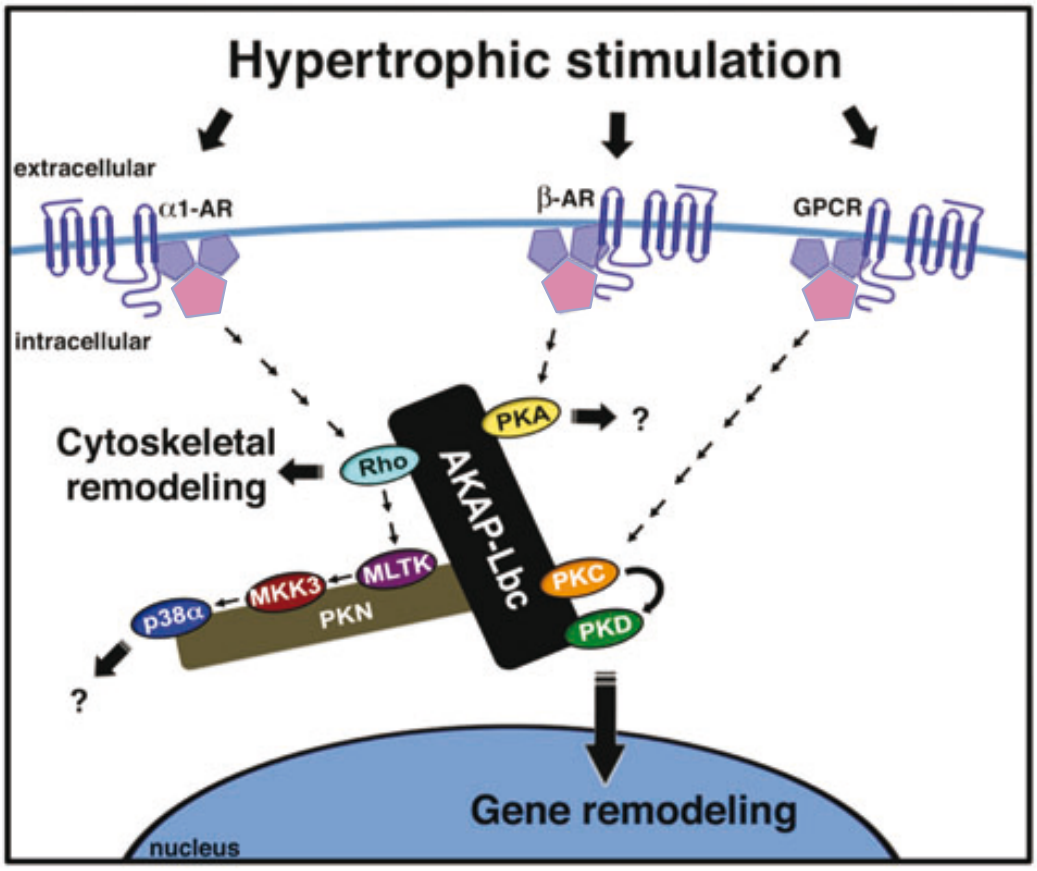

Figure 2. AKAP-Lbc coordinates hypertrophic signaling to promote cytoskeletal and gene remodeling. AKAP-Lbc is present in the cytoplasm, displaying a cytoskeletal and perinuclear localization. This anchoring protein serves as a scaffold for PKA, PKD, and its upstream activating kinase PKC. By bringing PKC and PKD into close proximity, AKAP-Lbc facilitates the phosphorylation and subsequent activation of PKD by PKC. Upon activation, PKD translocates to the nucleus promoting hypertrophic gene expression through phosphorylation of a histone deacetylase (HDAC5), leading to HDAC5 nuclear export and derepression of Mef2 transcription. AKAP-Lbc is a GEF for Rho. The Rho-GEF activity of AKAP-Lbc is stimulated via the $G\alpha_{12}$ family of heterotrimeric G-proteins in response to α1-adrenergic receptor (AR) activation. GEF activity can be inactivated by an AKAP-Lbc anchored PKA-dependent mechanism. AKAP-Lbc also coordinates a p38α MAPK complex, downstream of Rho, composed of PKNα, MLTK, MKK3, and p38α. Presently, the downstream targets and functional consequences of this AKAP-Lbc-associated signaling cascade are unknown.

interleukin-6 (IL-6), through an NF-κB –dependent mechanism and induces expression of fetal genes and subsequent cardiac myocyte hypertrophy (del Vescovo et al., 2013).

Rho promotes the activation of mitogen-activated protein kinase (MAPK) pathways downstream of α1-adrenergic receptors (α1-ARs) (Charron et al., 2001; Maruyama et al., 2002). It has been reported that AKAP-Lbc organizes a p38 MAPK complex with the RhoA effector PKNα and the MAPKs: MKK3, MLTK, and p38α. Thus, AKAP-Lbc forms a signaling complex that specifically promotes Rho-dependent activation of p38 following α1-AR stimulation (Cariolato et al., 2011). The p38 MAPK family has been implicated in several signaling pathways including promotion of proliferation, growth, inflammation, and contraction, through cytokine and G protein–coupled receptors. Specifically, α1-AR activation of p38α can regulate smooth muscle cell contractility and promote cardiomyocyte sarcomere remodeling during cardiac hypertrophy (Srinivasan et al., 2008; Cariolato et al., 2011). Recent studies were conducted using transgenic mice overexpressing a molecular inhibitor, which prevents the interaction between AKAP-Lbc and the p38-activating complex. These mice were subjected to aortic banding-induced pressure overload in order to induce compensetory cardiac hypertrophy. Interestiingly, results from this work suggest that disrupting formation of the AKAP-Lbc-p38 signaling complex suppresses hypertrophic induction in this model (Perez Lopez et al., 2013). Additionally, disruption of this interaction promotes ventricular dilation, activation of stress genes, and increased myocardial apoptosis. Attenuation of hypertrophy in this instance is

due to decreased phosphorylation of ribosomal protein S6 and 4E-binding protein 1, resulting in a reduced capacity for protein synthesis (Perez Lopez et al., 2013). Inactivation of AKAP-Lbc-associated Rho-GEF activity occurs via AKAP-Lbc-bound PKA phosphorylation and recruitment of 14-3-3 proteins (Diviani et al., 2004; Jin et al., 2004). Binding of 14-3-3 proteins promotes dissociation of the p38α activation module (Diviani et al., 2001) and may explain the inhibitory action of 14-3-3 on the p38 pathway. Together, these data indicate that AKAP-Lbc integrates signaling pathways that can regulate either activation or inhibition of p38α.

In addition to Rho-mediated myocardial hypertrophy, AKAP-Lbc also promotes the initiation of a developmental gene reprogramming paradigm (often termed the fetal gene response). Activation of these “fetal” cardiac genes accompanies cardiac hypertrophy, and promotes changes in contraction, metabolism, and calcium handling (Chien et al., 1999; Frey et al. 2003; Heineke et al., 2006; Hill et al., 2008; Barry et al., 2010). AKAP-Lbc facilitates the activation of PKD1 in response to hypertrophic stimuli (phenylephrine and endothelin), through recruitment of its upstream activator, PKC (Carnegie et al., 2004). Activated PKD can then phosphorylate class II histone deacetylases to promote their nuclear export, leading to the derepression of the transcription factor MEF2. Cardiomyocyte hypertrophy and tissue remodeling is then induced through MEF2-dependent transcription of hypertrophic genes (Vega et al., 2004; Carnegie et al., 2008). Although these experiments were performed in neonatal rat ventricular myocytes, investigation of human heart tissue samples provides

some further support for this mechanism. AKAP-Lbc mRNA was increased 2 ± 0.5-fold in samples obtained from patients exhibiting cardiac hypertrophy when compared to normal age-matched control samples (Carnegie et al., 2008). Thus, further research is required to determine if AKAP-Lbc may possibly be a valid biomarker for cardiac hypertrophy. Furthermore, gene-trap mice expressing a truncated version of AKAP-Lbc that abolishes PKD1 binding to AKAP-Lbc exhibited an accelerated progression to heart failure in two different models of pathological hypertrophy – transverse aortic banding (TAC)-induced pressure overload and angiotensin and phenylephrine infusion (Taglieri et al., 2014). These mice display reduced levels of PKD1 activation and histone deacetylase 5 (HDAC5) phosphorylation, attenuated cardiomyocyte hypertrophy, increased deposition of collagen, and increased apoptosis when compared to wild-type control mice (Taglieri et al., 2014). These findings are consistent with a reduced compensatory hypertrophy phenotype that leads to accelerated progression of cardiac dysfunction and heart failure in these mice (Taglieri et al., 2014). These data demonstrate that AKAP-Lbc-PKD1 signaling is critical in promoting hypertrophy.

AKAP-Lbc also acts to promote stress fiber formation downstream of Rho in NIH3T3 fibroblasts (Diviani et al., 2001) although the significance of this activity in cardiac cytoskeletal remodeling in response to stress is currently unknown. Follwing stimulation with lysophosphatidic acid (LPA), $G\alpha_{12}$ activates AKAP-Lbc. Subsequently, AKAP-Lbc-associated GEF activity facilitates the activation of Rho, which can then induce actin stress fiber formation and focal

adhesion assembly (Diviani et al., 2001). Interestingly, it has been reported that AKAP-Lbc may also function in a cardioprotective role. Anchoring of PKA by AKAP-Lbc has been shown to mediate activation and phosphorylation of Hsp20 on Ser16, thus facilitating the anti-apoptotic effects of the Hsp (Edwards et al., 2012).

E. AKAP-Lbc Interacting Proteins

1. Protein Kinase D (PKD)

Protein kinase D (PKD) is an evolutionarily conserved serine/threonine protein kinase consisting of three isoforms – PKD1, PKD2, and PKD3, each encoded by distinct genes. PKD consists of an N-terminal regulatory domain and a C-terminal catalytic domain (Valverde et al., 1994; Johannes et al., 1994). Isoforms of the PKD family have a high degree of sequence homology, sharing greater than 90% identical sequence within the kinase domain. Additionally, PKD1, PKD2, and PKD3 all possess similar protein structures. The N-terminal regulatory domain inhibits the activity of the C-terminal kinase domain. Additionally, the regulatory domain of PKD facilitates its association with intracellular and plasma membranes (Iglesias et al, 1998). In addition to a pleckstrin homology domain, the N-terminal domain contains 2 zinc finger–like motifs that allow PKD to bind diacylglycerol (DAG) and phorbol esters (Valverde et al., 1994; Van Lint et al., 1995). Binding in these instances activates PKD *in vitro* (Van Lint et al., 1995). Another mechanism of PKD activation involves

phosphorylation of the activation loop of the PKD kinase domain by PKC (Zugaza et al., 1996; Zugaza et al., 1997). The critical sites of are Ser744 and Ser748, and their phosphorylation has been accepted as the primary mechanism by which PKD is activated (Iglesias et al., 1998; Waldron et al., 2001; Waldron et al., 2003).

In response to specific stimuli, alternative mechanisms of PKD activation have been reported. For instance, following treatment with genotoxic agents, caspase 3 mediates cleavage of the PKD regulatory domain (Endo et al., 2000). Moreover, reports have indicated that in response to oxidative stress, Src and Abl kinases mediate phosphorylation of Tyr463 within the PH domain (Storz et al., 2003). Experimental data indicate that in response to oxidative stress stimuli, Tyr95 is phosphorylated. Predictive modeling suggests that this phosphorylated tyrosine residue may create a docking site for PKCδ, which subsequently phosphorylates Ser744/Ser748 in the PKD activation loop (Storz et al., 2007) (Fig. 3).

There are several lines of evidence that indicate the growing importance of PKD-mediating signaling in the heart. Reports have implicated regulatory roles for PKD in the processes of cardiac remodeling, hypertrophy, and myocardial contraction. PKD1 is the primary PKD isoform expressed in neonatal rat ventricular myocytes. In these cells, mRNA transcripts of PKD1, but not PKD2 or PKD3, are detectable by conventional methods (Haworth et al., 2000). Other reports have shown that increased PKD activity in cardiac myocytes enhances phosphorylation of cardiac troponin I at Ser24 and phosphorylation of cardiac

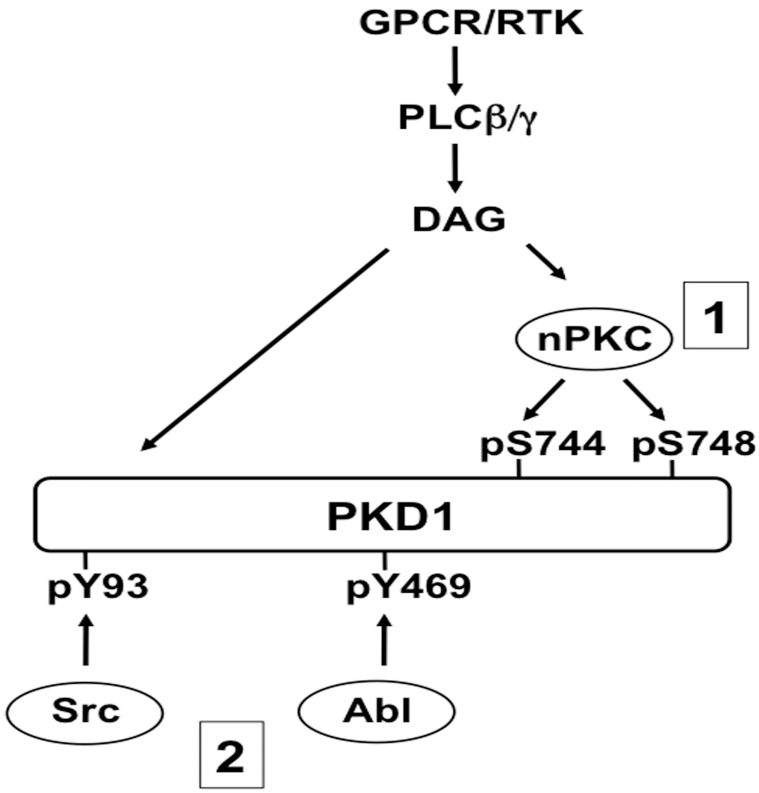

Figure 3. PKD1 activation. 1) PKD1 is phosphorylated and activated by PKC isoforms through a canonical pathway. G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) activate phospholipase C (PLC) to generate diacylglycerol (DAG). DAG in turn activates PKC which phosphorylates the activation loop of PKD1 at residues S744 and S748. **2)** Tyrosine phosphorylation of PKD1 by Src and Abl at residues Y93 and Y95 induces a conformational change promoting PKC recruitment, leading to phosphorylation and activation of PKD1.

myosin binding protein C (cMyBP-C) at Ser304. Phosphorylation of these proteins reduces myofilament Ca^{2+} sensitivity and enhances the rate of cross-bridge cycle, thus indicating that perturbed PKD activity affects the contractility of the heart (Bardswell et al., 2010; Cuello et al., 2007).

As previously stated, PKD directly phosphorylates class II HDAC5, which is a well-characterized suppressor of cardiac hypertrophy (Vega et al., 2004). This PKD-mediated phosphorylation of HDAC5 triggers its nuclear export thereby negating its ability to inhibit the induction of cardiac hypertrophy (Sucharov et al., 2006; Vega et al., 2004). AKAP-Lbc, which is upregulated under hypertrophic conditions, has been shown to scaffold activated PKD and mediate the phosphorylation and nuclear export of HDAC5 (Carnegie et al., 2008). Additionally, rats that exhibit spontaneously hypertensive heart failure have significantly levels of PKD expression and activity that are significantly elevated when compared to wild-type controls (Harrison et al., 2006). Furthermore, this study demonstrated that transgenic mice expressing a cardiac-specific constitutively active PKD mutant developed cardiac hypertrophy. Additionally these mice exhibited myocardial wall thinning, ventricular chamber dilation, and deterioration of contractility (Harrison et al., 2006). These findings suggest that sustained elevation of PKD activity is sufficient to induce cardiac hypertrophy and remodeling.

2. Src homology 2 (SH2) domain-containing phosphatase 2 (Shp2)

Src homology 2 (SH2) domain-containing phosphatase 2 (Shp2) is a protein tyrosine phosphatase (PTP) that is ubiquitously expressed. Encoded by the *PTPN11* gene, Shp2 contains tandem N-terminal SH2 domains and a C-terminal catalytic PTP domain (Fig. 4). Confirmation of Shp2's crystal structure along with supporting biochemical data have helped clarify the mechanism by which Shp2 activity is regulated. In its basal state, Shp2 is inactive. The N-SH2 domain folds back into the catalytic pocket of the C-terminal PTP domain, thereby blocking access by potential substrates, and inhibiting Shp2 PTP activity (Hof et al., 1998). Binding of phosphotyrosyl residues to the SH2 domains induces a conformational change, where the closed Shp2 structure is unfolded. This change in conformation allows substrates access to the catalytic pocket of the PTP domain (Hof et al., 1998). Shp2 interacts with a diverse range of tyrosine-phosphorylated proteins, including cytokine receptors, scaffold proteins, and receptor tyrosine kinases (RTK). Through these interactions, Shp2 critically regulates several important cellular processes including proliferation, differentation, and apoptosis.

Several lines of evidence demonstrate that Shp2 is a positive regulator of many intracellular signaling pathways. For example, Shp2 is required for full activation of the ERK/MAPK pathway by integrins, cytokine receptors, and most RTKs (Lutticken et al., 1994; Milarski et al., 1994; Welham et al., 1994; Fukada et al., 1996; Ugi et al., 1996). Although the role of Shp2 in this pathway has been extensively studied, the precise mechanism by which Shp2 mediates the activation of the MAPK pathway remains controversial. Shp2 can bind directly to

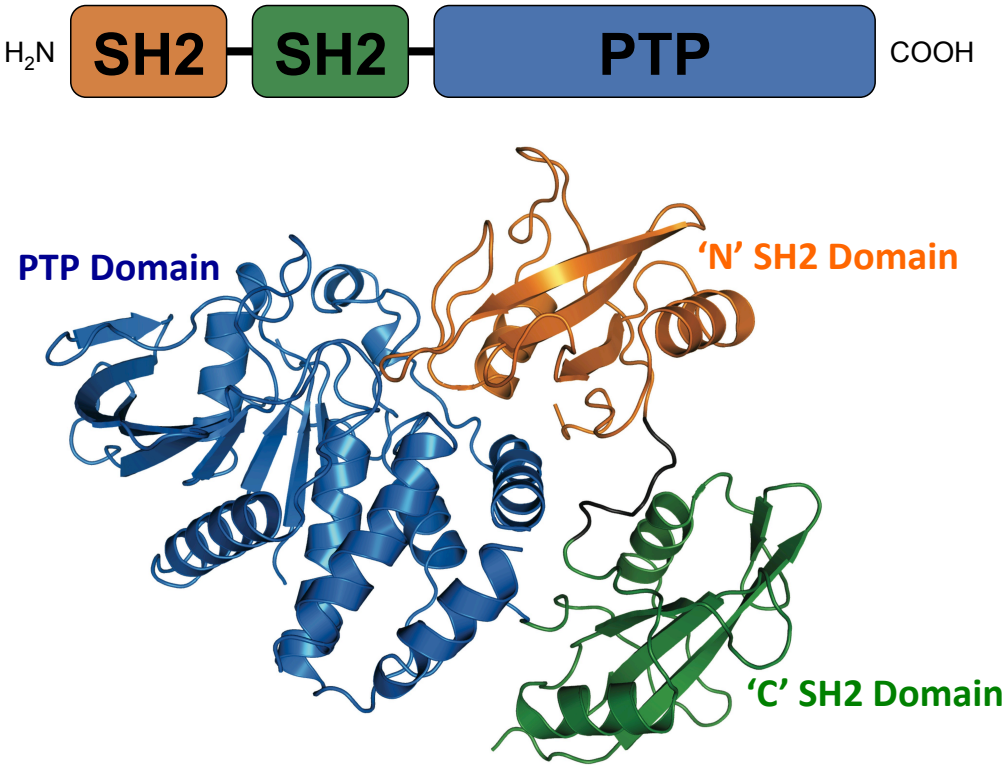

Figure 4. Structure of Shp2. Cartoon representation of the linear domain map (top) and crystal structure of full-length Shp2 (bottom) (PDB ID 2SHP). Shp2 features tandem SH2 domains, 'N' SH2 (orange) and 'C' SH2 (green), and a protein tyrosine phosphatase (PTP) domain (blue).

some RTKs, however, the most common method of Shp2 activation is through the binding of scaffold proteins. Cells expressing dominant negative Shp2 (Noguchi et al., 1994) or mouse embryonic fibroblasts in which *PTPN11* exon 3 has been deleted (Shi et al., 2000) both exhibit diminished activation of Ras, suggesting that Shp2 functions upstream of Ras in this pathway. However, cells expressing a catalytically inactive mutant of Shp2 demonstrate dysregulated ERK/MAPK signaling downstream of Ras, even in the presence of a constitutively active Ras. These findings suggest that Shp2 may also act in parallel to and/or downstream of Ras (Yamauchi et al., 1995).

Shp2 also has a crucial role in the functional regulation of other signaling pathways including NF-kB (You et al., 2001), JAK/STAT (Shi et al., 1998; You et al., 1999), and RhoA (Kontaridis et al., 2004) signaling. Additionally, Shp2 regulates the phosphoinositide 3-kinase (PI3K)/Akt pathway following growth factor stimulation. The role of Shp2 in this pathway is dependent upon the type of growth factor. In response to epidermal growth factor (EGF), Shp2 inhibits PI3K/Akt activation through binding and dephosphorylation of the p85 binding sites within GAB1 (Zhang et al., 2002). In contrast, following stimulation with growth factors such as platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF), Shp2 promotes PI3K/Akt activity (Zhang et al., 2002). Although Shp2 regulation of Akt has been extensively studied, the basis for this differential regulation is not clear.

Mutations within Shp2 have significant functional and biological consequences. Heterozygous missense mutations in *PTPN11,* which result in

loss-of-function of the phosphatase, are observed in up to 90% of LEOPARD Syndrome (LS) patient cases. LS is a rare autosomal dominant disorder, and its name is an acronym for its presenting symptoms of multiple Lentigines, ECG conduction abnormalities, Ocular hypertelorism, Pulmonic stenosis, Abnormalities of genitalia, Retardation of growth, and sensorineural Deafness (Digilio et al., 2002; Legius et al., 2002). Missense *PTPN11* mutations that cause gain-of-function alleles are responsible for nearly 50% of Noonan Syndrome (NS) cases (Tartaglia et al., 2001). Unlike LS, NS is fairly common (~1:1000–1:2500 live births) but is also characterized by multiple, variably penetrant defects, including congenital heart disease, facial dysmorphia, and short stature (Noonan 1968; Nora et al., 1974).

In NS patients, the primary cardiac disorder is pulmonary stenosis resulting from dysplastic valve leaflets. However, stenosis of other valves as well as septal defects has been observed (Marino et al., 1999; Yoshida et al., 2004). Additionally, hypertrophic cardiomyopathy (HCM) has been reported in cases of NS patients with *PTPN11* mutations, suggesting that increased Shp2 activity contributes to HCM. However, this manifestation is uncommon, occuring in only ~8% of NS patients (Sznaier et al., 2007). Conversely, the primary cardiac manisfestion of LS is HCM, presenting in ~80% of patient cases. Valvular defects similar to NS are also commonly observed in LS patients (Limongelli et al., 2007).

Because NS and LS have present with similar phenotypic characteristics in patients, they were presumed to share a common mechanism of

pathogenesis. However, identification of the point mutations and characterization of biochemical properties of Shp2 associated with each disorder revealed stark differences (Hanna et al., 2006; Kontaridis et al., 2006; Tartaglia et al., 2006). Most NS mutations are located within the portion of the N-SH2 domain which folds back and allows for the inhibitory intramolecular amino acid residue interactions with the PTP domain. These mutations disrupt Shp2's ability to retain the closed, inactive conformation. As a result, NS mutations result in gain-of-function alleles, which possess enhanced basal phosphatase activity (Keilhack et al., 2005).

In contrast, LS mutations affect residues within the PTP domain that are critical for phosphatase activity (Hanna et al., 2006; Kontaridis et al., 2006; Tartaglia et al., 2006). LS-associated mutants demonstrate decreased and/or absent PTP catalytic activity that results in loss-of-function of the phosphatase. These mutations result in impaired agonist-evoked ERK activation, but enhaced signaling through Akt/mTOR, FAK, Stat, and JNK pathways. However, like the mutants associated with NS, LS mutations also disrupt N-SH2-PTP domain interactions, suggesting that both NS and LS mutants may out-compete WT Shp2 for binding to RTKs and/or scaffold proteins (Kontaridis et al., 2006; Marin et al., 2011).

In response to cardiac stress, AKAP-Lbc plays a critical albeit poorly understood role in the development of cardiac hypertrophy and remodeling. The goal of my dissertation studies was to identify novel signaling components associated with AKAP-Lbc and to define the cardiac role of AKAP-Lbc and its

associated signaling components. I have identified the protein tyrosine phosphatase Shp2 as a novel component of the AKAP-Lbc singaling complex. Furthermore, my data indicate that AKAP-Lbc integrates PKA, Shp2, and PKD1 signaling in the heart and that AKAP-Lbc-associated Shp2 activity is reduced in hypertrophic hearts in response to chronic β-adrenergic stimulation and PKA activation. Thus, inhibition of Shp2 activity through AKAP-Lbc-anchored PKA is a previously unrecognized mechanism that may promote cardiac hypertrophy.

II. SRC HOMOLOGY 2 DOMAIN-CONTAINING PHOSPHATASE 2 (SHP2) IS A COMPONENT OF THE A-KINASE ANCHORING PROTEIN (AKAP)-LBC COMPLEX AND IS INHIBITED BY PROTEIN KINASE A (PKA) UNDER PATHOLOGICAL HYPTERTROPHIC CONDITIONS IN THE HEART

(This research was originally published in The Journal of Biological Chemistry and used by permission. Burmeister, B. T., Taglieri, D. M., Wang, L., and Carnegie, G. K. (2012) Src homology 2 domain-containing phosphatase 2 (Shp2) is a component of the A-kinase-anchoring protein (AKAP)-Lbc complex and is inhibited by protein kinase A (PKA) under pathological hypertrophic conditions in the heart. J Biol Chem 287, 40535-40546. © the American Society for Biochemistry and Molecular Biology.)

A. INTRODUCTION

Organization of the bewildering array of cell signaling proteins into coherent networks is facilitated by scaffold proteins (Shi et al., 2011). A-kinase-anchoring proteins (AKAPs) are a diverse family of scaffold proteins that form multiprotein complexes functioning to integrate cAMP signaling with other pathways (Wong et al., 2004; Beene et al., 2007; Carnegie et al., 2009). All members of the AKAP family possess a conserved PKA-anchoring domain as well as binding sites for other signaling components (Newlon et al., 2001; Pidoux et al., 2010). For example, to ensure that signaling is tightly regulated, AKAPs coordinate both signal activators (*e.g.* protein kinases) and signal terminators (*e.g.* protein phosphatases) (Scillace et al., 1999; Alto et al., 2002; Cardone et

al., 2004). Here, we report an interaction of the protein-tyrosine phosphatase, Shp2 (*PTPN11*) with AKAP-Lbc (also termed *AKAP13*) and demonstrate that AKAP-Lbc integrates PKA and Shp2 signaling in the heart.

Localized regulation and integration of signal transduction are important for proper cardiac function, and perturbation of this leads to heart failure. We are now just beginning to understand the spatiotemporal aspects of AKAP-mediated signaling in the heart. Multiple cardiac AKAPs have been characterized and shown to perform a critical role in mediating the effects of neurohumoral stimulation on the heart by integrating PKA activity with additional enzymes (Mauban et al., 2009; Diviani et al., 2011; Carnegie et al., 2011).

A recent proteomic study suggests that differential expression of AKAPs coupled with alterations in the AKAP "interactome" may be critical factors in heart failure (Aye et al., 2012). Indeed, we have demonstrated previously that AKAP-Lbc expression is up-regulated under hypertrophic conditions in rats as well as in human heart failure samples, promoting cardiac hypertrophy through a protein kinase D1 (PKD1)-mediated mechanism (Carnegie et al., 2008).

Cardiac hypertrophy is initially a beneficial, compensatory process, decreasing wall stress and increasing cardiac output and stroke volume. However, prolonged hypertrophy is maladaptive, transitioning to decompensation and cardiac failure. Multiple pathological hypertrophic pathways converge on a set of transcriptional regulators, promoting initiation of a developmental gene-reprogramming paradigm (often termed the fetal gene response). These "fetal"

cardiac genes encode proteins involved in contraction, calcium handling, and metabolism, and their activation accompanies cardiac hypertrophy (Frey et al., 2003; Heineke et al., 2006).

In addition to binding PKD1, AKAP-Lbc acts as Rho-guanine nucleotide exchange factor, implicated in cardiomyocyte hypertrophy (Appert-Collin et al., 2007), possibly through a p38α MAPK pathway (Cariolato et al., 2011). AKAP-Lbc also anchors protein kinase A (PKA) and protein kinase C (PKCα and PKCη isoforms) (Carnegie et al., 2004), and our previous data suggest a role for PKA in hypertrophic signaling (Carnegie et al., 2008), but downstream pathways are not clear.

Interestingly, Shp2 is also implicated in the modulation of myocyte size, cardiomyopathy, and heart failure (Nakamura et al., 2007; Krenz et al., 2008; Nakamura et al., 2009). LEOPARD syndrome patients most commonly manifest hypertrophic cardiomyopathy due to mutations in the *PTPN11* gene encoding Shp2 that generally results in impaired Shp2 catalytic activity (Kontaridis et al., 2006; Stewart et al., 2010). Here, we observe similar results, showing diminished Shp2 activity associated with AKAP-Lbc in hypertrophic heart samples induced by chronic isoproterenol treatment to activate PKA. Mechanistically, our data suggest that AKAP-Lbc facilitates the phosphorylation of Shp2 by PKA, acting to inhibit Shp2 PTP activity, which may in turn promote cardiac hypertrophy.

B. EXPERIMENTAL PROCEDURES

1. Antibodies and Reagents

Anti-V5-agarose, anti-FLAG-agarose, and anti-FLAG antibody were from Sigma. Anti-V5 antibody (mouse, 1:5000) and purified recombinant PKD1 were from Invitrogen. Anti-Shp2 antibody (rabbit, 1:1000) was from Santa Cruz and was also kindly provided by Gen-Sheng Feng (University of California San Diego) (rabbit, 1:20,000). Anti-phospho-PKA substrate (RRXS*/T*) antibody (1:1000) was from Cell Signaling Technology. Purified recombinant PKA was from Promega.

2. Bacterial and Mammalian Expression Constructs

cDNA for Shp2 expression was kindly provided by Gen-Sheng Feng. Bimolecular fluorescence complementation (BiFC) plasmids were originally from Chang-Deng Hu (Purdue University). Full-length AKAP-Lbc was expressed as a RFP and V5-tagged protein in pcDNA3.1, or as FLAG-AKAP-Lbc in pEGFP-N1. pEGFP-N1-AKAP-Lbc-ΔPKA expresses a mutant version of AKAP-Lbc that cannot bind the PKA-RII regulatory subunit (Diviani et al., 2001).

3. Bacterial Expression

GST-AKAP-Lbc fusion proteins were produced in BL21(DE3)pLys as described previously (Carnegie et al., 2004).

4. Transfections, Co-immunoprecipitations, and Pulldowns

HEK293 cells were transfected and lysed as described previously (Carnegie et al., 2004). For phosphorylation experiments, the phosphatase inhibitor microcystin-LR was included (100 nm). Lysates were incubated on ice for 10 min and centrifuged at 20,000 × *g* for 15 min at 4 °C. Cleared lysates were incubated with antibodies for 1 h at 4 °C with rocking, followed by precipitation of antibody-antigen complexes with protein A/G-agarose. Immunoprecipitates were washed 5 × 1 ml in lysis buffer, eluted in SDS-PAGE sample buffer, and separated by SDS-PAGE. GST pulldowns were performed similarly, except that protein complexes were isolated by incubation with glutathione-Sepharose for 1 h at 4 °C. For endogenous protein co-immunoprecipitations (co-IPs) from heart, frozen mouse hearts were homogenized by Polytron in 20 mm HEPES, 150 mm NaCl, 5 mm EDTA, 1% Triton X-100, 0.5%, and protease inhibitors. The heart extract was clarified by centrifugation at 20,000 × *g* for 20 min and then used for IP.

5. Mass Spectrometry

Proteomics and informatics services were provided by the CBC-UIC Research Resources Center Mass spectrometry, Metabolomics and Proteomics Facility, established in part by a grant from The Searle Funds at the Chicago Community Trust to the Chicago Biomedical Consortium.

6. BiFC

BiFC expression constructs consisting of the N terminus or C terminus of Venus (VN and VC, respectively) were originally provided by Chang-Deng Hu. AKAP-Lbc was cloned into the VN vector with the Venus fragment fused to the N terminus. Shp2 was cloned into the VC vector with the Venus fragment at the C terminus. Cells were transiently transfected and then imaged ~20 h after transfection so that the proteins were not highly overexpressed. CFP was expressed along with the BiFC constructs, as a marker for transfected cells.

7. Confocal Microscopy

Confocal images were acquired using a Carl Zeiss LSM 510 mounted on an Axiovert 100 M microscope. Images were obtained using a 514-nm argon laser for YFP and 458 nm for CFP with a Plan-Apochromat 63×/1.4 oil immersion objective lens. A 531–595-nm wavelength bandpass filter was used for YFP emission, and a 470–500-nm wavelength bandpass filter was used for CFP, with a pinhole of 0.7 Airy units, which provides a *z* resolution of ~0.6 μm.

8. *In Vitro* PTP Activity Assay

Following immunoprecipitation of either AKAP-Lbc or Shp2, immune complexes were washed five times with IP buffer (10 mm sodium phosphate buffer, pH 6.95, 150 mm NaCl, 5 mm EDTA, 5 mm EGTA, 1% Triton X-100)

before being resuspended in phosphatase assay buffer (50 mm HEPES, 100 mm NaCl, 5 mm DTT, 2 mm Na_2EDTA, 0.01% Brij-35, pH 7.5). The phosphatase assay was carried out in a total reaction volume of 50 µl using 30 µm fluorescein diphosphate as substrate. After a 20-min incubation at 30 °C, supernatant was transferred to a 96-well plate, and phosphatase activity was measured using a PHERAstar FS microplate reader, with excitation at 485 nm and emission at 520 nm.

For calibration of PTP activity using this assay, T cell PTP (New England Biolabs) was serially diluted and used for assay as described above. Fluorescence intensity was measured for known amounts of enzyme ranging from 0 to 500 milliunits of specific activity. One unit is defined as the amount of enzyme that hydrolyzes 1 nmol of *p*-nitrophenyl phosphate (50 mm) in 1 min at 30 °C in a total reaction volume of 50 µl.

9. *in vitro* Shp2 Phosphorylation

Immunoprecipitated Shp2 was phosphorylated *in vitro* in kinase assay buffer (25 mm Tris, pH 7.5, 0.1 mm EGTA, 0.1 mm Na_3VO_4, 0.03% Brij-35, 10 mm $MgAc_2$, 100 mm ATP, 1 mCi of [γ-^{32}P]ATP) supplemented with bacterially purified recombinant PKA C-subunit (0.2 mg), or recombinant PKD1 (0.2 mg), for 20 min at 30 °C. Reactions were terminated by washing twice with fresh kinase buffer prior to resuspension in Laemmli sample buffer or PTP activity assay.

10. Chronic Infusion of Isoproterenol and Measurement of Cardiac Hypertrophy in Mice

16-week-old, male FVB mice received continuous subcutaneous administration of isoproterenol (25 μg/g per day for 30 days) or saline control via minipump (Alzet) implantation. Hearts were assessed before and after treatment by M-mode echocardiography using a VisualSonics Vevo 770 ultrasound instrument.

11. Histology

Hearts were removed from mice, and a small section of the left ventricle was fixed in paraformaldehyde and embedded in paraffin. Paraffin-embedded sections were stained with hematoxylin and eosin.

12. Statistical Analysis of Data

All data are expressed as means ± S.E. Differences in quantitative variables were examined by one-way analysis of variance (ANOVA) or an unpaired two-tailed *t* test. A p value < 0.05 was considered significant (*), a p value < 0.01 was considered very significant (**), and a p value < 0.001 was considered extremely significant (***). All analyses were performed using InStat.

C. RESULTS

1. Shp2 Is a Novel Component of the AKAP-Lbc Complex.

The AKAP-Lbc signaling complex is composed of multiple protein kinases (Smith et al., 2010; Cariolato et al., 2011); therefore, we wondered whether AKAP-Lbc may also bind protein phosphatases. To identify novel binding partners, we performed multiple proteomic screens using GST-tagged AKAP-Lbc fragments to purify associated proteins, which were identified by tandem MS. This approach identified the tyrosine phosphatase Shp2 as an AKAP-Lbc associated protein (Fig. 5A). Binding of Shp2 to a family of immobilized GST-AKAP-Lbc fragments detected an interaction with a central portion of AKAP-Lbc (residues 1388–1922; Fig. 5B). The AKAP-Lbc-Shp2 interaction was validated in HEK293T cells by co-precipitation of endogenous Shp2 with AKAP-Lbc (Fig. 6A). In addition, endogenous Shp2 was co-purified with endogenous AKAP-Lbc from heart extract and vice versa (Fig. 6, B and C).

To visualize the interaction of AKAP-Lbc and Shp2 inside cells, we used BiFC. This technique has several advantages over more traditional immunostaining or fluorescent protein localization. In particular, the BiFC method enables us to specifically visualize the Shp2-AKAP-Lbc interaction at potentially low levels of protein expression, without background (i.e. fluorescence is observed only for Shp2-AKAP-Lbc complex formation) (Kerppola et al., 2006; Shyu et al., 2008). A nonfluorescent fragment (VN) from a split Venus fluorescent protein was fused to AKAP-Lbc (VN-AKAPLbc), and a nonfluorescent VC

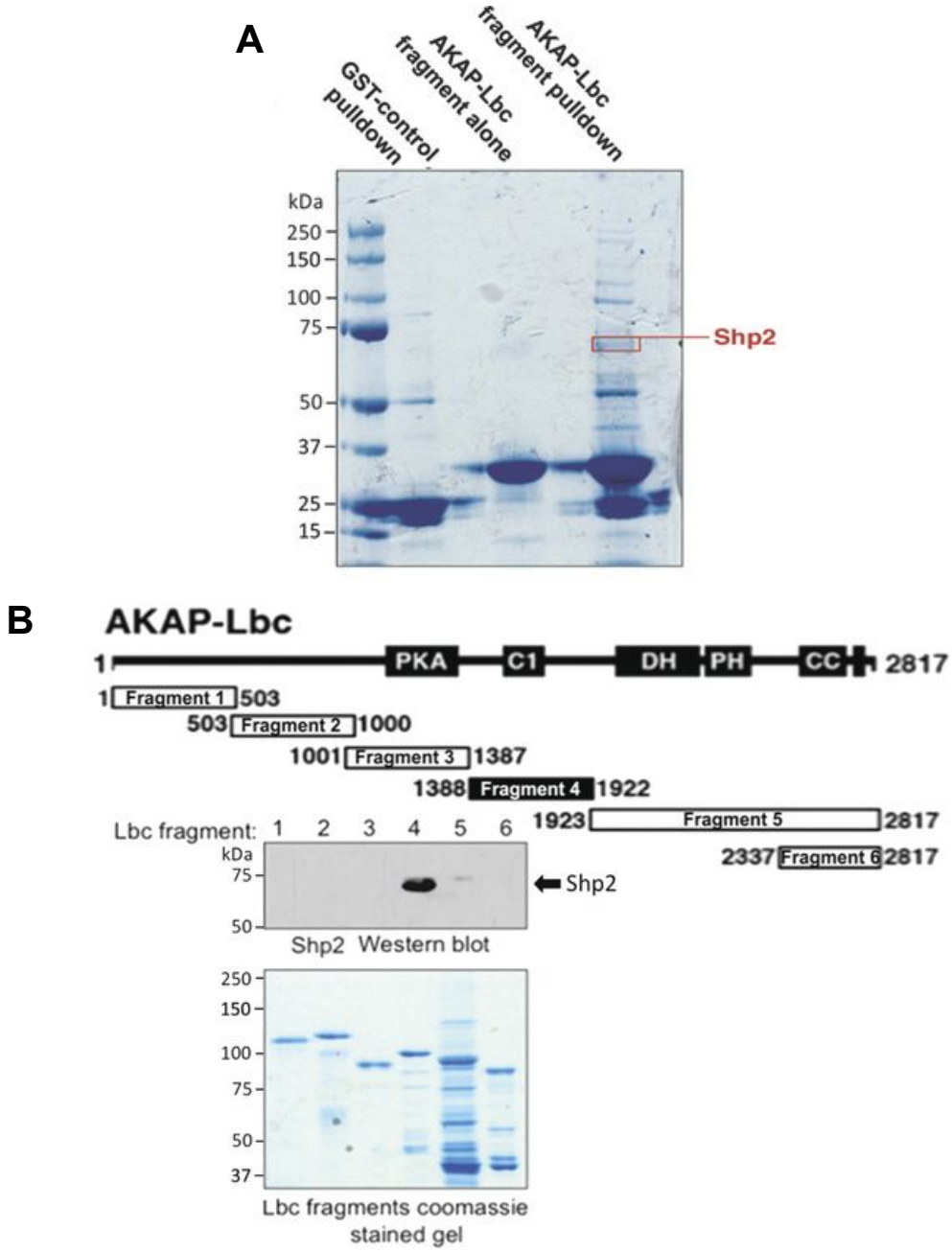

Figure 5. Mapping of Shp2 binding to AKAP-Lbc. ***A)*** Identification of Shp2 co-purifying with AKAP-Lbc by mass spectrometry. Heart extract was used for GST-AKAP-Lbc fragment (amino acid residues 1756–1801) pulldowns (2 mg of heart extract per pulldown). The resulting material was eluted from the GST-agarose and concentrated. Proteins were resolved by SDS-PAGE and Coomassie staining and identified by tandem MS. ***B)*** Shp2 binds to amino acid residues 1388–1922 of AKAP-Lbc. Diagram shows GST-AKAP-Lbc fragments used for pulldown experiments. *Shaded area* indicates Shp2 binding fragment as

determined by immunoblot detection of Shp2 co-purifying with AKAP-Lbc fragments by GST pulldown from heart extract. Coomassie-stained gel indicates equal expression of the AKAP-Lbc fragments that were used for GST pulldown.

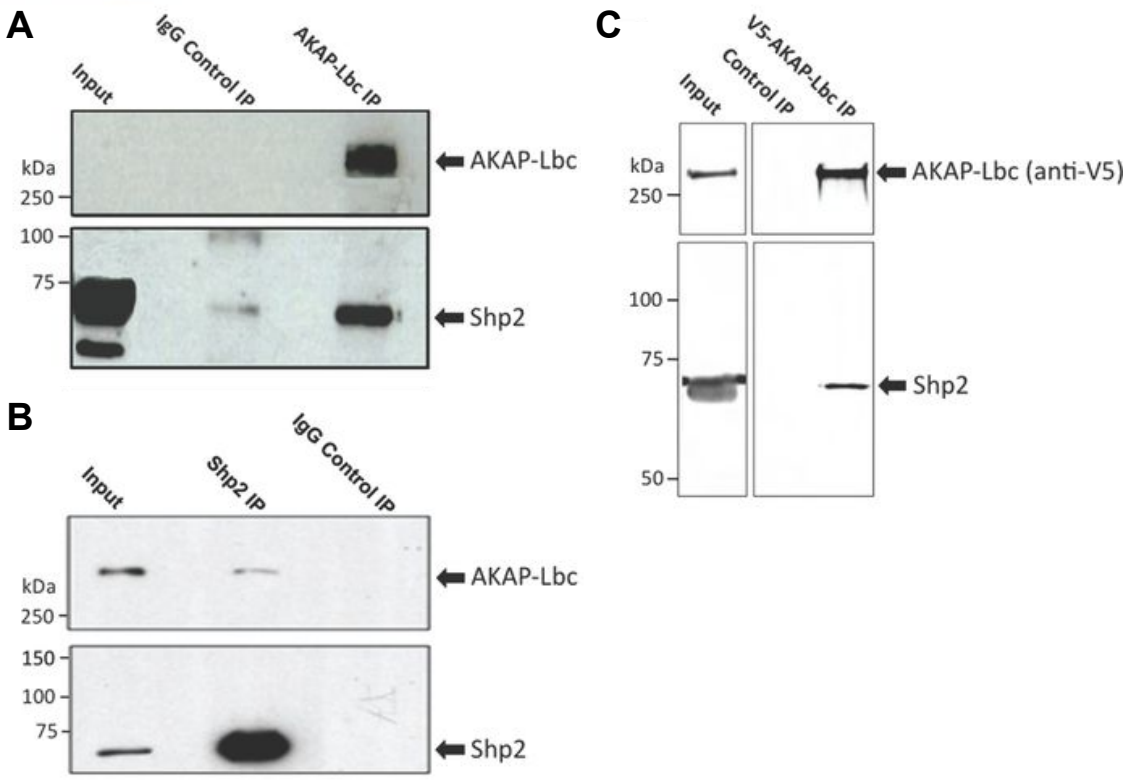

Figure 6. Shp2 interacts with AKAP-Lbc. ***A)*** Co-IP of endogenous Shp2 with endogenous AKAP-Lbc in the heart. AKAP-Lbc was immunoprecipitated from mouse heart extract (5 mg of total protein). Parallel control IgG IPs were carried out using an equal amount of heart extract. IPs were washed, and the bound proteins were separated by SDS-PAGE and transferred to nitrocellulose. Detection of AKAP-Lbc and co-purifying Shp2 were carried out by immunoblotting. ***B)*** Co-IP of endogenous AKAP-Lbc with endogenous Shp2 in heart. Shp2 was immunoprecipitated from mouse heart extract, and detection of AKAP-Lbc and co-purifying Shp2 were carried out by immunoblotting (as described for *A*). ***C)*** Co-IP of endogenous Shp2 with V5-AKAP-Lbc. HEK293 cells were transfected for expression of V5-tagged AKAP-Lbc. AKAP-Lbc was immunoprecipitated with anti-V5-agarose from cell lysates. Parallel control IPs were performed using an equal amount of lysate where V5-AKAP-Lbc was not expressed (*Control IP*). IPs were washed, and the bound proteins were separated by SDS-PAGE and transferred to nitrocellulose. Detection of Shp2 and AKAP-Lbc was carried out by immunoblotting.

fragment was fused to Shp2 fragment (VC-Shp2). Interaction of AKAP-Lbc and Shp2 brings the two nonfluorescent fragments into close proximity, thereby reconstituting a functional fluorescent protein, resulting in fluorescence (Fig. 7A). Results in Fig. 7 show that Shp2 specifically interacts with AKAP-Lbc in the cytoplasm of HEK293T cells (Fig. 7B). In Fig. 7C (merged image), yellow indicates overlap of AKAP-Lbc-Shp2 complex BiFC signal (green) with α-actinin (red), suggesting that a portion of the AKAP-Lbc-Shp2 complex has a sarcomeric and sarcolemmal localization in cardiac myocytes. We did not observe significant differences in AKAP-Lbc-Shp2 complex formation and localization by BiFC in myocytes under basal and agonist treatment. No fluorescence was observed when VN-AKAP-Lbc was expressed alone or with a noninteracting control VC-protein (VC-PI3K). Similarly, no fluorescence was observed with expression of VC-Shp2 alone or with a control VN-protein (VN-Rab5). Western blot analysis of protein expression confirmed that all proteins were similarly expressed and were not degraded. Collectively, our biochemical studies and imaging data demonstrate that Shp2 is a component of the AKAP-Lbc signaling complex.

2. PTP Activity Is Associated with AKAP-Lbc.

Immunoprecipitation of endogenous AKAP-Lbc from mouse heart extract followed by *in vitro* PTP activity assay demonstrates that tyrosine phosphatase activity co-purifies with AKAP-Lbc (Fig. 8). Shp2 IPs were used as a positive control in these experiments, and sodium orthovanadate was used to inhibit

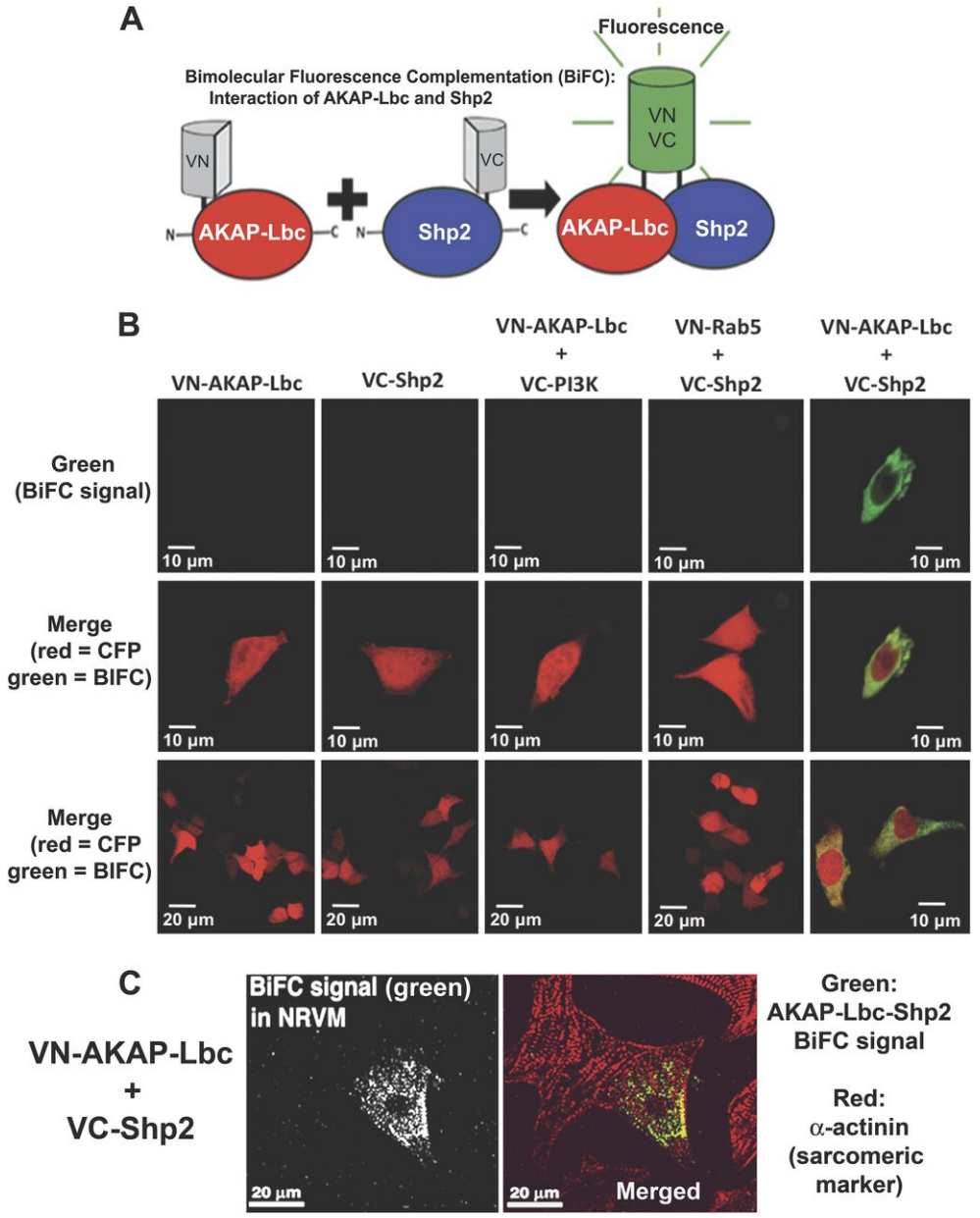

Figure 7. Visualization of AKAP-Lbc-Shp2 interaction inside cells. *A)* Schematic diagram illustrates the BiFC assay. The Venus (*green*) fluorescent protein is split into two nonfluorescent halves which are fused to AKAP-Lbc and Shp2. Specific protein-protein interaction results in a functional Venus (*green*) fluorescent protein. ***B)*** HEK293 cells were co-transfected with VN-AKAP-Lbc and VC-Shp2 and CFP (pseudo-colored *red*), as a marker for transfected cells. Cells were fixed 20 h after transfection and imaged. No BiFC (*green*) fluorescence is observed in cells expressing either VN-AKAP-Lbc or VC-Shp2 alone or when VN-AKAP-Lbc is co-expressed with a noninteracting control VC-protein (VC-

PI3K). Similarly, no BiFC (*green*) fluorescence is observed when VC-Shp2 is co-expressed with a control VN-protein (VN-Rab5), whereas specific interaction of VC-Shp2 with VN-AKAP-Lbc results in fluorescence. Equal protein expression was determined by Western blotting (data not shown). ***C)*** Neonatal rat ventricular cardiac myocytes were electroporated for expression of VN-AKAP-Lbc and VC-Shp2. After 48 h, cells were fixed, permeabilized, and immunostained for the sarcomeric marker protein α-actinin (*red* in *merged image*). BiFC signal is shown in *green* in the *merged image*.

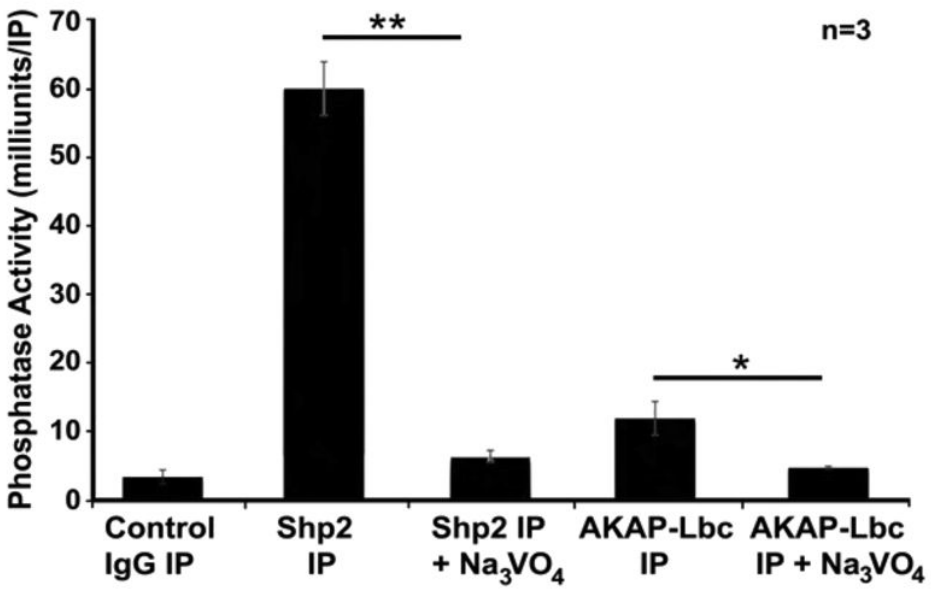

Figure 8. Measurement of PTP activity co-purifying with AKAP-Lbc. Measurement of PTP activity co-purifying with AKAP-Lbc from mouse heart. AKAP-Lbc complexes were isolated from mouse heart extract using anti-AKAP-Lbc antibody. Immunoprecipitates were washed, and PTP activity was measured by a fluorometric *in vitro* assay using fluorescein diphosphate as substrate. Sodium orthovanadate (100 μm working concentration) was added to inhibit PTP activity, confirming that released phosphate was due to phosphatase activity and not proteolytic activity. Parallel negative control IgG IP assays and positive control Shp2 IP assays were also were carried out. Results indicate mean PTP activity per IP ± S.E. (*error bars*). All assays were performed in triplicate for three independent experiments.

tyrosine phosphatase activity, demonstrating that we are specifically measuring PTP activity. Additionally, our results show that PTP activity is associated with V5-tagged AKAP-Lbc, expressed in HEK293T cells (Fig. 9A). Using two different antibodies in these experiments (anti-AKAP-Lbc and anti-V5) gives us confidence that the phosphatase activity measured is specifically associated with AKAP-Lbc. Importantly, we also carried out measurements of AKAP-Lbc-associated PTP activity from samples where Shp2 had been previously immunodepleted. Results presented in Fig. 9A show a dramatic reduction of PTP activity associated with AKAP-Lbc, suggesting that we are measuring Shp2 activity in the AKAP-Lbc complex. Western blots indicating corresponding levels of AKAP-Lbc and Shp2 from samples used in these assays are shown in Fig. 9B.

3. Shp2 Is a PKA Substrate.

Our results demonstrate that Shp2 is a component of the AKAP-Lbc complex; however, the function and regulation of Shp2 in the AKAP-Lbc complex are unclear. To determine whether Shp2 is a substrate for either PKA and/or PKD1, we performed *in vitro* phosphorylation assays using [γ-^{32}P]MgATP with immunopurified Shp2 and purified recombinant PKA and PKD1 (Fig. 10A). Following SDS-PAGE and transfer to nitrocellulose, autoradiography results demonstrate that Shp2 is phosphorylated by PKA but not by PKD1. PKD1 autophosphorylation as well as a positive control using HDAC5 (a well characterized PKD1 substrate) indicates that the PKD1 was active in this assay

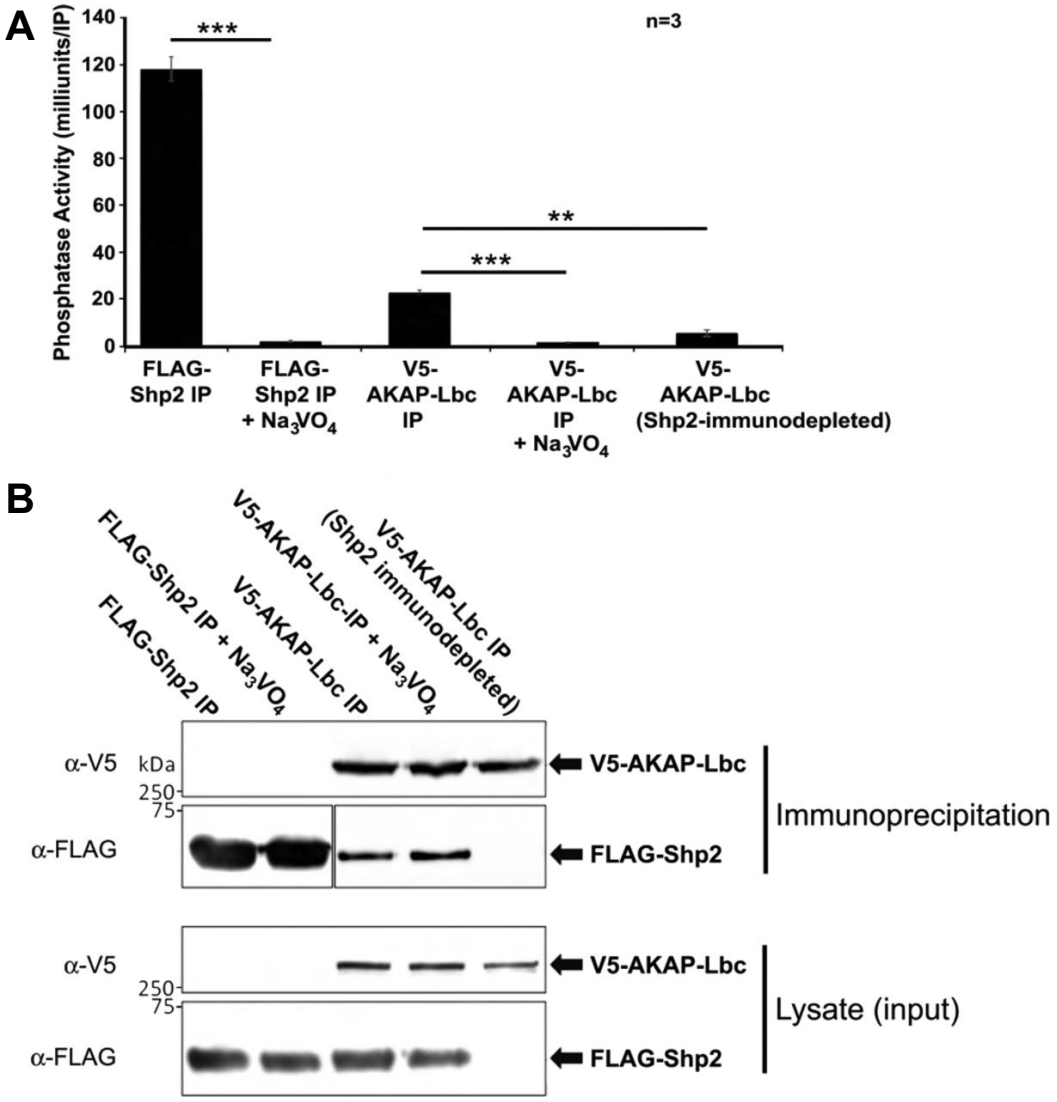

Figure 9. Measurement of Shp2 activity co-purifying with AKAP-Lbc. ***A)*** Measurement of PTP activity co-purifying with AKAP-Lbc expressed in HEK293 cells. V5-AKAP-Lbc complexes were isolated from HEK293 cells using anti-V5-agarose. Immunoprecipitates from cell lysates (2 mg of total protein) were washed, and PTP activity was measured as described above. As a control, PTP activity co-purifying with AKAP-Lbc expressed in HEK293 cells previously immunodepleted for Shp2 was measured. Results presented show mean PTP activity per IP ± S.E. after control IgG IP background activity has been subtracted. All assays were performed in triplicate for three independent experiments. Differences in quantitative variables were examined by ANOVA. p = 0.05 is considered significant (*), p = 0.01 is considered very significant (**), and p = 0.001 is considered extremely significant (***). ***B)*** Western blots showing corresponding levels of AKAP-Lbc and Shp2 in samples used for PTP activity measurement in Fig. *A*.

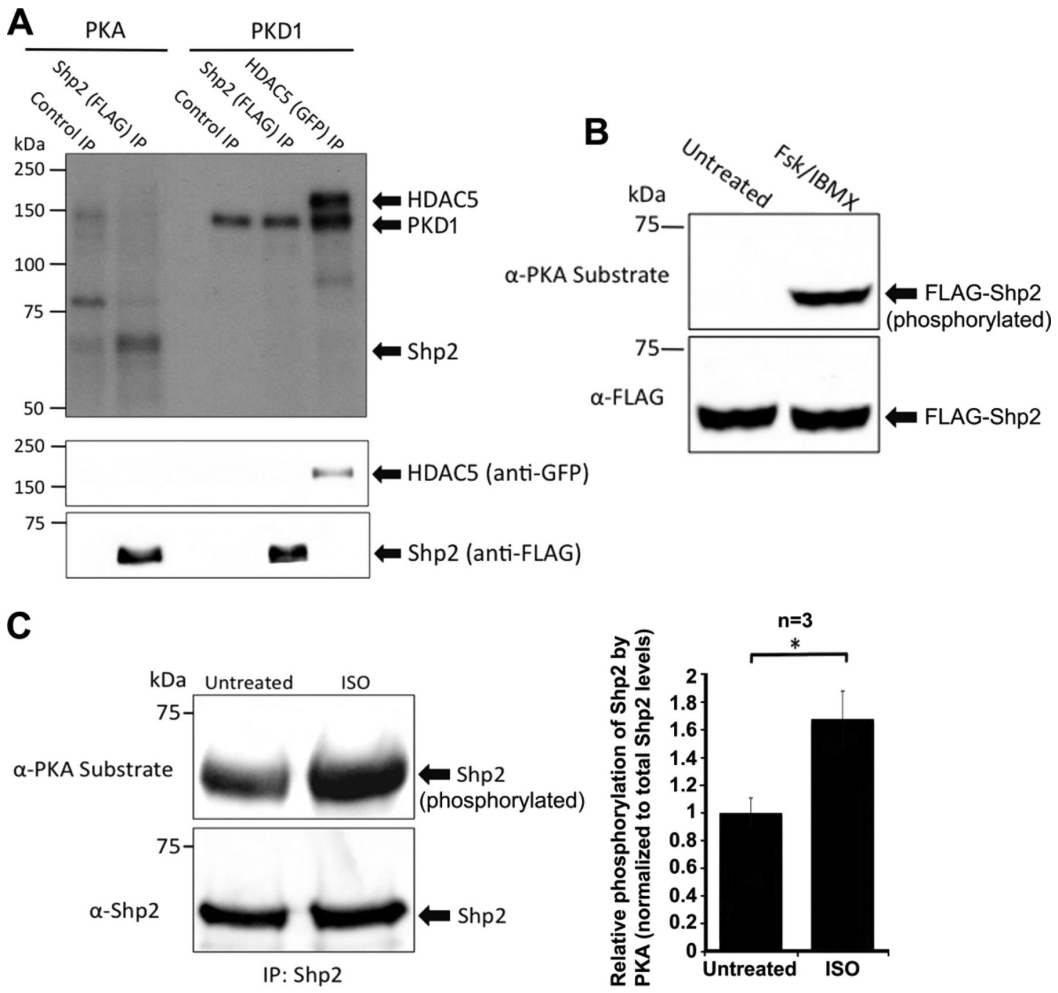

Figure 10. Shp2 is a PKA, but not a PKD1 substrate. ***A)*** PKA, but not PKD1, phosphorylates Shp2 *in vitro*. Immunoprecipitated FLAG-tagged Shp2 from HEK293 cells was phosphorylated *in vitro* in kinase assay buffer supplemented with [γ-^{32}P]ATP and bacterially purified recombinant PKA catalytic subunit (0.2 mg), or recombinant PKD1 (0.2 mg). Reactions were for 20 min at 30 °C and were terminated by washing twice with fresh kinase buffer prior to resuspension in Laemmli sample buffer. Incorporation of phosphate was determined by autoradiography following SDS-PAGE and transfer to nitrocellulose. Immunoblotting confirmed similar levels of Shp2 in both the PKA and PKD1 phosphorylation reactions. A control FLAG-IP was also carried out using cell lysate where FLAG-Shp2 was not expressed. ***B)*** Activation of PKA promotes Shp2 phosphorylation in HEK293 cells. Cells were treated with either DMSO (untreated) or forskolin (20 μm) and IBMX (75 μm) for 20 min to activate PKA. Western blotting using an anti-PKA-phosphosubstrate antibody was carried out following immunoprecipitation of FLAG-Shp2, SDS-PAGE, and transfer to nitrocellulose. The immunoblot was stripped and then probed for total levels of Shp2 with anti-FLAG antibody. ***C)*** Activation of PKA promotes Shp2

phosphorylation in cardiac myocytes. Neonatal rat ventricular myocytes were treated with either DMSO (untreated) or isoproterenol (10 μm for 20 min) to activate PKA. Western blotting using an anti-PKA-phosphosubstrate antibody was carried out following immunoprecipitation of endogenous Shp2, SDS-PAGE, and transfer to nitrocellulose. The immunoblot was stripped and then probed for total levels of Shp2 with anti-Shp2 antibody. The relative increase in Shp2 phosphorylation by PKA in response to isoproterenol was quantified over three independent experiments.

(Fig. 10A, top panel). Western blotting for Shp2 confirmed that equivalent levels of Shp2 were present in both the PKA and PKD1 reaction (Fig. 10A, bottom panel).

To determine whether PKA phosphorylates Shp2 *in vivo*, we expressed FLAG-Shp2 in HEK293T cells and then treated these cells with forskolin and IBMX to activate PKA, prior to lysis. Following immunoprecipitation of Shp2, SDS-PAGE and transfer to nitrocellulose, we performed Western blotting using an anti-PKA-phosphosubstrate antibody. This antibody recognizes PKA substrate phosphorylation with the consensus sequence R-R-X -pS/pT. Results in Fig. 10B show that Shp2 phosphorylation is barely detected under basal conditions, whereas Shp2 is phosphorylated in response to PKA activation. Similarly, Shp2 is phosphorylated by PKA in cardiac myocytes in response to isoproterenol stimulation (Fig. 10C). Overall, these results indicate that Shp2 is a PKA substrate.

4. PKA Phosphorylation of Shp2 Inhibits Its PTP Activity in the AKAP-Lbc Complex.

Next, we determined whether PKA activation and subsequent Shp2 phosphorylation modulate Shp2 activity. HEK293T cells were untreated or treated with forskolin/IBMX for 20 min prior to lysis, AKAP-Lbc immunoprecipitation, and PTP assay. Activation of PKA reduces AKAP-Lbc-associated PTP activity, suggesting that PKA inhibits Shp2 activity in the AKAP-

Lbc complex (Fig. 11A). The levels of AKAP-Lbc and associated Shp2 in these assays were determined by Western blotting, indicating similar levels of AKAP-Lbc and co-immunoprecipitating Shp2 in all conditions (Fig. 11B). Together, these results indicate that PKA does not affect the association of Shp2 with AKAP-Lbc, but acts to inhibit Shp2 catalytic activity in the AKAP-Lbc complex.

For direct evidence that PKA phosphorylation of Shp2 inhibits Shp2 catalytic activity we phosphorylated Shp2 *in vitro* with purified PKA and then performed a PTP activity assay. Results indicate that Shp2 is phosphorylated (Fig. 11D) and displays reduced PTP activity compared with untreated Shp2 (Fig. 11C).

Next, we tested whether AKAP-Lbc facilitates PKA phosphorylation of Shp2 by anchoring PKA.Weused a mutant form of AKAP-Lbc that cannot bind PKA, termed AKAP-Lbc-ΔPKA. The AKAP-Lbc-ΔPKA mutant has two amino acid substitutions (A1251P/I1260P) in the PKA-RII binding region that disrupt the amphipathic helix structure required for hydrophobic binding to the regulatory subunit of PKA, thereby disrupting PKA association (Diviani et al., 2001). HEK293T cells expressing wild-type AKAP-Lbc or the AKAP-Lbc-ΔPKA mutant were untreated or treated with forskolin/IBMX for 20 min prior to lysis, AKAPLbc immunoprecipitation, and PTP assay. Activation of PKA significantly reduces wild-type AKAP-Lbc (WT-AKAP-Lbc)-associated PTP activity, but not PTP activity associated with the AKAP-Lbc-ΔPKA mutant (Fig. 12A). Western blot analysis demonstrates equal expression of both WT-AKAP-Lbc and AKAPLbc-ΔPKA and equal co-immunoprecipitation of Shp2 (Fig. 12B). Using the PKA-

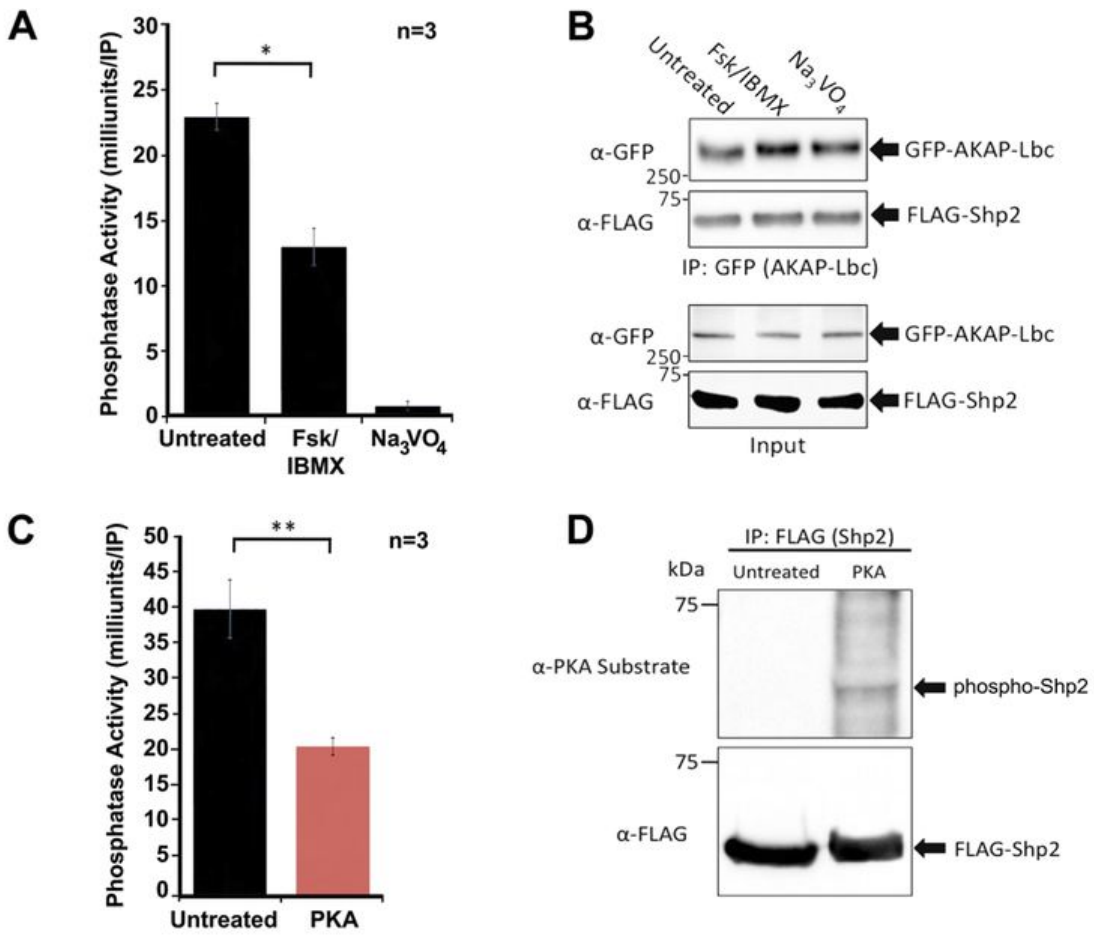

Figure 11. Shp2 activity in the AKAP-Lbc complex is inhibited by PKA. ***A)*** PKA activation promotes a decrease in Shp2 activity in the AKAP-Lbc complex. HEK293 cells transfected for expression of AKAP-Lbc and FLAG-Shp2 were treated for 20 min with either DMSO (untreated), or with forskolin (20 μm) and IBMX (75 μm) to activate PKA. Subsequent AKAP-Lbc immunoprecipitations were used for *in vitro* measurement of PTP activity. All assays were performed in triplicate for three independent experiments. Sodium orthovanadate was used in all assays to determine that PTP activity was being measured. ***B)*** Western blot loading controls demonstrate even expression of AKAP-Lbc and Shp2. To confirm even levels of AKAP-Lbc and co-immunoprecipitating Shp2 under all conditions, each IP was equally divided into four tubes; three tubes were used for PTP assay, and the fourth was used for SDS-PAGE and Western blotting, probed with anti-GFP antibodies for AKAP-Lbc detection and anti-FLAG antibodies for Shp2 detection. ***C)*** *In vitro* phosphorylation of Shp2 by PKA inhibits Shp2 activity. Immunoprecipitated Shp2 was phosphorylated *in vitro* in kinase assay buffer supplemented with bacterially purified recombinant PKA C-subunit (0.2 mg) for 20 min at 30 °C. Parallel control reactions were performed where no PKA was added. Reactions were terminated by washing twice with fresh kinase buffer prior to PTP activity assay. All assays were performed in triplicate for three

independent experiments. ***D)*** Western blot loading controls confirm phosphorylation of Shp2 by PKA and that equal amounts of Shp2 were present in all assay conditions.

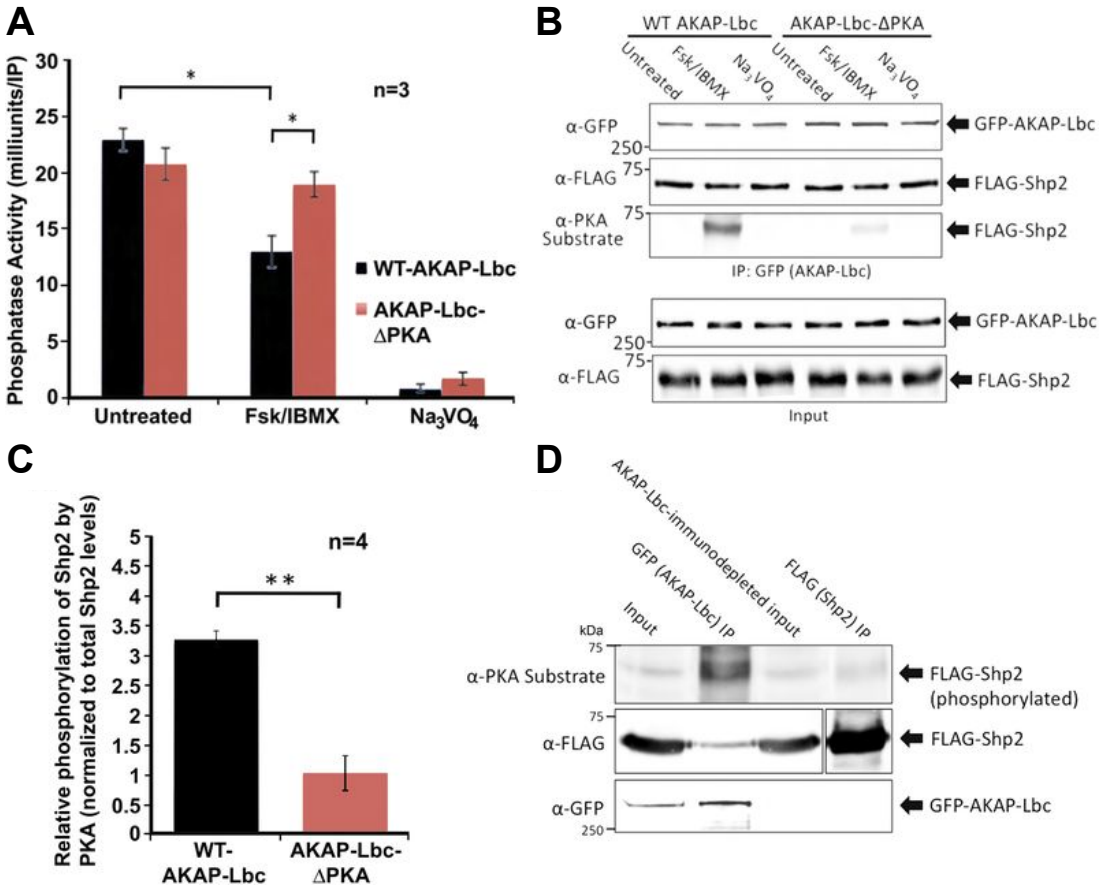

Figure 12. Shp2 activity in the AKAP-Lbc complex is inhibited by PKA. ***A)*** AKAP-Lbc-anchored PKA promotes a decrease in Shp2 activity. HEK293 cells were transfected for the expression of wild-type (WT)-AKAP-Lbc and FLAG-Shp2 or AKAP-Lbc-ΔPKA (a mutant that cannot bind PKA) and FLAG-Shp2. Prior to lysis, cells were treated for 20 min with either DMSO (untreated), or with forskolin/IBMX to activate PKA. AKAP-Lbc was immunoprecipitated, and immune complexes were used for *in vitro* measurement of PTP activity. All assays were performed in triplicate for three independent experiments. ***B)*** Western blot loading controls demonstrate comparable expression of AKAP-Lbc-WT, AKAP-Lbc-ΔPKA, and associated Shp2 for all assays; however, PKA phosphorylation of Shp2 (assessed using the anti-PKA-phosphosubstrate antibody) is reduced in cells expressing AKAP-Lbc-ΔPKA compared with AKAP-Lbc-WT. ***C)*** Shp2 phosphorylation by AKAP-Lbc-anchored PKA is quantified using ImageJ software. ***D)*** Shp2 not associated with AKAP-Lbc is minimally phosphorylated by PKA, in contrast to Shp2 in the AKAP-Lbc complex. Prior to lysis, HEK293 cells expressing GFP-AKAP-Lbc and FLAG-Shp2 were treated for 20 min with forskolin/IBMX to activate PKA. AKAP-Lbc was immunoprecipitated, and subsequent AKAP-Lbc-immunodepleted lysate was used for immunoprecipitation of Shp2. Immune complexes were subjected to SDS-PAGE and transfer to

nitrocellulose. Immunoblotting was performed with anti-PKA-phosphosubstrate antibody to determine the extent of Shp2 phosphorylation. Membrane was probed with anti-FLAG and anti-GFP antibodies to confirm the levels of Shp2 and AKAP-Lbc in each experimental condition. A shorter exposure is shown for total FLAG-Shp2 in the FLAG (Shp2) IP, compared with the other lanes (indicated by separation of the two exposures), due to relative high levels of total FLAG-Shp2 immunoprecipitated.

phosphosubstrate antibody we examined the extent of Shp2 phosphorylation in Shp2 associated with either WT-AKAP-Lbc or the AKAP-Lbc-ΔPKA mutant. Results show that PKA phosphorylation of Shp2 associated with AKAP-Lbc is barely detectable under basal conditions. In response to forskolin/IBMX treatment, Shp2 phosphorylation is significantly diminished when Shp2 is co-expressed with the AKAP-Lbc-ΔPKA mutant compared with the WT-AKAP-Lbc complex (Fig. 12, B and C). By carrying out an additional experiment, where we precleared AKAP-Lbc (and associated Shp2) from cell lysate prior to Shp2 immunoprecipitation, our results demonstrate that the Shp2 not associated with AKAP-Lbc is minimally phosphorylated by PKA, in contrast to the Shp2 that is associated with AKAP-Lbc (Fig. 12D). Collectively, these data suggest that AKAP-Lbc plays an important role in the regulation of Shp2 activity by facilitating phosphorylation of Shp2 by PKA. In summary, phosphorylation of Shp2 by AKAP-Lbc-anchored PKA inhibits Shp2 activity and does not affect the association of Shp2 with AKAP-Lbc.

5. Shp2 PTP Activity Is Depressed in Cardiac Hypertrophy Induced by Isoproterenol

Both AKAP-Lbc and Shp2 are implicated in pathological cardiac hypertrophy and heart failure. We have previously demonstrated that AKAP-Lbc-anchored PKA plays a role in the induction of hypertrophy (Carnegie et al., 2008), and recent publications demonstrate that Shp2 loss of function or

knockout in mice results in hypertrophic cardiomyopathy (Kontaridis et al., 2008; Marin et al., 2008; Ishida et al., 2011;).

Therefore, we hypothesized that inhibition of Shp2 activity in the AKAP-Lbc complex by PKA may be a previously unrecognized factor in the induction of cardiac hypertrophy. To test this hypothesis, we induced cardiac hypertrophy in mice by chronic infusion of isoproterenol (25 μg/g per day for 30 days) using a minipump (Taglieri et al., 2011). Heart morphology and function were assessed immediately by echocardiography prior to minipump implantation and after 30 days. Additionally, a small section of the left ventricle was sectioned and stained with hematoxylin. Results indicate that isoproterenol treatment significantly induced cardiac hypertrophy compared with control animals (infused with saline). Using these samples we observed that PTP activity associated with AKAP-Lbc is significantly decreased in hypertrophic heart compared with control heart (Fig. 13A and Table 1). Consistent with previous reports (Appert-Collin et al., 2007; Carnegie et al., 2008), we observed a 1.4 ± 0.1-fold increase in AKAP-Lbc expression in hypertrophic hearts; therefore, PTP activity was normalized to AKAP-Lbc expression for all IP assays. No change in Shp2 expression levels under hypertrophic conditions was observed. As in previous experiments, the levels of Shp2 co-immunoprecipitating with AKAP-Lbc were consistent in hypertrophic and control conditions, and importantly, Shp2 is phosphorylated by PKA under isoproterenol-induced hypertrophic conditions (Fig. 13B). Overall, these results suggest that chronic activation of PKA in the heart promotes the inhibition of Shp2 activity associated with AKAP-Lbc. This is a previously

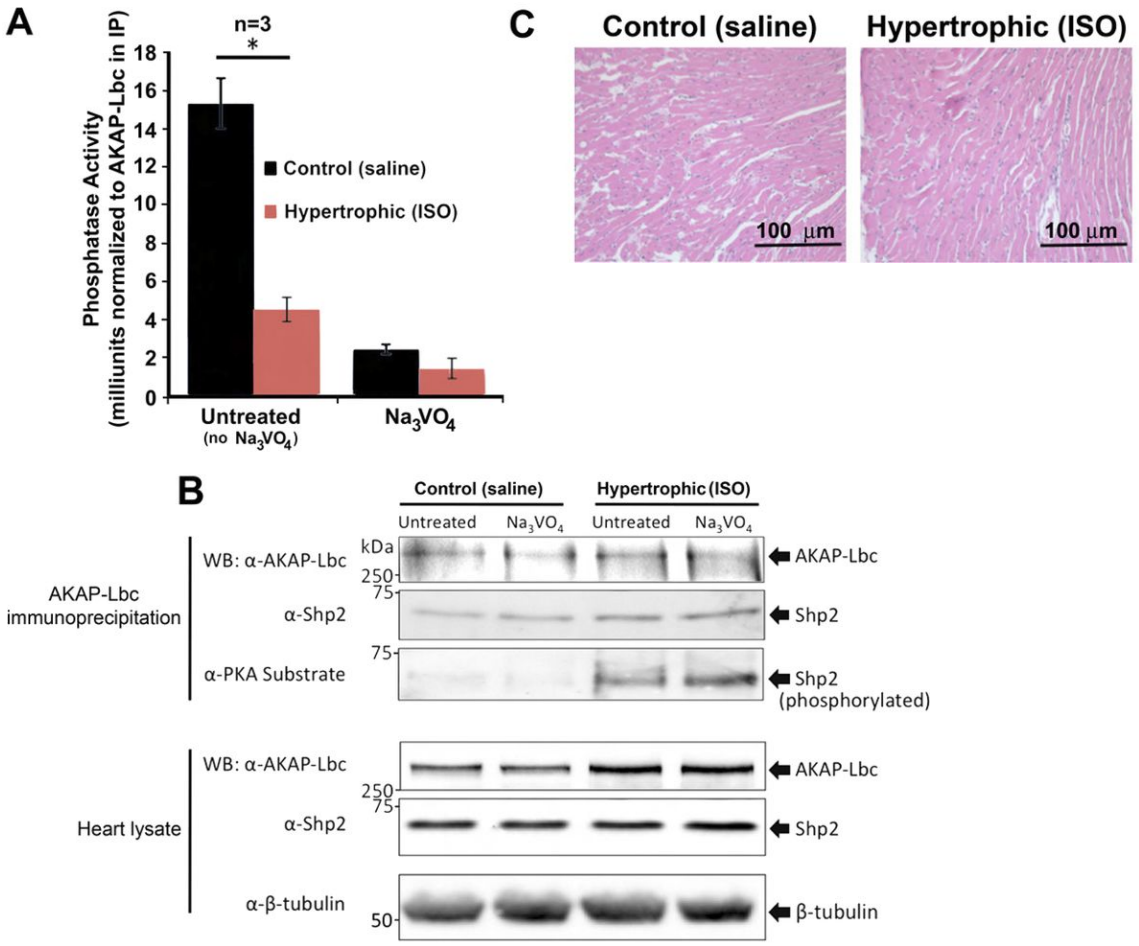

Figure 13. Shp2 activity is decreased in isoproterenol-induced hypertrophic hearts. ***A)*** AKAP-Lbc complexes were isolated from healthy (*Control*; saline-treated) and hypertrophic (*ISO*-treated) mouse heart extract by immunoprecipitation using anti-AKAP-Lbc antibody. Immunoprecipitates were washed, and PTP activity was measured as described previously. Results presented show PTP activity ± S.E. measured in triplicate from three hypertrophic hearts and three age-matched control healthy hearts. Control IgG IP background activity has been subtracted, and the PTP activity was normalized to AKAP-Lbc expression in the immunoprecipitation. Untreated refers to no Na_3VO_4. ***B)*** Western blotting of AKAP-Lbc and associated Shp2 levels in the IPs used for PTP assay. *Bottom panels* show levels of AKAP-Lbc and Shp2 in heart lysate used for IP. ***C)*** Histological analysis of mouse hearts corresponding to samples used for PTP assay. Hematoxylin/Eosin staining of left ventricle sections indicates isoproterenol-induced hypertrophic myocytes (Table 1). Echocardiography characteristics indicate ventricular dilation and hypertrophy.

Table 1. Echocardiographic characteristics of WT mice receiving saline solution (CTRL) or Isoproterenol (ISO) for 8 days

	Before CTRL (N = 8	CTRL (N = 8)	Before ISO (N = 8)	ISO (N = 5)
LV FS, %	31.71 ± 2.87	38.24 ± 4.77	35.46 ± 4.39	57.42 ± 3.38 *
EF, %	59.76 ± 4.02	67.82 ± 5.93	63.93 ± 5.56	87.56 ± 2.33 *
LV mass, mg	117.50 ± 11.95	125.26 ± 4.41	117.50 ± 11.95	177.30 ± 8.52 *
LVAWd (mm)	0.87 ± 0.09	0.87 ± 0.06	0.97 ± 0.04	1.24 ± 0.08 *
LVPWd (mm)	0.82 ± 0.05	0.80 ± 0.05	0.77 ± 0.04	1.11 ± 0.12 *

Abbreviations = HR: Heart rate; LV FS: left ventricular fractional shortening; LV ESV: left ventricular end-systolic volume; LV EDV: left ventricular end-diastolic volume; LV mass: left ventricular mass; LVAWD: left ventricular anterior wall thickness during diastole; LVPWd: left ventricular posterior wall thickness during diastole. * $p < 0.05$ vs. all other groups.

unrecognized mechanism that may contribute to the induction of cardiac hypertrophy (depicted in Fig. 14).

D. DISCUSSION

Here we identify the tyrosine phosphatase Shp2 as a previously unappreciated component of the AKAP-Lbc signaling complex. Our mapping experiments show that Shp2 binds predominantly to a central region of AKAP-Lbc encompassing amino acid residues 1388–1923. We show that Shp2 is a PKA substrate and that PKA phosphorylation of Shp2 inhibits its PTP activity. Furthermore, by using a mutant form of AKAP-Lbc that is unable to bind PKA, we demonstrate that AKAP-Lbc-anchored PKA modulates Shp2 phosphorylation and activity in the AKAP-Lbc complex.

Because AKAP-Lbc also scaffolds and promotes the activation of PKD1, we examined whether PKD1 could phosphorylate Shp2. Our results demonstrate that although PKA and PKD1 are basophilic protein kinases with similar substrate specificity that can phosphorylate shared cardiac substrates, PKD1 does not phosphorylate Shp2. These results indicate that inhibition of Shp2 is specific to PKA signaling and does not occur downstream of PKD. We are currently mapping the site(s) of PKA phosphorylation in Shp2. By performing *in vitro* PKA phosphorylation reactions using fragments of Shp2, our current data indicate multiple sites of phosphorylation.

Given the important cardiac roles of both AKAP-Lbc and Shp2, we

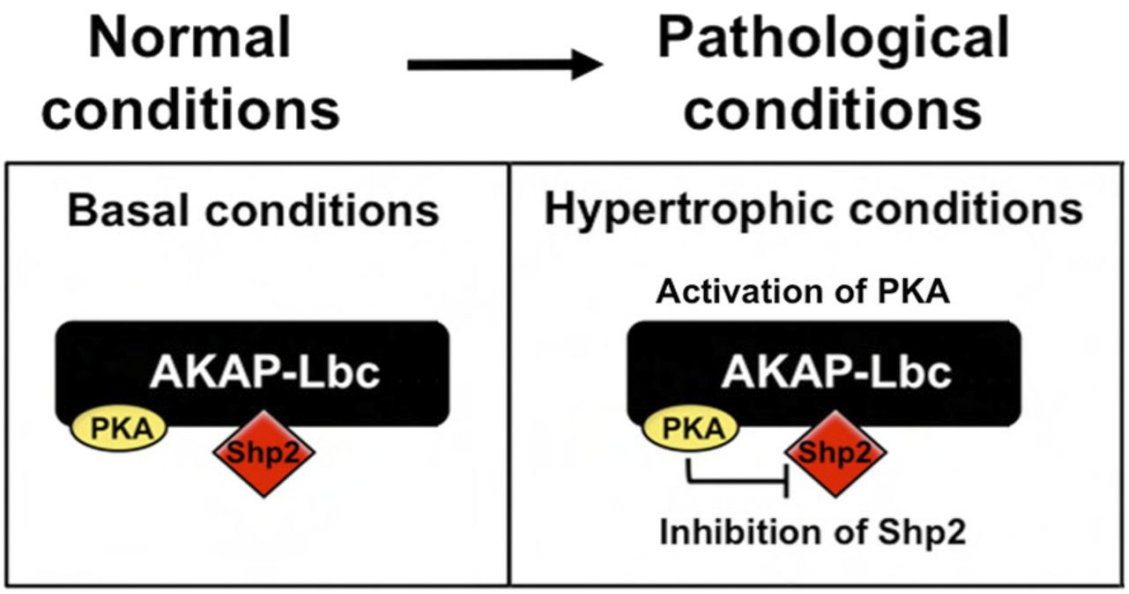

Figure 14. Model showing the role of AKAP-Lbc in the regulation of Shp2 activity by PKA. AKAP-Lbc assembles a signaling complex composed of PKA and Shp2 in cardiac myocytes. Stress conditions (*e.g.* chronic activation of the β-adrenergic receptor) lead to PKA activation, thereby promoting inhibition of Shp2 activity, which may contribute to the induction of cardiac hypertrophy.

performed experiments to investigate the AKAP-Lbc-Shp2 interaction in the heart. Several lines of evidence suggest that AKAP-Lbc interacts with Shp2 in the heart, which may be important for regulation of cardiac function. First, we demonstrate the co-IP of endogenous Shp2 with endogenous AKAP-Lbc from heart extract. Second, we are able to visualize this interaction using a BiFC approach, indicating a cytoplasmic Shp2-AKAP-Lbc interaction, with possible localization at the plasma membrane and association with the cytoskeleton. Third, our *in vitro* PTP activity assay data indicate that Shp2-PTP activity is associated with endogenous AKAP-Lbc in the heart.

Mutations resulting in loss of Shp2 catalytic activity are associated with congenital heart defects and cardiac hypertrophy (Kontaridis et al., 2004; Stewart et al., 2010). AKAP-Lbc is also implicated in cardiac hypertrophic signaling, and through knockdown/rescue experiments we previously showed that AKAP-Lbc-tethered PKA is important for the induction of cardiac myocyte hypertrophy (Carnegie et al., 2008); however, the mechanism of PKA action is unknown. To induce PKA activation and cardiac hypertrophy *in vivo*, mice were subjected to chronic β-adrenergic stimulation through isoproterenol infusion. Under these hypertrophic conditions, our results indicate that AKAP-Lbc-associated Shp2 activity is reduced. Thus, while induction of hypertrophy is a multifaceted process, inhibition of Shp2 activity through enhanced PKA signaling in response to chronic β-adrenergic stimulation may be a previously unrecognized mechanism promoting compensatory cardiac hypertrophy. Importantly, our Shp2 activity data using a form of AKAP-Lbc that is unable to bind PKA (AKAP-Lbc-

ΔPKA) supports a model where AKAP-Lbc facilitates the phosphorylation and inhibition of Shp2 through PKA (Fig. 12). Interestingly, we and others have previously observed up-regulation of AKAP-Lbc expression under hypertrophic conditions (Appert-Collin et al., 2007; Carnegie et al., 2008).

Experiments presented here were performed using a model of compensated hypertrophy, and from our echocardiography data it is clear that the hearts are not in the decompensatory phase leading to heart failure. Therefore it will be of interest to determine Shp2 activity at different stages progressing to heart failure.

III. PROTEIN KINASE A (PKA) PHOSPHORYLATION OF SHP2 INHIBITS ITS PHOSPHATASE ACTIVITY AND MODULATES LIGAND SPECIFICITY

(Portions of this research were originally published in The Journal of Biological Chemistry and used by permission. Burmeister, B. T., Wang, L., Gold, M. G., Skidgel, R. A., O'Bryan, J. P., and Carnegie, G. K. (2015) Protein kinase A (PKA) phosphorylation of Shp2 inhibits its phosphatase activity and modulates ligand specificity. J Biol Chem. © the American Society for Biochemistry and Molecular Biology.)

A. INTRODUCTION

Localized regulation and integration of intracellular signal transduction is critical for cardiac function. A pivotal mechanism that regulates signal transduction pathways is the formation of complexes between signaling molecules and scaffold proteins (Scott et al., 2009). A-kinase anchoring proteins (AKAPs) are a diverse family of scaffold proteins that provide a framework for the formation of multi-enzyme signaling complexes that integrate cAMP signaling with other pathways (Wong et al., 2004; Carnegie et al., 2009). All members of the AKAP family possess a conserved protein kinase A (PKA) amphipathic anchoring helix (Gold et al., 2006; Kinderman et al., 2006) as well as binding sites for additional signaling components (Pidoux et al., 2010). Importantly, these scaffold proteins target unique signaling complexes to discrete subcellular locations, thereby generating substrate specificity (Wong et al., 2004). In the heart, AKAPs play an essential role by integrating PKA signaling with additional

enzymes to modulate physiological and pathophysiological processes, including cardiac remodeling and the development of heart failure (Mauban et al., 2009; Carnegie & Burmeister 2011).

Spatiotemporal regulation of signal transduction is integral for proper cardiac function and perturbation of this regulation can lead to heart failure. AKAPs play a crucial role in directing PKA to several substrates important for cardiac function (Pidoux et al., 2010; Diviani 2008; Welch et al., 2010). Previous studies have shown that differential expression of AKAPs may be a critical factor in the development of heart failure (Aye et al., 2012). One such AKAP, AKAP-Lbc, is encoded by the *AKAP13* gene long transcript and is predominantly expressed in the heart (Diviani et al., 2001). Its expression in the rat heart is induced under hypertrophic conditions suggesting an important role in cardiac hypertrophy (Carnegie et al., 2008). A similar increase in AKAP-Lbc expression was observed in heart samples from patients with hypertrophic cardiomyopathy compared to control, age-matched healthy samples (Carnegie et al., 2008). Furthermore, our previous work revealed that AKAP-Lbc promotes cardiac hypertrophy through activation of a protein kinase D1 (PKD1)-mediated signaling pathway (Carnegie et al., 2008).

Cardiac myocytes primarily respond to increased workload by increasing in size (hypertrophy). Cardiac hypertrophy is a means to decrease ventricular wall tension and increase cardiac output and stroke volume. Initially, hypertrophy is a beneficial, compensatory process. However, prolonged hypertrophy is maladaptive, with the myocardium transitioning to decompensation and cardiac

failure. Multiple pathological hypertrophic pathways converge on a set of transcriptional regulators to activate hypertrophic gene expression. Initiation of this developmental gene-reprogramming paradigm is often termed the fetal gene response (Depre et al., 1998). These “fetal” cardiac genes encode proteins involved in contraction, calcium handling, and metabolism, and their activation accompanies cardiac hypertrophy (Frey et al., 2003; Heineke & Molentin 2006). Thus, defining the signaling events orchestrated by AKAP-Lbc may lead to the identification of new pharmacological approaches for the treatment of heart failure.

In addition to demonstrating that AKAP-Lbc mobilizes a pro-hypertrophic signaling pathway through PKD1, we have also demonstrated the importance of AKAP-Lbc-tethered PKA in the induction of cardiac hypertrophy through knockdown/rescue experiments (Carnegie et al., 2008). Furthermore, we have shown that the protein tyrosine phosphatase Shp2 (PTPN11) interacts with AKAP-Lbc and demonstrated that AKAP-Lbc integrates PKA and Shp2 signaling in the heart (Burmeister et al., 2012). Interestingly, Shp2 is also associated with the modulation of myocyte size, cardiomyopathy, and heart failure (Nakamura et al., 2007; Krenz et al., 2008; Nakamura et al., 2009). LEOPARD Syndrome patients most commonly manifest congenital heart defects and cardiac hypertrophy due to mutations in the *PTPN11* gene encoding Shp2 that generally result in impaired Shp2 catalytic activity (Kontaridis et al., 2006; Stewart et al., 2010). Furthermore, cardiomyocyte-specific genetic deletion of Shp2 results in rapid development of dilated cardiomyopathy (Kontaridis et al., 2008). Previously,

we observed diminished Shp2 activity associated with AKAP-Lbc following chronic isoproterenol treatment, which activates PKA and induces cardiac hypertrophy (Burmeister et al., 2012). However, prior to this study, the mechanism by which AKAP-Lbc inhibits Shp2 was unknown. Here, we report that AKAP-Lbc facilitates PKA phosphorylation of Shp2 at amino acid residues T73 and S189, thereby inhibiting its protein tyrosine phosphatase (PTP) activity and disrupting its binding to tyrosine-phosphorylated ligands. Although induction of cardiac hypertrophy is a multifaceted process, inhibition of Shp2 activity through enhanced PKA signaling represents a previously unrecognized mechanism contributing to cardiac hypertrophy.

B. EXPERIMENTAL PROCEDURES

1. Antibodies and Reagents

Anti-GFP antibody (mouse, 1:1000) was from Clontech. Anti-FLAG M2 antibody (mouse, 1:1000) and anti-α-actinin (mouse, 1:500) were from Sigma. Anti-phospho-PKA substrate (RRXS*/T*) antibody (rabbit, 1:1000) was from Cell Signaling Technology. Anti-phosphotyrosine antibody, clone 4G10 (mouse, 1:1000) and anti-GST (mouse, 1:1000) were from EMD Millipore. Wild-type Shp2 mammalian expression constructs were kindly provided by Dr. Gen-Sheng Feng (UCSD). Mammalian expression constructs for GAB1, GAB2, and constitutively active Src (Y527F) were kindly provided by Dr. Andrei Karginov (UIC).

2. Bacterial Expression

GST and GST-Shp2-SH2 domain proteins were expressed as N-terminal GST-tagged fusions using the pGEX-4T1 vector (Amersham) in bacteria (DH5α) and purified by Glutathione Sepharose chromatography (GE Healthcare). Bacterial cultures were grown overnight at 37°C. The following day bacterial cultures were diluted 1:10, grown to an OD_{600} of 0.6, and induced with 0.1mM IPTG for 3 hrs at 37°C. Cells were resuspended in MTPBS (16 mM Na_2HPO_4, 4 mM NaH_2PO_4-H_2O, 150 mM NaCl, 50 mM EDTA, 1% TritonX-100, pH 7.3), sonicated, and then insoluble debris removed by centrifugation. The supernatant was then incubated with Glutathione Sepharose beads (GE Healthcare) for 1 hr at 4°C. Beads were then washed 3x with MTPBS.

3. Transfections, Co-immunoprecipitations, and Pulldowns

HEK293T cells were transfected and lysed in cell lysis buffer (10 mM sodium phosphate buffer (pH 6.95), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100) as described previously (Carnegie et al., 2004). For phosphorylation experiments, the phosphatase inhibitor microcystin-LR was included (100 nM). Lysates were incubated on ice for 10 min and centrifuged at 20,000 × *g* for 15 min at 4°C. Cleared lysates were incubated with antibodies for 1 h at 4°C with rocking, followed by precipitation of antibody-antigen complexes with protein A/G-Agarose. Immunoprecipitates were washed 5 × 1 ml in lysis buffer, eluted in SDS-PAGE sample buffer, and separated by SDS-PAGE. GST pulldowns were performed similarly, except that protein complexes were isolated by incubation with glutathione-Sepharose for 1 h at 4°C.

4. Cell Culture, Immunocytochemistry, and Cell Imaging

Preparation of primary neonatal rat ventricular myocytes (NRVM) and confocal microscopy experiments were as described (Carnegie et al., 2008). NRVM were electroporated using a modified Amaxa Nucleofector protocol. After incubation at 37°C for 48 hr post-electroporation, cells were washed twice with PBS and fixed in 3.7% paraformaldehyde in PBS followed by staining for α-actinin. Secondary antibodies used were from Invitrogen. Confocal images were acquired using a Carl Zeiss LSM 510 mounted on an Axiovert 100 M microscope. Images were obtained using a 488 nm argon laser for GFP and 561 nm for RFP with a Plan-Apochromat 63X/1.4 oil immersion objective lens. Cell area of α-actinin and GFP double positive myocytes was quantified using ImageJ as previously described (Carnegie et al., 2008).

5. *In Vitro* PTP Activity Assay

Following immunoprecipitation of either AKAP-Lbc or Shp2, immune complexes were washed five times with IP buffer (10 mM sodium phosphate buffer, pH 6.95, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100) before being resuspended in phosphatase assay buffer (50 mM HEPES, 100 mM NaCl, 5 mM DTT, 2 mM Na_2EDTA, 0.01% Brij-35, pH 7.5). The phosphatase assay was carried out in a total reaction volume of 50 µl using 30 µM fluorescein diphosphate as substrate. After a 20-min incubation at 30 °C, supernatant was transferred to a 96-well plate, and phosphatase activity was measured using a PHERAstar FS microplate reader, with excitation at 485 nm and emission at 520

nm. For calibration of PTP activity using this assay, T cell PTP (New England Biolabs) was serially diluted and used for assay as described above. Fluorescence intensity was measured for known amounts of enzyme ranging from 0 to 500 milliunits of specific activity. One unit is defined as the amount of enzyme that hydrolyzes 1 nmol of *p*-nitrophenyl phosphate (50 mM) in 1 min at 30°C in a total reaction volume of 50 µl.

6. Structural Analysis

Structural alignments were performed using the secondary-structure matching superimpose tool in Coot (Emsley et al., 2010). Three-dimensional cartoon representations of SHP-2 were generated using Pymol (Schrodinger LLC).

7. Statistical Analysis of Data

All data are expressed as means ± S.E. Differences in quantitative variables were examined by one-way analysis of variance (ANOVA) or an unpaired two-tailed *t* test. A p value < 0.05 was considered significant. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$. All analyses were performed using InStat.

C. RESULTS

1. AKAP-Lbc-Associated Shp2 Activity Is Increased Under Basal Conditions.

We previously demonstrated that Shp2 is a component of the AKAP-Lbc

complex (Burmeister et al., 2012). Under conditions of enhanced PKA signaling, such as chronic β-adrenergic stimulation or forskolin (FSK)/IBMX treatment, AKAP-Lbc facilitates PKA phosphorylation of Shp2, which inhibits its PTP activity (Burmeister et al., 2012). To determine whether association with AKAP-Lbc affects the specific activity of Shp2 under basal conditions, we performed *in vitro* PTP activity assays and compared the specific activity of total Shp2 vs Shp2 associated with AKAP-Lbc. FLAG-tagged Shp2 was co-expressed in HEK293T cells with either FLAG-GFP or GFP-AKAP-Lbc (Fig. 15). Following immunoprecipitation, PTP activity was measured (Fig. 15A) and normalized to the amount of Shp2 in the immunoprecipitate (IP) (Fig. 15B). Similarly, GFP-tagged AKAP-Lbc was immunoprecipitated and PTP activity was assayed and normalized to the amount of Shp2 present in the IP. The specific PTP activity of AKAP-Lbc-associated Shp2 was increased by 4-fold ($p = 0.045$) compared to the total Shp2 immunoprecipitated from whole cell lysate.

2. PKA Phosphorylation of Sites T73 and S189 Inhibits Shp2 Activity.

To determine which amino acid residues within Shp2 are phosphorylated by PKA, we first identified potential candidate sites based on the consensus PKA phosphorylation motif (R-R-X-pS/pT) using Scansite 2.0 (Obenauer et al., 2003). We identified three candidate PKA phosphorylation sites (T73, S189, and S326) which were individually mutated to alanine. Wild-type (WT) or mutant Shp2 was co-expressed with AKAP-Lbc in HEK293T cells and then cells were treated with either vehicle or FSK/IBMX for 20 min. Following immunoprecipitation of AKAP-

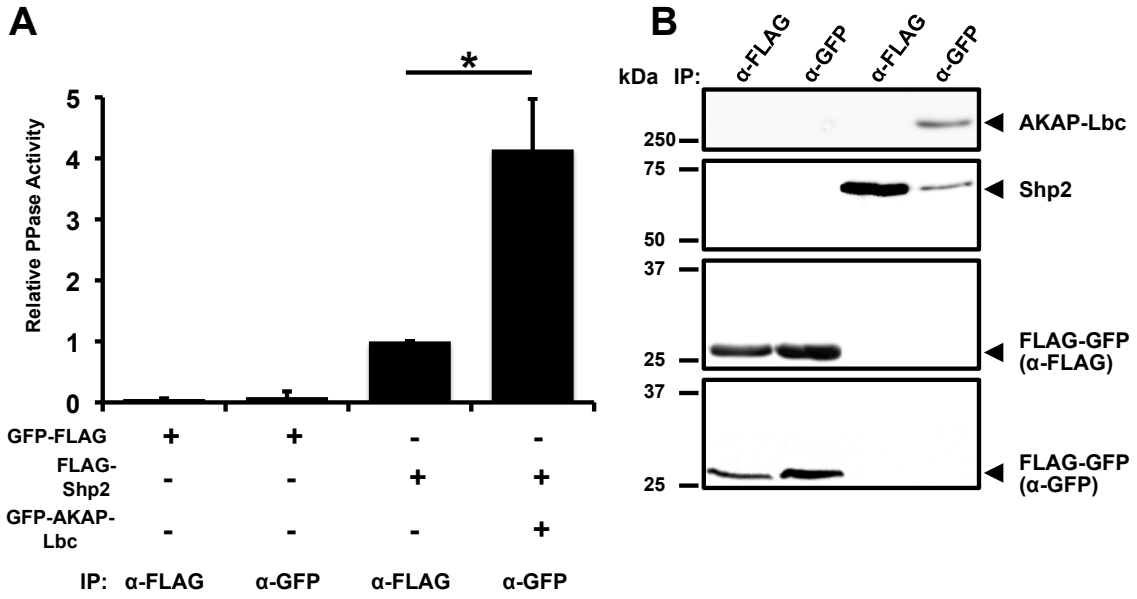

Figure 15. The specific activity of AKAP-Lbc-associated Shp2 is increased under basal conditions. ***A)*** HEK293T cells were transfected with FLAG-Shp2, GFP-AKAP-Lbc plus FLAG-Shp2, or FLAG-GFP vector as control. Shp2 or AKAP-Lbc was isolated from cell lysates using anti-FLAG-antibody or anti-GFP-antibody respectively. Immunoprecipitates were washed, and PTP activity was measured by a fluorometric *in vitro* assay. Parallel control anti-FLAG and anti-GFP IPs were assayed using an equal amount of lysate (2 mg) from cells expressing FLAG-GFP vector. Results are mean protein tyrosine phosphatase (PTP) activity per IP ± S.E. after normalizing to the amount of Shp2 in the IP. Band intensities were quantified using ImageJ and were within the linear dynamic range of detection. All assays were performed in triplicate for three independent experiments. Differences in quantitative variables were examined by ANOVA. ***B)*** Western blots showing corresponding levels of Shp2, AKAP-Lbc, and control FLAG-GFP vector in samples used for PTP activity measurement in Fig. A.

Lbc, phosphorylation of Shp2 was examined using an antibody that recognizes the consensus PKA phosphorylation sites R-R-X-pS/pT. Treatment with FSK/IBMX resulted in PKA-mediated phosphorylation of Shp2. Importantly, phosphorylation was diminished in the T73A and S189A mutants but not in the S326A mutant (Fig. 16A).

To determine the effect of PKA phosphorylation on Shp2 activity, we measured the PTP activity of WT, T73A, S189A, and S326A versions of Shp2 in AKAP-Lbc immunoprecipitates (Fig. 16B). Under basal conditions, no change in AKAP-Lbc-associated Shp2 activity was observed after mutation of the candidate sites. Treatment with FSK/IBMX inhibited AKAP-Lbc-associated WT Shp2 activity as well as that of the S326A mutant. However, mutation of either T73 or S189 to Alanine (A) reduced this inhibition by 2-fold ($p = 0.035$) and 1.7-fold ($p = 0.044$), while mutation of S326 to Alanine did not alter the inhibition of Shp2 following PKA activation. This difference in Shp2 activity is not due to changes in the association of the Shp2 mutants with AKAP-Lbc as equivalent amounts of each Shp2 protein were present in the AKAP-Lbc IPs (Fig. 16A).

The combined mutation of T73A and S189A in Shp2 eliminated PKA-induced phosphorylation of Shp2 (Fig. 16C). Consistent with the lack of PKA phosphorylation, the phosphatase activity of this phosphodefective Shp2 mutant was unaffected following PKA activation (Fig. 16D). We also generated a phosphomimetic version of Shp2 by mutation of T73 and S189 to Aspartate (D). Although PKA-induced phosphorylation of the Shp2 T73D/S189D mutant was mutant was similar to that of WT Shp2 following PKA activation, regardless of

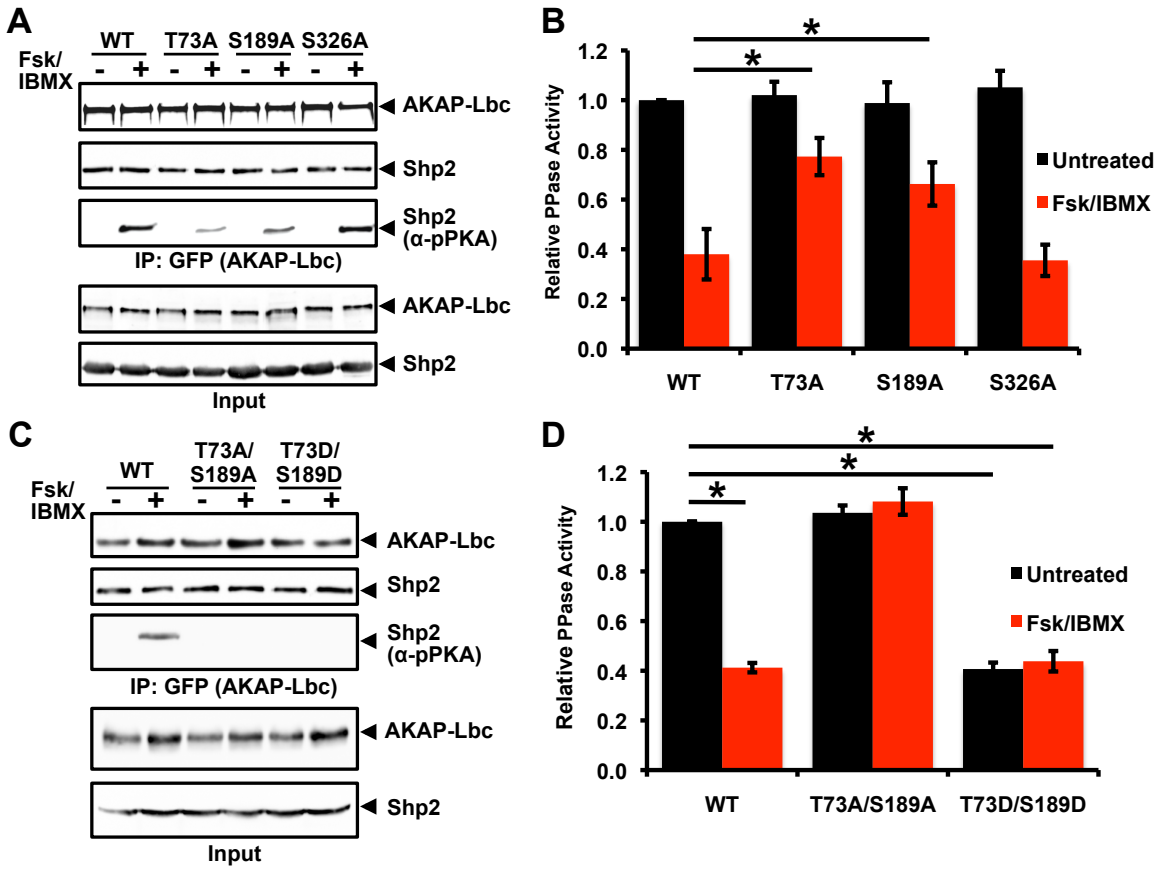

Figure 16. Phosphorylation of Shp2 residues T73 and S189 by PKA regulates Shp2 activity. ***A)*** Scansite (scansite.mit.edu) was used to identify consensus PKA phosphorylation (RRXpS/T) sites in Shp2, yielding three predicted residues: T73, S189, and S326. HEK293T cells were transfected with wild-type or S/T to A mutant Shp2 expression constructs. Prior to lysis, cells were treated for 20 min with either DMSO (vehicle), or with forskolin (20 μM) and IBMX (75 μM) to activate PKA. AKAP-Lbc was isolated from cell lysates using anti-GFP-antibody and immune complexes were used for *in vitro* measurement of PTP activity. Western blot loading controls demonstrate comparable expression of GFP-AKAP-Lbc and associated FLAG-Shp2-WT and FLAG-Shp2 mutants for all assays. Blots were probed with anti-PKA-phospho-substrate antibody to examine the extent of Shp2 phosphorylation. ***B)*** Quantification of 3 independent *in vitro* PTP assays. ***C)*** HEK293T cells were transfected with AKAP-Lbc and FLAG-Shp2-WT or FLAG-Shp2 T73A/S189A (PKA phospho-deficient) or FLAG-Shp2 T73D/S189D (PKA phosphomimetic) expression vectors. Prior to lysis, cells were treated for 20 min with either DMSO (vehicle), or with forskolin/IBMX to activate PKA. AKAP-Lbc was isolated from cell lysates using anti-GFP-antibody and immune complexes were used for *in vitro* measurement of PTP activity. Western blot loading controls demonstrate comparable expression of AKAP-Lbc and associated Shp2-WT and Shp2 mutants for all assays. Blots were probed with anti-PKA-phospho-substrate antibody to examine the extent of Shp2 phosphorylation. ***D)*** Quantification of 3 independent *in vitro* PTP assays.

also impaired, the AKAP-Lbc associated PTP activity of this phosphomimetic whether PKA was activated in cells (Fig. 16D). Thus, the T73A/S189A mutations block PKA mediated phosphorylation and inhibition of AKAP-Lbc associated Shp2 whereas the T73D/S189D phosphomimetic mutations lead to impaired AKAP-Lbc associated Shp2 activity under basal conditions.

3. Shp2 T73D/S189D SH2 Domains Display Altered Ligand Specificity.

The location of the PKA phosphorylation sites in relation to Shp2's N-terminal (N) and C-terminal (C) SH2 domains and the PTP domain are shown in context of the structure of Shp2 (Fig. 17). The hydroxyl group of the T73 side-chain participates in a helix capping interaction with the main-chain amide group of E76 at the N-terminus of helix αB in the 'N' SH2 domain. Similarly, the hydroxyl group of the S189 side-chain forms a helix cap with the main-chain amide of D192 at the N-terminus of helix αB in the 'C' SH2 domain. Sequence alignment of the 'N' and 'C' SH2 domains demonstrates that T73 and S189 are located at analogous positions within their respective SH2 domains (Fig. 18A). The two amino acids are structurally equivalent in that both form the first position of an N-capping motif that precedes helix αB of the SH2 domain (Fig. 18B). The most elegant way to explain the effects of dual T73/S189 phosphorylation on Shp2 activity is that phosphorylation in both cases would be expected to break the N-capping interaction and lead to destabilization/melting of helix αB. A stable αB helix is required for phosphotyrosine recruitment (Fig. 18C). Therefore, PKA phosphorylation may reduce Shp2 activation by reducing the binding affinity of

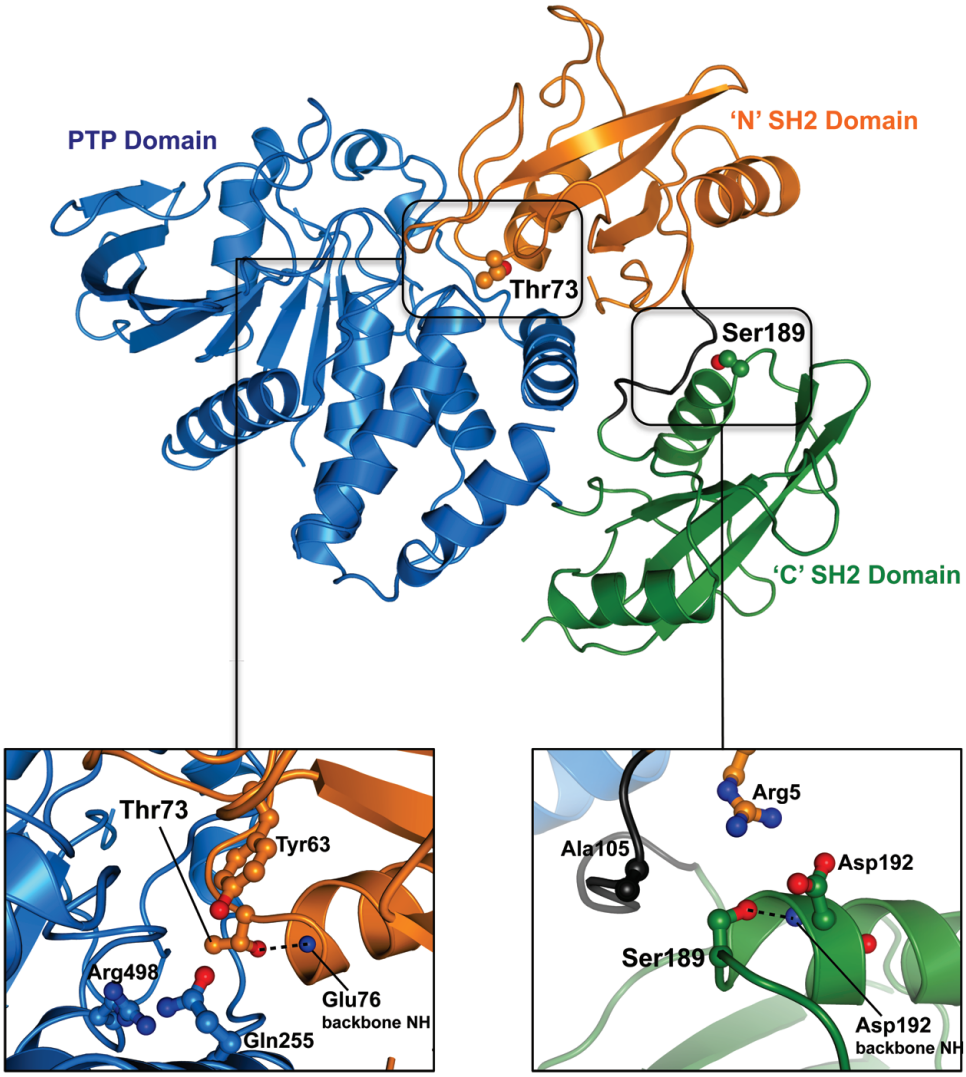

Figure 17. Modeling PKA phosphorylation of Shp2. Cartoon representation of the full-length Shp2 structure (PDB ID 2SHP) showing the location of two PKA phosphorylation sites in relation to the first 'N' SH2 domain (orange), second 'C' SH2 domain (green), and the protein tyrosine phosphatase (PTP) domain (blue). The left-hand close-up box shows the location of Thr73. The hydroxyl group of the Thr73 side-chain participates in a helix capping interaction with the main-chain amide group of Glu76 at the N-terminus of helix αB in the 'N' SH2 domain. Three residues are in the vicinity that have the potential to interact with phospho-Thr73: Arg498 and Gln255 in the PTP domain, and Tyr63 in the 'N' SH2 domain. The right-hand close-up box shows the location of Ser189. The hydroxyl group of the Ser189 side-chain forms a helix cap with the main-chain amide of Asp192 at the N-terminus of helix αB in the 'C' SH2 domain. Phosphorylation at Ser189 likely de-stabilizes two interactions in addition to disrupting the helix cap: a hydrophobic packing interaction between Ser189 and Ala105; and a salt-bridge between Arg5 and Asp192.

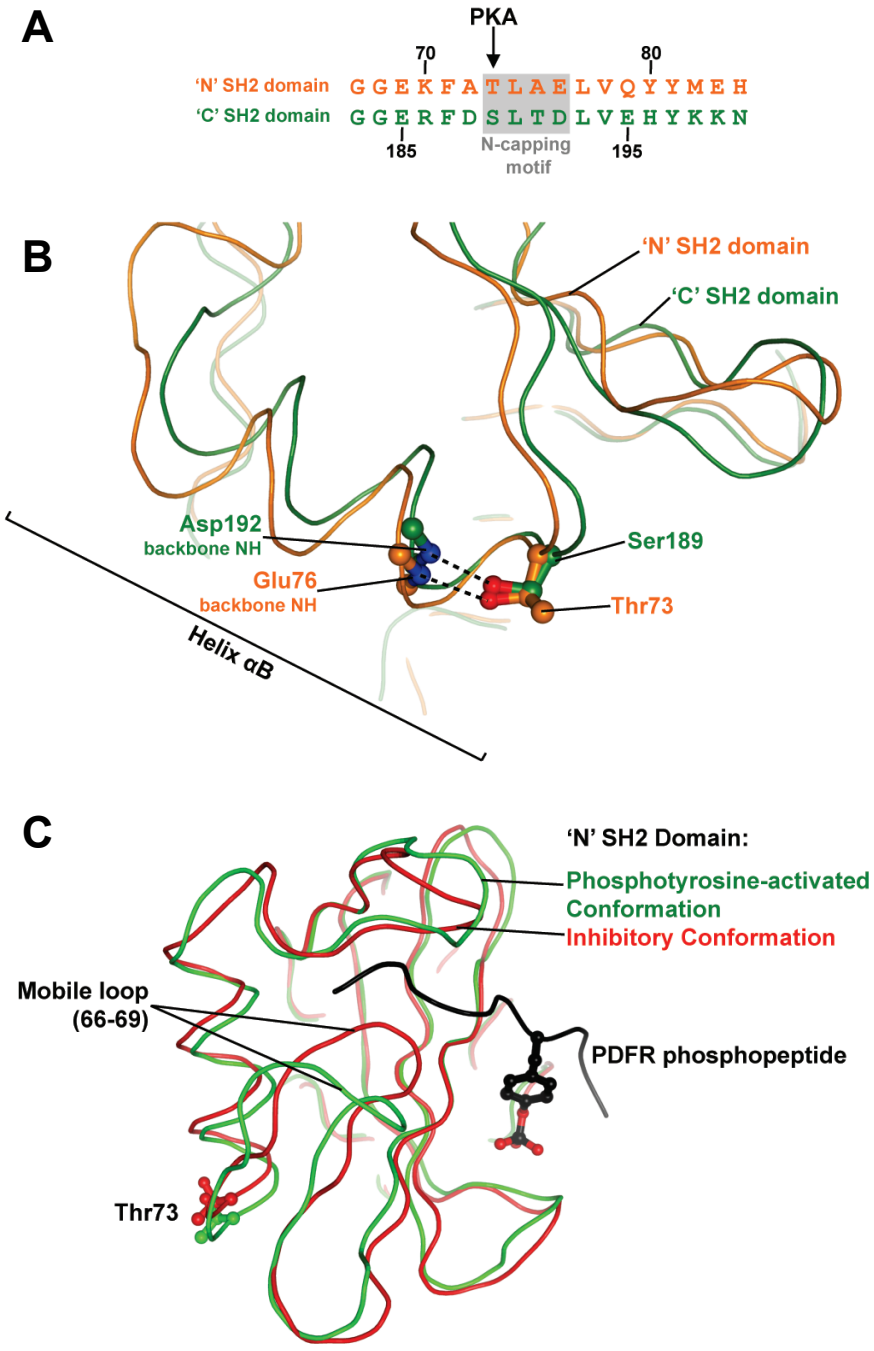

Figure 18. Modeling PKA phosphorylation of Shp2. ***A)*** Sequence alignment of the 'N' and 'C' SH2 domains indicates that Thr73 and Ser189 are at equivalent positions within their respective SH2 domains. Both amino acids form the first position of an N-capping motif that precedes helix αB. ***B)*** Superposition of the 'N' (orange) and 'C' (green) SH2 domains further demonstrates the structural equivalence of the two amino acids. Phosphorylation in both cases can be expected to break the N-capping interaction and lead to destabilization of helix αB. ***C)*** Superposition of the SHP-2 'N' SH2 domain in the presence (green, PDB

ID 1AYA) and absence (red, PDB ID 2SHP) of phosphotyrosine ligand (black) indicates that Ser/Thr at the N-terminal cap of helix αB is not highly dynamic. Furthermore, phosphorylation of Thr73/Ser189 will not sterically hinder recruitment of phospholigand.

phosphotyrosine ligands to each SH2 domain by the same mechanism.

To determine if PKA phosphorylation of Shp2 disrupts binding to tyrosine-phosphorylated ligands, we performed *in vitro* pulldown assays using bacterially-expressed WT or mutant (T73A/S189A or T73D/S189D) GST-tagged Shp2 SH2 domains. The scaffolding adaptor proteins Grb2-associtated-binding protein 1 (GAB1) and Grb2-associated-binding protein 2 (GAB2), as well as PKD1 were co-expressed with constitutively active Src tyrosine kinase in HEK293T cells. Lysates from these cells were subsequently used as inputs for pull-down with GST-SH2. The pull-down experiments demonstrated that the T73A/S189A double mutation has a negligible effect on Shp2 SH2 binding to GAB1 (Fig. 19A & B), GAB2 (Fig. 19C & D), or PKD1 (Fig. 19E & F). Conversely, the T73D/S189D double mutation reduced Shp2 binding to GAB1 by 5.7-fold (Fig. 19A & B; $p = 0.011$), to GAB2 by 2.7-fold (Fig. 19C & D; $p = 0.025$), and to PKD1 by 1.7-fold (Fig. 19E & F; $p = 0.028$).

4. Shp2 Interacts with PKD1.

The Shp2-PKD1 interaction was validated in HEK293 cells by co-precipitation of transfected PKD1 with Shp2 (Fig. 20A) and vice versa. Additionally, edogenous Shp2 was co-purified with endogenous PKD1 from mouse heart extract (Fig. 20B).

5. Shp2 Dephosphorylates PKD1 *In Vitro.*

It has been reported that the tyrosine kinases Src and Abl phosphorylate

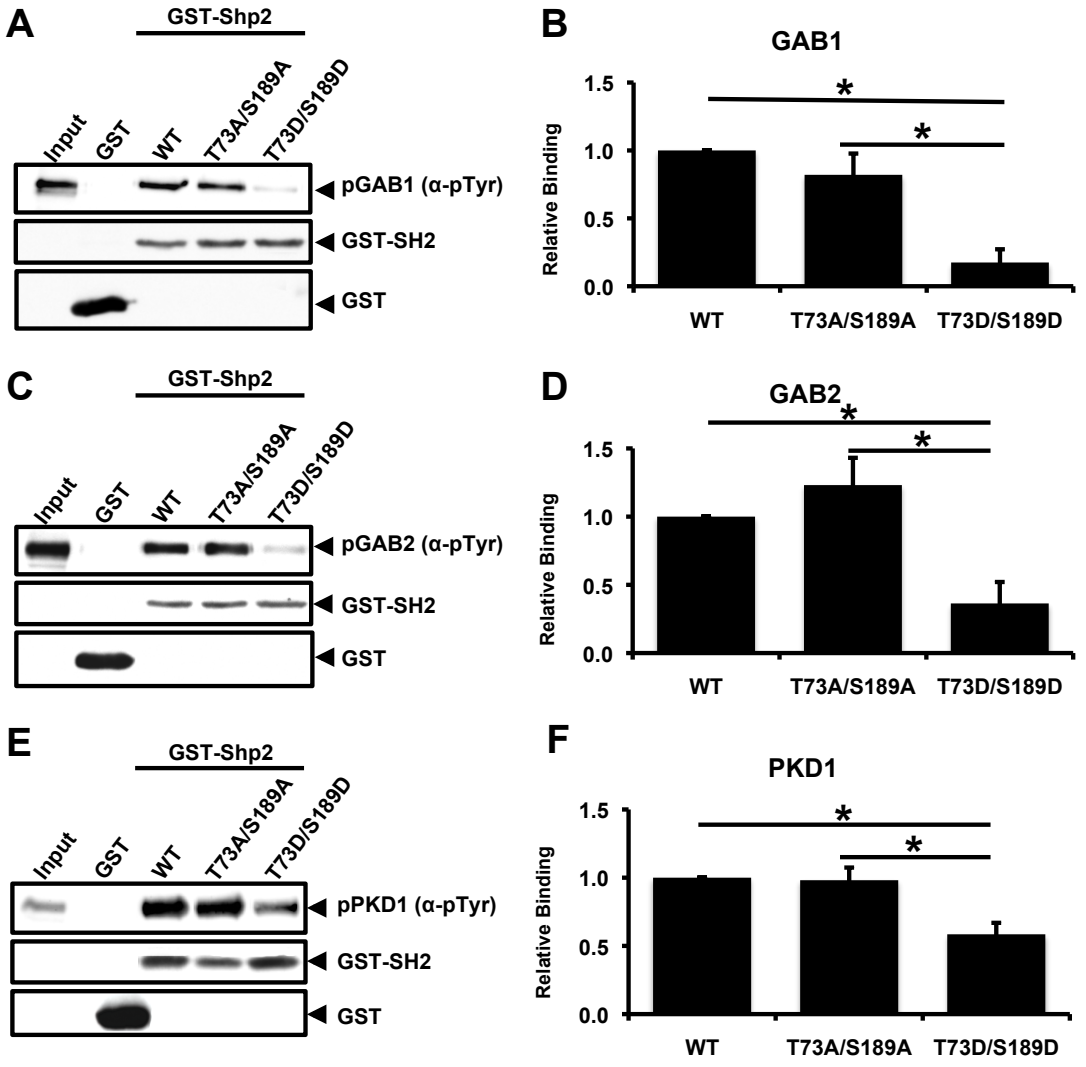

Figure 19. Mutation of residues T73 and S189 to Asp disrupts Shp2 binding to tyrosine-phosphorylated ligands. ***A)*** Association between GAB1 and wild-type Shp2 (WT) or PKA phospho-deficient Shp2 (T73A/S189A) or PKA phosphomimetic Shp2 (T73D/S189D) SH2 domains was assessed by pulldown assays using glutathione-Sepharose bead-bound bacterial fusion proteins. GST-Shp2 SH2 proteins were incubated with lysates from HEK293T cells transfected with Venus-GAB1 and constitutively active Src expression vectors. Proteins bound to beads were immunoblotted for phosphotyrosine (α-4G10) and GST. ***B)*** Band intensities were quantified using ImageJ, and relative binding was determined from the ratios of phosphorylated Venus-GAB1 to GST-Shp2 SH2 input. ***C)*** Association between GAB2 and wild-type Shp2 or mutant Shp2 SH2 domains was assessed as above. GST-Shp2 SH2 proteins were incubated with lysates from HEK293T cells transfected with Venus-GAB2 and constitutively active Src expression vectors. Proteins bound to beads were immunoblotted for

phosphotyrosine and GST. ***D)*** Band intensities were quantified using ImageJ, and relative binding was determined from the ratios of phosphorylated Venus-GAB2 to GST-Shp2 SH2 input. ***E)*** Association between PKD1 and wild-type Shp2 or mutant Shp2 SH2 domains was assessed. GST-Shp2 SH2 proteins were incubated with lysates from HEK293T cells transfected with GFP-PKD1 and constitutively active Src expression vectors. Proteins bound to beads were immunoblotted for phosphotyrosine and GST. ***F)*** Band intensities were quantified using ImageJ, and relative binding was determined from the ratios of phosphorylated GFP-PKD1 to GST-Shp2 SH2 input.

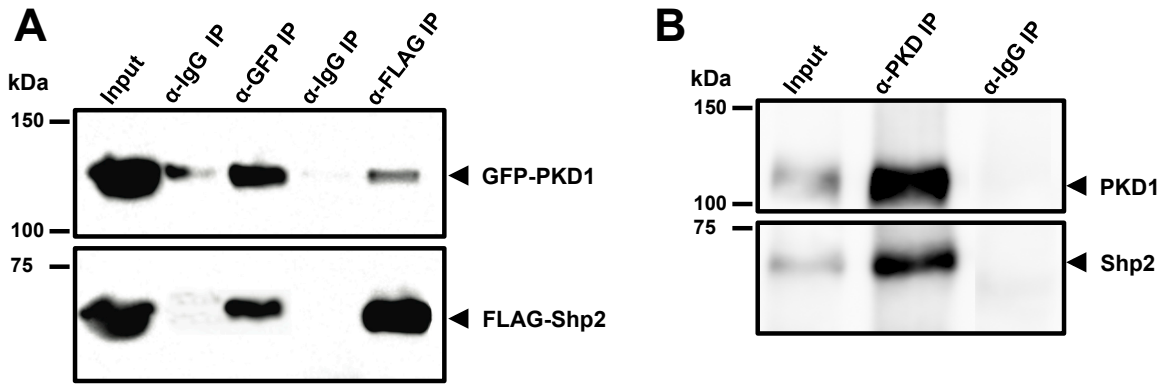

Figure 20. Shp2 co-immunoprecipitates with PKD1. ***A)*** Co-IP of transfected FLAG-Shp2 with GFP-PKD1. HEK293 cells were transfected for expression of FLAG-Shp2 and GFP-PKD1. Shp2 or PKD1 was immunoprecipitated with anti-FLAG or anti-GFP antibody from cell lysates, respectively. Parallel control IPs were performed using anti-IgG antibody from equal amount of lysate. IPs were washed, and the bound proteins were separated by SDS-PAGE and transferred to nitrocellulose. Detection of Shp2 and PKD1 was carried out by immunoblotting. ***B)*** Co-IP of endogenous Shp2 with endogenous PKD1 in the heart. PKD1 was immunoprecipitated from mouse heart extract (5 mg of total protein). Parallel control IgG IPs were carried out using an equal amount of heart extract. IPs were washed, and the bound proteins were separated by SDS-PAGE and transferred to nitrocellulose. Detection of PKD1 and co-purifying Shp2 were carried out by immunoblotting.

PKD1 (Storz et al., 2004a; Storz et al., 2004b; Doppler et al., 2007) suggesting that tyrosine phosphorylation within the pleckstrin homology (PH) domain of PKD1 induces a conformational change, promoting PKCδ recruitment and leading to activation of PKD1. To determine if PKD1 is a substrate for Shp2, we performed an *in vitro* dephosphorylation assay. Following *in vitro* phosphorylation with purified recombinant Src and Abl, PKD1 incubated with constitutively active Shp2 showed decreased levels of tyrosine phosphorylation compared to PKD1 incubated with WT Shp2 (Fig. 21). Conversely, incubation of PKD1 with dominant negative Shp2 enhanced tyrosine phosphorylation of PKD1.

6. Shp2-T73A/S189A Mutant Inhibits Isoproterenol-Induced Tyrosine Phosphorylation of PKD1 in the AKAP-Lbc Complex.

To determine if isoproterenol (ISO)-stimulated PKA activation results in enhanced tyrosine phosphorylation of PKD1 by inhibiting Shp2 in the AKAP-Lbc complex, we utilized HEK293 cells stably expressing the β1-adrenergic receptor (Rapacciuolo et al., 2003). These cells were transiently transfected with vectors encoding AKAP-Lbc, PKD1, and either Shp2 WT, Shp2-T73A/S189A, or Shp2-T73D/S189D. Analysis of AKAP-Lbc-associated PKD1 tyrosine phosphorylation revealed that expression of the Shp2-T73A/S189A mutant abolished the effect of ISO stimulation on PKD1 phosphorylation (Fig. 22). In contrast, expression of the Shp2-T73D/S189D mutant enhanced basal tyrosine phosphorylation of PKD1 in the AKAP-Lbc complex by 2-fold ($p = 0.014$), and treatment with ISO did not further increase the level of PKD1 phosphorylation (Fig. 22).

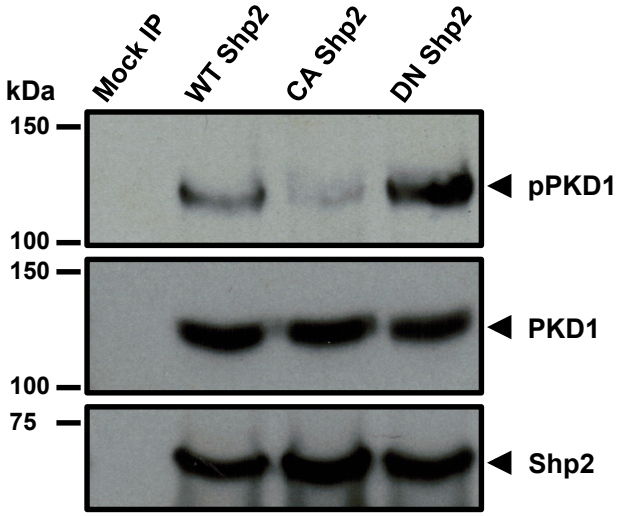

Figure 21. Shp2 dephosphorylates PKD1 *in vitro.* PKD1 was immunoprecipitated from transfected HEK293 cell lysates and phosphorylated *in vitro* using purified Src , Abl, and [γ-^{32}P]. Phosphorylated PKD1 was incubated with FLAG-tagged wild-type (WT), consitutively active (CA) or catalytically inactive, dominant negative (DN) Shp2 purified from HEK293 cells by anti-FLAG IP. PKD1 phosphorylation was measured by autoradiography (top panel).

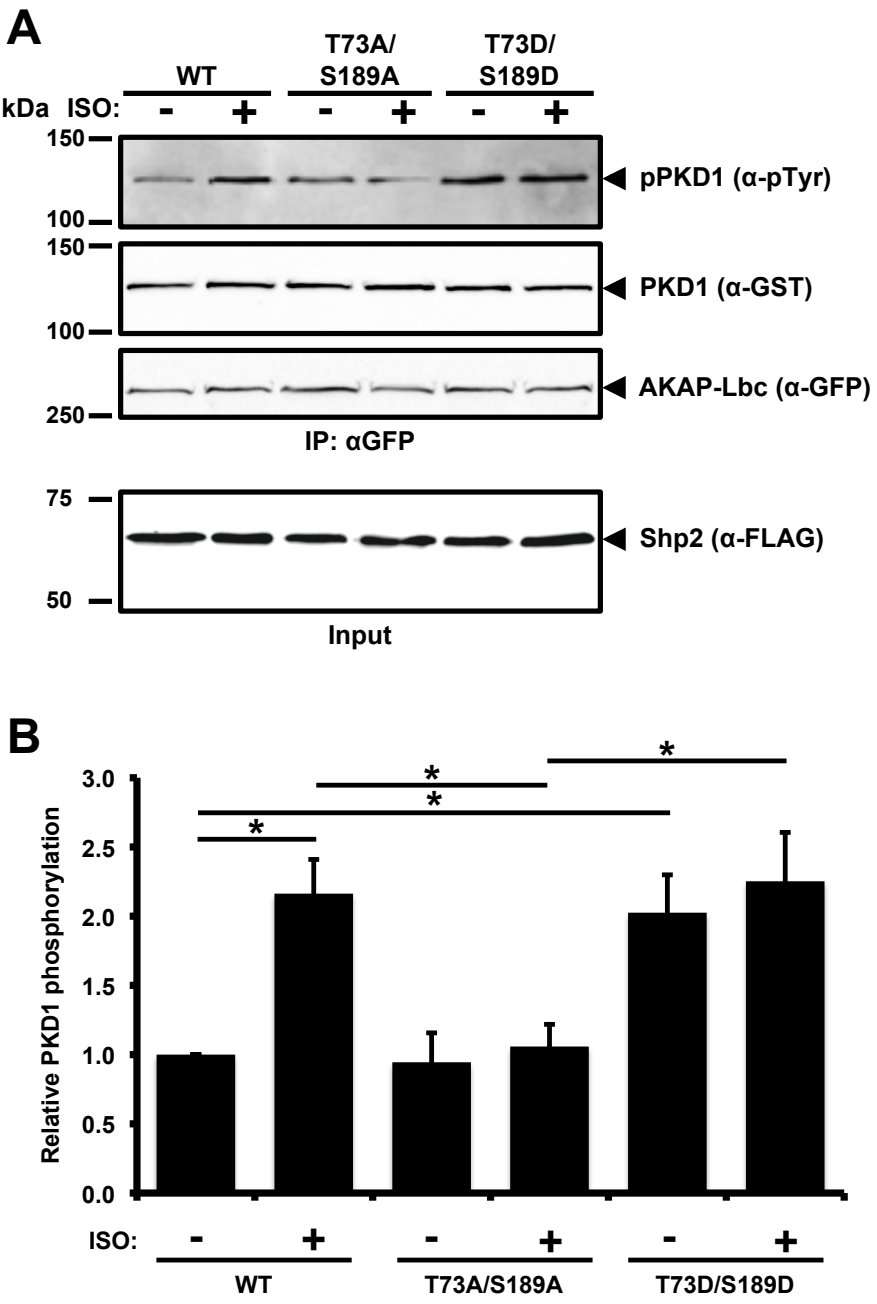

Figure 22. Inhibition of Shp2 by PKA enhances tyrosine phosphorylation of PKD1 in the AKAP-Lbc complex. ***A)*** HEK293 cells stably expressing the β1-adrenergic receptor were transfected with GFP-AKAP-Lbc, GST-PKD1 and either FLAG-Shp2-WT or FLAG-Shp2 T73A/S189A or FLAG-Shp2 T73D/S189D expression vectors. Prior to lysis, cells were treated for 20 min with either DMSO (vehicle) or isoproterenol (ISO) (10 μM) to activate PKA. AKAP-Lbc was isolated from cell lysates using anti-GFP-antibody. IPs were washed, and the bound proteins were separated by SDS-PAGE and transferred to nitrocellulose. Detection of tyrosine-phosphorylated GST-PKD1, total GST-PKD1, GFP-AKAP-

Lbc, and FLAG-Shp2 was carried out by immunoblotting. ***B)*** Band intensities were quantified using ImageJ from 4 independent experiments, and relative tyrosine-phosphorylation of GST-PKD1 was determined from the ratio of tyrosine-phosphorylated GST-PKD1 to total GST-PKD1 in the IP.

7. Overexpression of Shp2 T73A/S189A Inhibits Isoproterenol-Induced Cardiac Hypertrophy.

Chronic β-Adrenergic receptor (β-AR) stimulation by ISO was used as an *in vitro* cellular model to study hypertrophic PKA signaling. Neonatal rat ventricular myocytes (NRVM) were transfected with GFP-tagged vectors expressing either Shp2-WT, Shp2-T73A/S189A, or Shp2-T73D/S189D. In myocytes expressing GFP-Shp2-WT, treatment with ISO increased cellular size by 1.8-fold (Fig. 23; $p < 0.001$). This hypertrophic effect of ISO was inhibited by expression of the Shp2-T73A/S189A. However, expression of the Shp2 phosphomimetic mutant, Shp2-T73D/S189D, increased myocyte size under basal conditions by 1.38-fold ($p = 0.011$), with no further increase in size after ISO treatment (Fig. 23).

D. DISCUSSION

We have previously demonstrated that Shp2 is a component of the AKAP-Lbc complex and that AKAP-Lbc facilitates PKA phosphorylation of Shp2 (Burmeister et al., 2012). Here, we identify two key amino acids in Shp2 that are phosphorylated by PKA, namely Thr73 and Ser189. We demonstrate that phosphorylation of these residues disrupts Shp2 interaction with tyrosine-phosphorylated ligands and inhibits its protein tyrosine phosphatase activity. Furthermore, our data indicate that phosphomimetic mutant of Shp2, Shp2-T73D/S189D, induces a significant increase in the size of neonatal rat cardiomyocytes in the absence of ISO stimulation.

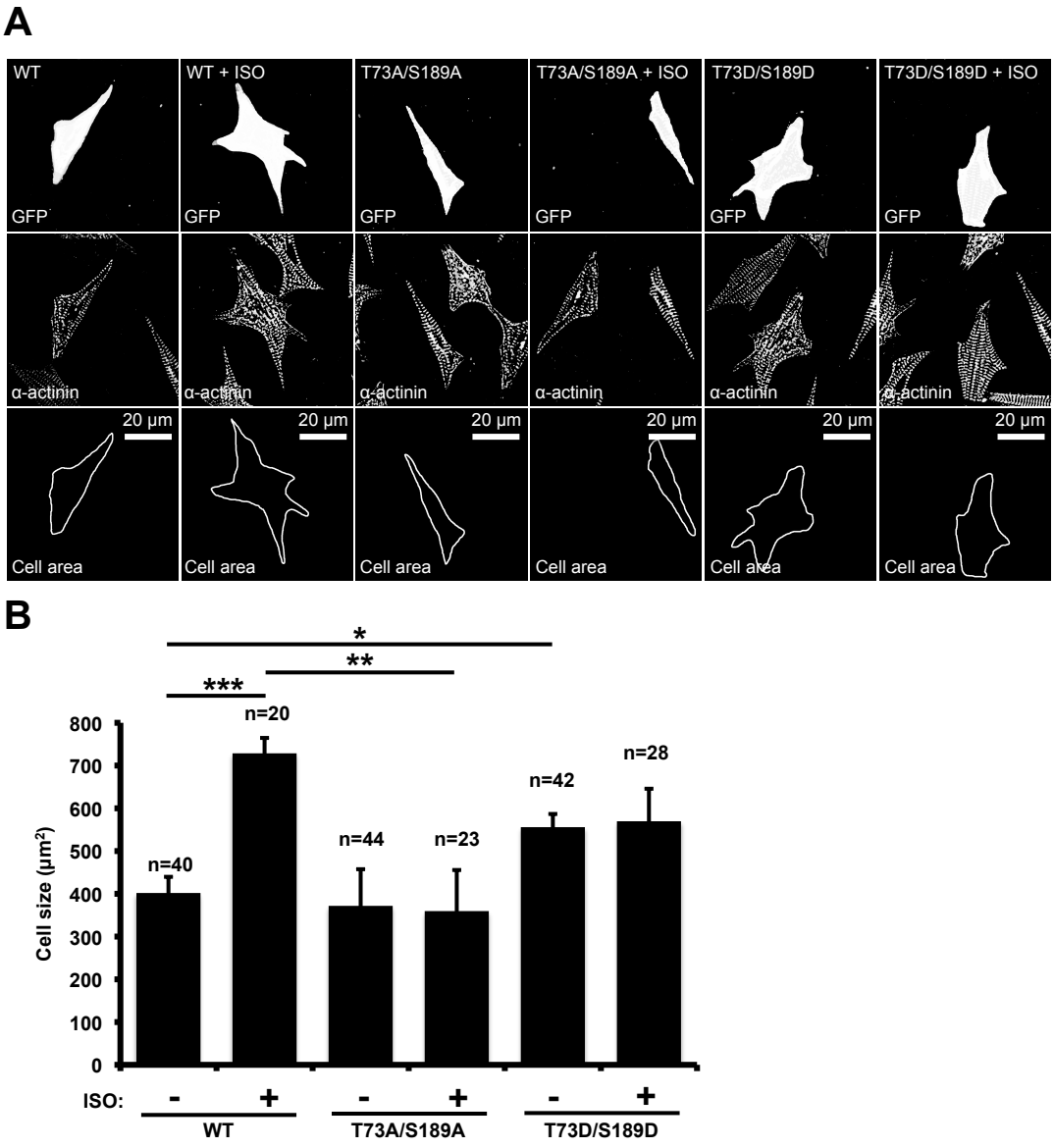

Figure 23. Inhibition of Shp2 by PKA promotes cardiac hypertrophy. ***A)*** NRVMs were transfected with a GFP-tagged wild-type Shp2 (WT) or PKA phospho-deficient Shp2 (T73A/S189A) or PKA phosphomimetic Shp2 (T73D/S189D) expression vector then treated with either DMSO or ISO for 48 hours to induce hypertrophy. Cells were fixed, permeabilized, and stained with α-actinin for cell imaging. ***B)*** Cardiomyocytes with both α-actinin staining and GFP expression were selected for cell size measurements and quantified. Data are expressed as mean ± S.E.M.; the number of cells counted is indicated.

Our previous mapping experiments have shown that Shp2 binds predominately to a central region of AKAP-Lbc (Burmeister et al., 2012). Under conditions of enhanced PKA activity, Shp2 association with the AKAP-Lbc complex facilitates PKA phosphorylation and inhibition of Shp2 activity (Burmeister et al., 2012). The present study demonstrates that under basal conditions, Shp2 specific activity is enhanced within the AKAP-Lbc complex, indicating that Shp2 binding to AKAP-Lbc increases its activity. Taken together, these findings suggest that the AKAP-Lbc complex plays a critical role in regulating Shp2 activity and may mediate a negative feedback mechanism under conditions of increased PKA activity.

Studies of AKAP-Lbc-associated activity of WT and Shp2 mutants revealed the criticality of residues 73 and 189. Mutation of T73/S189 to Ala or Asp abolished phosphorylation by PKA. Additionally, the T73A/S189A mutations block PKA-mediated inhibition of Shp2 activity, whereas the phosphomimetic mutations (T73D/S189D) lead to impaired AKAP-Lbc associated Shp2 activity under basal conditions. Together these results indicate that sites T73 and S189 are phosphorylated by PKA, inhibiting Shp2 activity. Further investigation of the mechanism of PKA-mediated Shp2 inhibition demonstrated that the T73A/S189A mutations do not significantly alter Shp2 SH2 binding to phosphorylated GAB1, GAB2 and PKD1. However, the phosphomimetic T73D/S189D mutations resulted in impaired phosphotyrosine binding by the Shp2 SH2 domains suggesting that PKA phosphorylation of Shp2 modifies its ligand specificity.

We propose that dual T73/S189 phosphorylation affects Shp2 activity by a

mechanism in which phosphorylation at both sites disrupts N-capping interactions with the SH2 domains leading to destabilization/melting of helix αB. A stable αB helix is required for phosphotyrosine recruitment by the SH2 domains. Therefore, PKA phosphorylation may inhibit Shp2 activation by reducing the binding affinity of phosphotyrosine ligands to each SH2 domain by a similar mechanism. Interestingly, Thr73 is inaccessible when Shp2 maintains an inactive conformation (Hof et al., 1998). Therefore, phosphorylation by PKA at this site would presumably only be possible following activation of the phosphatase which we demonstrated herein occurs within the AKAP-Lbc complex under basal conditions.

Another potential contributory mechanism is that phospho-Thr73 interacts with a phosphobinding site on the PTP domain including residues Arg498 and Gln255, thereby stabilizing Shp2 in its inactive conformation. Additionally, phosphorylation of Ser189 may disrupt interactions that link the ‘N’ and ‘C’ SH2 domains, to reduce cooperative binding of phosphotyrosine ligands to the two SH2 domains.

Interestingly, mutation of residue Thr73 to Ile has been observed in Noonan Syndrome and juvenile myelomonocytic leukemia patients (Kratz et al., 2005). The T73I mutation leads to higher basal Shp2 activity by shifting the basal equilibrium constant toward the active state. Activation by insulin receptor substrate 1 (IRS-1) is not greatly affected (Keilhack et al., 2005). This suggests that the branched aliphatic side-chain of Ile cannot be accommodated in the pocket of the phosphatase domain in proximity to Tyr63, Arg498 and Gln255.

Both branches of the Ile side-chain extend to the delta position, whereas both Thr and phospho-Thr have one branch terminating earlier with a methyl group at the gamma position. Ile73 thereby likely disrupts packing between the N-terminal SH2 domain and the phosphatase domain.

PKA, AKAP-Lbc, and Shp2 play prominent roles in signaling mechanisms leading to the induction of cardiac hypertrophy. Mutations resulting in loss of Shp2 catalytic activity are associated with congenital heart defects and cardiac hypertrophy (Kontaridis et al., 2006; Stewart et al., 2010). AKAP-Lbc is also implicated in cardiac hypertrophic signaling and we have shown that AKAP-Lbc-tethered PKA is important for the induction of cardiomyocyte hypertrophy (Carnegie et al., 2008). Consistent with the observation that mice deficient in PKA-Cβ are resistant to hypertrophic stimuli (Enns et al., 2010), current data from our lab indicate specific PKA inhibition abolishes ISO-induced cardiomyocyte hypertrophy, demonstrating the primary contribution of PKA. The results of this study demonstrate that the hypertrophic effect of ISO is inhibited by expression of the Shp2 T73A/S189A mutant, while expression of the T73D/S189D mutant significantly increases myocyte size in the absence of ISO stimulation. These results suggest that PKA phosphorylation of Shp2 at Thr73 and Ser189 contributes to β-AR-induced cardiomyocyte hypertrophy.

We previously demonstrated that AKAP-Lbc facilitates the activation of PKD1 in response to hypertrophic stimuli (Carnegie et al., 2004). Activated PKD1 then phosphorylates class II histone deacetylases to promote their nuclear export, leading to the derepression of the transcription factor MEF2, resulting in

cardiac hypertrophy and tissue remodeling through MEF2-dependent transcription of hypertrophic genes (Carnegie et al., 2008). Others have shown that PKD1 conditional knockout mice are resistant to ISO-dependent hypertrophy (Fielitz et al., 2008). Moreover, it has been reported that tyrosine phosphorylation within the PH domain of PKD1 enhances its catalytic activity (Doppler et al., 2007). Here, we demonstrate that ISO stimulates tyrosine phosphorylation of AKAP-Lbc-associated PKD1 in HEK293 cells stably expressing the β1-adrenergic receptor. The Shp2 T73A/S189A mutant inhibits ISO-induced PKD tyrosine phosphorylation whereas the Shp2 phosphomimetic mutant increases the basal tyrosine phosphorylation of PKD1 in the AKAP-Lbc complex. Taken together, our data suggest that Shp2 in the AKAP-Lbc complex maintains PKD in the dephosphorylated state and that upon PKA activation, AKAP-Lbc facilitates PKA phosphorylation and inhibition of Shp2, thereby allowing for enhanced PKD1 tyrosine phosphorylation and activation.

In conclusion, we have identified a novel mechanism of Shp2 inhibition that may promote cardiac hypertrophy. AKAP-Lbc facilitates PKA phosphorylation of Shp2, which inhibits Shp2 phosphatase activity. We have identified two critical amino acids in Shp2 that are phosphorylated by PKA. We demonstrate that dual phosphorylation of T73/S189 disrupts Shp2 interaction with tyrosine-phosphorylated ligands and inhibits its protein tyrosine phosphatase activity. Interestingly, we and others have observed up-regulation of AKAP-Lbc expression under hypertrophic conditions (Carnegie et al., 2008; Burmeister et al., 2012; Appert-Collin et al., 2007). We speculate that this increase in AKAP-

Lbc levels may facilitate the integration of PKA, Shp2, and PKD1 signaling in the heart (Fig. 24). Although induction of cardiac hypertrophy is a multifaceted process, inhibition of Shp2 activity through AKAP-Lbc-anchored PKA is a previously unrecognized mechanism that may promote cardiac hypertrophy in response to chronic β-adrenergic stimulation.

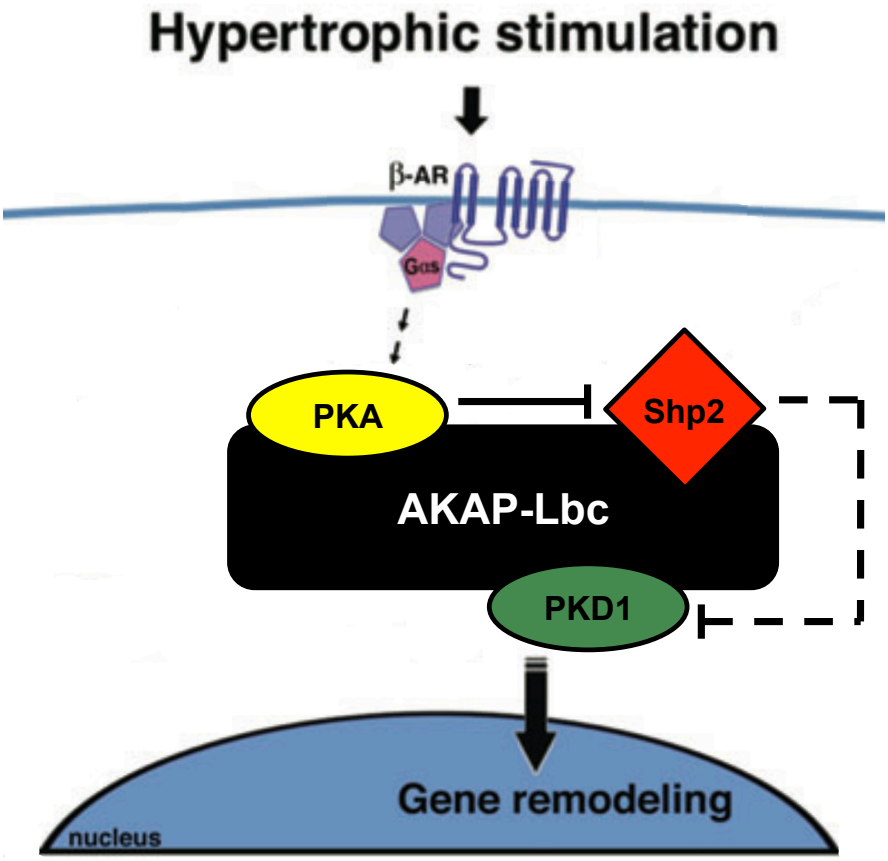

Figure 24. Model showing the role of AKAP-Lbc in the regulation of Shp2 activity and cardiac hypertrophy. AKAP-Lbc coordinates a signaling complex composed of PKA, PKD1, and Shp2 in cardiac myocytes. Chronic activation of the β-adrenergic receptor leads to PKA activation, thereby promoting phosphorylation and inhibition of Shp2 activity. We propose that Shp2 dephosphorylates PKD1 and that inhibition of Shp2 by PKA enhances PKD1 activation and cardiac remodeling.

IV. CONCLUSIONS AND FUTURE DIRECTIONS

Scaffold proteins are vital components of signal transduction within the cell. These proteins facilitate cellular processes by assembling multiprotein complexes that can be rapidly activated, and in many instances, scaffolds are required for complete activation of a signaling pathway. Additionally, scaffolds are capable of preventing crosstalk between related signaling pathways. The focus of this dissertation has been on elucidating the mechanism by which the scaffold protein AKAP-Lbc integrates PKA and Shp2 signaling in the heart to promote cardiac hypertrophy. Therefore, it is important to note that the relative expression of scaffolding proteins is a key variable that modulates signal output. Indeed, for any scaffold there exists a level of expression that achieves optimal signal transduction. A signature characteristic of a scaffold protein is a "bell-shaped" protein titration curve (Levchenko et al., 2000). Different expression levels of AKAP-Lbc may result in varied distributions of AKAP-Lbc and therefore different regulation of signal transduction pathways.

Outside of the results found in this dissertation, surprisingly little is known about how Shp2 activity and localization are affected by phosphorylation during the development of hypertrophy and transition to heart failure. Moreover, my studies were primarily conducted using overexpression systems in HEK293 cells. Therefore, it will be critical to investigate the proposed signaling mechanism using *in vivo* models of hypertrophy. Mice subjected to chronic isoproterenol or angiotensin II infusion or transverse aortic constriction (TAC) to induce hypertrophy and heart failure could be utilized to correlate and precisely establish

how pathological conditions modulate Shp2 activity, localization, and phosphorylation, thereby promoting heart failure. It will be important to determine how each of these aspects is affected during the development of hypertrophy and transition to heart failure. Based on published data and my own experimental experience, mice could be sacrificed at specific time points to investigate Shp2 under conditions of compensatory hypertrophy (1 month), decompensatory hypertrophy (4 months), and heart failure (6 months).

We have identified two key amino acids in Shp2, T73 and S189, that we believe are phosphorylated by PKA. Mutation of these residues abolishes PKA phosphorylation of Shp2; however, these results do not demonstrate that these residues are directly phosphorylated by PKA. Therefore to validate the identified phosphorylation sites, phosphorylation analysis by mass spectrometry should be utilized. Furthermore, to verify these phosphorylation sites in the context of full-length endogenous Shp2, it would be beneficial to develop phospho-specific antibodies to T73 and S189 in Shp2. These antibodies would be extremely useful in subsequent experiments to determine the phosphorylation state and localization of endogenous Shp2 by PKA under pathological conditions leading to heart failure.

Recently, several lines of evidence have implicated PKD1 [the predominant cardiac PKD isoform (Haworth et al., 2000; Bossuyt et al., 2008;)] in critical aspects of cardiac signaling. Under pathological conditions, PKD1 plays an essential part in the alteration of gene expression, remodeling processes, and induction of hypertrophy that underlies heart failure. PKD1 is also pivotal in the

regulation of Ca^{2+}-handling (Koncz et al., 2009; Goodall et al., 2010; Aita et al., 2011), sarcomeric dynamics, and cardiac contractility (Cuello et al., 2007; Bardswell et al., 2010). Interestingly, PKD1 may function in a cardioprotective manner under conditions of oxidative stress, such as myocardial ischemia (Storz et al., 2003; Xiang et al., 2011; Steler et al., 2012). Despite identification of these important cardiac functions, how PKD1 is regulated in the heart is not well known, and it is unclear how PKD1 activity is spatially regulated, terminated, or basally inhibited.

Based on my supporting data, I propose that Shp2 is a “gatekeeper” for PKD1 activation. I hypothesize that Shp2 dephosphorylates PKD1 and is a critical negative regulator of PKD1 activity and that under pathological conditions, Shp2 is inhibited by PKA phosphorylation and/or oxidation by reactive oxygen species, promoting PKD1 activation and cardiac remodeling. AKAP-Lbc functions as a scaffold for PKD1 and Shp2 and may facilitate this interaction, forming a signaling complex that regulates activation of PKD1 and thus cardiac hypertrophy and remodeling.

PKD1 is activated through two different pathways, as shown in Figure 3. These two modes of PKD1 activation explain, in part, the contextual and spatial activation of PKD1, leading to specific downstream signaling. However, key questions remain: 1) How is PKD1 activity regulated in response to different stimuli to generate different (patho)physiological responses? 2) How is PKD1 activity targeted to specific subcellular locations and substrates to regulate distinct (patho)physiological processes? 3) How is PKD1 activity inhibited or

"shut off?" In answer, I propose that Shp2 binds PKD1 phospho-tyrosine residues and dephosphorylates it, leading to PKD1 inactivation. Therefore, it would be interesting to investigate the concept that under pathological conditions of oxidative stress that favor cardiac hypertrophy and remodeling, reduced Shp2 activity (due to catalytic site oxidation and/or phosphorylation by PKA) promotes PKD1 activation: PKD1 becomes tyrosine phosphorylated and activated due to inhibition of Shp2 coupled with stimulation of Src/Abl kinase activity. Specifically, the correlation between PKD1 activity and Shp2 activity under normal and pathological conditions should be determined. Additionally, determining the effect of PKD1 inhibition by Shp2 on localized PKD1 activity and downstream signaling pathways in the heart would be very informative in further defining this signaling mechanism. It would be possible to use phospho-specific antibodies to determine the phosphorylation state of previously identified PKD1substrates, including: L-type calcium channel CaV1.2 (S1884) – relevant in regulation of excitation-contraction (EC) coupling (Aita et al., 2011); phospholamban (S16 and T17) and phospholemman (S63 and S68) – relevant in regulation of Ca^{2+}-handling (Chu et al., 2000; Hagemann et al., 2000; Song et al., 2005; Han et al., 2006); phospho-cTnI (S13/23) and phospho-MyBP-C (S273, S282, and S302) – relevant in regulation of contractility (Cuello et al., 2007; Bardswell et al., 2010); slingshot protein phosphatase (SSH1L) (S937 and S978) – involved in regulation of actin dynamics; may be relevant in regulation of contractility and cardioprotection in response to oxidative stress (Eiseler et al., 2009); Hsp27 (S15 and S82), NFκB/IKKβ (S181) – relevant to cardioprotection in response to oxidative stress

(Storz et al., 2003; Stetler et al., 2012); HDAC5 (S498) – relevant to the induction of cardiac hypertrophy (Vega et al., 2004).

In addition to the characterization PKA-AKAP-Lbc-Shp2 signaling in the heart, it would be fascinating to investigate this mechanism in other cell types. Of particular interest would be a possible role for AKAP-Lbc-Shp2 signaling in the pathophysiology of cancer and tumorigenesis. Cancer is a classification of diseases caused by uncontrolled cell growth and division, which triggers invasion of cells and damage of surrounding tissues (Hanahan et al., 2000). One cause of this dysregulation leading to disease is indiscriminant protein phosphorylation by protein kinases (Scott et al., 2009, Smith et al., 2011). Therefore, precise regulation of kinase signaling is critical for regulating normal cellular processes and maintaing the integrity of signal transduction pathways. One method of achieving the sort of regulation is through preassembled kinase signaling cascades. For instance, the ERK1/2 cascade relays signaling from growth factor receptos in order to promote proliferation of the cell through a Ras-dependent pathway. Activated Ras sebsuquently stimulates Raf kinase, which can then phosphorylate and activate MEK. This signal continues through the cascade, with MEK phosphorylating and activating ERK. ERK, the terminal kinase in the pathway, then phosphorylates effector proteins, resulting in changes in localization, activity, transcription, and translation of downstream targets (Kolch et al., 2005).

Several lines of evidence suggest that modulating the ERK cascade activity can be accomplished by recruiting additional regulatory enzymes. One

manner of accomplishing this modulation is through the establishment of multi-protein networks that are mediated by anchoring and scaffold proteins (Scott et al., 2009; Kolch et al., 2005). A number of scaffolds that regulate MEK/ERK signalling have been identified, including KSR-1 (kinase suppressor of Ras 1), β-arrestins, MP1 (MEK partner-1), MEK kinase 1, and IQGAP1 (Morrison et al., 2003; Kolch et al., 2005). KSR-1 interacts with AKAP-Lbc to form a larger signaling complex that integrates signals from cAMP with the ERK network. AKAP-Lbc binds to KSR-1 and mediates KSR-1 phosphorylation by PKA, leading to continued activation of ERK1/2 (Smith et al., 2010). Interestingly, cAMP elevation inhibits growth factor-dependent ERK signaling in some cell types (Dumaz et al., 2005), yet enhances signal transduction of the Raf/MEK/ERK cascade in others (Smith et al., 2010). PKA and its substrates in the AKAP-Lbc signaling network may play a role in coordinating these divergent responses.

Mutations in Shp2 account for ~35% of juvenile myelomonocytic leukemia (JMML) patient cases and are the primary cause of this disease. However, Shp2 mutations are also seen in patients with B-cell acute lymphoblastic leukemia (B-ALL), acute myelogenous leukemia (AML), and childhood myelodysplastic syndrome (Tartaglia et al., 2003; Bentires-Alj et al., 2004; Loh et al., 2004; Tartaglia et al., 2004). In rare cases, Shp2 mutations are found in solid tumors. Documented instances occurred in patients with melanoma, neuroblastoma, and lung and colon cancers (Bentires-Alj et al., 2004; Nomdedeu et al., 2005).

JMML is a rare myeloproliferative and myelodysplastic disease that affects young children. Its symptoms include macrocytic anemia and expansion and

tissue infiltration of myeloid cells (Emanuel et al., 1996). Mutations associated with JMML are primarily located in the N-SH2 domain of Shp2 and are gain-of-function mutations that interfere with the autoinhibitory interaction with the PTP domain of Shp2 (O'Reilly et al., 2000). Genetic mutations in Shp2, Ras, and the Ras GAP, NF1, are responsible for 85% of JMML cases. This finding suggests that hyperactivation of the MAPK/ERK pathway is critical for the development of JMML (Tartaglia et al., 2003).

Mice expressing mutations in Shp2 that are associated with JMML patients have been generated to investigate the role of Shp2 in tumorigenesis. Knock-in mice that conditionally express the Shp2 D61Y mutant allele in hematopoietic cells evoked a myeloproliferative disorder that resulted in lethality around 45 weeks after induction (Chan et al., 2009). Phenotypes of these mice featured myeloid expansion and tissue infiltration by granulocytes and macrophages, hepatosplenomegaly, anemia, and colony formation by bone marrow spleen cells. Additionally, the Lin(-)Sca1(+)cKit(+) stem cell compartment showed increased stem cell factor-induced colony formation and enhanced levels of Akt and Erk activation (Chan et al., 2009). Moreover, *in vitro* studies using bone marrow-derived hematopoietic progenitors from mice expressing JMML-associated Shp2 mutations (D61Y or E76K) promoted both cell-cycle progression and survival following stimulation with granulocyte-macrophage colony-stimulating factor. These mutations are also associated with increased ERK and Akt signaling (Yang et al., 2008).

The ultimate goal of my experiments was to rigorously define the cardiac

role of AKAP-Lbc and associated signaling components Overall, my data indicate that AKAP-Lbc integrates PKA and Shp2 signaling in the heart and that AKAP-Lbc-associated Shp2 activity is reduced in hypertrophic hearts in response to chronic β-adrenergic stimulation and PKA activation. While induction of cardiac hypertrophy is a complex and multifaceted process, inhibition of Shp2 activity through AKAP-Lbc-anchored PKA is a previously unrecognized mechanism that may promote cardiac hypertrophy. Although there are still many questions to address, it is my hope that the characterization of this signaling mechanism may prove to one day be therapeutically beneficial. It will be important to establish whether interfering with the ability of AKAP-Lbc to integrate signaling will mitigate pathological cardiac hypertrophy, remodeling and ischemic heart disease.

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VI. APPENDIX

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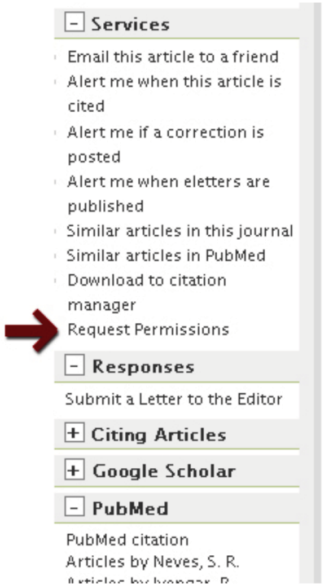

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Student Email Address: bburmei2@uic.edu
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VII. VITA

BRIAN BURMEISTER

EDUCATION:
University of Illinois at Chicago, *Chicago, IL* May 2015
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University of Iowa, *Iowa City, IA* May 2008
B.S. Biochemistry

RESEARCH EXPERIENCE:
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Investigating the coordination of signal transduction by scaffolding and subcellular targeting of proteins and subsequent regulation of cardiovascular disease pathogenesis

Research Assistant May 2008-Aug. 2009
University of Iowa Department of Dermatology, Iowa City, IA
Investigated the role of Collagen XVII in keratinocyte proinflammatory signaling pathways in blistering skin diseases

HONORS AND AWARDS
American Heart Association Pre-doctoral Fellowship, Jan. 2014-May 2015
TL1 Education for Clinical and Translational Scientists Pre-doctoral Fellowship, Aug. 2013-Present
American Society for Biochemistry and Molecular Biology Travel Award, Apr. 2013
UIC CCTS Pre-doctoral Translational Research Award, Apr. 2012-Aug. 2013
UIC Department of Cardiology NIH T32 Training Grant, Aug. 2011-Aug. 2013
Pharmacological Sciences Training Program Grant, Aug. 2010-Aug. 2011

PEER-REVIEWED PUBLICATIONS:
Burmeister BT, Wang L, Gold MG, Skidgel RA, O'Bryan JP, Carnegie GK. Protein Kinase A (PKA) phosphorylation of Shp2 inhibits its phosphatase activity and modulates ligand specificity. *J Biol Chem.* 2015 May 8;290(19):12058-67.

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Van den Bergh F, Eliason SL, **Burmeister BT**, Giudice GJ. Collagen XVII (BP180) modulates keratinocyte expression of the proinflammatory chemokine, IL-8. *Exp Dermatol*. 2012 Aug.; 21(8):605-11.

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CONFERENCE PRESENTATIONS:

"PKA regulates Shp2 activity: A role in pathological cardiac hypertrophy and heart failure."
Invited seminar at Association for Clinical and Translational Science Annual Meeting, Washington, D.C., April 2014

"A-kinase anchoring protein (AKAP)-Lbc coordinates protein kinase A (PKA) phosphorylation and inhibition of Src homology 2 domain-containing phosphatase 2 (Shp2)."
Research presented at Experimental Biology Annual Meeting, Boston, MA, April 2013 National Pre-doctoral Meeting, Rochester, MN, May 2013
Chicago Symposium on Cell Signaling, Chicago, IL, May 2013
American Heart Association Research Symposium, Chicago, IL, Sept. 2013
International Meeting on Anchored cAMP Signaling Complexes, Denver, CO, October 2013

"Regulation of protein kinase D (PKD) by the tyrosine phosphatase Shp2."
Research presented at Chicago Symposium on Cell Signaling, Chicago, IL, May, 2012