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# Effects of PHLPP1 Removal in the Heart

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

By

Courtney Moc

Committee in charge:

Nicole H. Purcell, Chair
Nigel M. Crawford, Co-Chair
Paul A. Price

2015

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Chair

University of California, San Diego

2015

## DEDICATION

I dedicate this Thesis to the foundation in my life: my family.

To my mom, thank you for everything you have done for me. Thank you for your love, support, guidance, and encouragement. You're my role model.

To my grandma and grandpa Moc, my second parents, for your love and care, and for raising me.

To my grandma and grandpa Lien, for your love and for raising my mom to be a strong, beautiful woman.

To my dad and brother Charley, for teaching me unconditional love.

To my step-dad Manuel and sister Natalie, for your support and for being sweet additions to my life.

To my Aunt Stella, my big sister, for your love, guidance, and welcoming arms.

To my Uncle David and Aunt Joanne, for your love and laughter, and for treating me like your own child.

And lastly,

To my love Andrew, whose smile and love has transformed my entire life, for bringing me so much happiness I never thought was possible.

Words cannot express how much appreciation I have for you all.
I love you and thank you so much.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| α-skActin | α-skeletal muscle actin |
| AGC | Protein kinase A, protein kinase G, protein kinase C |
| Akt/PKB | Protein kinase B |
| AMVM | Adult mouse ventricular myocytes |
| ANF | Atrial natriuretic factor |
| Angp2 | Angiopoietin 2 |
| β-MHC | Beta myosin heavy chain |
| BAD | Bcl-xL/Bcl-2 associated death promoter |
| BAEC | Bovine aortic endothelial cells |
| Bcl-2 | B-cell lymphoma 2 |
| bFGF | Basic fibroblast growth factor |
| BNP | Brain natriuretic peptide |
| BW | Body weight |
| C | Carboxyl |
| CA-Akt | Constitutively active Akt (E40K mutant Akt) |
| Casp3 | Caspase 3 |
| Casp9 | Caspase 9 |
| Col1a | Collagen 1a |
| Col3a | Collagen 3a |
| ERK1/2 | Extracellular-signal regulated kinase 1/2 |
| FOXO3 | Forkhead box O3 |
| FS | Fractional shortening |
| GSK3 | Glycogen synthase kinase 3 |
| HET | Heterozygous |
| HM | Hydrophobic motif |
| HW | Heart weight |
| IGF-1 | Insulin growth factor-1 |
| KD | Kinase domain |
| KO | Knock-out |
| LRR | Leucine-rich repeat |
| LW | Lung weight |
| MAPK | Mitogen-activated protein kinase |
| MDM2 | Mouse double minute 2 |
| $Mg^{+2}$ | Magnesium |
| $Mn^{+2}$ | Manganese |
| Mst1 | Mammalian sterile 20-like 1 |
| mTORC1 | Mammalian target of rapamycin complex 1 |
| Myr-Akt | Myristoylated Akt (membrane-targeted Akt) |
| N | Amino |
| NRVM | Neonatal rat ventricular myocytes |
| p70S6K | 70-kDa ribosomal protein S6 kinase |

| | |
|---|---|
| PDK1 | Phosphoinositide-dependent kinase 1 |
| PDZ | PSD95, Dlg1, Zo-1 |
| PH | Pleckstrin homology domain |
| PHLPP | Pleckstrin homology domain leucine-rich repeat protein phosphatase |
| PI3K | Phosphatidylinositol 3-kinase |
| $PIP_2$ | Phosphatidylinositol 4,5-bisphosphate |
| $PIP_3$ | Phosphatidylinositol 3,4,5-triphosphate |
| PKC | Protein kinase C |
| PP2A | Protein phosphatase 2A |
| PP2C | Protein phosphatase 2C |
| PPM | Protein phosphatase magnesium/manganese dependent |
| PRAS40 | Proline-rich Akt/PKB substrate of 40 kDA |
| PTEN | Phosphatase and tensin homolog |
| RA | Ras association |
| Rheb | Ras homolog enriched in brain |
| SCN | suprachiasmatic nucleus |
| SCOP | suprachiasmatic nucleus circadian oscillatory protein |
| Ser473 | Serine-473 |
| TAC | Transverse aortic constriction |
| Thr308 | Threonine-308 |
| TL | Tibia length |
| TORC2 | Target of rapamycin complex 2 |
| TSC2 | Tuberous sclerosis complex 2 |
| VEGFa | Vascular endothelial growth factor a |
| WT | Wild-type |

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## ACKNOWLEDGEMENTS

The past two years have been challenging, yet the most rewarding years of my life. Transitioning from a plant biology lab to pharmacology research, this thesis is a result of the help from many generous people. I would like to thank Dr. Nicole H. Purcell for taking in this lost bird under her wings, to mentor and guide me to where I am today. I am extremely fortunate to be given this opportunity to advance my education under you. Thank you for always being so bighearted with your love, care, and understanding. Furthermore, I want to thank my other committee members: Dr. Nigel M. Crawford whose patience, mentorship, and support, has provided me guidance to my passion and direction in life and Dr. Paul A. Price for his insight and time. Each professor was extremely busy yet still set aside time to meet and discuss my research with me.

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Chapter 2 is a reprint with minor modifications of the material as it appears in Moc C, Taylor AE, Zambrano CM, Barlow MS, Zhang X, Gustafsson AB, and Purcell NH. Physiological activation of Akt by PHLPP1 deletion protects against pathological hypertrophy. *Cardiovascular Research* 2015;105(2):160-170. The thesis author was the author of this manuscript. Dr. Nicole H. Purcell directed and supervised the research, which forms the basis for this chapter.

Lastly, I would like to thank my family and friends for their love and support. My family is my foundation to all my success. I especially want to thank Andrew Willeford who constantly supports and encourages me and for believing in me when I sometimes do not believe in myself.

**ABSTRACT OF THE THESIS**

Effects of PHLPP1 Removal in the Heart

By

Courtney Moc

Master of Science in Biology

University of California, San Diego, 2015

Professor Nicole H. Purcell, Chair
Professor Nigel M. Crawford, Co-Chair

The serine/threonine kinase Akt is involved in many cellular processes including growth, survival, metabolism, protein synthesis, and angiogenesis. In particular, the activation of Akt induces cardiac hypertrophy, a process in which the heart undergoes cell enlargement in order to increase or maintain cardiac function. Akt activation causes

physiological hypertrophy whereas prolonged Akt activation contributes to pathological hypertrophy and the development of heart failure. Extensive research has focused on regulating Akt activation; however, the mechanism of inactivation by phosphatases is largely unknown. A newly discovered phosphatase, PHLPP1 (PH domain leucine-rich repeat protein phosphatase) was found to dephosphorylate Akt. To determine the effect of PHLPP1 removal in the heart, we used global PHLPP1 knock-out (KO) mice. At baseline, wild-type (WT) and PHLPP1 KO exhibit no differences in heart size up to one year of age. However, PHLPP1 deletion increased Akt activity in the heart and cardiomyocytes. In a model for exercise-induced hypertrophy, loss of PHLPP1 resulted in increased heart size and myocyte cell area compared to WT. In contrast, in a model of pathological hypertrophy induced by transverse aortic constriction (TAC), compared to WT, PHLPP1 KO exhibited an attenuated increase in heart size, myocyte cell area, hypertrophic gene expression, fibrosis, and cell death. Furthermore, KO mice had a sustained increase in angiogenic marker expression and capillary density compared to WT. We demonstrate that removal of PHLPP1, which increases Akt activity in the heart to physiological levels without affecting cell size, increases angiogenesis and attenuates pathological hypertrophy following TAC.

# CHAPTER 1

# INTRODUCTION

## *1.1 Cardiovascular Diseases and Hypertrophy*

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the United States accounting for over 600,000 deaths in 2013.[1] Within CVD, heart failure is the largest contribution to these deaths.[2] In response to pathophysiological stress (i.e. pressure or volume overload such as hypertension, atherosclerosis, and valve defects), the heart undergoes cell enlargement in order to increase or maintain cardiac function. Cardiac hypertrophy is characterized by an increase in cardiomyocyte size, not number, with a growth in length and/or width to increase cardiac output and decrease ventricular wall tension. Maladaptive or pathological hypertrophy is characterized by ventricular remodeling and wall dilation, increase in fibrosis, and re-expression of fetal genes (i.e. atrial natriuretic factor, brain natriuretic peptide, α-skeletal muscle actin, and β-myosin heavy chain).[2-4] While hypertrophy is a compensatory mechanism, a prolonged pathologic response increases the risk of developing heart failure. In contrast, adaptive or physiological hypertrophy is induced by normal postnatal development, exercise, or pregnancy. It is characterized by an increase in cell diameter of the cardiac muscle cell, normal or enhanced contractile function, and induction of coronary angiogenesis (Scheme 1).[4-6] Angiogenesis increases overall capillary density through the formation of new capillaries from pre-existing capillaries.[5,6] This occurs through induction of growth factors such as vascular endothelial growth factor a (VEGFa) and angiopoietin 2 (Angp2).[5]

Cardiac hypertrophy is activated by many stimuli including hormones (i.e. insulin, angiotensin I, and growth hormone) and cytokines (i.e. insulin-like growth factor I and leukemia inhibitory factor).[7] These stimuli activate hypertrophic signaling pathways such as Akt, protein kinase C (PKC), calcineurin-NFAT, and mitogen-activated protein kinases (MAPK).[2,4,8] Akt, which is important for many cellular functions including cell growth, angiogenesis, and cell survival, is a pivotal regulator of stress signaling in cardiomyocytes. Therefore, understanding how Akt is regulated is critical for treatment of heart failure.

### *1.2 Akt Signaling*

Akt, which is also called protein kinase B (PKB), is a serine/threonine kinase involved in regulating cell survival, growth, metabolism, and cell cycle regulation.[5] Akt is a member of the AGC (protein kinase A, protein kinase G, protein kinase C) protein kinase family.[9,10] Akt has a pleckstrin homology (PH) domain in the amino (N) terminus, a central catalytic kinase domain (KD), and hydrophobic motif (HM) in the carboxyl (C) terminus.[5] There are three isoforms of Akt – Akt1, Akt2, and Akt3. Each isoform has different effects due to its location within the cell and expression level in tissue. In mice, knock-out (KO) studies revealed that Akt1 is important for cell growth and it is ubiquitously expressed, Akt2 which is highly expressed in insulin-responsive tissues (i.e. liver, kidneys, and muscles), displayed a diabetic phenotype when deleted, and Akt3 which is highly expressed in the brain was found to be involved in postnatal brain development since the size of the brains were smaller in the KO mice.[11-14] While all three isoforms are found in the heart, Akt1 and Akt2 are the predominant.[6,14]

Activation of Akt by phosphorylation is primarily regulated by phosphatidylinositol 3-kinase (PI3K) and phosphoinositide-dependent protein kinase 1 (PDK1). Under stimulation produced by a ligand, hormone, cytokine, or small molecule, PI3K catalyzes phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to phosphatidylinositol 3,4,5-triphosphate ($PIP_3$).[7] $PIP_3$ recruits the PH domain of Akt and PDK1 to the plasma membrane resulting in close proximity of both kinases.[5,7] Activated PDK1 phosphorylates Akt at one of its regulatory phosphorylation sites, threonine-308 (Thr308) at the activation loop on its KD and a variety of kinases phosphorylate Akt at the second regulatory phosphorylation site, serine-473 (Ser473) on its HM.[5-7] Both phosphorylation sites are required for full activity of Akt.[12] Once phosphorylated, Akt translocates to multiple sites within the cell where it phosphorylates downstream targets (Scheme 2).[12]

As mentioned, Akt phosphorylates many downstream targets; including tuberous sclerosis complex 2 (TSC2), mammalian target of rapamycin complex 1 (mTORC1), glycogen synthase kinase 3 (GSK3), 70-kDa ribosomal protein S6 kinase (p70S6K), mouse double minute 2 (MDM2), mammalian sterile 20-like 1 (Mst1), and caspase 9 (Casp9), which are responsible for various cellular functions within the cell.[7,12] The main mechanism for cell growth is through mTORC1, as it increases protein synthesis. When Akt phosphorylates and inactivates TSC2, this in turn activates Rheb (Ras homolog enriched in brain) to activate mTORC1. It has been recently demonstrated that Akt directly activates mTORC1 by phosphorylating PRAS40 (proline-rich Akt/PKB substrate of 40 kDa) which removes it from binding to mTORC1.[12] mTORC1 activates p70S6K, which in turn phosphorylates ribosomal protein S6, a component of the 40S ribosomal subunit. This activation increases protein translation and ribosomal biogenesis thereby

stimulating metabolism.[7,12] Furthermore, when Akt activates MDM2, it binds to p53 to repress its apoptotic effects and prevent cell cycle arrest.[7] Akt also promotes cell survival by negatively regulating the function or expression of several pro-apoptotic proteins such as GSK3, BAD and Mst1.[12] GSK3 is responsible for glycogen metabolism, gene transcription, protein translation, and cell apoptosis. Once Akt phosphorylates GSK3 on Ser9, this leads to repression of both alpha and beta GSK3, therefore, increasing cell survival.[7] Through pro-apoptotic B-cell lymphoma 2 (Bcl-2) family members such as Bcl-xL/Bcl-2 associated death promoter (BAD), Akt prevents the activation of Casp9, which is involved in the apoptosis-signaling cascade. Phosphorylation of BAD on Ser136 halts apoptosis events and promotes cell survival. Similarly, phosphorylation on Thr387 of Mst1 diminishes apoptotic cleavage by caspases. In addition, phosphorylated Mst1 phosphorylates forkhead box O3 (FOXO3) thereby halting apoptosis (Scheme 3).[15]

Based on the importance of Akt in cellular processes such as cell survival and normal cell growth, it is important to regulate and balance Akt activity. Akt signaling is terminated in two ways: removal of phosphatase and tensin homolog (PTEN) which inactivates $PIP_3$ signaling thus preventing Akt phosphorylation and activation as well as direct dephosphorylation of activated Akt. Akt is known to be down-regulated by two phosphatases: protein phosphatase 2A (PP2A) on the KD of Akt and pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) on its HM. PP2A has been shown to be less selective in its dephosphorylation than PHLPP. Both sites on Akt are required to be phosphorylated for full activation therefore dephosphorylation of one site results in partial activity.[7,12] Thus regulating phosphorylation by phosphatases is crucial for controlling Akt activity.

***1.3 Novel Protein Phosphatase: PHLPP***

The novel serine/threonine protein phosphatase, named after its domain composition, pleckstrin homology domain leucine rich repeat protein phosphatase (PHLPP), was discovered by the Newton lab at UCSD using a database search for proteins containing a PH and phosphatase domain.[16,17] Notably, however, almost a decade ago this same gene was isolated based on discovery of an mRNA that oscillated in a circadian-rhythm-dependent manner in the brain. The predicted protein was named SCOP (suprachiasmatic nucleus (SCN) circadian oscillatory protein), and corresponds to one of the splice variants of PHLPP, PHLLP1β.[16-18] SCOP was found to negatively regulate K-Ras-ERK1/2 pathway, which plays an important role in memory.[18] The PHLPP family is comprised of three members: PHLPP1α, PHLPP1β, and PHLPP2. PHLPP1α and PHLPP1β are splice variants from the same gene located at chromosome 18q21.33 while PHLPP2 is located at chromosome 16q22.3.[16,17,19-21] All three members have identical domain structure with a PH domain, leucine-rich repeat (LRR) segments, a protein phosphatase 2C (PP2C) domain, and ending with a PDZ (PSD95, Dlg1, Zo-1) ligand at the C-terminus. Uniquely, both PHLPP1β and PHLPP2 have a Ras-association (RA) domain at the N-terminus, preceding the PH domain (Scheme 4).[21] Thus, the molecular weight of PHLPP1α, PHLPP1β, and PHLPP2 in *Mus musculus* is ~150kDa, ~180kDa, and ~170kDa, respectively.[16,18] Cellular localization studies revealed that both PHLPP1 and PHLPP2 are present throughout the cell.[16,21]

Recent studies have found that PHLPP protein levels are regulated through degradation and biosynthesis by two feedback loops.[21] In cancer cells, Akt through GSK3β promotes the stability of PHLPP. When GSK3β is active, it phosphorylates

PHLPP creating a phosphodegron that is recognized by a E3 ubiquitin ligase, β-TrCP, which ubiquitinates and later degrades PHLPP. Also, it was demonstrated that the levels of PHLPP1 and β-TrCP are inversely proportional in the cell. However, when Akt phosphorylates GSK3β, this inhibits its activity, resulting in suppression of GSK3β-dependent degradation of PHLPP.[21,22] Furthermore, in cancer cells, mTORC1 through p70S6K regulates the protein expression of PHLPP. It was found that treatment with rapamycin, an inhibitor of mTORC1, resulted in a decrease of PHLPP expression without affecting mRNA levels or protein degradation. Interestingly, the addition of constitutively activated p70S6K countered the rapamycin affect and increased PHLPP protein expression, suggesting p70S6K is responsible for the increased protein expression (Scheme 5).[21,23]

### ***1.4 PHLPP and its function***

PHLPP are members of the PP2C subclass of protein phosphatase magnesium/manganese ($Mg^{+2}$/$Mn^{+2}$) -activated (PPM) family of serine/threonine phosphatases and are insensitive to phosphatase inhibitors such as okadaic acid and calyculin A.[21] In addition to requiring $Mg^{+2}$ or $Mn^{+2}$ for its catalytic activity, the PDZ ligand domain of PHLPP is essential for its ability to dephosphorylate Akt.[21] Overexpression of PHLPP in lung cancer cells showed a decrease in Akt phosphorylation specifically at Ser473 in comparison to cells without PHLPP.[17] This suggests that PHLPP dephosphorylates Akt only at its HM. Through mutational studies of its domains, it was found that the PDZ binding motif of PHLPP is necessary for its ability to dephosphorylate and regulate Akt.[17]

Although PHLPP1 and PHLPP2 were shown to dephosphorylate Ser473 on Akt, knockdown studies in cancer cells have shown differential regulation of the phosphorylation state of specific Akt isoforms, thereby affecting the phosphorylation of different downstream targets.[16,19] Through co-immunoprecipitation and knockdown studies in lung and breast cancer cells, it was determined that PHLPP1 has specificity towards Akt2 and Akt3 whereas PHLPP2 dephosphorylates Akt1 and Akt3.[16,19,24] Furthermore, this selectivity influenced downstream Akt targets: PHLPP1 affected GSK3α while PHLPP2 affected p27, and both PHLPP isoforms affected GSK3β and TSC2.[16,19,24] However, knockdown of PHLPP1 in cardiomyocytes demonstrated an equal increase in Ser473 phosphorylation as well as activity of both Akt1 and Akt2.[20] Moreover, studies involving PHLPP1 KO mice do not show specificity towards Akt isoforms in the heart or brain suggesting that this may be a cell-type specific event.[20,25]

It was determined in cancer cells that overexpression of PHLPP promoted apoptosis and suppressed proliferation through directly dephosphorylating Akt.[17] Besides Akt, PHLPP targets other AGC kinases such as protein kinase C (PKC), p70S6K, and Mst1 (Scheme 5).[2,19,21,26] Overexpression of PHLPP1 or PHLPP2 in kidney cells dephosphorylated PKC βII at its HM thereby destabilizing PKC.[26] Furthermore, a knockdown of both PHLPP isoforms resulted in an increase in PKC βII expression in colon cancer and normal breast epithelial cell lines.[26] Unlike cancer cells, knockdown of PHLPP1 in primary cardiomyocytes or astrocytes did not change the level of PKC isoforms.[20,25] Likewise, this was observed in the hearts and brains of PHLPP1 KO mice.[20,25]

In cancer cells, PHLPP promotes apoptosis by dephosphorylating and regulating the function or expression of several proteins including p70S6K and Mst1. As mentioned above, p70S6K initiates protein translation, and is tightly regulated by mTORC1. Studies in cancer cells have shown that PHLPP directly dephosphorylates Thr229 and Thr289 of p70S6K independent of Akt activation thereby repressing its function.[21,27] Also, Mst1 is a pro-apoptotic kinase and is directly phosphorylated by Akt, which in turn blocks its activity. It has been demonstrated that Mst1 is dephosphorylated directly at Thr387 by PHLPP resulting in its activation, which in turn targets p38 and JNK to induce apoptosis.[9,21,28]

### ***1.5 Akt and Hypertrophy in the Heart***

Studies using overexpression of Akt or isoform specific KO mouse models demonstrated Akt as a critical regulator of physiological and pathological hypertrophy.[4,5] Cardiac-specific overexpression of activated PI3K (p110α isoform), constitutively active Akt (E40K mutant Akt or CA-Akt), and myristoylated Akt (membrane-targeted Akt or myr-Akt) have all consistently demonstrated cardiac hypertrophy.[5,11,29,30] Cardiac-specific overexpression of CA-Akt1 led to a modest hypertrophic growth of cardiomyocytes and the heart did not exhibit pathological hypertrophy or contractile dysfunction.[31] In contrast, myr-Akt mice showed evidence of gross cardiac hypertrophy with some mice characterized with massive cardiac dilatation followed by death.[7,29,31,32] Furthermore, cardiac-specific inducible myr-Akt1 transgenic mice demonstrated that short-term Akt activation was associated with modest heart growth in size with preserved contractility and reversible hypertrophy while long-term Akt activation was correlated with

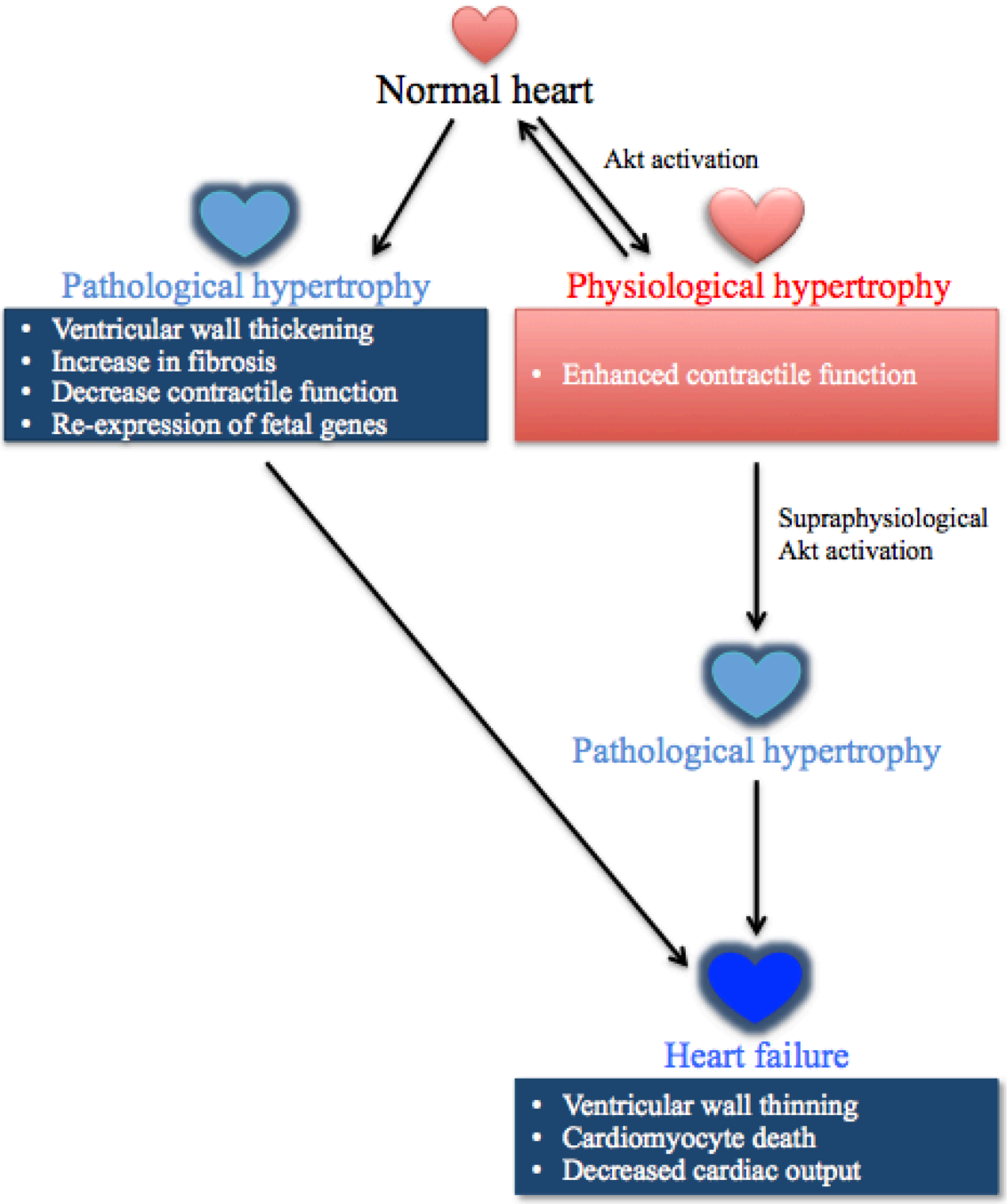
irreversible excessive cardiac hypertrophy and contractile dysfunction.[11,32] However, under normal stimulation Akt first associates with the membrane and upon translocation accumulates in the nucleus. Overexpression of a nuclear-targeted Akt demonstrated a profound inhibition of apoptotic activity and no evidence of hypertrophic growth, though, there was an increase in cardiomyocyte number.[6,33] These studies show the impact of Akt signaling on cardiac hypertrophy, specifically the importance of the duration and localization of Akt.

Studies using Akt KO mice models demonstrated impaired heart development. Akt1 KO mice developed enhanced cardiac growth in response to pathological stimuli (pressure overload) while being resistant to physiological stimuli (swimming training-induced hypertrophy), suggesting that Akt signaling actively suppresses signaling pathways that promote pathological hypertrophy while is responsible for physiological growth.[34] Akt2 KO mice demonstrated an increase in cardiomyocyte death following myocardial infarction.[14] The less studied Akt in the cardiovascular field is Akt3. Akt3 KO mice showed no apparent cardiac differences, having normal heart growth with no change in cardiomyocyte size at baseline.[13] These studies suggest the importance of Akt signaling in proper development and cardiac function.

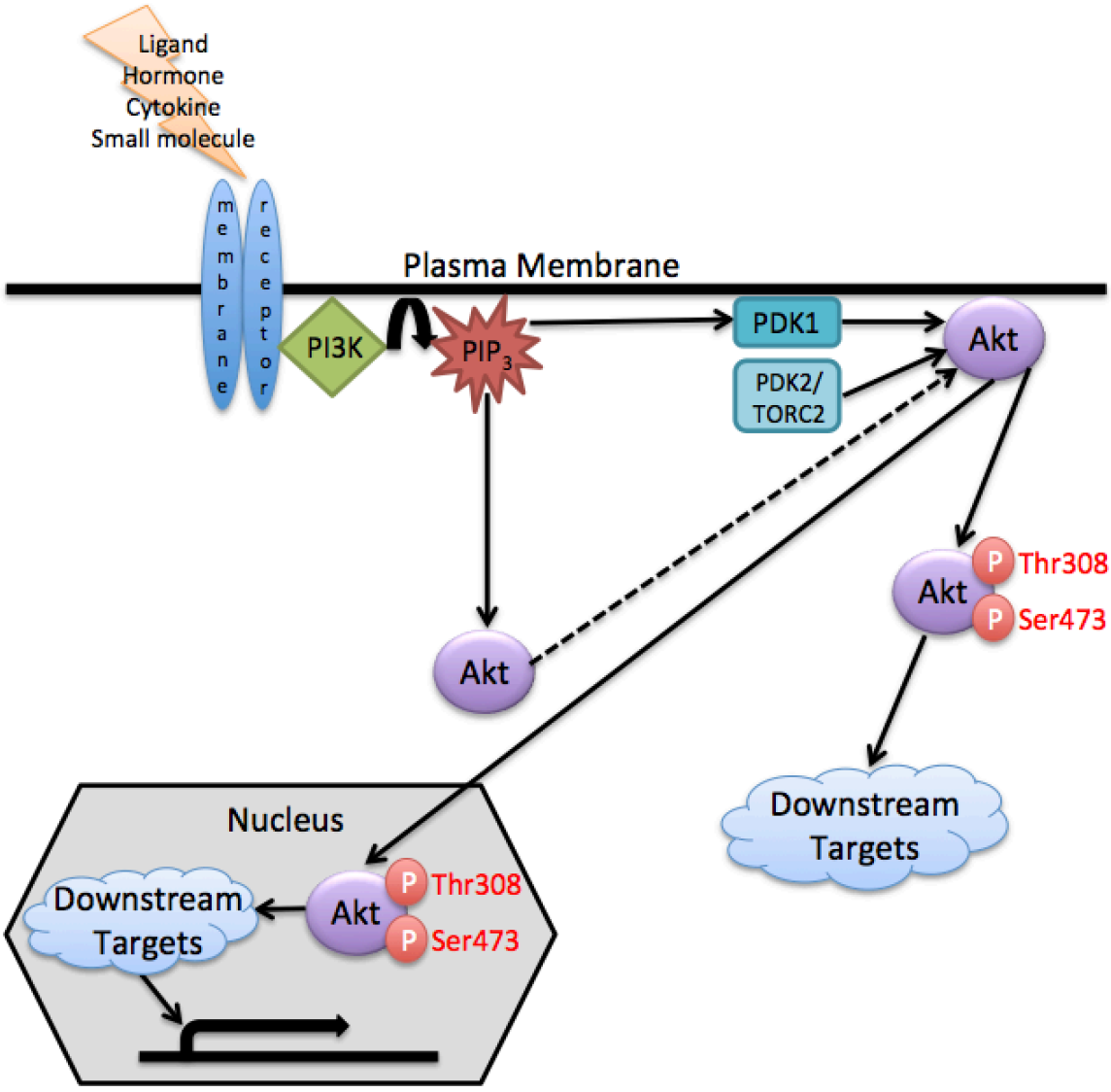
Given the importance of Akt in cell growth, survival, and cell cycle regulation, it is apparent the need for increased research efforts to determine the signaling pathways which regulate Akt following pathological and physiological hypertrophic responses in the heart. Since activation of Akt plays a pivotal role in cardiac growth, and our lab has demonstrated that removal of PHLPP1 in cardiomyocytes accentuates Akt activity, we wanted to determine the cardiac phenotype of global PHLPP1 KO mice. Mice will be

subjected to physiological and pathological stresses to determine whether increased Akt activity by PHLPP1 removal augments cardiac growth. These studies will allow us to understand the role of PHLPP1 in the heart and provide insight to future experimental direction. Ultimately our findings may lead us to therapeutic strategies for treating cardiac hypertrophy and heart failure.

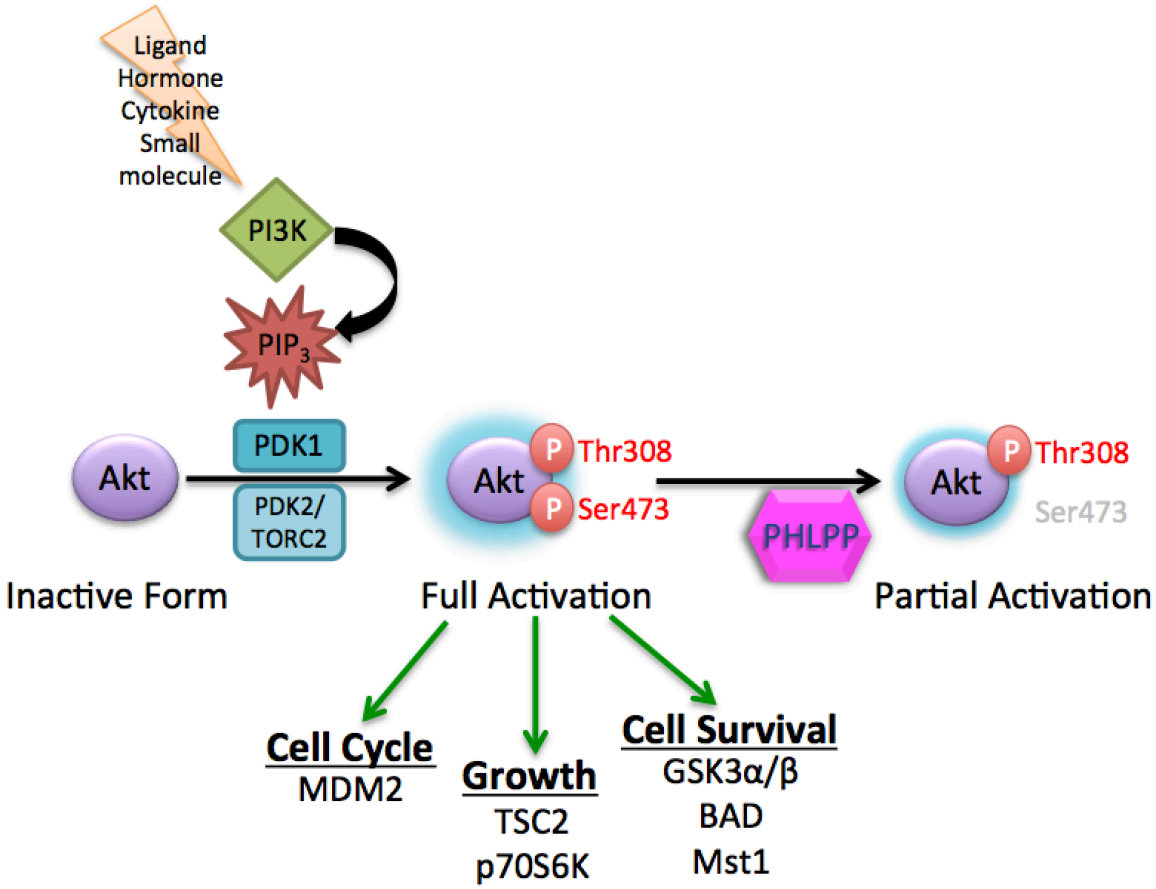
***1.6 Schemes***



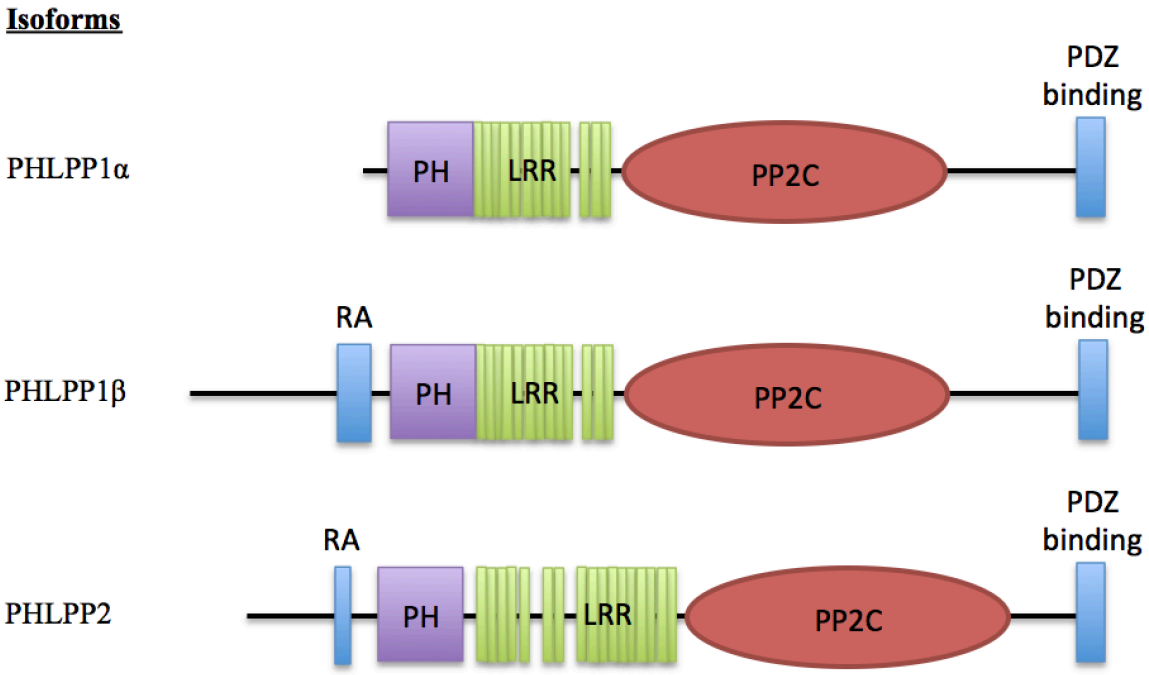

**Scheme 1: Types of hypertrophy.** Stresses such as, hypertension, atherosclerosis, and valve defects causes the heart to undergo irreversible remodeling known as pathological hypertrophy which eventually leads to heart failure. However, exercise or pregnancy causes a physiological hypertrophy through activation of the Akt signaling pathway, although continuous supraphysiological activation leads to pathological changes and eventually heart failure.



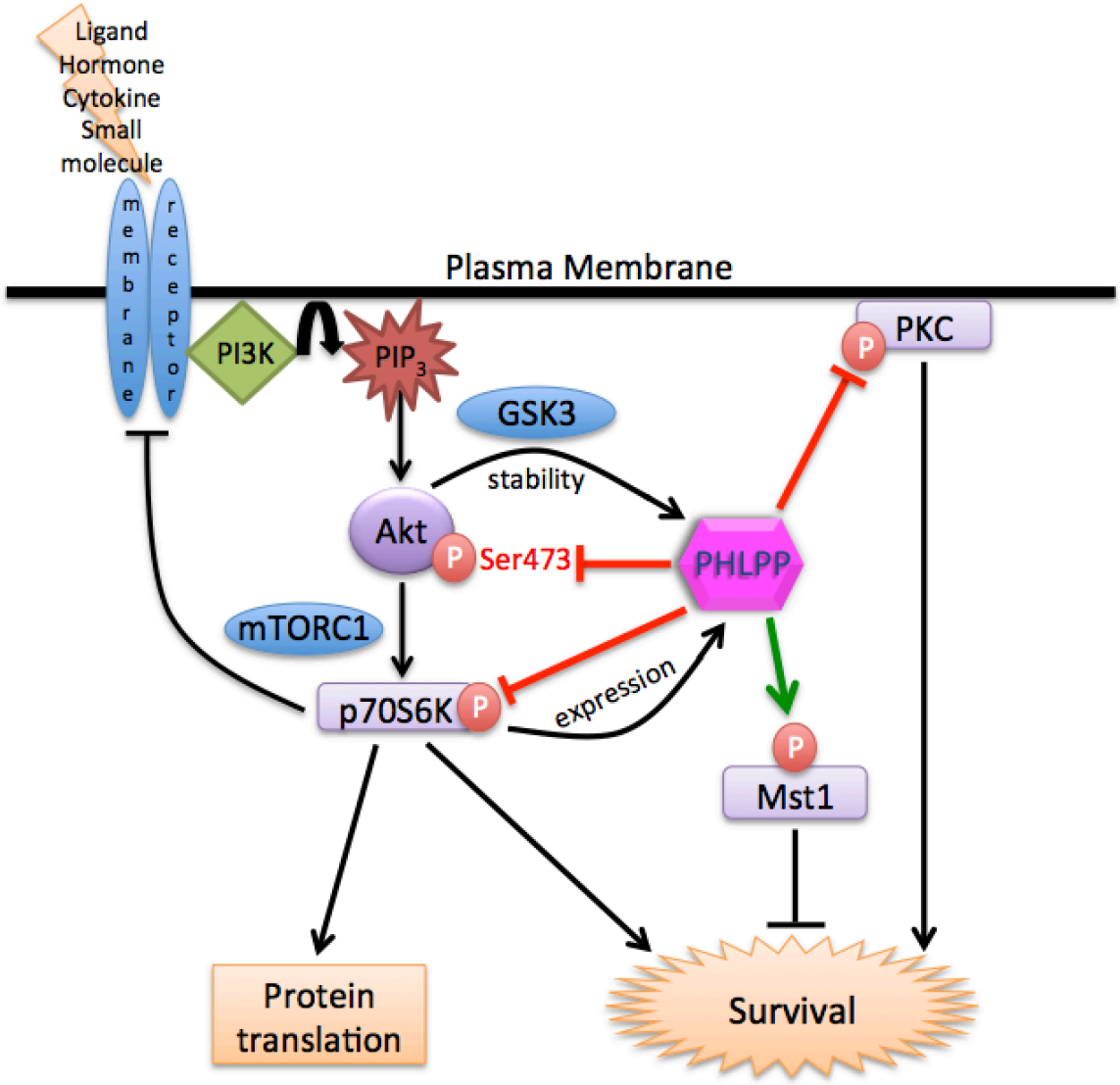

**Scheme 2: Akt signaling pathway.** Various stimuli bind receptors on the cell membrane to activate phosphatidylinositol 3-kinase (PI3K), which increases phosphatidylinositol 3,4,5-triphosphate ($PIP_3$) levels. $PIP_3$ recruits molecules with PH domains to the plasma membrane such as Akt and phosphoinositide-dependent kinase 1 (PDK1). Akt becomes phosphorylated at threonine-308 (Thr308) and serine-473 (Ser473) by PDK1 and PDK2/TORC2, respectively. Activated Akt translocates from the cytosol to the nucleus and various compartments within the cell to phosphorylate its downstream targets.




**Scheme 3: Activation of Akt targets.** Akt is fully active when it is phosphorylated at both threonine-308 (Thr308) and serine-473 (Ser473). Activated Akt directly phosphorylates downstream targets involved in preventing cell cycle arrest (MDM2) and promoting growth (TSC2 and p70S6K). Activated Akt phosphorylates and negatively regulates GSK3, BAD, and Mst1 to increase cell survival. PHLPP specifically dephosphorylates Akt at the Ser473 site, resulting in a partially active Akt.




**Scheme 4: Schematic of PHLPP isoforms.** All three isoforms contain a pleckstrin homology (PH) domain, leucine-rich repeat (LRR) segments, a protein phosphatase 2C (PP2C) domain, and ending with a PDZ (PSD95, Dlg1, Zo-1) ligand at the carboxyl terminus. Both PHLPP1β and PHLPP2 have a Ras-association (RA) domain at the amino terminus, preceding the PH domain. PHLPP1α and PHLPP1β are splice variants of the same gene located at chromosome 18q21.33 whereas PHLPP2 is located at chromosome 16q22.3. The molecular weight of PHLPP1α, PHLPP1β, and PHLPP2 in *Mus musculus* is ~150kDa, ~180kDa, and ~170kDa, respectively.




**Scheme 5: PHLPP signaling pathway.** PHLPP dephosphorylates several AGC kinases. PHLPP inactivates targets involved in protein translation and cell survival (Akt, p70S6K, and PKC) while it activates Mst1 to promote apoptosis. There are two negative feedback loops controlling PHLPP levels: high Akt activity suppresses GSK-3 mediated PHLPP degradation and p70S6K activity increases PHLPP expression levels.

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# CHAPTER 2

## Effects of PHLPP1 Removal in the Heart

### *2.1 Abstract*

**Aims** To examine the role of physiological Akt signalling in pathological hypertrophy through analysis of PHLPP1 (PH domain leucine-rich repeat protein phosphatase) knock-out (KO) mice.

**Methods and results** To investigate the *in vivo* requirement for 'physiological' control of Akt activation in cardiac growth, we examined the effect of deleting the Akt phosphatase, PHLPP, on the induction of cardiac hypertrophy. Basal Akt phosphorylation increased nearly two-fold in the cardiomyocytes from PHLPP1 KO mice and physiological hypertrophy induced by swimming exercise was accentuated as assessed by increased heart size and myocyte cell area. In contrast, the development of pathophysiological hypertrophy induced by pressure overload and assessed by increases in heart size, myocyte cell area, and hypertrophic gene expression was attenuated. This attenuation coincided with decreased fibrosis and cell death in the KO mice. Cast moulding revealed increased capillary density basally in the KO hearts, which was further elevated relative to wild-type mouse hearts in response to pressure overload. *In vitro* studies with isolated myocytes in co-culture also demonstrated that PHLPP1 deletion in cardiomyocytes can enhance endothelial tube formation. Expression of the pro-angiogenic factor VEGF was also elevated basally and accentuated in response to transverse aortic constriction in hearts from KO mice.

**Conclusion** Our data suggest that enhancing Akt activity by inhibiting its PHLPP1-mediated dephosphorylation promotes processes associated with physiological hypertrophy that may be beneficial in attenuating the development of pathological hypertrophy.

## *2.2 Introduction*

In response to diverse stressors, the adult heart undergoes hypertrophic enlargement, that is, a compensatory process that attempts to maintain or augment pump function. Normal hypertrophy observed in trained athletes or following postnatal development is referred to as 'physiological' hypertrophy.[1-4] In contrast, cardiac hypertrophy observed under conditions such as hypertension or myocardial infarction is referred to as 'pathological' hypertrophy since it is associated with cardiac dysfunction that ultimately transitions to heart failure.[5,6] Pathological growth is characterized by re-expression of a foetal gene programme, including β-myosin heavy chain (β-MHC) and α-skeletal actin (α-skActin), as well as by up-regulation of the natriuretic peptides, atrial natriuretic peptide, and brain natriuretic peptide (BNP).[7] Endocrine, paracrine, and autocrine regulatory factors directly influence cardiac hypertrophy and apoptosis through their actions on membrane receptors, including G-protein-coupled receptors, which transduce signals involving enzymes such as mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), calcineurin-NFAT, and Akt.[6-8,10]

Numerous studies have implicated Akt in regulating cardiac growth,[11-13] contractile function,[11,14-16] and angiogenesis.[17-19] Dysregulation of Akt activity has been associated with cancer, diabetes, and cardiovascular disease.[8,20,21] There are three

isoforms of Akt (Akt1, Akt2, and Akt3) encoded by different genes and expressed in a tissue-specific manner in mammalian cells. The Akt isoforms share similar structures, but their physiological functions *in vivo* are non-redundant as indicated by distinct phenotypes in isoform-specific gene-targeted mice.[22-24] Recently, a novel protein phosphatase PHLPP [PH domain leucine-rich repeat (LRR) protein phosphatase] was discovered, which dephosphorylates Akt on its hydrophobic motif, contributing to termination of Akt signalling. The PHLPP family of phosphatases is comprised of three members, PHLPP1α and PHLPP1β (splice variants of the same gene) and PHLPP2. The PHLPP isoforms have an identical domain structure in which a PH domain is followed by a region of LRRs, a PP2C phosphatase domain, and a C-terminal PDZ ligand domain.[25-26] In addition, PHLPP1β and PHLPP2 contain a Ras-association domain (RA domain) preceding the PH domain.[26-27] Cellular localization studies reveal that PHLPP1 and PHLPP2 are present throughout the cell[25-26] and both are expressed in cardiomyocytes.[28] PHLPP has also been found to dephosphorylate the hydrophobic motif of conventional and novel PKC isoforms *in vitro*, affecting PKC stability and thus the level of expression.[29] However, studies in which PHLPP was removed in cardiomyocytes and the heart demonstrated that the expression of PKC was unaffected.[28] Recently, other substrates with hydrophobic motifs have been identified as substrates for dephosphorylation by PHLPP. One such protein, S6K, is involved in protein translation and its activity is reduced by dephosphorylation by PHLPP.[30] Also, Mst1, which is a pro-apoptotic kinase, is a direct target of PHLPP through dephosphorylation of its inhibitory site Thr387. However, since Akt down-regulates Mst1 by phosphorylating its inhibitory site to cause protection, PHLPP therefore can regulate Mst1 indirectly by limiting Akt

activity.[31] Overall, PHLPP limits second messenger signalling and opposes cell growth and survival.

Physiological hypertrophy is initiated by various stimuli including mechanical stretch, endocrine, and hormonal factors.[2,3,32] Importantly, during physiological hypertrophy, there is no significant interstitial fibrosis or cardiac dysfunction nor is there up-regulation of foetal gene expression.[3] Akt activation is required for physiological growth of the heart,[12] and cardiac-specific overexpression of a constitutively active Akt mutant increases angiogenesis and cardiac mass and has been shown to be cardioprotective following pressure overload.[33-34] However, prolonged expression of activated Akt, which is membrane targeted, has negative inotropic effects and leads to decompensation and heart failure.[35] The location of constitutively activated Akt accounts for the development of pathophysiology since nuclear or SR targeted Akt has no deleterious effect on cell size in the heart and improves cardiac function and reduces apoptosis under pathological stress.[36-38] The level of Akt activity in these transgenic model systems is supraphysiological thus sustained high levels of kinase activity and its location could account for the development of deleterious effects. The effects of more physiological spatiotemporal increases in Akt signalling on cardiovascular growth and function have not been directly examined. Global deletion of PHLPP1 elevates basal and transiently accentuates agonist-induced Akt activation in the heart.[28] Using this genetic model, we demonstrate that a modest increase in Akt activity in the heart does not affect basal cardiomyocyte growth. In the face of stress induced by pressure overload, however, hypertrophy is diminished while in contrast the physiological hypertrophic response to swimming is accentuated. We suggest that removing the normal break on Akt activation

imposed by the PHLPP1 phosphatase accentuates physiological Akt activation, which exerts salutary effects in protecting the heart against pathological stress.

## ***2.3 Materials and Methods***

### *2.3.1 Animals*

All procedures were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of California San Diego. Generation of *global* homozygous Black Swiss/SV129 PHLPP1 knock-out (KO) mice has been previously described.[28,39] All experiments were performed on age-matched male wild-type (WT) and KO littermates.

### *2.3.2 Hypertrophy studies*

Pathological hypertrophy was induced by transverse aortic constriction (TAC) in male 8- to 10-week-old WT and KO mice as previously described.[40,41] Mice were anaesthetized under 2% isoflurane, intubated, and ventilated. Mice were euthanized by cervical dislocation and analysed following 1 day or 2 weeks TAC. For functional assessment following pressure overload, non-invasive echocardiography was performed to calculate fractional shortening and pressure gradients as described previously.[40,41] Echocardiographic assessment of function following TAC was performed in a blinded fashion. Swimming for 20 days as a model of exercise-induced hypertrophy was also performed.[41] Swimming mice were monitored for equal exertion. Upon completion of the study, mice were euthanized by cervical dislocation and cardiac phenotype analysed.

*2.3.3 Immunoblotting*

Hearts were obtained from WT and KO mice at baseline or subjected to TAC or exercise for various times. Hearts were removed directly and immediately frozen in liquid nitrogen till further processing. Adult mouse ventricular myocytes (AMVMs) were isolated from WT and KO mice as previously described.[28] Tissue or cells were homogenized in lysis buffer[28] and protein concentrations determined by Bradford assay. Total and phosphorylated proteins were determined by western blot as previously described.[28] The PHLPP1 and PHLPP2 antibodies used were from Bethyl Laboratories (Montgomery, TX, USA). Total Akt, phosphorylated (phopsho)-Akt (Ser473 and Thr308), phospho-MDM2, phospho-GSK3α/β, phospho-ERK1/2, phospho-Mst1(Thr183), phospho-p70S6kinase, cleaved caspase 9, cleaved caspase 3, VEGF-a, actinin, and GAPDH antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). Total pan PKC, PKCα, and PKCδ were from Santa Cruz Biotechnology (Dallas, TX, USA). Fold changes were determined by densitometry, normalized to accompanying GAPDH or actinin blots, and changes were expressed as relative values compared with WT samples.

*2.3.4 Akt kinase assay*

Extracts (150 μg) from WT and KO hearts were incubated with an antibody to Akt1 overnight. Akt catalytic activity was assessed using a non-radioactive Akt kinase assay kit following the manufacturer's protocol (Cell Signaling Technology).[28] The change in catalytic activity of Akt was determined by densitometry of a phosphorylated

GSK-3 substrate and normalized to total immunoprecipitated Akt. The activity of Akt was expressed as fold change relative to WT or control samples.

*2.3.5 Immunohistochemistry*

Hearts were collected at the indicated times as previously described,[41] fixed in 3.5% paraformaldehyde, and embedded in paraffin. Serial 5 μm sections were cut and stained with haematoxylin and eosin (H&E) to look at gross morphology or Masson's trichrome for collagen deposition. For assessment of cross-sectional area, tissue was stained with TRITC-labelled wheat germ agglutinin (WGA; Sigma, St Louis, MO, USA) and nuclei with DAPI (Vector Laboratories, Burlingame, CA, USA). Co-staining for capillaries was performed with anti-CD31 (EMD Millipore, Billerica, MA, USA). For cellular death associated with TAC, apoptosis was detected using a TRITC deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) protocol (Roche, San Francisco, CA, USA) and co-stained with DAPI for nuclear visualization.[39] In brief, an operator blinded to the treatment set counted cell death, capillary-to-myocyte ratio, and measured and quantified fibrosis and the cellular area across cells with central nuclei using the ImageJ software (version 1.40 g, NIH, Bethesda, MD, USA).

*2.3.6 RNA isolation and qPCR*

Total RNA was isolated using a micro-RNA isolation kit (Invitrogen, Carlsbad, CA, USA) from WT and KO hearts following TAC. cDNA was synthesized using the Verso cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) according to

the manufacturer's protocol. Amplification of genes involved in cardiac hypertrophy and angiogenesis [atrial natriuretic factor (ANF), BNP, α-skActin, β-MHC, collagen 1a (Col1a), collagen 3a (Col3a), VEGF-a, angiopoietin 2 (Angp2), and GAPDH] was analysed using specific probe sets from Applied Biosystems (Invitrogen). The fold change was calculated as previously described[42] and data were normalized to WT Sham.

*2.3.7 Microfil injection and coronary cast moulding*

Corrosion casting was used to evaluate the intact three-dimensional vascular network in the heart. The microfil injection was based on the previously described protocol.[43] The microfilm resin was prepared according to the protocol in Batson's no. 17 plastic replica and corrosion kit (Polysciences, Inc., Warrington, PA, USA). The hearts were visualized on a dissecting microscope with a CCD camera (×2 magnifications).

*2.3.8 Endothelial tube formation assay*

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1-day-old rat pups that were euthanized by $CO_2$ followed by decapitation as previously described.[28] Isolated cells ($0.15 \times 10^6$/well) were plated on Matrigel pre-coated 24-well plates. Following 24 h of plating, cardiomyocytes were transfected with either scrambled control or a pre-designed rat PHLPP1 ON-TARGET*plus* siRNA as previously described.[28,44] After overnight incubation, cells were washed and cultured for another 24 h in serum-free media. Bovine aortic endothelial cells (BAECs; Lonza, Walkersville, MD, USA) were plated on top of NRVMs ($0.75 \times 10^5$/well) in the presence of 5% foetal bovine serum and 3 ng/mL of basic fibroblast growth factor (bFGF). Tube formation was

visualized 6 h later. An operator blinded to the treatment counted six fields photographed at random on an Olympus light microscope (×10 magnification) and the number of tubes/field was calculated.

*2.3.9 Statistical analysis*

Researchers were blinded to the treatment group during analyses. Data are represented as mean ± SEM. Differences are considered statistically significant ($P < 0.05$) assessed using unpaired Student's *t*-test (for two groups), one-way (for multiple comparisons), or two-way ANOVAs (for multiple comparisons involving two variables) with *post hoc* Tukey analysis using the GraphPad Prism software (GraphPad, La Jolla, CA, USA).

## ***2.4 Results***

*2.4.1 Deletion of PHLPP1 increases Akt activity, but does not affect cardiac size or contractile function*

Heart extracts from WT, heterozygous, and PHLPP1 KO mice were analysed for PHLPP expression. PHLPP1 protein was decreased by 25% in heterozygous mice and undetectable in the hearts of null mice (*Figure 1A*). No compensatory change in PHLPP2 expression was evident (*Figure 1A*). Removal of PHLPP1 did not affect total Akt expression; however, phosphorylation of Akt at Ser473 was increased nearly two-fold in AMVMs from the PHLPP1 KO (*Figure 1B*) without a change in Thr308 phosphorylation as shown previously.[28] Removal of PHLPP1 did not alter basal cardiomyocyte size. Additionally, analysis of 1-year-old PHLPP1 KO mice showed no significant difference

in cardiac function (*Figure 1C*) and no overt change in overall heart weight (*Figure 1D* and see Supplementary material online, *Table S1*) compared with WT.

*2.4.2 PHLPP1 KO mice have an accentuated response to exercise-induced hypertrophy*

Since Akt activation is widely recognized as a mediator of physiological growth of the heart, as occurs during exercise and in response to hormones such as IGF-1,[12,32,45,46] we investigated the effect of PHLPP1 removal on physiological hypertrophy. WT and KO mice were subjected to forced swimming for 20 days and heart size assessed (*Figure 2A*). The ability of exercise to induce hypertrophy was found to be significantly accentuated (39 vs. 23% increase) in PHLPP KO vs. WT mice, respectively (*Figure 2B*). Histological assessment of cardiomyocyte size confirmed greater physiological hypertrophy in KO vs. WT mouse hearts following exercise (*Figure 2C*). PHLPP KO mice also showed increased phosphorylation of Akt as well as several downstream targets (i.e. MDM2, p70S6Kinase, and ERK) relative to WT mice after 3 days swim (*Figure 2D*, left panel), which were normalized at 20 days swim (*Figure 2D*, right panel).

*2.4.3 Attenuation of pressure overload-induced hypertrophy in PHLPP1 gene-targeted mice*

In contrast to the documented role of Akt in physiological hypertrophy, the role of Akt in the development of pathological hypertrophy is controversial. To determine how PHLPP1 removal and subsequent changes in Akt phosphorylation would affect the development of pathological hypertrophy, WT and KO mice were subjected to pressure overload by TAC. The pressure gradients across the aortic constriction were shown to be

equivalent and no significant differences in contractile function assessed by echocardiography were observed for either group following 2 weeks TAC (see Supplementary material online,*Table S2*). PHLPP1 KO mice did, however, show a moderate but significant attenuation of hypertrophy, as demonstrated by a smaller increase in heart weight/body weight ratio than in WT mice (*Figure 3A*, 29 vs. 46% increase, respectively). Histological examination of the hearts also revealed a significantly reduced increase in cardiomyocyte cell size following TAC in PHLPP1 KO hearts compared with WT (*Figure 3B*). Finally, up-regulation of hypertrophic genes (α-skActin, β-MHC, ANF, and BNP) by TAC was reduced in the KO mouse heart (*Figure 3C*).

*2.4.4 Akt activation in response to pressure overload*

As previously reported, PHLPP1 KO hearts have increased basal Akt Ser473 phosphorylation (*Figure 4A* and *C*) and Akt1 activity (*Figure 4B*).[28] TAC significantly increased Akt Ser473 phosphorylation between 1 and 14 days TAC in PHLPP1 KO, but not in WT, mice (*Figure 4A*). Akt1 kinase activity also remained significantly greater in PHLPP1 KO vs. WT animals subjected to TAC (*Figure 4B*). All Akt targets examined (i.e. GSK3α/β, MDM2, p70S6Kinase, and ERK1/2) had enhanced phosphorylation in the PHLPP1 KO vs. WT hearts at 1 day TAC (*Figure 4C*, left panel), which were equalized by 14 days (*Figure 4C*, right panel).

*2.4.5 PHLPP1 KO mice have decreased fibrosis and cell death following TAC*

Since removal of PHLPP1 decreased pathological hypertrophy and activation of foetal genes, we examined cellular fibrosis and cell survival in WT and KO hearts following 2 weeks TAC. Remarkably, PHLPP1 KO hearts showed little fibrosis (*Figure 5A*) as demonstrated by Mason's trichrome staining and changes in Col1a or Col3a gene expression (*Figure 5B*). There was also significantly less cell death following 2 weeks TAC in the PHLPP1 KO mice as assessed by TUNEL staining (*Figure 5C*), and levels of cleaved caspase 9 and caspase 3 were significantly reduced (*Figure 5D*). In addition, activation of Mst1, a pro-apoptotic protein, which is inhibited by Akt activity, was reduced in the KO hearts relative to WT mice following TAC (*Figure 5D*).

*2.4.6 PHLPP1 KO mice have increased capillary density*

Since it has been demonstrated that increased Akt activity in the heart can increase angiogenesis[33,37] and recent evidence suggests that angiogenesis can contribute to hypertrophy development,[35,48] we explored the possibility that changes in angiogenesis might contribute to the altered cardiac growth response observed in the PHLPP1 KO mouse heart. To this end, cast moulding was used to look for possible changes in heart vessel structure. At baseline, the KO mice had an evident increase in capillary bed density compared with WT mice (*Figure 6A*). To determine the cause of the increase in capillary density, the expression of VEGFa, a protein that can stimulate angiogenesis, was examined. VEGFa protein was significantly elevated in PHLPP1 KO compared with WT mouse hearts at baseline (*Figure 6B*). We then examined the effect of TAC on angiogenesis in WT vs. KO mice as assessed by the ratio of capillaries to myocytes in the

heart. KO mice had a significant increase in the ratio of capillary to myocyte at baseline compared with WT mice and following 2 weeks TAC displayed a significantly greater increase than that of their WT counterparts (*Figure 6C*). Furthermore, the expression of mRNA for Angp2 and VEGFa, markers of angiogenesis, were increased in KO, but not in WT, mice following 2 weeks TAC (*Figure 6D*).

To determine whether paracrine signalling from cardiomyocytes in PHLPP KO mice could affect capillary density, we carried out *in vitro* capillary tube formation experiments. Neonatal rat cardiomyocytes treated with scrambled or PHLPP1 siRNA were co-cultured with BAECs. siRNA-mediated knockdown of PHLPP1 in cardiomyocytes increased the number of tubes formed following serum stimulation compared with scrambled siRNA-treated cells (*Figure 7A*). Without BAECs, siRNA-mediated knockdown of PHLPP1 increased VEGFa mRNA expression compared with siRNA-scrambled control in the presence of serum (*Figure 7B*), suggesting that increased Akt in cardiomyocytes stimulates the production of angiogenic factors leading to increased angiogenesis.

## *2.5 Figures*

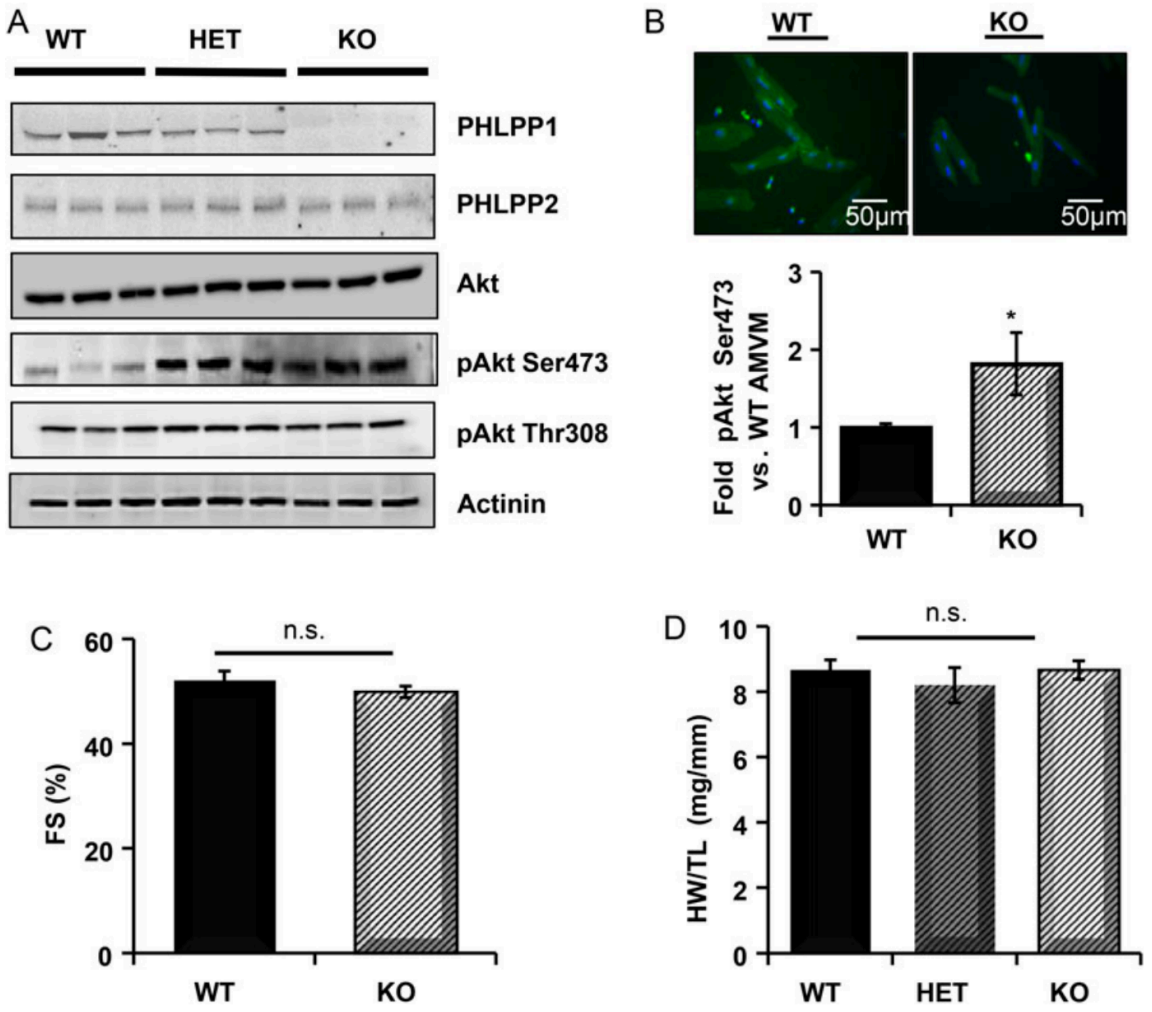



**Figure 1: Analysis of cardiac changes in PHLPP1 KO mice.** (*A*) Heart extracts (25 µg) from WT, heterozygous PHLPP1 (HET), and PHLPP1 KO mice were analysed for PHLPP isoform expression and Akt phosphorylation ($n = 3$). (*B*) Isolation of AMVMs and quantitation of Ser473 phosphorylation on Akt from WT and KO mice. (The graph represents $n = 4$ independent isolations WT/KO; $^*P < 0.05$, ×32 magnification.) (*C*) Percent fractional shortening (FS) measurement by non-invasive echocardiography of WT and KO mice at 12 months of age ($n = 9$). (*D*) Gravimetric analysis of heart weight-to-tibia length (HW/TL) ratio of WT, HET, and KO mice at 12 months of age ($n = 9$). n.s.: non-significant.

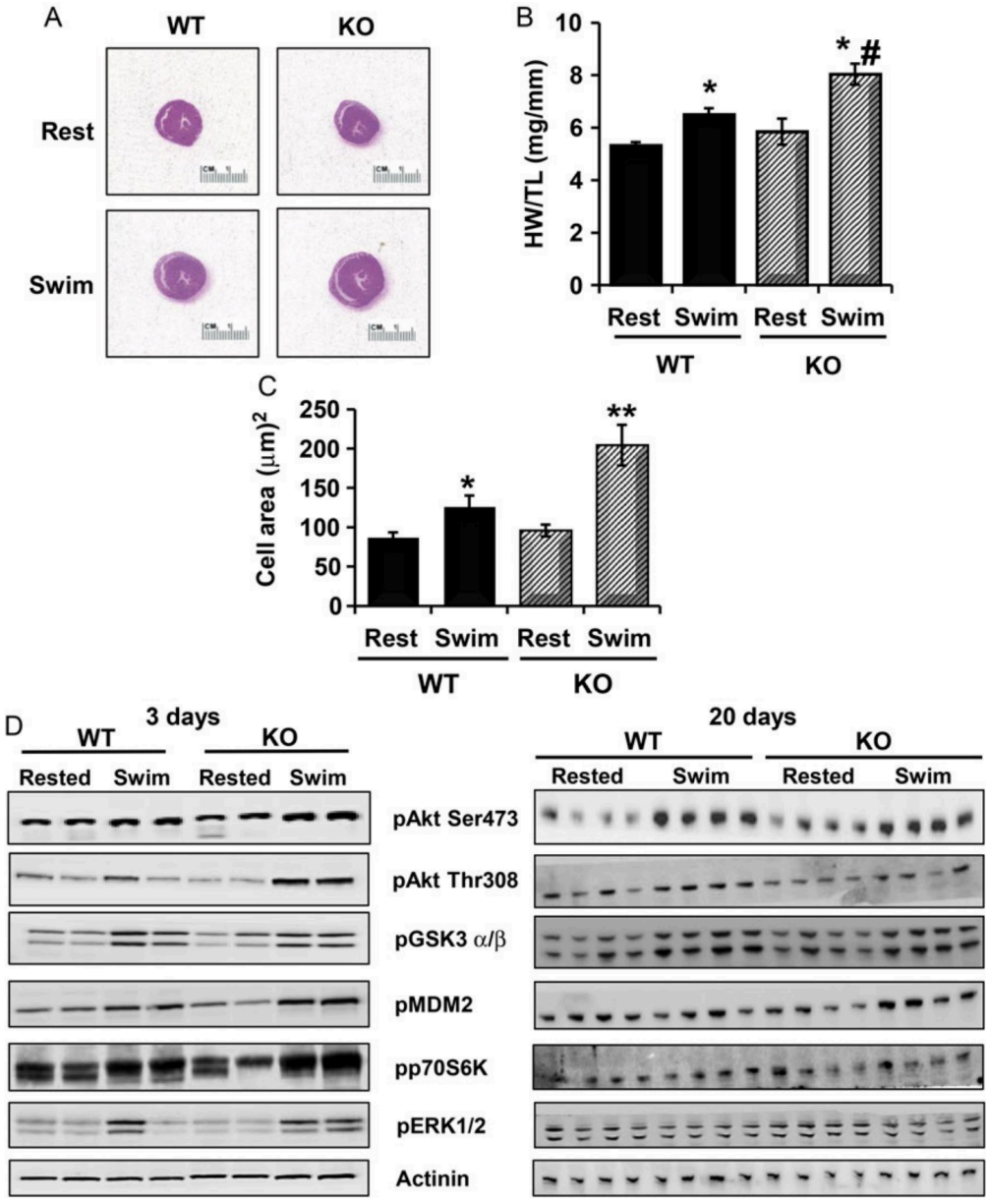



**Figure 2: Removal of PHLPP1 increases the physiological response of the heart to exercise.** WT and KO mice (12 weeks of age) were subjected to forced swimming for 20 days. (*A*) Representative H&E staining of WT and KO hearts at rest and following Swim. (*B*) Heart weight (HW) to tibia length (TL) of WT and KO mice at rest or following swim were analysed. (The graph represents $n = 9$ independent samples WT/KO; $*P<0.05$ compared with WT rest, $^{\#}P<0.05$ compared with WT swim.) (*C*) Measurement of myocyte cross-sectional area from ventricles of WT and KO mice at rest and following 20 days swim. (500 cells were counted, $n = 4$ WT/KO per group; $*P<0.05$ or $**P<0.01$ compared with WT rest.) (*D*) Protein analysis of Akt phosphorylation and downstream targets by western blotting in WT and KO heart extracts (15 μg) following 3 days ($n = 2$) and 20 days swim ($n = 4$). Actinin was blotted as a loading control.

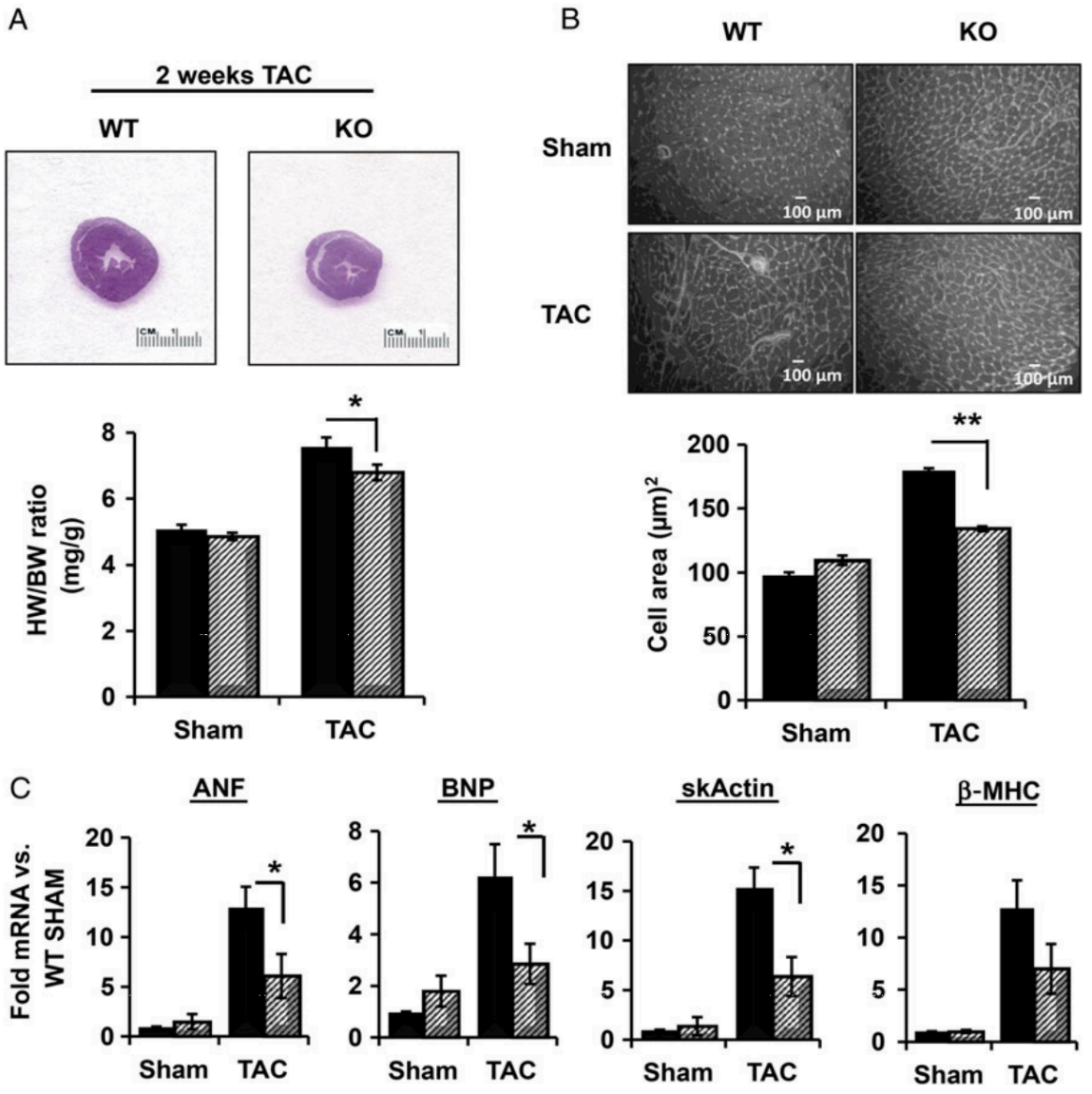



**Figure 3: Attenuation of pathological hypertrophy induced by pressure overload in PHLPP1 KO mice.** WT (black) and KO (striped) mice (8 weeks old) were subjected to pressure overload by TAC for 2 weeks. (*A*) Representative H&E staining of WT and PHLPP1 KO hearts following TAC and graph of heart weight-to-body weight ratio. (The graph represents $n = 15$ independent samples WT/KO; *$P<0.05$ compared with WT TAC.) (*B*) Measurement of myocyte cross-sectional area from ventricles of WT (black) and KO mice (striped). Representative WGA staining for WT and KO hearts following TAC and the quantified graph represents cell area analysis. (500 cells were counted, $n = 3$ WT/KO per group; **$P<0.01$ compared with WT TAC.) (*C*) Isolation of RNA from WT (solid) and KO (striped) hearts and the relative mRNA fold changes in hypertrophic gene expression. (The graphs represent $n = 8$ independent samples; *$P<0.05$ compared with WT TAC.) ANF: atrial natriuretic factor; BNP: brain natriuretic peptide; skActin: α-skeletal muscle actin; β-MHC: beta myosin heavy chain.

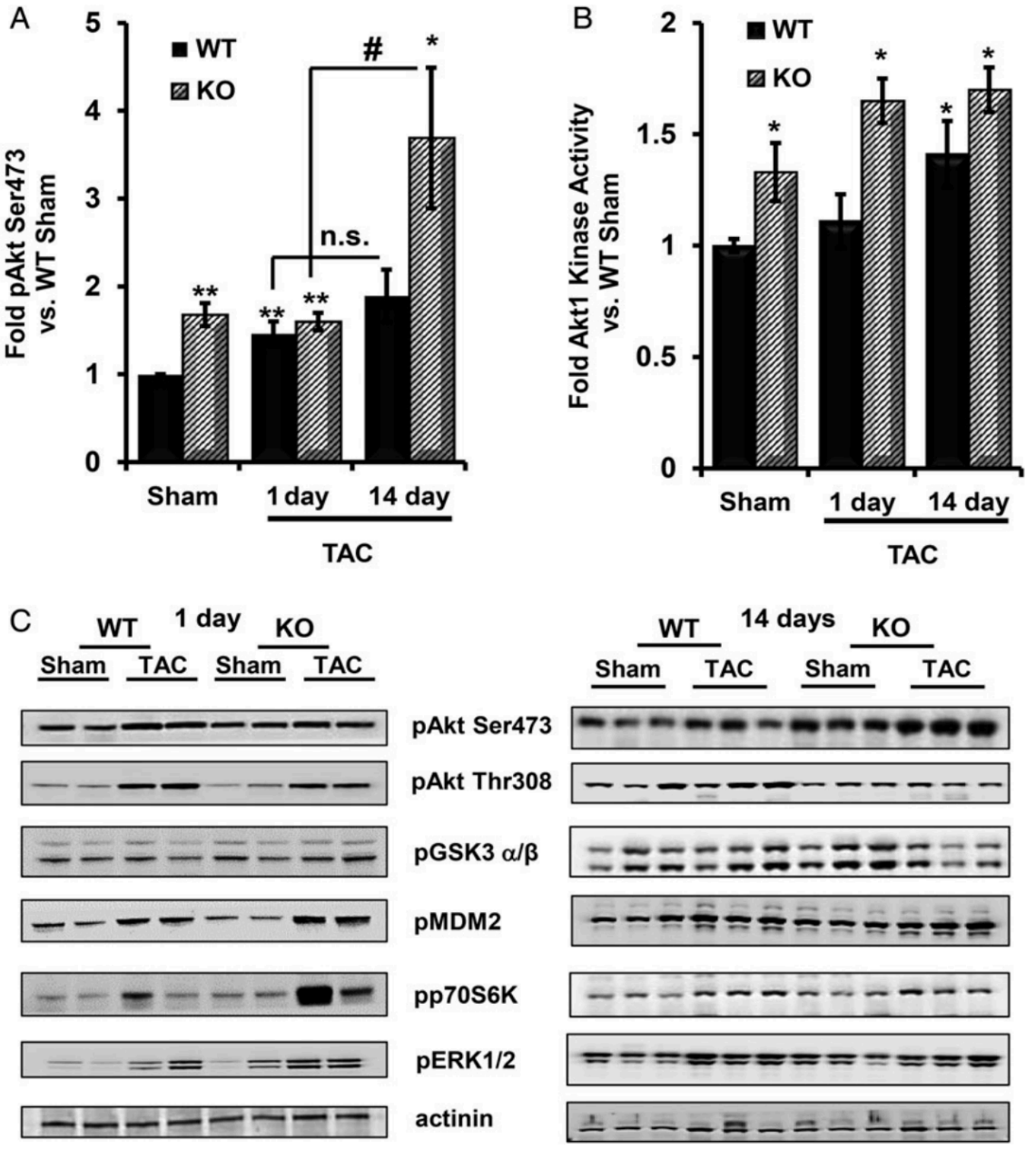



**Figure 4: Akt activation and downstream targets following pressure overload in the PHLPP1 KO mice.** WT and KO mice (12 weeks old) were subjected to TAC. (*A*) Quantification of Akt phosphorylation at Ser473 in WT and KO hearts following 1 and 14 days TAC. (The graph represents $n = 5$ hearts WT/KO; $^{*}P<0.05$ and $^{**}P<0.01$ compared with WT Sham and $^{\#}P<0.05$ compared with 1 day TAC.) (*B*) Fold change in Akt1 catalytic activity in WT and KO cardiac extracts following 1 and 14 days TAC. (The graphs represents $n = 4$ WT/KO, $^{*}P<0.05$ compared with WT Sham.) (*C*) Phosphorylation of Akt and its downstream targets in heart extracts (15 μg) from WT and KO mice following 1 day ($n = 2$) and 14 days TAC ($n = 3$). Actinin was blotted as a loading control.

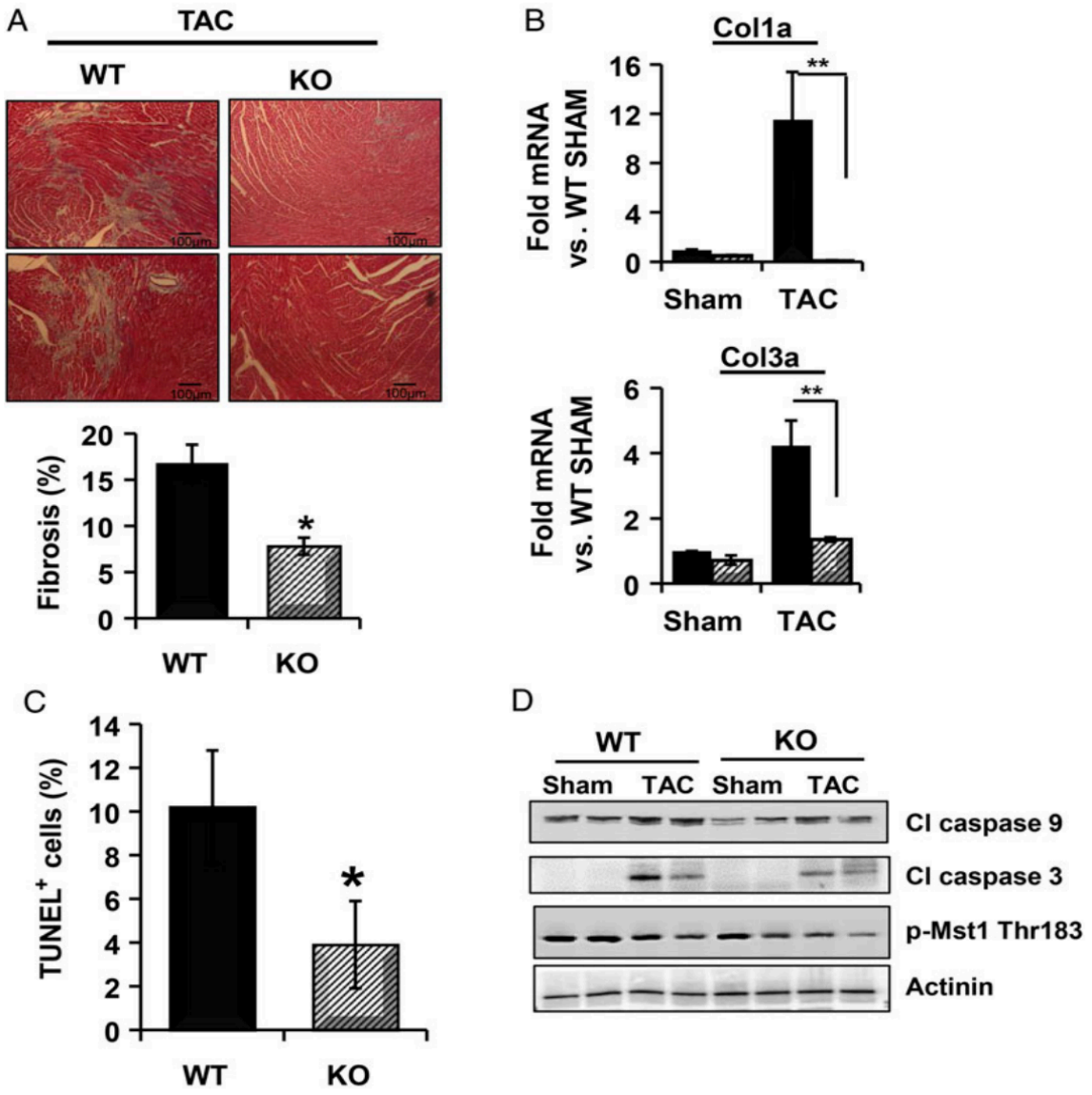



**Figure 5: PHLPP1 KO mice have reduced fibrosis and cell death following TAC.** (*A*) Representative Masson's trichrome-stained sections of WT and KO mice following 14 days TAC (×10 magnification). Percent fibrosis was calculated in WT and KO hearts from trichrome-stained sections using the Image J software. (The graph represents $n = 5$ hearts WT/KO; $^{*}P<0.05$ compared with WT TAC.) (*B*) Isolation of RNA from WT (solid) and KO (striped) hearts subjected to 2 weeks TAC and the relative mRNA fold changes in collagen gene expression. (The graphs represent $n= 8$ independent samples; $^{**}P<0.01$ compared with WT TAC.) Col1a: collagen 1a and Col3a: Col3a. (*C*) Cell death following 2 weeks TAC was determined in WT and KO hearts by TUNEL staining. The graph represents four hearts and six areas analysed ($^{*}P<0.05$ compared with WT TAC). (*D*) Western blot analysis of cleaved caspase 3, 9, and Mst1 phosphorylation at Thr183 from WT and KO hearts ($n = 2$) following TAC. Actinin was blotted as a loading control.

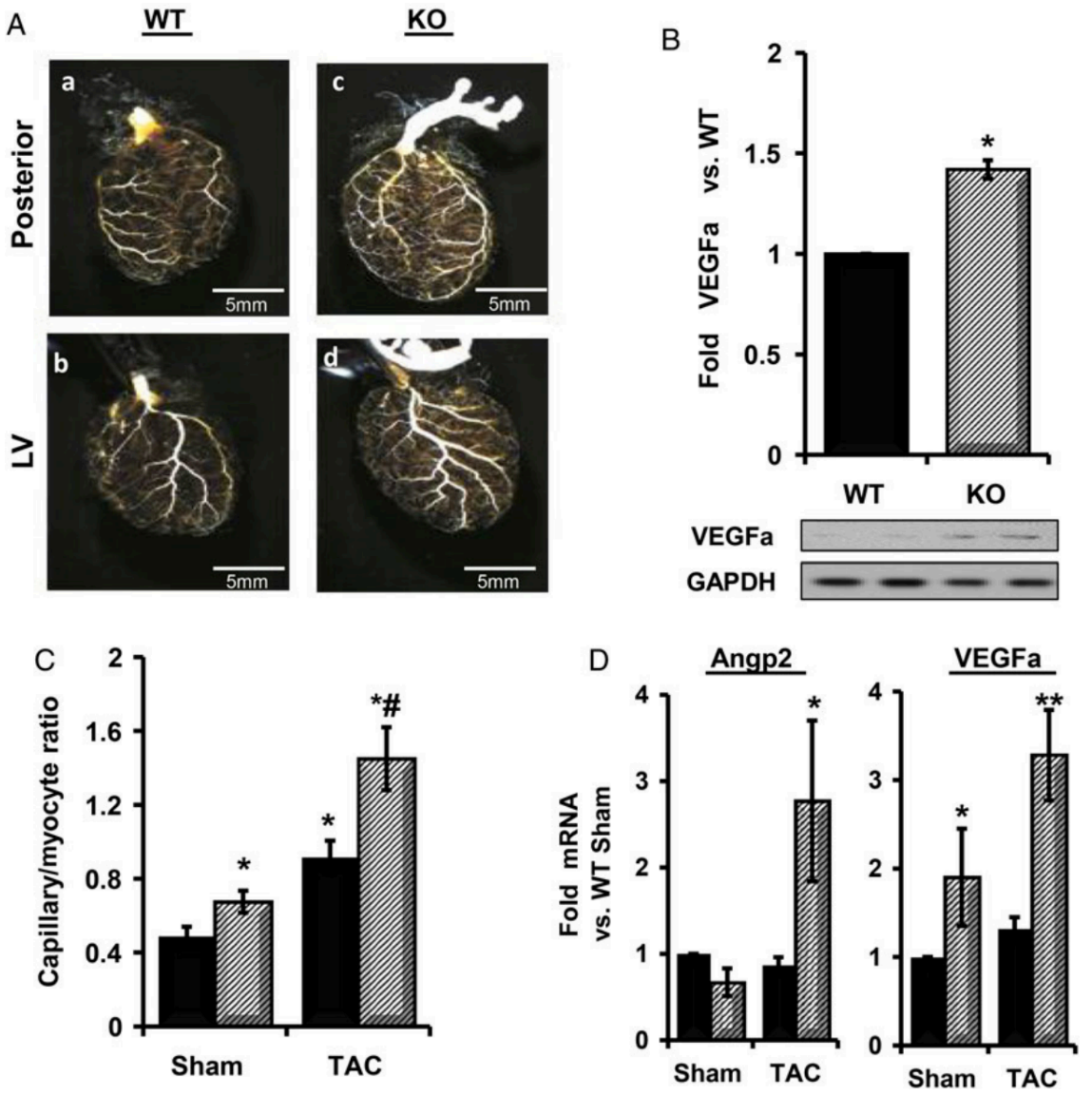



**Figure 6: Increased angiogenesis in PHLPP1 KO mice.** WT and KO mice (8 weeks of age) were analysed by (*A*) microfil polymer injection and corrosion casting of hearts for vasculature changes [posterior (a and c) and left ventricle (LV) view (b and d), ×2 magnification]. (*B*) Quantification and representative western blot of VEGFa levels at baseline in WT and PHLPP1 KO hearts. GAPDH was blotted for normalization ($n = 4$ hearts WT/KO; $^{*}P<0.05$ compared with WT baseline). (*C*) Quantification of capillary density between WT (solid) and KO (striped) mice following 2 week TAC. (The graph represents $n = 3$ independent hearts WT/KO; $^{*}P<0.05$ compared with WT Sham; $^{\#}P<0.05$ compared with WT TAC.) (*D*) Isolation of RNA from WT (solid) and KO (striped) hearts subjected to 2 weeks TAC. The relative mRNA fold changes in angiogenesis markers. (The graphs represent $n = 8$ independent samples; $^{*}P<0.05$ and $^{**}P<0.01$ compared with WT Sham.) Angp2: angiopoietin 2; VEGFa: vascular endothelial growth factor a.

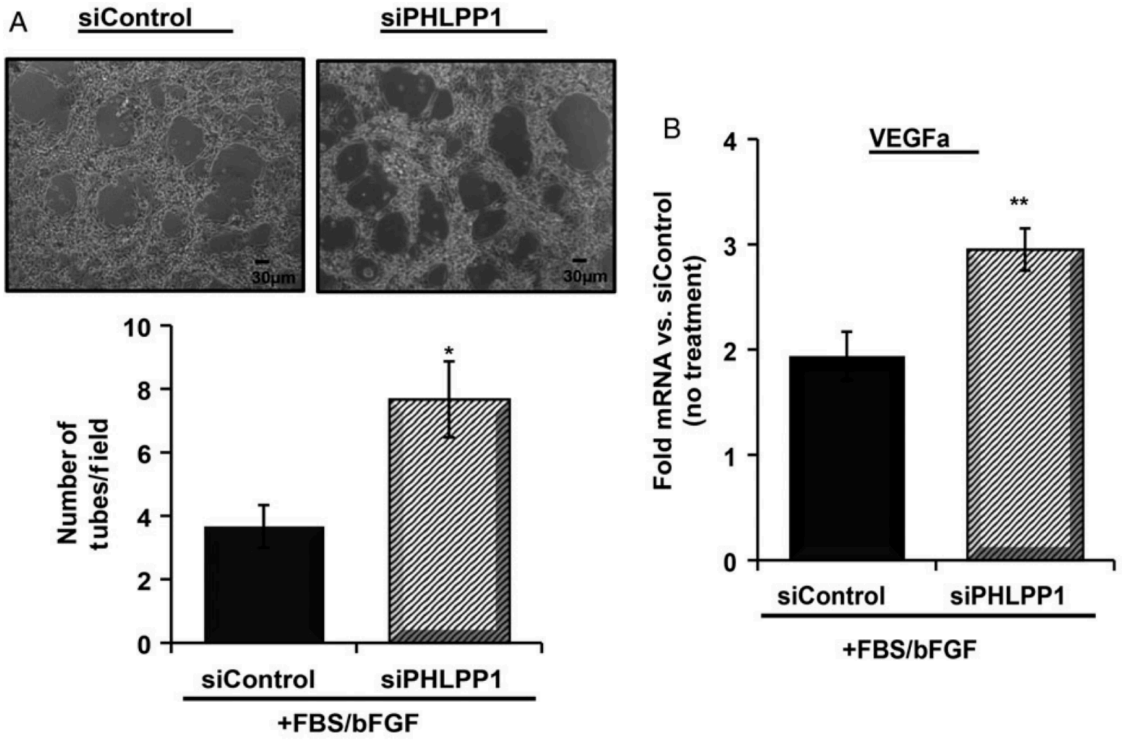
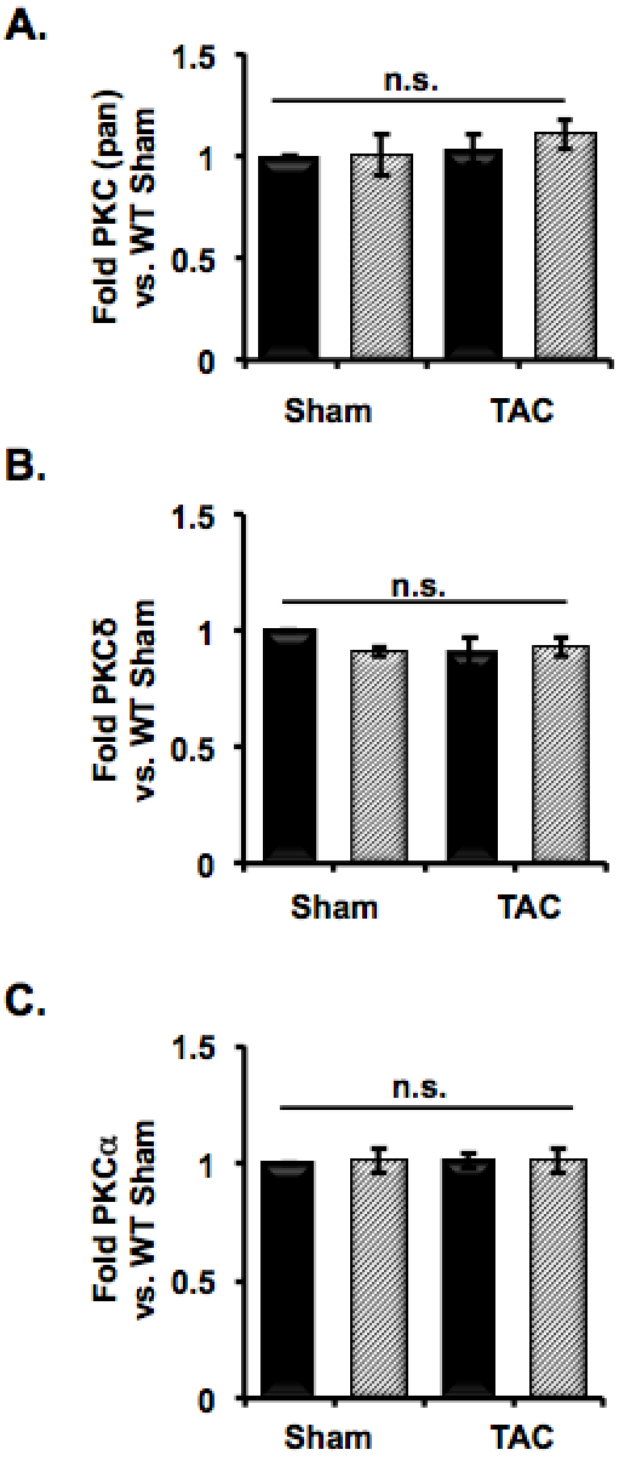



**Figure 7: Loss of PHLPP1 in cardiomyocytes increases endothelial cell tube formation.** NRVMs were transfected with scrambled control or PHLPP1 siRNA. Following 48 h, (*A*) BAECs were co-cultured with the NRVMs in the presence of serum (5%) and bFGF (3 ng/mL) for 6 h. Bright field images were taken at ×10 magnification. Quantification of tubes per field was determined. (The graph represents $n = 3$ independent experiments, six fields per experiment; $*P<0.05$ compared with siControl.) (*B*) Isolation of RNA from NRVMs with scrambled control or PHLPP1 siRNA with or without serum (5%) and bFGF (3 ng/mL) for 6 h (no BAEC). The relative mRNA fold changes in VEGFa were determined compared with siControl (no treatment). [The graph represents $n = 6$ independent experiments; $**P<0.01$ compared with siControl (treated).]




**Supplemental Figure 1**: WT (solid black bars) and KO (striped bars) mice were subjected to 2 weeks TAC and the level of PKC (A) pan (α,β,γ) (B) delta (δ) and (C) apha (α) was determined. Each graph represents n=4 independent animals and fold was normalized to WT Sham operated mice. n.s.=non-significant

## *2.6 Tables*

**Supplemental Table 1:** Gravimetric and echocardiographic analysis for male Wild-type (WT) and PHLPP1 knock-out (KO) mice at 1 year of age. There are no significant differences between the groups. (BW= body weight, HW=heart weight, LW=lung weight, TL= tibia length, FS= fractional shortening)

| 1 year Male | BW (g) | HW (mg) | LW (mg) | TL (mm) | HW/BW (mg/g) | HW/TL (mg/mm) | LW/BW (mg/g) | FS (%) |
|---|---|---|---|---|---|---|---|---|
| **WT (n=7)** | 37.54 ± 0.75 | 185.7 ± 7.60 | 255 ± 11 | 21.4 ± 0.3 | 4.98 ± 0.25 | 8.67 ± 0.30 | 11.88 ± 0.32 | 52.14 ± 1.73 |
| **KO (n=14)** | 37.07 ± 0.79 | 191.9 ± 6.20 | 246 ± 10 | 22.2 ± 0.4 | 5.18 ± 0.12 | 8.67 ± 0.29 | 11.12 ± 0.49 | 50 ± 1.07 |

**Supplemental Table 2:** Gravimetric and echocardiographic analysis for male WT and PHLPP1 KO mice (8-12 weeks of age) following 2 weeks TAC. Abbreviations are described in Table 1. († p< 0.05 vs. Sham, ‡ p<0.05 vs. WT TAC)

| 8-12 weeks Male | HW (mg) | BW (g) | TL (mm) | HW/BW (mg/g) | HW/TL (mg/mm) | FS (%) | Gradient (mmHg) |
|---|---|---|---|---|---|---|---|
| **WT SHAM (n=15)** | 141.37 ± 6.1 | 27.26 ± 0.98 | 20.3 ± 0.28 | 5.19 ± 0.13 | 6.96 ± 0.31 | 49 ± 1.4 | n.s. |
| **WT TAC (n=15)** | 207.7 ± 10.9 | 27.41 ± 0.96 | 19.6 ± 0.28 | 7.58 ± 0.28† | 10.60 ± 0.46† | 44 ± 1.8 | 55.5 ± 5.5 |
| **KO SHAM (n=12)** | 135.8 ± 4.7 | 27.16 ± 0.71 | 20.4 ± 0.19 | 5.0 ± 0.11 | 6.65 ± 0.26 | 50.7 ± 1.7 | n.s. |
| **KO TAC (n=12)** | 183.4 ± 7.1 | 28.35 ± 0.51 | 20.5 ± 0.26 | 6.46 ± 0.22† ‡ | 8.94 ± 0.36 † ‡ | 48.4 ± 1.2 | 64.5 ± 9.7 |

## *2.7 Discussion*

Despite intensive research into the role of Akt activation in the heart, its importance in regulating pathophysiological hypertrophy remains controversial. Modulation of PHLPP, which can directly dephosphorylate and inactivate Akt,[25,26,28] provides a mechanism for fine tuning this pathway and testing the physiological role of Akt in cardiac growth. We demonstrate that gene-targeted removal of PHLPP1 results in a very modest basal Akt activation (~25% increase)[28] without affecting cell size and accentuates the response to physiological stimulation of Akt as occurs with exercise training or IGF[32] (*Figures 1*, *2*, and *4B*). Based on several *in vivo* findings and as discussed in Introduction, the intensity and duration of Akt activation is a critical determinant of its role in physiological and pathological hypertrophy.[33-35,49-51] Hormones such as insulin and IGF-1, which are highly effective activators of the Akt signalling pathway,[45,46] enhance physiological growth of the heart in both rodents and humans. Conversely, removal of Akt1 in the heart inhibits exercise-induced cardiac hypertrophy.[12] Our finding that removal of PHLPP1 accentuates physiological hypertrophy induced by swimming (*Figure 2*) suggests that inhibition of PHLPP1 could be beneficial in augmenting physiological adaption of the heart following exercise.

Supraphysiological levels and localization of Akt in the heart lead to dysregulation of the signalling pathway and its targets, which ultimately culminates in pathological hypertrophy and failure.[33-35,49,50] The Akt signalling pathway is not, however, typically considered as an intrinsic maladaptive regulator of pathological hypertrophy. Since the magnitude and duration of Akt activity is clearly relevant to its effect on cardiomyocyte growth following pressure overload, we reasoned that more

subtle changes in Akt activation kinetics might actually have salutary effects on pathological hypertrophy development. In support of this possibility, the hypertrophic response to TAC was blunted in PHLPP1 KO compared with WT mice (*Figure 3A*). Removal of PHLPP1 significantly blunted the increase in cardiomyocyte size (*Figure 3B*), attenuated the re-expression of hypertrophic markers (*Figure 3C*), and increased Akt Ser473 phosphorylation. Akt enzyme activity assays using Akt1 and Akt2 antibodies demonstrated that Akt1 activity was increased in PHLPP 1 KO mice at baseline and following pressure overload (*Figure 4A* and *B*), whereas Akt 2 was not (data not shown).

The finding that removal of PHLPP1 accentuates Akt activation and decreases cell death after TAC is consistent with the well-documented anti-apoptotic effect of Akt on cardiomyocytes both *in vitro*[28,52] and *in vivo*.[16,22,33,53] PHLPP1 KO mice challenged with TAC have increased phosphorylation of Akt downstream targets that are important for cell survival, and maintenance of cardiac function (*Figure 4C*) as well as decreased TUNEL-positive cells (*Figure 5C*) and phosphorylation of pro-apoptotic targets (*Figure 5D*). In contrast, WT and PHLPP1 KO mice showed no significant differences in PKC isoform expression (see Supplementary material online, *Figure S1*) or Raf-1 phosphorylation (data not shown), both of which have been shown to be direct PHLPP targets in other systems.[54-55] There was also no difference in dephosphorylated NFAT between WT and KO mice following 1 day TAC (data not shown), suggesting no underlying defect in the calcineurin signalling pathway[10] in PHLPP1 KO mice.

Blunted hypertrophic growth in the presence of increased Akt activity would not, however, be expected to result from increased cardiomyocyte survival. We determined

that removal of PHLPP1 increased the baseline capillary density in the heart compared with WT mice (*Figure 6A* and *C*). The observation that the KO heart had increased VEGFa mRNA (*Figure 6D*) and protein expression (*Figure 6B*) at baseline is of particular interest as Akt activation within cardiomyocytes has been found to enhance expression of VEGFa,[56,57] which has in turn been demonstrated to increase coronary angiogenesis.[35,58] Thus, an increase in Akt activity in cardiomyocytes from PHLPP1 KO is likely to contribute to the increased capillary density found in the heart. Since removal of PHLPP1 is global in our mouse, our finding that knockdown of PHLPP1 specifically in cardiomyocytes *in vitro* increases their ability to cause endothelial cell tube formation in a myocyte/endothelial cell co-culture system supports our notion (*Figure 7A* and *B*).

Under hypertrophic stress, the heart increases capillary density to meet the demand of oxygen required for growth. WT mice increase their capillary density in response to TAC-induced pressure overload; however, the KO further increases the capillary-to-myocyte ratio compared with their WT counterparts (*Figure 6C*) and angiogenic marker expression (*Figure 6D*). Through co-culture experiments, we have established the possibility that increases in capillary density may in part be due to paracrine signalling from cardiomyocytes lacking PHLPP1 to surrounding endothelial cells within the heart (*Figure 7A* and *B*). We hypothesize that the increased capillary density provides more oxygen to meet cellular demands during hypertrophic growth, which would ultimately allow for reduced overall cell enlargement. In support of our findings, it has been recently demonstrated that inhibition of apoptosis during pressure overload preserves cardiac function through decreased fibrosis and is associated with increased overall angiogenesis and decreased hypertrophy.[48] Our previous finding that *ex*

*vivo* perfused hearts from PHLPP1 KO mice were protected from oxidative damage could be explained by increased capillary density as well as by the protection of cardiomyocytes by increased Akt activity.[28]

Our work is the first to show that removal of the serine/threonine phosphatase PHLPP1, which increases Akt activity in the heart to physiological levels without affecting cell size, increases basal and TAC-induced angiogenesis and ultimately blunts the overall development of pathological hypertrophy in response to pressure overload-induced stress. These findings are consistent with the data of Shiojima *et al*.,[16] demonstrating that activation of inducible expression of Akt following long-term TAC improves cardiac function, and the suggestion that this occurs through inhibition of cell death. Whether removal of PHLPP1, which activates Akt and inhibits cell death, would also rescue the development of heart failure following long-term TAC or myocardial infarction remains to be determined. Future studies using cardiac-specific removal of PHLPP1 will be necessary to determine whether increasing Akt activity in cardiomyocytes is sufficient to affect angiogenesis or whether endothelial or other cells in which PHLPP1 is deleted are also involved. In either case, our work raises the possibility that PHLPP1 inhibition would have therapeutic potential for protecting the heart against stress.

## ***2.8 Acknowledgements***

Chapter 2 is a reprint with minor modifications of the material as it appears in Moc C, Taylor AE, Zambrano CM, Barlow MS, Zhang X, Gustafsson AB, and Purcell NH. Physiological activation of Akt by PHLPP1 deletion protects against pathological hypertrophy. *Cardiovascular Research* 2015;105(2):160-170. The thesis author was the author of this manuscript. Dr. Nicole H. Purcell directed and supervised the research, which forms the basis for this chapter.

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# CHAPTER 3

# CONCLUSIONS

## *3.1 Summary of Findings*

PHLPP is a ubiquitously expressed phosphatase that dephosphorylates Akt specifically at Ser473 leading to inactivation of Akt. To characterize the effects of PHLPP1 removal on the heart, global PHLPP1 KO mice were generated and compared to WT mice following stress. PHLPP1 KO demonstrated an increase in basal Ser473 phosphorylation and modest increase in Akt activity in the heart compared to WT. This increase of about 25% in Akt is similar to a more physiological activation. Previous studies have shown supraphysiological increases in Akt activity at baseline is associated with cardiac hypertrophy.[1-4] However, we observed no change in cardiac size and contractile function upon measuring the total heart weight to tibia length ratio and fractional shortening between WT and KO up to one year, respectively. This discrepancy between an increase in Akt activity in the heart and no cardiomyocyte size changes raises a question as to whether the Akt signaling pathway is still intact.

Akt activation is widely recognized for induction of physiological growth of the heart during exercise and from stimulus such as insulin growth factor-1 (IGF-1).[5-7] We therefore examined the hearts response in PHLPP1 KO mice to exercise-induced hypertrophy (i.e. swimming). Consistent with the studies, our findings demonstrate that removal of PHLPP1 accentuates exercise-induced cardiac hypertrophy compared to WT. Our data suggests that the Akt pathway is intact and an increase in basal Akt activity by PHLPP1 removal in the heart accentuates physiological growth following exercise.

Activation of Akt has been shown to be cardioprotective, hence we questioned what would happen to the hearts of PHLPP1 KO mice following pathological stress. As mentioned in the Introduction, the duration and intensity of Akt activation impacts Akt signaling.[8-11] Previous studies in the heart demonstrated that exogenous Akt activation led to either reversible physiological hypertrophy or irreversible pathological hypertrophy.[8-12] Thus, the role of Akt activation remains controversial in the development of cardiac hypertrophy. Since removal of PHLPP1 mildly accentuated endogenous Akt activity, we would assume that there would be a more subtle increase in cardiac hypertrophy at baseline. To our surprise this was not the case and there was no change in cardiac size observed in the KO mice at baseline. What was more interesting was that following a pathological stress such as pressure overload (TAC), PHLPP1 KO mice demonstrated an attenuated hypertrophic response as measured by a decrease in myocyte cell area as compared to WT. Moreover, previous studies have found that pathological stress such as pressure overload leads to induction of fetal genes, cardiac fibrosis, and cardiomyocyte apoptosis.[2,13,14] However, we found that 2 weeks post TAC, PHLPP1 KO mice had decreased expression of hypertrophic markers and fibrotic genes in comparison to WT. Similarly, apoptosis in PHLPP1 KO was significantly reduced compared to WT following TAC. Interestingly, we found that there was a decrease in phosphorylation of Mst1 at Thr183, which is a pro-apoptotic site,[15] in the PHLPP1 KO mice following TAC. However, it is unclear whether PHLPP1 is regulating Mst1 directly or indirectly through Akt. In other systems, since PHLPP1 deletion increased phosphorylation of Raf-1 at Ser338, which is important for induction of oncogenic functions and can directly regulate PKC stability, a pro-survival pathway, we wanted to determine if other pathways may be

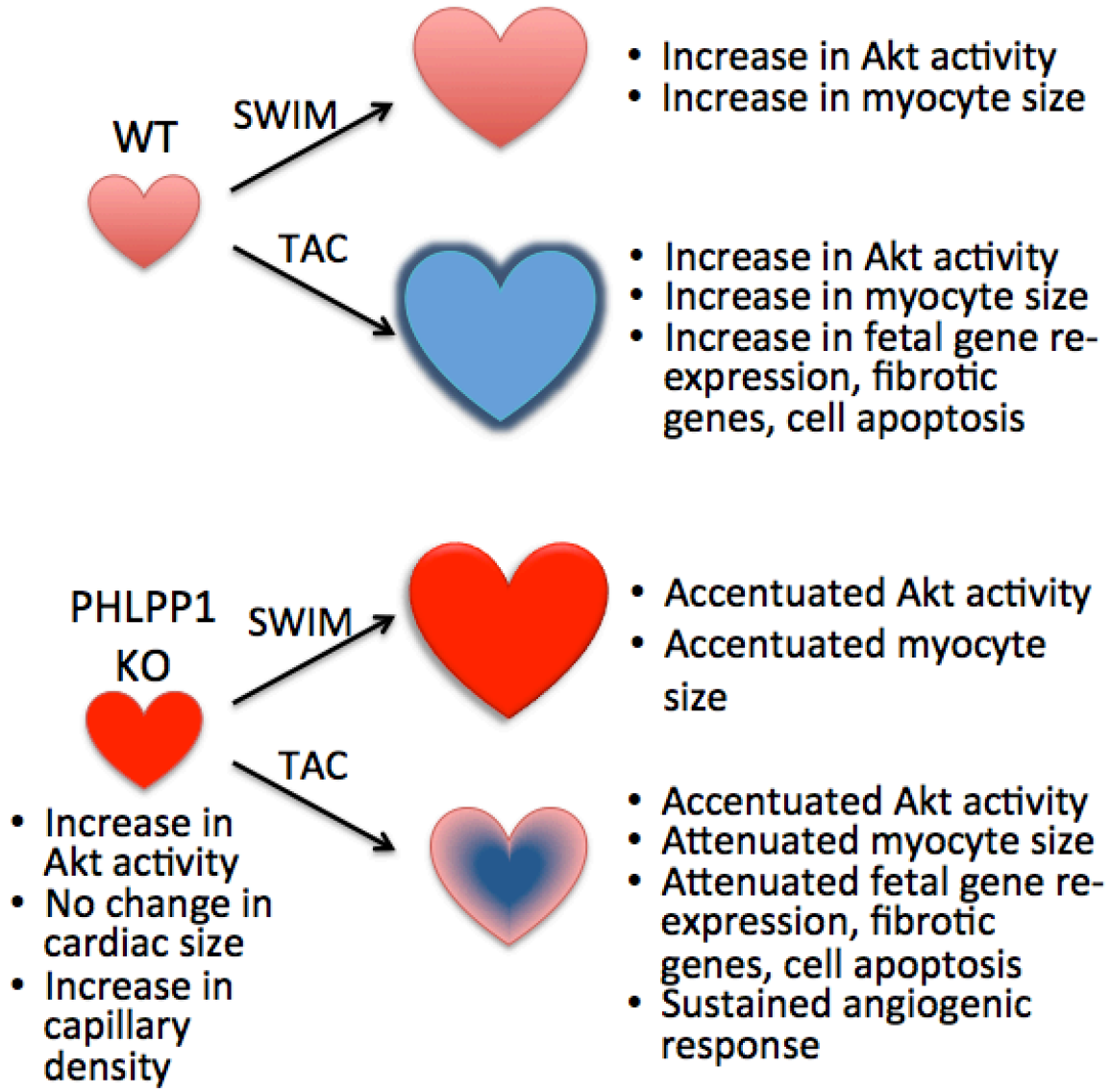
responsible for the cardiac protection.[1,16,17] However, our studies demonstrate that there were no changes in Raf-1 phosphorylation nor PKC isoform expression between PHLPP1 KO and WT in the heart following TAC. This suggests that removal of PHLPP1 does not regulate other pathways such as Raf1 or PKC to account for the cardiac protection.

We demonstrated that Akt1 activity was increased following TAC but not Akt2. These findings are consistent with studies that demonstrate the importance of Akt1 in cell size changes.[4,18-22] However to our surprise, increased Akt activity did not produce an enlargement of the heart but rather an attenuated hypertrophic response following TAC. Thus, we examined other possible mechanisms that could account for the attenuated hypertrophic response. Shiojima *et al.* suggested that an increase in Akt activity in cardiomyocytes leads to a coordinated growth of cardiomyocytes and coronary angiogenesis, which is cardiac protective following hypertrophic stress. Perhaps this could explain why the PHLPP1 KO mice had an attenuation of pathological hypertrophy. Therefore, we examined angiogenesis in the PHLPP1 KO mice and found compared to WT an increase in the protein and mRNA levels of VEGFa at baseline and following TAC, which are angiogenic markers found to be associated with enhanced capillary changes.[4,11] Thus, the enhanced capillary density in the PHLPP1 KO could help maintain cardiac function following TAC short-term and limit the hypertrophic growth of cardiomyocytes. Further analysis *in vitro* demonstrated that removal of PHLPP1 in cardiomyocytes increases paracrine signaling to the neighboring endothelial cells which may contribute to the increased capillary density seen in the heart as well as from changes in the endothelial cells themselves. Our findings agree with Shiojima *et al.* whom suggested that cardiomyocytes could signal to endothelial cells and increase

angiogenesis. Interestingly, Shiojima *et al.* demonstrated that short-term activation of Akt in the heart showed an increase in the protein levels of angiogenic markers whereas long-term activation showed attenuation.[23] Thus, we are unclear whether in our model system there will be a sustained angiogenic response to long-term TAC to induce cardiac protection.

In conclusion, under pathological stress induced by pressure overload, PHLPP1 KO mice had an attenuated response to pathological hypertrophy compared to WT mice. Our findings demonstrate for the first time that the removal of the Akt phosphatase PHLPP1, which increases Akt activation to physiological levels at baseline, does not affect basal cardiomyocyte growth but increased capillary density may be protective against pressure overload induced pathological changes in the heart (Scheme 6).

***3.2 Scheme***




**Scheme 6: Summary of hypertrophic response of PHLPP1 KO mice.** PHLPP1 KO displayed no difference in cardiac size at baseline compared to WT mice. However, PHLPP1 KO hearts were determined to have an increase in Akt activity and capillary density at baseline. Physiological hypertrophy induced by swimming was accentuated in the PHLPP1 KO mice compared to WT. Following pathological hypertrophy induced by pressure overload (TAC), WT mice demonstrated an increase in myocyte size, fetal gene expression, fibrosis, and cell death in the heart, while PHLPP1 KO had an attenuation of these measures. Furthermore, PHLPP1 KO mice following TAC exhibited a sustained angiogenic response compared to WT.

### *3.3 Future Studies*

The novel role of PHLPP1 on Akt signaling and its role in cardiac hypertrophy has just begun to be understood. Future aims should be directed at long-term effects of PHLPP1 removal on cardiac hypertrophy and heart failure. Preliminary work suggests that removal of PHLPP1 attenuates pressure-overload-induced pathological hypertrophy. The results were from two-week TAC, which is a hypertrophic but not failure model. As mentioned in the Introduction, the duration of Akt activation affects the type of hypertrophy. It could be possible at this time point that physiological Akt activation is cardioprotective whereas long-term activation of Akt may lead to pathological hypertrophy and eventually heart failure. Thus, the use of long-term pressure overload to discover whether PHLPP1 is still cardioprotective should be pursued.

Another future aim should be directed at cardiac-specific removal of PHLPP1. While global PHLPP1 gave us preliminary data about PHLPP1 function in respect to pathological and physiological hypertrophy, in general, global KO has its limits, as it could be possible that other cells types are compensating for the seen phenotype. As a result, cardiac-specific PHLPP1 KO can bypass this and the results found are more reliably associated with the inactivation of PHLPP1 in the cardiomyocytes. This can eliminate the compensatory mechanisms and strictly identify PHLPP1 effects in the heart.

Lastly, our work demonstrated the global deletion of PHLPP1 attenuated pathological hypertrophy induced by pressure overload. It would be interesting to examine whether PHLPP1 could be a therapeutic target and delete it following pathological hypertrophy to prevent failure. If PHLPP1 removal following injury protects

the heart, this would suggest the possibility of drug targeting PHLPP1 as an intervention for heart failure. The use for cardiac-specific inducible PHLPP1 KO would be useful for this aim.

Currently, PHLPP2 KO mice have been generated and are being characterized in the laboratory. Since studies using siRNA to remove PHLPP2 in cardiomyocytes suggest that it does not regulate Akt activity, future studies to determine the function of PHLPP2 in the heart are ongoing. It will be interesting to see how removal of PHLPP2 affects the heart and whether it has any effect on PHLPP1. Any additional data on the role of PHLPP1 and PHLPP2 in the heart brings us closer to understanding the complex signal transduction mechanisms involved in regulating cardiac hypertrophy and failure. As a result, this will give scientists and physicians the advantage to be able to develop specific and effective therapies against heart failure.

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