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Mechanisms of HDAC2 Function in the Regulation of Adult Cardiac Hypertrophy and Embryonic Myocyte Proliferation

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Abstract
HDACs can modify the structure and function of chromatin to regulate gene expression and can also modify many non-histone proteins that regulate cell function and signaling. HDACs have attracted increasing interest because they are involved in a variety of physiologic and pathophysiologic processes including stem cell maintenance, differentiation, cancer, inflammation and cardiac diseases. In the absence of Hdac2, mice display myocyte hyper-proliferation during embryonic development and resistance to cardiac hypertrophy in adulthood. This doctoral dissertation examines the function of two Hdac2 downstream targets, Inpp5f and Gata4, in regulating adult cardiac hypertrophy and embryonic myocyte proliferation, respectively. My results suggest that Inpp5f is an important endogenous modulator of the cardiac response to stress. I also performed studies to show that, in the absence of Hdac2 in the embryonic heart, hyper-acetylated Gata4 is responsible for myocyte hyper-proliferation. These findings provide a framework for understanding the role of Hdac2 in embryonic heart development and adult cardiac diseases.

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MECHANISMS OF HDAC2 FUNCTION IN THE REGULATION OF ADULT CARDIAC HYPERTROPHY AND EMBRYONIC MYOCYTE PROLIFERATION

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ABSTRACT

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HDACs can modify the structure and function of chromatin to regulate gene expression and can also modify many non-histone proteins that regulate cell function and signaling. HDACs have attracted increasing interest because they are involved in a variety of physiologic and pathophysiologic processes including stem cell maintenance, differentiation, cancer, inflammation and cardiac diseases. In the absence of Hdac2, mice display myocyte hyper-proliferation during embryonic development and resistance to cardiac hypertrophy in adulthood. This doctoral dissertation examines the function of two Hdac2 downstream targets, Inpp5f and Gata4, in regulating adult cardiac hypertrophy and embryonic myocyte proliferation, respectively. My results suggest that Inpp5f is an important endogenous modulator of the cardiac response to stress. I also performed studies to show that, in the absence of Hdac2 in the embryonic heart, hyper-acetylated Gata4 is responsible for myocyte hyper-proliferation. These findings provide a framework for understanding the role of Hdac2 in embryonic heart development and adult cardiac diseases.

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Chapter 1 Introduction

In this dissertation, I explore the mechanisms of histone deacetylase 2 (Hdac2) in regulating adult cardiac hypertrophy and embryonic cardiomyocyte proliferation. Previous work suggests that Hdac2 promotes hypertrophy associated with increased phosphoinositide 3-kinase (PI3K)/Akt signaling and Hdac2 negatively regulates the expression of a novel phosphoinositide 5-phosphatase, inositol polyphosphate-5-phosphatase 5 F (Inpp5f) (Trivedi et al., 2007). The activity of this protein is specific for PtdIns(4,5)P2 and PtdIns(3,4,5)P3 (Minagawa et al., 2001). In chapter 2, I describe experiments to study the role of Inpp5f in regulating hypertrophy. These studies led to the discovery that Inpp5f is an important endogenous modulator of cardiac responses to stresses and it regulates the hypertrophy process through regulating the PI3K/Akt pathway. Another interesting phenotype of Hdac2 knockout mice is hyper-proliferation of myocytes during embryonic development. In chapter 3, I investigated the role of Hdac2 in regulating GATA-binding factor 4 (Gata4), a crucial regulator of cardiomyocytes proliferation (Rojas et al., 2008; Zeisberg et al., 2005). This introductory chapter will summarize our current knowledge of PI3K/Akt signaling during cardiac hypertrophy, Gata4 function during heart development and other areas of interest related to my thesis project.

1.1 Cardiac hypertrophy

Cardiovascular disease remains the number one cause of mortality in the Western world, with heart failure representing the fastest growing subclass over the past decade (Heineke and Molkentin, 2006; Hobbs, 2004; Kannel, 2000; Levy et al., 2002). For example, there are approximately 5 million Americans currently diagnosed with heart failure, which is characterized by a 5-year survival rate of approximately 50%, resulting in a total economic impact as high as 100 billion dollars per year (Heineke and Molkentin, 2006).

Cardiac hypertrophy is an early milestone during the clinical course of heart failure and an important risk factor for subsequent cardiac morbidity and mortality (Hunter and Chien, 1999). During cardiac hypertrophy, individual myocytes grow in length and/or width as a means of increasing cardiac pump function and decreasing ventricular wall tension (Heineke and Molkentin, 2006). Although hypertrophy is initially beneficial, permitting enhanced cardiac output, it can ultimately become deleterious and result in cardiomyopathy, heart failure and sudden death (McKinsey and Olson, 1999). It can occur in response to normal physiological stimuli, as during pregnancy and exercise, or may be associated with disease-inducing stimuli leading to cardiac dilatation and congestive heart failure (Frey and Olson, 2003). The defining features of hypertrophy are an increase in cardiomyocyte size, enhanced protein synthesis, and a higher organization of the sarcomere. It is not accompanied by cell proliferation or cardiac cell renewal. The changes in cellular phenotype are preceded

and accompanied by the reinduction of the so-called fetal gene program, including natriuretic peptide precursor type A (Nppa) (encoding atrial natriuretic factor, ANF), Myh7(encoding β-myosin heavy chain), and Nppb (encoding BNP) (Frey and Olson, 2003).

Cardiac hypertrophy requires coupling of intracellular signal transduction systems with transcription factors that activate and maintain the hypertrophic program (McKinsey and Olson, 1999). Over the past years, signaling pathways involving growth factors, G proteins, mitogen-activated protein kinases and calcium-responsive phosphatases have emerged as critical regulators of cardiac hypertrophy (Dorn and Force, 2005; Gupta et al., 2007; McKinsey and Olson, 1999). The PI3K/Akt signaling network is of particular interest because many components in this pathway have been shown to modulate myocyte survival, hypertrophy, contractility, electrophysiology, metabolism, mechanotransduction and coronary angiogenesis (Oudit and Penninger, 2009). The following sections will describe these in detail and discuss the experimental data supporting a role for the PI3K/Akt pathway in the hypertrophic process.

Another particularly important phenomenon involved in gene expression is histone acetylation/deacetylation which is mediated by histone acetyl transferase (HAT) and HDAC (Ekwall, 2005). The balance between HAT and HDAC seems to play an especially important role in the control of gene expression, which, according to recent evidence, will also affect the hypertrophic process (Antos et al., 2003; Chang

et al., 2004; Kong et al., 2006; Kook et al., 2003; Montgomery et al., 2007; Trivedi et al., 2007; Zhang et al., 2002).

Despite all the current knowledge of hypertrophy, the cellular mechanisms that regulate the hypertrophic response to agonists or to stretch remain poorly understood, and treatments to combat hypertrophy are also lacking. My study will be directly relevant for the development of new therapies for cardiac hypertrophy and heart failure that target intrinsic abnormalities in signaling pathways and transcriptional programs within the diseased cardiac myocyte.

1.2 Overview of the PI3K/Akt signaling pathway

The PI3K/Akt signaling network (outlined in Figure 1.1) is crucial to widely divergent physiological processes that include cell cycle progression, differentiation, transcription, translation, apoptosis and metabolism (Martelli et al., 2006). PI3K can be activated by several receptor tyrosine kinases (RTKs), such as the insulin growth factor-1 (IGF-1) receptor (Walsh, 2006), as well as G protein-coupled receptors (GPCRs), including α- (Schluter et al., 1998) and β2-adrenergic receptors (Chesley et al., 2000; Zhu et al., 2001). Upon ligand binding, PI3K is translocated to the membrane, where it converts the plasma-membrane lipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2, PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3, PIP3). PIP3 then recruits both Akt/protein kinase B (PKB) and its

activator phosphoinositide-dependent protein kinase-1 (PDK1) to the membrane by binding to their pleckstrin homology (PH) domains, leading to phosphorylation and activation of Akt (Matsui et al., 2003). Activation of Akt then leads to activation of mammalian target of rapamycin (mTOR), a central regulator of protein synthesis. Akt also phosphorylates and inhibits a kinase, glycogen synthase kinase 3β (Gsk3β) (Dorn and Force, 2005).

The PI3K/Akt pathway is highly regulated. The termination of PI3K/Akt signaling by degradation of PtdIns(3,4,5)P3 can be mediated by at least two different types of phosphatases, 3-ptases (inositol polyphosphate 3-phosphatases), which converts PtdIns(3,4,5)P3 back to PtdIns(4,5)P2, and 5-ptases (inositol polyphosphate 5-phosphatases), which generates PtdIns(3,4)P2 (Ooms et al., 2009).

1.3 Inositol polyphosphate phosphatases

Phosphoinositides (PIs) are quantitatively minor phospholipids of cell membranes but their metabolism is highly active and accurately controlled by specific PI kinases and phosphatases (Pendaries et al., 2003). The importance of these PI phosphatases in cell regulation is illustrated by their involvement in human diseases such as cancer and diabetes (Blero et al., 2007) (Table 1.1). Cellular levels of PtdIns(3,4,5)P3 are about 50 nM in unstimulated cells but increase 40 fold within seconds to within 2 μM following agonist stimulation (Insall and Weiner, 2001;

Stephens et al., 1993). PtdIns(3,4,5)P3 is dephosphorylated within seconds by phosphatases. The most well-studied PtdIns(3,4,5)P3 3-ptase is PTEN (phosphatase and tensin homolog deleted on chromosome ten) (Cantley, 2002; Oudit et al., 2004), while the 5-ptases are a large family comprising 10 mammalian and 4 yeast members (Astle et al., 2006).

1.3.1 PTEN

PTEN was first identified as tumor suppressor gene localized on chromosome 10q23 (Li et al., 1997; Steck et al., 1997). Human germline mutations in PTEN are associated with several rare autosomal dominant cancer predisposition syndromes including Cowden disease (Knobbe et al., 2008; Liaw et al., 1997; Marsh et al., 1998) (Table 1.1). Mice homozygous for targeted deletions within the PTEN gene (PTEN -/-) die during embryonic development (between Days 6.5 and 9.5), while heterozygous mice develop normally but are prone to a wide range of tumor types including cancers of the breast, thyroid, endometrium and prostate as well as T cell lymphomas (Di Cristofano et al., 1998; Podsypanina et al., 1999; Stambolic et al., 1998; Suzuki et al., 1998). PTEN +/- mice also develop signs of autoimmune disease (Di Cristofano et al., 1999).

The main physiological substrates of PTEN are PtdIns(3,4,5)P3 and possibly PtdIns(3,4)P3. PTEN is considered the principal regulator of basal cellular PtdIns(3,4,5)P3 levels (Leslie and Downes, 2002). A lot of studies using PTEN null cell lines have been done to study the effects of PTEN activity on cellular

PtdIns(3,4,5)P3 (Leslie and Downes, 2002; Maehama and Dixon, 1998; Stambolic et al., 1998; Sun et al., 1999). Deletion of PTEN in murine fibroblasts and embryonic stem cells results in greatly elevated unstimulated PtdIns(3,4,5)P3 levels (Stambolic et al., 1998; Sun et al., 1999). Similar results have also been obtained with human tumor cell lines lacking PTEN, and PtdIns(3,4,5)P3 levels are reduced dramatically upon re-expression of the phosphatase (Taylor et al., 2000). HPLC analysis of labeled cellular PIs has also shown that PtdIns(3,4)P2 levels appear similarly high and decrease with PTEN re-expression (Taylor et al., 2000). In addition to this direct evidence, a wealth of data has shown greatly increased activity of the downstream kinase Akt in many PTEN null cell types, and that this activity is reduced by expression of PTEN (Haas-Kogan et al., 1998; Li and Sun, 1998; Li et al., 1998; Myers et al., 1998; Nakashima et al., 2000; Persad et al., 2000; Ramaswamy et al., 1999; Stambolic et al., 1998; Sun et al., 1999; Wu et al., 1998). Cardiac specific PTEN knockout mice have also been generated (Crackower et al., 2002) and the detailed phenotypes will be reviewed later.

1.3.2 Inositol polyphosphate 5-phosphatases

1.3.2.1 Src homology 2 (SH2) domain-containing inositol polyphosphophate 5-phosphatases 1 and 2 (SHIP and SHIP2)

SHIP and SHIP2 are capable of removing the 5-phosphate from PtdIns (3,4,5)P3 to yield PtdIns (3,4)P2. SHIP is expressed predominantly in hematopoietic cells where it is an important negative regulator of cytokine signaling. SHIP-/- mice have shorter life span associated with massive myeloid cell infiltration of the lungs

and numerous hematopoietic abnormalities (Helgason et al., 1998; Liu et al., 1998). SHIP2 is more widely expressed, most highly in brain, skeletal muscle and heart (Astle et al., 2006). It plays an important role in insulin signaling and obesity regulation (Grempler et al., 2007; Sleeman et al., 2005). When exposed to a normal diet, SHIP2-/- mice exhibit a basal metabolic rate similar to wild type controls However, SHIP2-/- mice are highly resistant to weight gain when placed on a high-fat diet. Under these conditions, SHIP2-/- mice demonstrate an increased metabolic rate and show no increase in serum lipids, insulin or glucose levels. The liver and skeletal muscle of the SHIP2-/- mice shows evidence of enhanced activation of the Akt signaling pathway (Sleeman et al., 2005). Genotypic analyses of diabetic rat and human subjects have found a significant association between SHIP2 gene polymorphisms and type 2 diabetes (Kagawa et al., 2005; Kaisaki et al., 2004; Marion et al., 2002).

1.3.2.2 Synaptojanin 1/2

Two synaptojanin isoforms (1 and 2) are 5-phosphatases which are able to hydrolyze PtdIns(3,4,5)P3 and PtdIns(4,5)P2 (McPherson et al., 1996; Woscholski et al., 1997). Synaptojanin 1 is a nerve terminal protein of 145 kDa that appears to participate with dynamin in synaptic vesicle recycling (McPherson et al., 1996). Synaptojanin 1-deficient mice die shortly after birth due to neurological defects (Cremona et al., 1999). Linkage analysis for bipolar disorder identified synaptojanin 1 as a candidate gene (Saito et al., 2001; Stopkova et al., 2004). The cDNA encoding

Synaptojanin 2 was identified by polymerase chain reaction using degenerate primers (Nemoto et al., 1997). It regulates the early stages of endocytosis. Depletion of synaptojanin 2 in A-549 cells causes a decrease in the number and size of vesicles at all stages of endocytosis (Rusk et al., 2003). Targeted depletion of synaptojanin 2 in glioblastoma cells also inhibits the formation of lamellipodia and invadopodia, and cell migration and invasion (Chuang et al., 2004).

Both Synaptojanin 1 and 2 have a Sac domain, which contains phosphatase activity (Guo et al., 1999). The Sac domain has approximately 400 amino acid residues and is defined by seven conserved motifs which appear to define the catalytic and regulatory regions of the phosphatase (Hughes et al., 2000). The highly conserved sequence RXNCXDCLDRTN in the sixth motif is proposed to be the catalytic core of the Sac domain phosphatases. The CX5R(T/S) motif within this sequence is also found in a number of metal-independent protein phosphatases and inositol polyphosphate phosphatases and is known to be a phosphatase catalytic site (Guo et al., 1999; Hughes et al., 2000). Interestingly, the Sac domain of the yeast synaptojanin-like protein Inp51p does not exhibit phosphatase activity, and the cysteine, arginine and threonine/serine residues are absent from the CX5R(T/S) motif of this protein, being replaced by alanine, lysine and proline respectively. Furthermore, mutations of the first conserved aspartate residue in the RXNCXDCLDRTN sequence as seen in the yeast sac1-8 and sac1-22 mutant alleles were demonstrated to inactivate the Sac1p functions (Hughes et al., 2000; Kearns et al., 1997; Zhong and Ye, 2003).

Together, this evidence suggests that the RXNCXDCLDRTN motif could well represent the catalytic core of the Sac phosphatases.

1.3.2.3 Inositol polyphosphate-5-phosphatase F (Inpp5f)

Inpp5f is a novel Sac domain-containing phosphoinositide 5-phosphatase whose activity is specific for PtdIns(4,5)P2 and PtdIns(3,4,5)P3 *in vitro* (Minagawa et al., 2001). As I will discuss later in the next chapter, I was able to identify the seven conserved motifs and the sixth conserved region of Inpp5f in both mouse and human has the highly conserved CX5R(T/S) motif, suggesting it is catalytically active. It is widely expressed in several tissues including brain, heart, skeletal muscle, kidney (Minagawa et al., 2001). However, its *in vivo* role has not been characterized prior to this study.

1.4 PI3K/Akt signaling in cardiac hypertrophy

The PI3K/Akt pathway is essential for both basal cell growth as well as adaptive (physiologic) and maladaptive (pathologic) hypertrophy (Oudit et al., 2004). Several transgenic and knockout models support a fundamental role of this pathway in the regulation of hypertrophy (Summarized in Table 1.2).

1.4.1 PI3K

PI3K inhibitors attenuate basal rates of protein synthesis in unstimulated cells and abolish increases in protein synthesis induced by insulin in neonatal cardiomyocytes (Pham et al., 2000). The complete knockout of p110α, the catalytic subunit of PI3Kα, was lethal at E9.5-E10.5 due to a severe proliferation defect (Bi et al., 1999) and therefore was of limited usefulness for cardiac studies. Cardiac specific over-expression of constitutively active PI3Kα (CA-PI3Kα) in mice results in enlarged hearts owing to increased cardiomyocyte size (Crackower et al., 2002; Shioi et al., 2000), whereas over-expression of dominant negative PI3Kα (DN-PI3Kα) produces smaller hearts with reduced cardiomyocyte size without affecting cell number (Crackower et al., 2002; McMullen et al., 2003; Shioi et al., 2000). These changes in heart size are associated with corresponding alterations in Akt, and p70S6-kinase (p70S6K) phosphorylation and activities. These studies suggest a positive role of PI3K in cardiac growth.

1.4.2 PDK1

PDK1 is a ubiquitously expressed 67 kDa kinase containing a N-terminal catalytic domain and a C-terminal PH domain (Storz and Toker, 2002; Toker and Newton, 2000). It is central in the PI3K signaling pathway and whole-body knockout of PDK1 is associated with embryonic lethality (Lawlor et al., 2002) while cardiac specific PDK1 ablation leads to reduced cardiomyocyte size and cardiomyopathy (Ito et al., 2009; Mora et al., 2003).

1.4.3 Akt/PKB

Akt is a serine/threonine protein kinase family comprising three closely related, highly conserved isoforms: Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ) (Chan et al., 1999; Oudit and Penninger, 2009). Akt2 and Akt3 show 81 and 83% amino acid identity, respectively, with Akt1 (Shioi et al., 2002). Only Akt1 and Akt2 are highly expressed in the heart (Dorn and Force, 2005). The activity of Akt is primarily controlled by PI3K and PTEN via the modulation of PtdIns(3,4,5)P3 levels (Maehama et al., 2001; Stambolic et al., 1998; Vanhaesebroeck et al., 2001)

Several Akt knockout and transgenic mouse models have been generated to study the physiological roles of Akt. Targeted disruption of the Akt1 gene in mice results in a growth retardation phenotype. They have a 20% reduction in body size, with a concomitant reduction in heart size (Chen et al., 2001; Cho et al., 2001b). More recently, Akt1 null mice were shown to be resistant to exercise-induced cardiac hypertrophy. Furthermore, adult murine cardiac myocytes are resistant to IGF-1 stimulated protein synthesis *in vitro* suggesting that Akt1 deficiency in myocytes account for the reduction in heart growth in this mouse model (DeBosch et al., 2006b). It may be useful to confirm these findings in a cardiac-specific Akt1 knockout model. Akt2 null mice exhibit insulin resistance and depending on genetic background, mild growth retardation (Cho et al., 2001a; Garofalo et al., 2003). Consistent with this, a mutation in the human AKT2 gene has been shown to be associated with autosomal dominant inheritance of severe insulin resistance and diabetes mellitus (George et al.,

2004). Akt2 null mice displayed normal cardiac growth responses to provocative stimulation, including ligand stimulation of cultured cardiomyocytes, pressure overload by transverse aortic constriction, and myocardial infarction. However, Akt2 is absolutely required for the maintenance of normal cardiac glucose metabolism and for cardiomyocyte survival in response to ischemic injury (DeBosch et al., 2006a). Akt3 null mice have smaller brain size due to the reduction in both cell size and cell number. However, they do not show the general growth retardation phenotype (Easton et al., 2005; Tschopp et al., 2005). Thus, data from the available Akt knockout models support a critical role specifically for Akt1 in normal growth of the heart. Akt1/Akt2 double-null animals die shortly after birth due to defects in multiple tissues including skeletal muscle, bone and skin (Peng et al., 2003). Akt1/Akt3 double-mutant mice die by E12 with more severe developmental defects in the cardiovascular and nervous systems (Yang et al., 2005). Thus, the phenotypes of these compound mutants suggest functional redundancy among the three Akt proteins.

In transgenic studies, myristoylated Akt1 (membrane-targeted Akt1, myr-Akt1) (Cook et al., 2002; Matsui et al., 2002; Shiojima et al., 2005) or a phosphorylation mimicking mutant of Akt1 (Akt1 T308D/S473D) (Shioi et al., 2002) or PH domain mutant of Akt1 (Akt1 E40K), which has increased affinity to phospholipids, (Condorelli et al., 2002; Kim et al., 2003) has been used as a constitutively active (CA) Akt1. One common phenotype associated with overexpression of CA-Akt1 in the heart is cardiac hypertrophy with an increase in myocyte size (Condorelli et al., 2002; Kim et al., 2003; Matsui et al., 2002; Shioi et al., 2002; Shiojima et al., 2005). And

the CA-Akt1 T308D/S473D mutant protein circumvented cardiac growth retardation induced by a KD-PI3K mutant protein (Shioi et al., 2002), suggesting Akt functions downstream of PI3K. In contrast, mice over-expressing kinase dead (KD)-Akt1 (K179M), in which the critical ATP binding site was mutated, has similar heart and myocyte size as their control littermates. However, The KD-Akt1 mutant protein attenuated the CA-PI3K-induced overgrowth of the heart (Shioi et al., 2002). Cardiac-specific myristoylated Akt3 transgenic mice also exhibited marked cardiac hypertrophy (Taniyama et al., 2005).

1.4.4 Gsk3β

Gsk3β was initially identified as a kinase that phosphorylates and inactivates glycogen synthase but is now recognized as a regulator of multiple processes including development, tumorigenesis, and metabolism (Doble and Woodgett, 2003). Gsk3β is constitutively active in unstimulated cells where it phosphorylates and negatively regulates most of its substrates. In response to RTK/GPCR activation, phosphorylation of the serine-9 residue in the N-terminal region of Gsk3β by Akt inhibits Gsk3β thereby leading to diverse effects (Doble and Woodgett, 2003; Toker and Cantley, 1997). Whole-body knockout of Gsk3β leads to embryonic lethality by E16.5 due to severe liver degeneration (Hoeflich et al., 2000). Cardiac specific overexpression of wildtype Gsk3β (Michael et al., 2004) or expression of Gsk3β (S9A) (Antos et al., 2002) reduces maturational growth of the heart, and reduces the cardiac hypertrophy induced by isoproterenol (ISO) infusion, transverse aortic constriction

(TAC) or overexpression of constitutively activated calcineurin (Antos et al., 2002), confirming that GSK-3β functions as a negative regulator of hypertrophy *in vivo*. Furthermore, inducible expression of Gsk3β (S9A) in the adult heart reduces the extent of established hypertrophy produced by TAC, as assessed by left ventricular weight/body weight (Sanbe et al., 2003). In contrast, cardiac overexpression of dominant-negative Gsk3β (K85M/K86I) results in hypertrophy (Hirotani et al., 2007).

Several potential mechanisms are proposed for the negative regulatory role of Gsk3β in cardiac hypertrophy. First, Gsk3β may attenuate cardiac hypertrophy by phosphorylating and inducing nuclear export of NFAT (nuclear factor of activated T-cells) (Heineke and Molkentin, 2006). Because calcineurin, a calcium-dependent phosphatase and a transducer of cardiac stress signals, induces of hypertrophy through dephosphorylation and nuclear import of NFAT (Molkentin et al., 1998), phosphorylation of NFAT by Gsk3β masks its nuclear localization sequence and antagonizes the hypertrophic effects of calcineurin. Indeed, constitutively active Gsk3β inhibits endothelin-1-induced NFAT nuclear import (Haq et al., 2000), and concomitant Gsk3β overexpression markedly reduces hypertrophy of calcineurin-transgenic mice (Antos et al., 2002). Second, because Gsk3β induces nuclear export of Gata4 (Morisco et al., 2001) and reduces transcriptional activity of myocardin (Badorff et al., 2005), both of which are transcription factors implicated in hypertrophic responses of myocytes (Perrino and Rockman, 2006; Pipes et al., 2006), Gsk3β may attenuate cardiac hypertrophy by suppressing the activities of these transcription factors. Third, Gsk3β inhibits initiation of protein translation by

negatively regulating eukaryotic translation initiation factor 2B (eIF2B) (Dorn and Force, 2005) and enhanced protein synthesis is an important feature during hypertrophy. In cultured cardiac myocytes, it was shown that phosphorylation of eIF2B by Gsk3β is critical for anti-hypertrophic effects of Gsk3β (Hardt et al., 2004). eIF2B phosphorylation is also increased in the heart of Gsk3β transgenic mice (Michael et al., 2004).

1.4.5 mTOR

mTOR is a 289 kDa evolutionarily conserved serine/threonine kinase that is inhibited by the drug rapamycin (Harris and Lawrence, 2003; Schmelzle and Hall, 2000). The best characterized function of mTOR in mammalian cells is regulation of translation (Inoki et al., 2005). Two most important downstream targets of mTOR are ribosomal S6 kinase (S6K) and eukaryote initiation factor 4E binding protein 1 (4EBP1), which are key regulators of protein translation (Inoki et al., 2005). Rapamycin has been shown to decrease agonist-induced hypertrophy *in vitro* (Sadoshima and Izumo, 1995) and load-induced hypertrophy in mice (Shioi et al., 2003). Cardiac hypertrophy induced by Akt overexpression is effectively blocked by rapamycin treatment (Shioi et al., 2002; Shiojima et al., 2005).

1.4.6 PTEN

The role of PTEN in cardiac hypertrophy has been studied in both cultured myocytes and mouse models. In neonatal cardiomyocytes, expression of DN-PTEN

increases protein synthesis and cell size associated with elevated level of phospho-Akt,, while overexpression of wildtype PTEN impaired these responses (Schwartzbauer and Robbins, 2001). Cardiac specific knockout of PTEN increases phospho-Akt, phospho-Gsk3β and phospho-p70S6K levels, resulting in increased cardiomyocyte and heart size. And co-expression of DN-p110α reverses the phenotype (Crackower et al., 2002).

1.5 Overview of histone deacetylases

Gene expression is regulated not only at the level of individual promoters, but also, at a more global level, by modulation of chromatin structure. Modification of core histones, around which DNA is wrapped, affects the packaging of nucleosomes and the accessibility of specific regions of DNA to transcription factors and the basal transcriptional machinery. Histone acetylation, mediated by HATs, results in chromatin relaxation and transcriptional activation (Gallinari et al., 2007), while HDACs induce deacetylation, chromatin condensation, and transcriptional repression (Ekwall, 2005; Johnson and Turner, 1999). HDACs lack intrinsic DNA binding ability and are recruited to target genes via their incorporation into large multiprotein transcriptional complexes as well as direct association with transcriptional activators or repressors (Grunstein, 1997). There are 11 HDACs encoded by mammalian genome, which can be classified into four families (class I, IIa, IIb and IV). The class I HDAC family consists of HDAC1, 2, 3 and 8, which are expressed ubiquitously and

consist mainly of a deacetylase domain with short amino- and carboxy-terminal extensions (Ekwall, 2005; Haberland et al., 2009). Class IIa HDACs (HDACs 4, 5, 7, and 9) have large N-terminal extensions in addition to the catalytic domain, and they have restricted express pattern. The extensions have conserved binding sites for the transcription factor myocyte enhancer factor 2 (MEF2) and the chaperone protein 14-3-3 (Haberland et al., 2009). HDAC5 and HDAC9 are highly expressed in muscle, heart and brain (Chang et al., 2004; Zhang et al., 2002). HDAC4 is mainly found in the brain and growth plates of the skeleton (Vega et al., 2004), and HDAC7 is expressed in endothelial cells and thymocytes (Chang et al., 2006). The class IIb family consists of HDAC6 and HDAC10. HDAC6 mainly exists in cytoplasm in mammalian cells (Zhang et al., 2008). Little is known about HDAC10. HDAC11 is the sole class IV HDAC, which has been shown to regulate the expression of interleukin 10 and immune tolerance (Villagra et al., 2009).

Mammalian HDACs are also able to deacetylate non-histone targets. Acetylation has been shown to affect protein stability, DNA binding, transcription activation, protein interactions, localization and enzymatic activity (Spange et al., 2009). For example, acetylation of p53 activates its sequence-specific DNA binding activity and consequently increases activation of its target genes (Gu and Roeder, 1997), while deacetylation of p53 by HDAC1 represses p53-dependent transcriptional activation and modulates p53-mediated cell growth arrest and apoptosis (Luo et al., 2000). As p53 acetylation might favor DNA binding and transcriptional activation, this mechanism has likewise been suggested for the transcription factors c-Myb (Sano

and Ishii, 2001; Tomita et al., 2000), GATA factors (Boyes et al., 1998; Hayakawa et al., 2004; Yamagata et al., 2000), E2F1 (Martinez-Balbas et al., 2000; Marzio et al., 2000), MyoD (Sartorelli et al., 1999) and many others.

1.6 HDACs in regulating cardiac hypertrophy

The roles of HDACs in cardiac hypertrophy have been investigated through several gene-targeting studies in the mouse. Mice lacking either HDAC5 or HDAC9 are normal at early age. However, by about 8 months of age, mutant animals develop spontaneous cardiac hypertrophy that appears to reflect sensitization to age-related cardiac insults. HDAC5 and HDAC9 mutant mice also develop grossly enlarged hearts in response to pressure overload or constitutive cardiac activation of calcineurin (Chang et al., 2004; Zhang et al., 2002). In HDAC9 knockout animals, MEF2 activity is increased (Zhang et al., 2002). It is proposed that HDAC5 and 9 can interact with MEF2 through the N terminal extension and thus lead to repression of hypertrophic genes, while class I HDACs lack this domain and fail to directly regulate the activity of MEF2 (Lemercier et al., 2000; Lu et al., 2000a; Lu et al., 2000b; Miska et al., 1999; Sparrow et al., 1999; Zhang et al., 2001). Besides MEF2, class II HDACs have been shown to associate with and repress the transcriptional activity of SRF, GATA, NFAT and myocardin, each of which have been shown to regulate cardiac hypertrophy (Frey and Olson, 2003).

However, chemical HDAC inhibitors including trichostatin A (TSA), actually prevent cardiac hypertrophy, leading to the conclusion that distinct HDACs play positive or negative roles in the control of cardiomyocyte hypertrophy (Antos et al., 2003; Kong et al., 2006). Consistent with this idea, HDAC2, a class I HDAC is important for inducing hypertrophy in homeodomain only protein (HOP) transgenic mice (Kook et al., 2003). Recently we have shown that global loss of HDAC2 results in partial perinatal lethality due to cardiac developmental defects that include enhanced cardiac myocyte proliferation. HDAC2 deficient mice were resistant to cardiac hypertrophy when exposed to hypertrophic stimuli (Trivedi et al., 2007). Resistance to hypertrophy was associated with increased expression of Inpp5f resulting in constitutive activation of Gsk3β via inactivation of Akt and Pdk1. While transgenic overexpression of HDAC2 in the heart reactivated fetal genes and induced cardiac hypertrophy. Inpp5f was significantly downregulated in HDAC2 transgenic heart (Trivedi et al., 2007).

1.7 GATA4 in heart development

In the later chapter of my thesis, I will describe some interesting HDAC2 knockout mice phenotypes which are related to GATA4 activity. The GATA family of transcription factors, GATA1-6, are known to be critical for embryonic development, cell growth, and differentiation (Viger et al., 2008). GATA transcription factors are defined by an evolutionarily conserved DNA binding domain consisting of

two zinc finger motifs that recognize the consensus-binding site WGATAR (Ko and Engel, 1993; Merika and Orkin, 1993). The GATA family can be separated into two subgroups based on spatial and temporal expression patterns. GATA1-3 are predominantly expressed in hematopoietic cell lineages and are essential for erythroid and megakaryocyte differentiation, the proliferation of hematopoietic stem cells, and the development of T lymphocytes (Weiss and Orkin, 1995). In contrast, GATA4-6 are expressed in several mesoderm and endoderm derived tissues such as the heart, gut and gonad (Charron and Nemer, 1999; Molkentin, 2000). GATA4 is one of the earliest genes expressed by specified cardiac precursors at the cardiac crescent stage of mouse development (Arceci et al., 1993; Kelley et al., 1993) and it has been extensively characterized as an essential regulator of cardiac development and differentiation, as well as in regulating survival and hypertrophic growth of the adult heart (Oka et al., 2007). GATA4 regulates the expression of genes that are important for cardiac contraction as well as the expression of other cardiac transcription factor genes, such as Mef2c, Hand2, and Nkx2-5 (Dodou et al., 2004; Lien et al., 1999; McFadden et al., 2000). Global loss of Gata4 in mice causes embryonic lethality at E9.5 as a result of severe defects in the extra-embryonic endoderm and aberrant heart and foregut morphogenesis (Kuo et al., 1997; Molkentin et al., 1997). Clinical studies have indicated a role in human congenital heart defects (CHD) (Garg et al., 2003; Pehlivan et al., 1999). Studies involving tissue-specific loss of Gata4 in murine cardiac myocytes have demonstrated a critical role for Gata4 in embryonic myocyte proliferation. Conditional inactivation of Gata4 using Nkx2-5 Cre resulted in

embryonic lethality around E11.5, with decreased cardiomyocyte proliferation and major defects in the development of the right ventricle (Zeisberg et al., 2005). Mice lacking Gata4 in the anterior heart field (AHF), for example, die by E13.5 due to significant right ventricular and interventricular septal myocyte proliferation defects. Gata4-null cardiomyocytes show down-regulation of a wide array of cell cycle associated genes, some of which are direct transcriptional targets of Gata4, including cyclinD2 and cdk4 (Rojas et al., 2008).

1.8 GATA4 post-translational modification and activity

GATA4 activity is modulated in response to a number of intracellular signaling pathways, and GATA4 protein is subject to post-translational modifications including phosphorylation, acetylation and sumoylation (Kawamura et al., 2005; Liang et al., 2001b; Wang et al., 2004). S105 phosphorylation by MEK1-ERK1/2 signaling enhances Gata4 DNA binding and transcription activation in cardiomyocytes (Kitta et al., 2003; Liang et al., 2001b). Gata4 is also activated by S261 phosphorylation via the cAMP/Protein Kinase signaling pathway in gonadal cells (Tremblay and Viger, 2003). Sumoylation is required for Gata4 to gain the ability to activate endogenous cardiac-specific genes (Wang et al., 2004). The histone acetyl transferase p300 is able to acetylate Lys311, 318, 320, and 322 of Gata4, resulting in enhanced DNA binding and transcriptional activity (Takaya et al., 2008). In transgenic mice, p300 over-expression in the heart induces Gata4 acetylation and cardiac hypertrophy (Miyamoto

et al., 2006; Yanazume et al., 2003a). Interestingly, p300 null mice die at E9.5 exhibiting defects in proliferation and cardiac development (Yao et al., 1998). Mechanisms for Gata4 deacetylation have, to our knowledge, not been previously described.

Table 1.1: The role of phosphatases in physiology and pathology in human and mice

Gene	Function	Substrate(s)	Human disease	Mice phenotype	References
PTEN	3-ptase	PtdIns(3,4)P2, PtdIns(3,4,5)P3	Cowden disease, Lhermitte-Duclos disease, Bannayan–Riley–Ruvalcaba syndrome, Cancer	Embryonic lethality of PTEN -/-. Tumorigenesis in PTEN +/-.	(Leslie and Downes, 2002; Pendaries et al., 2003
SHIP	5-ptase	PtdIns(3,4,5)P3	Acute Myelogenous Leukemia	Myeloproliferative phenotype with progressive splenomegaly, massive myeloid infiltration of the lungs, shortened lifespan of knockout mice. Severe osteoporotic phenotype.	(Takeshita et al., 2002) Helgason et al., 1998; Liu et al., 1998
SHIP2	5-ptase	PtdIns(3,4,5)P3	Type 2 diabetes	highly resistant to weight gain when placed on a high-fat diet	Grempler et al., 2007; Sleeman et al., 2005
Synaptojanin 1	5-ptase	PtdIns(4,5)P2, PtdIns(3,4,5)P3	Bipolar disorder	die shortly after birth due to neurological defects	Cremona et al., 1999; Saito et al., 2001; Stopkova et al., 2004
Synaptojanin 2	5-ptase	PtdIns(4,5)P2, PtdIns(3,4,5)P3	N/A	N/A	
Inpp5f	5-ptase	PtdIns(4,5)P2, PtdIns(3,4,5)P3	N/A	Cardiac specific KO is sensitive to hypertrophic stimuli	Zhu et al., 2009

Table 1.2: Gene-targeted and transgenic models with altered PI3K/Akt signaling in the heart

Genotype	Phenotype	References
Whole-body p110α KO	E9.5-E10.5 lethality	Bi et al., 1999
Cardiac-specific CA-PI3Kα	Increased heart and cardiomyocyte size	Crackower et al., 2002; Shioi et al., 2000
Cardiac specific DN-PI3Kα	Reduced heart and cardiomyocyte size	Crackower et al., 2002; McMullen et al., 2003; Shioi et al., 2000
Whole body PDK1 KO	E9.5 lethality	Lawlor et al., 2002
Cardiac-specific PDK1	Cardiomyopathy, reduced cardiomyocyte size, increased sensitivity to hypoxia, increased cardiomyocyte apoptosis	Ito et al., 2009; Mora et al., 2003
Whole body Akt1 KO	Growth retardation, normal glucose tolerance, defective in physiological hypertrophy	Chen et al., 2001; Cho et al., 2001b; DeBosch et al., 2006
Whole body Akt2 KO	Insulin resistance, mild growth retardation, normal cardiac growth	Cho et al., 2001a; Garofalo et al., 2003; DeBosch et al., 2006a
Whole body Akt3 KO	Reduced brain size	Easton et al., 2005; Tschopp et al., 2005
Cardiac-specific CA-Akt1 (myr-Akt1)	Cardiac hypertrophy, normal myocardial contractility	Matsui et al., 2002; Shiojima et al., 2005
Cardiac-specific CA-Akt1 (Akt1 T308D/S473D)	Cardiac hypertrophy, reduced myocardial contractility	Shioi et al., 2002
Cardiac-specific CA-Akt1 (Akt1 E40K)	Cardiac hypertrophy, enhanced myocardial contractility	Condorelli et al., 2002; Kim et al., 2003
Cardiac-specific KD-Akt1 (Akt1 K179M)	attenuated the CA-PI3K-induced overgrowth of the heart, normal myocardial contractility	Shioi et al., 2002
Cardiac-specific CA-Akt3 (myr-Akt1)	Cardiac hypertrophy, reduced myocardial contractility	Taniyama et al., 2005
Whole body Gsk3β KO	E16.5 lethality	Hoeflich et al., 2000
Cardiac-specific Gsk3β	Reduced maturational growth of the heart	Michael et al., 2004
Cardiac-specific CA Gsk3β (Gsk3β S9A)	Impaired hypertrophic responses	Antos et al., 2002

Cardiac-specific inducible Gsk3β (Gsk3β S9A)	Reversal of pre-existing hypertrohpy	Sanbe et al., 2003
Cardiac-specific DN Gsk3β (K85M/K86I)	Cardiac hypertrophy with improved contractility	Hirotani et al., 2007
Whole body knockin of Gsk3β (Gsk3β S9A)	Reduced hypertrophy	Matsuda et al., 2008
Cardiac-specific CA-mTOR	Normal response to hypertrophic stimuli	Shen et al., 2008
Cardiac-specific KD-mTOR	Normal response to hypertrophic stimuli, reduced function	Shen et al., 2008
Muscle-specific PTEN KO	Increased heart and cardiomyocyte size; reduced myocardial contractility	Crackower et al., 2002, Oudit et al., 2008

Figure 1.1: Overview of the PI3K/Akt pathway.

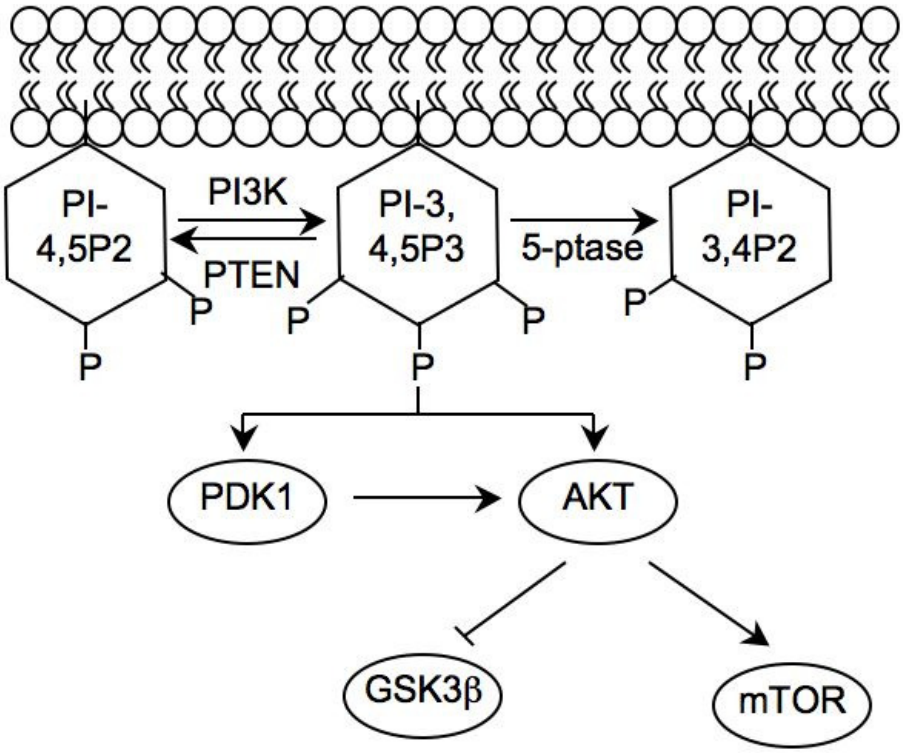


Figure 1.1: Overview of the PI3K/Akt pathway.

Upon activation, PI3K translocates to the cell membrane, converting PIP2 to PIP3. The PH domain of both Akt and its activator PDK1 associate with PIP3 allowing PDK1 to activate Akt. Activation of Akt then leads to activation of mTOR, a central regulator of protein synthesis. Akt also phosphorylates and inhibits a kinase, Gsk3β. Since Gsk3β inhibits protein translation and a number of transcription factors believed to play roles in the induction of the hypertrophic program of gene expression, inhibition of Gsk3β promotes both protein synthesis and gene transcription.

Chapter 2 Inpp5f is a Polyphosphoinositide Phosphatase that Regulates Cardiac Hypertrophic Responsiveness

Significant portions of this chapter have been published in *Circulation Research* (Zhu et al., 2009).

2.1 Summary

Cardiac hypertrophy occurs in response to a variety of extrinsic and intrinsic stimuli that impose increased biomechanical stress. The PI3K/Akt pathway has previously been strongly associated with hypertrophic signaling in the heart, and with the control of cell size in multiple contexts. This pathway is tightly regulated by many factors, including a host of kinases and phosphatases that function at multiple steps in the signaling cascade. For example, the PTEN tumor suppressor protein is a phosphoinositide 3-phosphatase that, by metabolizing PtdIns(3,4,5)P_3, acts in direct antagonism to growth factor stimulated PI3K. Inhibition of PTEN leads to cardiomyocyte hypertrophy. Here, I characterize another polyphoinositide phosphatase, inositol polyphosphate-5-phosphatase F (Inpp5f), which has recently been implicated in regulation of cardiac hypertrophy. Like PTEN, this phosphatase can degrade PtdIns(3,4,5)P_3 and thus modulates the PI3K/Akt pathway. *Inpp5f* knockout mice have augmented hypertrophy and reactivation of the fetal gene program in response to stress when compared to wild type littermates. Furthermore,

cardiac over-expression of Inpp5f in transgenic mice reduces hypertrophic responsiveness. These results suggest that Inpp5f is a functionally important endogenous modulator of cardiac myocyte size and of the cardiac response to stress.

2.2 Introduction

Cardiovascular disease remains the number one cause of mortality in the Western world, with heart failure representing the fastest growing subclass over the past decade (Heineke and Molkentin, 2006). In many cases, cardiac hypertrophy precedes cardiac dilation and heart failure. Cardiac hypertrophy can occur in response to normal physiological stimuli or may be maladaptive leading to cardiac dilatation and congestive heart failure. However, the cellular mechanisms that regulate the hypertrophic response to agonists or to stretch remain poorly understood, and the transition from hypertrophy to failure is also ill-defined. On the other hand, evidence accumulating over the last few decades confirms that intercellular signaling pathways and gene expression are fundamentally altered in states of hypertrophy and failure and a thorough understanding of these changes will provide therapeutic targets (Dorn and Force, 2005).

Recently, we showed that Hdac2 deficient mice are resistant to cardiac hypertrophy when exposed to hypertrophic stimuli (Trivedi et al., 2007). Resistance to hypertrophy in *Hdac2*$^{-/-}$ mice is associated with increased expression of the gene

encoding inositol polyphosphate-5-phosphatase f (Inpp5f) and constitutive activation of Gsk3β via inactivation of Akt and Pdk1. Conversely, transgenic over-expression of Hdac2 in the heart reactivates fetal genes and induces cardiac hypertrophy. In these transgenic hearts, *Inpp5f* is significantly down-regulated, Akt is activated, and Gsk3ß is inactive (Trivedi et al., 2007). Further studies suggest that Hdac2 is a direct regulator of *Inpp5f* (Trivedi et al., 2007). Thus, I hypothesized that Inpp5f might functionally contribute to cardiac regulation of the Akt/Gsk3β pathway in response to stress.

Inpp5f is one of several polyphosphoinositide phosphatases that have been identified and partially characterized (Astle et al., 2007). Prior work demonstrates that Inpp5f can degrade both phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2, PIP2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3, PIP3) by removing the 5' phosphate from the inositol ring(Minagawa et al., 2001). Although the PIP3 3' phosphatase PTEN has previously been shown to regulate cardiac myocyte hypertrophy and Akt signaling in the heart (Crackower et al., 2002; Oudit et al., 2004), a functional role for a PIP3 5' phosphatase in the heart has been less clear. However, in various non-cardiac model systems, both 3' and 5' inositol phosphatases are important regulators of PIP3 activity and downstream signaling. For example, the SH2 domain-containing inositol 5'-phosphatase (SHIP) and PTEN both play important roles to regulate PIP3 and Akt in immune cells (Kashiwada et al., 2007).

In order to determine the role of Inpp5f in the adult heart, I have created both

gain- and loss-of-function models. Our results indicate that Inpp5f transgenic mice are unable to reactivate fetal genes or to exhibit normal hypertrophic responses to adrenergic agonists, while Inpp5f knockout mice exhibit augmented hypertrophy and exaggerated reactivation of the fetal gene program under stress.

2.3 Results

2.3.1 Inpp5f regulates the PI3K/Akt pathway *in vitro*

Inpp5f removes the 5-phosphate from PIP3 (Minagawa et al., 2001) and thus I predicted that Inpp5f is able to affect PI3K/Akt pathway activity. To test this, I performed both transfection overexpression and siRNA knockdown experiments in H9C2 cells, a cardiomyocyte cell line. Transfection of *Inpp5f* resulted in down-regulation of pAkt and pGsk3β (Fig 2.1A). This effect was readily apparent despite an estimated transfection efficiency of 50%. The decrease in Akt and Gsk3β phosphorylation induced by *Inpp5f* overepression was reversed by co-transfection with a constitutively activated *Akt* (*caAkt*) (Fig 2.1A), suggesting that Akt is downstream of *Inpp5f* and is capable of regulating Gsk3β. Knockdown of *Inpp5f* in H9C2 cells with siRNA resulted in enhanced Akt phosphorylation (Fig 2.1B). Thus, Inpp5f is capable of modulating the Akt-Gsk3β pathway *in vitro*.

2.3.2 Generation of Inpp5f antibody

To our knowledge, there are no commercially available Inpp5f antibodies. In order to facilitate the study, I chose two epitope antigens (one from N-terminus and the other from C-terminus of the protein) for antibody generation based on hydrophilicity, orientation, flexibility and antigenecity analysis. I cloned these two regions into pGEX-2T vectors separately. The plasmids were transformed into BL21 bacteria and glutathione S-transferase (GST) fusion proteins were extracted, purified and confirmed by Coomassie blue staining (Fig 2.2A). The fusion proteins were then sent to Cocalico Biologicals, Inc (Reamstown, PA) for injection into rabbit. By computer prediction, the mouse Inpp5f protein is 129 kD. To confirm this prediction, I made an N-terminal FLAG-tagged Inpp5f and a C-terminal MYC-tagged Inpp5f, transfected into Cos7 cells and probed the cell proteins with either anti-FLAG antibody or the anti-MYC antibody (Fig 2.2B). A predominant 130 kD band is observed in Fig 2.2B. The rabbit anti-sera after the second injection was examined on both FLAG-tagged Inpp5f containing vector and empty vector transfected cells. The result shows that all four antibodies could recognize a band approximately 130 kD in the Inpp5f expressing cells which was not detected in the control transfected cells (Fig 2.2C). Therefore, all 4 antibodies could recognize a protein of the predicted molecular weight. Since the antibody #2086 gives less background, it was chosen for future studies.

2.3.3 Inactivation of Inpp5f

In order to produce mice lacking *Inpp5f*, we identified a gene-trap embryonic stem (ES) cell line in which the endogenous *Inpp5f* locus has been disrupted within intron 6, upstream of the exons encoding the catalytic phosphatase domain. This gene-trap insertion is predicted to result in the production of a fusion protein composed of the amino-terminal region of Inpp5f fused to ß-geo (Fig 2.3A). Heterozygous gene-trap ES cells were injected into blastocysts to create chimeric mice, which were bred to produce germ-line offspring. Resulting heterozygous mice were inter-crossed to produce homozygous *Inpp5f*$^{-/-}$ offspring, which were born in expected Mendelian ratios (Table 2.1), survived into adulthood and appeared healthy. Genotype was confirmed by Southern blot (Fig 2.3B) and by PCR (Fig 2.3C). I confirmed the lack of full length *Inpp5f* transcripts by real-time PCR using mRNA from adult *Inpp5f*$^{-/-}$ and wild-type hearts (Fig 2.3D). Western blot using antibody recognizing the C-terminus of Inpp5f demonstrated a lack of protein expression in the knockout (Fig 2.3E). Thus, I conclude that I have generated a null allele for *Inpp5f*.

2.3.4 Further characterization of the Inpp5f genomic locus

Since the insertion is predicted to result in a fusion protein with the N-terminal region of Inpp5f fused to β-geo, I sought to detect the β-gal activity in the knockouts. I performed β-gal staining in E13.5 embryos as well as in several tissues of adult mice

including the brain, liver and heart. However, I did not detect any β-gal staining (data not shown). To exclude the possibility that the level of expression is too low to detect, I utilized a sensitive β-galactosidase assay (Shaper et al., 1994; Young et al., 1993).In the protein extracts from the Z/EG reporter mouse tail, which constitutively express lacZ under the control of the CMV promoter (Novak et al., 2000), I was able to detect large amounts of β-galactosidase activity. However, analysis of tissues from knockout mice yielded results similar to those of the wildtypes (data not shown) and they are far below the positive control.

I sought to further analyze the Inpp5f genomic locus to make sure that the β-geo cassette is inserted in the right locus and the mice are true knockouts. First of all, I designed several primers to map the exact locus where the cassette is inserted (Fig 2.4A). As seen in Fig 2.4B, the cassette is somewhere between primer R3 and R4 within intron 6. I cloned the PCR product from FR3 in Fig 2.4A and sequencing suggests that the insertion is at the 1752nd nucleotide within intron 6, which has 3291 nucleotides total. PCR from cDNA from the heart shows the existence of the fusion transcript of the first 6 exons and β-geo (Fig 2.4C, D). Further in depth analysis of the *Inpp5f* genomic locus affected by the gene-trap revealed that the insertion event was accompanied by a loss of endogenous genomic sequence spanning exons 7-13 (Fig 2.5). Thus, the affected allele cannot produce a full-length protein or one with functional phosphatase activity. There are several possibilities why the fusion transcript is produced but no β-galactosidase activity is detected. Perhaps the fusion protein is not stable. It is also possible that large deletion of gemonic DNA results in a

loss of some important enhancer elements that are required for efficient tissue-specific transcription.

2.3.5 Inpp5f null hearts appear normal

We examined cardiac histology, size and function in *Inpp5f*$^{-/-}$ and littermate control mice. H&E staining of hearts was performed at E14.5, P0 and 2 months of age. At these time points, we could not identify any differences and *Inpp5f* null mice appear normal by gross and histological assessment (Fig 2.6A). We measured cardiac weight in relation to total body weight to determine relative cardiac size. At both 2 and 9 months of age, there was no difference in heart to body weight ratio between wild type and *Inpp5f*$^{-/-}$ littermates (Fig 2.6B, C).

In order to evaluate the functional impact of the loss of Inpp5f, we performed echocardiography on Inpp5f$^{-/-}$ and wild type litter mates at 2 and 9 months (Table 2.2). We did not observe any significant difference in the inter-ventricular septum (IVS), left ventricular posterior wall (LVPW) and the left ventricular internal diameter (IVID) measurements at either end-diastole or end-systole. Left ventricular (LV) ejection fraction (EF) and fractional shortening (FS) also was not different between groups. Thus, under basal conditions, *Inpp5f*$^{-/-}$ mice appear to have normal cardiac form and function.

2.3.6 Inpp5f regulates stress-induced hypertrophy

Next, we sought to determine if adult mice lacking *Inpp5f* would be more susceptible to agonist-induced cardiac hypertrophy. Wild-type and *Inpp5f*$^{-/-}$ litter mates at 2 months of age were treated with a constant infusion of saline or isoproterenol (ISO) delivered by osmotic mini-pump for 14 days. Animals lacking Inpp5f showed an increase in ISO-induced cardiac hypertrophy as measured by either the heart to body weight ratio or the heart weight to tibia length ratio (Fig 2.7A). In response to ISO, *Inpp5f*$^{-/-}$ heart also showed more potent reactivation of the fetal program of gene expression than wild type litter mates; transcripts for *Nppa* (encoding atrial natriuretic factor, ANF), *Myh7* (encoding β-MHC) and *Nppb* (encoding BNP) increased more dramatically in the knockout hearts (Fig 2.7B). Cellular hypertrophy, as revealed by wheat germ agglutinin (WGA) staining followed by quantification, was more pronounced in the *Inpp5f*$^{-/-}$ hearts compared to wild-type hearts after ISO (Fig 2.7C) (256.5 $\pm$ 21.8 μm^2 for *Inpp5f* $^{-/-}$ mice treated with ISO, n = 446 cells from 3 hearts; 194.8 $\pm$ 6.8 μm^2 for wild type mice treated with ISO, n = 514 from 3 hearts). Patchy areas of fibrosis were more evident in ISO-treated *Inpp5f*$^{-/-}$ hearts when compared to controls (Fig 2.7D) (Quantification of fibrotic area was 7.3% for *Inpp5f* $^{-/-}$ mice, 25 sections, three hearts; 1.8% for wild type mice, 27 sections, three hearts). We also observed more apoptotic cells in the *Inpp5f*$^{-/-}$ hearts after ISO treatment compared to wild type (0.37% of 16,804 cells from 4 *Inpp5f*$^{-/-}$ hearts, 0.24% of 21,558 cells from 5 wild type hearts, $p = 0.01$). Functional assessment by echocardiography after 2 weeks of ISO treatment revealed relative preservation fractional shortening

and ejection fraction, with thickening of the posterior wall in *Inpp5f*$^{-/-}$ animals at this time point (Table 2.3).

Neonatal myocytes isolated from *Inpp5f*$^{-/-}$ and wild type littermates revealed similar levels of Akt and Gsk3ß phosphorylation under basal conditions (Fig 2.7E). Treatment with ISO resulted in increased phosphorylation of Akt and Gsk3β in knockout cells when compared to controls (Fig. 2.7E), while no significant differences in Erk activation were noted (data not shown). IGF-1 treatment of myocytes revealed similar results (data not shown). Taken together, our data suggest that cardiac myocytes lacking Inpp5f are sensitive to stress-induced cardiac hypertrophy and activation of the Akt pathway.

2.3.7 PIP3 levels are altered in Inpp5f null mice

Although a prior report has indicated that Inpp5f exhibits 5-phosphatase activity when PtdIns(3,4,5)P3 and PtdIns(4,5)P2 are used as substrates *in vitro* (Minagawa et al., 2001), I sought to determine directly if loss of Inpp5f alters endogenous cardiac PIP3 levels *in vivo*. Endogenous PtdIns(3,4,5)P3 levels are low, and I am unaware of prior studies that directly measure levels of this important signaling molecule *in vivo* in genetically engineered animals. Therefore, I took two approaches simultaneously. First, I pursued collaboration with Dr Andrew Quong at the Thomas Jefferson University to measure the PIP3/PIP2 levels in the lipid extraction from adult knockout,

Tg and wildtype hearts by mass spectrometry. Our preliminary results (Fig 2.8A) with wild type hearts suggest that accurate measurements would be possible though significant optimization is required. The major problem is that signals are weak and they don't separate well enough from other signals. All the major signals turned out to be glycerophosphocholine, sphingomyelin, glycerophosphoethanolamide and glycerophosphatidic acids. As an alternative approach, I adapted an ELISA-based sensitive assay that has been used for cultured cells and compared PIP3 levels in control and *Inpp5f*$^{-/-}$ hearts before and after ligand stimulation intended to augment PIP3 levels. Heart tissue from 4 mice age 3-5 weeks was combined for each condition, and some samples were treated *ex vivo* with IGF-1, a potent activator of PI3K and AKT in the heart. Our data shows that PIP3 levels in *Inpp5f*$^{-/-}$ mice are ~1.6 fold greater than wild type littermates. However, upon IGF-1 stimulation, PIP3 levels in the knockout hearts increased to 4.9 fold those of stimulated wild type littermates (Fig 2.8B). Thus, loss of Inpp5f sensitizes the heart to hypertrophic stimulation.

2.3.8 Cardiac-specific Inpp5f transgenic mice

I used the well-characterized α-myosin heavy chain (α-MHC) promoter to direct cardiac-restricted expression of Inpp5f in transgenic mice (Fig 2.9A). I utilized two distinct strategies. First, I generated Flag-tagged Inpp5f. However, epitope tagging always has the possibility of affecting protein function. Therefore, I also generated a transgenic construct in which I included an IRES-GFP sequence after the

Inpp5f coding region. With this construct, I would be able to easily determine if the transgene is specifically expressed in the heart (via GFP expression) and accurate transgenic protein levels would be determined by the Inpp5f antibody I generated. 2 transgenic lines were generated for the Flag-tagged transgenic mice and 6 lines were generated for the IRES-GFP transgenic mice in order to control for effects mediated by sites of insertion. With both two strategies, I was able to confirm the germline transmission of the transgene by PCR (Fig 2.9B). For the Flag-tagged Inpp5f construct, I was also able to determine the transgene mRNA level by real-time PCR (data not shown) and verify the transgenic expression by Western blotting (Fig 2.9C) which revealed similar levels of protein expression in the two independent lines. However, for the IRES-GFP construct, Real-time PCR for Inpp5f gene didn't show any increase in the mRNA level (data not shown) and Western Blot for GFP didn't show detectable expression either (Fig 2.9D), which may be due to gene silencing, recombination, etc. I analyzed the phenotypes using the two lines of Flag-Inpp5f transgenic mice.

2.3.9 Inpp5f transgenic mice are resistant to stress-induced hypertrophy

Adult Inpp5f transgenic mice appeared healthy. Heart to body weight ratios of transgenic and wild type littermates were not significantly different at P60 (Fig 2.10A). Control and Inpp5f transgenic littermates at 2 months of age were treated with a constant infusion of saline or ISO for 14 days. As predicted, wild-type mice

exhibited cardiac hypertrophy, as revealed by an increase in both the heart to body weight ratio and the heart weight to tibia length ratio (Fig 2.10A). Hypertrophy of Inpp5f transgenic mice in response to ISO was significantly blunted (Fig 2.10A). Likewise, reactivation of the fetal gene program that accompanied hypertrophy in control animals was markedly attenuated in transgenic animals (Fig 2.10B). After treatment with ISO, we noted activation of the Akt pathway with enhanced phosphorylation of Akt and Gsk3ß in the control hearts (Fig 2.10C). However, ISO-induced increases in phospho-Akt and phospho-Gsk3ß were blunted in transgenic hearts.

2.3.10 Inpp5f has a Sac domain and it has the conserved catalytic motifs

As discussed in the introduction, Inpp5f has a Sac domain, which is approximately 400 amino acid residue in length and defined by seven conserved motifs which appear to define the catalytic and regulatory regions of the phosphatase(Hughes et al., 2000). The highly conserved sequence RXNCXDCLDRTN in the sixth motif is proposed to be the catalytic core of the SAC domain phosphatases. The SAC domain of the yeast synaptojanin-like protein Inp51p does not exhibit phosphatase activity, and the cysteine, arginine and threonine/serine residue are absent from CX5R(T/S) motif of this protein. In order to examine whether this novel protein also has these seven conserved motifs and the active catalytic core, I downloaded the sac1p and the Inp51p protein sequences in S.cerevisiea from NCBI,

and aligned these sequences together with the human and mouse Inpp5f sequences by CLUSTALW(Chenna et al., 2003). Sac1p exhibits phosphatase activity and actually it is the first protein in which the Sac domain is identified(Guo et al., 1999; Hughes et al., 2000). I identified seven highly conserved motifs and the sixth conserved region of Inpp5f in both mouse and human has the highly conserved CX5R(T/S) motif (Fig 2.11).

2.3.11 Interaction of Inpp5f with PDK1

I have showed that one mechanism for Inpp5f to regulate PI3K/Akt pathway is by dephosphorylating PIP3. As an attempt to look for other mechanisms how Inpp5f might affect this pathway, I identified a potential PDK1 interaction domain. I hypothesized that Inpp5f would interact with PDK1 and down-regulate its activity. I tested the interaction by co-transfecting Flag tagged Inpp5f and Myc tagged PDK1 and performing co-immunoprecipitation. As shown in Fig 2.12, I didn't detect any interaction between Inpp5f and PDK1. Some possibilities exist. First, PDK1 might interact with Inpp5f only under certain conditions, such as activation of the PI3K/Akt pathway. Second, some post-translational modifications might be required for the interaction, such as phosphorylation, acetylation, etc. Further experiment could be performed to detect the interaction under other conditions.

2.3.12 Genetic epistasis analysis

We have demonstrated that Hdac2 promotes cardiac hypertrophy and Inpp5f is up-regulated in Hdac2 knockout hearts, both during late gestation and in the adult while it is down-regulated in Hdac2 transgenic mice that over-express Hdac2 in the heart. Hdac2 can deacetylate histones at the Inpp5f upstream genomic region, consistent with direct regulation (Trivedi et al., 2007). My data also suggests that Inpp5f transgenic mice were unable to reactivate fetal genes or to exhibit normal hypertrophic responses and the transgenic hearts have decreased levels of phospho-AKT and phospho-GSK3β. In contrast, Inpp5f knockout mice have augmented hypertrophy associated with reactivation of the fetal gene program. However, it remains unclear to what extent Hdac2 regulates hypertrophy via Inpp5f as opposed to other, yet to be identified, mechanisms. To examine this, I crossed Hdac2+/- mice to Inpp5f-/- mice, and the resulting Hdac2+/-, Inpp5f+/- offspring were intercrossed to produce Hdac2-/-, Inpp5f-/- animals. I expect that the absence of Inpp5f will restore hypertrophic responsiveness in Hdac2 knockout mice. Table 2.4 shows the genotyping result I have obtained so far. I have not detected any double nulls, which could be either due to limited numbers or embryonic lethality. More genotyping and further analysis are needed to investigate this question.

Similarly, my work has also supported a role of Akt in the phenotypes I observed in Inpp5f knockout and transgenic mice. Deletion of Inpp5f leads to higher level of phospho-Akt and reactivation of fetal gene programs, which results in

sensitivity to hypertrophic stimuli. To further test the genetic epstasis of this pathway, I crossed Akt+/- mice, a general gift from Dr. Morris J. Birnbaum, to Inpp5f-/- mice, and then I crossed the resulting Akt+/-, Inpp5f+/- offspring to produce Akt-/-, Inpp5f-/- animals. Table 2.5 displays the genotyping result. Likewise, more numbers are necessary before firm conclusions can be made.

2.4 Discussion

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

Inpp5f encodes a 5' PIP3 phosphatase that is predicted to reduce PIP3 levels and subsequent activation of Akt and downstream signals. Our biochemical findings in Inpp5f transgenic and knockout hearts are consistent with this mode of action. The

Akt signaling network in the adult heart has been extensively examined and has been shown to contribute to both adaptive (physiologic) and maladaptive (pathologic) hypertrophy(Oudit et al., 2004). *Akt1*$^{-/-}$ mice have a 20% reduction in body size, with a concomitant reduction in heart size(Cho et al., 2001b) and they are defective in exercise-induced cardiac hypertrophy(DeBosch et al., 2006b). Cardiac specific over-expression of constitutively active or dominant negative forms of Akt lead to larger or smaller hearts respectively(Condorelli et al., 2002; Matsui et al., 2002; Shioi et al., 2002). Enhanced Akt activity is associated with increased p70S6 kinase activity and increased phospho-Gsk3β(Matsui et al., 2002; Shioi et al., 2002). Cardiac-specific over-expression of constitutively active Gsk3β is associated with reduced agonist and pressure-overload hypertrophy confirming that Gsk3β functions as a negative regulator of hypertrophy in vivo(Zhang et al., 2002). Thus, the alterations in Akt and Gsk3β phosphorylation that we observed *in Inpp5f* knockout and transgenic hearts are consistent with a model in which Inpp5f regulates PIP3 levels, Akt and Gsk3β activity and subsequent hypertrophic responsiveness.

The inositol polyphosphate 5-phosphatases are a large family of enzymes comprising at least 10 mammalian and 4 yeast members(Astle et al., 2006). Inpp5f has a Sac phosphatase domain which exhibits phosphatidylinositol polyphosphate phosphatase activity(Minagawa et al., 2001). The Sac domain is approximately 400 amino acid residue in length and defined by seven conserved motifs which appear to define the catalytic and regulatory regions of the phosphatase(Hughes et al., 2000). The highly conserved sequence RXNCXDCLDRTN in the sixth motif is proposed to

be the catalytic core of the SAC domain phosphatases. The CX5R(T/S) motif within this sequence is also found in a number of metal-independent protein phosphatases and inositol polyphosphate phosphatases and is known to be a phosphatase catalytic site(Guo et al., 1999; Hughes et al., 2000). Interestingly, the SAC domain of the yeast synaptojanin-like protein Inp51p does not exhibit phosphatase activity, and the cysteine, arginine and threonine/serine residue are absent from CX5R(T/S) motif of this protein, being replaced by alanine, lysine and proline respectively. Furthermore, mutations of the first conserved aspartate residue in the RXNCXDCLDRTN sequence as seen in the yeast *sac*1-8 and *sac*1-22 mutant alleles were demonstrated to inactivate the Sac1p functions(Hughes et al., 2000; Kearns et al., 1997; Zhong and Ye, 2003).

Some 5-phosphatases, such as the Src homology 2 (SH2) domain-containing inositol polyphosphophate 5-phosphatases 1 and 2 (Ship and Ship2) have been extensively characterized(Backers et al., 2003; Dyson et al., 2005; Harris et al., 2008; Krystal et al., 1999; Rohrschneider et al., 2000). Ship is expressed predominantly in hematopoietic cells where it is an important negative regulator of cytokine signaling. *Ship*$^{-/-}$ mice have a short life span associated with massive myeloid cell infiltration of the lungs and numerous hematopoietic abnormalities(Helgason et al., 1998; Liu et al., 1998). Ship2 is more widely expressed, with high expression in brain, skeletal muscle and heart(Astle et al., 2006). It plays an important role in insulin signaling and obesity regulation(Grempler et al., 2007; Sleeman et al., 2005). Ship2 has been reported to be a negative regulator of Akt activation. Although loss of Ship2 is not sufficient to activate Akt, the absence of Ship2 allows for greater activity upon Akt

stimulation(Rommel et al., 2001; Wada et al., 2001). These findings are reminiscent of our results with regard to Inpp5f-mediated regulation of basal and agonist-induced activation of Akt in the heart.

Although PTEN and the 5-phosphatases can all degrade PIP3, the degradation products are distinct. Whereas PTEN converts PIP3 to PI(4,5)P2, the 5-phosphatases convert PIP3 to PI(3,4)P2, which can function as a second messenger (Dowler et al., 2000). Therefore, the production of PI(3,4)P2 from PIP3 by Inpp5f and Ship phosphatases may function in part via active signaling, whereas PTEN action appears to be mediated through loss of active PIP3 signaling(Leslie and Downes, 2002). For example, PI(3,4)P2 has been shown to activate reactive oxygen species (ROS)(Brown et al., 2003) and the generation of ROS is a process that is increasingly recognized as an important contributor to depressed cardiac function and maladaptive remodelling(Takimoto and Kass, 2007). Thus, this process could also contribute to the phenotype we observed. Moreover, PI(3,4)P2 activity has been shown to correlate with Akt phosphorylation and activity(Ma et al., 2008). Inpp5f can also dephosphorylate PI(4,5)P2(Minagawa et al., 2001), and depletion of PI(4,5)P2 may contribute to cardiomyocyte apoptosis and subsequent heart failure(Halstead et al., 2005). Thus, regulation of PI(4,5)P2 levels may contribute to the mechanism by which Inpp5f regulates hypertrophy.

Under the sedentary conditions, we found that *Inpp5f* null mice have slightly higher level of cardiac PIP3, though phospho-Akt and phospho-Gsk3β are unaltered

and the mice do not show abnormal hypertrophy. However, upon IGF-1 stimulation, PIP3 levels in the knockout mice increase dramatically and they are more sensitive to hypertrophic stimuli. Hence, unlike PTEN, which alters the basal level of PIP3(Leslie and Downes, 2002), Inpp5f appears to modulate PIP3 levels primarily upon agonist-induced stimulation.

Our prior studies have suggested that *Inpp5f* is repressed at the transcriptional level by Hdac2. *Inpp5f* transcripts are elevated in Hdac2 knockout hearts and are diminished by over-expression of Hdac2 in the heart. siRNA-mediated knockdown of Hdac2 or chemical Hdac inhibition in H9C2 myocytes results in increased levels of *Inpp5f* and decreased phosphorylation of Akt and Gsk3ß. Although Hdac2 may modulate cardiac myocyte size and hypertrophic signaling via multiple pathways, our analysis of *Inpp5f* knockout and transgenic mice are consistent with a functional relationship between Hdac2 and Inpp5f in the setting of hypertrophic stimulation.

Taken together, the work presented here suggests that Inpp5f functions as a negative regulator of cardiac hypertrophy and Akt signaling. Loss of Inpp5f sensitize the heart's response to hypertrophic stimuli by modulating PI(3,4,5)P3 levels.

2.5 Materials and methods

***Inpp5f*$^{-/-}$ knockout mice**. An Inpp5f gene-trap ES clone was obtained from BayGenomics (now the International Gene Trap Consortium, ES clone no. XL0571).

The *Inpp5f* genomic locus is interrupted by insertion of the pGT01xf vector, which integrated into intron 6. Chimeric mice were produced by blastocyst injection according to standard protocols. Mice were genotyped by Southern blotting analysis of ScaI digested genomic DNA. *Inpp5f*$^{+/+}$ and *Inpp5f*$^{-/-}$ mice were subsequently genotyped by PCR using:

Wt F: 5' -AAAGCAGGTTGTGAAGTGGAGCTG- 3',

Wt R: 5' -TGCTCTTGTCATCATCCGAGGACT- 3',

Mut F: 5' -ATATTGAAACCCACGGCATGGTGC- 3' and

Mut R: 5' -TTTGATGGACCATTTCGGCACAGC- 3' primers. The mutant mice have one band at 323 bp, and the wild type mice have one band at 1039 bp.

The loss of Inpp5f was confirmed by qRT-PCR using

5' -ACAGGAGAAAGGAAGTTGGCAGGA -3' and

5' -AGCTTCATGCTCCTTCTCCTTGGT -3' primers, and by Western blot analysis using a rabbit polyclonal antibody targeted to the C terminus of Inpp5f (generated by our lab, see below).

Inpp5f-transgenic mice. A cDNA encoding human Flag-tagged Inpp5f was cloned into an expression plasmid containing the Myh6 (encoding α-Mhc) promoter(Heine et al., 2005), and transgenic mice were generated by standard techniques. Genotyping was performed by PCR analysis of genomic DNA using 5'-TCTTCCAAGCCAAGGACCACTACA- 3' and 5' -TCTGCACGGAATTGGTCAGGTCAT- 3', and cardiac specific expression of Inpp5f

was revealed by qRT-PCR using 5' -ACAGGAGAAAGGAAGTTAGCAGGA- 3' and 5' -AGCTTCATGTTCTTTCTCCTTGGT- 3' primers and Western blot analysis using antibodies to Flag (Sigma, F3165) or Inpp5f (generated by our lab, see below).

Inpp5f antibody generation. GST-Inpp5f fusion protein was expressed in bacteria, purified by standard techniques and used to immunize rabbits (Cocalico Biologicals Inc). A region of mouse *Inpp5f* cDNA encoding 61 C-terminal amino acids was amplified by PCR using primers 5'-CGGACTGGCTTCACAAAGCCCA-3', 5'-AGGAGGCGTCTGTCCCATTGGT-3' and cloned into the pGEX-2T vector. The GST fusion protein was expressed in BL21 bacteria, extracted and purified using Glutathione Sepharose 4B (GE Healthcare) according to the manufacturer's instructions. Purified protein was used to immunize rabbits (Cocalico Biologicals Inc).

Western blotting. Tissue lysates were prepared in lysis buffer consisting of 20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 1 μg/ml leupeptin, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM β-glycerophosphate. 1mM PMSF was added before use. Samples were separated by SDS-PAGE and transferred to PVDF membranes. We used antibodies to Gapdh (1:5,000 dilution, Chemicon, MAB374), phospho-Gsk3β (Ser 9), total Gsk3β, phospho-Akt (Ser 473), total Akt (1:1000 dilution, Cell Signaling, #9336, #9315, #4058, #9272). Primary antibody binding was visualized by using the Western Breeze

Kit (Invitrogen) according to the manufacturer's instructions. Inpp5f antibody was purified by Melon Gel IgG Spin Purification kit (Thermo Scientific) and then diluted 1:100 in 5% milk.

Treatment with isoproterenol. Isoproterenol (Sigma, I5627) was delivered by implanting a micro-osmotic pump (Alzet, Durect; model 1002) subcutaneously under pentobarbital anesthesia. ISO (30 mg/kg/d) or vehicle (Dulbecco's phosphate buffered saline, Gibco) was infused subcutaneously for 14 days as described previously[13].

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

β-galactosidase assay: β-galactosidase activity is measured by Galacto-Light (Tropix, BL100G). Activity was expressed as units/mg protein.

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

Quantitative real time PCR. Total RNA was isolated from dissected mouse hearts using Trizol (Invitrogen). RNA was reverse-transcribed using random hexamers and

the Superscript First Strand Synthesis Kit (Invitrogen). Gene expression was then evaluated by qRT-PCR (ABI PRISM 7900) using the SYBR Green (Applied Biosystems). Signals were normalized to their corresponding Gapdh controls and the ratios expressed as fold changes compared to wild-type. PCR conditions and primer set sequences are available upon request.

IGF-1 treatment. Hearts from one-month old mice were minced and incubated with 100nM Insulin like growth factor 1 (IGF-1) (Sigma, I8779) for 30 min at 37^{o}C followed by lipid extraction.

PIP3 ELISA. Mouse heart tissue was pulverized under liquid nitrogen using a mortar and pestle, followed by lipid extraction and ELISA assays as per manufacture's instruction (Echelon Biosciences Inc, K-2500). ELISA measurements were performed in triplicate after combining tissue from 4 hearts. .

***Inpp5f*$^{-/-}$ knockout mice**. *Inpp5f*$^{+/+}$ and *Inpp5f*$^{-/-}$ mice were genotyped by PCR using:

Wt F: 5' -AAAGCAGGTTGTGAAGTGGAGCTG- 3',

Wt R: 5' -TGCTCTTGTCATCATCCGAGGACT- 3',

Mut F: 5' -ATATTGAAACCCACGGCATGGTGC- 3' and

Mut R: 5' -TTTGATGGACCATTTCGGCACAGC- 3' primers.

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

The loss of Inpp5f was confirmed by qRT-PCR using

5' -ACAGGAGAAAGGAAGTTGGCAGGA -3' and

5' -AGCTTCATGCTCCTTCTCCTTGGT -3' primers.

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

Inpp5f antibody generation. The primers for amplifying mouse *Inpp5f* cDNA are 5'-CGGACTGGCTTCACAAAGCCCA-3', 5'-AGGAGGCGTCTGTCCCATTGGT-3'.

Statistic analysis. All data are expressed as the mean ± SD. Student's t test was used to compare heart to body weight ratios and heart weight to tibia length ratios. Probability values <0.05 were considered statistically significant.

Table 2.1: Loss of Inpp5f does not lead to embryonic or perinatal lethality.

Genotype (offspring of heterozygous crosses)	P0~P21	
	Expected	**Observed**
Inpp5f $^{+/+}$	105	123
Inpp5f $^{+/-}$	210	192
Inpp5f $^{-/-}$	105	105
Total	**420**	**420**
χ^2=4.629, 2 DOF, p=0.10		

Reproduced from (Zhu et al., 2009).

Table 2.2 Inpp5f$^{-/-}$ mice have normal cardiac function by echocardiography.

	2 months			9 months		
	wild type (n=3)	*Inpp5f* KO (n=3)	*p* Value	wild type (n=4)	*Inpp5f* KO (n=4)	*p* Value
IVSd (mm)	0.69±0.06	0.66±0.03	ns	0.66±0.06	0.67±0.07	ns
IVSs (mm)	1.13±0.12	1.00±0.04	ns	0.96±0.10	1.02±0.09	ns
LVPWd (mm)	0.69±0.06	0.64±0.07	ns	0.65±0.03	0.70±0.06	ns
LVPWs (mm)	1.02±0.13	0.90±0.07	ns	0.95±0.14	1.04±0.07	ns
LVIDd (mm)	3.92±0.16	3.76±0.13	ns	4.13±0.22	4.13±0.48	ns
LVIDs (mm)	2.57±0.10	2.54±0.17	ns	2.98±0.12	2.92±0. 40	ns
LVEF (%)	64±2	60±4	ns	56±4	57±4	ns
LVFS (%)	34±1	31±3	ns	29±3	29±2	ns

ns: not significant

Reproduced from (Zhu et al., 2009). This data was produced in collaboration with L.Yuan at the Physiology Core of the Cardiovascular Institute at the University of Pennsylvania.

Table 2.3: Echocardiography of wild type and Inpp5f-/- mice after 2 weeks of ISO infusion.

	2 months		
	wild type (n=5)	*Inpp5f* KO (n=5)	*p* Value
IVSd (mm)	0.69+0.10	0.78±0.15	ns
IVSs (mm)	1.01±0.17	1.16±0.25	ns
LVPWd (mm)	0.65±0.06	0.74±0.06	0.04
LVPWs (mm)	0.98±0.13	0.98±0.16	ns
LVIDd (mm)	3.97±0.36	3.96±0.45	ns
LVIDs (mm)	2.80±0.45	2.76±0.60	ns
LVEF (%)	57±12	59±12	ns
LVFS (%)	30±8	31±8	ns

Reproduced from (Zhu et al., 2009).

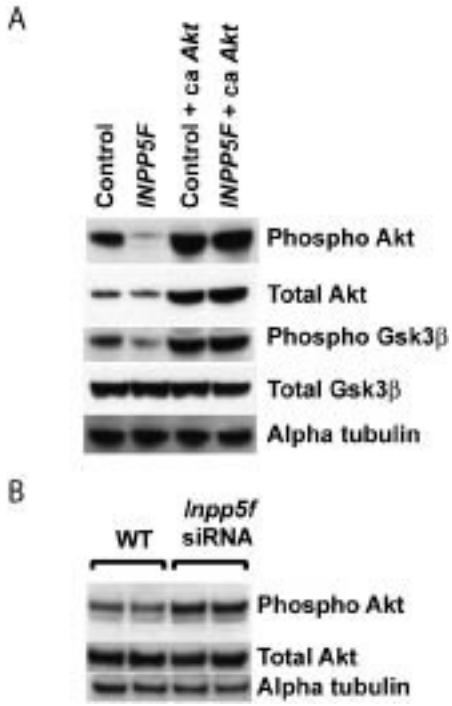
Table 2.4: Genotyping result from Hdac2+/-, Inpp5f+/- intercross (N=75).

Genotype	N
Hdac2 +/+ Inpp5f +/+	8
Hdac2 +/+ Inpp5f +/-	10
Hdac2 +/+ Inpp5f -/-	7
Hdac2 +/- Inpp5f +/+	9
Hdac2 +/- Inpp5f +/-	24
Hdac2 +/- Inpp5f -/-	11
Hdac2 -/- Inpp5f +/+	4
Hdac2 -/- Inpp5f +/-	2
Hdac2 -/- Inpp5f -/-	0

Table 2.5: Genotyping result from Akt+/-, Inpp5f+/- intercross (N=86).

Genotype	N
Akt +/+ Inpp5f +/+	10
Akt +/+ Inpp5f +/-	13
Akt +/+ Inpp5f -/-	6
Akt +/- Inpp5f +/+	10
Akt +/- Inpp5f +/-	21
Akt +/- Inpp5f -/-	15
Akt -/- Inpp5f +/+	5
Akt -/- Inpp5f +/-	6
Akt -/- Inpp5f -/-	0

Figure 2.1: Inpp5f regulates PI3K/Akt pathway in vitro.



Reproduced from (Trivedi et al., 2007).

Figure 2.1: Inpp5f regulates PI3K/Akt pathway in vitro.

(A) Overexpression of Inpp5f in H9C2 cells resulted in decreased phosphorylation of Gsk3β and Akt. Phosphorylated and total Gsk3β and Akt were measured by Western Blot with or without transfection of *Inpp5f* and *caAkt* expression vectors. (B) H9C2 cells were treated with siRNA to knockdown Inpp5f, and total and phosphorylated Akt levels were measured by Western Blot.

Figure 2.2: Generation and confirmation of Inpp5f antibodies.

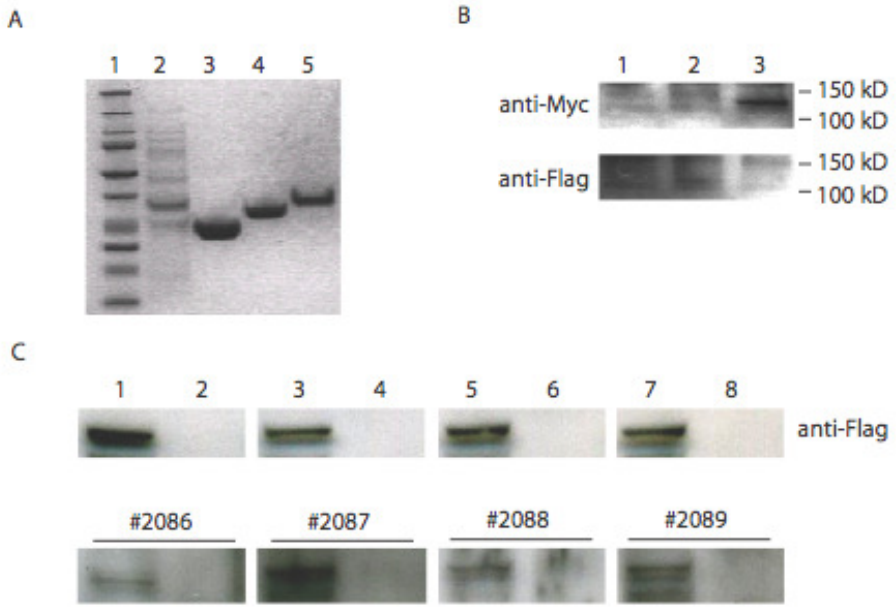
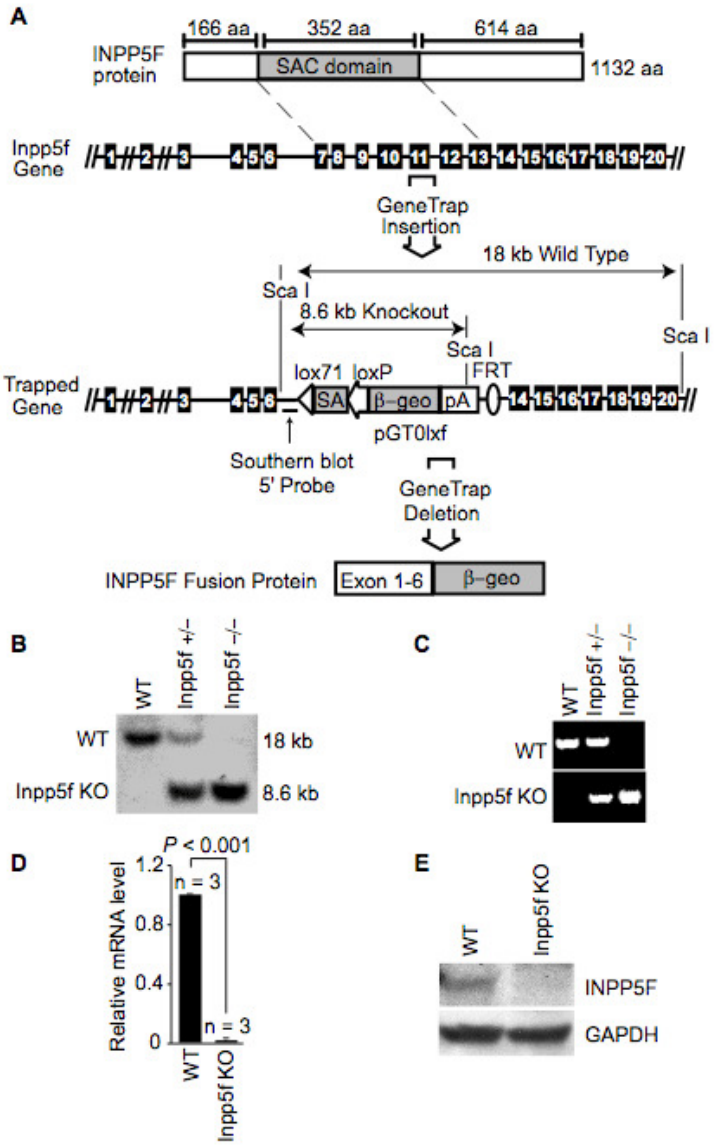


Figure 2.2: Generation and confirmation of Inpp5f antibodies.

A. Coomassie blue staining of the GST fusion protein. Lane 1, maker; Lane 2, unpurified protein; Lane 3, GST only; Lane 4, GST fused with epitope N; Lane 5, GST fused with epitope C. B. Tranfection of Cos7 cells with epitope tagged plasmid to determine the size of Inpp5f protein. Lane 1, control cell protein; Lane 2, FLAG-tagged Inpp5f transfected cell protein; Lane 3, MYC-tagged Inpp5f transfected cell protein. C. Examination of 4 sera on both Flag-tagged Inpp5f containing plasmid (Lane 1, 3, 5, 7) and empty vector transfected cells (Lane 2, 4, 6, 8).

Figure 2.3: Inactivation of Inpp5f.



Reproduced from (Zhu et al., 2009).

Figure 2.3: Inactivation of Inpp5f.

(A) Schematic representation of Inpp5f protein (top) and gene structure. The wild type and gene trap alleles are shown; exons are represented by black boxes. The sizes of the expected restriction fragments recognized by the Southern probe (indicated) are shown. (B) Southern blot of adult mice tail DNA resulting from a cross between *Inpp5f*$^{+/-}$heterozygotes. (C) PCR genotyping of offspring resulting from a cross between *Inpp5f* $^{+/-}$heterozygotes. (D) Real-time quantitative PCR of mRNA from adult wild-type and *Inpp5f*$^{/-}$ hearts shows the absence of *Inpp5f* mRNA in the knockout hearts. (E) Western blot of wild-type and *Inpp5f*$^{/-}$ adult heart tissue shows loss of Inpp5f protein in mutant hearts.

Figure 2.4: Confirmation of genetrap insertion and existence of fusion mRNA.

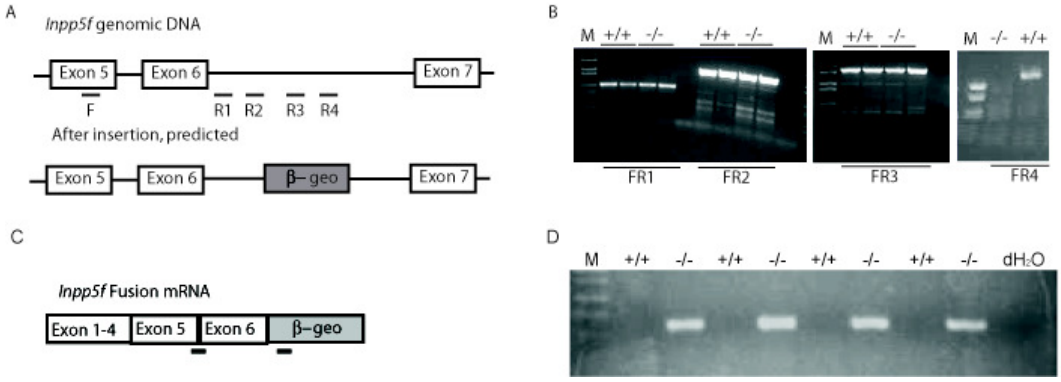
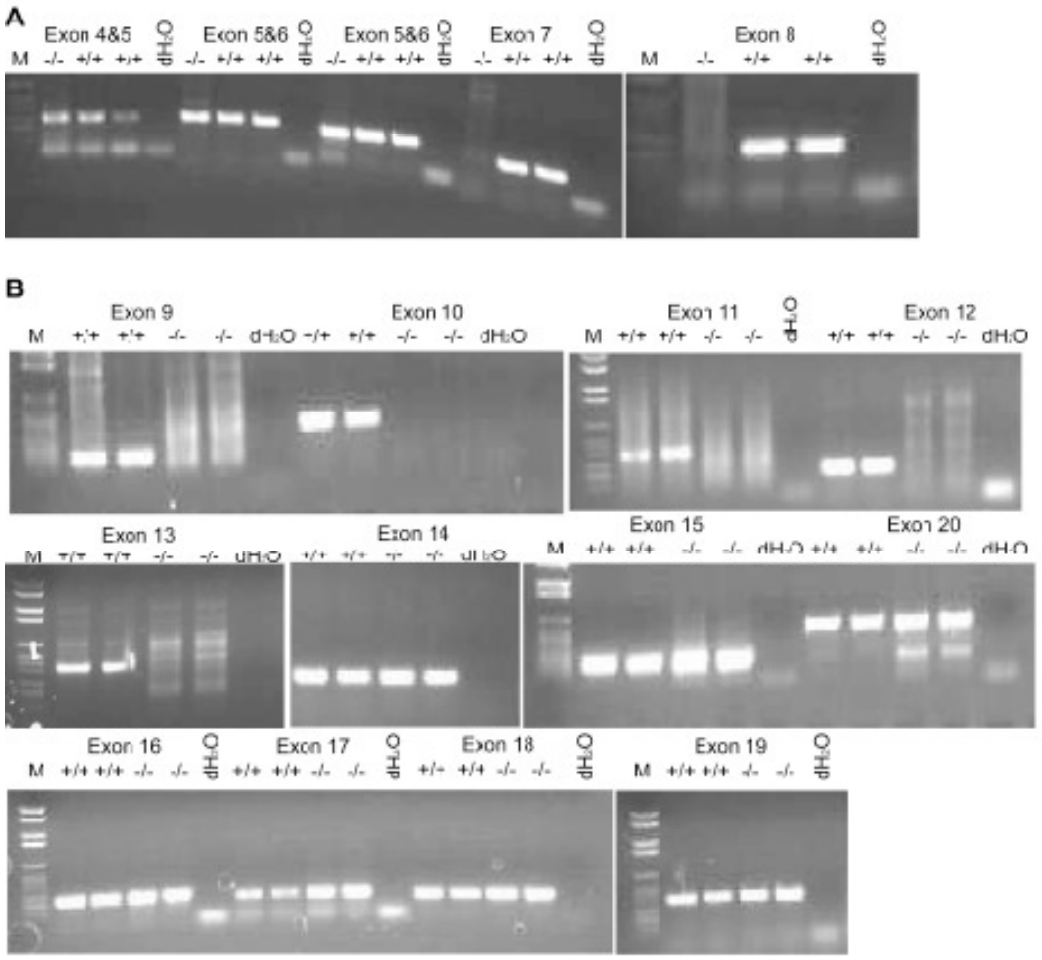


Figure 2.4: Confirmation of genetrap insertion and existence of fusion mRNA.

(A) Schematic of Inpp5f genomic DNA and the predicted genomic locus following the genetrap cassette insertion. The primers for PCR are shown. (B) PCR results from knockout and wildtype mice using 4 different reverse primers are shown. (C) Schematic representation of the Inpp5f fusion mRNA transcript. The primers for the Real-time PCR are shown. (D) Real-time RCR of mRNA from four individual knockout mice and their wild type littermate controls using primers shown in (C).

Figure 2.5: Examination of the endogenous Inpp5f genomic sequence.

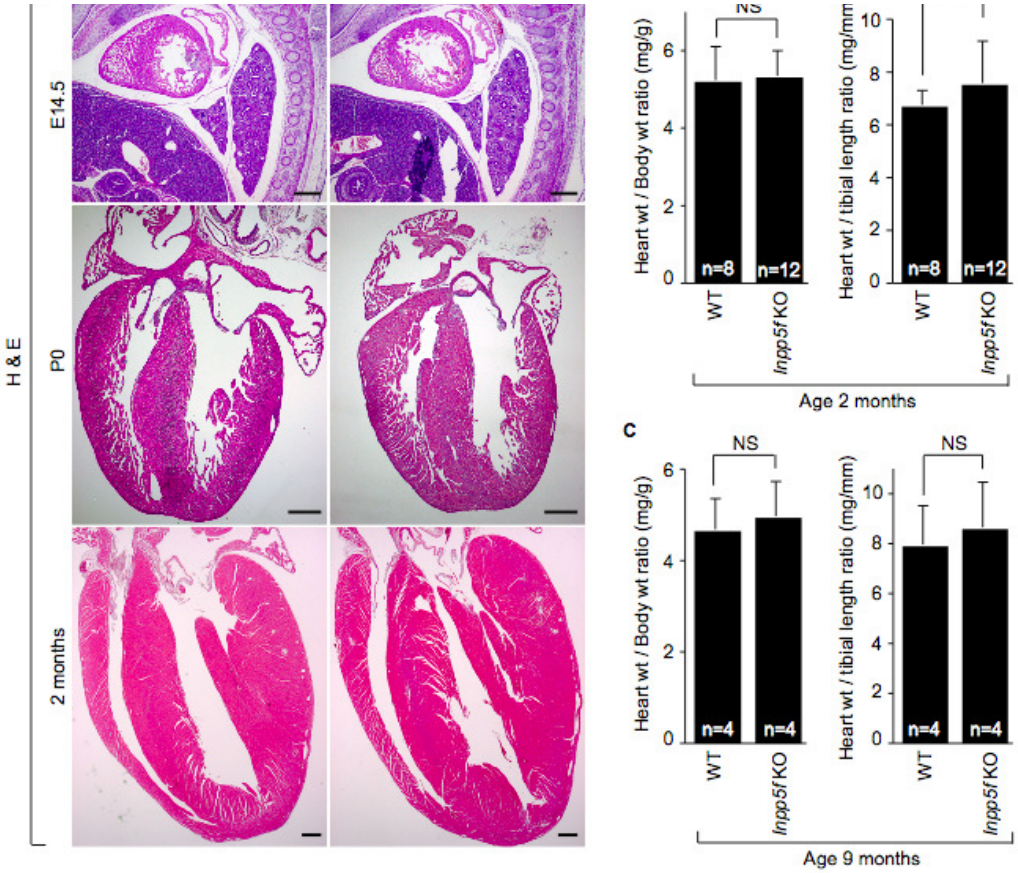


Reproduced from (Zhu et al., 2009).

Figure 2.5: Examination of the endogenous Inpp5f locus and the targeted locus.

Primers specific for each exon (4-20) were used to determine if the genomic sequence corresponding to these exons was present in the wildtype and homozygous mutant genome. PCR results show that exon 7 to 13 are deleted in the Inpp5f homozygous mutant genome.

Figure 2.6: Inpp5f $^{-/-}$ hearts appear normal.

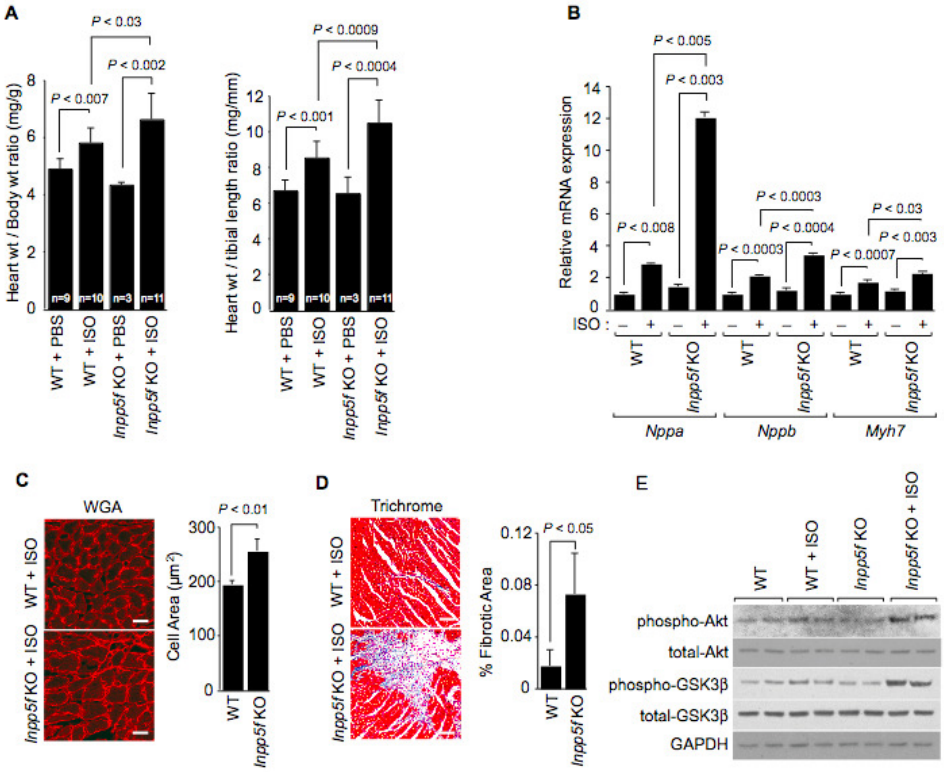


Reproduced from (Zhu et al., 2009).

Figure 2.6: *Inpp5f* $^{-/-}$ hearts appear normal.

(A) H&E-stained sections of wild-type and *Inpp5f*$^{-/-}$ hearts at E14.5, E16.5 and P65 (scale bar, 400 µm) reveals normal heart structure. (B) Heart to body weight and heart to tibia length ratios of *Inpp5f*$^{+/+}$ (n = 8) and *Inpp5f*$^{-/-}$ (n = 12) mice at 2 month of age. (C) Heart to body weight and heart to tibia length ratios of *Inpp5f*$^{+/+}$ (n = 4) and *Inpp5f*$^{-/-}$ (n = 4) mice at 9 month are shown. NS: not significant.

Figure 2.7: *Inpp5f*-deficient mice are susceptible to agonist-induced cardiac hypertrophy.

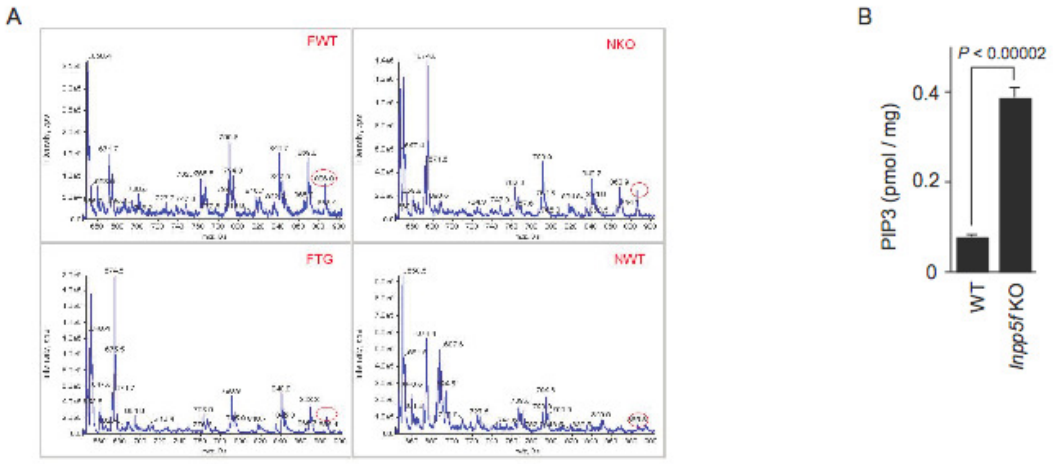


Adapted and modified from (Zhu et al., 2009).

Figure 2.7: *Inpp5f*-deficient mice are susceptible to agonist-induced cardiac hypertrophy.

(A) Wild-type and *Inpp5f*$^{-/-}$ littermates were subjected to chronic infusion of saline or ISO for 14 days. Heart weight to body weight and heart weight to tibia length ratios (± s.d.) were determined. (B) Transcripts for *Nppa*, *Nppb* and *Myh7* were detected by real-time quantitative PCR in hearts from wild-type and *Inpp5f*$^{-/-}$ mice following chronic infusion of saline or ISO. The values are expressed as the fold change in transcript abundance (± s.d.) compared to wild-type mice. (C) Wheat germ agglutinin (WGA) staining shows increased myocyte size in *Inpp5f*$^{-/-}$ compared to wild-type hearts (scale bar, 25 μm). (D)Trichrome staining demonstrates more fibrosis (blue) in *Inpp5f*$^{-/-}$ hearts treated with ISO (scale bar, 150 μm,) compared to control. (E) Western blots from neonatal cardiomyocytes treated with or without 10 μM ISO for 10 min are shown; Gapdh is shown as a loading control.

Figure 2.8: PIP3 levels are altered in the Inpp5f knockout mice.

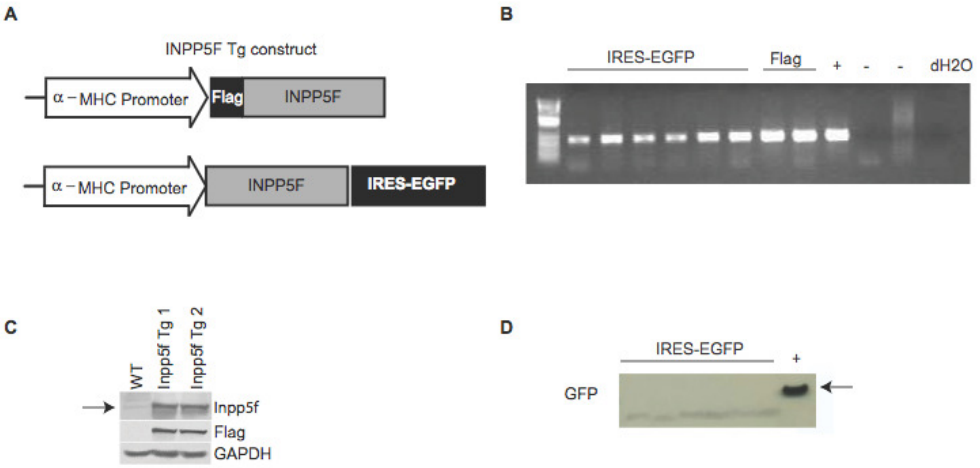


Adapted and modified from (Zhu et al., 2009). The mass spec data was obtained in collaboration with Dr Andrew Quong at Tomas Jefferson University.

Figure 2.8: PIP3 levels are altered in the Inpp5f knockout mice.

(A) Mass spec results from lipid extracts of 4 heart samples. Peak 886 (red circle) represents PIP3. (B) PIP3 levels, normalized to heart weight, in the *Inpp5f*$^{-/-}$ and wildtype hearts after 30 min of 100 nM IGF-1 stimulation were assayed as indicated in "Materials and Methods".

Figure 2.9: Generation of Inpp5f transgenic mice.

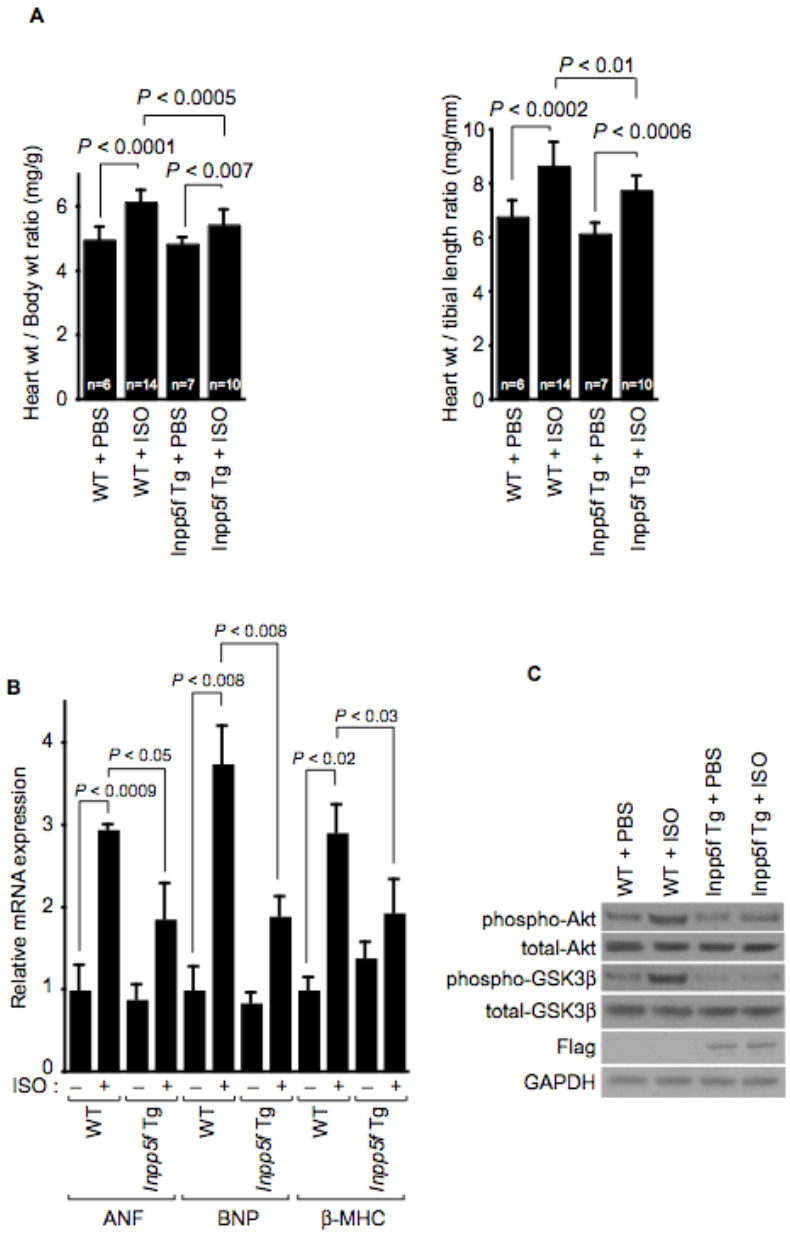


Adapted and modified from (Zhu et al., 2009).

Figure 2.9: Generation of Inpp5f transgenic mice.

(A) Schematic representation of the *Inpp5f* transgenic constructs. (B) PCR genotyping result for both constructs. 6 lines for IRES-EGFP and 2 lines for Flag construct are shown. 1 positive control and 2 negative controls are also shown. (C) Western blot analysis of myocardium from 2-month-old wild-type and *Inpp5f*-Tg mice. Transgenically expressed *Inpp5f* was epitope-tagged and was detected using an Inpp5f antibody and a Flag antibody. Arrow: Inpp5f protein. (D) Western Blot to detect the expression of GFP. Heart protein lysates from 6 lines of IRES-EGFP were extracted and probed with GFP antibody. The last lane is a positive control showing the expression of GFP.

Figure 2.10: *Inpp5f*-Tg mice are resistant to agonist-induced cardiac hypertrophy.



Adapted and modified from (Zhu et al., 2009).

Figure 2.10: *Inpp5f*-Tg mice are resistant to agonist-induced cardiac hypertrophy. (A) Wild-type and *Inpp5f*-Tg littermates were subjected to chronic infusion of saline or ISO for 14 days. Heart weight to body weight and heart weight to tibia length ratios (± s.d.) were determined. (B) Transcripts for *Nppa*, *Nppb* and *Myh7* were detected by real-time quantitative PCR from hearts of wild-type and *Inpp5f*-Tg mice following chronic infusion of saline or ISO. The values are expressed as the fold change in transcript abundance (± s.d.) compared to wild-type mice. (C) Western blots of myocardium from 2-month-old wild-type and *Inpp5f*-Tg mice subjected to infusion of saline or ISO for 14 days. Gapdh is shown as a loading control.

Figure 2.11: Inpp5f has a Sac domain and it has the conserved catalytic motifs.

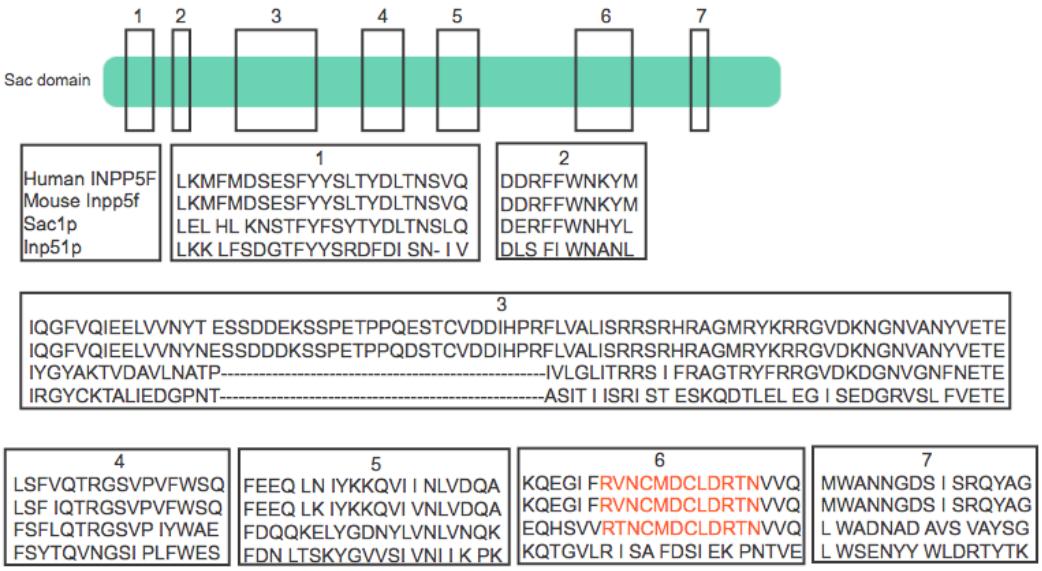


Figure 2.11: Inpp5f has a Sac domain and it has the conserved catalytic motifs.

The Sac domain contains seven highly conserved motifs, including the putative catalytic RXNCXDCLDRTN motif (conserved motif 6, shown in red). These are shown for the human Inpp5f, mouse Inpp5f and the yeast Sac domain-containing proteins Sac1p and Inp51p.

Figure 2.12: Inpp5f does not interact with PDK1.

Figure 2.12: Inpp5f does not interact with PDK1 . (A) Western blot shows the expression of two proteins in transfected Cos7 cells. (B) The proteins were extracted from transfected Cos7 cells. Co-immunoprecipitation was performed with an anti-Myc antibody. Inpp5f did not interact with PDK1 when immunoprecipated with an anti-Myc antibody and detected with anti-Flag antibody. Input control is shown on the left. Immunoblot (IB) of Myc control is shown at the bottom.

Chapter 3 Regulation of Gata4 deacetylation and stability by Hdac2

Some of this Chapter has been published in (Trivedi et al.).

3.1 Summary

In chapter 2, I examined the effects of gain and loss of Inpp5f in the heart, which might function downstream of Hdac2. However, Hdac2 nulls also display enhanced cardiac myocyte proliferation during embryonic stages, which I did not observe in Inpp5f mutant mice. This suggests that mechanisms unrelated to Inpp5f might be responsible for the embryonic phenotypes of Hdac2 nulls. In this chapter, I describe that Hdac2 and Gata4 physically interact and I map the Gata4 interaction domain. I also investigate the function of acetylation on Gata4 stability.

3.2 Introduction

Gata4 is one of the earliest genes expressed by specified cardiac precursors at the cardiac crescent stage of the mouse development (Arceci et al., 1993; Kelley et al., 1993). Global loss of Gata4 in mice causes embryonic lethality at E9.5 as a result of severe defects in the extra-embryonic endoderm and aberrant heart and foregut morphogenesis (Kuo et al., 1997; Molkentin et al., 1997). Studies involving tissue-specific loss of Gata4 in murine cardiac myocytes have demonstrated a critical role for Gata4 in embryonic myocyte proliferation (Zeisberg et al., 2005). Mice lacking

Gata4 in the anterior heart field (AHF), for example, die by E13.5 due to significant right ventricular and interventricular septal myocyte proliferation defects (Rojas et al., 2008). Gata4-null cardiomyocytes show down-regulation of a wide array of cell cycle associated genes, some of which are direct transcriptional targets of Gata4, including cyclinD2 and cdk4 (Rojas et al., 2008).

Recently, we have shown that global loss of HDAC2 results in partial perinatal lethality due to cardiac developmental defects that include enhanced cardiac myocyte proliferation (Trivedi et al., 2007). Microarray and RT-PCR analysis suggested that several cell cycle genes were up-regulated in Hdac2 null hearts at E16.5, including transcripts of known Gata4 targets, cyclin-D2 and cdk4. Interestingly, Gata4 protein levels were unaltered in the knockout hearts (unpublished data).

Gata4 transcriptional activity is known to be regulated by post-translational modification including acetylation, phosphorylation and sumoylation (Kawamura et al., 2005; Liang et al., 2001a; Wang et al., 2004). For example, the histone acetyl transferase (HAT) p300 is able to acetylate Lys311, 318, 320, and 322 of Gata4, resulting in enhanced DNA binding and transcriptional activity. In transgenic mice, p300 over-expression in the heart induces Gata4 acetylation and cardiac hypertrophy (Miyamoto et al., 2006; Yanazume et al., 2003a). Interestingly, p300 null mice die at E9.5 exhibiting defects in proliferation and cardiac development (Yao et al., 1998). Mechanisms for Gata4 deacetylation have, to our knowledge, not been previously described. This leads us to hypothesize that Hdac2 might deacetylate Gata4 and thus

regulates it transcriptional activity. As an initial step to test this hypothesis, I investigated the interaction between Gata4 and Hdac2. My results suggest that Gata4 is able to interact with Hdac2 and the lysine rich domain of is responsible for the interaction.

The present study focused on the role of Hdac2 during embryonic cardiac development. However, our prior studies have implicated important roles for Hdac2 in the adult heart (Kook et al., 2003; Trivedi et al., 2007). Gata4 also functions in homeostasis of the adult myocardium, and alterations in transcriptional activity, including those regulated by p300-mediated acetylation of Gata4, affect adult cardiac hypertrophy (Dai and Markham, 2001; Hirai et al., 2004; Yanazume et al., 2003a; Yanazume et al., 2003b). However, the model presented here to explain the roles of Hdac2 and Gata4 in regulation of embryonic myocardial proliferation cannot easily explain the roles for these proteins during adult cardiac hypertrophic processes. Hdac2 deficiency results in resistance to cardiac hypertrophy. Cardiac hypertrophy is generally associated with enhanced Gata4 activity, which drives cardiac structural gene expression. A simple extension of the model presented here would suggest that loss of Hdac2 would result in enhanced Gata4 activity, which is not consistent with the adult phenotypes. However, we have noted a dramatic loss of Gata4 protein in newborn mice lacking Hdac2 (unpublished data), suggesting that hyper-acetylation results in protein degradation, with resulting loss of Gata4 activity. This would be consistent with the adult phenotypes we have observed. Actually, a previous study has provided evidence that acetylated GATA-1 is targeted for degradation via the

ubiquitin/proteasome pathway and activation by acetylation simultaneously marks GATA-1 for degradation (Hernandez-Hernandez et al., 2006). The other part of my study is to elucidate the mechanisms by which acetylation of Gata4 leads to degradation. My preliminary results suggest that deacetylation of Gata4 by Hdac2 stabilizes Gata4.

3.3 Results

3.3.1 Hdac2 and Gata4 physically interact

Other Gata factors have been shown to be able to recruit Hdacs to regulate gene expression (Cantor and Orkin, 2005; Hong et al., 2005; Svensson et al., 1999; Tsang et al., 1997). For example, Hdac3 interacts with Gata2 to regulate transcriptional repression in hematopoietic progenitor cells (Ozawa et al., 2001). Gata1 recruits Hdac5 leading to reduced Gata1 transcriptional activity (Watamoto et al., 2003). In order to test if Gata4 and Hdac2 can physically interact with each other, I transfected 293 cells with HA-tagged Gata4. Total lysates were immunoprecipitated by anti-HA antibody to immunoprecipitate Gata4 and Western blot analysis was performed with Hdac2 antibody. The data suggest that Gata4 and Hdac2 can interact (Fig 3.1).

3.3.2 The interaction between Hdac2 and Gata4 requires the lysine rich domain of Gata4

The protein structure for Gata4 has been widely studied. It contains a domain of two adjacent zinc fingers (Cys-X2-Cys-X17-Cys-X2-Cys) that directs preferential binding to the nucleotide sequence element 5'-(A/T)GATA(A/G)-3' of target gene promoters (Pikkarainen et al., 2004). A lysine rich motif is present in between the two zinc fingers and two separate transcriptional activation domains are present within the N terminus of the protein (Morrisey et al., 1997). In order to identify the Hdac2 interaction domain in Gata4, eight Gata4 N-terminal deletion constructs and four C-terminal deletion constructs were made (Fig 3.2A). Expression analysis shows that three of the N-terminal deletion constructs and all four C-terminal deletion constructs expressed (Fig 3.2B). Co-immunoprecipitation experiments showed that all seven expressed constructs were able to interact with Hdac2 (Fig 3.3). Because all constructs have a common region amino acid 241-274 (Fig 3.2A), I hypothesized that this is the region that is responsible for the interaction. I then tried to optimize the transfection conditions for two N-terminal deletion constructs which do not contain region 241-274 (Δ265, Δ294). However, by adjusting the DNA/superfect ratio and incubation time, I failed to detect any expression (data not shown). Similarly, an N-terminal deletion construct with the first 240 aa did not express either (data not shown). I then made an internal deletion construct in which amino acids 241-274 were absent using a technique called splicing overlap extension PCR (Fig 3.4A). I confirmed the expression of the protein by Western blot (Fig 3.4B). However, the

expression level of this construct is much lower than the full length construct. I optimized the transfected DNA amount, DNA/superfect ratio as well as incubation time. Unfortunately, I found that these factors did not improve the expression level (Fig 3.5). Therefore, I diluted the full length Gata4 transfected cell lysate using the untransfected lysate so that the expression levels of the full length Gata4 and the internal deletion are similar (Fig 3.6A). This construct was unable to bind Hdac2 (Fig 3.6B). Therefore, our data suggest that Gata4 is able to interact with Hdac2 and the lysine rich motif 241-274 in Gata4 is responsible for this interaction.

3.3.3 Deacetylation of Gata4 by Hdac2 stabilizes Gata4

I hypothesized that acetylation of Gata4 positively signals ubiquitination and degradation in the heart. I first transfected H9c2 cells, a cardiomyocyte cell line, with HA-tagged Gata4 and then treated them with cycloheximide (CHX) after 48 hours to inhibit further protein synthesis. I measured Gata4 decay by Western blots and found that after 9 hrs, the protein disappeared by half and after 28 hrs, it was almost completely degraded (Fig 3.7). However, one caveat for this approach is that the protein half-life is measured when overall protein synthesis is abrogated and thus may not reflect the actual turnover rate under normal growth conditions. The stability and abundance of the proteolytic enzymes themselves might also be affected, which further complicates the accurate measurement of protein turnover rate. Therefore, I also used pulse chase as an alternative method, which imposes minimal disruption

with normal cell growth and metabolism. H9c2 cells were transfected with HA-Gata4 and cells were metabolically labeled for 30 min with [^{35}S] methionine/cysteine. The cells were harvested after 0, 2, 6, 11h of chase. This experiment gave me the time frame to measure Gata4 stability. The result suggested that after 2 h of chase, the amount of Gata4 showed a reduction of ~70% (Fig 3.8A). I then hypothesized that co-transfection with Hdac2 leads to increased Gata4 stability. Western blot showed over-expression of Hdac2 in the co-transfected cells (Fig 3.8C), while Gata4 is expressed at equal amount (data not shown). Preliminary pulse chase results suggest that Hdac2 stabilizes Gata4 though this experiment needs to be repeated for statistical analysis (Fig 3.8B).

3.4 Discussion

In this chapter, I demonstrated that Gata4 is able to interact with Hdac2 and the lysine rich domain located between the two zinc fingers is necessary for interaction. My preliminary results also suggest that deacetylation of Gata4 by Hdac2 might play a role in Gata4 stability.

Gata4 contains two distinct zinc finger domains and a C-terminal nuclear localization sequence that together constitute the DNA binding and protein-protein interaction domain (Molkentin, 2000). Gata4 also contains two transcriptional activation domains in the N-terminus (Pikkarainen et al., 2004). Gata4 has been shown to cooperate with a number of other transcription factors and co-activators. Specifically, the C-terminal zinc finger domain has been found to interact physically with several other factors to regulate cardiac gene promoters including p300, dHAND, Nkx-2.5 and NFAT (Dai et al., 2002; Dai and Markham, 2001; Lee et al., 1998; Molkentin et al., 1998). However, a co-repressor protein FOG-2 (friend of Gata-2) interacts with N-terminal zinc finger, and Gata6 may require both zinc fingers for their interaction (Charron et al., 1999; Lu et al., 1999; Svensson et al., 2000; Svensson et al., 1999; Tevosian et al., 1999). My studies identify the lysine rich domain located between the two zinc fingers as the region necessary for interaction with Hdac2.

Previous reports have indicated that Gata factors can recruit Hdacs to target genes, either by directly interacting or by associating with Fog proteins that can

recruit the NuRD complex containing Hdac1 and Hdac2 (Cantor and Orkin, 2005; Hong et al., 2005; Svensson et al., 1999; Tsang et al., 1997). For example, interaction of Hdac3 with Gata2 leads to transcriptional repression of Gata2 in hematopoietic progenitor cells (Ozawa et al., 2001). Similarly, Hdac5 interacts with Gata1 and represses its transcriptional activity (Watamoto et al., 2003). In each case, Hdac function suppresses Gata dependent transcriptional activity. Although it was generally assumed that Hdac-mediated transcriptional repression of Gata activity was mediated by histone deacetylation and chromatin remodeling, it will be interesting to re-address this question in light of our results to determine if other Gata proteins are regulated by Hdacs via direct deacetylation.

Acetylation has been shown regulate protein stability in two opposing ways. Lysines are targets for both acetylation and ubiquitination. Therefore, acetylation can stabilize proteins by preventing subsequent ubiquitination (Kouzarides, 2000) as demonstrated for both p53 (Li et al., 2002) and Smad7 (Gronroos et al., 2002). However, the opposite possibility also exists: acetylation at some lysines might positively signal ubiquitination at other lysines to thus cause protein loss. For example, acetylated GATA-1 is targeted for degradation via the ubiquitin/proteasome pathway and activation by acetylation simultaneously marks GATA-1 for degradation (Hernandez-Hernandez et al., 2006). Therefore, a lysine mutated construct of Gata4 is of limited use in studying the effect of acetylation on Gata4 stability. In my study, I used Hdac2 co-transfection with Gata4. Alternative methods such as p300 co-transfection and Hdac2 inhibitor can also be performed to examine the effect of

acetylation on Gata4 stability.

3.5 Materials and methods

Immunoprecipitation. 293 cells were homogenized in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM DTT) containing 1 mM PMSF, phosphatase inhibitors (Sigma), and protease inhibitors mixture (Sigma). The samples were sonicated five times for 3 s. The lysate was collected by centrifugation at 16,000 X g for 10 min at 4°C. Pre-cleared lysates with beads were incubated with primary antibodies for 16 h at 4°C. After incubation for 1 h at 4°C with beads, immunocomplexes were collected, washed three times with immunoprecipitation buffer at 4°C, and applied to 4-12% SDS-polyacrylamide gels for Western blot analysis.

Western blotting. Tissue lysates were prepared in lysis buffer consisting of 20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 1 μg/ml leupeptin, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM β-glycerophosphate. 1mM PMSF was added before use. Samples were separated by SDS-PAGE and transferred to PVDF membranes. We used antibodies to Gapdh (1:5,000 dilution, Chemicon, MAB374), HA (1:1000 dilution, SantaCruz) and Hdac2 (1:1000 dilution, Invitrogen). Primary antibody binding was visualized by using the Western Breeze Kit (Invitrogen) according to the manufacturer's instructions.

Transient transfections. H9c2 myoblasts and 293 cells were transfected with SuperFect reagent (Qiagen) as per manufacturer's protocol.

Plasmids and deletion constructs cloning. Plasmids for expression of Hdac2 and Gata4 are previously described (Durocher et al., 1997; Kook et al., 2003; Trivedi et al., 2007). Various deletion mutants of HA tagged Gata4 cDNA were created by PCR amplification, sequence verified, and subcloned into the pGC eukaryotic expression vector. Primers sequences for Gata4 deletion constructs are as follows:

N terminal deletion constructs:

Reverse primer is 5'-TTACGCGGTGATTATGTCCC-3'

Forward primer:

Δ80: 5'-GCCACCatgTACCCATACGATGTTCCAGATTACGCTGGGACCCAGCAGGGTAG-3'

Δ129: 5'-GCCACCatgTACCCATACGATGTTCCAGATTACGCTGCAGCCTACAGCAGTAGC-3'

Δ183: 5'-GCCACCatgTACCCATACGATGTTCCAGATTACGCTTTCGACAGCCCAGTCCTG-3'

Δ209:

5'-

GCCACCatgTACCCATACGATGTTCCAGATTACGCTTTCTCAGAAGGCAGAG

AGTGT-3'

Δ240: 5'-

GCCACCatgTACCCATACGATGTTCCAGATTACGCTGGCCTCTACCACAAGAT

GAAT-3'

Δ265:

5'-

GCCACCatgTACCCATACGATGTTCCAGATTACGCTGTAGGCCTCTCCTGTGC

C-3'

Δ294:

5'-

GCCACCatgTACCCATACGATGTTCCAGATTACGCTGGCCTCTACATGAAGCT

CC-3'

Δ329:

5'-

GCCACCatgTACCCATACGATGTTCCAGATTACGCTCCAGCAGGTCCTCCTGG

-3'

C terminal deletion constructs:

Forward primer is 5'-

GCCACCatgTACCCATACGATGTTCCAGATTACGCTTACCAAAGCCTGGCTAT

G-3'

Reverse primer:

Δ391-440: 5'-TTAGCCGGACACAGTACTGAATGT-3'

Δ337-440: 5'-TTACTCGCCAGGAGGACCTG-3'

Δ311-440: 5'-TTACCGCATTGCAAGAGGCCT-3'

Δ275-440: 5'-TTACTGGCAGTTGGCACAGGA-3'

Splicing overlap extension PCR. The PCR methods are previously described (Warrens et al., 1997). Primers sequences for the splicing overlap extension PCR are as follows:

Gata4 primer A: 5'-ATG TAC CAA AGC CTG GCT AT-3'

Gata4 primer B hybrid: 5'-CAG TGT GGT GGT GGT AGT ACA GGC GTT GCA TAG GTA-3'

Gata4 primer C hybrid: 5'-TAC CTA TGC AAC GCC TGT ACT ACC ACC ACC ACA CTG-3'

Gata4 primer D: 5'-TTA CGC GGT GAT TAT GTC CC-3'

Pulse chase. Cells were starved for 30 min in 2 ml of methionine/cysteine-free medium (Invitrogen) at 37^{o}C, metabolically labeled for 30 min with 125 μCi/ml of ^{35}S methionine/cysteine (pulse), washed free of unbound radioactive amino acids, and incubated in prewarmed complete medium (chase). At the indicated time points, the cells were disrupted in ice-cold lysis buffer and immunoprecipitated with anti-HA antibody. Proteins were subjected to SDS-PAGE. Labeled proteins were visualized

and quantitatively analyzed with phosphor imager.

Figure 3.1: Hdac2 and Gata4 physically interact.

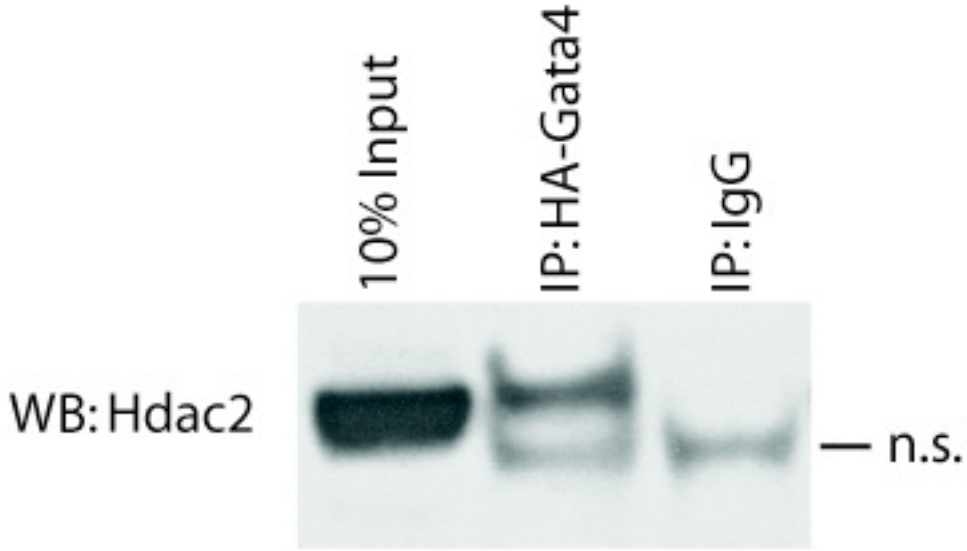
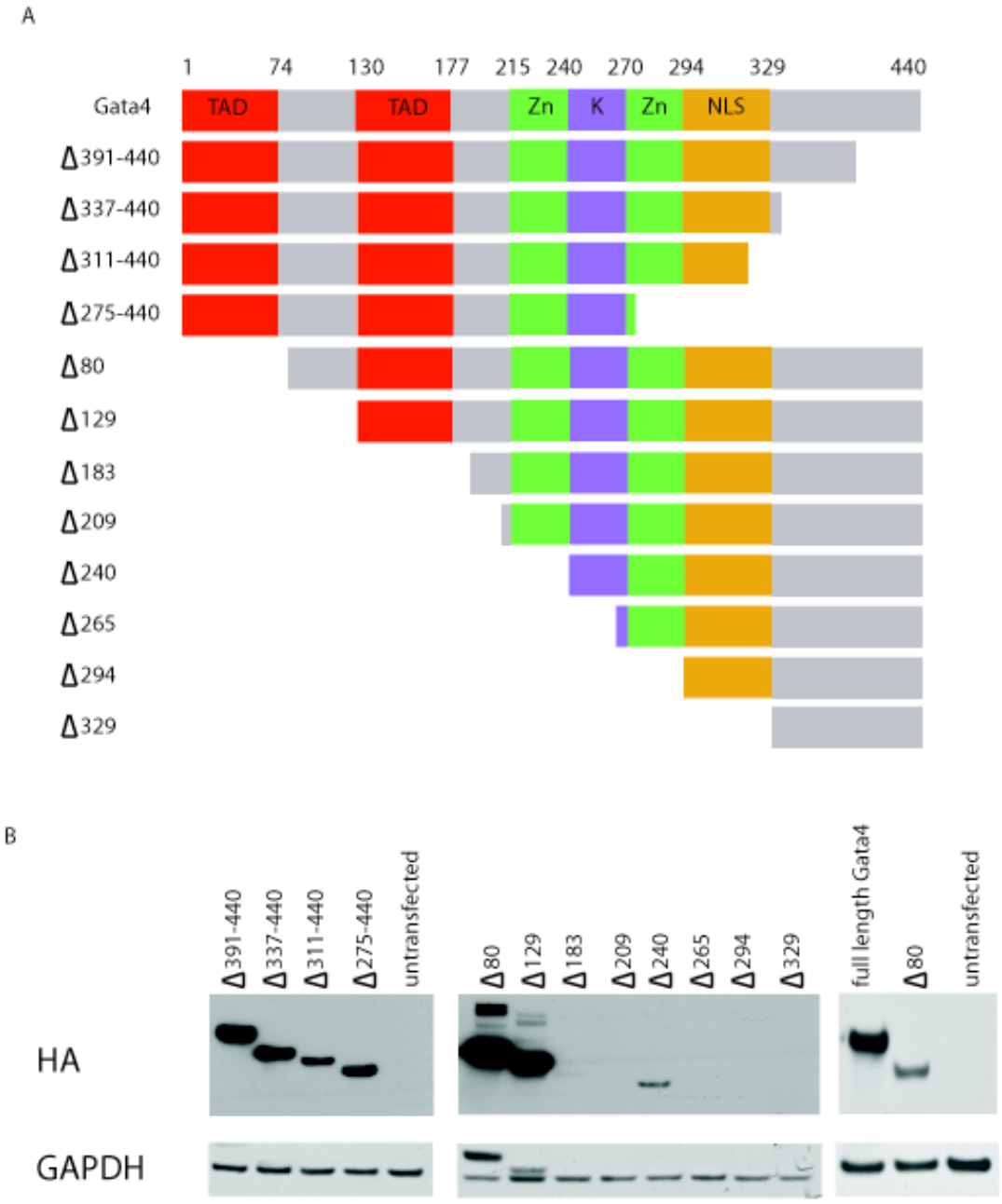


Figure 3.1: Hdac2 and Gata4 physically interact.

293 cells were transfected with HA-Gata4. Immunoprecipitation were performed by anti- HA antibody to immunoprecipitate Gata4 and western blot analysis was performed with Hdac2 antibody to detect the endogenous Hdac2. n.s. non-specific band.

Figure 3.2: Generation of 12 Gata4 deletion constructs.

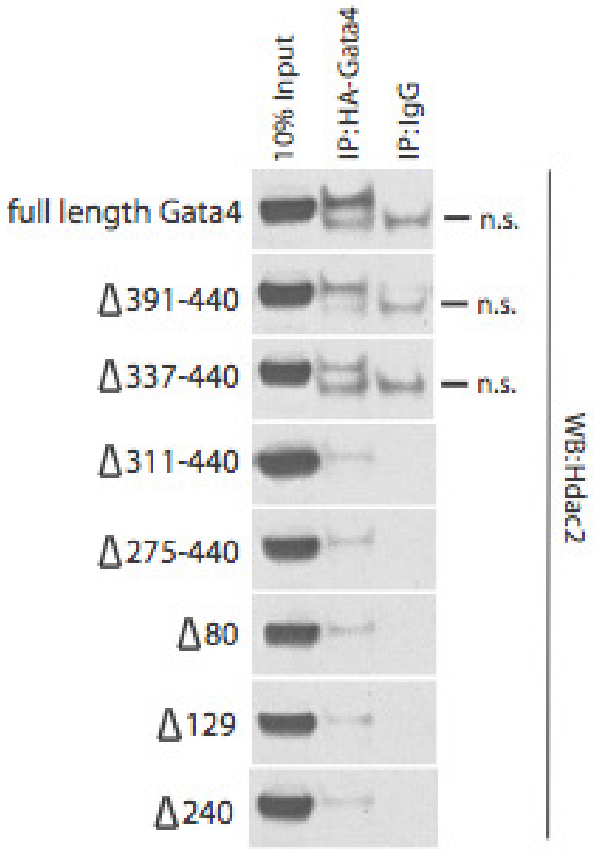


Adapted and modified from (Trivedi et al.).

Figure 3.2: Generation of 12 Gata4 deletion constructs.

(A) Schematic representation of Gata4 and deletion constructs. (B) Western blot was performed to examine the expression of the 12 deletion constructs. 7 constructs were expressed.

Figure 3.3: Hdac2 is able to interact with all seven deletion constructs.



Adapted and modified from (Trivedi et al.).

Figure 3.3: Hdac2 is able to interact with all seven deletion constructs. Total lysates from Gata4 or various Gata4 deletion constructs transfected 293 cells were immunoprecipitated with anti-HA antibody and Western blot analysis was performed with anti-Hdac2 antibody to detect endogenous Hdac2. n.s. - non-specific.

Figure 3.4: Generation of a Gata4 internal deletion construct Δ241-274.

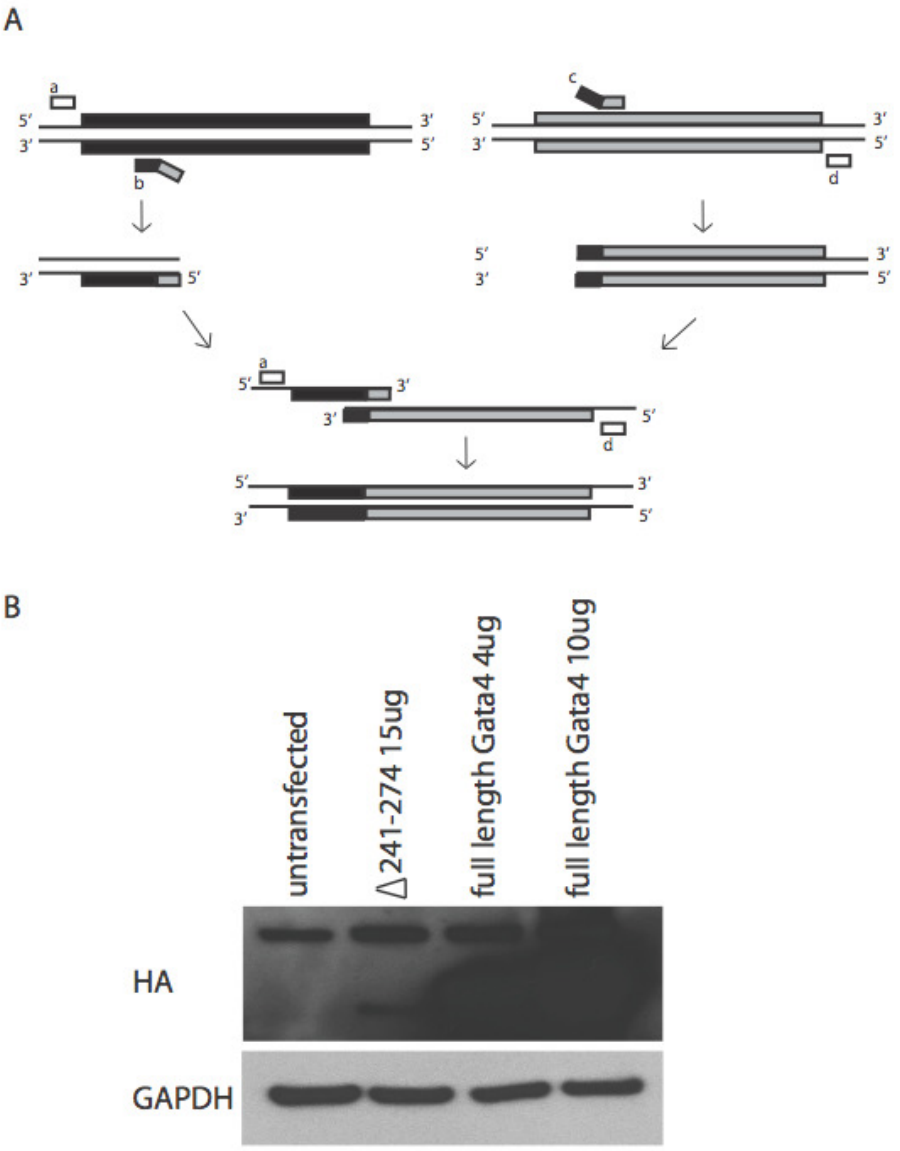
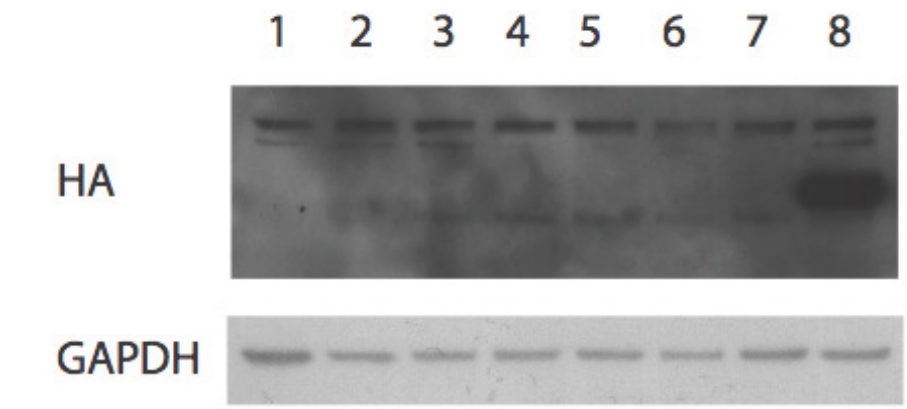


Figure 3.4: Generation of a Gata4 internal deletion construct Δ241-274. (A) The schematic representation of splicing overlap extension PCR. The details are discussed in the materials and methods section. cDNAs are represented lined as bars. Modified from (Warrens et al., 1997). (B) The internal deletion construct is expressed though at a much lower level as detected by Western blot. The H9c2 cells were transfected with different amounts of DNA as indicated in the figure.

Figure 3.5: Optimization of Δ241-274 construct transfection conditions.



	Lanes	DNA transfected/ug	DNA/Superfect	hours after transfection
Δ241-274	1	0	N/A	24
	2	10	1:2	24
	3	10	1:6	24
	4	10	1:10	24
	5	15	1:6	24
	6	20	1:6	24
	7	10	1:6	48
Gata4 full length	8	4	1:6	48

Figure 3.5: Optimization of Δ241-274 construct transfection conditions. DNA amounts, DNA/Superfect ratios and incubation times were adjusted to increase the construct expression.

Figure 3.6: The internal deletion construct Δ241-274 was unable to interact with Hdac2.

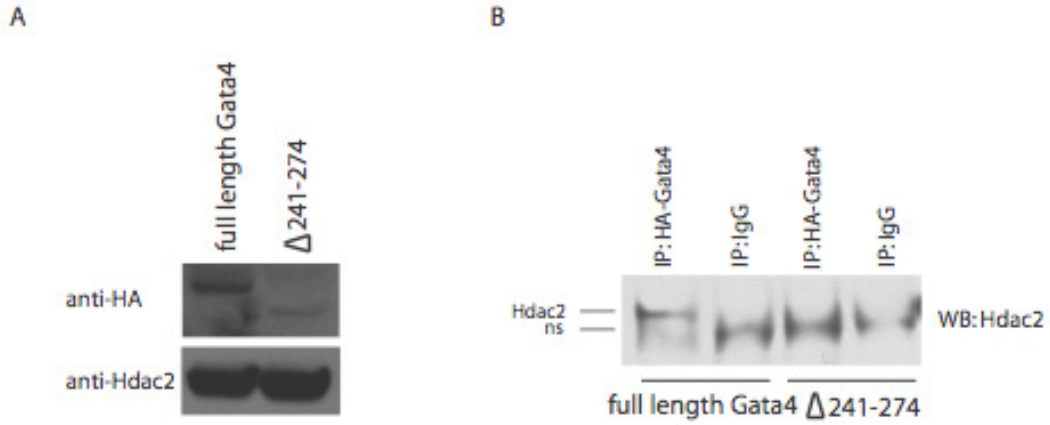


Figure 3.6: The internal deletion construct Δ241-274 was unable to interact with Hdac2. (A) Gata4 full length construct transfected cell lysate was diluted 50 fold with untransfected lysate. 50 µg total protein was loaded to compare the level of Gata4 and Hdac2. (B) Total lysates from Gata4 or the internal deletion construct Δ241-274 transfected 293 cells were immunoprecipated with anti-HA antibody and Western blot analysis was performed with anti-Hdac2 antibody to detect endogenous Hdac2. ns-non specific.

Figure 3.7: Blockage of protein synthesis leads to Gata4 degradation.

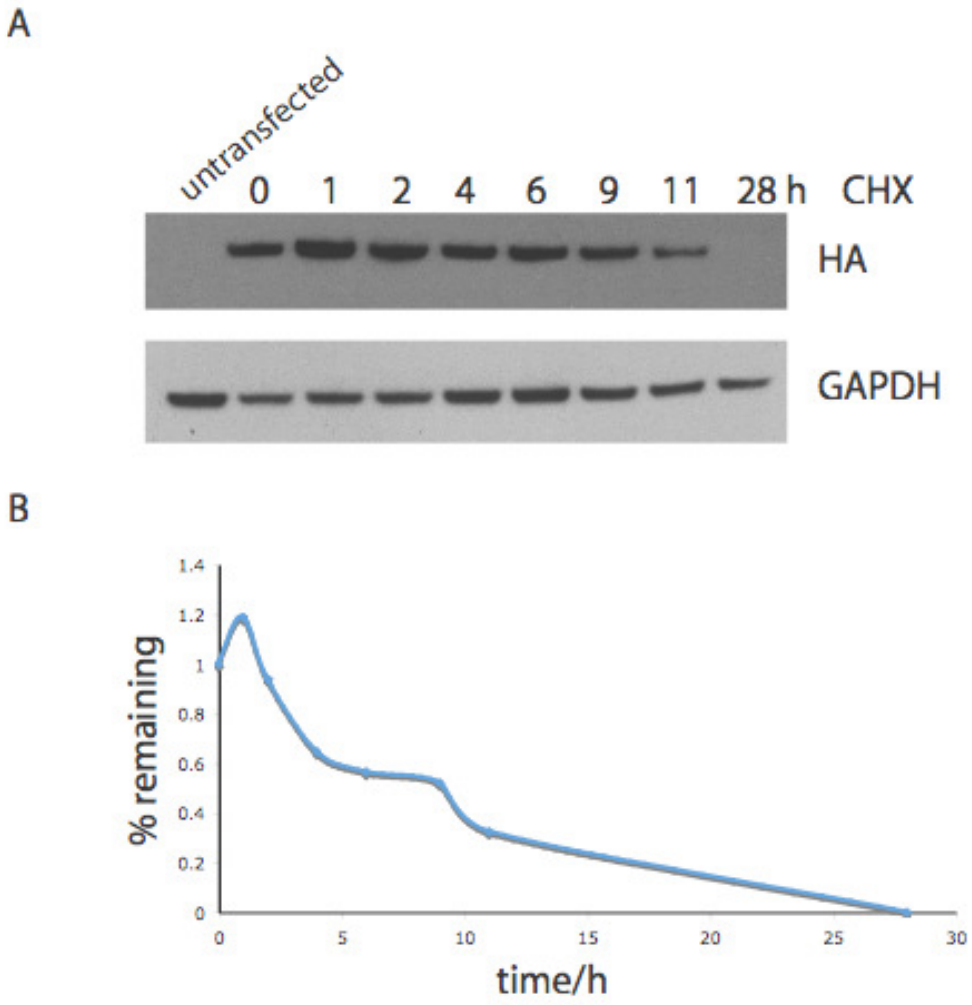


Figure 3.7: Blockage of protein synthesis leads to Gata4 degradation.

(A) H9c2 cells were transfected with HA-Gata4. 48 hrs after transfection, the cells were treated with 10 μg/ml CHX for indicated time periods. The cells were lysed and the protein lysates were subjected to SDS-PAGE. Western blot was performed using anti-HA antibody. (B) Quantification of the remaining protein amount.

Figure 3.8: Co-transfection with Hdac2 stabilizes Gata4.

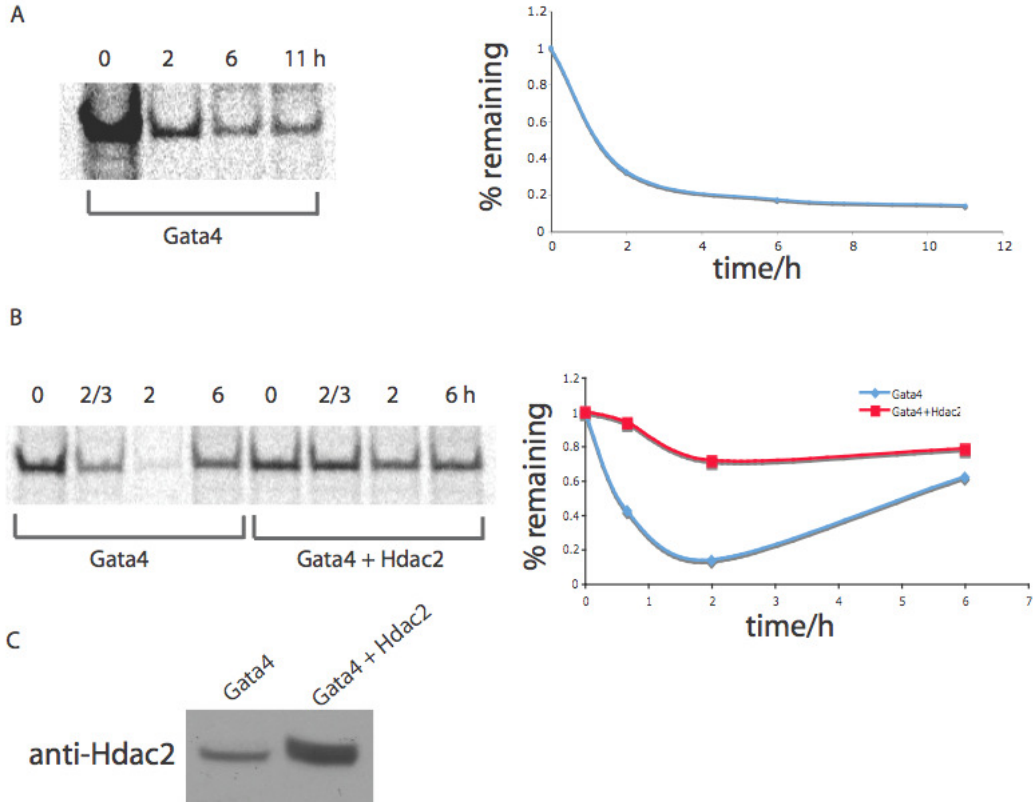


Figure 3.8: Co-transfection with Hdac2 stabilizes Gata4. (A) H9c2 cells were transfected with HA-Gata4. 48 hrs after transfection, the cells were pulse labeled with ^{35}S and then collected at indicated time points. The protein lysates were immunoprecipitated with anti-HA antibody and proteins were subjected to SDS-PAGE. Labeled proteins were visualized with phosphor imager. The quantification is shown on the right. (B) H9c2 cells were transfected with either HA-Gata4 alone or HA-Gata4 and Hdac2 both. Pulse chase experiment was performed similarly. (C) Western blot confirms the over-expression of Hdac2 in the Hdac2 and Gata4 co-transfected cells.

Chapter 4 Conclusions and Future Directions

4.1 Summary

In this dissertation I have investigated the potential mechanisms of Hdac2 in the regulation of adult cardiac hypertrophy and embryonic myocyte proliferation. In chapter 2, I described the function of a poorly described PIP3 5' phosphatase Inpp5f in cardiac hypertrophic responsiveness. Our prior work (Trivedi et al., 2007) had implicated this gene in cardiac hypertrophy because it is regulated by Hdac2, and Hdac2 deficiency protects against hypertrophy by modulating the Akt pathway. My work directly tests the functions of Inpp5f via both gain and loss of function studies. I describe the knockout mouse, and transgenic mice over-expressing Inpp5f in the heart. In both cases, the mice are normal under basal conditions, but the response to hypertrophic stimuli are abnormal in terms of cardiac size, myocyte size and gene expression. This work has important clinical implications. The lack of basal defects suggests that this phosphatase may be a tractable target for therapeutic intervention for heart failure. In chapter 3, I studied the potential mechanisms of how Hdac2 might regulate myocyte proliferation during embryonic development. My preliminary results suggest that Gata4 may be responsible for the hyper-proliferation defects in Hdac2 null mice. Hdac2 is able to interact with Gata4. However, since Gata4 also functions in homeostasis of the adult myocardium and cardiac hypertrophy is generally associated with enhanced Gata4 activity, the model presented in chapter 3 cannot easily explain its role during adult cardiac hypertrophy. Therefore, in chapter 3,

I also sought to study the effects of acetylation on Gata4 stability. My preliminary result suggests that deacetylation stabilizes Gata4. In the absence of Hdac2, Gata4 is hyper-acetylated leading to more proliferation during embryonic development. However, hyper-acetylation also marks Gata4 for degradation, which explains the significant down-regulation of Gata4 levels after birth. Many critical questions still remain for Inpp5f, Gata4 and Hdac2 and I discuss below several aspects that I feel worth further investigation.

4.2 Future directions

4.2.1 Inpp5f, a tumor suppressor?

My results suggest that Inpp5f negatively regulates the PI3K/Akt pathway *in vivo*. The PI3K/Akt pathway activation initiates a signal transduction cascade that promotes cell growth, survival and metabolism. The abnormal activation of this pathway has been validated by epidemiological and experimental studies as an essential step toward the initiation and maintenance of human tumors (Garcia-Echeverria and Sellers, 2008). PTEN, a negative regulator of this pathway, acts as a tumor suppressor. It is mutated or lost in both heritable and spontaneous cancers. Germline mutations in PTEN cause autosomal dominant hamartoma tumor syndromes, whereas sporadic missense mutations occur frequently in central nervous system (20%), endometrial (39%), colorectal (9%), skin (17%), prostate (14%) and breast (6%) cancers. Monoallelic loss of PTEN contributes to tumor growth in the context of

other somatic mutations, and PTEN protein levels correlate with disease severity (Yuan and Cantley, 2008). When I first got the Inpp5f null mice, I expected that they would develop some kind of tumors as they age. However, I did not observe any sign of cancer in these mice. PTEN is considered the principal regulator of basal cellular PtdIns(3,4,5)P3 levels (Leslie and Downes, 2002). Other PIP3 phosphotases only allow for greater pathway activity upon Akt stimulation. For example, a single somatic missense mutation in the phosphatase domain of SHIP1 has been identified in patients with acute myeloid leukemia which reduced the catalytic activity of SHIP1. The basal level of Akt phosphorylation was comparable between leukemia cells with and without the mutation. However, leukemia cells with this mutation showed enhanced Akt phosphorylation following stimulation (Luo et al., 2003). Besides, Ship2 has been reported to be a negative regulator of Akt activation. Although loss of Ship2 is not sufficient to activate Akt, the absence of Ship2 allows for greater activity upon Akt stimulation (Rommel et al., 2001; Wada et al., 2001). Therefore, it is very possible that unlike PTEN, which as a stringent regulator attenuates PtdIns(3,4,5)P3 signaling, the 5-phosphatases could function as more subtle modulators of the PtdIns(3,4,5)P3 signals. It would be interesting to see if Inpp5f increases tumor development in a Pten null or heterozygous background. Other 5' phosphatases may be able to compensate for Inpp5f under the relatively sedentary conditions under non-stressed situations. It would also be informative to test if loss of Inpp5f in combination with loss of Ship1 and/or Ship2 would result in cancer.

4.2.2 Inpp5f, a poorly characterized 5' phosphatase

In chapter 2, I demonstrated that Inpp5f was able to degrade PIP3 in the heart. I also identified seven highly conserved motifs and the sixth conserved region of Inpp5f in both mouse and human has the highly conserved CX5R(T/S) motif. It would be interesting to see if mutations in the cysteine, arginine and threonine/serine residues could disrupt its enzymatic activity. We could either transfect the mutated Inpp5f and measure Akt level or measure PIP3 levels directly by ELISA.

4.2.3 To what extent does Hdac2 regulate hypertrophy through Inpp5f?

In chapter 2, I elucidated the role of Inpp5f in regulating cardiac hypertrophy by utilizing a gene-trap Inpp5f global knockout mouse model and a cardiac specific over-expression mouse model. My data suggests that Inpp5f transgenic mice were unable to reactivate fetal genes or to exhibit normal hypertrophic responses while Inpp5f knockout mice have augmented hypertrophy associated with reactivation of the fetal gene program. Biochemical analysis indicated that these responses were mediated by the PI3K/Akt pathway. Furthermore, we have also shown that Hdac2 promotes cardiac hypertrophy (Trivedi et al., 2007) and that Inpp5f is up-regulated in Hdac2 knockout hearts, both during late gestation and in the adult while it is down-regulated in Hdac2 transgenic mice that over-express Hdac2 in the heart. Hdac2 can deacetylate histones at the Inpp5f upstream genomic region, consistent with direct regulation

(Trivedi et al., 2007). However, it remains unclear whether Hdac2 regulates hypertrophy partially or entirely via Inpp5f. I performed some preliminary crosses with Hdac2+/- and Inpp5f -/- to generate Hdac2-/-Inpp5f-/-. Future experiments can be done to see if these double knockout mice respond differently to hypertrophic stimuli from the Hdac2 nulls. I predict that these double knockout mice will be relatively sensitive to hypertrophy and the PI3K/Akt pathway will be activated compared with the Hdac2-/-. Similarly, Inpp5f transgenic mice can be crossed to Hdac2 transgenic mice to determine if the double transgenic mice are less susceptible to hypertrophic stimuli than the Hdac2 transgenic mice.

4.2.4 Does Inpp5f function upstream of Akt?

My results suggest that loss of Inpp5f leads to hyper-activation of the PI3K/Akt pathway upon stimulation while over-expression of Inpp5f results in down-regulation of the pathway activity in the heart. I performed Akt+/- and Inpp5f -/- crosses to address the question if Inpp5f really functions upstream of Akt in vivo. Future experiments can be done to analyze the double knockout phenotypes. I expect that the double knockout would be relatively resistant to hypertrophy compared with Inpp5f single nulls.

4.2.5 Does Gata4 need Friend of GATA (Fog) to recruit Hdac2?

Most of the protein-protein interactions of GATA factors are mediated by its C-terminal zinc finger, including the interaction with p300, Nkx2.5, NFAT, while the N-terminal zinc finger interacts with Fog transcription factors (Pikkarainen et al., 2004). My study identifies the lysine rich domain located between the two zinc fingers as the region necessary for interaction with Hdac2. It is unknown whether this interaction is direct or requires other co-factors. Gata factors can recruit Hdacs to target genes, either by directly interacting or by associating with Fog (Friend of Gata) proteins that can recruit the NuRD complex containing Hdac1 and Hdac2 (Cantor and Orkin, 2005; Hong et al., 2005; Svensson et al., 1999; Tsang et al., 1997). The lysine rich domain we identified is very close to the region where Fog interacts with Gata. Therefore, it would be interesting to investigate if Gata4 requires Fog to interact with Hdac2. Several mutation screens have been done to identify point mutations in Gata-1 that disrupt binding to FOG-1 (Crispino et al., 1999; Crispino and Orkin, 2002) and these residues are highly conserved among all vertebrate GATA factors (Cantor and Orkin, 2005). Experiments can be performed using in vitro translated Gata4 and Hdac2 proteins to test if the interaction is direct or not. Co-IP can also be done in a Fog expressing cell line using mutated Gata4 that cannot recruit Fog and to see if the mutant Gata4 can interact with Hdac2 or not.

4.2.6 Can Hdacs deacetylate other Gata members?

Other Gata family members have also been shown to recruit Hdacs to mediate transcriptional repression. For example, Hdac3 associates with and represses Gata2 in hematopoietic progenitor cells (Ozawa et al., 2001). Gata1 interaction with Hdac5 results in reduced Gata1 transcriptional activity (Watamoto et al., 2003). In each case, Hdac function suppresses Gata dependent transcriptional activity. Although it was generally assumed that Hdac-mediated transcriptional repression of Gata activity was mediated by histone deacetylation and chromatin remodeling, it will be interesting to re-address this question in light of my results to determine if other Gata proteins are regulated by Hdacs via direct deacetylation.

4.2.7 To what extent does Hdac2 regulates cardiomyocyte proliferation through Gata4 acetylation?

In chapter 3, I showed that Hdac2 interacted with Gata4. Our lab also demonstrates that Hdac2 is able to deacetylate Gata4 and in Hdac2 null hearts Gata4 acetylation is increased. Acetylated Gata4 has enhanced DNA binding and transcriptional ability (Takaya et al., 2008). Several studies have demonstrated a critical role for Gata4 in embryonic myocyte proliferation (Rojas et al., 2008; Zeisberg et al., 2005). All this evidence suggests that Hdac2 inhibits cardiomyocyte proliferation through deacetylating Gata4. However, we did not perform the definitive

experiments to test this concept. Future work could address this, by crossing Hdac2 nulls with cardiac specific Gata4 nulls. We could expect the myocytes from double nulls have less proliferation rates than Hdac2 nulls. A cardiac specific Gata4 acetylation deficient (by mutating lysine to alanine) knockin mouse model could also be generated. These mice can then be crossed with Hdac2 knockouts. Since the mutated Gata4 could not be acetylated, loss of Hdac2 would not lead to more myocyte proliferation.

4.3 Concluding remarks

The studies presented here define the mechanisms of Hdac2 in regulating embryonic heart development and adult heart hypertrophy. I identified two targets of Hdac2, Inpp5f and Gata4, and I investigated how they contribute to the phenotypes of Hdac2 nulls. Hdac2 regulates Inpp5f through modifying the histones of its promoter while it regulates Gata4 through acetylating the Gata4 protein itself. The characterization of Hdacs and HATs and their various activities has attracted increasing investigative interest because of their importance in stem and progenitor cell biology and in cellular reprogramming. It would be exciting to see how these findings will contribute to the study of embryology to human medicine.

References

Antos, C.L., T.A. McKinsey, M. Dreitz, L.M. Hollingsworth, C.L. Zhang, K. Schreiber, H. Rindt, R.J. Gorczynski, and E.N. Olson. 2003. Dose-dependent blockade to cardiomyocyte hypertrophy by histone deacetylase inhibitors. *J Biol Chem*. 278:28930-7.

Antos, C.L., T.A. McKinsey, N. Frey, W. Kutschke, J. McAnally, J.M. Shelton, J.A. Richardson, J.A. Hill, and E.N. Olson. 2002. Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. *Proc Natl Acad Sci U S A*. 99:907-12.

Arceci, R.J., A.A. King, M.C. Simon, S.H. Orkin, and D.B. Wilson. 1993. Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol Cell Biol*. 13:2235-46.

Astle, M.V., K.A. Horan, L.M. Ooms, and C.A. Mitchell. 2007. The inositol polyphosphate 5-phosphatases: traffic controllers, waistline watchers and tumour suppressors? *Biochem Soc Symp*:161-81.

Astle, M.V., G. Seaton, E.M. Davies, C.G. Fedele, P. Rahman, L. Arsala, and C.A. Mitchell. 2006. Regulation of phosphoinositide signaling by the inositol polyphosphate 5-phosphatases. *IUBMB Life*. 58:451-6.

Backers, K., D. Blero, N. Paternotte, J. Zhang, and C. Erneux. 2003. The termination of PI3K signalling by SHIP1 and SHIP2 inositol 5-phosphatases. *Adv Enzyme Regul*. 43:15-28.

Badorff, C., F.H. Seeger, A.M. Zeiher, and S. Dimmeler. 2005. Glycogen synthase kinase 3beta inhibits myocardin-dependent transcription and hypertrophy induction through site-specific phosphorylation. *Circ Res*. 97:645-54.

Bi, L., I. Okabe, D.J. Bernard, A. Wynshaw-Boris, and R.L. Nussbaum. 1999. Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase. *J Biol Chem*. 274:10963-8.

Blero, D., B. Payrastre, S. Schurmans, and C. Erneux. 2007. Phosphoinositide phosphatases in a network of signalling reactions. *Pflugers Arch*. 455:31-44.

Boyes, J., P. Byfield, Y. Nakatani, and V. Ogryzko. 1998. Regulation of activity of the transcription factor GATA-1 by acetylation. *Nature*. 396:594-8.

Brown, G.E., M.Q. Stewart, H. Liu, V.L. Ha, and M.B. Yaffe. 2003. A novel assay system implicates PtdIns(3,4)P(2), PtdIns(3)P, and PKC delta in intracellular production of reactive oxygen species by the NADPH oxidase. *Mol Cell*. 11:35-47.

Cantley, L.C. 2002. The phosphoinositide 3-kinase pathway. *Science*. 296:1655-7.

Cantor, A.B., and S.H. Orkin. 2005. Coregulation of GATA factors by the Friend of GATA (FOG) family of multitype zinc finger proteins. *Semin Cell Dev Biol*. 16:117-28.

Chan, T.O., S.E. Rittenhouse, and P.N. Tsichlis. 1999. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem*. 68:965-1014.

Chang, S., T.A. McKinsey, C.L. Zhang, J.A. Richardson, J.A. Hill, and E.N. Olson. 2004. Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. *Mol Cell Biol*. 24:8467-76.

Chang, S., B.D. Young, S. Li, X. Qi, J.A. Richardson, and E.N. Olson. 2006. Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10. *Cell*. 126:321-34.

Charron, F., and M. Nemer. 1999. GATA transcription factors and cardiac development. *Semin Cell Dev Biol*. 10:85-91.

Charron, F., P. Paradis, O. Bronchain, G. Nemer, and M. Nemer. 1999. Cooperative interaction between GATA-4 and GATA-6 regulates myocardial gene expression. *Mol Cell Biol*. 19:4355-65.

Chen, W.S., P.Z. Xu, K. Gottlob, M.L. Chen, K. Sokol, T. Shiyanova, I. Roninson, W. Weng, R. Suzuki, K. Tobe, T. Kadowaki, and N. Hay. 2001. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev*. 15:2203-8.

Chenna, R., H. Sugawara, T. Koike, R. Lopez, T.J. Gibson, D.G. Higgins, and J.D. Thompson. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res*. 31:3497-500.

Chesley, A., M.S. Lundberg, T. Asai, R.P. Xiao, S. Ohtani, E.G. Lakatta, and M.T. Crow. 2000. The beta(2)-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G(i)-dependent coupling to phosphatidylinositol

3'-kinase. *Circ Res*. 87:1172-9.

Cho, H., J. Mu, J.K. Kim, J.L. Thorvaldsen, Q. Chu, E.B. Crenshaw, 3rd, K.H. Kaestner, M.S. Bartolomei, G.I. Shulman, and M.J. Birnbaum. 2001a. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science*. 292:1728-31.

Cho, H., J.L. Thorvaldsen, Q. Chu, F. Feng, and M.J. Birnbaum. 2001b. Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem*. 276:38349-52.

Chuang, Y.Y., N.L. Tran, N. Rusk, M. Nakada, M.E. Berens, and M. Symons. 2004. Role of synaptojanin 2 in glioma cell migration and invasion. *Cancer Res*. 64:8271-5.

Condorelli, G., A. Drusco, G. Stassi, A. Bellacosa, R. Roncarati, G. Iaccarino, M.A. Russo, Y. Gu, N. Dalton, C. Chung, M.V. Latronico, C. Napoli, J. Sadoshima, C.M. Croce, and J. Ross, Jr. 2002. Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice. *Proc Natl Acad Sci U S A*. 99:12333-8.

Cook, S.A., T. Matsui, L. Li, and A. Rosenzweig. 2002. Transcriptional effects of chronic Akt activation in the heart. *J Biol Chem*. 277:22528-33.

Crackower, M.A., G.Y. Oudit, I. Kozieradzki, R. Sarao, H. Sun, T. Sasaki, E. Hirsch, A. Suzuki, T. Shioi, J. Irie-Sasaki, R. Sah, H.Y. Cheng, V.O. Rybin, G. Lembo, L. Fratta, A.J. Oliveira-dos-Santos, J.L. Benovic, C.R. Kahn, S. Izumo, S.F. Steinberg, M.P. Wymann, P.H. Backx, and J.M. Penninger. 2002.

Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways. *Cell*. 110:737-49.

Cremona, O., G. Di Paolo, M.R. Wenk, A. Luthi, W.T. Kim, K. Takei, L. Daniell, Y. Nemoto, S.B. Shears, R.A. Flavell, D.A. McCormick, and P. De Camilli. 1999. Essential role of phosphoinositide metabolism in synaptic vesicle recycling. *Cell*. 99:179-88.

Crispino, J.D., M.B. Lodish, J.P. MacKay, and S.H. Orkin. 1999. Use of altered specificity mutants to probe a specific protein-protein interaction in differentiation: the GATA-1:FOG complex. *Mol Cell*. 3:219-28.

Crispino, J.D., and S.H. Orkin. 2002. The use of altered specificity mutants to probe specific protein-protein interactions involved in the activation of GATA-1 target genes. *Methods*. 26:84-92.

Dai, Y.S., P. Cserjesi, B.E. Markham, and J.D. Molkentin. 2002. The transcription factors GATA4 and dHAND physically interact to synergistically activate cardiac gene expression through a p300-dependent mechanism. *J Biol Chem*. 277:24390-8.

Dai, Y.S., and B.E. Markham. 2001. p300 Functions as a coactivator of transcription factor GATA-4. *J Biol Chem*. 276:37178-85.

DeBosch, B., N. Sambandam, C. Weinheimer, M. Courtois, and A.J. Muslin. 2006a. Akt2 regulates cardiac metabolism and cardiomyocyte survival. *J Biol Chem*. 281:32841-51.

DeBosch, B., I. Treskov, T.S. Lupu, C. Weinheimer, A. Kovacs, M. Courtois, and A.J.

Muslin. 2006b. Akt1 is required for physiological cardiac growth. *Circulation*. 113:2097-104.

Di Cristofano, A., P. Kotsi, Y.F. Peng, C. Cordon-Cardo, K.B. Elkon, and P.P. Pandolfi. 1999. Impaired Fas response and autoimmunity in Pten+/- mice. *Science*. 285:2122-5.

Di Cristofano, A., B. Pesce, C. Cordon-Cardo, and P.P. Pandolfi. 1998. Pten is essential for embryonic development and tumour suppression. *Nat Genet*. 19:348-55.

Doble, B.W., and J.R. Woodgett. 2003. GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci*. 116:1175-86.

Dodou, E., M.P. Verzi, J.P. Anderson, S.M. Xu, and B.L. Black. 2004. Mef2c is a direct transcriptional target of ISL1 and GATA factors in the anterior heart field during mouse embryonic development. *Development*. 131:3931-42.

Dorn, G.W., 2nd, and T. Force. 2005. Protein kinase cascades in the regulation of cardiac hypertrophy. *J Clin Invest*. 115:527-37.

Dowler, S., R.A. Currie, D.G. Campbell, M. Deak, G. Kular, C.P. Downes, and D.R. Alessi. 2000. Identification of pleckstrin-homology-domain-containing proteins with novel phosphoinositide-binding specificities. *Biochem J*. 351:19-31.

Durocher, D., F. Charron, R. Warren, R.J. Schwartz, and M. Nemer. 1997. The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *Embo J*. 16:5687-96.

Dyson, J.M., A.M. Kong, F. Wiradjaja, M.V. Astle, R. Gurung, and C.A. Mitchell. 2005. The SH2 domain containing inositol polyphosphate 5-phosphatase-2: SHIP2. *Int J Biochem Cell Biol*. 37:2260-5.

Easton, R.M., H. Cho, K. Roovers, D.W. Shineman, M. Mizrahi, M.S. Forman, V.M. Lee, M. Szabolcs, R. de Jong, T. Oltersdorf, T. Ludwig, A. Efstratiadis, and M.J. Birnbaum. 2005. Role for Akt3/protein kinase Bgamma in attainment of normal brain size. *Mol Cell Biol*. 25:1869-78.

Ekwall, K. 2005. Genome-wide analysis of HDAC function. *Trends Genet*. 21:608-15.

Frey, N., and E.N. Olson. 2003. Cardiac hypertrophy: the good, the bad, and the ugly. *Annu Rev Physiol*. 65:45-79.

Gallinari, P., S. Di Marco, P. Jones, M. Pallaoro, and C. Steinkuhler. 2007. HDACs, histone deacetylation and gene transcription: from molecular biology to cancer therapeutics. *Cell Res*. 17:195-211.

Garcia-Echeverria, C., and W.R. Sellers. 2008. Drug discovery approaches targeting the PI3K/Akt pathway in cancer. *Oncogene*. 27:5511-26.

Garg, V., I.S. Kathiriya, R. Barnes, M.K. Schluterman, I.N. King, C.A. Butler, C.R. Rothrock, R.S. Eapen, K. Hirayama-Yamada, K. Joo, R. Matsuoka, J.C. Cohen, and D. Srivastava. 2003. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature*. 424:443-7.

Garofalo, R.S., S.J. Orena, K. Rafidi, A.J. Torchia, J.L. Stock, A.L. Hildebrandt, T. Coskran, S.C. Black, D.J. Brees, J.R. Wicks, J.D. McNeish, and K.G. Coleman. 2003. Severe diabetes, age-dependent loss of adipose tissue, and

mild growth deficiency in mice lacking Akt2/PKB beta. *J Clin Invest*. 112:197-208.

George, S., J.J. Rochford, C. Wolfrum, S.L. Gray, S. Schinner, J.C. Wilson, M.A. Soos, P.R. Murgatroyd, R.M. Williams, C.L. Acerini, D.B. Dunger, D. Barford, A.M. Umpleby, N.J. Wareham, H.A. Davies, A.J. Schafer, M. Stoffel, S. O'Rahilly, and I. Barroso. 2004. A family with severe insulin resistance and diabetes due to a mutation in AKT2. *Science*. 304:1325-8.

Grempler, R., D. Zibrova, C. Schoelch, A. van Marle, J.F. Rippmann, and N. Redemann. 2007. Normalization of prandial blood glucose and improvement of glucose tolerance by liver-specific inhibition of SH2 domain containing inositol phosphatase 2 (SHIP2) in diabetic KKAy mice: SHIP2 inhibition causes insulin-mimetic effects on glycogen metabolism, gluconeogenesis, and glycolysis. *Diabetes*. 56:2235-41.

Gronroos, E., U. Hellman, C.H. Heldin, and J. Ericsson. 2002. Control of Smad7 stability by competition between acetylation and ubiquitination. *Mol Cell*. 10:483-93.

Grunstein, M. 1997. Histone acetylation in chromatin structure and transcription. *Nature*. 389:349-52.

Gu, W., and R.G. Roeder. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell*. 90:595-606.

Guo, S., L.E. Stolz, S.M. Lemrow, and J.D. York. 1999. SAC1-like domains of yeast SAC1, INP52, and INP53 and of human synaptojanin encode

polyphosphoinositide phosphatases. *J Biol Chem*. 274:12990-5.

Gupta, S., B. Das, and S. Sen. 2007. Cardiac hypertrophy: mechanisms and therapeutic opportunities. *Antioxid Redox Signal*. 9:623-52.

Haas-Kogan, D., N. Shalev, M. Wong, G. Mills, G. Yount, and D. Stokoe. 1998. Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. *Curr Biol*. 8:1195-8.

Haberland, M., R.L. Montgomery, and E.N. Olson. 2009. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat Rev Genet*. 10:32-42.

Halstead, J.R., K. Jalink, and N. Divecha. 2005. An emerging role for PtdIns(4,5)P2-mediated signalling in human disease. *Trends Pharmacol Sci*. 26:654-60.

Haq, S., G. Choukroun, Z.B. Kang, H. Ranu, T. Matsui, A. Rosenzweig, J.D. Molkentin, A. Alessandrini, J. Woodgett, R. Hajjar, A. Michael, and T. Force. 2000. Glycogen synthase kinase-3beta is a negative regulator of cardiomyocyte hypertrophy. *J Cell Biol*. 151:117-30.

Hardt, S.E., H. Tomita, H.A. Katus, and J. Sadoshima. 2004. Phosphorylation of eukaryotic translation initiation factor 2Bepsilon by glycogen synthase kinase-3beta regulates beta-adrenergic cardiac myocyte hypertrophy. *Circ Res*. 94:926-35.

Harris, S.J., R.V. Parry, J. Westwick, and S.G. Ward. 2008. Phosphoinositide lipid phosphatases: natural regulators of phosphoinositide 3-kinase signaling in T lymphocytes. *J Biol Chem*. 283:2465-9.

Harris, T.E., and J.C. Lawrence, Jr. 2003. TOR signaling. *Sci STKE*. 2003:re15.

Hayakawa, F., M. Towatari, Y. Ozawa, A. Tomita, M.L. Privalsky, and H. Saito. 2004. Functional regulation of GATA-2 by acetylation. *J Leukoc Biol*. 75:529-40.

Heine, H.L., H.S. Leong, F.M. Rossi, B.M. McManus, and T.J. Podor. 2005. Strategies of conditional gene expression in myocardium: an overview. *Methods Mol Med*. 112:109-54.

Heineke, J., and J.D. Molkentin. 2006. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol*. 7:589-600.

Helgason, C.D., J.E. Damen, P. Rosten, R. Grewal, P. Sorensen, S.M. Chappel, A. Borowski, F. Jirik, G. Krystal, and R.K. Humphries. 1998. Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span. *Genes Dev*. 12:1610-20.

Hernandez-Hernandez, A., P. Ray, G. Litos, M. Ciro, S. Ottolenghi, H. Beug, and J. Boyes. 2006. Acetylation and MAPK phosphorylation cooperate to regulate the degradation of active GATA-1. *Embo J*. 25:3264-74.

Hirai, M., K. Ono, T. Morimoto, T. Kawamura, H. Wada, T. Kita, and K. Hasegawa. 2004. FOG-2 competes with GATA-4 for transcriptional coactivator p300 and represses hypertrophic responses in cardiac myocytes. *J Biol Chem*. 279:37640-50.

Hirotani, S., P. Zhai, H. Tomita, J. Galeotti, J.P. Marquez, S. Gao, C. Hong, A. Yatani, J. Avila, and J. Sadoshima. 2007. Inhibition of glycogen synthase kinase 3beta

during heart failure is protective. *Circ Res*. 101:1164-74.

Hobbs, R.E. 2004. Guidelines for the diagnosis and management of heart failure. *Am J Ther*. 11:467-72.

Hoeflich, K.P., J. Luo, E.A. Rubie, M.S. Tsao, O. Jin, and J.R. Woodgett. 2000. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature*. 406:86-90.

Hong, W., M. Nakazawa, Y.Y. Chen, R. Kori, C.R. Vakoc, C. Rakowski, and G.A. Blobel. 2005. FOG-1 recruits the NuRD repressor complex to mediate transcriptional repression by GATA-1. *Embo J*. 24:2367-78.

Hughes, W.E., F.T. Cooke, and P.J. Parker. 2000. Sac phosphatase domain proteins. *Biochem J*. 350 Pt 2:337-52.

Hunter, J.J., and K.R. Chien. 1999. Signaling pathways for cardiac hypertrophy and failure. *N Engl J Med*. 341:1276-83.

Inoki, K., M.N. Corradetti, and K.L. Guan. 2005. Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet*. 37:19-24.

Insall, R.H., and O.D. Weiner. 2001. PIP3, PIP2, and cell movement--similar messages, different meanings? *Dev Cell*. 1:743-7.

Ito, K., H. Akazawa, M. Tamagawa, K. Furukawa, W. Ogawa, N. Yasuda, Y. Kudo, C.H. Liao, R. Yamamoto, T. Sato, J.D. Molkentin, M. Kasuga, T. Noda, H. Nakaya, and I. Komuro. 2009. PDK1 coordinates survival pathways and beta-adrenergic response in the heart. *Proc Natl Acad Sci U S A*. 106:8689-94.

Johnson, C.A., and B.M. Turner. 1999. Histone deacetylases: complex transducers of

nuclear signals. *Semin Cell Dev Biol*. 10:179-88.

Kagawa, S., T. Sasaoka, S. Yaguchi, H. Ishihara, H. Tsuneki, S. Murakami, K. Fukui, T. Wada, S. Kobayashi, I. Kimura, and M. Kobayashi. 2005. Impact of SRC homology 2-containing inositol 5'-phosphatase 2 gene polymorphisms detected in a Japanese population on insulin signaling. *J Clin Endocrinol Metab*. 90:2911-9.

Kaisaki, P.J., M. Delepine, P.Y. Woon, L. Sebag-Montefiore, S.P. Wilder, S. Menzel, N. Vionnet, E. Marion, J.P. Riveline, G. Charpentier, S. Schurmans, J.C. Levy, M. Lathrop, M. Farrall, and D. Gauguier. 2004. Polymorphisms in type II SH2 domain-containing inositol 5-phosphatase (INPPL1, SHIP2) are associated with physiological abnormalities of the metabolic syndrome. *Diabetes*. 53:1900-4.

Kannel, W.B. 2000. Vital epidemiologic clues in heart failure. *J Clin Epidemiol*. 53:229-35.

Kashiwada, M., P. Lu, and P.B. Rothman. 2007. PIP3 pathway in regulatory T cells and autoimmunity. *Immunol Res*. 39:194-224.

Kawamura, T., K. Ono, T. Morimoto, H. Wada, M. Hirai, K. Hidaka, T. Morisaki, T. Heike, T. Nakahata, T. Kita, and K. Hasegawa. 2005. Acetylation of GATA-4 is involved in the differentiation of embryonic stem cells into cardiac myocytes. *J Biol Chem*. 280:19682-8.

Kearns, B.G., T.P. McGee, P. Mayinger, A. Gedvilaite, S.E. Phillips, S. Kagiwada, and V.A. Bankaitis. 1997. Essential role for diacylglycerol in protein transport

from the yeast Golgi complex. *Nature*. 387:101-5.

Kelley, C., H. Blumberg, L.I. Zon, and T. Evans. 1993. GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. *Development*. 118:817-27.

Kim, Y.K., S.J. Kim, A. Yatani, Y. Huang, G. Castelli, D.E. Vatner, J. Liu, Q. Zhang, G. Diaz, R. Zieba, J. Thaisz, A. Drusco, C. Croce, J. Sadoshima, G. Condorelli, and S.F. Vatner. 2003. Mechanism of enhanced cardiac function in mice with hypertrophy induced by overexpressed Akt. *J Biol Chem*. 278:47622-8.

Kitta, K., R.M. Day, Y. Kim, I. Torregroza, T. Evans, and Y.J. Suzuki. 2003. Hepatocyte growth factor induces GATA-4 phosphorylation and cell survival in cardiac muscle cells. *J Biol Chem*. 278:4705-12.

Knobbe, C.B., V. Lapin, A. Suzuki, and T.W. Mak. 2008. The roles of PTEN in development, physiology and tumorigenesis in mouse models: a tissue-by-tissue survey. *Oncogene*. 27:5398-415.

Ko, L.J., and J.D. Engel. 1993. DNA-binding specificities of the GATA transcription factor family. *Mol Cell Biol*. 13:4011-22.

Kong, Y., P. Tannous, G. Lu, K. Berenji, B.A. Rothermel, E.N. Olson, and J.A. Hill. 2006. Suppression of class I and II histone deacetylases blunts pressure-overload cardiac hypertrophy. *Circulation*. 113:2579-88.

Kook, H., J.J. Lepore, A.D. Gitler, M.M. Lu, W. Wing-Man Yung, J. Mackay, R. Zhou, V. Ferrari, P. Gruber, and J.A. Epstein. 2003. Cardiac hypertrophy and histone deacetylase-dependent transcriptional repression mediated by the

atypical homeodomain protein Hop. *J Clin Invest*. 112:863-71.

Kouzarides, T. 2000. Acetylation: a regulatory modification to rival phosphorylation? *Embo J*. 19:1176-9.

Krystal, G., J.E. Damen, C.D. Helgason, M. Huber, M.R. Hughes, J. Kalesnikoff, V. Lam, P. Rosten, M.D. Ware, S. Yew, and R.K. Humphries. 1999. SHIPs ahoy. *Int J Biochem Cell Biol*. 31:1007-10.

Kuo, C.T., E.E. Morrisey, R. Anandappa, K. Sigrist, M.M. Lu, M.S. Parmacek, C. Soudais, and J.M. Leiden. 1997. GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev*. 11:1048-60.

Lawlor, M.A., A. Mora, P.R. Ashby, M.R. Williams, V. Murray-Tait, L. Malone, A.R. Prescott, J.M. Lucocq, and D.R. Alessi. 2002. Essential role of PDK1 in regulating cell size and development in mice. *Embo J*. 21:3728-38.

Lee, Y., T. Shioi, H. Kasahara, S.M. Jobe, R.J. Wiese, B.E. Markham, and S. Izumo. 1998. The cardiac tissue-restricted homeobox protein Csx/Nkx2.5 physically associates with the zinc finger protein GATA4 and cooperatively activates atrial natriuretic factor gene expression. *Mol Cell Biol*. 18:3120-9.

Lemercier, C., A. Verdel, B. Galloo, S. Curtet, M.P. Brocard, and S. Khochbin. 2000. mHDA1/HDAC5 histone deacetylase interacts with and represses MEF2A transcriptional activity. *J Biol Chem*. 275:15594-9.

Leslie, N.R., and C.P. Downes. 2002. PTEN: The down side of PI 3-kinase signalling. *Cell Signal*. 14:285-95.

Levy, D., S. Kenchaiah, M.G. Larson, E.J. Benjamin, M.J. Kupka, K.K. Ho, J.M.

Murabito, and R.S. Vasan. 2002. Long-term trends in the incidence of and survival with heart failure. *N Engl J Med*. 347:1397-402.

Li, D.M., and H. Sun. 1998. PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. *Proc Natl Acad Sci U S A*. 95:15406-11.

Li, J., L. Simpson, M. Takahashi, C. Miliaresis, M.P. Myers, N. Tonks, and R. Parsons. 1998. The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. *Cancer Res*. 58:5667-72.

Li, J., C. Yen, D. Liaw, K. Podsypanina, S. Bose, S.I. Wang, J. Puc, C. Miliaresis, L. Rodgers, R. McCombie, S.H. Bigner, B.C. Giovanella, M. Ittmann, B. Tycko, H. Hibshoosh, M.H. Wigler, and R. Parsons. 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science*. 275:1943-7.

Li, M., J. Luo, C.L. Brooks, and W. Gu. 2002. Acetylation of p53 inhibits its ubiquitination by Mdm2. *J Biol Chem*. 277:50607-11.

Liang, Q., L.J. De Windt, S.A. Witt, T.R. Kimball, B.E. Markham, and J.D. Molkentin. 2001a. The transcription factors GATA4 and GATA6 regulate cardiomyocyte hypertrophy in vitro and in vivo. *J Biol Chem*. 276:30245-53.

Liang, Q., R.J. Wiese, O.F. Bueno, Y.S. Dai, B.E. Markham, and J.D. Molkentin. 2001b. The transcription factor GATA4 is activated by extracellular signal-regulated kinase 1- and 2-mediated phosphorylation of serine 105 in cardiomyocytes. *Mol Cell Biol*. 21:7460-9.

Liaw, D., D.J. Marsh, J. Li, P.L. Dahia, S.I. Wang, Z. Zheng, S. Bose, K.M. Call, H.C. Tsou, M. Peacocke, C. Eng, and R. Parsons. 1997. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat Genet*. 16:64-7.

Lien, C.L., C. Wu, B. Mercer, R. Webb, J.A. Richardson, and E.N. Olson. 1999. Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. *Development*. 126:75-84.

Liu, Q., F. Shalaby, J. Jones, D. Bouchard, and D.J. Dumont. 1998. The SH2-containing inositol polyphosphate 5-phosphatase, ship, is expressed during hematopoiesis and spermatogenesis. *Blood*. 91:2753-9.

Lu, J., T.A. McKinsey, R.L. Nicol, and E.N. Olson. 2000a. Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. *Proc Natl Acad Sci U S A*. 97:4070-5.

Lu, J., T.A. McKinsey, C.L. Zhang, and E.N. Olson. 2000b. Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. *Mol Cell*. 6:233-44.

Lu, J.R., T.A. McKinsey, H. Xu, D.Z. Wang, J.A. Richardson, and E.N. Olson. 1999. FOG-2, a heart- and brain-enriched cofactor for GATA transcription factors. *Mol Cell Biol*. 19:4495-502.

Luo, J., F. Su, D. Chen, A. Shiloh, and W. Gu. 2000. Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature*. 408:377-81.

Luo, J.M., H. Yoshida, S. Komura, N. Ohishi, L. Pan, K. Shigeno, I. Hanamura, K.

Miura, S. Iida, R. Ueda, T. Naoe, Y. Akao, R. Ohno, and K. Ohnishi. 2003. Possible dominant-negative mutation of the SHIP gene in acute myeloid leukemia. *Leukemia*. 17:1-8.

Ma, K., S.M. Cheung, A.J. Marshall, and V. Duronio. 2008. PI(3,4,5)P3 and PI(3,4)P2 levels correlate with PKB/akt phosphorylation at Thr308 and Ser473, respectively; PI(3,4)P2 levels determine PKB activity. *Cell Signal*. 20:684-94.

Maehama, T., and J.E. Dixon. 1998. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem*. 273:13375-8.

Maehama, T., G.S. Taylor, and J.E. Dixon. 2001. PTEN and myotubularin: novel phosphoinositide phosphatases. *Annu Rev Biochem*. 70:247-79.

Marion, E., P.J. Kaisaki, V. Pouillon, C. Gueydan, J.C. Levy, A. Bodson, G. Krzentowski, J.C. Daubresse, J. Mockel, J. Behrends, G. Servais, C. Szpirer, V. Kruys, D. Gauguier, and S. Schurmans. 2002. The gene INPPL1, encoding the lipid phosphatase SHIP2, is a candidate for type 2 diabetes in rat and man. *Diabetes*. 51:2012-7.

Marsh, D.J., P.L. Dahia, V. Coulon, Z. Zheng, F. Dorion-Bonnet, K.M. Call, R. Little, A.Y. Lin, R.A. Eeles, A.M. Goldstein, S.V. Hodgson, A.L. Richardson, B.G. Robinson, H.C. Weber, M. Longy, and C. Eng. 1998. Allelic imbalance, including deletion of PTEN/MMACI, at the Cowden disease locus on 10q22-23, in hamartomas from patients with Cowden syndrome and germline PTEN mutation. *Genes Chromosomes Cancer*. 21:61-9.

Martelli, A.M., M. Nyakern, G. Tabellini, R. Bortul, P.L. Tazzari, C. Evangelisti, and L. Cocco. 2006. Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutical implications for human acute myeloid leukemia. *Leukemia*. 20:911-28.

Martinez-Balbas, M.A., U.M. Bauer, S.J. Nielsen, A. Brehm, and T. Kouzarides. 2000. Regulation of E2F1 activity by acetylation. *Embo J*. 19:662-71.

Marzio, G., C. Wagener, M.I. Gutierrez, P. Cartwright, K. Helin, and M. Giacca. 2000. E2F family members are differentially regulated by reversible acetylation. *J Biol Chem*. 275:10887-92.

Matsui, T., L. Li, J.C. Wu, S.A. Cook, T. Nagoshi, M.H. Picard, R. Liao, and A. Rosenzweig. 2002. Phenotypic spectrum caused by transgenic overexpression of activated Akt in the heart. *J Biol Chem*. 277:22896-901.

Matsui, T., T. Nagoshi, and A. Rosenzweig. 2003. Akt and PI 3-kinase signaling in cardiomyocyte hypertrophy and survival. *Cell Cycle*. 2:220-3.

McFadden, D.G., J. Charite, J.A. Richardson, D. Srivastava, A.B. Firulli, and E.N. Olson. 2000. A GATA-dependent right ventricular enhancer controls dHAND transcription in the developing heart. *Development*. 127:5331-41.

McKinsey, T.A., and E.N. Olson. 1999. Cardiac hypertrophy: sorting out the circuitry. *Curr Opin Genet Dev*. 9:267-74.

McMullen, J.R., T. Shioi, L. Zhang, O. Tarnavski, M.C. Sherwood, P.M. Kang, and S. Izumo. 2003. Phosphoinositide 3-kinase(p110alpha) plays a critical role for the induction of physiological, but not pathological, cardiac hypertrophy. *Proc*

Natl Acad Sci U S A. 100:12355-60.

McPherson, P.S., E.P. Garcia, V.I. Slepnev, C. David, X. Zhang, D. Grabs, W.S. Sossin, R. Bauerfeind, Y. Nemoto, and P. De Camilli. 1996. A presynaptic inositol-5-phosphatase. *Nature*. 379:353-7.

Merika, M., and S.H. Orkin. 1993. DNA-binding specificity of GATA family transcription factors. *Mol Cell Biol*. 13:3999-4010.

Michael, A., S. Haq, X. Chen, E. Hsich, L. Cui, B. Walters, Z. Shao, K. Bhattacharya, H. Kilter, G. Huggins, M. Andreucci, M. Periasamy, R.N. Solomon, R. Liao, R. Patten, J.D. Molkentin, and T. Force. 2004. Glycogen synthase kinase-3beta regulates growth, calcium homeostasis, and diastolic function in the heart. *J Biol Chem*. 279:21383-93.

Minagawa, T., T. Ijuin, Y. Mochizuki, and T. Takenawa. 2001. Identification and characterization of a sac domain-containing phosphoinositide 5-phosphatase. *J Biol Chem*. 276:22011-5.

Miska, E.A., C. Karlsson, E. Langley, S.J. Nielsen, J. Pines, and T. Kouzarides. 1999. HDAC4 deacetylase associates with and represses the MEF2 transcription factor. *Embo J*. 18:5099-107.

Miyamoto, S., T. Kawamura, T. Morimoto, K. Ono, H. Wada, Y. Kawase, A. Matsumori, R. Nishio, T. Kita, and K. Hasegawa. 2006. Histone acetyltransferase activity of p300 is required for the promotion of left ventricular remodeling after myocardial infarction in adult mice in vivo. *Circulation*. 113:679-90.

Molkentin, J.D. 2000. The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. *J Biol Chem*. 275:38949-52.

Molkentin, J.D., Q. Lin, S.A. Duncan, and E.N. Olson. 1997. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev*. 11:1061-72.

Molkentin, J.D., J.R. Lu, C.L. Antos, B. Markham, J. Richardson, J. Robbins, S.R. Grant, and E.N. Olson. 1998. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell*. 93:215-28.

Montgomery, R.L., C.A. Davis, M.J. Potthoff, M. Haberland, J. Fielitz, X. Qi, J.A. Hill, J.A. Richardson, and E.N. Olson. 2007. Histone deacetylases 1 and 2 redundantly regulate cardiac morphogenesis, growth, and contractility. *Genes Dev*. 21:1790-802.

Mora, A., A.M. Davies, L. Bertrand, I. Sharif, G.R. Budas, S. Jovanovic, V. Mouton, C.R. Kahn, J.M. Lucocq, G.A. Gray, A. Jovanovic, and D.R. Alessi. 2003. Deficiency of PDK1 in cardiac muscle results in heart failure and increased sensitivity to hypoxia. *Embo J*. 22:4666-76.

Morisco, C., K. Seta, S.E. Hardt, Y. Lee, S.F. Vatner, and J. Sadoshima. 2001. Glycogen synthase kinase 3beta regulates GATA4 in cardiac myocytes. *J Biol Chem*. 276:28586-97.

Morrisey, E.E., H.S. Ip, Z. Tang, and M.S. Parmacek. 1997. GATA-4 activates transcription via two novel domains that are conserved within the GATA-4/5/6

subfamily. *J Biol Chem*. 272:8515-24.

Myers, M.P., I. Pass, I.H. Batty, J. Van der Kaay, J.P. Stolarov, B.A. Hemmings, M.H. Wigler, C.P. Downes, and N.K. Tonks. 1998. The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proc Natl Acad Sci U S A*. 95:13513-8.

Nakashima, N., P.M. Sharma, T. Imamura, R. Bookstein, and J.M. Olefsky. 2000. The tumor suppressor PTEN negatively regulates insulin signaling in 3T3-L1 adipocytes. *J Biol Chem*. 275:12889-95.

Nemoto, Y., M. Arribas, C. Haffner, and P. DeCamilli. 1997. Synaptojanin 2, a novel synaptojanin isoform with a distinct targeting domain and expression pattern. *J Biol Chem*. 272:30817-21.

Novak, A., C. Guo, W. Yang, A. Nagy, and C.G. Lobe. 2000. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis*. 28:147-55.

Oka, T., J. Xu, and J.D. Molkentin. 2007. Re-employment of developmental transcription factors in adult heart disease. *Semin Cell Dev Biol*. 18:117-31.

Ooms, L.M., K.A. Horan, P. Rahman, G. Seaton, R. Gurung, D.S. Kethesparan, and C.A. Mitchell. 2009. The role of the inositol polyphosphate 5-phosphatases in cellular function and human disease. *Biochem J*. 419:29-49.

Oudit, G.Y., and J.M. Penninger. 2009. Cardiac regulation by phosphoinositide 3-kinases and PTEN. *Cardiovasc Res*. 82:250-60.

Oudit, G.Y., H. Sun, B.G. Kerfant, M.A. Crackower, J.M. Penninger, and P.H. Backx.

2004. The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. *J Mol Cell Cardiol*. 37:449-71.

Ozawa, Y., M. Towatari, S. Tsuzuki, F. Hayakawa, T. Maeda, Y. Miyata, M. Tanimoto, and H. Saito. 2001. Histone deacetylase 3 associates with and represses the transcription factor GATA-2. *Blood*. 98:2116-23.

Pehlivan, T., B.R. Pober, M. Brueckner, S. Garrett, R. Slaugh, R. Van Rheeden, D.B. Wilson, M.S. Watson, and A.V. Hing. 1999. GATA4 haploinsufficiency in patients with interstitial deletion of chromosome region 8p23.1 and congenital heart disease. *Am J Med Genet*. 83:201-6.

Pendaries, C., H. Tronchere, M. Plantavid, and B. Payrastre. 2003. Phosphoinositide signaling disorders in human diseases. *FEBS Lett*. 546:25-31.

Peng, X.D., P.Z. Xu, M.L. Chen, A. Hahn-Windgassen, J. Skeen, J. Jacobs, D. Sundararajan, W.S. Chen, S.E. Crawford, K.G. Coleman, and N. Hay. 2003. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. *Genes Dev*. 17:1352-65.

Perrino, C., and H.A. Rockman. 2006. GATA4 and the two sides of gene expression reprogramming. *Circ Res*. 98:715-6.

Persad, S., S. Attwell, V. Gray, M. Delcommenne, A. Troussard, J. Sanghera, and S. Dedhar. 2000. Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. *Proc Natl Acad Sci U S A*. 97:3207-12.

Pham, F.H., P.H. Sugden, and A. Clerk. 2000. Regulation of protein kinase B and 4E-BP1 by oxidative stress in cardiac myocytes. *Circ Res*. 86:1252-8.

Pikkarainen, S., H. Tokola, R. Kerkela, and H. Ruskoaho. 2004. GATA transcription factors in the developing and adult heart. *Cardiovasc Res*. 63:196-207.

Pipes, G.C., E.E. Creemers, and E.N. Olson. 2006. The myocardin family of transcriptional coactivators: versatile regulators of cell growth, migration, and myogenesis. *Genes Dev*. 20:1545-56.

Podsypanina, K., L.H. Ellenson, A. Nemes, J. Gu, M. Tamura, K.M. Yamada, C. Cordon-Cardo, G. Catoretti, P.E. Fisher, and R. Parsons. 1999. Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *Proc Natl Acad Sci U S A*. 96:1563-8.

Ramaswamy, S., N. Nakamura, F. Vazquez, D.B. Batt, S. Perera, T.M. Roberts, and W.R. Sellers. 1999. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proc Natl Acad Sci U S A*. 96:2110-5.

Rohrschneider, L.R., J.F. Fuller, I. Wolf, Y. Liu, and D.M. Lucas. 2000. Structure, function, and biology of SHIP proteins. *Genes Dev*. 14:505-20.

Rojas, A., S.W. Kong, P. Agarwal, B. Gilliss, W.T. Pu, and B.L. Black. 2008. GATA4 is a direct transcriptional activator of cyclin D2 and Cdk4 and is required for cardiomyocyte proliferation in anterior heart field-derived myocardium. *Mol Cell Biol*. 28:5420-31.

Rommel, C., S.C. Bodine, B.A. Clarke, R. Rossman, L. Nunez, T.N. Stitt, G.D.

Yancopoulos, and D.J. Glass. 2001. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol*. 3:1009-13.

Rusk, N., P.U. Le, S. Mariggio, G. Guay, C. Lurisci, I.R. Nabi, D. Corda, and M. Symons. 2003. Synaptojanin 2 functions at an early step of clathrin-mediated endocytosis. *Curr Biol*. 13:659-63.

Sadoshima, J., and S. Izumo. 1995. Rapamycin selectively inhibits angiotensin II-induced increase in protein synthesis in cardiac myocytes in vitro. Potential role of 70-kD S6 kinase in angiotensin II-induced cardiac hypertrophy. *Circ Res*. 77:1040-52.

Saito, T., F. Guan, D.F. Papolos, S. Lau, M. Klein, C.S. Fann, and H.M. Lachman. 2001. Mutation analysis of SYNJ1: a possible candidate gene for chromosome 21q22-linked bipolar disorder. *Mol Psychiatry*. 6:387-95.

Sanbe, A., J. Gulick, M.C. Hanks, Q. Liang, H. Osinska, and J. Robbins. 2003. Reengineering inducible cardiac-specific transgenesis with an attenuated myosin heavy chain promoter. *Circ Res*. 92:609-16.

Sano, Y., and S. Ishii. 2001. Increased affinity of c-Myb for CREB-binding protein (CBP) after CBP-induced acetylation. *J Biol Chem*. 276:3674-82.

Sartorelli, V., P.L. Puri, Y. Hamamori, V. Ogryzko, G. Chung, Y. Nakatani, J.Y. Wang, and L. Kedes. 1999. Acetylation of MyoD directed by PCAF is necessary for the execution of the muscle program. *Mol Cell*. 4:725-34.

Schluter, K.D., Y. Goldberg, G. Taimor, M. Schafer, and H.M. Piper. 1998. Role of

phosphatidylinositol 3-kinase activation in the hypertrophic growth of adult ventricular cardiomyocytes. *Cardiovasc Res*. 40:174-81.

Schmelzle, T., and M.N. Hall. 2000. TOR, a central controller of cell growth. *Cell*. 103:253-62.

Schwartzbauer, G., and J. Robbins. 2001. The tumor suppressor gene PTEN can regulate cardiac hypertrophy and survival. *J Biol Chem*. 276:35786-93.

Shaper, N.L., A. Harduin-Lepers, and J.H. Shaper. 1994. Male germ cell expression of murine beta 4-galactosyltransferase. A 796-base pair genomic region, containing two cAMP-responsive element (CRE)-like elements, mediates male germ cell-specific expression in transgenic mice. *J Biol Chem*. 269:25165-71.

Shioi, T., P.M. Kang, P.S. Douglas, J. Hampe, C.M. Yballe, J. Lawitts, L.C. Cantley, and S. Izumo. 2000. The conserved phosphoinositide 3-kinase pathway determines heart size in mice. *Embo J*. 19:2537-48.

Shioi, T., J.R. McMullen, P.M. Kang, P.S. Douglas, T. Obata, T.F. Franke, L.C. Cantley, and S. Izumo. 2002. Akt/protein kinase B promotes organ growth in transgenic mice. *Mol Cell Biol*. 22:2799-809.

Shioi, T., J.R. McMullen, O. Tarnavski, K. Converso, M.C. Sherwood, W.J. Manning, and S. Izumo. 2003. Rapamycin attenuates load-induced cardiac hypertrophy in mice. *Circulation*. 107:1664-70.

Shiojima, I., K. Sato, Y. Izumiya, S. Schiekofer, M. Ito, R. Liao, W.S. Colucci, and K. Walsh. 2005. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. *J Clin Invest*. 115:2108-18.

Sleeman, M.W., K.E. Wortley, K.M. Lai, L.C. Gowen, J. Kintner, W.O. Kline, K. Garcia, T.N. Stitt, G.D. Yancopoulos, S.J. Wiegand, and D.J. Glass. 2005. Absence of the lipid phosphatase SHIP2 confers resistance to dietary obesity. *Nat Med*. 11:199-205.

Spange, S., T. Wagner, T. Heinzel, and O.H. Kramer. 2009. Acetylation of non-histone proteins modulates cellular signalling at multiple levels. *Int J Biochem Cell Biol*. 41:185-98.

Sparrow, D.B., E.A. Miska, E. Langley, S. Reynaud-Deonauth, S. Kotecha, N. Towers, G. Spohr, T. Kouzarides, and T.J. Mohun. 1999. MEF-2 function is modified by a novel co-repressor, MITR. *Embo J*. 18:5085-98.

Stambolic, V., A. Suzuki, J.L. de la Pompa, G.M. Brothers, C. Mirtsos, T. Sasaki, J. Ruland, J.M. Penninger, D.P. Siderovski, and T.W. Mak. 1998. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell*. 95:29-39.

Steck, P.A., M.A. Pershouse, S.A. Jasser, W.K. Yung, H. Lin, A.H. Ligon, L.A. Langford, M.L. Baumgard, T. Hattier, T. Davis, C. Frye, R. Hu, B. Swedlund, D.H. Teng, and S.V. Tavtigian. 1997. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet*. 15:356-62.

Stephens, L.R., T.R. Jackson, and P.T. Hawkins. 1993. Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-trisphosphate: a new intracellular signalling system? *Biochim Biophys Acta*. 1179:27-75.

Stopkova, P., J. Vevera, I. Paclt, I. Zukov, and H.M. Lachman. 2004. Analysis of SYNJ1, a candidate gene for 21q22 linked bipolar disorder: a replication study. *Psychiatry Res*. 127:157-61.

Storz, P., and A. Toker. 2002. 3'-phosphoinositide-dependent kinase-1 (PDK-1) in PI 3-kinase signaling. *Front Biosci*. 7:d886-902.

Sun, H., R. Lesche, D.M. Li, J. Liliental, H. Zhang, J. Gao, N. Gavrilova, B. Mueller, X. Liu, and H. Wu. 1999. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. *Proc Natl Acad Sci U S A*. 96:6199-204.

Suzuki, A., J.L. de la Pompa, V. Stambolic, A.J. Elia, T. Sasaki, I. del Barco Barrantes, A. Ho, A. Wakeham, A. Itie, W. Khoo, M. Fukumoto, and T.W. Mak. 1998. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. *Curr Biol*. 8:1169-78.

Svensson, E.C., G.S. Huggins, F.B. Dardik, C.E. Polk, and J.M. Leiden. 2000. A functionally conserved N-terminal domain of the friend of GATA-2 (FOG-2) protein represses GATA4-dependent transcription. *J Biol Chem*. 275:20762-9.

Svensson, E.C., R.L. Tufts, C.E. Polk, and J.M. Leiden. 1999. Molecular cloning of FOG-2: a modulator of transcription factor GATA-4 in cardiomyocytes. *Proc Natl Acad Sci U S A*. 96:956-61.

Takaya, T., T. Kawamura, T. Morimoto, K. Ono, T. Kita, A. Shimatsu, and K. Hasegawa. 2008. Identification of p300-targeted acetylated residues in

GATA4 during hypertrophic responses in cardiac myocytes. *J Biol Chem*. 283:9828-35.

Takeshita, S., N. Namba, J.J. Zhao, Y. Jiang, H.K. Genant, M.J. Silva, M.D. Brodt, C.D. Helgason, J. Kalesnikoff, M.J. Rauh, R.K. Humphries, G. Krystal, S.L. Teitelbaum, and F.P. Ross. 2002. SHIP-deficient mice are severely osteoporotic due to increased numbers of hyper-resorptive osteoclasts. *Nat Med*. 8:943-9.

Takimoto, E., and D.A. Kass. 2007. Role of oxidative stress in cardiac hypertrophy and remodeling. *Hypertension*. 49:241-8.

Taniyama, Y., M. Ito, K. Sato, C. Kuester, K. Veit, G. Tremp, R. Liao, W.S. Colucci, Y. Ivashchenko, K. Walsh, and I. Shiojima. 2005. Akt3 overexpression in the heart results in progression from adaptive to maladaptive hypertrophy. *J Mol Cell Cardiol*. 38:375-85.

Taylor, V., M. Wong, C. Brandts, L. Reilly, N.M. Dean, L.M. Cowsert, S. Moodie, and D. Stokoe. 2000. 5' phospholipid phosphatase SHIP-2 causes protein kinase B inactivation and cell cycle arrest in glioblastoma cells. *Mol Cell Biol*. 20:6860-71.

Tevosian, S.G., A.E. Deconinck, A.B. Cantor, H.I. Rieff, Y. Fujiwara, G. Corfas, and S.H. Orkin. 1999. FOG-2: A novel GATA-family cofactor related to multitype zinc-finger proteins Friend of GATA-1 and U-shaped. *Proc Natl Acad Sci U S A*. 96:950-5.

Toker, A., and L.C. Cantley. 1997. Signalling through the lipid products of

phosphoinositide-3-OH kinase. *Nature*. 387:673-6.

Toker, A., and A.C. Newton. 2000. Cellular signaling: pivoting around PDK-1. *Cell*. 103:185-8.

Tomita, A., M. Towatari, S. Tsuzuki, F. Hayakawa, H. Kosugi, K. Tamai, T. Miyazaki, T. Kinoshita, and H. Saito. 2000. c-Myb acetylation at the carboxyl-terminal conserved domain by transcriptional co-activator p300. *Oncogene*. 19:444-51.

Tremblay, J.J., and R.S. Viger. 2003. Transcription factor GATA-4 is activated by phosphorylation of serine 261 via the cAMP/protein kinase a signaling pathway in gonadal cells. *J Biol Chem*. 278:22128-35.

Trivedi, C.M., Y. Luo, Z. Yin, M. Zhang, W. Zhu, T. Wang, T. Floss, M. Goettlicher, P.R. Noppinger, W. Wurst, V.A. Ferrari, C.S. Abrams, P.J. Gruber, and J.A. Epstein. 2007. Hdac2 regulates the cardiac hypertrophic response by modulating Gsk3 beta activity. *Nat Med*. 13:324-31.

Trivedi, C.M., W. Zhu, Q. Wang, C. Jia, H.J. Kee, L. Li, S. Hannenhalli, and J.A. Epstein. Hopx and Hdac2 interact to modulate Gata4 acetylation and embryonic cardiac myocyte proliferation. *Dev Cell*. 19:450-9.

Tsang, A.P., J.E. Visvader, C.A. Turner, Y. Fujiwara, C. Yu, M.J. Weiss, M. Crossley, and S.H. Orkin. 1997. FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. *Cell*. 90:109-19.

Tschopp, O., Z.Z. Yang, D. Brodbeck, B.A. Dummler, M. Hemmings-Mieszczak, T.

Watanabe, T. Michaelis, J. Frahm, and B.A. Hemmings. 2005. Essential role of protein kinase B gamma (PKB gamma/Akt3) in postnatal brain development but not in glucose homeostasis. *Development*. 132:2943-54.

Vanhaesebroeck, B., S.J. Leevers, K. Ahmadi, J. Timms, R. Katso, P.C. Driscoll, R. Woscholski, P.J. Parker, and M.D. Waterfield. 2001. Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem*. 70:535-602.

Vega, R.B., K. Matsuda, J. Oh, A.C. Barbosa, X. Yang, E. Meadows, J. McAnally, C. Pomajzl, J.M. Shelton, J.A. Richardson, G. Karsenty, and E.N. Olson. 2004. Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell*. 119:555-66.

Viger, R.S., S.M. Guittot, M. Anttonen, D.B. Wilson, and M. Heikinheimo. 2008. Role of the GATA family of transcription factors in endocrine development, function, and disease. *Mol Endocrinol*. 22:781-98.

Villagra, A., F. Cheng, H.W. Wang, I. Suarez, M. Glozak, M. Maurin, D. Nguyen, K.L. Wright, P.W. Atadja, K. Bhalla, J. Pinilla-Ibarz, E. Seto, and E.M. Sotomayor. 2009. The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance. *Nat Immunol*. 10:92-100.

Wada, T., T. Sasaoka, M. Funaki, H. Hori, S. Murakami, M. Ishiki, T. Haruta, T. Asano, W. Ogawa, H. Ishihara, and M. Kobayashi. 2001. Overexpression of SH2-containing inositol phosphatase 2 results in negative regulation of insulin-induced metabolic actions in 3T3-L1 adipocytes via its 5'-phosphatase catalytic activity. *Mol Cell Biol*. 21:1633-46.

Walsh, K. 2006. Akt signaling and growth of the heart. *Circulation*. 113:2032-4.

Wang, J., X.H. Feng, and R.J. Schwartz. 2004. SUMO-1 modification activated GATA4-dependent cardiogenic gene activity. *J Biol Chem*. 279:49091-8.

Warrens, A.N., M.D. Jones, and R.I. Lechler. 1997. Splicing by overlap extension by PCR using asymmetric amplification: an improved technique for the generation of hybrid proteins of immunological interest. *Gene*. 186:29-35.

Watamoto, K., M. Towatari, Y. Ozawa, Y. Miyata, M. Okamoto, A. Abe, T. Naoe, and H. Saito. 2003. Altered interaction of HDAC5 with GATA-1 during MEL cell differentiation. *Oncogene*. 22:9176-84.

Weiss, M.J., and S.H. Orkin. 1995. GATA transcription factors: key regulators of hematopoiesis. *Exp Hematol*. 23:99-107.

Woscholski, R., P.M. Finan, E. Radley, N.F. Totty, A.E. Sterling, J.J. Hsuan, M.D. Waterfield, and P.J. Parker. 1997. Synaptojanin is the major constitutively active phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase in rodent brain. *J Biol Chem*. 272:9625-8.

Wu, X., K. Senechal, M.S. Neshat, Y.E. Whang, and C.L. Sawyers. 1998. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. *Proc Natl Acad Sci U S A*. 95:15587-91.

Yamagata, T., K. Mitani, H. Oda, T. Suzuki, H. Honda, T. Asai, K. Maki, T. Nakamoto, and H. Hirai. 2000. Acetylation of GATA-3 affects T-cell survival and homing to secondary lymphoid organs. *Embo J*. 19:4676-87.

Yanazume, T., K. Hasegawa, T. Morimoto, T. Kawamura, H. Wada, A. Matsumori, Y. Kawase, M. Hirai, and T. Kita. 2003a. Cardiac p300 is involved in myocyte growth with decompensated heart failure. *Mol Cell Biol*. 23:3593-606.

Yanazume, T., T. Morimoto, H. Wada, T. Kawamura, and K. Hasegawa. 2003b. Biological role of p300 in cardiac myocytes. *Mol Cell Biochem*. 248:115-9.

Yang, Z.Z., O. Tschopp, N. Di-Poi, E. Bruder, A. Baudry, B. Dummler, W. Wahli, and B.A. Hemmings. 2005. Dosage-dependent effects of Akt1/protein kinase Balpha (PKBalpha) and Akt3/PKBgamma on thymus, skin, and cardiovascular and nervous system development in mice. *Mol Cell Biol*. 25:10407-18.

Yao, T.P., S.P. Oh, M. Fuchs, N.D. Zhou, L.E. Ch'ng, D. Newsome, R.T. Bronson, E. Li, D.M. Livingston, and R. Eckner. 1998. Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. *Cell*. 93:361-72.

Young, D.C., S.D. Kingsley, K.A. Ryan, and F.J. Dutko. 1993. Selective inactivation of eukaryotic beta-galactosidase in assays for inhibitors of HIV-1 TAT using bacterial beta-galactosidase as a reporter enzyme. *Anal Biochem*. 215:24-30.

Yuan, T.L., and L.C. Cantley. 2008. PI3K pathway alterations in cancer: variations on a theme. *Oncogene*. 27:5497-510.

Zeisberg, E.M., Q. Ma, A.L. Juraszek, K. Moses, R.J. Schwartz, S. Izumo, and W.T. Pu. 2005. Morphogenesis of the right ventricle requires myocardial expression of Gata4. *J Clin Invest*. 115:1522-31.

Zhang, C.L., T.A. McKinsey, S. Chang, C.L. Antos, J.A. Hill, and E.N. Olson. 2002.

Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. *Cell*. 110:479-88.

Zhang, C.L., T.A. McKinsey, J.R. Lu, and E.N. Olson. 2001. Association of COOH-terminal-binding protein (CtBP) and MEF2-interacting transcription repressor (MITR) contributes to transcriptional repression of the MEF2 transcription factor. *J Biol Chem*. 276:35-9.

Zhang, Y., S. Kwon, T. Yamaguchi, F. Cubizolles, S. Rousseaux, M. Kneissel, C. Cao, N. Li, H.L. Cheng, K. Chua, D. Lombard, A. Mizeracki, G. Matthias, F.W. Alt, S. Khochbin, and P. Matthias. 2008. Mice lacking histone deacetylase 6 have hyperacetylated tubulin but are viable and develop normally. *Mol Cell Biol*. 28:1688-701.

Zhong, R., and Z.H. Ye. 2003. The SAC domain-containing protein gene family in Arabidopsis. *Plant Physiol*. 132:544-55.

Zhu, W., C.M. Trivedi, D. Zhou, L. Yuan, M.M. Lu, and J.A. Epstein. 2009. Inpp5f is a polyphosphoinositide phosphatase that regulates cardiac hypertrophic responsiveness. *Circ Res*. 105:1240-7.

Zhu, W.Z., M. Zheng, W.J. Koch, R.J. Lefkowitz, B.K. Kobilka, and R.P. Xiao. 2001. 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*. 98:1607-12.