Exploring the regulation of PGC-1α – Investigating the Gly482Ser Polymorphism of PGC-1α and the PGC-1α4 isoform

Naveen P. Khan

Faculty of Medicine
Division of Experimental Medicine
McGill University
Montreal, Quebec, Canada

Supervisor: Dr. Jennifer L. Estall
Unite de mecanismes moleculelaires du diabete
Institut de recherches cliniques de Montreal (IRCM)

A thesis submitted to McGill University in partial fulfillment of the requirements of the Master of Science degree

Table of Contents 1

Abstract 4

Résumé 6

Acknowledgements 8

List of Abbreviations 9

Chapter 1 - General Introduction 12

Diabetes: A global health crisis of growing concern 13
Type 1 Diabetes 14
Hallmarks of Type 2 Diabetes 16
Hereditary components of Type 2 Diabetes risk 18
Primer to PGC-1α 20
Association of PGC-1α with diabetes 22
Figure 1 - PGC-1α is linked through various tissues with diabetes risk and glucose homeostasis 24
Isoforms of PGC-1α 25
PGC-1α structure and Post-Translational Modifications 26
Figure 2 - Structure of PGC-1α and post translational modifications 29

Chapter 2 - The impact of the Gly482Ser Polymorphism on the PGC-1α Protein 31

Section I - Introduction 31
The PGC-1α Gly482Ser polymorphism and the link with Type 2 Diabetes 32
Table 1 - Risk of diabetes associated with the Gly482Ser polymorphism varies with ethnicity 33
Figure 3 - The Gly482Ser Polymorphism 36
The impact of the Gly482Ser polymorphism on fitness and other metabolic factors 37
Figure 4 - Summary of various phenotypes associated with the Gly482Ser polymorphism 42
Interaction with other polymorphisms associated with diabetes risk 43
Influence on responsiveness to treatment 43
Table 2 - Summary of interactions of Gly482Ser PGC-1α with other polymorphsims 44
Beta cells and the Gly482Ser SNP 46
Studies addressing the functional impact of the Gly482Ser SNP 47
Table 3 - Summary of mechanistic studies 48
Objectives 50
Figure 5 - Known domains of interaction of PGC-1α 51

52

Section II - Methods
Cell culture and treatments 53
Western blot 53
RNA isolation and cDNA synthesis 53
Quantitative Real-Time PCR 54
Site-directed mutagenesis 54
Statistical Analysis method 55

Section III - Results 56
482Gly hPGC-1α protein is more stable than 482Ser hPGC-1α 57
Figure 6 - Lower protein levels of PGC-1α 482Ser detected than PGC-1α 482Gly 58
Proteosomal Inhibitor MG132 restored 482Ser protein 59
Figure 7 - Treatment with proteosome inhibitor MG132 recovers PGC-1α 482Ser 60
482Gly and 482Ser variants have different half-lives 61
Figure 8 - PGC-1α 482Ser has a shorter half-life than PGC-1α 482Gly 62
In silico determination that the Serine at position 482 is a putative phospho-site 63
Figure 9 - In silico analysis to predict putative post translational modification 64
hPGC-1α 482Asp exhibits reduced stability similar to hPGC-1α 482Ser 65
Sirt1 and Tfam are differentially regulated by the two variants 66
Figure 10 Phosphomimic mutant of PGC-1α at amino acid 482 is less stable 64
Figure 11 - Sirt1 and Tfam are differently regulated by 482Gly and 482Ser PGC-1α 67

Section IV - Discussion 70
Summary of Results 71
Difference in stability of PGC-1α due to the Gly482Ser polymorphism 72
Gly482Ser as a putative phosphorylation site of PGC-1α 74
Differential coactivator function of Gly482Ser PGC-1α 76

Chapter 3 - Localization of PGC-1α4 in response to TNFα 81

Section I - Introduction 81
An introduction to PGC-1α4 82
Hepatocytes and inflammation 84
Significance of protein localization 85
Figure 12 - Summary of differences in protein sequence of PGC-1α1, PGC-1α4 and NT-PGC-1α 86
Objectives 87

Section II - Methods 88
Cell culture and treatments 89
Western blot 89
RNA isolation and cDNA synthesis 89

Quatitative Real Time PCR 90
Immunoflourescence 90
Cellular Fractionation 91
Statistical Analysis method 91

Section III - Results 92
Figure 13- PGC-1α4 is predominantly cytoplasmic in H2.35 cells 93
PGC-1α4 is primarily localized in the cytoplasm in hepatocytes 94
PGC-1α1 and PGC-1α4 are stabilized by short treatment with TNFα 95
Figure 14 - TNFα stabilizes protein of both isoforms 96
PGC-1α4 localization is influenced by TNFα 97
Figure 15 - PGC-1α4 localization is influenced by TNFα 98
Cytochrome C is differentially regulated by the two isoforms in hepatocytes 99
Figure 16 - Influence of overexpression of PGC-1α1 and PGC-1α4 on endogenous genes 101

Section IV - Discussion 102
Summary of results 103
Localization of PGC-1α4 104
Influence of TNFα on the protein and coativator function of PGC-1α4 106

Chapter 4 - Perspectives and Conclusions 109

Summary of conclusions 110
The link between the Gly482Ser polymorphism and PGC-1α and diabetes 111
Figure 17 - Potential effect of the Gly482Ser polymorphism of PGC-1α in beta cells 115
The localization of PGC-1α4 and its translocation on TNFα treatment 116
Figure 18 – Potential effect of TNFα on PGC-1α4 119
Perspectives 120

References 121

Appendix 137
Primers used for qPCR 138
Primers used for cloning 139

Abstract

The peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α) co-activates various genes essential to oxidative metabolism, mitochondrial biogenesis and function, reactive oxygen species detoxification and angiogenesis. PGC-1α is expressed in various metabolically active tissues, and its expression is reduced in diabetic patients. This transcriptional co-activator has several isoforms with overlapping functions; a more recently described truncated isoform, PGC-1α4, plays a distinct but complementary role to canonical PGC-1α1, and its role and characteristics in different tissues are yet to be explored.

The Gly482Ser polymorphism of PGC-1α is associated with an increased risk of developing diabetes, as well as reduced fitness and increased adiposity, and it is associated with reduced PGC-1α mRNA expression in beta cells. Prior studies are discordant, having restricted their investigation of this polymorphism to its impact on co-activator function on one or two promoters. We hypothesized that since PGC-1α drives its own expression, this polymorphism reduces the stability of the PGC-1α stability. In our studies, we used human PGC-1α constructs with a Glycine or a Serine at amino acid 482. Lower levels of the Serine variant translated protein were detected by Western blot compared with the Glycine variant, despite equal mRNA transcript levels. Both translated products were restored to equal levels by MG132, and cycloheximide chase demonstrated that the 482Ser PGC-1α protein has a shorter half-life than 482Gly PGC-1α. We also observed through qPCR on a range of typical endogenous PGC-1α targets that this polymorphism differentially regulates some but not all gene targets. We also investigated the novel isoform PGC-1α4, which our lab previously found inhibited the cleaving of caspase-3, and exhibited anti-apoptotic

properties. Our immunofluorescence data indicated that PGC-1α4 was localized in the cytoplasm and that it shifted to the nucleus in response to TNFα treatment. Using cell fractionation, we noted that not only does the nuclear content of PGC-1α4 increase on treatment with TNFα, but the cytoplasmic content does as well.

Altogether, our results indicate a novel site of regulation on the canonical PGC-1α protein that is associated with human disease, and elucidate a distinct response to TNFα treatment by one of its isoforms, adding nuance to our understanding of this isoform's anti-apoptotic properties, and how its activity is regulated.

Résumé

La protéine Peroxisome Proliferator –activated receptor 1 gamma Co-activator 1 alpha (PGC-1α) contrôle l'expression d'une variété de gènes essentiels au métabolisme oxydatif, à la fonction et la biogénèse mitochondriale, à la détoxification des espèces réactives de l'oxygène et à l'angiogénèse. PGC-1α est exprimé dans différents tissues métaboliquement actifs et son expression est diminuée chez les patients diabétiques. Ce co-activateur transcriptionnel a plusieurs isoformes possédant des fonctions qui se chevauchent. Un nouvel isoforme tronqué, PGC-1α4, a été récemment décrit comme ayant un rôle distinct mais complémentaire à l'isoforme canonique PGC-1α1 mais sa fonction dans différents tissues n'a jamais été investiguée. Le polymorphisme Gly482Ser de PGC-1α est associé avec une augmentation du risque d'être diagnostiqué diabétique, une diminution de la capacité aérobique et ainsi qu'une adiposité plus importante. Enfin ce polymorphisme est associé avec une expression génique réduite de PGC-1α dans les cellules béta du pancréas. Jusqu'à présent, la littérature est réduite sur l'effet de ce polymorphisme sur la fonction de co-activatrice de PGC-1α. Puisque PGC-1α augmente sa propre expression, nous avons fait l'hypothèse que ce polymorphisme diminue la stabilité de la protéine PGC-1α. Dans nos études, nous avons utilisés des plasmides exprimant l'acide aminé Glycine ou Serine en position 482. Des niveaux d'expressions protéiques diminués du variant Serine, comparé au variant glycine, ont été observés par immunoblot malgré des niveaux d'expression génique équivalents. Les deux variants sont observés au même niveau d'expression protéique après traitement par le MG132 ou cycloheximide, démontrant que la demi-vie du variant Ser482 PGC-1α est plus courte que celle du variant Gly482. Nous avons aussi

mesuré par PCR semi quantitative que ce polymorphisme régule différemment des gènes cibles canoniques de PGC-1α. Nous avons investigué le rôle de PGC-1α4, que notre laboratoire a démontré comme ayant des propriétés anti-apoptotiques en inhibant le clivage de Caspase-3. Nos données d'immunofluorescence ont démontrées le contraire, indiquant que PGC-1α4 est importée du cytoplasme vers le noyau après traitement. Par fractionnement cellulaire, nous montrons que le contenu nucléaire et cytoplasmique en PGC-1α4 augmente après traitement par TNFα.

Nos résultats indiquent un nouveau site de régulation de la protéine PGC-1α, associé à différentes maladies, et montrent une réponse distincte au TNFα par un des ses isoformes, éclairant notre compréhension de la régulation de ses propriétés anti-apoptotiques.

Acknowledgements

I wish to express my sincerest gratitude to my supervisor Dr. Jennifer Estall for extending the opportunity to work in her laboratory. She has truly been the definition of a mentor, and has motivated, supported, trained and encouraged me throughout the Master's research and writing process. I would also like to extend my gratitude to my committee members, Dr. Javier Di Noia, Dr. Mathieu Ferron, Dr. Simon Wing and Dr. Louise Larose for their input on my project, and support throughout.

My project would not have been possible without the support of the Estall lab members. Thank you Dr. Daniel Oropeza for making the human Gly482Ser polymorphism constructs, for teaching me to fear the ultra centrifuge, and for being a constant source of wisdom. Aurèle, thank you so much for translating my abstract, having the answer to everything, managing every crisis and being super cool. Roxy Vandenbeek was essential to the completion of the phospomimetic experiments, and is an all round stellar human being – sorry I made you endure the cell fractionation optimization process. Dr. Nathalie Jouvet, without whom I would not have even known how to transfect INS-1 cells, thank you for all your advice and help with trouble-shooting. Mélissa Léveillé, thank you for helping me overcome my phobia of qPCR, even though it took so many tries. I will miss our coffee runs. To Clémence Meunier and Dr. Cristina Bosoi, thank you for your invaluable input and help. I wish to thank Dr. Dominic Fillion for his training and help with microscopy, and the molecular biology core team of the IRCM, who have always been incredibly helpful.

To my mother, the first scientist I knew, thank you for being an inspiration, and keeping me afloat. To my father and sister, thank you for your constant support and perspective. And to my husband, thank you for making everything so easy.

List of Abbreviations

482Gly	PGC-1α with a Glycine at amino acid 482
482Ser	PGC-1α with a Serine at amino acid 482
ACBP-1C	acyl-coA binding protein 1
Akt	Protein Kinase B
AMPK	AMP-activated protein kinase
BMI	Body Mass Index
Ca^{2+}	Calcium ion
CaMKIV	Calcium/Calmodulin-dependent protein kinase type IV
cDNA	complementary DNA
CKII	Casein Kinase II
Clk2	CDC-like Kinase II
COX1V	CytochromeC Oxidase Subunit IV
CREB	cAMP response elemen-element binding protein
CytoC	Cytochrome C
DGAT2	Diacylglycerol O-Acyltransferase 2
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECL	Enhanced Chemiluminescence Susbtrate
ERRα	Estrogen-related receptor alpha
FOXO1	Forkhead box protein O1
FPG	Fasting Plasma Glucose Level
FXR	Farnesoid X receptor
GalNAc	*N*-Acetylgalactosamine
GCN5	General Control of Amino Acid synthesis protein 5
Gly	Glycine
GS3Kβ	Glycogen synthase kinase 3 beta
GSIS	Glucose Stimulated Insulin Secretion
GST	Glutathione S-transferase

GWAS	Genome Wide Association Studies
HeLa	Henrietta Lacks cervical cancer cells
HNF4α	Hepatocyte nuclear factor 4 alpha
HOMA-%beta	Homeostasis Model Assessment beta cell function
hPGC-1α	Human PGC-1α
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IAT	Impaired Aerobic Threshold
INS1	Insulin secreting beta cell derived line, Rat
iPSC	induced pluripotent stem cells
KCQN1	Potassium channel, voltage gated KQT-like subfamily Q member 1
LT	Lactate Threshold
MED1	Mediator complex subunit 1
MEF2C	Myocyte Enhancer Factor 2C
MKK6E	Constitutively active mitogen-activated protein kinase kinase 6
MODY	Mature Onset Diabetes
mRNA	Messenger Ribonucleic Acid
mtDNA	Mitochondrial deoxyribonucleic acid
NEFA	Non-essential Fatty acids
NRF-1	Nuclear respiratory factor 1
O-GlcNAc	O-linked N-acetylglucosamine
OGTT	Oral Glucose Tolerance Test
OPTIMEM	Improved Minimal Essential Medium
p38 MAPK	p38 Mitogen-activated protein kinase
PCOS	Polycystic Ovarian Syndrome
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PINS	Post Prandial Serum Insulin
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha gene
PPARα	Peroxisome proliferator-activated receptor alpha
PPARδ	Peroxisome proliferator-activated receptor delta

PPARϒ	Peroxisome proliferator-activated receptor gamma
PPARϒ2	Peroxisome proliferator-activated receptor gamma 2
PPRE	Peroxisome proliferator-activated receptor response element
PPRE-Luc	Peroxisome proliferator-activated receptor response element - Luciferase
PRMT1	Protein Arginine N-methyltransferase 1
PVDF	Polyvinylidene fluoride
qPCR	quantitaive real-time PCR
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RRM	RNA recognition motifs
RS	Argenine and serine reside-rich domain
RXRα	Retinoid X Receptor, Alpha
SAM	San Antonio Metabolism study
SCFcdc4	SKP1-CUL1-F-box protein cell division control protein 4
Ser	Serine
siRNA	Short interfering bibonucleic acid
Sirt1	Sirtuin 1
SLIM	Study on lifestyle intervention and impaired glucose tolerance Maastricht study
SNP	Single nucleotide polymorphism
SOD2	Superoxide Dismutase 2, Mitochondrial
STOP-NIDDM	Study to Prevent Non-insluin Dependent Diabetes Mellitus
SUMO	Small Ubiquitin-Like Modifier
T2D	Type 2 Diabetes
Tfam	Transcription factor A, mitochondrial
Tfam-Luc	Transcription factor A, mitochondrial - Luciferase
TZD	Thiazolidinedione
V(O2max)	Maximal Oxygen Uptake
VAGES	Veterans Administration Genetic Epidemiology Study

Chapter One

General Introduction

Diabetes: A global health crisis of growing concern

Diabetes is presently the most common metabolic disorder, with 387 million people living with diabetes, and an average prevalence across regions of 8.3% [1]. Annual estimates of the growth of this non-communicable disease have surpassed each other with every progressing year, and the present number is predicted to balloon by a further 205 million by 2035 [1]. The extent and reach of this disease is worsened by the fact that an estimated 46.3% of cases are not yet diagnosed [1].

Diabetes has long been viewed as a disease of affluence and advanced age. In recent years there has been a rapid and startling change in the demographic worst affected by the disease, with 77% of cases belonging to lower or middle-income countries [1]. While the prevalence of diabetes is still highest in North America, in absolute terms, South East Asia and the Western Pacific are leading global numbers of people living with diabetes. The incidence of diabetes in the paediatric population is also rising, with cases such as that of a 3-year-old toddler being diagnosed and treated for type 2 diabetes (T2D) catching the attention and alarm of the general public [2].

The current cost of this disease is high, and is expected to increase at an unprecedented rate. Presently, 1 in every 9 dollars allocated to healthcare is spent on diabetes, leading to an absolute spending of US$612 billion; every 7 seconds, a person dies as a consequence of diabetes, with 4.9 million deaths in 2014 [1]. It is expected that the greatest burden of future cases and costs will be borne by low-income countries, as cases are expected to increase by 108% in these regions, as compared with high income countries, that anticipate an increase of 28% [1]. Of even further concern to these countries is the prediction that the

highest proportion of future cases will come from the under-50 age group [1]. Lower income countries present the most difficult challenge in terms of health care expansion to manage this health crisis.

These sobering estimates point to an essential need for early, concerted detection and treatment, improved prevention, and a better understanding of diabetes.

Type 1 Diabetes

Globally, 10% of cases of diabetes are classified as type 1, and unlike type 2 diabetes (T2D) lifestyle is not a causative factor. Type 1 diabetes (T1D) is a chronic autoimmune disease, where insulin deficiency arises from the immune insult on the beta-cells of the islets of Langerhans [3]. It is not yet certain what causes this autoimmune insult, although both genetics and microorganisms have been implicated [4]. The strongest and earliest association with susceptibility is with the HLA region on chromosome region on 6p21, also known as insulin-dependent diabetes mellitus locus or IDDM1 [5, 6]. Lesser risk is associated with the IDDM2 locus on chromosome 11 where the insulin gene is found [7, 8], PTNPN22 that encodes the lymphoid protein tyrosine phosphatase (LYP) [9, 10] which is also associated with other autoimmune diseases [11], interleukin (IL)-2 receptor-a (IL2RA) [12-14], and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) in the IDDM12 region [15, 16], a protein essential to the negative regulation of immune responses, also associated with various other autoimmune diseases [17].

Autoantibodies are found before the manifestation of T1D, suggesting a diabetogenic event far earlier than clinical diagnosis [4]. There is a search for a possible environmental insult

triggering the autoimmune attack on the beta cells, bolstered with the rising incidence of T1D [18]. The enterovirus coxsackievirus is major candidate in at least a subset of cases [19], with higher antibody titers found in recent onset patients as compared to healthy controls [20], as well as a higher abundance of enterovirus found in intestinal biopsy samples [21]. Bacteria [22], and more specifically the balance of intestinal microbiota [23] have also been implicated, as well as environmental triggers such as cow's milk [24] and wheat proteins [25] that may promote cross reactivity with beta cell antigens, while vitamin D reduces incidence [26] by modulating immune activation. However in all these cases, where animal models indicate an adverse effect of these triggers, there is limited evidence of similar effect on human risk of T1D.

T1D diabetes also differs from T2D in the mode of treatment. While preventative trials have been attempted, such as the Diabetes Prevention trial (DPT-1) [27], they have so far failed to offer evidence of complete prevention of the disease. Insulin continues to be the major treatment for T1D. Future therapies include insulin substitution with molecules such as SmartInsulin, so insulin is only released in the presence of a particular glucose concentration [4], immune modulators to allow tolerance of self antigens on the beta cells [28], compounds to enhance beta cells mass or function [29], and cytokines based therapeutics intended to interfere with the advent of the disease [30-32].

Hallmarks of Type 2 Diabetes

Traditionally, Type 2 Diabetes (T2D) is viewed to correspond with the obesity "epidemic". However, in Asia, obesity and diabetes do not always correlate quite so neatly; for example, obesity rates in India are low while the country presents the second highest number of cases of diabetes in the world. Furthermore, it has been reported in Asian populations, diabetes risk increases at a lower BMI than in Europeans [33].

Diabetes has long been characterized with increased hepatic gluconeogenesis and reduced responsiveness of peripheral tissue to insulin (insulin resistance), leading to increased hyperglycemia and eventual beta cell failure in the pancreas. However, other tissues are now being considered for the role they play in the progression of the disease: enhanced lipolysis in the adipose cell, incretin resistance and deficiency in the gastrointestinal tract, hyperglucagonemia by alpha cell in the islets of Langerhans, insulin resistance in the brain and augmented glucose reabsorption in the kidneys [34].

The progression of diabetes is thought to begin with insulin resistance. Insulin resistance is demonstrated in the liver through increased gluconeogenesis even under the condition of hyperinsulinemia [35], and presents in muscle and peripheral tissues as a failure to take up glucose in response to insulin [36-38]. The increase in basal hepatic glucose production from 2.0 mg/kg in a healthy state to 2.5 mg/kg in T2D is an outcome of insulin resistance and enhanced sensitivity to glucagon and lipotoxicity that increases expression and activity of PEPCK and pyruvate carboxylase [39] and glucotoxixity that increases glucose-6-phosphotase activity [40]. Meanwhile, euglycemic clamp studies demonstrate that muscle insulin resistance contributes to 85-90% of failure to clear glucose in type 2 diabetic

patients [41]. It has also been found under hyperinsulinemic euglycemic clamp conditions that obese and diabetic individuals are unable to clear intracellular glucose, implying a defect in glucose phosphorylation besides impairment of glucose uptake into cells [36].

Beta cells are able to sustain insulin production to maintain a state of normoglyecmia; however, with increased burden, beta cells begin to fail and hyperglycemia sets in leading to diabetes [34, 42]. It is reported that the relative influence of insulin resistance and beta cell dysfunction on the progression of the disease varies between ethnic groups [42].

The contribution of the rate of beta cell dysfunction to the progression of the disease is particularly striking, as it is now understood that beta cell failure is much worse by full onset of diabetes than was previously understood. In 2009, the research group of Dr. Ralph DeFronzo [34] demonstrated how patients from the San Antonio Metabolism (SAM) study and Veterans Administration Genetic Epidemiology Study (VAGES) in the upper tertile of impaired glucose tolerance had already lost 80-85% of beta cell function [34]. It has also been reported in autopsy studies that pancreatic tissue from obese individuals with impaired fasting glycemia and T2D had 40 and 63% reduction in beta cell volume respectively, as compared with lean individuals [43]. This underscores yet again the need for timely intervention in subjects at risk, as well as the need for therapies to focus on protecting the beta cell.

Hereditary components of Type 2 Diabetes risk

It is estimated that as many as 183 million, or half of all people with diabetes, are not aware they have the disease [1]. Type 2 diabetes has long been associated with a sedentary lifestyle, and risk factors include obesity, body fat distribution, poor diet, lack of physical activity, as well as age, sex, ethnicity, previous gestational diabetes or history of elevated fasting glucose, hypertension, chronic stress and dyslipidemia. There is also evidence of family based clustering of this disease and a hereditary component to disease risk. It has been found that in monozygotic twins, the concordance of diabetes is up to 70%, whereas the concordance in dizygotic twins is below 20% [44]. Offspring of one parent with diabetes have a lifetime risk of 40%, and this risk increases when both parents are diabetic [45]. Furthermore, it has been found that the relative risk of diabetes doubles when a first-degree relative has the disease. Despite this evidence for the genetic component of diabetes, there is still a lack of understanding and certainty over genetic markers; thus far, only 10% of the heritability has been explained by the findings of Genome Wide Association Studies (GWAS) [46]. As yet, there is no genetic test for the disease.

There are powerful advantages to understanding the genetics of diabetes, not just limited to predicting an individual's predisposition to the disease. Elucidating the genes connected with diabetes will help to map out disease mechanisms and the precise steps in the progression of the disease, as well as assigning new targets for treatment and prevention. With the genetics of diabetes more fully understood, there is a potential for the development of more personalized medicine for diabetics and those with impaired glucose tolerance (IGT)[47].

Presently, our knowledge on the genetics of diabetes is limited. This limitation is primarily due to the very complex and heterogeneous nature of diabetes itself. An accurate diagnosis of diabetes can present a challenge, and is achieved after excluding other diseases associated with chronic hyperglycemia, such as type 1 diabetes, MODY, and gestational diabetes. Accurately assessing for T2D itself in a non-diabetic cohort is dubious in the absence of an oral glucose tolerance test (OGTT) [48]. As a complex traits disease, cohorts and risk must be stratified based on many factors such as age, obesity, ethnicity, other metabolic disease factors; on the other hand, studies investigating diabetes risk factors may miss factors associated with obesity and dyslipidemia that would contribute to the eventual development of diabetes.

Risk loci identified thus far are linked with low effect, particularly in comparison with clinical risk factors that are already established. Identified genes have to be validated for their influence on the aetiology of the disease and specific contribution to risk. Many target loci identified by GWAS do not occur within the coding or proximal regulatory region of known genes, or lie in introns, thus finding data to support the usefulness of these variants in future screening of the disease or better understanding the aetiology of diabetes is challenging [47]. However, future identification of risk alleles will be bolstered by improved methods in screening. New approaches benefit from enhanced accuracy and precision of diabetes diagnosis, and new sequencing techniques and genotyping methods such as custom made exome sequencing chips and the Illumina array, respectively, will help identify rare variants with large effects [49]. There is also a shift towards investigating non-European populations, offering a view into differences in allele frequencies between ethnic groups that might not have been apparent through Caucasian-centric GWA studies

[48, 49]. For example, the KCQN1 gene was identified in East Asian populations, where the gene occurred at a frequency of 30-40% as compared with 10% in European populations [50]. Further nuance is offered by the investigation of structural variation and epigenetics, and how these might influence diabetes risk.

Thus, while there is as yet limited data from genetic studies that can influence clinical management of the disease at present, there is a strong need for translational research to elucidate functional effects of genes identified by GWAS.

Introduction to PGC-1α

The Peroxisomal Proliferator Gamma Coactivator -1α (PGC-1α) was discovered through a yeast two-hybrid screen seeking out interacting factors of PPARϒ in brown adipose tissue on cold stimulation [51]. Human PGC-1α was cloned and mapped soon after from the kidney [52]. This coactivator has since been detected in various metabolically active tissues including neurons, brain and adipose tissue, and has been shown to regulate several metabolic intracellular pathways.

PGC-1α is a particularly versatile coactivator. While originally identified as a coactivator of PPARϒ, it has since been demonstrated to enhance the activity of PPARα [53], and associates with many transcription factors required in oxidative metabolism [54], mitochondrial biogenesis [55], reactive oxygen species (ROS) homeostasis as well as insulin and glucagon signalling pathways [56, 57]. PGC-1α is also known to interact with several nuclear receptor families such as mineralocorticoid, vitamin D, thyroid hormone, and liver X receptors [58]. The coactivator further associates with unliganded receptors

such as the Hepatocyte nuclear factor α (HNFα) [59], Farnesoid X receptor (FXR) [60] and Estrogen related receptor α (ERRα) [61].

Besides its role as a coactivator, PGC-1α is also predicted to influence mRNA biogenesis and processing, due to the presence of arginine-serine rich domains (RS) and RNA recognition motif (RRM) in its sequence, and due to the interaction of its C terminus with MED1 [62].

There is a complex level of control over PGC-1α transcription, protein stability and activity, further underscoring the importance of this coactivator in transducing metabolic signals within cells. PGC-1α expression levels respond to cold, exercise, insulin, cytokines and glucagon. At the transcriptional level, PGC-1α is induced in response to calcium signalling through activation of Calcium/calmodulin dependent protein kinase IV (CaMK1V) and Ca^{2+} [63]. Its expression is also enhanced by the activation of cAMP response element binding protein (CREB) [64], p38 mitogen activated protein kinase (p38 MAPK) [65], and by Myocyte Enhancer Factor 2C (MEF2C) (as this is a target of PGC-1α activation, this leads to a feed forward loop) [66]. The control of PGC-1α by stimuli is often in a very tissue specific manner [64, 67-70], so different factors exert distinct extents of influence depending on the tissue.

At the protein level, PGC-1α is under complex control through phosphorylation, acetylation, SUMOylation, methylation, and ubiquitination. Kinases including AMP-activated protein kinase (AMPK) and p38 MAPK enhance PGC-1α activity or stabilize the protein; on the other hand, Akt [71] and Cdc-like kinase 2 (Clk2) [72] diminish PGC-1α activity, while phosphorylation by Glycogen synthase kinase 3β (GSK3β) targets the PGC-1α protein for subsequent ubiquitination and degradation [73]. Degradation of the PGC-1α protein is controlled by ubiquitination by the action of Skp1/Cullin/F-box cell division control 4

(SCF^{CDC4})[74]. The activity of PGC-1α protein is also kept in strict regulation based on bioenergetics status by general control of amino acid synthesis 5 (GCN5) [75] and silence information regulator 2-like 1 (Sirt1) [76], through acetylation and deacetylation respectively.

Association of PGC-1α with diabetes

PGC-1α is expressed, and plays a significant role, in various tissues closely linked with type 2 diabetes (T2D). The coactivator's role relevant to glucose homeostasis and possible diabetes risk in the various tissues is discussed here briefly.

Fat PGC-1α was first identified in brown adipose tissue, where it is expressed in response to cold. PGC-1α overexpression in white adipocytes leads to their "beige-ing", that is, the cells develop features of brown fat by increasing expression of UCP1 and fatty acid oxidation enzymes [77]. This coactivator is believed to contribute to insulin sensitizing in adipose tissue as its expression is increased in adipocytes on treatment with the Thiazolidinedione (TZD) derived rosiglitazone, coupled with an increase in mitochondrial mass and energy expenditure [78].

Beta cells Earlier on, PGC-1α was considered detrimental to beta cell function, as PGC-1α levels are elevated in Zucker diabetic fatty rats (the rat model of obesity and hypertension with the recessive trait of the leptin receptor) and ob/ob mice (mice with a mutation leaving them unable to produce leptin, resulting in excessive eating and obesity) that have impaired beta cell function and are models of type 2 diabetes. Adenoviral overexpression in primary mouse islets blunts insulin secretion in mice [79]. However, it was more recently

reported that overexpression of PGC-1α in adult β cells has no effect on β cell function [80]. Beta cell specific knockout of PGC-1s in mice resulted in diminished insulin secretion (GSIS)[81], largely attributed to the loss of fatty acid potentiation of insulin secretion. Our lab further found that as a consequence of the knockdown, beta cells had an increased intracellular accumulation of acyl-glycerols and cholesterol esters, due to disrupted lipolytic enzyme expression [81].

Interestingly, when human islets from type 2 diabetics and healthy controls are compared, the islets of T2D patients express significantly lower PGC-1α mRNA [82]. Furthermore, when PGC-1α is knocked down by siRNA in healthy islets, insulin secretion (GSIS) is diminished without influencing glucagon expression [82]. Genomic DNA from human islets showed 10-fold higher DNA methylation on the PPARGC1A promoter in diabetic islets as compared to non-diabetic islets [82].

Liver PGC-1α is essential in mediating the response to glucagon and insulin in the liver. It is induced by glucagon in the liver, and induces gluconeogenic enzymes such as PEPCK to increase glucose output [83]. As described before, the activity of PGC-1α is strictly regulated by acetylation and deacetylation in response to the bioenergetics status of the cell. Mice heterozygous for PGC-1α in the liver display aberrant lipid metabolism, and develop insulin resistance on a high fat diet, underscoring the importance of this gene in metabolism in the liver [84].

Muscle Essential for glucose metabolism in the muscle, PGC-1α expression is increased in response to exercise [85], and promotes glucose entry [66, 86] as well as the expression of nuclear genes necessary for mitochondrial biogenesis and proliferation[54, 55, 87]. In transgenic mice, overexpression of PGC-1α leads to fiber-type switch, from fast-twitch, type

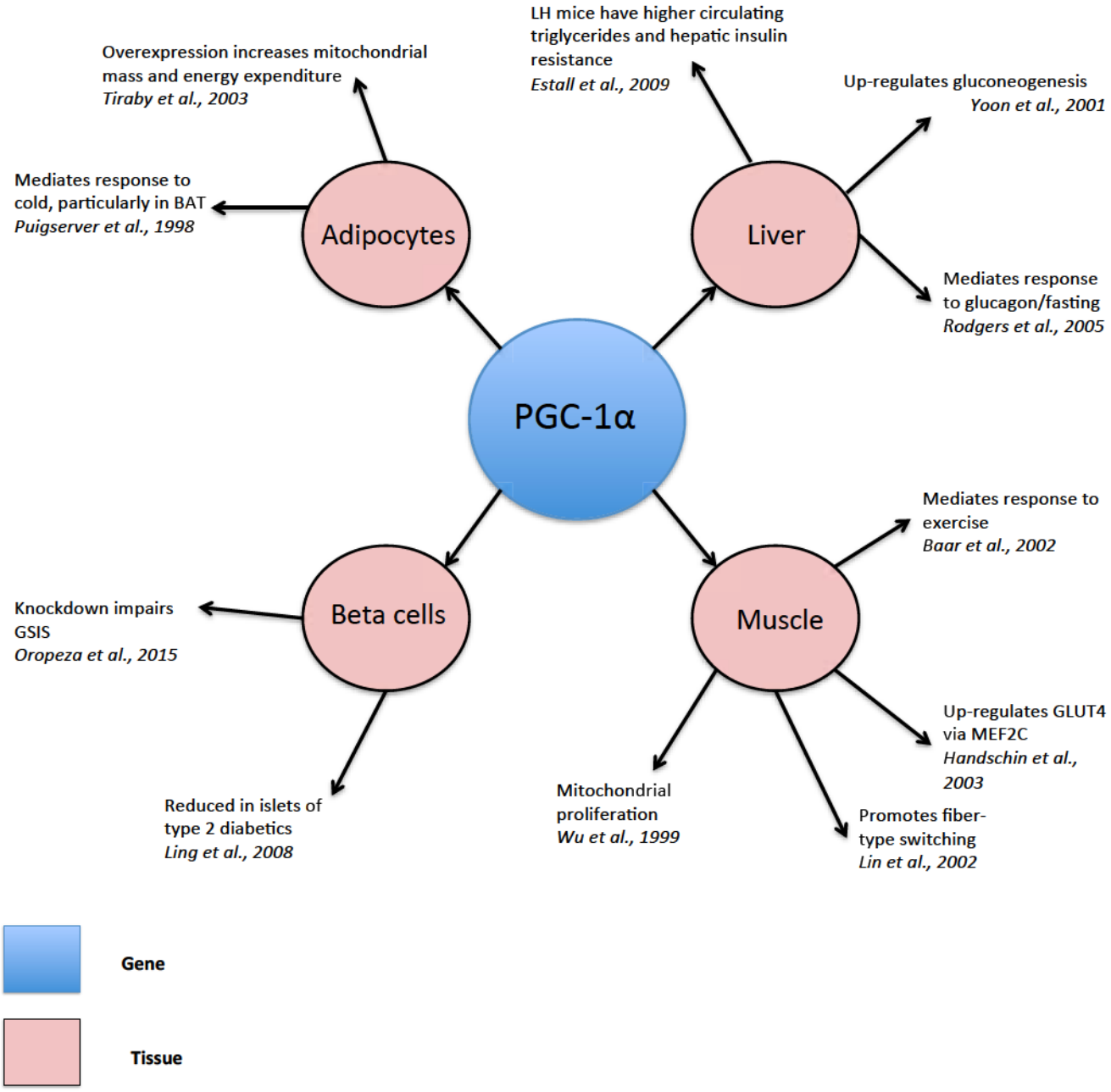

Figure 1: PGC-1α is linked through various tissues with diabetes risk and glucose homeostasis

The role played by PGC-1α in diabetes is of considerable interest. Multiple studies have explored this role in tissues that are relevant to metabolic disease. The results of these studies have been summarized here.

LH – liver heterozygotes of PGC-1α, BAT – brown adipose tissue, GLUT4 – glucose transporter type 4, MEF2C – myocyte enhancer factor 4, GSIS – glucose stimulated insulin secretion

II muscle fibres to slow twitch, type I fibres [88]. PGC-1α is also reportedly induced by insulin, and reduced with age [89]. PGC-1α expression levels are reduced in the skeletal muscle of diabetic and non-diabetic subjects with a family history of diabetes [90], and the expression of oxidative phosphorylation genes regulated by PGC-1α is also reduced significantly in the muscle of diabetic subjects [91]. In muscle-specific PGC-1α knockout mice, young mice have reduced fasting glucose and insulin, though upon aging these mice exhibit impaired glucose tolerance due to diminished mitochondrial oxidative capacity, resulting in an increase in fat mass, insulin resistance and systemic inflammation in the white adipose tissue and liver [92].

Isoforms of PGC-1α

The canonical PGC-1α, now known as PGC-1α1, is 797 amino acids long in mice and 798 amino acids in humans, consists of 13 exons and is expressed by a proximal promoter found directly 5' upstream of exon 1 [51]. Another isoform generated by this proximal promoter is spliced to generate a stop codon after exon 6, resulting in a 270 amino acid protein known as NT-PGC-1α.

An alternative promoter, known as the distal promoter, found approximately 14 kb upstream of exon 1, is highly responsive to stimuli such as exercise [93, 94]. The transcripts expressed by this promoter differed from the canonical transcript at exon 1, dubbed exon 1b, and based on alternative splicing the transcripts were named as follows: PGC-1α-b, with a 12 amino acid exon 1, or PGC-1α-c, with a 3 amino acid exon 1. This alternate promoter also generates isoforms of NT-PGC-1α with these iterations of exons 1b [93, 95]. Other

shorter transcripts also possess exon 1b, such as the 379 amino acid PGC-1α2 and the 370 amino acid PGC-1α3 [95].

A novel isoform was identified specific to the liver [96], and does not possess 127 amino acids at the N terminus as the transcriptional start site (TSS) is present in intron 2, resulting in yet another exon 1, exon 1L. This isoform was discovered in human liver, but not reported in other mammals. In the brain, yet another distinct TSS was identified for isoforms of PGC-1α, found 587kb upstream of exon 2 [97]. This transcript yields several brain specific isoforms, with 5 novel exon 1 variants.

The isoforms of PGC-1α vary in induction of expression as well as the tissue in which they are expressed [95]. However, most of these isoforms are found to broadly overlap with the canonical PGC-1α1 in coactivator function [95]. In 2012, a novel isoform of PGC-1α, PGC-1 α4, was reported, which co-activates a particularly distinct set of genes from the canonical protein, including IGF1 [95, 98].

PGC-1α structure and Post-Translational Modifications

Phosphorylation - PGC-1α is extensively regulated by phosphorylation. AMPK increases PGC-1α transcription, and augments the co transcriptional activity of PGC-1α by phosphorylating threonine-177 (Figure 2) and serine-538 [99]. Akt inhibits PGC-1α activity by phosphorylating the protein at serine-570 in response to insulin signalling [71]. Alternately, insulin signalling also stabilizes protein kinase Clk2, which phosphorylates PGC-1α in its arginine-serine rich (RS) region, curtailing its co transcriptional activity towards FOXO1, inhibiting gluconeogenesis [72]. PGC-1α is also phosphorylated at two

sites in the RS domain by the S6 kinase following feeding, specifically curtailing PGC-1α coactivation of gluconeogenic genes without influencing activation of mitochondrial and fatty acid oxidation genes by blocking the interaction of PGC-1α with HNF4a without impacting interaction with ERRα and PPARα [100]. In muscle cells, cytokine mediated phosphorylation of PGC-1α at threonine-262, serine-265 and threonine-298 by p38 MAP kinase enhance its stability, and also prevents its interaction with corepressor p160MBP in myoblasts, increasing PGC-1α coactivation activity [65]. PGC-1α degradation is promoted by phosphorylation by glycogen synthase kinase 3β (GSK3β) at threonine-295 following oxidative stress [73]. The truncated isoform of PGC-1α, NT-PGC-1α is also reported to be phosphorylated by protein kinase A (PKA) at serine 194, serine 241 and threonine-256, which blocks the nuclear export of PGC-1α by disrupting binding to the nuclear exporter chromosome region maintenance 1 (CRM1) [101]. This increases nuclear accumulation of NT-PGC-1α, which lacks the NLS signal of full length PGC-1α and can be found in both the cytosol and nucleus; thereby, the activity of PKA increases NT-PGC-1α coactivated transcription [101].

Acetylation - PGC-1α is acetylated at residues throughout its entire protein sequence by GCN5 [102] and is deacetylated by Sirt1 [103]. Sirt1 utilizes the coenzyme nicotinamide adenine dinucleotide (NAD+), linking its activity with energy status of the cell [104]. During fasting, exercise, redox stress, Sirt1 activity is enhanced, resulting in the deacetylation of PGC-1α, which leads to an increase in its coactivation activity. In the muscle, this results in an escalation in mitochondrial metabolism [105, 106], while in the liver, PGC-1α regulated

gluconeogenic gene expression levels correspond to Sirt1 knockdown and overexpression [103, 107].

Conversely, in a high-energy state, GCN5 is induced, acetylating PGC-1α [75, 102]. PGC-1α is also known to interact with acetyl transferases p300, SRC-1, and SRC-3, but it is not known to be directly acetylated by these [108, 109].

Ubiquitination – When phosphorylated by GSK3B and p38 MAPK, PGC-1α can bind the E3 ubiquitin ligase SCF^{cdc4}, resulting in its ubiquitylation and degradation [74]. Cdc4 levels decrease under conditions of oxidative stress, causing increases in PGC-1α protein and resultant gene expression [74]. While GSK3β phosphorylation is known to eventually result in the degradation of PGC-1α [73], the entire mechanism by which p38 MAPK regulates PGC-1α stability is not completely elucidated, as this kinase is also reported to stabilize the protein [65].

Others – PGC-1α is activated by methylation at arginine 665, 667 and 669 by protein arginine methyltransferase 1 (PRMT1), a nuclear receptor coactivator, increasing its activity resulting in enhanced expression of mitochondrial biogenesis genes [110]. It is also targeted by the *O*-linked *N*-acetylglucosamine (O-GlcNAc) transferase at serine 333 in the regulatory domain that binds p160MBP [111]. The lysine residue 183 found in the activation domain of PGC-1α is also targeted for small ubiquitin-like modifier (SUMO) 1 protein, inhibiting its coactivation activity, possibly by increasing interaction with corepressor RIP140 [112]. This is an interesting level of regulation, as this lysine is also a target of acetylation [103].

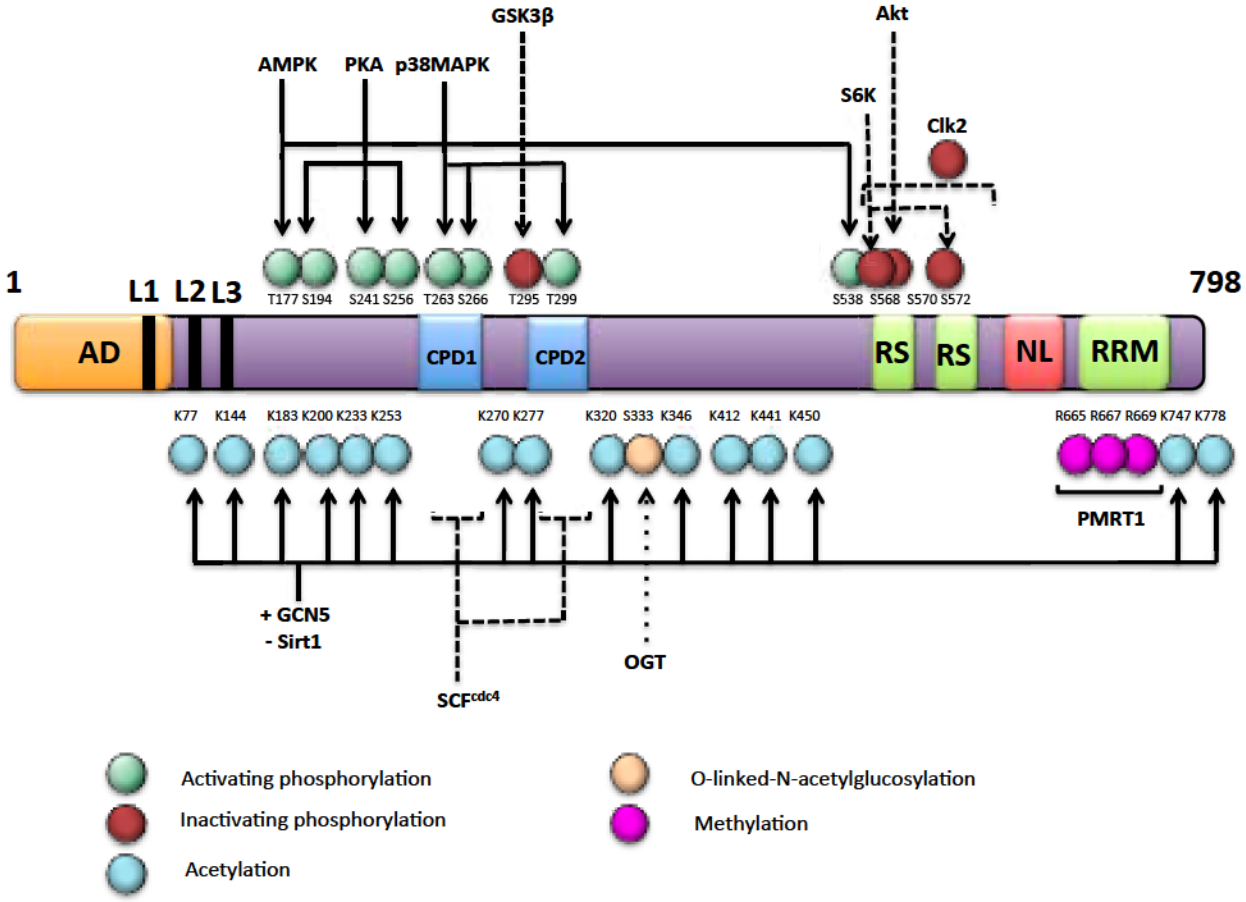

Figure 2 – Structure of PGC-1α and post-translational modifications

All residues indicated reflect the human sequence of the protein, except the acetylated residues. AD – Activation Domain, L1-3 – LXXLL motifs, CPD1,2 – CDC4 phosphodegron, RS – argenine-serine rich domains, NL – nuclear localization signal, RRM – RNA recognition motif

Figure adapted from Soyal, 2006 and Fernandez-Marcos and Auwerx, 2011

Structure – PGC-1α binds with histone acetyl transferases (HAT) including steroid receptor coactivator- 1 (SRC-1) and CREB binding protein (CREB)/p300 [109] at its N terminus, in the absence of innate HAT activity. Three LXXLL motifs (L1, L2 and L3) are found in the N terminal domain [113]. These signatures motifs are defined features of nuclear proteins, with the L standing for leucine and the X any amino acid, and they support the interaction of nuclear receptor proteins with different factors [58]. The L2 motif and N terminal domain are responsible for several PGC-1α-nuclear receptor interactions, such as estrogen receptor α (ERα) [51, 114]. Estrogen Related Receptor ϒ (ERRY) binds to both L2 and L3 [115], while ERRα binds with L3 [116]. Downstream of the L3 motif is a negative regulatory region targeted for phosphorylation by p38 MAPK [65], and which associates with PPARγ, FXR and nuclear respiratory factors NRF-1 and NRF-2 [113]. The MADS box transcription enhancer factor interacts with the central region of PGC-1α (amino acids 400-500), leading to the enhanced expression of GLUT4 [117].

Downstream of this central region, PGC-1α interacts with FOXO1 [56] and Hepatocyte nuclear factor 4 alpha (HNF4α) [59], important transcription factors for metabolism. PGC-1α contains two RS domains and an RNA recognition motif (RRM) in its C terminal domain [113]. The RS domains interact with the spliceosome [118] and TRAP220, necessary for chromatin remodelling and pre initiation complex formation [119]; the RRM domain is known to play a role in transcriptional elongation [120]. Furthermore, the RS and RRM domains flank PGC-1α's nuclear localization signal [73].

Chapter Two

The Impact of the Gly482Ser Polymorphism on the PGC-1α Protein

Section I : Introduction

The PGC-1α Gly482Ser polymorphism and the link with Type 2 Diabetes

In 1998, an autosomal genomic scan linked gene locus 4p15-q12 with increased fasting plasma insulin concentration in Pima Indians, a domain that includes the PPARGC1A gene [121]. Three years later, Ek et al., screened seven PGC-1α polymorphisms for their association with type 2 diabetes (T2D) in Danish Caucasians [122]. Out of those, the Gly482Ser polymorphism (Figure 3) showed significant association with the disease, with the Serine allele occurring most frequently among type 2 diabetic patients as compared with glucose tolerant controls. This group reported a 1.34 genotype relative risk of T2D [122]. These findings were of particular interest at the time as the Pro12Ala polymorphism of PPARΥ was one of the few susceptibility gene variants consistently reported to be associated with reduced risk of diabetes [123, 124], and PGC-1α is a co activator of PPARΥ [51].

Soon after, Hara and colleagues reported the link in a Japanese population. They investigated three "common" variations in the coding region of PGC-1α in a population of over 900 subjects: IVS4-11T>C, Thr394Thr and Gly482Ser. They found that following adjustment for BMI, age, and gender, heterozygotes and homozygotes for the Serine allele had higher fasting insulin as well as higher insulin resistance [125]. While this group did not report an increased incidence of the Serine allele in subjects with diabetes, they found that the haplotype of Thr394Thr- Gly482Ser was associated with T2D [125].

A subsequent study in Pima Indians determined that subjects with the Gly/Gly genotype had lower mean insulin secretory response as well as altered lipid oxidation. These

Nationality/ Ethnicity	Effect shown	Reference
Pima Indians	Gly homozygotes have lower mean insulin secretory response; polymorphism does not associate with T2D	[125]
Danish Caucasians	1.34 relative risk of diabetes for carriers of the Ser allele	[121]
Canadians, Germans, Austrians, Finnish, Swedish, Danes	1.6 fold risk of conversion from impaired glucose tolerance to T2D in carriers of Ser allele	[126]
Finnish	Haplotypes including 482Ser PGC-1α associate with higher OGTT in children of T2D parents	[127]
Italians	Ser allele associates with insulin resistance in obese subjects	[128]
Dutch	Ser allele associates with reduced blood glucose in non-obese, and higher blood glucose in obese	[129]
North Indians (Kashmir, Punjab and Jammu)	Ser allele associates with a 2.04 fold higher risk of T2D	[132]
North Indians (Kashmir, Punjab and Jammu)	Being homozygous for 482Gly PGC-1α associated with >4 fold protection against diabetes	[133]
Southern Indians (Chennai)	The Gly482Ser PGC-1α polymorphism does not associate with diabetes	[131]
Kurdish Iranians	Ser allele significantly associates with susceptibility for T2D, odds ratio 5.23	[134]
Northern Chinese	Ser allele associates with a 1.852 fold increase in T2D risk among men	[135]
Japanese	Ser allele associated with higher fasting insulin, higher insulin resistance; part of haplotype associated with T2D	[124]

Table 1 – Risk of diabetes associated with the Gly482Ser polymorphism varies with ethnicity

Much like the variability of diabetes risk itself with ethnicity, the risk associated with the Gly482Ser polymorphism varies significantly between ethnic groups.

individuals were also found to have a larger mean subcutaneous adipocyte size, along with higher mean plasma free fatty acid concentration levels. However, these individuals have higher carbohydrate oxidation as well, and their BMI was not considered as a variable in this study [126].

The Study to Prevent Non-Insulin Dependent Diabetes Mellitus (STOP-NIDDM) trial involved subjects from Canada, Germany, Austria, Finland, Sweden and Denmark, and was designed to assess acarbose intervention in cases with impaired glucose tolerance. Acarbose is an anti-diabetic drug that works as a starch blocker by inhibiting the intestinal enzyme alpha glucosidase. In this cohort, from multiple populations, the Gly482Ser polymorphism was associated with a 1.6 fold risk of conversion to T2D [127]. In a separate study on Finnish subjects, haplotypes including this polymorphism were associated with elevated glucose levels in oral glucose tolerance tests (OGTT) in offspring of type 2 diabetic patients [128].

Studies have also looked into the added effect of this polymorphism on established clinical risk factors. In an Italian cohort of obese Caucasians (BMI>28kg/m^2) that pooled both normal glucose tolerance (NGT) and impaired glucose tolerance (IGT) subjects, Fanelli et al., reported that the Serine genotype (both heterozygous and homozygous) influences insulin resistance in carriers of the polymorphism, decreasing insulin sensitivity independently from age, gender, BMI, HDL-cholesterol and triglycerides. This effect was diminished in significance though still present in individuals with impaired glucose tolerance (IGT), suggesting that in this cohort, the polymorphism had less of an effect than other factors considered that are associated with IGT [129]. These carriers also reported a higher homeostatic model assessment beta cell function (HOMA-beta%), which was

expected to be a result of the higher insulin resistance [129]. BMI influences glucose levels associated with this polymorphism, as observed in a study carried out on 3244 participants in the Doetinchem cohort (subjects aged 20-59, all from the town of Doetinchem in rural Netherland), where individuals with a BMI lower than 25kg/m^2 that are carriers of the Serine allele have lower non-fasting blood glucose levels [130]. However, in obese participants, this relation is inversed, and carriers of the Serine variant have higher glucose levels. A similar trend was reported in participants of the study on lifestyle intervention and impaired glucose tolerance Maastricht (SLIM), although this was not found to be statistically significant. The SLIM trial was a randomized controlled lifestyle intervention over 3 years and included both diet counselling as well as a regimen consisting of both resistance and aerobic exercise [131]. It is thought that PGC-1α's prominent roles in both the liver and muscle could explain this inversion with BMI [130]. In the liver PGC-1α upregulates gluconeogenesis [64, 83], contributing to an increase in blood glucose, whereas in the muscle, PGC-1α is essential for glucose uptake through its recruitment of GLUT4 transporters [85, 117], and the subsequent oxidation of glucose through its control of mitochondrial genes [54, 55, 90, 91]. Muscle specific knockouts of PGC-1α in mice contribute to insulin resistance [92] . Hence it is hypothesized that due to the 482Ser polymorphism of PGC-1α, in lean individuals, diminished gluconeogenesis in the liver explains the lower blood glucose, whereas in obese individuals who are already susceptible to insulin resistance, a reduction in muscle sensitivity to insulin would result in higher blood glucose levels [130].

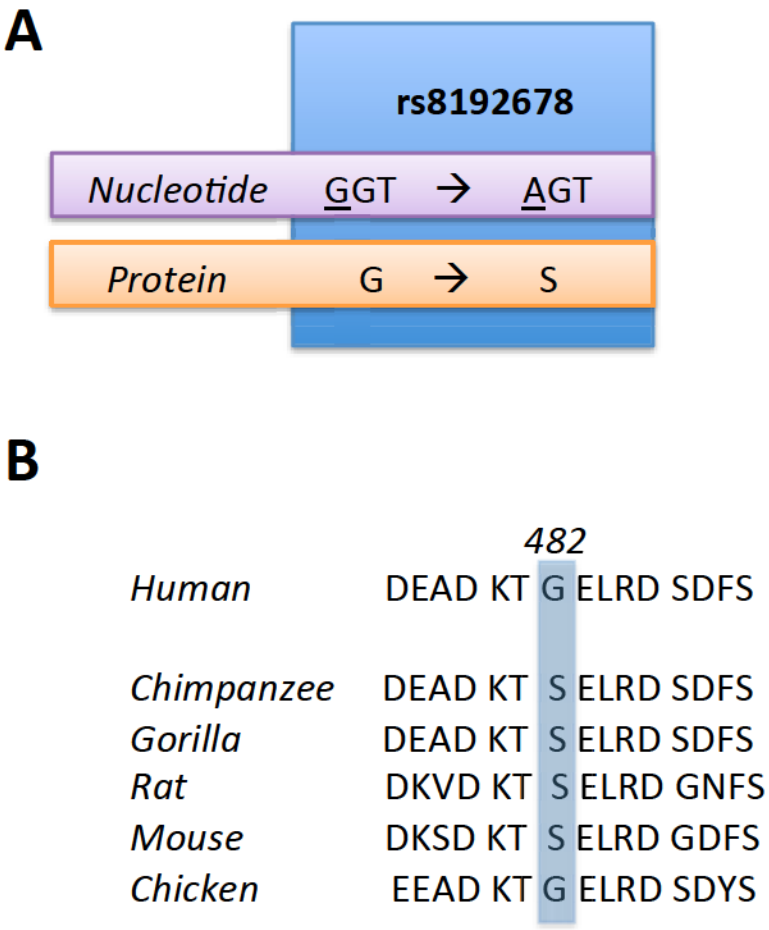

Figure 3: The Gly482Ser polymorphism

A) The mutation in the coding sequence is a result of a G → A nucleotide switch at position 1444. A serine at position 482 is the minor allele in most human populations genotyped for this polymorphism.

B) Genotypes reported for various other mammalian species possess a serine at the position homologous to amino acid 482, including greater apes, and mice. One species reported to also possess a Glycine at the corresponding position is chicken.

(Protein sequences were retrieved from ensembl.org, Comparative Genomics – Gene Tree)

It is interesting to note that while most studies focus on Caucasian subjects, the relative risk associated with T2D for the Gly482Ser PGC-1α polymorphism is modest in these populations when compared with relative risk determined in other populations, such as Indians [132-134], Iranians [135] and Chinese [136]. This has been summarized in Table 1.

The impact of the Gly482Ser polymorphism on fitness and other metabolic factors

A number of studies have established a striking trend of overrepresentation of the Glycine allele among elite athletes from multiple nationalities [137-140], and the Glycine allele is reported to improve fitness standards following fitness interventions in otherwise sedentary individuals [141, 142]. The Serine allele has also been associated with hypertension [143-145], left ventricular dysfunction [146, 147], higher BMI [128, 148] and altered NEFA clearance [149] (Figure 4).

Fitness

When comparing the allele frequency among world-class Spanish male endurance athletes with unfit United Kingdom Caucasian controls, the Serine allele is reported to be significantly lower among the athletes – 29.1% as compared to 40% [137]. Athletes in this study had considerably higher V(O2max) and lower BMI. These finding are not confounded by nationality, as the frequency of the Serine allele is similar between individuals not selected for fitness, found at a frequency of 36.9% among the Spanish cohort as compared to 37.5% in UK Caucasians [137]. When comparing Israeli endurance athletes, sprinters and healthy controls, endurance athletes have a significantly lower frequency of the Serine allele in contrast with both sprinters and healthy controls [138]. The frequency differences

were similar to those reported in the Spanish-UK study, with the Serine allele reported at a 25% frequency among endurance athletes and 43% among healthy controls. Strikingly, stratifying subjects based on competitiveness illustrates that top-level athletes (who represented Israel in world track and field championships or the Olympics) have an even lower frequency of the Serine allele when compared with national level athletes; there were no Serine homozygotes identified among either the national or top level endurance athletes, as opposed to the 13% frequency among sprinters [138]. The reduced frequency of the Serine allele is also found among Polish and Russian elite endurance and strength-endurance athletes when compared with unfit individuals [140]. A study on Turkish endurance athletes and controls reported the same trend, and further determined that velocity and maximal oxygen consumption are associated with the Glycine allele [139]. Attempts to construct an "optimal" polygenic profile for world-class athletes, including this polymorphism however have not reached significant conclusions [150, 151].

Interest in the Gly482Ser polymorphism is not limited to elite athletes; it also associates with the degree of improvement in metabolic measures following fitness interventions in "normal" individuals who are not elite athletes. In the Tuebingen Lifestyle Program, participants at risk of diabetes (family history of the disease, BMI > 27 kg/m^2 or history of IGT or gestational diabetes) received dietary counselling to control calorie intake, and were expected to perform a minimum of 3 hours of moderate sports every week, with endurance exercise encouraged in particular. Following 9 months on this program, carriers of the Serine allele had lower improvement in individual aerobic threshold and insulin sensitivity [141]. However, this trend suggesting the Serine allele is associated with reduced endurance capacity is not supported in all populations; in Chinese men, there is no

significant association between this polymorphism and maximum aerobic capacity (VO2max) or response to endurance training [152]; in Japanese men the contrary was reported, and the Serine allele is associated with a higher lactate threshold (LT), translating to higher aerobic capacity [153].

Supporting the role of this polymorphism in fitness, the Serine variant of PGC-1α has reduced interaction with myocyte enhancer factor 2C (MEF2C) as compared to the Glycine variant [154], which would impede on the expression of GLUT4 [117] in the muscle as well as fiber-type switching [88]. Furthermore, it was determined that individuals with the Serine allele do not show the same increase in slow contracting oxidative fibres following 10 week endurance training (by cycling with heart rate at 70-90% VO_{2peak}) as Glycine carriers [142]. However, this study did not find any difference in capillary supply, mitochondrial density, mitochondrial enzyme activities or cellular lipid content between genotypes, which are also controlled by PGC-1α.

Metabolic factors: insulin resistance, adiposity and BMI

The Diabetes Prevention Program (DPP) assessed the effect of metformin and lifestyle changes (weight loss and increased physical activity) on diabetes development. Non-diabetic people with IGT and high fasting glucose were recruited, and changes in metabolic traits (including triacylglycerols, fasting glucose and insulin, post glucose challenge glucose and insulin), adipose tissue and weight were quantified, following an intervention on either placebo, metformin or lifestyle change [155, 156]. The Serine allele is associated significantly with increased subcutaneous adiposity and worsening of insulin resistance after 1 year [157]. The Serine allele is also associated with an increase in percentage body fat, insulin and homeostatic model assessment of insulin resistance (HOMA-IR) in Korean

children between the ages of 10 and 12, although effect sizes on insulin and HOMA-IR are diminished on adjustment for other factors (age, sex, body fat) [158]. A higher waist-to-hip ratio is linked with the Serine allele in Chinese non-diabetic subjects; the Serine allele is further associated with higher oxidative stress (measured by serum thiobarbituric acid reactive substance levels) in response to hyperglycemia [159]. In the Diabetes Control and Complications Trial (DCCT), comparing efficacy of intensive insulin therapy over conventional therapy for type 1 diabetes, the frequency of the Serine allele increased in participants with greater weight gain following insulin therapy [160]. On the other hand, Serine carriers benefit more from caloric restrictions, as a low calorie diet was associated with a greater diminishing of insulin resistance that was found to be elevated prior to the study [161]. Inactive, elderly men who are carriers of the Serine allele are at higher risk for obesity as compared with inactive elderly men who are Glycine homozygotes, or active elderly men [153, 162]. The polymorphism has not been linked with increased body fat in Indians [163] nor in the Maori, though this association was found in Tongans [148].

Differences in total cholesterol and low-density lipoprotein are also associated with this polymorphism in Caucasians [164], though not in Mongolians [165]. Interestingly, Serine carriers benefit from bariatric surgery significantly more than Glycine homozygotes do, with improvements in both metabolic and inflammatory markers, such as C-reactive protein, Serum interleukin-6, and intima media thickness of the carotid artery [166].

Hypertension and left ventricular dysfunction

PGC-1α plays several important roles in the cardiovascular system, implicated in coactivating genes essential to angiogenesis, fatty acid oxidation, oxidative phosphorylation, mitochondrial biogenesis and protection against reactive oxygen species

(ROS). Knockout mice lacking PGC-1α suffer more severe left ventricular (LV) hypertrophy [167] and rapid heart failure [168] in response to transverse aortic constriction (TAO), a model of overload-induced cardiac stress [168], and develop pronounced cardiac dysfunction with age as their hearts are unable to increase work output to stimuli due to diminished ATP production [169]. The association of the Gly482Ser polymorphism of PGC-1α with hypertension is conflicting. In diabetic [144] or young [170] Caucasian men, the Serine allele is linked with increased risk of hypertension, whereas in middle aged Danish Caucasians it is linked with a reduced risk of hypertension [143, 145]. In connection with the purported link with hypertension, the Serine allele is also associated with increased indices of left ventricular dysfunction in patients with hypertension [147] but also decreases the risk of left ventricular dysfunction in men in a randomly selected group [146].

Polycystic Ovarian Syndrome

Finally, it is interesting to consider the possible link with Polycystic Ovarian Syndrome (PCOS), given the association of this polymorphism with insulin resistance. This Serine allele is not found to correlate with PCOS in Korean [171] and Chinese women [172], though a correlation is found with PCOS in Caucasian women [173]. This polymorphism is not associated with risk of gestational diabetes [174].

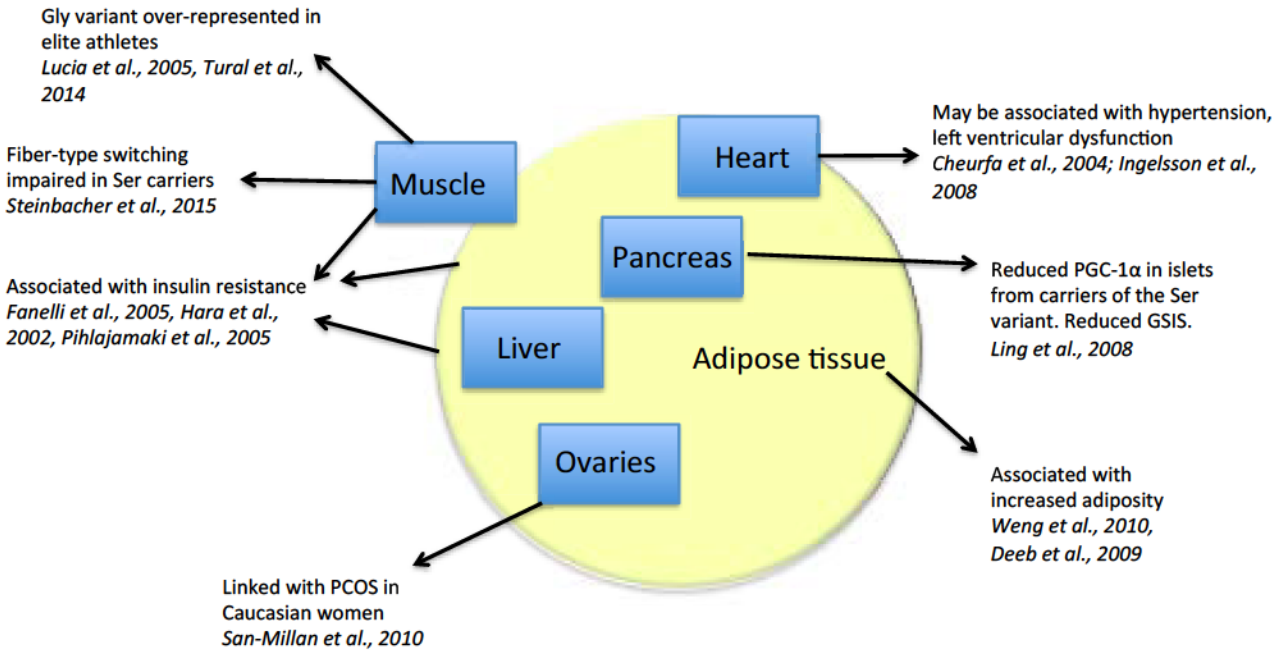

Figure 4 – Summary of the various phenotypes associated with the Gly482Ser polymorphism
The Gly482Ser polymorphism has been linked with a variety of phenotypes associated with the metabolic syndrome in various tissues. These associations are particularly striking given the important role PGC-1α expression plays in the functions of these tissues and organs.

GSIS – glucose stimulated insulin secretion

Interaction with other polymorphisms associated with diabetes risk

The STOP NIDDM trial determined that while there was no interaction on effect size between the PPARϒ Pro12Ala [175] and PGC-1α Gly482Ser polymorphisms [127], there was an additive effect on the risk of developing diabetes from impaired glucose tolerance when considering the Gly482Ser polymorphism of PGC-1α with PPARδ SNPs rs6902123 and rs3734254 [176], which independently increase overall adiposity [177].

The Gly482Ser polymorphism has an additive effect on individual aerobic threshold (IAT) and insulin sensitivity in response to a fitness intervention, together with rs2267668 of PPARδ [141]. Both minor alleles were associated with diminished improvement following the fitness intervention, both independently as well as additively (Table 2).

Influence on responsiveness to treatment

An exciting outcome of studying the genetics of diabetics is the discovery of genotype-influenced responses to treatment. This offers the prospect of personalizing treatment regimens, in addition to expanding our understanding of the biological mechanism of the genetic factor. It has been found in various populations that the Gly482Ser polymorphism can influence a patient's response to common diabetes treatments [127]. The STOP-NIDDM trial investigated the impact of acarbose intervention in cases with impaired glucose tolerance. Acarbose assists in the management of glucose levels by curbing post-prandial

Polymorphism (gene)	Independent effect	Interaction with Gly482Ser PGC-1α	Reference
rs6902123 (PPARδ)	2.7 fold increase in risk of diabetes; induces changes in adiposity, hepatic fat storage, muscle mass	Both increase risk of conversion from IGT to T2D (additive)	[175,176]
rs3734254 (PPARδ)	Unknown	Both part of haplotype that associates with 2.5 fold higher risk of T2D (additive)	[175]
rs2267668 (PPARδ)	Induces changes in adiposity, hepatic fat storage, muscle mass	Both associate with change in fitness and insulin sensitivity	[140]
rs1801282 (PPARγ)	Women in acarbose group with Pro12Pro genotype were more likely to develop diabetes (OR 2.89)	No interaction; only carriers of 482Ser responded to acarbose treatment not Pro12Pro or Pro12Ala	[126]
rs659366 (UCP2)	Minor allele is protective against diabetes risk	Interaction in both increased risk (major UCP2 allele with 482Ser) and protection against T2D (minor allele with 482Gly)	[133]

Table 2 – Summary of interactions of Gly482Ser PGC-1α with other polymorphisms

IGT – Impaired glucose tolerance, T2D – type 2 diabetes, OR – odds ratio, rs – reference SNP cluster ID, PPARδ – peroxisome proliferator-activated receptor delta, PPARγ - peroxisome proliferator-activated receptor gamma, UCP2 – mitochondrial uncoupling protein 2

hyperglycaemia, and so is useful in both patients with diabetes as well as individuals presenting hyperglycemia. The STOP-NIDDM cohort determined that only carriers of the Serine allele responded to acarbose intervention, not Glycine homozygotes [127]. This was particularly striking as it was also shown that these Serine carriers had a 1.6 fold higher risk of developing diabetes without the medication. The authors of this paper propose that acarbose protects against post-prandial hyperglycemia induced ROS [127], a function of PGC-1α by the generation of ROS-detoxifying enzymes [178-180].

The STOP-NIDDM trial mostly focused on Caucasian subjects, albeit from multiple global populations. Ke-Han Zhang and colleagues reported that Chinese patients diagnosed with T2D and possessing the Glycine allele responded better to rosiglitazone, an insulin sensitizer and PPARY agonist, with fasting plasma glucose levels (FPG) and post prandial serum insulin (PINS) reduced most effectively in patients homozygous for the Glycine allele [181]. This study also determined a modestly higher frequency of the Serine allele in the T2D patient cohort as compared with healthy control cases ($p< 0.002$) [181]. A Chinese study published the same year looked into another TZD, pioglitazone. While this study was able to conclude a difference in response to treatment attributable to the Pro12Ala polymorphism of PPARY, no significant difference was determined for the Gly482Ser genotype [182]. This study's cohort had a far higher allele frequency for Serine as compared to frequencies commonly reported in studies investigating this polymorphism. TZD derivatives rosiglitazone and pioglitazone overlap in only a limited number of genes regulated (41 activated, 57 repressed), whereas several genes influenced by these drugs differ, with 25 genes activated and 83 repressed by rosiglitazone but not pioglitazone and 12 activated and 13 repressed by pioglitazone but not rosiglitazone [183].

Beta cells and the Gly482Ser SNP

Beta cell dysfunction following chronic hyperglycemia is a hallmark of type 2 diabetes. Loss of PGC-1α diminishes glucose stimulated insulin secretion in mice [81]. At this time, only one study has investigated the impact of the Gly482Ser polymorphism in human islets [82]. This study found that islets with the Serine allele lowers PPARGC1A expression as well as reduced glucose stimulated insulin secretion, even in non-diabetic carriers. While basal insulin secretion, as well as the insulin response to arginine or glibenclamide was not significantly associated with genotype, insulin response to 16.7 mmol/l glucose was reduced in non-diabetic islets with the Serine allele compared with Glycine homozygotes islets [82]. These findings in non-diabetic islets are especially striking as PGC-1α mRNA was reduced by 90% in patients with diabetes, and these islets had reduced insulin secretion [82]; knocking down PGC-1α by siRNA in islets from healthy donors reduced insulin secretion by 41% without affecting glucagon levels [82]. Furthermore, multivariate regression analysis linked disease status with PPARGC1A expression levels, independently of BMI or age.

Studies addressing the functional impact of the Gly482Ser SNP

Functional studies exploring the impact of this SNP on PGC-1α function are in disagreement (Table 3). In 2006, Choi et al. published that the 482Gly variant had decreased capacity as a coactivator on the Tfam promoter [184]. In promoter-reporter studies conducted on Chang human hepatocytes, reduced PPRE-Luc and Tfam-Luc activity in response to transfection with PGC-1α 482Gly compared to 482Ser was reported. Consistent with this in vitro data, subjects with the 482Gly SNP had mitochondrial DNA (mtDNA) reduced by 20% in peripheral blood leukocytes collected from Korean subjects, both those presenting T2D as well as healthy subjects [184].

A study investigating the effect of the PGC-1α Gly482Ser polymorphism on Acyl Co-A Binding Promoter using an ACBP-1C-SEAP promoter reporter construct in HepG2 cells reported no difference attributable to the polymorphism [185]. These results were an interesting contrast to the previous study, as the ACBP-1C promoter includes a PPRE [185]. This study further determined no difference in functionality between the Glycine and Serine variant when the constructs were co-transfected with functional partners PPARγ2/RXRα [185]. Another study on Japanese subjects with diabetes attempted to explain why men with diabetes and the Serine variant possessed lowered plasma adiponectin level, even after adjustment for BMI and age [186]. They were unable to identify a functional difference in coactivation due to the polymorphism in their promoter-reporter experiments on transfected HeLa cells [186]. The adiponectin gene promoter, again, contains the PPRE.

Gene/ Promoter	Effect	Reference
PPRE	482Gly PGC-1α has reduced coactivator activity	[183]
Tfam	482Gly PGC-1α has reduced coactivator activity	[183]
ACBP-1C	No difference in coactivation activity due to polymorphism	[184]
Adiponectin	No difference in coactivation activity due to polymorphism	[185]
PEPCK	482Ser PGC-1α has reduced coactivator activity	[186]

Table 3 – Summary of mechanistic studies investigating functional impact of the Gly482Ser polymorphism

PPRE – peroxisome proliferator response element, Tfam – Transcrption factor A, mitochondria, ACBP-1C – Acyl-CoA Binding Protein 1A, PEPCK – phosphoenolpyruvate carboxykinase

Chen and colleagues reported in 2013 that the Serine variant induced PEPCK less than the Glycine variant, and further found that hepatocyte fat deposition was reduced to a greater extent in H4IIE (cultured hepatocyte) cells overexpressing the Glycine variant of PGC-1α as opposed to the Serine variant [187]. The PEPCK promoter also contains the PPRE. The expression of PEPCK is rapidly induced by TZD treatment, with the greatest induction from troglitazone, followed by rosiglitazone and pioglitazone [183], a comparison not yet assessed for the Gly482Ser polymorphism. However, PGC-1α is thought to co-activate PEPCK through other transcription factors, such as FOXO1, GR and HNF4α [56, 64].

The contention over the influence of this polymorphism on the downstream expression of genes containing PPRE elements specifically is particularly interesting given the role of PPARY ligands such as thiazolidinedione (TZD) in the treatment of diabetes. These genes are also of interest given the difference in response to rosiglitazone (a derivative of TZD) reported in Asian populations, where Glycine homozygotes benefited more than Serine carriers [181]. However, it is clear that functional studies published to date have been quite limited in the scope of the impact on PGC-1α function they aim to assess, leaving much unknown about the biological and functional importance of the Gly482Ser polymorphism (Figure 5).

Objectives

The Gly482Ser polymorphism of PGC-1α is associated with type 2 diabetes in several populations, and also with metabolic capacity. Various studies attempting to study the functional importance of this polymorphism offer limited elucidation of the biological consequences of this on the PGC-1α protein or its function. In our present study we hypothesize that this polymorphism PGC-1α affects protein stability. Our objective is to determine 1) the impact of this amino acid substitution on the protein stability of PGC-1α, 2) determine whether this site is modified by PTM and 3) determine whether the substitution confers differential gene regulation resulting from the polymorphism.

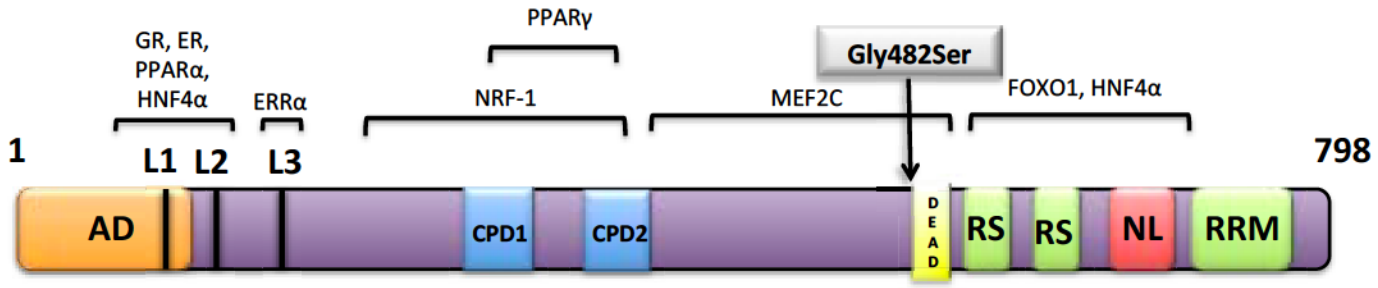

Figure 5 – Known domains of interaction with other proteins on the PGC-1α protein sequence, and their positions relative to the Gly482Ser polymorphism

Modified from Soyal et al., 2006

AD – activation domain, L1-L3 – LXXLL motifs 1-3, CPD1 – CDC4 phosphodegron, DEAD – DEAD box motif, RS – arginine-serine domain, NL – nuclear localization signal, RRM – RNA recognition motif, GR – glucocorticoid receptor, ER – estrogen receptor, PPARα, peroxisome proliferator activated receptor α, HNF4α – hepatocyte nuclear factor 4 α, ERRα – estrogen-related receptor α, NRF-1 – nuclear respiratory factor 1, PPARϒ - peroxisome proliferator activated receptor ϒ, MEF2C – myocyte enhancer factor 2, FOXO1 – forkhead box class O 1.

Chapter Two

Section II: Methods

Cell culture and treatments*:* INS1 cells were cultured in RPMI 1640 (Wisent) medium with 10% heat inactivated FBS (Wisent), 1% penicillin and streptomycin (Wisent), and 1x supplement (10mM HEPES, 1mM 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 reduced serum media. After 4 hours, the transfection media was replaced with normal INS1 media without penicillin and streptomycin, according to the 2012 Lipofectamine 2000 protocol. Cells were left for 24-48 hours (as specified) at 37°C with 5% CO_2. Cycloheximide chase experiments were done with 50 mg/ml of cycloheximide (Sigma-Aldrich), and MG132 (Sigma-Aldrich) used at a concentration of 10 μM.

Western Blot: Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) plus protease cocktail inhibitor (Calbiochem). Protein concentration was estimated using DC assay. Equal amounts of protein (20-40 μg) were loaded onto 10% polyacrylamide gels, and were transferred onto polyvinylidene diflouride (PVDF) membranes (GE healthcare) overnight. PGC-1α isoforms were detected using anti-PGC-1α mouse mAb (4Cl.3) antibody (1:1000, Calbiochem). PGC1α primary antibody was diluted in 2% milk and membranes incubated overnight at 4°C. Goat anti-mouse IgG antibody conjugated to HRP (1:5000) was used as a secondary antibody. The ECL detection system was used to detect a signal from the blots.

RNA isolation and cDNA synthesis: Total RNA from INS1 cells was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol.

For cDNA synthesis, 1 µg of RNA from INS1 cells was incubated with 1 U/ml DNAse1 at 37°C for 15 minutes followed by 15 minutes at 65°C for DNAse1 heat inactivation. Total RNA in 20 µl volume 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 subjected to qPCR.

Quantitative Real-Time PCR: cDNA underwent amplifications for the gene of interest and for the endogenous control hypoxanthine-guanine phosphoribosyl transferase (HPRT). 5 µl reactions were set up in a 384 well plate, using Power SYBR green PCR Master Mix (Life Technologies). The cycling program occurred in two steps, a polymerase activation step for 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 min of 60°C using the Viia 7 system from Life Technologies. Data was normalized to the endogenous control and relative mRNA expression was determined using the ΔΔCt method. Results were graphed using Graphpad Prism.

Site directed mutagenesis: The New England Biolabs Q5 Site Directed Mutagenesis Kit was used to generate mutants with an Aspartate and an Alanine at position 482. Mutagenesis was carried out according to the manufacturers instructions, and primers were designed using NEBaseChanger, the NEB online primer design software, and PCR conditions were also defined by this program. Primers have been listed in Table 1 in the Appendix. Human PGC-1α –482Ser in pcDNA 3.1(+) was used as the template.

Statistical analysis method: GraphPad Prism was used to calculate statistical significance by two-way ANOVA for mRNA expression, and results were expressed as means ± standard deviation (SD). Statistical significance was defined as $p < 0.05$.

Chapter Two

Section III: Results

482Gly hPGC-1α protein is more stable than 482Ser hPGC-1α

Studies attempting to elucidate the stability, and various degradation pathways, of PGC-1α have focused on mouse PGC-1α [188, 189], and not the human sequence. Presently only Serine has been reported at amino acid 481 in mice. The role of the amino acid 482 (or the corresponding amino acid 481 in mice) has yet to be understood. Most studies attempting to investigate this polymorphism have focused on the impact on co-activator function on select promoters. The domain containing amino acid 482 has not yet been assigned a functional role in either human or mouse PGC-1α.

Human constructs of PGC-1α that happened to have a Glycine at amino acid 482 were modified by site directed mutagenesis to introduce a Serine at this position by overlap-extension PCR previously by Dr. Daniel Oropeza. We verified the sequences of our constructs using Sanger sequencing. These constructs were transfected in equal amounts (1 μg per well) in equally confluent INS-1 cells. Western blots were probed for PGC-1α, and the 482Gly protein was detected at a level 10 times higher than the 482Ser following 48 hours of expression (Figure 6A, B).

To determine if this difference occurred at the mRNA or protein level, qPCR was performed on RNA derived from cells in these experiments. Since our hPGC-1α primers were not designed to detect rat PGC-1α, we normalized levels to C_t's determined for endogenous rat PGC-1α RNA expression in pcDNA 3.1+ (control) transfected cells. We found both constructs to be similarly over expressed (Figure 6C), and that there was no statistical difference between the overexpression of the constructs expressing 482Gly or 482Ser *PPARGC1A* at the RNA level.

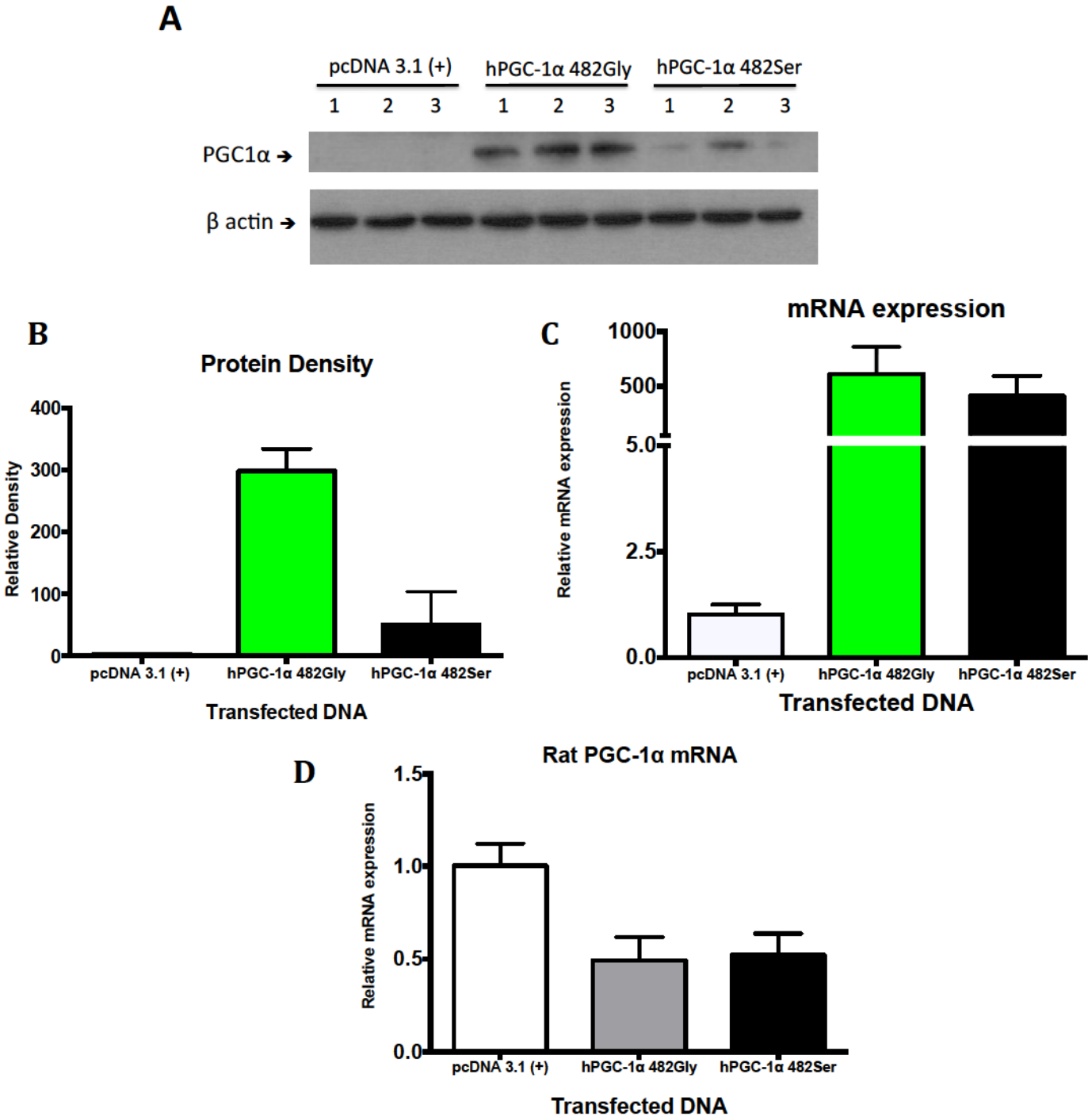

Figure 6 – Lower protein levels of PGC-1α 482Ser detected than PGC-1α 482Gly
INS 1 cells were transiently transfected with the following constructs: empty vector pcDNA 3.1+ (3.1+), hPGC-1α 482Gly (Gly) and hPGC-1α 482Ser (Ser). Following 48 hours, cells were harvested for protein, and lysates were subjected to Western blot and mRNA quantification

A) Western blot for PGC-1α on cell lysates overexpressing respective constructs, representative of 3 independent experiments
B) Western blot quantification by ImageJ software (n=3)
C) mRNA expression of transfected constructs analyzed by quantitative real-time PCR (n=6)
D) Gene expression of endogenous rat PGC-1α measured by quantitative real-time PCR using primers specific for rat PGC-1α (n=6)

PGC-1α has been reported to increase its own expression [66], so we checked the endogenous rat PGC-1α expression in the INS-1 cells to rule out differential up-regulation of rat PGC-1α in the INS-1s by the two constructs. We observed that overexpressing PGC-1α actually reduced the expression of endogenous PGC-1α mRNA in our samples with rat specific PGC-1α primers, and noted no discernible difference between the two constructs (Figure 6D). Taken together, we were able to conclude that differences in the expression of hPGC-1α 482Gly and 482Ser variants were restricted to the protein level, as we did not observe any differences in endogenous or transcribed RNA from overexpression in our experiments.

Proteosomal inhibitor MG132 restored the 482Ser protein

Our results suggested a difference in protein stability between the 482Gly and 482Ser variants of hPGC-1α. We used the proteosomal inhibitor MG132 to test whether 482Ser protein expression would be restored to a level equivalent to that of 482Gly by blocking protein degradation.

Protesomal inhibitor MG132 is a peptide aldehyde that strongly inhibits the peptidase activities of proteasomes and calpain activity [190]. We restricted MG132 treatment to 3 hours, and used 10 μM MG132 to limit toxicity to cells.

As hypothesized, we were able to stabilize the hPGC-1α protein of both variants 482Gly and 482Ser to equivalent levels, following 48 hours of expression (Figure 7). The quantity of

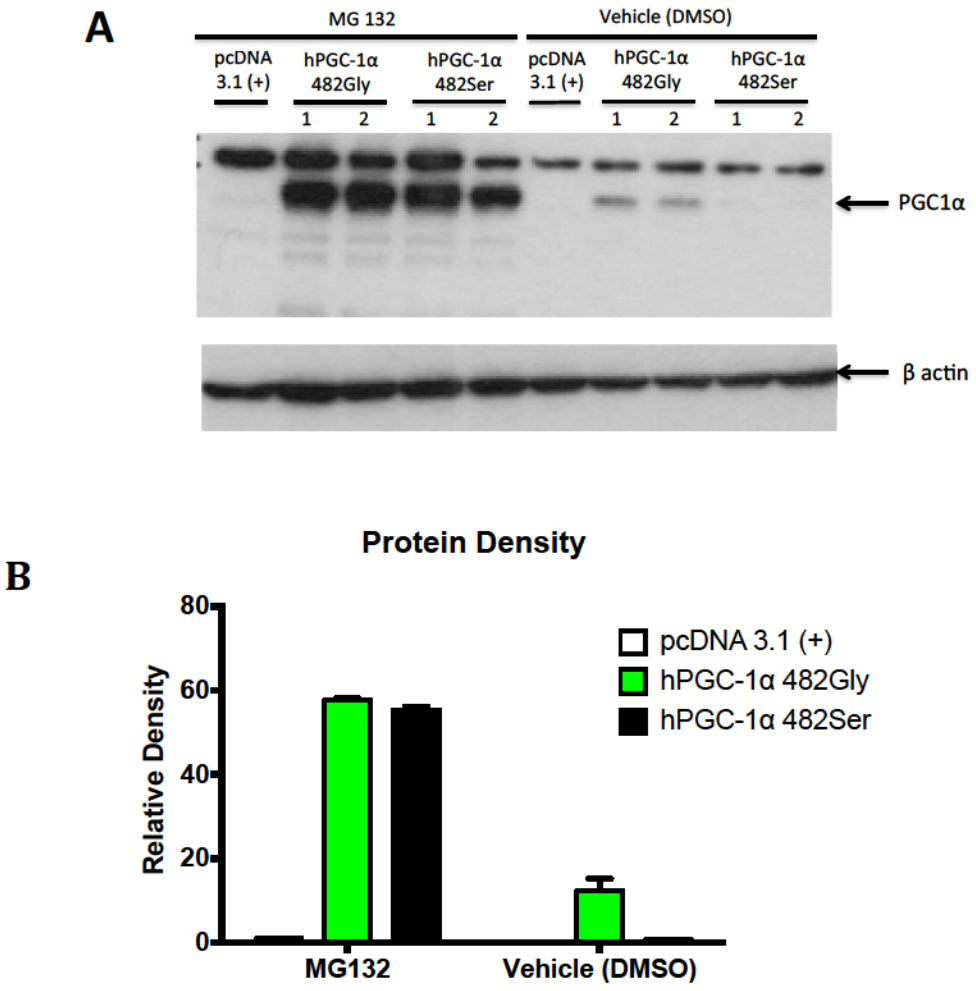

Figure 7 – Treatment with proteasome inhibitor MG132 recovers PGC-1α 482Gly and 482Ser to equivalent levels

INS1 cells were transfected with constructs expressing hPGC-1α 482Gly, hPGC-1α 482Ser and pcDNA 3.1+ as a control. After 48 hours, they were treated with 10 uM MG132, or DMSO (vehicle) as a control.

A) Western blot for PGC-1α on transfected and treated cell lysates, representative of 3 independent experiments

B) Quantification of Western blot by Image J; densities are relative to pcDNA3.1 (+) (MG132 treated), normalized to β actin

PGC-1α recovered for both constructs was much higher than vehicle (DMSO) treated samples, which is consistent with PGC-1α being targeted by the ubiquitin proteasome pathway [188]. Nonetheless, as we show in the DMSO treated samples there is a 5-fold difference between vehicle-treated (DMSO) treated samples, with 482Ser protein level decreased, as compared with no significant difference between the MG132 treated samples. With this data, we were also able to rule out defects in translation.

482Gly and 482Ser variants have different half-lives

Previously published calculations of PGC-1α half-life used the mouse PGC-1α protein [65, 188, 189], with a Serine at amino acid 481 [51]. To the best of our knowledge, this is the first study to determine the half-life of human PGC-1α, and compare it for the two variants 482Gly and 482Ser PGC-1α.

For the purpose of our study we used cycloheximide to determine the half-life of each variant. Cycloheximide is an inhibitor of protein translation in eukaryotes, and it functions by blocking the translocation step in elongation by blocking a motif in the E-site of 60S ribosome [191]. Thus, the half-life of the protein under study can be calculated by monitoring the disappearance of the protein over time.

In a cycloheximide chase experiment we compared protein persistence at the following time points 0, 0.5, 1, 2, 3 hours following addition of cycloheximide. We hypothesized that the 482Gly protein would have a longer half-life than the 482Ser protein based on the higher levels of protein detected after 48 hours (Figure 6A).

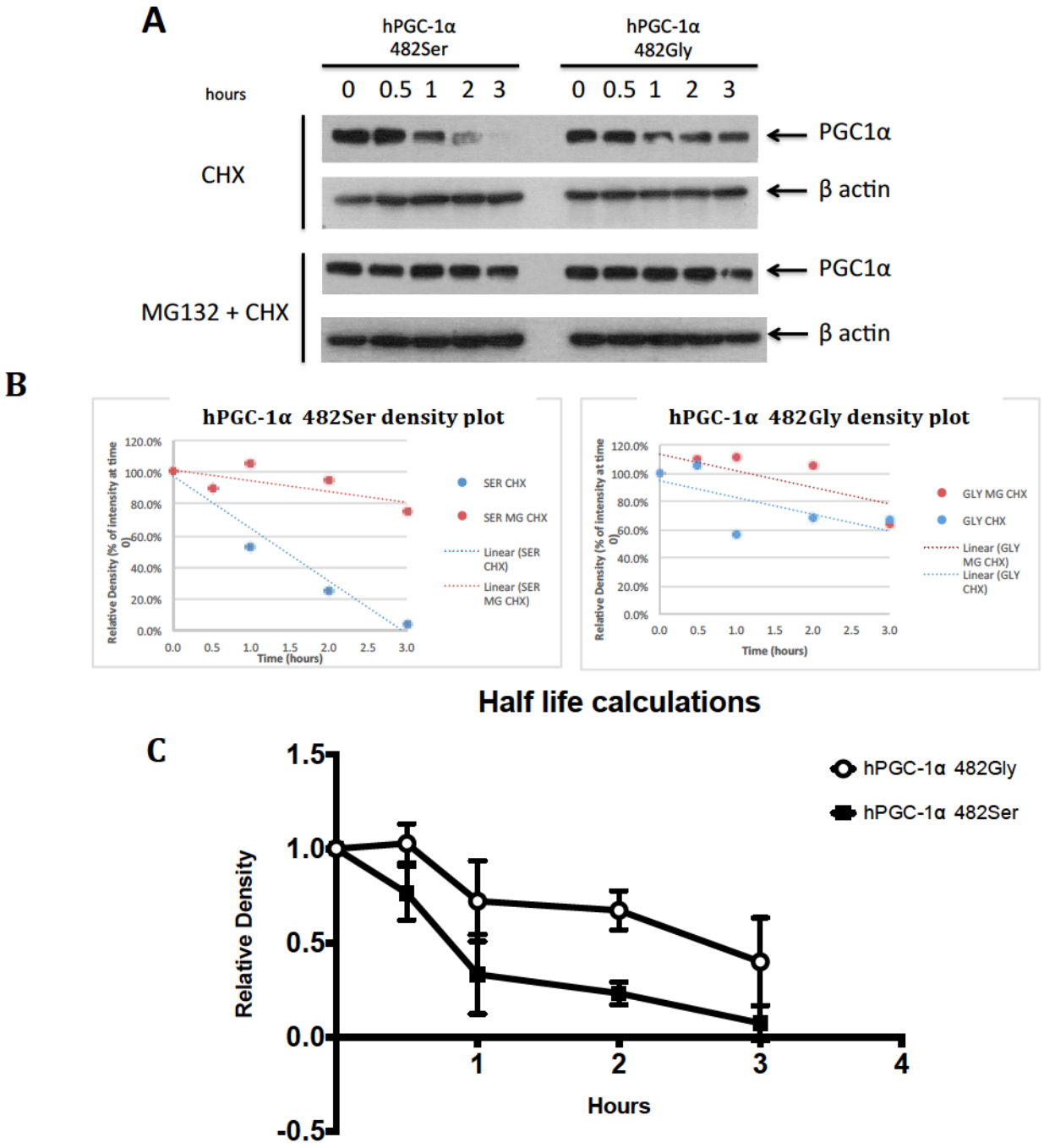

Figure 8. PGC-1α 482Ser has a shorter half-life than PGC-1α 482Gly

INS 1 cells were transiently transfected for 24 hours with hPGC1α constructs with a Glycine (Gly) or Serine (Ser) at amino acid 482. Wells were then treated with 50μg/ml of cycloheximide (Sigma) for the hours indicated. At the same time, analogous wells were treated with both cycloheximide and MG132 (10μM)

A) Western blot for PGC-1α on cell lysates from the cycloheximide chase, representative of 3 independent experiments

B) Quantification of Western blot of cycloheximide chase by Image J. Protein density relative to density at 0 hours, normalized to β actin.

C) 3 independent experiments were quantified by Image J and plotted to calculate half lives of the respective proteins. Points indicate mean values, and bars indicate standard deviation (n=3).

As shown in Figure 8A and B, 482Gly protein persists following cycloheximide treatment for longer than the 482Ser protein. In samples treated with MG132 along with cycloheximide, we again noted that inhibition of the proteasome stabilized protein from both variants similarly. (Figure 8A, B)

In order to calculate the half-life of the respective proteins, we quantified western blots from 3 independent experiments and plotted relative protein levels versus time, as shown in Fig 8C. The half-life of 482Ser was found to be close to 0.7 hours, while 482Gly was 2.5 hours. It is notable that mouse PGC-1α has a Serine at position 481 (that corresponds to amino acid 482 in humans), and our half-life value for 482Ser PGC-1α agrees with that determined by Trausch-Azhar et al. in 2010 [189] for mouse PGC-1α. Taken together with our previous data, this supports the idea that amino acid 482 of PGC-1α may play a role in the degradation of human PGC-1α. The question, however, remains of how this degradation is mediated and how the Serine residue impacts this mechanism.

In silico determination that the Serine at position 482 is a putative phospho-site

Due to the difference in stability, we hypothesized that amino acid 482 (specifically Serine) is a target for post-translational modification. As discussed before, the Serine at position 482 is reported for most species other than humans and chickens. It was also discussed by Nitz et al. that the amino acid modifications at position 482 are not predicted to inherently destabilize any tertiary structures in the protein [192]. We ran the sequences of our constructs through online programs that predict phosphorylation and O-glycosylation (Figure 9), as Serine is a common amino acid for these post-translational modifications.

Sequence flanking Gly482Ser
DEAD KT S ELRD

Server	Predicted Kinase	Score	Consensus Sequence
NetPhos 2.0/ NetPhosK	CKII	0.756	SXXE/D, SXE/D, S/D
GPS 3.0	Akt/AKT1/AGC	1.683	RXXS/T

Server	Score
NetOGlyc 4.0	0.77592

Figure 9 – In silico analysis to predict putative post-translational modification on Serine at amino acid 482 in PGC-1α
The entire PGC-1α sequence was entered into servers that use neural networks to predict post-translational modifications based on similarity to known consensus sequences. Scores are assigned that must exceed the threshold determined by the neural network to be a predicted site (threshold is 0.5 for NetPhos 2.0, NetPhosK and NetOGlyc 4.0). The sequence flanking the Gly482Ser polymorphism, and the consensus sequences for the predicted kinases are included for reference.

CKII – casein kinase 2, Akt – Protein kinase B, AGC – Protein kinase A, G and C family, S – Serine, E – Glutamic acid, D – Aspartate, T – Threonine, X – any amino acid, A – Alanine, R - – Arginine, K – Lysine, L – Leucine

The NetPhos 2.0 program (http://www.cbs.dtu.dk/services/NetPhos/), a server that uses a neural network to predict phosphorylation sites in eukaryotic proteins, predicted that the Serine at position 482 was likely phosphorylated with a score 0f 0.756, agreeing with a previously published prediction [113]; NetPhosK 1.0, a server that uses a neural network to generate kinase specific phosphorylation site predictions in eukaryotic proteins, predicted that this kinase was CKII (http://www.cbs.dtu.dk/services/NetPhosK/)

We further analyzed our sequence using GPS 3.0 (http://gps.biocuckoo.org/), another kinase specific phosphorylation site prediction software, that predicted that the Serine at amino acid 482 was an Akt phosphorylation target. Akt is known to phosphorylate PGC-1α in response to insulin stimulation at position Serine-570 and inhibit PGC-1α activity.

Finally we ran our sequence through YinOYang 1.2,

(http://www.cbs.dtu.dk/services/YinOYang/) which predicted that the Serine at position 482 was not a likely O-β-GlcNAc attachment site. It is, however, a predicted GalNAc O-glycosylation site (http://www.cbs.dtu.dk/services/NetOGlyc/) (Figure 9).

hPGC-1α 482Asp exhibits reduced stability similar to hPGC-1α 482Ser

Since in silico analysis predicted amino acid 482 to be a putative phosphorylation site, to verify this possibility we generated mutants at the site 482, including a "phosphomimic" (Aspartate at the 482 site), and a non-phosphorylatable mutant (Alanine at the position 482). The polar group in the Aspartate mimics the phosphorylated Serine, while the Alanine, though similar in size to Serine with three carbons, does not possess the exposed –OH group that is targeted by kinases. We hypothesized that the phosphomimic protein

would be less stable than the alanine mutant, and that protein persistence of the phopshomimic would be similar to that of 482Ser PGC-1α while the alanine mutant would be similar to that of hPGC-1α 482Gly.

We overexpressed these constructs in the same vector for 24 hours, and treated INS-1 cells with cycloheximide for 3 hours to evaluate the degradation kinetics of the mutants and compared them to the Glycine and Serine variants. In the untreated samples, we again found considerably less protein for the 482Asp construct, similar to the 482Ser construct. On the other had, the 482Ala protein was detected at higher levels, similar to 482Gly (Figure 10). Protein persistence following 3-hour treatment with cycloheximide also suggested the alanine mutant was more stable than the Aspartate and Serine mutant. While phosphomimics do offer some insight into the possible effect of phosphorylation, they imply constitutive phosphorylation, and thus may not be entirely biologically comparable. However, these experiments support the hypothesis that post-translational modification of the 482 site of PGC-1α by phosphorylation may regulate the stability of the protein.

Sirt1 and Tfam are differentially regulated by the two variants

Previous studies assessing the functional impact of the Gly482Ser polymorphism have focused on differential coactivation of target genes by either variant [185, 187, 193]. We also investigated influences on gene coactivation by the polymorphism, and collected RNA from INS1 cells transiently overexpressing PGC-1α 482Ser and 482Gly for 24 hours or 48 hours, and subjected it to qPCR to investigate if the Glycine and Serine variant exhibit differential regulation of target genes, after verifying that there was no significant

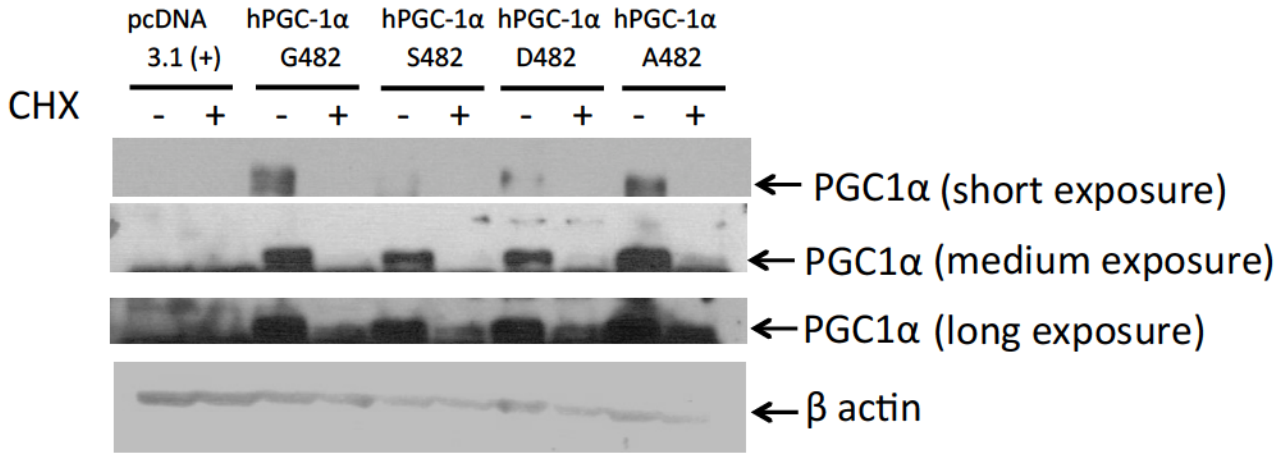

Figure 10. Phosphomimic mutants of PGC-1α at amino acid 482 are less stable
Site directed mutagenesis was used to introduce an aspartate at amino acid 482 (D482), as a phosphomimic and an alanine at amino acid 482, as a non-phosphorylatable mutant. INS-1 cells were transfected with either of these two mutant constructs of PGC-1α or the 482Gly (G482) or 482Ser (S482) PGC-1α constructs, and allowed to express for 24 hours, before treatment with 50 μg/ml cycloheximide (CHX) for 3 hours or vehicle (DMSO). Cell lysates collected from this experiment were then subjected to western blot probed for PGC-1α.

difference in the overexpression of either construct (Figure 11A and D). Genes selected for analysis were transcription factors known to be co-activated by PGC-1α, and target genes downstream of these.

At 24 hours Cytochrome C, Nrf-1 and PPARα were modestly induced, but presented no significant differences between the 482Gly and 482Ser PGC-1α. Downstream targets *Dgat2* and COXIV were also induced, but there were no significant differences between the variants. In contrast, Sirt1 ($p<0.0001$) and Tfam ($p=0.0007$) were upregulated considerably, and to different extents based on the variant overexpressed (Figure 11B), with the Glycine variant increasing Sirt1 and Tfam 7-8 fold, while the Serine variant inducing these 12 and 11 fold respectively. The greater induction of Tfam agrees with previously published data that determined increased activity of the Serine variant on the Tfam promoter [193]. However, to our knowledge, this is the first report of the increase of Sirt1 with PGC-1α overexpression in beta cells (earlier reports have assessed PGC-1α/Sirt1 interaction in the liver [103] at the protein level). Interestingly, we did not see this induction following 48 hours overexpression of the variants (Figure 11E), suggesting a feedback mechanism or transient regulation, or that the transcripts of Sirt1 and Tfam may be very unstable.

Furthermore, at both 24 hours and 48 hours, we found that ERRα and SOD2 were less induced by the Serine variant as compared with the Glycine variant, albeit this difference was not statistically significant (Figure 11C,F). It is interesting to note that there is no consistent trend across all of PGC-1α's targets in the beta cell depending on the polymorphism; this indicates various thresholds of activation needed for each of these genes, and also underscores the more complex nature of gene regulation.

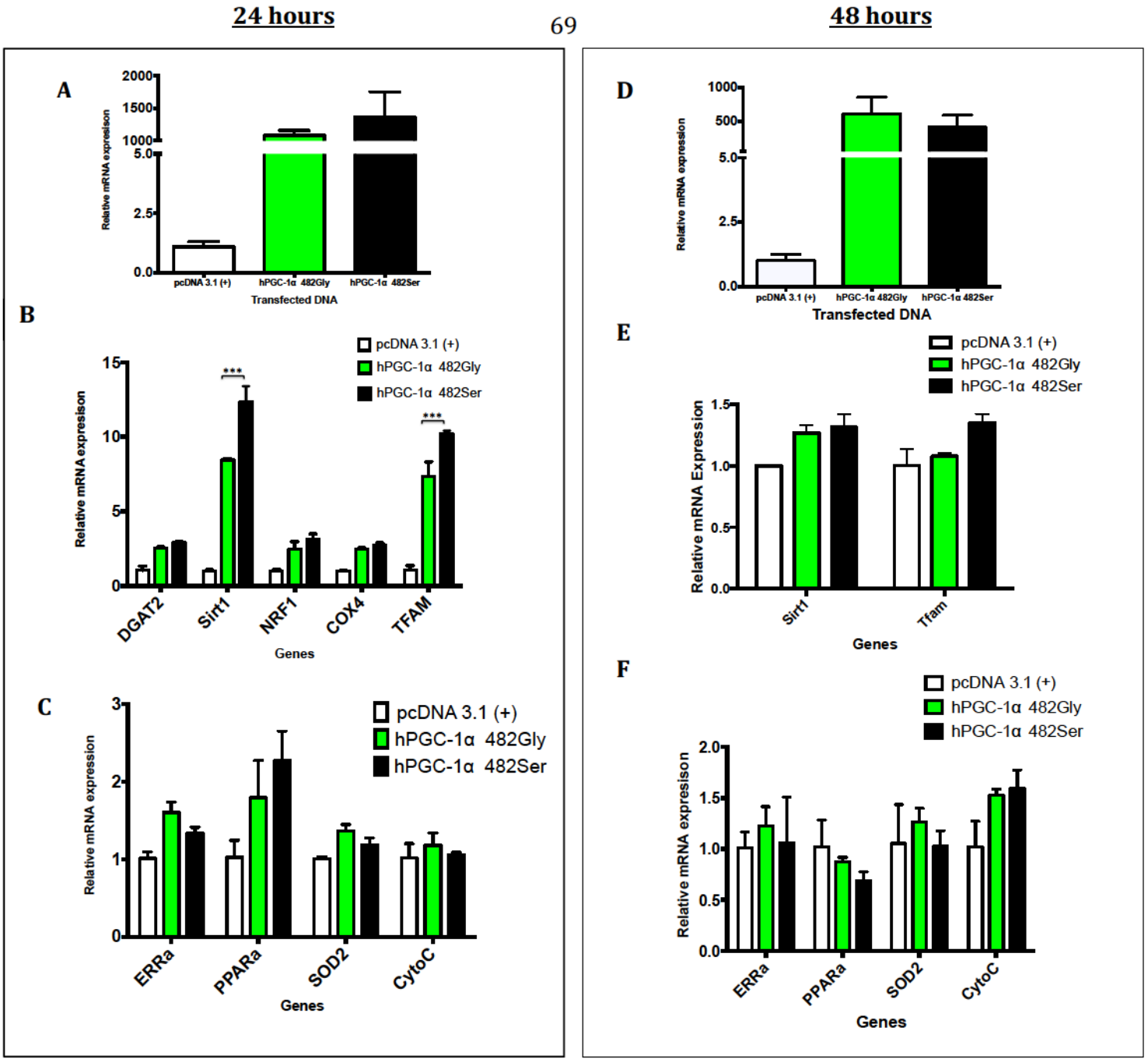

Figure 11 – Sirt1 and Tfam are differently regulated by 482Gly and 482Ser PGC-1α after 24 hours

INS-1 cells overexpressing the hPGC-1α 482Gly, hPGC-1α 482Ser, or empty vector pcDNA 3.1(+) as a control were harvested for RNA after 24 and 48 hours. Quantitative real-time PCR was performed with primers selected to assess transfection (A,D) and gene targets of interest (B,C,E,F) at the respective time points (n=4-6).

**** $p<0.0001$, ***$p<0.001$. Results are plotted as means with bars representing S.D.

Chapter Two

Section IV: Discussion

Summary of Results

In this study we aimed to investigate the influence of the Gly482Ser polymorphism on the human PGC-1α1 protein. This polymorphism has been associated with diabetes and metabolic disorders for over two decades in large population studies; as yet there is no consensus on the influence of this substitution on the structure or co-activator function of the PGC-1α1 protein. In INS-1 cells, an immortalized rat beta cell line, we show that the 482Ser PGC-1α protein was less stable than the 482Gly PGC-1α protein, and confirmed this finding by stabilizing both to similar levels with the proteasome inhibitor MG132, and determined the half-lives of the protein arising from each polymorphism. This difference in stability between two proteins differing only at one amino acid suggests that the amino acid 482 of human PGC-1α may be a site of potential post-translational modification. To further elucidate the possible modifications at this site, we analyzed our sequences by Servers designed to predict putative phosphorylation sites as well as kinases targeting the site. The two kinases that came up in our in silico search, Akt and CKII, have not been reported to target exon 8 of PGC-1α previously. To validate that the Serine at amino acid 482 may be phosphorylated, we designed "phosphomimic" mutants, designed to mimic either a phosphorylated Serine (Aspartate) or non-phosphorylatable Serine (Alanine) at position 482. The 482Asp mutant was less stable than the 482Ala PGC-1α and expressed protein levels similar to the 482Ser protein. In contrast, the 482Gly and 482Ala proteins had similar stability, supporting our hypothesis. We further observed that certain canonical PGC-1α genes (Tfam and Sirt1) were differentially regulated by the two polymorphisms, while most gene targets of PGC-1α coactivation showed no difference.

Difference in stability of PGC-1α due to the Gly482Ser polymorphism

We show in this study that the 482Ser PGC-1α is less stable than the 482Gly, a novel finding that is particularly interesting given that this polymorphism has as yet only been reported in humans and chickens [113]. This raises the question as to why the 482Ser variant occurs less frequently in humans, when it is found in most other animal species; if the Serine variant was selected against, what advantage does the Glycine variant offer? Four studies investigating the influence of this polymorphism on PGC-1α have focused on the differing effects on coactivator function; however, this specific domain of PGC-1α is not generally associated with transcription factor binding. In fact, this is a relatively uncharacterized domain of the protein and our data shows for the first time that this region may be important for controlling degradation of the PGC-1α protein.

We consistently observed differences in protein persistence, and were able to rescue both proteins to equivalent levels with MG132, suggesting that the site regulates the targeting of PGC-1α to the proteasome. A caveat to this conclusion would be that the proteasome targets various other targets on PGC-1α, as well as other regulators of PGC-1α, thus perhaps differences in stability were masked on treatment with MG132. A possible experiment to further clarify this would be to determine differences in PGC-1α ubiquitination by coexpressing our constructs with a tagged ubiquitin construct. Following immunoprecipitation, increased ubiquitin associated with the 482Ser PGC-1α protein as compared with 482Gly PGC-1α would support our hypothesis that this specific site mediates the degradation of PGC-1α.

Most previous studies designed to elucidate mechanisms regulating PGC-1α stability, post-translational modification and function used the mouse PGC-1α as a template. This means that most reports have focused on PGC-1α with a Serine at the homologous position to amino acid 482 in humans, since this is the only form as yet found in mice. These values have varied between publications – in 2007 Trausch-Azhar et al., reported the PGC-1α's half-life was 0.3h in HL1 mouse atrial cardiac myocytes [189], whereas in 2001 Puigserver et al., reported that PGC-1α had a half-life of 2.27 hours in C2C12 mouse muscle cells [65]. It is interesting to note that the half-life of the human 482Ser PGC-1α calculated in our present study (0.7 hours) is within the range reported by earlier studies [189], whereas the human 482Gly protein has a considerably longer half-life. This, in addition to the fact that most other species possess a Serine at this position (Figure 3B), supports the idea that the Serine at position 482 may be a site of regulation that was lost with the Glycine substitution. Unfortunately little is known about the 3 dimensional structure of PGC-1α [61, 194, 195], limiting our speculation as to how modification of polar amino group that could be targeted by a kinase may influence the structure of PGC-1α. Previous studies that have tried to predict possible effects of switching the amino acid at position 482 on the secondary protein structure by in silico analysis have reported no possible effects [185]. This agrees with our hypothesis that this site is the target of a possible post-translational modification, a very interesting area of future study in a protein intricately regulated by post-translational modifications.

Gly482Ser as a putative phosphorylation site of PGC-1α

Previous studies assessing kinase targeting of PGC-1α have not offered any evidence that this site is phosphorylated. Furthermore, studies investigating ubiquitination of PGC-1α following phosphorylation by GSK3B did not identify exon 8 or amino acid 482 as a putative target [73, 74]. Of the predicted kinases, Akt and CKII, Akt has been studied before with PGC-1α as a substrate [71], whereas CKII has not. Akt phosphorylates PGC-1α in response to insulin signalling; however, PGC-1α stability has not been reported to be affected by insulin signalling, though its co-activator activity is repressed by phosphorylation at amino acid 570 [71]. CKII kinase activity has not been evaluated on PGC-1α; however, the substrate of this kinase are ubiquitous and its regulation multifaceted. This includes association with the cell cycle, which PGC-1α has not been linked with yet. It is notable that while the prediction scores we derived for these kinases are well above the threshold, they are still quite modest and only predictions based on consensus sequences.

In order to determine the kinase targeting amino acid 482, an unbiased approach may be more favourable given that we do not have overwhelming evidence to support either of these candidates. Using a candidate-based approach to identifying the kinase would be time-consuming and possibly biased. To this end, a high throughput kinase screen designed to cover over 90% of the human kinome may offer the most reliable identification of the kinase. Such a screen would entail an in vitro approach of co-expressing the GST-tagged substrate and the selected kinase, and determine which kinase is suitable candidate [196, 197]. Identification of a candidate kinase will then have to be validated in vivo, as the

screen conditions (for example, the cell type used to co-express the substrate and kinase, or the ectopic expression of the kinase) may not be relevant to the physiological context of interest, and because co-expressing the kinase with a substrate of interest encourages non-specificity of kinase activity, although it is reported that substrates in this screen are usually only targeted by kinases of the same family [197], limiting non-specificity. Multiplexing substrates also helps in ruling out background kinase activity [197]. The developers of this screen recommend confirming results either by using a non-phosphorylatable mutant of the substrate, or knocking down the kinase identified by the screen in the cell of interest to see if phosphorylation of the protein of interest is abrogated [197].

Before initiating a kinase screen, however, we must first have evidence that the amino acid 482 of human PGC-1α is indeed phosphorylated. Phosphomimic experiments offer a view into the effect of constitutively phosphorylated proteins, and can support our hypothesis. However, there are limitations to this approach, not least of which is that proteins are not usually in a constant state of phosphorylation. Such experiments lose the nuance of a biological system, where a kinase is regulated by specific signals within cells. Furthermore, Aspartate and phosphorylated Serine though similar are not identical structurally or in their charge. Given the lack of published information of PGC-1α's three-dimensional structure we cannot be sure that this experiment does not itself introduce an artefact. Nonetheless, the use of site directed mutagenesis to generated mimics of phosphoserine (and other phosphorylated amino acids) is established throughout scientific literature, since about ten years after site directed mutagenesis was first described in 1978 [198]. Published evidence shows that a positive result using this technique is very often

confirmed by other methods (such as metabolic labelling). However, a negative result (a phosphomimic not having the same phenotype as the Serine/Thr) does not necessarily confirm that the site is not phosphorylated [199].

To conclusively confirm that this site is phosphorylated, or if there are any other modifications of interest in this region, the PGC-1α protein could be subjected to Mass-Spectrometry analysis. We have attempted on numerous occasions to immunoprecipitate the overexpressed human PGC-1α variants to limited success. Unfortunately, existing commercial antibodies are not "clean" enough, and when samples are silver stained following immunoprecipitation, a discrete band cannot be isolated for Mass-Spectrometry. We have also attempted to use a FLAG N-terminal tag, again to limited success. It appears that translation may begin from an alternative start-codon that is very close to the predicted and published start codon, thus, the FLAG may be eliminated following translation. We are in the process of creating a PGC-1α protein with a C terminal V5 tag. Tagging PGC-1α offers its own caveats as well, since both the N and C terminals are implicated in the degradation of PGC-1α [74, 188, 189], and the nuclear localization signal (NLS) is found in the C terminal domain. Both tagged PGC-1α variants will have to be compared with untagged variants to ensure that the tag does not produce an artefact.

Differential coactivator function of the Gly482Ser PGC-1α

Studies investigating differential effects of this polymorphism on PGC-1α coactivaor function are in disagreement. The findings we report in this study may offer some insight into the basis of this disagreement, as we see differential effects of the polymorphism on

certain gene targets of PGC-1α, but not others. Furthermore, we also note a temporal effect of the protein on these targets. Other studies looked into the effect of the Gly482Ser polymorphism on coactivator targets in hepatocytes, and phenotypes associated with these by using promoter-reporter assays with overexpression of the variants, limiting the scope of their investigation to one or two targets. Our study differs with these in that our experimental model was a rat insulinoma immortalized line, and that we looked at the effect of over-expressing the variants on a variety of endogenous targets. Some of these targets are co-activated by PGC-1α and some genes are affected downstream, and were selected to address differences in co-activation efficiency arising out of differences in stability between the two variants. Nonetheless, we did indeed see that there was a difference in Tfam regulation based on the polymorphism, as the Serine variant induced Tfam to a higher level than the Glycine variant. We also noted that Sirt1 induction was greater with the Serine variant as compared with the Gly; the induction we observed in this study is particularly interesting as this is the first report of Sirt1 being regulated by PGC-1α in beta cells. The importance of the Sirt1/PGC-1α is known in liver cells where Sirt1-controlled de-acetylation of PGC-1α under fasting conditions activates gluconeogenic and fatty acid oxidation genes [200]. In beta cells, Sirt1 promotes insulin secretion by repressing uncoupling protein 2 (UCP2) [201]. The role played by Sirt1/PGC-1α interaction is not yet known in beta cells, but is very interesting, given that PGC-1α induces UCP2 [202], and Sirt1 is necessary for β cells to detect fluctuations in blood glucose [203]. It is intriguing to note that the polymorphism -866G/A in UCP2 that increases the risk of developing diabetes is significantly associated with risk polymorphisms of PGC-1α, including Gly482Ser, whereas the protective genotype of both UCP2 and PGC-1α are

additively protective against the risk of developing diabetes [134]. UCP2 protects beta cells against reactive oxygen species (ROS) [204]. We may speculate that the greater induction of Sirt1 by 482Ser PGC-1α could repress UCP2 leaving the beta cells more susceptible to damage by ROS under conditions of hyperglycemia, while the more rapid degradation of 482Ser PGC-1α would result in limited induction of UCP2 when needed to protect the beta cells. It would be interesting to see how UCP2 is affected by either Glycine or Serine variant of PGC-1α, and their differential regulation of Sirt1, and how beta cells manage ROS with the overexpression of either variant. Differences in ROS detoxification would also be interesting given the differential regulation by the Gly482Ser polymorphism of Tfam. While the beta cell specific knockout of Tfam has not been described, mice with Tfam knocked out in the adipose tissue display decreased complex 1 activity and increased uncoupled respiration, leading to increased energy expenditure and insulin sensitivity, and reduced adipose tissue mass and hepatosteatosis [205].

ERRα is an effector of PGC-1α and mediates the expression of OXPHOS genes and genes necessary for mitochondrial biogenesis [206]; SOD2 plays an essential role in the detoxification of ROS in the mitochondria. The impaired induction of both by the Serine variant could potentially contribute to beta cell dysfunction; however, the reduction noted in this study was not statistically significant. Other typical targets of PGC-1α, such as CytoC, PPARα, DGAT2 and COX4, while upregulated by overexpression of human PGC-1α, did not differ between the polymorphisms. It is notable that the trend in the differences is not simply that all genes that differ between the constructs are less induced by the 482Ser PGC-1α. This may have been expected based on our finding that 482Ser PGC-1α is less stable, given that all PGC-1α activity has been understood in the context of protein-protein

interactions. We cannot extrapolate a specific trend from our results to help explain the possible pathway influenced by this polymorphism; ERRα and Tfam are both essential to the mitochondria, the former mediating mitochondrial biogenesis [206], the latter mitochondrial transcription [207], and deviate in opposite trends. However, it interesting to note the differences in Sirt1 upregulation as Sirt1 is itself involved in the regulation of PGC-1α. Sirt1 activates PGC-1α through its deacetylase function, and works antagonistically with the histone acetyltransferase GCN5. It has been noted that when Sirt1 activates PGC-1α in response to oxidative stress, GSK3B is also activated and inhibits PGC-1α activity by promoting its proteosomal degradation following phosphorylation on Threonine-295, thereby curtailing the effect of Sirt1 on PGC-1α activation [70]. It may be speculated that in response to this surge in activation of Sirt1, and thus an enhancement in the activation of PGC-1α, an antagonistic mechanism may be activated as part of negative feedback. Since the 482Ser PGC-1α induces Sirt1 significantly more, this feedback mechanism may degrade the hPGC-1α protein more rapidly. This study was limited to determining any perturbations in predicted or known PGC-1α targets at two set time points; it would be interesting to measure Sirt1 induction in a time dependent manner and assess if Sirt1 is upregulated more rapidly in the 482Ser overexpressing cells as compared with the 482Gly, and if this induction is curtailed faster in 482Ser samples than 482Gly.

The assessment of perturbations here was restricted to a pre-selected list of canonical PGC-1α target genes. For a more complete view of pathways responding differently to the polymorphism it may be more valuable to use a microarray or RNA-seq and assess pathways at multiple downstream steps. Another limitation of our study was the use of rat insulinoma cells to study a human protein; while there is great homology between rat and

human PGC-1α (94%), the protein sequences are not identical, particularly in the precise domain flanking the 482 amino acid position. Further investigations on the Gly482Ser polymorphism may benefit from the use of human beta cell lines that are now available, and are being validated. In addition to the advantage of studying a human system, these cells have also been engineered to switch between immortal and “natural” states, offering an intriguing insight into the protein biology without the caveat of using an immortalized line [208].

Chapter 3

Localization of PGC-1α4 in response to TNFα

Section I: Introduction

An introduction to PGC-1α4

PGC-1α4 was cloned and characterized by Ruas et al. from mouse skeletal muscle in 2012 [95]. It possesses the same alternative exon 1 as PGC-1α2 (exon 1b), and is truncated after exon 6 due to a 31-nucleotide insert between exon 6 and 7 that introduces an early stop codon. This protein is only 266 amino acids long. This group reported that PGC-1α4 mRNA was expressed in the skeletal muscle, heart, brain and brown adipose tissue.

A particularly striking property of this truncated isoform is the set of novel gene targets it regulates. In the muscle, it does not influence typical canonical PGC-1α1 mitochondrial targets such as Cytochrome C and cytochrome c oxidase subunit 5B (*CoxVb*), or others such as carnitine palmitoyltransferase 1 (CPT1) and medium-chain acyl-coA dehydrogenase (MCAD) [95]. On the other hand, PGC-1α4 induces insulin-like growth factor (IGF1) while repressing myostatin. Myotubes transduced with adenovirus over-expressing PGC-1α4 exhibit pronounced hypertrophy, while knocking down PGC-1α4 blunts skeletal muscle hypertrophy. The hypertropic action of PGC-1α4 is independent of estrogen-related receptors [95]. Transgenic mice overexpressing PGC-1α4 in myotubes are also resistant to cancer-induced cachexia [95].

PGC-1α induces vascular endothelial growth factor (VEGF) under conditions of hypoxia [209], without up-regulating mitochondrial genes [210] that are canonical targets of PGC-1α1 regulation. This distinction is achieved by the hypoxic induction of both truncated isoforms, PGC-1α4 and NT-PGC-1α, and their knockdown abolishes the increase in expression of VEGF in response to hypoxia [210]. Conversely, transgenic mice expressing

PGC-1α4 under the control of the MEF2C enhancer/myogenin promoter in skeletal muscle [95] have significant induction of VEGF and angiogenesis, without an increase in mitochondrial genes [210].

The novel roles of PGC-1α4 reported in muscle open a new area of investigation into its distinct functionality in different tissues, and how it modulates the PGC response to various stimuli. Our lab has previously found that, as in myotubes [95], the sets of genes regulated by PGC-1α1 and PGC-1α4 in hepatocytes overlap very little (unpublished data). On TNFα treatment, genes induced by both PGC-1α1 and PGC-1α4 increased from 4 to 2166, and genes regulated specifically by PGC-1α4 increased from 20 to 1208, while gene set enrichment revealed that only PGC-1α4's regulated set of genes were involved in anti-apoptotic pathways (unpublished data). Our lab further discovered that PGC-1α4 inhibited caspase 3 cleavage following TNFα treatment in hepatocytes (unpublished data), and a cytokine cocktail (TNFα, IL1β and IFN-Y) treatment in INS1 cells [211], as compared with GFP expressing controls and PGC-1α1 expressing cells. The reduction in the apoptotic index (assessed by the cell death ELISA kit) was also confirmed by cell death ELISA, that measured cytoplasmic histone-associated DNA fragments after apoptosis (Sigma), establishing yet another distinct property of PGC-1α4 as an anti-apoptotic agent (unpublished data). The mechanism by which PGC-1α4 inhibits apoptosis is presently under investigation.

Hepatocytes and inflammation

It is now firmly established in the field of endocrinology that inflammation plays a profound role in the development of diabetes and the progression of complications. The first molecular link established between inflammation and obesity was found to be TNFα. This cytokine (and adipokine) is overexpressed in the adipose tissues and muscle of obese humans [212, 213], evocative of the overexpression found in mouse models of obesity [214]. TNFα was determined to play a prominent role in the development of insulin resistance, as the addition of the cytokines prevents insulin action, and TNFα deficient obese models of mice [215] are protected from the development of insulin resistance.

Inflammation contributes to liver damage in type 2 diabetic patients. Inflammatory cytokines contribute to the development of hepatic fibrosis, including Non-Alcoholic Steatohepatitis (NASH) [216]. This link is particularly pronounced in type 2 diabetes; a distinct set of genes is expressed in NASH patients with T2D compared to NASH patients without T2D [216]. Apoptosis is a key feature of liver diseases such as NASH, and is increased with greater insulin resistance [217], becoming the primary facilitator of liver injury and liver fibrosis [218]. The role of hepatocyte apoptosis has also been implicated in a plethora of other liver diseases, ranging from ischemia-reperfusion injury to acute liver failure. The main executioners of the extrinsic pathway of apoptosis in hepatocytes are considered to be TNFα and FasL [219]. . Enhanced levels of adipokines such as TNFα are also thought to mediate the progression of NASH to hepatocellular carcinoma.

Besides the role TNFα plays in liver damage in the metabolic disorder, it also mediates the liver injury sustained by the action of viruses such as HCV and HBV [220], and alcohol

[221]. Hepatocellular apoptosis plays a key role in the development of fibrosis as a result of these factors as well [222].

Given the established role of the hepatocellular apoptosis in human liver disease, it is of great interest to study mechanisms of apoptosis and the prevention of apoptosis in response to cytokines such as TNFα.

Significance of protein localization

In 2007, Sano et al., showed that in the absence of MG132, PGC-1α was diffused throughout the nucleus [188], and that on proteasome inhibition PGC-1α remained sequestered in the nucleus in poly-ubiquinated aggregates. The NLS of PGC-1α is found in the C terminal domain between the RS and RRM domains, so the exclusion of the C terminus abrogates the protein sequestering in the nucleus; the mutant PGC-1α 1-565 was found throughout the nucleus and cytoplasm, whereas the mutant PGC-1α 1-292 was found entirely within the cytoplasm. These C terminal mutants were also more stable than full length PGC-1α.

Studies focusing on the isoform NT-PGC-1α report that while the protein has a greater cytoplasmic concentration as compared with nuclear [223], the regulation of these compartment shares is dynamic and shifts based on stimuli to the cell, with PKA activation reportedly serving to increase the nuclear content of NT-PGC-1α [101]. NT-PGC-1α and PGC-1α4 only differ in their exon 1, and are both truncated in exon 6, so the NLS of PGC-1α that is found in the C terminus is absent in these isoforms (Figure 12). Sano et al., had not reported mislocalization of the N terminal deletion mutants of PGC-1α, although they did

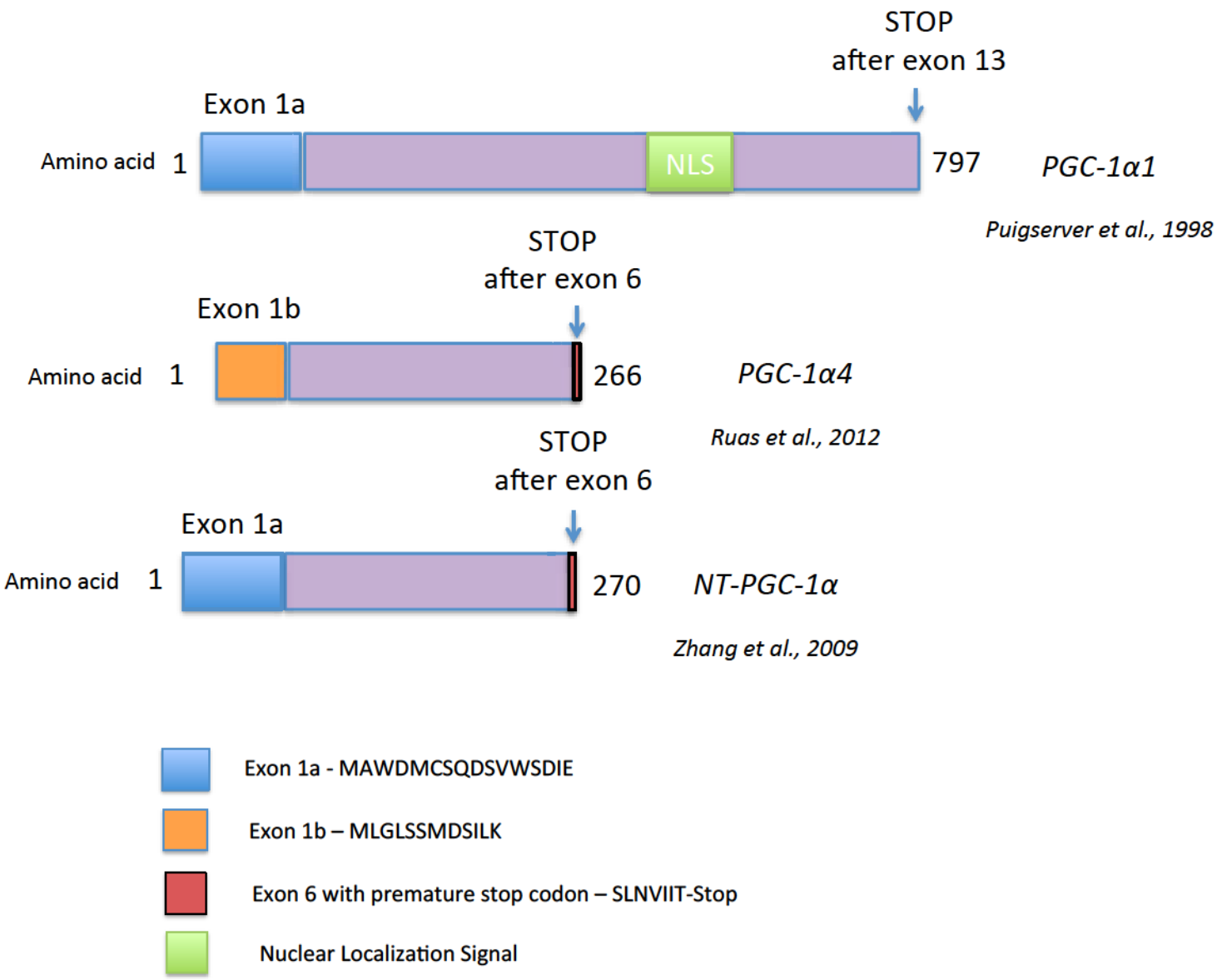

Figure 12: Summary of differences in protein sequence of PGC-1α1, PGC-1α4 and NT-PGC-1α
Due to the alternative promoter, PGC-1α4 possesses a distinct exon 1 from PGC-1α1 and NT-PGC-1α. Both PGC-1α4 and NT-PGC-1α have an insertion in exon 6 that results in an early stop codon, resulting in the truncated protein sequence, that does not include the Nuclear Localization Signal (NLS) found in the canonical PGC-1α1 within the RS (arginine-serine rich) and RRM (RNA recognition motif) domain towards the C terminus.
Figure modified from Martinez-Redondo, Pettersson and Ruas, 2015

find that these proteins tend to aggregate in nuclear speckles [188], so differences in localization due to exon 1b were not expected between the two truncated isoforms. However, when Ruas et al., described PGC-1α4 in 2012, the protein's localization was reported to be nuclear in muscle cells [95], in spite of the absence of the C terminal NLS.

Given the novel roles played by PGC-1α4 as compared to the canonical PGC-1α1 and its anti-apoptotic properties in response to TNFα treatment, it would be interesting to investigate if the localization of this protein is influenced by external signals.

Objectives

PGC-1α4 is a novel isoform of PGC-1α, and possesses unique properties including the induction of novel targets such as IGF-1 [95] and mediates angiogenesis in hypoxic conditions [210] in muscle; it is yet to be explored how this truncated protein behaves distinctly in different tissues. It has been reported that PGC-1α4 curbs TNFα mediated cell death in unpublished data by a previous graduate student [211] but it is not yet understood how. Intracellular localization of the protein can offer a wealth of information on the function of a protein, and help work out the mechanisms of distinct properties.

In this study, we aim to 1) determine PGC-1α4 localization in hepatocytes, and 2) determine if it is influenced by TNFα treatment.

Chapter Three

Section II: Methods

Cell culture and treatments: H2.35 cells were cultured in DMEM (Wisent) medium with 5% FBS (Wisent), 1% penicillin and streptomycin (Wisent) and 1 uM Dexamethasone. Cells were transfected overnight with Lipofectamine 2000 (2.5 µg DNA and 6.5 µl lipofectamine) in normal medium, and then maintained in starvation medium overnight before TNFα treatment. Cells were treated with 50 ng/ml of TNFα for 3 hours before harvesting.

Western Blot: Cells were either fractionated (as described in cell fractionation in this section) or were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) plus protease cocktail inhibitor (Calbiochem) as indicated in the experiment. Following protein concentration determination by the DC assay, 20 µg of protein (or diluted, as indicated in the legend) were loaded onto 10% polyacrylamide gels, and then transferred overnight onto polyvinylidene diflouride (PVDF) membranes (GE healthcare). Besides the PGC-1α primary antibody (Calbiochem, ST1202), additional antibodies used include the anti-beta tubulin (Antibody ID AB_2315513) from Developmental Studies Hybridoma Bank (DSHB) was used as a control for the cytoplasmic fraction. Anti-ChK2 (Checkpoint kinase 2) antibody was from Cell signalling (#2662S) and used as loading control for the nuclear fraction.

RNA isolation and cDNA synthesis: Total RNA from H2.35 cells was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol.

For cDNA synthesis, 1 µg of RNA from H2.35 cells was incubated with 1 U/ml DNAse1 at 37°C for 15 minutes followed by 15 minutes at 65°C for DNAse1 heat inactivation. Total RNA in a volume of 20 µl was reverse transcribed with 50 U Multiscribe reverse

transcriptase (Applied Biosystems) and 20 U RNAse inhibitor (Biobasic). cDNA was produced 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: cDNA was subjected to amplifications for the gene of interest and for the endogenous control hypoxanthine-guanine phosphoribosyl transferase (HPRT). 5 µl reactions were set up in a 384 well plate, using Power SYBR green PCR Master Mix (Life Technologies). The cycling program occurred in two steps, a polymerase activation step for 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 min of 60°C using the Viia 7 system from Life Technologies. Data was normalized to the endogenous control and relative mRNA expression was determined using the ΔΔCt method. Results were graphed using Graphpad Prism

Immunofluorescence: H2.35 cells were allowed to attach to poly-L-lysine coated coverslips in 24 well plates overnight (20,000 per well). Following transfection and TNFα treatment, cells were fixed with 4% paraformaldehyde after being rinsed with PBS. Cells were permeabilized with 0.5% Triton X-100, and the blocked with 1% BSA for 1 hour. Cells were then incubated with primary anti-V5 antibody overnight. An Alexa 488 or Alexa 555 conjugated secondary antibody was used, as indicated in legend. Cell nuclei were stained with Hoescht before coverslips were mounted onto glass slides using Dako Fluorescent Mounting Media. Images were taken using an LSM 700 confocal microscope.

Cellular Fractionation: H2.35 cells were plated in 6 well plates, and allowed to settle overnight. Cells were transfected overnight the next day, and subsequently serum-starved overnight. Cells were treated with TNFα for 3 hours or treatment media without TNFα, and then trypsinized. All subsequent steps were carried out on ice. Cells were washed with cold PBS, spun down, and then washed again. After PBS was removed from the cell pellet, cells were resuspended with Buffer A, containing 0.1% NP-40 subsitute (Bioshop), 20 mM HEPES, 3mM $MgCl_2$, 0.25 M sucrose, 3mM 2-mercaptoethanol, protease inhibitor cocktail (Calbiochem). Cells were incubated for 5 minutes, and then spun down at 3000 g for 5 minutes. The supernatant was removed and kept as the cytoplasmic fraction (spun down once more to remove any debris); the pellet was washed and incubated for 5 minutes with Buffer A to remove cytoplasmic contamination. After spinning at 3000 g for 5 minutes, Buffer A was removed and the nuclear pellet resuspended in RIPA buffer with protease inhibitor cocktail for 30 min, then sonicated briefly. Following sonication, the nuclear fraction was spun down at 15,000 rpm for 20 minutes, and the supernatant pulled off as the nuclear fraction.

Statistical analysis method: GraphPad Prism was used to calculate statistical significance by two-way ANOVA for mRNA expression, and results were expressed as means ± standard deviation (SD). Statistical significance was defined as $p < 0.05$.

Chapter Three

Section III: Results

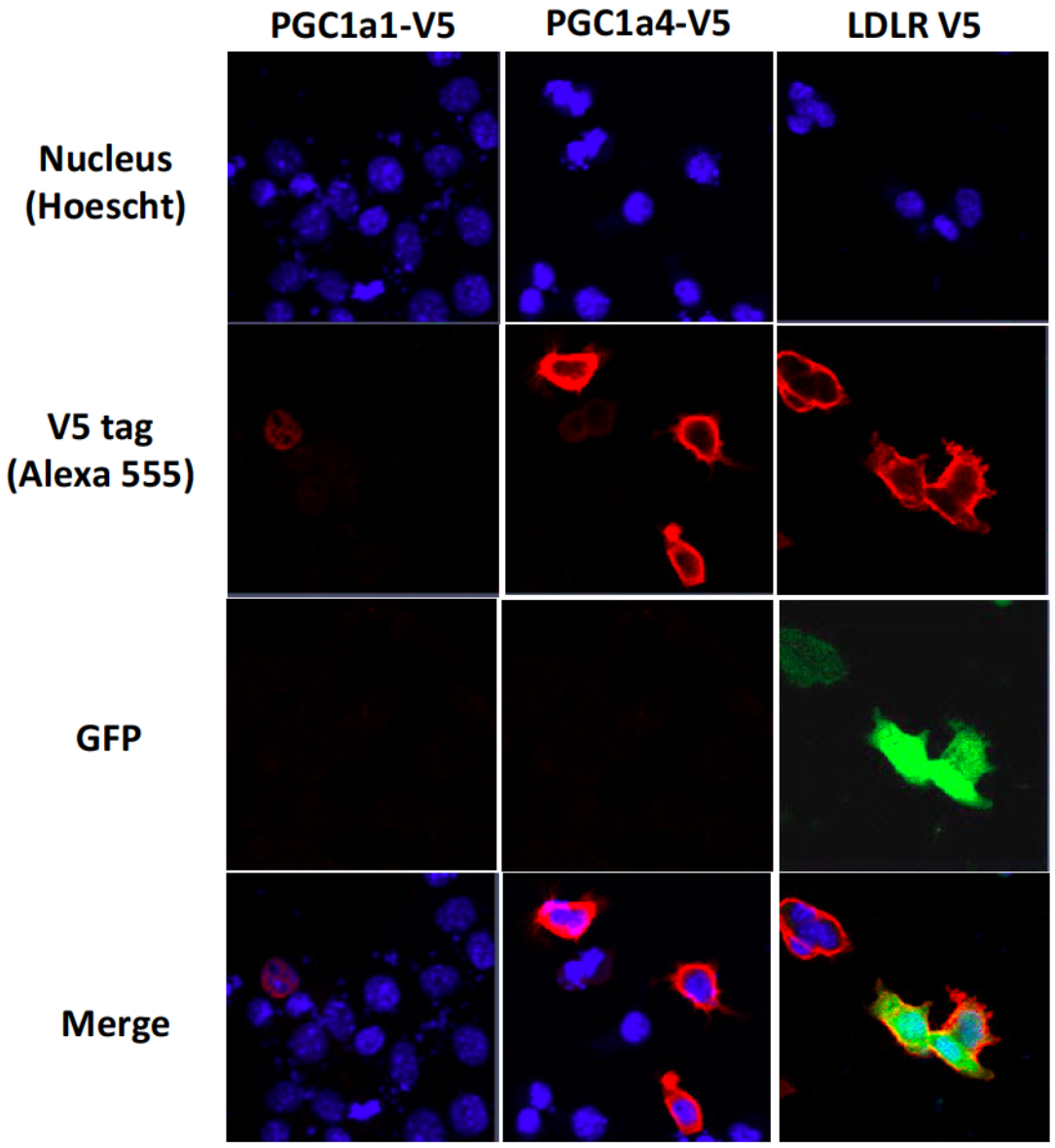

Figure 13. PGC-1α4 is predominantly cytoplasmic in H2.35 cells
Immunofluorescence images of PGC-1a4 localizing in the cytoplasm. PGC-1a1 is localized in the nucleus.
Controls include a negative control for the secondary antibody, and a positive control for the V5 antibody (LDLR-V5). The plasmid of the positive control co expresses GFP, thus verifying the localization of the Alexa 555.
(Magnification 40x) Experiments were done in biological triplicates; images are representative of three independent experiments.

PGC-1α4 is primarily localized in the cytoplasm in hepatocytes

In 2012 Ruas et al. published that like PGC-1α1, PGC-1α4 is also primarily localized in the nucleus of primary myotubes [95]. This is consistent with the protein's role as a transcription factor. We used H2.35 cells, a mouse hepatocyte cell line, and optimised our protocol to stain nuclear and cytoplasmic proteins. We overexpressed V5 tagged PGC-1α1 and PGC-1α4 to verify their localization; the proteins had to be overexpressed as presently available antibodies are not sensitive enough to detect the endogenous protein. In untreated, transfected cells, over-expressed PGC-1α1 is entirely sequestered within the nucleus, as previously shown [188]. However, the over-expressed PGC-1α4 protein was found mostly excluded from the nucleus and localized predominantly in the cytoplasm (Figure 13).

We verified our protocol with a positive control, transfecting H2.35 cells with pIRES2-LDLR-V5-eGFP, and found that only cells expressing the GFP (and hence, the V5 tagged LDLR) stained with Alexa 555. Alexa 555 staining for V5-tagged LDLR was restricted to the cytoplasmic membrane.

These results also agree with the greater abundance of PGC-1α4 protein we see on Western, as the signal from PGC-1α4 was considerably stronger as compared with PGC-1α1. This is most likely due to protein stability, similar to NT-PGC-1α [223]. Pulse chase experiments could determine whether this is due to enhanced protein stability.

This localization raises many questions about the trafficking of PGC-1α4 to the nucleus, and its role as a transcriptional coactivator. Specifically, there is the question of what conditions allow PGC-1α4 to reach the nucleus, or what alternate mechanism allows it to exert its

coactivator functions (for example, via other downstream effectors). This data also agrees with our lab's unpublished microarray data that few genes are induced with PGC-1α4 overexpression in hepatocytes in the absence of TNFα.

PGC-1α1 and PGC-1α4 are stabilized by short treatment with TNFα

Our lab has previously observed that overexpression of PGC-1α4 in INS 1 cells [211] as well as in hepatocytes (unpublished data) curtails caspase 3 cleavage and inhibits apoptosis. We found that in response to TNFα treatment for 3 hours, both PGC-1α4 and PGC-1α1 were stabilized at the protein level (Figure 14A-C). We verified that between the cytokine untreated and treated samples, there was no difference at the RNA level with primers that targeted exon 2 of PGC-1α1 and PGC-1α4 (Figure 14 D). Furthermore, we also used primers designed to target the V5 end of our constructs and verified that at the mRNA level there was no difference between treated and untreated cells, and our construct was not inadvertently responding to the cytokine treatment as pcDNA 3.1 (-) possesses a CMV promoter [224](Figure 14 E, data only shown for a4-V5 primer).

It is interesting to note this difference in protein levels with a shorter cytokine treatment, as after a longer treatment of 8 hours we have observed that PGC-1α1 and PGC-1α4 both drop at the protein level (unpublished, [211]). We found a reduction in endogenous PGC-1α mRNA detected by our exon 2 primers (Fig 14D). This agrees with earlier published reports that found that TNFα subdues PGC-1α transcription [225].

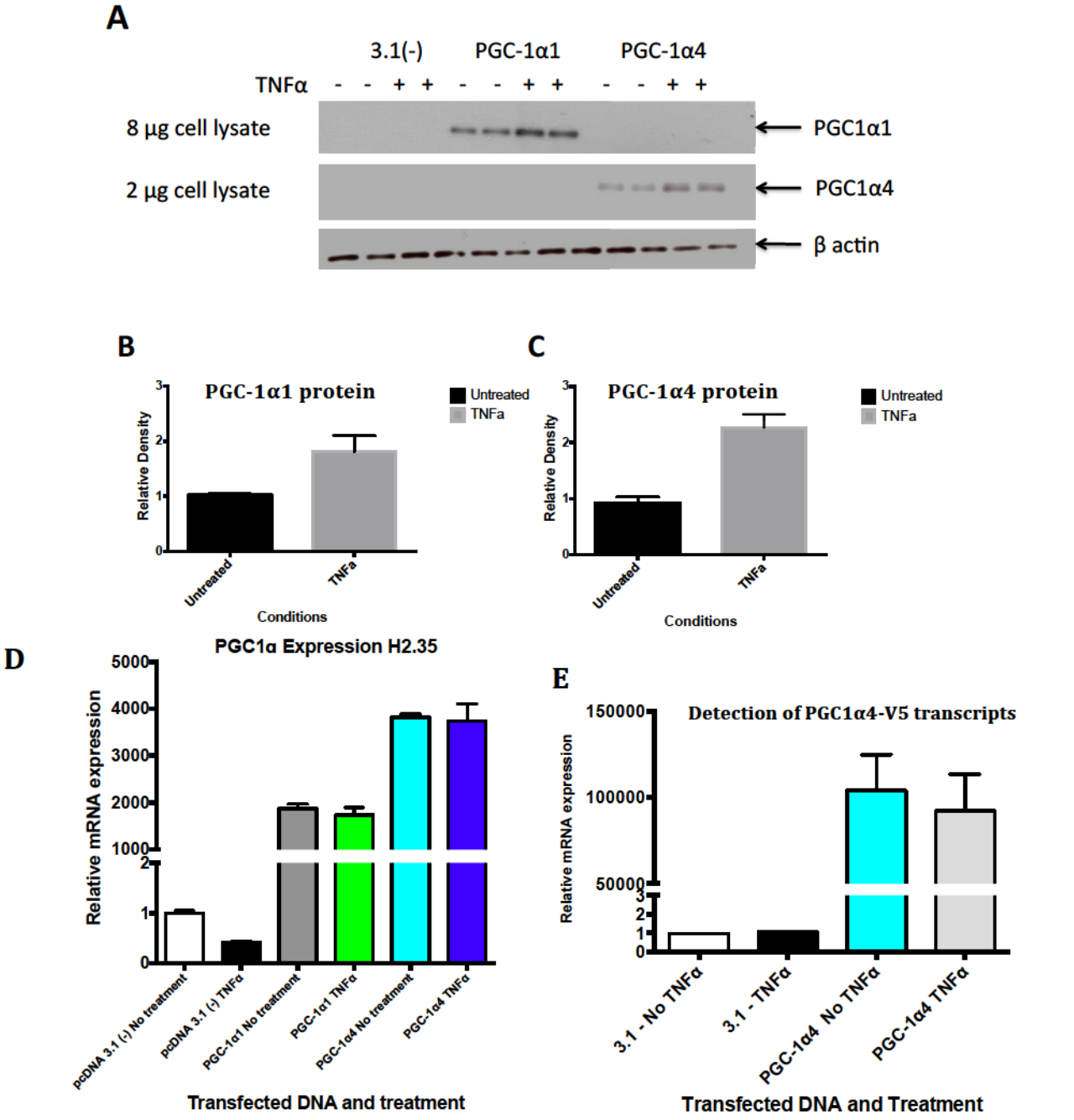

Figure 14. TNFα stabilizes protein of both isoforms

H2.35 were transiently transfected for 24 hours with the indicated constructs. The cells were serum-starved overnight, and then treated with TNFα (50 ng/ml) for 3 hours in treatment media.

A) Western blot indicating protein levels for both PGC-1α1 and PGC-1α4, representative of 3 independent experiments
B) Western blot quantification of PGC-1α1 and C) PGC-1α4 by ImageJ
D) qPCR to measure exon 2 of PGC-1α, found in both PGC-1α1 and PGC-1α4 (n=4)
E) qPCR data using primers designed to detect PGC-1-α4-V5. (n=4)

Bars indicate mean values, and error bars stand for standard deviation.

PGC-1α4 localization is influenced by TNFα

Our lab has microarray data demonstrating that PGC-1α4 has an increased set of distinct coactivator targets from PGC-1α1 on TNFα treatment (unpublished data). Our lab has also shown that on TNFα treatment, PGC-1α4 exhibits anti apoptotic properties [211]. On the basis of these findings, we hypothesized that PGC-1α4 localization could be modified with cytokine treatment, and that the protein would translocate to the nucleus to exert its anti-apoptotic function.

To investigate this, we observed H2.35 cells using immunofluorescence after a 3-hour treatment with TNFα (or control) treatment. We noted no differences in cellular localization of PGC-1α1, which remained confined within the nucleus following TNFα treatment (Figure 15A). We did however note that there seemed to be an increase in the nuclear content of PGC-1α4 (figure 15A).

To verify our immunofluorescence data, we further performed cell fractionation to compare the nuclear and cytoplasmic contents of PGC-1α4 (Figure 15B). We were able to detect PGC-1α4 in the nuclear fraction of untreated cells and this amount was enhanced upon treatment with TNFα. However, we also noticed an enhancement in the cytoplasmic fraction of PGC-1α4 with cytokines treatment (Figure 15B). PGC-1α1 was detected in the nucleus when overexpressed; however a band of the exact same size was also detected in the cytoplasmic fraction (Figure 15B)

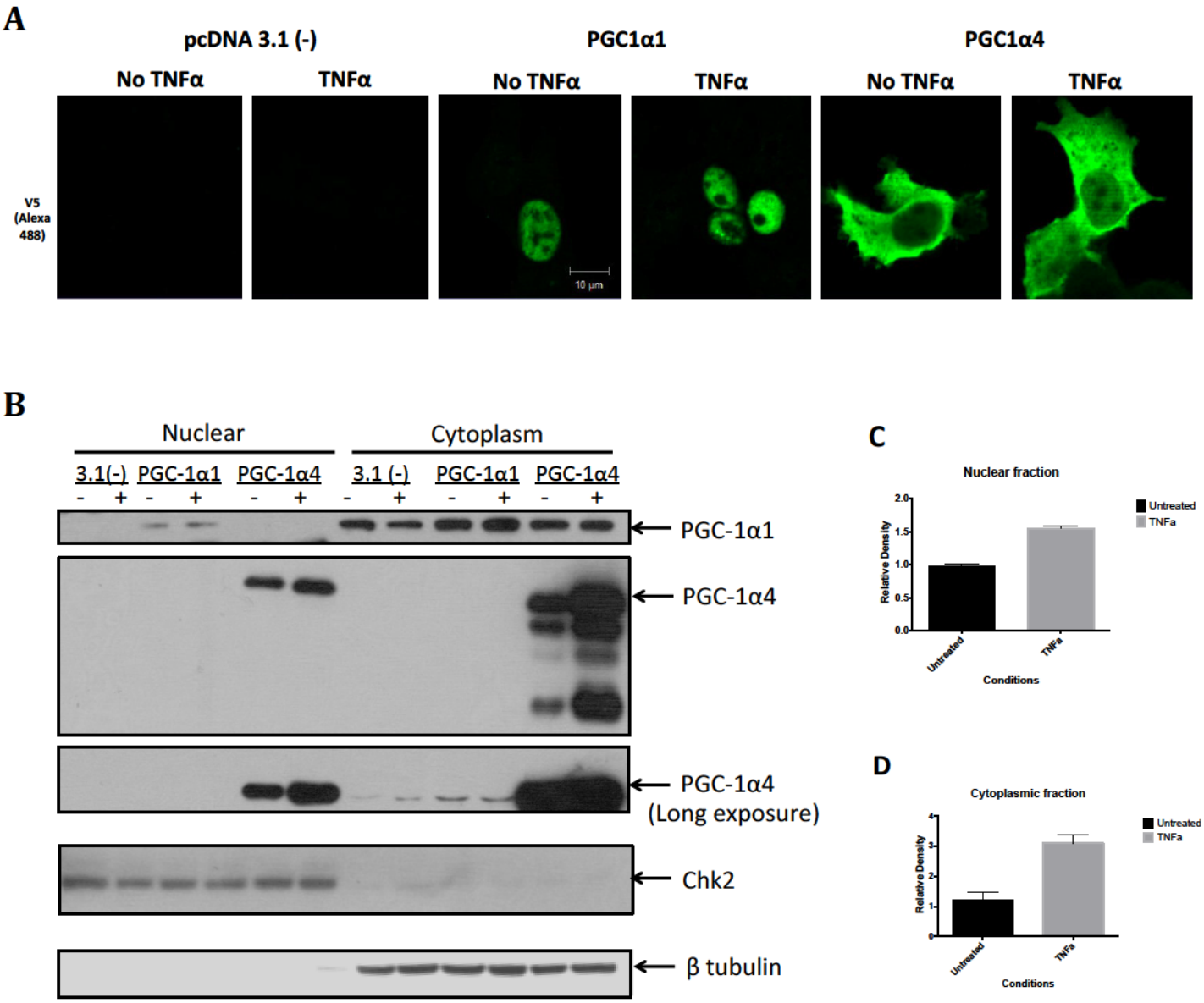

Figure 15. PGC1α4 increases nuclear localization in response to cytokine treatment
H2.35 cells were transiently transfected for 48 hours with PGC-1α1, PGC-1α4 or the control pcDNA 3.1(-) and then treated with TNFα (50 ng/ml) for 3 hours.

A) Immunofluorescence was used to determine the subcellular localization of these proteins, 63X magnification in oil. Representative of 3 independent experiments, done with biological triplicates
B) Western blot of cell fractionation of transfected H2.35 cells. Representative of 3 independent fractionation experiments, with biological duplicates.
C) And D) Image J quantification of western blots; density is relative PGC-1α4 density in untreated samples, normalized to Chk2 and β tubulin respectively. (n=4)

Cytochrome C is differentially regulated by the two of PGC-1α isoforms in hepatocytes

Both PGC-1α1 and PGC-1α4 protein were stabilized by TNFα following 3 hours of treatment (Figure 14A), which could increase target gene activation. In spite of sharing most of their sequence until exon 6, where PGC-1α4 is truncated, and differing only in exon 1, PGC-1α1 and PGC-1α4 have very little overlap in co-activator targets [95]. This was observed in our qPCR analysis that found that while Cytochrome C was upregulated in the presence of overexpressed PGC-1α1 and was even further enhanced on TNFα treatment ($p=0.04763$), this upregulation was not seen in PGC-1α4 treated cells (Figure 16 A), as was reported before in myocytes [95]. There was no difference in Cytochrome C levels between control and PGC-1α4 overexpressing cells, with and without TNFα (Figure 14).

We detected chemokine (C-C motif) ligand 2 CCL2, (a cytokine up-regulated by TNFα) [226] levels using qPCR and found that in response to TNFα treatment, CCL2 levels increased just over 20 fold, and there were no significant differences between our empty vector control, PGC-1α1 over expressing cells and PGC-1α4 overexpressing cells (Figure 16 B). While this is an expected response to TNFα treatment, it is contrary to previous reports that overexpression of PGC-1α curtailed the TNFα mediated induction of CCL2 [227].

Mitochondria fission and fusion may function as a survival mechanism in response to inflammatory stimulus [228]. It was previously believed that mitochondrial fission mediated by dynamin-related protein 1 (Drp-1) lead to the release of cytochrome C into the cytoplasm, and was a necessary first step in programmed cell death [229]. However, the present view of the relationship between mitochondrial dynamics and apoptosis is more

complex, as evidence has emerged that mitochondrial fission may not be necessary for initiation of apoptosis through mitochondrial outer membrane permeabilization (MOMP) [230], or that it may even curtail MOMP [231]. Cells also counter stress by fusion of the mitochondria, termed stress induced mitochondrial hyperfusion (SIMH), and this is believed to be beneficial for cell survival [232, 233]. PGC-1α has also been reported to activate Mfn2 [234]. We were unable to quantifiably discriminate between differences of mitochondrial fission and fusion in our immunofluorescence experiments (data not shown), due to technical limitations. Instead, we decided to investigate perturbations in Mfn1, Mfn2, and Opa1, involved in mitochondrial fusion, and Drp1, involved in fission. To address whether PGC-1α4 could be acting as a coactivator on gene targets controlling mitochondrial dynamics known to influence apoptosis, we hypothesized that it may inhibit mitochondrial fission, or promote mitochondrial fusion. However, we found no significant differences in these expression levels of these genes on overexpression of either PGC-1α1 or PGC-1α4; furthermore, we did not detect differences in these genes between untreated and cytokine treated controls (Figure 16C), though this may be due to the limited replicates we had for this experiment.

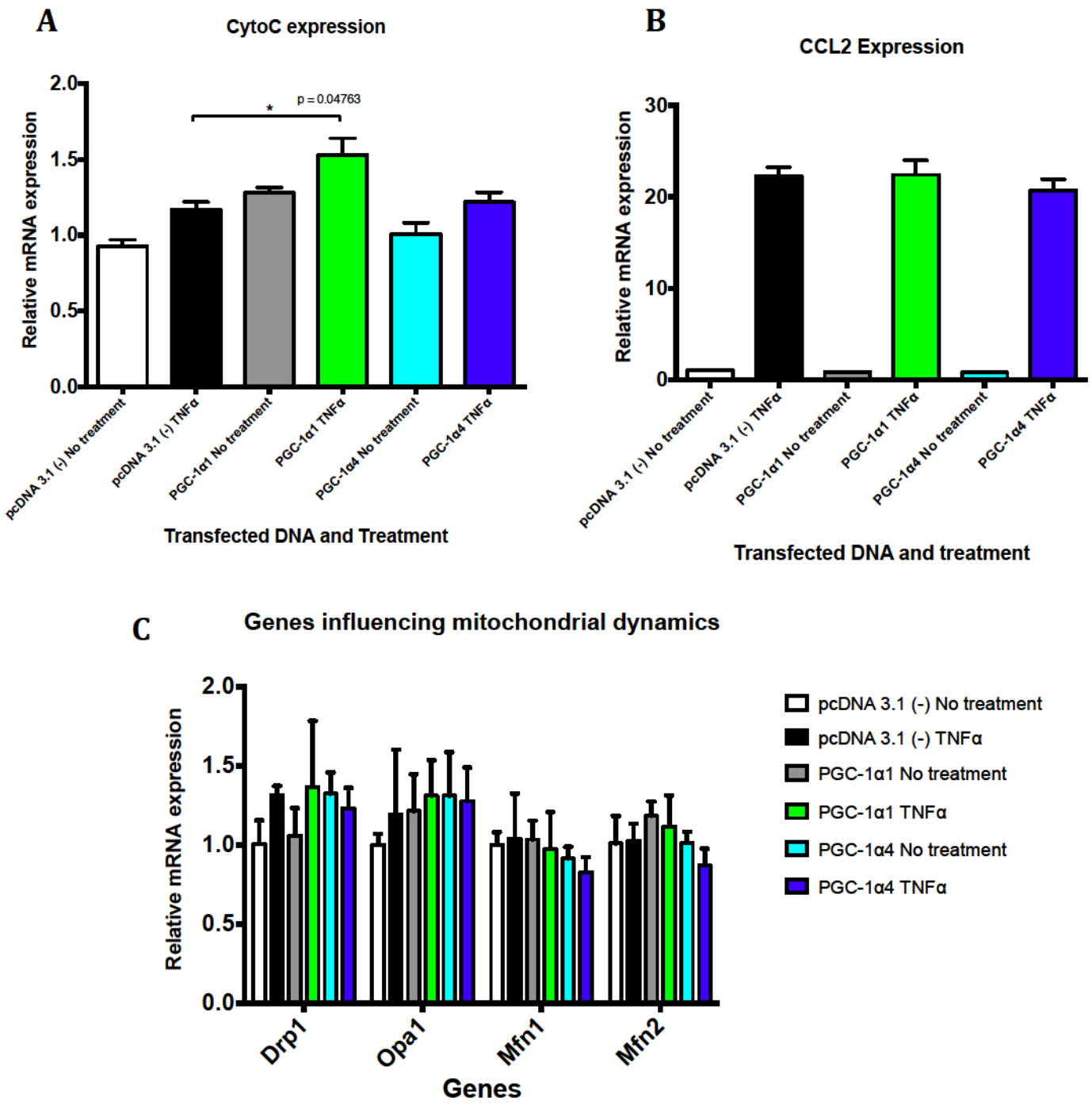

Figure 16. Influence of overexpression of PGC-1α1 and PGC-1α4 on endogenous genes

H2.35 overexpressing PGC-1α1, PGC-1α4 or control pcDNA 3.1- were treated with TNFα (50 ng/ml) for 3 hours. Perturbations in endogenous gene expression were assessed via quantitative real-time PCR A) Cytochrome C, B) CCL2 and C) mitochondrial fission and fusion genes, Drp1, Opa1, Mfn1 and Mfn2. (n=4, except Drp1, n=2)
Bars indicate mean values, and error bars stand for standard deviation. * indicates $p < 0.05$

Chapter Three

Section IV: Discussion

Summary of results

PGC-1α4 is an isoform of PGC-1α1, truncated at exon 6 and differing in exon 1, that is regulated by an alternative promoter. It has previously been found that PGC-1α4 possesses anti apoptotic properties [211] protecting against cytokine-mediated cell death, and that its coactivator function differs significantly from PGC-1α1 [95]. Through this study, we aimed to further understand these properties of PGC-1α4, and work out a possible mechanism of its action.

A previous study by Ruas et al., published in 2012 reported that PGC-1α4, like PGC-1α1 is localized in the nucleus [95]. We hypothesized that in order to inhibit the cleavage of caspase 3, PGC-1α4 would translocate from the nucleus to the cytoplasm where caspase 3 is cleaved. However, contrary to the previous report, we found that PGC-1α4 was predominantly localized in the cytoplasm. This was intriguing, as our lab had previously seen that PGC-1α4 regulates few genes in the absence of a stimulus; however, on TNFα treatment the number of genes induced by PGC-1α4 surges significantly (unpublished data). We proceeded to investigate any shift in protein localization due to TNFα treatment for three hours. Through immunofluorescence, we noted that there was an enhancement of nuclear content of PGC-1α4, while there was no difference in the localization of the PGC-1α1 localization. In order to confirm and quantify this observation, we subjected cells overexpressing these constructs to cellular fractionation, and probed western blots for the nuclear and cytoplasmic fractions of PGC-1α. We found that while the nuclear fraction for PGC-1α4 did indeed increase following 3 hours of TNFα treatment, so did the cytoplasmic fraction. This agreed with whole cell assessment of PGC-1α4 protein content following

three hours of TNFα treatment, which also increased. Finally, we sought to determine any differences in PGC-1α4 coactivator function that may help illuminate on its inhibition of apoptosis. We found no differences in the expression of mitochondrial fission and fusion genes Drp1, Opa1, Mfn1 or Mfn2, nor did we note any difference in the upregulation of CCL2 in response to TNFα treatment. While there is a difference in Cytochrome C regulation between the isoforms PGC-1α1 and PGC-1α4, there was no difference between the control and PGC-1α4 overexpressing cells.

Localization of PGC-1α4

When Ruas et al. first identified PGC-1α4, it was reported to be localized in the nucleus in myotubes [95]. The Nuclear Localization Signal (NLS) of PGC-1α1 is found between amino acids 565-798 [188]. Like PGC-1α4, NT-PGC-1α does not possess this sequence, as it truncated upstream at amino acid 266, and this isoform was also reported to be localized in the cytoplasm. Our finding that PGC-1α4 is predominantly localized in the cytoplasm may differ from the earlier report of nuclear localization [95] due to the difference in method between this present study and the Ruas et al. study. While this group did use adenoviral overexpression in its immunofluorescence experiments, the PGC-1α4 protein was not tagged, and they used a mouse monoclonal PGC-1α antibody for immunocytochemistry experiments. In our experiments, we used a C terminal V5-Tag on our constructs, and detected this tag in our immunofluorescence experiments, as we have experienced that available PGC-1α antibodies are not very specific. To further confirm our data, an N terminus tagged PGC-1α4 may also be used, to ensure that the sequence of the tag is not

influencing the localization. It may be further noted that while Ruas et al., investigated PGC-1α4 in myotubes, we are investigating PGC-1α4 in a hepatocyte cell line, leaving room for differences to arise due to cell specific mechanisms regulating PGC-1α4 localization.

It is interesting that PGC-1α4 is localized in the cytoplasm as it does play a strong and distinct role as a coactivator from PGC-1α1 [95]. Addressing possible factors influencing the nuclear content of the protein would help us understand previous unpublished data that treatment with cytokines significantly increased genes upregulated by PGC-1α4, while in the absence of a stimulus few genes were induced by it.

Though the immunofluorescence data here suggests that TNFα enhances the nuclear content of PGC-1α4, the cellular fractionation data adds a possible confounding factor – the cytoplasmic fraction is also increased. It may be possible that the increase in nuclear content following cytokine treatment is an artefact of our overexpression model, and that upon stabilizing the protein following TNFα treatment more of the protein mislocalizes to the nucleus, as opposed to via a regulatory translocation mechanism. To rule out this issue, investigation of the response of endogenous PGC-1α4 to TNFα is necessary. We detected a band running at the weight of PGC-1α4 in the negative control (pcDNA3.1 (-), Figure 15 B) in the cytoplasmic fraction, and noted that it too increased in density upon TNFα treatment, but did not detect this in the nuclear treatment. There are two caveats to this observation, however; foremost, we see that the greatest subcellular share of PGC-1α4 is found in the cytoplasm, so perhaps the nuclear share was not detected at this exposure. Secondly, PGC-1α antibodies can be quite "dirty" as can be seen in the nuclear and cytoplasmic fractions of PGC-1α1 (Figure 15 B). PGC-1α1 is reported to be exclusively nuclear [188] (and Figure 13), yet we detected a strong band at the exact weight of PGC-1α1 the cytoplasmic fraction.

Without an Sh knockdown of PGC-1α4 to verify that this "endogenous" band, we cannot be certain that the "endogenous PGC-1α4" is in fact PGC-1α4.

It was also interesting to note the difference in the bands detected on the Western between the two fractions. Whereas in the nucleus, we detect one discrete band, we detect 4 bands in the cytoplasmic fraction, though both fractions were treated with the exact same protease inhibitor cocktail, PGC-1α1 is believed to degrade in the nucleus [188], and C-terminal truncated mutants stabilize in the cytoplasm. With the little known about PGC-1α4, it is possible these are degradation products in vivo (before harvest), and that the degradation of this isoform of PGC-1α is primarily cytoplasmic. Alternately, this raises the possibility of cleaved versions of the protein that exist only in the cytoplasm.

Additionally, we were unable to investigate mitochondrial localization of PGC-1α4 in the confines of our experiments. A fractionation protocol designed to isolate the mitochondrial fraction would offer more nuance to the exploration of PGC-1α4 localization. Alternately, more sophisticated immunofluorescence experiments may elucidate this, through MitoTracker Orange staining of mitochondria and subsequent co-localization with the signal from PGC-1α4 assessed by calculating the Pearson's coefficient using ImageJ.

Influence of TNFα on the protein and coactivator function of PGC-1α4

It was interesting to find that PGC-1α4 was stabilized by the treatment by TNFα. It has been noted before that while TNFα treatment itself reduces transcription of PGC-1α [235](shown in our results as well), p38 MAPK activity stabilizes the protein by phosphorylating it at amino acids 263, 266 and 299. PGC-1α4 only contains 2 of these sites,

and the activity of p38 MAPK on PGC-1α4 has not been assessed before; however, it is possible that p38 MAPK can stabilize PGC-1α4 as it does PGC-1α1. Our lab has previously noted that in INS1 cells [211] PGC-1α4 protein is reduced following prolonged (8 hour) cytokine exposure. It is possible that the protein is stabilized in the short term (3-hour) and then degraded at a later time point. However, our experiments only examined a single time point, and we did not conduct experiments to assess the influence of cytokines on the half-life of PGC-1α4. Determining if it is indeed p38 MAPK that stabilizes PGC-1α4 or another subcellular actor would help elucidate the role this protein plays in hepatocytes in response to the TNFα signal.

As reported before in muscle [95], we noted that in hepatocytes Cytochrome C, though induced on treatment with cytokines and on overexpression of canonical PGC-1α1, was not significantly up-regulated by PGC-1α4. We also noted no differences in the up-regulation of CCL2 in response to TNFα treatment, so the direct response to TNFα treatment between our samples is the same. We found no significant differences in mitochondrial fission and fusion genes. It is established that the response to an apoptotic or stress signal like TNFα is mitochondrial fission, promoting the release of Cytochrome C into the cytoplasm. It would be more informative to look at the actual protein expression of these genes as opposed to mRNA levels; furthermore, it would be more illustrative to observe the actual mitochondrial dynamics through microscopy. Mitochondrial fusion can be elegantly monitored by using mitochondrial–targeted GFP that activates with a two-photon laser, and observing diffusion of this GFP through the mitochondrial network since fusion results in the mixing of mitochondrial content [236]. Fragmentation of the mitochondrial network may be more simply measured using a mitochondria specific dye such as MitoTracker, and

analyzing images of stained mitochondria using ImageJ that assesses “shape descriptor” parameters to determine circularity and aspect ratio of the mitochondrial network, thus the branching and shape of the mitochondria.

Chapter 4

Perspectives and Conclusion

Summary of conclusions

PGC-1α is master regulator of metabolism [113] and mitochondrial function and biogenesis [57]. This protein is thus of great interest in several metabolic [90] and inflammatory diseases [237]. The Gly482Ser polymorphism of PGC-1α is connected with type 2 diabetes [122, 143], fitness [141, 142] and adiposity [157]. Investigations into the functional contribution of this polymorphism have been limited in scope to its influence in co-activator function on one or two promoters [143, 184-187]. We have determined in this study that the Gly482Ser polymorphism influences the degradation of PGC-1α, that this may be a putative phosphorylation site, and that the Serine and Glycine variant differently induce Sirt1 and Tfam. The mechanism by which this polymorphism influences the stability of PGC-1α is yet to be known, but we have preliminary evidence that it is a phosphorylation site. Once demonstrated, we will be tasked with determining the kinase and further mechanisms of regulation.

PGC-1α acts distinctly through various isoforms, and the PGC-1α4 isoform in particular shows great independence from the canonical sequence in its downstream effectors [95]. Our lab has previously determined that in INS-1 cells [211] and hepatocytes (unpublished data), PGC-1α4 exhibits anti-apoptotic properties in the presence of cytokines. In this study we report that PGC-1α4 is localized in the cytoplasm, and that on treatment with the cytokine TNFα, the PGC-1α4 shifts to the nucleus. This raises the question about the mechanism by which this translocation is mediated, and its functional significance.

The link between the Gly482Ser polymorphism of PGC-1α and diabetes

It has been challenging to narrow down the context in which the Gly482Ser polymorphism should be studied, given the long list of human studies connecting it to various phenotypes and risk factors. If the 482Ser PGC-1α is similarly less stable in cells besides the beta cell, such as myocytes, adipocytes and hepatocytes, this may explain the lengthy cohort of risk factors linked to this polymorphism, such as reduced responsiveness to fitness interventions [141, 142], increased adiposity [157, 159] and insulin resistance [125, 129, 141, 158, 161]

The decreased stability of 482Ser PGC-1α would explain why Serine carriers (individuals for heterozygous or homozygous for the 482Ser PGC-1α polymorphism) are less responsive to fitness interventions [142], and why the Glycine allele is overrepresented in endurance athletes [137, 139]. PGC-1α drives development of type 1 (slow twitch) muscle fibres, necessary for endurance, in conjunction with myocyte enhancer factor-2 (Mef2) [238]. PGC-1α also exerts control over lactate homeostasis, by promoting the expression of lactate dehydrogenase (LDH) B, and limiting LDH A, through the estrogen-related receptor α (ERRα), thereby inhibiting the rise of blood lactate during exercise [239]. Indeed, Glycine carriers may even be viewed as simply having advantage over Serine carriers, as PGC-1α persists in their myocytes for longer or at a higher level in response to the same exercise stimulus [240]. Our findings also agree with reports that Serine carriers are more insulin resistant [125] as PGC-1α expression is lowered in diabetics and individuals with insulin resistance, as well as in their offspring [90], and the muscle specific knockout of PGC-1α

contributes to insulin resistance when combined with age [92]. It was previously discussed that the Serine allele modulation of blood glucose is influenced by body mass index, with reduced glycaemia at lower BMI and higher glycaemia at higher BMI in comparison with Glycine allele homozygotes [130]. Intriguingly, another group published in 2009 that transgenic mice with whole body overexpression of human PGC-1α (and its regulatory sequences) had improved muscle insulin sensitivity, while restricting insulin sensitivity of the liver as gluconeogenesis was promoted (because of PEPCK and G6Pase) [241]. These finding may be linked if we consider the view that the Glycine variant of PGC-1α is "stabilized", particularly in contrast with the mouse PGC-1α, that possesses a Serine at the homologous position. This paradoxical contribution to insulin sensitivity played by PGC-1α may also play a part in the varying risk this polymorphism poses by ethnicity, as different communities may be influenced by the liver or muscle functions of PGC-1α differently based on lifestyle and cultural factors such as daily activity and diet, and may also explain why Serine allele homozygotes, while more susceptible to insulin resistance than Glycine carriers when obese, benefit most from caloric restriction [161].

Our findings also offer context to increased adiposity witnessed in Serine carriers. PGC-1α plays a powerful role in adipose tissue, having been first identified in metabolically active brown fat [51], and modulating the switch from fat storing to fat oxidizing cells [77]; it has further been noted that expression of PGC-1α was lowered by three-fold in morbidly obese subjects when compared with lean individuals [242], and that PGC-1α controls lipolysis enzyme expression [81]. A less stable Serine variant of PGC-1α could thus contribute to greater fat storage, promoting adiposity.

Returning to the beta cells, the decreased stability of 482Ser PGC-1α may explain the findings of the 2008 Ling et al., study [82]. This group reported a reduction in endogenous PGC-1α mRNA amongst carriers of the Serine allele. As PGC-1α is involved in a positive feedback loop with itself [66], it is possible that with the reduced stability of the 482Ser PGC-1α protein, over time there is a lower endogenous expression of PGC-1α. This group also reported lower insulin secretion in islets from carriers of the Serine allele. A previous study found that adenoviral overexpression of PGC-1α in isolated rat islets decreases glucose stimulated insulin secretion, without influencing basal secretion, due to the modification in expression of several metabolic genes [79]. Glucose-6-phosphatase (G6P) expression was increased through forkhead protein box O1 (FOXO1), while glucokinase and GLUT2 expression was diminished, limiting increases in ATP levels on glucose stimulation, and ultimately suppressing membrane depolarization in the β cell [79]. However, another recently published study found that β cell specific PGC-1 knockout of the isoforms PGC-1α and PGC-1β had diminished insulin secretion on treatment with high glucose and palmitate, without impacting mitochondrial content or function [81]. Insulin secretion was instead limited due to the diminished lipid metabolism for energy, and the decreased expression of lipolytic enzymes ATGL and HSL [81] that are required for insulin secretion [243-246]. In our experiments, we did not measure insulin secretion in INS1 cells overexpressing our human PGC-1α constructs. Given that the previously mentioned studies were conducted in mice that carry a Serine at amino acid 482, and our observation that these polymorphisms induces Sirtuin 1 (Sirt1) and mitochondrial factor A (Tfam) to different extents, it would be interesting to determine whether the Gly482Ser polymorphism influences glucose-stimulated insulin secretion in rat cells, echoing

observations made in humans [82]. Both constructs can be overexpressed in beta cells, and any resultant influence on insulin secretion compared between polymorphisms. However, based on the differences between overexpression of PGC-1α and its knockout in beta cells, it may be useful to also use a model that avoids overexpression such as a genetically modified line of rodent beta cells with a glycine amino acid substituted at position 482, or equivalent experiments in a human beta cell line modified to express either variant.

The specific role of PGC-1α in the context of diabetes is complicated given its different roles in different tissues, and how these roles contribute to glycaemia, fitness and metabolism. Studies often disagree when contrasting overexpression and knockdown of the gene, as in the case of insulin secretion discussed above. The results of our study open a new aspect to understanding the role of PGC-1α in the human metabolic disease. There is much to be understood on the molecular level of what the instability of 482Ser PGC-1α, or perhaps enhanced stability of the 482Gly PGC-1α, means in various tissues implicated in diabetes. Presently, studies have been very restricted in their scope when assessing any differential effects of this polymorphism, and it may be advantageous to expand from the single promoter reporter construct approach by exploring more globally the effect of this polymorphism on PGC-1α stability by RNA-seq, in various tissues. Our findings may support early screening of individuals with the Serine allele, as it has been established that these patients are more likely to: progress from hyperglycemia to established diabetes [127]; become hyperglycemic with increased BMI [129]; respond less to fitness

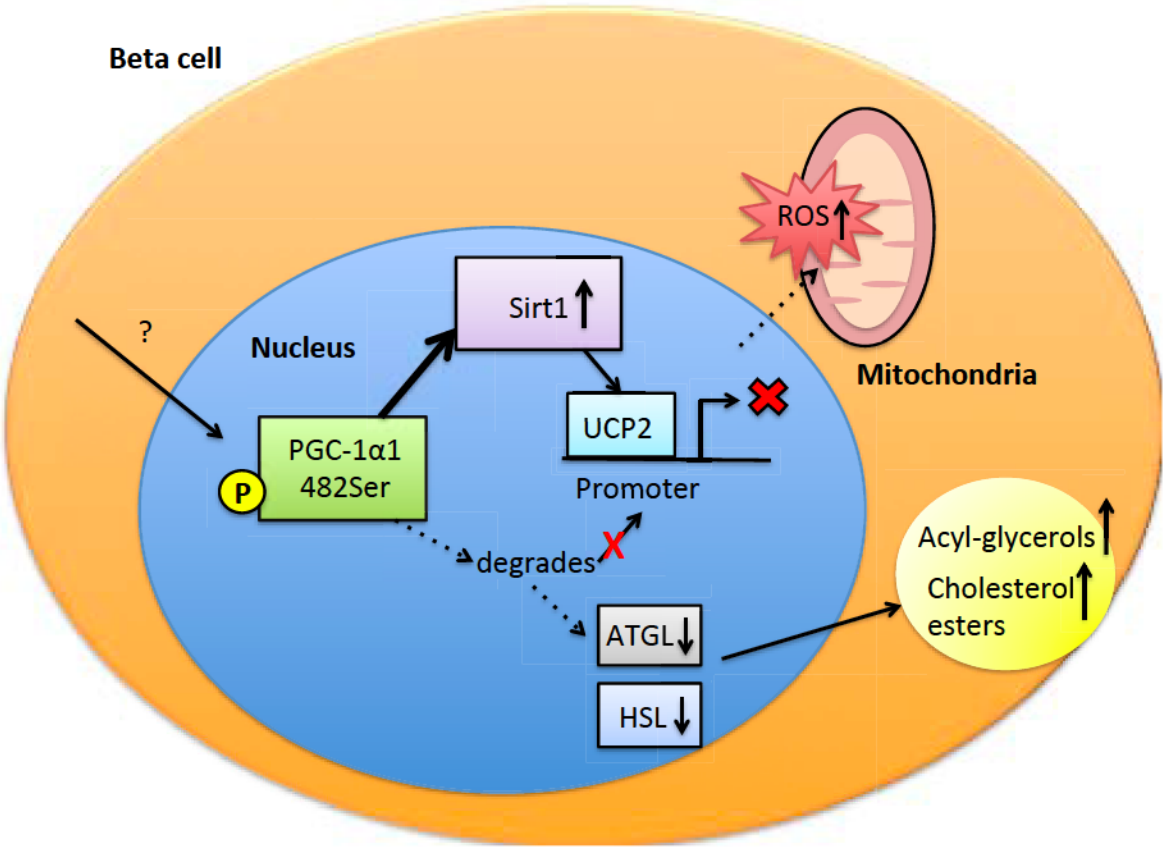

Figure 17 – Suggested effect of Gly482Ser polymorphism of PGC-1α in the beta cell

482Ser PGC-1α stimulates Sirt1 expression, and increased Sirt1 might inhibit UCP2 expression (Bordone et al., 2005). This reduction in UCP2 may result in increased ROS production (Basu Ball et al., 2011); indeed the increased Tfam we detect may be the cell countering the inhibition of UCP2, rather than a direct effect of the less stable variant of PGC-1α.
The more rapidly induced PGC-1α may also result in limited induction of lipolysis genes, leading to an accumulation of acyl-glycerols and cholesterol esters in the beta cell (Oropeza et al., 2015). Both pathways would contribute to beta cell dysfunction.
The rapid degradation also means that PGC-1α cannot drive its own expression in a feed forward loop.
The kinase acting on the serine at amino acid 482 of PGC-1α, and the stimulus that drives this kinase is yet to be determined.

ROS – reactive oxygen species, UCP2 – uncoupling protein 2, Sirt1 – Sirtuin 1, ATGL – adipose triglyceride lipase, HSL – hormone sensitive lipase

interventions [141]; and respond best to management of post prandial hyperglycemia [127]. While this gene will certainly not be the sole diagnostic for diabetes risk, particularly given its high frequency (up to 46% carriers, according to dbSNP) (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=rs8192678 last accessed April 8th, 2016 [247]), it may prove to be helpful in designing approaches to manage hyperglycaemia prior to diabetes, or even in designing targeted therapies for metabolic disease.

The localization of PGC-1α4 and its translocation on TNFα treatment

PGC-1α4 has been a particularly fascinating isoform since it was first described in 2012, when it was found to regulate 519 genes separately from PGC-1α1 in myotubes, as opposed to just 98 genes regulated by both [95]. Our lab further found that in hepatocytes, on TNFα stimulus, there was a surge in genes up regulated by PGC-1α4 (unpublished data). The mechanism by which PGC-1α4 regulates its target genes, and downstream effect of its activity are of considerable interest.

Perhaps the foremost question that arises from our discovery that PGC-1α4 is primarily cytoplasmic is what function, if any, it may exert in the cytoplasm. Investigations into the function of NT-PGC-1α, the other truncated PGC-1α isoform also localized in the cytoplasm, have continued to focus on the role it may play as a co-activator of mitochondrial function in the nucleus [101, 248, 249]. Based on earlier studies using Gal4-DBD fusion constructs of PGC-1α_{120}, PGC-1α_{170} and PGC-1α_{284}, it has been proposed that this isoform has more potent transactivation properties than full length canonical PGC-1α [53, 109]. It is also suggested that NT-PGC-1α may be sequestered out of the nucleus in the absence of a

translocation signal, to limit co-activator activity [101]. PGC-1α4, truncated at amino acid 266, contains these active domains (amino acids 1-284), and is also missing part of the negative regulatory region [113] where p38 mitogen activated protein kinase (p38 MAPK) acts [65], so the exclusion from the nucleus in the absence of a translocation signal may follow the same regulation rationale. On the other hand, PGC-1α4 may have a functional role in the cytoplasm as well. One group has detected PGC-1α outside the nucleus (using, separately, Santa Cruz antibodies that detect amino acid 1-300 of the canonical human PGC-1α sequence, and the C terminus) associated with Sirt1 in the mitochondria, possibly directly regulating mitochondrial biogenesis as it is found on the region of mtDNA recognized by mitochondrial transcription factor A (Tfam) [250]. It may be possible that PGC-1α4's localization in the cytoplasm affords it similar interaction with organelles embedded in the cytoplasm, particularly the mitochondria.

The mechanism of the shift of PGC-1α4 to the nucleus also remains to be explained. It is interesting to note that the p65 subunit of NF-kappaB is reported to bind to PGC-1α, and this binding increases on treatment with TNFα [250]. This binding is directly dependent on the post-translational modification of the p65 subunit, which is the limiting factor, and serves to suppress PGC-1α function [251]. Notably, the LXXLL motifs are required for this association [251], and this domain is found within PGC-1α4. It is well known that NF-kB translocation between the nucleus and cytoplasm is dynamically regulated [252], and that on treatment with TNFα, it rapidly shuttles to the nucleus [253, 254]. We can thus speculate that PGC-1α4, on increased binding with NF-κB in the presence of TNFα, shuttles into the nucleus in tandem, in addition to possible stabilization by phosphorylation by p38

MAPK. This must be confirmed with co-immunoprecipitation studies to address any association, and translocation, and it would further be of interest to investigate if NF-kB represses PGC-1α4 as it does the canonical PGC-1α, or what other factor is transporting PGC-1α4 into the nucleus. Furthermore, it is yet to be confirmed that p38 MAPK does indeed phosphorylate the PGC-1α4 amino acids 263 and 266, as it does in canonical PGC-1α.

Our lab has previously reported that PGC-1α4 overexpression diminishes caspase-3 cleavage in response to cytokines in β cells [211], and the cytoplasmic localization reported here supports this, as caspase-3 is cleaved in cytoplasm [255, 256], before translocating to the nucleus to cleave downstream nuclear effectors [257, 258]. We have yet to determine, however, if it then continues to play an anti-apoptotic role in the nucleus, as a co-activator or whether it interacts with caspase 3 in the cytoplasm at all. More generally, our findings also raise questions about what other factors modulate PGC-1α4 localization, the role this translocation would play in different tissues, and how the function of PGC-1α4 in the nucleus and in the cytoplasm is divided. Perhaps PGC-1α4 was previously reported to be localized in the nucleus [95] in myotubes as it is restricted there in muscle, while in hepatocytes the localization is shifted between the cytoplasm and nucleus. These questions all delineate intriguing paths of future study.

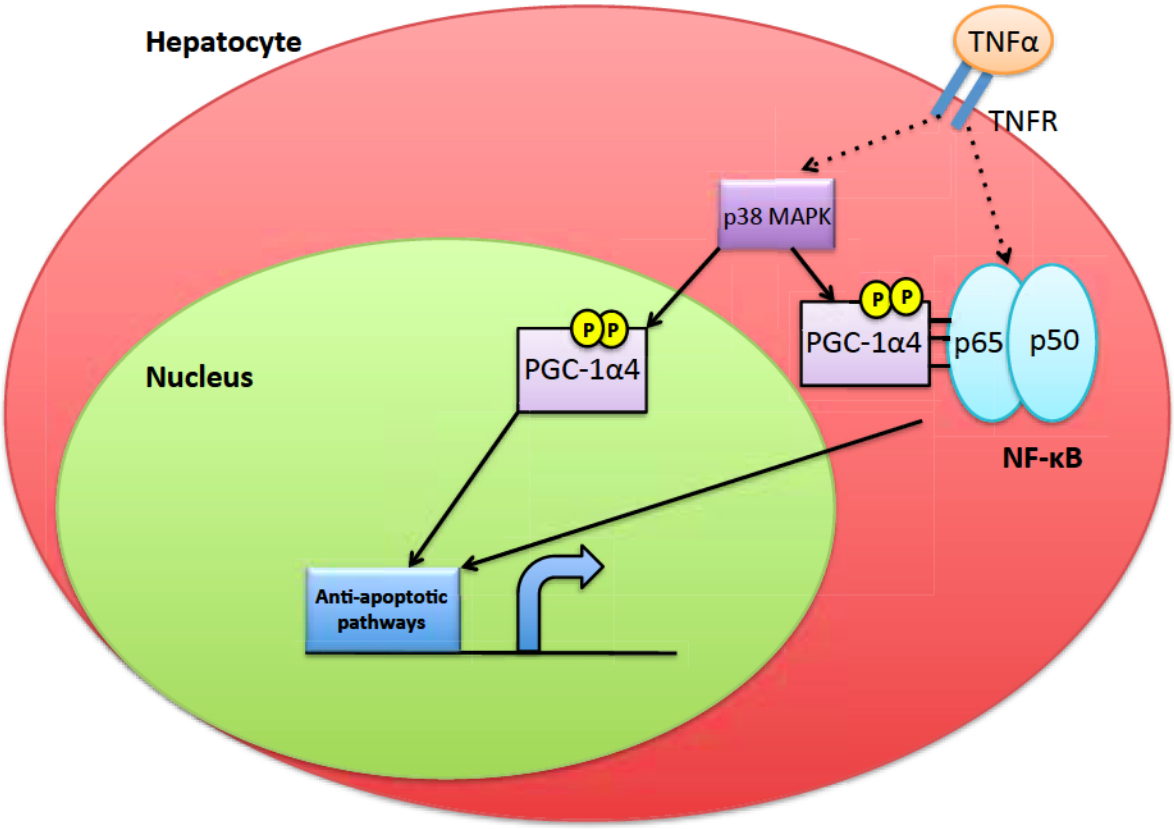

Figure 18 - Suggested response of PGC-1α4 to TNFα

In response to the TNFα stimulus, NF-κB could interact with PGC-1α4 in the LXXLL domains. This may in parallel to or after possible p38 MAPK phosphorylation at amino acid 263 and 266. p38 MAPK may also act on any PGC-1α4 in the nucleus to stabilize it.
With this increased accumulation of PGC-1α4, there is a surge in PGC-1α4 regulated genes, including anti-apoptotic genes specifically induced by this isoform.

TNFα – Tumor Necrosis Factor α, TNFR – Tumor Necrosis Factor Receptor, NF-κB – Nuclear factor light chain enhancer of activated B cells, p38 MAPK – p38 mitogen activated protein kinase

Perspectives

Elucidation of the mechanisms behind the findings reported here may have significant therapeutic benefits, besides significantly expanding on our understanding of PGC-1α, its isoforms and their regulation. Through further investigation, we may better understand the risk posed by the Gly482Ser polymorphism of PGC-1α. We may also learn how to manage the carrier phenotype that includes insulin resistance and adiposity, reduced response to fitness interventions, as well as greater responsiveness to caloric restrictions and drugs to manage post-prandial hyperglycaemia. Elucidation of PGC-1α4's functional response to TNFα may also be beneficial, as we may then utilize our knowledge of the anti-apoptotic properties of this isoform. This elucidation may help to help treat or manage diseases arising from chronic inflammation, such as diabetes, NASH, cancer, cardiovascular and neurological diseases – diseases all rooted in tissues and organs PGC-1α is expressed in.

References

1. Guariguata, L., et al., *Global estimates of diabetes prevalence for 2013 and projections for 2035.* Diabetes Research and Clinical Practice. **103**(2): p. 137-149.
2. *Abstracts of 51st EASD Annual Meeting.* Diabetologia, 2015. **58**(1): p. 1-607.
3. Padgett, L.E., et al., *The role of reactive oxygen species and proinflammatory cytokines in type 1 diabetes pathogenesis.* Ann N Y Acad Sci, 2013. **1281**: p. 16-35.
4. Van Belle, T.L., K.T. Coppieters, and M.G. Von Herrath, *Type 1 Diabetes: Etiology, Immunology, and Therapeutic Strategies.* Physiological Reviews, 2011. **91**(1): p. 79-118.
5. Nerup, J., et al., *HL-A antigens and diabetes mellitus.* Lancet, 1974. **2**(7885): p. 864-6.
6. Singal, D.P. and M.A. Blajchman, *Histocompatibility (HL-A) Antigens, Lymphocytotoxic Antibodies and Tissue Antibodies in Patients with Diabetes Mellitus.* Diabetes, 1973. **22**(6): p. 429-432.
7. Bennett, S.T., et al., *Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus.* Nat Genet, 1995. **9**(3): p. 284-92.
8. Kennedy, G.C., M.S. German, and W.J. Rutter, *The minisatellite in the diabetes susceptibility locus IDDM2 regulates insulin transcription.* Nat Genet, 1995. **9**(3): p. 293-8.
9. Bottini, N., et al., *A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes.* Nat Genet, 2004. **36**(4): p. 337-8.
10. Smyth, D., et al., *Replication of an Association Between the Lymphoid Tyrosine Phosphatase Locus (LYP/PTPN22) With Type 1 Diabetes, and Evidence for Its Role as a General Autoimmunity Locus.* Diabetes, 2004. **53**(11): p. 3020-3023.
11. Bottini, N., et al., *Role of PTPN22 in type 1 diabetes and other autoimmune diseases.* Semin Immunol, 2006. **18**(4): p. 207-13.
12. Lowe, C.E., et al., *Large-scale genetic fine mapping and genotype-phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes.* Nat Genet, 2007. **39**(9): p. 1074-82.
13. Vella, A., et al., *Localization of a type 1 diabetes locus in the IL2RA/CD25 region by use of tag single-nucleotide polymorphisms.* Am J Hum Genet, 2005. **76**(5): p. 773-9.
14. Qu, H.-Q., et al., *Toward Further Mapping of the Association Between the IL2RA Locus and Type 1 Diabetes.* Diabetes, 2007. **56**(4): p. 1174-1176.
15. Nisticò, L., et al., *The CTLA-4 Gene Region of Chromosome 2q33 Is Linked to, and Associated with, Type 1 Diabetes.* Human Molecular Genetics, 1996. **5**(7): p. 1075-1080.
16. Ueda, H., et al., *Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease.* Nature, 2003. **423**(6939): p. 506-11.
17. Waterhouse, P., et al., *Lymphoproliferative Disorders with Early Lethality in Mice Deficient in Ctla-4.* Science, 1995. **270**(5238): p. 985-988.
18. Gale, E.A.M., *The Rise of Childhood Type 1 Diabetes in the 20th Century.* Diabetes, 2002. **51**(12): p. 3353-3361.
19. Filippi, C.M. and M.G. von Herrath, *Viral Trigger for Type 1 Diabetes: Pros and Cons.* Diabetes, 2008. **57**(11): p. 2863-2871.

20. Gamble, D.R., et al., *Viral Antibodies in Diabetes Mellitus.* BMJ, 1969. **3**(5671): p. 627-630.
21. Oikarinen, M., et al., *Detection of enteroviruses in the intestine of type 1 diabetic patients.* Clin Exp Immunol, 2008. **151**(1): p. 71-5.
22. Bach, J.F., *The effect of infections on susceptibility to autoimmune and allergic diseases.* N Engl J Med, 2002. **347**(12): p. 911-20.
23. Vaarala, O., M.A. Atkinson, and J. Neu, *The "Perfect Storm" for Type 1 Diabetes: The Complex Interplay Between Intestinal Microbiota, Gut Permeability, and Mucosal Immunity.* Diabetes, 2008. **57**(10): p. 2555-2562.
24. Karjalainen, J., et al., *A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus.* N Engl J Med, 1992. **327**(5): p. 302-7.
25. Klemetti, P., et al., *T-cell reactivity to wheat gluten in patients with insulin-dependent diabetes mellitus.* Scand J Immunol, 1998. **47**(1): p. 48-53.
26. Mathieu, C., et al., *Vitamin D and diabetes.* Diabetologia, 2005. **48**(7): p. 1247-57.
27. Barker, J.M., K.K. McFann, and T. Orban, *Effect of oral insulin on insulin autoantibody levels in the Diabetes Prevention Trial Type 1 oral insulin study.* Diabetologia, 2007. **50**(8): p. 1603-6.
28. Bresson, D., et al., *Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs.* J Clin Invest, 2006. **116**(5): p. 1371-81.
29. Suarez-Pinzon, W.L., J.R. Lakey, and A. Rabinovitch, *Combination therapy with glucagon-like peptide-1 and gastrin induces beta-cell neogenesis from pancreatic duct cells in human islets transplanted in immunodeficient diabetic mice.* Cell Transplant, 2008. **17**(6): p. 631-40.
30. Rothe, H., et al., *IL-18 Inhibits Diabetes Development in Nonobese Diabetic Mice by Counterregulation of Th1-Dependent Destructive Insulitis.* The Journal of Immunology, 1999. **163**(3): p. 1230-1236.
31. Christen, U., et al., *A Dual Role for TNF-α in Type 1 Diabetes: Islet-Specific Expression Abrogates the Ongoing Autoimmune Process When Induced Late but Not Early During Pathogenesis.* The Journal of Immunology, 2001. **166**(12): p. 7023-7032.
32. Zheng, X.X., et al., *Favorably tipping the balance between cytopathic and regulatory T cells to create transplantation tolerance.* Immunity, 2003. **19**(4): p. 503-14.
33. Yoon, K.-H., et al., *Epidemic obesity and type 2 diabetes in Asia.* The Lancet. **368**(9548): p. 1681-1688.
34. DeFronzo, R.A., *From the Triumvirate to the Ominous Octet: A New Paradigm for the Treatment of Type 2 Diabetes Mellitus.* Diabetes, 2009. **58**(4): p. 773-795.
35. DeFronzo, R.A., E. Ferrannini, and D.C. Simonson, *Fasting hyperglycemia in non-insulin-dependent diabetes mellitus: Contributions of excessive hepatic glucose production and impaired tissue glucose uptake.* Metabolism - Clinical and Experimental. **38**(4): p. 387-395.
36. Pendergrass, M., et al., *Muscle glucose transport and phosphorylation in type 2 diabetic, obese nondiabetic, and genetically predisposed individuals.* American Journal of Physiology - Endocrinology and Metabolism, 2007. **292**(1): p. E92-E100.
37. Groop, L.C., et al., *Glucose and free fatty acid metabolism in non-insulin-dependent diabetes mellitus. Evidence for multiple sites of insulin resistance.* The Journal of Clinical Investigation, 1989. **84**(1): p. 205-213.

38. Ferrannini, E., et al., *The disposal of an oral glucose load in patients with non-insulin-dependent diabetes.* Metabolism - Clinical and Experimental. **37**(1): p. 79-85.
39. Gastaldelli, A., et al., *Influence of obesity and type 2 diabetes on gluconeogenesis and glucose output in humans: a quantitative study.* Diabetes, 2000. **49**(8): p. 1367-1373.
40. Clore, J.N., J. Stillman, and H. Sugerman, *Glucose-6-phosphatase flux in vitro is increased in type 2 diabetes.* Diabetes, 2000. **49**(6): p. 969-974.
41. DeFronzo, R.A., et al., *Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus.* The Journal of Clinical Investigation, 1985. **76**(1): p. 149-155.
42. Diamond, M.P., et al., *Reciprocal Variations in Insulin-Stimulated Glucose Uptake and Pancreatic Insulin Secretion in Women With Normal Glucose Tolerance.* Journal of the Society for Gynecologic Investigation, 1995. **2**(5): p. 708-715.
43. Butler, A.E., et al., *β-Cell Deficit and Increased β-Cell Apoptosis in Humans With Type 2 Diabetes.* Diabetes, 2003. **52**(1): p. 102-110.
44. Newman, B., et al., *Concordance for type 2 (non-insulin-dependent) diabetes mellitus in male twins.* Diabetologia, 1987. **30**(10): p. 763-8.
45. Groop, L., et al., *Metabolic Consequences of a Family History of NIDDM (The Botnia Study): Evidence for Sex-Specific Parental Effects.* Diabetes, 1996. **45**(11): p. 1585-1593.
46. *Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes.* Nat Genet, 2012. **44**(9): p. 981-990.
47. McCarthy, M.I., *Genomics, Type 2 Diabetes, and Obesity.* New England Journal of Medicine, 2010. **363**(24): p. 2339-2350.
48. Lyssenko, V. and L. Groop, *Genome-wide association study for type 2 diabetes: clinical applications.* Curr Opin Lipidol, 2009. **20**(2): p. 87-91.
49. Lyssenko, V. and M. Laakso, *Genetic Screening for the Risk of Type 2 Diabetes: Worthless or valuable?* Diabetes Care, 2013. **36**(Supplement 2): p. S120-S126.
50. Unoki, H., et al., *SNPs in KCNQ1 are associated with susceptibility to type 2 diabetes in East Asian and European populations.* Nat Genet, 2008. **40**(9): p. 1098-1102.
51. Puigserver, P., et al., *A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis.* Cell, 1998. **92**(6): p. 829-39.
52. Esterbauer, H., et al., *Human peroxisome proliferator activated receptor gamma coactivator 1 (PPARGC1) gene: cDNA sequence, genomic organization, chromosomal localization, and tissue expression.* Genomics, 1999. **62**(1): p. 98-102.
53. Vega, R.B., J.M. Huss, and D.P. Kelly, *The Coactivator PGC-1 Cooperates with Peroxisome Proliferator-Activated Receptor α in Transcriptional Control of Nuclear Genes Encoding Mitochondrial Fatty Acid Oxidation Enzymes.* Molecular and Cellular Biology, 2000. **20**(5): p. 1868-1876.
54. 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.
55. Wu, Z., et al., *Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1.* Cell, 1999. **98**(1): p. 115-24.
56. Puigserver, P., et al., *Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction.* Nature, 2003. **423**(6939): p. 550-5.

57. Austin, S. and J. St-Pierre, *PGC1α and mitochondrial metabolism – emerging concepts and relevance in ageing and neurodegenerative disorders.* Journal of Cell Science, 2012. **125**(21): p. 4963-4971.
58. Heery, D.M., et al., *A signature motif in transcriptional co-activators mediates binding to nuclear receptors.* Nature, 1997. **387**(6634): p. 733-6.
59. Rhee, J., et al., *Regulation of hepatic fasting response by PPARgamma coactivator-1alpha (PGC-1): requirement for hepatocyte nuclear factor 4alpha in gluconeogenesis.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 4012-7.
60. Zhang, Y., et al., *Peroxisome proliferator-activated receptor-gamma coactivator 1alpha (PGC-1alpha) regulates triglyceride metabolism by activation of the nuclear receptor FXR.* Genes Dev, 2004. **18**(2): p. 157-69.
61. Kallen, J., et al., *Evidence for ligand-independent transcriptional activation of the human estrogen-related receptor alpha (ERRalpha): crystal structure of ERRalpha ligand binding domain in complex with peroxisome proliferator-activated receptor coactivator-1alpha.* J Biol Chem, 2004. **279**(47): p. 49330-7.
62. Wallberg, A.E., et al., *Coordination of p300-Mediated Chromatin Remodeling and TRAP/Mediator Function through Coactivator PGC-1α.* Molecular Cell. **12**(5): p. 1137-1149.
63. Wu, H., et al., *Regulation of mitochondrial biogenesis in skeletal muscle by CaMK.* Science, 2002. **296**(5566): p. 349-52.
64. Herzig, S., et al., *CREB regulates hepatic gluconeogenesis through the coactivator PGC-1.* Nature, 2001. **413**(6852): p. 179-83.
65. 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.
66. Handschin, C., et al., *An autoregulatory loop controls peroxisome proliferator-activated receptor γ coactivator 1α expression in muscle.* Proceedings of the National Academy of Sciences, 2003. **100**(12): p. 7111-7116.
67. Akimoto, T., B.S. Sorg, and Z. Yan, *Real-time imaging of peroxisome proliferator-activated receptor-gamma coactivator-1alpha promoter activity in skeletal muscles of living mice.* Am J Physiol Cell Physiol, 2004. **287**(3): p. C790-6.
68. 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.
69. 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.
70. Fernandez-Marcos, P.J. and J. Auwerx, *Regulation of PGC-1α, a nodal regulator of mitochondrial biogenesis.* The American Journal of Clinical Nutrition, 2011. **93**(4): p. 884S-890S.
71. Li, X., et al., *Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1[agr] transcription coactivator.* Nature, 2007. **447**(7147): p. 1012-1016.
72. Rodgers, J.T., et al., *Cdc2-like Kinase 2 Is an Insulin-Regulated Suppressor of Hepatic Gluconeogenesis.* Cell Metabolism. **11**(1): p. 23-34.
73. Anderson, R.M., et al., *Dynamic regulation of PGC-1alpha localization and turnover implicates mitochondrial adaptation in calorie restriction and the stress response.* Aging Cell, 2008. **7**(1): p. 101-11.

74. 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.
75. Kelly, T.J., et al., *GCN5-mediated Transcriptional Control of the Metabolic Coactivator PGC-1β through Lysine Acetylation.* Journal of Biological Chemistry, 2009. **284**(30): p. 19945-19952.
76. Nemoto, S., M.M. Fergusson, and T. Finkel, *SIRT1 Functionally Interacts with the Metabolic Regulator and Transcriptional Coactivator PGC-1α.* Journal of Biological Chemistry, 2005. **280**(16): p. 16456-16460.
77. Tiraby, C., et al., *Acquirement of brown fat cell features by human white adipocytes.* J Biol Chem, 2003. **278**(35): p. 33370-6.
78. Wilson-Fritch, L., et al., *Mitochondrial remodeling in adipose tissue associated with obesity and treatment with rosiglitazone.* J Clin Invest, 2004. **114**(9): p. 1281-9.
79. Yoon, J.C., et al., *Suppression of beta cell energy metabolism and insulin release by PGC-1alpha.* Dev Cell, 2003. **5**(1): p. 73-83.
80. Valtat, B., et al., *Fetal PGC-1α Overexpression Programs Adult Pancreatic β-Cell Dysfunction.* Diabetes, 2013. **62**(4): p. 1206-1216.
81. 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.
82. Ling, C., et al., *Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion.* Diabetologia, 2008. **51**(4): p. 615-22.
83. Yoon, J.C., et al., *Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1.* Nature, 2001. **413**(6852): p. 131-8.
84. Estall, J.L., et al., *Sensitivity of lipid metabolism and insulin signaling to genetic alterations in hepatic peroxisome proliferator-activated receptor-gamma coactivator-1alpha expression.* Diabetes, 2009. **58**(7): p. 1499-508.
85. Baar, K., et al., *Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1.* Faseb j, 2002. **16**(14): p. 1879-86.
86. Bryant, N.J., R. Govers, and D.E. James, *Regulated transport of the glucose transporter GLUT4.* Nat Rev Mol Cell Biol, 2002. **3**(4): p. 267-77.
87. Gleyzer, N., K. Vercauteren, and R.C. Scarpulla, *Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators.* Mol Cell Biol, 2005. **25**(4): p. 1354-66.
88. Lin, J., et al., *Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres.* Nature, 2002. **418**(6899): p. 797-801.
89. Ling, C., et al., *Multiple environmental and genetic factors influence skeletal muscle PGC-1alpha and PGC-1beta gene expression in twins.* J Clin Invest, 2004. **114**(10): p. 1518-26.
90. 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.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8466-71.
91. Mootha, V.K., et al., *PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes.* Nat Genet, 2003. **34**(3): p. 267-73.

92. Sczelecki, S., et al., *Loss of Pgc-1α expression in aging mouse muscle potentiates glucose intolerance and systemic inflammation.* American Journal of Physiology - Endocrinology and Metabolism, 2014. **306**(2): p. E157-E167.
93. Chinsomboon, J., et al., *The transcriptional coactivator PGC-1α mediates exercise-induced angiogenesis in skeletal muscle.* Proceedings of the National Academy of Sciences, 2009. **106**(50): p. 21401-21406.
94. Miura, S., et al., *Isoform-specific increases in murine skeletal muscle peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) mRNA in response to beta2-adrenergic receptor activation and exercise.* Endocrinology, 2008. **149**(9): p. 4527-33.
95. Ruas, J.L., et al., *A PGC-1alpha isoform induced by resistance training regulates skeletal muscle hypertrophy.* Cell, 2012. **151**(6): p. 1319-31.
96. Felder, T.K., et al., *Characterization of Novel Peroxisome Proliferator-activated Receptor γ Coactivator-1α (PGC-1α) Isoform in Human Liver.* The Journal of Biological Chemistry, 2011. **286**(50): p. 42923-42936.
97. Soyal, S.M., et al., *A greatly extended PPARGC1A genomic locus encodes several new brain-specific isoforms and influences Huntington disease age of onset.* Hum Mol Genet, 2012. **21**(15): p. 3461-73.
98. Martinez-Redondo, V., A.T. Pettersson, and J.L. Ruas, *The hitchhiker's guide to PGC-1alpha isoform structure and biological functions.* Diabetologia, 2015. **58**(9): p. 1969-77.
99. Jager, S., et al., *AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha.* Proc Natl Acad Sci U S A, 2007. **104**(29): p. 12017-22.
100. Lustig, Y., et al., *Separation of the gluconeogenic and mitochondrial functions of PGC-1{alpha} through S6 kinase.* Genes Dev, 2011. **25**(12): p. 1232-44.
101. Chang, J.S., et al., *Regulation of NT-PGC-1alpha subcellular localization and function by protein kinase A-dependent modulation of nuclear export by CRM1.* J Biol Chem, 2010. **285**(23): p. 18039-50.
102. Lerin, C., et al., *GCN5 acetyltransferase complex controls glucose metabolism through transcriptional repression of PGC-1alpha.* Cell Metab, 2006. **3**(6): p. 429-38.
103. Rodgers, J.T., et al., *Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1.* Nature, 2005. **434**(7029): p. 113-8.
104. Houtkooper, R.H., et al., *The Secret Life of NAD(+): An Old Metabolite Controlling New Metabolic Signaling Pathways.* Endocr Rev, 2010. **31**(2): p. 194-223.
105. Canto, C., et al., *AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity.* Nature, 2009. **458**(7241): p. 1056-60.
106. Gerhart-Hines, Z., et al., *Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha.* Embo j, 2007. **26**(7): p. 1913-23.
107. Rodgers, J.T. and P. Puigserver, *Fasting-dependent glucose and lipid metabolic response through hepatic sirtuin 1.* Proc Natl Acad Sci U S A, 2007. **104**(31): p. 12861-6.
108. Coste, A., et al., *The genetic ablation of SRC-3 protects against obesity and improves insulin sensitivity by reducing the acetylation of PGC-1{alpha}.* Proc Natl Acad Sci U S A, 2008. **105**(44): p. 17187-92.

109. Puigserver, P., et al., *Activation of PPARgamma coactivator-1 through transcription factor docking.* Science, 1999. **286**(5443): p. 1368-71.
110. Teyssier, C., et al., *Activation of nuclear receptor coactivator PGC-1α by arginine methylation.* Genes & Development, 2005. **19**(12): p. 1466-1473.
111. Housley, M.P., et al., *A PGC-1α-O-GlcNAc Transferase Complex Regulates FoxO Transcription Factor Activity in Response to Glucose.* Journal of Biological Chemistry, 2009. **284**(8): p. 5148-5157.
112. Rytinki, M.M. and J.J. Palvimo, *SUMOylation attenuates the function of PGC-1alpha.* J Biol Chem, 2009. **284**(38): p. 26184-93.
113. Soyal, S., et al., *PGC-1α: a potent transcriptional cofactor involved in the pathogenesis of type 2 diabetes.* Diabetologia, 2006. **49**(7): p. 1477-1488.
114. Tcherepanova, I., et al., *Modulation of estrogen receptor-alpha transcriptional activity by the coactivator PGC-1.* J Biol Chem, 2000. **275**(21): p. 16302-8.
115. Liu, D., Z. Zhang, and C.T. Teng, *Estrogen-related receptor-gamma and peroxisome proliferator-activated receptor-gamma coactivator-1alpha regulate estrogen-related receptor-alpha gene expression via a conserved multi-hormone response element.* J Mol Endocrinol, 2005. **34**(2): p. 473-87.
116. Ichida, M., S. Nemoto, and T. Finkel, *Identification of a specific molecular repressor of the peroxisome proliferator-activated receptor gamma Coactivator-1 alpha (PGC-1alpha).* J Biol Chem, 2002. **277**(52): p. 50991-5.
117. Michael, L.F., et al., *Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1.* Proc Natl Acad Sci U S A, 2001. **98**(7): p. 3820-5.
118. Shen, H., J.L. Kan, and M.R. Green, *Arginine-serine-rich domains bound at splicing enhancers contact the branchpoint to promote prespliceosome assembly.* Mol Cell, 2004. **13**(3): p. 367-76.
119. Ge, K., et al., *Transcription coactivator TRAP220 is required for PPAR gamma 2-stimulated adipogenesis.* Nature, 2002. **417**(6888): p. 563-7.
120. Monsalve, M., et al., *Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1.* Mol Cell, 2000. **6**(2): p. 307-16.
121. 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.
122. 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**(12): p. 2220-6.
123. 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.
124. Stumvoll, M. and H. Haring, *The peroxisome proliferator-activated receptor-gamma2 Pro12Ala polymorphism.* Diabetes, 2002. **51**(8): p. 2341-7.
125. 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.
126. 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.
127. 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.
128. 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.
129. 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.
130. Povel, C.M., et al., *Glucose levels and genetic variants across transcriptional pathways: interaction effects with BMI.* Int J Obes, 2010. **34**(5): p. 840-845.
131. Roumen, C., et al., *Impact of 3-year lifestyle intervention on postprandial glucose metabolism: the SLIM study.* Diabetic Medicine, 2008. **25**(5): p. 597-605.
132. Vimaleswaran, K.S., et al., *Peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α) gene polymorphisms and their relationship to Type 2 diabetes in Asian Indians.* Diabetic Medicine, 2005. **22**(11): p. 1516-1521.
133. 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.
134. Rai, E., et al., *Interaction between the UCP2–866G/A, mtDNA 10398G/A and PGC1α p.Thr394Thr and p.Gly482Ser polymorphisms in type 2 diabetes susceptibility in North Indian population.* Human Genetics, 2007. **122**(5): p. 535-540.
135. Shokouhi, S., et al., *Association between PGC-1alpha gene polymorphisms and type 2 diabetes risk: a case-control study of an Iranian population.* Can J Diabetes, 2015. **39**(1): p. 65-72.
136. 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.
137. Lucia, A., et al., *PPARGC1A genotype (Gly482Ser) predicts exceptional endurance capacity in European men.* J Appl Physiol (1985), 2005. **99**(1): p. 344-8.
138. 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.
139. Tural, E., et al., *PPAR-alpha and PPARGC1A gene variants have strong effects on aerobic performance of Turkish elite endurance athletes.* Mol Biol Rep, 2014. **41**(9): p. 5799-804.
140. Maciejewska, A., et al., *The PPARGC1A gene Gly482Ser in Polish and Russian athletes.* J Sports Sci, 2012. **30**(1): p. 101-13.
141. Stefan, N., et al., *Genetic variations in PPARD and PPARGC1A determine mitochondrial function and change in aerobic physical fitness and insulin sensitivity during lifestyle intervention.* J Clin Endocrinol Metab, 2007. **92**(5): p. 1827-33.
142. Steinbacher, P., et al., *The single nucleotide polymorphism Gly482Ser in the PGC-1alpha gene impairs exercise-induced slow-twitch muscle fibre transformation in humans.* PLoS One, 2015. **10**(4): p. e0123881.
143. Andersen, G., et al., *PGC-1alpha Gly482Ser polymorphism associates with hypertension among Danish whites.* Hypertension, 2005. **45**(4): p. 565-70.

144. 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.
145. Oberkofler, H., et al., *Peroxisome proliferator-activated receptor-gamma coactivator-1 gene locus: associations with hypertension in middle-aged men.* Hypertension, 2003. **41**(2): p. 368-72.
146. Ingelsson, E., et al., *The PPARGC1A Gly482Ser polymorphism is associated with left ventricular diastolic dysfunction in men.* BMC Cardiovasc Disord, 2008. **8**: p. 37.
147. Rojek, A., et al., *Impact of the PPARGC1A Gly482Ser polymorphism on left ventricular structural and functional abnormalities in patients with hypertension.* J Hum Hypertens, 2014. **28**(9): p. 557-63.
148. Myles, S., et al., *Testing the thrifty gene hypothesis: the Gly482Ser variant in PPARGC1A is associated with BMI in Tongans.* BMC Med Genet, 2011. **12**: p. 10.
149. Franks, P.W., et al., *PPARGC1A coding variation may initiate impaired NEFA clearance during glucose challenge.* Diabetologia, 2007. **50**(3): p. 569-73.
150. Ruiz, J.R., et al., *Is there an optimum endurance polygenic profile?* The Journal of Physiology, 2009. **587**(Pt 7): p. 1527-1534.
151. Muniesa, C.A., et al., *World-class performance in lightweight rowing: is it genetically influenced? A comparison with cyclists, runners and non-athletes.* Br J Sports Med, 2010. **44**(12): p. 898-901.
152. He, Z., et al., *Is there an association between PPARGC1A genotypes and endurance capacity in Chinese men?* Scand J Med Sci Sports, 2008. **18**(2): p. 195-204.
153. Nishida, Y., et al., *Association between the PPARGC1A polymorphism and aerobic capacity in Japanese middle-aged men.* Intern Med, 2015. **54**(4): p. 359-66.
154. Zhang, S.L., et al., *Association between peroxisome proliferator-activated receptor-gamma coactivator-1alpha gene polymorphisms and type 2 diabetes in southern Chinese population: role of altered interaction with myocyte enhancer factor 2C.* Chin Med J (Engl), 2007. **120**(21): p. 1878-85.
155. Group, D.P.P.R., *Reduction in the Incidence of Type 2 Diabetes with Lifestyle Intervention or Metformin.* New England Journal of Medicine, 2002. **346**(6): p. 393-403.
156. The, D.P.P.R.G., *The Diabetes Prevention Program: recruitment methods and results.* Controlled Clinical Trials, 2002. **23**(2): p. 157-171.
157. 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.
158. 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.
159. Weng, S.W., et al., *Gly482Ser polymorphism in the peroxisome proliferator-activated receptor gamma coactivator-1alpha gene is associated with oxidative stress and abdominal obesity.* Metabolism, 2010. **59**(4): p. 581-6.
160. Deeb, S.S. and J.D. Brunzell, *The role of the PGC1alpha Gly482Ser polymorphism in weight gain due to intensive diabetes therapy.* PPAR Res, 2009. **2009**: p. 649286.
161. 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.

162. 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.
163. Vimaleswaran, K.S., et al., *Effect of polymorphisms in the PPARGC1A gene on body fat in Asian Indians.* Int J Obes (Lond), 2006. **30**(6): p. 884-91.
164. Mirzaei, K., et al., *An exonic peroxisome proliferator-activated receptor-gamma coactivator-1alpha variation may mediate the resting energy expenditure through a potential regulatory role on important gene expression in this pathway.* J Nutrigenet Nutrigenomics, 2012. **5**(2): p. 59-71.
165. Su, X.L., et al., *Association between peroxisome proliferator-activated receptor gamma coactivator-1 alpha polymorphism and hypertension in Mongolians in Inner Mongolia.* Genet Mol Res, 2011. **10**(4): p. 3930-6.
166. Geloneze, S.R., et al., *PGC1alpha gene Gly482Ser polymorphism predicts improved metabolic, inflammatory and vascular outcomes following bariatric surgery.* Int J Obes (Lond), 2012. **36**(3): p. 363-8.
167. Lu, Z., et al., *PGC-1 alpha regulates expression of myocardial mitochondrial antioxidants and myocardial oxidative stress after chronic systolic overload.* Antioxid Redox Signal, 2010. **13**(7): p. 1011-22.
168. Arany, Z., et al., *Transverse aortic constriction leads to accelerated heart failure in mice lacking PPAR-γ coactivator 1α.* Proceedings of the National Academy of Sciences, 2006. **103**(26): p. 10086-10091.
169. Arany, Z., et al., *Transcriptional coactivator PGC-1α controls the energy state and contractile function of cardiac muscle.* Cell Metabolism, 2005. **1**(4): p. 259-271.
170. Vimaleswaran, K.S., et al., *The Gly482Ser genotype at the PPARGC1A gene and elevated blood pressure: a meta-analysis involving 13,949 individuals.* J Appl Physiol (1985), 2008. **105**(4): p. 1352-8.
171. Chae, S.J., et al., *Peroxisome proliferator-activated receptor-gamma and its coactivator-1alpha gene polymorphisms in Korean women with polycystic ovary syndrome.* Gynecol Obstet Invest, 2010. **70**(1): p. 1-7.
172. Wang, Y., et al., *Polymorphisms of the peroxisome proliferator-activated receptor-gamma and its coactivator-1alpha genes in Chinese women with polycystic ovary syndrome.* Fertil Steril, 2006. **85**(5): p. 1536-40.
173. San-Millan, J.L. and H.F. Escobar-Morreale, *The role of genetic variation in peroxisome proliferator-activated receptors in the polycystic ovary syndrome (PCOS): an original case-control study followed by systematic review and meta-analysis of existing evidence.* Clin Endocrinol (Oxf), 2010. **72**(3): p. 383-92.
174. Leipold, H., et al., *Peroxisome proliferator-activated receptor gamma coactivator-1alpha gene variations are not associated with gestational diabetes mellitus.* J Soc Gynecol Investig, 2006. **13**(2): p. 104-7.
175. *Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls.* Nature, 2007. **447**(7145): p. 661-78.
176. Andrulionytė, L., et al., *Single Nucleotide Polymorphisms of PPARD in Combination With the Gly482Ser Substitution of PGC-1A and the Pro12Ala Substitution of PPARG2 Predict the Conversion From Impaired Glucose Tolerance to Type 2 Diabetes: The STOP-NIDDM Trial.* Diabetes, 2006. **55**(7): p. 2148-2152.

177. Thamer, C., et al., *Variations in PPARD determine the change in body composition during lifestyle intervention: a whole-body magnetic resonance study.* J Clin Endocrinol Metab, 2008. **93**(4): p. 1497-500.
178. St-Pierre, J., et al., *Suppression of Reactive Oxygen Species and Neurodegeneration by the PGC-1 Transcriptional Coactivators.* Cell, 2006. **127**(2): p. 397-408.
179. St-Pierre, J., et al., *Bioenergetic Analysis of Peroxisome Proliferator-activated Receptor γ Coactivators 1α and 1β (PGC-1α and PGC-1β) in Muscle Cells.* Journal of Biological Chemistry, 2003. **278**(29): p. 26597-26603.
180. Valle, I., et al., *PGC-1α regulates the mitochondrial antioxidant defense system in vascular endothelial cells.* Cardiovascular Research, 2005. **66**(3): p. 562-573.
181. Zhang, K.-H., et al., *Effects of the Peroxisome Proliferator Activated Receptor-γ Coactivator-1α (PGC-1α) Thr394Thr and Gly482Ser Polymorphisms on Rosiglitazone Response in Chinese Patients With Type 2 Diabetes Mellitus.* The Journal of Clinical Pharmacology, 2010. **50**(9): p. 1022-1030.
182. Hsieh, M.-C., et al., *Common polymorphisms of the peroxisome proliferator-activated receptor–γ (Pro12Ala) and peroxisome proliferator-activated receptor–γ coactivator–1 (Gly482Ser) and the response to pioglitazone in Chinese patients with type 2 diabetes mellitus.* Metabolism, 2010. **59**(8): p. 1139-1144.
183. Sears, D.D., et al., *Selective Modulation of Promoter Recruitment and Transcriptional Activity of PPARγ.* Biochemical and biophysical research communications, 2007. **364**(3): p. 515-521.
184. 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**(3): p. 708-12.
185. 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.
186. 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.
187. Chen, Y., et al., *Gly482Ser mutation impairs the effects of peroxisome proliferator-activated receptor gamma coactivator-1alpha on decreasing fat deposition and stimulating phosphoenolpyruvate carboxykinase expression in hepatocytes.* Nutr Res, 2013. **33**(4): p. 332-9.
188. 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.
189. Trausch-Azar, J., et al., *Ubiquitin Proteasome-dependent Degradation of the Transcriptional Coactivator PGC-1α via the N-terminal Pathway.* The Journal of Biological Chemistry, 2010. **285**(51): p. 40192-40200.
190. Johnson, M. *MATER METHODS 2012;2:133*. last modified : 2015-01-26; original version : 2012-10-25
11th November 2015]; Available from: http://www.labome.com/method/Proteasome-Inhibitors.html.
191. Schneider-Poetsch, T., et al., *Inhibition of Eukaryotic Translation Elongation by Cycloheximide and Lactimidomycin.* Nature chemical biology, 2010. **6**(3): p. 209-217.

192. Nitz, I., et al., *Analysis of PGC-1α variants Gly482Ser and Thr612Met concerning their PPARγ2-coactivation function.* Biochemical and Biophysical Research Communications, 2007. **353**(2): p. 481-486.
193. 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.
194. Takacs, M., et al., *The asymmetric binding of PGC-1alpha to the ERRalpha and ERRgamma nuclear receptor homodimers involves a similar recognition mechanism.* PLoS One, 2013. **8**(7): p. e67810.
195. Greschik, H., et al., *Communication between the ERRalpha homodimer interface and the PGC-1alpha binding surface via the helix 8-9 loop.* J Biol Chem, 2008. **283**(29): p. 20220-30.
196. Fu, A. and R.A. Screaton, *Using kinomics to delineate signaling pathways: control of CRTC2/TORC2 by the AMPK family.* Cell Cycle, 2008. **7**(24): p. 3823-8.
197. Reeks, C. and R.A. Screaton, *Identification of Kinase-substrate Pairs Using High Throughput Screening.* J Vis Exp, 2015(102): p. e53152.
198. Hutchison, C.A., 3rd, et al., *Mutagenesis at a specific position in a DNA sequence.* J Biol Chem, 1978. **253**(18): p. 6551-60.
199. Paleologou, K.E., et al., *Phosphorylation at Ser-129 but Not the Phosphomimics S129E/D Inhibits the Fibrillation of α-Synuclein.* Journal of Biological Chemistry, 2008. **283**(24): p. 16895-16905.
200. Rodgers, J.T., et al., *Metabolic adaptations through the PGC-1α and SIRT1 pathways.* FEBS Letters, 2008. **582**(1): p. 46-53.
201. Bordone, L., et al., *Sirt1 Regulates Insulin Secretion by Repressing UCP2 in Pancreatic ? Cells.* PLoS Biol, 2005. **4**(2): p. e31.
202. Nakatani, T., et al., *Mechanism for peroxisome proliferator-activated receptor-alpha activator-induced up-regulation of UCP2 mRNA in rodent hepatocytes.* J Biol Chem, 2002. **277**(11): p. 9562-9.
203. Luu, L., et al., *The loss of Sirt1 in mouse pancreatic beta cells impairs insulin secretion by disrupting glucose sensing.* Diabetologia, 2013. **56**(9): p. 2010-20.
204. Produit-Zengaffinen, N., et al., *Increasing uncoupling protein-2 in pancreatic beta cells does not alter glucose-induced insulin secretion but decreases production of reactive oxygen species.* Diabetologia, 2007. **50**(1): p. 84-93.
205. Vernochet, C., et al., *Adipose-Specific Deletion of TFAM Increases Mitochondrial Oxidation and Protects Mice against Obesity and Insulin Resistance.* Cell Metabolism, 2012. **16**(6): p. 765-776.
206. Schreiber, S.N., et al., *The estrogen-related receptor alpha (ERRalpha) functions in PPARgamma coactivator 1alpha (PGC-1alpha)-induced mitochondrial biogenesis.* Proc Natl Acad Sci U S A, 2004. **101**(17): p. 6472-7.
207. Litonin, D., et al., *Human mitochondrial transcription revisited: only TFAM and TFB2M are required for transcription of the mitochondrial genes in vitro.* J Biol Chem, 2010. **285**(24): p. 18129-33.
208. Scharfmann, R., et al., *Development of a conditionally immortalized human pancreatic β cell line.* The Journal of Clinical Investigation, 2014. **124**(5): p. 2087-2098.

209. Arany, Z., et al., *HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1[agr].* Nature, 2008. **451**(7181): p. 1008-1012.
210. Thom, R., et al., *Hypoxic Induction of Vascular Endothelial Growth Factor (VEGF) and Angiogenesis in Muscle by Truncated Peroxisome Proliferator-activated Receptor γ Coactivator (PGC)-1α.* The Journal of Biological Chemistry, 2014. **289**(13): p. 8810-8817.
211. Sczelecki, S., *Understanding the Function of PGC-1α Isoforms in ß-cell Survival and Diabetes*. 2013, McGill University Department of Medicine McGill Theses.
212. Hotamisligil, G.S., et al., *Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance.* J Clin Invest, 1995. **95**(5): p. 2409-15.
213. Saghizadeh, M., et al., *The expression of TNF alpha by human muscle. Relationship to insulin resistance.* J Clin Invest, 1996. **97**(4): p. 1111-6.
214. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, *Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance.* Science, 1993. **259**(5091): p. 87-91.
215. Uysal, K.T., et al., *Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function.* Nature, 1997. **389**(6651): p. 610-4.
216. Estep, J.M., et al., *Expression of cytokine signaling genes in morbidly obese patients with non-alcoholic steatohepatitis and hepatic fibrosis.* Obes Surg, 2009. **19**(5): p. 617-24.
217. Civera, M., et al., *Relationship between insulin resistance, inflammation and liver cell apoptosis in patients with severe obesity.* Diabetes Metab Res Rev, 2010. **26**(3): p. 187-92.
218. Bataller, R. and D.A. Brenner, *Liver fibrosis.* Journal of Clinical Investigation, 2005. **115**(2): p. 209-218.
219. Hatano, E., *Tumor necrosis factor signaling in hepatocyte apoptosis.* Journal of Gastroenterology and Hepatology, 2007. **22**: p. S43-S44.
220. Chen, Y.L., C.K. Yu, and H.Y. Lei, *Propionibacterium acnes induces acute TNFalpha-mediated apoptosis of hepatocytes followed by inflammatory T-cell-mediated granulomatous hepatitis in mice.* J Biomed Sci, 1999. **6**(5): p. 349-56.
221. Dai, L.L., et al., *Synthesis of endotoxin receptor CD14 protein in Kupffer cells and its role in alcohol-induced liver disease.* World J Gastroenterol, 2003. **9**(3): p. 622-6.
222. Wang, K., *Molecular mechanisms of hepatic apoptosis.* Cell Death Dis, 2014. **5**: p. e996.
223. Zhang, Y., et al., *Alternative mRNA Splicing Produces a Novel Biologically Active Short Isoform of PGC-1α.* The Journal of Biological Chemistry, 2009. **284**(47): p. 32813-32826.
224. Ritter, T., et al., *Stimulatory and inhibitory action of cytokines on the regulation of hCMV-IE promoter activity in human endothelial cells.* Cytokine, 2000. **12**(8): p. 1163-70.
225. Palomer, X., et al., *TNF-alpha reduces PGC-1alpha expression through NF-kappaB and p38 MAPK leading to increased glucose oxidation in a human cardiac cell model.* Cardiovasc Res, 2009. **81**(4): p. 703-12.
226. Satriano, J.A., et al., *Oxygen radicals as second messengers for expression of the monocyte chemoattractant protein, JE/MCP-1, and the monocyte colony-stimulating factor, CSF-1, in response to tumor necrosis factor-alpha and immunoglobulin G.*

Evidence for involvement of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase. Journal of Clinical Investigation, 1993. **92**(3): p. 1564-1571.
227. Kim, H.J., et al., *Effects of PGC-1alpha on TNF-alpha-induced MCP-1 and VCAM-1 expression and NF-kappaB activation in human aortic smooth muscle and endothelial cells.* Antioxid Redox Signal, 2007. **9**(3): p. 301-7.
228. Suen, D.-F., K.L. Norris, and R.J. Youle, *Mitochondrial dynamics and apoptosis.* Genes & Development, 2008. **22**(12): p. 1577-1590.
229. Frank, S., et al., *The Role of Dynamin-Related Protein 1, a Mediator of Mitochondrial Fission, in Apoptosis.* Developmental Cell, 2001. **1**(4): p. 515-525.
230. Parone, P.A., et al., *Inhibiting the Mitochondrial Fission Machinery Does Not Prevent Bax/Bak-Dependent Apoptosis.* Molecular and Cellular Biology, 2006. **26**(20): p. 7397-7408.
231. Arnoult, D., *Mitochondrial fragmentation in apoptosis.* Trends in Cell Biology, 2007. **17**(1): p. 6-12.
232. van der Bliek, A.M., Q. Shen, and S. Kawajiri, *Mechanisms of Mitochondrial Fission and Fusion.* Cold Spring Harbor Perspectives in Biology, 2013. **5**(6).
233. Tondera, D., et al., *SLP‐2 is required for stress‐induced mitochondrial hyperfusion.* The EMBO Journal, 2009. **28**(11): p. 1589-1600.
234. Soriano, F.X., et al., *Evidence for a mitochondrial regulatory pathway defined by peroxisome proliferator-activated receptor-gamma coactivator-1 alpha, estrogen-related receptor-alpha, and mitofusin 2.* Diabetes, 2006. **55**(6): p. 1783-91.
235. Tang, K., P.D. Wagner, and E.C. Breen, *TNF-α-mediated reduction in PGC-1α may impair skeletal muscle function after cigarette smoke exposure.* Journal of Cellular Physiology, 2010. **222**(2): p. 320-327.
236. Twig, G., et al., *Fission and selective fusion govern mitochondrial segregation and elimination by autophagy.* The EMBO Journal, 2008. **27**(2): p. 433-446.
237. Ryan, Scott D., et al., *Isogenic Human iPSC Parkinson's Model Shows Nitrosative Stress-Induced Dysfunction in MEF2-PGC1α Transcription.* Cell. **155**(6): p. 1351-1364.
238. Lin, J., et al., *Transcriptional co-activator PGC-1[alpha] drives the formation of slow-twitch muscle fibres.* Nature, 2002. **418**(6899): p. 797-801.
239. Summermatter, S., et al., *Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A.* Proceedings of the National Academy of Sciences, 2013. **110**(21): p. 8738-8743.
240. Pilegaard, H., B. Saltin, and P.D. Neufer, *Exercise induces transient transcriptional activation of the PGC-1α gene in human skeletal muscle.* The Journal of Physiology, 2003. **546**(3): p. 851-858.
241. 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.
242. Semple, R.K., et al., *Expression of the thermogenic nuclear hormone receptor coactivator PGC-1[alpha] is reduced in the adipose tissue of morbidly obese subjects.* Int J Obes Relat Metab Disord, 2003. **28**(1): p. 176-179.

243. Fex, M., et al., *A beta cell-specific knockout of hormone-sensitive lipase in mice results in hyperglycaemia and disruption of exocytosis.* Diabetologia, 2009. **52**(2): p. 271-80.
244. Peyot, M.L., et al., *Adipose triglyceride lipase is implicated in fuel- and non-fuel-stimulated insulin secretion.* J Biol Chem, 2009. **284**(25): p. 16848-59.
245. Mulder, H., et al., *Inhibition of lipase activity and lipolysis in rat islets reduces insulin secretion.* Diabetes, 2004. **53**(1): p. 122-8.
246. Tang, T., et al., *Desnutrin/ATGL activates PPARdelta to promote mitochondrial function for insulin secretion in islet beta cells.* Cell Metab, 2013. **18**(6): p. 883-95.
247. Sherry, S.T., et al., *dbSNP: the NCBI database of genetic variation.* Nucleic Acids Research, 2001. **29**(1): p. 308-311.
248. Chang, J.S. and H.-J. Jun, *Regulation of Hepatic Gluconeogenesis by NT-PGC-1α, the Short Isoform of PGC-1α.* The FASEB Journal, 2015. **29**(1 Supplement).
249. Wen, X., et al., *Effect of exercise intensity on isoform-specific expressions of NT-PGC-1 alpha mRNA in mouse skeletal muscle.* Biomed Res Int, 2014. **2014**: p. 402175.
250. Aquilano, K., et al., *Peroxisome Proliferator-activated Receptor γ Co-activator 1α (PGC-1α) and Sirtuin 1 (SIRT1) Reside in Mitochondria: POSSIBLE DIRECT FUNCTION IN MITOCHONDRIAL BIOGENESIS.* Journal of Biological Chemistry, 2010. **285**(28): p. 21590-21599.
251. Alvarez-Guardia, D., et al., *The p65 subunit of NF-kappaB binds to PGC-1alpha, linking inflammation and metabolic disturbances in cardiac cells.* Cardiovasc Res, 2010. **87**(3): p. 449-58.
252. Birbach, A., et al., *Signaling molecules of the NF-kappa B pathway shuttle constitutively between cytoplasm and nucleus.* J Biol Chem, 2002. **277**(13): p. 10842-51.
253. Ding, G.J.F., et al., *Characterization and Quantitation of NF-κB Nuclear Translocation Induced by Interleukin-1 and Tumor Necrosis Factor-α: DEVELOPMENT AND USE OF A HIGH CAPACITY FLUORESCENCE CYTOMETRIC SYSTEM.* Journal of Biological Chemistry, 1998. **273**(44): p. 28897-28905.
254. Fuseler, J.W., et al., *Analysis and quantitation of NF-kappaB nuclear translocation in tumor necrosis factor alpha (TNF-alpha) activated vascular endothelial cells.* Microsc Microanal, 2006. **12**(3): p. 269-76.
255. Luo, M., et al., *Nuclear entry of active caspase-3 is facilitated by its p3-recognition-based specific cleavage activity.* Cell Res, 2010. **20**(2): p. 211-222.
256. William C. Earnshaw, Luis M. Martins, and S.H. Kaufmann, *Mammalian Caspases: Structure, Activation, Substrates, and Functions During Apoptosis.* Annual Review of Biochemistry, 1999. **68**(1): p. 383-424.
257. Kamada, S., et al., *Nuclear Translocation of Caspase-3 Is Dependent on Its Proteolytic Activation and Recognition of a Substrate-like Protein(s).* Journal of Biological Chemistry, 2005. **280**(2): p. 857-860.
258. Takemoto, K., et al., *Spatio-temporal activation of caspase revealed by indicator that is insensitive to environmental effects.* The Journal of Cell Biology, 2003. **160**(2): p. 235-243.

Appendix

Primers used for qPCR

Primer ID	Sequence
a4 V5 F	GCT CAA GCC AAA CCA ACA ACT T
a4 V5 R	TAG AAT CGA GAC CGA GGA GAG
COXIV F	AGT TTA ACG AGA GCT TCG CCG AGA
COXIV R	AGC CGA GTG AAG CCA ATG AAG AAC
DGAT2 F	ATCTTCTCTGTCACCTGGCT
DGAT2 R	ACCTTTCTTGGGCGTGTTCC
Drp1For	CAGGAATTGTTACGGTTCCCTAA
Drp1Rev	CCTGAATTAACTTGTCCCGTGA
ERRa F	CGG TGT GGC ATC CTG TGA
ERRa R	CTC CCC TGG ATG GTC CTC TT
Exon2 F	GCT CAT TGT TGT ACT GGT TGG ATA TG
Exon2 R	TGA TGT GAA TGA CTT GGA TAC AGA CA
hrmPGC1a F	CCT TAT TTT CTC AAA GAC CC
hrmPGC1a R	GGA TCT TGA AGA GGA TCT AC
human PGC1a F	GGA CAT GTG CAA CCA GGA CT
human PGC1a R	CAC TTG AGT CCA CCC AGA AAG CT
mCyto C F	GCAAGCATAAGACTGGACCAAA
mCyto C R	TGTTGGCATCTGTGTAAGAGAATC
Mfn1For	CCTACTGCTCCTTCTAACCCA
Mfn1Rev	AGGGACGCCAATCCTGTGA
Mfn2For	CCTACTGCTCCTTCTAACCCA
Mfn2Rev	AGGGACGCCAATCCTGTGA
Nrf1 Set 4 Forward Primer	AAC GGA AAC GGC CTC ATG TGT TTG
Nrf1 Set 4 Reverse Primer	AGA GTA CAA TCG CTT GCT GTC CCA
Opa1For	CCTACTGCTCCTTCTAACCCA
Opa1Rev	AGGGACGCCAATCCTGTGA
rat PGC1a R	CCG TTT TGG AAT TGA CTG ACT GA
ratPGC1a F	GGC GGG AGC AAT CTG AGT TA
rPPARa f	GGCTGAGAAGACGCTTGTGGCC
rPPARa r	CCGTGAGCTCGGTGACGGTCTCC
SIRT1 F	CAG TGT CAT GGT TCC TTT GC
SIRT1 R	CAC CGA GGA ACT ACC TGA T
SOD2-F	GGCCAAGGGAGATGTTACAAC
SOD2-R	GCAACTCTCCTTTGGGTTCTC
TFAM F	GGT ATG GAG AAG GAG GCC CGG C
TFAM R	CGA ATC ATC CTT TGC CTC CTG GAA GC

Primers used for cloning

Primer ID	Sequence
V5 tagging	
a1 Xho1 FWD	CTC GAG GCC AGC ATG GCT TGG GAC ATG T
a1 Age 1 Rev	ACC GGT CCT GCG CAA GCT TCT CTG TCT CTG AGC
a4 Xho1 FWD	CTC GAG GCC ACC ATG TTG GGA TTG TCA T
a4 Age1 Rev	ACC GGT TAA AAA CAA ATT TGG TGA C
Phosphomimic	
Asp482hPGC1a NEB F	AGA CAA GAC CGA TGA ACT GAG GGA CAG
Asp482 PGC1a NEB R	GCT TCG TCG TCA AAA ACA G
Ala482 hPGC1a NEB F	AGA CAA GAC CGC TGA ACT GAG GGA CAG
Ala482 hPGC1a NEB R	GCT TCG TCG TCA AAA ACA G