Isoform-specific Roles of Prolyl-Hydroxylases in the Regulation of β-cell Insulin Secretion during Diet-Induced Obesity in Males

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Type 2 diabetes affects approximately 480 million individuals worldwide and is associated with impaired tissue insulin sensitivity and β-cell dysfunction. Although there has been much research into nutrient-regulated insulin secretion and the progression to β-cell dysfunction in type 2 diabetes, the story is still incomplete. Hypoxia-inducible factor prolyl 4-hydroxylases (PHDs) are α-ketoglutarate dioxygenases commonly known to regulate hypoxia-inducible factor-1α (HIF-1α). Unique expression profiles of PHD1, PHD2 and PHD3 isozymes suggest isoform-specific roles in α-ketoglutarate-sustained insulin secretion. Our laboratory recently showed a role for β-cell PHD1 and PHD3 in insulin secretion, and previous research suggests that PHD2 may play a role in obesity-induced metabolic dysfunction. This thesis focuses on possible roles that β-cell PHDs may play in moderating the interrelationship between defective nutrient-sustained insulin secretion and obesity-induced β-cell dysfunction.

We placed β-cell-specific PHD1, PHD2 or PHD3 knockout mice on a high-fat diet to explore the roles of PHD isoforms in regulating β-cell function under diet-induced obesity. β-cell-specific PHD1 knockout mice did not display any unique obesity-induced metabolic phenotypes compared to high-fat diet-fed control mice. β-cell-specific PHD3 knockout mice on the high-fat diet experienced increased weight gain compared to high-fat diet-fed control mice. However, despite increased fasting blood glucose levels, they showed no exacerbated impairments to *in vivo* glucose homeostasis and plasma lipid profiles. β-cell-specific PHD2 knockout mice resisted high-fat diet-induced obesity and showed improved *in vivo* glucose homeostasis combined with minor alterations in their plasma lipid profile. The lack of obesity-induced metabolic dysfunction in β-cell-specific PHD2 knockout mice could be explained by enhanced β-cell mass and *ex vivo* glucose-stimulated insulin secretion. Overall, β-cell-specific PHD2 knockout mice have

ameliorated glucose homeostasis and β-cell function during obesity, potentially due to PHD2's role in discouraging HIF-1α stability during metabolic stress.

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A1C	Glycated haemoglobin
ATP/ADP	Adenosine triphosphate/Adenosine diphosphate
AUC	Area under the curve
cDNA	Complementary deoxyribonucleic acid
CHOW	Control diet
CLAMS	Comprehensive laboratory animal monitoring system
CYP	Cyclophilin
D	Diazoxide
DMαKG	Dimethyl α-ketoglutarate
DMM	Dimethyl malate
GLUT	Glucose transporter type
HFD	High-fat sucrose diet
HG	High glucose
HIF-1	Hypoxia-inducible factor-1
Ins-1/2	Insulin-1/2 promoter
K_{ATP}	ATP-sensitive potassium channel
KCl	Potassium chloride
KO	Knockout
KRB	Krebs-ringer bicarbonate
LDHA	Lactate dehydrogenase A
LG	Low glucose
LoxP	locus of X-over P1
mRNA	Messenger ribonucleic acid
Nnt	Nicotinamide nucleotide transhydrogenase
OCR	Oxygen consumption rate
PBS	Phosphate buffer solution
PC	Pyruvate carboxylase
PCR	Polymerase chain reaction
PDK1	Pyruvate dehydrogenase kinase 1
Pdx1	Pancreatic and duodenal homeobox
PFKM	Phosphofructokinase
PHD	Hypoxia-inducible factor prolyl 4-hydroxylase

PKM2	Pyruvate kinase – muscle isoform 2
PPARγ	Peroxisome proliferator-activated receptor-γ
RPMI	Roswell Park Memorial Institute
SEM	Standard error of the mean
TCA	Tricarboxylic acid cycle
V_{CO2}	Volume of oxygen used
V_{O2}	Volume of carbon dioxide produced
WT	Wild-type

Chapter 1: Introduction

1.1 Diabetes Mellitus

Diabetes mellitus is a prevalent group of metabolic disorders, affecting 537 million individuals worldwide.[1] Individuals with the disease are diagnosed with unregulated high blood glucose levels due to a lack of circulating insulin or missing insulin action (Table 1).[2,3] High glucose levels can increase the amount of glycated haemoglobin (A1C) in red blood cells, but glycated haemoglobin levels can be misleading with certain medical conditions, age and ethnicity.[3] Chronic hyperglycemia resulting from diabetes mellitus can deteriorate an individual's quality of life as it can cause life-threatening complications like kidney disease, cardiovascular disease, vision loss and nerve damage.[4] In 2021, the international diabetes foundation reported 6.7 million deaths from diabetic complications.[1] Aside from personal burdens, diabetes mellitus is a financial burden to global healthcare. Taxing symptom management and treating diabetic complications have cost the global healthcare system equivalent to $1 trillion in 2021.[1]

Table 1. Clinical diagnostic criteria for prediabetes and diabetes

Diagnostic criteria	Prediabetes	Diabetes
Fasting blood glucose	6.1-6.9 mmol/L	> 7.0 mmol/L
Glycated haemoglobin (A1C)	6.0-6.4%	> 6.5%
Oral glucose tolerance test: 2-hour blood glucose	7.8-11.0 mmol/L	> 11.1 mmol/L
Random blood glucose		> 11.1 mmol/L

There are various clinical classifications of diabetes mellitus, all classified according to the unique pathological routes for the development of the disorder. This includes type 1 diabetes, type 2 diabetes, gestational diabetes, and diabetes resulting from genetic, endocrine or exocrine defects.[2] Type 1 diabetes is the autoimmune destruction of β-cells diagnosed by the presence of pancreatic islet antibodies.[2] Gestational diabetes is commonly known as temporary hyperglycemia resulting from pregnancy.[2] However, there may be an increased risk of developing type 2 diabetes post-delivery.[2] Type 2 diabetes is the focus of this project.

1.1.1 Type 2 diabetes

Despite the various clinical classifications of diabetes mellitus, the rapid incline of urbanization and the adoption of Western lifestyles have caused type 2 diabetes to become more prevalent.[5] Type 2 diabetes currently accounts for 90% of all diabetes mellitus cases and is commonly connected with poor nutrition and consistent weight gain.[6] Impaired insulin sensitivity, pancreatic β-cell dysfunction, reduced β-cell mass, and imbalanced lipid portfolios associate with type 2 diabetes.[6-8] Common lipid portfolios include high plasma triglyceride levels, low high-density lipoprotein cholesterol levels, and small low-density lipoproteins susceptible to glycation and oxidation.[8] Diagnosis of type 2 diabetes is not straightforward. A combination of glycated haemoglobin levels and fasting blood glucose levels or 2-hour glucose levels post-oral glucose tolerance test detects individuals who develop low insulin sensitivity.[9] Plasma samples negative for pancreas islet autoantibodies can rule out type 1 diabetes.[9] Additional DNA testing focused on β-cell genetic defects can highlight individuals with intact insulin sensitivity who are at risk for type 2 diabetes.[9]

Although multi-omics have discovered novel circulating biomarkers to increase our understanding of its pathological events, the various environmental and molecular factors create

challenges for dissecting the mechanisms of β-cell dysfunction in type 2 diabetes.[6,10-12] This project was designed to explore one possible avenue of the interrelationship between defects in anaplerotic-sustained insulin secretion and diet-induced β-cell dysfunction to understand a glimpse of type 2 diabetes pathophysiology. The following sections will provide more background on anaplerotic-regulated insulin secretion and the hypoxia sensing pathway in β-cells to highlight where hypoxia-inducible factor prolyl 4-hydroxylases (PHDs) could fit into this scheme.

1.1.2 Current pharmacological agents for type 2 diabetes

Therapeutics for individuals diagnosed with type 2 diabetes vary and depend on age, health, and other medical conditions. Changes to lifestyle habits and diet remain the first line of treatment for type 2 diabetes as it avoids drug side effects.[13] However, in cases where lifestyle changes cannot regulate blood glucose levels, there have been continuous attempts to develop new pharmacological agents.[13] Due to the challenging nature of type 2 diabetes pathogenesis, pharmaceutical development is focused on multiple targets. Current pharmacological agents for type 2 diabetes focus on either lowering blood glucose levels or regulating weight to prevent the onset of other medical complications (Table 2).[14,15] Insulin injections are used in rare cases where pharmacological agents cannot lower blood glucose levels in individuals with type 2 diabetes.[15] Understanding β-cell dysfunction during metabolic stress could supplement future pharmaceutical agents by designing drugs that regulate β-cell function in these conditions.

Table 2. Pharmaceutical agents for type 2 diabetes

Class	Drug	Mode of action
Biguanides	Metformin	Enhances sensitivity to insulin in peripheral tissues
Incretins	Gliptins	Promote incretin activity to stimulate insulin secretion
	Glucagon-like peptide 1 receptor agonists	
Sodium-glucose cotransporter-2 inhibitors	Gliflozins	Prevent glucose reabsorption in the kidneys
Insulin secretagogues	Sulfonylureas	Activates the K_{ATP} channel-dependent pathway to stimulate insulin secretion
Thiazolidinediones	Glitazones	Enhance insulin sensitivity in peripheral tissues
Weight loss	Orlistat	Inhibits fatty acid absorption and enhances incretin levels

1.1.3 Types of insulin

In 1922, Dr. Banting and Dr. Best first discovered the use of bovine insulin in individuals with diabetes.[16] Since then, purified extracted bovine and porcine insulin has been the front-line medication to improve the quality of life of individuals with diabetes by alleviating symptoms.[16] Research heavily focused on modifying animal insulin to design an extended-acting version of insulin to eliminate the need for multiple injections.[16] In the 1980s, recombinant DNA technology was used to generate biosynthetic human insulin, eliminating the need for animal-sourced insulin.[16] For the last 30 years, pharmaceutical companies have designed various insulin analogues that are classified based on how fast insulin can reach the bloodstream, the peak time and the duration of insulin's activity after injection (Table 3).[16,17]

Depending on lifestyle, age, and metabolism, individuals with type 2 diabetes may only require one type of insulin or a mixture of multiple types.[17]

Table 3. Types of insulin

	Types of insulin	Time to reach the bloodstream	Peak time	Total duration
Mealtime insulins	Rapid-acting	15 minutes	1-2 hours	3-5 hours
	Short-acting	30 minutes	2-3 hours	6 hours
Basal insulins	Intermediate-acting	1-3 hours	5-8 hours	18 hours
	Long-acting	1.5 hours	No peak	24 hours

1.2 The importance of insulin for nutrient homeostasis

Insulin is a crucial regulator for whole-body carbohydrate and lipid homeostasis. After a meal, the digestion of carbohydrates increases blood glucose levels, which signals insulin secretion from pancreatic β-cells.[18] Although both glucose and fatty acids are essential cellular energy sources, insulin will dictate glucose use over fatty acids.[19] Insulin will bind to the insulin receptor on insulin-sensitive cells, which triggers a phosphorylation cascade, ultimately leading to glucose transporter type 4 (GLUT4) translocation to the plasma membrane (Figure 1).[20] GLUT4 promotes the uptake of glucose to generate and store cellular energy.[20]

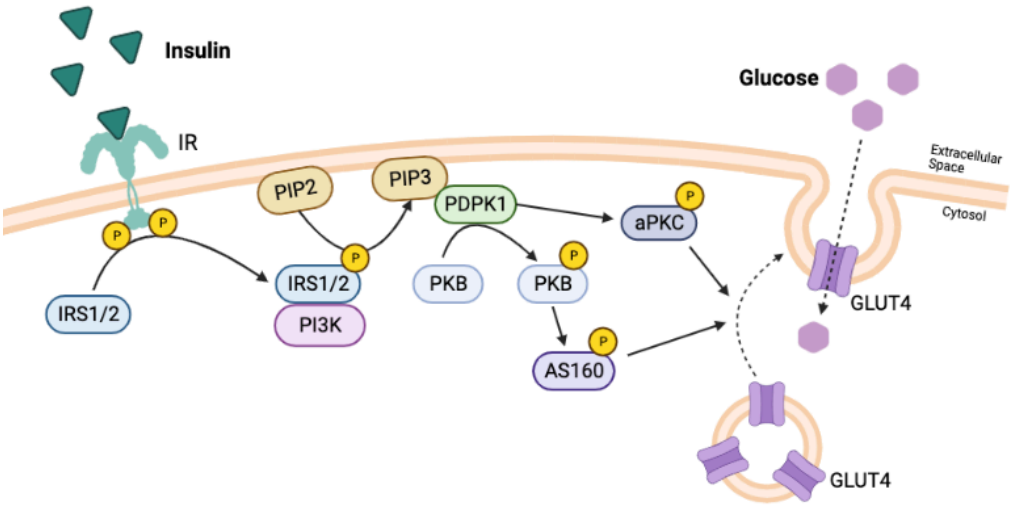


Figure 1. The insulin signalling cascade. Activation of the insulin receptor (IR) transduces signals for the phosphorylation of protein kinase B (PKB) and translocation of GLUT4 to the plasma membrane. IRS1/2, insulin receptor substrate 1/2; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-bisphosphate; PDPK1, phosphoinositide-dependent protein kinase-1; AS160, akt substrate 160; aPKC, atypical protein kinase C. Figure was created with BioRender.com.

Adipose tissue is the primary compartment for triglyceride storage. Insulin inhibits lipolysis and promotes lipogenesis and triglyceride storage (Figure 2).[21] The uncontrolled release of fatty acids and adipokines during impaired insulin sensitivity may cause nutrient excess after a meal.[22,23] Excess fatty acids may interfere with insulin signalling, whereas adipokines could disrupt insulin secretion.[22-24] Adipose tissue is most likely the last organ to develop defects in insulin sensitivity.[25,26]

The liver is the main site for glycogen storage and will release glucose during periods of starvation to prevent hypoglycemia.[21] When glucose is abundant, insulin will inhibit glycogenolysis and gluconeogenesis in the liver (Figure 2).[21] A decrease in insulin sensitivity

may release additional glucose, exacerbating glucose levels after a meal. Impaired insulin sensitivity generally starts in the liver.[26]

Skeletal muscle primarily focuses on glucose uptake to promote insulin-stimulated glucose clearance.[21] Glucose uptake triggers a fuel switch from fatty acids to glucose.[27] Majority of glucose is oxidized to generate fuel, and the remaining is shuttled to glycogen synthesis (Figure 2).[21,27] A loss of insulin signalling in skeletal muscles may alter lipid metabolism and encourage lipid accumulation.[27] Adipose tissue glucose uptake can overcompensate impaired skeletal muscle, preventing immediate glucose intolerance.[21]

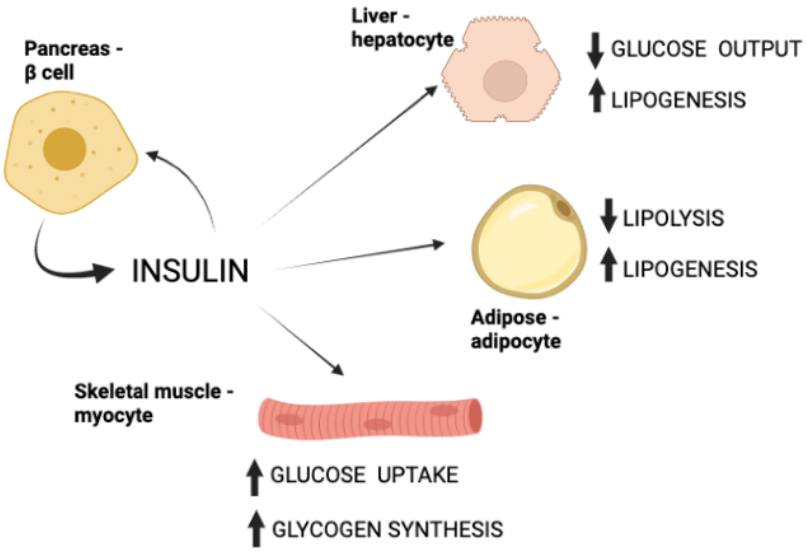


Figure 2. Insulin-regulated nutrient metabolism in adipose tissue, the liver, and skeletal muscles. In response to glucose, pancreatic β-cells release insulin. Insulin signalling in adipocytes, hepatocytes, and skeletal myocytes will regulate nutrient homeostasis after a meal. Figure was created with BioRender.com.

1.3 Pancreatic β-cell

Insulin-secreting β-cells are found in a cluster of other hormone-secreting cells called the islet of Langerhans, which are dispersed amongst acinar cells.[28] Despite islets only compromising 2% of the pancreas, they carry out significant exocrine functions.[29] Islet cells show interdependency through hormonal communication, but the arrangement of hormone-secreting cells within islets differs between humans and rodents (Figure 3).[28] The proportion of β-cells in human islets is similar to α-cells (glucagon-secreting cells), whereas rodent islets are mostly comprised of β-cells.[28]

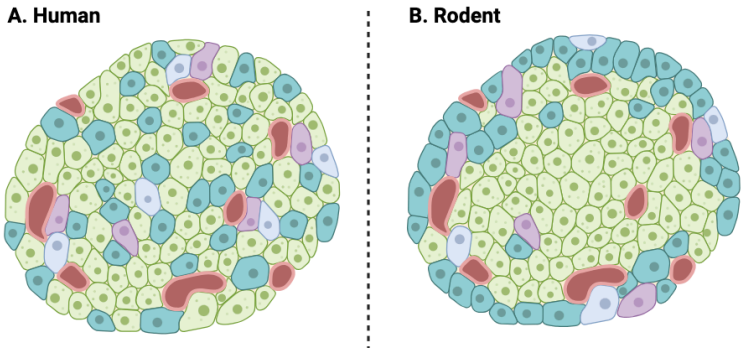


Figure 3. Composition of hormone-secreting cells in human and rodent islets. (A) Human islets have no concrete pattern of hormone-secreting cells. (B) β-cells (dark green) inhabit the middle of rodent islets and are surrounded by α-cells (light green). Blood vessels are shown in red. Figure was created with BioRender.com.

Islets are highly vascularized, allowing β-cells to sense blood glucose levels quickly.[30] β-cells are uniquely equipped with insulin-independent glucose transporters; GLUT2 in rodents or GLUT1/GLUT3 in humans.[31,32] Glucokinase, a unique hexokinase isozyme, commits glucose to glycolysis once it enters the cell.[33] There are two phases to β-cell insulin secretion. During the first phase, insulin is released rapidly in response to glucose metabolism postprandial.[34] The second phase will sustain insulin release over several hours.[34] Insulin

release from β-cells is regulated via two pathways, the K_{ATP} channel-dependent and the K_{ATP} channel-independent pathway.

1.3.1 The K_{ATP} channel-dependent pathway for the acute secretion of insulin

The K_{ATP} channel-dependent pathway will trigger glucose-stimulated insulin secretion (Figure 4). Once inside the cell, glucose is metabolized into pyruvate by glycolysis.[35] Pyruvate will enter the tricarboxylic acid (TCA) cycle to produce reducing equivalents for the mitochondria electron transport chain.[35] The electrons from reducing equivalents will drive oxidative phosphorylation, consuming oxygen and producing adenosine triphosphate (ATP).[35] ATP will travel to the cytosol and close the ATP-sensitive potassium (K_{ATP}) channel.[36] Repressed K+ efflux will depolarize the cell membrane and open the voltage-gated Ca^{2+} channel.[37] Ca^{2+} influx promotes insulin granule exocytosis.[37,38] This mechanism continues to be revised despite its discovery decades ago.[39]

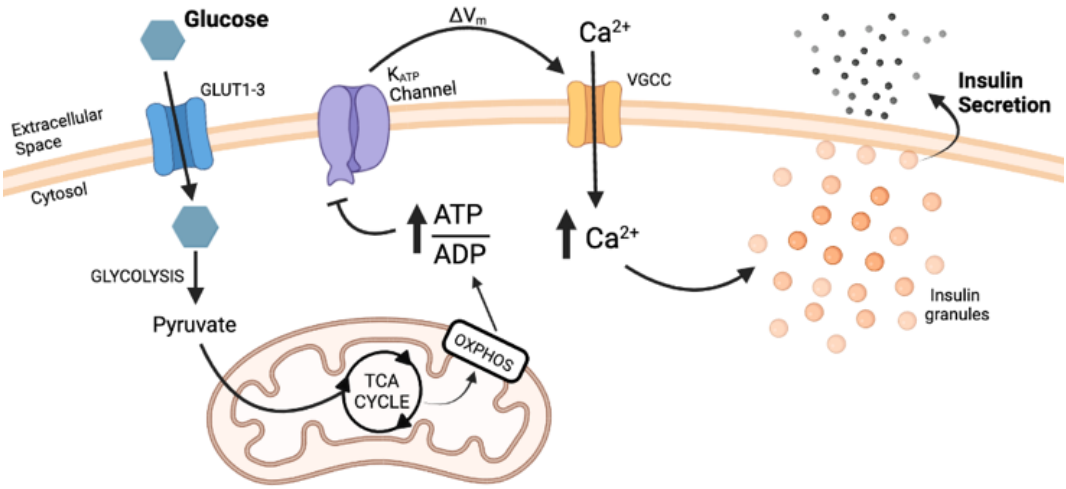


Figure 4. β-cell glucose-stimulated insulin secretion. (i) Glucose metabolism eventually results in ATP synthesis. (ii) ATP will close the K_{ATP} channel leading to (iii) Ca^{2+} influx and (iv) insulin granule exocytosis. OXPHOS, oxidative phosphorylation; VGCC, voltage-gated Ca^{2+} channel; ΔVm, membrane potential. Figure was created with BioRender.com.

1.3.2 The K_{ATP} channel-independent pathway for sustained insulin secretion

In addition to the K_{ATP} channel-dependent pathway, the K_{ATP} channel-independent pathway facilitates the second phase of sustained insulin secretion. In the K_{ATP} channel-independent pathway, glucose metabolism stimulates anaplerosis, an increase in mitochondrial TCA cycle intermediates.[40-44] Intermediates will travel to the cytosol to generate coupling factors, which sustain insulin release by promoting insulin granule exocytosis (Figure 5).[40-44] Coupling factors are defined as intermediates or cofactors that connect nutrient metabolism to insulin secretion.

1.3.3 The role of anaplerotic-derived α-ketoglutarate in β-cell function

Pyruvate carboxylase is highly expressed in β-cells and encourages the production of anaplerotic-derived coupling factors.[45,46] One theory regarding how anaplerosis produces coupling factors involves a mechanism known as pyruvate cycling. Pyruvate cycling includes three cycles; the pyruvate-malate, the pyruvate-citrate and the pyruvate-isocitrate cycle (Figure 5).[42,47-49] In the three pathways, pyruvate is shuttled into the TCA cycle, generating TCA cycle intermediates; malate, citrate and isocitrate. Specific carriers will shuttle malate, citrate and isocitrate into the cytosol.[50] In a series of enzymatic reactions, the cytosolic intermediates will regenerate pyruvate and produce coupling factors for insulin secretion.[42,47-49] In addition to pyruvate cycling, the metabolism of cytosolic isocitrate can generate α-ketoglutarate, another potential coupling factor.[51]

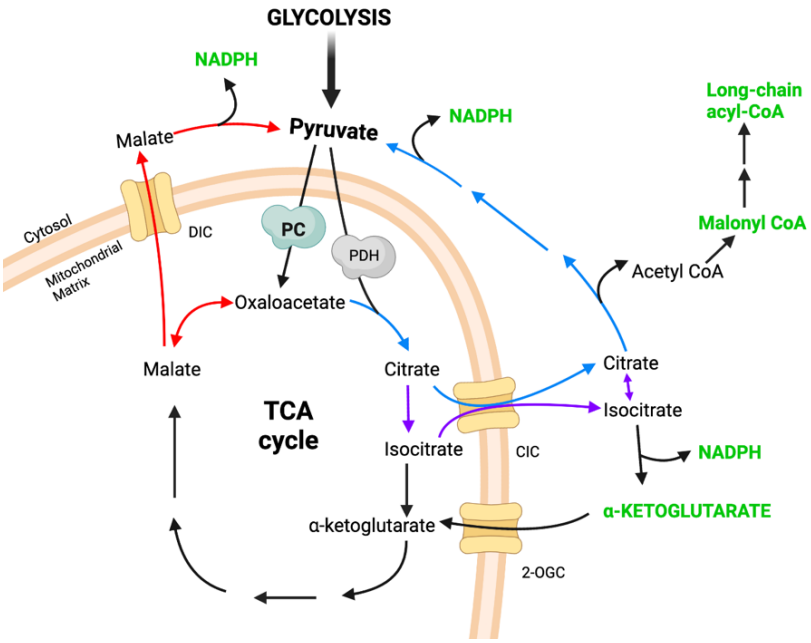


Figure 5. Mitochondria pyruvate cycling produces coupling factors for sustaining insulin secretion. The pyruvate-malate (red), pyruvate-citrate (blue), and pyruvate-isocitrate (purple) cycles replenish pyruvate in β-cells. Cytosolic reactions generate nutrient-derived coupling factors (green), signals that sustain insulin secretion in the K_{ATP} channel-independent pathway. PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; DIC, dicarboxylate carrier; CIC, citrate/isocitrate carrier; 2-OGC, 2-oxoglutarate carrier; NADPH, nicotinamide adenine dinucleotide phosphate. Figure was created with BioRender.com.

The complete mechanism of how α-ketoglutarate can act as a coupling factor for regulated insulin secretion is still being investigated.[47,51-53] Older studies proposed glutamate dehydrogenase would metabolize cytosolic α-ketoglutarate to glutamate.[54] Glutamate would then enter insulin granules and promote their exocytosis.[55] The fact that glutamate dehydrogenase favours α-ketoglutarate production challenged this idea.[55] Studies have since turned to other α-ketoglutarate-dependent enzymes. PHDs can metabolize α-ketoglutarate to hydroxylate target proteins, which may play a role in sustaining insulin secretion.[52]

1.4 The hypoxia sensing pathway

Poor blood circulation or low oxygen content in the blood will result in hypoxia.[56] Chronic hypoxia plays a role in the pathogenesis of various diseases and can be associated with type 2 diabetes.[57] Increased body fat inhibits vascularization, whereas an oversupply of plasma nutrients will increase the demand for mitochondria oxygen in nutrient metabolism.[56,58,59]

Long-term cellular hypoxia will induce hypoxia-inducible factors to coordinate a cellular response and promote survival in these conditions.[59] Hypoxia-inducible factor-1α (HIF-1α) will dimerize with HIF-1β to form a complete transcription factor.[59,60] The transcription factor will recognize the hypoxia-responsive element in genes for metabolic reprogramming and cell proliferation (Figure 6A).[61,62] HIF-1α/HIF-1β is suggested to upregulate gene expression for promoting glycolysis and lipid storage in anaerobic survival.[63-68] When oxygen is available, PHDs will hydroxylate HIF-1α, targeting it for ubiquitin-mediated degradation by the von Hippel-Lindau protein (Figure 6B).[61,69]

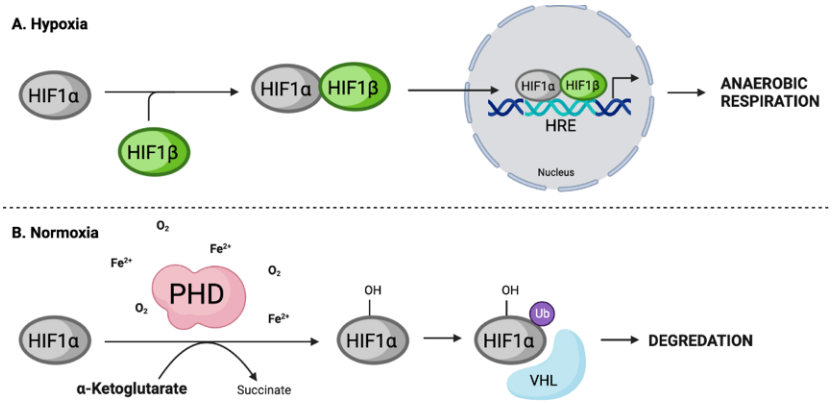


Figure 6. HIF-1α regulation in the hypoxia sensing pathway. (A) In hypoxic conditions, HIF-1α dimerizes with HIF-1β and binds to the hypoxia response element (HRE) of genes for anaerobic respiration. (B) Normoxic conditions will target HIF-1α for ubiquitin(Ub)-mediated degradation by the von Hippel-Lindau (VHL) complex. Figure was created with BioRender.com.

1.4.1 The role of the hypoxia sensing pathway in β-cell function

Studies suggested that HIF-1α is necessary for β-cell function.[70] In normal conditions, the upregulation of HIF-1α impaired β-cell function, but increased HIF-1α stability during diet-induced obesity improved β-cell function.[70-72] The dysregulation of HIF-1α stability by PHDs may explain one of the many reasons for β-cell dysfunction in type 2 diabetes.

1.5 PHD isoforms

PHDs may moderate the interrelationship between defective nutrient-sustained insulin secretion and dysregulated metabolic reprogramming in β-cells. PHDs metabolize α-ketoglutarate and oxygen to hydroxylate target protein at their proline residues.[73] There are multiple suggested protein targets for PHDs, for example, pyruvate kinase – muscle isoform 2 (PKM2) and acetyl-CoA carboxylase (ACC2).[74-82] There are three PHD isozymes that function independently; PHD1 (46 kDa), PHD2 (43 kDa) and PHD3 (27 kDa), coded by genes *Egln2, Egln1* and *Egln3,* respectively.[83] At the genomic level, *Egln1, Egln2* and *Egln3* lie on human chromosomes 1, 19 and 14, with all three genes containing five sequencing exons.[83] Each isoform has a unique messenger RNA (mRNA) and protein expression pattern, which suggests distinct functions and confirms the importance of studying isoforms separately (Table 4).

Table 4. Distinct expression patterns of PHD isoforms

PHD isoform	mRNA expression	Protein expression	Inferred function
PHD1	Nucleus [84]	Highly expressed in placenta and testis[85]	- Angiogenesis - Cell proliferation
PHD2	Cytosol [84]	- Equally expressed in all tissues[85] - Upregulated in hypoxia[84]	Regulate oxidative stress
PHD3	Nucleus & cytosol [84]	- Highly expressed in the heart, brain, skeletal muscle, and kidneys[85] - Upregulated in hypoxia[84]	Regulate oxidative stress

1.5.1 The role of PHDs in nutrient homeostasis during metabolic stress

Studies directed at understanding nutrient dysregulation during type 2 diabetes explored the roles of PHDs in diet-induced obese mouse models. Mice exposed to PHD inhibitors displayed *in vivo* protection against diet-induced obesity, improved insulin sensitivity, and regulated β-cell insulin secretion.[86-88] Despite the promising results of PHD inhibitors *in vivo*, the lack of isoform specificity makes it challenging to determine isoform-specific roles in diet-induced metabolic dysfunction. Thankfully, Cre-LoxP (loxP - locus of X-over P1) technology has generated isoform-specific PHD whole-body or tissue-specific knockout mice to study PHD isoforms separately (Table 5).[89] One caveat with the adipocyte knockout mouse in Table 5 is that PHD2 may also be absent in the central nervous system.[90,91]

Table 5. Metabolic phenotypes of whole-body and tissue-specific PHD knockout (KO) mice during diet-induced obesity

Animal model	Metabolic phenotype
Whole-body KO	
PHD1 KO	No improvements or deterioration to metabolic parameters [92]
PHD2 hypomorphic*	- Protected against obesity [86] - Improved glucose tolerance - Improved insulin sensitivity
PHD3 KO	Only explored in conjunction with low-density lipoprotein receptor deficiency [93]
Tissue-specific KO	
Hepatocyte PHD3 KO	- Improved glucose tolerance [94] - Improved fasting blood glucose and plasma insulin levels - Increased HIF-2α stability
Adipocyte PHD2 KO	- Improved glucose tolerance [90,95] - Reduced adipocyte size and lipid accumulation in adipocytes - Increased HIF-1α stability and glycolysis gene expression in adipocytes

* Note: A whole-body PHD2 knockout mouse is embryonic lethal.[96]

The metabolic phenotypes of whole-body and tissue-specific PHD-deficient mice suggest a primary role for PHD2 and a minor role for PHD3 in diet-induced metabolic dysfunction. Studies showed that **only** PHD2 inhibited HIF-1α stability, blocking metabolic reprogramming and anaerobic respiration during metabolic stress.[94,95] For specific cell types, such as adipocytes, metabolic reprogramming to anaerobic respiration may be preferred for regulated cellular function during metabolic stress. On the other hand, PHD3 targeted HIF-2α, a transcription factor that targets genes for maintaining insulin signalling and promoting angiogenesis.[62,94] Cells like hepatocytes may prefer intact insulin signalling rather than metabolic reprogramming to regulate cell function during metabolic stress.[97] β-cell PHD

isoforms have yet to be explored in obesity-induced metabolic dysfunction. Since β-cell function heavily relies on regulated nutrient metabolism, they may prefer metabolic reprogramming to prevent permanent damage in nutrient excess.

1.6 The role of PHDs in β-cell insulin secretion (our laboratory's recent findings)

Previous studies with β-cell models have highlighted the importance of cytosolic α-ketoglutarate in sustaining β-cell insulin secretion.[42,47,51] Our laboratory explored the roles of PHDs, α-ketoglutarate-dependent enzymes, in β-cell models. PHD inhibitors blunted glucose-stimulated insulin secretion, suggesting a potential mechanism for α-ketoglutarate-sustained insulin secretion.[52,53] Small interfering RNA in rat insulinoma cells and β-cell-specific PHD knockout mice explored β-cell PHD isoforms separately (Table 6).[53]

Table 6. Protein expression of PHD isoforms in the pancreas

PHD isoform	Protein expression[53]		Inferred role in β-cell function during normal conditions[53]
	Acinar tissue	Islet cells	
PHD1	High expression	Cytosol	- Encourage glucose sensing or glycolysis - Regulate β-cell apoptosis
PHD2	Expressed	Cytosol & nucleus	No significant function confirmed
PHD3	No expression	Cytosol	- Facilitate TCA flux - Regulate β-cell apoptosis

Despite normal *in vivo* glucose homeostasis, only β-cell-specific PHD1 or PHD3 knockout islets had impaired glucose-stimulated insulin secretion *ex vivo* and reduced β-cell mass.[53] β-cell-specific PHD1 knockout islets displayed no defects in calcium signalling or anaplerosis, suggesting a role for PHD1 in early mechanisms such as glucose sensing or glycolysis.[53] Whereas insulin secretion from β-cell-specific PHD3 knockout islets was partially rescued by

anaplerosis stimulators, suggesting a role for PHD3 in regulating the TCA cycle.[53] β-cell PHD2 knockout islets showed no significant impairments in glucose-stimulated insulin secretion or changes to β-cell mass. These results suggest critical roles for PHD1 and PHD3 in β-cell function, with potential roles in obesity-induced β-cell dysfunction to be explored.

β-cell PHD2 absence may follow the same resistance against obesity-induced metabolic dysfunction shown in the existing literature (Table 5).[86,95] Nasteska and colleagues recently investigated the role of PHD3 in β-cell lipid metabolism during metabolic stress. The presence of PHD3 sustained β-cell glucose sensing by presumably inhibiting fatty acid β-oxidation during nutrient excess.[98]

1.7 Rationale

Diabetes mellitus is one of the longest-standing diseases recognized since the first century, and its complete pathophysiology remains a mystery.[99] Although the discovery of exogenous insulin in 1922 by Dr. Banting and Dr. Best alleviates symptoms and has improved the quality of life of individuals with diabetes, it does not present a cure.[16] Other therapeutics such as, islet transplants for type 1 diabetes have limitations, whereas oral drugs for managing type 2 diabetes may have side effects.[16,100-102] The lack of efficacy for current therapeutics can be associated with the limited knowledge of nutrient-regulated insulin secretion.

PHDs have two defining characteristics that highlight a potential role in moderating the interrelationship between defective anaplerosis-sustained insulin secretion, obesity-induced β-cell dysfunction, and type 2 diabetes:

i) PHDs regulate nutrient homeostasis during diet-induced obesity.[86-88,90,94,95,97]

ii) PHDs regulate nutrient-sustained insulin secretion.[52,53]

Studies have shown adipocyte PHD2 and hepatocyte PHD3 play a role in diet-induced metabolic dysfunction.[86,90,94,95] Since skeletal muscle, adipose tissue, the liver, and the pancreas regulate nutrient homeostasis, defining the roles of β-cell PHDs in diet-induced obesity will supplement whole-body PHD knockout studies.[86,92] PHDs have not been explored in skeletal muscles yet.

Existing literature has also suggested general PHD inhibitors as potential therapeutics for metabolic dysfunction.[87,103,104] General PHD inhibitors include iron chelators or α-ketoglutarate analogues, both can interfere with other iron-dependent pathways or α-ketoglutarate enzymes.[52,103-106] It may also not be beneficial to inhibit all three PHD isozymes during metabolic dysfunction. Our laboratory recently suggested that β-cell PHD1 and PHD3 are necessary for β-cell function.[53] The design of PHD isoform-targeted therapeutics can avoid inhibiting essential enzymes involved in regulating tissue-specific functions and eliminate the downfalls associated with general PHD inhibitors *in vivo*.

1.8 Objectives and Hypothesis

The interrelationship between nutrient-sustained insulin secretion, obesity-induced β-cell dysfunction and type 2 diabetes is unclear. This thesis aims to investigate the isoform-specific roles of β-cell PHDs in nutrient-sustained insulin secretion during diet-induced obesity in male mice. Our laboratory has previously investigated β-cell-specific PHD1, PHD2 or PHD3 knockout mice, highlighting a role for PHD1 and PHD3 in α-ketoglutarate-sustained insulin secretion in normal conditions. β-cell PHD1 and PHD3 may target non-HIF-1α proteins to sustain insulin secretion during metabolic stress. Therefore, β-cell-specific PHD1 or PHD3 knockout mice will show impaired glucose homeostasis during diet-induced obesity. Whereas, β-cell PHD2 may primarily regulate HIF-1α stability, interfering with β-cell function during

diet-induced obesity. β-cell-specific PHD2 knockout mice will be protected from obesity-induced impairments in glucose homeostasis and β-cell function.

Objective 1: Investigate *in vivo* metabolic consequences of diet-induced obesity in β-cell-specific PHD1, PHD2 or PHD3 knockout male mice to determine isoform-specific roles in glucose homeostasis.

Objective 2: Characterize diet-induced obesity effects on islets of β-cell-specific PHD2 knockout male mice *ex vivo.*

Hypothesis: We hypothesized that PHD2 plays a negative role, whereas PHD1 and PHD3 play a positive role in regulating β-cell response to metabolic stress.

Chapter 2: Methodologies

2.1 Animal model

2.1.1 Loss-of-function model

A loss-of-function mouse model investigated the *in vivo* roles of β-cell PHD isoforms during diet-induced obesity. Cre-LoxP recombination technology disrupts *Egln2*, *Egln1* or *Egln3* genes in mouse pancreatic β-cells, diminishing PHD1, PHD2 or PHD3 protein expression, respectively. According to the Basic Local Alignment Search Tool, mouse PHD1 (43 kDa), PHD2 (45 kDa), and PHD3 (27 kDa) share 84%, 90% and 97% similarity, respectively, at the protein level with human isoforms.

2.1.2 Animal housing and handling

The animal facility at the University of Waterloo housed mice in a controlled temperature (20-23°C) and humidity (40-60%) environment with 12-hour light and dark cycles. Mice used for experimentation were individually caged and provided ab libitum food and water. For *in vivo* procedures, mice were handled according to the Canadian Council of Animal Care and approved by the Office of Research Ethics at the University of Waterloo (AUPP - 41354).

2.1.3 β-cell-specific PHD isoform knockout mice

Cre-LoxP technology with two genetically modified mouse lines generated β-cell-specific PHD1, PHD2 or PHD3 knockout mice:

i) PHD1, PHD2, or PHD3 floxed mice

Dr. Guo-Hua Fong (University of Connecticut Health Center) gifted C57Bl/6 mice homozygous for floxed PHD1, PHD2, and PHD3. Floxed mice have *LoxP* sites inserted into introns surrounding exon 2 of the *Egln1* (PHD2) and *Egln3* (PHD3) gene, or exon 3 of *Egln2* (PHD1) gene (Figure 7).[96] PHD floxed mice were backcrossed for ten generations with C57Bl/6N mice (Charles River Laboratories).

ii) Insulin-1 (Ins-1) Cre-recombinase mice

C57Bl/6N PHD floxed mice were backcrossed for ten generations with Ins-1 Cre-recombinase mice (Jackson Laboratory; mixed 6J/6N genetic background). According to the expression patterns of Ins-1, pancreatic β-cells will solely express Cre-recombinase (Figure 7).[107] It has been shown that Ins-1 Cre-recombinase mice do not express Cre-recombinase in any other organs.[107] Offspring of backcrossed mice were heterozygous for floxed PHD1 ($PHD1^{+/fl}$), PHD2 ($PHD2^{+/fl}$), or PHD3 ($PHD3^{+/fl}$) with β-cell Cre-recombinase expression (Ins-1 Cre^{+}). Continuous breeding between heterozygous mice promoted Mendelian inheritance.

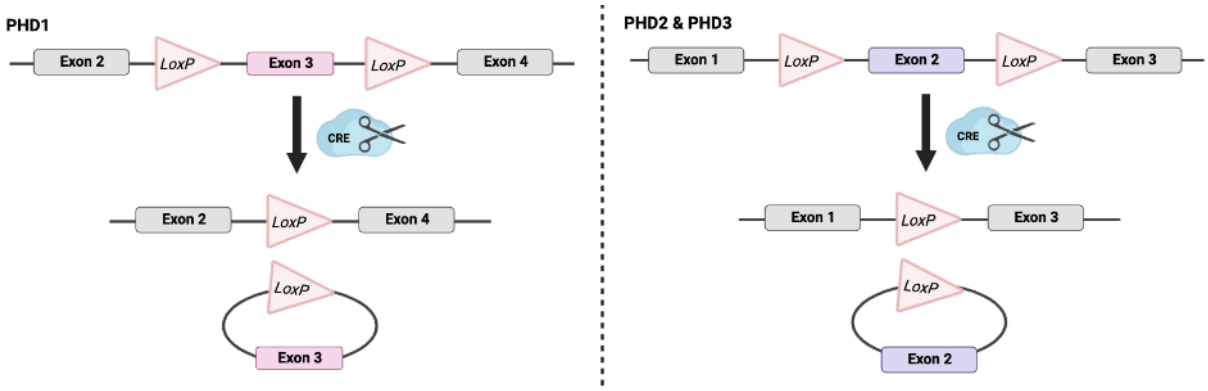


Figure 7. Schematic of Cre-LoxP technology for PHD knockout mice. Cre-recombinase regulated by the insulin-1 (Ins-1) promoter recognized and cleaved DNA in β-cells at *LoxP* sites. DNA ligase rejoined the cleaved DNA, ultimately deleting the flanked exon of the target PHD gene. Figure was created with BioRender.com.

2.1.4 Genotyping

i) DNA extraction

Ear tissue was collected and digested for DNA extraction using MagMax DNA Multi-Sample Ultra 2.0 Kit (Applied Biosystems) with Versa 10 Automated Workstation (Aurora Biomed Inc., Canada). Ear tissue was digested at 70°C for 18 hours in MagMax Tissue DNA

Extraction buffer and Proteinase K to remove residual protein contamination. The addition of Ribonuclease A after digestion removed residual RNA contamination. The tissue lysate was vortexed in MagMax Cell Lysis/Magnetic Bead Solution, where magnetic bead DNA extraction technology isolated genomic DNA. 80% ethanol removed any remaining contamination, and MagMax elution buffer eluted genomic DNA from the magnetic beads.

ii)Polymerase chain reaction (PCR)

Extracted genomic DNA was prepared with Phusion Plus PCR Master Mix (Applied Biosystems) containing DNA polymerase and corresponding PHD primers. A PCR temperature profile according to the manufacturer's instructions accommodated the maximum efficiency of the DNA polymerase and the primer's melting temperature (Tm) to promote annealing (Table 7). PHD1, PHD2 and PHD3 primers were previously designed to identify homozygous floxed mice (Table 8).[53] They detected the presence of *LoxP* sites surrounding the targeted exon in genomic DNA.

Table 7. PCR thermocycling profiles for genotyping primers

<table>
<tr><th rowspan="3">Primers</th><th colspan="4">Thermocycling profile</th></tr>
<tr><th rowspan="2">Step 1 (1 cycle)</th><th colspan="2">Step 2 (30 cycles)</th><th rowspan="2">Step 3 (1 cycle)</th></tr>
<tr><th>Denaturation</th><th>Annealing</th><th>Extension</th></tr>
<tr><td>PHD1</td><td rowspan="4">98°C for 30 secs</td><td rowspan="4">98°C for 10 secs</td><td rowspan="4">60°C for 10 secs</td><td>72°C for 30 secs</td><td rowspan="4">72°C for 5 mins</td></tr>
<tr><td>PHD2</td><td rowspan="2">72°C for 45 secs</td></tr>
<tr><td>PHD3</td></tr>
<tr><td>Ins-1 Cre</td><td>72°C for 20 secs</td></tr>
</table>

Table 8. List of genotyping PCR primers

Gene	Primers (T_m)
PHD1 WT – 506bp Floxed – 606bp	Forward: 5' GGA GCT GGA GTT CTA GGT CAG GTT 3' (59.9°C) Reverse: 5' TGA GAC CAG GCA GAG GGA GTT 3' (60.1°C)
PHD2 WT – 1440bp Floxed – 1940bp	Forward: 5' GTG TAC CTC AAC CTC CGC CG 3' (60.3°C) Reverse: 5' AGG GGA TTT GTA GTT GGC CG 3' (57.5°C)
PHD3 WT – 1227bp Floxed – 1727bp	Forward: 5' CTC AGA CCC CCT AAG TAT GT 3' (52.9°C) Reverse: 5' CCA CGT TAA CTC TAG AGC CAC TGA 3' (58.0°C)
Ins-1 Cre 370bp	Forward: 5' ACC TGA AGA TGT TCG CGA TTA TCT 3' (56.1°C) Reverse: 5' ACC GTC AGT ACG TGA GAT ATC TT 3' (55.0°C)

PCR samples were loaded onto a 1.2% agarose gel (0.002% ethidium bromide) and ran with a 0.5X tris-borate-ethylenediaminetetraacetic acid (0.5M tris base, 0.5M boric acid, 1mM ethylenediaminetetraacetic acid) running buffer. DNA fragments were visualized with ultraviolet light and compared to a standard 100 base pair (bp) DNA ladder (Invitrogen) (Figure 8).

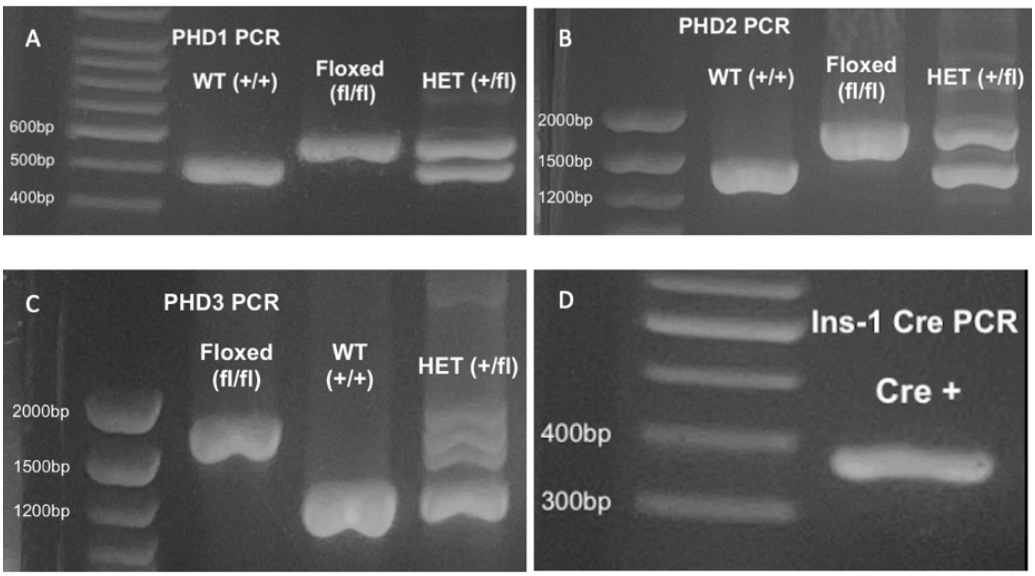


Figure 8. PCR genotyping for (A) PHD1, (B) PHD2, (C) PHD3, and (D) Ins-1 Cre-recombinase. The recombination cassette in floxed genes generated a difference in fragment sizes between wild-type (WT) and floxed (fl) mice. Cre-recombinase confirmed a β-cell knockout in floxed mice (PHD1$^{fl/fl}$, Ins-1 Cre^{+}; PHD2$^{fl/fl}$, Ins-1 Cre^{+}; PHD3$^{fl/fl}$, Ins-1 Cre^{+}). HET, heterozygous; Cre, cre-recombinase.

2.1.5 Animal controls

Wild-type mice were homozygous for the non-floxed PHD target gene, thus unaffected by β-cell Cre-recombinase (Figure 9). Whereas floxed mice were homozygous for floxed PHD1, PHD2, or PHD3. β-cell Cre-recombinase deleted the target PHD gene, generating β-cell-specific PHD1 (PHD1$^{fl/fl}$, Ins-1 Cre^{+}), PHD2 (PHD2$^{fl/fl}$, Ins-1 Cre^{+}), or PHD3 (PHD3$^{fl/fl}$, Ins-1 Cre^{+}) knockout mice. Wild-type mice (PHD1$^{+/+}$, Ins-1 Cre^{+}; PHD2$^{+/+}$, Ins-1 Cre^{+}; PHD3$^{+/+}$, Ins-1 Cre^{+}) acted as controls. Data collected from wild-type mice were pooled together since there were no phenotypic differences across control mice.[53]

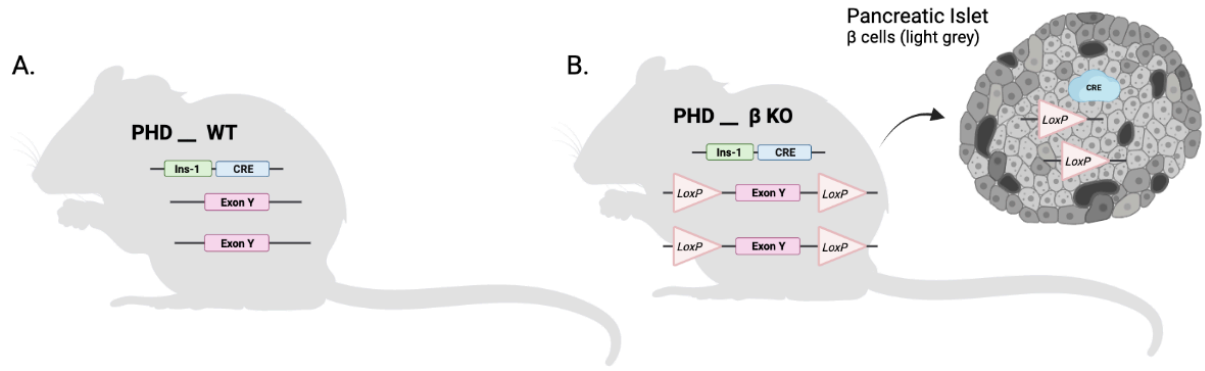


Figure 9. Genetics of (A) wild-type (WT) and (B) β-cell single-isoform PHD knockout (KO) mice. Cre-recombinase regulated by the Ins-1 promoter was only expressed in β-cells. (A) Non-floxed PHD genes in wild-type mice were unaffected by β-cell Cre-recombinase (B) Cre-recombinase deleted floxed genes in β-cells, generating a β-cell-specific knockout mouse. Figure was created with BioRender.com.

2.1.6 Diet-induced obesity

Three-month-old male wild-type and β-cell-specific PHD1, PHD2 or PHD3 knockout mice were placed on a ten-week diet; either a control diet (CHOW - diet number 2018; Teklad Diets, Madison, WI, USA) or a high-fat sucrose diet (HFD - diet number D12331 Research Diets Inc., New Brunswick, NJ, USA) to promote diet-induced obesity (Table 9, Figure 10). The high-fat sucrose diet will be referred to as "high-fat diet" for the remainder of this thesis.

Table 9. Composition of calories in control and high-fat diets

Macronutrients	Control diet	High-fat diet
Fat	18 kcal%	58 kcal%
Carbohydrates	58 kcal% (Sucrose not specified)	25.5 kcal% (Sucrose – 52% of total carbohydrates)
Protein	24 kcal%	16.5 kcal%

Weight gain and food intake were measured every two weeks. Once the diet was completed, *in vivo* and *ex vivo* experiments began.

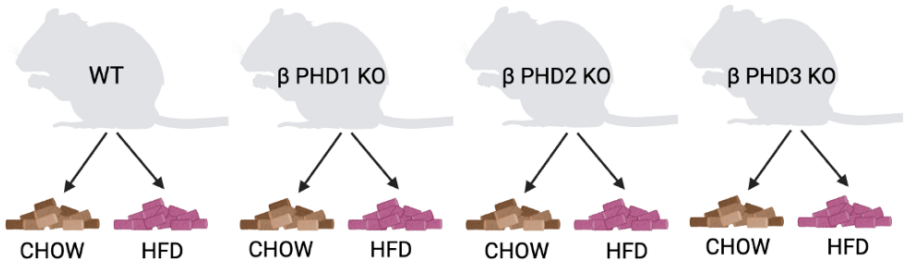


Figure 10. Schematic of the eight treatment groups. Mice of four different genotypes were placed on a ten-week diet, either the control diet (CHOW) or the high-fat diet (HFD). WT, wild-type; KO, knockout. Figure was created with BioRender.com.

2.2 Objective 1: Investigate *in vivo* metabolic consequences of diet-induced obesity in β-cell-specific PHD1, PHD2 or PHD3 knockout male mice

One challenge with evaluating *in vivo* insulin-regulated nutrient homeostasis is circadian-regulated metabolism. Since mice are nocturnal, the circadian clock induces the expression of glucose metabolism and insulin secretion genes during dark phases (7 am - 7 pm) to stimulate β-cell function.[108] Due to fluctuations of β-cell function at different times during the day, sample collection at similar time points ensured data reproducibility.

2.2.1 Intraperitoneal glucose tolerance test

Wild-type and β-cell-specific PHD1, PHD2, or PHD3 knockout mice were fasted for 16 hours (5:00 pm – 9:00 am). Fasted basal (0 minutes) blood glucose levels were collected from the tail vein using a Contour NEXT Glucometer (Bayer, Germany). Mice received an intraperitoneal injection of glucose (1.5 g of glucose/kg of body weight) prepared in phosphate

buffer solution (PBS). Blood glucose levels were measured at 10, 20, 30, 60 and 120 minutes post-injection to evaluate *in vivo* whole-body glucose tolerance.[53]

2.2.2 Plasma insulin during glucose tolerance test

Approximately 50 µL of tail vein blood was collected into a heparin-coated capillary tube at fasted basal time, 10, and 30 minutes post-glucose injection. Centrifugation isolated plasma from red blood cells. A Rat/Mouse Insulin Enzyme-linked Immunosorbent Assay Kit (Millipore Canada) measured plasma insulin levels following the manufacturer's protocol.[53] The assay captured insulin with an anti-rat insulin antibody, and a polyclonal biotinylated antibody detected the bound insulin. Horseradish peroxidase and 3,3',5,5'-tetramethylbenzidine were added to quantify the amount of bound biotinylated antibodies. The peroxidase reaction produced a precipitate that was quantified at 450 nm.

2.2.3 Insulin tolerance test

An insulin tolerance test assessed *in vivo* insulin sensitivity. Wild-type and β-cell-specific PHD1, PHD2, or PHD3 knockout mice were fasted for four hours (9:00 am – 1:00 pm). A shorter fasting time avoids hypoglycemia and the counterregulatory effects of glucagon. Pancreatic α-cells can release glucagon during hypoglycemia to trigger hepatic glucose output.[109]

Fasted basal (0 minutes) blood glucose levels were collected from the tail vein using a Contour NEXT Glucometer (Bayer, Germany). Mice received an intraperitoneal injection of rapid-acting insulin (1.2 units/kg of body weight; Novo Nordisk, Denmark) prepared in PBS.[53] Rapid-acting insulin accesses the bloodstream approximately 15 minutes post-injection.[17] Blood glucose levels were measured at 10, 20, 30, 45, 60, 90, and 120 minutes post-injection.

2.2.4 Plasma fatty acid and triglyceride levels

Mice were fed or fasted for four hours (9:00 am - 1:00 pm), and approximately 60 µL of tail vein blood was collected at 1:00 pm into heparin-coated capillary tubes. Centrifugation isolated plasma from red blood cells. A Free Fatty Acid Quantification Assay Calorimetric Kit (Abcam) broke down plasma fatty acids into CoA derivatives.[110] Whereas a Triglyceride Quantification Assay (Abcam) broke down triglycerides into glycerol.[110] CoA derivatives and glycerol were quantified at 570 nm.

2.2.5 Whole-body nutrient metabolism - Comprehensive Laboratory Animal Monitoring System (CLAMS)

Mice were placed individually in metabolic chambers to acclimatize for 24 hours with unlimited food and water. Once mice were acclimatized, CLAMS (Oxymax, Columbus Instruments, Ohio, USA) collected data from each chamber every 30 minutes over 24 hours. The chambers measured the volume of oxygen used (V_{O2} - mL/kg/min), the volume of carbon dioxide produced (V_{CO2} - mL/kg/min), and locomotive activity. Values for V_{O2} and V_{CO2} calculated the respiratory exchange ratio, whole-body lipid oxidation and carbohydrate oxidation.[53,110] All measurements were normalized to the animal's body weight. Each mouse underwent two trials, and each 30-minute measurement was averaged.

The following calculations assumed protein oxidation was insignificant: [111]

1. Respiratory exchange ratio = V_{CO2} / V_{O2}
2. Lipid oxidation rate (g/min/kg) = $(1.67 \times V_{O2}) – (1.67 \times V_{CO2})$
3. Carbohydrate oxidation rate (g/min/kg) = $(4.55 \times V_{CO2}) - (3.21 \times V_{O2})$

2.3 Objective 2: Characterization of diet-induced obesity effects on β-cell-specific PHD2 knockout male islets *ex vivo*

2.3.1 Immunostaining of β-cell mass, proliferation, and apoptosis

i) Pancreas tissue processing

Pancreas tissue was dissected from mice, weighed, and fixed in neutral buffered formalin (Epredia) for approximately 24 hours. A Leica Automatic Benchtop Tissue Processor (Leica Biosystems, Germany) dehydrated (70-100% ethanol), cleared (xylene), and embedded fixed tissue in paraffin wax. A microtome sectioned (5 μm) wax tissue blocks at three levels separated by 100 μm. Tissue sections were mounted onto Leica X-tra slides (Leica Biosystems), a positively charged slide to keep the tissue from falling off, and baked for 48 hours at 40°C.

ii) Chromogenic immunohistochemistry staining

Chromogenic immunohistochemistry staining assessed β-cell mass using specific antigen biomarkers. A series of xylene and ethanol incubations rehydrated the paraffin-embedded tissue slides. Antigen retrieval (10 mM sodium citrate, 0.05% tween20, pH 6.0) was required to unmask cross-linked antigens. Two buffers blocked the tissue to eliminate non-specific reactions during staining. 3% hydrogen peroxide is an endogenous peroxidase and alkaline phosphatase-blocking buffer, and SuperBlock (Thermo Scientific) blocked non-specific antibody binding. Tissue slides were double stained with two sets of primary and enzyme-conjugated secondary antibodies (Table 10). The first set of primary and secondary antibodies targeted glucagon to evaluate α-cell mass. The second set of primary and secondary antibodies targeted insulin to determine β-cell mass. No challenges were seen with immunohistochemistry colocalization; since glucagon resides in α-cells, it did not overlap with cytosolic insulin in β-cells.[53]

A double staining system using horseradish peroxidase- and alkaline phosphatase-conjugated secondary antibodies visualized antigen biomarkers upon substrate addition (Vector Laboratories) (Table 10).[53] Hematoxylin, a nuclear stain, counterstained tissue for pancreas visualization. A series of ethanol and xylene incubations dehydrated the stained tissue, followed by mounting with a coverslip using Leica Surgipath Micromount (Leica Biosystems). Aperio ScanScope software (Leica Biosystems, Germany) imaged and analyzed slides according to the manufacturer's protocol.

Table 10. List of chromogenic immunohistochemistry antibodies to detect α- and β-cell mass

	Islet characteristic	**Primary antibody**	**Secondary antibody**	**Substrate**	**Coloured precipitate**
1st set of antibodies	α-cell mass	Anti-glucagon (1:1000; Abcam 92517)	Anti-rabbit IgG - Alkaline Phosphatase (Vector Laboratories MP5401)	Permanent red (Vector Laboratories SK5105)	Red
2nd set of antibodies	β-cell mass	Anti-insulin (1:1000; Abcam 6995)	Anti-mouse IgG - Horseradish Peroxidase (Vector Laboratories MP2400)	3,3'-Diaminobenzidine (Vector Laboratories SK4105)	Brown

ii) Immunofluorescent staining

Immunofluorescent staining assessed pancreas composition of islet sizes, β-cell proliferation and apoptosis using specific antigen biomarkers. The same method of rehydration, antigen retrieval and blocking in immunohistochemistry staining was used for immunofluorescent staining. The only exception was that slides were not blocked for

endogenous peroxidase and alkaline phosphatase. Tissue slides were double-stained with different primary and fluorescent secondary antibodies (Table 11). The first set of primary and secondary antibodies targeted Ki67 or cleaved caspase-3. Whereas the second set of primary and secondary antibodies targeted insulin to determine β-cell proliferation or apoptosis, respectively. Target antigens with overlapping intracellular locations may pose a challenge with chromogenic immunohistochemistry visualization.[112] Nuclear Ki67 and cleaved caspase-3 may slightly colocalize with cytosolic insulin in β-cells, therefore visualization of Ki67 and cleaved caspase-3 was better achieved using fluorescence.[53,113,114]

Stained tissue slides were mounted with ProLong Diamond Mountant with SYTOX deep red (Invitrogen), a nuclear stain visualized at an excitation of 628 nm and emission of 692 nm. EVOS microscope and Celleste Image Analysis software (Invitrogen, Massachusetts, USA) imaged and analyzed slides. Celleste software counted Ki67 or cleaved caspase-3 positive β-cell nuclei according to a designed "parent-child" protocol. Islets were additionally binned into size categories: neogenic (<11 cells), medium (11-50 cells) and large (>50 cells).[115-118]

Table 11. List of immunofluorescent antibodies to detect β-cell proliferation and apoptosis

	1st set of antibodies			**2nd set of antibodies**		
β-cell characteristic	**Primary antibody**	**Secondary antibody**	**Excitation/ emission (nm)**	**Primary antibody**	**Secondary antibody**	**Excitation/ emission (nm)**
β-cell apoptosis	Anti-caspase-3 (1:100; Cell Signaling 9579)	Anti-rabbit IgG - Alexa Fluor Plus 555 (1:500; ThermoFisher A32732)	531/593	Anti-insulin (1:600; Dako A0564)	Anti-guinea Pig IgG – Alexa Fluor 488 (1:500; Jackson ImmunoResearch 705-545-148)	470/525
β-cell proliferation	Anti-Ki67 (1:50; Abcam 16667)					

2.3.2 β-cell function

Controlling variability amongst isolated islets can be a challenge. Islets vary in size, β-cell composition, and β-cell function.[119] There are many ways to normalize, but normalization is not always helpful since islet size and β-cell function may be unassociated.[119] An equal mixture of islet sizes was visually collected to consider islet variation.[119] For example, a sample of 10 islets consisted of two large, four medium, and four small islets.

2.3.2.1 Islet isolation

A SomnoSuite Low-Flow Anaesthesia system (Kent Scientific, Connecticut, USA) sedated mice with an isoflurane inhalant anaesthetic (Fresenius Kabi). An induction chamber with 500 mL/min airflow and an anaesthesia concentration of 3.0% initially induced mice. Once non-responsive, mice were transferred to a nose cone to establish a surgical plane. Sutures clamped off the bile duct at the sphincter of Oddi and the junction between the cystic and hepatic bile duct. The upper portion of the common bile duct was cannulated to perfuse the pancreas with approximately 3 mL of digestion buffer (TM Research Grade Liberase - 0.125 Wunsch units/mL, Collagenase I, and Collagenase II diluted in Hank's Balance Salt Solution; Roche).[53] The dissected pancreas was digested in a water bath (37°C) with gentle shaking for 20-25 minutes. Basic Roswell Park Memorial Institute (RPMI) media washed tissue lysate, and islets were manually picked into islet growth media (10% fetal bovine serum, 200 mM glutamine, 10000 U/mL penicillin G, 10000 µg/mL streptomycin, and 25 µg/mL amphotericin B in RMPI media).[53] Islets were incubated in growth media overnight at 37°C and 5% CO_2 to recover from stress brought on by digestion.

2.3.2.2 Glucose-stimulated insulin secretion assay

A glucose-stimulated insulin secretion assay detected defects in high-glucose insulin secretion capacity. Islets were pretreated with low glucose (LG, 2 mM) Krebs-ringer bicarbonate (KRB – 139 mM sodium chloride, 4.7 mM potassium chloride, 1.5 mM potassium phosphate, 1.16 mM magnesium sulfate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5 mM sodium bicarbonate, 3.11 mM calcium chloride, 0.2% bovine serum albumin, pH 7.4) buffer in two 30-minute incubations.[53] Ten stimulated islets per genotype were incubated in 500μL of designed KRB buffer treatments for one hour. The treatments listed in Table 12 were used to dissect the pathways involved in regulating insulin secretion and highlight defects associated with impaired glucose-stimulated insulin secretion.[46,53,110,120]

Table 12. List of treatments for glucose-stimulated insulin secretion assays

Treatments	Roles in regulating insulin secretion	Impaired insulin secretion	
		Defects	Metabolic pathway
Low glucose (LG, 2 mM)			
High glucose (HG, 10mM)			
LG + diazoxide (200 µM) + potassium chloride (30 mM)	**Diazoxide:** Opens the K_{ATP} channel **Potassium chloride:** Depolarizes plasma membrane	- Calcium influx/signalling - Insulin granule exocytosis	K_{ATP} channel-dependent pathway
HG + diazoxide + potassium chloride		- Anaplerosis - TCA cycle	K_{ATP} channel-independent pathway
HG + dimethyl malate (10 mM)* + dimethyl α-ketoglutarate (10 mM)*	**Malate & α-ketoglutarate:** Anaplerosis substrates	- Anaplerosis - TCA cycle	K_{ATP} channel-independent pathway

*Adding a dimethyl group to malate and α-ketoglutarate allowed permeability to the plasma membrane. It was cleaved off after entering the cell.

After the one-hour incubation, 300µL of supernatant was collected. A rat radioimmunoassay (Millipore Canada) quantified insulin content in the supernatant according to the manufacturer's protocol.[53] The assay incubated a fixed amount of rat insulin antibody with the insulin sample, which occupied the antibody binding sites. Adding I^{125}-labeled insulin occupied the leftover antibody binding sites. Fully occupied antibodies were precipitated from the solution, and a Wizard Gamma Counter (PerkinElmer, Massachusetts, USA) measured sample radioactivity. Measured I^{125} counts per minute indirectly measured the amount of insulin secreted from islets.

2.3.2.3 Expression profile of genes influencing β-cell glucose metabolism

Real-time PCR evaluated the expression of genes in glucose metabolism to evaluate the degree of anaerobic reprogramming in obesity-exposed islets (Table 13).[63-68] A RNeasy Plus Kit (Qiagen) extracted RNA from 100 isolated islets according to the manufacturer's protocol.[53] Guanidine isothiocyanate and β-mercaptoethanol lysed cells and inactivated ribonucleases. Genomic DNA was removed using a silica-membrane spin column, and a second silica-membrane spin column isolated RNA from the cell lysate using centrifugation. An iScript cDNA Synthesis Kit (Bio-Rad) was used to synthesize complementary DNA (cDNA) from isolated RNA.[53] Approximately 50 ng of cDNA was added to a mixture of SsoFast EvaGreen Real-Time PCR Supermix (Bio-Rad) and predesigned primers (Table 13).[53,121] The Real-Time PCR machine software (Applied Biosystems, Massachusetts, USA) quantified gene expression as a percentage of a housekeeping control gene, cyclophilin (CYP).[122]

Table 13. List of real-time PCR primers for genes influencing β-cell glucose metabolism

Gene	Protein function	Primer sequence (Tm)
GLUT2	β-cell glucose transport	Forward: 5' ACC TTG GCT TTC ACT GTC TT 3' (54.4°C) Reverse: 5' GAC TAT GTG AGC AGA TCC TTC AG 3' (54.7°C)
PFKM	Catalyzes the third reaction in glycolysis	Forward: 5' GGA GAT GCC CAA GGT ATG AA 3' (54.4°C) Reverse: 5' CCT CCG ATG ACA CAC AGA 3' (53.9°C)
PKM2	Catalyzes the last reaction in glycolysis	Forward: 5' CGC AAC ACT GGC ATC ATT T 3' (54.2°C) Reverse: 5' GAT CTC AGG TCC CTT TGT ATC C 3' (55.4°C)
LDHA	Catalyzes lactate synthesis from pyruvate	Forward: 5' TGG CTT GTG CCATCA GTA TC 3' (55.1°C) Reverse: 5' CTT GCA GTG TGG ACT GTA CTT 3' (55.8°C)
PC	Catalyzes oxaloacetate synthesis from pyruvate	Forward: 5' GAT GCT GAA GTT CCA AAC AGT TC 3' (54.5°C) Reverse: 5' GGT AGG CTT CAT CAG CTT TCT 3' (54.6°C)
PDK1	Inactivates pyruvate dehydrogenase	Forward: 5' ACT TCT ATG CGC GCT TCT C 3' (55.2°C) Reverse: 5' CAA CTC CTG AAG GCT TTG GAT A 3' (54.7°C)
CYP	Assembles multidomain proteins	Forward: 5' GGA CAG GGA AAT CAA CTC AGA AG 3' (55.4°C) Reverse: 5' CTT ATC AAT GGC CAA CAG AGG G 3' (55.8°C)

2.3.2.4 β-cell HIF-1α protein expression

Since PHDs inhibit HIF-1α stability, western blotting was used to evaluate β-cell HIF-1α protein expression.[94-96] Isolated islets (130-140 islets) were sonicated in Pierce radioimmunoprecipitation assay lysis buffer (Thermo Scientific) supplemented with HALT protease and phosphatase inhibitor cocktail (Thermo Scientific) to protect proteins during protein extraction.[123] Centrifugation removed the remaining cellular debris. Islet protein samples were quantified using a Pierce bicinchoninic acid calorimetric assay (Thermo Scientific) at 562 nm.

Approximately 25-30 μg of protein was heated (70°C) in BOLT lithium dodecyl sulphate (with Coomassie dye) and BOLT-reducing dithiothreitol buffers (Invitrogen).[53] The buffers provided denaturing and reducing conditions, which allows protein separation based on

molecular weight during gel electrophoresis. Islet protein samples, MagicMark XP protein standard (Invitrogen), and PageRuler pre-stained protein ladder (Thermo Scientific) were loaded onto a BOLT 10% bis-tris polyacrylamide gel (Invitrogen) and ran with a 2-(*N*-morpholino) ethane sulfonic acid and sodium dodecyl sulfate running buffer (Invitrogen) at 180V. Once proteins were separated, proteins were semi-dry transferred onto a polyvinylidene fluoride membrane using a Power Blotter (Invitrogen, Massachusetts, USA) according to the manufacturer's protocol.[53]

Once the transfer was complete, total protein per well was detected using a no-stain protein labelling reagent (Invitrogen), which forms covalent bonds with lysine residues. iBright imaging software (Invitrogen) normalized protein content across wells to accurately quantify the target proteins. A loading control (housekeeping protein) can get oversaturated in a concentrated protein sample.[124] Therefore, a total protein stain better reflected quantitative differences.

After total protein normalization, SuperBlock (Thermo Scientific), a glycoprotein-blocking buffer, blocked the membrane to prevent the non-specific binding of antibodies. The membrane was probed with primary and secondary antibodies using an iBind Western Device (Invitrogen, Massachusetts, USA) according to the manufacturer's protocol (Table 14).[123] The iBright Imaging software (Invitrogen, Massachusetts, USA) detected fluorescent bands and confirmed the observed molecular weight of HIF-1α. Total protein normalization analysis quantified the relative amounts of HIF-1α.

Table 14. Western blot antibodies for detecting β-cell HIF-1α

Target protein	Actual molecular weight (kDa)	Primary antibody	Secondary antibody
HIF-1α	93	HIF-1α Polyclonal (1:100, Proteintech 20960-1-AP)	Anti-rabbit IgG – Alexa Fluor Plus 800 (1:2000; ThermoFisher A32735)

2.4 Statistical analysis

Previous studies and A priori power analysis using G*Power (Heinrich-Heine University, Germany) determined the required sample sizes for *in vivo* and *ex vivo* analysis. GraphPad Prism 9 (Massachusetts, USA) was used to perform statistical analyses. Data was presented as the mean $\pm$ standard error of the mean (SEM). A 1- or 2-way analysis of variance with Tukey's and Dunnett's post hoc tests evaluated statistical significance.[53] Tukey's test compared all means, whereas Dunnett's test compared means to controls. A p-value of <0.05 was considered significant.

Chapter 3: Results

3.1 Objective 1: Investigate *in vivo* metabolic consequences of diet-induced obesity in β-cell-specific PHD1, PHD2 or PHD3 knockout male mice

Metabolic consequences of obese β-cell-specific PHD1, PHD2 or PHD3 knockout mice were investigated to determine isoform-specific roles of β-cell PHDs in glucose homeostasis during diet-induced obesity. *In vivo* investigation involved analysis of weight gain, glucose tolerance, plasma insulin levels, insulin sensitivity, plasma lipid levels and whole-body nutrient metabolism.

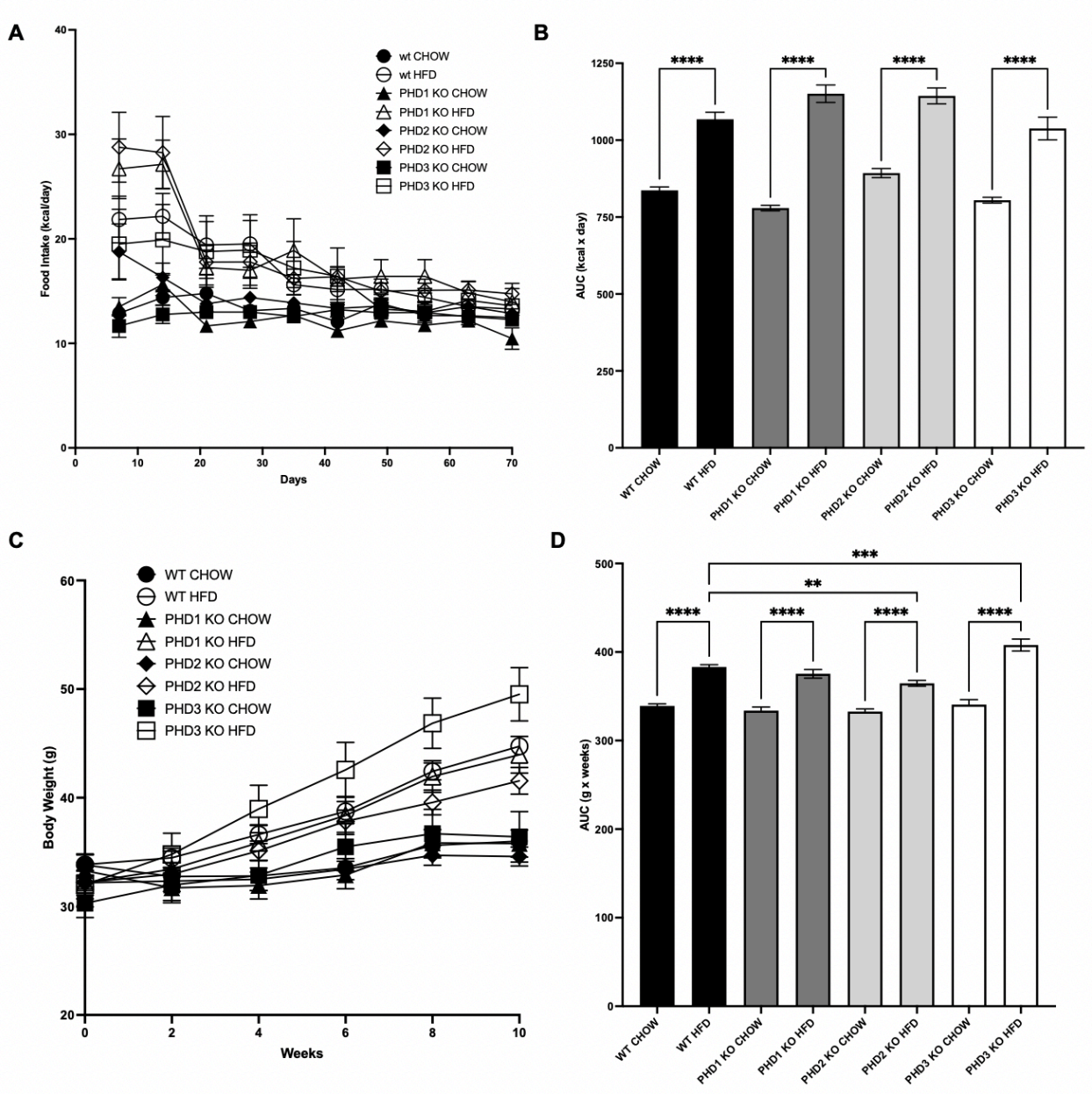
3.1.1 The absence of β-cell PHD2 restricts high-fat diet-induced weight gain, but a lack of β-cell PHD3 enhances weight gain and hepatomegaly

Mice were placed on a ten-week control or high-fat diet to induce obesity and metabolic stress. Food intake was significantly greater for all mice on the high-fat diet and did not differ between genotypes (Figures 11A-B). High-fat diet-fed mice appear to intake more during the first couple weeks (days 7-14), but intake decreases and becomes similar to control-fed mice around day 49 (Figure 11A). Body weights between high-fat diet-fed and control diet-fed mice begin to diverge at week two (Figure 11C). Despite genotypes, all high-fat diet-fed mice gained significant weight over control diet-fed mice (Figure 11D). However, β-cell-specific PHD2 knockout mice on the high-fat diet gained significantly less weight than high-fat diet-fed control mice, and β-cell-specific PHD3 knockout mice on the high-fat diet gained significantly more weight than high-fat diet-fed control mice (Figure 11D).

Pancreas weights were not significantly altered between any mice (Figure 11E). β-cell-specific PHD2 knockout mice appear to have slightly larger pancreas on the high-fat diet, but the difference was insignificant. Despite high-fat diet-induced weight gain in all genotypes

(Figure 11D), the percentage of body weight made up from the pancreas was significantly lower only in high-fat diet-fed control mice (Figure 11F).

Except for β-cell-specific PHD3 knockout mice, liver weights also did not change between genotypes on the high-fat diet (Figure 11G). High-fat diet-fed β-cell-specific PHD3 knockout mice experienced hepatomegaly, showing slightly enlarged livers only compared to control diet-fed control mice (Figure 11G). Since high-fat diet-fed β-cell-specific PHD3 knockout mice showed significantly enhanced weight gain, the enlarged livers did not alter the percentage of body weight made up from the liver (Figures 11D and H). High-fat diet-fed control and β-cell-specific PHD2 knockout mice also appear to have slightly larger livers than their respective genotype on the control diet, but the difference was insignificant (Figure 11G). Overall, the trends of high-fat diet-fed mice suggest that β-cell PHD2 promotes high-fat diet-induced obesity without influencing pancreas or liver sizes. Whereas β-cell PHD3 controls obesity and liver size on the high-fat diet.


A
Food Intake (kcal/day)
Days
wt CHOW
wt HFD
PHD1 KO CHOW
PHD1 KO HFD
PHD2 KO CHOW
PHD2 KO HFD
PHD3 KO CHOW
PHD3 KO HFD
B
AUC (kcal x day)
****
WT CHOW
WT HFD
PHD1 KO CHOW
PHD1 KO HFD
PHD2 KO CHOW
PHD2 KO HFD
PHD3 KO CHOW
PHD3 KO HFD
C
Body Weight (g)
Weeks
WT CHOW
WT HFD
PHD1 KO CHOW
PHD1 KO HFD
PHD2 KO CHOW
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PHD3 KO CHOW
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AUC (g x weeks)
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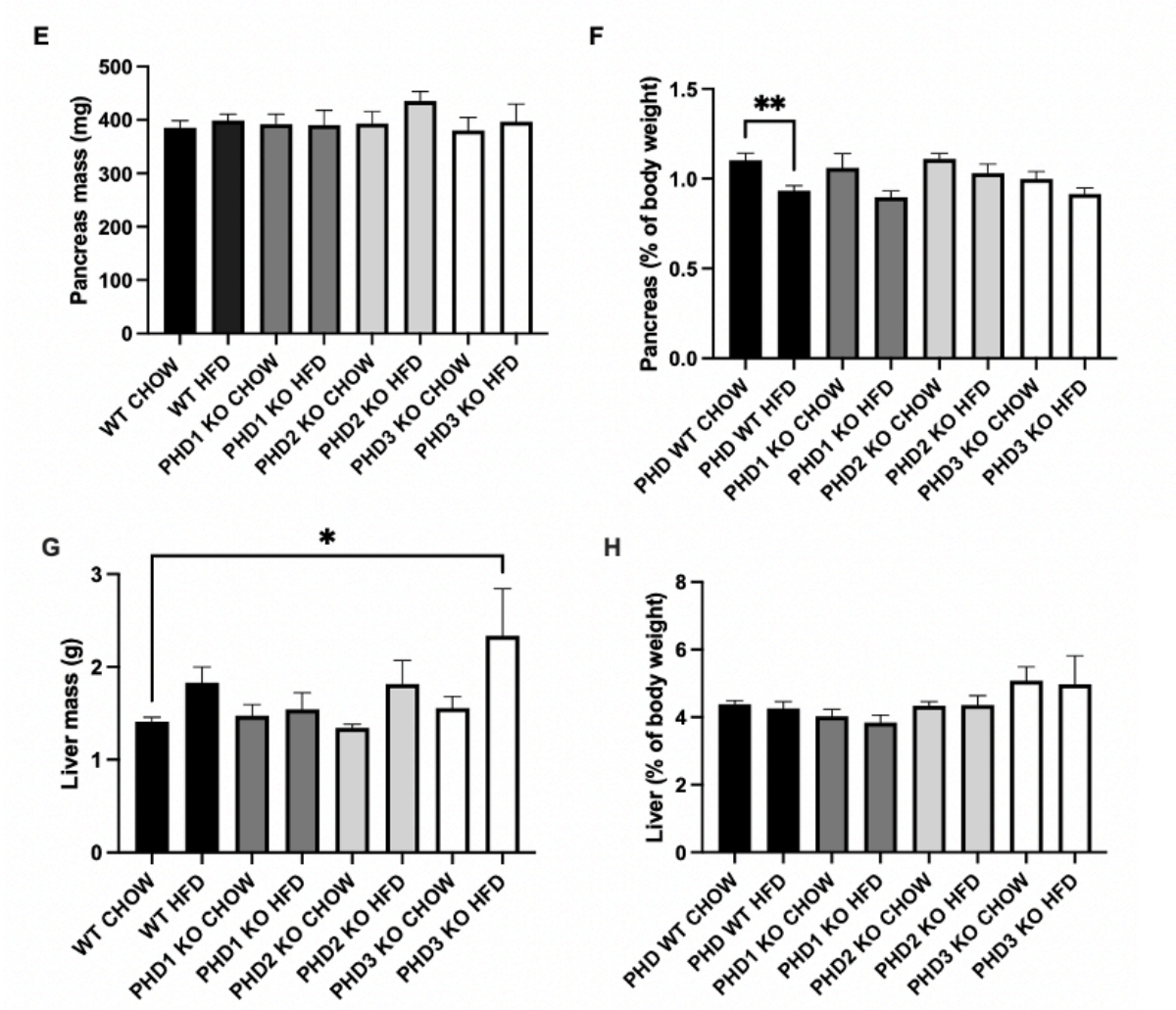

Figure 11. Effects of a high-fat diet on (A) food intake, (C) body, (E) pancreas and (G) liver weights in control (wild-type) and β-cell-specific PHD knockout mice. (B) AUC of (A). (D) AUC of (C). Data are mean ± SEM (n = 9 to 22 per treatment). (F) Pancreas weight as a percentage of body weight. (H) Liver weight as a percentage of body weight. Data are mean ± SEM (n = 6 to 9 per treatment). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

3.1.2 A lack of β-cell PHD2 and PHD3 reduces plasma insulin levels during diet-induced obesity, but the absence of β-cell PHD2 improves glucose tolerance

Fasted mice were injected with glucose (1.5 g of glucose/kg of body weight) to observe obesity-induced changes in glucose tolerance. Plasma insulin levels were quantified to assess β-cell response to high glucose levels *in vivo* during obesity. All mice had similar fasting glucose levels except high-fat diet-fed β-cell-specific PHD3 knockout mice (Figure 12A). β-cell-specific PHD3 knockout mice on the high-fat diet showed significantly elevated fasting blood glucose levels only compared to control diet-fed control mice, suggesting these mice may be in the early stages of type 2 diabetes development.[3] All genotypes displayed significantly increased fasting plasma insulin levels on the high-fat diet, except β-cell-specific PHD2 knockout mice (Figure 12B). β-cell-specific PHD2 knockout and β-cell-specific PHD3 knockout mice on the high-fat diet had lower fasting plasma insulin levels than high-fat diet-fed control mice.

Except for β-cell-specific PHD2 knockout mice, all genotypes on the high-fat diet showed impaired glucose tolerance. High-fat diet-fed control, β-cell-specific PHD1 knockout and β-cell-specific PHD3 knockout mice experienced elevated blood glucose levels than control diet-fed mice after 60 minutes post injection (Figure 12C). Higher glucose curves resulted in a significantly larger area under the curve compared to its respective genotype on the control diet (Figures 12C and E). Blood glucose levels of high-fat diet-fed β-cell-specific PHD2 knockout mice were comparable to control diet-fed mice at all time points (Figure 12C). The area under the glucose curve for high-fat diet-fed β-cell-specific PHD2 knockout mice was significantly lower than high-fat diet-fed control mice (Figure 12E). β-cell-specific PHD2 knockout mice

showed similar glucose tolerance on either diet but experienced improved glucose tolerance on the high-fat diet compared to all high-fat diet-fed genotypes.

All genotypes on the high-fat diet, except for β-cell-specific PHD3 knockout mice, had significantly increased plasma insulin levels at all time points during the glucose tolerance test (Figures 12D and F). Interestingly, β-cell-specific PHD2 knockout mice had a smaller increase in plasma insulin levels during the glucose tolerance test when on the high-fat diet (Figures 12D and F). The resulting area under the plasma insulin curve for high-fat diet-fed β-cell-specific PHD2 knockout and β-cell-specific PHD3 knockout mice were significantly lower than high-fat diet-fed control mice (Figure 12F). Plasma insulin levels of high-fat diet-fed β-cell-specific PHD3 knockout mice during the glucose tolerance test were comparable to all genotypes on the control diet (Figure 12D). High-fat diet-fed β-cell-specific PHD3 knockout mice also showed a significantly lower area under the plasma insulin curve than all genotypes on the high-fat diet, except for β-cell-specific PHD2 knockout mice (Figure 12F). Overall, data suggests that PHD2 impairs glucose homeostasis during diet-induced obesity, whereas PHD3 may play a protective role.

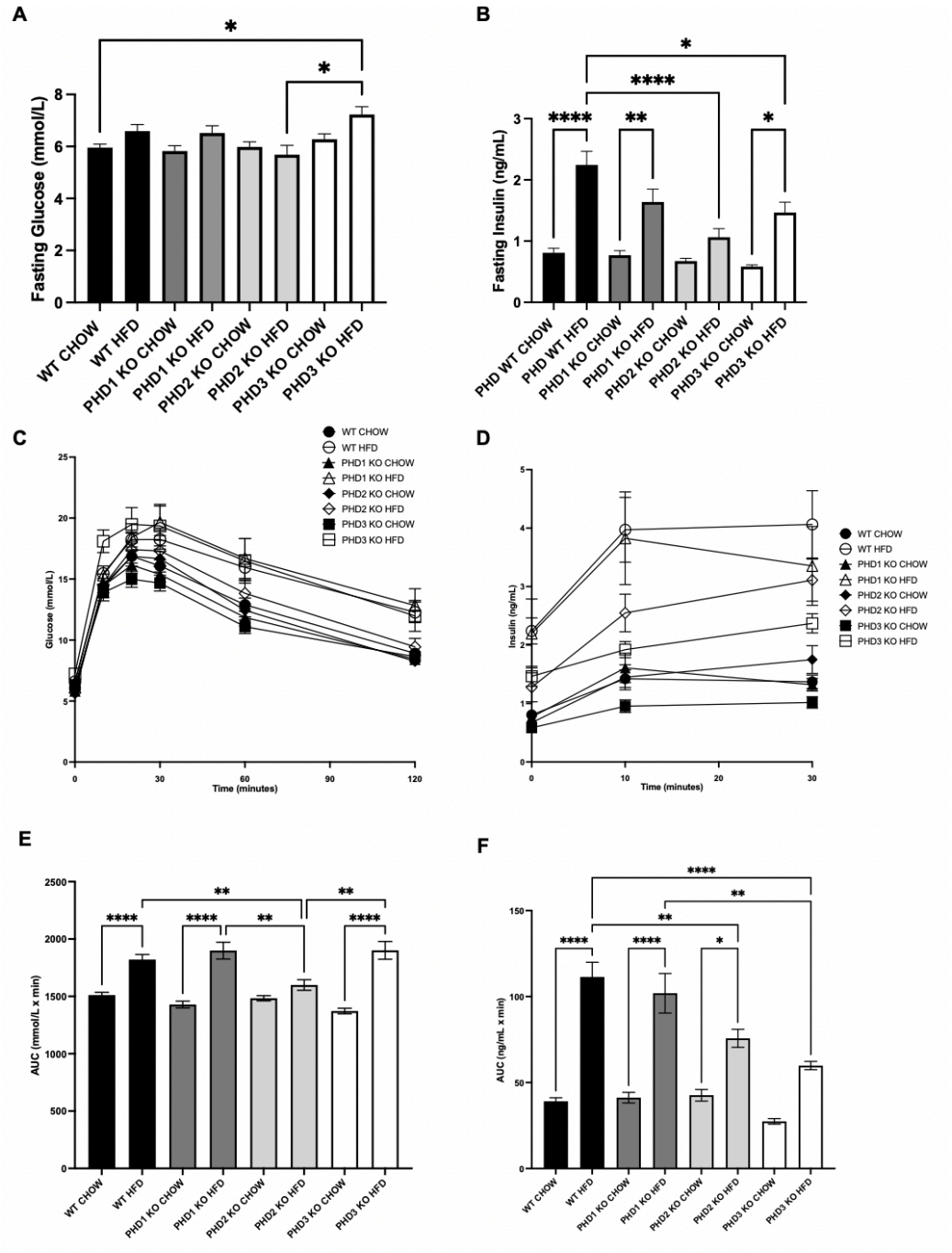

Figure 12. Effects of diet-induced obesity on (C) glucose tolerance and (D) plasma insulin levels during a glucose tolerance test in control (wild-type) and β-cell-specific PHD knockout mice. (A) Fasting blood glucose levels. (B) Fasting plasma insulin levels. (E) AUC of (C). (F) AUC of (D). Data are mean ± SEM (n = 10 to 22 per treatment). **P* < 0.05, ***P* < 0.01, *****P* < 0.0001. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

3.1.3 β-cell PHD2 absence improves insulin sensitivity during diet-induced obesity

Since most high-fat diet-fed genotypes displayed elevated plasma insulin levels during the glucose tolerance test, fasted mice were injected with insulin (1.2 units of insulin/kg of body weight) to assess insulin sensitivity. Glucose curves between genotypes were difficult to differentiate (Figure 13A). Thus, comparisons relied on analysis from the area under the glucose curve. Only control and β-cell-specific PHD1 knockout mice experienced impaired insulin sensitivity from diet-induced obesity. The area under the glucose curve for high-fat diet-fed control and β-cell-specific PHD1 knockout mice significantly increased compared to their respective genotypes on the control diet (Figure 13B). β-cell-specific PHD2 knockout or β-cell-specific PHD3 knockout mice experienced unaltered insulin sensitivity on either diet. Interestingly, high-fat diet-fed β-cell-specific PHD2 knockout experienced improved insulin sensitivity compared to high-fat diet-fed control mice. The insulin tolerance test suggests that β-cell PHD2 may encourage the loss of insulin sensitivity during metabolic stress.

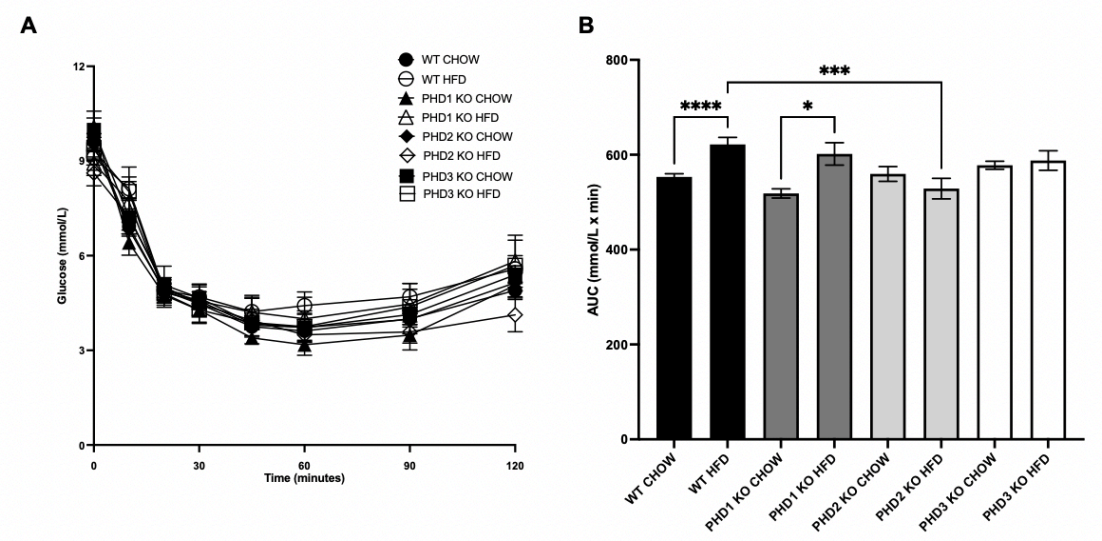

Figure 13. Effects of diet-induced obesity on (A) insulin tolerance in control (wild-type) and β-cell-specific PHD knockout mice. (B) AUC of (A). Data are mean ± SEM (n = 10 to 22 per treatment). *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

3.1.4 The absence of β-cell PHD2 elevates fed plasma free fatty acid levels

Plasma free fatty acid and triglyceride levels were measured in the fed and fasted state to assess obesity-altered dyslipidemia, which is commonly associated with impaired insulin sensitivity.[8] Despite genotypes, control diet-fed mice had comparable fasting fatty acid and triglyceride levels with similar patterns of increased fasting lipid levels on the high-fat diet (Figures 14A-B). However, elevated fasted plasma fatty acid levels in high-fat diet-fed β-cell-specific PHD1 and β-cell-specific PHD2 knockout mice were insignificant (Figure 14A). All high-fat diet-fed mice had similar fasting fatty acid and triglyceride levels (Figures 14A-B).

Fed fatty acid levels were comparable in all control diet-fed mice except for a significant elevation in β-cell-specific PHD2 knockout mice (Figure 14C). Control diet-fed mice also showed similar fed plasma triglyceride levels, but control diet-fed β-cell-specific PHD3

knockout mice displayed significantly lower plasma triglyceride levels than β-cell-specific PHD1 knockout and β-cell-specific PHD2 knockout mice on the control diet (Figure 14D). β-cell-specific PHD1 knockout and β-cell-specific PHD3 knockout mice appear to show similar patterns as control mice of increased fed plasma fatty acid and triglyceride levels on the high-fat diet (Figures 14C-D). Fed plasma fatty acid levels in high-fat diet-fed β-cell-specific PHD2 knockout mice were significantly higher than all genotypes on the high-fat diet (Figure 14C). Fasted and fed lipid profiles suggest that β-cell PHD3 disturbs control diet-fed plasma triglyceride levels, whereas β-cell PHD2 may regulate fed plasma fatty acid levels. Elevated fed and fasted plasma triglyceride levels of control mice and β-cell-specific PHD3 knockout suggest dyslipidemia associated with diet-induced type 2 diabetes.[8]

Control mice on the control diet showed similar plasma fatty acid levels between the fed and fasted state, yet fed plasma triglyceride levels were higher than in the fasted state (Figures 14E-F). On the high-fat diet, control mice experienced elevated fed plasma fatty acid and triglyceride levels over the fasted state (Figures 14E-F). β-cell-specific PHD1 knockout mice on both diets showed comparable trends as control mice of plasma fatty acid and triglyceride levels between the fed and the fasted state (Figures 14E-F). However, the elevated fed plasma fatty acid levels over the fasted state of high-fat diet-fed β-cell-specific PHD1 knockout mice were insignificant (Figure 14E). Both control diet-fed and high-fat diet-fed β-cell-specific PHD2 knockout mice exhibited higher fed plasma fatty acid and triglyceride levels than in the fasted state (Figures 14E-F). Despite diets, β-cell-specific PHD3 knockout mice had unaltered plasma fatty acid levels between the fed and fasted state (Figure 14E). High-fat diet-fed β-cell-specific PHD3 knockout mice only experienced elevated fed plasma triglyceride levels over the fasted state (Figure 14F). Ultimately, plasma triglyceride levels were higher in the fed state than

in the fasted state for most genotypes. However, plasma fatty acid levels remained relatively unaltered between the fed and the fasted states, except in β-cell-specific PHD2 knockout mice and high-fat diet-fed control mice.

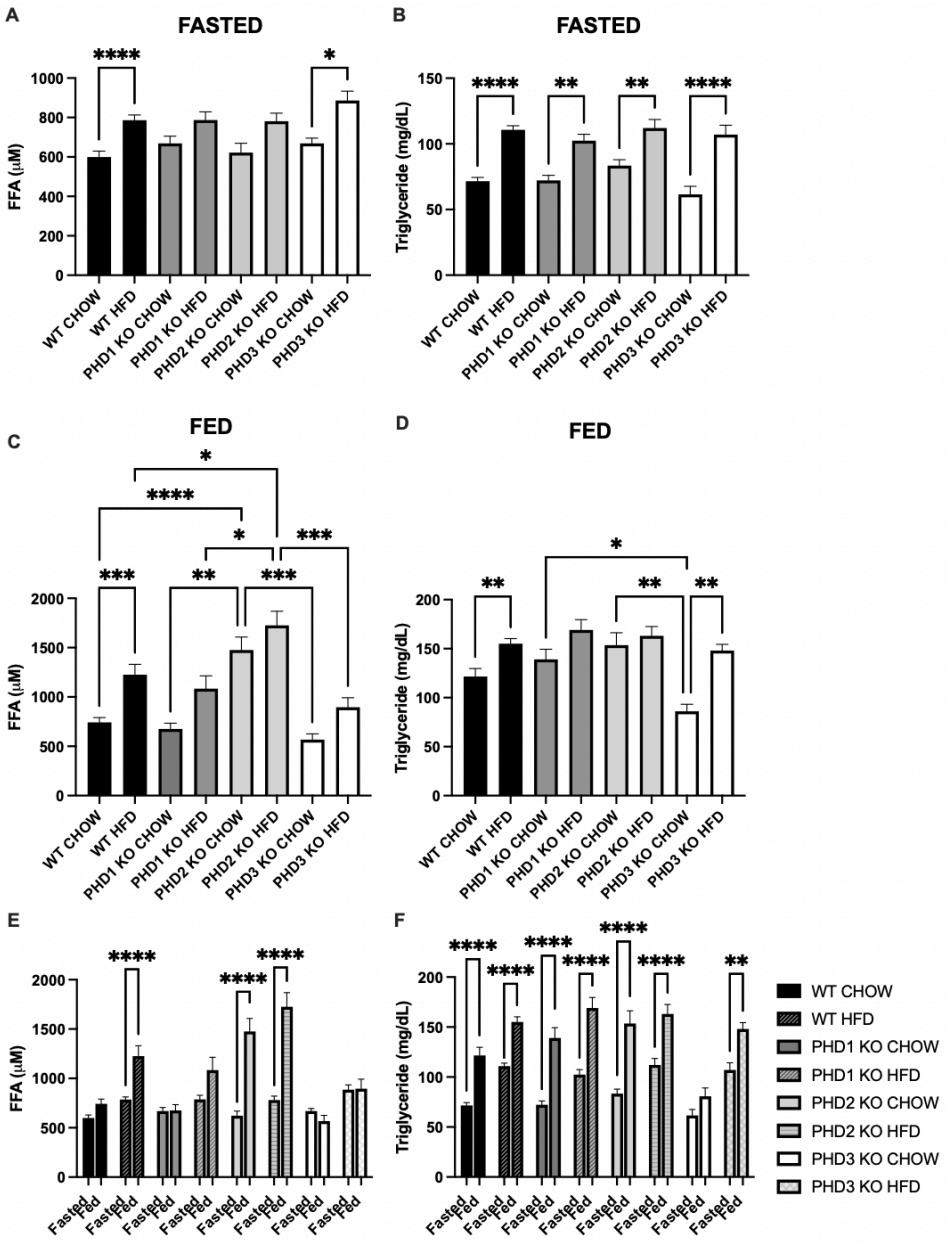

Figure 14. Effects of diet-induced obesity on plasma free fatty acid (FFA) and triglyceride levels in control (wild-type) and β-cell-specific PHD knockout mice. (A) Fasted plasma fatty acid levels. (B) Fasted plasma triglyceride levels. (C) Fed plasma fatty acid levels. (D) Fed plasma triglyceride levels. (E) Fasted vs. fed plasma fatty acid levels. (E) Fasted vs. fed plasma triglyceride levels. Data are mean ± SEM (n = 9 to 18 per treatment). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

3.1.5 A lack of β-cell PHD2 reduces activity levels in diet-induced obesity, but β-cell PHDs do not impact whole-body carbohydrate or lipid oxidation

Due to altered weight gain on the high-fat diet between genotypes, locomotive activity was assessed using CLAMS. As expected, locomotive activity decreased for all genotypes on the high-fat diet (Figure 15). Interestingly, control diet-fed and high-fat diet-fed β-cell-specific PHD2 knockout mice showed more sedentary behaviour. High-fat diet-fed β-cell-specific PHD1 knockout mice showed more active behaviour with slightly higher activity levels than control mice on the high-fat diet. Control diet-fed β-cell-specific PHD3 knockout mice showed higher activity levels than control-diet-fed control mice.

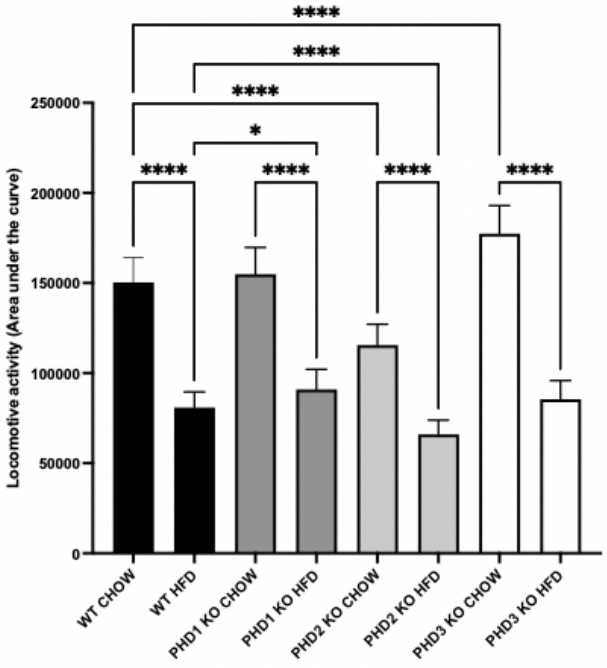

Figure 15. Effects of diet-induced obesity on locomotive activity in control (wild-type) and β-cell-specific PHD knockout mice. Data are mean ± SEM (n = 11 to 24 per treatment). *$P < 0.05$, ****$P < 0.0001$. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

Respiratory exchange ratio, whole-body lipid and carbohydrate oxidation rates were also calculated by CLAMS to assess how high-fat diet-fed mice cope with nutrient excess through altered metabolism. The respiratory exchange ratio for all genotypes on the high-fat diet decreased from 0.9 on the control diet to approximately 0.8 (Figure 16A). There was no significant difference in the respiratory exchange ratio between genotypes on the high-fat diet (Figure 16B). A respiratory exchange ratio closer to 0.7 indicates a metabolic preference for lipid energy sources, most likely due to the abundance of circulating fatty acids and triglycerides.[110]

All genotypes on the high-fat diet experienced comparable *in vivo* carbohydrate oxidation rates around 0.023 g/min/kg (Figure 16C). Rates remained relatively unaltered throughout the light (resting phase) and dark cycle (active phase). *In vivo* carbohydrate oxidation rates for most genotypes on the control diet was around 0.049 g/min/kg during the light cycle and increased to around 0.070 g/min/kg during the dark cycle. In addition, all genotypes on the control diet experienced comparable unaltered *in vivo* lipid oxidation rates of around 0.007 g/min/kg (Figure 16E). Most high-fat diet-fed genotypes experienced an *in vivo* lipid oxidation rate of around 0.018 g/min/kg in the light cycle, increasing to around 0.024 g/min/kg during the dark cycle. β-cell-specific PHD3 knockout mice on the high-fat diet experienced an overall trend of a lower *in vivo* lipid oxidation rate at approximately 0.013 g/min/kg, which increased to 0.018 g/min/kg during the dark cycle.

Following a lower respiratory exchange ratio, all genotypes on the high-fat diet had significantly decreased whole-body carbohydrate oxidation and increased lipid oxidation *in vivo* compared to their respective genotypes on the control diet (Figures 16D and F). Interestingly, β-cell-specific PHD2 knockout and β-cell-specific PHD3 knockout mice showed

slight exceptions to this. *In vivo* carbohydrate oxidation for high-fat diet-fed β-cell-specific PHD2 knockout mice did not significantly decrease compared to β-cell-specific PHD2 knockout mice on the control diet (Figure 16D). In contrast, β-cell-specific PHD3 knockout mice did not show significantly increased *in vivo* lipid oxidation on the high-fat diet (Figure 16F). Overall, all genotypes on the high-fat diet experienced lower activity levels, but high-fat diet-fed genotypes showed comparable respiratory exchange ratios combined with similar carbohydrate and lipid oxidation rates.

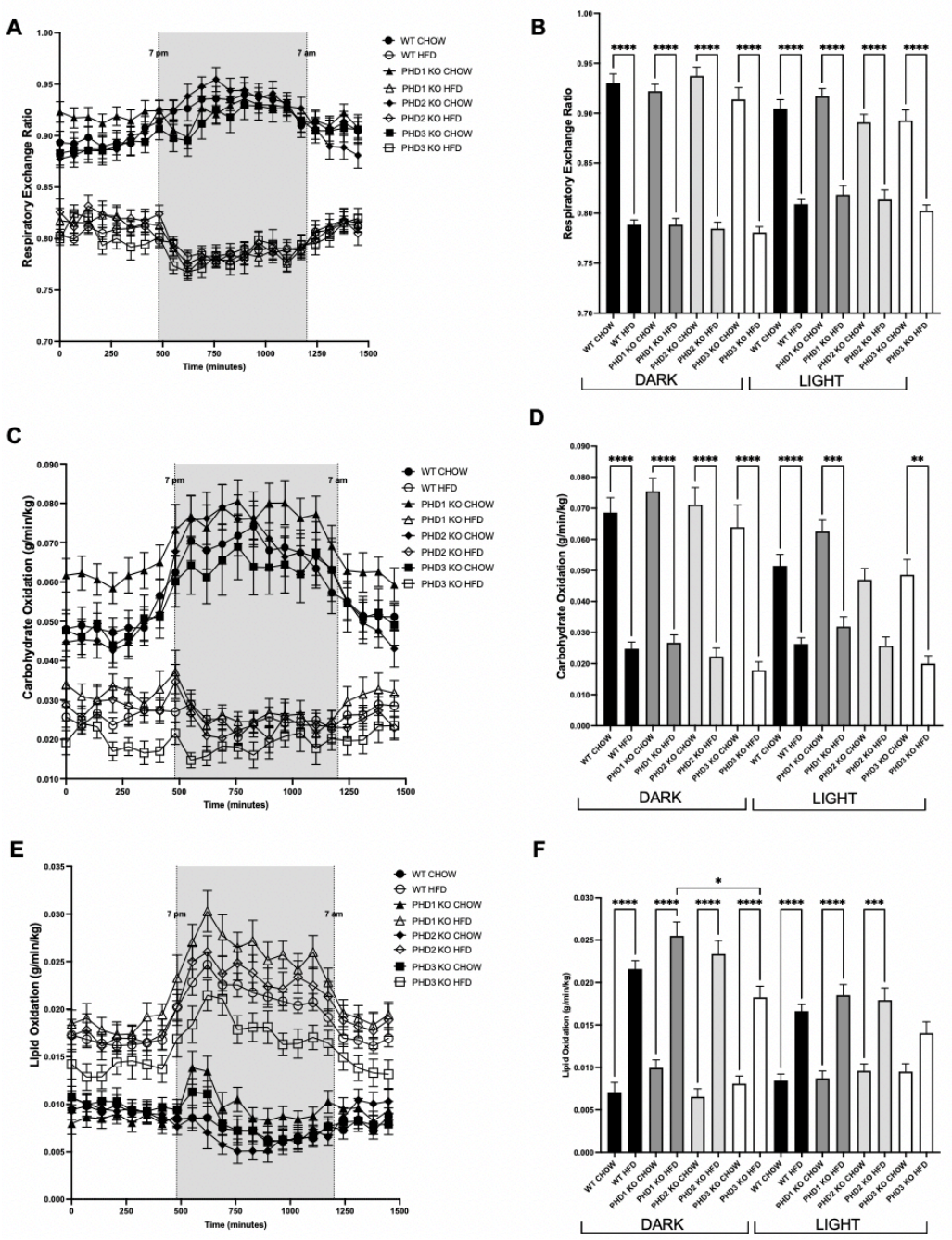

Figure 16. Indirect calorimetry measurements of (A) respiratory exchange ratio, (C) whole-body carbohydrate oxidation, and (E) whole-body lipid oxidation in control (wild-type) and β-cell-specific PHD knockout mice. (B) AUC of (A). (D) AUC of (C). (F) AUC of (E). Data are mean ± SEM (n = 11 to 24 per treatment). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

3.2 Objective 2: Characterization of diet-induced obesity effects on β-cell-specific PHD2 knockout male islets *ex vivo*

Only β-cell-specific PHD2 knockout mice were protected from developing metabolic consequences in glucose homeostasis associated with diet-induced obesity. Compared to high-fat diet-fed control mice, high-fat diet-fed β-cell-specific PHD2 knockout mice experienced a smaller weight gain, improved glucose tolerance, improved insulin sensitivity and lower plasma insulin levels during a glucose tolerance test. Despite improved glucose homeostasis during diet-induced obesity, β-cell-specific PHD2 knockout mice displayed reduced activity levels and elevated fed plasma fatty acid levels. β-cell mass and function were analyzed *ex vivo* attempting to understand the unique metabolic phenotype of high-fat diet-fed β-cell-specific PHD2 knockout mice.

3.2.1 PHD2 absence enhances β-cell mass during diet-induced obesity

Chromogenic immunohistochemistry stains visualized β- and α-cell mass to assess altered cell mass in response to chronic metabolic stress (Figure 17A). β-cell mass between control diet-fed control islets and β-cell-specific PHD2 knockout islets was similar (Figure 17B). Although insignificant, high-fat diet-exposed control islets appear to have decreased β-cell mass compared to control diet-exposed control islets. β-cell-specific PHD2 knockout islets exposed to the high-fat diet showed significantly increased β-cell mass compared to high-fat diet-fed control islets. A lack of significant difference in α-cell mass suggests α-cell mass did not change between islets (Figure 17C). Thus, PHD2 encourages decreased β-cell mass during chronic nutrient excess without affecting α-cell mass.

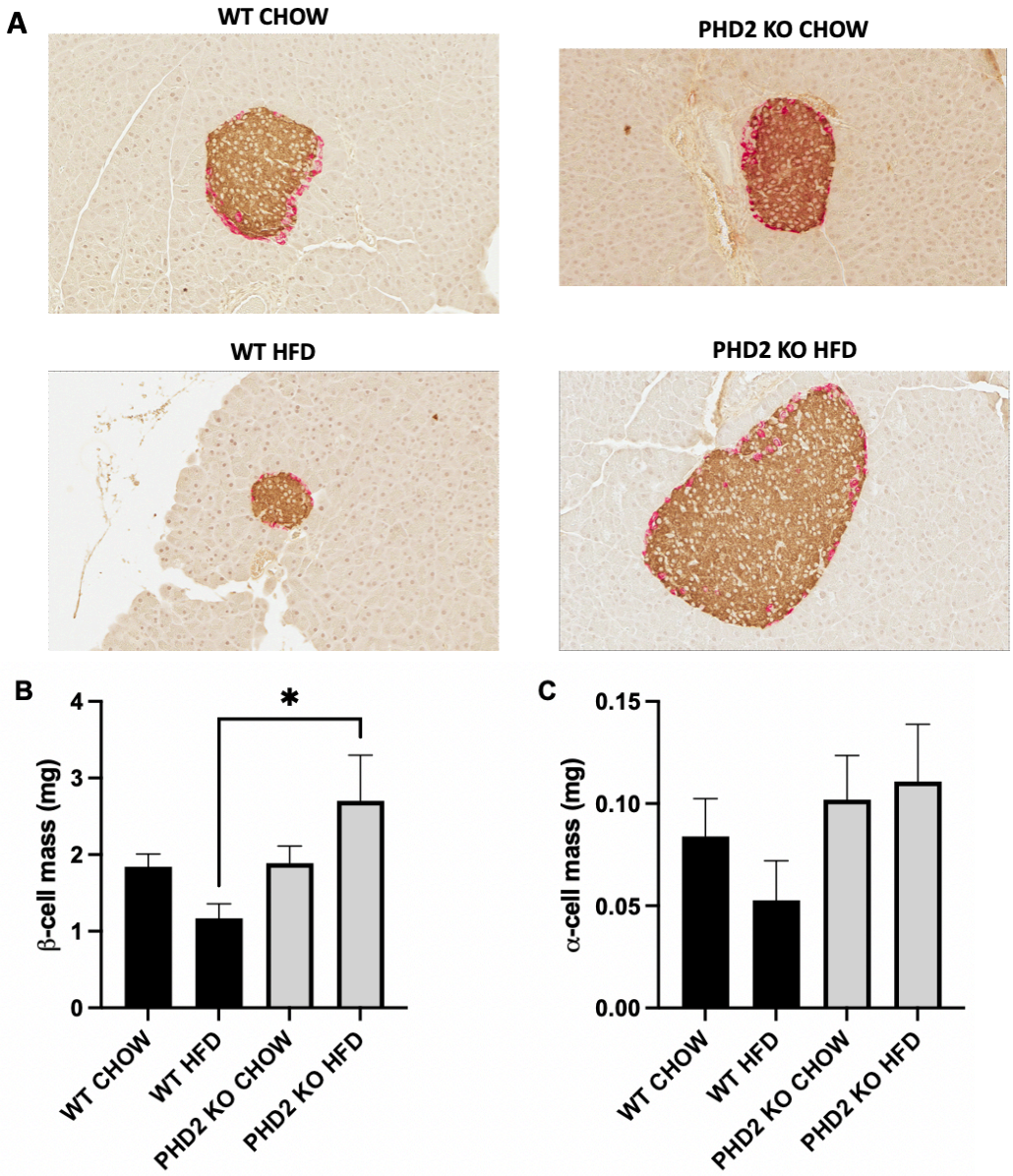

Figure 17. Effects of diet-induced obesity on (B) β-cell mass and (C) α-cell mass in control (wild-type) and β-cell-specific PHD2 knockout islets. (A) Chromogenic visualization of β-cells (dark brown) and α-cells (red). Pancreas were counterstained with hematoxylin (light brown). Data are mean ± SEM (n = 6 mice per treatment). *$P < 0.05$. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

3.2.2 A lack of β-cell PHD2 increases the large islet population and decreases the neogenic population during diet-induced obesity

Isolated sectioned pancreas were stained using immunofluorescence to visualize islet morphometrics and understand changes in β-cell mass (Figure 18A). Islets were classified by size: neogenic, medium or large. The medium islet population remained relatively similar between all pancreas (Figure 18B). High-fat diet-exposed β-cell-specific PHD2 knockout pancreas had a smaller population of neogenic islets compared to control diet-exposed and high-fat diet-exposed control pancreas. The large islet population of high-fat diet-exposed β-cell-specific PHD2 knockout pancreas was significantly elevated compared to high-fat diet-exposed control pancreas. Significant changes in the composition of neogenic and large islets per pancreas suggest β-cell PHD2 ultimately discourages the generation of large islets to hinder compensatory growth of β-cell mass during metabolic stress (Figure 17B).

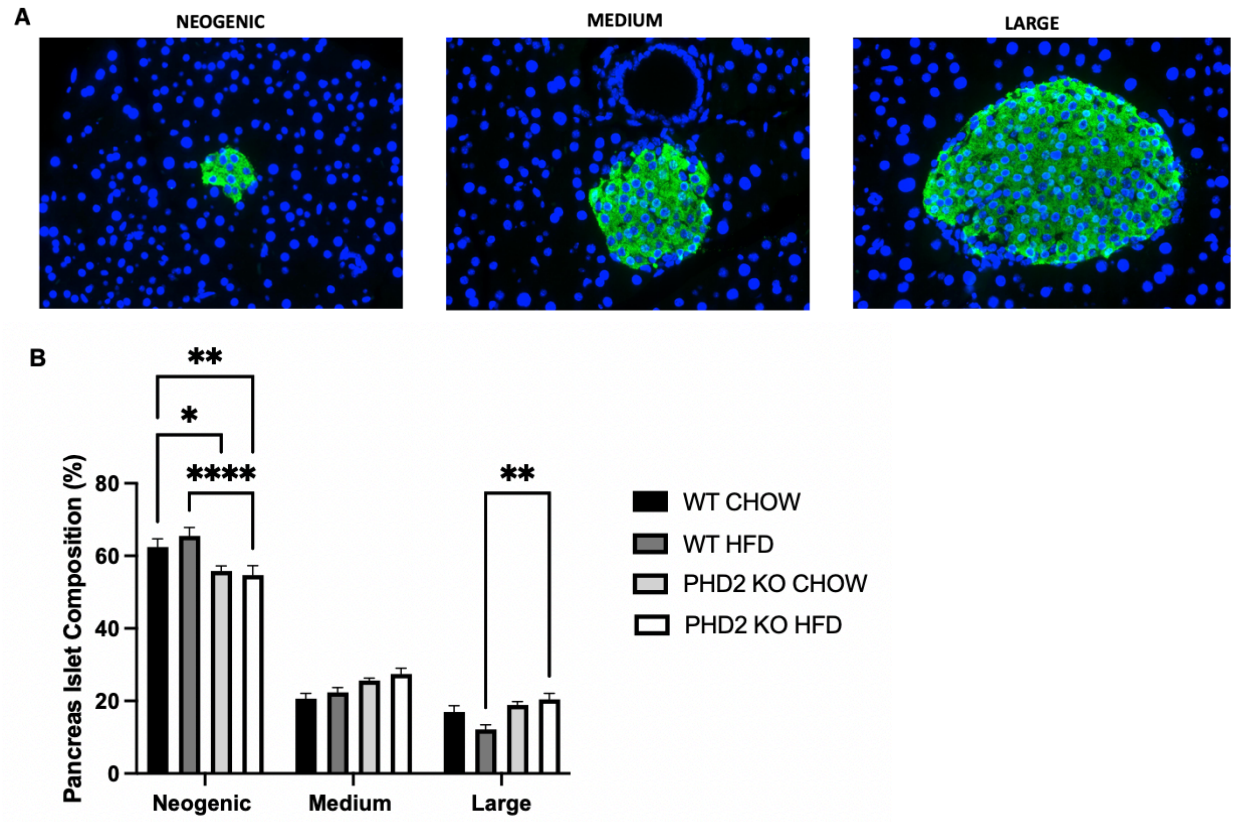

Figure 18. Effects of diet-induced obesity on (B) islet size in control (wild-type) and β-cell-specific PHD2 knockout islets. (A) Immunofluorescent visualization of islet sizes: neogenic (<11 nuclei), medium (11-50 nuclei) and large (>50 nuclei). Green – insulin, Blue – nuclei. Data are mean ± SEM (n = 6 per treatment). *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

3.2.3 PHD2 absence does not impact β-cell apoptosis but elevates β-cell proliferation in neogenic and large islets during diet-induced obesity

In addition to islet morphometrics, pancreas sections were stained with immunofluorescent antibodies to visualize Ki67 and cleaved caspase-3, assessing β-cell proliferation and apoptosis, respectively (Figure 19A). Overall β-cell proliferation was relatively unaltered between control diet-exposed control islets, high-fat diet-exposed control islets, and control diet-exposed β-cell-

specific PHD2 knockout islets (Figure 19B). High-fat diet-exposed β-cell-specific PHD2 knockout islets showed elevated β-cell proliferation compared to control islets exposed to the control and high-fat diet. More specifically, neogenic and large islets of high-fat diet-exposed β-cell-specific PHD2 knockout pancreas experienced significantly enhanced β-cell proliferation (Figure 19C).

Only high-fat diet-exposed control islets experienced altered β-cell apoptosis, most likely attributed to changes in medium islet β-cell apoptosis (Figures 19D-E). β-cell apoptosis decreased in medium islets of high-fat diet-exposed control pancreas compared to control diet-exposed control pancreas (Figure 19E). Medium islets of high-fat diet-exposed β-cell-specific PHD2 knockout pancreas showed similar β-cell apoptosis as control diet-exposed control and β-cell-specific PHD2 knockout pancreas. Neogenic and large islet β-cell apoptosis remained relatively unaltered between all pancreas. Although insignificant, large islet β-cell apoptosis appears to be reduced in high-fat diet-exposed control pancreas and control-diet exposed β-cell-specific PHD2 knockout pancreas compared to control pancreas exposed to the control diet. Together, the results suggest a negative role for PHD2 in regulating β-cell proliferation in neogenic and large islets, leading to reduced β-cell mass during metabolic stress (Figure 17B).

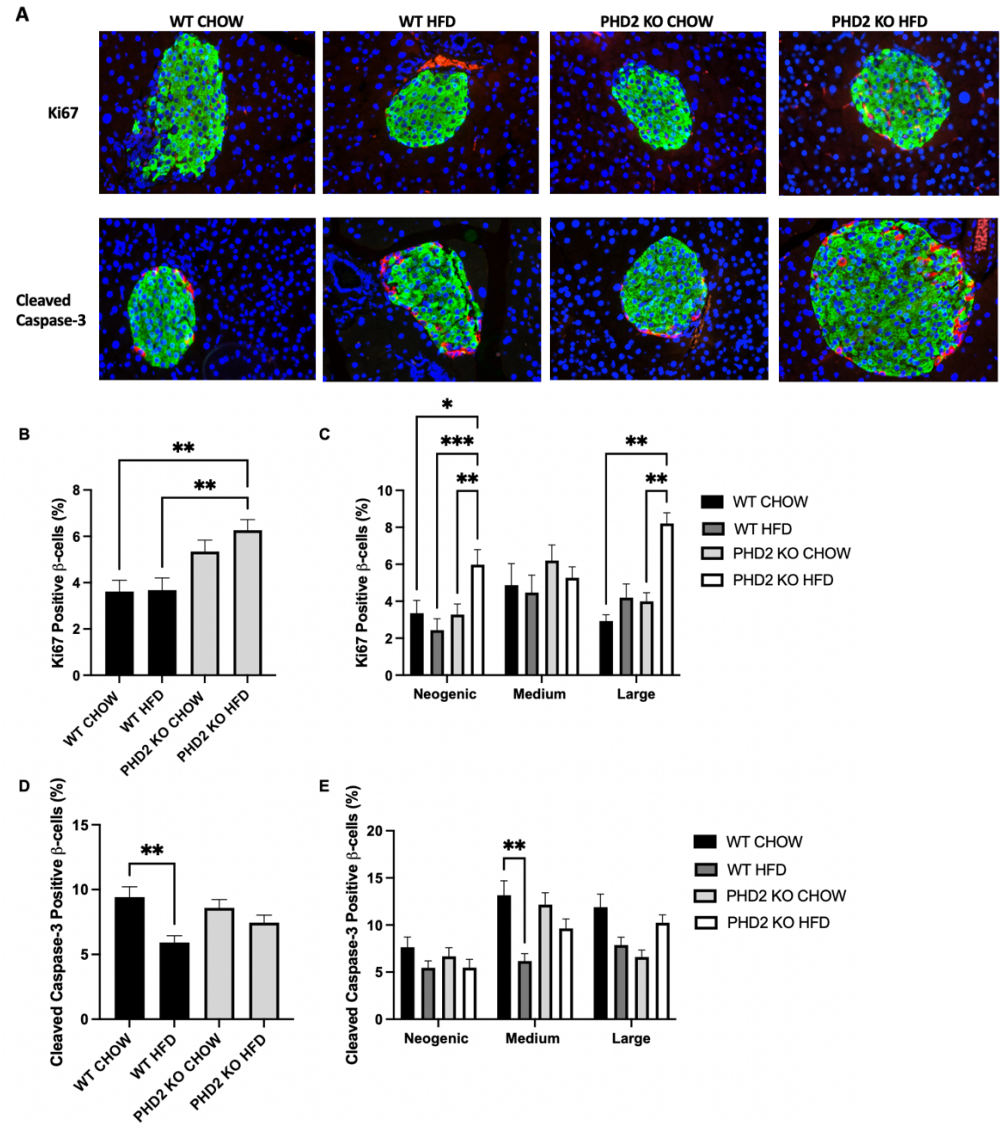

Figure 19. Effects of diet-induced obesity on (B) β-cell proliferation and (D) apoptosis in control (wild-type) and β-cell-specific PHD2 knockout islets. (A) Immunofluorescent visualization of β-cell proliferation and apoptosis. Nuclear Ki67 (red) indicates proliferation, and cleaved caspase-3 (red) indicates apoptosis. Positive β-cells were identified by colocalization of Ki67 or cleaved caspase-3 with insulin (green) surrounded nuclei (blue). (C) β-cell proliferation and (E) apoptosis according to islet size. Data are mean ± SEM (n = 6 mice per treatment, approximately 10,000-32,000 insulin-positive β-cells counted). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

3.2.4 A lack of β-cell PHD2 enhances high glucose-stimulated insulin secretion during diet-induced obesity

Isolated islets were stimulated *ex vivo* with low (2 mM) and high glucose (10 mM) to observe the β-cell's ability to secrete insulin after exposure to diet-induced obesity. Basal insulin secretion in response to low glucose was relatively unaltered between all islets (Figure 20). Although insignificant, control diet-exposed β-cell-specific PHD2 knockout islets and high-fat diet-exposed control islets appear to have lower basal insulin secretion than control diet-exposed control islets.

Control diet-exposed control and β-cell-specific PHD2 knockout islets experienced similar high glucose insulin secretion (Figure 20). In response to high glucose, high-fat diet-exposed control islets displayed impaired insulin secretion compared to control islets exposed to the control diet. However, high-fat diet-exposed β-cell-specific PHD2 knockout islets showed enhanced high glucose insulin secretion compared to all islets. The absence of β-cell PHD2 significantly rescued obesity-impaired insulin secretion, suggesting that PHD2 interferes with glucose-stimulated insulin secretion during chronic metabolic stress.

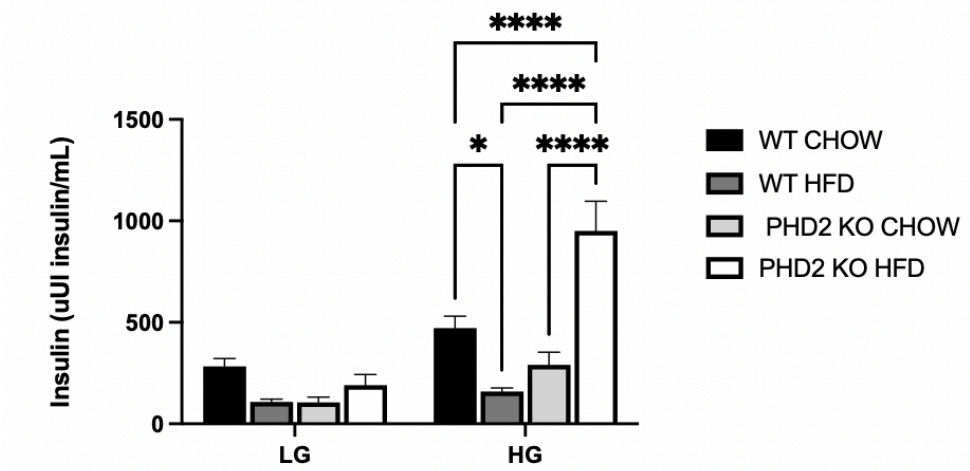

Figure 20. Effects of diet-induced obesity on insulin secretion in control (wild-type) and β-cell-specific PHD2 knockout islets in response to low glucose (LG; 2 mM) and high glucose (HG; 10 mM). Data are mean ± SEM (n = 17-28 per treatment). *$P < 0.05$, ****$P < 0.0001$. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

3.2.5 β-cell PHD2 absence improves the K_{ATP} channel-dependent and -independent pathways of glucose-stimulated insulin secretion during diet-induced obesity

The K_{ATP} channel-dependent and -independent pathways were assessed to dissect the mechanism of enhanced *ex vivo* insulin secretion of high-fat diet-exposed β-cell-specific PHD2 knockout islets. Isolated islets exposed to diazoxide (200 μM) clamps the K_{ATP} channels open, and potassium chloride (30 mM) depolarizes the membrane to maintain high intracellular calcium levels. Impaired insulin secretion in response to low glucose, potassium chloride and diazoxide suggests calcium signalling defects in the K_{ATP} channel-dependent pathway. Whereas reduced insulin secretion in response to high glucose, potassium chloride and diazoxide suggests anaplerosis defects in the K_{ATP} channel-independent pathway.

As expected, control and β-cell-specific PHD2 knockout islets exposed to the control diet showed comparable insulin secretion in response to low or high glucose with potassium

chloride and diazoxide (Figure 21). High-fat diet-exposed control islets appear to have reduced low glucose, potassium chloride and diazoxide-stimulated insulin secretion compared to control diet-exposed control islets, but the change was insignificant. In response to high glucose with potassium chloride and diazoxide, high-fat diet-exposed control islets experienced a significant decrease in insulin secretion compared to control diet-exposed control islets. Thus, obesity-impaired *ex vivo* insulin secretion (Figure 20) was mostly attributed to impaired anaplerosis (Figure 21).

In response to either low or high glucose, potassium chloride and diazoxide, high-fat diet-exposed β-cell-specific PHD2 knockout islets had comparable insulin secretion to control and β-cell-specific PHD2 knockout islets exposed to the control diet (Figure 21). However, high-fat diet-exposed β-cell-specific PHD2 knockout islets secreted significantly more insulin compared to high-fat diet-exposed control islets in response to low or high glucose with potassium chloride and diazoxide. Together, results indicate that β-cell PHD2 absence improves calcium signalling in the K_{ATP} channel-dependent pathway and restores anaplerosis in the K_{ATP} channel-independent pathway of glucose-stimulated insulin secretion during chronic metabolic stress. Therefore, β-cell PHD2 may interfere with the K_{ATP} channel-dependent and -independent pathways during diet-induced obesity.

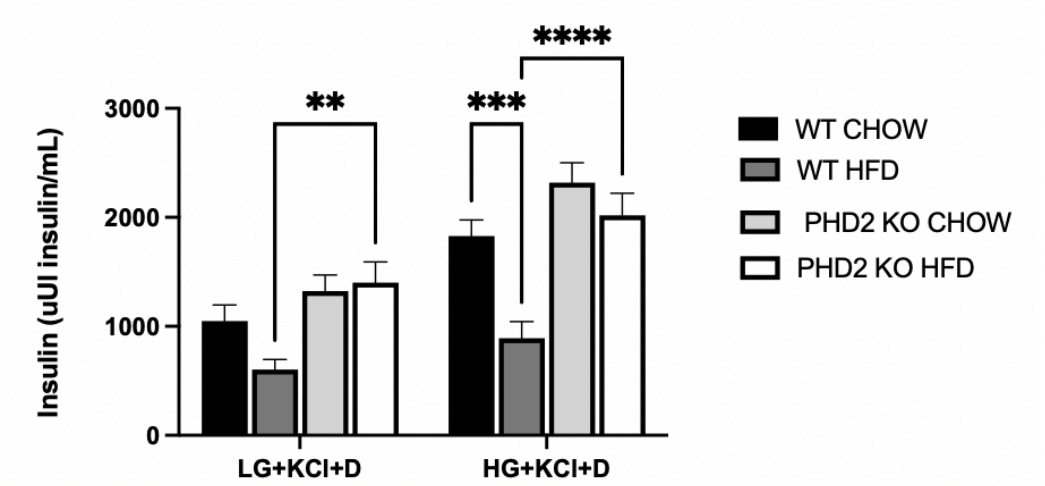

Figure 21. Effects of potassium chloride (KCl; 30 mM) and diazoxide (D; 200 µM) treatments on insulin secretion in control (wild-type) and β-cell-specific PHD2 knockout islets exposed to the high-fat diet. Data are mean ± SEM (n = 21-29 per treatment). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet; LG, low glucose (2 mM); HG, high glucose (10 mM).

3.2.6 Anaplerosis substrates impair insulin secretion in β-cells lacking PHD2 exposed to diet-induced obesity

Islets exposed to membrane-permeable anaplerosis substrates promote TCA flux and can rescue impaired high glucose-stimulated insulin secretion associated with K_{ATP} channel-independent pathway defects. Control diet-exposed control and β-cell-specific PHD2 knockout islets showed similar insulin secretion in response to high glucose and anaplerosis substrates (Figure 22). Promoting anaplerosis did not rescue impaired insulin secretion of control islets exposed to the high-fat diet (Figures 20 and 22). High-fat diet-exposed control islets secreted significantly less insulin in response to high glucose and anaplerosis substrates than control islets exposed to the control diet (Figure 22). Interestingly, despite intact anaplerosis (Figure 21), high-fat diet-exposed β-cell-specific PHD2 knockout islets also followed a similar

reduction of insulin secretion in response to anaplerosis substrates (Figure 22). Insulin secretion in response to high glucose and anaplerosis substrates was comparable between control and β-cell-specific PHD2 knockout islets exposed to the high-fat diet. Although β-cells lacking PHD2 exposed to diet-induced obesity showed enhanced *ex vivo* glucose-stimulated insulin secretion (Figure 20), they may not be able to cope with amplified TCA flux (Figure 22).

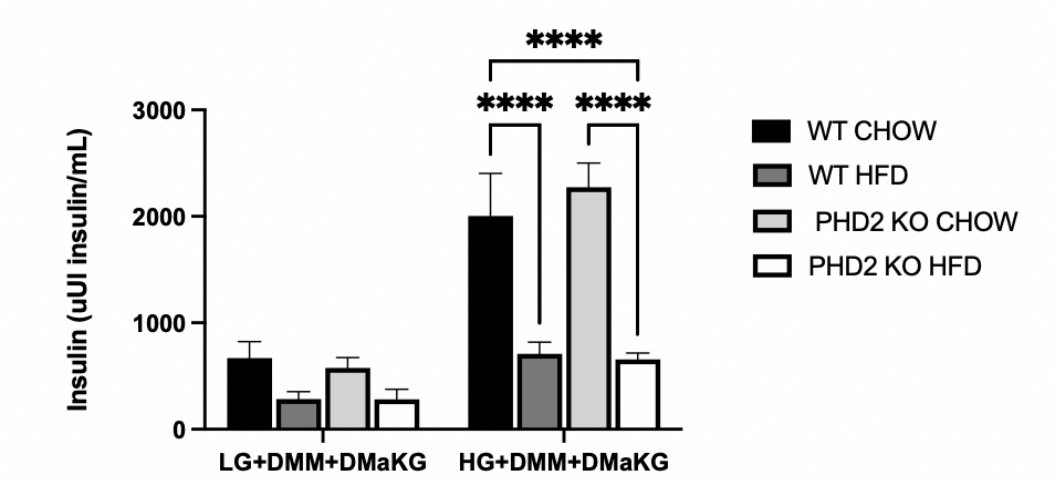

Figure 22. Effects of dimethyl malate (DMM; 10 mM) and dimethyl α-ketoglutarate (DMaKG; 10 mM) treatments on insulin secretion in control (wild-type) and β-cell-specific PHD2 knockout islets exposed to the high-fat diet. Data are mean ± SEM (n = 20-30 per treatment). ****$P < 0.0001$. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet; LG, low glucose (2 mM); HG, high glucose (10 mM).

3.2.7 Absence of PHD2 encourages β-cell *PFKM* expression during diet-induced obesity

To further dissect the enhanced β-cell function of high-fat diet-exposed β-cell-specific PHD2 knockout islets, the degree of anaerobic reprogramming was evaluated using an expression profile of genes influencing β-cell glucose metabolism. Expression of *glucose transporter 2* (*GLUT2*), *phosphofructokinase (PFKM)*, *pyruvate kinase (PKM2), pyruvate carboxylase (PC)*, *pyruvate dehydrogenase kinase (PDK1)*, and *lactate dehydrogenase (LDHA)*

was analyzed in isolated islets. GLUT2 is an insulin-independent glucose transporter and its gene expression was unaltered between all islets (Figure 23A). PFKM and PKM2 participate in glycolysis, where PFKM is a crucial rate-limiting enzyme at the beginning of glycolysis. *PFKM* expression appears slightly elevated in high-fat diet-exposed control islets and control diet-exposed β-cell-specific PHD2 knockout islets (Figure 23B). However, only increased *PFKM* expression in high-fat diet-exposed β-cell-specific PHD2 knockout islets compared to control diet-exposed control islets was significant. On the other hand, *PKM2* expression was unaltered between all islets (Figure 23C).

PC, PDK1, and LDHA regulate the metabolic fate of pyruvate. β-cells express PC to encourage pyruvate entry into the TCA cycle for glucose-stimulated insulin secretion, whereas PDK1 indirectly discourages TCA flux. LDHA regulates pyruvate fates only under anaerobic conditions. Islets displayed no significant changes in *PC*, *PDK1,* and *LDHA* expression (Figures 23D-F). *PDK1* expression appears elevated in high-fat diet-exposed β-cell-specific PHD2 knockout islets compared to control diet-exposed control islets, but the change was insignificant (Figure 23F). The overall lack of change in the gene expression profile suggests that PHD2 does not significantly alter glucose metabolism during metabolic stress. β-cell PHD2 may only interfere with glycolytic *PFKM* expression during diet-induced obesity.

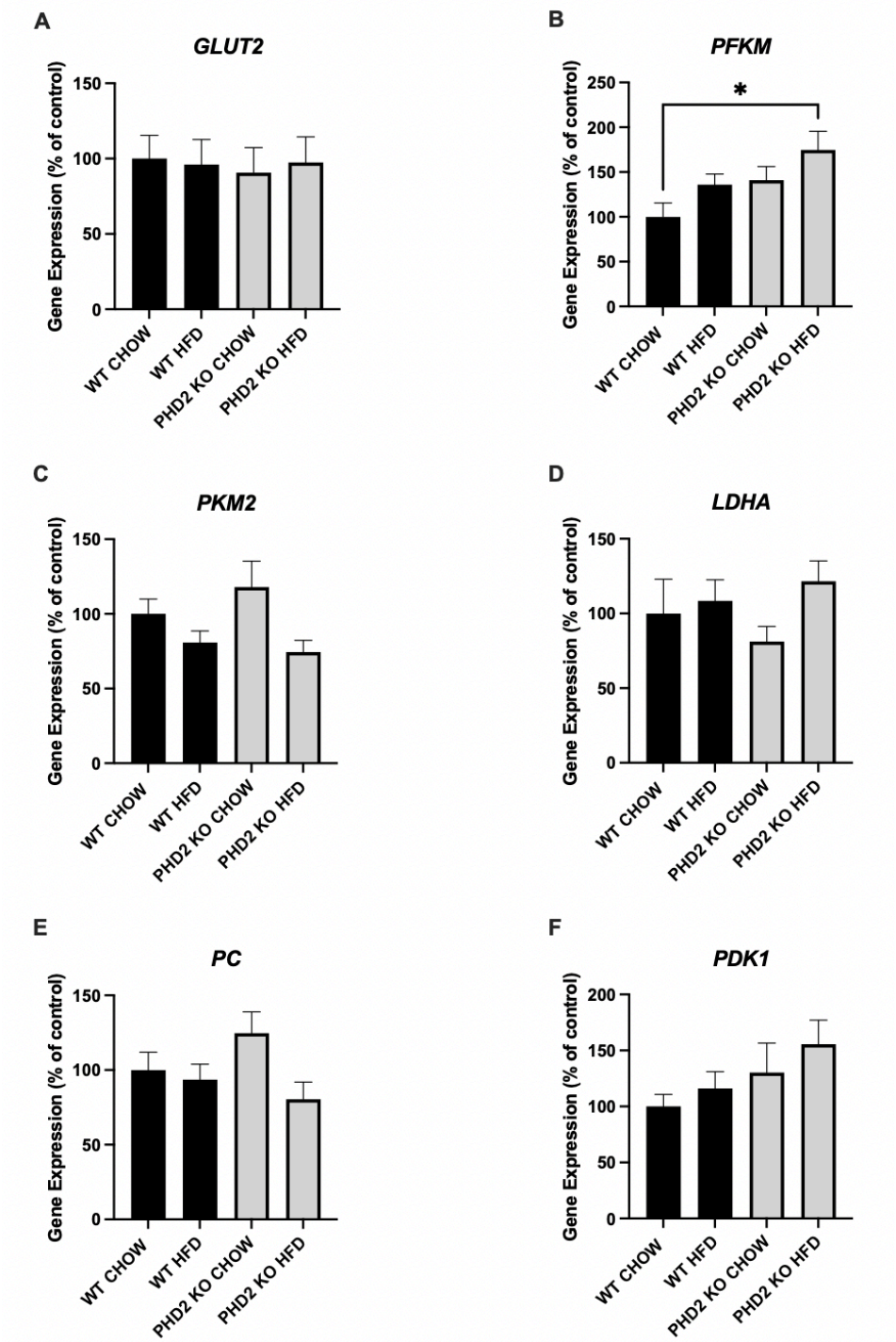

Figure 23. Effects of diet-induced obesity on gene expression in control (wild-type) and β-cell-specific PHD2 knockout islets. Gene expression was corrected by cyclophilin, an internal housekeeping control gene. Data are mean ± SEM (n = 8-14 per treatment). *$P < 0.05$. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

3.2.8 A lack of PHD2 encourages β-cell HIF-1α stability in diet-induced obesity

Since HIF-1α is a commonly known target of PHDs, western blots quantified the relative protein expression of β-cell HIF-1α in isolated islets (Figure 24A, Appendix Figure 31).[61,69] Increased HIF-1α stability could partially explain the enhanced β-cell function of high-fat diet-exposed β-cell-specific PHD2 knockout islets.[70] Control islets exposed to the control or high-fat diet and control diet-exposed β-cell-specific PHD2 knockout islets showed similar HIF-1α protein expression (Figure 24B). However, high-fat diet-exposed β-cell-specific PHD2 knockout islets experienced increased HIF-1α expression compared to control and β-cell-specific PHD2 knockout islets exposed to the control diet. Enhanced HIF-1α expression in high-fat diet-exposed β-cell-specific PHD2 knockout islets suggests that PHD2 prevents HIF-1α stability only during chronic metabolic stress.

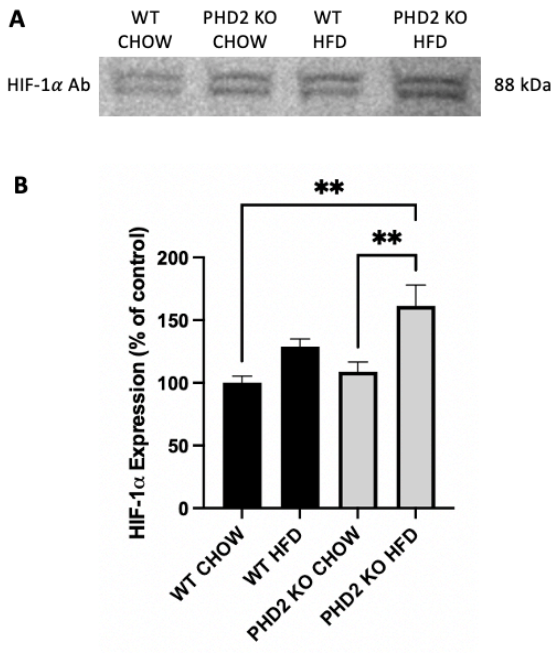

Figure 24. Effects of diet-induced obesity on HIF-1α protein expression in control (wild-type) and β-cell-specific PHD2 knockout islets. (A) HIF-1α was detected at 88 kDa. (B) HIF-1α protein expression was normalized to the total protein loaded per sample. Data are mean ± SEM (n = 5 per treatment). $^{**}P < 0.01$. Ab, antibody; WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

Chapter 4: Discussion

4.1 The "Surwit" modelled high-fat diet promoted obesity-induced metabolic dysfunction in β-cell-specific PHD knockout mice on a C57Bl/6N genetic background

4.1.1 A C57Bl/6N genetic background allows for β-cell dysfunction as a result of diet-induced obesity

Various mouse strains have been explored to understand the effects of metabolic stress on glucose homeostasis. A study comparing genetic backgrounds showed similar diet-induced weight gain patterns but different degrees of glucose tolerance.[125,126] The C57Bl/6 genetic background is more prone to diet-induced obesity and insulin sensitivity impairments, predisposing these mice to diabetes.[127-131] The development of human type 2 diabetes shows a similar progression, where genetic factors and weight gain from nutrient-excessive diets predispose humans to type 2 diabetes.[127] Other diabetes mouse models, like the genetically modified obese mouse (C57Bl/6J ob/ob) and the diabetes mouse (C57Bl/6J db/db), explain some of the genetic influences in the pathophysiology since these models can develop diabetes on a chow diet.[132,133] These mouse models also show neuroendocrine abnormalities, creating a limitation in the application to human type 2 diabetes.[132,133]

Diet-induced obesity studies with C57Bl/6J and 6N mice vary from 12-24 weeks on a high-fat diet, where diabetic phenotypes have been observed after 15 weeks.[110,127] Hyperglycemia may occur after one week of a high-fat diet, but hyperinsulinemia may require significant weight gain after 11 weeks on a high-fat diet.[134,135] Acute high-fat diet studies with C57Bl/6N mice have also shown forms of metabolic dysfunction. A six-week 45% high-fat diet showed impaired glucose tolerance, and an eight-week 60% high-fat diet promoted obesity and impaired β-cell insulin secretion.[136,137] Although studies vary between 6J and 6N mice, studies

suggest they have similar relative impairments in glucose and insulin tolerance in response to diet-induced obesity.[137] With obesity-associated diabetic phenotypes and β-cell dysfunction occurring between 8 to 11 weeks on a high-fat diet, a ten-week high-fat diet was implemented to assess mice in the early stages of obesity-induced type 2 diabetes development. Since control mice displayed significant weight gain, reduced insulin sensitivity, elevated plasma triglyceride levels, and β-cell dysfunction on the high-fat diet, the ten-week diet intervention was sufficient for predisposing mice to type 2 diabetes.

Although C57Bl/6J mice were predominantly used for diet-induced metabolic dysfunction research, studies reveal an insulin secretion defect in the genetic background.[138,139] The 6J substrain has a deletion of the *nicotinamide nucleotide transhydrogenase (Nnt)* gene, impairing oxidized nicotinamide adenine dinucleotide replenishment in the TCA cycle.[138] Compared to C57Bl/6N mice, the 6J substrain shows impaired glucose-stimulated insulin secretion, suggesting the *Nnt* deletion could generate confounding associations between diet-induced obesity and β-cell dysfunction.[140] The mouse model in the project was designed with a C57Bl/6N genetic background to properly investigate the effects of diet-induced obesity on β-cell function in β-cell-specific PHD knockout islets. Control islets displayed significantly impaired *ex vivo* glucose-stimulated insulin secretion on the high-fat diet, suggesting that β-cell dysfunction can be confidently associated with diet-induced obesity on the C57Bl/6N genetic background.

4.1.2 The "Surwit" high-fat diet promoted diet-induced obesity

Fat is supposedly the main driver for diet-induced obesity and glucose intolerance in C57Bl/6 mice.[141] However, standard high-fat diets are palmitate dense, a saturated fatty acid that generates β-cell dysfunction after long-term exposure.[130,142] This project investigated the

effects of diet-induced obesity on nutrient homeostasis in β-cell-specific PHD knockout mice. Thus, the focus was not on the consequential effects of dietary fat. The high-fat sucrose diet used in this project was modelled after the "Surwit" diet, which has been widely implemented in diet-induced obesity and type 2 diabetes studies.[143] The diet's fat content is dense in lauric acid, a saturated fatty acid typically directed to storage, thus ideal for inducing obesity.[143,144] Fortunately, lauric acid and the sucrose content in the "Surwit" high-fat diet do not directly interfere with β-cell function.[130,143] The sucrose content in the "Surwit" high-fat diet encourages a higher feed efficiency, efficiently inducing weight gain without increasing high-fat diet-induced caloric intake.[141] Although feed efficiency was not analyzed, all genotypes on the high-fat diet experienced significant weight gain than control diet-fed mice, suggesting the high-fat sucrose diet successfully promoted obesity.

Diet matching is beneficial in high-fat diet studies investigating the consequential effects of dietary fat. Ideally, matching all non-variable ingredients in the control and experimental diets avoids confounding conclusions of altered metabolic phenotypes caused by excess fat intake. The low-fat grain-based chow control diet used in this project did not match the sucrose content in the experimental diet since this project focused on diet-induced obesity. Previous studies revealed that low-fat (10.5 kcal %) and high-sucrose (60 kcal%) matching diets impair glucose-stimulated insulin secretion, potentially creating a challenge in identifying true β-cell dysfunction in diet-induced obese β-cell-specific PHD knockout mice.[143] One concern about the grain-based chow diet is the fibre content. However, high fibre content typically only imposes huge variations in gut microbiota research.[145] Our laboratory has experience evaluating diet-induced obesity in the chosen mouse model and diets, helping generate reproducible data and comparison between control groups across studies.[110,146]

4.2 Objective 1: Investigate *in vivo* metabolic consequences of diet-induced obesity in β-cell-specific PHD1, PHD2 or PHD3 knockout male mice

4.2.1 Summary

Only β-cell-specific PHD2 knockout and β-cell-specific PHD3 knockout mice showed unique diet-induced obese metabolic phenotypes. Diet-induced weight gain was hindered in β-cell-specific PHD2 knockout mice, ultimately showing improved metabolic dysfunction on the high-fat diet. High-fat diet-fed β-cell-specific PHD2 knockout mice showed reduced hyperinsulinemia during a glucose challenge (Figure 25B). Regulated *in vivo* insulin secretion during nutrient excess may avoid loss of insulin sensitivity. Despite improved glucose and insulin tolerance on the high-fat diet, β-cell-specific PHD2 knockout mice experienced high fed plasma fatty acid levels. Future steps to evaluate adiposity, tissue-specific lipolysis and lipogenesis, and lipid storage could explain reduced weight gain and elevated plasma fatty acid levels in high-fat diet-fed β-cell-specific PHD2 knockout mice.

Despite increased weight gain on the high-fat diet, β-cell-specific PHD3 knockout mice displayed similar glucose intolerance to control mice. However, metabolic consequences in β-cell-specific PHD3 knockout mice on the high-fat diet may arise from hypoinsulinemia (Figure 25C). A study suggested that a lack of β-cell PHD3 encourages fatty acid oxidation, interfering with glucose-stimulated insulin secretion.[98] Lower plasma insulin levels during a glucose challenge may not sufficiently allow glucose uptake in hepatocytes and myocytes, ultimately leading to elevated blood glucose levels. Further analysis of adiposity, adipocyte glucose uptake and hepatocyte lipid storage could possibly explain increased weight gain and elevated liver mass in high-fat diet-fed β-cell-specific PHD3 knockout mice.

Our last study suggested β-cell dysfunction in the absence of PHD1, but β-cell-specific PHD1 knockout mice followed similar metabolic phenotypes as control mice on the high-fat diet.[53] A glucose challenge highlighted high plasma insulin levels, most likely compensating for insulin sensitivity impairments (Figure 25A). Impaired insulin sensitivity ultimately disrupts nutrient homeostasis, explaining impaired glucose tolerance and elevated fasted plasma triglyceride levels in high-fat diet-fed β-cell-specific PHD1 knockout mice.

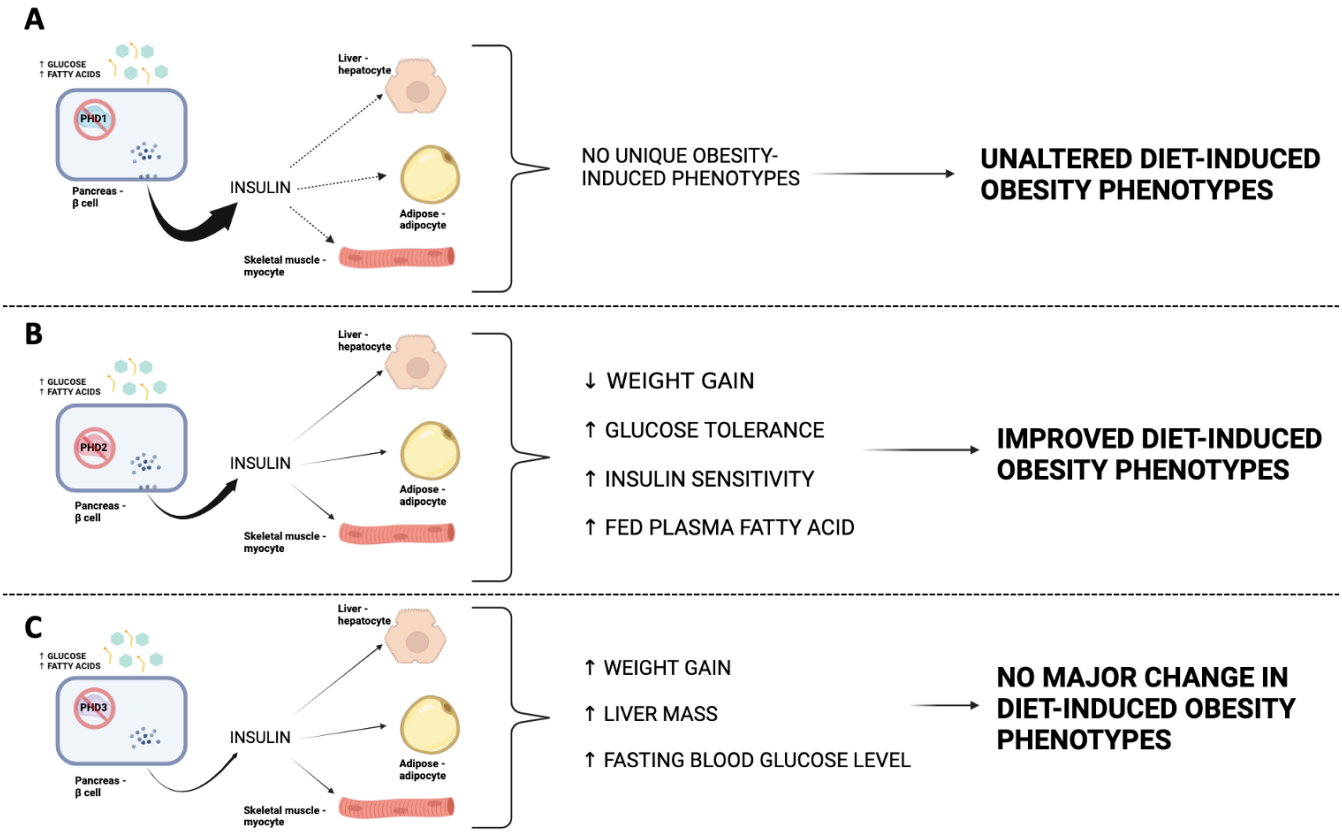

Figure 25. Metabolic phenotypes of (A) β-cell-specific PHD1 knockout mice, (B) β-cell-specific PHD2 knockout mice or (C) β-cell-specific PHD3 knockout mice on the high-fat diet. (A) High-fat diet-fed β-cell-specific PHD1 knockout mice displayed hyperinsulinemia, loss of insulin sensitivity and high plasma glucose and fatty acid levels like control mice on the high-fat diet. Dashed lines present impaired insulin sensitivity. (B) β-cell-specific PHD2 knockout mice were protected against diet-induced obesity and maintained normal glucose homeostasis despite higher fed plasma fatty acid levels. (C) High-fat diet-fed β-cell-specific PHD3 knockout mice showed increased weight gain accompanied by larger livers. Despite elevated fasting blood glucose levels and lower plasma insulin levels, β-cell-specific PHD3 knockout mice experienced comparable glucose intolerance as control mice on the high-fat diet. Figure was created with BioRender.com.

4.2.2 β-cell PHD2 and PHD3 influence diet-induced obesity independent of activity levels and whole-body nutrient metabolism

Diet-induced obesity is commonly associated with sedentary lifestyles but is also associated with increased subcutaneous and visceral fat depots.[110,129,141,147] Enhanced adiposity results from an increased number of adipocytes, adipocyte growth from accumulated lipid storage, or adipose tissue inflammation.[90,95,129] Although adipose weights were not measured, all genotypes on the high-fat diet showed similar caloric intake and experienced significant weight gain, supporting diet-induced obesity.[127,141] However, β-cell-specific PHD2 knockout mice displayed reduced diet-induced weight gain, and β-cell-specific PHD3 knockout mice showed increased diet-induced weight gain.

Activity levels did not support altered diet-induced weight gain in β-cell-specific PHD2 knockout and β-cell-specific PHD3 knockout mice. Sedentary behaviour would partly explain increased diet-induced weight gain, but activity levels between β-cell-specific PHD3 knockout and control mice on the high-fat diet were comparable.[147] Whereas, high-fat diet-fed β-cell-specific PHD2 knockout mice showed more sedentary behaviour, contradicting reduced diet-induced weight gain.[147] Control and β-cell-specific PHD1 knockout mice experienced similar diet-induced weight gain despite slightly higher activity levels in high-fat diet-fed β-cell-specific PHD1 knockout mice. It would be interesting to measure subcutaneous and visceral fat depots to see if altered diet-induced weight gain in β-cell-specific PHD2 knockout and β-cell-specific PHD3 knockout mice is associated with different adiposity patterns.[110,129,141]

Like whole-body PHD2 hypomorphic mice, β-cell PHD2 knockout mice were resistant to diet-induced obesity.[90] Improved diet-induced obesity was previously associated with adipocyte hypotrophy and elevated adipose lipolysis.[90] Additional analysis of adiposity and adipose

lipolysis is required to make similar associations in high-fat diet-fed β-cell-specific PHD2 knockout mice. Hypothetically, adipocyte hypotrophy and elevated adipose lipolysis could support elevated fed plasma fatty acid levels in high-fat diet-fed β-cell-specific PHD2 knockout mice.[90] However, elevated adipocyte lipolysis was also associated with increased whole-body lipid oxidation.[148] Unaltered whole-body lipid oxidation between high-fat diet-fed control and β-cell-specific PHD2 knockout mice may challenge this hypothesis.

Increased weight gain in high-fat diet-fed β-cell-specific PHD3 knockout mice may be associated with changes in *in vivo* lipid homeostasis, but whole-body lipid oxidation and plasma lipid profiles were similar to control mice on the high-fat diet.[21] Although adiposity was not analyzed, one hypothesis could be that high-fat diet-fed β-cell-specific PHD3 knockout mice may be experiencing enhanced adiposity, possibly from adipocyte hypertrophy.[90,95,129] Exponentiated adipocyte hypertrophy was previously associated with blunted basal adipocyte lipolysis and reduced ectopic lipid deposition.[95,149] Unfortunately, high-fat diet-fed β-cell-specific PHD3 knockout mice did not show reduced plasma fatty acid levels compared to control mice on the high-fat diet. Additionally, hepatomegaly in high-fat diet-fed β-cell-specific PHD3 knockout mice may be associated with liver inflammation and ectopic hepatocyte lipid storage.[150] Hepatomegaly did not significantly influence body weight since the percentage of liver weight to body weight in high-fat diet-fed β-cell-specific PHD3 knockout mice did not increase. Overall, further investigation into adiposity, adipocyte lipolysis and hepatocyte lipid storage is required to understand increased diet-induced weight gain in high-fat diet-fed β-cell-specific PHD3 knockout mice.

4.2.3 β-cell PHD2 and PHD3 play a role in *in vivo* glucose homeostasis during diet-induced obesity

β-cell mass may increase initially during nutrient excess, encouraging enhanced insulin secretion *in vivo* to regulate nutrient homeostasis.[134,137] However, obesity-induced adipose inflammation and adipocyte lipolysis inhibit insulin signalling and eventually interfere with insulin secretion.[22-24] In cases of impaired insulin sensitivity, β-cells compensate by encouraging hyperinsulinemia.[134] Eventually, impaired whole-body insulin sensitivity or β-cell dysfunction promotes hyperglycemia, altering *in vivo* glucose homeostasis.[2,3] Only β-cell-specific PHD2 knockout mice and β-cell-specific PHD3 knockout mice displayed unique patterns of *in vivo* glucose homeostasis during diet-induced obesity. Comparable glucose intolerance, high plasma insulin levels, and loss of insulin sensitivity between high-fat diet-fed control and β-cell-specific PHD1 knockout mice are supported by similar diet-induced weight gain.

β-cell-specific PHD2 knockout mice on the high-fat diet experienced reduced diet-induced weight gain, aligning with improved glucose and insulin tolerance. Improved obesity may delay or prevent the subsequent loss of insulin sensitivity, ultimately sustaining glucose tolerance in high-fat diet-fed β-cell-specific PHD2 knockout mice.[3,24] Lower plasma insulin levels during a glucose tolerance test in high-fat diet-fed β-cell-specific PHD2 knockout mice support sustained insulin sensitivity, suggesting a resistance against hyperinsulinemia.[134] Previous studies suggested that PHD2 interferes with adaptive anaerobic glycolysis during diet-induced obesity, which was associated with glucose intolerance.[90,95] However, β-cell-specific PHD2 knockout islets exposed to the high-fat diet only experienced elevated *phosphofructokinase*

(*PFKM)* expression (objective 2). Characterization of β-cell mass and function *ex vivo* was conducted in objective 2, shedding more light on β-cell function *in vivo*.

Our recent study showed impaired *ex vivo* glucose-stimulated insulin secretion in β-cell-specific PHD1 knockout and β-cell-specific PHD3 knockout islets.[53] Insulin secretion defects did not exacerbate obesity-induced glucose intolerance in β-cell-specific PHD1 knockout or β-cell-specific PHD3 knockout mice. As expected, glucose intolerance in high-fat diet-fed β-cell-specific PHD1 knockout mice was associated with a loss in insulin sensitivity and glucose-challenged hyperinsulinemia.[3,134] However, glucose intolerance in high-fat diet-fed β-cell-specific PHD3 knockout mice was associated with elevated fasting blood glucose levels, consistent with the development of diabetes.[3] Despite increased diet-induced weight gain, high-fat diet-fed β-cell-specific PHD3 knockout mice maintained insulin sensitivity.[24] It would be interesting to see if an additional two weeks of the high-fat diet would exacerbate obesity-induced glucose intolerance and promote loss of insulin sensitivity in β-cell-specific PHD3 knockout mice.

PHD3 may play a role in β-cell nutrient metabolism to regulate glucose homeostasis during metabolic stress.[98] In nutrient-excessive conditions, PHD3 prevents β-cell fatty acid oxidation to maintain glucose-linked insulin secretion.[98] β-cell-specific PHD3 knockout islets exposed to the high-fat diet show signs of impaired glucose-stimulated insulin secretion *ex vivo* (Appendix Figure 28), which could be associated with unregulated fatty acid oxidation in β-cells.[98] β-cell dysfunction could explain lower plasma insulin levels during a glucose challenge, possibly supporting normal insulin sensitivity in high-fat diet-fed β-cell-specific PHD3 knockout mice. However, obesity-impaired insulin sensitivity may also occur at various stages.[26] A lack of glucose uptake in insulin-insensitive myocytes could be compensated by increased glucose

uptake in insulin-sensitive adipocytes.[151] Enhanced adipocyte glucose uptake may be associated with increased diet-induced weight gain in β-cell-specific PHD3 knockout mice.[151] It would be beneficial to analyze organ-specific insulin signalling and adipocyte glucose uptake to highlight any progressions of obesity-impaired insulin sensitivity in high-fat diet-fed β-cell-specific PHD3 knockout mice.[152]

4.2.4 β-cell PHD3 protects against diabetic dyslipidemia during diet-induced obesity

Unregulated lipid homeostasis in obesity-induced type 2 diabetes is often associated with elevated plasma free fatty acid and triglyceride levels.[8,153,154] Diabetic dyslipidemia includes high plasma triglyceride levels and low high-density lipoprotein cholesterol levels.[8] Control mice experienced elevated fed and fasted plasma lipid levels on the high-fat diet, consistent with diet-induced obesity.[8,153,154] Elevated plasma lipid levels are supported by loss of *in vivo* insulin sensitivity, reduced *in vivo* glucose tolerance, and β-cell dysfunction *ex vivo* (objective 2), suggesting high-fat diet-fed control mice are progressing towards type 2 diabetes.[6,8] However, normal fasting blood glucose levels in high-fat diet-fed control mice do not yet support obesity-induced diabetes.[3] Considering similar diet-induced weight gain and *in vivo* glucose homeostasis, high-fat diet-fed β-cell-specific PHD1 knockout mice displayed comparable fasted and fed plasma lipid levels as high-fat diet-fed control mice. Elevated plasma triglyceride levels hint at dyslipidemia in high-fat diet-fed control and β-cell-specific PHD1 knockout mice. Lower high-density lipoprotein cholesterol levels would also be necessary to support diabetes-associated dyslipidemia.[8]

Despite increased diet-induced weight gain, high-fat diet-fed β-cell-specific PHD3 knockout mice also showed comparable plasma lipid levels as high-fat diet-fed control mice. However, the plasma lipid profile of high-fat diet-fed β-cell-specific PHD3 knockout mice was

associated with *in vivo* glucose intolerance, β-cell dysfunction *ex vivo* (Appendix Figure 28), and elevated fasting blood glucose levels, supporting type 2 diabetes.[3,6] Elevated plasma triglyceride levels combined with type 2 diabetes would also suggest diabetic dyslipidemia.[8] Additional analysis of plasma high-density lipoprotein cholesterol levels and plasma glycated haemoglobin in high-fat diet-fed β-cell-specific PHD3 knockout mice would confirm diabetic dyslipidemia and type 2 diabetes.[3,8]

High-fat diet-fed β-cell-specific PHD2 knockout mice displayed significantly elevated fed plasma fatty acid levels but comparable plasma triglyceride levels to high-fat diet-fed control mice. Again, elevated plasma lipid levels should suggest progression of type 2 diabetes, but high-fat diet-fed β-cell-specific PHD2 knockout mice experienced improved *in vivo* glucose homeostasis and enhanced β-cell function *ex vivo* (objective 2).[8,153,154] The question that remains is: how do β-cell-specific PHD2 knockout mice cope with elevated plasma lipid levels without experiencing obesity-induced metabolic dysfunction?

The lipid profile of β-cell-specific PHD2 knockout mice suggests that β-cell PHD2 regulates plasma free fatty acid levels. Plasma free fatty acid levels are tightly regulated by *de novo* fatty acid synthesis, triglyceride storage and lipolysis.[155] Since food intake was comparable between genotypes on the control or high-fat diet, ingested lipids may not be responsible for changes in fed plasma fatty acid levels in β-cell-specific PHD2 knockout mice.[155,156] Elevated fed plasma fatty acid levels, yet normal fasted plasma fatty acid levels, may suggest elevated basal fatty acid synthesis or lipolysis in the fed state, followed by elevated triglyceride storage in the fasted state.[21,155] Since control diet-fed β-cell-specific PHD2 knockout mice showed normal whole-body lipid oxidation rates, storage of triglycerides may deplete the elevated fed plasma fatty acid levels, generating comparable fasted plasma fatty acid

levels as control diet-fed control mice.[155] Similar fatty acid patterns between high-fat diet-fed control and β-cell-specific PHD2 knockout mice may hint at β-cell PHD2 regulating plasma fatty acid levels during diet-induced obesity in a similar fashion. Differentially regulated fatty acid homeostasis may help β-cell-specific PHD2 knockout mice resist diet-induced obesity. Additional investigation into tissue-specific lipid metabolism is required to support this hypothesis.

4.3 Objective 2: Characterization of diet-induced obesity effects on β-cell-specific PHD2 knockout male islets *ex vivo*

4.3.1 Summary

To understand resistance against obesity-induced metabolic dysfunction in β-cell-specific PHD2 knockout mice, β-cell mass and function of isolated islets were assessed *ex vivo*. Obesity-exposed β-cell-specific PHD2 knockout islets displayed enhanced β-cell mass and glucose-stimulated insulin secretion *ex vivo*, combined with elevated HIF-1α protein expression. Enhanced β-cell mass and function provide insight as to why high-fat diet-fed β-cell-specific PHD2 knockout mice experienced improved glucose homeostasis during diet-induced obesity.

PHD2 may encourage β-cell neogenesis but ultimately interferes with β-cell proliferation during diet-induced obesity. Despite a smaller neogenic islet population, obesity-exposed β-cell-specific PHD2 knockout islets showed elevated β-cell proliferation and an increased large islet population, aligning with enhanced β-cell mass (Figure 26A). Although HIF-1α activity and downstream genes were not assessed, HIF-1α stability in obesity-exposed β-cell-specific PHD2 knockout islets may eventually repress *pancreatic and duodenal homeobox 1 (Pdx1)* expression.[90,157-159] *Pdx1* gene expression is essential for β-cell neogenesis, thus the smaller neogenic islet population in obesity-exposed β-cell-specific PHD2 knockout islets may be

associated with downregulated *Pdx1*.[159-161] β-cell proliferation in obesity-exposed β-cell-specific PHD2 knockout islets may be associated to non-HIF-1α proliferative pathways.[160,162]

β-cell PHD2 may disrupt calcium signalling in the K_{ATP} channel-dependent pathway and anaplerosis in the K_{ATP} channel-independent pathway during diet-induced obesity. Obesity-exposed β-cell-specific PHD2 knockout islets showed normal calcium signalling and anaplerosis, explaining intact glucose-stimulated insulin secretion *ex vivo* during metabolic stress (Figure 26B). High-fat diet-exposed β-cell-specific PHD2 knockout islets did not experience major changes in the expression of genes influencing β-cell glucose metabolism, except for significantly elevated *phosphofructokinase* (*PFKM)* expression. Lipid metabolism was not analyzed in isolated islets, but increased *PFKM* expression in glycolysis may suggest enhanced dihydroxyacetone phosphate shuttling to the glycerolipid/free fatty acid cycle.[163] Promoting the glycerolipid/free fatty acid cycle may favour lipid-derived coupling factor generation, potentially hinting at one mechanism of enhanced glucose-stimulated insulin secretion *ex vivo* from obesity-exposed β-cell-specific PHD2 knockout islets.[163]

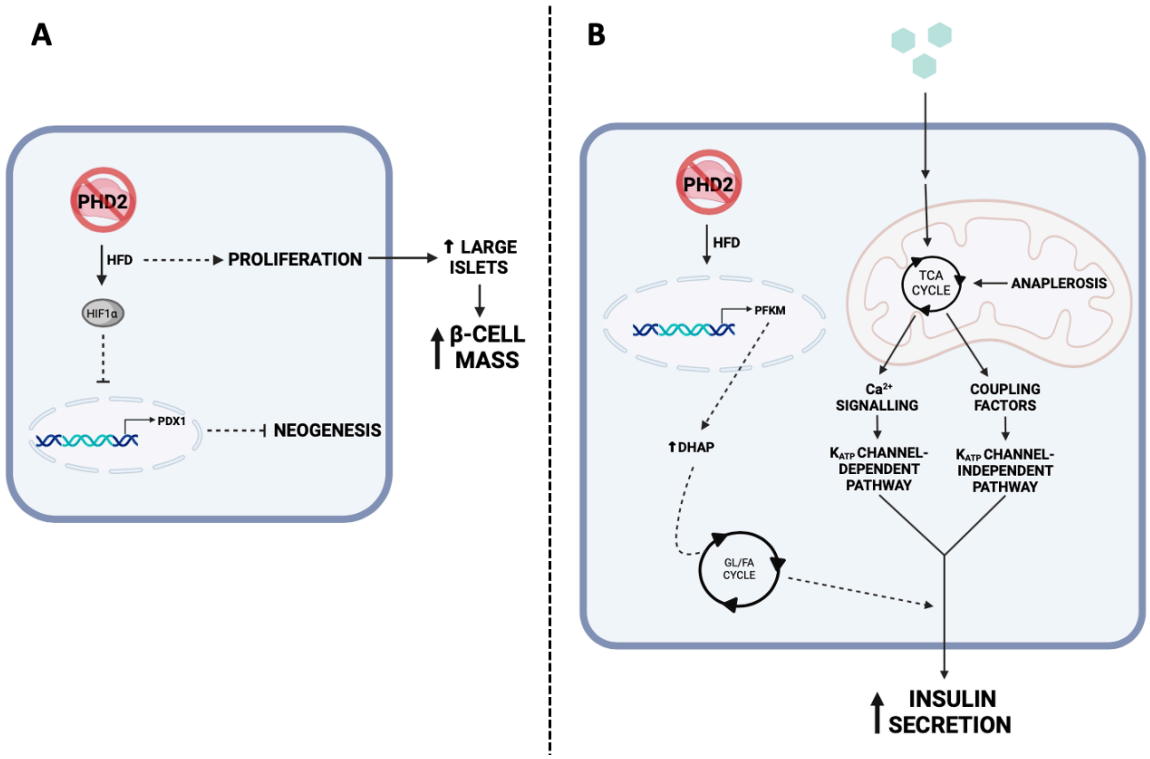

Figure 26. Proposed mechanisms of enhanced (A) β-cell mass and (B) glucose-stimulated insulin secretion in PHD2 knockout β-cells exposed to obesity. Dashed arrows present hypothesized mechanisms since pathways were not analyzed in this project. (A) HIF-1α stability in PHD's absence may prevent *pancreatic and duodenal homeobox 1 (Pdx1)* expression, inhibiting β-cell neogenesis during obesity. More importantly, a lack of PHD2 encourages unknown mechanisms of β-cell proliferation during obesity, supporting the large islet population and enhancing β-cell mass. (B) β-cells lacking PHD2 have intact K_{ATP} channel-dependent and -independent pathways, sustaining glucose-stimulated insulin secretion during metabolic stress. Upregulated *phosphofructokinase (PFKM)* participates in glycolysis and generates dihydroxyacetone phosphate (DHAP), which is shuttled into the glycerolipid/free fatty acid (GL/FA) cycle. An amplified glycerolipid/free fatty acid cycle generates lipid-derived coupling factors for β-cell insulin secretion. HFD, high-fat diet; TCA, tricarboxylic acid cycle. Figure was created with BioRender.com.

4.3.2 PHD2 promotes β-cell neogenesis but ultimately interferes with β-cell proliferation to hinder β-cell mass during diet-induced obesity

Nutrient excess may initially trigger β-cell neogenesis and islet proliferation, promoting an increase in β-cell mass and insulin content to regulate high nutrient levels.[134,164-166] Post-mortem analysis of pancreas from obese humans had 50% greater β-cell mass than healthy humans.[167] However, chronic metabolic stress may eventually impair islet neogenesis, trigger β-cell apoptosis or transdifferentiation into other islet cells, ultimately reducing β-cell mass.[110,165-168] Pancreas from individuals with type 2 diabetes showed a 40% decrease in β-cell mass due to higher apoptosis rates.[167] Insignificantly diminished β-cell mass in obesity-exposed control islets may suggest progression toward type 2 diabetes.[167] Since β-cell apoptosis was decreased in obesity-exposed control islets, an additional two weeks of diet intervention may trigger β-cell apoptosis and significantly reduce β-cell mass.[110]

β-cell-specific PHD2 knockout islets displayed enhanced β-cell mass during diet-induced obesity. β-cell mass itself cannot explain the improved metabolic phenotype of high-fat diet-fed β-cell-specific PHD2 knockout mice because not all β-cells may be functionally mature.[169] However, increased β-cell mass in obesity-exposed β-cell-specific PHD2 knockout pancreas was complimented with enhanced *ex vivo* glucose-stimulated insulin secretion, hinting that the majority of β-cell mass may be functionally mature. Implementing a β-cell maturation biomarker in immunohistochemistry stains could have visualized "functional" β-cell mass.[169] β-cell mass can increase through neogenesis, proliferation or transdifferentiation into β-cells.[170] Higher rates of β-cell proliferation most likely generated a greater population of large islets, enhancing β-cell mass in high-fat diet-exposed β-cell-specific PHD2 knockout islets. A

decrease in the neogenic population and a lack of change in α-cell mass rule out neogenesis or α-cell to β-cell transdifferentiation, respectively.

Various studies suggest condition-specific roles for PHD2 in cell neogenesis and proliferation. PHD2 promoted stress-induced neogenesis, yet inhibited stress-induced cell proliferation.[90,158,161,171,172] The absence of PHD2 encourages HIF-1α stability, which represses the expression of transcription factor *peroxisome proliferator-activated receptor-γ (PPARγ)*.[90,157,158] PPARγ interfered with β-cell proliferation in normal conditions, but a lack of PPARγ prevented compensatory β-cell growth during diet-induced obesity.[162] Enhanced β-cell proliferation and mass in obesity-exposed β-cell-specific PHD2 knockout islets suggests that PHD2 absence may stimulate other proliferative pathways independent of PPARγ during metabolic stress.[160,162] Downstream target genes of PPARγ include *pancreatic and duodenal homeobox 1 (Pdx1)*, a critical gene for β-cell neogenesis.[159-161] Although *Pdx1* expression was not analyzed, obesity-exposed β-cell-specific PHD2 knockout islets displayed elevated HIF-1α expression and a slightly smaller neogenic islet population, hinting at impaired β-cell neogenesis.[90,158] Despite possibly impaired β-cell neogenesis, increased β-cell proliferation in neogenic islets of obesity-exposed β-cell-specific PHD2 knockout mice may maintain the small islet population since small islets are essential for overall islet function.[173,174]

Contradicting results do not provide a lot of certainty as to whether PHD2 affects β-cell proliferation during normal conditions. Our last study showed that PHD2 encouraged β-cell proliferation and apoptosis during normal conditions without affecting β-cell mass.[53] As expected, control diet-exposed β-cell-specific PHD2 knockout islets experienced unaltered β-cell mass, but β-cell proliferation and apoptosis were also unaltered. Unfortunately, our last study did not assess islet sizes, making it difficult to compare proliferative and apoptotic trends

in β-cell-specific PHD2 knockout islets exposed to normal conditions between studies.[53] β-cell-specific PHD2 knockout mice experienced slight differences in diet compositions and age between the two studies, factors that could affect β-cell proliferation.[175-177] Additional observations are required to suggest whether age or slight changes in body weight affected β-cell proliferation during normal conditions in β-cell-specific PHD2 knockout mice.

4.3.3 β-cell PHD2 encourages K_{ATP} channel-dependent and -independent pathway impairments during diet-induced obesity

Under normal conditions, β-cell PHD2 does not affect *ex vivo* glucose-stimulated insulin secretion.[53] In our last study, β-cell-specific PHD2 knockout islets showed normal glucose-stimulated insulin secretion *ex vivo,* accompanied by normal calcium signalling and anaplerosis.[53] Control diet-exposed β-cell-specific PHD2 knockout islets showed similar results. Unaltered *pyruvate carboxylase (PC)*, *pyruvate dehydrogenase kinase (PDK1),* and *lactate dehydrogenase (LDHA)* expression in control diet-exposed β-cell-specific PHD2 knockout islets suggest unaltered pyruvate flux into the TCA cycle, ultimately supporting normal anaplerosis.[42,47-49]

Various diet-induced obesity studies have shown high glucose-impaired insulin secretion.[178-180] Studies used C57Bl/6J mouse models and varied in high-fat diet compositions, but the association between obesity and reduced insulin secretion was similar. Our laboratory highlighted obesity-induced K_{ATP} channel-independent pathway defects from impaired anaplerosis in rat insulin-2 (Ins-2) promoter Cre-recombinase C57Bl/6N mice.[110] Obesity-exposed control islets in this project displayed comparably impaired anaplerosis. However, unlike our last diet-induced obesity study, anaplerosis substrates did not rescue high glucose-impaired insulin secretion.[110] Our previous study implemented a longer diet intervention and a

different Cre-recombinase mouse model.[110] Ins-2 Cre deleter mice as β-cell knockout models can additionally express Cre-recombinase in the central nervous system and the hypothalamus, possibly presenting confounding obesity-altered insulin secretion results.[181,182] Additional controls, such as mice without Ins-2 Cre-recombinase expression, could have confidently eliminated the possibility of confounding results in this study.

Most importantly, β-cell PHD2 may encourage K_{ATP} channel-dependent and -independent pathway impairments during diet-induced obesity. β-cell-specific PHD2 knockout islets displayed improved calcium signalling and avoided anaplerosis defects during metabolic stress, showing enhanced glucose-stimulated insulin secretion *ex vivo*. Shockingly, anaplerosis substrates impaired high-glucose insulin secretion, suggesting that additional observations are required to understand the capacity of anaplerosis in obesity-exposed β-cell-specific PHD2 knockout islets. Previous studies strongly suggest that metabolic stress shifts glucose metabolism away from oxidative phosphorylation in PHD2's absence.[61,90,94,95,163] Thus, obesity-exposed β-cell-specific PHD2 knockout islets may not be able to withstand upregulated TCA flux from anaplerosis substrate treatment.

As explained in section 4.3.2, PHD2's role in regulating HIF-1α may hint at downregulated *peroxisome proliferator-activated receptor-γ (PPARγ)* expression in obesity-exposed β-cell-specific PHD2 knockout islets.[90,157,158] Transcription factor PPARγ also regulates the expression of genes for mitochondrial biogenesis.[183] Although β-cell *PPARγ* expression was not analyzed in obesity-exposed β-cell-specific PHD2 knockout islets, reduced mitochondria biogenesis could partially explain why these islets have impaired insulin secretion in response to treatment with anaplerosis substrates. Further observations, like oxygen consumption during anaplerosis-stimulated insulin secretion and mitochondrial clustering, could be enlightening.

4.3.4 β-cell PHD2 may interfere with glycolysis by restricting *PFKM* expression during diet-induced obesity

One study observed downregulated *glucose transporter 2* (*GLUT2)* and *pyruvate carboxylase* (*PC)* gene expression in a diet-induced obese mouse model.[184] Whereas, type 2 diabetes was associated with reduced *GLUT2* expression and increased *lactate dehydrogenase* (*LDHA)* expression in β-cells.[185-188] Diet-induced obese control mice experienced type 2 diabetes phenotypes such as β-cell dysfunction *ex vivo*, impaired insulin sensitivity and elevated plasma triglyceride levels. However, unaltered β-cell *GLUT2*, *PC,* and *LDHA* expression combined with normal fasting blood glucose levels indicate that the length of diet intervention may not yet support obesity-induced type 2 diabetes in high-fat diet-fed control mice.

In normal conditions, β-cell PHD2 may not affect the expression of genes influencing glucose metabolism.[53] Similar to our last study, control diet-exposed β-cell-specific PHD2 knockout islets showed unaltered *pyruvate kinase (PKM2)* expression.[53] However, during obesity-induced metabolic stress, PHD2 may hinder HIF-1α-upregulated expression of glycolytic genes, preventing the diversion of glucose metabolism away from oxidative phosphorylation.[86,90,189,190] In various studies, PHD2 absence encouraged stress-induced gene expression of *GLUT2, phosphofructokinase (PFKM), pyruvate dehydrogenase (PDK1),* and *lactate dehydrogenase (LDHA).*[86,90,189,190] Obesity-exposed β-cell-specific PHD2 knockout islets only showed elevated *PFKM* expression. Since phosphofructokinase is a rate-limiting enzyme for glycolysis, elevated *PFKM* expression in β-cell-specific PHD2 knockout islets during metabolic stress may hint at upregulated glycolysis and dihydroxyacetone phosphate production.[163] Dihydroxyacetone phosphate is a substrate of glycerol production and a metabolite for the glycerolipid/free fatty acid cycle.[163]

Shuttling glucose carbons to the glycerolipid/free fatty acid cycle mitigates mitochondrial dysfunction and endoplasmic reticulum stress from increased oxidative phosphorylation flux.[191] The glycerolipid/free fatty acid cycle can also generate lipid-derived insulin secretion coupling factors, sustaining the K_{ATP} channel-independent pathway.[163] A recent study showed that promoting the maximum capacity of the glycerolipid/free fatty acid cycle preserved β-cell secretory function in high nutrient conditions.[192] On the other hand, β-cell glycerol release through glycerol-3-phosphate phosphatase encourages glucose detoxification and avoids stress-induced β-cell apoptosis.[193] Glycerol release and the glycerolipid/free fatty acid cycle were not analyzed in this project. However, elevated gene expression in the glycerolipid/free fatty acid cycle and of glycerol-3-phosphate phosphatase could partially explain enhanced *ex vivo* glucose-stimulated insulin secretion in obesity-exposed β-cell-specific PHD2 knockout islets.[192,193]

The literature strongly supports stress-induced upregulated *PDK1* expression in PHD2's absence.[86,90,189,190] Obesity-exposed β-cell-specific PHD2 knockout islets experienced insignificantly elevated *PDK1* expression. Additional samples may be required to exhibit statistical significance. Pyruvate dehydrogenase kinase inhibits pyruvate dehydrogenase, an enzyme that generates acetyl CoA for the TCA cycle.[194] Pyruvate dehydrogenase and pyruvate carboxylase equally shuttle pyruvate into the TCA cycle.[195] However, pyruvate carboxylase contributes more carbons to the TCA cycle.[195] Compensatory upregulation of *PC* may maintain glucose-stimulated insulin secretion during pyruvate dehydrogenase inhibition.[196] Despite intact anaplerosis and glucose-stimulated insulin secretion in high-fat diet-exposed β-cell-specific PHD2 knockout islets, unaltered *PC* and insignificantly elevated *PDK1* expression may support anaplerosis substrate-impaired insulin secretion in these islets.[196]

Pyruvate dehydrogenase-generated acetyl-CoA is also shuttled to the cytoplasm through the citrate/isocitrate carrier shuttle for fatty acid synthesis.[49,194] Insignificantly increased *PDK1* expression may hint at altered fatty acid synthesis in obesity-exposed β-cell-specific PHD2 knockout islets.[49,194] Fortunately, glutamine can also mediate fatty acid synthesis.[197] Studies have associated HIF-1α with upregulated fatty acid synthesis, fatty acid uptake and lipid droplet triglyceride storage.[63,198-202] β-cell fatty acid uptake was associated with high nutrient-induced β-cell dysfunction, while maximal triglyceride storage was associated with preserved β-cell function in high nutrient conditions.[192,203,204] Elevated HIF-1α stability in obesity-exposed β-cell-specific PHD2 knockout islets may be associated with upregulated fatty acid synthesis, enhancing lipid storage to protect β-cell function during metabolic stress.[63,192,198,200-202] Additional experiments investigating lipid homeostasis in obesity-exposed β-cell-specific PHD2 knockout islets are necessary to support this hypothesis.

4.3.5 β-cell PHD2 primarily targets HIF-1α during diet-induced obesity

During metabolic stress, HIF-1α binds to HIF-1β, targeting the hypoxia response element to upregulate gene expression for glycolysis and lipid storage in anaerobic reprogramming.[63-68] HIF-1α is associated with upregulated *PKM2, PFKM, PDK1* and *LDHA* expression, with similar gene expression trends also observed in PHD2 knockout studies.[80,90,205-207] Obesity-exposed β-cell-specific PHD2 knockout islets displayed elevated HIF-1α stability, but only showed upregulated *PFKM* expression. Observing protein-DNA binding of HIF-1α at the hypoxia response element is necessary to suggest HIF-1α-altered *PFKM* gene expression in high-fat diet-exposed β-cell-specific PHD2 knockout islets.

Elevated HIF-1α expression was only observed in obesity-exposed β-cell-specific PHD2 knockout islets, supplementing studies that show PHD2 primarily regulates HIF-1α.[94,95] HIF-

1α expression in obesity-exposed β-cell-specific PHD1 knockout and β-cell-specific PHD3 knockout islets is currently comparable to control diet-exposed control islets (Appendix Figure 31). Additional samples of β-cell-specific PHD1 knockout and β-cell-specific PHD3 knockout islets are required to ensure HIF-1α expression is statistically unaltered. In β-cells lacking PHD2, PHD3 may play a minor role in regulating HIF-1α activity. A study showed that PHD3 fosters co-activator binding to HIF-1α, thus encouraging HIF-1α-regulated transcription.[80] β-cell-specific double PHD knockout or β-cell-specific triple PHD knockout mice could investigate compensational functions of β-cell PHDs. The generation of these models is in progress.

β-cell PHD3 may focus on regulating HIF-2α, inducing genes for vascularization, antioxidants, and insulin signalling during metabolic stress.[62,94,164,208-210] Vasodilation during metabolic stress improves islet microcirculation for glucose sensing.[164,208-210] Regulated β-cell antioxidant levels prevent the elevation of reactive oxygen species, avoiding mitochondria damage in metabolic stress.[210] β-cell insulin signalling is crucial for metabolic stress-induced compensatory β-cell growth, enhancing insulin content to compensate for nutrient excess.[211] Analyzing HIF-2α protein expression in high-fat diet-exposed β-cell-specific PHD3 knockout islets could support a role for β-cell PHD3 in HIF-2α regulation.

Even if obesity-exposed β-cell-specific PHD3 knockout islets experience elevated HIF-2α stability, they showed impaired glucose-stimulated insulin secretion *ex vivo* (Appendix Figure 28). In addition to HIF-2α, β-cell PHD3 may also regulate non-HIF-1α targets involved in insulin secretion.[53,98] It would be interesting to see if high-fat diet-exposed β-cell-specific PHD3 knockout islets display elevated HIF-2α protein expression and increased β-cell mass regardless

of impaired glucose-stimulated insulin secretion.[94,211] β-cell mass analysis of β-cell-specific PHD3 knockout pancreas exposed to diet-induced obesity is still in progress.

Is HIF-1α stability more important than HIF-2α for β-cell function during metabolic stress, or vice versa? HIF-1α is necessary for β-cell function, while both β-cell HIF-1α and HIF-2α are important for regulating *in vivo* glucose homeostasis during diet-induced obesity.[70,210] Only HIF-1α upregulation in β-cells improved obesity-induced metabolic dysfunction, whereas β-cell HIF-2α upregulation had no effect.[70,212] Diet-induced obesity promoted *HIF-1α* and *HIF-2α* expression in β-cells, but *HIF-2α* expression was favoured.[72,208,210] β-cell HIF-1α and HIF-2α may encourage complementary and differential transcriptional regulation to overcome metabolic stress. β-cell HIF-1α primarily regulates glycolysis, whereas β-cell HIF-2α regulates the cellular redox state and vasodilation.[70,164,210] Neither HIF-1α nor HIF-2α showed any effects on β-cell apoptosis during diet-induced obesity.[70,212] Associations between HIF-1α stability and lipid metabolism have yet to be analyzed in β-cells.

Chapter 5: Conclusions

5.1 Final remarks

The well-known K_{ATP} channel-dependent pathway of insulin secretion was discovered 40 years ago.[213] Islet biologists are still trying to define mechanisms for the K_{ATP} channel-independent pathway. The importance of anaplerotic-derived coupling factors for β-cell function may suggest anaplerosis defects in obesity-induced β-cell dysfunction.[42,47-49] Anaplerotically-derived α-ketoglutarate is still being investigated as a coupling factor in β-cell insulin secretion.[47,51-53] β-cell PHD1 and PHD3 may suggest mechanisms of α-ketoglutarate in anaplerotic-sustained insulin secretion through non-HIF-1α proteins during metabolic stress (Figure 27).[52,53] Whereas β-cell PHD2 targets HIF-1α for degradation, suggesting a mechanism of α-ketoglutarate in metabolic stress-impaired insulin secretion (Figure 27).[86,90,95] *In vivo* glucose homeostasis and β-cell function *ex vivo* of diet-induced obese β-cell-specific PHD2 knockout mice confirmed the hypothesis for β-cell PHD2. *In vivo* glucose homeostasis of diet-induced obese β-cell-specific PHD1 knockout and β-cell-specific PHD3 knockout mice only partially support the hypothesis for β-cell PHD1 and PHD3.

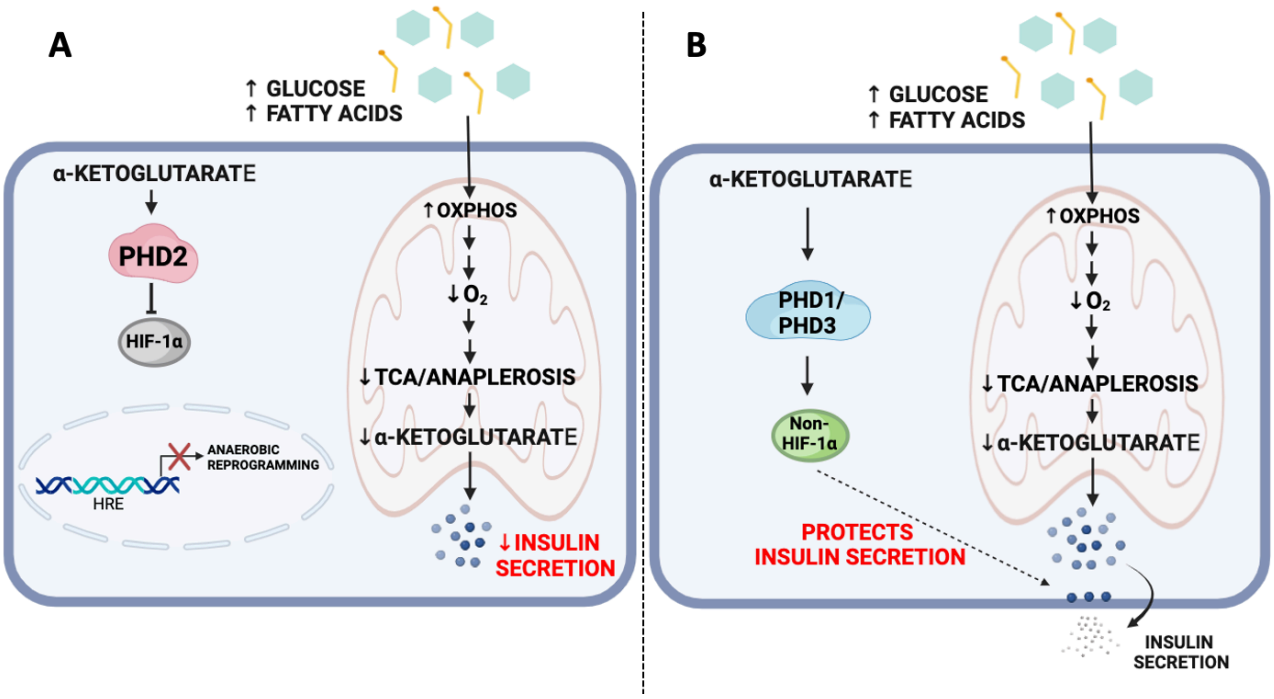

Figure 27. A proposed mechanism of PHD1, PHD2, or PHD3 in β-cell function during diet-induced obesity. (A) PHD1 or PHD3 will target non-HIF-1α proteins, sustaining glucose-linked insulin secretion during obesity-impaired anaplerosis. (B) PHD2 promotes HIF-1α degradation, encouraging defective anaplerosis in obesity-induced metabolic stress. OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid cycle; HRE, hypoxia-responsive element. Figure was created with BioRender.com.

β-cell-specific PHD2 knockout mice resisted against high-fat diet-induced obesity, obesity-induced glucose intolerance and hyperinsulinemia. Despite significant weight gain, elevated fasting blood glucose levels, and hypoinsulinemia, diet-induced obese β-cell-specific PHD3 knockout mice sustained insulin sensitivity and obesity-induced glucose intolerance was not exacerbated. β-cell-specific PHD1 knockout mice did not display any unique obesity-induced metabolic phenotypes. *Ex vivo* characterization of obesity-exposed β-cell-specific PHD2 knockout islets showed enhanced β-cell mass and glucose-stimulated insulin secretion,

clarifying improved metabolic phenotypes of β-cell-specific PHD2 knockout mice during obesity. Despite normal anaplerosis, elevated HIF-1α stability in obesity-exposed β-cell-specific PHD2 knockout islets may encourage alterations in nutrient metabolism to enhance β-cell function during metabolic stress. *Ex vivo* characterization of β-cell mass and function is required to understand obesity-induced metabolic phenotypes of β-cell-specific PHD1 knockout and β-cell-specific PHD3 knockout mice.

5.2 Limitations & future studies

Pitfalls for the project lie in the mouse model, islet physiology and β-cell maturation. In the past, β-cell Cre-recombinase deleter models have caused some challenges for β-cell research. Cre-recombinase itself has impacted glucose homeostasis, therefore skewing data interpretation.[214] Insulin-1 (Ins-1) Cre-recombinase mice are currently the superior β-cell-specific knockout mouse model. A comparison between control and Ins-1 Cre-recombinase mice did not show significant differences in fed-glycemia, glucose tolerance, and body weight in males or females.[107] Unfortunately, Cre-recombinase is still susceptible to DNA methylation silencing.[107,215] Thankfully, our last study showed minimal islet mRNA and protein expression of PHD1, PHD2, or PHD3 in isolated islets, confirming Cre-recombinase mediated β-cell knockout mice.[53] Supplementary data from our recent study did not show significant differences in glucose homeostasis between Ins-1 Cre-recombinase mice, PHD floxed mice (without Ins-1 Cre-recombinase), and C57Bl/6N mice.[53]

Secondly, β-cell function can fluctuate during the day due to circadian-mediated regulation.[108] Since mice are nocturnal, the circadian clock focuses on β-cell growth and repair during the day.[108] Therefore, experiments conducted during the day should consider that mouse β-cells may not be at insulin secretion capacity. *In vivo* nutrient measurements and *ex vivo* islet

isolations were conducted at similar times to ensure reproducible and comparable data. Lastly, anti-insulin antibodies visualized β-cells in immunohistochemistry and immunofluorescent stains. An insulin biomarker cannot differentiate between immature non-functional and mature functional β-cells.[169] Unfortunately, any observations of β-cell characteristics cannot be assumed to mature functional β-cells. An additional late maturation biomarker should have been used during staining protocols to eliminate non-functional β-cells from the analysis.[169]

Future steps in extending this project include *ex vivo* characterization of β-cell mass and function of obesity-exposed β-cell-specific PHD1 knockout and β-cell-specific PHD3 knockout islets. Investigating β-cell function *ex vivo* could supplement the unique obesity-induced metabolic consequences of β-cell-specific PHD3 knockout mice. Other potential areas of future research that stem from this project are summarized in Table 15.

Table 15. Possible areas for future research

Area for investigation	Reasoning	Possible experiments	Goal
Obesity-exposed β-cell-specific PHD2 knockout islets: - β-cell lipid metabolism	HIF-1α may upregulate fatty acid synthesis and lipid storage[63,198-202]	**Lipid storage:** -Measure islet triglyceride content -Immunofluorescent visualization of β-cell lipid droplets **Glycerolipid/free fatty acid cycle:** -Islet transcriptomics and proteomics **Fuel preference:** -Compare glucose and fatty acid oxidation in islets using Seahorse Flux Analyzer	Highlight possible mechanisms of enhanced *ex vivo* glucose-stimulated insulin secretion
Diet-induced obese β-cell-specific PHD knockout female mice: - Glucose homeostasis	Sex differences in estrogen – females respond to obesity differently than males[216]	**Ten-week high-fat diet:** Similar methods as objective 1	Determine sex-specific roles of β-cell PHDs during diet-induced obesity
β-cell-specific double or triple PHD knockout mice: - Glucose homeostasis - β-cell function	PHDs may have compensational functions[80]	**No diet intervention:** Similar methods as objective 1 and objective 2	Further investigate isoform-specific roles of β-cell PHDs

5.3 Significance

β-cell PHDs may have a possible role in moderating the interrelationship between defective anaplerotic-sustained insulin secretion, diet-induced β-cell dysfunction, and type 2 diabetes.[52,53,86-88,90,94,95,97] The long-term goal of deciphering β-cell mechanisms is to find

various targets that regulate β-cell function during stress. Existing literature suggests PHD inhibitors as potential therapeutics for metabolic dysfunction.[87,103,104] Non-specific PHD inhibitors include iron chelators or α-ketoglutarate analogues. Both can interfere with other iron-dependent pathways or α-ketoglutarate enzymes.[52,103-106] It may not be beneficial to inhibit all three PHD isozymes since PHDs play unique roles in β-cells. Our laboratory recently showed that eliminating β-cell PHD1 and PHD3 decreased β-cell mass and impaired *ex vivo* β-cell function in normal conditions.[53] Whereas, this project showed that eliminating β-cell PHD2 during metabolic stress enhanced β-cell mass and function *ex vivo*, improving high-fat diet-induced obesity, glucose tolerance, and insulin sensitivity *in vivo.* Now that we know that only β-cell PHD2 plays a role in obesity-induced metabolic dysfunction, an ideal type 2 diabetes therapeutic should inhibit PHD2 only. Targeted therapeutics could eliminate the downfalls associated with non-specific PHD inhibitors *in vivo*.[52,103-106]

This project also presents opportunities for islet biologists to explore non-HIF-1α targets of β-cell PHD1 and PHD3 to build on β-cell mechanisms. Based on previous literature and the results of this project, β-cell PHD2 primarily targets HIF-1α suggesting β-cell PHD1 and PHD3 target non-HIF-1α proteins.[62,74-82,94,95] Extending PHD mechanisms of β-cell regulation during normal conditions or metabolic stress could provide additional therapeutic targets for sustaining β-cell function in type 2 diabetes. New therapeutics will benefit the oral drug inventory and improve the efficacy of islet transplants. Multiple therapeutic targets that may reverse type 2 diabetes would tackle the diabetes epidemic and alleviate healthcare and personal burdens.[1,4] The global prevalence of type 2 diabetes confirms the urgent need to understand islet biology and β-cell function fully.[1,6]

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Appendix

A.1 A lack of β-cell PHD3 does not affect impaired high glucose-stimulated insulin secretion during diet-induced obesity

β-cell function was briefly assessed in obesity-exposed β-cell-specific PHD3 knockout islets. Isolated islets were subjected to low (2 mM) and high (10 mM) glucose *ex vivo,* and an insulin radioimmunoassay quantified secreted insulin. β-cell-specific PHD3 knockout islets exposed to the control or high-fat diet experienced an insignificant reduction of basal insulin secretion compared to control-diet-exposed control islets (Figure 28). However, control diet-exposed β-cell-specific PHD3 knockout islets showed normal insulin secretion in response to high glucose. Whereas insulin secretion in response to high glucose from obesity-exposed control and β-cell-specific PHD3 knockout islets was significantly lower than control diet-exposed control islets. High glucose insulin secretion was comparable between obesity-exposed control and β-cell-specific PHD3 knockout islets. The analysis is still in progress and requires additional samples to confirm significant and insignificant changes between treatments.

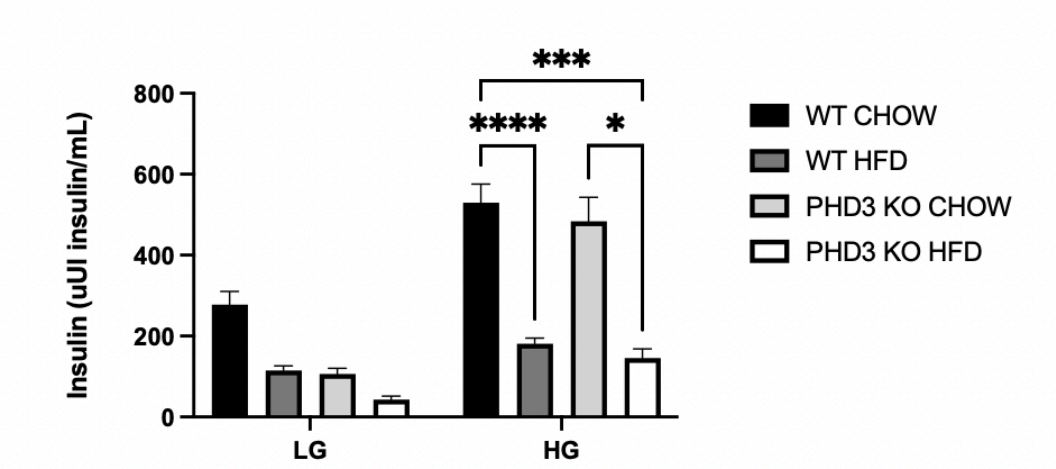

Figure 28. Effects of diet-induced obesity on insulin secretion in control (wild-type) and β-cell-specific PHD3 knockout islets in response to low glucose (LG; 2 mM) and high glucose (HG; 10 mM). Data are mean ± SEM (n = 12-25 per treatment). **P* < 0.05, ****P* < 0.001, *****P* < 0.0001. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.

A.2 β-cell PHD3 absence improves the K_{ATP} channel-independent pathway of glucose-stimulated insulin secretion during diet-induced obesity

Incubating isolated β-cell-specific PHD3 knockout islets with diazoxide (200 μM) and potassium chloride (30 mM) assessed potential defects in the K_{ATP} channel-dependent and -independent pathways. Insulin secretion in response to low glucose, diazoxide and potassium chloride was comparable between all islets, suggesting no defects in calcium signalling (Figure 29). Although insignificant, insulin secretion in response to low glucose, diazoxide and potassium chloride from obesity-exposed β-cell-specific PHD3 knockout islets appears lower than control diet-exposed control islets. The small sample set may not yet show significant differences between islet groups.

β-cell-specific PHD3 knockout islets exposed to the control diet show enhanced insulin secretion in response to high glucose, diazoxide and potassium chloride compared to control diet-exposed control islets (Figure 29). The current sample set may contain outliers, thus additional samples are still required. Most importantly, a lack of β-cell PHD3 may improve defective anaplerosis in diet-induced obesity. Obesity-exposed β-cell-specific PHD3 knockout islets secreted more insulin in response to high glucose, diazoxide and potassium chloride than obesity-exposed control islets. Insulin secretion in response to high glucose, diazoxide and potassium chloride was comparable between control diet-exposed control islets and obesity-exposed β-cell-specific PHD3 knockout islets.

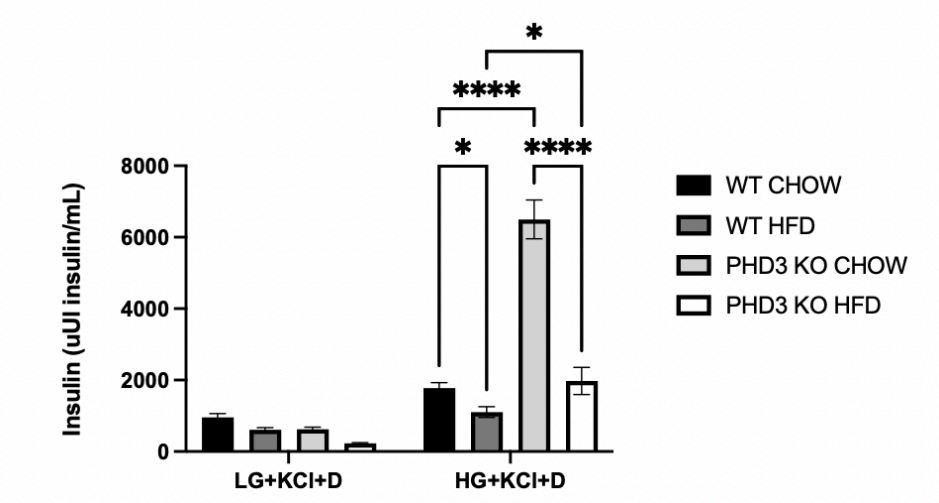

Figure 29. Effects of potassium chloride (KCl; 30 mM) and diazoxide (D; 200 µM) treatments on insulin secretion in control (wild-type) and β-cell-specific PHD3 knockout islets exposed to diet-induced obesity. Data are mean ± SEM (n = 10-20 per treatment). *$P < 0.05$, ****$P <$ 0.0001. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet; LG, low glucose (2 mM); HG, high glucose (10 mM).

A.3 Anaplerosis substrates partially rescue impaired insulin secretion from obesity-exposed β-cells lacking PHD3

Isolated β-cell-specific PHD3 knockout islets were also incubated with anaplerosis substrates; dimethyl malate (10 mM) and dimethyl α-ketoglutarate (10 mM). Anaplerosis substrates can rescue impaired glucose-stimulated insulin secretion if impairments are a result of K_{ATP} channel-independent pathway defects. Anaplerosis substrates did not affect insulin secretion from β-cell-specific PHD3 knockout islets exposed to the control diet. Insulin secretion in response to anaplerosis substrates between control diet-exposed control and β-cell-specific PHD3 knockout islets was similar (Figure 30). Compared to control diet-exposed control islets, basal insulin secretion with anaplerosis substrates was similarly impaired in obesity-exposed control and β-cell-specific PHD3 knockout islets. However, anaplerosis substrates may partially rescue impaired high glucose insulin secretion in obesity-exposed β-cell-specific PHD3 knockout islets. Insulin secretion in response to high glucose and anaplerosis substrates from high-fat diet-exposed β-cell-specific PHD3 knockout islets did not significantly decrease compared to control diet-exposed control islets.

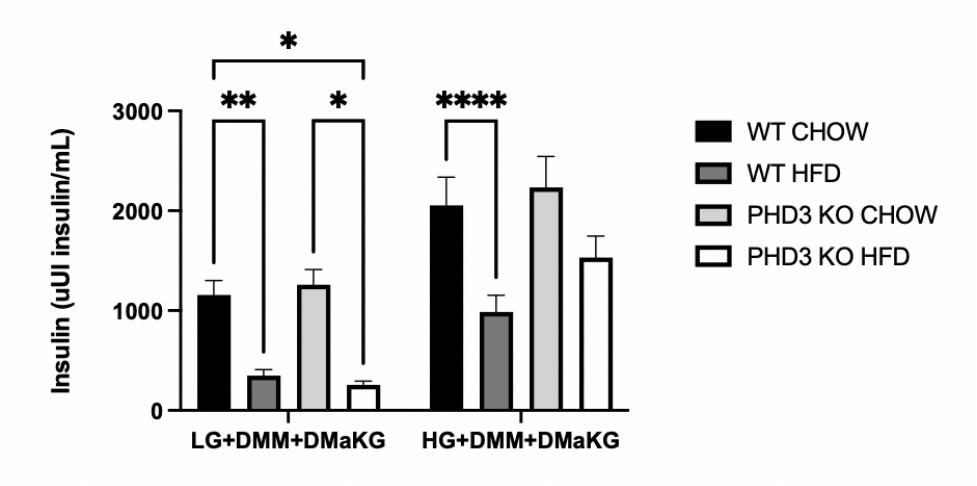

Figure 30. Effects of dimethyl malate (DMM; 10 mM) and dimethyl α-ketoglutarate (DMaKG; 10 mM) treatments on insulin secretion in control (wild-type) and β-cell-specific PHD3 knockout islets exposed to diet-induced obesity. Data are mean ± SEM (n = 10-17 per treatment). **P* < 0.05, ***P* < 0.01, *****P* < 0.0001. WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet; LG, low glucose (2 mM); HG, high glucose (10 mM).

A.4 A lack of PHD1 or PHD3 does not affect β-cell HIF-1α stability during diet-induced obesity

Western blots briefly quantified relative HIF-1α protein expression in β-cell-specific PHD1 knockout or β-cell-specific PHD3 knockout islets exposed to diet-induced obesity. HIF-1α expression was unaltered between all islet groups, suggesting that β-cell PHD1 and PHD3 may preferentially target non-HIF-1α proteins (Figure 31). Additional samples are required to confirm statistical insignificance.

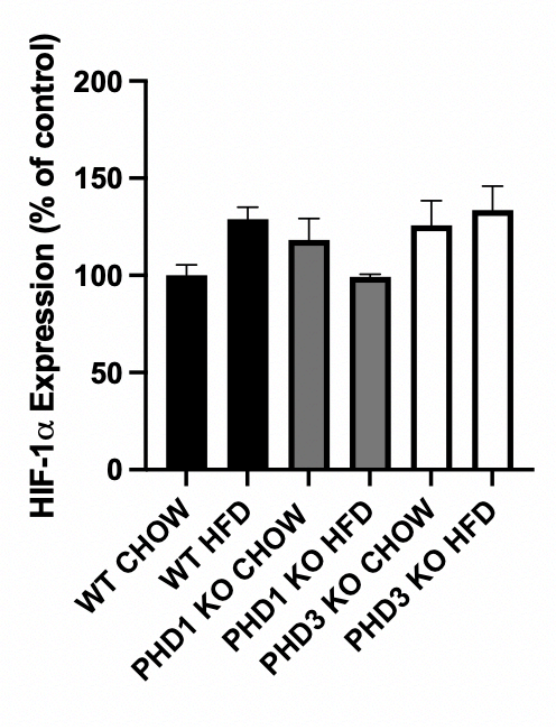

Figure 31. Effects of diet-induced obesity on HIF-1α protein expression in control (wild-type) and β-cell-specific PHD1 or PHD3 knockout islets. HIF-1α protein expression was normalized to the total protein loaded per sample. Data are mean ± SEM (n = 3-5 per treatment). WT, wild-type; KO, knockout; CHOW, control diet; HFD, high-fat diet.