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Dr. Sabire Ozcan, Major Professor

Dr. Michael Mendenhall, Director of Graduate Studies

REGULATION OF GLUCOSE HOMEOSTASIS BY THE PHLPP1 PHOSPHATASE

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By

Kara LeAnn Larson

Lexington, Kentucky

Director: Dr. Sabire Özcan

Associate Professor of Molecular and Cellular Biochemistry

Lexington, Kentucky

2014

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ABSTRACT OF DISSERTATION

REGULATION OF GLUCOSE HOMEOSTASIS BY THE PHLPP1 PHOSPHATASE

Type 2 Diabetes Mellitus is a metabolic disease that affects one in ten people in the United States. It is caused by a combination of genetics and lifestyle factors. Disease progression begins with insulin resistance in peripheral tissues followed by pancreatic beta-cell failure. The mechanisms behind disease progression are not completely understood. PH domain leucine rich repeat protein phosphatase 1 (PHLPP1) is a known regulator of Akt and other members of the AGC kinase family. Akt has been established to play a role in numerous metabolic signaling pathways, including insulin action. It is hypothesized that as a regulator of Akt, PHLPP1 would have an important function in glucose homeostasis. Glucose tolerance tests performed on 8-week old *Phlpp1*$^{-/-}$ mice revealed no significant difference in glucose tolerance compared to wild type, however these mice did exhibit increased fasting blood glucose levels. Glucose tolerance tests were repeated at 20 weeks on the same mice and, interestingly, they displayed impaired glucose tolerance compared to wild type. Insulin tolerance tests showed that 8-week old mice have increased insulin sensitivity, however, the 20-week old mice were insulin-resistant compared to control animals. The 20-week old knockout mice also had significantly higher fasting blood glucose levels compared to 8-week old mice. To determine if the increased fasting blood glucose levels are due to increased hepatic glucose output, pyruvate tolerance tests were performed on both the 8 & 20 week old mice. Old mice displayed significantly increased hepatic glucose production compared to wild type. Echo-MRI done on 24-week old mice showed significantly increased fat mass and decreased lean mass in the *Phlpp1*$^{-/-}$ mice compared to wild type littermates. Western blot analysis of liver samples from 32-week old *Phlpp1*$^{-/-}$ mice indicates loss of Akt signaling accompanied by a decrease in IRS2 protein levels, a common indicator of insulin resistance. These data suggest that *Phlpp1*$^{-/-}$ mice mimic the development of type 2 diabetes in humans, and provide a unique animal model to study the progression of type 2 diabetes and diabetes-associated complications.

KEYWORDS: Type 2 diabetes mellitus, PHLPP, Akt, glucose, animal model

Kara LeAnn Larson

July 30, 2014

REGULATION OF GLUCOSE HOMEOSTASIS BY THE PHLPP1 PHOSPHATASE

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Chapter 1

Introduction

Maintaining normoglycemia and proper energy balance

For proper function and survival it is necessary to maintain normal glucose homeostasis and energy balance. To do this, organisms employ a tightly-regulated network of various organs, tissues, and cell types to meet energy demand and utilize energy supply. Glucose is typically the main source of energy and is used for the production of ATP. For mammals, the interaction between cells in the pancreas, liver, skeletal muscle, brain, and adipose tissue via the secretion and uptake of various hormones is what regulates this energy balance (**Figure 1.1)**. These hormones, when bound to receptors in the target tissues, communicate the storage or utilization of glucose to meet the demands of the organism. It is necessary to maintain a narrow range of blood glucose levels (80-120 mg/dL), and to do this the body fluctuates between glucose storage, utilization, and production [1]. This process is particularly crucial for the brain as it cannot metabolize other forms of energy, and cannot function correctly when blood glucose levels fall too low [2]. Dysregulation of the processes necessary for maintaining glucose homeostasis can lead to the development of various metabolic disorders including type 2 diabetes mellitus [3].

The role of the endocrine pancreas in regulating glucose homeostasis

The pancreas is the key regulator in glucose metabolism. It contains two types of tissue: exocrine and endocrine, with endocrine tissue being important for glucose homeostasis. Pancreatic endocrine tissue is made up of cells clusters called Islets of

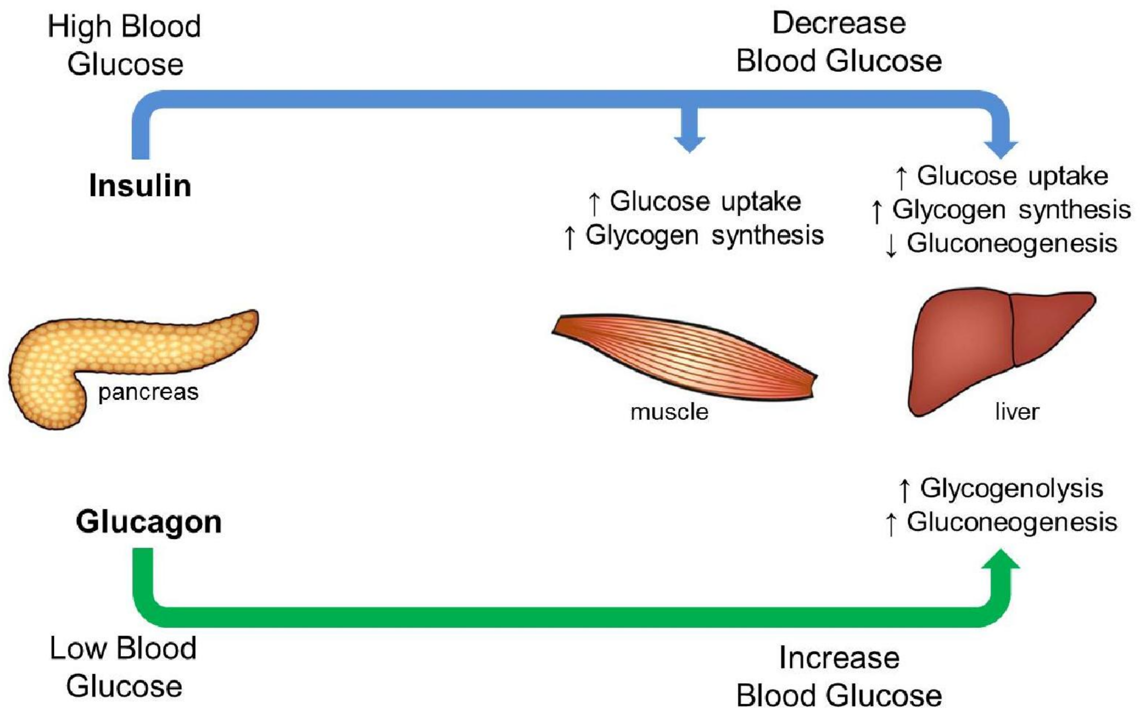

Figure 1.1: Regulation of Glucose Homeostasis

When blood glucose levels are high, pancreatic beta cells release insulin into the bloodstream where it travels to the peripheral tissues. In the muscle, insulin promotes muscle glucose uptake and turns on glycogen synthesis. Insulin also promotes glucose uptake and turns on glycogen synthesis in the liver as well as inhibits gluconeogenesis. This results in the overall lowering of blood glucose levels. Conversely, when blood glucose levels are low, pancreatic alpha-cells secrete glucagon, which travels to the liver where it turns on glycogenolysis, or the breakdown of glycogen to glucose. When glycogen stores are depleted, gluconeogenesis is turned on. Gluconeogenesis is the production of glucose from pyruvate, glycerol, lactate, or certain amino acids. Hepatic glucose production is necessary to maintain blood glucose levels during times of fasting.

Langerhans. Islets make up about 2-3% of the total pancreas and are found uniformly dispersed throughout the exocrine tissue. Islets themselves consist of five different cell types with each cell type responsible for the secretion of a different hormone. Epsilon cells, PP cells, and delta cells combine to compose up to 16% of the total islet. These three cell types are responsible for the secretion of ghrelin, somatostatin, and pancreatic polypeptide, respectively. These hormones are involved in promoting hunger (ghrelin) and regulating the release of other pancreatic and gastrointestinal hormones (somatostatin and pancreatic polypeptide). The other two cell types, alpha cells and beta cells, are the most important for the regulation of blood glucose levels and overall glucose homeostasis.

Alpha cells make up a large portion of the islet at 15-20%. Alpha cells secrete the hormone glucagon to stimulate the release of glucose stores in the liver. This is done during times of fasting when glucose is scarce. Glucagon travels to the liver where it promotes glycogenolysis or gluconeogenesis, which is the production of glucose from either glycogen or pyruvate. Glucagon can also promote the production of ketone bodies when carbohydrate stores are exhausted.

65-80% of the islet is made up of beta cells [4]. Their primary function is the production and secretion of insulin. Insulin is the hormone responsible for lowering blood glucose levels during post-prandial increases. The beta cell is able to quickly take up glucose and release insulin into the bloodstream [5, 6]. Insulin then travels to the peripheral tissues where it signals for the uptake of glucose thus lowering blood glucose levels. It also signals to turn off hepatic glucose production, as it is unnecessary to produce glucose when blood glucose levels are already elevated. While many

hormones are necessary for the maintenance of glucose homeostasis, insulin is typically considered the most important.

Muscle Glucose Uptake

Once in the bloodstream, insulin travels to various tissues including skeletal muscle. It binds to the insulin receptor in the cell membrane, which then sets of a signaling cascade that ends in the rapid translocation of the glucose transporter, GLUT4, to the plasma membrane. Under conditions of low insulin, GLUT4 is sequestered in intracellular vesicles, however, upon insulin binding to the insulin receptor, vesicles containing GLUT4 fuse with the plasma membrane effectively inserting the transporter into the membrane [7, 8]. This allows for the facilitated diffusion of circulating glucose in the bloodstream to enter the muscle cell down its concentration gradient [9, 10]. Once in the cell, glucose is converted to glucose-6-phosphate (G6P) via hexokinase. G6P then enters glycolysis for the purpose of ATP production or is used for synthesis of glycogen, a storage form of glucose that acts as an immediate reserve source of energy for the muscle cell in times of need.

Hepatic Glucose Metabolism

The liver plays multiple roles in maintaining normal glucose homeostasis. During the postabsorptive state, when blood glucose levels are low and insulin secretion is at basal levels, liver glucose uptake is almost completely abolished. This is true for other insulin-sensitive tissues as well. During this time the majority of glucose is taken up by the brain. In order to maintain normal blood glucose levels, and therefore healthy brain function during this circumstance, the liver produces and releases glucose through the

processes of glycogenolysis and gluconeogenesis. Glucose production in liver is triggered by a combination of the fall of insulin levels as well as the secretion of glucagon from pancreatic alpha cells [11]. Glycogenolysis is the production of glucose from glycogen, a polymeric storage form of glucose. Glycogen breakdown is the primary method of energy production during times of fasting. Once glycogen stores run low, the liver switches to gluconeogenesis, the production of glucose from various carbon substrates including lactate, glycerol, and certain amino acids.

Conversely, when blood glucose levels are high, the liver acts as the principle site of glucose deposition absorbing up to one third of an oral glucose load [12]. Insulin is secreted from the pancreatic beta cell into the bloodstream where it first meets the liver. Insulin binding to its receptor promotes glucose conversion to glucose-6-phosphate (G6P) effectively trapping glucose within the hepatocyte [13]. Similarly to muscle, G6P can then be used for the production of ATP via glycolysis or for glycogen production. Concurrently, insulin signaling in the liver attenuates glucose production so as to reduce post-prandial rises in blood glucose levels.

Type 2 Diabetes Mellitus

Type 2 diabetes mellitus is a metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion and insulin action. It is currently estimated to affect almost 30 million people in the United States and up to 350 million worldwide and will likely reach pandemic levels by the year 2030 [14, 15]. Many complications and conditions are associated with type 2 diabetes including high blood pressure, high cholesterol, heart disease, blindness, kidney disease, nerve damage, amputations, and pregnancy complications. Treatment costs for diabetes and its related complications

topped $245 billion dollars in the United States in 2012 [14]. Taken together, these facts indicate a need for a better understanding of the disease in order to facilitate the discovery of novel treatments and preventions.

There is an established progression in the development of type 2 diabetes mellitus in patients. The disease is caused by a combination of genetic factors as well as lifestyle choices. A diet high in fat and carbohydrates maintained over a long period of time is typically associated with the development of diabetes. The disease begins with the onset of insulin resistance, which occurs when tissues that are typically responsive to insulin become insensitive to its effects. This means that muscle and liver cells are no longer able to take up glucose and that hepatic glucose production cannot be suppressed. The loss of insulin signaling in these tissues can be compensated for a period of time through an increase in beta-cell mass and function resulting in an increase in insulin production and secretion [16]. However, prolonged exposure to high glucose causes a decline in beta-cell function and number [17]. Loss of beta-cell compensation results in uncontrolled hyperglycemia due to a combination of insulin resistance and hypoinsulinemia [18].

Pleckstrin Homology Domain Leucine-Rich Repeat Protein Phosphatase

*P*leckstrin *h*omology domain *l*eucine-rich repeat *p*rotein *p*hosphatase (PHLPP) is a serine-threonine protein phosphatase in the PP2C-type family that exhibits tumor suppressor function in many cancers [19]. It was named for its protein domains as it contains a PH domain, a leucine-rich repeat region, a phosphatase domain, and a PDZ domain (**Figure 1.2)**. PHLPP was initially discovered as being regulated in a circadian manner in the suprachiasmatic nucleus and was named SCOP or *s*uprachiasmatic nucleus *c*ircadian *o*scillatory *p*rotein. It was later determined to play a role in the light-

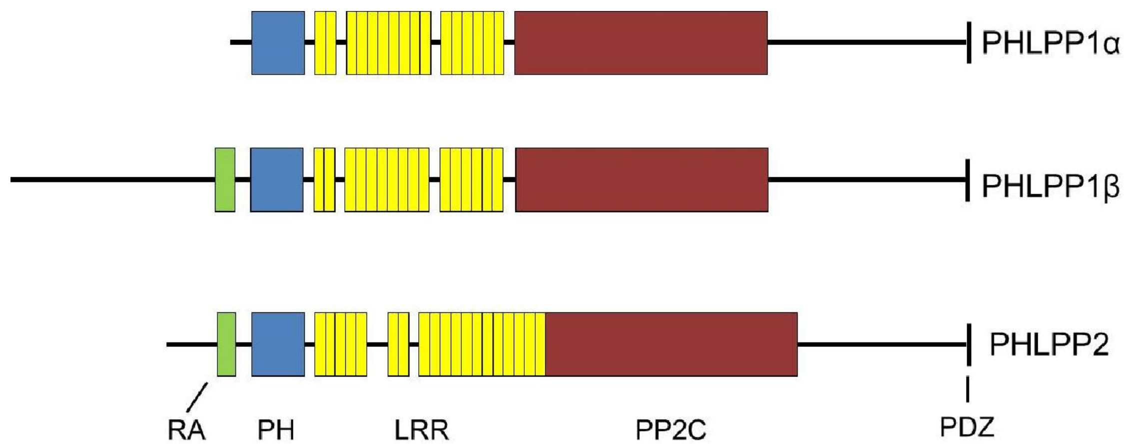

Figure 1.2: PHLPP domain structure

There are two PHLPP isoforms: PHLPP1 and PHLPP2. PHLPP1 consists of two different splice variants. All PHLPP proteins contain a pleckstrin homology (PH) domain, a leucine rich repeat region (LRR), a PP2C phosphatase domain, and a PDZ binding motif. PHLPP1β and PHLPP2 also contain a Ras association (RA) domain. (Modified from [20])

induced resetting of the circadian clock [21, 22]. PHLPP has been found to be a regulator of three different AGC kinases. It was uncovered as a phosphatase for Akt through a rational search for a protein containing both a PH domain as well as a phosphatase domain. It was shown to dephosphorylate Akt at serine 473 reducing its catalytic activity thereby reducing proliferation and promoting apoptosis. The second AGC kinase it was found to dephosphorylate is PKCβII at serine 660. This causes the destabilization of PKC which shunts the kinase to degradation pathways [23]. Lastly, it negatively regulates S6K through dephosphorylation of threonine 389, reducing its activity and thereby reducing overall protein translation [24]. Outside of AGC kinases, PHLPP has been shown to regulate Mst1 and Raf1. Mst1 is proapototic and when dephosphorylated at threonine 387 by Akt is inactivated. PHLPP dephosphorylates this site inducing apoptosis and arresting cell growth. Mst1 activation is further enhanced by PHLPP as PHLPP inactivates Akt thus reducing Akt's inhibitory effects on Mst1 [25]. Most recently, PHLPP has been shown to regulate Raf1, a downstream effector of the ERK pathway. PHLPP dephosphorylation of Raf1 reduces its activity causing an increase in the invasive and migratory abilities of cells as well as activates the epithelial-mesenchymal transition, an important process in cancer metastasis [26]. These are the only confirmed targets of PHLPP, but seeing as how there are more than 100,000 phosphorylated serine and threonine sequences in cells, and fewer than 40 known Ser/Thr phosphatases it is highly likely that PHLPP has other targets [27].

While it is known that PHLPP is a regulator of several proteins, it has primarily been studied as a negative regulator of Akt. Akt, also known as protein kinase B, is a central signaling molecule in many cellular processes including glucose metabolism, apoptosis, and cell proliferation. Akt is a part of the PI3K cascade, which is activated when a growth factor binds to a receptor tyrosine kinase at the cell surface. The

receptor activates PI3K, which then converts phosphatidyl inositol 4,5-bisphosphate (PIP_2) to phosphatidyl inositol 1,4,5-triphosphate (PIP_3). This lipid recruits PDK1 and Akt to the plasma membrane. PDK1 phosphorylates Akt at threonine 308 and the mTORC2 complex phosphorylates Akt at serine 473, a key residue in its catalytic domain. Until the discovery of PHLPP it was unknown how Akt was dephosphorylated and therefore negatively regulated. It was later determined that there are two PHLPP isoforms, and that PHLPP1 differentially regulates Akt2 and Akt3 whereas PHLPP2 regulates Akt1 and Akt3 [28]. PHLPP1 was further determined to consist of two splice variants: PHLPP1α and PHLPP1β with PHLPP1β corresponding to the original SCOP protein discovered in the brain.

PHLPP1/2 have been principally studied for their role in cancer. PHLPP1 is found at a locus that frequently undergoes a loss of heterozygosity in colon cancer and PHLPP2 is at a locus that undergoes a loss of heterozygosity in breast, ovarian, prostate, and hepatocellular cancers [29]. PHLPP overexpression has been demonstrated to increase apoptosis in non-small cell lung cancer and breast cancer cells and to inhibit tumor growth in glioblastoma and colon cancer cells in xenografted nude mice [19, 28, 30]. Conversely, decreased expression of PHLPP has been linked to the metastatic potential of breast cancer cells [31].

PHLPP and Type 2 Diabetes Mellitus

As PHLPP has mainly been studied for its role in cancer, not much is known about its function in metabolism and type 2 diabetes. There have only been two studies looking at a connection between PHLPP expression and diabetes or obesity in human patients. The first study found that Akt serine 473 phosphorylation was decreased after

insulin stimulation in muscle cells of type 2 diabetic patients versus non-diabetic patients. PHLPP1 mRNA expression levels were increased in these patients suggesting that the increase in PHLPP1 is the likely reason for the decrease in Akt phosphorylation and subsequent inactivation [32]. A second study found that PHLPP1 protein levels were increased in the adipose tissue of obese patients compared to non-obese patients and that PHLPP1 abundance positively correlated with BMI. This correlated with a decrease in basal Akt Ser473 phosphorylation levels. Furthermore, a twofold increase in PHLPP1 levels were found in the skeletal muscle of obese patients, however, there were no differences between obese patients that had normal fasting glucose versus impaired fasting glucose. Lastly, they determined that PHLPP1 protein levels were increased upon insulin treatment in HepG2 cells and that overexpressing PHLPP1 in HepG2 cells reduces insulin-stimulated glycogen content compared to control cells [33].

Akt signaling in the pancreatic beta- cell, muscle, and liver

Since not a great deal is known about PHLPP and its role in glucose homeostasis, it is important to look at Akt to determine what function PHLPP may be playing through its negative regulation of Akt. In all gluco-regulatory tissues Akt is downstream of the insulin receptor in the PI3K cascade. When insulin binds to the insulin receptor (IR) at the cell membrane, IRS proteins binds to the IR, which act as an adaptor molecule for PI3K. Upon activation, PI3K phosphorylates PIP_2 to PIP_3. The PH domains in Akt and PDK1 bind to PIP_3 at the plasma membrane. Akt is activated through phosphorylation, and it then moves throughout the cell to mediate downstream signaling which can vary depending on the cell type. In the muscle, Akt activation leads to the translocation of GLUT4 to the plasma membrane allowing for glucose uptake [34]. Therefore, insulin signaling activates Akt, increasing muscle glucose uptake thereby

lowering blood glucose levels (**Figure 1.3**). In the liver, Akt activation leads to phosphorylation and inactivation of Foxo1. Foxo1 is a transcription factor that when not phosphorylated, is active and present in the nucleus where it is responsible for the transcription of genes necessary for gluconeogenesis. When Foxo1 is phosphorylated it is unable to enter the nucleus and therefore gluconeogenic gene transcription is turned off leading to suppression of gluconeogenesis [35]. Therefore, upon insulin signaling, Akt is activated and gluconeogenesis is suppressed. At the same time, Akt phosphorylation and inactivation of GSK3β promotes glycogen synthesis effectively shunting the glucose taken up by the hepatocyte towards storage as glycogen (**Figure 1.4)** [36]. The role of Akt in the beta-cell is less clear. It has been shown to play a role in cell size, cell survival, and proliferation which can be important for expanding beta-cell mass during times of increasing insulin resistance [37]. Additionally, there have been studies suggesting that Akt plays a role in glucose stimulated insulin secretion. Transgenic mice overexpressing Akt had a six times higher beta-cell mass and increased insulin secretion [38]. Conversely, transgenic mice expressing a kinase-dead mutant of Akt exhibited a decrease in glucose-stimulated insulin secretion (**Figure 1.5)** [39].

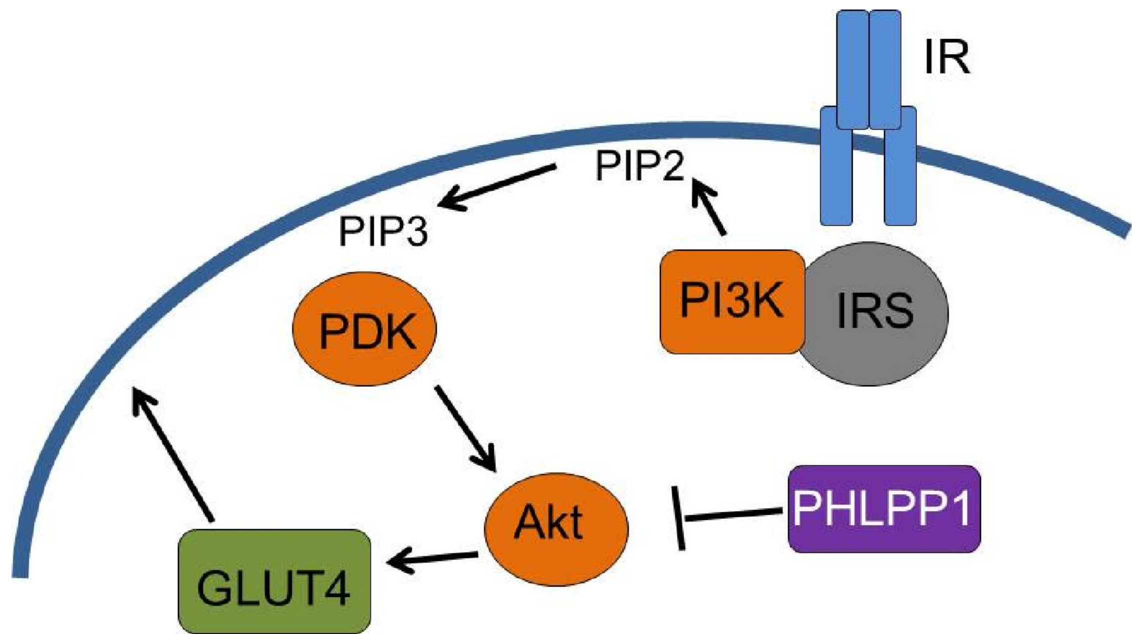

Figure 1.3: Akt signaling in the muscle cell

Insulin binds to and activates the insulin receptor (IR), which causes tyrosine phosphorylation of IRS protein. The IRS proteins act as adaptor molecules for PI3K. Once PI3K is bound, it converts PIP_2 to PIP_3. PDK1 and Akt bind to PIP_3 through their PH domains. Akt is phosphorylated and activated resulting in the subsequent translocation of GLUT4 to the plasma membrane. GLUT4 takes up glucose from the bloodstream thereby lowering blood glucose levels.

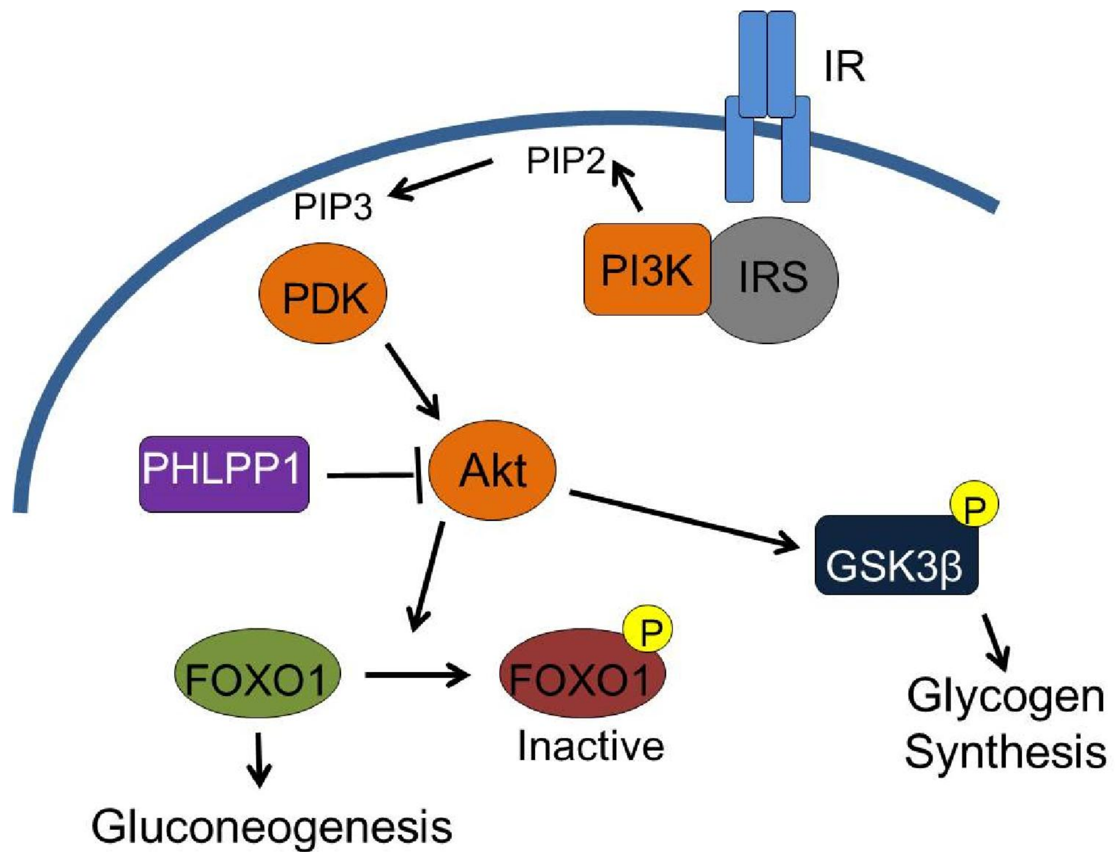

Figure 1.4: Akt signaling in the liver

Activation of the insulin signaling pathway in liver leads to phosphorylation of Foxo1 by Akt. Foxo1 is a transcription factor that normally translocates to the nucleus and activates the transcription of gluconeogenic genes. Upon phosphorylation by Akt, Foxo1 is excluded from the nucleus thus leading to suppression of gluconeogenesis. Concurrently, Akt phosphorylates GSK3β, which is an inhibitor of glycogen synthesis. Upon phosphorylation, it is inactivated allowing for glycogen synthesis to take place.

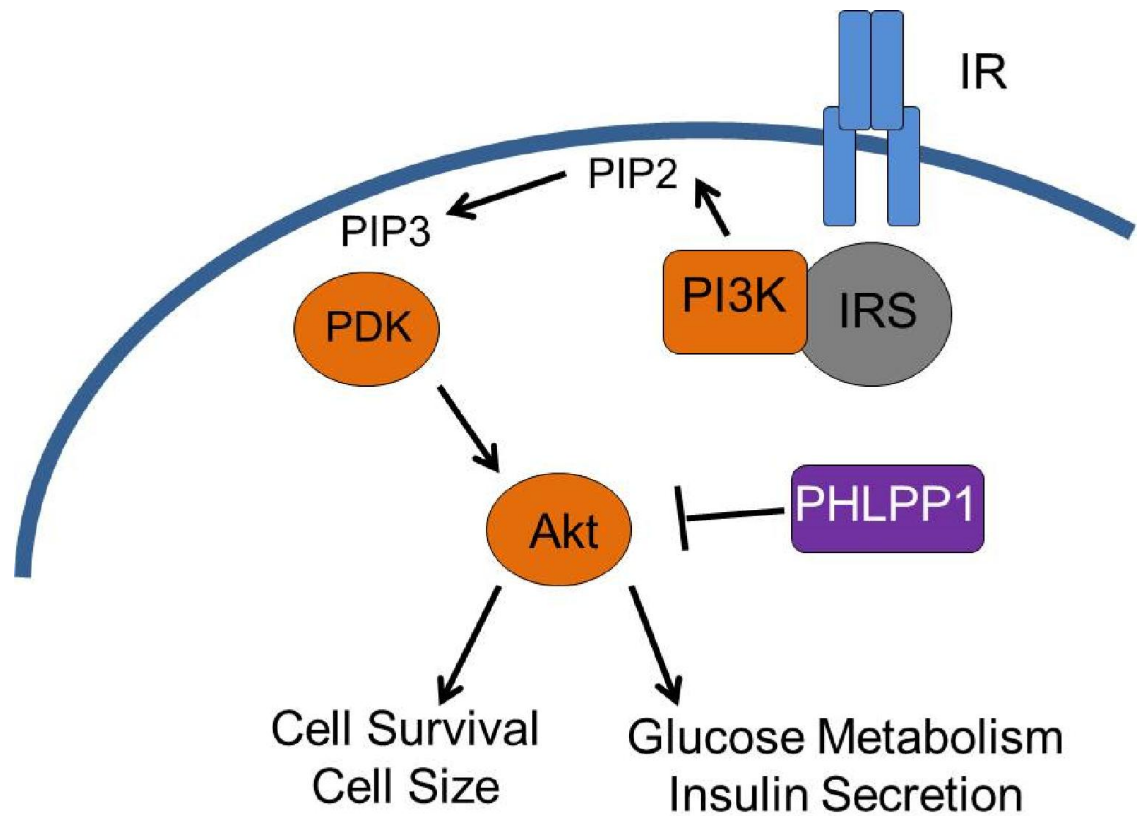

Figure 1.5: Akt signaling in the pancreatic beta-cell

Insulin signaling in the pancreatic beta cell leads to activation of Akt, which has been shown to promote cell survival and to increase cell size as well play a role in glucose stimulated insulin secretion.

Feedback Regulation of PHLPP via Akt and S6K

There are two known feedback loops that regulate PHLPP. The first is a feedback loop from Akt that controls PHLPP degradation. GSK3β is a kinase inhibited via phosphorylation by Akt. GSK3β phosphorylates PHLPP1 targeting it for ubiquitination and degradation. Therefore, if PHLPP1 levels are low, Akt will be highly active causing a decrease in GSK3β activity leading to decreased PHLPP1 degradation subsequently increasing PHLPP1 activity. This negative feedback loop is lost in many cancer cell lines. In cancer cells, PHLPP1 is still phosphorylated by GSK3β, however, it can no longer interact with the E3 ligase necessary for ubiquitination due to mislocalization of the ligase, thus Akt can no longer regulate PHLPP1 protein levels [40].

There is a second feedback loop involving the direct dephosphorylation of S6K by PHLPP. It is well established that there is a negative feedback loop from S6K to IRS1 (insulin receptor substrate 1), an adaptor protein involved in the insulin receptor-PI3K-Akt pathway. S6K phosphorylates IRS1 targeting it for degradation and attenuating signaling through this pathway. This negative feedback loop between S6K and the IRS proteins has been shown to play a role in insulin resistance as prolonged insulin signaling highly activates S6K resulting in degradation of the IRS proteins [41]. This uncouples the insulin receptor (IR) from PI3K signaling rendering a cell unable to respond to insulin signal. When PHLPP activity is high, S6K activity is decreased resulting in a decrease of IRS1 degradation (**Figure 1.6**). This activates PI3K signaling, increasing flux through the insulin receptor pathway. However, loss of PHLPP expression has a similar effect to prolonged insulin signaling. Low PHLPP activity results in high S6K activation which decreases IRS1 protein levels and decreases

insulin-dependent activation of Akt thus promoting the uncoupling of the IR from the PI3K pathway [24].

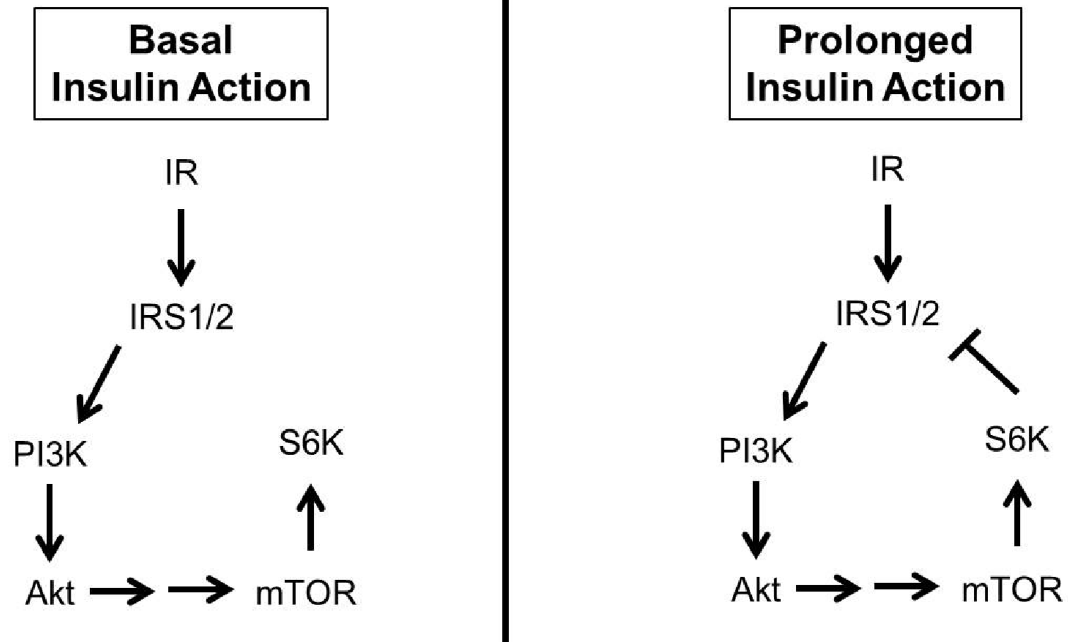

Figure 1.6: Feedback regulation between S6K and IRS

Basal insulin signaling results in activation of the PI3K pathway. However, during instances of prolonged insulin action, such as when a patient is developing type 2 diabetes, S6K is highly activated resulting in the phosphorylation of the IRS proteins targeting them for degradation by the proteasome. This effectively uncouples IR activation from PI3K signaling. This feedback regulation between S6K and IRS has been indicated to play role in insulin resistance. Loss of PHLPP signaling has been shown to play a similar role as prolonged insulin action, resulting in high S6K activation and the degradation of IRS. (Modified from [41])

Akt and Type 2 Diabetes Mellitus

As Akt is downstream of the insulin receptor it has been found to play a role in many processes important for glucose metabolism including GLUT4 translocation, stimulation of glycogen synthesis, increasing the rate of glycolysis, and inhibiting gluconeogenesis [42]. There are three Akt isoforms all exhibiting non-redundancy in their function and expression. Akt1 is ubiquitously expressed and has been indicated in controlling organism size, adipogenesis, and skeletal muscle differentiation. Akt3 is mainly expressed in neurons and is required for normal brain size. Akt2 has been found to have increased expression in insulin-responsive tissues, and is required for maintaining glucose homeostasis [43].

There have been two studies looking at the effects of the loss of Akt2 expression in mice. The first study determined that Akt2 deficient mice exhibited fasting and fed hyperglycemia with compensatory hyperinsulinemia. These mice displayed both glucose and insulin intolerance with an inability to suppress hepatic glucose production. These mice also exhibited an increase in islet mass and number but the increase was not as dramatic as what has been seen in other mouse models of insulin resistance possibly due to the disruption in Akt2 signaling. This resulted in an inadequate compensatory hyperinsulinemia in response to the hyperglycemia [44].

The second study used a different Akt2 knockout mouse model. Similarly to the first study, the mice exhibited fasting and fed hyperglycemia and glucose intolerance. Unlike the previous study, these mice displayed growth deficiency along with lipoatrophy that progressed with age. Interestingly, the male mice exhibited three different phenotypes when it came to plasma insulin levels. One subset were found to be

hyperinsulinemic followed by a decline in insulin levels with corresponding hyperglycemia. A second subset exhibited steadily rising hyperinsulinemia corresponding with steadily rising hyperglycemia. Meanwhile, a third subset displayed hypoinsulinemia along with extreme hyperglycemia. Ultimately, 75% of males displayed an extreme diabetic phenotype by 5-8 months of age [45].

Loss of Akt2 activity has also been found to lead to severe type 2 diabetes mellitus in humans. During a screen for gene mutations in patients with severe insulin resistance, one patient was found to have a missense mutation in the catalytic domain of Akt2. The patient, along with three relatives, was found to be heterozygous for the mutation. All exhibited extreme hyperinsulinema and three of the four developed diabetes in their thirties. The original patient was found to have severe insulin resistance in both the muscle and the liver. Similarly to the Akt2 knockout mice, the patient also had decreased body fat compared to predicted total body fat for her height and weight [46].

Significance of this Study

PHLPP phosphatase has been demonstrated to function as a tumor suppressor in many cancers through its negative regulation of Akt, however, PHLPP's role in other disease states is mostly unknown. We know that Akt is upstream of GLUT4 in the IR/PI3K pathway in muscle promoting glucose uptake upon insulin stimulation. In the liver, Akt is responsible for turning off glucose production via the inactivation of Foxo1 as well as for promotion of glycogen storage through phosphorylation of GSK3β. In the pancreatic beta-cell Akt plays a role in cell size and proliferation in response to increasing hyperglycemia as well as a role in promoting insulin secretion. Based on

these data combined with the effects seen upon disruption of Akt expression in both mice and humans, we hypothesize that PHLPP also functions in the regulation of glucose metabolism. As Akt2 is the isoform necessary to maintain normoglycemia, we focused on PHLPP1 as it specifically targets Akt2. We used *Phlpp1* whole body knockout mice to study its role in glucose homeostasis. The obtained data suggest that that loss of *Phlpp1* expression in mice leads to development of type 2 diabetes mellitus.

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Chapter 2

Materials and Methods

Animals: All animals were housed in a specific pathogen-free animal facility at the University of Kentucky with 12-hour light-dark cycle. All animal procedures were reviewed and approved by the University of Kentucky Institutional Animal Care and Use Committee. *Phlpp1* null mice on 129Sv/C57BL6 background as described previously [22] were backcrossed with C57BL/6NJ mice (Jackson Laboratories, Bar Harbor, ME) for five generations. Heterozygous mating pairs were used to generate *Phlpp1*$^{-/-}$ and wild type animals.

Genotyping: All animals were genotyped by taking tail snips at 21 days old. Tail snips were put into 300μL DNA extraction buffer (10mM NaOH, 0.1mM EDTA) and heated for 10 minutes at 95°C. 6μL of each sample were combined with 10μL GoTaq® Green Master Mix 2x (Promega), 2μL forward primer, 1μL reverse primer 1, and 1μL reverse primer 2. (All primer sequences are listed in Table 2.1). Samples were run on a Stratagene Robocycler 96. Cycling conditions are as follows:

1 cycle	1 minute at 95°C
35 cycles	30 seconds at 95°C
	1 minute at 54°C
	1 minute at 72°C
1 cycle	5 minutes at 72°C

All samples were visualized on a 2% agarose gel with EZ-Vision® Three DNA Dye & Buffer, 6x (Amresco).

Tolerance Tests: All tolerance tests were carried out with males and followed the standard operating procedures established previously [47]. For the glucose tolerance tests (GTT), mice were fasted for 6 hours, weighed, and then i.p. injected with glucose (2g/kg, Sigma). For the insulin tolerance tests (ITT) mice were fasted for 4 hours, weighed and then i.p. injected with insulin (0.75units/kg, Humulin R, Eli Lilly). For the pyruvate tolerance tests (PTT), mice were fasted for 16 hours, weighed, and then i.p. injected with pyruvate (2g/kg, Thermo Scientific). All blood was collected via the tail vein and blood glucose levels were measured using a NovaMax Plus glucometer post-injection at the times indicated.

Measurement of Insulin Secretion: To measure glucose stimulated insulin secretion, blood was collected from male mice via the tail vein into microvette lithium-heparin tubes (Sarstedt) at time 0 and 15 minutes post glucose injection (2g/kg). Blood samples were mixed with 50µL of 50mM EDTA in 1x PBS and centrifuged for 5 minutes at 5000 rpm at 4°C. The supernatant (plasma) was removed and kept for insulin measurement. To determine plasma insulin concentrations, insulin ELISAs were performed according to manufacturer's instructions (Ultra-sensitive Mouse Insulin ELISA Kit, Crystal Chem). ELISAs were read on the µQuant spectrophotometer (Bio-Tek Instruments) at an absorbance of 450nm.

Measurement of Pancreatic Beta-Cell Mass: Beta-cell mass and islet area were measured in four non-consecutive pancreas sections per mouse stained for insulin using Image J (National Institutes of Health, Bethesda, MD) as described previously [48]. Pancreata were removed, weighed, fixed in Bouin's solution and embedded in paraffin. Islet number was quantified manually in insulin stained sections in a blinded fashion using an Olympus CH2 upright microscope with an intraocular calibrated grid. Beta-cell

mass was estimated as the product of the relative cross-sectional area of beta-cell per total pancreas, and the weight of the pancreas by examining pancreata from five wild type and *Phlpp1*$^{-/-}$ animals.

Echo-MRI: To determine body composition, live, unanesthetized mice were measured using an EchoMRI-5000 whole-body composition analyzer (Echo Medical System, Houston, TX). The machine uses magnetic resonance relaxometry.

Indirect Calorimetry: Food consumption, body temperature, animal activity, O_2 consumption, and CO_2 production was measured using the TSE LabMaster system by the staff of the COBRE Metabolic Core at the University of Kentucky. Echo-MRI was performed before and after putting the animals into the cages for measurement of lean and fat mass in order to monitor changes in body composition during indirect calorimetry.

Isolation and Purification of Islets: Pancreatic islets were isolated from mice for making protein extracts. Mice were euthanized via CO_2 followed by cervical dislocation. Pancreata were removed aseptically via injection of Hank's Balanced Salt Solution (HBSS, Lonza) containing 0.5mg/mL collagenase type V (Sigma). Pancreata were separated from the stomach and duodenum and immediately placed in a solution of 0.5mg/mL collagenase in HBSS. They were then incubated at 37°C with frequent agitation to facilitate digestion. Digestion was stopped by adding chilled HBSS with 10% fetal bovine serum (FBS). Samples were then centrifuged at 1000 rpm at room temperature for 2 minutes. The supernatant was aspirated away and the pellet was resuspended in 20mL HBSS with 10% FBS. After resuspension samples were spun again at 1000 rpm at room temperature for 2 minutes. This was repeated two more

times. The pellet was resuspended in 20mL HBSS. An equal volume of room temperature histopaque-1077 (Sigma) was gently underlaid beneath the resuspended islets. This was centrifuged for 10 minutes at 1800 rpm at room temperature with the centrifuge brake turned off. The islet layer (interphase) was transferred to a new tube. 10mL of HBSS with 10% FBS was added to the tube containing the histopaque layer. Both tubes were then centrifuged at 1800 rpm for 2 minutes at room temperature. The supernatant was aspirated away, and the pellets were resuspended in 20mL HBSS with 10% FBS. The samples were centrifuged at 1800 rpm for 2 minutes at room temperature. The supernatant was aspirated away and the pellets were used for protein extract preparation.

Liver collection: Mice were euthanized using CO_2 followed by cervical dislocation. Livers were removed aseptically, cut into thirds, wrapped in foil, and flash frozen in liquid nitrogen. They were stored at -80°C until used for protein extracts or RNA isolation. For staining, the isolated livers were immediately put into 10% formalin, incubated overnight at room temperature, and then paraffin embedded.

Western Blot Analysis: For islet samples, lysis buffer (50mM Tris, 20% glycerol, 150mM NaCl, 0.5% NP-40, 5mM $MgCl_2$, and 0.2mM EDTA) containing 1mM PMSF, 5μg/mL aprotinin, 1μg/mL leupeptin, 1μg/mL pepstatin, 1μL/mL phosphatase inhibitor cocktail #1 and #2 (Sigma) was added to pelleted islets, vortexed and incubated on ice for 30 minutes. For liver samples, pieces of liver were added to a Dounce homogenizer containing 3mL of the above-described lysis buffer and homogenized until completely liquefied. Lysates were then transferred to microcentrifuge tubes and incubated on ice for 30 minutes. After incubation all samples were centrifuged for 15 minutes at 10,000 rpm at 4°C. Supernatants were transferred to new tubes and pellets were discarded. All

samples were subject to BCA analysis for determination of protein concentration. Most tissue samples had to be diluted (1:3 for islets, 1:5 for liver) in lysis buffer before BCA measurement. 100μg of protein sample were boiled at 100°C for 10 minutes in 4x SDS sample buffer (200mM Tris-Cl pH 6.8, 400mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol) and 0.4M DTT. Proteins were separated using 8% SDS-PAGE . Protein was transferred to nitrocellulose at 30 volts overnight at 4°C. The nitrocellulose membrane was stained with Ponceau red for protein visualization, washed with 1x TTBS (20mM Tris pH 7.4, 150mM NaCl, 0.1 % Triton X-100), blocked with blocking buffer (1xTTBS supplemented with 5% milk or 5% BSA), and incubated in primary antibody (diluted in blocking buffer) overnight at 4°C. After washing in 1X TTBS three times for 5 minutes each, the membrane was incubated with secondary antibody (anti-mouse or anti-rabbit) conjugated with horseradish peroxidase (diluted in blocking buffer) for 2 hours at room temperature. After washing with 1X TTBS for 15 minutes four times, the membrane was exposed to ECL reagent (Dura, Thermo Scientific) and film for various time points. β-Actin was used as loading control. All antibodies, along with supplier, catalog number, species, and dilution are listed in Table 2.2.

Bicinchoninic Acid (BCA) Assay: BCA assays were carried out according to manufacturer's specifications (Pierce). All samples were diluted in water and run as triplicate. BSA protein standard (0, 1, 2, 4, 8, and 16 μg/μL) was used to construct a linear standard curve and equation which was used to determine concentration using optical density (OD) at 562nM using the μQuant Spectrophotometer (Bio-Tek Instruments).

RNA Extraction from Liver: For 1 g of tissue, use 10-20 mL Trizol (Life Technologies). Sample volume should not exceed 10% of the volume of Trizol being used. The

samples were incubated in Trizol for 5 minutes at room temperature after homogenization in a Dounce homogenizer. 0.2mL chloroform was added for every 1mL of Trizol used. Samples were shaken vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. They were then centrifuged for 15 minutes at 12,000 x g at 4°C. The aqueous phase was transferred to a fresh microcentrifuge tube. Since the liver has a high content of polysaccharides, 0.25mL isopropanol was added to the aqueous phase followed by 0.25mL high salt precipitation solution (0.8M sodium citrate and 1.2M NaCl) per 1mL Trizol used. The resulting solution was mixed, incubated at room temperature for 10 minutes and centrifuged for 10 minutes at 12,000 x g at 4°C. The supernatant was removed and the pellet was washed with 1mL 75% ethanol for every 1mL Trizol. The sample was mixed by flicking and inverting the tube and centrifuged at 7,500 x g for 5 minutes at 4°C. The RNA was air dried and re-dissolved in RNase-free water. Samples were sometimes incubated at 55-60°C for 10-15 minutes to dissolve RNA. RNA concentration was measured using the NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Periodic Acid Schiff Staining: Paraffin embedded liver sections from randomly fed *Phlpp1*$^{+/+}$ and *Phlpp1*$^{-/-}$ mice were stained for glycogen using the Periodic Acid Schiff Staining Kit (Sigma). Slides were first deparaffinized and hydrated by immersing in: xylene for 6 minutes at room temperature (two times), absolute ethanol for 5 minutes at room temperature, 95% ethanol for 4 minutes at room temperature, 70% ethanol for 3 minutes at room temperature and distilled water for 1 minute at room temperature (two times). Periodic acid Schiff staining was then performed according to manufacturer's specifications. Slides were then dehydrated and mounted by immersing in: 70% ethanol for 3 minutes at room temperature, 95% ethanol for 3 minutes at room temperature, absolute ethanol for 3 minutes at room temperature, and xylene for 3 minutes at room

temperature. Two drops of mounting media (Gel/Mount Aqueous Mounting Medium, Biomeda Corporation) were placed on top of the tissue and coverslips were placed over each section. The media was allowed to set for one hour and the edges of the coverslips were sealed with clear nail polish. Staining was visualized using a Nikon Eclipse E600.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR): RNA (1µg) isolated from liver was treated with DNase I (Sigma) according to manufacturer's specifications. cDNA was made by adding 4µL qScript cDNA SuperMix (Quanta Biosciences) and 5µL RNase/DNase-free water to the tubes containing the DNase treated RNA. The reaction was then run on a Stratagene Robocycler 96 according to the qScript cDNA kit specifications.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR): qRT-PCR was carried out using PowerSYBR® Green PCR Master Mix (Applied Biosystems) according to manufacturer's specifications on a Stratagene Mx3005P machine (Agilent Technologies). All samples were run in duplicate. See Table 2.1 for all primer sequences. Average Ct values were used to calculate relative levels of target genes between genotypes. Rpl13a was used as a control and all target gene Ct values were normalized to Rpl13a transcript levels. The delta-delta Ct method of quantitation was used to determine approximate fold difference [49].

Statistical Analysis: All data was analyzed using the students paired t-test. All experiments analyzed for significance were performed a minimum of three times. A p-value of < 0.05 was deemed statistically significant. Error bars represent ± SD.

Table 2.1: Primers used for genotyping and qRT-PCR

Primer:	Designation:	Sequence:
Phlpp1 KO43 (forward)	OS512	5'-TAGGAGAGACTAGTGACATC-3'
Phlpp1 KO44 (reverse #1)	OS513	5'-TGAGCTTATACGCTGTGATGC-3'
Phlpp1 KO56 (reverse #2)	OS514	5'-AGCCGATTGTCTGTTGTGC-3'
PEPCK (forward)	Pck2F	5'-GTGGAAGGTCGAATGTGTGG-3'
PEPCK (reverse)	Pck2R	5'-TAAACACCCCCATCGCTAGT-3'
Glucose-6-phosphatase (forward)	G6pc2F	5'-GTGTTTGAACGTCATCTTGTG-3'
Glucose-6-phosphatase (reverse)	G6pc2R	5'-TTAGTAGCAGGTAGAATCCAA-3'
Fructose-1,6-bisphosphatase (forward)	Fbp1F	5'-GTAACATCTACAGCCTTAATGAG-3'
Fructose-1,6-bisphosphatase (reverse)	Fbp1R	5'-CCAGAGTGCGGTGAATATC-3'
Glycogen Synthase (forward)	Glycogen Synthase Forward Prim	5'-ACCAAGGCCAAAACGACAG-3'
Glycogen Synthase (reverse)	Glycogen Synthase Reverse Prim	5'-GGGCTCACATTGTTCTACTTGA-3'
Glycogen Phosphorylase (forward)	Glycogen Phosphorylase Forward	5'-CACCTGCACTTCACTCTGGTC-3'
Glycogen Phosphorylase (reverse)	Glycogen Phosphorylase Reverse	5'-TTGGGACACTTGTCGTAGTAGT-3'
Rpl13a (forward)	Rpl13a Forward primer	5'-CTGTGAAGGCATCAACATTTCTG-3'
Rpl13a (reverse)	Rpla13a Reverse primer	5'-GACCACCATCCGCTTTTTCTT-3'

Table 2.2: Primary Antibodies

Antibody:	Type:	Company and Catalog Number:	Dilution:
PHLPP1	Rabbit polyclonal	Bethyl Labs, A300-660A	1:500
PHLPP2	Rabbit polyclonal	Bethyl Labs, A300-661A	1:1,000
phospho-Akt (Ser473)	Rabbit polyclonal	Cell Signaling, 9271S	1:500
Akt	Rabbit polyclonal	Cell Signaling, 9272S	1:1,000
β-Actin	Mouse monoclonal	Sigma, A5441	1:10,000
IRS2	Rabbit polyclonal	Cell Signaling, 3089S	1:1,000
phospho-p70S6K (Thr389)	Rabbit polyclonal	Cell Signaling, 9205S	1:500
p70S6K	Rabbit polyclonal	Cell Signaling, 9202	1:1,000

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Chapter 3

The role of PHLPP1 in overall glucose homeostasis and energy balance

Introduction

The PI3K/Akt pathway is extremely important in the maintenance of glucose homeostasis. It is found downstream of the insulin receptor and so plays an essential role in insulin-sensitive tissues. In the muscle, Akt is important for the translocation of GLUT4 to the plasma membrane to allow for muscle glucose uptake [34]. In the liver, Akt is responsible for turning off gluconeogenesis via Foxo1 and for activation of glycogen synthesis via downregulation of GSK3β [35, 36]. Additionally, in the pancreatic-beta cell, Akt plays a role in cell size and proliferation as well as in glucose-stimulated insulin secretion [37-39]. Loss of Akt expression has been linked with a type 2 diabetes phenotype in both mice and humans. In mice, loss of Akt2 leads to hyperglycemia with compensatory hyperinsulinemia along with glucose and insulin intolerance [44]. Another study found that loss of Akt2 expression in mice caused glucose intolerance, lipoatrophy, and fed and fasting hyperglycemia. A subset of these mice developed a decline in insulin secretion indicating beta-cell failure. Overall 75% of the male mice in this study developed a severe diabetic phenotype [45]. In humans, a missense mutation in the catalytic domain of Akt2 found during a screen of diabetic patients was associated with severe insulin resistance, extreme hyperinsulinemia, and a decrease in body fat [46].

*P*leckstrin *h*omology domain *l*eucine-rich repeat *p*rotein *p*hosphatase (PHLPP) is a negative regulator of Akt via dephosphorylation of serine 473 in its catalytic domain. Loss of serine 473 phosphorylation attenuates Akt signaling. PHLPP regulation of Akt has been highly studied in cancer as Akt is a central signaling molecule in preventing

apoptosis and promoting cell proliferation [19]. There are two PHLPP isoforms and both are found at loci frequently lost in cancers [29]. PHLPP's role in other disease states is not well known. There have been two studies looking at PHLPP expression in obese and/or diabetic patients. The first found that *Phlpp* mRNA expression is increased in the muscle tissue of type 2 diabetic patients compared to non-diabetic patients with a concomitant decrease in Akt ser473 phosphorylation [32]. The second study determined that PHLPP1 protein levels are increased in adipose tissue of obese patients and that *Phlpp1* expression positively correlates with BMI [33].

We hypothesized that since Akt has been shown to play a role in blood glucose regulation and overall homeostasis, that PHLPP would also have a regulatory function in glucose homeostasis. This study focused on the PHLPP1 isoform as it specifically regulates Akt2, which is important for blood glucose regulation [28]. All of the experiments were carried out using whole body *Phlpp1* knockout mice. All experimental details are described in Chapter 2.

Results

Phlpp1$^{-/-}$ mice display significantly reduced body weight when young and increased when older due to differences in fat mass

Phlpp1$^{-/-}$ mice were generated using heterozygous pairings to produce both wild type and null offspring. When weaning at three weeks of age, we observed that the *Phlpp1*$^{-/-}$ mice were noticeably smaller than their wild type littermates. We began measuring their body weight at four weeks of age and every four weeks thereafter to quantify any differences. At four and eight weeks of age the *Phlpp1*$^{-/-}$ mice weighed

significantly less than their wild type counterparts. Beginning at twelve weeks of age the weight of the *Phlpp1*$^{-/-}$ mice was not significantly different from wild type. This remained true until 24 weeks of age when the *Phlpp1*$^{-/-}$ mice began to weigh significantly more than the control animals. The increase in weight was sustained until 36 weeks when we discontinued measurement (**Figure 3.1)**.

To determine whether the differences in weight between *Phlpp1*$^{-/-}$ and wild type mice were due to changes in fat mass we performed Echo-MRI. Echo-MRI has the ability to measure fat and lean mass, as well as free and total water in live, unanesthetized mice. Echo-MRI uses nuclear magnetic resonance (NMR) to measure the differences in proton spins in the various soft tissue environments. The proton signals being measured are related to the properties of the material being scanned, therefore, the machine is able to tell the difference between fat and lean mass. We performed Echo-MRI on 8-week old wild type and *Phlpp1*$^{-/-}$ mice. As expected, the *Phlpp1*$^{-/-}$ mice had significantly decreased amounts of fat mass compared to wild type controls. Interestingly, they also exhibited significantly decreased amounts of lean mass. Since the *Phlpp1*$^{-/-}$ gained weight faster than their wild type littermates as they aged, we also measured lean and fat mass in 24-week old mice, the age at which the weight of the *Phlpp1*$^{-/-}$ mice began to increase. The 24-week old *Phlpp1*$^{-/-}$ mice had significantly increased amounts of fat mass compared to wild type, however, they maintained their decrease in lean mass (**Figure 3.2)**.

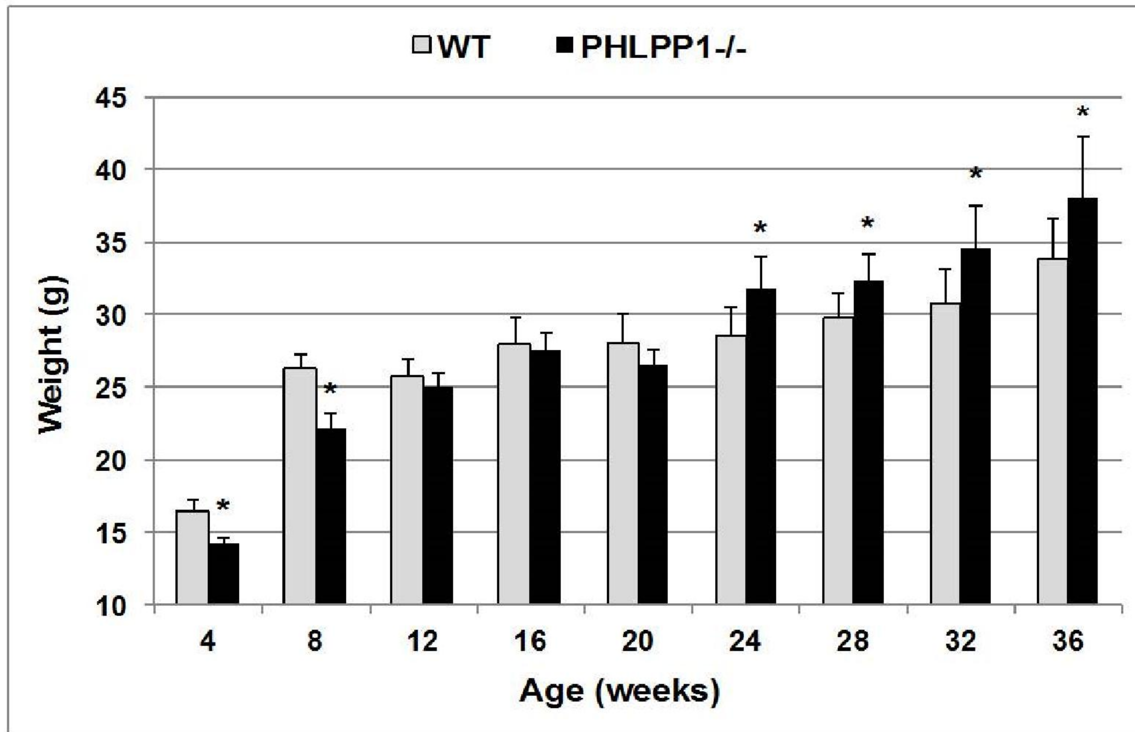

Figure 3.1: *Phlpp1*$^{-/-}$ mice weigh significantly less when young and significantly more when old

Wild type and *Phlpp1*$^{-/-}$ mice were weighed beginning at 4 weeks and every 4 weeks subsequently for 36 weeks. The results are mean values (n=10) ± SD. *p < 0.05

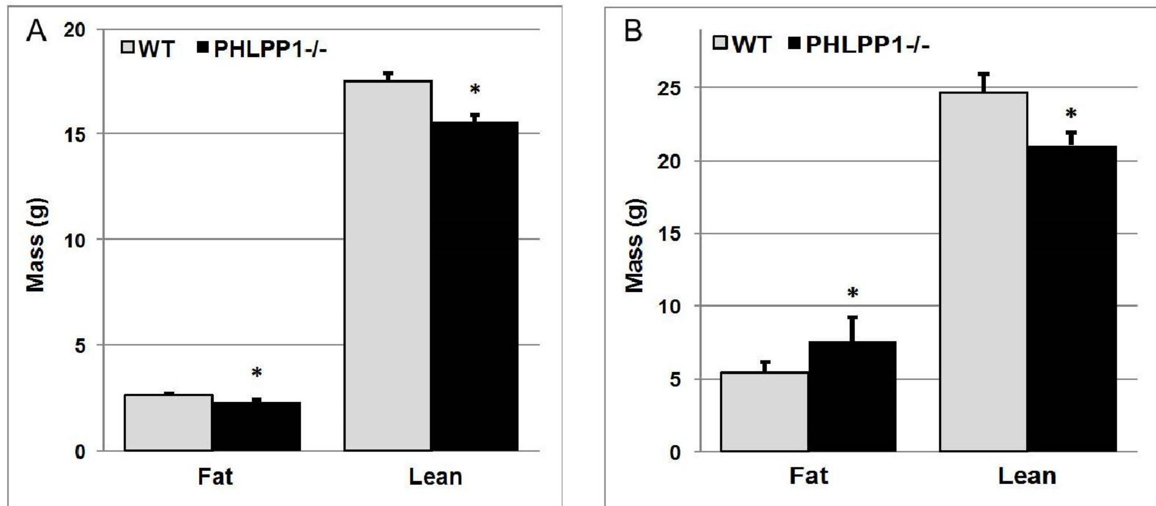

Figure 3.2: *Phlpp1*$^{-/-}$ mice exhibit significantly decreased fat mass at 8 weeks and significantly increased fat mass at 24 weeks

Echo-MRI was used to measure fat and lean mass of live, unanesthetized *Phlpp1*$^{-/-}$ and wild type mice at **(A)** 8 weeks old and **(B)** 24 weeks of age. The results are mean values (n=14 for WT, n=12 for *Phlpp1*$^{-/-}$) ± SD. *$p < 0.05$

***Phlpp1$^{-/-}$* mice have increased fasting blood glucose levels but normal glucose tolerance**

Abnormal fasting blood glucose levels can be a good indicator of disruptions to overall glucose homeostasis. Increased fasting blood glucose may not signify that a patient currently has type 2 diabetes, however it does predispose a patient to the disease. The liver is mainly responsible for maintaining glucose levels during times of fasting, and it does this by producing glucose via glycogenolysis or gluconeogenesis. Hepatic glucose production is turned off by insulin, but if the liver becomes insulin resistant, then glucose production becomes uncontrolled, and fasting glucose levels are increased. Since insulin resistance is a necessary step in the development of type 2 diabetes, changes in fasting blood glucose levels can be an early indicator of the disease progression.

To determine if *Phlpp1*$^{-/-}$ mice display any changes in fasting blood glucose levels, we fasted eight week old wild type and *Phlpp1*$^{-/-}$ littermates overnight for 16 hours and measured blood glucose levels in the morning using a glucometer. Interestingly, *Phlpp1*$^{-/-}$ mice displayed significantly increased fasting blood glucose levels compared to wild type. We repeated the measurement using 24-week old mice and found that the *Phlpp1*$^{-/-}$ mice still exhibited a significant increase in fasting levels, and this increase was more pronounced compared to the 8-week old mice (**Figure 3.3a)**. To get a better idea of overall glucose homeostasis, we measured blood glucose levels of *Phlpp1*$^{-/-}$ mice and wild type controls fed *ad libitum*. At eight weeks of age, there was no significant difference, however, 24-week old *Phlpp1*$^{-/-}$ displayed significantly increased blood glucose levels compared to wild type (**Figure 3.3b**.)

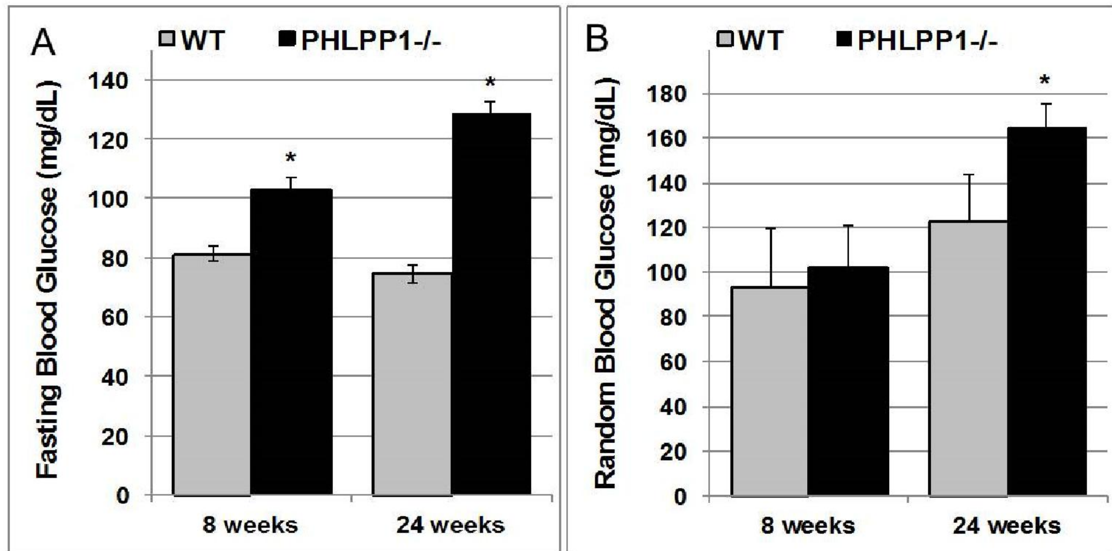

Figure 3.3: *Phlpp1$^{-/-}$* mice display increased fasting blood glucose levels at both 8 and 24 weeks and increased random blood glucose at only 24 weeks

(A) Fasting blood glucose (n=10) and **(B)** blood glucose of animals fed *ad libitum* (n=10) was measured in 8- and 24-week old *Phlpp1$^{-/-}$* and wild type mice using blood collected via the tail vein. Results are mean values ± SD. *$p < 0.05$

A glucose tolerance test is used to measure glucose clearance from the bloodstream. Once a glucose load has been either ingested or injected, blood glucose levels are measured for up to two hours. If blood glucose levels do not return to normal levels after the two-hour period, then this indicates impaired glucose tolerance (IGT). IGT can be an indicator of reduced glucose uptake due to insulin resistance, impaired glucose-stimulated insulin secretion, or hepatic insulin resistance causing uncontrolled glucose output. IGT typically prefaces the onset of type 2 diabetes.

To determine if the *Phlpp1*$^{-/-}$ mice had impaired glucose tolerance, we performed glucose tolerance tests (GTT) on 8-week old *Phlpp1*$^{-/-}$ and wild type littermates. Mice were fasted overnight for 16 hours, weighed, and then i.p. injected with 2 g/kg glucose. Fasting glucose was measured at time 0, and blood glucose levels were measured every 15 minutes post-injection for two hours. Despite the differences in fasting glucose levels, the *Phlpp1* null mice showed a slight increase in glucose tolerance, however, this was not statistically significant (**Figure 3.4**).

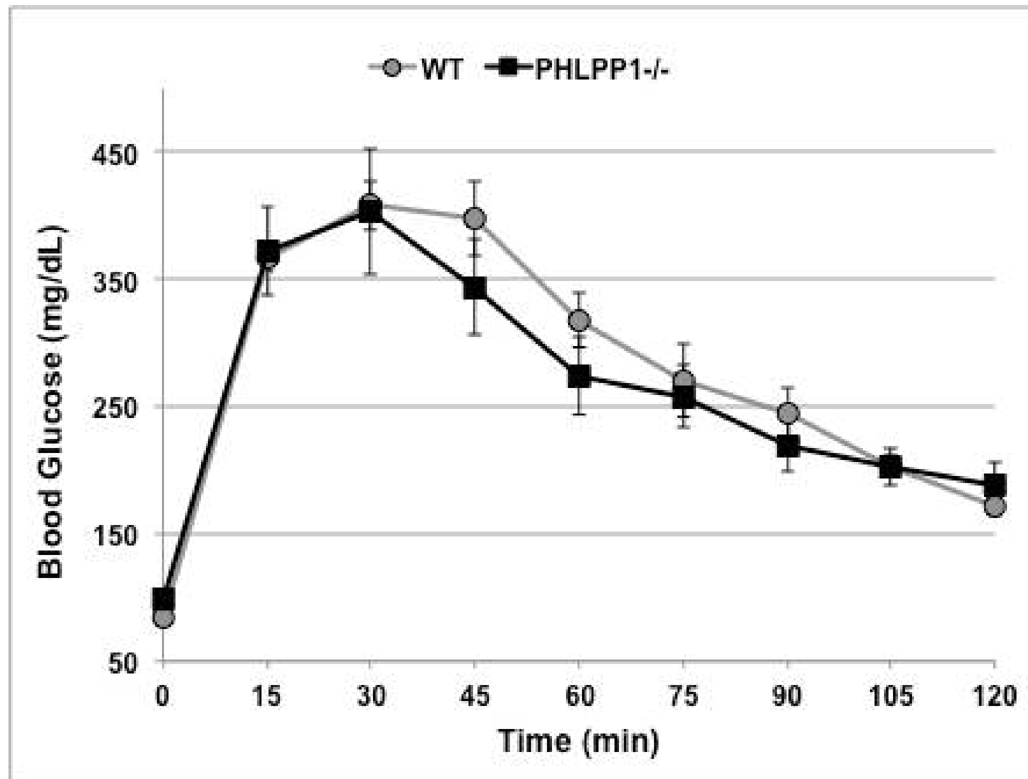

Figure 3.4: *Phlpp1*$^{-/-}$ mice display normal glucose tolerance

8-week old wild type and *Phlpp1*$^{-/-}$ mice were fasted overnight and then weighed. Blood glucose was measured at time 0, and mice were injected with 2g/kg glucose. Blood glucose was measured every 15 minutes post-injection for 2 hours. Blood was collected via the tail vein and measured using a glucometer. The results are mean values (n= 10) ± SD.

***Phlpp1* null mice have slightly increased insulin tolerance and hepatic glucose production**

An insulin tolerance test (ITT) is similar to a glucose tolerance test in that it measures glucose clearance over time. In an ITT, however, insulin is injected rather than glucose, and it is used to measure insulin sensitivity. When injected with an insulin bolus, blood glucose levels should decrease until counter-regulatory responses begin. If blood glucose levels do not decrease normally, it can be inferred that the organism has changes in insulin sensitivity. If blood glucose levels decrease more quickly, then an organism is considered more sensitive to insulin. Conversely, if blood glucose levels do not fall as quickly, an organism is likely insulin insensitive or insulin resistant whether in the muscle, liver, or both.

To measure insulin tolerance in *Phlpp1* null mice, we fasted 8-week old mice for 4 hours, weighed them, measured glucose levels at time 0, and then i.p. injected them with 0.75 units/kg insulin. We measured blood glucose levels post-injection for two hours. *Phlpp1*$^{-/-}$ mice have a slight increase in insulin sensitivity compared to wild type littermates, but the most striking differences occurred between 75 and 120 minutes post insulin injection (**Figure 3.5a**). The half-life of insulin in mice is ten minutes, therefore differences after 30 minutes are likely not due to insulin action [50]. The differences seen in the latter half of the ITT are likely due to counter-regulatory responses. In mice, once blood glucose levels fall to around 80 mg/dL, counter-regulatory hormones, including glucagon from the pancreas, are released [51]. Therefore, it is likely the differences seen 75 minutes and later are due to alterations in counter-regulation.

Because the 8-week old mice exhibited increased fasting blood glucose levels we performed a pyruvate tolerance test (PTT) in order to determine if the mice had increased rates of hepatic glucose production. The liver is responsible for maintaining blood glucose levels during times of fasting via glycogenolysis and gluconeogenesis to produce glucose. Pyruvate is the starting material for gluconeogenesis. Injection of pyruvate and then measurement of the increase of blood glucose levels post-injection can indicate the flux through the gluconeogenic pathway. If blood glucose levels increase quickly, that is suggestive of increased rates of hepatic glucose production via gluconeogenesis.

To perform the PTT, we fasted 8-week old *Phlpp1*$^{-/-}$ and wild type mice overnight for 16 hours to deplete glycogen stores. We weighed the mice, measured fasting glucose levels, and then i.p. injected with 2 g/kg pyruvate. We then measured blood glucose levels every 15 minutes post-injection for 90 minutes. *Phlpp1* null mice had slightly increased glucose production compared to wild type controls. This could be contributing factor to the increased fasting blood glucose levels seen in the *Phlpp1*$^{-/-}$ mice (**Figure 3.5b)**.

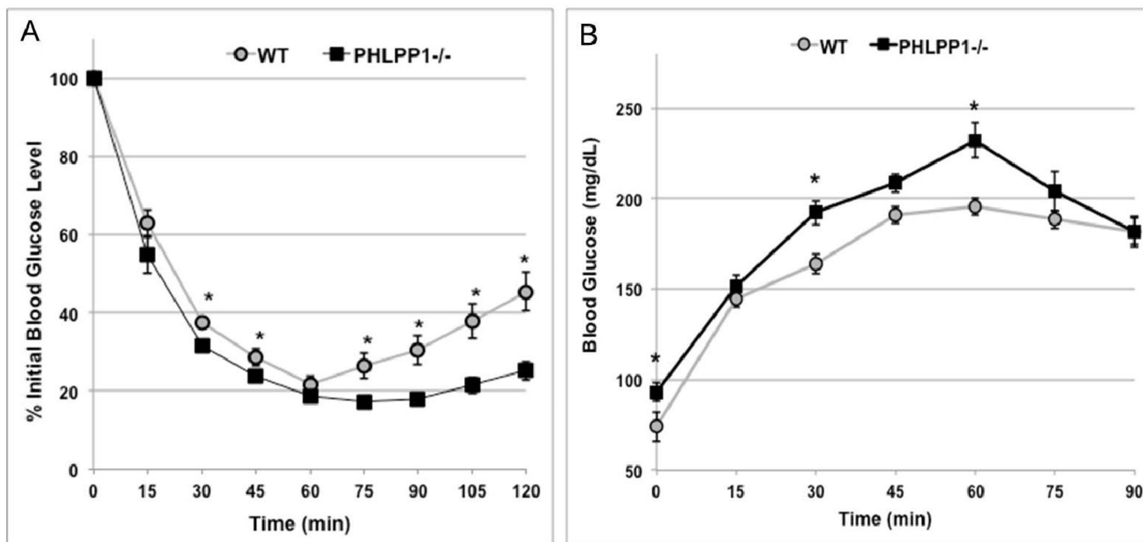

Figure 3.5: 8-week old *Phlpp1*$^{-/-}$ mice exhibit slightly increased insulin sensitivity and glucose production

(A) 8-week old wild type and *Phlpp1*$^{-/-}$ mice were fasted for 4 hours and then weighed. Basal glucose levels were measured at time 0, and then mice were injected with 0.75u/kg insulin. Glucose levels were measured every 15 minutes post-injection for 2 hours. **(B)** Mice were fasted for 16 hours and then weighed. Basal glucose levels were measured at time 0, and then injected with 2 g/kg pyruvate. Glucose levels were measured every 15 minutes post-injection for 90 minutes. Blood was collected via the tail vein using a glucometer. The results are mean values (n=14 for WT, n=12 for *Phlpp1*$^{-/-}$) ± SD. *$p < 0.05$

Young *Phlpp1*$^{-/-}$ mice compensate for insulin resistance with hyperinsulinemia

Akt has been indicated to play a role in insulin secretion as well as beta-cell expansion. This idea, along with our previous data that *Phlpp1* null mice exhibit increased fasting blood glucose but normal glucose tolerance led us to determine whether insulin secretion and beta-cell mass is affected in the *Phlpp1*$^{-/-}$ mice. To measure glucose stimulated insulin secretion in eight week old mice, we collected blood samples from 16 hour fasted *Phlpp1*$^{-/-}$ and wild type mice at baseline (time 0) and 15 minutes after injection with glucose. Plasma was separated from the whole blood and insulin levels were measured using an insulin ELISA. *Phlpp1* null mice exhibited significantly increased plasma insulin levels compared to control mice. Wild type mice displayed a four-fold increase in plasma insulin levels 15 minutes post glucose injection. *Phlpp1*$^{-/-}$ mice exhibited an eight fold increase in plasma insulin levels at baseline compared to wild type (**Figure 3.6**). While the *Phlpp1*$^{-/-}$ mice did exhibit an increase in plasma insulin levels after injection with the glucose bolus, it was not significantly higher compared to control. This is likely due to the already high baseline levels of insulin leaving less insulin available for secretion upon glucose stimulation.

One cause of increased insulin secretion is an increase in beta-cell mass and islet number. This typically happens in response to developing insulin resistance in the peripheral tissues. Increasing beta-cell mass in the pancreas can help to overcome insulin insensitivity as an increased number of beta cells will produce and secrete higher amounts of insulin. To measure beta-cell mass and islet area, pancreata from ten week old *Phlpp1*$^{-/-}$ and wild type mice were fixed, paraffin-embedded, and sectioned. Sections were stained for insulin and beta-cell mass was estimated. *Phlpp1*$^{-/-}$ displayed a modest

but significant increase in both beta-cell mass and islet area (**Figures 3.7a & 3.7b)**. (Insulin staining and measurement of beta-cell mass and islet area was done by Juan Carlos Alvarez-Perez in the laboratory of Adolfo Garcia-Ocaña, Icahn School of Medicine at Mount Sinai, New York) This corresponds well with the increase in glucose stimulated insulin secretion seen in Figure 3.5. Considering the increased fasting blood glucose levels along with hyperinsulinemia, the data so far suggests that the *Phlpp1*$^{-/-}$ mice are insulin resistant, but are able to compensate in part through increased insulin secretion.

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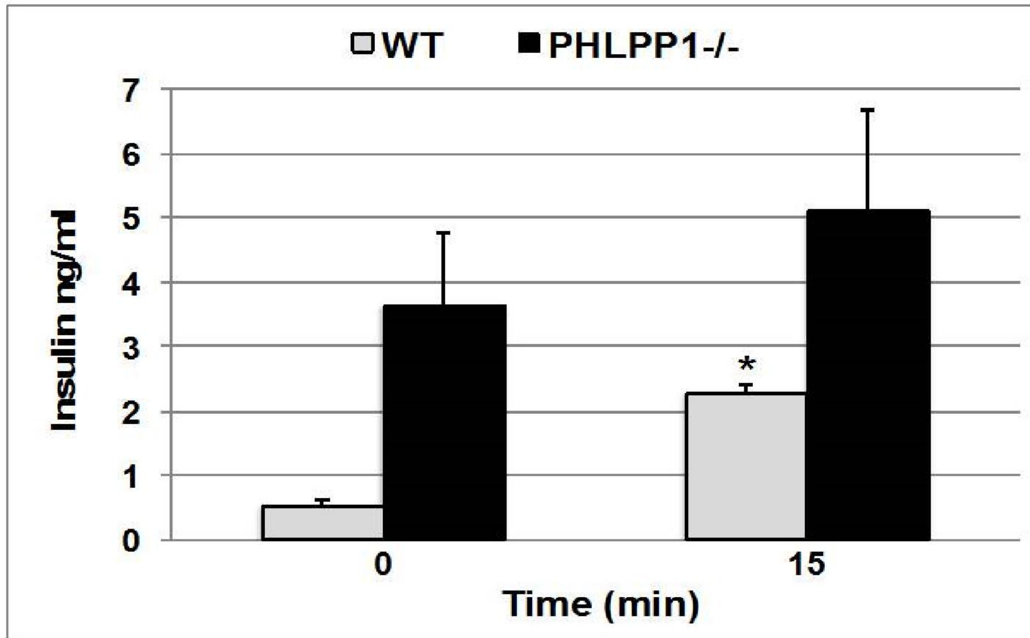

Figure 3.6: Basal plasma insulin levels are significantly increased in *Phlpp1*$^{-/-}$ mice

Wild type and *Phlpp1*$^{-/-}$ mice were fasted for 16 hours, weighed, and then injected with 2 g/kg glucose. Blood samples were taken via tail vein before glucose injection and 15 minutes post-injection. Plasma was separated from whole blood through centrifugation. Insulin levels were measured with ELISA. The results are mean values (n=5) ± SD.*$p < 0.05$.

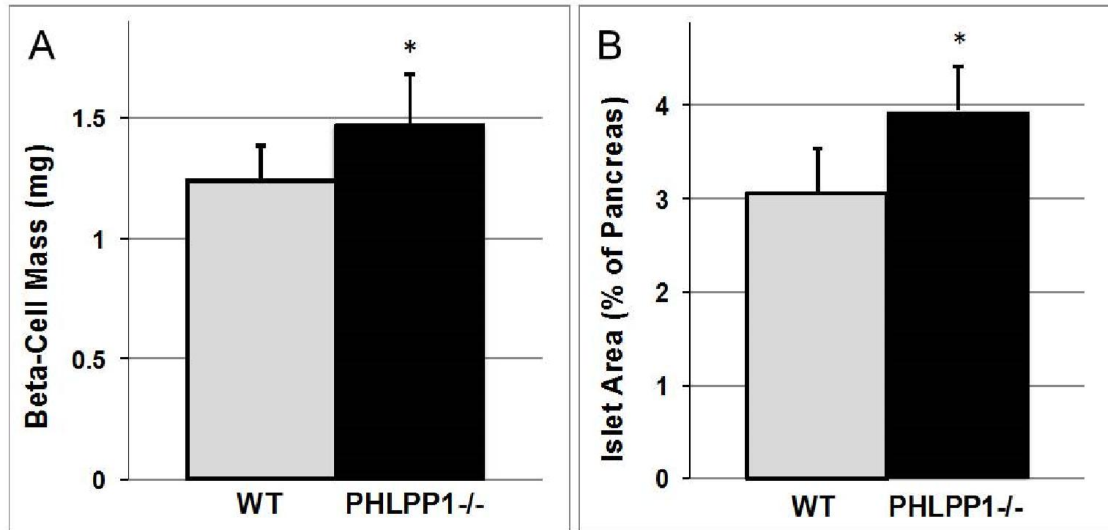

Figure 3.7: 10-week old *Phlpp1*$^{-/-}$ mice exhibit increased beta-cell mass and islet area

Pancreata were isolated from 10-week old wild type and *Phlpp1*$^{-/-}$ mice, paraffin embedded, sectioned, and stained for insulin. Beta-cell mass was measured in four non-consecutive pancreas sections per mouse. Islet number was manually quantified and beta-cell mass was measured using ImageJ. (Staining and determination of beta-cell mass was done by Juan Carlos Alvarez-Perez in the laboratory of Adolfo Garcia-Ocaña, Icahn School of Medicine at Mount Sinai, New York.) The results are mean values (n=5) ± SD. *$p < 0.05$

Phlpp1$^{-/-}$ mice become glucose-intolerant as they age

The development of diabetes begins with insulin resistance in the peripheral tissues. This means that the muscle and liver are unable to respond to insulin normally and therefore, muscle is unable to take up glucose as efficiently leaving blood glucose levels increased. Insulin resistance in liver causes uncontrolled glucose production and output, which also causes blood glucose levels to increase. However, this can be overcome with amplified insulin secretion from the beta cells, and an organism will present with normal glucose tolerance. Our previous data indicated that the *Phlpp1* null mice had increased fasting blood glucose levels, but normal glucose tolerance. They also exhibited increased plasma insulin levels correlated with increased beta-cell mass leading to the conclusion that the *Phlpp1*$^{-/-}$ mice were insulin resistant but compensated with hyperinsulinemia. We saw that the 24-week old *Phlpp1*$^{-/-}$ mice not only maintained increased fasting glucose levels, but they were more pronounced compared to the 8-week old mice. As aging is a cause of type 2 diabetes, it is highly probable that the *Phlpp1* null mice were developing uncontrolled blood glucose levels due to increasing insulin resistance and failing beta-cell compensation.

To determine if the *Phlpp1*$^{-/-}$ mice develop glucose intolerance as they age, we repeated the glucose tolerance test (GTT) on 20-week old mice. In contrast to the 8-week old mice, the *Phlpp1*$^{-/-}$ mice exhibited decreased glucose tolerance and had increased area under the curve (AUC) for glucose compared to wild type (**Figures 3.8a & 3.8b)**. This suggested that while these mice could compensate for insulin resistance when they were young through hyperinsulinemia resulting in normal glucose tolerance,

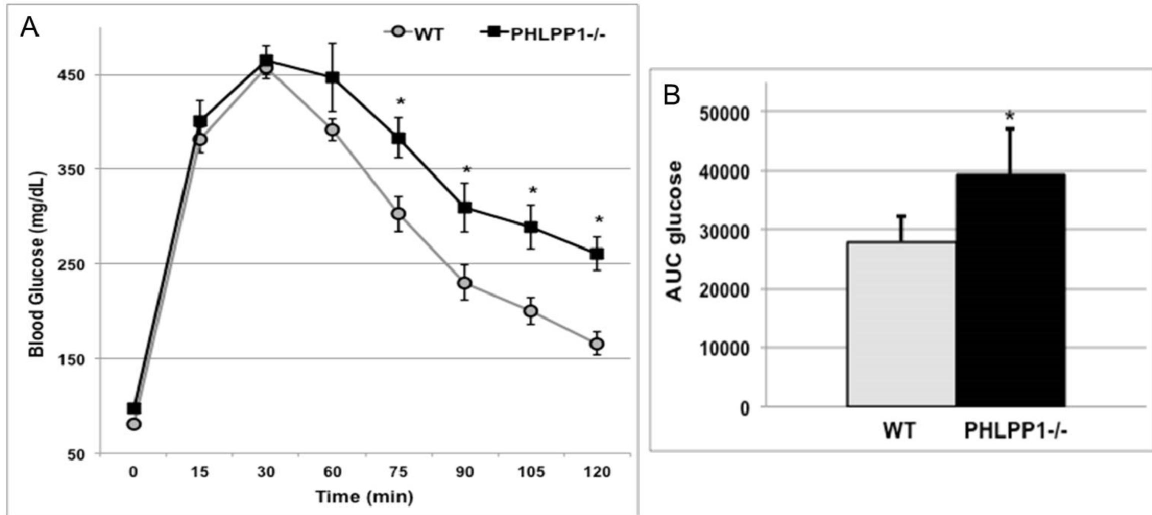

Figure 3.8: 20-week old *Phlpp1*$^{-/-}$ mice exhibit impaired glucose tolerance

(**A**) A glucose tolerance test was performed on 20-week old wild type and *Phlpp1*$^{-/-}$ mice. Mice were fasted overnight and then weighed. Basal glucose levels were measured at time 0, and then mice were injected with 2 g/kg glucose. Blood glucose levels were measured every 15 minutes post-injection for 2 hours. Blood was collected via the tail vein and measured with a glucometer. **(B)** AUC glucose was calculated for the GTT. The results are mean values (n = 14 for WT, n = 12 for *Phlpp1*$^{-/-}$) ± SD. *$p < 0.05$

as they aged, they were no longer able to normally regulate blood glucose levels resulting in glucose intolerance.

To determine if the changes in glucose tolerance in the 20-week old *Phlpp1*$^{-/-}$ mice were due to changes in glucose stimulated insulin secretion from the pancreatic-beta cells, plasma insulin was measured at basal levels (time 0) and 15 minutes after injection of a glucose bolus. Basal plasma insulin levels were similar between *Phlpp1*$^{-/-}$ and wild type mice (**Figure 3.9**). In response to a glucose bolus, control mice exhibited a fourfold increase in plasma insulin levels, whereas *Phlpp1*$^{-/-}$ displayed only a twofold increase. Compared to the 8-week old mice, the 20-week old mice had reduced plasma insulin levels (**Figure 3.5 vs. Figure 3.9**). This indicates that the *Phlpp1* null mice are no longer able to compensate for insulin resistance due to insufficient insulin secretion. This accounts for the decreased glucose tolerance in the *Phlpp1*$^{-/-}$ mice. Loss of beta-cell compensation in response to insulin insensitivity is the final step in the development of type 2 diabetes mellitus.

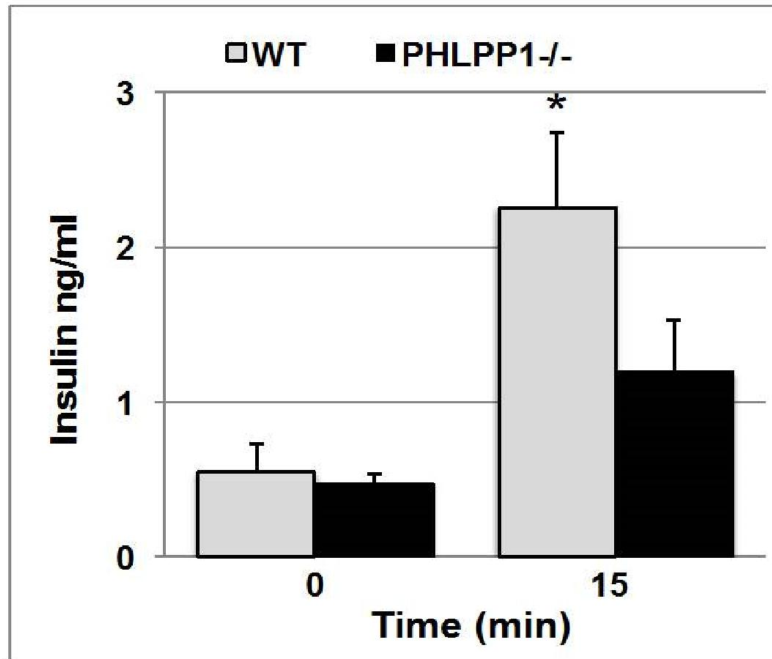

Figure 3.9: Plasma insulin is decreased in 22-week old *Phlpp1*$^{-/-}$ mice

22-week old wild type and *Phlpp1*$^{-/-}$ mice were fasted for 16 hours, weighed, and then injected with 2 g/kg glucose. Blood samples were taken via tail vein before glucose injection and 15 minutes post-injection. Plasma was separated from whole blood through centrifugation. Insulin levels were measured with ELISA. The results are mean values (n=5) ± SD.*$p < 0.05$.

24-week old *Phlpp1* null mice are insulin resistant and exhibit increased rates of glucose production

The loss of glucose tolerance in the aged *Phlpp1*$^{-/-}$ suggests that they had become insulin resistant. To determine if they had lost the ability to respond to insulin we performed an insulin tolerance test on 20-week old *Phlpp1*$^{-/-}$ and wild type controls. The mice displayed significantly increased blood glucose levels and decreased insulin sensitivity with an increased AUC for insulin suggesting that these mice are insulin resistant (**Figures 3.10a & 3.10b)**. Insulin resistance is a hallmark of type 2 diabetes.

To determine if the aged mice had changes in glucose production during fasting, 24-week old *Phlpp1*$^{-/-}$ and wild type controls were fasted overnight for 16 hours and injected with 2 g/kg pyruvate, the substrate for gluconeogenesis. *Phlpp1*$^{-/-}$ mice exhibited increased rates of glucose production and increased AUC compared to wild type mice (**Figures 3.11a & 3.11b**). Since fasting glucose levels were already significantly increased for the *Phlpp1*$^{-/-}$ mice, it is important to look at the rate of glucose production, which can be represented as the slope between time 0 and time 15. *Phlpp1*$^{-/-}$ mice produced glucose at a rate of 4.6 mg/dL min^{-1} while the wild type mice produced glucose at a rate of 3.26 mg/dL min^{-1}. This increase in glucose production correlates with the increased fasting blood glucose levels seen in the 24-week old *Phlpp1*$^{-/-}$ mice.

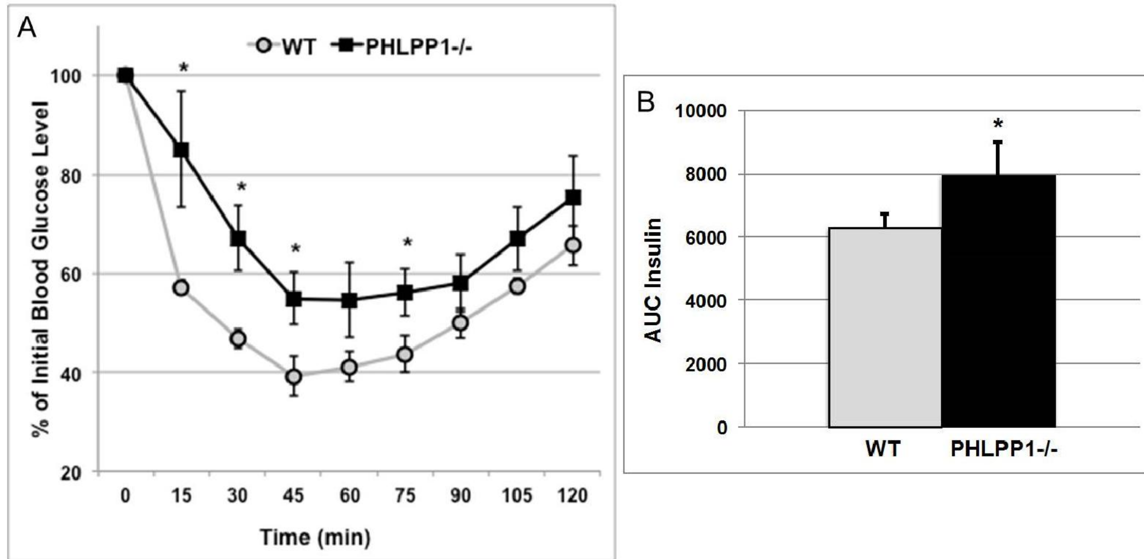

Figure 3.10: 20-week old *Phlpp1*$^{-/-}$ mice exhibit decreased insulin tolerance

(A) 20-week old wild type and *Phlpp1*$^{-/-}$ mice were fasted for four hours and then weighed. Basal glucose levels were measured at time 0, and then mice were injected with 0.75u/kg insulin. Glucose levels were measured every 15 minutes post-injection for 2 hours. **(B)** Area under the curve was calculated. The results are mean values (n = 14 for WT, n = 12 for *Phlpp1*$^{-/-}$) ± SD. *p < 0.05

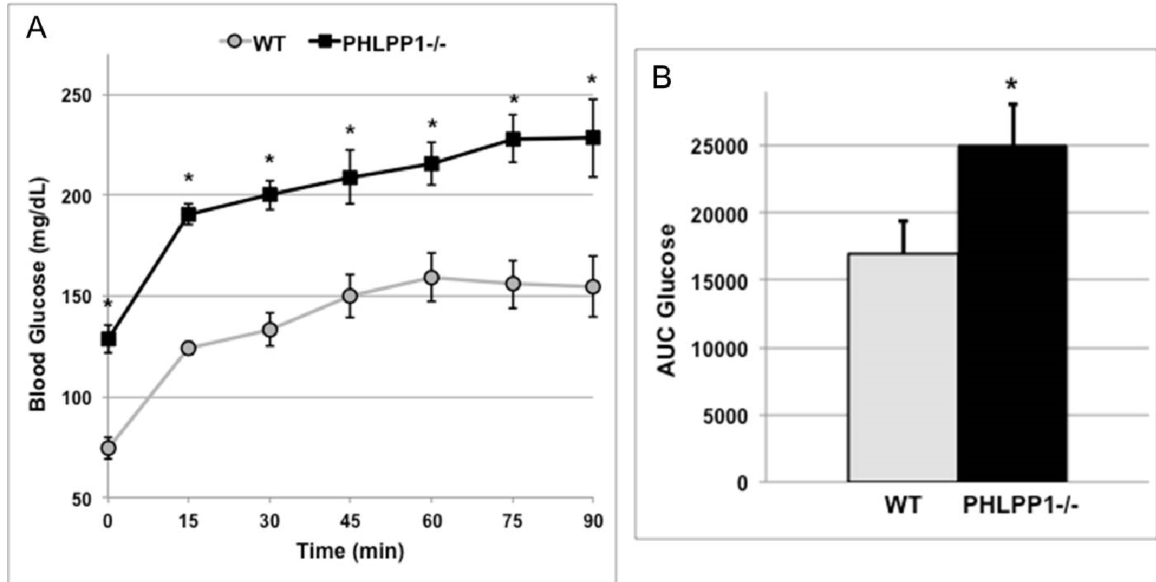

Figure 3.11: 24-week old *Phlpp1*$^{-/-}$ mice display increased glucose production

24-week old mice were fasted for 16 hours and then weighed. Basal glucose levels were measured at time 0, and then injected with 2 g/kg pyruvate. Glucose levels were measured via the tail vein using a glucometer. The results are mean values (n=14 for WT, n=12 for *Phlpp1*$^{-/-}$) ± SD. *$p < 0.05$

***Phlpp1$^{-/-}$* mice lose less body weight during an overnight fast compared to wild type mice**

It has been indicated in previous studies that overnight fasting in mice promotes glucose utilization. This is different from in humans where overnight fasting has been shown to impair glucose utilization. This difference is likely due to the fact that mice are most active at night, and it is the time when food intake is highest [47]. Therefore, overnight fasting in mice is useful for determining changes in glucose utilization. Because a long fast leads to such an increase in glucose utilization, mice that have been fasted overnight have been shown to lose up to 15% of their body weight [47]. When measuring *Phlpp1$^{-/-}$* and wild type mice after an overnight fast, it was noticed that *Phlpp1$^{-/-}$* lost less weight than their wild type littermates. In fact, the *Phlpp1$^{-/-}$* lost only 5% of their body weight while the wild type mice lost over 10% (**Figure 3.12**). This suggests that the *Phlpp1$^{-/-}$* mice have decreased insulin-stimulated glucose utilization. This new data supports our previous data suggesting that the *Phlpp1$^{-/-}$* suffer from insulin resistance. Insulin resistance would cause a loss of insulin-stimulated glucose utilization thus leading to a smaller loss of body weight when fasted overnight.

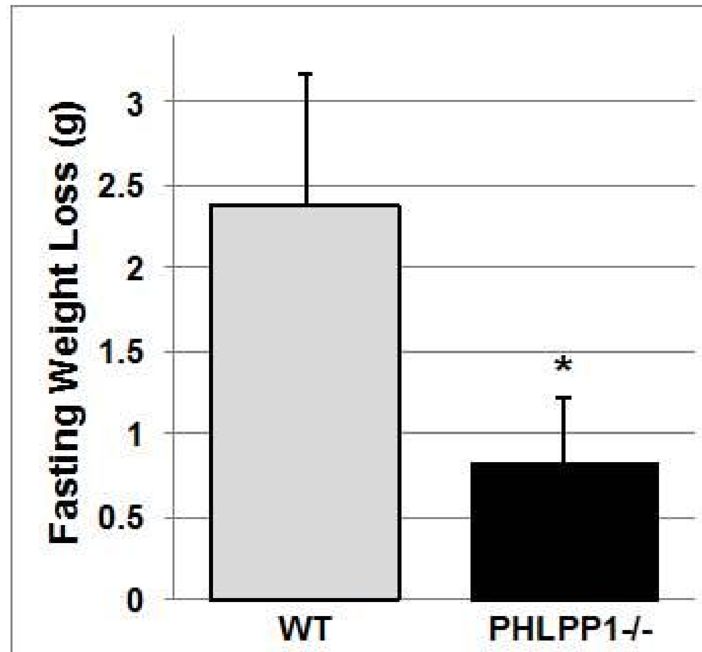

Figure 3.12: *Phlpp1*$^{-/-}$ mice lose less body weight during a long fast

20-week old wild type and *Phlpp1*$^{-/-}$ mice were weighed, fasted for 16 hours, and then weighed again. The results are mean values (n=10) ± SD. $*p < 0.05$

Phlpp1$^{-/-}$ mice display no significant differences in food intake, activity, O_2 consumption, energy expenditure, or respiratory exchange rate

Many of the differences seen in the *Phlpp1*$^{-/-}$ mice could be due to changes in food intake and activity rates. In order to determine whether the *Phlpp1* null mice displayed differences in these areas we used indirect calorimetry. Indirect calorimetry measurements give a general metabolic phenotype of a set of mice. To do the experiments, we used the TSE LabMaster system in the metabolic core at the University of Kentucky. For this experiment, mice were put into individual cages with special food and watering systems that keep track of food and water intake in real time. Mice were allowed to acclimate to the new cages for one week before measurements were taken. The cage system measures food intake, water intake, O_2 consumption, CO_2 production, and activity. From those measurements, energy expenditure and the respiratory exchange ratio can be calculated [52]. For this experiment we used 16-week old *Phlpp1*$^{-/-}$ and wild type controls and took measurements for three full days. There was no significant difference in cumulative food intake between wild type and *Phlpp1* null mice (**Figure 3.13a**). Nor was there any difference in the total activity of the mice (**Figure 3.13b**). There was also no difference in O_2 (vO_2) consumption (**Figures 3.13c**).

Energy expenditure is calculated using vO_2, vCO_2, and various physiological constants. The *Phlpp1*$^{-/-}$ mice exhibited no significant difference in energy expenditure compared to wild type mice in either the dark or light cycle (**Figure 3.14a**). Respiratory exchange ratio (RER) is the ratio of vCO_2 to vO_2 and is a function of macronutrient utilization. The closer an RER is to 0.7, the more lipid is being used for an energy source. For example, during times of fasting, when carbohydrate stores are scarce, lipids become the energy source and RER drops to 0.7. *Phlpp1*$^{-/-}$ mice exhibit no

difference in RER compared to wild type mice indicating that they have no change in macronutrient utilization (**Figure 3.14b**).

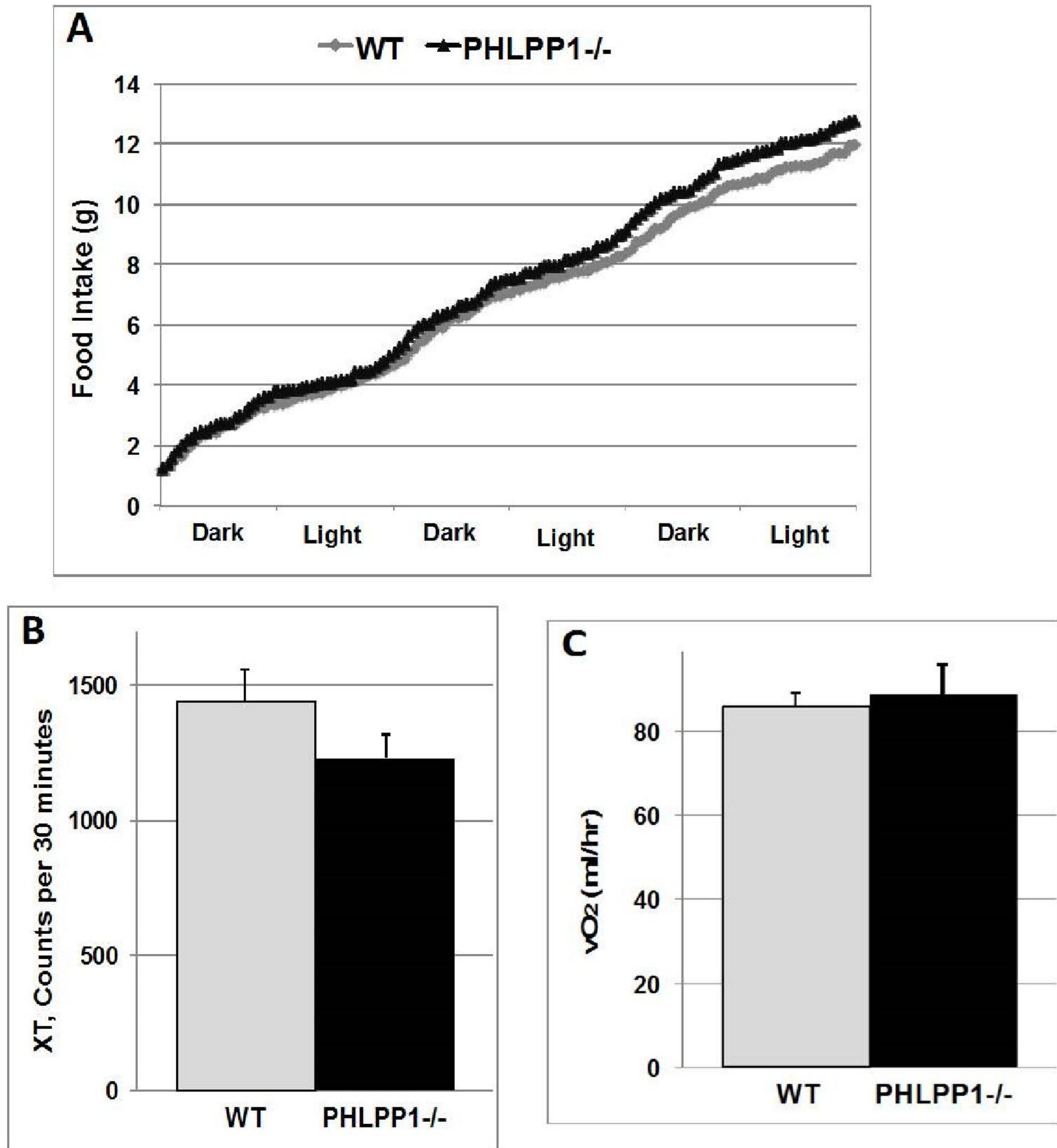

Figure 3.13: *Phlpp1$^{-/-}$* mice exhibit no difference in food intake, activity, or O_2 production

16-week old wild type and *Phlpp1$^{-/-}$* mice were monitored in a TSE LabMaster system in order to obtain a broader metabolic phenotype of the null mice. *Phlpp1$^{-/-}$* mice exhibited no difference in **(A)** cumulative food intake, **(B)** total activity, or **(C)** oxygen consumption. The results are mean values (n=5 for WT, n=4 for *Phlpp1$^{-/-}$*) ±SD.

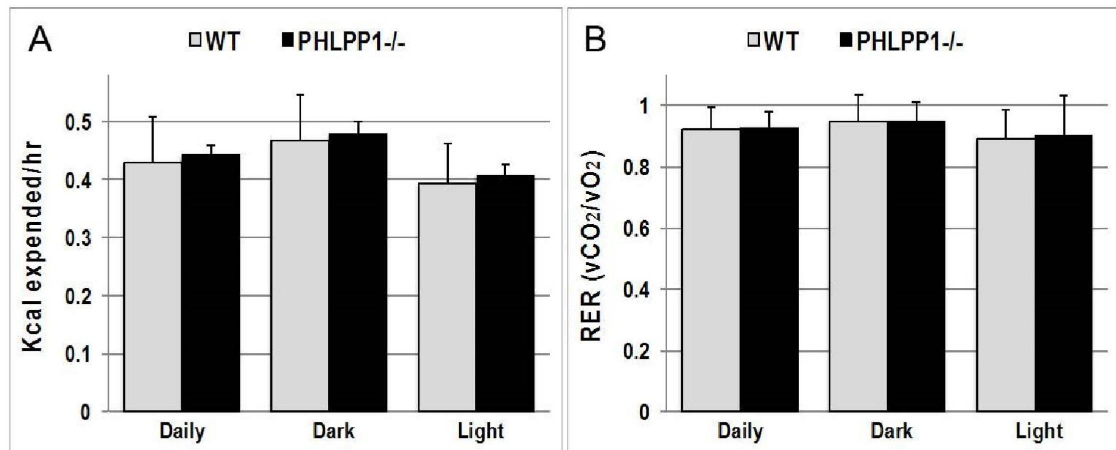

Figure 3.14: *Phlpp1*$^{-/-}$ mice exhibit no difference in energy expenditure or respiratory exchange ratio (RER)

16-week old wild type and *Phlpp1*$^{-/-}$ mice were monitored in a TSE LabMaster system in order to obtain a broader metabolic phenotype of the null mice. *Phlpp1*$^{-/-}$ mice exhibited no difference in **(A)** energy expenditure or **(B)** respiratory exchange ratio. Both were calculated by the software using collected data and physiological constants. The results are mean values (n=5 for WT, n=4 for *Phlpp1*$^{-/-}$) ± SD.

Conclusions

We set out to determine if PHLPP1 plays a role in glucose homeostasis. We hypothesized that due to PHLPP's known regulation of Akt that it would likely function in regulating blood glucose levels. Through the use of *Phlpp1* null mice we determined that loss of *Phlpp1* causes increased fasting blood glucose levels that become more pronounced through aging (**Figure 3.1**). The young mice exhibit normal glucose tolerance (**Figure 3.2**) but display hyperinsulinemia suggesting that they are insulin resistant but compensate with increased glucose-stimulated insulin secretion from the pancreatic beta cells (**Figure 3.6**). As the *Phlpp1* null mice age, they become glucose intolerant (**Figure 3.8**) as well as insulin intolerant (**Figure 3.10**). The old mice exhibit decreased plasma insulin (**Figure 3.9**) secretion indicating that the beta-cells can no longer compensate for the insulin resistance. Our next step in this study was to determine the molecular mechanisms behind the physiological changes seen in the *Phlpp1*$^{-/-}$ mice.

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Chapter 4

The effects of the loss of *Phlpp1* on the Akt signaling pathway

Introduction

PHLPP1 dephosphorylates Akt causing attenuation of Akt signaling. In all gluco-regulatory tissues, Akt is downstream of the insulin receptor (IR) in the PI3K cascade. When insulin binds to the IR at the cell membrane, IRS proteins bind to the IR. The IRS proteins act as adaptor molecules for PI3K. Upon activation, PI3K phosphorylates PIP_2 to PIP_3 which act as lipid second messengers recruiting PDK and Akt to the plasma membrane. Once at the membrane, PDK and Akt are activated through phosphorylation. Akt then moves throughout the cell to mediate downstream signaling. In the muscle, Akt activation leads to the translocation of GLUT4, a glucose transporter, to the plasma membrane allowing for glucose uptake (**See figure 1.3**). Therefore, insulin binds to its receptor promoting glucose uptake out of the bloodstream thereby lowering blood glucose levels [34]. In the liver, Akt activation leads to phosphorylation and inactivation of Foxo1. Foxo1 is a transcription factor responsible for the expression of key gluconeogenic genes. When Foxo1 is non-phosphorylated, it is active and present inside the nucleus, turning on gene transcription, thus turning on gluconeogenesis. When Akt is active, Foxo1 is phosphorylated and unable to enter the nucleus thereby gluconeogenesis is not activated. Additionally, Akt signals through the inactivation of GSK3β to promote glycogen synthesis effectively shunting the glucose taken up by the hepatocyte towards storage as glycogen (**See figure 1.4**) [35, 36]. The role of Akt in the beta-cell is less clear. It has been indicated to play a role in cell size, cell survival, and proliferation which are important for expanding beta-cell mass during

times of increasing insulin resistance [37]. There have also been studies indicating that Akt plays a role in glucose-stimulated insulin secretion (**See figure 1.5**) [38, 39].

We would expect that a loss of *Phlpp1* would lead to an increase of phosphorylated Akt leading to a subsequent increase in the downstream signaling effects of Akt. As we saw with the data described in Chapter 3, this direct effect does not appear to be taking place in the *Phlpp1* null mice. The mice develop a type 2 diabetes phenotype as they age, which is not what we would have expected if Akt signaling is enhanced. The regulation of glucose homeostasis and the development of type 2 diabetes involves many cell and tissue types so it is necessary to look at the signaling mechanisms in each separately in order to get a better idea of what effects are caused by the loss of *Phlpp1*. We focused on the liver and the pancreatic islets since from a young age, the *Phlpp1*$^{-/-}$ mice exhibited increased fasting blood glucose levels, typically attributed to the liver, and because the mice displayed hyperinsulinemia associated with an increase in beta-cell mass followed by a loss of glucose-stimulated insulin secretion.

When examining PHLPP signaling it is important to remember that PHLPP has been implicated in certain feedback loops involving its direct targets and downstream effectors. One of these feedback loops was hypothesized to have an effect in insulin signaling as it involves the degradation of IRS proteins. PHLPP has been shown to directly dephosphorylate S6K [24]. S6K phosphorylates IRS targeting it for degradation by the proteasome [41]. High flux through S6K, as seen during prolonged insulin signaling, causes a loss of IRS expression leading to an uncoupling of the PI3K pathway from insulin signaling. This leads to a loss of Akt activation [24, 41]. Loss of PHLPP has

been shown to have a similar effect as prolonged insulin signaling: increased S6K activation, IRS degradation, and dampening of Akt activation [29].

Results

Akt phosphorylation is increased in *Phlpp1*$^{-/-}$ pancreatic islets compared to wild type but decreases during aging

To determine what effect the loss of *Phlpp1* has on Akt phosphorylation, islets were isolated under basal conditions from 6-week old and 30-week old *Phlpp1*$^{-/-}$ and wild type mice. Protein lysates were made and run on an SDS-PAGE gel. Phosphorylated-Akt (ser473) was measured using Western blotting. In 6-week old mice, loss of *Phlpp1* led to an increase in phosphorylated Akt levels. 30-week old *Phlpp1* null mice also exhibited an increase in Akt phosphorylation levels compared to wild type but a 40% decrease compared to 6-week old mice (**Figure 4.1**). The increase in Akt phosphorylation is expected with the loss of *Phlpp1* expression. Additionally, Akt activity is frequently increased in beta cells during insulin resistance-induced beta-cell expansion [53].

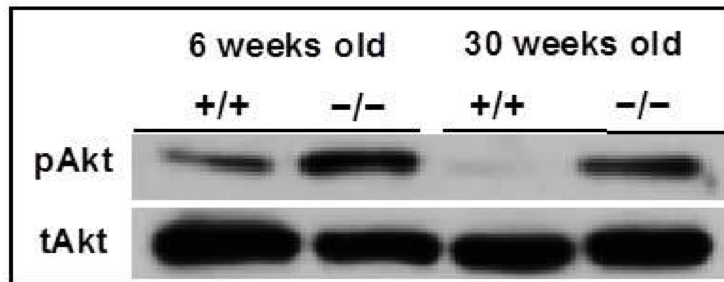

Figure 4.1: Akt phosphorylation is increased in the islets of *Phlpp1*$^{-/-}$ mice

Islets were purified from pancreata taken from 6-week old and 30-week old wild type and *Phlpp1*$^{-/-}$ mice. Islets were pooled from 3-5 mice for preparation of lysates. Lysates were run on an 8% SDS-PAGE gels and then used for Western blot analysis using antibodies for phosphorylated Akt (serine 473) and total Akt.

S6K phosphorylation is increased and IRS2 expression is decreased in aged *Phlpp1*$^{-/-}$ pancreatic islets

To determine if S6K phosphorylation and therefore IRS2 expression is affected, we blotted for both using the same islet lysates as used in Figure 4.1. Phosphorylated S6K was increased in the *Phlpp1*$^{-/-}$ mice at both 6 and 30 weeks of age, with higher phosphorylation levels appearing in the aged mice (**Figure 4.2**). IRS2 levels are unchanged between the 6-week old *Phlpp1*$^{-/-}$ and wild type mice, however, IRS2 is decreased in the 30-week old *Phlpp1*$^{-/-}$ compared to control. This is likely due to the increase in S6K activation in these islets. Since S6K is regulated directly by PHLPP1, but also is a downstream effector of Akt, the loss of *Phlpp1* could be increasing S6K phosphorylation through either mechanism.

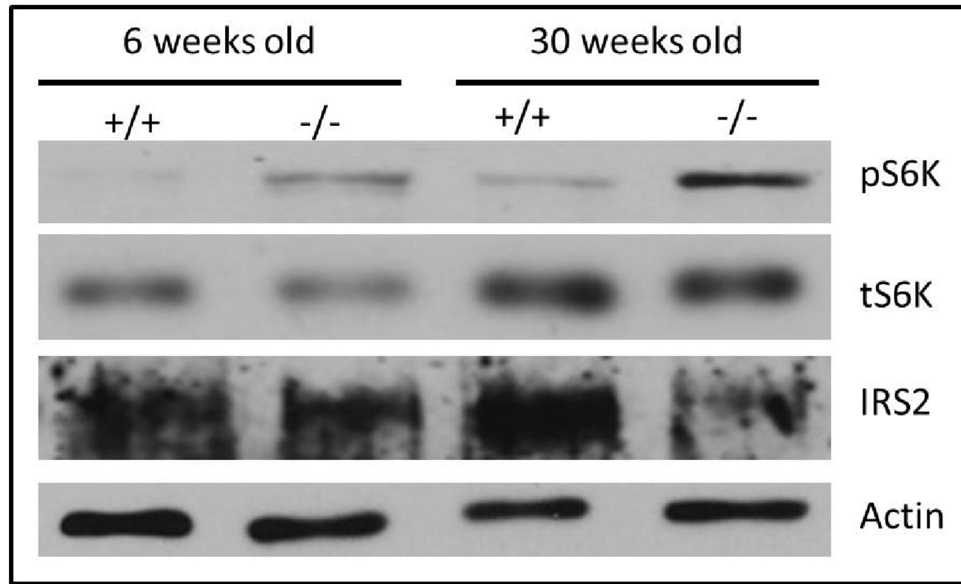

Figure 4.2: S6K phosphorylation is increased in *Phlpp1*$^{-/-}$ 6- and 30-week old mice while IRS2 expression is decreased in 30-week old *Phlpp1*$^{-/-}$ mice

Islets were purified from pancreata taken from 6- week old and 30-week old wild type and *Phlpp1*$^{-/-}$ mice. Islets were pooled from 3-5 mice for preparation of lysates. Lysates were run on an 8% SDS-PAGE gels and then used for Western blot analysis using antibodies for phosphorylated S6K (threonine 389), total S6K, and IRS2. All samples were normalized to actin.

Akt phosphorylation decreases in livers of *Phlpp1*$^{-/-}$ mice with aging

Phlpp1$^{-/-}$ mice exhibited increased fasting blood glucose from a young age that became more pronounced through aging. Fasting blood glucose is primarily maintained by the liver which produces glucose through glycogenolysis and gluconeogenesis. Because we saw such increases in fasting glucose levels, we focused on the liver to determine what may be causing these changes. We isolated livers from 6- and 30-week old *Phlpp1*$^{-/-}$ and wild type mice that had been fasted for 16 hours. Protein lysates were made and run on an SDS-PAGE gel. Western blotting revealed that in the 6-week old mice, Akt phosphorylation was lower in the *Phlpp1*$^{-/-}$ compared to wild type mice, but it was not statistically significant (**Figure 4.3a**). Interestingly, in the 30-week old *Phlpp1*$^{-/-}$ mice Akt ser473 phosphorylation was drastically downregulated compared to control mice (**Figure 4.3b**). Using Image J for quantification it was determined that Akt phosphorylation was significantly decreased (**Figure 4.3c**). With the loss of *Phlpp1* we would have expected an increase in Akt phosphorylation rather than a decrease, however, it is possible that the feedback loop involving S6K and IRS2 is being induced thus dampening Akt activation (See Figure 1.6).

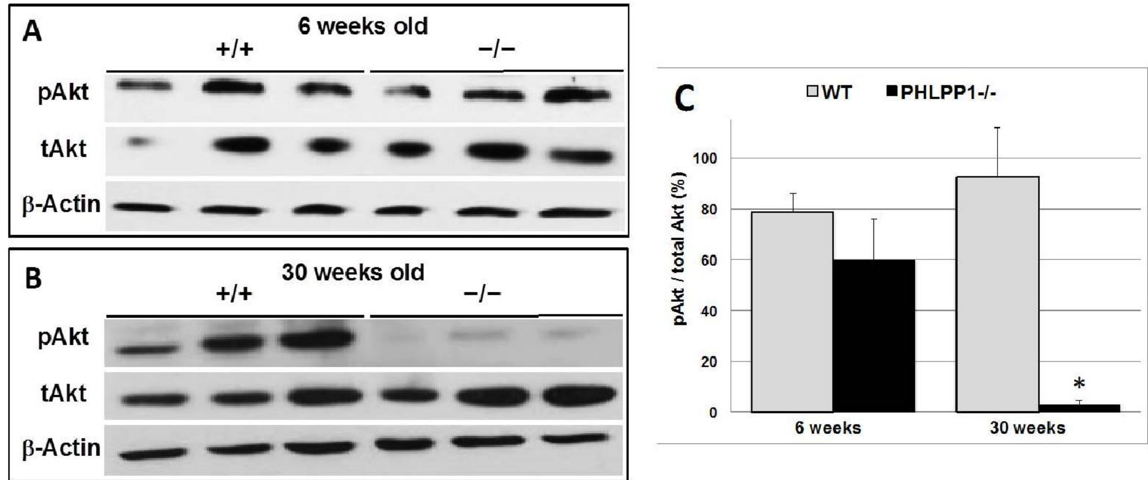

Figure 4.3: Akt phosphorylation is decreased in the livers of 6-week and 30-week old *Phlpp1*$^{-/-}$ mice

Livers were removed from **(A)** 6- and **(B)** 30-week old wild type and *Phlpp1*$^{-/-}$ mice that had been fasted for 16 hours. Lysates were made from individual livers via homogenization. 3 samples for each genotype were run on 8% SDS-PAGE gels and transferred for Western blot analysis. Samples were blotted for phosphorylated Akt (serine 473), total Akt, and Actin. **(C)** Bands were quantified using Image J.

S6K activity is increased in aged *Phlpp1*$^{-/-}$ mice resulting in IRS2 degradation

To determine if the feedback loop between S6K and IRS2 is being induced causing the loss of Akt phosphorylation, Western blotting was performed using lysates from 30-week old *Phlpp1*$^{-/-}$ and wild-type livers under basal conditions. S6K phosphorylation was increased in the *Phlpp1* null mice compared to controls. IRS2 expression was decreased in these mice suggesting that an increase in S6K activity is causing the phosphorylation and degradation of IRS2 (**Figure 4.4**). This would lead to a decoupling of the insulin receptor from the PI3K pathway causing a decrease in Akt phosphorylation and activation. We hypothesized that this loss of Akt activation would lead to an increase in gluconeogenesis due to the loss of Akt's inhibition on Foxo1, the transcription factor necessary for the expression of key gluconeogenic genes (see Figure 1.4). Increased flux through Foxo1 would lead to an increase in hepatic glucose production, increasing fasting blood glucose levels just as is seen in the *Phlpp1*$^{-/-}$ mice.

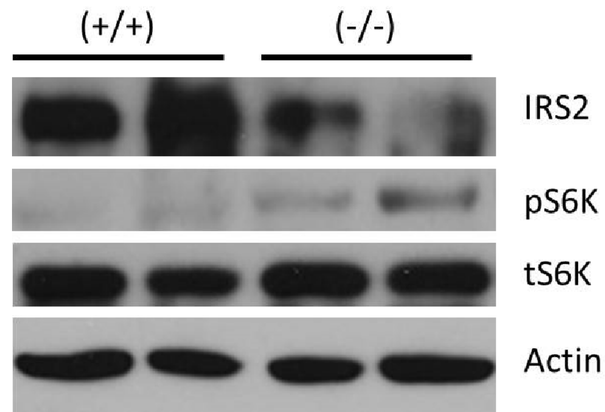

Figure 4.4: 30-week old *Phlpp1*$^{-/-}$ mice display increased S6K phosphorylation and decreased IRS2 expression as part of a negative feedback loop

Livers were removed from 30-week old wild type and *Phlpp1*$^{-/-}$ mice that had been fasted for 16 hours. Lysates were made from individual livers via homogenization. 3 samples for each genotype were run on 8% SDS-PAGE gels and transferred for Western blot analysis. Samples were blotted for phosphorylated S6K (threonine 389), total S6K, IRS2, and actin.

Increased fasting blood glucose levels in *Phlpp1*$^{-/-}$ mice is not due to changes in glycogen metabolism or transcriptional regulation of gluconeogenic genes

There are two modes through which the liver produces glucose during times of fasting. The first is through glycogenolysis, or the breakdown of glycogen to glucose [54]. The second is through gluconeogenesis, or the conversion of pyruvate to glucose through several successive reactions. In a mouse, glycogen stores are depleted after 4-6 hours of fasting [47]. Once glycogen is gone, the liver switches to gluconeogenesis for glucose production. The *Phlpp1*$^{-/-}$ mice exhibited increased fasting blood glucose levels after 16 hour fasts leading us to hypothesize that the increased glucose production was due to gluconeogenesis. To confirm this, we fasted 6-week old *Phlpp1*$^{-/-}$ and wild type mice for four hours and 16 hours and then measured their blood glucose levels. We found that there was no difference in blood glucose levels after four hours of fasting, however, just as we had seen previously there was a significant increase in *Phlpp1*$^{-/-}$ glucose levels after a 16 hour fast indicating that increased gluconeogenesis is responsible (**Figure 4.5a**). To further confirm that glycogen metabolism is not affected in these mice, livers from *Phlpp1*$^{-/-}$ and wild type mice were paraffin embedded, sectioned, and stained using Periodic Acid Schiff staining in order to visualize glycogen content of the livers. There was no obvious difference in glycogen levels between wild type and *Phlpp1* null mice confirming the hypothesis that the changes in fasting blood glucose levels were not due to changes in glycogen metabolism (**Figure 4.5b**).

Foxo1 is a transcription factor that upregulates gluconeogenesis through the transcription of the gluconeogenic genes phosphoenolpyruvate carboxykinase (*Pck1*), fructose-1,6-bisphosphatase (*Fbp1*), and glucose-6-phosphatase (*G6pc*). These genes are the typical regulatory points for gluconeogenesis. To determine if fasting blood

glucose levels are increased in *Phlpp1*$^{-/-}$ mice due to increased expression of *Pck1*, *Fbp1*, and *G6pc*, RNA was extracted from 30-week old *Phlpp1*$^{-/-}$ and control livers. The RNA was then converted to cDNA in a reverse transcriptase reaction and used in quantitative PCR to determine the relative expression levels of each gene. We found that there was no significant difference in the expression in the *Pck1*, *G6pc*, or *Fbp1* genes (**Figure 4.6**). We additionally examined the expression of the glycogen metabolism genes glycogen synthase (*Gys2*) and glycogen phosphorylase (*Pygl*) and found no significant difference compared to wild type (**Figure 4.6**). This was surprising as the liver is the primary producer of glucose during times of fasting, however, it is not the only site of glucose production as the kidney has been shown to contribute substantially to glucose production [55].

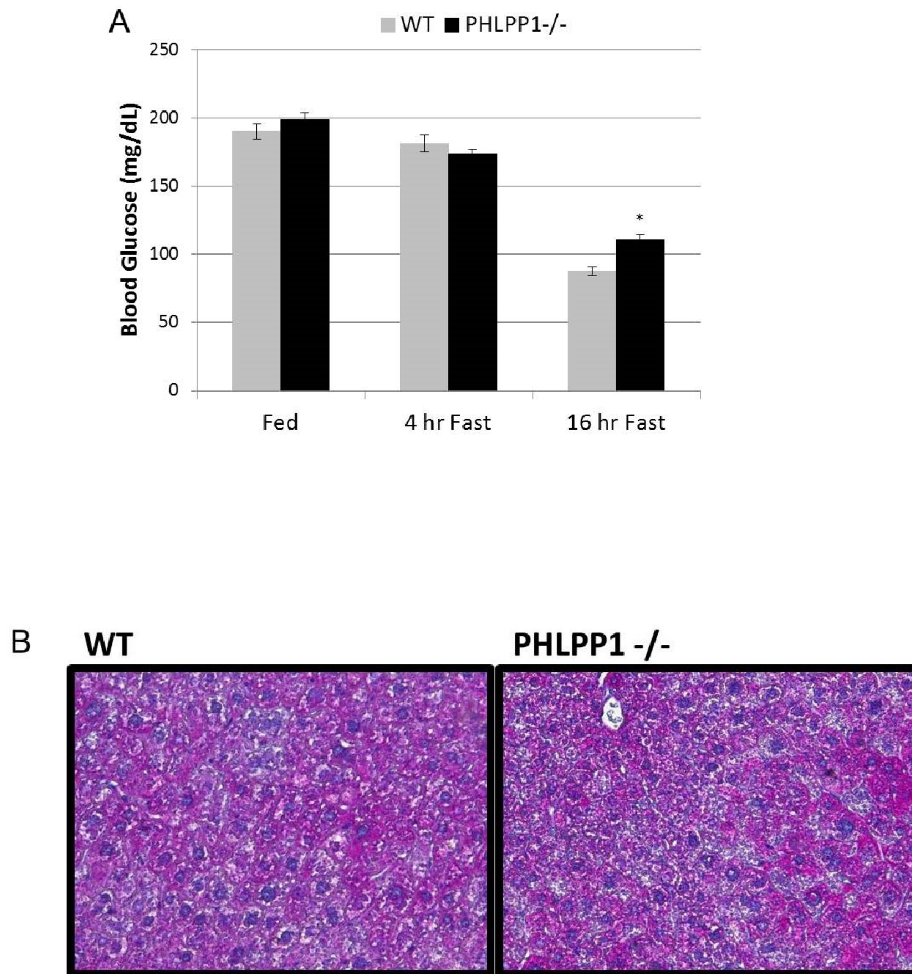

Figure 4.5: Fasting glucose in *Phlpp1*$^{-/-}$ mice is only increased after a 16-hour fast and is not due to changes in glycogen metabolism

(A) 6-week old wild type and *Phlpp1*$^{-/-}$ mice were fasted for 4 hours, 16 hours, or allowed to feed *ad libitum.* Blood was collected via the tail vein and glucose levels were measured using a glucometer. The results are mean values (n= 14 for WT, n= 12 for *Phlpp1*$^{-/-}$) ± SEM. *$p < 0.05$ **(B)** Livers were removed from wild type and *Phlpp1*$^{-/-}$ mice, paraffin embedded, section and stained for glycogen using a Periodic acid Schiff staining kit (Sigma). Images are representatives of stained sections (n=3).

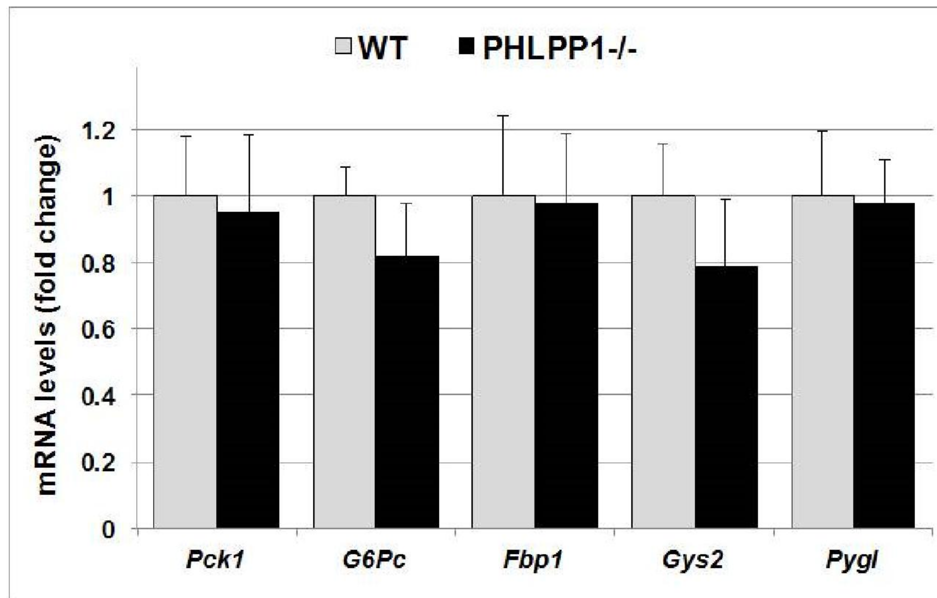

Figure 4.6: Expression of genes required for gluconeogenesis and glycogenolysis is not altered in *Phlpp1*$^{-/-}$ versus wild type mice

Livers were removed from 16 hour fasted 30-week old wild type and *Phlpp1*$^{-/-}$ mice. RNA was extracted from livers using Trizol. RNA samples were used in reverse transcriptase and subsequent qPCR reactions. Phosphoenolpyruvate carboxykinase (*Pck1*), glucose-6-phosphatase (*G6pc*), fructose-1,6-bisphosphatase (*Fbp1*) glycogen synthase (*Gys2*) and glycogen phosphorylase (*Pygl*) expression was measured. All gene expression was normalized to Rpl13a expression levels. The results are mean values (n=5) ± SD.

Conclusions

We determined that the loss of *Phlpp1* has different effects in islets versus liver. In islets, we saw an increase in Akt phosphorylation compared to wild type controls, however, the level of Akt phosphorylation did decrease during aging (**Figure 4.1**). Conversely, Akt phosphorylation was drastically reduced in the aged *Phlpp1*$^{-/-}$ livers compared to controls (**Figure 4.3**). Furthermore, S6K activity was increased resulting in the loss of IRS2 expression as it has been shown that S6K phosphorylation of IRS2 targets it for degradation (**Figure 4.4**). The loss of IRS2 uncouples the PI3K/Akt pathway from insulin signaling resulting in an attenuation of Akt activity. It has been shown in previous studies that prolonged insulin signaling causes this uncoupling effect, and that loss of *Phlpp1* expression has a similar result [24, 41]. Therefore, we hypothesized that the loss of *Phlpp1* in the livers of the null mice caused an increase in S6K activity resulting in IRS2 degradation and a decrease in Akt activity. This decrease in activity would lead to an increase in hepatic glucose production. However, upon examination of gluconeogenic gene expression using qPCR, we found that there were no significant differences between *Phlpp1*$^{-/-}$ and wild type mice (**Figure 4.6**) leading us to conclude that the increase in glucose production during times of fasting is coming from elsewhere, perhaps the kidney.

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Chapter 5

Discussion and Future Directions

Type 2 diabetes mellitus is a metabolic disease that develops through aging. It begins with the onset of insulin resistance that is compensated for by increased insulin secretion from the pancreatic-beta cell resulting in normal glucose tolerance but hyperinsulinemia. Eventually the beta-cells can no longer keep up with the increasing need for insulin and begin to fail. Loss of beta-cell compensation leads to uncontrolled hyperglycemia due to a combination of insulin resistance and hypoinsulinemia. Akt has been indicated in many studies to play a role in regulating glucose homeostasis via the insulin receptor-PI3K pathway. Loss of Akt2 in mice leads to hyperglycemia, glucose intolerance, and in some mice, severe diabetes [44, 45]. PHLPP dephosphorylates Akt in its catalytic domain leading to attenuation of Akt signaling. As Akt is a key regulator in cell proliferation and apoptosis pathways, PHLPP has been mainly studied for its role as a tumor suppressor in many cancers. There have only been two studies examining the role of PHLPP in type 2 diabetes. Both found that *Phlpp* expression is increased in the muscle of type 2 diabetic patients with a concomitant decrease in Akt ser473 phosphorylation [32]. Additionally, it was found that *Phlpp* expression is increased in the adipose tissue of obese patients, and that its expression is positively correlated with BMI [33]. Considering that PHLPP is a known regulator of Akt, and that Akt plays such a role in regulating blood glucose levels, we hypothesized that PHLPP also functions in blood glucose homeostasis. We utilized whole body *Phlpp1*$^{-/-}$ mice to determine the role of *Phlpp1* in glucose homeostasis. We metabolically characterized the *Phlpp1* null mice and have determined that they develop type 2 diabetes as they age.

Loss of *Phlpp1* causes increased fasting blood glucose levels

When we began our characterization of the *Phlpp1*$^{-/-}$ mice we discovered that at 8 weeks of age they exhibited increased fasting blood glucose levels compared to their wild type littermates. When we measured their fasting blood glucose levels again at 24 weeks of age, we found that they maintained their increase in fasting blood glucose, and it was more pronounced compared to 8 weeks. Interestingly, the 8-week old mice exhibited normal glucose tolerance despite the change in fasting blood glucose levels. By 24 weeks of age, however, the *Phlpp1*$^{-/-}$ mice exhibited impaired glucose tolerance along with decreased insulin sensitivity. Pyruvate tolerance tests revealed a slight increase in glucose production in 8-week old *Phlpp1*$^{-/-}$ mice and a greater increase in the 24-week old mice. This led us to hypothesize that the increased fasting blood glucose levels were due to increased rates of hepatic glucose production as the liver is the central producer of glucose during times of fasting. We concluded that the changes in fasting blood glucose levels were not due to changes in glycogen metabolism after observing that the fasting levels were only increased after a 16-hour fast, when glycogen stores are scarce and this was confirmed with PAS staining and qPCR analysis of the expression of glycogen synthase and glycogen phosphorylase. During such a long fast, gluconeogenesis is responsible for glucose production. The transcription of the gluconeogenic genes phosphoenolpyruvate carboxykinase (*Pck1*), glucose-6-phosphatase (*G6pc*), and fructose-1,6-bisphosphatase (*Fbp1*) is a key regulatory point in turning gluconeogenesis on or off via the transcription factor Foxo1. Akt is upstream of Foxo1, and when Akt is turned off, Foxo1 is present in the nucleus, turning on the transcription of *Pck1*, *G6pc*, and *Fbp1*, thus turning on gluconeogenesis. Insulin signals through PI3K to activate Akt and turn off gluconeogenesis when blood glucose levels are high. During type 2 diabetes this becomes misregulated leaving the transcription of

these genes on. We hypothesized that the transcription of the gluconeogenic genes are upregulated in the livers of *Phlpp1*$^{-/-}$ mice. We performed qPCR to quantitate the mRNA expression of these genes and found that there was no difference in expression between wild type and *Phlpp1* null mice. This indicates that the increase in fasting blood glucose levels seen in the *Phlpp1*$^{-/-}$ mice is not due to increased rates of hepatic gluconeogenesis.

It is possible that the increased fasting blood glucose levels are due to renal gluconeogenesis. Glucose production in the kidney is important during prolonged fasting and has been shown to play an important role in the regulation of glucose homeostasis [56]. There is no consensus on the contribution of the kidney to total gluconeogenesis, likely due to the differences in methodologies used to measure renal glucose output, however, studies have indicated that the kidneys of patients undergoing liver transplantation can increase glucose release to compensate for 50-100% of normal hepatic glucose output [55]. Additionally, it is uncommon for patients with extreme hepatic malfunction to develop hypoglycemia, and this is attributed to increases in renal glucose output. Therefore, it is possible that the increase in fasting blood glucose levels in the *Phlpp1*$^{-/-}$ mice is due to an increase in renal glucose output.

There was a recent study that looked at the expression levels of *Pck1 and G6pc* in rodent models of fasting hyperglycemia and in patients with type 2 diabetes mellitus. In the rodent models, they found that there was no change in the expression of *Pck1* or *G6pc* despite having clear increases in fasting blood glucose levels. They examined gluconeogenesis using radio-labeled alanine, a gluconeogenic precursor, and found that rate of gluconeogenesis was increased despite the lack of increase in gluconeogenic gene expression. They also examined the expression of these genes in the livers of

type 2 diabetic patients. They found that similarly to the rodent models, there was no change in the expression of the either *Pck1* or *G6pc* despite the patients exhibiting fasting hyperglycemia. There was no difference in expression between patients taking medication and those who weren't [57].

Additionally, there are other modes in which the gluconeogenesis pathway may be regulated. Pyruvate carboxylase (PC), and fructose-1,6-bisphosphatase (FBPase), other enzymes within the pathway, can be regulated allosterically. PC is regulated by acetyl Co-A, whereas FBPase is subject to regulation by fructose-2,6-bisphosphatase and AMP [58, 59]. Furthermore, the flux through gluconeogenesis can be increased through an increase in substrates, such as glycerol and amino acids [60, 61]. Therefore, in the *Phlpp1*$^{-/-}$ animals, we are seeing an increase in gluconeogenesis causing an increase in fasting blood glucose levels. This increase is not due to a change in the transcription of the key gluconeogenic genes *Pck1, Fbp1*, or *G6pc*, but may be caused by other means such as increased gluconeogenesis in the kidney, allosteric regulation of key gluconeogenic enzymes, or an increased flux through the pathway due to an increase in substrates. Furthermore, it has been shown in various mouse models and in human T2DM patients that increases in fasting blood glucose levels are not always correlated with an increase in the expression of these genes suggesting there may be other regulatory mechanisms taking place that are as of yet, not understood.

Akt phosphorylation is decreased in the livers of *Phlpp1*$^{-/-}$ mice

We examined the status of Akt phosphorylation in the livers of *Phlpp1*$^{-/-}$ mice in order to better understand the phenotype seen in these mice. Western blot analysis of 6-week old mice discovered that *Phlpp1*$^{-/-}$ mice had decreased Akt phosphorylation

compared to wild type controls. PHLPP dephosphorylates Akt thus the loss of *Phlpp1* expression should lead to an increase in Akt phosphorylation and activation. We examined Akt phosphorylation in 30-week old *Phlpp1*$^{-/-}$ mice and found that Akt phosphorylation was again decreased in these mice and the decrease was more pronounced in the older mice. Therefore Akt phosphorylation and activation is deceased in the young *Phlpp1*$^{-/-}$ mice and it decreases even further as they age.

We hypothesized that the loss of Akt phosphorylation was due to the induction of a negative feedback loop that has been previously shown to cause a decrease in Akt phosphorylation. During normal signaling, insulin binds to the insulin receptor (IR) activating the IRS proteins which serve as an adaptor protein for PI3K. PI3K activation leads to the conversion of PIP_1 to PIP_2. This lipid second messenger recruits PDK1 and Akt to the plasma membrane where Akt is phosphorylated and activated. Akt activation leads to many downstream effects including the activation of mTOR kinase. mTOR phosphorylates and activates S6K, a kinase important for protein synthesis and cell proliferation. It is also known to phosphorylate the IRS proteins targeting them for degradation by the proteasome. It has been shown that during times of prolonged signaling, such as in a hyperinsulinemic setting, S6K becomes highly activated leading to the loss of IRS protein levels. Since the IRS proteins are upstream of the PI3K/Akt pathway, loss of IRS leads to a decrease in the activation of this pathway effectively uncoupling insulin signaling from the PI3K/Akt pathway. This has been indicated as a main cause of insulin resistance in type 2 diabetes [41]. High insulin signaling over time would lead to a loss of Akt signaling thus attenuating any of its downstream effects, many of which have shown to be important for regulating blood glucose levels.

Loss of *Phlpp* expression has been shown to have the same effect as prolonged insulin signaling. PHLPP has been indicated to dephosphorylate and inactivate S6K directly and loss of PHLPP's regulation of S6K leads to uncontrolled S6K activity inducing the negative feedback loop described above [24]. To determine if this negative feedback loop is being induced in the livers of the *Phlpp1*$^{-/-}$ mice, we performed Western blot analysis to look at S6K phosphorylation and IRS2 protein levels. We found that S6K phosphorylation was increased in the *Phlpp1*$^{-/-}$ mice and that IRS2 protein levels were decreased leading us to believe that this negative feedback look was being induced. This led to a loss of PI3K activation thus leading to a decrease in Akt activation. Whether this is due to PHLPP's direct regulation of S6K or through its dephosphorylation of Akt, as loss of *Phlpp1* expression would increase the flux through S6K, is unknown.

This induction of the negative feedback loop and loss of Akt signaling would mean that the liver in the *Phlpp1*$^{-/-}$ mice is insulin resistant. It appears that the feedback loop is induced at a young age which could be a cause of the increased fasting blood glucose levels if the liver is indeed the source of those increased levels, but as discussed above, that is unclear since there was no difference in the expression of key gluconeogenic genes. The role of the liver in contributing to the disruptions to overall glucose homeostasis in these mice needs to be further investigated.

Akt phosphorylation is increased in the islets of *Phlpp1*$^{-/-}$ mice

The *Phlpp1*$^{-/-}$ mice displayed normal glucose tolerance when they were young despite having increased fasting blood glucose levels. This was attributed to an increase in glucose-stimulated insulin secretion from the pancreatic beta-cell. When we measured plasma insulin levels in the 6-week old mice, the *Phlpp1*$^{-/-}$ mice exhibited an

8-fold increase in insulin levels at basal conditions. Additionally, they exhibited a significant increase in beta-cell area and islet mass compared to control mice. When they aged, however, their glucose tolerance decreased. When plasma insulin levels were measured in the older mice, it was found that at basal levels, plasma insulin concentrations in the *Phlpp1*$^{-/-}$ mice were similar to that of wild type mice. However, upon glucose stimulation, plasma insulin levels only increased by twofold in the *Phlpp1* null mice compared to a fourfold increase upon stimulation in the wild type mice. This suggests that the *Phlpp1*$^{-/-}$ mice are no longer able to compensate for insulin resistance due to beta-cell failure.

In order to examine the signaling mechanisms promoting this phenotype, we looked at Akt phosphorylation levels in the islets of *Phlpp1*$^{-/-}$ and wild type mice. In 6-week old mice, Akt phosphorylation was increased in the null mice compared to controls. This is expected as a loss of PHLPP signaling would increase Akt phosphorylation and activation. In the islets of 30-week old mice we found that Akt phosphorylation was still increased in the *Phlpp1*$^{-/-}$ mice, but had declined by about 40% compared to the 6-week old mice. To determine whether the S6K/IRS2 feedback loop was being induced in the *Phlpp1*$^{-/-}$ islets we blotted for phosphorylated S6K and IRS2 protein levels in islets from both 6-week old and 30-week old mice. We found that S6K phosphorylation was increased in the *Phlpp1*$^{-/-}$ mice with a more pronounced increase appearing in the 30-week old mice. IRS2 protein levels were decreased in the 30-week old *Phlpp1* null mice, but not the 6-week old mice. This is likely due to the increase in S6K activity in those mice. It does not appear, however, that the negative feedback loop is being fully induced in the *Phlpp1*$^{-/-}$ islets, as there is only a slight decrease in both IRS2 expression and Akt phosphorylation in the 30-week old null islets.

The increase in Akt phosphorylation that is seen in the young mice likely contributes to the increase in beta-cell mass and subsequent increase in glucose-stimulated insulin secretion seen in those mice. The underlying mechanisms behind why glucose-stimulated insulin secretion is seemingly lost in the 30-week old mice needs to be further investigated including measuring beta-cell mass and islet area in those mice. As to why the feedback loop is induced in the liver but not the islets is not known, however, it has been shown that the feedback loop is not always induced and depends on cell type [19, 24].

PHLPP1 is involved in the light- induced resetting of the circadian clock

PHLPP is most highly expressed in brain and was first discovered in the suprachiasmatic nucleus (SCN). It was shown to be regulated in a circadian manner, and then was later discovered to play a role in the light-induced resetting of the circadian clock. It was found that *Phlpp1*$^{-/-}$ mice display a delayed shortening of tau, or circadian period length, after a light stimulus. Light is one of the many stimuli that can induce resetting of the circadian clock, and it was found that loss of PHLPP1 plays a role in adapting to these external cues. It was suggested that PHLPP functions in the SCN where it fine tunes the circadian clock, allowing it to adapt to external cues. Loss of *Phlpp1* expression causes a disruption to the resetting that takes place after an external cue.

It has been established that disruptions to circadian rhythms are associated with metabolic syndrome, obesity, and diabetes. Many studies have looked at the effects shiftwork has in humans and have found that workers develop increased body weight, hyperleptinemia, and elevated insulin secretion. The clock genes *Period, Clock,* and

Bmal1 have been studied using transgenic mice, and all mouse models developed disruptions in metabolism including obesity, hyperglycemia, and disrupted insulin responsiveness. Specifically, knockout of *Bmal1* led to severe insulin resistance, hyperglycemia, and changes in fat accumulation. Furthermore, it has been determined that mice exhibit a circadian rhythm of insulin action, specifically, that mice are most resistant to insulin during the phase of relative inactivity [62]. As PHLPP is a known regulator of the resetting of the circadian clock it is possible that the loss of *Phlpp1* expression promotes insulin resistance due to a disruption of proper circadian rhythms. This would need to be further studied in order to determine its contributions to the overall metabolic phenotype of the *Phlpp1*$^{-/-}$ mice.

Conclusions

Phlpp1$^{-/-}$ mice exhibit increased fasting blood glucose levels from a young age but normal glucose tolerance due to an increase in glucose-stimulated insulin secretion. As they age, glucose-stimulated insulin secretion decreases due to beta-cell failure leading to a decrease in glucose tolerance. Therefore, *Phlpp1*$^{-/-}$ mice develop type 2 diabetes by progressing through steps similar to those as humans providing a unique animal model for studying the disease. Further studies need to be carried out to obtain a better understanding of the contributions of individual tissues to the phenotype of the *Phlpp1*$^{-/-}$ mice and to understand the signaling mechanisms behind the development of type 2 diabetes. Understanding how PHLPP1 contributes to regulation of glucose homeostasis may contribute to the development of novel treatments for the disease.

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Appendix

List of Abbreviations

AGC: PKA, PKG, PKC kinase family

AMP: Adenosine monophosphate

ATP: Adenosine triphosphate

AUC: Area under the curve

BCA: Bicinchoninic acid assay

BMI: Body mass index

cDNA: Complimentary deoxyribonucleic acid

COBRE: Centers of biomedical research excellence

Ct: Threshold cycle

DNA: deoxyribonucleic acid

DTT: Dithiothreitol

ECL: Enhanced chemiluminescence

EDTA: ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

ERK: Extracellular-signal regulated kinase

Fbp1: Fructose-1,6-bisphosphatase 1

FBS: Fetal bovine serum albumin

Foxo1: Forkhead box protein O1

G6P: Glucose-6-phosphate

G6pc: Glucose-6-phosphatase, catalytic subunit

GLUT4: Glucose transporter 4

GSK3β: Glycogen synthase kinase β

GTT: Glucose tolerance test

Gys2: Glycogen synthase 2

IGT: Impaired glucose tolerance

I.P.: Intraperitoneal

IR: Insulin receptor

IRS: Insulin receptor substrate

ITT: Insulin tolerance test

HBSS: Hank's balanced salt solution

LRR: Leucine rich repeat region

Mst1: Macrophage stimulating 1

mRNA: Messenger RNA

MRI: Magnetic resonance imaging

mTOR: Mammalian target of rapamycin

mTORC2: Mammalian target of rapamycin complex 2

OD: Optical density

PAS: Periodic acid Schiff

PBS: Phosphate buffered saline

PC: Pyruvate carboxylase

Pck1: Phosphoenolpyruvate carboxykinase 1

PCR: Polymerase chain reaction

PDK1: Pyruvate dehydrogenase kinase isozyme 1

PDZ: PSD95, Dlg1, zo-1

PH: pleckstrin homology

PHLPP: Pleckstrin homology domain leucine rich repeat protein phosphatase

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

PIP_2: Phosphatidylinsolitol-4,5-bisphosphate

PIP_3: Phosphatidylinositol-3,4,5-triphosphate

PKB: protein kinase B

PKC: protein kinase C

PMSF: Phenylmethylsulfonylfluoride

PP2C: Protein phosphatase 2C

PTT: Pyruvate tolerance test

Pygl: Glycogen phosphorylase, liver

qPCR: quantitative polymerase chain reaction

qRT-PCR: quantitative real time- polymerase chain reaction

RA: Ras associated

RER: Respiratory exchange ratio

RNA: Ribonucleic acid

Rpl13a: 60s ribosomal protein L13a

RT-PCR: Reverse transcription- polymerase chain reaction

S6K: Ribosomal protein S6 kinase

SCN: Suprachiasmatic nucleus

SD: Standard deviation

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

SEM: Standard error of the mean

Ser: Serine

SCOP: Suprachiasmatic nucleus oscillating protein

T2DM: Type 2 diabetes mellitus

Thr: Threonine

TTBS: Tris buffered saline + 0.1% Tween

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Vita

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Education

Purdue University, West Lafayette, IN 2005-2009

B.S. in Biochemistry

Minor in Spanish

Awards and Fellowships

Kentucky Opportunity Fellowship, **UK** 2012-2013

Research Challenge Trust Fund Award, **UK** 2011-2012

Max Steckler Fellowship, **UK** 2011-2012

Graduate School Fellowship, **UK** 2009-2010

Dean’s List, **Purdue** 2009

Ag Research Fund Scholarship, **Purdue** 2009

J. Kelly & Margaret Ritchey O’Neall Memorial Scholarship, **Purdue** 2006-2009

Walter Pugsley Scholarship, **Purdue** 2005-2009

Floyd & Nellie Elliot Scholarship, **Purdue** 2005-2008

Research & Professional Experience

Graduate Student, University of Kentucky 2010-2014

Mentor: Sabire Özcan, Ph.D.

Undergraduate Researcher, Purdue University 2007-2009

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Biological Science Aide, U.S. Department of Agriculture-ARS 2007-2009

Laboratory of Joseph Anderson, Ph.D.

Intern, Procter & Gamble 2008

Student Participant, Research & Engineering Apprenticeship Program 2004

Ball State University

Teaching Experience

University of Kentucky, Lexington, KY Spring 2011

Teaching Assistant, BCH 401G

Publications

Larson KL, Liu, J, Alvarez-Perez JC, Garcia-Ocaña A, Gao T, Özcan S. Phlpp1 null mice develop age-dependent type 2 diabetes. *In review- J. Endocrinol*

Niedowicz DM, Reeves VL, Platt TL, Kohler K, Beckett TL, Powell DK, Lee TL, Sexton TR, Song E, Brewer LD, Latimer CS, Kraner SD, **Larson KL**, Özcan S, Norris CM, Hersh LB, Porter NM, Wilcock DM, and Murphy MP. Acta Neuropathol Commun 2(1): 64 Obesity and diabetes cause dementia in the absence of accelerated β-amyloid deposition in a novel murine model of mixed or vascular dementia.

Stanford JC, Morris AJ, Sunkara M, Popa GJ, **Larson KL**, Özcan S. 2012. J. Biological Chemistry (16) 13457-64. Sphingosine-1-Phosphate (S1P) regulates glucose-stimulated insulin secretion in pancreatic beta cells.

Kaitchuck RH, Hill RL, Corn AP, Gevirtz J, **Levell KL**, Valenti TL. 2006. JAAVSO (34) 165-176. A photometric study of the contact binary system FU Draconis.

Presentations

Biochemistry Department Student Seminar Series (Oral Presentation)
October 2013, University of Kentucky, Lexington, KY

Biochemistry Department Retreat (Oral Presentation)
June 2013, University of Kentucky; Carrollton, KY
2ND PLACE AWARD WINNER

Barnstable Brown Obesity & Diabetes Research Day (Poster Presentation)
May 2013, University of Kentucky; Lexington, KY

Biochemistry Department Student Seminar Series (Oral Presentation)
March 2013, University of Kentucky; Lexington, KY

Student Data Club (Oral Presentation)
November 2012, University of Kentucky; Lexington, KY

Midwest Islet Club (Oral Presentation)

May 2012, University of Pittsburgh; Pittsburgh, PA

Biochemistry Department Retreat (Poster Presentation)

June 2012, University of Kentucky, Carrollton, KY

Barnstable Brown Obesity & Diabetes Research Day (Poster Presentation)

May 2012, University of Kentucky; Lexington, KY

Biochemistry Department Student Seminar Series (Oral Presentation)

April 2012, University of Kentucky; Lexington, KY

Barnstable Brown Obesity & Diabetes Research Day (Poster Presentation)

May 2011, University of Kentucky; Lexington, KY

Biochemistry Department Retreat (Poster Presentation)

May 2011, University of Kentucky; Corbin, KY

American Society for Biochemistry and Molecular Biology (Poster Presentation)

April 2011, Walter E. Washington Convention Center; Washington, DC

Biochemistry Department Student Seminar Series (Oral Presentation)

January 2011, University of Kentucky; Lexington, KY

Undergraduate Research & Poster Sympoiusm (Poster Presentation)

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Professional Memberships

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