The molecular basis of increased diabetes susceptibility in carriers of the PGC-1α (482Ser) risk allele

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PREFACE

IRCM - Proteomics Discovery Platform

I want to specifically acknowledge the contributions of Denis Faubert who helped develop our mass spectrophotometry protocol and completed the LC-MS/MS procedure and analyzed our data, and to Josée Champagne who helped me with the band excision procedure.

IRCM – Microinjection Services

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ABSTRACT - ENGLISH

A Gly482Ser single nucleotide polymorphism within the coding region of human peroxisome proliferator activated receptor (PPAR)-γ coactivator 1 (PGC-1α) is associated with type 2 diabetes susceptibility (T2D) and other metabolic diseases; however, the functional significance of the Gly482Ser polymorphism has yet to be characterized. By cycloheximide chase, we determined that a serine residue at amino acid position 482 decreases protein stability, compared to the 482Gly counterpart when over expressed in INS-1 β-cells. Replacing this residue with either an alanine residue (phosphorylation-null) or an aspartate residue (phosphomimetic) led to protein stabilization and destabilization, respectively, suggesting that the 482Ser site is phosphorylated. We identified, by *in vitro* kinome screen, that MARK4 phosphorylates a PGC-1α 482Ser-containing peptide, and that inhibition of MARK4 kinase stabilizes the full length PGC-1α 482Ser variant similarly to the 482Gly variant. Accordingly, coactivation of several PGC-1α target genes is impaired by the PGC-1α 482Ser variant. These data provide evidence that there is a phosphorylation event at the 482Ser site and that this post-translational modification regulates stability of the protein. We have also used CRISPR/Cas9 technology to generate mouse lines that possess the analogous mouse PGC-1α 481Gly allele. These mouse lines will allow us to test our *in vitro* findings in an *in vivo* model. Taken together, our data suggests that links between the Gly482Ser SNP variants and metabolic disease may be due to reduced protein stability, and thus, coactivator function. Understanding how the variant affects PGC-1α stability and function under its endogenous promoter may help to design novel therapeutics to treat those who possess this SNP and are at heightened risk of diabetes complications.

ABSTRACT – FRANÇAIS

Le polymorphisme d'un seul nucléotide Gly482Ser dans la région codante du co-activateur du récepteur gamma activé par les proliférateurs de peroxysomes humain (PPAR)-y 1 (PGC-1α) est associé à une prédisposition au diabète de type 2 (DT2) et à d'autres maladies métaboliques. Cependant, la fonction du polymorphisme Gly482Ser demeure inconnue. Après traitement au cycloheximide, nous avons déterminé qu'un résidu sérine à la position 482 (482Ser) diminue la stabilité de la protéine comparativement au résidu 482Gly et ce lorsque surexprimé dans la lignée cellulaire pancréatique INS-1. Le remplacement de ce résidu par un résidu alanine (phosphorylation nulle) ou un résidu aspartate (phosphomimétique) conduit respectivement, suggérant que le site 482Ser est phosphorylé. Nous avons identifié par criblage de kinases in vitro, que MARK4 phosphoryle un peptide contenu dans PGC-1α 482Ser et que l'inhibition de la kinase MARK4 stabilise la variante de PGC-1α 482Ser de manière similaire au variant de 482Gly. En conséquence, la co-activation de plusieurs gènes cibles de PGC-1α est altérée par le variant PGC-1α 482Ser. Ces données prouvent qu'il existe un événement de phosphorylation sur le site 482Ser et que cette modification post-traductionnelle régule la stabilité de la protéine. Nous avons également utilisé la technologie CRISPR / Cas9 pour générer des lignées de souris possédant l'allèle PGC-1α 481Gly analogue de la souris. Ces lignées de souris nous permettront de tester nos résultats in vitro dans un modèle in vivo. Nos données suggèrent que les liens entre les variants du SNP Gly482Ser et la maladie métabolique peuvent être causés par la stabilité réduite de la protéine, et donc, de la fonction de co-activateur. Comprendre comment le variant affecte la stabilité et la fonction de PGC-1α sous son promoteur endogène peut

aider à concevoir de nouveaux agents thérapeutiques pour traiter ceux qui possèdent ce polymorphisme d'un seul nucléotide et qui sont plus à risque de complications du diabète.

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LIST OF ABBREVIATIONS

482Ser	PGC-1α with a serine at amino acid 482
570Ser	PGC-1α with a serine at amino acid 570
ACBP-1	Acyl-CoA binding protein 1
Akt	Protein kinase B
AMPK	5' AMP-activated protein kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
cDNA	Complementary-dioxyribonucleic acid
Clk2	CDC-like kinase 2
CLOCK	clock circadian regulator
CPT1	Carnitine palmitoyltransferase 1
DNA	Dioxyribonucleic acid
ERRα	Estrogen-related receptor alpha
FBS	Fetal bovine serum
FFA	Free fatty acid
Glut4	Glucose transporter type 4
Gly482	PGC-1α with a glycine at amino acid 482
Gpx1	Glutathione Peroxidase 1
GSK-3β	Glycogen synthase kinase 3 beta
GST	Glutathione S-transferase
GST	Glutathione S-transferase
GWAS	Genome wide association study
HDL	High density lipoprotein
HDR	Homology directed repair
HDR	homology directed repair
HFD	High fat diet
HGP	Hepatic glucose production
HOMA-%B	Homeostatic model assessment - steady state β cell function
HOMA-IR	Homeostatic model assessment - insulin resistance
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HTT	Huntingtin
IP	Immunoprecipitation
MARK4	MAP/Microtubule affinity-regulating kinase 4
mRNA	Messenger-ribonucleic acid

mtDNA	Mitochondrial dioxyribonucleic acid
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
Ndufs	NADH-ubiquinone oxidoreductase
NEFA	Non-esterified fatty acid
NHEJ	Non-homologous end joining
ob/ob	obese/obese
OXPHOS	Oxidative phosphorylation
p38 MAPK	p38 mitogen-activated protein kinase
PCR	Polymerase Chain Reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1α	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PGC-1β	peroxisome proliferator-activated receptor gamma, coactivator 1 β
PNPLA3	Patalin-like phospholipase domain-containing protein 3
PPAR	peroxisome proliferator-activated receptor
PPARγ	peroxisome proliferator-activated receptor gamma
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha gene
PPRE	Peroxisome proliferator activated receptor response element
PTM	Post-translational modification
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SCF	SKP1-CUL1 F-box protein
SCF	Skp1-Cullin-F-box ligase
Sdhα	Succinate hehydrogenase complex Flavoprotein subunit A
SDS	Sodium dodecyl sulfate
Ser	Serine
siRNA	Short interfering ribonucleic acids
SNP	Single nucleotide polymorphism
SOD2	Superoxide dismutase 2
ssODN	Single stranded oligodeoxynucleotides
ssODN	single stranded oligodeoxynucleotides

T2D	Type 2 diabetes
Tfam	Transcription factor A, mitochondrial
TG	Triglyceride
Thr	Threonine
WFS1	Wolfram syndrome 1

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GENERAL INTRODUCTION

1 – Type 2 Diabetes

1.1 – Obesity and Type 2 Diabetes

Over the past 25 years, the number of people affected by Type 2 diabetes (T2D) has nearly quadrupled, growing from 108 million in 1980 to 422 million measured in 2014. Not only has the global prevalence of diabetes nearly doubled during this time, but now even younger generations are becoming affected by the disease [1]. It has been suggested that the increase in T2D among children and adolescents may be attributed to higher degrees of obesity, rather than overall diabetes prevalence alone [2].

This climb in obesity is reflective of socioeconomic changes that encourage increased caloric intake and decreased energy expenditure (i.e. lack of exercise). Diabetes often manifests itself in hyperglycemia; typical fasting blood glucose should range between 3.9-5 mmol/l, whereas those with T2D are often greater than 7 mmol/l. Hyperglycemia arises when peripheral tissues become insulin resistant, the liver increases glucose production and/or pancreatic β-cells fail to produce enough insulin to maintain euglycemia. Obesity, a very common condition that often accompanies T2D, is typically associated with chronic, low grade systemic inflammation which is the result of elevated levels of pro-inflammatory cytokines originating from adipose and/or liver tissues [3]. Upon hyperplasia of adipose tissue, inflammatory cytokine and circulating free fatty acid (FFA) levels can rise and start to accumulate in metabolically active tissues, including muscle and liver [4]. Ultimately, this can impair insulin signaling and mitochondrial function in these tissues, which can, in turn, impair glucose uptake and contribute to systemic insulin resistance [4, 5]. A consequence of increased intracellular lipids

accumulating in the liver is increased hepatic glucose production (HGP) [6]. Unwarranted HGP can result in compensatory hyperinsulinemia, which drives insulin resistance and dysregulation of lipid metabolism [6, 7]. In an attempt to maintain normoglycemia, β-cells upregulate insulin secretion [8]. Unfortunately, this high level of insulin secretion is only sustainable for so long before β-cells begin to fail, allowing T2D to progress. Currently, there remains a lack of consensus whether impaired insulin secretion is the consequence of dysfunctional β-cells, the consequence of dysregulated glucose and lipid metabolism in other metabolic tissues, or a combination of the two.

1.2 – The Genetic Basis of Diabetes

Metabolic syndrome is a broad spectrum of diseases that are typically characterized by several risk factors such as: hypertension, abdominal obesity, hyperglycemia, abnormal triglyceride and cholesterol levels and low high-density lipoprotein (HDL) levels. Presence of one, or more of these risk factors often gives rise to increased susceptibility to diseases including, but not limited to, diabetes, fatty liver disease and/or cardiovascular disease. Metabolic disease has long been associated with poor diet, age, health and a sedentary life. Although these lifestyle factors contribute significantly to disease susceptibility and development of the metabolic syndrome, the influence of genetics is widely accepted. In type 2 diabetes (T2D), there is substantial evidence supporting the role of genetic factors in the susceptibility and pathogenesis of the disease as well as individual response to treatment. For example, when a single parent has T2D, there is 40% chance of diabetes in offspring and there is a 70% concordance of incidence amongst monozygotic twins [9] compared to 10% between

dizygotic twins [10]. While these strong associations provide convincing evidence for a strong genetic basis, efforts to identify specific risk genes have proven challenging. There also remains a lack of agreement and certainty pertaining to the majority of genetic risk markers for diabetes and to what degree environment affects the epigenetic regulation of said markers [11].

There exists a lack of agreement and certainty surrounding genetic risk markers for diabetes, with less than 10% of heritability currently explained by findings of Genome Wide Association Studies (GWAS) [3 4]. While GWAS have significantly advanced our knowledge of genetic factors and molecular pathways influencing diabetes risk, major roadblocks exist in our ability to translate this knowledge from bench to bedside. Single nucleotide polymorphisms (SNPs) associated with increased disease risk often lie in uncharacterized genomic regions or have unknown/unpredictable effects on protein function, limiting usefulness of this information for prevention or therapeutic targeting. Furthermore, each SNP in isolation has arguably minor effects on risk and “genetic risk signatures” are difficult to prove experimentally and do not seem to increase our ability to better predict risk over traditional methods [12]. To date, there are at least 80 gene variants associated to diabetes risk [12, 13]. Many of their known or predicted target pathways are linked to pancreatic β-cell biology or insulin sensitivity, yet functional consequences of most variants on these pathways have yet to be elucidated. Even in cases where the predicted gene target is clear, the basic biology of many encoded proteins is still unknown. Although largely unbiased in their design, GWAS studies have limitations. It is widely accepted that diabetes risk is influenced by ethnicity; however, GWAS for this disease are mostly limited to Caucasian populations of European decent

or Asian populations of Japanese or Chinese decent [12]. More recent efforts incorporating multi-ethnic analyses have confirmed many of the previously identified genes and have identified new candidates that appear specific for certain genetic backgrounds [14]. Unfortunately, the goal to identify genes influencing worldwide diabetes risk often leads to variants that are found only in isolated populations being ignored or undervalued. The influence of sex on diabetes risk is also well established, with males being more susceptible than women prior to menopause; however, for the most part, males and females are grouped together in these large-scale genomic analyses.

Further complexity introduced by geographical, cultural, environmental and sociological (i.e. gender) influences undoubtedly have effects on outcomes in these large-scale epidemiological analyses. In the age of personalized medicine, understanding biological roles of gene variants, and how they are influenced by environment, could have more value over standardized lists of “risk-associated” and “protective” genes. While GWAS is a powerful tool, sources of genetic risk (not determined significant by GWAS) have been identified and confirmed through more classical means involving fundamental research, clinical studies and/or linkage analysis. One of the targets identified by these alternative methods is the gene encoding PGC-1α, *Ppargc1a.*

2 – PGC-1α: A versatile transcriptional coactivator of metabolism

2.1 – PGC-1α discovery and characterization

In 1998, an autosomal genomic scan of 363 non-diabetic Pima Indians designed to reveal genetic loci linked to pre-diabetic traits identified gene locus 4p15-q12 to be associated with increased fasting insulin [15], an established hallmark of T2D. This domain of human chromosome 4 contains approximately 44 known genes, including clock circadian regulator (*CLOCK*), huntingtin (*HTT*), Wolfram syndrome 1 (*WFS1*), and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (*PPARGC1A*). Deeper analysis of a subset of genes involved in energy metabolism revealed that a common polymorphism within the coding region of *PPARGC1A* (rs8192678, 1444G>A, Gly482Ser) is associated with acute measures of insulin secretion [16]. This SNP encoding a single nucleotide difference in the mRNA sequence, results in either a serine or a glycine amino acid residue being encoded at the 482 position of human Peroxisome proliferator-activated receptor (PPAR) Gamma Coactivator 1-alpha (PGC-1α).

Around the same time, the PGC-1α protein was identified in a yeast two-hybrid screen searching for PPARγ-interacting factors in brown adipose tissue [17]. Subsequent functional studies revealed that PGC-1α is a powerful transcriptional coactivator of gene pathways controlling mitochondrial biogenesis and oxidative capacity [18]. In the following years, the importance of PGC-1α in controlling multiple aspects of mitochondrial structure and biology became well established, often leading to the protein being referred to as a master regulator of mitochondrial function [19].

PGC-1α is a large, uniquely structured protein with activation and transcription factor binding domains largely concentrated in its N-terminal regions [20], as well as RNA

arginine-serine (RS) rich domains that can interact with RNA processing factors [21]. The coactivator is expressed in many metabolically active tissues including muscle, neurons, adipose tissue, liver, and heart [22]. In addition to PPARγ, PGC-1α enhances the activity of other PPARs [23, 24] and associates with transcription factors required for oxidative metabolism, mitochondrial biogenesis, reactive oxygen species (ROS) homeostasis, insulin and glucagon signalling [22]. PGC-1α is involved in glucose uptake by skeletal muscle through regulation of *Glut4* expression and translocation and impacts metabolism of glucose and lipids in liver by stimulating transcription of several enzymes required for hepatic gluconeogenesis and fatty acid oxidation [25-27]. In mouse muscle, PGC-1α is required to maintain expression of mitochondrial genes, oxidative phosphorylation, and exercise capacity [28, 29]. While in the β-cell and adipose tissue, PGC-1α is dispensable for maintenance of mitochondria, but significantly impacts insulin secretion and insulin sensitivity, respectively [30-32]. These examples highlight the highly tissue-specific nature of PGC-1α and its considerable involvement in whole-body metabolism.

2.2 – The PGC-1α Gly482Ser polymorphism

Since the discovery of PGC-1α's link to both mitochondrial function and diabetes risk, interest in this protein and the effects of genetic variation on metabolic health have grown. While the PGC-1α Gly482Ser SNP has yet to be identified in GWAS for metabolic disease, it does interact with the widely known PPARγ Pro12Ala GWAS-associated risk allele [33] to influence fasting and post-prandial insulin, as well as homeostatic model assessment of insulin resistance (HOMA-IR) [34]. With PPARγ as an established downstream target of PGC-1α, the identification of two genetic variants converging on a

single signaling pathway is compelling evidence for the role of altered PPAR function in diabetes pathogenesis. Accordingly, PPARγ agonists (e.g. TZDs) remain some of the most highly effective insulin sensitizing drugs.

In 2001, a clinical study investigating the influence of seven PGC-1α genetic variants in Danish Caucasians independently identified significant associations between T2D incidence and the PGC-1α Gly482Ser polymorphism, with a 1.34 relative risk of disease for carriers of the 482Ser allele [35]. With growing knowledge of its biological importance in adaptive mitochondrial metabolism, this early clinical evidence linking the PGC-1α SNP to diabetes aligned well with the then emerging hypothesis that mitochondrial dysfunction could underlie diabetes pathology [36].

While a second set of analyses failed to reproduce the association of the PGC-1α Gly482Ser SNP with T2D in Pima Indians, non-diabetic Pima Indians carrying one or two serine-containing alleles have higher insulin secretion 3- and 30-minutes following glucose infusion with equal blood glucose levels [16], suggesting increased insulin resistance. They also had lower FFA, smaller adipocyte size, and higher rates of lipid oxidation even in the presence of clamped insulin [16], further suggesting that the PGC-1α 482Ser allele reduces insulin effectiveness, possibly in a dominant manner. Consistently, obese Caucasians of Italian decent with a PGC-1α 482Ser allele also have decreased insulin sensitivity (by HOMA-IR and increased fasting insulin) independent of age, sex, BMI, HDL-cholesterol or triglycerides and regardless of hetero- or homozygosity at the gene locus [37]. Japanese subjects with one or two PGC-1α 482Ser containing alleles also present increased fasting insulin and insulin resistance following adjustment for BMI, age, and sex in a population of over 900 subjects [38].

Interestingly, incidence of the serine-containing allele alone is not consistently elevated in subjects with diabetes, pointing to the importance of other genetic and environmental factors that influence associated risk. Haplotypes containing the PGC-1α Gly482Ser and other polymorphisms (e.g. a synonymous PGC-1α Thr394Thr variant) are significantly associated with T2D incidence [38, 39] and impaired oral glucose tolerance in offspring of type 2 diabetic subjects [40], suggesting an additive or modifying role for the PGC-1α Gly482Ser polymorphism in regulating insulin sensitivity. Consistent with this hypothesis, a study of 3244 participants aged 20-59 (Netherlands) demonstrates that carriers of the serine allele with a BMI less than 25 kg/m^2 have lower non-fasting blood glucose [41], while the relationship becomes inversed when BMI is >25, with obese carriers of the serine allele having significantly higher fasting glucose. While these observations suggest that the PGC-1α Gly482Ser polymorphism has a more modifying than causative role in diabetes pathogenesis, genetic variation of this locus has significant implications on overall risk assessment, disease severity, and treatment strategy in risk allele carriers.

2.3 – Effects of the Gly482Ser polymorphism on metabolism

Adiposity and BMI

While investigating effects of metformin and lifestyle interventions (i.e. weight loss and increased physical activity) on T2D development in patients with high fasting glucose and impaired glucose tolerance, it was found that the PGC-1α Gly482Ser polymorphism independently associates with increased adiposity [42]. PGC-1α 482Ser allele carriers have elevated baseline HOMA-IR and subcutaneous adiposity, but this association is no longer statistically significant following adjustment for BMI [42]. The PGC-1α 482Ser allele is also associated with elevated body fat mass in Korean children of normal body weight [43] and in overweight, non-diabetic Chinese adults [44]. Moreover, an increase in total body fat mass, hip circumference, BMI, and body fat ratio is observed in PGC-1α 482Ser/482Ser homozygotes of Mexican-Mestizo decent [45]. Excessive weight gain is also associated with the PGC-1α 482Ser polymorphism in males with type 1 diabetes receiving intensive diabetes therapy [46]. On the contrary, there is no association between the SNP and obesity in Danish Caucasians [47] or in a population of Asian Indians [48]. In Pima Indians, fasting nonesterified fatty acids (NEFA) levels are lower for PGC-1α 482Ser carriers [16] suggesting that adipose tissue lipolysis might be impaired and obese Caucasian carriers of the PGC-1α 482Ser allele have reduced clearance of NEFA following oral glucose challenge [49]. Elevated post-prandial NEFA can inhibit insulin signaling and glucose disposal and promote lipoprotein secretion from liver, which both contribute to T2D pathogenesis.

Taken together, available data suggest that the PGC-1α Gly482Ser polymorphism negatively impacts adipose tissue biology or function in certain populations; and

specifically, the 482Ser-containing allele confers higher risk of obesity. Thus, links of this SNP to increased T2D susceptibility may simply be due to influence on adiposity. This may also explain why carriers of the PGC-1α 482Ser allele seem to benefit more from interventions aimed at weight loss, including caloric restriction [50], bariatric surgery [51], and acarbose treatment [52], than 482Gly allele carriers. Serine-allele carriers are less responsive to the beneficial effects of rosiglitazone on post-prandial insulin, HDL, blood glucose (fasting and post-prandial), and HOMA-IR [53].

Insulin Secretion

PGC-1α levels in the endocrine pancreas may directly regulate β-cell function. High levels of PGC-1α are detected in islets of Zucker diabetic fatty rats and *ob*/*ob* mice, two animal models of T2D [54]. However, overexpression of PGC-1α in mouse islets within physiological levels *in vivo* does not impair β-cell function in adult mice [55] and β-cell specific knockout of both PGC-1α and PGC-1β *diminishes* glucose stimulated insulin secretion [31]. These data in mice are consistent with data in humans, suggesting that PGC-1α gene expression in the pancreatic islets directly correlates with insulin secretion [56]. However, despite effects on glucose-stimulated insulin secretion, decreased PGC-1α in β-cells does not impact whole body glucose tolerance in lean or obese mice [31]. Therefore, low PGC-1α in β-cells may not play a predominant role in increased diabetes risk associated with gene variation, but likely is additive or synergistic with effects on adiposity and insulin sensitivity in other tissues. Functional studies addressing the impact of the PGC-1α Gly482Ser polymorphism in β-cells will be discussed later, in Chapter 1.

Liver Metabolism

In addition to T2D, the PGC-1α Gly482Ser polymorphism is associated with non-alcoholic fatty liver disease (NAFLD). Fatty liver disease is a significant risk factor for diabetes, increasing chances of developing T2D by two to three-fold [57]. With recent interest in metabolic liver disease as a predisposing factor to diabetes, NAFLD risk genes may also be of interest in predicting diabetes risk. The PGC-1α Gly482Ser polymorphism increases risk of NAFLD in obese Taiwanese children (OR 1.74), compared to controls homozygous for the PGC-1α 482Gly variant [58]. In obese Taiwanese adults, having at least one 482Ser-containing allele is an independent risk factor for non-alcoholic steatohepatitis (NASH, a disease associated with higher steatosis and ballooning degeneration of liver cells) and carries an additive effect of the PGC-1α Gly482Ser with the PNPLA3 rs738409 SNP on NASH incidence [59]. In contrast, newly diagnosed German subjects with type 1 diabetes homozygous for the PGC-1α Gly482 allele have lower hepatic adenosine triphosphate, suggesting impaired mitochondrial metabolism [60]. These opposing findings provide further evidence that environmental and/or lifestyle factors influence SNP impact on metabolic health and that tissue-specific effects that may be additive.

2.4 – The impact of sex and ethnicity on the PGC-1α Gly482Ser polymorphism and diabetes

Sex-specific effects

Accumulating evidence suggests PGC-1α expression and activity may be significantly influenced by sex. The PGC-1α 482Ser allele is associated with reduced risk of diastolic dysfunction (OR 0.13-0.19) in Swedish men [61], but no effect is observed in women of either study. In contrast, a significant *increase* in arterial hypertension prevalence is reported for only male French Caucasians with a PGC-1α 482Ser allele [62]. In Japanese males with type 2 diabetes, having one or two PGC-1α 482Ser alleles lowers circulating adiponectin (an anti-diabetic adipokine), while PGC-1α genotype has no influence on adiponectin concentrations in females. In contrast, only PGC-1α 482Ser/482Ser Japanese women trended toward having higher fasting plasma glucose [63]. While seemingly contradictory results are presented in these studies (protective versus detrimental effects of variant and varying influences of sex), it is interesting to note that that the French and Japanese subjects also had T2D, suggesting that additional lifestyle and genetic factors play a modifying role in PGC-1α activity. In support of this, sedentary Swedish males carrying a PGC-1α 482Ser allele have a greater risk of developing obesity with age; but again, no association with obesity, age or activity is reported for the Swedish females [64].

There is compelling evidence that sex has a significant effect on the outcome of this SNP on metabolic disease; often having more significant outcomes in one sex over another that are significantly influenced by environmental factors such as activity, diet, and age. While clear metabolic differences in clinical studies suggest sex is an important

factor influencing PGC-1α activity, very few studies considering sex as a variable attempt to explain these sexually dimorphic metabolic phenotypes.

Ethnicity as a risk factor

The PGC-1α protein, and the domain containing the GlySer482 SNP in particular, are highly conserved across species [65]. Interestingly, while the 482Ser-containing allele is arguably the "minor allele" in many human populations [65], most other vertebrates only have a serine residue in this position. These limited data suggest the PGC-1α 482Gly variant may have appeared later in evolution and became enriched in humans due to selective pressure, or it was selected against in other species. A higher than expected prevalence of PGC-1α 482Gly/482Gly might suggest that this allele provides some selective advantage to certain groups. In support of this theory, the PGC-1α 482Gly variant is found over represented in elite endurance athletes of Russian [66], German [67], Turkish [68] and Israeli [69] decent. However, if the PGC-1α 482Ser variant is truly a strong risk allele for metabolic disease in humans, why does this variant remain so prevalent in humans and why is it not selected against in other species?

A plausible explanation might be that the onset of metabolic disease often occurs years after reproductive age, providing no resistance to the allele being passed onto future progeny. However, if the SNP has no influence prior to disease onset, one would expect classical mendelian ratios of SNP prevalence, which is not the case in most populations evaluated. The majority of published clinical studies on this SNP were performed on subjects of European descent, where the prevalence of the PGC-1α 482Gly/482Gly phenotype averages around 50%, with 482Ser/482Ser homozygotes

detected at rates of 10-15% [70]. Interestingly, the prevalence of each variant seems to vary greatly depending on geographical location [71]. Sampling data suggest that PGC-1α 482Ser “risk” allele prevalence can reach >80% in some Polynesian island nations of the South Pacific, while many areas within Africa, Papua New Guinea and Indonesia have frequencies less than 3% (based on data from the Human Genome Diversity Cell Line Panel) [71]. These large variant frequency differences between populations led to a theory that the 482Ser allele may be considered a “thrifty gene, providing advantages to species that depend on fat storage capacity for survival (e.g. during periods of famine or hibernation in rodents) [71]. However, in times of relative food abundance, having the serine variant may contribute to obesity and increased metabolic disease prevalence.

Recently, this hypothesis was challenged, as statistical testing does not find evidence for departure from natural evolution for this locus in a range of Polynesian, Asian, European or African populations [72]. Additional evidence against the thrifty gene hypothesis comes from the fact that relative risk of T2D associated with the PGC-1α 482Ser allele is not the same across populations of humans that have recently become exposed to relatively similar diets and lifestyles. In Caucasian populations, although significant, the risk of T2D is only modestly increased (e.g. odds ratio ranging between 1.1 – 1.8), while odds ratios for T2D risk increase greatly in PGC-1α 482Ser carrying subjects of Northern Indian (OR 2.04-3.19), Iranian (OR 9.0), Chinese (OR 1.64-1.85) and Tunisian decent (OR 1.17–2.98) [73-77]. Thus, it is plausible that differences in disease risk between ethnicities linked to this polymorphism are due to additive or synergistic effects with other genetic modifiers or environmental factors specific to

geographical region. This is supported by evidence that haplotypes containing both the Gly482Ser of PGC-1α and the Pro12Ala of PPARγ have a greater risk of diabetes [52]

3 – The effect of polymorphism on PGC-1α structure and function

3.1 - PGC-1α Stability

It has been shown in previous studies that there is a 5-fold increase in PGC-1α protein in nuclear extracts following a one hour treatment with the proteasomal inhibitor MG132 [78]. Given this data, we hypothesize that the PGC-1α 482Ser polymorphism increases T2D (and other metabolic disease) susceptibility in some populations due to impacts on protein stability. Because lower PGC-1α expression is commonly associated with poor health outcomes [27], decreased protein stability in carriers of the PGC-1α 482Ser allele may result in impaired coactivator activity, therefore increasing T2D and metabolic syndrome risk.

In vitro analyses show no differences in the ability of the PGC-1α 482Gly and 482Ser variants to induce adiponectin promoter activity following ectopic expression in HeLa cells to equal protein levels [63]. Equivalent coactivator activity for the variants was also demonstrated on the ACBP-1C promoter in HepG2 cells [79]. These data suggest that the polymorphism may not have any direct effects on PGC-1α coactivator function. However, coactivator activity for the PGC-1α 482Ser variant is reduced on the PEPCK and CPT1α promoters in HepG2 cells [80], yet increased compared to the PGC-1α 482Gly variant on the TFAM promoter in HeLa cells [81]. Thus, cell environment as well as the gene target may be determining factors for observed differences in variant activity.

In agreement with these findings, we have shown that the half-life of the PGC-1α Ser482 variant is shorter than the 482Gly variant in cultured liver cells, corresponding with reduced coactivator activity on target genes involved in ROS detoxification [82]. A difference in protein stability was also found when the two variants were overexpressed in INS-1 β-cells (unpublished data). It is possible that the differences in protein stability could be attributed, at least in part, to differences in mRNA expression, as has been shown in muscle [83] and islets [30] of serine allele carriers with T2D. These studies provided the first piece of evidence suggesting that the PGC-1α Gly482Ser polymorphism destabilizes PGC-1α mRNA and protein. Yet despite clear associations of 482Ser with low PGC-1α expression, it is not clear how this occurs or whether the polymorphism also affects coactivator activity in β-cells.

3.2 – Post-translational modifications of PGC-1α

PGC-1α transcription, protein stability and activity are very precisely controlled. PGC-1α expression levels change rapidly in response to physiological stressors or increased energy demand (e.g. cold, exercise, fasting, inflammation) [84], and the protein is quickly degraded [78]. Influences of external stimuli on PGC-1α activity, mechanisms controlling mRNA transcription and protein stability are also tissue-specific [19, 85-87]. For example, PGC-1α expression in cardiomyocytes is constitutively elevated to support the high level of β-oxidation [22]; whereas in liver tissue, PGC-1α is basally much lower, but can be rapidly induced in response to fasting to drive gluconeogenesis [27].

An additional level of complexity to PGC-1α regulation is added by extensive post-translational modification (PTMs). Phosphorylation, acetylation, ubiquitination and methylation all affect PGC-1α stability and activity [19]. For example, phosphorylation by Akt at the 570Ser residue enhances PGC-1α activity, whereas Clk2 phosphorylation of serine residues in the SR region (spanning residues 564-635) decreases coactivator activity [19]. Coactivator activity can also be increased by stabilizing the protein itself. For instance, p38 MAPK phosphorylates PGC-1α at Ser/Thr residues and increases the half-life of the protein by approximately 3 times (6.27 h vs. 2.38 h) [88]. Conversely, there are other cases where phosphorylation has the opposite effect. There are phosphorylation events that generate phosphodegrons, defined as short motifs that once phosphorylated, serve as markers for protein ubiquitination [89]. An example of this is the phosphorylation of PGC-1α at Thr295 by glycogen synthase kinase 3β (GSK-3β), which serves as a marker for ubiquitination by Skp1-Cullin-F-box ligase (SCF^{Cdc4}) and leads to subsequent proteosomal degradation [90, 91]. To this end, it is shown that following 1 h treatment with the proteasomal inhibitor MG132, there is a 5-fold increase in PGC-1α protein in the nucleus [78]. This shows that PGC-1α levels and/or activity are regulated by the ubiquitin proteasome pathway. With these data in mind, we therefore propose the model that phosphorylation of the PGC-1α 482Ser residue leads to decreased protein stability and thus, impairs coactivator function.

Chapter 1: The Gly482Ser polymorphism affects PGC-1α stability and function in INS-1 β-cells

It is widely accepted that decreases in transcription of metabolic and mitochondrial genes contribute to decreased oxidative phosphorylation, decreased lipid oxidation and increased ROS presence within tissues [92]. A common consequence of such dysregulation is obesity, progressive insulin resistance and ultimately T2D [93]. Muscle and liver tissues, as well as pancreatic islets from patients with T2D have low *PPARGC1A* gene expression that is associated with insulin resistance and glucose intolerance [30, 94, 95]. Interestingly, *PPARGC1A* gene expression is regulated by both insulin secretion-enhancing and -impairing factors in the pancreas. *PPARGC1A* expression is regulated by GLP-1 and cAMP which enhance insulin secretion, as well as glucocorteroids, obesity, and glucolipotoxicity, which are known to impair glucose-stimulated insulin secretion [30, 31, 96-98]. Over expression of PGC-1α does not impair insulin secretion in adult mice [55] and β-cell specific knockout of both PGC-1α and PGC-1β diminishes glucose stimulated insulin secretion [31]. Moreover, siRNA knockdown of PGC-1α in human islets reduces the insulin stimulation index, thereby demonstrating a causal link between PGC-1α levels and β-cell function [30]. These data in mice are consistent with data in humans, suggesting that PGC-1α gene expression in the pancreatic islets directly correlates with β-cell function [56], and that decreases in PGC-1α may be associated with increased T2D susceptibility. One mechanism by which PGC-1α expression can be reduced is through primary sequence polymorphisms, such as the Gly482Ser polymorphism.

Upon further analyzing the relationship between the PGC-1α Gly482Ser polymorphism and important metabolic parameters, a relationship between the polymorphism and β-cell function becomes apparent [16, 30, 37]. It is shown that there is

a 90% reduction in *PPARGC1A* gene expression in islets of patients with T2D compared to those without diabetes, and that isolated islets from carriers of the PGC-1α 482Ser allele also have significantly lower gene expression and insulin secretion compared to isolated islets from those who are homozygous for the 482Gly allele [30]. In contrast, other studies show that non-diabetic PGC-1α 482Ser carriers have improved β-cell function [37]. However, it is not clear whether this is a primary effect of the polymorphism improving β-cell function or increased insulin secretion secondary peripheral insulin resistance.

Although a majority of the above-mentioned studies were conducted using mouse and human models, our lab has produced similar findings in the INS-1 rat insulinoma β-cell line; we observed that cAMP similarly induced PGC-1α and that PGC-1α knockdown by shRNA also impaired expression of genes involved in oxidative phosphorylation in INS-1 β-cells [31]. Since we can reproduce, in INS-1 β-cells, the original finding that the human PGC-1α 482Ser decreases PGC-1α protein and we are interested in the role of PGC-1α in β-cells, we chose to pursue our studies using the INS-1 cell line.

CHAPTER 1 - OBJECTIVES

Many studies show that there is a correlation between the PGC-1α Gly482Ser polymorphism and increased type 2 diabetes susceptibility [35, 37, 38]. However, little is known about the polymorphism and studies focusing on the functional significance of the Gly482Ser polymorphism are sparse, particularly concerning its function within the β-cell. Based on previous unpublished findings by another student in our lab suggesting that the PGC-1α 482Ser variant is less stable than the 482Gly variant in INS-1 β-cells [99], we hypothesized that a phosphorylation event at the 482Ser could be responsible for PGC-1α destabilization. The objectives of this study were to 1) ascertain whether the PGC-1α 482Ser residue is a phospho-site, 2) if so, elucidate the kinase responsible for the phosphorylation and 3) assess any effects of the PGC-1α Gly482Ser polymorphism on coactivator activity.

CHAPTER 1 – METHODS

Cell culture and treatments*:* INS-1 β-cells were cultured in RPMI 1640 (Wisent) medium with 10% Heat Inactivated FBS (Wisent), 1% penicillin and streptomycin (Wisent), and 1x supplement (10 mM HEPES, 1 mM sodium pyruvate, 50 μM β- mercaptoethanol). Cells were plated in 6 well plates the night before experiments. Cells were transfected when 80-90% confluent, with Lipofectamine 2000 (2.5 μl per well) and 1 μg of DNA, with OPTIMEM (or Extreme-DMEM) reduced serum media. After 4 h, the transfection media was replaced with normal INS-1 media. Cells were left for 24 h (as specified) at 37°C with 5% CO_2. Cycloheximide chase experiments were done with 50 mg/ml of cycloheximide (Sigma-Aldrich), MG132 (Sigma-Aldrich) used at a concentration of 10 mg/ml, actinomycin D (Sigma-Aldrich) at 1 μg/ml and MRT67307 (Sigma-Aldrich) at 1 μg/ml for the indicated periods of time.

Western Blot: Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease cocktail inhibitor (Calbiochem), and phosphatase inhibitor (Roche). Protein concentration was estimated using DC assay on BSA standards. Equal amounts of protein (40 μg) were loaded onto 10-12% polyacrylamide gels and were transferred onto polyvinylidene diflouride (PVDF) membranes (GE healthcare). PGC-1α was detected using anti-PGC-1α mouse mAb antibody (1:1000, Millipore) diluted in 2% milk. Membranes were incubated overnight at 4°C. Anti-mouse IgG antibody conjugated to HRP (1:5000, Biorad) was used to label protein/1° antibody complexes. HRP signal detection was performed using the ECL detection system.

Immunoprecipitation & Mass Spectrophotometry: INS-1 β-cells that were cultured in RPMI 1640 (as mentioned above) were transfected at 70% confluency with human FLAG-PGC-1α Gly482 and 482Ser –expressing plasmids. Media was replaced after 4 h and cells were harvested after 24 h. Cells were lysed and proteins were quantified as described above. 1000 μg of protein was incubated with 1) IgG-conjugated to anti-IgG agarose beads to minimize non-specific interactions for the immunoprecipitation, and then 2) with anti-FLAG M2 monoclonal antibody (1 mg/ml, Sigma) conjugated to anti-IgG agarose beads (Sigma). The proteins were immunoprecipitated overnight at 4°C on a rotator and then washed 4 times using wash buffer (1% Triton X-100, 20 mM Tris-HCl, 150 mM NaCl, 0.1% SDS). The immunoprecipitated protein was eluted from the beads using 5X sample buffer (1 M Tris pH 6.8, 20% SDS, Glycerol, 2.5% Bromophenol blue, 1M DTT) and loaded into a 10% SDS-PAGE gel that was 0.75 mm thick.

Silver Staining: The gel containing the resolved immunoprecipitated human FLAG- PGC-1α Gly482 and Ser482 proteins was incubated in fixing solution (50% methanol, 10% acetic acid in water), rinsed in waster and then incubated in reducing solution (0.2 g/L sodium thiosulfate in water). Next, the gel was incubated in silver nitrate (2 g/L) and then developed in developing solution (30 g/L sodium carbonate, formaldehyde, 10 mg/L sodium thiosulfate) until desired intensity. The reaction was terminated by addition of 1% acetic acid solution. Bands that appeared at the expected height for PGC-1α were excised, digested by Trypsin or LysC enzymes and subsequently analyzed by LC-MS/MS.

Adenovirus production: Plasmids expressing the human FLAG-PGC-1α Gly482 and 482Ser were enzymatically digested and sub-cloned into the pShuttle-CMV plasmid (Agilent). pShuttle-CMV plasmids containing correct FLAG-PGC-1α Gly482 and 482Ser sequences were linearized and transformed into BJ5183-AD-1 bacteria using electroporation. Plasmids from single colonies expressing the recombinant FLAG-PGC-1α Gly482 and 482Ser plasmids were transformed into DH5α bacterial cells and purified using the Maxiprep kit (QIAGEN). Adenivurs producing 293-Ad cells were each transfected with the recombinant FLAG-PGC-1α Gly482 or 482Ser plasmids. The cells were amplified until 100% of the 293-Ad cells were infected (measured by GFP positive cells). Virus was purified from the cells using $CsCl_2$ density gradients, and subsequently stored at -80°C.

Single-step affinity purification: At 70% confluency INS-1 cells were infected with FLAG-hPGC-1α 482Gly and Ser482 constructs using Adenovirus. Media was replaced after 24 h and cells were harvested after 48 h. Cells were pelleted at 4°C and then loaded into a syringe so they could be prepared for cryolysis using a PM-100 grinder. 10-15 g of cell powder was added to extraction buffer and subsequently, the mixture was added to magnetic beads (Dynabeads by ThermoFisher Scientific) conjugated to anti-FLAG M2 monoclonal antibody. The proteins were immunoprecipitated for 30 minutes at 4°C on a rotator, and then washed 3x in fresh extraction buffer without Tween. FLAG-PGC-1α proteins were digested on the beads using 500 ng of LysC for 4 hours and overnight, both at 37°C. Phosphopeptides were enriched using ZipTips (EMD Millipore).

Kinome Screen: 2 sets of annealed oligos, encoding the protein: N - QAVFDDEADKT[G/S]ELRDSDFSNEQ – C, were cloned into the GST-encoding pgex 4t1 plasmid. Single BL-21 bacterial colonies containing the GST-fusion were incubated overnight at 37°C in LB broth containing 100 μg/ml ampicillin (Amp). Next, 500 ml of LB-Amp with 0.1 mM IPTG was added to the flask to induce GST-peptide expression. After 4 hours, the cells were pelleted by centrifugation at 400 rpm and stored at -80°C. The pellets were resuspended in 1X PBS and then sonicated until the mixture was no longer viscous. The sample was centrifuged and the supernatant retained for fusion peptide purification using the GST Fusion Protein Purification Kit (Genscript) as per manufacturers instruction in the TM0185 manual. Crude, input, wash and sample aliquots were retained and assessed using coommassie staining of a 10% SDS-PAGE gel.

The GST-tagged 482Ser peptides were separately incubated with 420 different purified human kinases and γ^{32}P-labelled ATP for 30 minutes. Autoradiography was used to detect the phosphorylated GST-482Ser peptides that were run on SDS-PAGE gels. MBP, a peptide that is readily phosphorylated by kinases was used as an internal control to ensure kinase activity and substrate specificity.

RNA isolation and cDNA synthesis: Total RNA from INS-1 cells was extracted using Trizol reagent (Invitrogen) as indicated by the manufacturer's protocol. For cDNA synthesis, 1 μg of RNA was incubated with 1 U/ml DNAse1 at 37°C for 15 minutes followed by 15 minutes at 65°C for inactivation of DNAse1. Total RNA in a total volume of 20 μl was reverse transcribed with 50 U Multiscribe reverse transcriptase (Applied Biosystems) and 20 U RNAse inhibitor (Biobasic). cDNA was synthesized at 25°C for 10 minutes, 37°C for

120 minutes and 85°C for 5 minutes. 80 μl of water (1:5 dilution) was added to each sample and was stored at -20°C. cDNA samples were further assessed with qPCR.

Quantitative Real-Time PCR: The gene of interest was amplified from cDNA; hypoxanthine-guanine phosphoribosyl transferase (HPRT) and/or 18S RNA genes were used as controls. 5 μl reactions were set up in a 384 well plate, using Power SYBR green PCR Master Mix (Life Technologies). cDNA amplified by 40 cycles of 15 seconds at 95°C and 1 min of 60°C using the Viia 7 (Life Technologies). Data was normalized to the endogenous control and relative mRNA expression was determined using the ΔΔCt method.

Statistics: Graphpad Prism was used for graphing results and calculating statistics. PGC-1α protein comparison statistics in Figure 2A were calculated using one-way ANOVA and all protein half-life estimations were calculated using non-linear regression. Time-course gene and protein expression statistics were calculated using 2-way ANOVA.

CHAPTER 1 - RESULTS

PPARGC1A mRNA stability is not affected by the polymorphism

We first examined whether the difference in *PPARGC1A* gene expression (482Gly -encoding homozygotes vs. 482Ser-encoding hetero- and homozygotes) that is reported by Ling et al. (2008) could be attributed to differences in mRNA stability [30]. We treated INS-1 β-cells over expressing either human 482Gly or 482Ser PGC-1α under control of the constitutive CMV promoter with a potent transcriptional inhibitor actinomycin D, and then assessed PGC-1α mRNA expression by qPCR by comparison of ct values to a standard curve. We observed that the half-lives of the 482Gly and Ser482 PGC-1α constructs (approximately 9 h and 6 h, respectively) were comparable to the reported PGC-1α (not specific for the Gly482Ser polymorphism) half-life in ES cells (ranges 5 h to 10 h) [100]. Although the half-life of the PGC-1α 482Ser-encoding variant was slightly less than that of the 482Gly-encoding variant, the difference between the two is not statistically significant (Figure 1A). *Myc* gene expression was used as a positive control for actinomycin D activity; indeed, the estimated half-life of the *Myc* gene was also similar to reported values in ES cells, which is approximately 1 h (Figure 1B) [100]. These findings suggest that the difference in PGC-1α protein stability observed in INS1 β-cells [99] was not caused by differences at the transcriptional level.

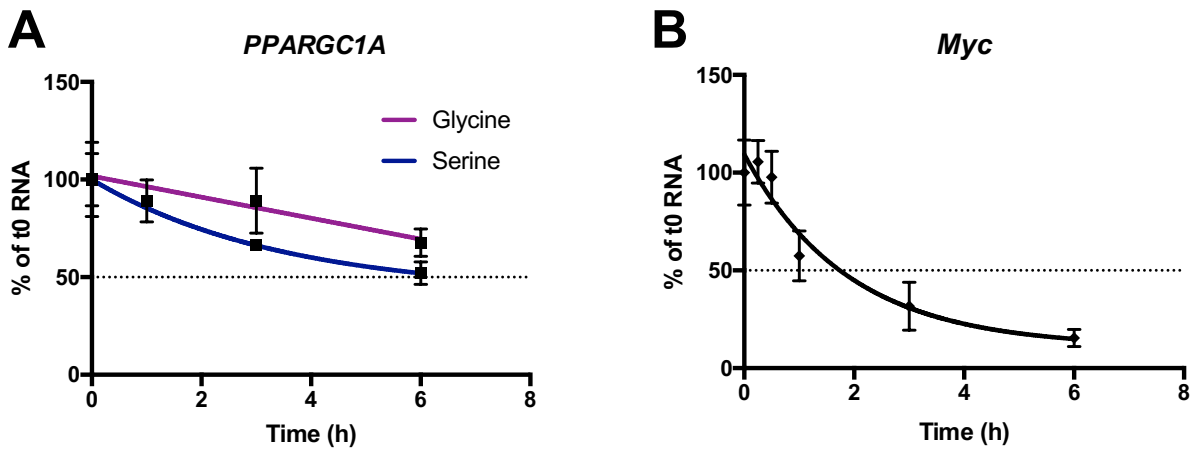


Figure 1: There is no difference in half-life between the 482Gly- and Ser-encoding human PPARGC1A transcripts. INS-1 cells over expressing the 482Gly- and Ser-encoding variants wer treated from 0 to 6 h with 1 µg/ml actinomycin D at 24 h after the start of transfection. Gene expressing was assessed using qRT-PCR. **A.** *PPARGC1A* amounts (in 1 µl) were quantified by comparison of ct values to a *PPARGC1A* standard curve. **B.** *Myc* was quantified as a fold change over the stable *18s* gene (in 1 µg total RNA). Expression levels for *Myc* are an average from the cells over expressing either *PPARGC1A* variant. Non-linear regression was used to estimate transcript half-life and error bars are representative of SEM for each data point. 2-way ANOVA was used to test for significance ($p>0.05$). Data are representative of 3 separate experiments.

Phosphorylation regulates PGC-1α degradation

Phosphorylation serves as a marker for ubiquitination [19] and, interestingly, this post-translational modification at other sites is implicated in the control of PGC-1α activity and stability [90]. *In silico* analysis performed by our lab predicts that the 482Ser residue may be a site of phosphorylation by CKII or Akt kinases [99]. Interestingly, phosphorylation by Akt at Ser570 in PGC-1α can inactivate PGC-1α, thereby impairing coactivator activity [101, 102]. PGC-1α phosphorylation by CKII has not been reported in the literature to the best of our knowledge. To test whether the 482Ser residue may be a site of phosphorylation, we generated human PGC-1α 482Asp (phospho-serine) and 482Ala (phospho-null serine) mutants. Following 24 h of expression, protein from INS-1 cells expressing human PGC-1α 482Gly, 482Ser, 482Ala, 482Asp or pcDNA 3.1+ vector-only control were analyzed by western blot. Cells expressing the 482Ser and 482Asp (phospho-mimicking serine) constructs appear to have lower protein levels compared to the 482Gly and 482Ala (phospho-null serine) constructs, despite equal total protein levels (Figure 2A). Of note, cells overexpressing both the 482Ala and 482Asp variants have lower overall PGC-1α protein expression compared to cells overexpressing the naturally occurring 482Gly and 482Ser variants. It is possible that these point mutations could also impair PGC-1α protein expression (or stability) similar to the 482Ser variant. Addition of MG132, a proteasomal inhibitor, uniformly stabilized PGC-1α levels by preventing proteasomal degradation, suggesting that the 482Ser variant destabilizes PGC-1α and that it is degraded via the ubiquitin proteasome pathway.

In a similar experiment to assess whether the phospho-null serine and phospho-mimicking serine had similar differences in protein half-life, INS-1 cells were transfected

with only the 482Ala and 482Asp constructs and treated with an inhibitor of protein synthesis, cycloheximide. As shown in Figure 2B, the half-life of PGC-1α is shorter in the cells over-expressing the 482Asp (0.8 h) compared to the 482Ala (2.5 h). These estimated protein half-lives resemble those of the 482Ser and 482Gly variants (0.7 h and 2.5 h, respectively), suggesting that this is a domain that directs proteasomal degradation of PGC-1α and that phosphorylation at this residue may regulate this process or other PTMs that affect protein stability.

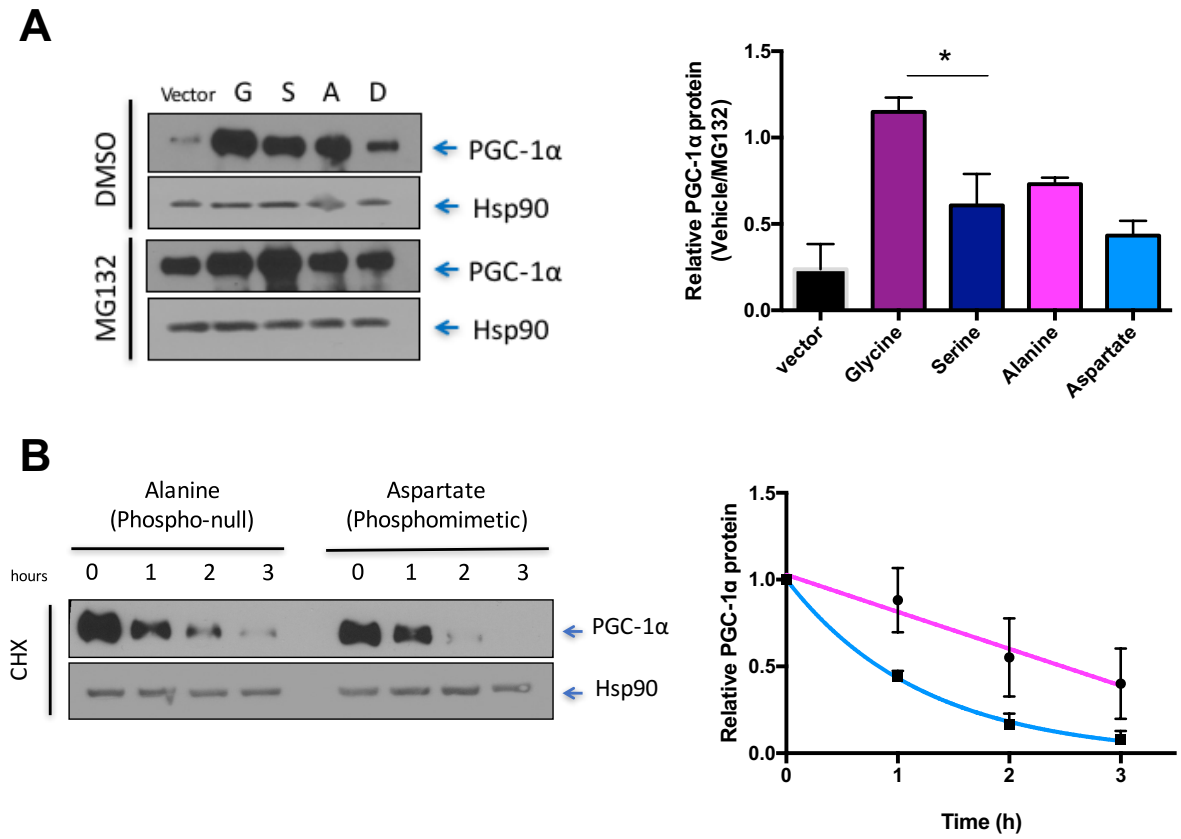


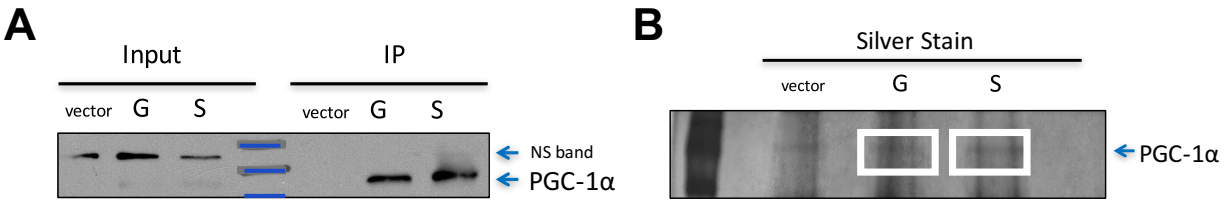
Figure 2: The PGC-1α 482Ser and 482Asp (phospho-mimetic) variants are less stable than their respective 482Gly and 482Ala (phospho-null mimetic) counterparts. Protein was harvested from INS-1 cells over expressing human PGC-1α 482Gly, 482Ser, 482Ala, 482Asp and pcDNA 3.1+ vector-only control. PGC-1α protein in 40 µg total protein was assessed by western blot. **A.** INS-1 cells were treated 3 h with 50 mg/ml MG132, a proteasomal inhibitor, of DMSO (vehicle only control), at 24 h after the start of transfection. Band densities are normalized to Hsp90 band intensity. one-way ANOVA was used to test for significance (* = $p<0.05$). Data are representative of 3 separate experiments. **B.** INS-1 cells were treated for 0 - 3 h with 50 µg/ml of cycloheximide (CHX), an inhibitor of translation, at 24 h past the start of transfection. Band densities are relative to the 0 h time point and normalized to Hsp90 band intensity. Non-linear regression was used to estimate protein half-life and error bars are representative of SEM. A paired t-test was used to test for significance ($p=0.03$). Data are representative of 3 separate experiments.

Ascertaining the PGC-1α 482Ser residue as a site of phosphorylation

Although we observe comparable fold differences (approximately 2x), in PGC-1α protein between the 482Gly and 482Ser variants and the 482Ala and 482Asp mimetics, it is still necessary to ascertain whether the protein is indeed phosphorylated at this residue. To do this, we choose to use LC-MS/MS (Liquid chromatography-tandem mass spectrometry).

We suspect that PGC-1α is of low abundance in INS1 β-cells, as detection of endogenous protein levels by WB has been quite challenging in the past. Moreover, there is often a more prominent non-specific band just above the PGC-1α that can impair PGC-1α detection by western blot (Figure 3A). In addition, if the 482Ser-phosphorylated form were unstable, it would be even less abundant, increasing difficulty of detection. Our initial strategy was to immunoprecipitate (IP) protein from INS-1 cells overexpressing human FLAG-PGC-1α 482Gly or 482Ser constructs. IP using anti-FLAG antibody conjugated to anti-mouse IgG agarose beads enriched PGC-1α and eliminated the non-specific band that appears above PGC-1α (Figure 3A). After validating the IP protocol, we silver stained the acrylamide gel to detect the resolved, immunoprecipitated human FLAG-PGC-1α 482Gly and 482Ser proteins. Silver staining was the most appropriate technique, as it offered much higher sensitivity compared to other protein staining techniques [103]. Although these bands were quite faint (Figure 3B), we were able to detect peptides, by data-mining, containing the human PGC-1α 482Gly and 482Ser sites using the LysC enzyme (Figure 3C), thus we will continue using this enzyme for future experiments. By this method, a PGC-1α 482Ser *phosphorylated* form was not detected by LC-MS/MS. Addition of MG132 to INS-1 cells over expressing the two human PGC-1α variants 3 h

before harvesting did not improve detection of a phosphorylated 482Ser–containing peptide. We also tried a backhanded approach of β-elimination to dephosphorylate the site, followed by Michael Addition to irreversibly modify the phospho-site for enhanced detection by LC-MS/MS; however, we were unable to re-solubilize dried PGC-1α protein to complete this reaction. Possible reasons why detection of a phosphorylated 482Ser residue has proven so challenging include low species abundance and/or difficulty in the actual detection of these phospho-peptides. Therefore, we decided to scale-up our approach to increase detection of PGC-1α overall.



C

Possible Fragments	Glycine hPGC-1α	Serine hPGC-1α
Trypsin enzyme		
T[G/S]ELR	X	X
HFGHPSQAVFDDEADKT[G/S]ELR	X	X
T[G/S]ELRDSDFSNEQFSK	X	X
LysC enzyme		
T[G/S]ELRDDFSNEQFSK	✓	✓ no phosphorylation

Figure 3: Peptides containing the Gly482Ser polymorphism are detected by LC-MS/MS. Protein was harvested from INS1 cells over expressing FLAG - PGC-1α-482Gly and Ser constructs. 800 µg of protein was immunoprecipitated using anti-FLAG antibody conjugated to anti-IgG agarose beads. 40 µg of protein was used for the input control. **A.** Western blot for PGC-1α using 40 µg total protein input and total immunoprecipitated protein. **B.** Silver stained 10% gel to detect immunoprecipitated PGC-1α protein for band excision and enzymatic digestion for LC-MS/MS analysis. **C.** Possible fragments generated by enzymatic digestion, and their corresponding results using Mascot for analysis and subsequent data mining for low abundance peptides. X means that there was no detection, and ? means that there was detection.

Designing a new strategy for detection of the phosphorylated form of 482Ser PGC-1α

As the protein half-life of the phospho-mimetics was so short, we hypothesized that if indeed the protein is phosphorylated at the 482 site, the resulting protein may be degraded quite quickly, decreasing our chance for detection. In order to increase likelihood of phosphorylated-482Ser PGC-1α detection, we decided to increase the amount of protein for LC-MS/MS analysis. The first step was to create human FLAG PGC-1α 482Gly and 482Ser-expressing adenovirus to increase the number of transfected cells (100% infection vs. 60-70% transfection) and decrease the cost of transfection reagents (approximately 40 10 cm^2 plates need to be infected). Adenovirus production involved cloning 482Gly or 482Ser FLAG-PGC-1α first into a shuttle vector, and then into the AdEasy vector so that virus can be produced in Ad-293 cells (Figure 4A). The resulting viruses were purified using $CsCl_2$ density gradients. The next steps will be to infect INS-1 β-cells with either 482Gly or 482Ser FLAG-human PGC-1α adenovirus to increase protein yield and thus the chance of detecting the 482Ser-phosphorylated form of PGC-1α. The phosphorylation status will be assessed using LC-MS/MS. All of these steps are summarized in Figure 4B and in the methods section.

An alternative approach to elucidating the 482Ser residue as a phospho-site could include use of Phos-tag gels, and looking for a shift of the PGC-1α protein. Unfortunately, the shift attributed to this single phosphorylation event may not be detected as PGC-1α is already a heavily phosphorylated protein.

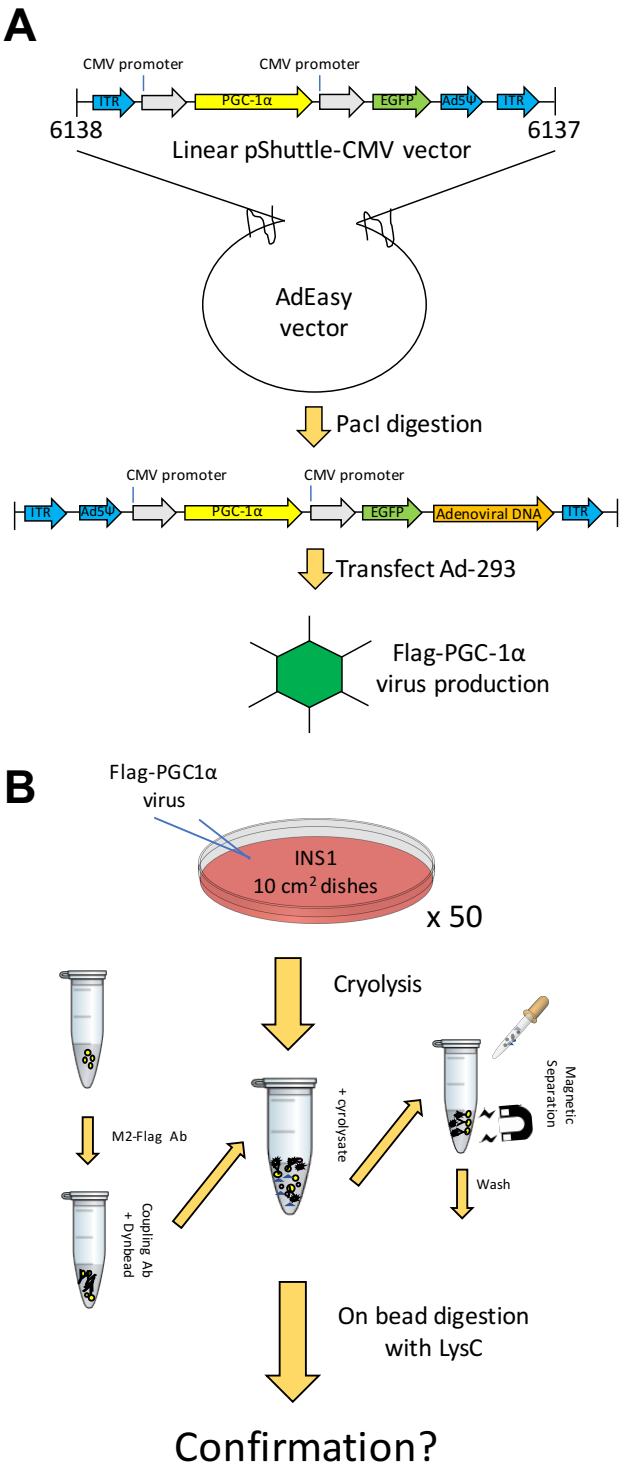


Figure 4: A visual summary of the strategy designed to increase the likelihood of detection of lower abundant species of PGC-1α. A. Cloning steps required to make the human FLAG-PGC-1α 482Gly and Ser expressing viruses. **B.** Strategy to scale up the FLAG-PGC-1α immunoprecipitation and detection of 482Ser-phosphorylated human PGC-1α.

Mark4 is a potential kinase for PGC-1α(482Ser) phosphorylation

As a complementary approach, an *in vitro* kinase assay was carried out in an attempt to determine whether the 482Ser residue is phosphorylated *in vitro* and to identify any potential kinases. First, we cloned and purified GST-tagged PGC-1α 482Ser peptides for the screen (Figure 5A). These purified peptides, along with γ^{32}P-labelled ATP, were incubated with 420 individually purified human kinases. Myelin basic protein (MBP), a heavily and easily phosphorylated protein, served as an internal control for function of the kinase assay and specificity of a specific kinase for the 482Ser-containing peptide. We identified two candidate kinases using film detection of γ^{32}phosphorylated peptides: Lyn Kinase and MARK4 (microtubule-associated affinity-regulating kinase 4) (Figure 5B). However, Lyn kinase is a Threonine/Tyrosine kinase that may have been detected because it phosphorylated a nearby residue such as the Thr481 that is immediately upstream the 482Ser residue. Other kinases were identified (BTK, RPS6KB2 and NEK2) however the internal control, MBP, was also heavily phosphorylated suggesting that these kinases lacked specificity for the GST-482Ser containing peptide (Supplementary Figure 1). Therefore, the remainder of the kinase study was focussed on MARK4. MARK4 is a member of the microtubule-associated affinity-regulating kinase family that is expressed in many tissue types [104]. In adipocytes, over expression of MARK4 initiates apoptosis and knockdown by shRNA against MARK4 increases PGC-1α protein levels [105, 106]. Additionally, MARK4 knock out mice have increased PGC-1α gene expression, along with improved glucose tolerance and are resistant to HFD-induced obesity [107]. MARK4 is an interesting candidate for this project because it affects not only PGC-1α gene and protein levels, but also, metabolism as a whole.

To test whether MARK4 activity regulates PGC-1α protein stability, we tried to make three shMark4 plasmids to knockdown rat MARK4 in the INS-1 β-cells. We co-transfected INS-1 cells with each of the shMark4 plasmids with either the human PGC-1α 482Gly and 482Ser-expressing plasmids. However, the most effective shMark4 plasmid only knocked down Mark4 by less than 30% (Supplementary Figure 2). Instead of testing more shMark4 plasmids, or purchasing siRNA against Mark4, we decided to test the efficacy of the chemical inhibitor MRT67307 which largely inhibits MARK4 (and other members of the MARK family to a lesser degree) while maintaining activity of other kinases, such as AMPK, that also phosphorylate PGC-1α [108]. In concordance with the reported PGC-1α Gly482 half-life (2.5 h) [99], we show in Figure 5C that treatment of INS-1 cells over expressing either human PGC-1α 482Gly or 482Ser variants with MRT67307 stabilized the 482Ser variant (3.1 h) similarly to the 482Gly variant (2.4 h). These findings provided us with evidence that MARK4 activity could affect PGC-1α degradation; however future experiments need to include a CHX control to ensure that cycloheximide is arresting PGC-1α translation and a control to test the efficacy of the inhibitor on MARK 4 activity.

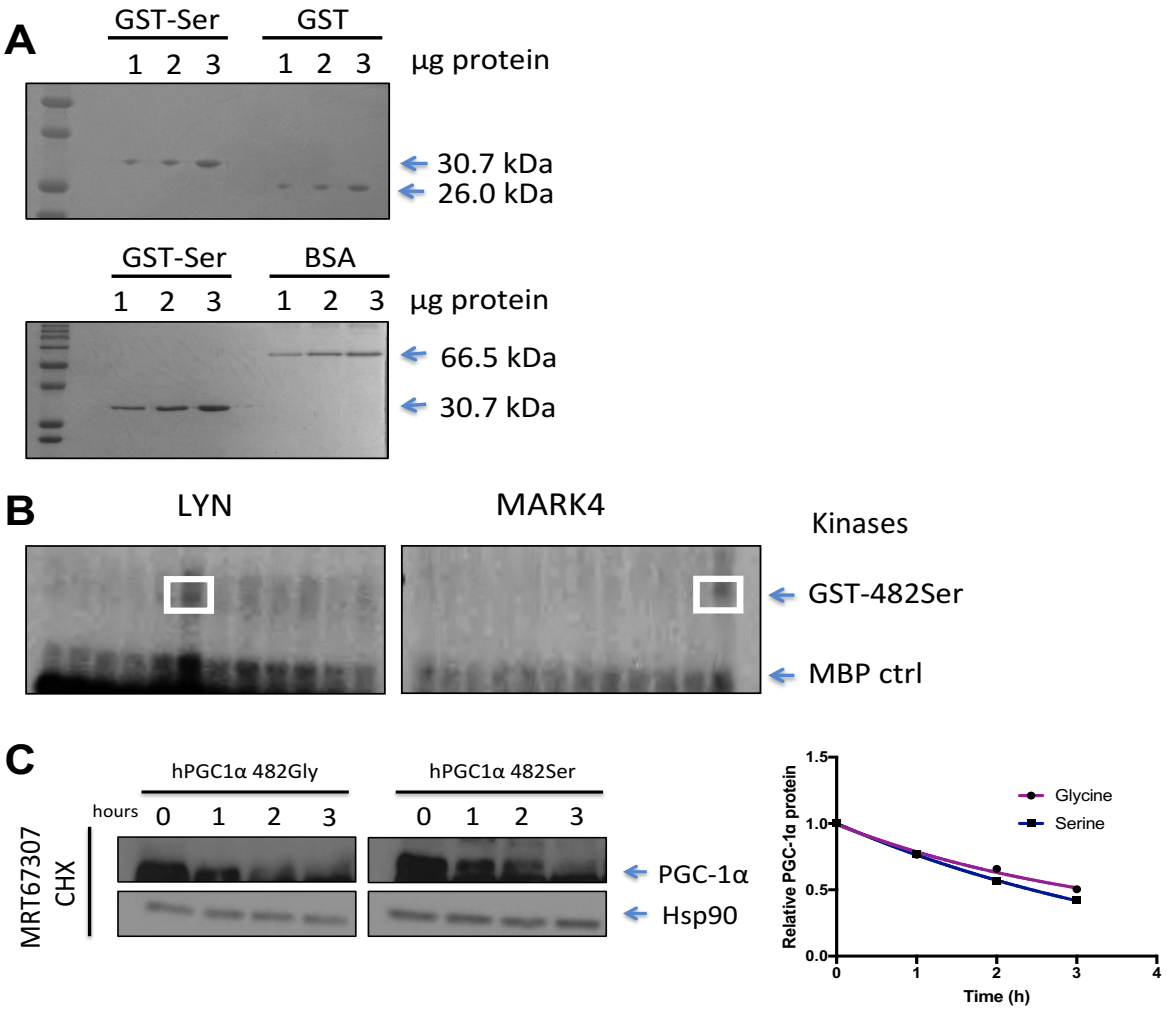


Figure 5: MARK4 kinase may be responsible for the phosphorylation of the human PGC-1α 482Ser residue. A. The GST-482Ser fusion peptide was over expressed and purified from BL21 bacterial cells. The peptide was run along side GST-only control (top), and BSA (bottom) and coommassie stained to ensure peptide expression and accurate protein quantification, respectively. **B.** The 482Ser-peptides were incubated separately with 420 different, purified kinases and γ^{32}phosphate ATP. The bands detected (white square) indicate a phosphorylated substrate. MBP was an internal control that is heavily phosphorylated to ensure assay function and kinase specificity. **C.** INS-1 cells over expressing the human PGC-1α 482Gly and Ser variants were treated with 500 nM MRT67307 from 0 to 3 h at 24 h after the start of transfection. Band densities are relative to the 0 h time point and normalized to Hsp90 band intensity. Non-linear regression was used to estimate protein half-life. Data are respresentative of 1 preliminary experiment.

The Gly482Ser polymorphism affects coactivator function

As a transcriptional coactivator, PGC-1α forms complexes with specific transcription factors to drive mRNA expression, regulate and integrate many metabolic processes [22]. Accordingly, we decided to investigate whether the PGC-1α Gly482Ser polymorphism affects gene expression of well-established downstream gene programs of the coactivator, including oxidative phosphorylation (OXPHOS), ROS detoxification genes and transcription factors. Unpublished data in our lab has shown that the PGC-1α Gly482 variant has increased coactivator function on *ERRα* [99], which can control expression of genes in the OXPHOS pathway to increase ATP production by the mitochondria [109] and *Sod2* [99], which is known to ameliorate cellular oxidative stress. We measured gene expression for OXPHOS genes that are known to co-regulate with one another in response to PGC-1α [94], as well as important genes involved in ROS detoxification, at 12 h intervals spanning from 0 to 48 h.

mRNA expression for PGC-1α was similar for the two constructs throughout the experiment, and peaked at 36 h post-transfection (Figure 6A). INS-1 cells over expressing the 482Ser variant, but not the 482Gly variant, have a decrease in expression of several PGC-1α target genes over the 48 h time course (Figure 6B): *Ndufs1, Ndufs5, Sdhα,* which are subunits of complexes 1 and 2 of the mitochondrial electron transport chain and therefore affect the OXPHOS pathway, and *Catalase* and *Gpx1,* which protect the cell from oxidative damage caused can be caused by ROS produced in mitochondria.

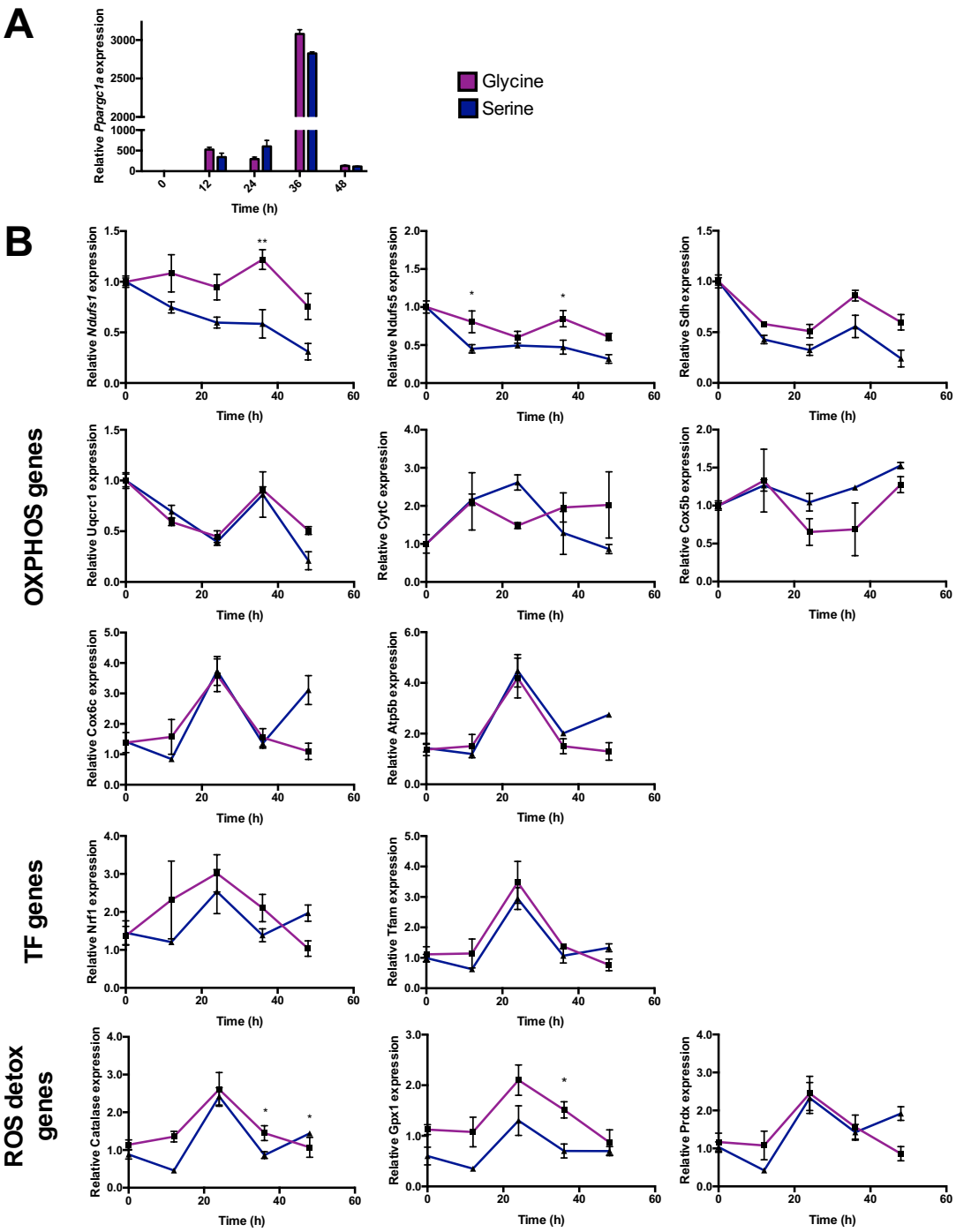


Figure 6: The Gly482Ser polymorphism affects coactivator function on several PGC-1α target genes. RNA was collected from INS1 cells over expressing humanPGC-1α 482Gly and Ser variants at 0, 12, 24, 36 and 48h after the start of transfection. Gene expression was assessed using qRT-PCR **A.** *PPARGC1A* was quantified as a fold change over *Hprt* (in 1µg total RNA).**B.** Target genes were quantified as a fold change over *Hprt* (in 1 µg total RNA). These data were normalized to the 0 h time point of the empty vector control (pcDNA3.1+)**.** Error bars are representative of SEM for each data point. 2-way ANOVA was used to test for significance (*=p<0.05). Data are representative of 3 separate experiments.

CHAPTER 1 – DISCUSSION

In summary, we have determined by cycloheximide chase that the Serine residue at amino acid position 482 of human PGC-1α decreases protein stability, compared to the 482Gly variant. Replacing this residue with either an Alanine residue (phosphorylation-null) or an Aspartate residue (phosphomimetic) led to protein stabilization and destabilization similar to the native 482Gly and Ser482 PGC-1α protein variants, respectively, when overexpressed in INS-1 β-cells. This regulation was also observed when constructs were expressed in HepG2 human hepatocytes [82], suggesting this mechanism is not β-cell specific. Addition of a proteasomal inhibitor (MG132) stabilized PGC-1α protein to comparable levels, suggesting that this amino acid modification plays an important role in PGC-1α degradation via the proteasome. Our data provide compelling evidence that there is a phosphorylation event at the 482Ser site and that this post-translational modification regulates stability of the protein. Although we were unable to include any conclusive evidence that the serine at position 482 of the full-length protein is indeed phosphorylated in cells, current efforts are focussed on ascertaining the phosphorylation status of the 482Ser residue by refined methods.

The differences in stability between the phosphomimetic and phospho-null constructs suggest that phosphorylation at the 482 site is sufficient for protein destabilization. This likely occurs because the negatively charged phosphate group modifies PGC-1α in such a way that this region becomes exposed and available for ubiquitination. A good example of this phenomenon is the phosphorylation of IκBα allows the region to be polyubiquitinated by a E3 ligase complex and degraded by the proteasome [110]. In fact, this is the mechanism by which GSK-3β also regulates PGC-

1α levels [90], thus it is possible that PGC-1α stability controlled by the 482 site is also regulated by a mechanism similar to this.

To test this hypothesis, we plan to co-transfect INS-1 cells with human FLAG-PGC-1α 482Gly or 482Ser PGC-1α constructs and hemagglutinin (HA)-tagged ubiquitin (Ub), with or without MG132 to prevent ubiquitinated PGC-1α degredation. Immunoprecipitation of FLAG-PGC-1α and subsequent immunoblotting for HA-Ub could reveal whether PGC-1α 482Ser is more heavily ubiquitinated compared to the 482Gly variant. It is possible that both variants are similar in terms of ubiquitination status, as studies show that PGC-1α is relatively unstable and that ubiquitin blots usually appear as smears [78, 88, 90]. Alternatively, we can over express our FLAG-tagged PGC-1α 482Gly and 482Ser truncated proteins (spans residues 390 to 565) constructs to hopefully discern a difference in ubiquitination that is specific to the PGC-1α 482 residue.

While the human Flag-PGC-1α Gly482 and 482Ser viruses were being cloned, amplified and purified for the modified LC-MS/MS protocol, we identified by kinome screen MARK4 as a potential kinase that phosphorylates the 482Ser residue in a 23 residue GST-tagged 482Ser PGC-1α peptide. MARK4 is an interesting candidate as it is part of the AMPK-related family of kinases that are shown to increase PGC-1α transcription [19, 105]. We hypothesize that decreased MARK4 activity will result in PGC-1α stabilization, which could have beneficial effects on metabolism. This is supported by studies showing that MARK4 deficient mice have improved insulin sensitivity, similar to what is observed in a PGC-1α transgenic mouse model that expresses human *PPARGC1A* by 2-fold. These humanized mice again demonstrate the highly tissue specific nature of PGC-1α where elevated *PPARGC1A* in the liver can be detrimental, or

beneficial in muscle [111]. However, there are studies showing that β-cell-specific overexpression of PGC-1α and adenovirus-mediated overexpression of PGC-1α in isolated mouse islets can impair insulin secretion [54, 55]. It will be interesting to determine whether stabilizing endogenous PGC-1α, rather than over expressing the protein to levels that are not physiologically relevant, could be the key to improving β-cell function and most importantly, whole body metabolism. Interestingly, in preliminary experiments we found that treatment with the chemical inhibitor MRT67307 along with cycloheximide increases the half-life of the PGC-1a 482Ser protein similar that of the 482Gly protein. It is possible that stabilizing the 482Ser variant by inhibiting MARK4-mediated protein degradation could lead to improved β-cell function, especially for those who with the 482Ser variant that have low PGC-1α expression and are obese and/or prediabetic [30, 56, 112].

Interestingly, adipocytes from mice fed a HFD for 10 weeks had an increase in *Mark4* expression, along with decreases in antioxidant activity [105]. Moreover, knock down of *Mark4* in cultured adipocytes increases PGC-1α protein and rescues antioxidant activity [105]. These studies, along with our data showing that inhibition of MARK4 activity stabilizes the human PGC-1α 482Ser variant, provide correlative evidence for a possible association between MARK4 activity, obesity and PGC-1α protein levels [106, 107]. Although the main focus of this project was to understand the impact of the Gly482Ser polymorphism in the β-cell, it would also be interesting to look into the effect of MARK4 knockdown on PGC-1α in other cell lines since we have also observed a difference in stability between the two PGC-1α variants in HepG2 liver cells [82].

Based on our data, it seems plausible that knocking down *Mark4* in INS-1 cells would stabilize the PGC-1α 482Ser variant and thus increase coactivation of PGC-1α target genes similar to that of the 482Gly variant. There are many kinases that phosphorylate PGC-1α in order to activate lipid and glucose gene programs, thus it is possible that MARK4 may act similarly [19]. It would be interesting to knock down *Mark4* and re-assess the effect of the polymorphism on PGC-1α stability as well as coactivator function.

The best model to assess the impact of the polymorphism on endogenous PGC-1α activity in tissues (islets, liver, muscle and/or adipose) would be by using a knock-in approach to humanize a model organism, such as a mouse or cell line. This could be done by using CRISPR/cas9 technology, which will be discussed in Chapter 2.Of note, high fat, high fructose-feeding may be required to tease out the differences in protein stability, as PGC-1α is increased under these conditions and may play a more important role in boosting metabolism under times of stress. Additionally, obesity is often a concurrent with impaired insulin secretion in human carriers of the PGC-1α 482Ser allele compared to patients homozygous for the 482Gly allele [31, 42]. We speculate that humanized mice homozygous for the PGC-1α 481Gly allele will be more resistant to high-fat/high-fructose challenge, with respect to insulin secretion and overall β-cell function.

Gain-of-function gene expression results in cell lines suggest that PGC-1α human PGC-1α 482Gly and 482Ser variants differentially regulate genes in the OXPHOS and ROS detoxification gene programs, where the 482Gly variant seems to have significantly enhanced coactivator activity over the 482Ser variant on many, but not all gene targets [63, 79, 80, 113]. In concordance with data presented in Figure 6, the human PGC-1α

482Gly variant improves coactivation of antioxidant genes *Sod2, Gpx1* and *Catalase* when overexpressed in HepG2 cells as well [82]. Although it seems unlikely that a single amino acid substitution could wholly alter the function of PGC-1α, there are 2 possible explanations for why carriers of the 482Ser allele have increased risk of metabolic disease. First, it is possible that decreased protein stability impairs the ability of PGC-1α as a coactivator in general. The first option could be assessing PGC-1α coactivation for each gene of interest using a luciferase assay, and the second by co-immunoprecipitation of PGC-1α with immunoblotting for transcription factors specific for those genes.

Taken together, we propose the following mechanism by which PGC-1α stability is regulated: the PGC-1α 482Ser residue is phosphorylated by the MARK4 kinase, which may trigger ubiquitination of nearby lysine residue(s), and ultimately, mark the protein for proteasomal degradation (Figure 7). The decreased PGC-1α stability could lead to decreased coactivation of PGC-1α target genes and thus, impaired cellular metabolism.

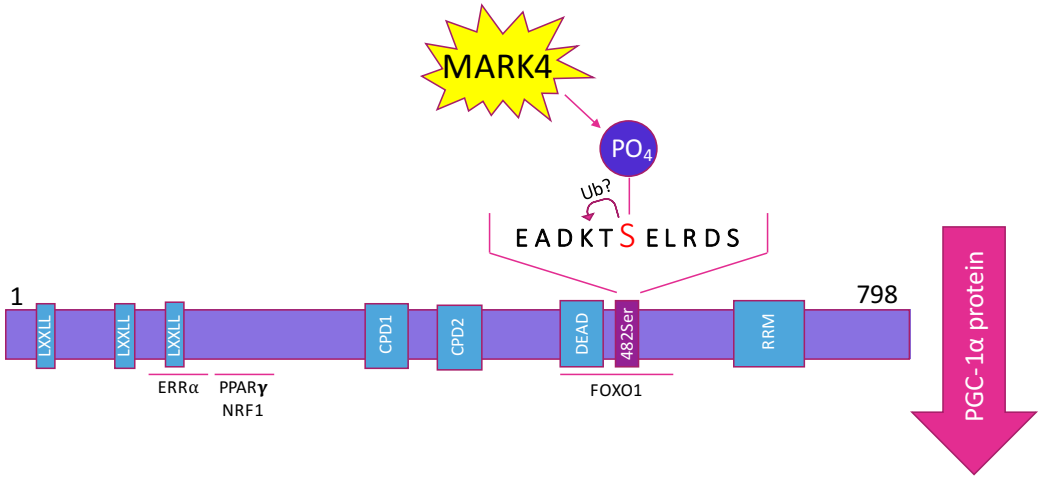


Figure 7: Proposed model for the destabilization of PGC-1α(482Ser) variant . The PGC-1α 482Ser residue is phosphorylated by the MARK4 kinase, which may trigger ubiquitination of nearby lysine residue(s), and ultimately, increasing protein proteasomal degradation

Chapter 2: Using CRISPR/Cas9 Technology to generate a humanized mouse line harbouring the a PGC-1α 481Gly allele

CHAPTER 2 – INTRODUCTION

CRISPR/Cas9 gene editing technology is becoming an increasingly valuable tool for scientists interested in studying the effects of genetic modifications. There exist two modes of gene editing: Non-homologous end joining (NHEJ) and homology directed repair (HDR). Typically, NHEJ is used for insertion and deletions whereas HDR is used for precise insertions and substitutions. To generate humanized mice harbouring the PGC-1α 481Gly allele, we needed to mutate a single nucleotide, adenine (AGT=Ser) to a guanine (GGT = Gly), thus we elected to use the HDR method.

First, the Cas9 protein requires a guide RNA molecule to direct the enzyme to the desired modification site on the genome. Typically, plasmids (i.e. px330) are modified to express both the Cas9 protein and its guide RNA. Cells and/or embryos are then transfected and/or injected with the Cas9 and the guide RNA expressing plasmid along with a long, single stranded oligodeoxynucleotide (ssODN) which has ~50bp homology arms and a template for the desired point mutation. When these plasmids are expressed, the guide RNA aligns with its complementary DNA sequence and allows the accompanying Cas9 protein to generate a double stranded break in the DNA. The ssODN serves as a template during DNA repair and the substitution mutation is completed. Although the workflow is relatively uncomplicated, the efficiency and the specificity can be quite variable [114].

Gene editing using CRISPR/Cas9 offers many benefits, which traditional methods do not. For cell-lines, plasmids that stably express protein-encoding genes were traditionally used to express mutant alleles; however, these genes are usually under the constitutive human cytomegalovirus (CMV) promoter, rather than the endogenous

promoter so they cannot be as precisely regulated by promoter binding elements (i.e. activators, repressors). For the CRISPR/Cas9 method in cell-lines, we can transfect cells with plasmids that co-express the Cas9 protein and guideRNA with GFP, and then separate single cell clones by fluorescence-assisted cell sorting (FACS) to ensure that we are only propagating cells that are expressing the Cas9 protein. This increases efficiency of the procedure and allows us to derive many clones from a single transfection.

Furthermore, in mice, the alternative to CRISPR/cas9 is homologous recombination. This process is much more time-consuming as injected ES cells require approximately 6 months before they can be implanted into pseudopregnant females, and during this time, there is no way to screen whether the desired modification is present in the mouse [115]. Using CRISPR/Cas9 technology, we can bypass the 6-month waiting period and genotype pups for the mutation as soon as they are available, thereby expediting the whole process. Moreover, we also are able to study the effects of gene mutations under the endogenous promoter, which makes this method superior to traditional homologous recombination.

We have identified, *in vitro*, that the PGC-1α 482Ser variant is less stable than the 481Gly variant when over expressed (by the CMV promoter) in INS-1 β-cells, and that a putative phosphorylation event at the 482Ser site by MARK4 kinase is the cause of increased protein destabilization. This decrease in protein stability is reflected in decreased coactivation of several PGC-1α targets. By generating an INS-1 cell line and mouse line that express the mutant 481Gly variant under the endogenous promoter we can test the functional consequences in an enhanced *in vitro* model and *in vivo,* respectively.

Previous studies show that low PGC-1α results in impaired insulin secretion by pancreatic β-cells and, in mouse models of low PGC-1α, there is impaired mitochondrial function in cultured hepatocytes as well as decreased expression of several PGC-1α mitochondrial target genes in white adipocytes [31, 32, 116]. Additionally, our *in vitro* data show that the human PGC-1α 482Ser variant is less stable when expressed in both INS-1 β-cell and HepG2 hepatocytes. We therefore hypothesize that the mouse PGC-1α protein will be stabilized in the humanized PGC-1a 481Gly-expressing mice and increase coactivation of PGC-1α target genes in response to nutritional cues (i.e. glucose and lipid challenges).

CHAPTER 2 – OBJECTIVES

Initially, our investigation into the functional consequences of the Gly482Ser polymorphism involved the over expression of human PGC-1α 482Gly and 482Ser constructs in INS-1 β-cells. However, this experiment was exceedingly time demanding and would not be beneficial for other projects in the lab. We therefore decided to focus our efforts on designing a strategy to express the mutant PGC-1α 481Gly allele in mice as it would allow us to test the consequences of the Gly481Ser polymorphism *in vivo,* provide us with a renewable source of primary cells, including pancreatic islets, hepatocytes, adipocytes and muscle tissue. Moreover, we will be able to assess the impact of the polymorphism under the endogenous PGC-1α promoter, rather than the commonly used, constitutively active CMV promoter. Therefore, the objective of this aspect of the project was to design an effective targeting strategy to use CRISPR/Cas9 to create the analogous 481Gly Gly to Ser mutation in the mouse genome, 2) create a β-cell line expressing either the PGC-1α 481Gly or 481Ser variants to study the effects of the variant on protein stability and function of the endogenous gene *in vitro*, and 3) use this strategy to generate a humanized mouse line expressing an allele analogous to the human 482Gly variant.

CHAPTER 2 - METHODS

Cell culture and treatments*:* INS-1 β-cells were cultured in RPMI 1640 (Wisent) medium with 10% Heat Inactivated FBS (Wisent), 1% penicillin and streptomycin (Wisent), and 1x supplement (10 mM HEPES, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol). Cells were plated in 6-well plates the night before experimentation. Cells were transfected when 80-90% confluent, with Lipofectamine 2000 (2.5 μl per well) and 1 μg of DNA, with OPTIMEM (or Extreme-DMEM) reduced serum media. After 4 h, the transfection media was replaced with normal INS-1 media. Cells were left for 24 h (as specified) at 37°C with 5% CO_2. The cycloheximide chase experiment was done with 50 mg/ml of cycloheximide (Sigma-Aldrich); cells were treated from 1 – 3 h.

Western Blot: Cells were lysed in RIPA buffer (50mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease cocktail inhibitor (Calbiochem), and phosphatase inhibitor (Roche). Protein concentration was estimated using DC assay on BSA standards. Equal amounts of protein (40 μg) were loaded onto a 10% polyacrylamide gel, and were transferred onto a PVDF membrane (GE healthcare). PGC-1α was detected using anti-PGC-1α mouse mAb antibody (1:1000, Millipore) diluted in 2% milk/PBST. The membrane was incubated overnight at 4°C. Anti-mouse IgG antibody conjugated to HRP (1:5000, Biorad) was used to label protein/1° antibody complexes. HRP signal detection was performed using the ECL detection system.

CRISPR cell-line: Two guide RNAs (gRNA) specific for the desired PGC-1α 1441 A to G mutation (Guide 1: 5’ – AGACAAGACCAGTGAACTAC – 3’ and Guide 2: 5’ –

TAGACAAGACCAGTGAACTA – 3') were cloned into the GFP and Cas9-encoding pSpCas9(BB)-2A-GFP plasmid [117]. When INS-1 cells were 80% confluent in 6-well plates, they were transfected with Lipofectamine 2000 (2.5 μl per well), 1 μg of plasmid DNA and 4.5 μl of 10 μM of ssODN stock (5' – CACTTCGGTCATCCCAGTCAAGCTGTTTTTGACGACAAAGTAGACAAGACCGGTGA ACTACGGGATGGCAACTTCAGTAATGAACAATTCTCCAAACTACCTGTGTTTATAAA – 3') with OPTIMEM reduced serum media. After 4 h, the transfection media was replaced with normal INS-1 media. Cells were left for 24 h (as specified) at 37°C with 5% CO_2. Cells were then scraped in 1X PBS, pelleted and resuspended in 250 μl of fresh 1X PBS. Using FACS, GFP-positive single cells expressing the plasmid were seeded into each well of 2 96-well plates. The cells were propagated individually until they were in 2X 6-well plates (one for maintaining the cell-line and the other for genotyping). The genomic DNA from each propagating cell line was purified using a genomic DNA purification kit (Qiagen). Next, the DNA region encoding the 1441 site was amplified by PCR F: 5' - TGCCACCACCAACAAAGAGG – 3' and R: 5' - TGAGCACCATTGGATCGCC – 3' to yield a 1250 bp product. This product was digested using Age1 restriction digest (ACCGGT).

CRISPR mice: Two guide RNAs (gRNA) specific for the desired PGC-1α 1441 A to G mutation (Guide 1: 5' – CAGACAAGACCAGTGAACTA – 3' and Guide 2: 5' – AAGACCAGTGAACTAAGGGA – 3') were cloned into the Cas9-encoding px330 plasmid. Embryos from B6C3F1 zygotes were microinjected (IRCM core facilities) with the two plasmids together, each expression a single guide with the ssODN (5' –

TGAACAAGCACTTCGGTCATCCCTGTCAAGCTGTGTTTGACGACAAATCAGACAAG ACCGGTGAACTAAGAGATGGCGACTTCAGTAATGAACAATTCTCCAAACTACCTGT GTTTATAA – 3') that acts as a template during homology directed repair (HDR). The embryos were implanted into pseudo-pregnant female mice. After weaning, the pups were screened using PCR F: 5'-GCTAATGGATCCTACATTTCTTTTTGTTTC -3' and R: 5'-GCAACTTGCCTCTTAGCGC-3' to amplify the mutated region from genomic DNA, and subsequent Age1 restriction digest (ACCGGT).

The resulting digested PCR products were resolved by gel electrophoresis on a 2% agarose gel.

Genotyping by Taqman Assay: Using a pre-made TaqMan genotyping kit (Applied Biosciences), we developed a protocol that could be used to genotype cDNA from over expressed mouse PGC-1α, endogenous mouse PGC-1α from Min6 mouse β-cells, mouse primary (adipose, muscle, liver and islets) cells, over expressed human PGC-1α, endogenous PGC-1α from human HepG2 hepatocytes, INS-1 rat β-cells and human islets. For each reaction in a 384-well plate, we used 2.5 μl of 2X TaqMan Universal PCR Master Mix, 0.25 μl of 20X working stock of SNP Genotyping Assay (as per manufacturers instruction) and 10 ng of DNA and water up to a total of 5 μl. DNA amplified by 40 cycles of 15 seconds at 92°C and 1 min of 60°C using the Viia 7 (Life Technologies). The allelic discrimination is visually represented in a scatter plot. The result is the relative fluorescence of the VIC (Gly-specific) and FAM (Ser-specific) dyes against the internal ROX dye control. An example of the scatter plot is shown in Supplementary Figure 3.

Statistics: Graphpad Prism was used for graphing results and calculating statistics. PGC-1α protein half-life estimation was calculated using non-linear regression. Time-course gene and protein expression statistics were calculated using 2-way ANOVA.

CHAPTER 2 - RESULTS

The mouse PGC-1α 481Ser is less stable than the humanized PGC-1α 481Gly protein

In order to determine whether mouse PGC-1α stability was affected by the Gly481Ser polymorphism, we tested whether the protein stability of mouse PGC-1α 481Ser and 481Gly (humanized variant) were similar to that of the human PGC-1α 482Gly and 482Ser variants. INS-1 cells were transfected with the mouse PGC-1α 481Ser and 481Gly constructs (or vector only control) and treated with an inhibitor of protein synthesis, cycloheximide (CHX). Addition of CHX decreased mouse PGC-1α protein overall but the rate of degradation was faster in the INS-1 cells over expressing the Ser481 PGC-1α (Figure 8A). Upon measuring the half-life for the two mouse PGC-1α variants, we see yet again in Figure 8B that the mouse PGC-1α 481Ser variant is less stable (1.1h) than the 481Gly variant (3.4h), which is similar to the differences in half-lives observed for the human homologs. We concluded from this experiment that the mouse could be a suitable model to study the Gly482Ser polymorphism *in vivo* and that mutation of the mouse 481Ser to a 481Gly will likely stabilize, and thus increase, endogenous PGC-1a levels in the entire mouse.

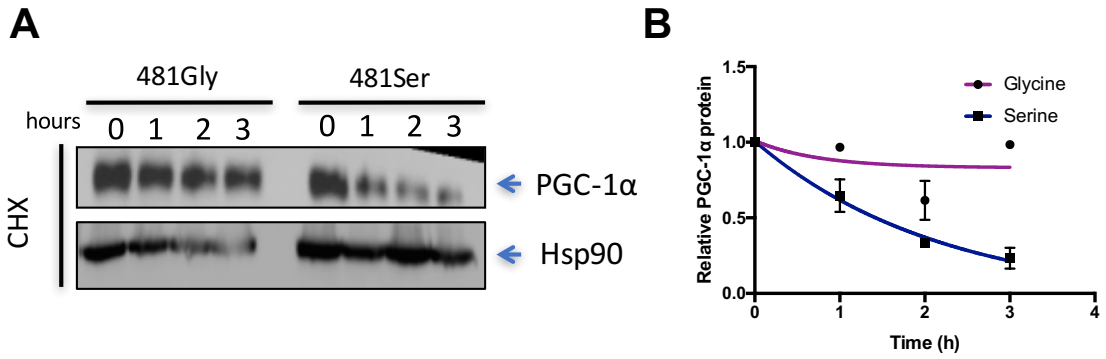


Figure 8: The mouse PGC-1α 481Ser variant is less stable than the mutant mouse PGC-1α 481Gly protein. INS-1 cells over expressing the mouse PGC-1α 481Gly and Ser constructs were treated for 0 to 3 h with 50 µg/ml of cycloheximide (CHX), an inhibitor of translation, at 24 h past the start of transfection. **A.** Western blot for PGC-1α (and Hsp90 control) using 40 µg total protein. Band densities are relative to the 0 h time point and normalized to Hsp90 band density. **B.** Half-life was determined using non-linear regression where 0 h was equal to 1.0, and error bars are representative of SEM for each data point. 2-way ANOVA was used to test for significance (p=0.01). Data are representative of 2 separate experiments.

Using CRISPR/Cas9 to generate a PGC-1α 481Gly-expressing INS-1 cell line

After determining that over expression of the Gly481Ser mouse PGC-1α polymorphism also impacted PGC-1α stability, we decided to test whether the stability of PGC-1α was similarly affected when the two 481Gly and 481Ser variants were expressed under the endogenous PGC-1α promoter. To do this, we transfected INS-1 β-cells with pSpCas9(BB)-2A-GFP plasmid expressing GFP, Cas9 protein and either guide 1 or guide 2. At 24 h post transfection, we seeded single GFP-expressing INS-1 β-cell clones into individual wells (Figure 9 A). The expression of the guide RNA and Cas9 are driven by the U6 promoter, which drives RNA polymerase III transcription; RNA polymerase III is desired as it starts and ends transcription of the guide without adding extra nucleotides, or post-transcriptional RNA modifications like the poly-A tail and 5' cap [118]. Moreover, the guides were designed such that they were immediately upstream of the Protospacer-adjacent motif (PAM) which is important for Cas9-gRNA complex binding to that specific region in the genome. At 24 h post-transfection, the cells expressing GFP (and consequently, Cas9 protein-gRNA complex) were sorted by FACS for GFP-positive cells. For guide 1, 0.28% cells expressed the plasmid and for guide 2, 0.26% of cells (Figure 9B).

From the 2 96-well plates, we obtained 36 clonal cell lines over the span of 3 months. Unfortunately, when they were genotyped using PCR amplification and restriction enzyme digestion, we had no 481Gly-positive cell lines, and some were mutated beyond the desired 1441 G to A mutation (Figure 9C). At this point, we decided to focus our efforts on developing a strategy to induce the 481Gly mutation in mice.

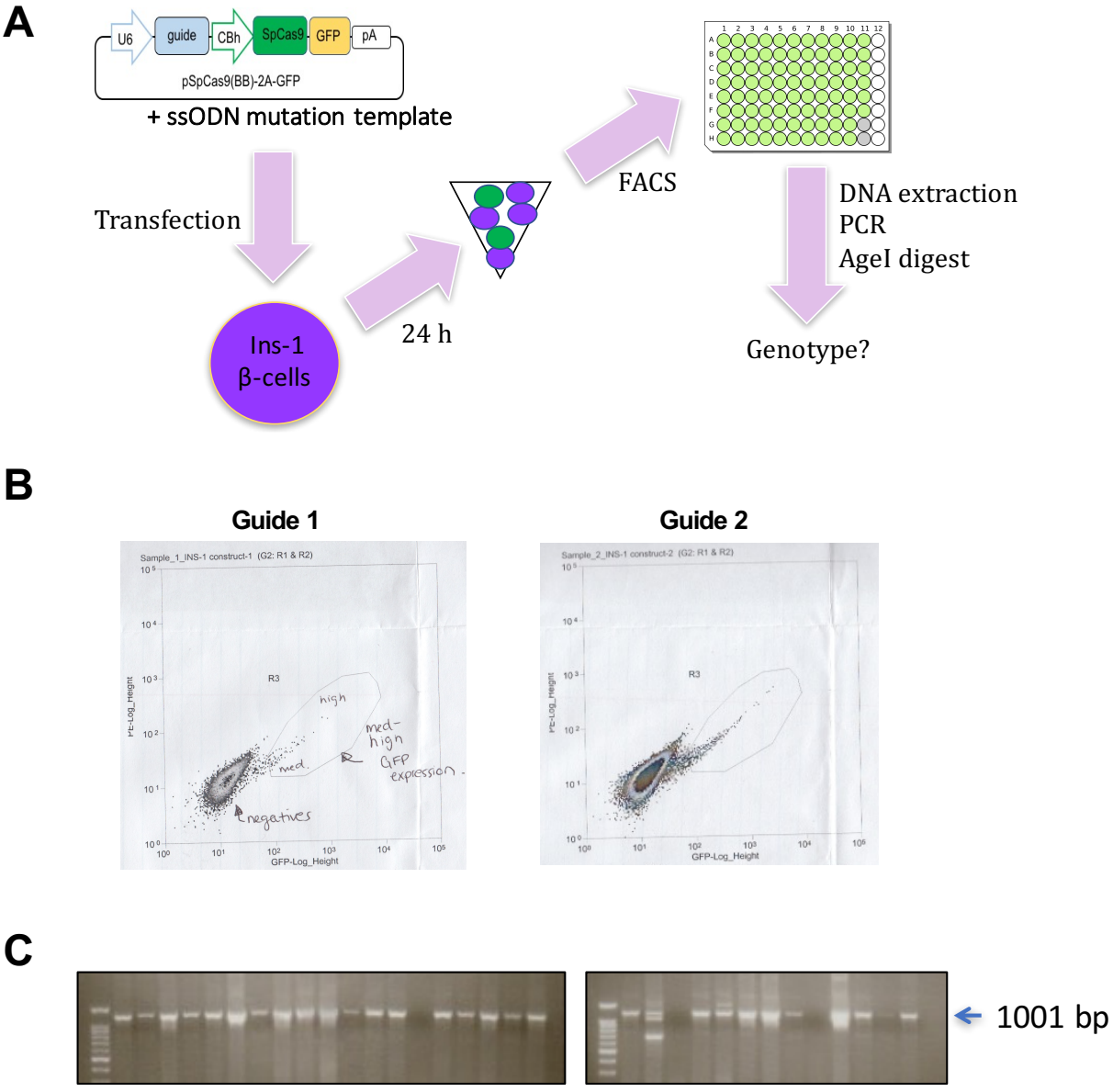


Figure 9: CRISPR strategy used generate a PGC-1α 481Gly-expressing INS-1 cell line. **A.** INS-1 cells were transfected with ssODN template and pSpCas9(BB)-2A-GFP plasmid expressing Cas9 + guideRNA. **A.** The cells expressing GFP-Cas9-guideRNA were sorted using FACS at 24 h past the start of transfection and seeded 1 cell per well in 96 well plates. **B.** The resulting FACS plot showing the population of cells that highly express the pSpCas9(BB)-2A-GFP plasmid. **C.** INS-1 cells were screened using PCR and subsequent Age1 digestion. The digestion patterns were visualized using gel electrophoresis.

Using CRISPR/Cas9 to generate a mouse line expressing the PGC-1α 481Gly protein

Although the CRISPR/Cas9 approach for INS-1 cells did not work, we decided to try to use the technology again but this time in mice. Developing mice harbouring the mutant PGC-1α 481Gly allele offered many more benefits than developing a 481Gly-positive cell line. Most importantly, they would be a renewable source of primary cells from all different tissue types (i.e. pancreas, liver, adipose and muscle). To do this, mouse embryos were microinjected with two px330 plasmids expressing Cas9 protein with guide 1 and Cas9 protein with guide 2. These microinjected embryos were implanted into pseudo pregnant females. We screened approximately 500 pups to finally obtain 8 founders, resulting in an efficiency of 2-3% which is over 10X lower than the 32% that was reported by Wang, B et al (2015) [119].

In order to screen the resulting pups (F0), we used the Taqman SNP genotyping kit to determine which mice, from the 500 pups, possessed the mutant PGC-1α 481Gly allele. To verify that these mice indeed possessed the mutant PGC-1α 481Gly allele, we TA cloned the PGC-1α SNP region into the pGEM-T Easy vector using the same genotyping primers that are described in the methods section. We transformed DH5α bacterial cells with the TA-cloned plasmids and purified plasmids from 6 individual bacterial colonies per mouse line. Presence of the PGC-1α 481Gly and 481Ser alleles was verified using Sanger Sequencing. From the verified pool of 8 founders, we selected 4 mice that we would backcross with our C57BL/6N wildtype mice. We selected a mixture of females (2) and males (2) to backcross to ensure transmission of the allele would not be affected by sex. Moreover, 3 of the 4 mice that were selected were from different micro-injection events to account for any variability in the injection process. The resulting

offspring (F1) were screened using PCR amplification and AgeI digestion (Figure 10B). Complete digestion (of all PCR product) occurs when the mouse has two copies of the mutant PGC-1α 481Gly allele and is thus homozygous, detection of only the full-length PCR product occurs when the mouse is homozygous for the 481Ser allele, and for heterozygotes, there is detection of both the full length and the digested PCR product.

From the F1 generation, we also crossed heterozygote siblings (ex: p338 M x p338 F) from each line for 5 different lines (p338, p481, p482, p154 and p57) to create pure mouse lines. Only 5 of the 8 were selected as founders of these lines tended to produce the most PGC-1α 481Gly-positive mice, thereby increasing our likelihood of quickly obtaining mice homozygous for the mutant PGC-1α 481Gly allele in the next generation. The siblings from these different lines are currently breeding and their resulting pups will create the "test pool" of mice for characterization (Figure 10C). We are interested to generate this test pool so we can test whether the mutant 481Gly mouse PGC-1α variant affects protein stability (481Ser homozygotes vs. 481Gly homozygotes) in isolated islets.

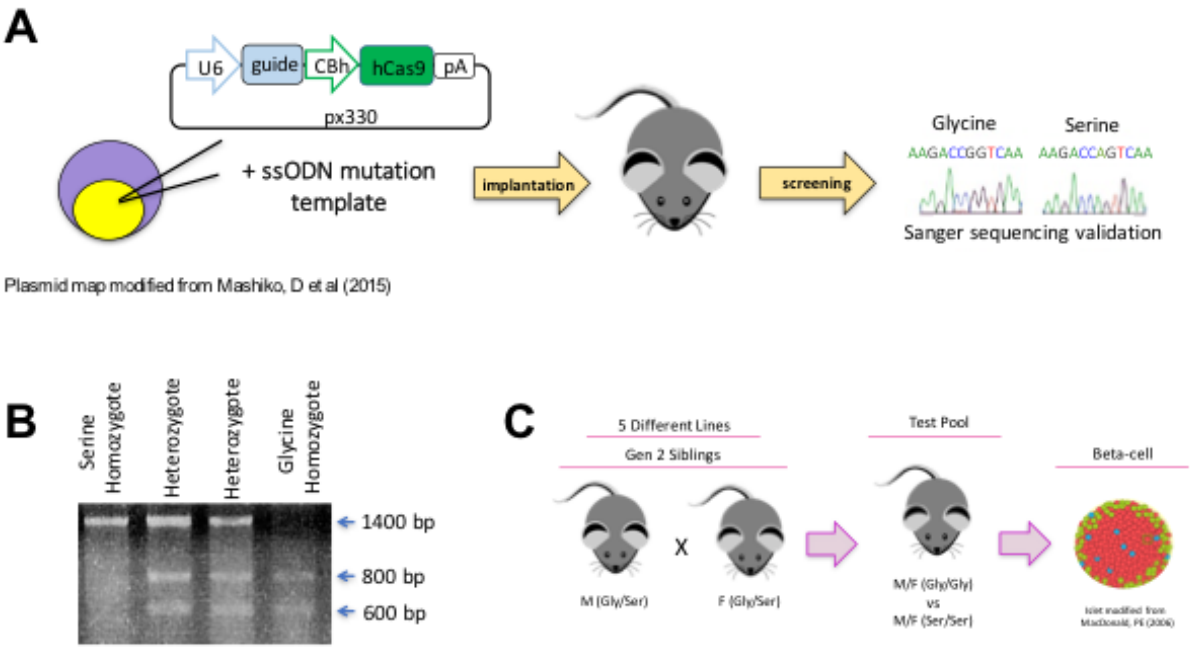


Figure 10: CRISPR strategy used for the generation of the humanized PGC-1α 481Gly mutant mouse line. A. Embryos from B6C3F1 females crossing B6C3F1 males were microinjected with ssODN template and px330 plasmids expressing Cas9 + guideRNA. The microinjected embryos were implanted into pseudo-pregnant female mice. The resulting pups were genotyped using sanger sequencing. **B.** Siblings from four founder lines were bred and the resulting pups were screened using PCR and subsequent Age1 digestion. **C.** The strategy used to assess the impact of the *481Gly* allele *in vivo*

Currently, three lines have produced PGC-1α 481Gly positive mice (Figure 11A), possibly because there is no germline transmission in these lines and mutations in the in the PGC-1α gene. The p57 line was removed because the PGC-1α PCR product was much larger than the anticipated size, indicating that the PGC-1α gene was dramatically mutated beyond the desired A-to-G nucleotide substitution. Although the PCR product for line p154 (not shown) was the correct size, this breeding pair failed to produce any 481Gly-carrying mice so this line was also removed from the test pool. We suspect that there was no germline transmission for these mice, so we have set up a new breeding cage with different p154 heterozygote males (2) and female (1) to try to obtain PGC-1α 481Gly-positive mice. Figure 9A shows the pedigree tree for the current test pool of mice along with the specific genotypes for each line, stratified by sex. The experimental ratio 2(Ser/Ser): 1(Ser/Gly): 0(Gly/Gly) was different than the expected 1(Ser/Ser):2(Gly/Ser):1(Gly/Gly) ratio (Figure 11B). From line p338 we obtained 15 481Ser homozygotes, 11 heterozygotes and 0 481Gly homozygotes (ratio: 1:1:0), from line p481 we obtained 11 481Ser homozygotes, 17 heterozygotes and 1 481Gly homozygote (ratio: 1:1:0*), and from line p482, we obtained 26 481Ser homozygotes, 5 heterozygotes and 0 481Gly homozygotes (ratio: 1:0:0); this is summarized in Figures 11A and B).

Figure 11C shows a summary of the backcrossed mice lines; currently we are breeding mice for the F3 generation.

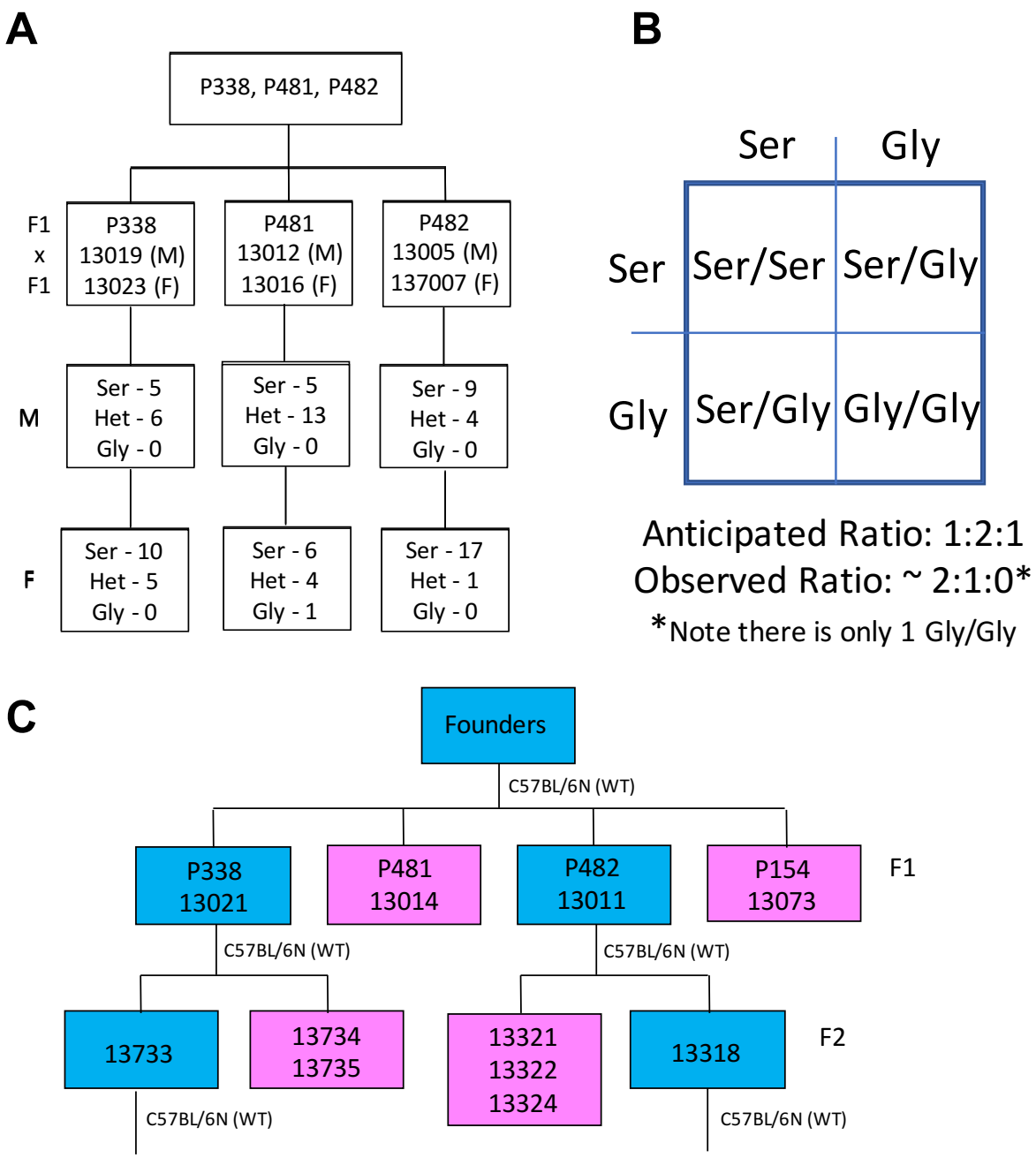


Figure 11: Pedigree charts summarizing the mice genotypes from the test pool and backcrossings. A. Three lines were capable of producing humanized 481Gly positive mice (Male and Female). **B.** Punnent square showing the anticipated genotypic ratio, and the observed ration. **C.** Four lines were backcrossed to select againtst off-target effects from the CRISPR/Cas9 procedure. Males in blue boxes, females in pink boxes.

CHAPTER 2 – DISCUSSION

In summary, we have shown that over expression of the mouse PGC-1α 481Gly and 481Ser variants in INS-1 β-cells affects mouse PGC-1α protein stability similarly to how the analogous amino acid substitution affects the human PGC-1α protein when over expressed in INS1 β-cells (Figure 8). Moreover, previous data shows low PGC-1α results in impaired insulin secretion by β-cells and, when you knock-out PGC-1α, there is impaired mitochondrial function in cultured primary hepatocytes as well as decreased expression of several PGC-1α mitochondrial target genes in white adipocytes [30-32, 116]. We therefore hypothesize that the mouse PGC-1α protein will be stabilized in the humanized PGC-1a 481Gly-expressing mice, resulting in mice that could be resistant to glucolipotoxicity-induced impaired insulin secretion, and/or insulin resistance in the liver and peripheral tissues.

Currently, there are only a handful of studies that look at the impact of whole body PGC-1α modification, and how expression of the mutant PGC-1α 481Gly allele affects metabolic function in mice as a whole [111] Unfortunately, many of these studies do not address the impact of PGC-1α in the pancreas, specifically. The first study that PGC-1α null mice are resistant to HFD-induced obesity and are insulin sensitive, likely due to increased energy expenditure and effects on the brain [116]. A later study used a different PGC-1α knock out mouse model that knocks of canonical PGC-1α while leaving other, shorter PGC-1α variants (i.e PGC-1α4 and NT-PGC-1α) unaffected. The truncated forms still have the LXXLL motifs necessary for interaction with nuclear receptor proteins [120]. These PGC-1α null mice remain insulin sensitive and glucose tolerant in response to HFD challenge, possibly because the N-terminus of PGC-1α can still effectively bind to nuclear

receptors and affect gene expression [120]. Most of the tissue abnormalities detected in these two models are concordant. According to Lin J, et al (2004), PGC-1α null mice only have *observable* abnormalities in brown adipose (lipid droplet accumulation) and brain tissues (lesions in the striatum), while isolated hepatocytes have impaired mitochondrial function and elevated expression of gluconeogenic genes [116]. In the second model of low PGC-1α, hepatic mitochondrial function is decreased and hepatocytes from fasted mice accumulate triglycerides (TG) and are steatotic [120]. They suggest that the steatosis observed in the livers of these mice *could* be attributed to the increase in TG production [120], possibly caused by the effects of other PGC-1α isoforms. With these data in mind, we expect that if the mutant PGC-1α 481Gly variant indeed stabilizes the protein, we could improve mitochondrial function as well as glucose and lipid metabolism of HFD-challenged mice.

Paradoxically, no significant difference in overall insulin sensitivity was found between mice slightly overexpressing human PGC-1α (2-fold), but when assessed on a tissue-by-tissue basis, hepatic insulin sensitivity was impaired while muscle insulin sensitivity was improved [111]. Although the transgenic mice show very little difference from the wildtype, with respect to overall insulin sensitivity, there are no data concerning pancreatic islets, or β-cells, which are responsible for regulating insulin secretion and thus also have an impact on diabetes development. With this in mind, the humanized CRISPR/cas9-generated 481Gly expressing mouse will allow us to compare and contrast, in greater detail, the effects of the mutant mouse PGC-1α 481Gly variant in multiple types. Our model is very beneficial as it allows us to investigate the effects of the polymorphism of the mouse protein under the endogenous mouse promoter.

Another interesting finding from our data was that crossing F1 heterozygotes did not result in the expected 1:2:1 ratio (Figure 11B). Instead, we observed a ratio of 2:1:0 (only 1 481Gly homozygote out of 29 progeny). It is likely that this experimental ratio is dissimilar to the predicted ratio due to off-target, and thus, deleterious effects of the CRISPR/Cas9 method itself and because these mice were only backcrossed once. Our next step will be to breed the males and females from one mouse line with the males and females from other lines to hopefully increase the likelihood of obtaining more 481Gly homozygotes. For our permanent mouse lines, we will be backcrossing at least 5 times (96.875% C57BL/6N) to select against any off-target CRISPR/Cas9 induced mutations (lethal or otherwise). Currently, we have pups from the 3rd backcross (87.5% C57BL/6N background).

For the scope of this project specifically, we are interested to assess the impact of the polymorphism in β-cells on function and health. We suspect that we were unable to generate an INS-1 cell line using CRISPR/Cas9 technology because the efficiency appears to be quite low (2-3%). If we wanted to pursue this model in the future, we will have to screen at least 100 individual clones in order to obtain at least 2-3 mutants.

The remainder of the discussion will be focussed on the CRISPR/Cas9 generated PGC-1α 481Gly-expressing mice. Assuming that the mouse PGC-1α 481Gly protein is stabilized, we will be able to compare static insulin secretion by islets in response to glucose, assess how mitochondrial function in response to different substrates is affected and whether cells have improved ROS detoxification. One caveat for this project is, in humans, the differences in PGC-1α expression and metabolic function between patients that are homozygous for the 482Gly allele and those who carry the 482Ser allele are only

observed when patients are overweight, or obese [42-45]. Therefore, it is possible that these mice will need to be challenged by a high fat, high fructose diet to increase mouse PGC-1α expression or activity to levels where the differences in metabolic function between the genotypes are more striking and/or relevant. However, it is important to remember that while both mice and humans share obesity as a risk factor for T2D; some genes (and SNPs) associated with obesity and glucose metabolism in humans may not necessarily be physiologically relevant in a mouse model [121]. In nature, the PGC-1α 481Gly allele does not naturally occur in mice, which may confound our study. Thus, we must consider that if there is no correlation observed in mice, it does not necessarily mean that the SNP is unimportant in humans.

GENERAL DISCUSSION

Peroxisome proliferator-activated receptor (PPAR)-γ coactivator 1 (PGC-1α) is a transcriptional coactivator that is known to regulate transcription of many metabolic processes. The single nucleotide polymorphism (SNP) within the coding region of human PGC-1α causing a missense mutation encoding either a Gly or Ser at the 482 site is associated with T2D susceptibility. The PGC-1α 482Ser variant increases the relative risk of T2D development in certain populations, and haplotypes containing this SNP have lower indices of β-cell function compared to other variants (including the 482Gly variant). Currently, studies investigating the differential effects of the polymorphism on coactivator activity at the molecular level are in disagreement. The aim of this project was to determine how PGC-1α coactivator function is affected by the Gly482Ser polymorphism in the β-cell.

Based on previous data from our lab, we hypothesized that phosphorylation of the human PGC-1α 482Ser residue leads to decreased coactivator stability and function. Creation of phosphomimetic constructs (Figure 2A-B) was our first piece of evidence that a phosphorylation event at the human PGC-1α 482Ser site could affect coactivator stability. These findings are concordance with the study by Olson et al (2008) that shows phosphorylation at specific residues can destabilize the protein, and lead to proteasomal degradation [90]. We then discovered that MARK4 could possibly phosphorylate the PGC-1α 482Ser residue using a peptide spanning PGC-1α residues 471 to 493 (Figure 5A). Moreover, we found that treatment with the MRT67307 chemical inhibitor of MARK4 stabilized the PGC-1α 482Ser, but not the 482Gly, variant (Figure 5C), although this was only a preliminary experiment and needs to be repeated with a CHX-only control to ensure

that the CHX is fully arresting translation. Nevertheless, this finding was exciting because MARK4 has already been linked to diabetes, obesity and inflammation [105-107]. A mechanism linking MARK4 activity and its association with PGC-1α has not been shown and, to the best of our knowledge, there are no studies investigating a relationship between MARK4 levels and T2D development in humans [105, 107]. Thus, findings from these experiments may open up a new avenue of research focussing on the impact of MARK4 on obesity, metabolism and T2D pathophysiology.

In vitro analyses attempting to elucidate the functional significance of the PGC-1α Gly482Ser polymorphism are sparse and inconclusive. In the HeLa human cell-line, the 482Gly variant has impaired coactivator activity on the Tfam promoter. Interestingly, when we over expressed the two human PGC-1α 482Gly and 482Ser in INS-1 cells, we found that there was no difference in *Tfam* gene expression. Moreover, at the 24 h post-transfection, *Tfam* expression is not increased, yet in the HeLa cells, PGC-1α activity on the Tfam promoter is greater than 2.5-fold above baseline despite similar PGC-1α protein levels [81]. These conflicting findings could mean that the PGC-1α Gly482Ser polymorphism affects coactivator function independently of its impact on protein stability. It is also shown that HepG2 hepatocytes over expressing the PGC-1α 482Ser variant have reduced activity on the PEPCK promoter, compared to the 482Gly variant [80]. Although we have not assessed the effect of the Gly482Ser polymorphism on PEPCK expression in β-cells, it would be interesting to determine, using our mouse model, how the PGC-1α polymorphism influences PEPCK expression.

Using CRISPR/Cas9 technology to generate mice that are homozygous for the PGC-1α(481Gly) variant offers many benefits for this project. It provides us with a

renewable source of primary cells (i.e. islets) to explore the impact of the polymorphism on metabolism, allows us to test the *in vitro* findings presented in Chapter 1 using an *in vivo* model. As mentioned above, we plan to assess the impact of the polymorphism not only on a molecular level (i.e PGC-1α protein stability, MARK4 impact on stability, differential coactivator function of established PGC-1α target genes) but on a physiological level as well.

While there are numerous human studies showing the human PGC-1α 482Ser allele is detrimental for T2D development and impaired β-cell function, other studies show that the effect of the allele is only detrimental when patients are obese [41]. A study by Franks et al (2014) found that the Gly482Ser PGC-1α polymorphism independently associates with increased adiposity and that 482Ser allele carriers have elevated baseline subcutaneous adiposity [42]. Although we find that low PGC-1α in β-cells is detrimental to insulin secretion, it is also possible that the effect of the PGC-1α Gly482Ser polymorphism primarily influences adiposity and insulin sensitivity in other tissues, and that impaired β-cell function secondary to insulin resistance [30, 31]. Perhaps adipose tissue would be more suitable for assessing the effects of the PGC-1α polymorphism at the molecular level. Furthermore, we can determine whether insulin-stimulated glucose uptake is also improved in adipocytes from humanized PGC-1α 481Gly-expressing mice fed a HFHF diet. It is shown that knocking out PGC-1α in white adipocytes decreases the expression of several PGC-1α mitochondrial target genes and thus, increases the prevalence of insulin resistance in these mice compared to their non-mutant counterparts [32].

We would speculate that mice homozygous for the potentially more stable humanized PGC-1α 481Gly variant would be less insulin resistant and have improved coactivation of mitochondrial genes controlling lipid and glucose metabolism. Consequently, we would expect that mice would have improved glucose (and lipid) metabolism which would place less of a burden on β-cells for maintaining glucose homeostasis. This hypothesis fits very nicely with clinical data showing that carriers of the PGC-1α 482Ser allele seem to benefit more from interventions aimed at weight loss, including caloric restriction [50], bariatric surgery [51], and acarbose treatment [52], than PGC-1α Gly482 allele carriers. There is also mouse data showing that decreased PGC-1α in β-cells does not impact whole body glucose tolerance in lean mice [31]. Therefore, low PGC-1α in β-cells may not play a predominant role in increased diabetes risk associated with gene variation, but is likely additive or synergistic with effects on adiposity and insulin sensitivity in other tissues.

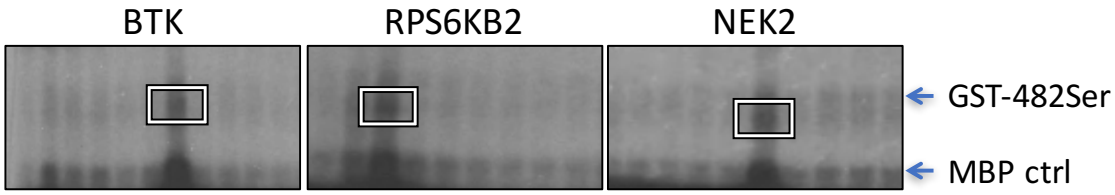
In terms of therapeutic relevance, stabilizing PGC-1α could improve metabolic function in many tissue types. Although our findings are only preliminary, we have identified a kinase whose inactivation may lead to stabilization of the PGC-1α 482Ser protein. Therefore, targeting this kinase may provide a therapeutic strategy to improve metabolic function in patients with the PGC-1α 482Ser allele who are obese and at risk of developing T2D.

CONCLUSIONS AND PERSPECTIVES

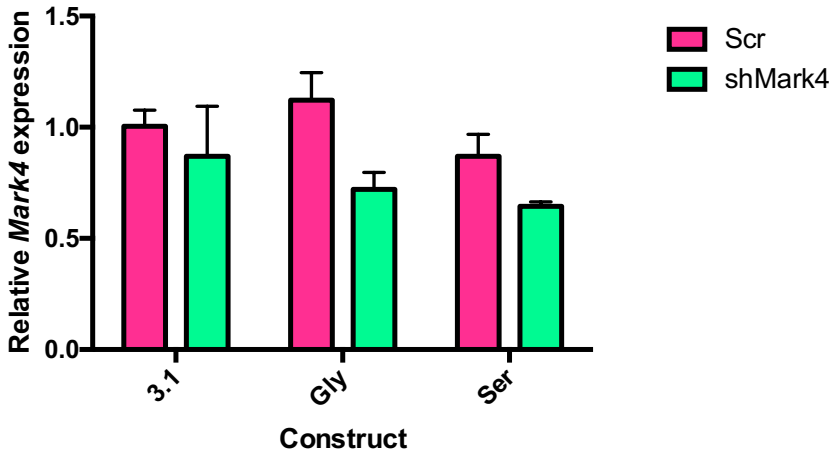
There are many clinical studies showing that there is a correlation between the Gly482Ser polymorphism and increased type 2 diabetes susceptibility, whereby the carriers of the 482Ser allele are shown to have reduced metabolic function, especially in those who are obese. However, little is known about the Gly482Ser substitution and studies focusing on the pathophysiology of the variant are far and few between, particularly concerning its function within the β-cell. Our data strongly suggest that substitution of a glycine for the serine residue in human and mouse PGC-1a creates a phosphorylation site and potentiates protein degradation. Our preliminary data suggest that the 482Ser variant of PGC-1α is a site of MARK4 phosphorylation, and that this phosphorylation event drives the protein destabilization, which may explain the observed impairment in coactivation of specific OXPHOS and antioxidant genes with the 482Ser variant. Using the CRISPR/Cas9-generated mice as described in chapter 2, we will investigate these theories *in vivo*, as well as the physiological relevance the polymorphism has on whole body metabolism, if any.

In summary, our study with this particular polymorphism of *PPARGC1A* highlights the importance of identifying the molecular consequences of gene mutations on protein stability and/or function in order to accurately identify potential targets for therapeutics and the target population that will most benefit from them. Furthermore, this work may facilitate simple genetic screening to identify subjects at heightened risk of diabetic complications and those most likely to benefit from therapies targeting this pathway.

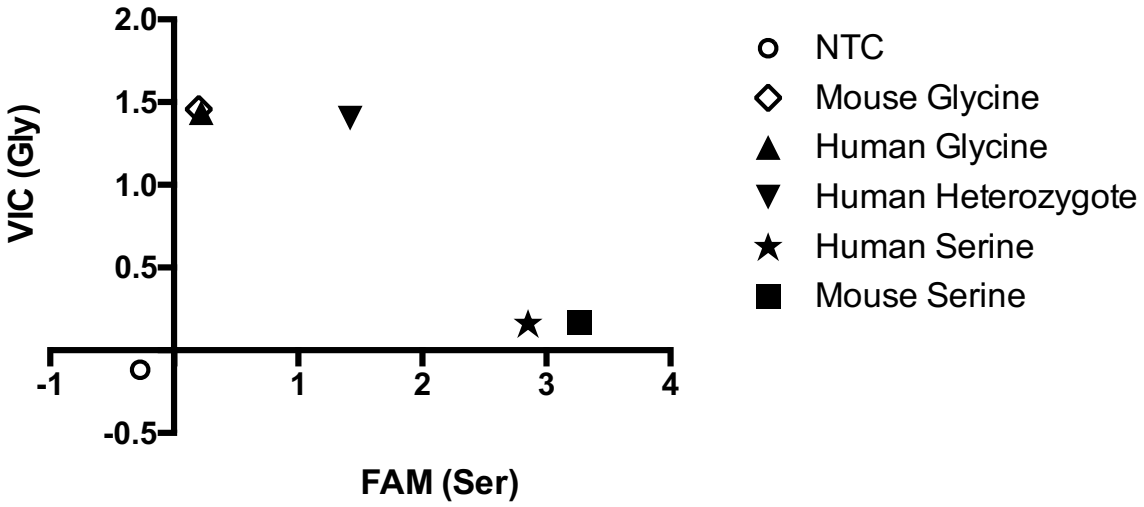
SUPPLEMENTARY FIGURES



Supplementary Figure 1 : BTK, RPS6KB2 and NEK2 kinases also phosphorylated the 482Ser residue. The GST-482Ser fusion peptide were incubated separately with 420 different kinases and γ^{32}phosphate ATP. The bands detected (white square) indicate a phosphorylated substrate. MBP was an internal control that is heavily phosphorylated to ensure assay function and kinase specificity. MBP bands are similarly dark compared to the GST-482Ser bands; BTK, RPS6KB2 and NEK2 are non-specific kinases for the GST-482Ser peptide.



Supplementary Figure 2 : shMARK4 constructs do not sufficiently knockdown MARK4 kinase. INS-1 cells over expressing the human PGC-1α 482Gly or Ser (or pcDNA 3.1+ empty vector control) with the shMARK4 plasmid did not have a significant decrease in *MARK4* expression compared to INS-1 cells over expressing the human PGC-1α 482Gly or Ser (or pcDNA 3.1+ empty vector control) with the scramble (Scr) plasmid.



Supplementary Figure 3: Developping a protocol to genotype different cell and tissue types for the PGC-1α Gly482Ser polymorphism. cDNA from INS-1 cells over expressing human PGC-1α 482Gly and Ser or mouse PGC-1α 481Gly and Ser variants was genotyped using the TaqMan Genotyping kit. The results are shown as the relative fluorescence of the VIC (Gly-specific) and FAM (Ser-specific) dyes against the internal ROX dye control.

REFERENCES

1. Organization., W.H., *Global report on diabetes*, in 2016, World Health Organization: France.
2. Reinehr, T., *Type 2 diabetes mellitus in children and adolescents.* World Journal of Diabetes, 2013. **4**(6): p. 270-281.
3. Ye, J., *Mechanisms of insulin resistance in obesity.* Frontiers of medicine, 2013. **7**(1): p. 14-24.
4. Bouzakri, K. and J.R. Zierath, *MAP4K4 gene silencing in human skeletal muscle prevents tumor necrosis factor-alpha-induced insulin resistance.* J Biol Chem, 2007. **282**(11): p. 7783-9.
5. Daniele, G., et al., *Chronic Reduction of Plasma Free Fatty Acid Improves Mitochondrial Function and Whole-Body Insulin Sensitivity in Obese and Type 2 Diabetic Individuals.* Diabetes, 2014. **63**(8): p. 2812-2820.
6. Rui, L., *Energy Metabolism in the Liver.* Compr Physiol, 2014. **4**(1): p. 177-97.
7. Savage, D.B., K.F. Petersen, and G.I. Shulman, *Disordered Lipid Metabolism and the Pathogenesis of Insulin Resistance.* Physiological reviews, 2007. **87**(2): p. 507-520.
8. Kahn, S.E., *The importance of the beta-cell in the pathogenesis of type 2 diabetes mellitus.* Am J Med, 2000. **108 Suppl 6a**: p. 2s-8s.
9. Barnett, A.H., et al., *Diabetes in identical twins. A study of 200 pairs.* Diabetologia, 1981. **20**(2): p. 87-93.
10. Newman, B., et al., *Concordance for type 2 (non-insulin-dependent) diabetes mellitus in male twins.* Diabetologia, 1987. **30**(10): p. 763-8.
11. Raciti, G.A., et al., *Understanding type 2 diabetes: from genetics to epigenetics.* Acta Diabetologica, 2015. **52**(5): p. 821-827.
12. Wang, X., et al., *Genetic markers of type 2 diabetes: Progress in genome-wide association studies and clinical application for risk prediction.* J Diabetes, 2016. **8**(1): p. 24-35.
13. Ali, O., *Genetics of type 2 diabetes.* World Journal of Diabetes, 2013. **4**(4): p. 114-123.
14. Replication, D.A.G. and A.G.E.N.T.D.C.S.A.T.D.C.M.A.T.D.C.T.D.G.E.b.N.-g.s.i.m.-E. Meta-analysis Consortium, *Genome-wide trans-ancestry meta-analysis provides insight into the genetic architecture of type 2 diabetes susceptibility.* Nature genetics, 2014. **46**(3): p. 234-244.
15. Pratley, R.E., et al., *An autosomal genomic scan for loci linked to prediabetic phenotypes in Pima Indians.* J Clin Invest, 1998. **101**(8): p. 1757-64.
16. Muller, Y.L., et al., *A Gly482Ser missense mutation in the peroxisome proliferator-activated receptor gamma coactivator-1 is associated with altered lipid oxidation and early insulin secretion in Pima Indians.* Diabetes, 2003. **52**(3): p. 895-8.
17. Puigserver, P., et al., *A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis.* Cell, 1998. **92**(6): p. 829-39.
18. Wu, Z., et al., *Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1.* Cell, 1999. **98**(1): p. 115-24.
19. Fernandez-Marcos, P.J. and J. Auwerx, *Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis.* Am J Clin Nutr, 2011. **93**(4): p. 884s-90.

20. Devarakonda, S., et al., *Disorder-to-order transition underlies the structural basis for the assembly of a transcriptionally active PGC-1alpha/ERRgamma complex.* Proc Natl Acad Sci U S A, 2011. **108**(46): p. 18678-83.
21. Wallberg, A.E., et al., *Coordination of p300-Mediated Chromatin Remodeling and TRAP/Mediator Function through Coactivator PGC-1α.* Molecular Cell, 2003. **12**(5): p. 1137-1149.
22. Finck, B.N. and D.P. Kelly, *PGC-1 coactivators: inducible regulators of energy metabolism in health and disease.* Journal of Clinical Investigation, 2006. **116**(3): p. 615-622.
23. Kleiner, S., et al., *PPAR{delta} agonism activates fatty acid oxidation via PGC-1{alpha} but does not increase mitochondrial gene expression and function.* J Biol Chem, 2009. **284**(28): p. 18624-33.
24. Vega, R.B., J.M. Huss, and D.P. Kelly, *The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes.* Mol Cell Biol, 2000. **20**(5): p. 1868-76.
25. Wu, H., et al., *PGC-1α, glucose metabolism and type 2 diabetes mellitus.* Journal of Endocrinology, 2016. **229**(3): p. R99-R115.
26. Wende, A.R., et al., *A Role for the Transcriptional Coactivator PGC-1α in Muscle Refueling.* Journal of Biological Chemistry, 2007. **282**(50): p. 36642-36651.
27. Rhee, J., et al., *Partnership of PGC-1alpha and HNF4alpha in the regulation of lipoprotein metabolism.* J Biol Chem, 2006. **281**(21): p. 14683-90.
28. Sczelecki, S., et al., *Loss of Pgc-1alpha expression in aging mouse muscle potentiates glucose intolerance and systemic inflammation.* Am J Physiol Endocrinol Metab, 2014. **306**(2): p. E157-67.
29. Correia, J.C., D.M. Ferreira, and J.L. Ruas, *Intercellular: local and systemic actions of skeletal muscle PGC-1s.* Trends Endocrinol Metab, 2015. **26**(6): p. 305-14.
30. Ling, C., et al., *Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion.* Diabetologia, 2008. **51**(4): p. 615-622.
31. Oropeza, D., et al., *PGC-1 coactivators in β-cells regulate lipid metabolism and are essential for insulin secretion coupled to fatty acids.* Molecular Metabolism, 2015. **4**(11): p. 811-822.
32. Kleiner, S., et al., *Development of insulin resistance in mice lacking PGC-1alpha in adipose tissues.* Proc Natl Acad Sci U S A, 2012. **109**(24): p. 9635-40.
33. Altshuler, D., et al., *The common PPARgamma Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes.* Nat Genet, 2000. **26**(1): p. 76-80.
34. Ruchat, S.M., et al., *Evidence for interaction between PPARG Pro12Ala and PPARGC1A Gly482Ser polymorphisms in determining type 2 diabetes intermediate phenotypes in overweight subjects.* Exp Clin Endocrinol Diabetes, 2009. **117**(9): p. 455-9.
35. Ek, J., et al., *Mutation analysis of peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1) and relationships of identified amino acid polymorphisms to Type II diabetes mellitus.* Diabetologia, 2001. **44**(12): p. 2220-2226.
36. Fosslien, E.E., *Mitochondrial medicine--molecular pathology of defective oxidative phosphorylation.* Annals of Clinical and Laboratory Science. **31**(1): p. 25-67.

37. Fanelli, M., et al., *The Gly482Ser missense mutation of the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 alpha) gene associates with reduced insulin sensitivity in normal and glucose-intolerant obese subjects.* Dis Markers, 2005. **21**(4): p. 175-80.
38. Hara, K., et al., *A genetic variation in the PGC-1 gene could confer insulin resistance and susceptibility to Type II diabetes.* Diabetologia, 2002. **45**(5): p. 740-3.
39. Esterbauer, H., et al., *Peroxisome Proliferator-Activated Receptor-γ Coactivator-1 Gene Locus.* Diabetes, 2002. **51**(4): p. 1281.
40. Pihlajamaki, J., et al., *Haplotypes of PPARGC1A are associated with glucose tolerance, body mass index and insulin sensitivity in offspring of patients with type 2 diabetes.* Diabetologia, 2005. **48**(7): p. 1331-4.
41. Povel, C.M., et al., *Glucose levels and genetic variants across transcriptional pathways: interaction effects with BMI.* Int J Obes (Lond), 2010. **34**(5): p. 840-5.
42. Franks, P.W., et al., *Common variation at PPARGC1A/B and change in body composition and metabolic traits following preventive interventions: the Diabetes Prevention Program.* Diabetologia, 2014. **57**(3): p. 485-90.
43. Ha, C.D., et al., *Relationship of PGC-1alpha gene polymorphism with insulin resistance syndrome in Korean children.* Asia Pac J Public Health, 2015. **27**(2): p. Np544-51.
44. Weng, S.-W., et al., *Gly482Ser polymorphism in the peroxisome proliferator–activated receptor γ coactivator–1α gene is associated with oxidative stress and abdominal obesity.* Metabolism - Clinical and Experimental. **59**(4): p. 581-586.
45. Vázquez-Del Mercado, M., et al., *The 482Ser of PPARGC1A and 12Pro of PPARG2 Alleles Are Associated with Reduction of Metabolic Risk Factors Even Obesity in a Mexican-Mestizo Population.* BioMed Research International, 2015. **2015**: p. 285491.
46. Deeb, S.S. and J.D. Brunzell, *The Role of the PGC1α Gly482Ser Polymorphism in Weight Gain due to Intensive Diabetes Therapy.* PPAR Research, 2009. **2009**: p. 649286.
47. Ambye, L., et al., *Studies of the Gly482Ser polymorphism of the peroxisome proliferator-activated receptor gamma coactivator 1alpha (PGC-1alpha) gene in Danish subjects with the metabolic syndrome.* Diabetes Res Clin Pract, 2005. **67**(2): p. 175-9.
48. Vimaleswaran, K.S., et al., *Effect of polymorphisms in the PPARGC1A gene on body fat in Asian Indians.* Int J Obes, 2006. **30**(6): p. 884-891.
49. Franks, P.W., et al., *PPARGC1A coding variation may initiate impaired NEFA clearance during glucose challenge.* Diabetologia, 2007. **50**(3): p. 569-573.
50. Goyenechea, E., et al., *Enhanced short-term improvement of insulin response to a low-caloric diet in obese carriers the Gly482Ser variant of the PGC-1alpha gene.* Diabetes Res Clin Pract, 2008. **82**(2): p. 190-6.
51. Geloneze, S.R., et al., *PGC1[alpha] gene Gly482Ser polymorphism predicts improved metabolic, inflammatory and vascular outcomes following bariatric surgery.* Int J Obes, 2012. **36**(3): p. 363-368.

52. Andrulionyte, L., et al., *Common polymorphisms of the PPAR-gamma2 (Pro12Ala) and PGC-1alpha (Gly482Ser) genes are associated with the conversion from impaired glucose tolerance to type 2 diabetes in the STOP-NIDDM trial.* Diabetologia, 2004. **47**(12): p. 2176-84.
53. Zhang, K.H., et al., *Effects of the peroxisome proliferator activated receptor-gamma coactivator-1alpha (PGC-1alpha) Thr394Thr and Gly482Ser polymorphisms on rosiglitazone response in Chinese patients with type 2 diabetes mellitus.* J Clin Pharmacol, 2010. **50**(9): p. 1022-30.
54. Yoon, J.C., et al., *Suppression of β Cell Energy Metabolism and Insulin Release by PGC-1α.* Developmental Cell, 2003. **5**(1): p. 73-83.
55. Valtat, B., et al., *Fetal PGC-1α Overexpression Programs Adult Pancreatic β-Cell Dysfunction.* Diabetes, 2013. **62**(4): p. 1206-1216.
56. Barroso, I., et al., *Meta-analysis of the Gly482Ser variant in PPARGC1A in type 2 diabetes and related phenotypes.* Diabetologia, 2006. **49**(3): p. 501-5.
57. Ballestri, S., et al., *Nonalcoholic fatty liver disease is associated with an almost twofold increased risk of incident type 2 diabetes and metabolic syndrome. Evidence from a systematic review and meta-analysis.* J Gastroenterol Hepatol, 2016. **31**(5): p. 936-44.
58. Lin, Y.C., et al., *A common variant in the peroxisome proliferator-activated receptor-gamma coactivator-1alpha gene is associated with nonalcoholic fatty liver disease in obese children.* Am J Clin Nutr, 2013. **97**(2): p. 326-31.
59. Tai, C.M., et al., *Derivation and validation of a scoring system for predicting nonalcoholic steatohepatitis in Taiwanese patients with severe obesity.* Surg Obes Relat Dis, 2016.
60. Gancheva, S., et al., *Variants in Genes Controlling Oxidative Metabolism Contribute to Lower Hepatic ATP Independent of Liver Fat Content in Type 1 Diabetes.* Diabetes, 2016. **65**(7): p. 1849.
61. Ingelsson, E., et al., *The PPARGC1A Gly482Ser polymorphism is associated with left ventricular diastolic dysfunction in men.* BMC Cardiovasc Disord, 2008. **8**: p. 37.
62. Cheurfa, N., et al., *The Gly482Ser polymorphism in the peroxisome proliferator-activated receptor-gamma coactivator-1 gene is associated with hypertension in type 2 diabetic men.* Diabetologia, 2004. **47**(11): p. 1980-3.
63. Okauchi, Y., et al., *PGC-1alpha Gly482Ser polymorphism is associated with the plasma adiponectin level in type 2 diabetic men.* Endocr J, 2008. **55**(6): p. 991-7.
64. Ridderstrale, M., et al., *Increased risk of obesity associated with the variant allele of the PPARGC1A Gly482Ser polymorphism in physically inactive elderly men.* Diabetologia, 2006. **49**(3): p. 496-500.
65. Soyal, S., et al., *PGC-1alpha: a potent transcriptional cofactor involved in the pathogenesis of type 2 diabetes.* Diabetologia, 2006. **49**(7): p. 1477-88.
66. Maciejewska, A., et al., *The PPARGC1Agene Gly482Ser in Polish and Russian athletes.* Journal of Sports Sciences, 2012. **30**(1): p. 101-113.
67. Norbert, S., et al., *Genetic Variations in PPARD and PPARGC1A Determine Mitochondrial Function and Change in Aerobic Physical Fitness and Insulin Sensitivity during Lifestyle Intervention.* The Journal of Clinical Endocrinology \& Metabolism, 2007. **92**(5): p. 1827-1833.

68. Tural, E., et al., *PPAR-α and PPARGC1A gene variants have strong effects on aerobic performance of Turkish elite endurance athletes.* Molecular Biology Reports, 2014. **41**(9): p. 5799-5804.
69. Eynon, N., et al., *Do PPARGC1A and PPARα polymorphisms influence sprint or endurance phenotypes?* Scandinavian Journal of Medicine & Science in Sports, 2010. **20**(1): p. e145-e150.
70. Kunej, T.T., *A Gly482Ser polymorphism of the peroxisome proliferator-activated receptor-gamma coactivator-1 (PGC-1) gene is associated with type 2 diabetes in Caucasians.* Folia biologica, 2004. **50**(5): p. 157-8.
71. Myles, S., et al., *Testing the thrifty gene hypothesis: the Gly482Ser variant in PPARGC1A is associated with BMI in Tongans.* BMC Medical Genetics, 2011. **12**: p. 10-10.
72. Cadzow, M., et al., *Lack of direct evidence for natural selection at the candidate thrifty gene locus, PPARGC1A.* BMC Medical Genetics, 2016. **17**: p. 80.
73. Bhat, A., et al., *PGC-1alpha Thr394Thr and Gly482Ser variants are significantly associated with T2DM in two North Indian populations: a replicate case-control study.* Hum Genet, 2007. **121**(5): p. 609-14.
74. Rai, E.E., *Interaction between the UCP2-866G/A, mtDNA 10398G/A and PGC1alpha p.Thr394Thr and p.Gly482Ser polymorphisms in type 2 diabetes susceptibility in North Indian population.* Human Genetics. **122**(5): p. 535-40.
75. Shokouhi, S., *Association between PGC-1alpha gene polymorphisms and type 2 diabetes risk: a case-control study of an Iranian population.* Canadian Journal of Diabetes. **39**(1): p. 65-72.
76. Sun, L., et al., *The Gly482Ser variant of the PPARGC1 gene is associated with Type 2 diabetes mellitus in northern Chinese, especially men.* Diabet Med, 2006. **23**(10): p. 1085-92.
77. Jemaa, Z., et al., *The Gly482Ser polymorphism of the peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) is associated with type 2 diabetes in Tunisian population.* Diabetes Metab Syndr, 2015. **9**(4): p. 316-9.
78. Sano, M., et al., *Intramolecular control of protein stability, subnuclear compartmentalization, and coactivator function of peroxisome proliferator-activated receptor gamma coactivator 1alpha.* J Biol Chem, 2007. **282**(35): p. 25970-80.
79. Nitz, I., et al., *Analysis of PGC-1alpha variants Gly482Ser and Thr612Met concerning their PPARgamma2-coactivation function.* Biochem Biophys Res Commun, 2007. **353**(2): p. 481-6.
80. Chen, Y., et al., *Gly482Ser mutation impairs the effects of peroxisome proliferator–activated receptor γ coactivator-1α on decreasing fat deposition and stimulating phosphoenolpyruvate carboxykinase expression in hepatocytes.* Nutrition Research, 2013. **33**(4): p. 332-339.
81. Choi, Y.S., et al., *Impaired coactivator activity of the Gly482 variant of peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1alpha) on mitochondrial transcription factor A (Tfam) promoter.* Biochem Biophys Res Commun, 2006. **344**.

82. Besse-Patin, A., et al., *Estrogen Signals through PPARG coactivator 1 alpha to Reduce Oxidative Damage Associated with Diet-induced Fatty Liver Disease.* Gastroenterology.
83. Ling, C., et al., *Multiple environmental and genetic factors influence skeletal muscle PGC-1α and PGC-1β gene expression in twins.* The Journal of Clinical Investigation, 2004. **114**(10): p. 1518-1526.
84. Melloul, D. and M. Stoffel, *Regulation of Transcriptional Coactivator PGC-1{alpha}.* Science of Aging Knowledge Environment, 2004. **2004**(9): p. pe9.
85. Akimoto, T., et al., *Exercise stimulates Pgc-1alpha transcription in skeletal muscle through activation of the p38 MAPK pathway.* J Biol Chem, 2005. **280**(20): p. 19587-93.
86. Akimoto, T., et al., *Skeletal muscle adaptation in response to voluntary running in Ca2+/calmodulin-dependent protein kinase IV-deficient mice.* Am J Physiol Cell Physiol, 2004. **287**(5): p. C1311-9.
87. Gomez-Ambrosi, J., G. Fruhbeck, and J.A. Martinez, *Rapid in vivo PGC-1 mRNA upregulation in brown adipose tissue of Wistar rats by a beta(3)-adrenergic agonist and lack of effect of leptin.* Mol Cell Endocrinol, 2001. **176**(1-2): p. 85-90.
88. Puigserver, P., et al., *Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1.* Mol Cell, 2001. **8**(5): p. 971-82.
89. Holt, L.J., *Regulatory modules: Coupling protein stability to phopshoregulation during cell division.* FEBS Letters, 2012. **586**(17): p. 2773-2777.
90. Olson, B.L., et al., *SCF(Cdc4) acts antagonistically to the PGC-1α transcriptional coactivator by targeting it for ubiquitin-mediated proteolysis.* Genes & Development, 2008. **22**(2): p. 252-264.
91. Anderson, R.M., et al., *Dynamic regulation of PGC-1α localization and turnover implicates mitochondrial adaptation in calorie restriction and the stress response.* Aging Cell, 2008. **7**(1): p. 101-111.
92. Rovira-Llopis, S., et al., *Mitochondrial dynamics in type 2 diabetes: Pathophysiological implications.* Redox Biology, 2017. **11**: p. 637-645.
93. Patti, M.E., et al., *Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1.* Proceedings of the National Academy of Sciences, 2003. **100**(14): p. 8466-8471.
94. Mootha, V.K., et al., *PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes.* 2003. **34**: p. 267.
95. Westerbacka, J., et al., *Genes Involved in Fatty Acid Partitioning and Binding, Lipolysis, Monocyte/Macrophage Recruitment, and Inflammation Are Overexpressed in the Human Fatty Liver of Insulin-Resistant Subjects.* Diabetes, 2007. **56**(11): p. 2759.
96. Hussain, M.A., et al., *Increased Pancreatic β-Cell Proliferation Mediated by CREB Binding Protein Gene Activation.* Molecular and Cellular Biology, 2006. **26**(20): p. 7747-7759.
97. Zhang, P., et al., *Free fatty acids increase PGC-1α expression in isolated rat islets.* FEBS Letters, 2005. **579**(6): p. 1446-1452.
98. Kim, J.W., et al., *Suppression of peroxisome proliferator-activated receptor gamma-coactivator-1alpha normalizes the glucolipotoxicity-induced decreased*

BETA2/NeuroD gene transcription and improved glucose tolerance in diabetic rats. Endocrinology, 2009. **150**(9): p. 4074-83.
99. Khan, N., *Exploring the regulation of PGC-1α-investigating the polymorphism of PGC-1α and the PGC-1α4 isoform*, in *Department of Medicine*. 2015, McGill University.
100. Sharova, L.V., et al., *Database for mRNA Half-Life of 19 977 Genes Obtained by DNA Microarray Analysis of Pluripotent and Differentiating Mouse Embryonic Stem Cells.* DNA Research, 2009. **16**(1): p. 45-58.
101. Shinoda, K., et al., *Phosphoproteomics Identifies CK2 as a Negative Regulator of Beige Adipocyte Thermogenesis and Energy Expenditure.* Cell metabolism, 2015. **22**(6): p. 997-1008.
102. Li, X., et al., *Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1alpha transcription coactivator.* Nature, 2007. **447**(7147): p. 1012-6.
103. Weiss, W., F. Weiland, and A. Gorg, *Protein detection and quantitation technologies for gel-based proteome analysis.* Methods Mol Biol, 2009. **564**: p. 59-82.
104. Hurov, J. and H. Piwnica-Worms, *The Par-1/MARK Family of Protein Kinases: From Polarity to Metabolism.* Cell Cycle, 2007. **6**(16): p. 1966-1969.
105. Liu, Z., et al., *Mark4 promotes oxidative stress and inflammation via binding to PPARγ and activating NF-κB pathway in mice adipocytes.* Scientific Reports, 2016. **6**: p. 21382.
106. Feng, M., et al., *Mark4 promotes adipogenesis and triggers apoptosis in 3T3-L1 adipocytes by activating JNK1 and inhibiting p38MAPK pathways.* Biology of the Cell, 2014. **106**(9): p. 294-307.
107. Sun, C., et al., *Inactivation of MARK4, an AMP-activated protein kinase (AMPK)-related kinase, leads to insulin hypersensitivity and resistance to diet-induced obesity.* J Biol Chem, 2012. **287**(45): p. 38305-15.
108. Clark, K., et al., *Phosphorylation of CRTC3 by the salt-inducible kinases controls the interconversion of classically activated and regulatory macrophages.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(42): p. 16986-16991.
109. Mootha, V.K., et al., *Erralpha and Gabpa/b specify PGC-1alpha-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle.* Proc Natl Acad Sci U S A, 2004. **101**(17): p. 6570-5.
110. Christian, F., E.L. Smith, and R.J. Carmody, *The Regulation of NF-κB Subunits by Phosphorylation.* Cells, 2016. **5**(1): p. 12.
111. Liang, H., et al., *Whole body overexpression of PGC-1α has opposite effects on hepatic and muscle insulin sensitivity.* American Journal of Physiology - Endocrinology and Metabolism, 2009. **296**(4): p. E945-E954.
112. Ek, J., et al., *Mutation analysis of peroxisome proliferator-activated receptor-gamma coactivator-1 (PGC-1) and relationships of identified amino acid polymorphisms to Type II diabetes mellitus.* Diabetologia, 2001. **44**.
113. Choi, Y.-S., et al., *Impaired coactivator activity of the Gly482 variant of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) on mitochondrial transcription factor A (Tfam) promoter.* Biochemical and Biophysical Research Communications, 2006. **344**(3): p. 708-712.

114. Graham, D.B. and D.E. Root, *Resources for the design of CRISPR gene editing experiments.* Genome Biology, 2015. **16**: p. 260.
115. Skarnes, W.C., *Is mouse embryonic stem cell technology obsolete?* Genome Biology, 2015. **16**(1): p. 109.
116. Lin, J., et al., *Defects in Adaptive Energy Metabolism with CNS-Linked Hyperactivity in PGC-1α Null Mice.* Cell, 2004. **119**(1): p. 121-135.
117. Ran, F.A., et al., *Genome engineering using the CRISPR-Cas9 system.* Nat Protoc, 2013. **8**(11): p. 2281-2308.
118. Schwartz, C.M., et al., *Synthetic RNA Polymerase III Promoters Facilitate High-Efficiency CRISPR–Cas9-Mediated Genome Editing in Yarrowia lipolytica.* ACS Synthetic Biology, 2016. **5**(4): p. 356-359.
119. Wang, B., et al., *Highly efficient CRISPR/HDR-mediated knock-in for mouse embryonic stem cells and zygotes.* Biotechniques, 2015. **59**(4): p. 201-2, 204, 206-8.
120. Leone, T.C., et al., *PGC-1α Deficiency Causes Multi-System Energy Metabolic Derangements: Muscle Dysfunction, Abnormal Weight Control and Hepatic Steatosis.* PLoS Biology, 2005. **3**(4): p. e101.
121. King, A.J.F., *The use of animal models in diabetes research.* British Journal of Pharmacology, 2012. **166**(3): p. 877-894.