BETA-ADRENERGIC RECEPTOR SIGNALING

IN GRAVIN KNOCKOUT MICE

A Dissertation Presented to the

Department of Pharmacological and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment of the

Requirement for the Degree

Doctor of Philosophy

In

Pharmacology

By

Ashley Guillory

December 2012

Beta-Adrenergic Receptor Signaling in Gravin Knockout Mice

_____*signature*_____
Ashley N. Guillory, B.S.

_____*signature*_____
Bradley K. McConnell, Ph.D.

_____*signature*_____
Mustafa Lokhandwala, Ph.D.

_____*signature*_____
Richard Bond, Ph.D.

_____*signature*_____
Adebayo Oyekan, Ph.D., D.V.M

_____*signature*_____
Ming-He Huang, M.D., Ph.D.

_____
F. Lamar Pritchard, Ph.D., R.Ph
Dean, College of Pharmacy
University of Houston

BETA-ADRENERGIC RECEPTOR SIGNALING

IN GRAVIN KNOCKOUT MICE

An Abstract Presented to the

Department of Pharmacological and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment of the

Requirement for the Degree

Doctor of Philosophy

In

Pharmacology

By

Ashley Guillory

December 2012

Abstract

Beta-adrenergic receptors (β-AR) signal through protein kinase A (PKA)-dependent phosphorylation, which affects calcium (Ca^{2+}) homeostasis to increase contractility. PKA binds to A-kinase anchoring proteins (AKAPs) which localize PKA and its substrates to defined subcellular locations. Gravin, an AKAP, targets PKA, protein kinase C (PKC), and other signaling molecules to the β_2-AR. Gravin mediates desensitization/resensitization of the receptor by facilitating its phosphorylation by PKA and PKC. However, the role of gravin in β-AR mediated regulation of cardiac function is unclear.

We measured various aspects of the β-AR signaling pathway in wild-type (WT) and gravin knockout (KO) mice after acute β-AR stimulation by the nonspecific agonist isoproterenol (ISO). Using echocardiographic analysis, we observed that left ventricular fractional shortening and cardiac output were increased in KO animals compared to WT animals before and after ISO treatment. cAMP production, PKA activity and phosphorylation of phospholamban and troponin I was comparable in WT and KO hearts regardless of treatment. However, basal cardiac myosin binding protein C phosphorylation at position 273 was significantly increased in KO versus WT hearts. Additionally, heat shock protein 20 (Hsp20) was significantly more phosphorylated in KO versus WT hearts, following ISO treatment.

We also measured the β-AR signaling pathway following chronic ISO stimulation. Using echocardiography analysis, we observed that cardiac contractility remained significantly increased in KO mice after chronic ISO treatment compared to WT mice. Additionally, the absence of gravin blocked the ISO-induced desensitization of β_1-ARs seen in chronically stimulated WT hearts while β_2-AR density was significantly increased in ISO treated KO hearts. Furthermore, we observed alterations in PKA phosphorylation of phospholamban, troponin I, myosin binding protein C and protein phosphatase-1 inhibitor-1 that may have a positive effect on cardiac function.

Our results demonstrate that gravin does play a substantial role in modulating β-AR signaling by increasing baseline cardiac function as well as continuing to augment contractility in response to acute and chronic β-AR stimulation. Furthermore, the absence of gravin appears to abrogate many of the maladaptive changes associated with chronic β-AR stimulation. Thus, we propose that gravin may be a viable therapeutic target to modulate cardiac function in disease states.

Table of Contents

LIST OF ABBREVIATIONS

AC	adenylyl cyclase
AKAP	A-kinase anchoring protein
β-AR	beta-adrenergic receptor
C	catalytic subunit of PKA
Ca^{2+}	calcium
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	3'-5'-cyclic adenosine monophosphate
cMyBPC	cardiac myosin binding protein C
CO	cardiac output
EC-coupling	excitation-contraction coupling
EF	ejection fraction
FS	fractional shortening
G_i	inhibitory GTP-binding protein
G_s	stimulatory GTP-binding protein2
GRK2	G-protein coupled receptor kinase 2
Hsp20	heat-shock protein 20
HW:BW	heart-to-body-weight ratio
IPP-1	protein phosphatase-1 inhibitor 1
ISO	isoproterenol

IVSd	intra-ventricular septum at diastole
IVSs	intra-ventricular septum at systole
KO	gravin knockout
LV	left ventricular
LVPWd	left ventricular posterior wall diameter during diastole
LVPWs	left ventricular posterior wall diameter during systole
NCX	sodium/calcium exchanger
PDE4D	phosphodiesterase type 4D
PKA	protein kinase A
PKC	protein kinase C
PLB	phospholamban
PP2B	protein phosphatase 2B
R	regulatory subunit of PKA
RyR	ryanodine receptor
SERCA2a	sarcoplasmic reticulum Ca^{2+} ATPase
SR	sarcoplasmic reticulum
SV	stroke volume
TnI	troponin I
WT	wild-type

LIST OF TABLES

LIST OF FIGURES

1. Introduction and Statement of Problem

Heart failure is the leading cause of death in the United States and is a major cause of death globally. The national cost of treating patients with heart failure was more than 37.2 billion in 2009 and is expected to increase exponentially as the percentage of the aged population increases.[1] Heart failure is defined as a chronic disease in which the heart is not able to pump sufficient oxygenated blood to the meet the demands of the body to maintain normal physiological function. This insufficiency is primarily due to reduced cardiac contractility. Both heart failure and aging is associated with reduced cardiac contractility.[2] Contractility can be enhanced via stimulation of β-adrenergic receptors (β-ARs). However, chronic β-AR stimulation, as occurs in heart failure, results in reduced responsiveness of the heart to stimulation due to downregulation and desensitization of the β-ARs.[3, 4]

The molecular mechanisms of cardiac dysfunction as a result of heart failure are multi-factorial and involve a plethora of kinases, phosphatases and other signaling molecules.[5] Desensitization of the β-ARs leads to significant disruption of the activity of downstream signaling molecules such as protein kinase A (PKA).[5] Reduced PKA activity alters its substrate phosphorylation: some substrates are hypophosphorylated while others are hyperphosphorylated. For example, in heart failure, PKA phosphorylation of phospholamban is decreased while PKA phosphorylation of the ryanodine receptor is increased.[6-8] Other kinases such as protein kinase C (PKC) and calcium/calmodulin

dependent kinase II (CaMKII) display enhanced activity following chronic β-AR stimulation.[5, 6, 9] Additionally, there is altered expression of proteins involved in calcium (Ca^{2+}) handling and β-AR receptor function such as G-protein coupled receptor kinase 2 (GRK2), the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) and the inhibitory GTP-binding protein (G_i).[10-13] Upregulation of phosphatase expression further disrupts the phosphorylation and subsequent action of key proteins involved in modulating cardiac contractility.[14] Taken together, these perturbations lead to alterations of Ca^{2+} homeostasis that further depress cardiac function.[15] In addition to depressed cardiac function, chronic β-AR stimulation causes the upregulation of the transcription of pro-hypertrophic genes. This increase in pro-hypertrophic signaling leads to gross morphological changes via hypertrophy and remodeling that exacerbate the diminished condition of the heart.[16-18]

Conventionally, inotropic therapies such as β-AR agonists and phosphodiesterase (PDE) inhibitors have been the method of choice for treating cardiac dysfunction. The mechanism of action of these therapies primarily focuses on increasing the availability of Ca^{2+} or the responsiveness of the myofilaments to Ca^{2+} by either increasing cAMP production or decreasing its breakdown. Although these therapies are initially beneficial, their side effects of tachycardia and ventricular arrhythmia often result in death.[3, 19-21] These effects are primarily due to sustained increases in intracellular Ca^{2+} levels, which cause cardiotoxicity and induce cardiomyocyte apoptosis.[22] Despite the usage of "newer" drugs such as angiotensin converting enzyme inhibitors and β-AR antagonists, patient

survival with long-term treatment is less than optimal. Furthermore, none of the current therapies rescue the failing heart and revert the maladaptive changes associated with cardiac dysfunction.[3] Thus, alternative treatments that can avoid some of the dangerous side effects seen with therapies such as β-AR agonism are desirable.

One such alternative target is to enhance the Ca^{2+} sensitivity of the myofilaments rather than increasing intracellular Ca^{2+} such as by altering PKA phosphorylation of myofilament proteins.[23] Gravin, an A kinase anchoring protein, integrates several signaling cascades thought to be involved in the reduction of contractility, the progression of heart failure and cardiac hypertrophy by scaffolding key components of these cascades such as PKA, PKC, GRK2 and PDE4D.[24, 25] Therefore our *central hypothesis* is that disruption of gravin/PKA interactions will increase cardiomyocyte signaling by blocking gravin regulated receptor desensitization in normal hearts and rescue the impaired contractile response seen in the presence of chronic β-AR stimulation. Furthermore, we propose the inhibition of gravin's association with the β_2-AR as a putative drug target for the treatment of reduced contractility.

2. Review of Literature

The heart is responsible for pumping oxygenated blood to supply oxygen and nutrients to the body's organs and peripheral tissues. This process is accomplished via the contraction of cardiomyocytes, which is initiated by action potentials conducted from the sinoatrial node. Excitation-contraction (EC) coupling is the process whereby excitation of myocytes, due to entry of an action potential, is translated into myocyte contraction. This coupling is required for the heart to expel oxygenated blood to perfuse the tissues of the body.[2]

Ca^{2+}, a second messenger whose entry into the cardiac myocyte, is essential for both the excitation of the cell and the resulting contraction. In fact, calcium directly activates the contraction machinery.[2] G-protein coupled receptors such as β-ARs play a prominent role in the modulation of Ca^{2+} entry and contraction. This modulation is accomplished via the activation of downstream signaling cascades that regulate Ca^{2+} homeostasis.[26] Changes in Ca^{2+} homeostasis result in contractile dysfunction and can lead to cardiac myopathy. Heart failure and arrhythmia are examples of pathological syndromes associated with mishandling of calcium.[27]

2.1 Excitation-Contraction Coupling.

Excitation-contraction coupling initiates with the depolarization of the myocytes. The depolarization activates voltage-gated L-type Ca^{2+} channels allowing influx of extracellular Ca^{2+} into the cytosol. As the intracellular Ca^{2+} level rises, it triggers the

release of Ca^{2+} from the sarcoplasmic reticulum (SR) via its binding to ryanodine receptors (RyR). Once the intracellular Ca^{2+} concentration is sufficiently increased, calcium binds to troponin C, which activates contraction of the myofilaments.[2] Ca^{2+} binding to troponin C changes the formation of the troponin complex, removing tropomyosin from covering the myosin binding site, which permits the cross-bridging of myosin to actin. Myosin binds to actin and slides the actin to shorten the sarcomere length and effectuate contraction. The strength of the contraction can be altered by changing the amplitude or duration of the calcium transient and/or by altering the sensitivity of the myofilaments to calcium.[2]

2.2 Relaxation.

In order for relaxation to occur, Ca^{2+} must be removed from the contraction mechanism. Contraction ends as the intracellular Ca^{2+} concentration is lowered to basal levels by four main mechanisms: reuptake of Ca^{2+} into the SR by the SERCA2a, efflux of Ca^{2+} through the sarcolemmal sodium/calcium exchanger (NCX), uptake of Ca^{2+} into the mitochondria via the mitochondria Ca^{2+} uniporter and efflux of Ca^{2+} through the sarcolemmal Ca^{2+}-ATPase. The majority of Ca^{2+}, approximately 98%, is removed via the SERCA2a and NCX. The mitochondrial Ca^{2+} uniporter and the sarcolemmal Ca^{2+}-ATPase are known as the slow systems and only remove 1-2% of the Ca^{2+}.[2]

2.3 Beta-adrenergic receptor signaling in the normal myocardium

EC-coupling can be modulated via agonist-induced receptor activation of kinase signaling cascades. β-AR signaling is the primary mediator of cardiac contractility and function as activation of β-ARs results in increased inotropy, chronotropy and lusitropy.[3] Norepinephrine and epinephrine released from the sympathetic nerve terminals and the adrenal gland, respectively, bind to β-ARs to activate their partnering GTP-binding proteins (G-proteins). Stimulatory G-proteins (G_s) enhance the activity of adenylyl cyclase (AC) thereby increasing the production of 3'-5'-cyclic adenosine monophosphate (cAMP). cAMP is the activator of PKA, which is the primary effector of β-AR signaling.[2]

2.3.1 Beta-adrenergic receptors

There are at least three subtypes of β-ARs that have been identified in the heart: β_1, β_2 and β_3. These subtypes differ in their quantities, ligand binding affinity, subcellular locations, coupling to G-proteins as well as in the signaling pathways activated. The total number of cardiac β-ARs consists of primarily β_1-AR (70-80% of the total), which couples to G_s to positively affect AC activity. Less than 30% of the total β-ARs are β_2-ARs, which can couple to both G_s and inhibitory G-proteins (G_i).[6] Coupling of the β_2-AR to G_i leads to a reduction in AC activity, activation of phosphatidylinositol 3'-kinase and the recruitment of phosphodiesterase to the plasma membrane, essentially the opposite of coupling to G_s.[28-30] β_3-ARs are present in the heart at very low levels (less than 10% of

total) and mediate negative inotropic effects through a G_i/nitric oxide pathway, although, there is some evidence that the receptor can also couple to G_s.[6, 31] Additionally, β_1-ARs have been shown to be evenly distributed along the plasma membrane while β_2-ARs are primarily found in the caveolae.[32] Furthermore, differential effects can be achieved with activation of β_1- versus β_2-adrenergic receptors. Activation of β_1-AR produces inotropic, chronotropic and lusitropic responses whereas activation of β_2-ARs specifically enhances calcium influx. This specific action of β_2-ARs appears to occur because the receptor can be found directly complexed with the L-type calcium channel along with Gs, adenylyl cyclase, PKA, and protein phosphatase 2A.[33]

Recent advances using specialized biosensors have indicated another major difference in β_1- versus β_2-AR signaling: the duration and reach of action. β_2-AR activation was sufficient to enhance PKA activity but this enhancement was very transient and not as long-lasting as that induced by β_1-AR activation. Additionally, selective β_2-AR activation was not sufficient to alter phosphorylation of phospholamban (PLB), a protein located at the SR, whereas β_1-AR activation enhanced phosphorylation of both PLB and troponin I (TnI).[34] These data confirms an earlier report in canine cardiomyocytes that, while activation of β_2-ARs enhanced heart rate and contractility, these changes were not reflected by increased PKA activity and substrate phosphorylation. Indeed, the authors determined that PLB, TnI and cardiac myosin binding protein C (cMyBPC) phosphorylation remained similar to basal levels.[35]

2.3.2 Protein kinase A

Protein kinase A is a threonine/serine kinase that phosphorylates a wide assortment of proteins. The kinase is a heterotetramer consisting of two catalytic (C) and two regulatory (R) subunits. There are three C subunit isoforms, α, β and γ, and four R subunit isoforms, RIα, RIβ, RIIα and RIIβ. Cα, Cβ, RIα and RIIα are the predominant isoforms and are expressed in most tissues.[36] Each regulatory subunit is capable of binding two cAMP molecules. Upon binding cAMP, the regulatory units activate and release the catalytic subunits. The freed catalytic subunits disperse to phosphorylate PKA's substrates.[36]

PKA substrate phosphorylation is required for many cellular processes including metabolism, gene expression and contraction.[37] In the heart, β-AR signaling activates PKA RIIα, which phosphorylates many of the components of the EC-coupling mechanism such as PLB, TnI and the RyR to modulate their function and/or activity.[38-41] The phosphorylation of these and other related proteins by PKA, increases the Ca^{2+} current, the content, uptake and release of Ca^{2+} from the SR and the dissociation of Ca^{2+} from the myofilaments to facilitate both the contraction and the relaxation of the heart.[2, 26]

2.3.3 Role of PKA in Excitation-Contraction Coupling.

PKA phosphorylation of PLB disinhibits the SERCA2a allowing faster reuptake of Ca^{2+} and dissociation of Ca^{2+} from myofilaments.[26] Additionally, PKA phosphorylation of TnI assists in the removal of calcium from the myofilaments and augments

relaxation.[42] Faster uptake of Ca^{2+} into the SR proportionally increases the speed of relaxation. However, this action of PKA simultaneously allows for greater load of Ca^{2+} into the SR, which suggests that there would be more Ca^{2+} released into the cytosol and thus stronger contraction when Ca^{2+} release is next triggered. Moreover, this increased SR Ca^{2+} load also increases the sensitivity of the RyR to the intracellular concentration of Ca^{2+}, which can lead to spontaneous SR Ca^{2+} release.[2]

PKA phosphorylation of TnI inhibits actin's myosin binding site and reduces myofilament Ca^{2+} sensitivity, which causes a reduction in force. Nevertheless, this reduction in Ca^{2+} sensitivity can be overcome by enhancing Ca^{2+} entry and release. PKA phosphorylation of L-type Ca^{2+} channels increases the opening probability of the channel as well as increases the Ca^{2+} influx through the channel. The role of PKA phosphorylation of the RyR is still controversial but some studies have shown that PKA phosphorylation can increase the open probability of the receptor. Additionally, RyR phosphorylation may also modulate the responsiveness of SR Ca^{2+} release to the influx of Ca^{2+} through L-type Ca^{2+} channels.[2]

PKA substrate phosphorylation not only initiates or activates signal transduction but is also responsible for termination of signal transduction. PKA can cause the activation of phosphodiesterases that reduce cAMP levels as well as phosphatases that dephosphorylate PKA's substrates[43].[44] Additionally, PKA has been shown to phosphorylate the β_2-AR, which has been shown to be involved in the switching of the

receptor's coupling from G_s to G_i. Therefore, PKA acts as a modulator of its own activity[28, 45, 46]

2.3.4 A kinase anchoring proteins

As mentioned previously, PKA activity is required for many aspects of normal cell function and phosphorylates a vast number of substrates. PKA is localized to its substrates and different intracellular compartments by A-kinase anchoring proteins (AKAPs) that assist in the specificity and diversity of PKA dependent signaling. Over 70 different AKAPs have been identified and, although there is very little sequence homology between them, all AKAPs have three main properties: 1) a conserved amphipathic helix that interacts with PKA's regulatory subunits; 2) unique targeting domain and 3) binding sites for PKA substrates.

Most AKAPs bind to the RII subunit of PKA but there are a few AKAPs that bind to the RI subunits as well. Moreover, autophosphorylation of the RII subunit results in an enhanced RII/AKAP interaction.[47] The targeting of the PKA/AKAP complex is a result of various types of protein and lipid interactions that occur through the unique targeting domains located inside of each AKAP, such as Dbl and Pleckstrin homology domains.[48] Furthermore, AKAPs are often complexed with a phosphatase that can dephosphorylate PKA substrates and/or a phosphodiesterase, which breaks down cAMP to stop its activation of PKA. AKAPs can also mediate the integration of PKA signaling with other signaling pathways by including signaling proteins of other pathways in their scaffold.

For example, several AKAPs such as gravin and AKAP-Lbc have been shown to contain protein kinase C (PKC) in their complexes, whose signaling often opposes that of PKA. Finally, AKAPs have also been shown to form even larger complexes with other scaffolding molecules, such as the membrane associated guanylate kinase scaffolding protein.[49] Indeed, *in vitro* work by C.C. Malbon's group demonstrates the ability of gravin to form homo-oligomers as well as hetero-oligomers with AKAP5.[50]

Of the 70 currently identified AKAPs, more than 14 of these are expressed in both rodent and human hearts including AKAP 15/18, mAKAP, yotiao and gravin. While all of these are found concurrently in the heart, each localizes PKA to a different area of the cell. Additionally, AKAPs are heavily involved in modulation of EC coupling, both in generating the cardiac action potential as well as in Ca^{2+} handling and the movement of Ca^{2+} in the cardiac myocyte.[51] For example, AKAP15/18 colocalizes with L-type Ca^{2+} channels and mediates PKA phosphorylation of the channel, which leads to enhanced channel activity and increased Ca^{2+} current.[52] AKAP79 can also engineer the phosphorylation of L-type Ca^{2+} channels and it is thought that AKAP79 can substitute for AKAP15/18. AKAP79 has also been shown to be involved in the local effects of periodic opening of the L-type Ca^{2+} channels to enhance vascular tone.[53] mAKAP is found on the nuclear envelope and the SR and is involved in regulating the activity of RyR to facilitate Ca^{2+} induced Ca^{2+} release from the SR for contraction.[54]

The importance of PKA/AKAP interactions in mediating cardiac function has been demonstrated through studies using Ht31, a peptide that competes with all AKAPs to bind to PKA RII subunits. The usage of Ht31 in cardiomyocytes resulted in altered phosphorylation of PKA substrates such as TnI and cMyBPC following β-AR stimulation with isoproterenol (ISO).[55] Additionally, McConnell et al., showed that Ht31 disruption of PKA/AKAP interactions resulted in reduced phosphorylation of PLB and the RyR. However, despite these changes in PKA substrate phosphorylation, contractility was enhanced in the presence of β-AR stimulation.[56] Furthermore, disruption of specific AKAP/PKA interactions has also been shown to alter β-AR mediated cardiac function. For example, disruption of AKAP15/18δ's interaction with PLB negatively affected β-AR stimulated phosphorylation of PLB and decreased Ca^{2+} re-uptake. Thus, alterations in PKA/AKAP interactions can have profound effects on β-AR signaling and cardiac function.

Additionally, recent studies have shown that disruption of normal AKAP complexes or function can play a role in the development of cardiac disease and dysfunction. Disruption of yotiao's interaction with KCNQ1, a voltage-gated potassium channel, has been implicated in the development of Long-QT syndrome, a disorder characterized by prolonged repolarization of the action potential, which can lead to arrhythmia and sudden cardiac death.[57] Another example of the importance of AKAPs in regulating normal

cardiac function can be found in the discovery of a single nucleotide polymorphism, Val-646, of D-KAP2 that significantly increases the risk of sudden cardiac death.[58]

2.3.5 Gravin

Gravin was first identified as an autoantigen recognized by myasthenia gravis sera and was later identified as an AKAP by the lab of John Scott.[59, 60] It is a large molecular weight protein (≈250 kDa) and is also referred to as AKAP12, AKAP250, Src-suppressed C kinase substrate depending upon the species in which it was identified. It is highly expressed in the bladder, brain, lung, testes and heart and is found primarily associated with the plasma membrane but can also be found circulating in the cytosol.[61, 24] In addition to PKA, gravin includes the tyrosine kinase Src, protein phosphatase 2B (PP2B) and PKC in its signaling complex and target them to the β_2-AR. Additionally, G-protein coupled receptor kinase 2 (GRK2), calcium/calmodulin dependent kinase II (CaMKII), β-arrestin and PDE4D (multiple isoforms; 3, 5 and 8) have also been postulated to form complexes with gravin. However, some of these interactions appear, particularly that of β-arrestin, to be transitory in nature. [62, 63]

Additionally, gravin is one of the first AKAPs determined to be or defined as multifunctional.[64] The majority of the current literature concerning gravin and its orthologues have been investigating the role of gravin in various forms of cancer. Indeed, reduction of gravin expression has been associated with cancer progression as gravin expression suppresses both tumor growth and metastasis. These studies have shown that

gravin plays a prominent role in regulating cell migration as well as the progression of the cell through the cell cycle.[65] In addition, gravin also plays a role in the angiogenesis and tight junction formation in the blood brain barrier and the regulation of cAMP accumulation in airway smooth muscle.[66, 67]

In the heart, gravin functions not only to assist in facilitating PKA's action but also to modulate the desensitization/resensitization cycle of the β_2-AR as gravin scaffolds proteins, such as PKA and PKC, that can phosphorylate and desensitize the β_2-AR. [24, 25, 60] Additionally, studies have shown that knockdown of gravin expression blocks the association of GRK2 and β-arrestin, proteins involved in β-AR desensitization, with the β_2-AR. [25, 63] Phosphorylation of the β_{2-}AR by GRK2, PKA or PKC leads to the association of β-arrestin, which in turn mediates sequestration of the receptor into clathrin coated pits. Dephosphorylation of the receptor causes the dissociation of β-arrestin and clathrin as well as the trafficking of the receptor back to the membrane. Thus, gravin, by complexing both kinases and phosphatases, facilitates both desensitization and resensitization of the receptor.

Furthermore, gravin binds to the β_2-AR along the C-terminal tail of the receptor and has been shown to remain complexed with the receptor throughout the desensitization/resensitization cycle. Fan et al., showed using confocal microscopy that gravin remained associated with the receptor during sequestration, which confirms earlier data from the same research group that β-arrestin and clathrin are transiently included in

the gravin scaffolding complex.[24] In addition to phosphorylating the β_2-AR, PKA and PKC can also directly phosphorylate gravin with varying results. PKA phosphorylation of gravin enhances its association with the β_2-AR while PKC phosphorylation reduces the affinity of gravin for the receptor.[63, 68]

2.4 Beta-adrenergic receptor signaling in the chronically stimulated myocardium

Chronic β-adrenergic receptor signaling occurs in pathophysiological states such as heart failure, which is the endpoint for many cardiac diseases. In heart failure, cardiac function is significantly diminished and the sympathetic nervous system tries to compensate for the lack by increasing the release of neurotransmitters such as norepinephrine. This compensatory mechanism is both beneficial and effective in the acute setting but prolonged exposure to β-AR stimulation results in alterations of PKA dependent signal transduction. The results of these alterations have a deleterious effect on both cardiac function and structure.[6]

2.4.1 β-adrenergic receptors with chronic stimulation

Chronic activation of β-ARs results in their desensitization and subsequent internalization, which prevents the receptors from responding to stimulation. It has been well documented in both animal models of heart failure and in human heart failure that β_1-AR density is drastically reduced and the remaining receptors, both β_1- and β_2-ARs are uncoupled from stimulatory G-proteins, essentially unavailable to mediate cardiac contractility.[4, 69] The reduction in β_1-AR density alters the ratio of β_1- to β_2-AR subtypes

from 70:30 to approximately 50:50.[70] Additionally, β_2-ARs become uncoupled from G_s and instead couple to G_i furthering negative inotropic signaling.[6] Expression levels of G_i have also been shown to be increased in human heart failure, which may contribute to the increased coupling of β_2-ARs to G_i.[71] Controversial findings have been reported concerning the effect of chronic β-AR stimulation on β_3-ARs. Fischer et al. reported that β_3-AR gene expression was unchanged in heart failure whereas Port et al. indicated the β_3-AR gene expression is increased.[18, 72] Thus, there is a reduction in total β-AR density as well as a loss of functional receptors, which results in diminished cardiac function. As would be expected, the changes in β-AR density and G-protein coupling produce maladaptive changes in the signaling pathway, which includes alterations in PKA substrate phosphorylation [2].

The increase of β-AR desensitization is mediated in part by an upregulation of GRK2 expression, which phosphorylates both β_1- and β_2-ARs.[10] Indeed, studies using βARKct, a peptide inhibitor of GRK2, showed that inhibition of GRK2 was sufficient to prevent the characteristic changes in β-AR signaling in animal models of heart failure.[73, 74] Additionally, PKA and PKC can also phosphorylate β_2-ARs to facilitate their desensitization and/or coupling to G_i. [75, 76] Furthermore, overstimulation of β_1-ARs has been associated with an increase in pro-apoptotic signaling, which would further worsen cardiac function.[77, 78]

2.4.2 PKA and AKAPs in chronic β-AR stimulation

Due to the downregulation of β_1-ARs, the uncoupling of β_2-ARs to G_s and the other changes that are occurring with the β-ARs, it comes as no surprise that chronic β-AR stimulation has a significant effect on PKA activity and function. Decreased phosphorylation of key EC-coupling proteins such as PLB and TnI are hallmark indicators of chronic β-AR stimulation and contribute to the alterations in Ca^{2+} handling that result in decreased cardiac function.[79, 80] PLB phosphorylation is decreased because of both decreased PKA phosphorylation as well as increased activity of protein phosphatase 1.[26]

In addition to changes in PKA phosphorylating its substrates, it has also been demonstrated, in human heart disease, that autophosphorylation of the RII subunit is reduced. Since this phosphorylation strengthens PKA/AKAP interactions, it is thought that the changes in substrate phosphorylation involve the disruption of AKAP signaling.[81] Although decreased β-AR mediated cAMP production decreases PKA activity, reduced PKA activity does not necessarily correlate with diminished PKA substrate phosphorylation. Indeed, some of PKA's substrates, such as RyR, become hyperphosphorylated in failing hearts.[7] The hyperphosphorylation of the RyR results in leak of Ca^{2+} from the SR. Combined with a decrease in phosphorylation of PLB, inhibiting re-uptake of Ca^{2+} into the SR by the SERCA2a, the Ca^{2+} leak through the RyR results in depletion of the SR Ca^{2+} store.[7, 79] The reduced SR load lowers the Ca^{2+}

transient upon the next depolarization of the cell further dampening cardiac contractility.[16] There are also alterations in the expression of other proteins involved in the movement of Ca^{2+}. In addition to increased inhibition by PLB, SERCA2a expression is significantly decreased in heart failure, which results in decreased Ca^{2+} re-uptake into the SR.[82] Conversely, there is increased expression of protein phosphatases increasing the dephosphorylation of the EC-coupling mechanism and augmenting the disruption of Ca^{2+} homeostasis.[16, 26]

2.4.3 Cardiac hypertrophy and remodeling

The activation of kinases and phosphatases such as PKA, PKC, and PP2B as a result of β-AR stimulation results in the activation of pro-hypertrophic genes. Activation of these genes causes cardiac remodeling, which is characterized by alterations in the cellular structure as well as in the size and shape of the heart. While cardiac remodeling and hypertrophy is thought to be initially compensatory, extensive morphological changes exacerbate cardiac dysfunction. For example, reduced PKA-dependent phosphorylation of PLB, cMyBPC and TnI in heart failure have been associated with increased ventricular remodeling. PP2B (also known as calcineurin) dephosphorylates the transcription factor NFAT causing its translocation to the nucleus. Once in the nucleus, NFAT causes the transcription of genes involved in hypertrophy and remodeling.[83] PKCα, the PKC isoform that binds to gravin, has also been implicated in prohypertrophic signaling as it activates extracellular signal regulated kinase (ERK1/2).[84]

Several AKAPs, including mAKAP and AKAP-lbc have been linked to the induction of cardiac hypertrophy as they are thought to mediate signaling that initiates hypertrophic gene expression primarily through activation of the mitogen activated protein kinase (MAPK) pathway.[85, 86] Conversely, AKAP1 inhibits PP2B activity, suggesting that the scaffolding protein is involved in anti-hypertrophic signaling. We hypothesize that gravin may also play a role in regulating hypertrophic/remodeling signaling since it contains several of the kinases and phosphatases involved in both the induction and inhibition of cardiac hypertrophy.

In order to test our hypotheses concerning the role of gravin in the modulation of β-AR signaling, gravin knockout mice (previously produced by Dr. McConnell) were subjected to acute and/or chronic β-AR stimulation. We measured various aspects of the β-AR signaling pathway such as cAMP production, PKA activity and PKA substrate phosphorylation in both of these settings.

3. Methods and Materials

3.1 Animals

Animal care was provided by AAALAC accredited facilities at either University of Houston (UH) or the Baylor College of Medicine (BCM). All the experimental methods and procedures have been IACUC approved (UH-ACP-11-032 and BCM-AN-5199). Gravin knockout mice were produced using gene trap technology to remove the gravin gene (NM_031185). In short, the gene trap vector, containing a splice-acceptor sequence, inserted into intron 2 of the gravin gene, deleting exons 3 and 4, which contain the binding sites for β_2-AR, PKA and PKC. Embryonic cells containing the gene trap vector were obtained from BayGenomics, microinjected into normal blastocysts and surgically implanted into foster mice at the University of Maryland School of Medicine Transgenic/Knockout Core Facility. Male mice were mated with female wild-type mice (C57BL/6) to produce heterozygous mice that were then mated to obtain wild-type and mutant littermates. For experimental studies, male gravin knock-out mice aged 10-12 weeks were compared with wild-type littermates.

3.2 Echocardiography measurements for acute β-adrenergic receptor stimulation experiments

Baseline measurements by echocardiography were obtained before intraperitoneal (i.p.) injection with the nonspecific β-AR agonist isoproterenol (ISO; 0.25μg/g) or the vehicle (ascorbic acid; 0.002%). Cardiac morphology and function were assessed by

serial M-mode echocardiography with a VisualSonics Vevo 770 High-Resolution In-Vivo Micro-Imaging System (VisualSonics In., Ontario, Canada) equipped with a 30 MHz microprobe. Ventricular measurements in M-mode were taken at baseline and 3 minutes after ISO injection with at least three readings per mouse.

3.3 Echocardiography measurements for chronic β-adrenergic receptor stimulation experiments

Baseline measurements by echocardiography were obtained before intraperitoneal (i.p.) injection with the nonspecific β-AR agonist isoproterenol (ISO; 0.25μg/g) or the vehicle (ascorbic acid; 0.002%). Following i.p. injection, mini-osmotic pumps (Alzet; model 2002) delivering ISO (15 mg/kg/day x 14 days) or vehicle were implanted subcutaneously in age-matched (16 to 20 weeks old) male mice anesthetized in a gas chamber with isoflurane (2.5% isoflurane, 97.5% O_2). Sufficient anesthesia was determined by a lack of response to toe pinch. Cardiac morphology and function were assessed by serial M-mode echocardiography with Vevo 770 Imaging System equipped with a 30 MHz microprobe. Ventricular measurements in M-mode were taken at 7-days and 14-days after the implantation with at least three readings per mouse.

3.4 Membrane Preparation

Animals were anesthetized with isoflurane (2.5% isoflurane, 97.5% O_2). Hearts were quickly explanted, washed in sterile PBS containing protease inhibitors and weighed. The left ventricles were isolated and flash frozen in LN_2. Membranes were prepared from

excised left ventricles from WT and gravin-KO mice. The hearts were quickly thawed at 30˚ C, roughly chopped, and then homogenized by a Polytron Homogenizer in lysis buffer (50mM HEPES, 150mM KCl, 5mM EDTA (pH 7.4), and 10µl/ml protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 800 x *g* for 15 min. The supernatant was centrifuged at 40,000 x *g* for 20 min. The resulting supernatant was removed and stored at -80˚C for kinase activity assays. The pellet was resuspended in binding buffer (50mM HEPES (pH 7.4), 5mM $MgCl_2$) and stored at -80˚C for use in radioligand binding assays and other biochemical assays. Protein concentration was estimated via a BCA assay kit (Pierce).

3.5 Radioligand Binding Assay

β-AR density was determined as previously described [87]. Briefly, 10µgs of membranes were incubated with varying concentrations of [^{125}I]-iodocyanopindolol (10pM-4nM; Perkin Elmer) at 37˚C for one hour. Reactions were terminated via harvesting by a Perkin Elmer harvester onto Whatman GF/B filters, and then washed three times with ice-cold binding buffer. The filters were dried then scintillation fluid was added and the radioactive count was determined by a TopCount Scintillation Counter (Perkin Elmer). Nonspecific binding was determined in the presence of 20µM alprenolol (Sigma). β_1-AR density was determined in the presence of 70 nM of the β_2-AR antagonist ICI 118,551 (Sigma). Similarly, β_2-AR density was determined in the presence of 300

nM of the β_1-AR antagonist CGP 20712A (Sigma). Each experiment was performed in triplicate. Bmax was normalized to milligrams of protein.

3.6 cAMP production

cAMP production was measured according to manufacturer's instructions using an enzyme immunoassay kit (Enzo LifeSciences) in membrane fractions as previously described [88]. This assay utilizes a polyclonal antibody that binds to a conjugate molecule that forms a covalent bond with the cAMP in the sample. Briefly, samples (100µl) are added to wells coated with a goat anti-rabbit IgG antibody. cAMP conjugated to alkaline phosphatase is then added in addition to a cAMP rabbit polyclonal antibody, followed by the addition of the pNpp substrate. The substrate changes color when catalyzed by the alkaline phosphatase conjugated to cAMP. The absorbance is read after the addition of stop solution at 405nm. The signal is inversely proportional to the cAMP concentration. Each experiment was performed in duplicate. cAMP production was normalized to milligrams of protein.

3.7 PKA Activity

PKA activity was determined via an enzyme immunoassay kit (Enzo LifeSciences) using cytosolic preparations according to the manufacturer's instructions. The assay used a synthetic peptide as the kinase substrate and a polyclonal antibody that recognized the phosphorylated form of the substrate. The substrate is pre-coated onto the wells of a 96-well plate. 30µls of sample was added then ATP was added to initiate reaction. Following

the addition of the polyclonal antibody and the peroxidase conjugated secondary antibody, the assay was developed with tetramethylbenzidine. The color development is proportional to the kinase activity. Color development was stopped by addition of acidic stop solution and the absorbance was measured at 405nm. Each experiment was performed in triplicate. Specific PKA activity was determined using 10μM PKI (Sigma).

3.8 PKC activity

PKC activity was determined via an enzyme immunoassay kit (Enzo LifeSciences) using cytosolic preparations according to the manufacturer's instructions. The assay used a synthetic peptide as the kinase substrate and a polyclonal antibody that recognized the phosphorylated form of the substrate. The substrate is pre-coated onto the wells of a 96-well plate. 30μls of sample was added then ATP was added to initiate reaction. Following the addition of the polyclonal antibody and the peroxidase conjugated secondary antibody, the assay was developed with tetramethylbenzidine. The color development is proportional to the kinase activity. Color development was stopped by addition of acidic stop solution and the absorbance was measured at 405nm. Each experiment was performed in triplicate.

3.9 Histological Analysis

Hearts were harvested and fixed in 10% formalin overnight, embedded in paraffin, sectioned at 5μm thickness and stained using hematoxylin and eosin (H&E) and Masson's Trichrome (MT) according to manufacturer's instructions. The sectional area

was measured with a microscope (Nikon Eclipse T*i*-U) and digital camera (Nikon Digital Sight DS-Qi1Mc). Photomicrographs of the sections were evaluated for morphology and cellular dimensions.

3.10 Immunoblot analysis

Immunoblot analysis was carried out as previously described using antibodies for GAPDH (Cell Signaling), SERCA2a (Cell Signaling), Calsequestrin 2 (Pierce antibodies), AKAP250 (Abcam), β_1-AR (Santa Cruz), β_2-AR (Santa Cruz), PDE4D (Pierce antibodies), total Hsp20 (Millipore), phospho-Hsp20 (Abcam), phospho-MyBPC (Ser-273), phospho-MyBPC (Ser-282), phospho-MyBPC (Ser-302) and total-MyBPC (generous gift from Sakthivel Sadayappan), total- and phospho-phospholamban (Upstate or Cell Signaling), $G_{\alpha i}$ (Cell Signaling), total- and phospho-protein phosphatase-1 inhibitor-1 (Santa Cruz Biotechnology), phospho- and total PKCα (Cell Signaling) and β_3-AR (Santa Cruz Biotechnology) [56]. Crude heart homogenates, membrane fractions or cytosolic fractions were resolved by SDS-PAGE gradient (4–12% Bis-Tris) gels, and then transferred to polyvinylidine difluoride membranes. Blots were then incubated overnight at 4˚C with primary antibodies. The blots were washed three times with TBS containing 0.1% Tween 20, and then probed with appropriate secondary antibodies (Cell Signaling). Blots were developed by an enhanced chemiluminescence kit (Pierce). Blots were stripped and re-probed with other antibodies when appropriate. NIH Image J was used for densitometric analysis of the immunoblots.

3.11 Statistical Analysis

Data were processed using Microsoft Excel and GraphPad Prism 5.0. All values are expressed as the mean ± S.E.M. Comparisons between two groups were determined using unpaired 2-tailed Student's *t* test. Analysis was performed using one-way ANOVA, followed by a Tukey's *post hoc* multiple comparison test when multiple groups were compared. *P* values of less than 0.05 were considered significant.

RESULTS

4. Effect of Acute β-Adrenergic Receptor Stimulation on Cardiac Function in Gravin Knockout Mice

4.1 Increased Cardiac Function in Gravin KO Mice.

Wild-type (WT) and gravin knockout (KO) mice were injected i.p. with isoproterenol (0.25μg/g). Echocardiography was used to assess cardiac dimensions and function in WT and KO mice at baseline and three minutes after ISO injection (Table 1). Representative M-mode echocardiograph images for WT and KO are shown in Figure 1. Left ventricular mass (corrected) for both WT and KO hearts was determined via echocardiography and normalized to body weight (mg/g). There was no significant difference in LV mass to body weight ratio between the WT and the KO mice (Figure 2A). Additionally, histological analysis did not reveal any differences between WT and gravin KO hearts (Figure 2B).

The cardiac dimensions intra-ventricular septum (IVS) and left ventricular (LV) posterior wall (PW) were not significantly different at diastole between the two groups (Table 1). However, both IVS and LVPW at diastole were significantly increased in both WT and KO mice following ISO injection compared to the vehicle treated animals. Conversely, IVS and LVPW at systole were significantly increased in the KO mice even at baseline despite the absence of stimulation (Table 1). With ISO stimulation, both IVS and LVPW thickness were increased in WT and KO hearts compared to their respective

controls. Additionally, both parameters were significantly increased in the KO ISO hearts compared to the WT ISO hearts, indicating that the absence of gravin improves contractile function (Table 1).

Cardiac contractility was also assessed by echocardiography at baseline and after ISO treatment in WT and KO mice. Heart rate was similar between WT and KO mice at baseline and both similarly increased with ISO treatment (WT baseline: 478.0±8.6; KO baseline: 467.0±6.4; WT ISO: 596.4±8.1; KO ISO: 570.7±16.4; bpm; Table 1). The cardiac contractility parameters LV ejection fraction (EF), LV fractional shortening (FS), cardiac output (CO) and stroke volume (SV) were significantly increased in KO mice even in the absence of ISO stimulation (Table 1). After ISO stimulation, these parameters increased in both treatment groups, versus their respective controls. LV fractional shortening was also significantly enhanced in the gravin-KO ISO mice compared to with WT ISO mice (WT baseline: 32.40±0.92; KO baseline: 40.02±1.04; WT ISO: 54.47±4.16; KO ISO: 68.03±2.67; %; $p<0.0001$). Similarly, stroke volume was also much greater in the gravin-KO ISO mice than the WT ISO mice (WT baseline: 40.63±3.79; KO baseline: 50.96±0.49; WT ISO: 47.62±1.16; KO ISO: 64.25±4.06; µl; $p=0.0056$). Cardiac output was also significantly increased in both the gravin-KO mice at baseline and following ISO stimulation when compared to their WT counterparts (WT baseline: 21.53±0.60; KO baseline: 25.55±0.50; WT ISO: 26.97±1.35; KO ISO:

32.84±1.39; ml/min; $p<0.0001$). These data indicate that the absence of gravin in the heart results in increased contractility both before and after β-AR stimulation.

4.2 β-AR Expression.

C.C. Malbon's group has definitively shown the role of gravin in β_2-AR's desensitization and resensitization cycle and that suppression of gravin expression results in decreased desensitization of the receptor.[25, 63] We examined via western blot whether gravin's absence altered the protein expression of either β_1-AR or β_2-AR. Our results indicate that β_1-AR protein expression levels were similar between WT and KO animals and that ISO stimulation does not alter the receptor's expression levels (Figure 3). Similarly, β_2-AR protein expression levels were similar between WT and KO animals both before and after ISO stimulation (Figure 4).

4.3 cAMP Production

β-ARs stimulate adenylyl cyclase to enhance the production of cAMP, which activates signaling cascades through PKA and other signaling molecules such as Epac1.[89] Since gravin's absence is expected to enhance β_2-AR signaling by blocking termination of its signaling through kinase phosphorylation, we expected that cAMP production would be altered in the gravin KO mice. However, our results indicate that cAMP production between WT and KO untreated mice is similar. ISO stimulation, as expected, increased cAMP production in both WT and KO mice but again there was no difference

between the two groups (Figure 5). Unpublished data by others in our group have shown that PDE activity is significantly increased in the KO mice at baseline compared to WT mice.[a] Additionally, ISO treatment further augments PDE activity in the KO mice. Thus, cAMP production may indeed be enhanced in KO mice due to increased β-AR activity but there is no net change in cAMP production due to similarly enhanced PDE activity.

4.4 ISO-stimulated Increases in PKA Activity and Substrate Phosphorylation.

Since PKA is a major effector of β-AR stimulation and its action to phosphorylate its substrates has profound effects on the regulation of contraction and relaxation[2], we evaluated PKA activity in vehicle and ISO treated WT and KO mice hearts. Additionally, we expected that PKA activity would be reduced in the KO mice hearts due to the increase of PDE activity. However, similar to what was seen in the cAMP production a comparable increase in PKA activity was seen in both WT and KO ISO stimulated hearts indicating the knockdown of gravin does not affect the activation of the kinase despite enhanced PDE activity (Figure 6). Since PKA activity was not altered in the gravin KO mice, we hypothesized that perhaps PKA localization is altered and that this alteration would affect PKA substrate phosphorylation. Thus, we measured several PKA substrates that are key components of the EC-coupling mechanism.

[a] Ashley N. Guillory, Xing Yin, Cori Wijaya, Andrea Diaz Diaz, Abeer R'ababah, Fatin Atrooz and Bradley McConnell. Acute beta adrenergic receptor stimulation on cardiac function in A kinase anchoring protein gravin knockout mice. (In preparation, 2012).

PKA-induced PLB phosphorylation was significantly increased in both WT and KO hearts following ISO infusion (Figure 7). When phosphorylated at Ser-16, PLB can no longer inhibit the sarcoplasmic reticulum Ca^{2+} ATPase, which is responsible for the uptake of Ca^{2+} into the sarcoplasmic reticulum following contraction. Reuptake of Ca^{2+} into the sarcoplasmic reticulum results in faster cardiac relaxation as well as increases the Ca^{2+} store available for release at the next depolarization of the cell.[2] Protein phosphatase-1 inhibitor 1 (IPP-1), when phosphorylated by PKA, inhibits protein phosphatase-1 (PP-1), which is primarily responsible for dephosphorylating key components of the EC coupling mechanism to attenuate contractility. PLB is a target of PP-1 and dephosphorylation of PLB results in the inhibition of SERCA2a to decrease Ca^{2+} reuptake into the SR.[90, 91] IPP-1 overexpression has been shown to augment cardiac function most likely by maintaining or increasing phospholamban phosphorylation to disinhibit SERCA2a.[91, 92] We observed that basal IPP-1 phosphorylation and expression was unchanged in the absence of gravin (Figure 8). Furthermore, isoproterenol treatment similarly increased IPP-1 phosphorylation in both WT and gravin-KO hearts (p-value=0.0013).

Similarly, TnI phosphorylation was also increased in the ISO infused hearts of both genotypes (Figure 9). TnI phosphorylation is also associated with augmenting relaxation of the heart as increased phosphorylation of this protein reduces the sensitivity of the myofilaments to Ca^{2+}. Since PKA phosphorylation of these two proteins were similar in

the WT and KO mice, these data indicate that gravin does not play an essential role in mediating their phosphorylation by PKA.

We also investigated whether gravin's absence affected other PKA substrates. Phosphorylation of heat shock protein 20 (Hsp20) by PKA has been shown to be cardioprotective in several pathophysiological disease states and Hsp20 is normally complexed with PDE4D, which reduces its phosphorylation.[93-95] Therefore, we hypothesized that gravin's absence would alter PDE4D localization and thus enhance Hsp20 phosphorylation.[62]. Although, phosphorylated Hsp20 was not detectable in either the WT or KO vehicle hearts, Hsp20 phosphorylation was significantly increased in the KO ISO group compared to the WT ISO (Figure 10).

cMyBPC has three major phosphorylation sites, Ser-273, Ser-282 and Ser-302. These sites can be phosphorylated by a variety of proteins including PKA, PKC, CaMKII and protein kinase D.[96-98] cMyBPC is involved in the modulation of myofilament sensitivity to Ca^{2+} and ablation of these three phosphorylation sites has been shown to have profound effects on cardiac function. [99-101] Similar to what was seen with PLB and TnI, phosphorylation of sites Ser-282 and Ser-302 in cMyBPC was significantly increased in both WT and KO hearts in the presence of ISO stimulation compared to their respective controls with no difference between the two ISO groups (Figure 11A-C). However, phosphorylation of site Ser-273 was significantly increased in the KO vehicle treated hearts compared to the WT vehicle treated hearts but was not further augmented

with ISO treatment. Following ISO stimulation, Ser-273 phosphorylation in WT hearts was increased to a level similar to that seen in the gravin-KO animals (Figure 11A, D). These data indicate that cMyBPC phosphorylation at Ser-273 may play a key role in enhancing cardiac function in KO mice. Additionally, the mechanism for the acute ISO stimulated increase in cardiac function in KO mice may involve both cMyBPC Ser-273 phosphorylation as well as enhanced Hsp20 phosphorylation.

To further characterize whether Ca^{2+} movement across the SR was altered in KO mice, we measured SERCA2a expression. SERCA2a is the main pump responsible for the reuptake of Ca^{2+} from the cytosol into the sarcoplasmic reticulum, which facilitates relaxation of the cardiac muscle.[102] Western blot analysis showed that removal of gravin did not affect SERCA2a expression (Figure 12). Additionally, isoproterenol treatment did not alter SERCA2a expression; it remained equal between WT and KO hearts regardless of treatment. Thus, SR Ca^{2+} uptake should not be altered in the KO mice.

5. Effect of Chronic β-Adrenergic Receptor Stimulation on Cardiac Function in Gravin Knockout Mice

5.1 Resistance to Hypertrophy in Response to Chronic ISO Stimulation.

To examine the role of gravin in mediating cardiac function in the presence of prolonged β-AR stimulation, mini-osmotic pumps delivering isoproterenol were implanted in WT and KO mice; mini-osmotic pumps delivering the vehicle (ascorbic acid) were used as controls (vehicle or sham). Echocardiography was used to assess cardiac dimensions and function at baseline, at 7-days, and at 14-days following chronic ISO stimulation (Table 2). A representative M-mode echocardiography image demonstrating these results on cardiac dimensions are shown in Figure 13.

ISO induced hypertrophy was analyzed in WT and KO mice following 14 days of ISO stimulation. As expected, heart-to-body-weight ratio (HW:BW) was significantly increased in WT mice in response to chronic ISO treatment. However, this increase in HW:BW due to chronic β-AR treatment was blocked in KO mice (Figure 14). In addition, the prominent histological abnormalities, including myocyte hypertrophy, myofibrillar disarray, and cardiac fibrosis, that was observed in WT mice in response to chronic ISO treatment at 14-days, was abrogated in KO mice (Figure 15).

In the absence of agonist stimulation at baseline, IVS and LVPW thickness during diastole were not significantly different between WT and KO mice (Table 2). However, diastolic IVS and LVPW thickness increased in WT mice in response to

chronic ISO treatment at both 7-days and 14-days, versus controls. This increase in diastolic IVS and LVPW was not seen in KO hearts treated with ISO for 14 days. In contrast, systolic IVS and LVPW dimensions at baseline increased in KO hearts versus WT hearts in the absence of stimulation (Table 2). Additionally, systolic IVS and LVPW thickness increased in WT mice in response to chronic ISO treatment at both 7-days and 14-days, versus controls. The increase in systolic IVS and LVPW thickness was further augmented in KO mice, as compared to WT mice in response to chronic ISO treatment, also indicating improved cardiac function during contraction in KO hearts versus WT hearts (Table 2). Taken together, these results suggest that ablation of gravin inhibits chronic ISO induced cardiac hypertrophy and LV remodeling.

5.2 Improved Cardiac Function in Gravin Knockout Mice Despite Prolonged ISO Stimulation.

At baseline, LVFS, SV, LVEF, and CO was increased in KO mice versus WT control mice (Table 2). 7-days following chronic ISO stimulation, these parameters increased in both WT and KO mice, versus their respective controls, but were significantly augmented in the KO mice (Table 2). Furthermore, LVFS, SV, LVEF, and CO did not continue to increase in WT mice, 14-days following chronic ISO stimulation, versus the WT controls. Instead, LVFS and LVEF declined in WT mice following 14-days chronic ISO treatment versus 7-days chronic ISO treatment, consistent with the detrimental response of chronic ISO stimulation on cardiac function over time (Table 2). In contrast, after 14-

days of chronic ISO stimulation, cardiac function continued to be significantly increased in KO mice, both compared to WT and KO controls, thus indicating the protective role of gravin's absence on cardiac contractility.

5.3 Normalization of β-AR density and affinity with chronic β-AR stimulation in gravin KO mice.

We hypothesized that the aforementioned increase in cardiac function despite chronic β-AR stimulation is due to the absence of gravin attenuating desensitization of the receptors and maintaining β-AR subtype ratio at normal levels. Therefore, we determined total β-AR density and affinity in control and ISO treated WT and KO mice as well as the density and affinity of the two major subtypes, β_1 and β_2, using radioligand binding assays.

As seen in Figure 16A, total β-AR density did not change between the four groups (WT vehicle: 177.6± 37.14; WT ISO: 187.5±24.4; KO vehicle: 181.8±32.7; KO ISO: 201.9±38.5; fmol/mg). Similarly, ISO treatment did not have a significant effect on total β-AR affinity although it appears to be slightly decreased in the WT ISO group and both KO groups (WT vehicle: 0.25±0.043; WT ISO: 0.32±0.0051: KO vehicle: 0.32±0.046; KO ISO: 0.33±0.062; nM; Figure 16B). We then examined the density and affinity of the β-AR subtypes to determine which was responsible for the decrease in total β-AR affinity. The β_1-AR density in the WT ISO group was significantly decreased compared to its control while there was no significant difference in β_1-AR density between the KO

treated and nontreated groups (WT vehicle: 175.8±27.1; WT ISO: 135±13; KO vehicle: 168±19; KO ISO: 167.7±17.0; fmol/mg; p=0.013; Figure 17A). Similarly, β_1-AR affinity (Figure 17B) was only significantly reduced in the WT ISO group (WT vehicle: 0.44±0.036; WT ISO: 0.93±0.20; KO vehicle: 0.60±0.12; KO ISO: 0.61±0.15; nM; p=0.024). Figure 18A shows that β_2-AR density was significantly increased in the KO ISO group compared to all of the other groups (WT vehicle: 52.38±4.649; WT ISO: 45.71±2.717; KO vehicle: 21.92±2.67; KO ISO: 75.07±4.811; fmol/mg; p=<0.0001). β_2-AR affinity was comparable among the four groups (WT vehicle: 0.16±0.025; WT ISO: 0.18±0.035; KO vehicle: 0.16±0.024; KO ISO: 0.16±0.033; nM; Figure 18B).

Previous studies have shown that β_3-AR mRNA expression is significantly increased in failing human hearts, which may enhance the depression of cardiac function. [103] However, others have shown that β_3-AR mRNA levels are not affected by the presence of HF. [72] Therefore, we measured β_3-AR protein expression levels via western blot to determine whether β_3-AR expression was altered in KO mice treated with ISO. However, we did not see a significant difference in β_3-AR expression among the four groups (Figure 19). Additionally, we measured the protein expression levels of G_i as it has been shown that G_i expression levels significantly increase in the presence of heart failure. [12, 71] As expected, the WT ISO group had significantly increased G_i levels (p-value<0.0001; Figure 20). However, this increase was abrogated in the KO ISO group. These results indicate that the absence of gravin largely attenuates the detrimental effect of chronic β-

AR stimulation on receptor density and affinity as well as blocks the upregulation of inhibitory G-protein expression.

5.4 cAMP production and PKA activity in WT and KO mice following chronic β-AR stimulation

β-ARs mediate the activation of PKA by increasing the production of cAMP in response to agonist stimulation.[2] Therefore, we measured the capability of WT and KO hearts, in the presence and absence of ISO, to produce cAMP under two conditions. cAMP accumulation in KO ISO treated mice was comparable to its control. In contrast, the WT ISO treated mice produced significantly less cAMP when compared to its control (WT vehicle: 3.29±0.023; WT ISO: 1.74±0.18; KO vehicle: 2.42±0.37; KO ISO: 2.48±0.65; pmol/mg; p=0.005; Figure 21). These data indicate that gravin-KO mice have normal or near-normal production of cAMP despite long-term β-AR stimulation. .

Since there were definitive changes in cAMP production between the WT and gravin-KO treatment groups, we next determined whether these changes were translated into alterations in PKA activity. Surprisingly, PKA activity was significantly reduced in both the WT ISO and KO ISO mice compared to their respective controls (WT vehicle: 0.44±0.009; WT ISO: 0.35±0.03; KO vehicle: 0.46±0.01; KO ISO: 0.35±0.03; pmol/mg; p=0.0043; Figure 22). Thus, despite the maintenance of cAMP production in gravin-KO ISO mice, PKA activity is still affected by prolonged β-AR stimulation. Our lab has shown that ISO significantly increases PDE activity in the KO mice compared to the WT

mice. Therefore, the reduction of PKA activity despite the maintenance of cAMP production in the KO mice is probably due to PDE breakdown of cAMP.

5.5 Differential effect of gravin's absence on PKA substrate phosphorylation.

Since we have observed increased contractility despite reduced activity of PKA in the KO ISO treated animals, we examined whether PKA phosphorylation of its classical substrates was similarly altered. Curiously, despite increased basal cardiac contractility, phosphorylation of PLB was similar between WT and KO vehicle treated animals. However, there was a significant increase in phospho-PLB in the KO ISO group compared to both the gravin-KO vehicle and the WT ISO groups (Figure 23). This increase in PLB phosphorylation was somewhat surprising so we investigated whether this was due to direct phosphorylation of PLB by PKA or indirectly through PKA's action on proteins involved in regulating PLB phosphorylation.

Although PLB can be directly phosphorylated by PKA, PLB phosphorylation is terminated by protein phosphatase 1, which is regulated by IPP-1, which in turn is activated by PKA [90, 91]. Therefore, we measured IPP-1 phosphorylation to see whether increased PLB phosphorylation was due to enhanced PKA phosphorylation of PLB or IPP-1. Indeed, we observed that IPP-1 phosphorylation was significantly reduced in WT ISO hearts compared to the WT control, KO control and the KO ISO hearts (Figure 24). Conversely, IPP-1 phosphorylation remained comparable in the KO hearts regardless of

the presence of ISO. These data indicate that the increased PLB phosphorylation in the KO ISO group is due in part to the maintenance of IPP-1 phosphorylation.

Since PKA phosphorylation can alter the Ca^{2+} sensitivity of the myofilaments, we measured several PKA substrates that are involved in modulating Ca^{2+} sensitivity. TnI phosphorylation was significantly increased in the WT ISO group compared to both its control and the KO ISO group (Figure 25). cMyBPC is a contractile protein whose phosphorylation has been shown to be critical for cardiac function[99]. We measured phosphorylation at three sites: Ser-273, Ser-282 and Ser-302. Ser-273 phosphorylation was significantly lower in both the KO control group and the KO ISO group compared to the WT control. While Ser-273 phosphorylation in WT ISO did not significantly increase compared to the WT control, it was significantly higher than the gravin-KO ISO group (Figure 26). Ser-282 phosphorylation was significantly higher in the WT ISO group versus the WT control. Additionally, Ser-282 phosphorylation was increased in vehicle treated KO mice. However, ISO stimulation did not further augment Ser-282 phosphorylation. Additionally, Ser-302 phosphorylation was significantly increased in both WT ISO and gravin-KO vehicle treated and ISO treated groups. These findings suggest that the absence of gravin has a profound effect on the action of PKA, that this effect is substrate-dependent and may be the result of altered localization of PKA.

5.6 PKC activity in WT and KO mice with chronic β-AR stimulation

Gravin also includes PKC in its scaffolding complex; specifically PKCα. Studies in various types of cancers have shown that binding of PKCα to gravin inhibits its activity.[104] Additionally, PKCα expression is upregulated in heart failure and is associated with the progression of heart failure especially the induction of hypertrophic genes.[84] As expected basal PKC activity was significantly increased in the KO animals. Additionally, PKC activity significantly increased in both WT and KO ISO groups compared to their respective controls. Furthermore, PKC activity was further enhanced in the KO ISO group compared to both the KO and the WT controls (WT vehicle: 0.051±0.010; WT ISO: 0.24±0.016; KO vehicle: 0.12±0.019; KO ISO: 0.21±0.015; pmol/mg; $p<0.0001$, Figure 27). Despite these overt changes in PKC activity, we did not see altered expression of PKCα in the KO mice, either before or after ISO treatment (Figure 28). Furthermore, PKCα phosphorylates IPP-1 at a different site from PKA: Ser-67 rather than Thr-35. Phosphorylation of Ser-67 of IPP-1, unlike the phosphorylation of Thr-35, was similar among the four groups (Figure 29). Further investigation is needed to determine the cause and the functional significance of the increased PKC activity in KO mice.

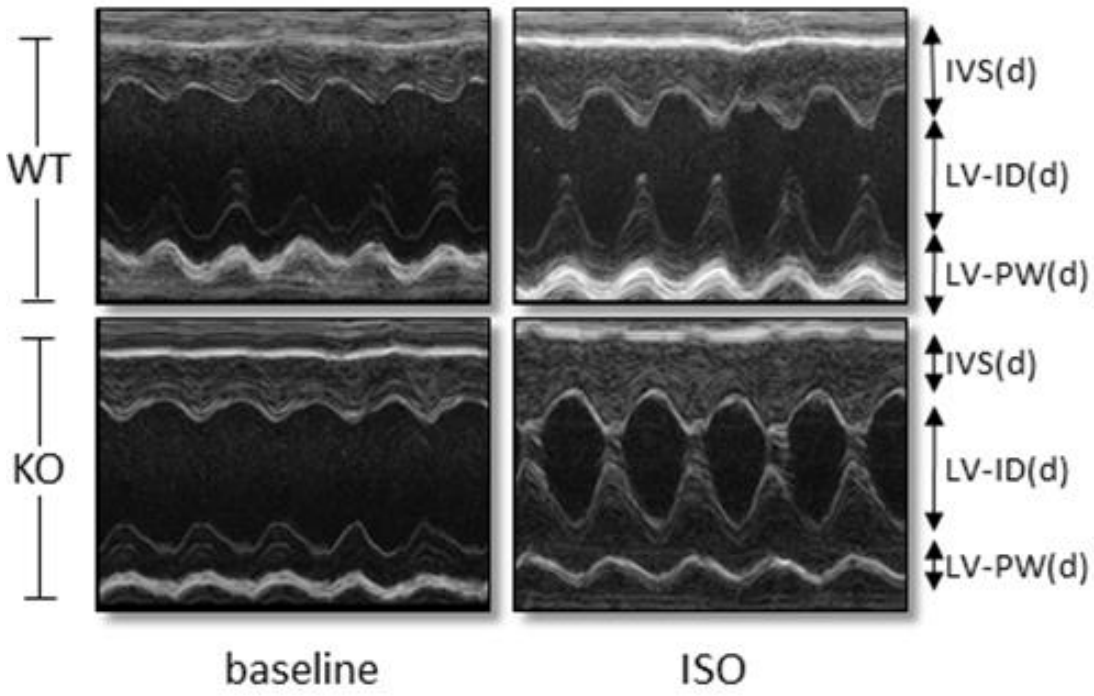

Figure 1: Representative transthoracic echocardiographic M-mode image of the LV from WT and KO hearts at baseline and following 3 minutes of ISO treatment (0.25µg/g). Baseline measurements, n=36 (WT) and n=37 (KO); all other measurements, n=6.

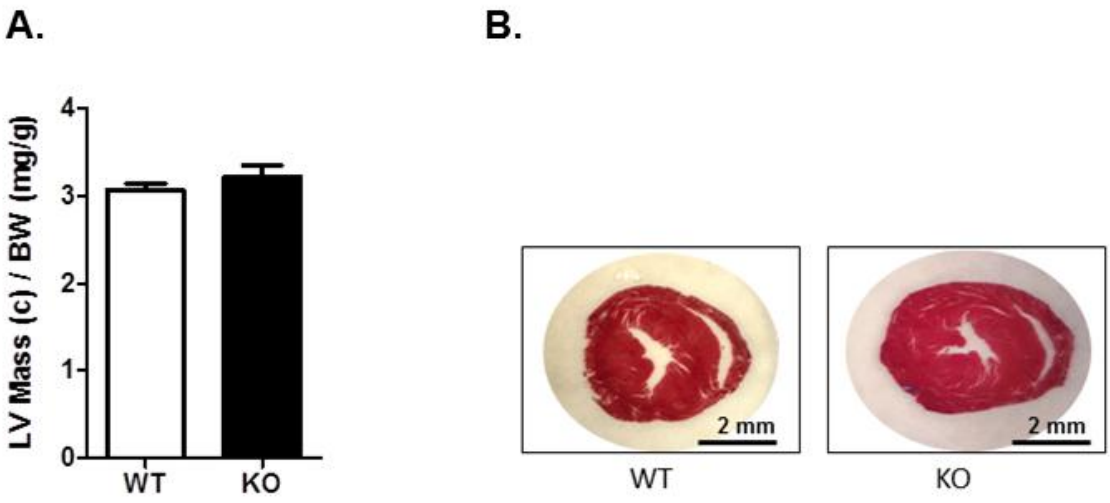

Figure 2: There is no difference in cardiac morphology in response to acute ISO stimulation between WT and KO mice. *(A)* LV mass (corrected) to body weight (BW) ratio (mg/g) illustrating cardiac size of WT and KO hearts following 3 minutes of ISO infusion. *(B)* Masson's Trichrome (MT) stained paraffin sections of WT and gravin-KO hearts. Data are expressed as the mean ± S.E.M.; n=6.

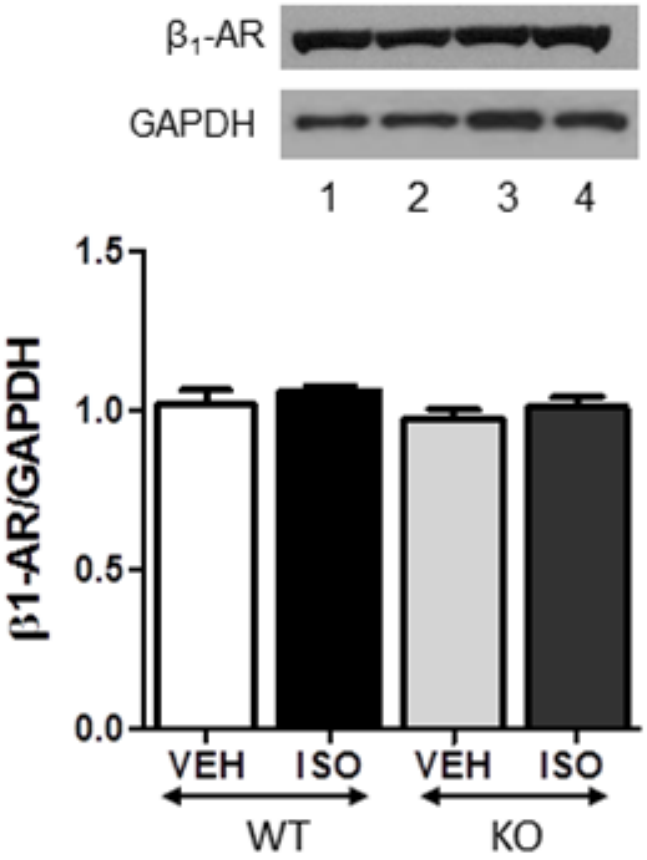

Figure 3: There are no changes in β_1-AR protein expression between WT and KO mice. Western blot analysis of β_1-AR protein expression in left ventricular membrane fractions isolated from WT and gravin-KO mice following acute vehicle or ISO infusion (10µg/g/min). The *upper panel* shows a Western blot with anti-β_1-AR antibody and the *lower panel* shows a Western blot with an antibody to GAPDH (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO).The *bar graphs* show the ratio of the receptor expression to GAPDH normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n=6.

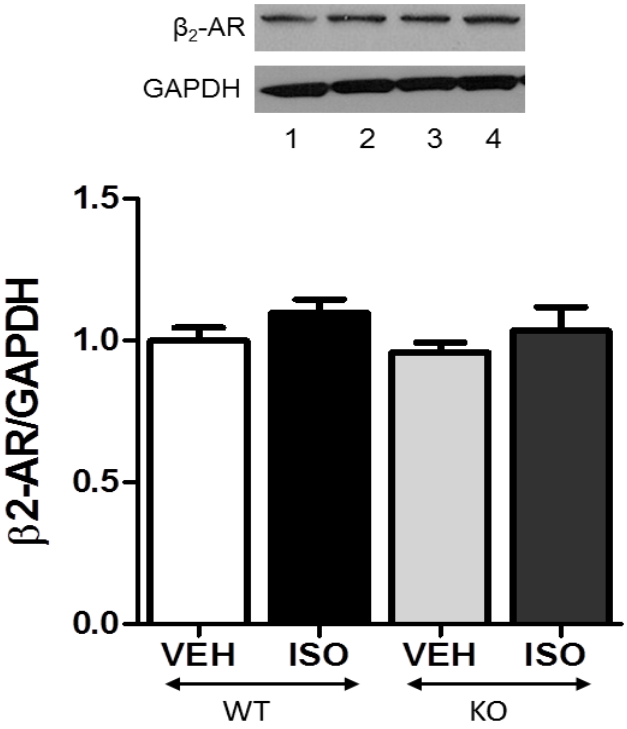

Figure 4: There are no changes in β_2-AR protein expression between WT and KO mice. Western blot analysis of β_2-AR protein expression in left ventricular membrane fractions isolated from WT and gravin-KO mice following acute vehicle or ISO infusion (10µg/g/min). The *upper panel* shows a Western blot with anti-β_2-AR antibody and the *lower panel* shows a Western blot with an antibody to GAPDH (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO).The *bar graphs* show the ratio of the receptor expression to GAPDH normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n=6.

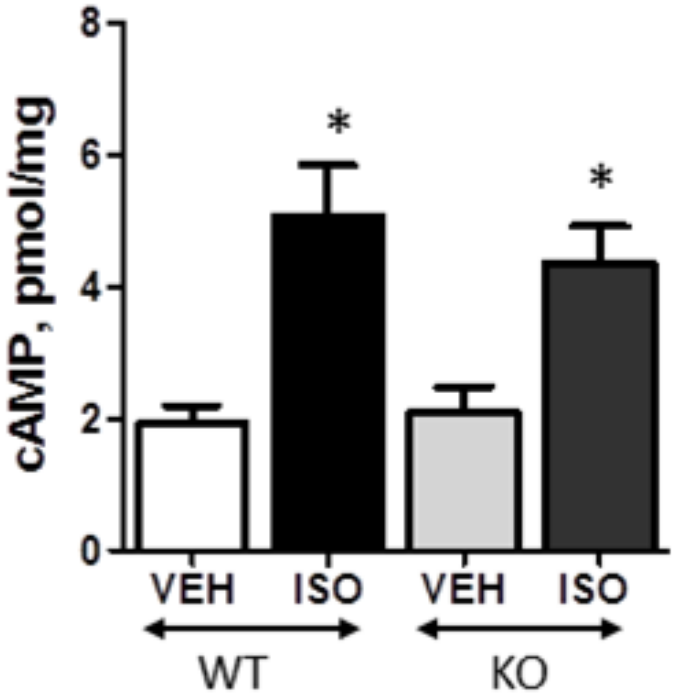

Figure 5: cAMP production remains similar between WT and KO mice. cAMP production in left ventricular membrane fractions isolated from WT and gravin-KO mice following acute vehicle or ISO infusion (10µg/g/min). Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P <0.05 vs. vehicle of same phenotype.

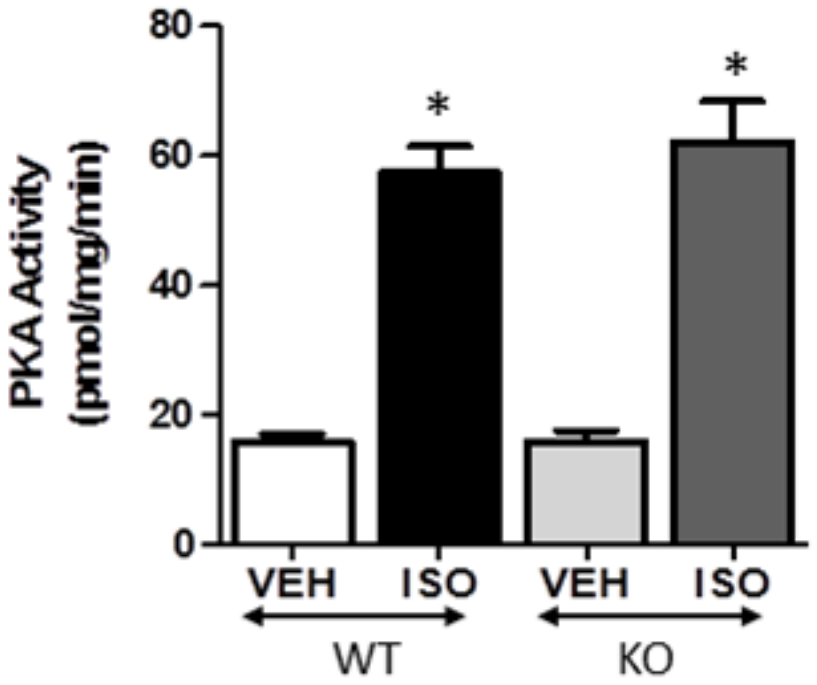

Figure 6: There is no difference in PKA activity between WT and KO mice. PKA activity in left ventricular cytosolic fractions isolated from WT and KO mice following acute vehicle or ISO infusion (10ug/g/min). Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P <0.05 vs. vehicle of same phenotype.

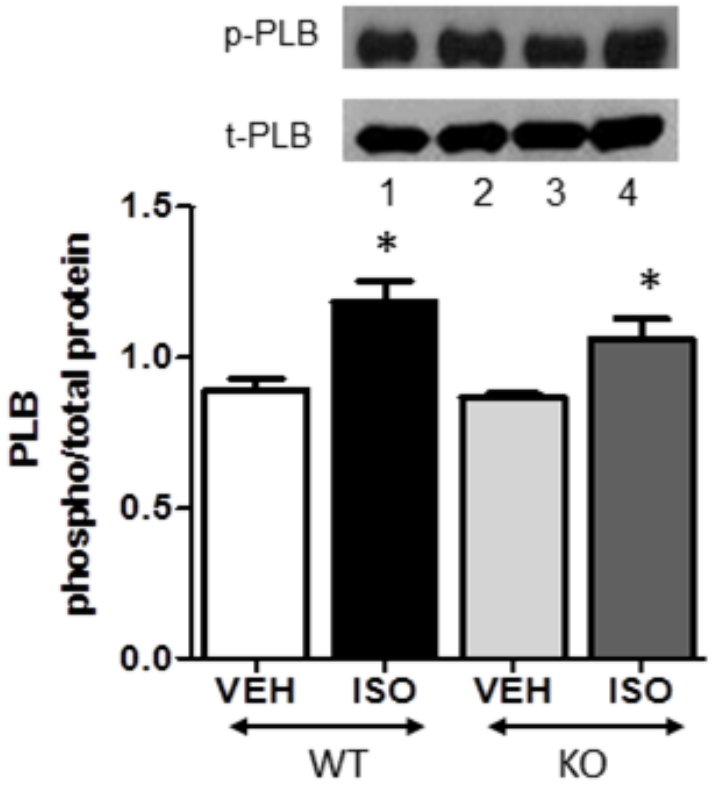

Figure 7: WT and KO mice have similar levels of PLB phosphorylation. Western blot analysis of PKA substrate phosphorylation of phospholamban (PLB) in left ventricular total homogenates isolated from WT and gravin-KO mice following acute vehicle or ISO infusion (10µg/g/min). The *upper panel* shows a Western blot with anti-phospho-protein antibody and the *lower panel* shows a Western blot with an antibody to total-protein (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO). The *bar graphs* show the ratio of phosphorylated to total protein normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P <0.05 vs. vehicle of same phenotype.

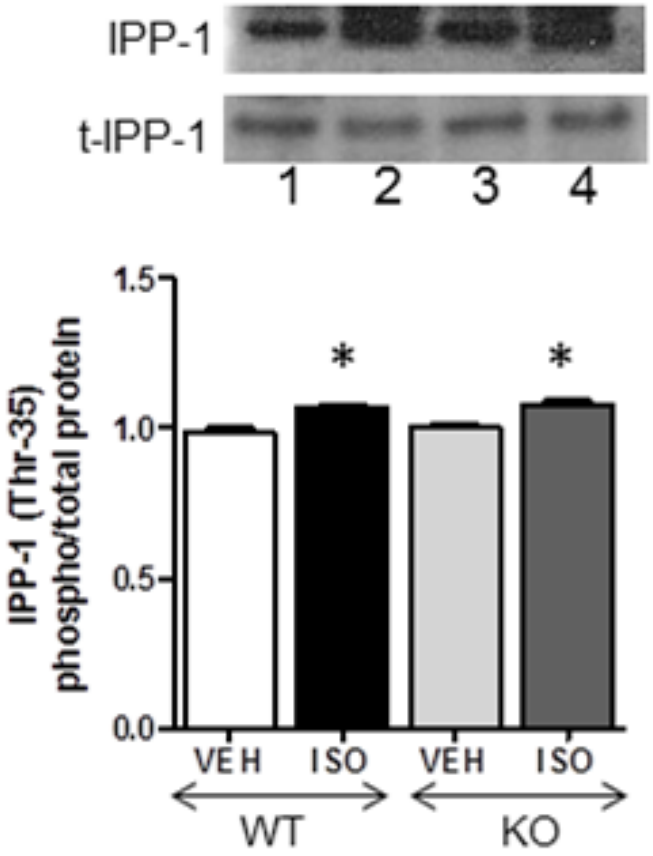

Figure 8: There is equal PKA phosphorylation of IPP-1 in WT and KO mice. Western blot analysis of PKA substrate phosphorylation of protein phosphatase 1 inhibitor 1 (IPP-1) in left ventricular total homogenates isolated from WT and gravin-KO mice following acute vehicle or ISO infusion (10µg/g/min). The *upper panel* shows a Western blot with anti-phospho-protein antibody and the *lower panel* shows a Western blot with an antibody to total-protein (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO). The *bar graphs* show the ratio of phosphorylated to total protein normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P <0.05 vs. vehicle of same phenotype.

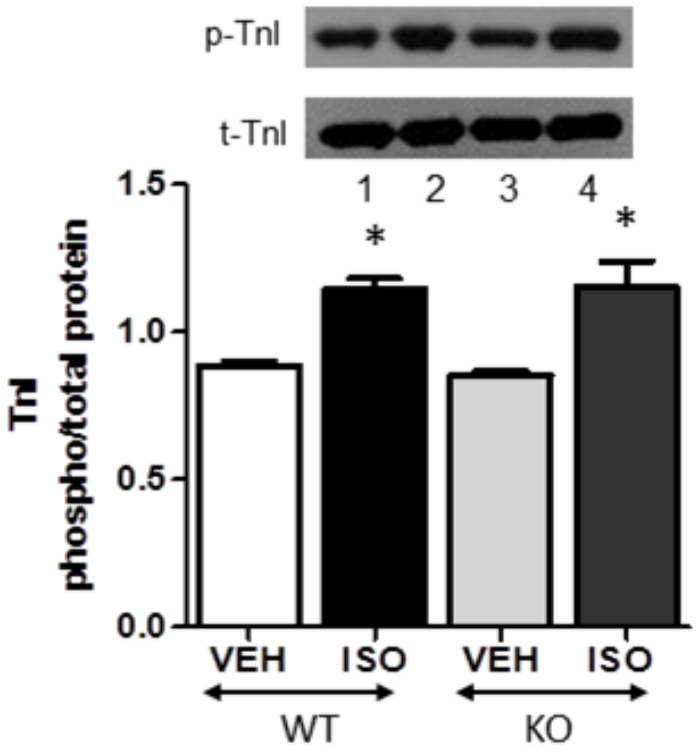

Figure 9: There is equal TnI phosphorylation in WT and KO mice. Western blot analysis of PKA substrate phosphorylation of Troponin I (TnI) in left ventricular total homogenates isolated from WT and gravin-KO mice following acute vehicle or ISO infusion (10µg/g/min). The *upper panel* shows a Western blot with anti-phospho-protein antibody and the *lower panel* shows a Western blot with an antibody to total-protein (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO). The *bar graphs* show the ratio of phosphorylated to total protein normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P <0.05 vs. vehicle of same phenotype.

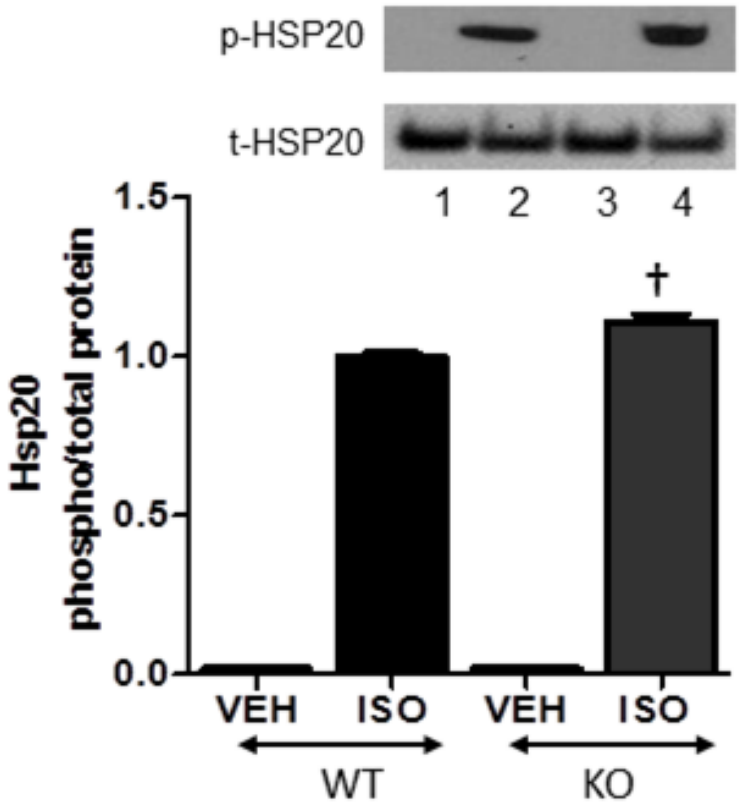

Figure 10: There is significantly increased Hsp20 phosphorylation in KO mice after acute ISO treatment compared to WT mice. Western blot analysis of PKA substrate phosphorylation of heat shock protein 20 (Hsp20) in left ventricular total homogenates isolated from WT and gravin-KO mice following acute vehicle or ISO infusion (10µg/g/min). The *upper panel* shows a Western blot with anti-phospho-protein antibody and the *lower panel* shows a Western blot with an antibody to total-protein (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO). The *bar graphs* show the ratio of phosphorylated to total protein normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; †$P<0.05$ vs. WT ISO.

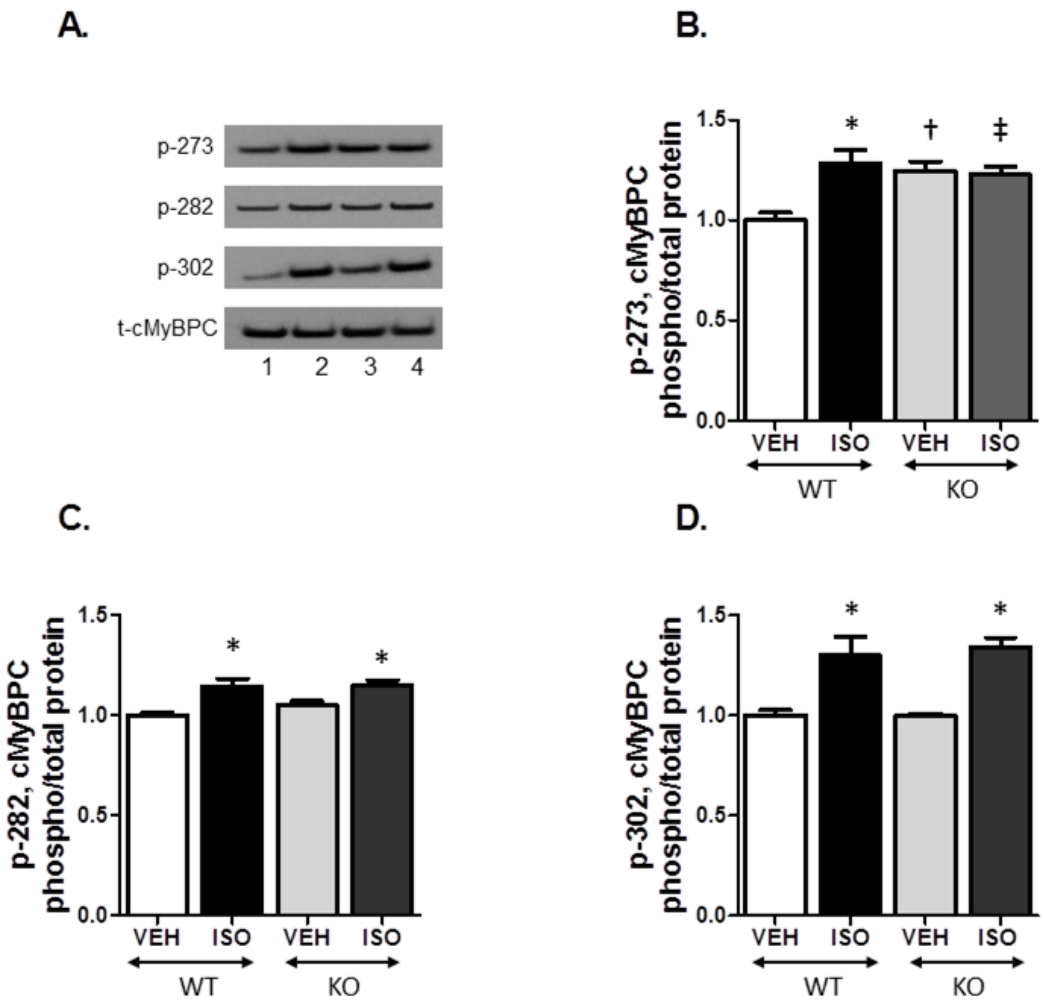

Figure 11: Phosphorylation of cMyBPC in response to acute ISO stimulation in WT and gravin-KO mice. *(A)* Representative western blots of the three phosphorylation sites of myosin binding protein C (MyBPC) as well as total MyBPC in left ventricular homogenates isolated from WT and gravin-KO mice following acute vehicle or ISO infusion (10µg/g/min; (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4:

KO ISO). (*B-D*) The *bar graphs* show the ratio of phosphorylated to total protein for p273, p282 and p302 respectively normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P <0.05 vs. vehicle of same phenotype; †P<0.05 either vehicle in WT vs. vehicle in KO mice or ISO in WT vs. ISO in KO mice; ‡P<0.05 either vehicle in WT vs. ISO in KO mice or ISO in WT vs. vehicle in KO mice.

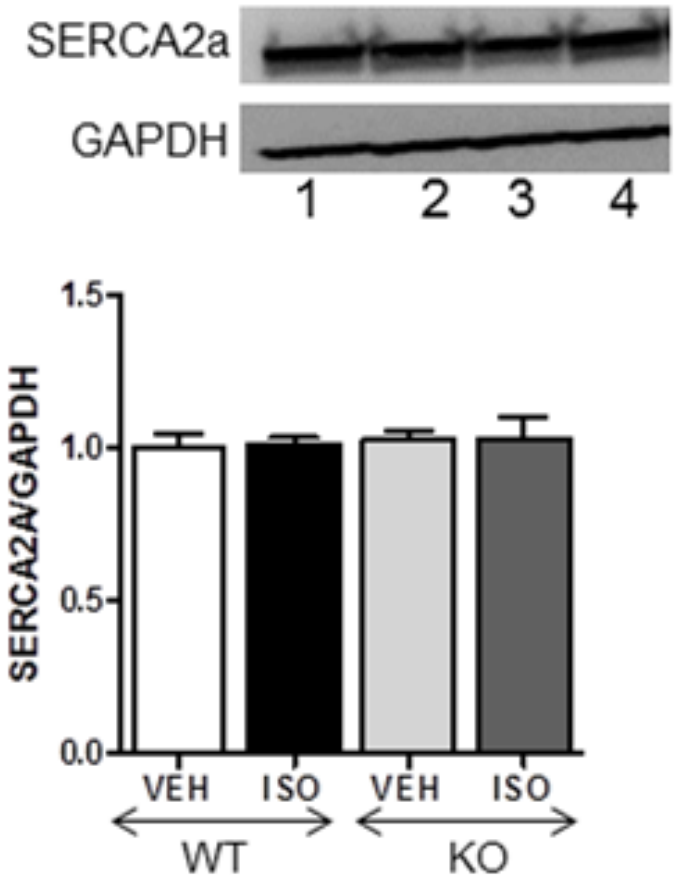

Figure 12: There is no difference in SERCA2a expression between WT and KO mice. Western blot analysis of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) in left ventricular total homogenates isolated from WT and gravin-KO mice following acute vehicle or ISO infusion (10µg/g/min). The *upper panel* shows a Western blot with anti-protein antibody and the *lower panel* shows a Western blot with an antibody to GAPDH (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO). The *bar graphs* show the ratio of phosphorylated to total protein normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples.

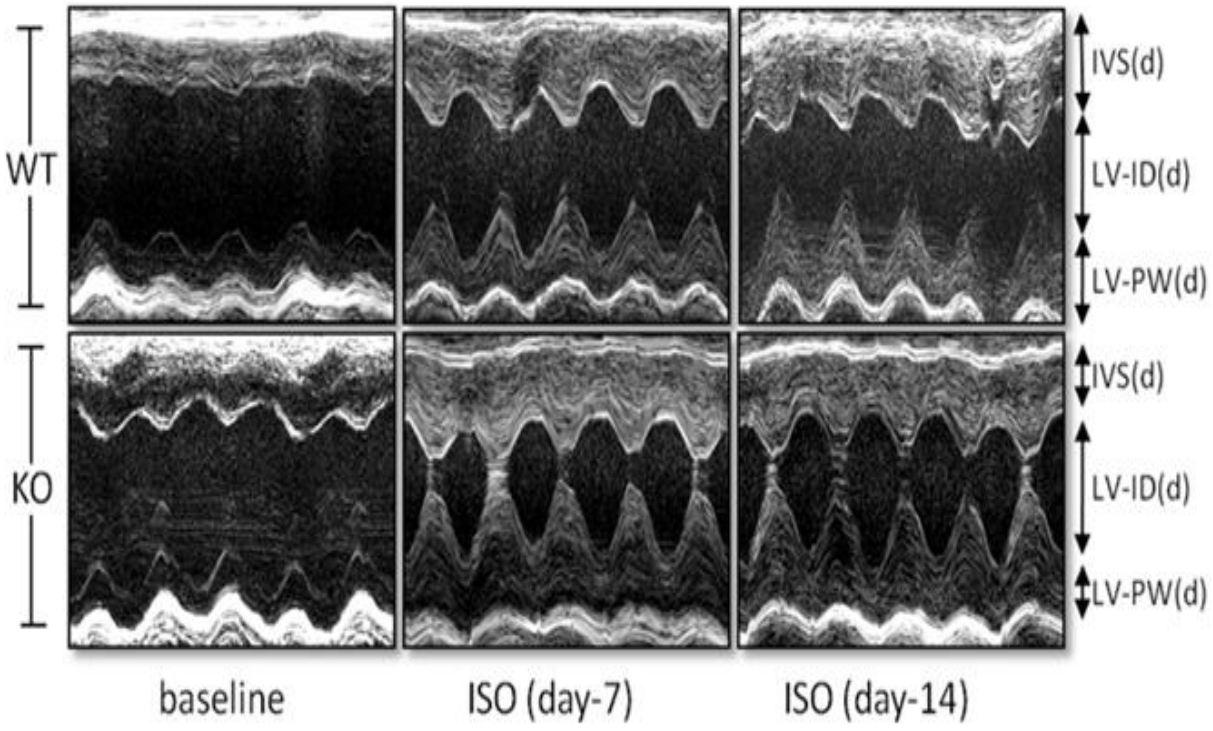

Figure 13: Representative transthoracic echocardiographic M-mode image of the LV from WT and KO hearts at baseline, following 7 days of ISO treatment and following 14 days of ISO treatment (15mg/kg/day). All measurements n=9.

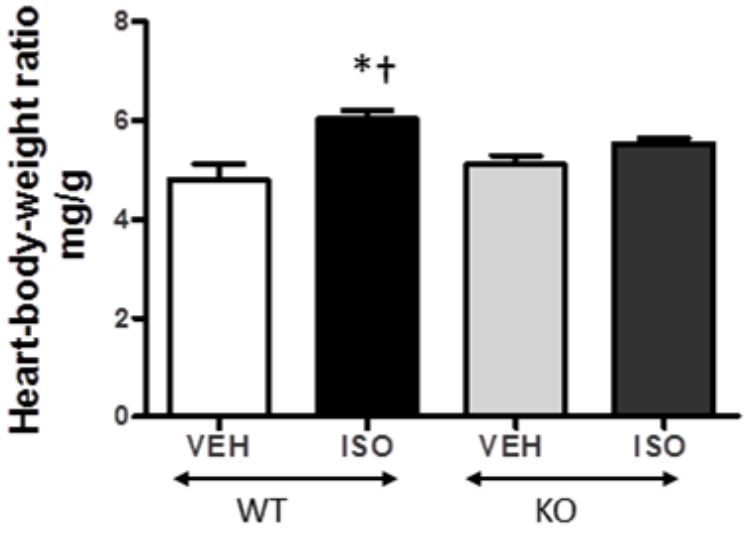

Figure 14: The absence of gravin blocks ISO-induced increases in heart-to-body-weight ratio. Heart-to-body-weight ratio of WT and KO hearts vehicle and ISO treated after 14 days of ISO treatment (15mg/kg/day). Data are expressed as mean ± S.E.M; n=10; *P <0.05 vs. vehicle of same phenotype; †P<0.05 ISO in WT vs. ISO in KO mice.

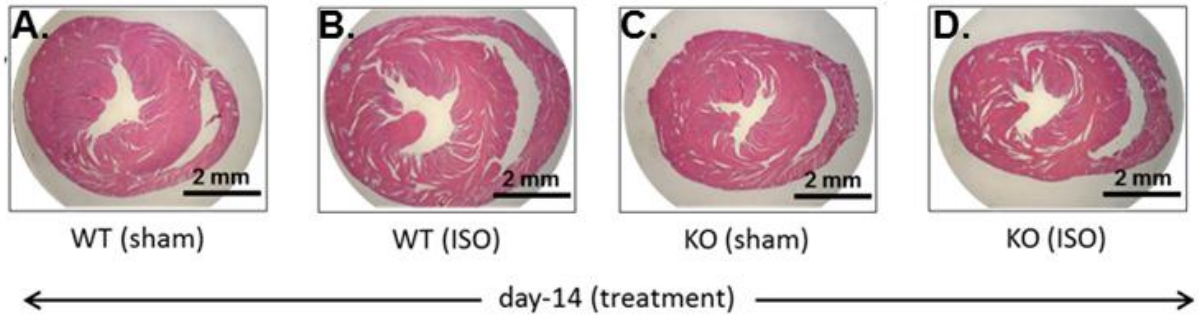

Figure 15: Cardiac morphology in response to chronic ISO stimulation in WT and KO mice. Masson's Trichrome (MT) stained paraffin sections of excised hearts following 14-days of ISO infusion. Sham mice were infused with control buffer (0.002% ascorbic acid).

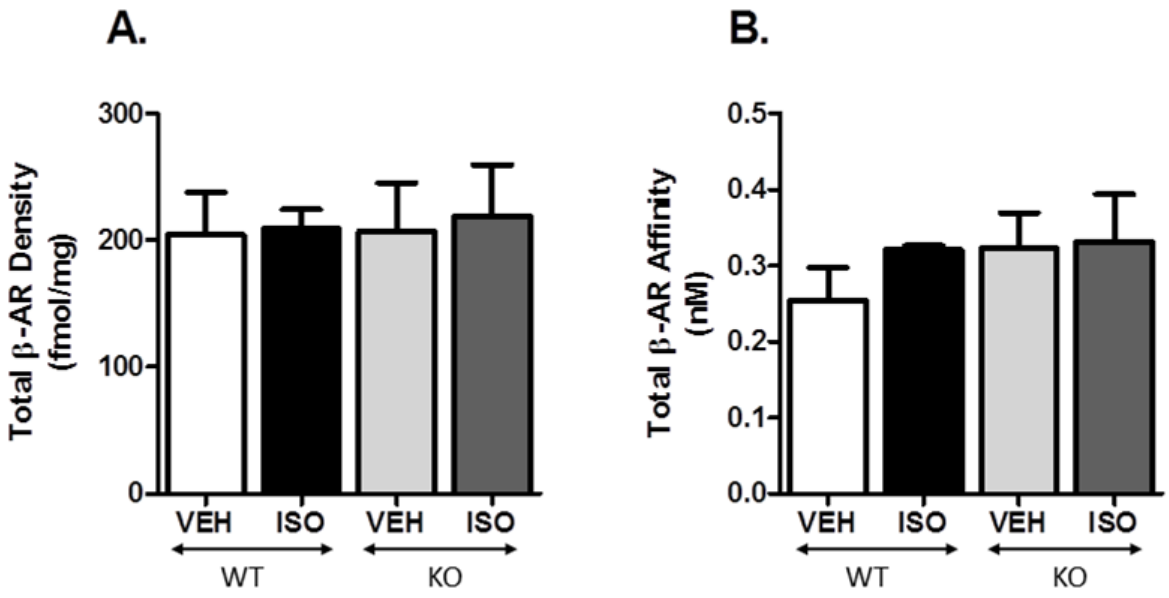

Figure 16: Total β-AR density and affinity following chronic ISO stimulation was similar between WT and KO mice. (*A*) β-adrenergic receptor density and (*B*) affinity was determined in left ventricular membrane fractions isolated from WT and gravin-KO mice following 14 days of ISO infusion. Saturation curves were made using [^{125}I]-cyanopindolol (10pM-4nM). Nonspecific binding was determined in the presence of 20μM alprenolol. Bmax (receptor density) was normalized to fmol/mg. Data expressed are means ± S.E.M.; n=5-10.

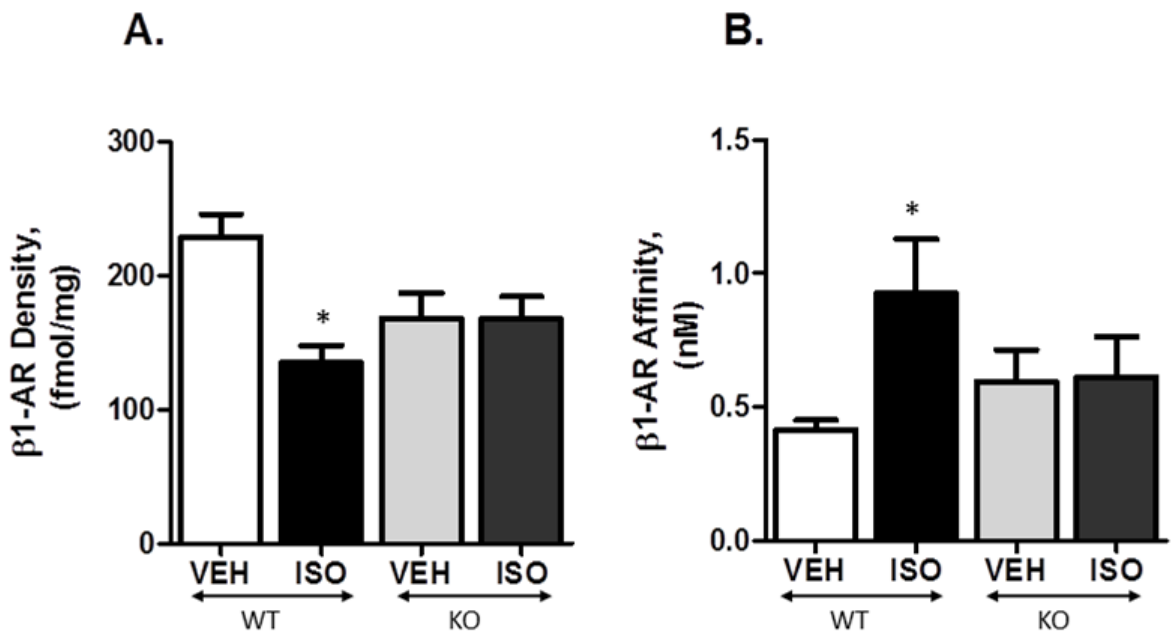

Figure 17: β_1-AR density and affinity was significantly reduced in WT mice but remained near normal levels in KO mice. (*A*) β_1–AR density and (*B*) affinity was measured in left ventricular membrane fractions isolated from WT and gravin-KO mice following 14 days of ISO infusion. Saturation curves were made using [^{125}I]-cyanopindolol (10pM-4nM). Nonspecific binding was determined in the presence of 20µM alprenolol. β_1-AR density was determined in the presence of 70nM ICI 118,551 to block β_2-AR. Bmax was normalized to fmol/mg. Data expressed are means ± SEM; n=5 to 10; *P <0.05 vs. vehicle of same phenotype.

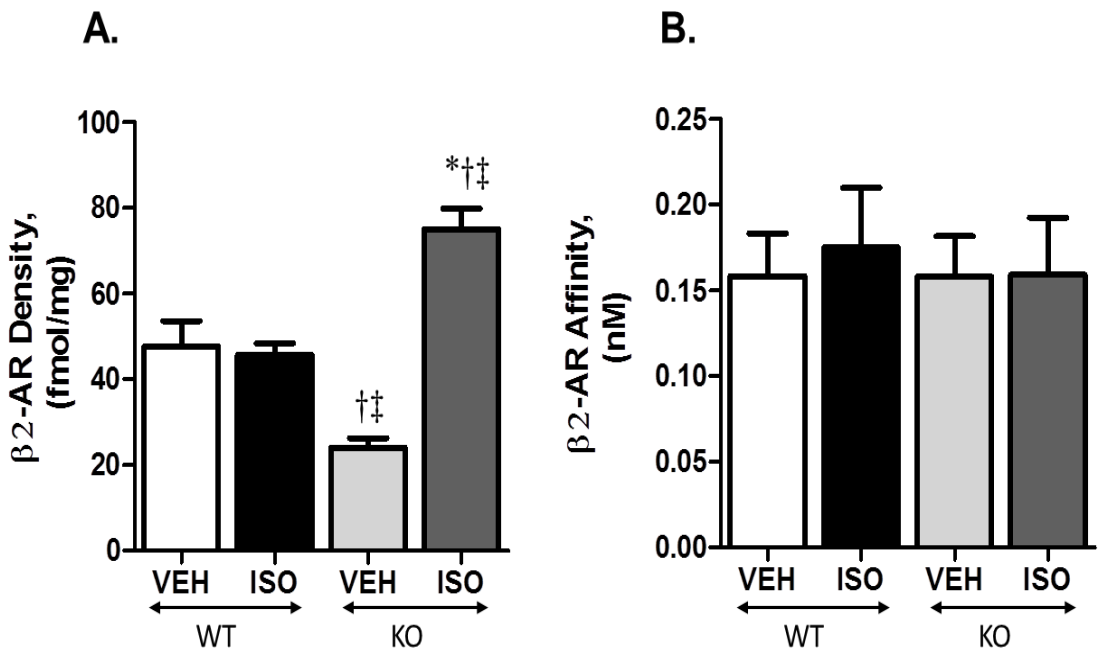

Figure 18: β_2-AR density was significantly increased in KO mice following chronic β-AR stimulation. β_2–AR density and affinity was measured in left ventricular membrane fractions isolated from WT and gravin-KO mice following 14 days of ISO infusion. Saturation curves were made using [^{125}I]-cyanopindolol (10pM-4nM). Nonspecific binding was determined in the presence of 20μM alprenolol. β_1-AR density was determined in the presence of 300μM CGP 20712A to block β_1-AR. Bmax was normalized to fmol/mg. Data expressed are means ± SEM; n=5 to 10; *P <0.05 vs. vehicle of same phenotype; †P<0.05 vs. WT vehicle; ‡P<0.05 vs. WT ISO.

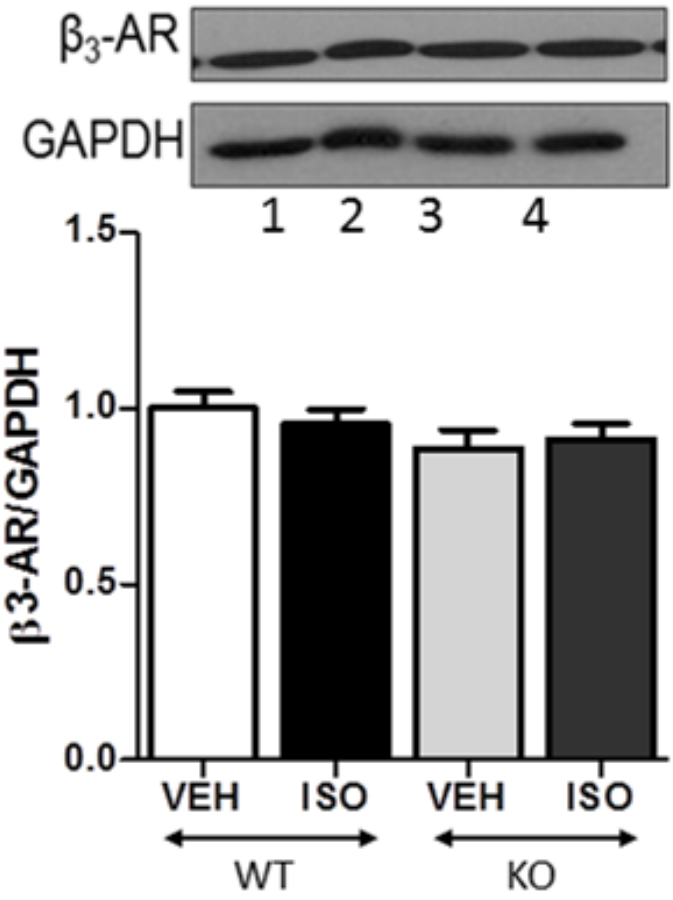

Figure 19: There is no difference in β_3-AR protein expression between WT and KO mice. Western blot analysis of total protein expression of β_3-AR in left ventricular membrane fractions isolated from WT and gravin-KO mice following 14 days of ISO infusion. For each substrate, the *upper panel* shows a Western blot with total protein antibody and the *lower panel* shows a Western blot for GAPDH. The *bar graphs* show the ratio of total protein to GAPDH normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples.

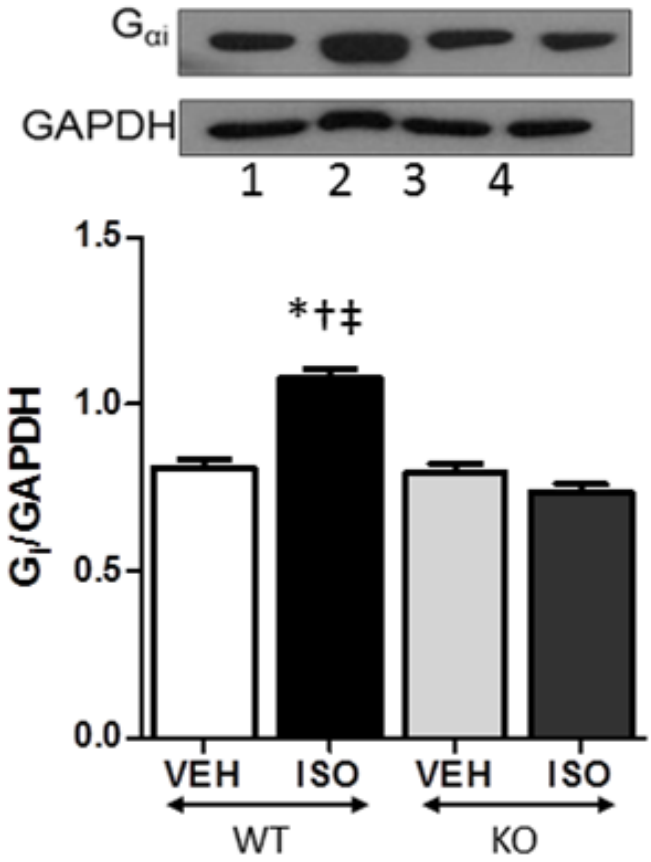

Figure 20: The absence of gravin blocks chronic ISO-induced upregulation of G_i expression. Western blot analysis of total protein expression of G_i in left ventricular membrane fractions isolated from WT and gravin-KO mice following 14 days of ISO infusion. For each substrate, the *upper panel* shows a Western blot with total protein antibody and the *lower panel* shows a Western blot for GAPDH. The *bar graphs* show the ratio of total protein to GAPDH normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05 vs. vehicle of same phenotype; †P<0.05 vs. KO ISO; ‡P<0.05 vs. KO vehicle.

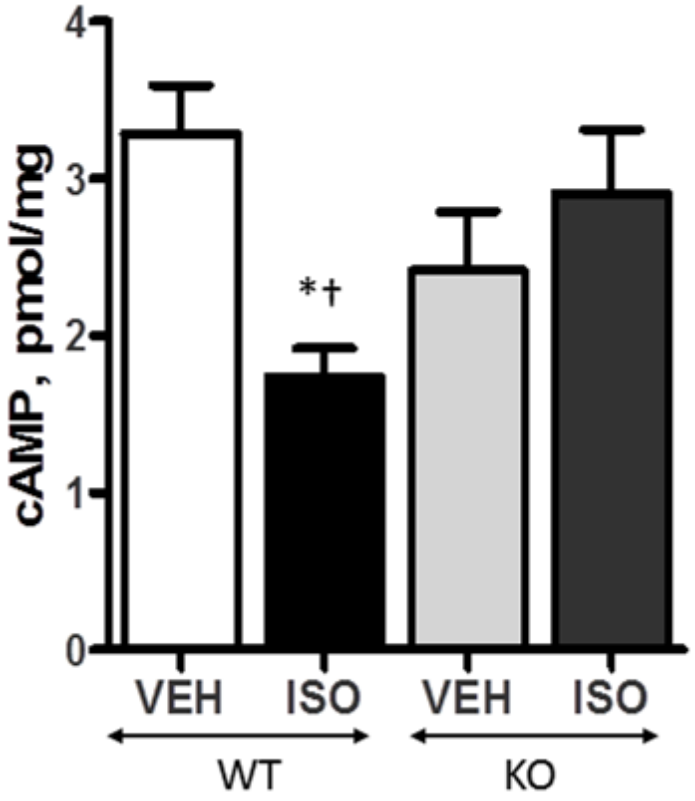

Figure 21: The absence of gravin blocks chronic ISO-induced decrease in cAMP production. cAMP production in left ventricular membrane fractions isolated from WT and gravin-KO mice following chronic vehicle or ISO treatment. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P <0.05 vs. vehicle of same phenotype; †P<0.05 vs. KO ISO.

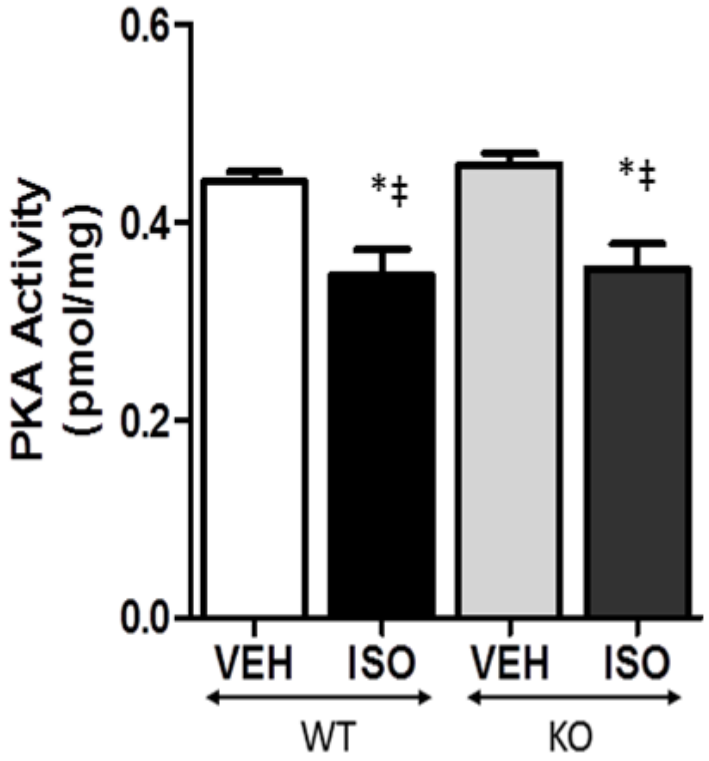

Figure 22: PKA activity is similarly decreased in WT and KO mice following chronic ISO stimulation. PKA activity in left ventricular cytosolic fractions isolated from WT and KO mice following chronic vehicle or ISO treatment. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P <0.05 vs. vehicle of same phenotype; ‡P<0.05 vs. vehicle of opposite phenotype.

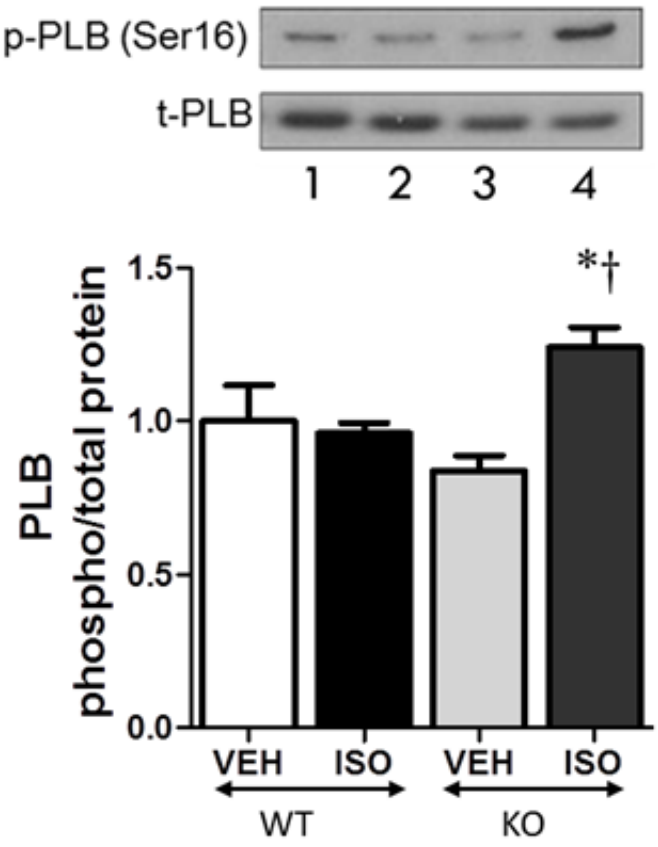

Figure 23: Gravin KO mice have significantly enhanced PLB phosphorylation following chronic ISO stimulation. Western blot analysis of PKA substrate phosphorylation of phospholamban (PLB) in left ventricular total homogenates isolated from WT and gravin-KO mice following chronic vehicle or ISO treatment. The *upper panel* shows a Western blot with anti-phospho-protein antibody and the *lower panel* shows a Western blot with an antibody to total-protein (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO). The *bar graphs* show the ratio of phosphorylated to total protein normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P <0.05 vs. vehicle of same phenotype; †P<0.05 vs. WT ISO.

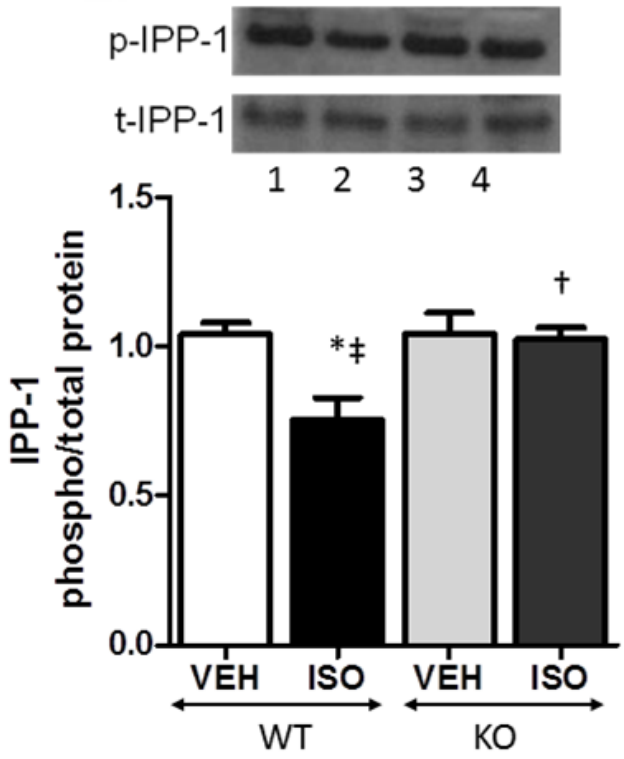

Figure 24: The absence of gravin blocks ISO-induced reduction of IPP-1 phosphorylation. Western blot analysis of PKA substrate phosphorylation of protein phosphatase 1 inhibitor 1 (IPP-1) in left ventricular total homogenates isolated from WT and gravin-KO mice following chronic vehicle or ISO stimulation. The *upper panel* shows a Western blot with anti-phospho-protein antibody and the *lower panel* shows a Western blot with an antibody to total-protein (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO). The *bar graphs* show the ratio of phosphorylated to total protein normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05 vs. vehicle of same phenotype; †P<0.05 vs. WT ISO; ‡P<0.05 vs. KO vehicle.

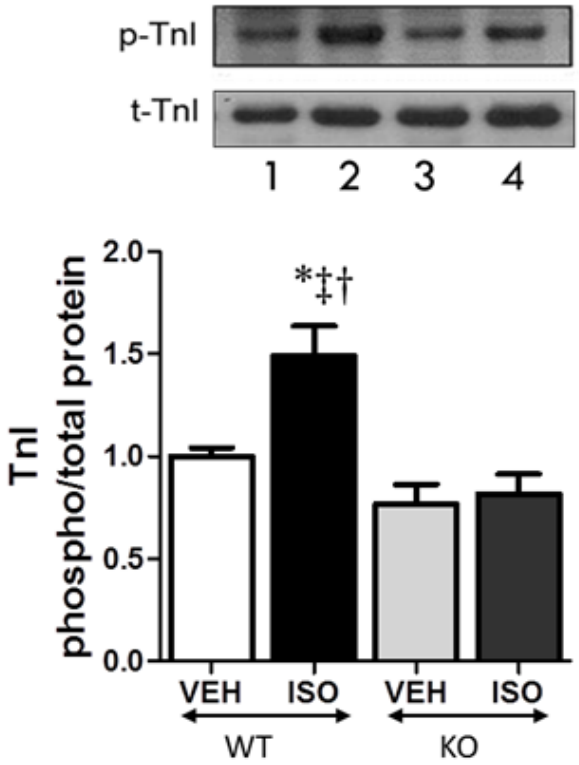

Figure 25: The absence of gravin blocks chronic ISO-induced increased TnI phosphorylation. Western blot analysis of PKA substrate phosphorylation of troponin I (TnI) in left ventricular total homogenates isolated from WT and gravin-KO mice following chronic vehicle or ISO stimulation. The *upper panel* shows a Western blot with anti-phospho-protein antibody and the *lower panel* shows a Western blot with an antibody to total-protein (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO). The *bar graphs* show the ratio of phosphorylated to total protein normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05 vs. vehicle of same phenotype; †P<0.05 vs. WT ISO; ‡P<0.05 vs. KO vehicle.

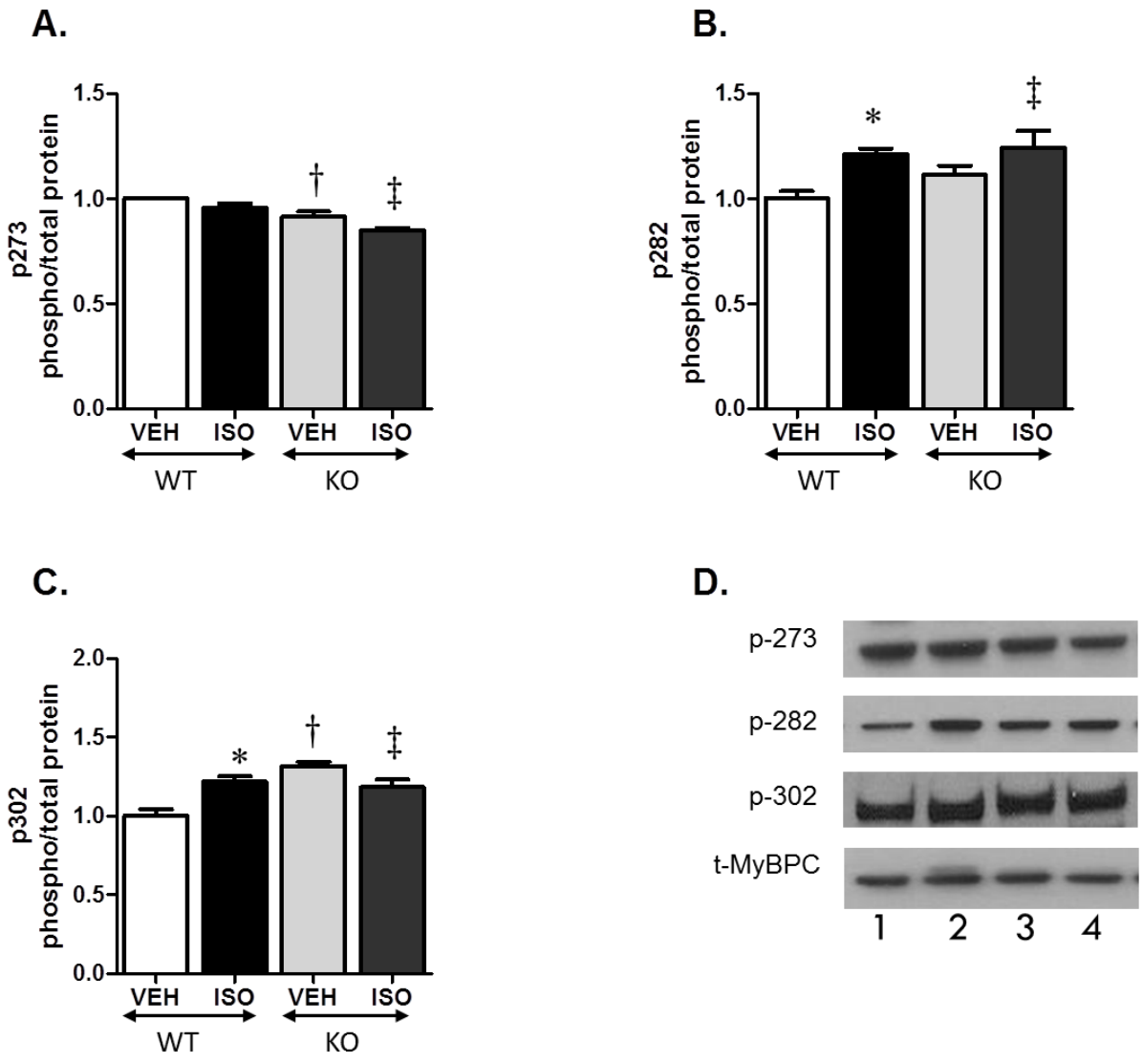

Figure 26: Alterations in cMyBPC phosphorylation between WT and KO mice. (*A-C*) The *bar graphs* show the ratio of phosphorylated to total protein for p273, p282 and p302 respectively normalized to WT vehicle. (*D*) Representative western blots of the three phosphorylation sites of myosin binding protein C (MyBPC;) as well as total MyBPC in

left ventricular homogenates isolated from WT and gravin-KO mice following chronic vehicle or ISO treatment (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO). Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P <0.05 vs. vehicle of same phenotype; †P<0.05 either vehicle in WT vs. vehicle in KO mice or ISO in WT vs. ISO in KO mice; ‡P<0.05 either vehicle in WT vs. ISO in KO mice or ISO in WT vs. vehicle in KO mice.

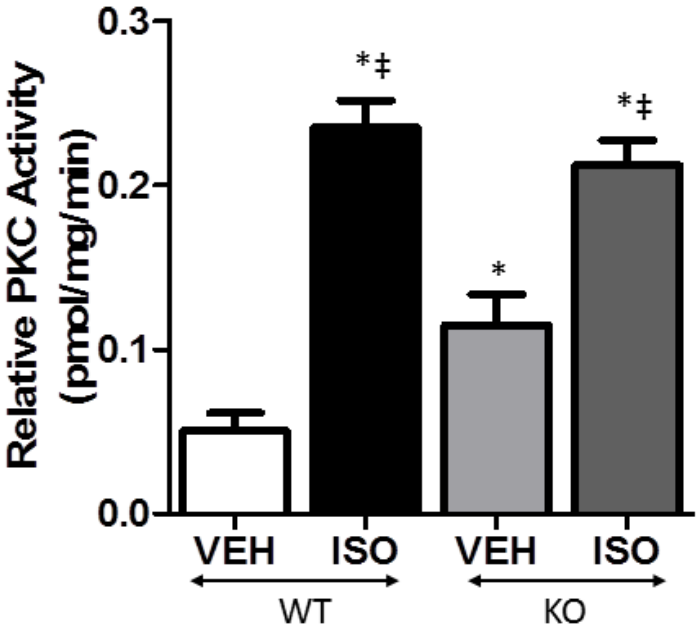

Figure 27: Gravin KO mice have significantly enhanced PKC activity. PKC activity in left ventricular cytosolic fractions isolated from WT and KO mice following chronic vehicle or ISO treatment. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P <0.05 vs. vehicle of same phenotype; ‡P<0.05 vs. vehicle of opposite phenotype.

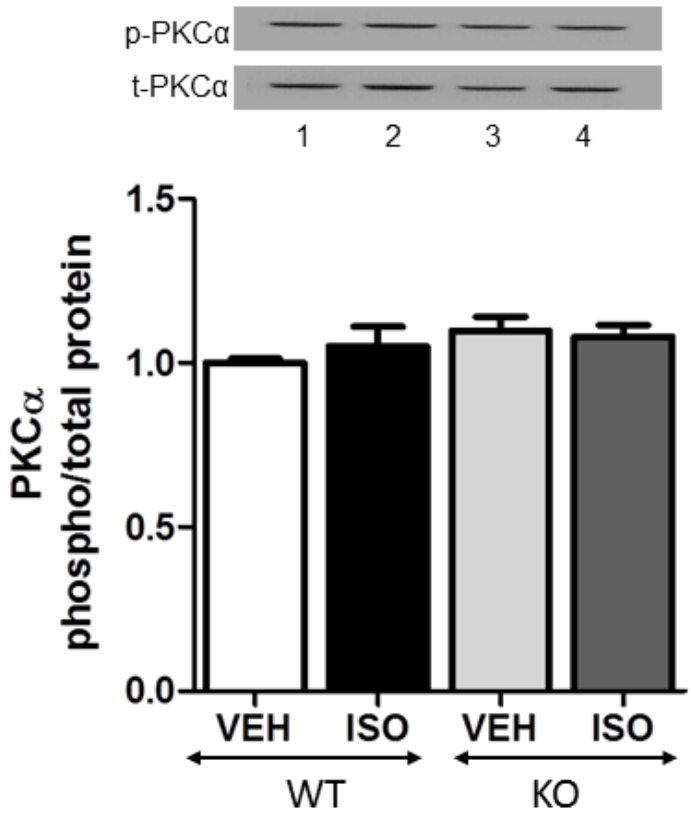

Figure 28: PKCα phosphorylation is unaltered between WT and KO mice regardless of ISO treatment. Western blot analysis of PKCα in left ventricular total homogenates isolated from WT and gravin-KO mice following chronic vehicle or ISO stimulation. The *upper panel* shows a Western blot with anti-phospho-protein antibody and the *lower panel* shows a Western blot with an antibody to total-protein (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO). The *bar graphs* show the ratio of phosphorylated to total protein normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples.

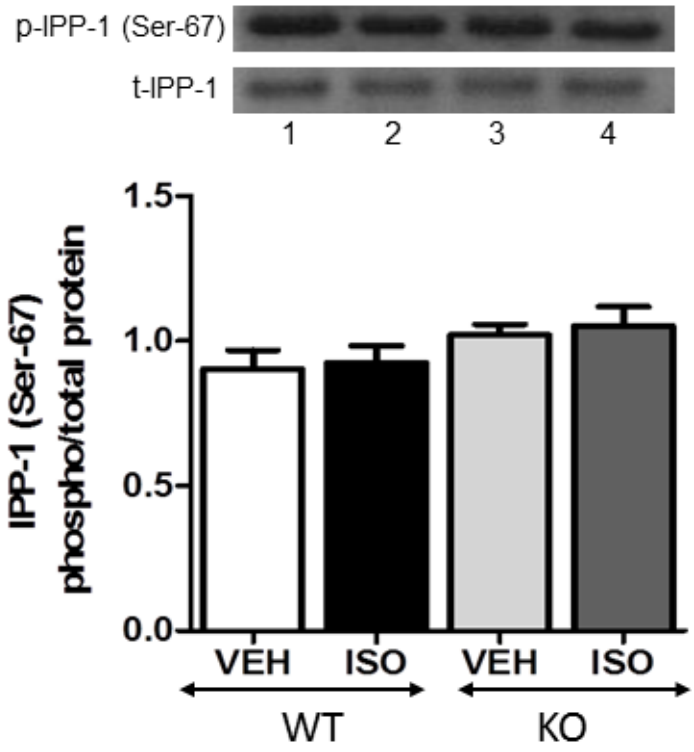

Figure 29: PKCα phosphorylation of IPP-1 is similar between WT and KO mice. Western blot analysis of PKCα phosphorylation of protein phosphatase 1 inhibitor 1 (IPP-1) in left ventricular total homogenates isolated from WT and gravin-KO mice following chronic vehicle or ISO stimulation. The *upper panel* shows a Western blot with anti-phospho-protein antibody and the *lower panel* shows a Western blot with an antibody to total-protein (Lane 1: WT VEH; Lane 2: WT ISO; Lane 3: KO VEH; Lane 4: KO ISO). The *bar graphs* show the ratio of phosphorylated to total protein normalized to WT vehicle. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples.

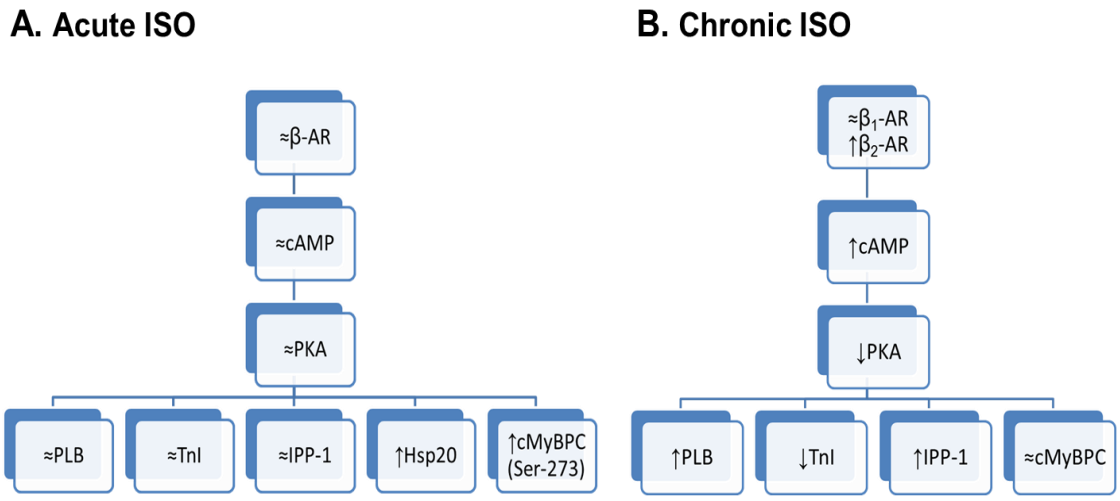

Figure 30: Summary of the effect of acute and chronic ISO treatment in KO mice. The changes listed are comparing KO mice to similarly treated WT mice. (*A)* Summary of changes induced by acute (0.25µg/g for 3 min) ISO stimulation in KO mice; (*B)* Summary of changes induced by chronic (15mg/kg/day for 14 days) ISO stimulation in KO mice. β-AR-beta adrenergic receptor; cAMP-3'-5'-cyclic adenosine monophosphate; PKA-protein kinase A; PLB-phospholamban; TnI-troponin I; IPP-1-protein phosphatase inhibitor 1; Hsp20-heat shock protein 20; cMyBPC-cardiac myosin binding protein C.

Table 1. Echocardiographic parameters of WT and Gravin-KO Mice

	WT Baseline	KO Baseline	WT Vehicle	KO Vehicle	WT ISO	KO ISO
HR (bpm)	478 ± 9	467 ± 6	467 ± 20	448 ± 20	596 ± 8*	571 ± 16*
IVSd (mm)	0.88 ± 0.02	0.87 ± 0.02	0.92 ± 0.03	0.93 ± 0.03	1.13 ± 0.15*‡	1.05 ± 0.22*‡
IVSs (mm)	0.96 ± 0.02	1.13 ± 0.02†	1.08 ± 0.05	1.30 ± 0.07†	1.12 ± 0.03*†	1.43 ± 0.08*†
LVPWd (mm)	0.81 ± 0.03	0.83 ± 0.04	0.78 ± 0.09	0.78 ± 0.07	1.10 ± 0.04*†	1.09 ± 0.04*†
LVPWs (mm)	1.18 ± 0.03	1.44 ± 0.04†	1.08 ± 0.09	1.30 ± 0.11†	1.84 ± 0.12*†	2.05 ± 0.10*†
SV (µL)	40.63 ± 0.79†	50.96 ± 0.49	43.94 ± 4.01	51.30 ± 2.36†	47.62 ± 1.16†‡	64.25 ± 4.06*†‡
EF (%)	58.86 ± 1.25	68.89 ± 1.25†	65.40 ± 4.15	75.42 ± 3.21†	87.81 ± 3.38*†	93.94 ± 1.28*†‡
CO (mL/min)	21.53 ± 0.60	25.55 ± 0.51†	19.82 ± 1.18	24.78 ± 1.00†	26.97 ± 1.35*†	32.84 ± 1.39*‡
LV FS (%)	32.40 ± 0.92	40.02 ± 1.04‡	33.89 ± 2.47	43.00 ± 1.47‡	54.47 ± 4.16*	68.03 ± 2.67*†‡

All values are expressed as mean ± SEM. KO, knockout; ISO, isoproterenol; HR, heart rate; IVS, intraventricular septum; LVPW, left ventricular posterior wall; SV, stroke volume; EF, ejection fraction; CO, cardiac output; *P <0.05 vs. baseline of same phenotype; †P<0.05 either baseline in WT vs. baseline in KO mice or ISO in WT vs. ISO in KO mice; ‡P<0.05 either baseline in WT vs. ISO in KO mice or ISO in WT vs. baseline in KO mice.

Table-2: ***Echocardiograph Parameters in WT and gravin-KO Mice in Response to Control (sham) or Chronic ISO Stimulation.***

	HR (bpm)	IV Sd (mm)	IV Ss (mm)	LV PWd (mm)	LV PWs (mm)	LV Vs (µl)	LV FS (%)	LV EF (µl)	SV (µl)	CO (ml/min)
Baseline										
WT (n=36)	483±9.71	0.89±0.02	0.96±0.02	0.81±0.03	1.18±0.03	29.4±1.99	32.40±0.92	58.9±1.25	40.63±0.79	21.53±0.6
KO (n=37)	454±6.8	0.87±0.02	1.13±0.02#	0.83±0.04	1.44±0.04#	22.77±1.36#	40.02±1.05	68.89±1.25	50.96±0.49	25.55±0.51
Day-7										
WT Sham (n=9)	498±24.01	0.93±0.03	0.92±0.03	0.84±0.07	1.07±0.02	33.03±0.9	31.13±1.24	60.3±2.62	38.97±3.17	22.62±0.97
KO Sham (n=9)	483±12.22	0.9±0.08	1.14±0.04#	0.82±0.05	1.46±0.03#	19.81±1.93#	40.66±1.26	69.65±1.46#	50.42±3.17	28.27±0.73
WT + ISO (n=9)	631±10.3*	1.16±0.04*	1.23±0.02*	1.19±0.06*	1.66±0.12*	11.62±1.29*	45.9±0.37*	80.13±1.60*	47.30±1.89	27.70±1.33*
KO + ISO (n=9)	631±17.82*	0.93±0.04#	1.40±0.07*#	0.84±0.07#	2.14±0.1*#	5.88±1.56*#	63.6±2.44*#	92.01±1.58*#	61.54±3.87*#	38.46±1.8*#
Day-14										
WT Sham (n=9)	498±31.84	0.92±0.03	0.96±0.05	0.79±0.05	0.96±0.03	33.93±4.23	30.64±1.29	58.76±1.91	38.81±2.23	20.76±0.89
KO Sham (n=9)	486±15.8	0.89±0.05	1.16±0.05#	0.8±0.03	1.43±0.04#	23.96±1.88#	37.33±1.46	67.68±1.82	49.33±2.22	25.17±0.5
WT + ISO (n=9)	607±11.93*	1.16±0.02*	1.09±0.05	1.21±0.09*	1.4±0.11*	29.67±6.24	33.01±3.31	63.6±5.00	42.15±1.53	24.43±0.99
KO + ISO (n=9)	617±24.79*	1.01±0.04#	1.38±0.08*#	0.8±0.05#	1.88±0.15*#	13.68±4.02*#	55.91±5.98*#	83.05±4.59*#	57.02±1.95	34.22±1.1*

BW indicates body weight; HR-heart rate; LVIDs-left ventricular internal diameter, systole; IVSs-interventricular septum, systole; IVSd-IVS, diastole; LVPWd-left ventricle posterior wall, diastole; LVPWs-LVPW, systole; LV Vd-LV volume, diastole ; LV Vs-LV volume, systole ; LV FS-LV fractional shortening; LV EF-LV ejection fraction; SV-stroke volume; CO-cardiac output; WT-wild type; KO-gravin deficiency; WT7d-WT treated on 7th day; WT 14d-WT treated on 14th day, ISO-isoproterenol; *p<0.05 vs respective sham; #p<0.05 vs WT baseline/7d/14d.

6. Discussion

6.1 Effect of acute β-AR stimulation on gravin knockout mice

Cardiac contractility is largely dependent upon the movement of Ca^{2+} in which homeostasis is itself regulated mainly through β-AR signaling.[3] Although cardiac contractility is mainly mediated through β_1-AR signaling, signaling through β_2-ARS coupled to G_s also has a positive inotropic effect. Additionally, signaling through β_2-AR has been shown to be beneficial both by increasing contraction in the normal heart and also promoting cell survival and cardioprotection in the injured or failing heart.[105] In the cardiomyocyte, stimulation of the β-adrenergic signaling pathway activates PKA which phosphorylates a number of substrates involved in cardiac contraction.

Disruption of PKA/AKAP interactions by addition of Ht31, a peptide that competes with AKAPs to bind the PKA regulatory subunits, has previously been shown to have a positive inotropic effect in the presence of β-AR stimulation.[56] In Ht31 over-expressed cardiomyocytes, baseline contractility remained unaltered but treatment with isoproterenol (10nM) resulted in enhanced contractility despite similar magnitudes of change in the intracellular Ca^{2+} transients in the WT and Ht31 over-expressed cardiomyocytes. PKA substrate phosphorylation was also significantly reduced in Ht31 cardiomyocytes.[55] However, there was greater cell shortening in the ISO treated Ht31 over-expressed cardiomyocytes. Additionally, LV ejection fraction and stroke volume were significantly increased in Ht31 over-expressed hearts following isoproterenol

treatment.[56] Gravin facilitates the phosphorylation of the receptor by both PKA and PKC as well as the recruitment of GRK2 and β-arrestin to the β_2-AR to initiate the desensitization/resensitization cycle and terminate the signaling of the receptor.[63] Thus, blockade of the termination of β_2-AR signaling would be expected to increase cardiac function. Accordingly, using gravin KO mice, we demonstrate that removal of this anchoring protein has increased cardiac function following acute stimulation by the nonspecific β-agonist isoproterenol.

Our results demonstrate that cardiac contractility is significantly increased in the gravin-KO mice, both before and after ISO stimulation (Table 1). Interestingly, despite enhanced contractility, cAMP production, PKA activity and PLB and TnI phosphorylation were similar between WT and KO hearts with or without ISO treatment (Figures 5-9). McConnell et al., have previously reported that global disruption of PKA/AKAP interactions resulted in a significant decrease of PKA phosphorylation of key proteins involved in excitation-contraction coupling, namely PLB, TnI and the ryanodine receptor, after ISO stimulation.[56] Since these changes were not mirrored in the KO mice, the data indicate that PKA phosphorylation of PLB and TnI is not dependent upon or facilitated by gravin. Therefore, the reduced PLB and TnI phosphorylation previously reported may be due to the disruption of PKA with another of the AKAPs present in the heart. However, it is also possible that one or more of these AKAPs are up-

regulated in the absence of gravin or that gravin's removal results in a larger pool of PKA available to AKAPs situated near PLB and TnI maintaining their phosphorylation.

Additionally, new technology using AKAR, a fluorescent biosensor, to investigate the spatial and temporal dynamics of PKA activation and signaling have shown that preferential stimulation of β_2-ARs results in a transient enhancement of PKA activity compared to the enhancement produced by stimulation of β_1-ARs.[34] Therefore, the increased cardiac function seen in the gravin KO mice may indeed be due to enhanced β_2-AR signaling which, because of its transient nature, may not have been detected using our current methods. Furthermore, β_2-AR stimulated PKA activity results in differential phosphorylation of PKA substrates and appears to depend upon the proximity of the substrates to the receptor.[34] Our current data corresponds with this concept as we did not see changes in phosphorylation of PLB, a sarcoplasmic reticulum protein but we did see slightly enhanced phosphorylation of Hsp20, a protein that has been shown to be localized primarily in the cytosol, in the KO ISO treated mice (Figure 10).[94] Previous studies have shown that Hsp20 phosphorylation is beneficial to the myocardium, therefore, this enhanced Hsp20 phosphorylation may contribute to the mechanism for increased cardiac function in the KO mice.[93]

IPP-1 can be phosphorylated by both PKA and protein kinase C but PKA phosphorylation of IPP-1 activates the protein, which then inactivates PP-1. PP-1 dephosphorylates many proteins involved in EC coupling including PLB, the inhibitor of

SERCA2a.[90, 91] IPP-1 null mice have been shown to have decreased cardiac function, increased protein phosphatase-1 activity and decreased phospholamban phosphorylation.[14, 92] Conversely, IPP-1 overexpression results in the augmentation of cardiac function even in the absence of β-adrenergic receptor stimulation.[92] Thus, PKA phosphorylation of IPP-1 is an important positive modulator of cardiac function. The absence of gravin did not affect IPP-1 expression, in the absence or presence of acute isoproterenol treatment, suggesting that PLB phosphorylation would be maintained as well (Figure 8). This maintenance of PLB phosphorylation enhances the activity of SERCA2a resulting in the increase of Ca^{2+} uptake into the SR and the facilitation of cardiac relaxation and contraction.

Further investigation into PKA substrates revealed alterations in protein phosphorylation that may also play a role in mediating the increased cardiac function seen in the ISO treated KO mice. cMyBPC is an important contractile protein whose phosphorylation has recently been implicated in the control of cardiac function. cMyBPC is phosphorylated by a variety of proteins at three major sites: Ser-273, Ser-282 and Ser-302. Although it is known that these phosphorylation sites are essential for both the activity of the protein as well as normal cardiac function, the effect of altered phosphorylation at individual sites is not clearly understood.[99, 100] Additionally, Sadayappan et al., reported that Ser-282 phosphorylation was a prerequisite for Ser-302 phosphorylation.[101] However, there is not much data concerning the functional role of

Ser-273 phosphorylation. Ser-273 phosphorylation was significantly increased in the gravin-KO mice compared to WT mice in the absence of ISO. Indeed, Ser-273 phosphorylation in the KO mice was comparable to that in the ISO treated WT mice regardless of the presence of ISO (Figure 11). We propose that this baseline increase in Ser-273 phosphorylation in the KO mice may help to explain the increased cardiac function seen in the KO mice as studies have shown that increased cMyBPC phosphorylation results in enhancement of the actin-myosin interaction resulting in the augmentation of the cross-bridge cycling rate and increased cardiac contractility.[106-108]

Studies performed upon the KO mice by our collaborators revealed that KO mice have reduced baseline and peak amplitude of Ca^{2+} compared to WT mice, which is similar to what was seen in cardiomyocytes expressing Ht31.[55] Additionally, there were indications that KO mice might have lower SR Ca^{2+} accumulation or content. However, our western blot analysis indicated that SERCA2a expression in KO hearts was equal to that of the WT (Figure 12). The SERCA2a is a pivotal protein in the modulation of both cardiac contractility and relaxation as its removal of Ca^{2+} from the cytosol into the SR facilitates relaxation and increased SR Ca^{2+} content results in the augmentation of subsequent contractions.[102] Alterations in SERCA2a expression in turn alters Ca^{2+} homeostasis, which translates into modification of cardiac contractility and relaxation. Additionally, mice with reduced SERCA2a expression more rapidly developed heart failure compared to WT mice while overexpression of SERCA2a enhances cardiac

function.[13, 109, 110] Our study shows that the absence of gravin does not affect SERCA2a expression. Furthermore, SERCA2a expression remained constant following acute isoproterenol treatment in both WT and gravin-KO mice hearts, indicating that Ca^{2+} reuptake into the SR is unaltered.

6.2 Effect of chronic β-AR stimulation on gravin knockout mice

β-AR stimulation initially enhances cardiac contractility but chronic stimulation triggers maladaptive responses that lead to decreased cardiac function and heart failure (HF). In the clinical setting, patients with HF experience increased sympathetic nervous system (SNS) activity in an effort to regulate cardiac function. The increase in SNS tone can result in cardiac arrhythmia, myocardial infarction and sudden cardiac death. β-AR antagonist treatment is used, when appropriate, to counteract the upregulation of SNS tone and reduce the progression of HF. However, the use of positive inotropic agents are limited by severe adverse effects such as sleep disturbances, cardiac arrhythmia and ventricular tachycardia.[3] Thus, alternate methods of attenuating the effects of chronic β-AR stimulation are of great importance. Our findings suggest that blockade of gravin may be an alternative mechanism of blocking the maladaptive effects of chronic β-AR stimulation.

The maintenance of cardiac function in the chronically treated KO mice appears to involve the reduction of overstimulation of PKA/Ca^{2+} signaling caused by chronic β-AR stimulation. PKA phosphorylation of its substrates in acute β-AR stimulation is an

important mediator of Ca^{2+} homeostasis and thus, cardiac contractility. In heart failure, PKA activation is significantly increased resulting in increased phosphorylation of its substrates. While increased phosphorylation of some of PKA's substrates may be beneficial for cardiac function, hyperphosphorylation of other substrates can be detrimental as PKA phosphorylates proteins that are involved in the termination of pro-contractility signaling.

In response to chronic β-AR stimulation, activation of PKA, GRK2 and β-arrestin lead to β-AR desensitization and downregulation.[6, 28, 111] Gravin assists in PKA phosphorylation of the receptor for desensitization as well as facilitates the recruitment of GRK2.[6, 112] Chronic β-AR stimulation results in a combination of β_1-AR downregulation, β_2-AR desensitization and increased β_2-AR signaling through G_i instead of G_s, resulting in a dramatic decrease in cardiac function.[6, 113] Gravin has been shown to play a major role in facilitating the desensitization of β-ARs. Additionally, it is well established from the literature that GRK2 directly phosphorylates occupied β-ARs and, with β-arrestin, causes receptor internalization and degradation[114]. GRK2 expression and activity are upregulated in failing heart which results in increased β-AR desensitization[115]. However, expression of a 184 amino acid C-terminus peptide inhibitor of GRK2 (βARK1ct) inhibited GRK-mediated receptor phosphorylation and HF reduction, resulting in enhanced basal and β-AR stimulated cardiac function[87].

Similarly, our lab has shown that KO ISO treated hearts had significantly lower levels of phosphorylated GRK2 and β-arrestin than the WT ISO treated hearts (unpublished data).[b] Furthermore, our findings show that while β_1-AR density and affinity was reduced in the WT ISO group, these changes were blocked in the KO mice (Figure 17). β_2-AR density was increased in both treatment groups but to a greater extent in the gravin-KO ISO group (Figure 18). These changes suggest that the absence of gravin may induce a similar scenario to that seen with βARK1ct treatment. Thus, blockade of GRK-mediated desensitization by the absence of gravin may play a role in the enhanced cardiac function seen in ISO treated gravin-KO mice. Additionally, our results indicate that gravin may also play a role in mediating β_1-AR desensitization instead of just affecting β_2-AR desensitization as has been previously described.[63]

While β_1- and β_2-ARs are the most prominent β-ARs in the heart, there are also a small percentage of β_3-ARs present. β_3-ARs have been shown to couple to G_i and activate the nitric oxide pathway which results in a negative inotropic response.[6] Fischer et al. demonstrated that mRNA levels of β_3-AR remained unchanged between failing and non-failing hearts.[72] Accordingly, we did not see any difference in β_3-AR protein expression among the four groups: WT and KO vehicles and ISO treated (Figure 19).

[b] Xing Yin, Ashley Guillory, Cori Wijaya, Abeer R'ababah, Quiying Fan and Bradley McConnell. Analysis of cardiac function in gravin (AKAP12) knockout mice with chronic beta-adrenergic receptor signaling. (In preparation 2012)

These findings indicate that increased cardiac function in gravin-KO mice is not due to upregulation of β_3-AR.

Changes in the level of G-protein expression have also been associated with chronic β-AR stimulation.[12, 71] Specifically, increased expression of G_i has been reported in HF, which results in increased coupling of this G-protein to the β_2-AR. Signaling through this G-protein antagonizes adenylyl cyclase activity resulting in reduced activation of PKA and β-AR-dependent augmentation of cardiac function.[29] Our data shows that chronic β-AR induced upregulation of G_i levels is blocked in KO animals. The lack of increased G_i levels in the KO ISO group suggests that coupling of G_i to the β_2-AR would be expected to be similar to that seen in the absence of HF (Figure 20). Additionally, normal G_i protein expression may contribute to maintaining cardiac function in the ISO treated KO mice.

Stimulation of β-ARs coupled to G_s results in the activation AC to increase the production of cAMP.[2] In HF, cAMP production levels are decreased, partially by the upregulation of $G_{\alpha i}$ expression, which inhibits AC.[116] Indeed, Bohm et al. showed that desensitization of adenylyl cyclase was associated with downregulation of β_1-ARs and increased G_i expression.[117] These findings are consistent with our data showing that cAMP production in the presence of ISO is significantly decreased in the WT ISO mice (Figure 21). Conversely, cAMP production in the KO ISO mice is comparable to that

produced in untreated WT mice. This data indicates that the absence of gravin abrogates the desensitization of adenylyl cyclase.

cAMP binds to the regulatory subunits of PKA to cause the release of the catalytic subunits, which are then free to phosphorylate PKA's various substrates.[36] Numerous reports have shown that PKA activity is significantly reduced in the presence of chronic β-AR stimulation. Accordingly, we determined that PKA activity was reduced in both treatment groups despite the rescued cAMP production seen in the KO ISO group (Figure 22). As previously mentioned, PDE activity is significantly increased in KO ISO treated animals compared to both nontreated WT and KO animals as well as ISO treated WT animals (unpublished data).[c] Therefore, the apparent inconsistencies between cAMP accumulation and PKA activity may primarily be due to the enhanced action of PDE.

Once activated, PKA phosphorylates a number of substrates involved in the regulation of cardiac function including phospholamban and troponin I.[31, 37] Phospholamban acts as a negative inhibitor of the SERCA2a and its phosphorylation relieves inhibition of the pump allowing for reuptake of Ca^{2+} into the SR. This facilitates relaxation of the heart as well as increases the Ca^{2+} store available for release upon the next contraction.[15] Acute β-AR stimulation greatly increases PLB phosphorylation and positively affects cardiac contractility. However, in heart failure, PLB phosphorylation is

[c] Ashley N. Guillory, Xing Yin, Cori Wijaya, Andrea Diaz Diaz, Abeer R'ababah, Fatin Atrooz and Bradley McConnell. Acute beta adrenergic receptor stimulation on cardiac function in A kinase anchoring protein gravin knockout mice. (In preparation, 2012).

decreased which inhibits the SERCA2a and leads to decreased Ca^{2+} store in the SR. While PLB phosphorylation was slightly decreased (not significant) in the WT ISO treated hearts, PLB phosphorylation was significantly increased in the KO ISO treated hearts which would lead to increased SR Ca^{2+} store and enhanced Ca^{2+} release from the SR via the RyR (Figure 23). We suggest that this increased SR Ca^{2+} content and subsequent increased SR Ca^{2+} released is partially responsible for the enhanced cardiac function seen in gravin-KO mice chronically treated with ISO.

IPP-1 is activated by PKA phosphorylation following β-AR stimulation. Activated IPP-1 inactivates protein phosphatase 1 to sustain PKA substrate phosphorylation.[90, 91] Increased IPP-1 phosphorylation has been associated with increased cardiac function, mainly through the enhancement of PLB phosphorylation and activity. Additionally, chronic β-AR stimulation has been shown to significantly reduce IPP-1 protein levels and phosphorylation.[118] This finding is corroborated by our data, which shows that IPP-1 phosphorylation was significantly reduced in the WT ISO groups. Conversely, this reduction was abrogated in the KO ISO group, which indicates that IPP-1 may play a critical role in the enhancement of cardiac function seen in the KO ISO mice (Figure 24). Therefore, the enhanced PLB phosphorylation seen in the KO ISO group is probably the result of a combination of increased PLB phosphorylation as well as the maintenance of IPP-1 phosphorylation levels.

Troponin I, another PKA substrate, is a key protein involved in modulating the Ca^{2+} sensitivity of the myofilaments. PKA-dependent TnI phosphorylation decreases myofilament Ca^{2+} affinity to facilitate muscle relaxation.[119] Therefore, a decrease in TnI phosphorylation would result in an increased Ca^{2+} affinity for troponin C to cause increased Ca^{2+} sensitivity for force development. TnI phosphorylation was not altered by chronic ISO treatment in the KO mice: observed as decreased TnI phosphorylation in response to chronic ISO, as compared to WT mice (Figure 25). This indicates that chronic β-AR stimulation in KO mice results in increased sensitivity of the myofibrillar apparatus to Ca^{2+}, and thus increased force development at any given intracellular free Ca^{2+} concentration.

cMyBPC is a thick filament protein whose importance in cardiac function has more recently come to light. Studies have shown that cMyBPC phosphorylation is required for normal cardiac function.[99] PKA phosphorylation of this protein, similar to TnI phosphorylation, reduces Ca^{2+} sensitivity of the myofilaments and facilitates relaxation.[99, 100] Total cMyBPC phosphorylation has been shown to be decreased in both human and mouse HF.[120] The absence of this downregulation of cMyBPC phosphorylation in the WT ISO hearts may be because, while 14 days of ISO treatment is sufficient to induce hypertrophic stress, it is not sufficient to send the hearts completely into HF (Figure 26). Also, studies showing the decrease in cMyBPC phosphorylation were performed using either surgical or genetic models of HF.[120] Although much is not known about the

functional significance of the three cMyBPC phosphorylation sites, phosphorylation of Ser-282 has been shown to facilitate the phosphorylation of the other sites.[101] Additionally, MyBPC can be phosphorylated by PKC, protein kinase D and CaMKII as well as PKA, making it difficult to interpret the functional relevance of phosphorylation.[96-98] Since gravin can coordinate the signaling of several of these kinases, further studies are needed to determine whether the changes seen in cMyBPC phosphorylation in the absence of gravin are PKA-dependent or are the result of altered activity of PKC or CaMKII.

Increased SNS tone that occurs in response to decreased contractility also results in the activation of pro-hypertrophic signals, such as that mediated through activation of ERK1/2, that, in the long-term, are detrimental to the heart. ERK1/2 signaling cascades can both regulate protein synthesis as well as activate transcription factors to stimulate pro-hypertrophic gene expression.[85, 121, 122] The activation of ERK1/2 and other pro-hypertrophic signaling cascades while initially compensatory, ultimately negatively impact cardiac function. Previous studies in our lab showed that ERK1/2 activation (as measured by its phosphorylation state) was significantly reduced in the KO ISO mice compared to the WT ISO mice (unpublished data).[d] This finding was further corroborated by our data reported here that showed a similar reduction of histological

[d] Xing Yin, Ashley Guillory, Cori Wijaya, Abeer R'ababah, Quiying Fan and Bradley McConnell. Analysis of cardiac function in gravin (AKAP12) knockout mice with chronic beta-adrenergic receptor signaling. (In preparation 2012)

abnormalities associated with cardiac hypertrophy as well as *in vivo* data displaying reduced cardiac remodeling parameters in the gravin-KO mice (Figures 14-15).

In summary, our findings indicate that gravin may play a significant role in β-AR-dependent signaling to modulate cardiac function both in the presence and absence of β-AR stimulation. We have shown that the removal of gravin results in increased basal cardiac function as well as increased cardiac function with chronic ISO stimulation. The effect of both acute and chronic ISO treatment in the KO mice has been summarized in Figure 30. The mechanism by which cardiac function is enhanced in gravin-KO mice following chronic ISO treatment appears to involve the normalization of the β-AR signaling pathway. Despite the presence of chronic β-AR stimulation, gravin-KO mice maintained near normal β-AR density and affinity. Maximal cAMP production via adenylyl cyclase in the gravin-KO ISO mice was comparable to that of WT. Despite reductions in PKA activity, PKA phosphorylation of substrates in the KO ISO group was also maintained at levels similar to that seen in untreated WT mice. Additionally, these alterations in PKA phosphorylation of substrates would be expected to augment cardiac function. Therefore, we propose the inhibition of gravin's protein/protein interactions as a novel therapeutic target for the treatment of disease states associated with chronic β-AR stimulation and desensitization of the β-AR signaling pathway leading to reduced cardiac function.

7. Summary and Conclusions

1. Gravin knockout mice have higher basal contractility.
2. Gravin knockout mice continue to have enhanced contractility following acute β-AR stimulation.
3. β-AR protein expression, cAMP accumulation and PKA activity is similar between WT and gravin knockout animals.
4. PLB and TnI phosphorylation is unaltered in gravin knockout mice before or after acute β-AR stimulation.
5. There is increased baseline phosphorylation of Ser-273 of cMyBPC in gravin knockout mice, which may contribute to enhanced basal contractility.
6. There is increased Hsp20 phosphorylation following β-AR stimulation in gravin knockout mice, which may contribute to enhanced contractility.
7. Gravin knockout mice have increased cardiac contractility compared to WT mice despite chronic β-AR stimulation.
8. The absence of gravin is sufficient to block chronic β-AR stimulation induced hypertrophy.
9. β-AR downregulation as a result of chronic β-AR stimulation is also blocked in the absence of gravin.
10. cAMP production levels are maintained in gravin knockout mice despite chronic β-AR stimulation.

11. PKA activity following chronic β-AR stimulation is reduced in gravin knockout mice, which may be due to increased PDE activity.
12. PLB phosphorylation is enhanced in gravin knockout mice following chronic β-AR stimulation, which may involve both direct phosphorylation of PLB by PKA as well as the maintenance of IPP-1 phosphorylation to inactivate protein phosphatase 1, the phosphatase responsible for dephosphorylating PLB.
13. TnI phosphorylation is normalized in the gravin knockout mice compared to the WT mice.
14. β-AR desensitization and downregulation is inhibited by the absence of gravin. Thus, β-AR signaling is normalized in the gravin knockout mice in the presence of chronic β-AR stimulation.

8. REFERENCES

1. Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB, Flegal K, *et al.* Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2009;**119**:480-486.
2. Bers DM. Cardiac excitation-contraction coupling. *Nature* 2002;**415**:198-205.
3. Katz AM, Lorell BH. Regulation of cardiac contraction and relaxation. *Circulation* 2000;**102**:IV69-74.
4. Bristow MR, Ginsburg R, Minobe W, Cubicciotti RS, Sageman WS, Lurie K, *et al.* Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts. *The New England journal of medicine* 1982;**307**:205-211.
5. El-Armouche A, Eschenhagen T. Beta-adrenergic stimulation and myocardial function in the failing heart. *Heart Fail Rev* 2009;**14**:225-241.
6. Lohse MJ, Engelhardt S, Eschenhagen T. What is the role of beta-adrenergic signaling in heart failure? *Circ Res* 2003;**93**:896-906.
7. Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Rosemblit N, *et al.* PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* 2000;**101**:365-376.

8. Bartel S, Stein B, Eschenhagen T, Mende U, Neumann J, Schmitz W, *et al.* Protein phosphorylation in isolated trabeculae from nonfailing and failing human hearts. *Molecular and cellular biochemistry* 1996;**157**:171-179.

9. Wang W, Zhu W, Wang S, Yang D, Crow MT, Xiao RP, *et al.* Sustained beta1-adrenergic stimulation modulates cardiac contractility by Ca2+/calmodulin kinase signaling pathway. *Circ Res* 2004;**95**:798-806.

10. Ungerer M, Bohm M, Elce JS, Erdmann E, Lohse MJ. Altered expression of beta-adrenergic receptor kinase and beta 1-adrenergic receptors in the failing human heart. *Circulation* 1993;**87**:454-463.

11. Choi DJ, Koch WJ, Hunter JJ, Rockman HA. Mechanism of beta-adrenergic receptor desensitization in cardiac hypertrophy is increased beta-adrenergic receptor kinase. *J Biol Chem* 1997;**272**:17223-17229.

12. Eschenhagen T, Mende U, Nose M, Schmitz W, Scholz H, Haverich A, *et al.* Increased messenger RNA level of the inhibitory G protein alpha subunit Gi alpha-2 in human end-stage heart failure. *Circ Res* 1992;**70**:688-696.

13. Schultz Jel J, Glascock BJ, Witt SA, Nieman ML, Nattamai KJ, Liu LH, *et al.* Accelerated onset of heart failure in mice during pressure overload with chronically decreased SERCA2 calcium pump activity. *Am J Physiol Heart Circ Physiol* 2004;**286**:H1146-1153.

14. Carr AN, Schmidt AG, Suzuki Y, del Monte F, Sato Y, Lanner C, *et al.* Type 1 phosphatase, a negative regulator of cardiac function. *Mol Cell Biol* 2002;**22**:4124-4135.

15. Bers DM. Altered cardiac myocyte Ca regulation in heart failure. *Physiology (Bethesda)* 2006;**21**:380-387.

16. Houser SR, Piacentino V, 3rd, Weisser J. Abnormalities of calcium cycling in the hypertrophied and failing heart. *J Mol Cell Cardiol* 2000;**32**:1595-1607.

17. Nunn C, Zou MX, Sobiesiak AJ, Roy AA, Kirshenbaum LA, Chidiac P. RGS2 inhibits beta-adrenergic receptor-induced cardiomyocyte hypertrophy. *Cell Signal* 2010;**22**:1231-1239.

18. Port JD, Bristow MR. Altered beta-adrenergic receptor gene regulation and signaling in chronic heart failure. *J Mol Cell Cardiol* 2001;**33**:887-905.

19. Hunt SA, Baker DW, Chin MH, Cinquegrani MP, Feldman AM, Francis GS, *et al.* ACC/AHA Guidelines for the Evaluation and Management of Chronic Heart Failure in the Adult: Executive Summary A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Revise the 1995 Guidelines for the Evaluation and Management of Heart Failure): Developed in Collaboration With the International Society for Heart and Lung Transplantation; Endorsed by the Heart Failure Society of America. *Circulation* 2001;**104**:2996-3007.

20. Packer M, Carver JR, Rodeheffer RJ, Ivanhoe RJ, DiBianco R, Zeldis SM, *et al.* Effect of oral milrinone on mortality in severe chronic heart failure. The PROMISE Study Research Group. *The New England journal of medicine* 1991;**325**:1468-1475.

21. Feldman AM, Bristow MR, Parmley WW, Carson PE, Pepine CJ, Gilbert EM, *et al.* Effects of vesnarinone on morbidity and mortality in patients with heart failure. Vesnarinone Study Group. *The New England journal of medicine* 1993;**329**:149-155.

22. Cerella C, Diederich M, Ghibelli L. The dual role of calcium as messenger and stressor in cell damage, death, and survival. *International journal of cell biology* 2010;**2010**:546163.

23. Hajjar RJ, Schwinger RH, Schmidt U, Kim CS, Lebeche D, Doye AA, *et al.* Myofilament calcium regulation in human myocardium. *Circulation* 2000;**101**:1679-1685.

24. Fan G, Shumay E, Wang H, Malbon CC. The scaffold protein gravin (cAMP-dependent protein kinase-anchoring protein 250) binds the beta 2-adrenergic receptor via the receptor cytoplasmic Arg-329 to Leu-413 domain and provides a mobile scaffold during desensitization. *J Biol Chem* 2001;**276**:24005-24014.

25. Shih M, Lin F, Scott JD, Wang HY, Malbon CC. Dynamic complexes of beta2-adrenergic receptors with protein kinases and phosphatases and the role of gravin. *J Biol Chem* 1999;**274**:1588-1595.

26. Dash R, Kadambi V, Schmidt AG, Tepe NM, Biniakiewicz D, Gerst MJ, *et al.* Interactions between phospholamban and beta-adrenergic drive may lead to cardiomyopathy and early mortality. *Circulation* 2001;**103**:889-896.

27. Pogwizd SM, Schlotthauer K, Li L, Yuan W, Bers DM. Arrhythmogenesis and contractile dysfunction in heart failure: Roles of sodium-calcium exchange, inward rectifier potassium current, and residual beta-adrenergic responsiveness. *Circ Res* 2001;**88**:1159-1167.

28. Daaka Y, Luttrell LM, Lefkowitz RJ. Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* 1997;**390**:88-91.

29. Perry SJ, Baillie GS, Kohout TA, McPhee I, Magiera MM, Ang KL, *et al.* Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. *Science* 2002;**298**:834-836.

30. Zhu W, Zeng X, Zheng M, Xiao RP. The enigma of beta2-adrenergic receptor Gi signaling in the heart: the good, the bad, and the ugly. *Circ Res* 2005;**97**:507-509.

31. Kaumann AJ, Molenaar P. Modulation of human cardiac function through 4 beta-adrenoceptor populations. *Naunyn Schmiedebergs Arch Pharmacol* 1997;**355**:667-681.

32. Rybin VO, Xu X, Lisanti MP, Steinberg SF. Differential targeting of beta -adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A

mechanism to functionally regulate the cAMP signaling pathway. *J Biol Chem* 2000;**275**:41447-41457.

33. Balijepalli RC, Foell JD, Hall DD, Hell JW, Kamp TJ. Localization of cardiac L-type Ca(2+) channels to a caveolar macromolecular signaling complex is required for beta(2)-adrenergic regulation. *Proc Natl Acad Sci U S A* 2006;**103**:7500-7505.

34. Soto D, De Arcangelis V, Zhang J, Xiang Y. Dynamic protein kinase a activities induced by beta-adrenoceptors dictate signaling propagation for substrate phosphorylation and myocyte contraction. *Circ Res* 2009;**104**:770-779.

35. Kuschel M, Zhou YY, Spurgeon HA, Bartel S, Karczewski P, Zhang SJ, *et al.* beta2-adrenergic cAMP signaling is uncoupled from phosphorylation of cytoplasmic proteins in canine heart. *Circulation* 1999;**99**:2458-2465.

36. Scott JD. Cyclic nucleotide-dependent protein kinases. *Pharmacol Ther* 1991;**50**:123-145.

37. Walsh DA, Van Patten SM. Multiple pathway signal transduction by the cAMP-dependent protein kinase. *FASEB J* 1994;**8**:1227-1236.

38. Keely SL. Activation of cAMP-dependent protein kinase without a corresponding increase in phosphorylase activity. *Research communications in chemical pathology and pharmacology* 1977;**18**:283-290.

39. Solaro RJ, Moir AJ, Perry SV. Phosphorylation of troponin I and the inotropic effect of adrenaline in the perfused rabbit heart. *Nature* 1976;**262**:615-617.

40. Yoshida A, Takahashi M, Imagawa T, Shigekawa M, Takisawa H, Nakamura T. Phosphorylation of ryanodine receptors in rat myocytes during beta-adrenergic stimulation. *J Biochem* 1992;**111**:186-190.

41. Hayes JS, Brunton LL. Functional compartments in cyclic nucleotide action. *Journal of cyclic nucleotide research* 1982;**8**:1-16.

42. Kentish JC, McCloskey DT, Layland J, Palmer S, Leiden JM, Martin AF, *et al.* Phosphorylation of troponin I by protein kinase A accelerates relaxation and crossbridge cycle kinetics in mouse ventricular muscle. *Circ Res* 2001;**88**:1059-1065.

43. MacKenzie SJ, Baillie GS, McPhee I, MacKenzie C, Seamons R, McSorley T, *et al.* Long PDE4 cAMP specific phosphodiesterases are activated by protein kinase A-mediated phosphorylation of a single serine residue in Upstream Conserved Region 1 (UCR1). *British journal of pharmacology* 2002;**136**:421-433.

44. Ahn JH, McAvoy T, Rakhilin SV, Nishi A, Greengard P, Nairn AC. Protein kinase A activates protein phosphatase 2A by phosphorylation of the B56delta subunit. *Proc Natl Acad Sci U S A* 2007;**104**:2979-2984.

45. Liu R, Ramani B, Soto D, De Arcangelis V, Xiang Y. Agonist dose-dependent phosphorylation by protein kinase A and G protein-coupled receptor kinase regulates beta2 adrenoceptor coupling to G(i) proteins in cardiomyocytes. *J Biol Chem* 2009;**284**:32279-32287.

46. Zamah AM, Delahunty M, Luttrell LM, Lefkowitz RJ. Protein kinase A-mediated phosphorylation of the beta 2-adrenergic receptor regulates its coupling to Gs and Gi. Demonstration in a reconstituted system. *J Biol Chem* 2002;**277**:31249-31256.

47. Zakhary DR, Fink MA, Ruehr ML, Bond M. Selectivity and regulation of A-kinase anchoring proteins in the heart. The role of autophosphorylation of the type II regulatory subunit of cAMP-dependent protein kinase. *J Biol Chem* 2000;**275**:41389-41395.

48. Colledge M, Scott JD. AKAPs: from structure to function. *Trends Cell Biol* 1999;**9**:216-221.

49. Colledge M, Dean RA, Scott GK, Langeberg LK, Huganir RL, Scott JD. Targeting of PKA to glutamate receptors through a MAGUK-AKAP complex. *Neuron* 2000;**27**:107-119.

50. Gao S, Wang HY, Malbon CC. AKAP12 and AKAP5 form higher-order hetero-oligomers. *J Mol Signal* 2011;**6**:8.

51. Mauban JR, O'Donnell M, Warrier S, Manni S, Bond M. AKAP-scaffolding proteins and regulation of cardiac physiology. *Physiology (Bethesda)* 2009;**24**:78-87.

52. Fraser ID, Tavalin SJ, Lester LB, Langeberg LK, Westphal AM, Dean RA, *et al.* A novel lipid-anchored A-kinase Anchoring Protein facilitates cAMP-responsive membrane events. *EMBO J* 1998;**17**:2261-2272.

53. Gao T, Yatani A, Dell'Acqua ML, Sako H, Green SA, Dascal N, *et al.* cAMP-dependent regulation of cardiac L-type Ca2+ channels requires membrane targeting of PKA and phosphorylation of channel subunits. *Neuron* 1997;**19**:185-196.

54. Kapiloff MS, Schillace RV, Westphal AM, Scott JD. mAKAP: an A-kinase anchoring protein targeted to the nuclear membrane of differentiated myocytes. *J Cell Sci* 1999;**112 (Pt 16)**:2725-2736.

55. Fink MA, Zakhary DR, Mackey JA, Desnoyer RW, Apperson-Hansen C, Damron DS, *et al.* AKAP-mediated targeting of protein kinase a regulates contractility in cardiac myocytes. *Circ Res* 2001;**88**:291-297.

56. McConnell BK, Popovic Z, Mal N, Lee K, Bautista J, Forudi F, *et al.* Disruption of protein kinase A interaction with A-kinase-anchoring proteins in the heart in vivo: effects on cardiac contractility, protein kinase A phosphorylation, and troponin I proteolysis. *J Biol Chem* 2009;**284**:1583-1592.

57. Chen L, Marquardt ML, Tester DJ, Sampson KJ, Ackerman MJ, Kass RS. Mutation of an A-kinase-anchoring protein causes long-QT syndrome. *Proc Natl Acad Sci U S A* 2007;**104**:20990-20995.

58. Tingley WG, Pawlikowska L, Zaroff JG, Kim T, Nguyen T, Young SG, *et al.* Gene-trapped mouse embryonic stem cell-derived cardiac myocytes and human genetics implicate AKAP10 in heart rhythm regulation. *Proc Natl Acad Sci U S A* 2007;**104**:8461-8466.

59. Gordon T, Grove B, Loftus JC, O'Toole T, McMillan R, Lindstrom J, *et al.* Molecular cloning and preliminary characterization of a novel cytoplasmic antigen recognized by myasthenia gravis sera. *J Clin Invest* 1992;**90**:992-999.

60. Nauert JB, Klauck TM, Langeberg LK, Scott JD. Gravin, an autoantigen recognized by serum from myasthenia gravis patients, is a kinase scaffold protein. *Current biology : CB* 1997;**7**:52-62.

61. Streb JW, Kitchen CM, Gelman IH, Miano JM. Multiple promoters direct expression of three AKAP12 isoforms with distinct subcellular and tissue distribution profiles. *J Biol Chem* 2004;**279**:56014-56023.

62. Willoughby D, Wong W, Schaack J, Scott JD, Cooper DM. An anchored PKA and PDE4 complex regulates subplasmalemmal cAMP dynamics. *EMBO J* 2006;**25**:2051-2061.

63. Lin F, Wang H, Malbon CC. Gravin-mediated formation of signaling complexes in beta 2-adrenergic receptor desensitization and resensitization. *J Biol Chem* 2000;**275**:19025-19034.

64. Carnegie GK, Means CK, Scott JD. A-kinase anchoring proteins: from protein complexes to physiology and disease. *IUBMB life* 2009;**61**:394-406.

65. Akakura S, Gelman IH. Pivotal Role of AKAP12 in the Regulation of Cellular Adhesion Dynamics: Control of Cytoskeletal Architecture, Cell Migration, and Mitogenic Signaling. *Journal of signal transduction* 2012;**2012**:529179.

66. Horvat SJ, Deshpande DA, Yan H, Panettieri RA, Codina J, Dubose TD, Jr., *et al.* A-kinase anchoring proteins regulate compartmentalized cAMP signaling in airway smooth muscle. *FASEB J* 2012;**26**:3670-3679.

67. Lee SW, Kim WJ, Choi YK, Song HS, Son MJ, Gelman IH, *et al.* SSeCKS regulates angiogenesis and tight junction formation in blood-brain barrier. *Nat Med* 2003;**9**:900-906.

68. Wang HY, Tao J, Shumay E, Malbon CC. G-Protein-coupled receptor-associated A-kinase anchoring proteins: AKAP79 and AKAP250 (gravin). *European journal of cell biology* 2006;**85**:643-650.

69. Feldman AM. Modulation of adrenergic receptors and G-transduction proteins in failing human ventricular myocardium. *Circulation* 1993;**87**:IV27-34.

70. Wallukat G, Podlowski S, Nissen E, Morwinski R, Csonka C, Tosaki A, *et al.* Functional and structural characterization of anti-beta1-adrenoceptor autoantibodies of spontaneously hypertensive rats. *Molecular and cellular biochemistry* 2003;**251**:67-75.

71. Feldman AM, Cates AE, Bristow MR, Van Dop C. Altered expression of alpha-subunits of G proteins in failing human hearts. *J Mol Cell Cardiol* 1989;**21**:359-365.

72. Fischer V, Gabauer I, Tillinger A, Novakova M, Pechan I, Krizanova O, *et al.* Heart adrenoceptor gene expression and binding sites in the human failing heart. *Ann N Y Acad Sci* 2008;**1148**:400-408.

73. Rockman HA, Chien KR, Choi DJ, Iaccarino G, Hunter JJ, Ross J, Jr., *et al.* Expression of a beta-adrenergic receptor kinase 1 inhibitor prevents the development of myocardial failure in gene-targeted mice. *Proc Natl Acad Sci U S A* 1998;**95**:7000-7005.

74. Harding VB, Jones LR, Lefkowitz RJ, Koch WJ, Rockman HA. Cardiac beta ARK1 inhibition prolongs survival and augments beta blocker therapy in a mouse model of severe heart failure. *Proc Natl Acad Sci U S A* 2001;**98**:5809-5814.

75. Hausdorff WP, Lohse MJ, Bouvier M, Liggett SB, Caron MG, Lefkowitz RJ. Two kinases mediate agonist-dependent phosphorylation and desensitization of the beta 2-adrenergic receptor. *Symposia of the Society for Experimental Biology* 1990;**44**:225-240.

76. Sibley DR, Strasser RH, Benovic JL, Daniel K, Lefkowitz RJ. Phosphorylation/dephosphorylation of the beta-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. *Proc Natl Acad Sci U S A* 1986;**83**:9408-9412.

77. Zhu WZ, Zheng M, Koch WJ, Lefkowitz RJ, Kobilka BK, Xiao RP. Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes. *Proc Natl Acad Sci U S A* 2001;**98**:1607-1612.

78. Communal C, Singh K, Sawyer DB, Colucci WS. Opposing effects of beta(1)- and beta(2)-adrenergic receptors on cardiac myocyte apoptosis : role of a pertussis toxin-sensitive G protein. *Circulation* 1999;**100**:2210-2212.

79. Waggoner JR, Kranias EG. Role of phospholamban in the pathogenesis of heart failure. *Heart failure clinics* 2005;**1**:207-218.

80. Bodor GS, Oakeley AE, Allen PD, Crimmins DL, Ladenson JH, Anderson PA. Troponin I phosphorylation in the normal and failing adult human heart. *Circulation* 1997;**96**:1495-1500.

81. Zakhary DR, Moravec CS, Bond M. Regulation of PKA binding to AKAPs in the heart: alterations in human heart failure. *Circulation* 2000;**101**:1459-1464.

82. Kiriazis H, Kranias EG. Genetically engineered models with alterations in cardiac membrane calcium-handling proteins. *Annual review of physiology* 2000;**62**:321-351.

83. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, *et al.* A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 1998;**93**:215-228.

84. Braz JC, Bueno OF, De Windt LJ, Molkentin JD. PKC alpha regulates the hypertrophic growth of cardiomyocytes through extracellular signal-regulated kinase1/2 (ERK1/2). *J Cell Biol* 2002;**156**:905-919.

85. Thorburn J, Frost JA, Thorburn A. Mitogen-activated protein kinases mediate changes in gene expression, but not cytoskeletal organization associated with cardiac muscle cell hypertrophy. *J Cell Biol* 1994;**126**:1565-1572.

86. Thorburn J, Xu S, Thorburn A. MAP kinase- and Rho-dependent signals interact to regulate gene expression but not actin morphology in cardiac muscle cells. *EMBO J* 1997;**16**:1888-1900.

87. Koch WJ, Rockman HA, Samama P, Hamilton RA, Bond RA, Milano CA, *et al.* Cardiac function in mice overexpressing the beta-adrenergic receptor kinase or a beta ARK inhibitor. *Science* 1995;**268**:1350-1353.

88. Salim S, Sinnarajah S, Kehrl JH, Dessauer CW. Identification of RGS2 and type V adenylyl cyclase interaction sites. *J Biol Chem* 2003;**278**:15842-15849.

89. Insel PA, Zhang L, Murray F, Yokouchi H, Zambon AC. Cyclic AMP is both a pro-apoptotic and anti-apoptotic second messenger. *Acta physiologica* 2012;**204**:277-287.

90. Herzig S, Neumann J. Effects of serine/threonine protein phosphatases on ion channels in excitable membranes. *Physiol Rev* 2000;**80**:173-210.

91. Oliver CJ, Shenolikar S. Physiologic importance of protein phosphatase inhibitors. *Front Biosci* 1998;**3**:D961-972.

92. El-Armouche A, Rau T, Zolk O, Ditz D, Pamminger T, Zimmermann WH, *et al.* Evidence for protein phosphatase inhibitor-1 playing an amplifier role in beta-adrenergic signaling in cardiac myocytes. *FASEB J* 2003;**17**:437-439.

93. Edwards HV, Scott JD, Baillie GS. PKA phosphorylation of the small heat-shock protein Hsp20 enhances its cardioprotective effects. *Biochem Soc Trans* 2012;**40**:210-214.

94. Pipkin W, Johnson JA, Creazzo TL, Burch J, Komalavilas P, Brophy C. Localization, macromolecular associations, and function of the small heat shock-related protein HSP20 in rat heart. *Circulation* 2003;**107**:469-476.

95. Sin YY, Edwards HV, Li X, Day JP, Christian F, Dunlop AJ, *et al.* Disruption of the cyclic AMP phosphodiesterase-4 (PDE4)-HSP20 complex attenuates the beta-agonist induced hypertrophic response in cardiac myocytes. *J Mol Cell Cardiol* 2011;**50**:872-883.

96. Bardswell SC, Cuello F, Rowland AJ, Sadayappan S, Robbins J, Gautel M, *et al.* Distinct sarcomeric substrates are responsible for protein kinase D-mediated regulation of cardiac myofilament Ca2+ sensitivity and cross-bridge cycling. *J Biol Chem* 2010;**285**:5674-5682.

97. Gautel M, Zuffardi O, Freiburg A, Labeit S. Phosphorylation switches specific for the cardiac isoform of myosin binding protein-C: a modulator of cardiac contraction? *EMBO J* 1995;**14**:1952-1960.

98. Mohamed AS, Dignam JD, Schlender KK. Cardiac myosin-binding protein C (MyBP-C): identification of protein kinase A and protein kinase C phosphorylation sites. *Arch Biochem Biophys* 1998;**358**:313-319.

99. Sadayappan S, Gulick J, Osinska H, Martin LA, Hahn HS, Dorn GW, 2nd, *et al.* Cardiac myosin-binding protein-C phosphorylation and cardiac function. *Circ Res* 2005;**97**:1156-1163.

100. Sadayappan S, Osinska H, Klevitsky R, Lorenz JN, Sargent M, Molkentin JD, *et al.* Cardiac myosin binding protein C phosphorylation is cardioprotective. *Proc Natl Acad Sci U S A* 2006;**103**:16918-16923.

101. Sadayappan S, Gulick J, Osinska H, Barefield D, Cuello F, Avkiran M, *et al.* A critical function for Ser-282 in cardiac Myosin binding protein-C phosphorylation and cardiac function. *Circ Res* 2011;**109**:141-150.

102. Kranias EG, Hajjar RJ. Modulation of Cardiac Contractility by the Phopholamban/SERCA2a Regulatome. *Circ Res* 2012;**110**:1646-1660.

103. Moniotte S, Kobzik L, Feron O, Trochu JN, Gauthier C, Balligand JL. Upregulation of beta(3)-adrenoceptors and altered contractile response to inotropic amines in human failing myocardium. *Circulation* 2001;**103**:1649-1655.

104. Guo LW, Gao L, Rothschild J, Su B, Gelman IH. Control of protein kinase C activity, phorbol ester-induced cytoskeletal remodeling, and cell survival signals by the scaffolding protein SSeCKS/GRAVIN/AKAP12. *J Biol Chem* 2011;**286**:38356-38366.

105. Pleger ST, Boucher M, Most P, Koch WJ. Targeting myocardial beta-adrenergic receptor signaling and calcium cycling for heart failure gene therapy. *J Card Fail* 2007;**13**:401-414.

106. Tong CW, Stelzer JE, Greaser ML, Powers PA, Moss RL. Acceleration of crossbridge kinetics by protein kinase A phosphorylation of cardiac myosin binding protein C modulates cardiac function. *Circ Res* 2008;**103**:974-982.

107. Flashman E, Redwood C, Moolman-Smook J, Watkins H. Cardiac myosin binding protein C: its role in physiology and disease. *Circ Res* 2004;**94**:1279-1289.

108. Stelzer JE, Patel JR, Moss RL. Protein kinase A-mediated acceleration of the stretch activation response in murine skinned myocardium is eliminated by ablation of cMyBP-C. *Circ Res* 2006;**99**:884-890.

109. He H, Giordano FJ, Hilal-Dandan R, Choi DJ, Rockman HA, McDonough PM, *et al.* Overexpression of the rat sarcoplasmic reticulum Ca2+ ATPase gene in the heart of transgenic mice accelerates calcium transients and cardiac relaxation. *J Clin Invest* 1997;**100**:380-389.

110. Baker DL, Hashimoto K, Grupp IL, Ji Y, Reed T, Loukianov E, *et al.* Targeted overexpression of the sarcoplasmic reticulum Ca2+-ATPase increases cardiac contractility in transgenic mouse hearts. *Circ Res* 1998;**83**:1205-1214.

111. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* 2002;**115**:455-465.

112. Tao J, Wang HY, Malbon CC. Protein kinase A regulates AKAP250 (gravin) scaffold binding to the beta2-adrenergic receptor. *EMBO J* 2003;**22**:6419-6429.

113. Dorn GW, 2nd. Medicine. Refugee receptors switch sides. *Science* 2010;**327**:1586-1587.

114. Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. *Annu Rev Biochem* 1998;**67**:653-692.

115. Iaccarino G, Tomhave ED, Lefkowitz RJ, Koch WJ. Reciprocal in vivo regulation of myocardial G protein-coupled receptor kinase expression by beta-adrenergic receptor stimulation and blockade. *Circulation* 1998;**98**:1783-1789.

116. Kirchhefer U, Schmitz W, Scholz H, Neumann J. Activity of cAMP-dependent protein kinase and Ca2+/calmodulin-dependent protein kinase in failing and nonfailing human hearts. *Cardiovasc Res* 1999;**42**:254-261.

117. Bohm M, Castellano M, Agabiti-Rosei E, Flesch M, Paul M, Erdmann E. Dose-dependent dissociation of ACE-inhibitor effects on blood pressure, cardiac hypertrophy, and beta-adrenergic signal transduction. *Circulation* 1995;**92**:3006-3013.

118. El-Armouche A, Gocht F, Jaeckel E, Wittkopper K, Peeck M, Eschenhagen T. Long-term beta-adrenergic stimulation leads to downregulation of protein phosphatase inhibitor-1 in the heart. *Eur J Heart Fail* 2007;**9**:1077-1080.

119. Zhang R, Zhao J, Mandveno A, Potter JD. Cardiac troponin I phosphorylation increases the rate of cardiac muscle relaxation. *Circ Res* 1995;**76**:1028-1035.

120. El-Armouche A, Pohlmann L, Schlossarek S, Starbatty J, Yeh YH, Nattel S, *et al.* Decreased phosphorylation levels of cardiac myosin-binding protein-C in human and experimental heart failure. *J Mol Cell Cardiol* 2007;**43**:223-229.

121. Gillespie-Brown J, Fuller SJ, Bogoyevitch MA, Cowley S, Sugden PH. The mitogen-activated protein kinase kinase MEK1 stimulates a pattern of gene

expression typical of the hypertrophic phenotype in rat ventricular cardiomyocytes. *J Biol Chem* 1995;**270**:28092-28096.

122. Yue TL, Gu JL, Wang C, Reith AD, Lee JC, Mirabile RC, *et al.* Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy. *J Biol Chem* 2000;**275**:37895-37901.