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The role of GPS2 in regulating lipid metabolism and inflammation in adipose tissue

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Dissertation

THE ROLE OF GPS2 IN REGULATING LIPID METABOLISM AND INFLAMMATION IN ADIPOSE TISSUE

by

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B.S., Syracuse University, 2008

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It is strange that only extraordinary (wo)men make the discoveries, which later appear so simple and easy.

-Georg C. Lichtenberg
DEDICATION

I would like to dedicate this work to my parents, Carl and Janice Cederquist, who always recognized my potential and inspired me to use it.
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This work would not have been possible without the guidance and support of many people. First, I would like to thank my thesis advisor, Dr. Valentina Perissi, for giving me the opportunity to perform my graduate work in her lab. She has been a wonderful mentor and I am extremely grateful for her guidance and support throughout this process. I also need to thank Dr. M. Dafne Cardamone and Dr. Claudia Lentucci for not only providing great scientific input as colleagues but for also being wonderful friends and a strong support system throughout my studies. I must also thank all past and present members of the Perissi Lab for providing an enthusiastic and entertaining lab environment and for which much of this work would not be possible without. I must also acknowledge my thesis committee in which every member has been an integral part of my success: Dr. Konstantin Kandror for serving as Chair and always offering helpful advice during meetings; Dr. Stephen Farmer for always inciting my passion for science, pushing me to think more critically, and giving me hope that there are many sunny vacations in my future; Dr. Susan Fried for technical advice and many insightful discussions especially when problems arose; and Dr. Andrew Henderson for embarking as part of a transcriptional project yet enthusiastically supporting my work when it did not follow that direction.

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THE ROLE OF GPS2 IN REGULATING LIPID METABOLISM AND INFLAMMATION IN ADIPOSE TISSUE

CARLY THERESA CEDERQUIST

Boston University School of Medicine, 2017

Major Professor: Valentina Perissi, Ph.D., Assistant Professor of Biochemistry

ABSTRACT

Type 2 diabetes is an increasingly prevalent disease posing great burdens to healthcare, in which obesity and adipose tissue (AT) dysfunction are central components of disease progression. Impairment of insulin signaling and inflammation in AT can trigger insulin resistance but an improved understanding of metabolic dysfunction during disease progression is necessary to identify novel targets for therapeutics. G-protein pathway suppressor 2 (GPS2) plays an important role as a mediator of lipid metabolism and inflammatory responses, both processes regulating insulin resistance, but GPS2 exact function in AT remains unknown. These data suggest GPS2 in AT plays an important role in systemic regulation of metabolic homeostasis and dissecting GPS2 function in AT is therefore critical for understanding how metabolic and inflammatory pathways are inter-regulated during the development of insulin resistance. These studies describe the characterization of the adipocyte-specific GPS2 knockout (GPS2-AKO) mouse model. A novel layer of regulation in the insulin signaling cascade was identified, based on ubiquitin conjugating enzyme E2N (Ubc13)-mediated K63 ubiquitination of protein kinase B (AKT). GPS2 was found to be a negative regulator of this pathway through inhibition of Ubc13. Loss of GPS2 promoted AKT hyperubiquitination and activation
and enhanced insulin signaling in adipocytes, a mechanism conserved in other cell types. Phenotypic characterization of GPS2-AKO mice showed they developed increased body weight and AT mass when fed chow diet. The white AT (WAT) was characterized by adipocyte hypertrophy, a result of impaired lipolysis and increased lipogenesis. Similarly, brown AT (BAT) acquired a WAT phenotype caused by increased lipid accumulation from compromised lipolysis and defective mitochondrial biogenesis. Despite significant increases in adiposity, GPS2-AKO mice had improved systemic insulin sensitivity due to enhanced insulin signaling and lipid storage capacity. Observations indicated GPS2-AKO mice on high fat diet (HFD) became excessively obese and inflamed, yet displayed reduced peripheral tissue lipid deposition and remained metabolically healthy. This work describes how GPS2 modulates systemic metabolism by regulating insulin signaling, lipid storage capacity, mitochondrial biogenesis and inflammation. Dissecting GPS2 function in AT provides insight into points of regulatory convergence among pathways connecting AT to systemic metabolic regulation, helping to uncover innovative targets for the treatment of metabolic disorders.
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<th>Description</th>
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<tbody>
<tr>
<td>Minute</td>
<td>..........................</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>4HO tam</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP Binding Cassette Subfamily A Member 1</td>
</tr>
<tr>
<td>ABCG1</td>
<td>ATP Binding Cassette Subfamily G Member 1</td>
</tr>
<tr>
<td>ACACA</td>
<td>Acetyl-CoA Carboxylase Alpha</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA Carboxylase</td>
</tr>
<tr>
<td>Acetyl-coa</td>
<td>Acetyl coenzyme A</td>
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<tr>
<td>ADIPO</td>
<td>Adipocyte</td>
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<tr>
<td>ADIPOQ</td>
<td>Adiponectin</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AGPAT2</td>
<td>1-Acylglycerol-3-Phosphate O-Acyltransferase 2</td>
</tr>
<tr>
<td>AKT/PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>Ap2</td>
<td>Fatty Acid Binding Protein 4</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>ARG1</td>
<td>Arginase 1</td>
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<tr>
<td>AT</td>
<td>Adipose tissue</td>
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</tbody>
</table>
ATF2 ................................................................. Activating Transcription Factor 2
ATGL ........................................................................ adipose triglyceride lipase
ATP ........................................................................... Adenosine triphosphate
ATP5A ................................................................. Mitochondrial ATP synthase alpha-subunit
B TUB ........................................................................ Beta Tubulin
BAT ........................................................................ brown adipose tissue
BCR ........................................................................ B cell antigen receptor
BLNK ........................................................................ B-cell linker 1
C terminal ................................................................ carboxyl terminal
c-jun ........................................................................ AP-1 transcription factor subunit
cAMP ........................................................................ Cyclic adenosine monophosphate
CAP .......................................................................... CBL associated protein
CBL .......................................................................... Casitas B-lineage Lymphoma
CD11B ........................................................................ Integrin alpha M
CD11C ........................................................................ Integrin alpha X
CD19 .......................................................................... Cluster of Differentiation 19
CD206 ........................................................................ mannose receptor
CD301 ...................................................................... macrophage galactose-type C-type lectin
CEBPβ ....................................................................... CCAAT/Enhancer Binding Protein Beta
ChIP .......................................................................... Chromatin Immunoprecipitation
chREBP .................................................................... Carbohydrate-responsive element-binding protein
CIDEA ....................................................................... Cell death activator CIDE-A
COX2 ............................................................................................................. Cytochrome c oxidase subunit 2
Cyclo A ............................................................................................................. Cyclophilin A
CYCLOA ............................................................................................................ Cyclophilin A
CYLD .................................................................................................................. Ubiquitin carboxyl-terminal hydrolase
CYP7a1 ........................................... Cytochrome P450 Family 7 Subfamily A Member 1
CYP8b1 ........................................... Cytochrome P450 Family 8 Subfamily B Member 1
DAG ....................................................................................................................... diacylglycerol
DAPI ..................................................................................................................... 4’,6-diamidino-2- phenylindole
DGAT1 .............................................................. Diacylglycerol O-Acyltransferase 1
DGAT2 .............................................................. Diacylglycerol O-Acyltransferase 2
DIO ................................................................................................................. Diet induced obesity
DIO2 .............................................................. Deiodinase, Iodothyronine, Type II
DL ......................................................................................................................... Deciliter
DNA ................................................................................................................... Deoxyribonucleic acid
DNL ...................................................................................................................... De novo lipogenesis
DUB .................................................................................................................... Deubiquitinase
E ......................................................................................................................... Embryonic
E ......................................................................................................................... glutamic acid
E1 ....................................................................................................................... Ubiquitin-activating enzyme
E2 ....................................................................................................................... Ubiquitin-conjugating enzyme
E3 ....................................................................................................................... Ubiquitin ligase enzyme
ECM .................................................................................................................... Extracellular matrix
EGF ................................................................. Epidermal growth factor
ELOVL3 ........................................................... ELOVL Fatty Acid Elongase 3
EPI .................................................................... Epididymal
ER ........................................................................ endoplasmic reticulum
ERα ................................................................. Estrogen receptor alpha
erbB ................................................................. Epidermal growth factor receptor
ERK ...................................................................... extracellular signal-related kinase
F4/80 ........................................ EGF-like module-containing mucin-like hormone receptor-like 1
FACS ................................................................. Fluorescence-activated cell sorting,
FASN .................................................................... Fatty acid synthase
FBP1 ................................................................. Fructose-Bisphosphatase 1
FFA ....................................................................... free fatty acid
FGF21 ............................................................... Fibroblast growth factor 21
FOXO1 ............................................................... Forkhead box protein O1
FXR ....................................................................... farnesoid X receptor
G ............................................................................. Grams
G3P ....................................................................... Glyceraldehyde 3-phosphate
G6PC ................................................................. Glucose-6-Phosphatase Catalytic Subunit
gDNA ...................................................................... Genomic DNA
GLUT4 ............................................................... Glucose transporter type 4
GPS2 .................................................................. G-Protein Pathway Supressor 2
GPS2-AKO ........................................................... adipocyte-specific GPS2 knockout mouse model
GPS2-BKO ........................................B cell-specific GPS2 knockout mouse model
GSK3β ..........................................................Glycogen synthase kinase 3 beta
GTT ................................................................Glucone tolerance test
H ...............................................................................Hour
H&E .........................................................................Hematoxylin and Eosin
H3K9 .....................................................................Histone H3 tri methyl K9
HA ................................................................................Human influenza hemagglutinin
HDAC .........................................................................Histone deacetylase
HDAC3 ..................................................................Histone deacetylase 3
HFD .............................................................................. high fat diet
HIF1α ........................................................................ Hypoxia-inducible factor 1-alpha
HSL ........................................................................... hormone-sensitive lipase
HSP70 ..................................................................Heat Shock Protein 70
i.p. ................................................................................ intraperitoneal injection
IGF ............................................................................... Insulin like growth factor
IgG ........................................................................... Immunoglobulin G
IKBα ........................................................................ Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKKβ ....................................................................... inhibitor of nuclear factor kappa-B kinase subunit beta
IL1β .............................................................................. Interleukin 1 beta
IL10 ........................................................................ Interleukin 10
Il6 ........................................................................... Interleukin 6
IP .............................................................................. Immunoprecipitation
IR ............................................................... insulin resistance
IRS-1 ............................................................... insulin receptor substrate 1
IRS-2 ............................................................... insulin receptor substrate 2
ISO ................................................................. Isoproterenol
ITT ................................................................. Insulin tolerance test
JNK ................................................................. c-Jun N-terminal kinase
K ................................................................. lysine
K11 ............................................................... Lysine 11 ubiquitin chain
K48 ............................................................... Lysine 48 ubiquitin chain
K63 Ub ........................................................... lysine 63 ubiquitination
KD ................................................................. Knock down
kDa ................................................................. kilodalton
KDM4A .......................................................... Lysine Demethylase 4A
KG ................................................................. Kilograms
KO ................................................................. Knockout
LIV ............................................................... Liver
LRH-1 ............................................................ liver receptor homolog-1
LXR ............................................................... Liver X receptor
M1 ................................................................. classically activated
M2 ................................................................. alternatively activated
MAC ............................................................. Macrophage
MAC2 ............................................................ Galectin-3
MAPK .............................................................. mitogen-activated protein kinase
MCP1 .............................................................. Monocyte chemotactic protein 1
mDNA .......................................................... Mitochondrial DNA
MetS ............................................................ metabolic syndrome
MG ............................................................ Millograms
MIN ............................................................ Minutes
mM ........................................................... Millimolar
MRI ........................................................... Magnetic resonance imaging
mRNA ........................................................... Messenger RNA
MTCO1 ........................................................ Mitochondrially Encoded Cytochrome C Oxidase 1
mTOR ........................................................ mammalian target of rapamycin
mTORC1 ...................................................... mTOR complex 1
mTORC2 ...................................................... mTOR complex 2
MYF5 ........................................................ Myogenic factor 5
N terminal ..................................................... amino terminal
NAFL ........................................................... non alcoholic fatty liver
NCoR ........................................................ nuclear receptor co-repressor 1
NDUFB8 ..................................................... NADH:Ubiquinone Oxidoreductase Subunit B8
NFκB ........................................................ nuclear factor kappa B
NG ............................................................ Nanograms
NK cell ........................................................ Natural killer cell
NLR receptor .............................................. nucleotide-binding oligomerization domain-like receptor
NOD receptor .... Nucleotide-binding oligomerization domain-containing protein receptor
NOS2 ................................................................................ Nitric Oxide Synthase 2
NS .................................................................................. Non Significant
O/N .................................................................................. Overnight
OB/OB .................................................................................. Leptin-deficient
OCR .................................................................................. Oxygen consumption rate
OTUB1 ........................................................ OTU Deubiquitinase, Ubiquitin Aldehyde Binding 1
P .................................................................................. Phosphate
P62 .................................................................................. Sequestosome-1
PAX7 .................................................................................. Paired Box 7
PCR .................................................................................. Polymerase Chain Reaction
PDK1 .................................................................................. Phosphoinositide-dependent kinase-1
PEPCK .................................................................................. Phosphoenolpyruvate carboxykinase
PG .................................................................................. Picograms
PGC1α ........................................... Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PH domain ......................................................................... Pleckstrin homology domain
PI3K .................................................................................. Phosphoinositide 3-kinase
PIP3 .................................................................................. Phosphatidylinositol (3,4,5)-trisphosphate
PKA .................................................................................. Protein Kinase A
PKC .................................................................................. protein kinase C
PPARα ........................................................ Peroxisome proliferator-activated receptor alpha
PPARγ ........................................................ peroxisome proliferator-activated receptor gamma
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PRAS40</td>
<td>Proline-rich Akt substrate of 40 kDa</td>
</tr>
<tr>
<td>PRDM16</td>
<td>PR domain containing 16</td>
</tr>
<tr>
<td>PRMT6</td>
<td>Protein Arginine Methyltransferase 6</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PTPases</td>
<td>protein tyrosine phosphatases</td>
</tr>
<tr>
<td>RAG1</td>
<td>Recombination Activating 1</td>
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<td>RAG2</td>
<td>Recombination Activating 2</td>
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<td>Ring Finger Protein 8</td>
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<td>Reactive oxygen species</td>
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<td>Ribosomal protein S6</td>
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<tr>
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<td>Real time Quantitative PCR</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SCD1</td>
<td>Stearoyl-CoA desaturase-1</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDH8</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the means</td>
</tr>
<tr>
<td>SENP1</td>
<td>SUMO1/Sentrin Specific Peptidase 1</td>
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</table>
Ser: Serine
SH2 domain: Src homology 2 domain
SHP: small heterodimer partner
SHP2: protein-tyrosine phosphatase 2C
SIAH2: Siah E3 Ubiquitin Protein Ligase 2
siCTRL: Control siRNA
siGPS2: GPS2 siRNA
siRNA: Small interfering RNA
siUbc13: Ubc13 siRNA
SKP2: S-Phase Kinase-Associated Protein 2, E3 Ubiquitin Protein Ligase
SMRT: nuclear receptor co-repressor 2
SREBP1: Sterol regulatory element-binding transcription factor 1
SUMO1: Small Ubiquitin-Like Modifier 1
SUMO2: Small Ubiquitin-Like Modifier 2
SVF: Stromal vascular fraction
T: Threonine
T2D: Type 2 Diabetes
TAX1: human T-cell leukemia virus type 1
TBL1: Transducin (beta)- 1X-linked
TBLR1: Transducin Beta Like 1 X-Linked Receptor 1
TBP: TATA-Box Binding Protein
TCA cycle: Tricarboxylic acid cycle
WT ............................................................ Weight

WT ..................................................................... Wild type
CHAPTER ONE - INTRODUCTION

Metabolic Syndrome and Type 2 Diabetes

*Hallmarks of disease progression and insulin resistance*

The metabolic syndrome (MetS) is broadly defined as a cluster of pathological conditions including hypertension, glucose intolerance, dyslipidemia, and excessive central body fat that when all occurring in combination increase an individual’s risk of heart disease, stroke, and diabetes mellitus\(^1,2\). The prevalence of MetS is increasing worldwide and becoming a serious health concern, especially in aging populations. Studies conducted to evaluate the occurrence of this syndrome determined that the overall prevalence of MetS in the United States in 2011-2012 was roughly 35% and close to 50% in adults over the age of 60\(^3\). Although various genetic components associated with MetS have been suggested by genome-wide association studies, typical risk factors mainly include sedentary lifestyle, increasing age, excessive caloric intake and obesity and non-alcoholic fatty liver (NAFL) disease\(^1,2\). The presence of MetS can lead to various complications and is highly predictive for prognoses of cardiovascular disease (CVD) and Type 2 diabetes mellitus (T2D)\(^1,2\). T2D, also known as adult onset diabetes, is a chronic condition in which the body cannot properly metabolize and store glucose, resulting in hyperglycemia\(^1,2,4-6\). This disease has become an epidemic in which there are approximately 150 million people worldwide with T2D and this figure is estimated to double by the year 2020\(^6\). In this condition, metabolic tissues of the body are unresponsive to the effects of insulin and \(\beta\) cells within the pancreas are no longer able to produce the appropriate amount of insulin necessary to maintain glucose homeostasis\(^1,2,4-\).
Insulin resistance is a hallmark and key component of the pathophysiology of these diseases.

Insulin resistance (IR) is clinically defined as the inability of a fixed amount of endogenous/exogenous insulin to increase glucose uptake and utilization. IR can manifest itself in many ways in MetS and T2D. Impaired insulin signaling in muscle, for example, causes extensive systemic insulin resistance as muscle is a central organ in regulating insulin-stimulated glucose disposal. Additionally, insulin resistance in the liver leads to increased hepatic glucose output that contributes to dyslipidemia because proper insulin action in the liver is important to suppress hepatic gluconeogenesis and glycogenolysis. Dysfunctional insulin signaling in adipose tissue also results in impaired fat tissue expansion, lipid spillover, and low-grade chronic inflammation, which can in turn promote IR in other tissues. The brain also plays a role in disease progression because IR in this tissue contributes to fasting hyperglycemia and compromised appetite regulation. The most notable risk factor for T2D and insulin resistance is obesity, specifically visceral adiposity. This disease can be managed; however, various complications can manifest that require constant clinical care resulting in enormous burdens for health care systems worldwide. This has triggered an immediate need for increased drug discovery and novel therapeutics for insulin resistance and T2D.

**Adipose tissue and its role in metabolism**

Although traditionally defined as a passive organ responsible for nutrient storage, insulation, and mechanical support, adipose tissue (AT) is now appreciated as an active endocrine organ highly involved in organismal metabolism. It is responsible for...
coordinating endocrine and paracrine metabolic cues from various tissues and is a professional secretory organ, releasing various adipokines and cytokines to regulate pathways involved in maintaining homeostasis during fasting and feeding. For example, AT can release adiponectin, an insulin sensitizing hormone, and leptin, a satiety hormone, as well as others to manage systemic nutritional status, food intake and energy expenditure\textsuperscript{7-10}. As a nutrient storage depot, AT is a vastly dynamic tissue with immense metabolic flexibility for expansion and contraction to influence systemic lipid homeostasis. Specifically, adipose tissue controls the surplus storage of energy in the form of neutral triglycerides (TG) via the process of de novo lipogenesis (DNL). When energy expenditure requirements increase or under low nutrient conditions, AT can breakdown TGs into free fatty acids (FFAs) and glycerol via lipolysis which are released into circulation to provide energy for other tissues\textsuperscript{7-9}.

Individual AT depots are complex and heterogeneous organs that are comprised of many cell types to aid in functional competency of the depot as a whole. These cells include adipocytes, adipocyte progenitors, fibroblasts and various vascular cells, and macrophages and numerous other immune cells\textsuperscript{11,12}. Many of these cell types allow AT depots to properly respond to nutritional cues. This occurs through various cellular and structural remodeling processes that include tissue expansion through hypertrophy and hyperplasia, recruitment of immune cells, and remodeling of the vasculature and ECM. These processes allow adequate oxygenation and mobilization of nutrients during remodeling to allow for lipid storage or the release of energy stores when necessary.
There are different subclasses of adipose tissue which are broadly divided into white and brown adipose tissues. The white adipose tissue (WAT) consists of large, unilocular lipid droplet filled adipocytes whose primary responsibility is nutrient storage\textsuperscript{13,14}. The brown adipose tissue (BAT), on the other hand, consists of multilocular lipid droplets, mitochondria-rich and UCP1 expressing adipocytes, whose primary function is involved in regulating energy expenditure and thermogenesis\textsuperscript{13,14}.

Additionally, the WAT depots can be subclassified based on the anatomical location of these depots. Two of the most notable and studied WAT depots are the visceral and subcutaneous depots, which differ in cellular, molecular and structural features involved in remodeling, adipogenic potential, vascularization, and immune cell populations. Interestingly, different adipose tissue depots correlate differently with risk factor for metabolic dysfunction. Visceral adipose tissue correlates with a higher incidence of Type 2 diabetes whereas subcutaneous adipose tissue does not assume this same correlation\textsuperscript{12,15}. While AT is required to maintain proper metabolism, an excessive accumulation of WAT in humans is the most predominant factor associated with insulin resistance and low-grade chronic inflammation resulting in whole body metabolic dysfunction. Although alternations in WAT mass occur in healthy individuals, it becomes clinically relevant when excessive WAT mass associates with various health problems.

**Obesity as a major risk factor for insulin resistance and T2D**

Obesity, which has become a pandemic, has been established as a profound factor contributing to insulin resistance and T2D. Obese AT releases increased amounts of FFAs, glycerol, adipokines, pro-inflammatory cytokines and other factors involved in the
Figure 1.1 – Mechanisms of obesity-induced insulin resistance.

Obesity drives defects in multiple tissues to induce systemic insulin resistance. Insulin resistant tissues propagate tissue defects resulting in metabolic dysfunction and T2D. This figure was adapted from: Oh da, Y. & Olefsky, J. M. G protein-coupled receptors as targets for anti-diabetic therapeutics. *Nat Rev Drug Discov* **15**, 161-172, doi:10.1038/nrd.2015.4 (2016).¹⁶
development of insulin resistance (Figure 1.1). Adipocytes have an immense capacity to synthesize and store TGs during feeding and hydrolyze and release TGs as FFAs and glycerol during fasting. During early stages of obesity, adipocytes are still able to maintain normal cycles of TG storage and lipolysis, albeit expressing higher levels of various enzymes involved in TG synthesis to manage the increased lipid load. However, as adiposity increases, the ability of adipocytes to regulate lipolysis, lipogenesis and insulin-stimulated glucose transport is greatly impaired. When adipocytes become too large they become dysfunctional and cannot properly store FFAs as neutral lipids. These fatty acids can be inappropriately stored in peripheral tissues as well as in circulation to drive insulin resistance and dysfunction of other tissues, most commonly the liver and skeletal muscle. Toxic lipid species can also increase and exacerbate both AT and systemic metabolic dysfunction. Under obese conditions adipocytes are unable to appropriately secrete adipokines and cytokines and respond to circulating metabolic signals. These dysfunctions elicit an inflammatory response to aid in the buffering of lipids and the remodeling of the tissue. If these inflammatory pathways in the AT are not under proper control they are thought to contribute to the progression of insulin resistance and T2D as well. As AT insulin signaling dysfunction is a principal mediator of many of these metabolic defects, this will be described in a subsequent introductory chapter.
Insulin signaling pathway in adipose tissue

Overview and pathway members

Insulin is an important anabolic hormone produced by β cells in the pancreas. It regulates whole body metabolism by modulating the uptake of circulating glucose, fatty acids and amino acids in target tissues\textsuperscript{20-24}. The insulin signaling cascade in adipocytes has been studied in many facets. Downstream mediators of this pathway have been shown to regulate glucose storage and uptake, protein synthesis and lipid synthesis. Insulin-mediated signal transduction and transcription is also responsible for regulation of pathways involved in growth and proliferation, gluconeogenesis and glycogen synthesis, lipolysis, cholesterol synthesis, apoptosis and autophagy\textsuperscript{20-24}.

Insulin exerts its actions by stimulating a complex signal transduction cascade activated upon binding to the insulin receptor (Figure 1.2). The insulin receptor belongs to a subfamily of receptor tyrosine kinases and is comprised of a tetrameric protein complex with two extracellular α and two transmembrane β subunits\textsuperscript{20-24}. These subunits function as allosteric enzymes where the α subunit inhibits the tyrosine kinase activity of the β subunit when ligand is not present. Upon insulin binding, the α subunit undergoes conformational changes to derepress the kinase activity of the β subunit. This immediately leads to phosphorylation of the β subunit, additional β subunit conformational changes and increased kinase activity of the receptor\textsuperscript{20-24}. The insulin receptor then phosphorylates a number of intracellular substrates to initiate a complex network of signaling cascades. Nine direct insulin receptor substrates have been
identified thus far: 4 IRS proteins, Gab-1, p60, Cbl, APS, and Shc isoforms\textsuperscript{20-24}. The phosphorylated tyrosine residues on these proteins act as docking sites for SH2 domain containing target proteins to further mediate downstream signaling kinase cascades. The main intracellular pathways activated by insulin include the PI3K/AKT pathway, the Ras/MAPK/ERK pathway, and the CAP/Cbl pathway\textsuperscript{20-24}. The PI3K/AKT pathway is responsible and necessary for many of the effects of insulin and is the primary insulin-stimulated pathway we have focused on in this thesis.

Keeping the insulin signaling cascade under tight regulation is reflected by the presence of many negative regulators. Inhibition of insulin receptor signaling can be accomplished by inhibitory serine phosphorylation events leading to interaction between the IRS proteins with 14-3-3 to turn off downstream signaling. Inhibition of this pathway also occurs through protein tyrosine phosphatases (PTPases) which catalyze the swift dephosphorylation of the receptor and its various substrates to similarly shut off insulin-mediated signals\textsuperscript{20-24}. Significant progress has been made to understand the molecular details of the insulin signaling pathway.

\textit{The role and regulation of AKT in the insulin signaling pathway}

Protein kinase B, AKT/PKB, is a serine/threonine kinase that has been identified as a critical component of multiple cellular pathways regulating cell growth, survival, proliferation, and metabolism. Particularly, regulation of insulin signaling in AT relies on the activation of AKT\textsuperscript{20-24}. Three isoforms of AKT have been identified, each with distinct functions. AKT2 is the predominant isoform involved in metabolism and regulation of insulin action on glucose transport, whereas AKT1 has been linked to cell
Figure 1.2 – The canonical insulin signaling pathway.

Insulin action results in the activation of multiple downstream signaling cascades to affect a variety of cellular processes. This figure was adapted from Siddle, K. Signalling by insulin and IGF receptors: supporting acts and new players. *J Mol Endocrinol* **47**, R1-10, doi:10.1530/JME-11-0022 (2011).25
growth, survival and apoptosis and AKT3 has a suggested function in brain development\textsuperscript{20,22,25-27}.

Although AKT isoforms have been shown to play different roles, all isoforms have a conserved structure that consists of an N terminal PH domain, a central kinase domain, and a C terminal regulatory domain comprised of a hydrophobic motif. The PH domain of AKT plays a critical role in recognition by upstream kinases as well as for the recruitment of AKT to the plasma membrane. This domain binds to membrane lipid products, such as the second messenger phosphatidylinositol (3,4,5)-triphosphate (PIP3) produced upon activation of the phosphatidylinositol 3-kinase (PI3K) by the insulin receptor\textsuperscript{24-26}. The kinase domain of AKT is similar to other ACG kinases, containing a conserved threonine residue that undergoes phosphorylation to activate the critical enzymatic activity of AKT\textsuperscript{24-26}. The C terminal hydrophobic motif also contains a conserved site of phosphorylation to allow for full activation of AKT\textsuperscript{24-26}.

Following AKT translocation to the membrane, the kinase is phosphorylated on two specific sites, threonine 308 in the catalytic domain by PDK1 and serine 473 in the C terminal hydrophobic domain by mTORC2. The phosphorylation events stabilize AKT in an active conformation, leading to the activation of an intricate network of downstream signaling cascades\textsuperscript{20,22,25-27}. Some key examples include AKT phosphorylation of AS160 involved in GLUT4 translocation, AKT phosphorylation of GSK3\(\beta\) to modulate glycogen and fatty acid synthesis, as well as AKT phosphorylation of kinases involved with mTORC1 to regulate growth and proliferation\textsuperscript{20,22,25-27}. Negative regulation of AKT can be achieved by the actions of PTEN which is able to negatively regulate intracellular
levels of PIP3 to inhibit AKT action as well as via negative regulation of upstream mediators in the insulin signaling cascade\textsuperscript{20,22,25-27}.

**Insulin signaling in the development of insulin resistance**

Defects in the insulin signaling cascade have been associated with the development of insulin resistance in adipose tissue, muscle, and liver. Studies in both mice and humans have shown that both genetics and environment have been found responsible for these defects. Various mutations in the insulin receptor, IRS proteins, PI3K activity and AKT have been identified and targeted deletion of these components have shown their ability to drive insulin resistant phenotypes\textsuperscript{20,22,25-27}. Patients with rare genetic defects in insulin receptor expression, binding, phosphorylation state and kinase activity have shown that these alterations can account for insulin resistance\textsuperscript{28}. Specific deletion of IRS proteins have revealed their importance in the development of insulin resistance as well. IRS-1 knockout mice show a phenotype of growth retardation, insulin resistance in peripheral tissues and impaired glucose tolerance whereas IRS-2 knockout mice present a phenotype of insulin resistance in peripheral tissues and modest defective growth in some tissues, mainly various regions of the brain\textsuperscript{29-32}. Mice with systemic knockout of AKT2 were also found to develop insulin resistance in muscle and liver\textsuperscript{33}. Elevated FFAs have been found associated with reduced insulin-stimulated IRS-1 phosphorylation as well as reduced PI3K activity\textsuperscript{34}. These and additional models have highlighted the importance of players in the insulin signaling cascade in the development of T2D.
Lipotoxicity induced by insulin resistance can lead to several impairments in the insulin signaling cascade as well. Impaired glucose uptake as a result of impaired insulin signaling acts as a feed forward mechanism to propagate the insulin resistant phenotype. Unrestricted circulation of FFAs can mediate many inhibitory effects on the insulin signaling pathway as well. \(^{20,22,25-27}\) It has been shown that fatty acids can also act as ligands to activate TLR signaling pathways to induce pro-inflammatory signals that impair insulin action through activation of JNK resulting in IRS-1 inactivating phosphorylation events. Sphingolipid ceramides have also been shown to inhibit insulin-stimulated AKT activation via activation of TNFα.\(^{20,22,25-27}\) Inflammatory effects on insulin signaling and insulin resistance will be discussed further in a subsequent section. Overall, tight regulation of insulin signaling is extremely important to maintain metabolic homeostasis and interrupted insulin-mediated signaling cascades result in insulin resistance and T2D.

**AKT regulation by ubiquitination in signaling pathways**

It is well appreciated that AKT activation requires its translocation to the plasma membrane and binding to membrane lipids via the PH domain. This was eloquently illustrated by showing that adding a myristylation residue to AKT could sufficiently drive its translocation to the membrane and induce its activation, regardless if upstream insulin signaling was not active\(^ {35}\). Despite these results, the exact cues that target and drive AKT movement to the membrane or other factors involved in the regulation of this translocation are less elucidated.
Recent work has revealed that non-proteolytic ubiquitination of AKT might be a contributing factor for the kinase recruitment to the plasma membrane, phosphorylation, and thus activation\textsuperscript{36-39}. Specifically, it was shown that upon IGF and IL1\(\beta\) stimulation, the E3 ubiquitin ligase, Traf6, promoted the ubiquitination of AKT\textsuperscript{40}. TRAF6-mediated K63 ubiquitination was required for AKT translocation to the plasma membrane, phosphorylation and activation upon growth factor stimulation (Figure 1.3). Lysine residues 8 and 14 within the PH domain of AKT were identified as ubiquitination sites necessary for AKT ubiquitination and activation\textsuperscript{40}. A similar mechanism was later observed in response to EGF stimulation in which another E3 ligase, Skp2, was responsible for the K63 ubiquitination of AKT\textsuperscript{41}. Again, the ubiquitination of AKT by Skp2 was required for AKT recruitment to the plasma membrane and in turn its phosphorylation and activation\textsuperscript{41}. In addition, deubiquitination of AKT via the actions of the deubiquitinase, CYLD, appeared to counteract AKT activation. CYLD deficiency led to hyperactive AKT membrane recruitment and activation in response to growth factor stimulation\textsuperscript{42}.

In humans AKT ubiquitination has been predominantly studied in the context of activating cancer mutations, such as that of oncogenic AKT1 mutant E17K, which drives increased ubiquitination and hyperactivation of AKT and downstream growth promoting pathways\textsuperscript{43}. However, there is one patient case study in which it was observed that the same mutation in AKT2 led to enhanced AKT activation, insulin signaling, hypoglycemia and hypoinsulinemia\textsuperscript{44,45}. Although no connections with non-proteolytic ubiquitination of AKT and insulin signaling have been made, these data suggest that
Figure 1.3 – Model of AKT ubiquitination.

Upon certain growth factor stimulation, AKT undergoes K63 ubiquitination, which results in membrane translocation and subsequent phosphorylation and activation. This figure was adapted from: Yang, W. L., Wu, C. Y., Wu, J. & Lin, H. K. Regulation of Akt signaling activation by ubiquitination. *Cell Cycle* 9, 487-497 (2010).
aberrant AKT ubiquitination could be similarly affecting insulin signaling and promoting metabolic dysfunction.

**Obesity-associated inflammation**

**Overview**

Over the past couple of decades, a great deal of epidemiological evidence has revealed a strong correlation between inflammation and obesity-induced insulin resistance\(^{46-56}\). Obesity-associated inflammation is now a widely recognized critical factor in the development of insulin resistance and T2D\(^{46-56}\). Although many studies have revealed a clear association between chronic, low-grade inflammation and activation of pro-inflammatory signaling cascades with decreased insulin sensitivity, the exact physiological events leading to the initiation of inflammatory signaling and obesity is not well understood. Interestingly, local inflammation in the adipose tissue has been shown to positively affect metabolic flexibility\(^{57}\). In agreement, therapeutic interventions using anti-inflammatory drugs have proven largely unsuccessful in treating metabolic diseases in clinical trials\(^{58}\). Thus, it appears inflammatory pathway contributions to inducing insulin resistance and vice versa are more complex than initially envisioned.

In the search for mechanisms responsible for the pathogenesis of obesity associated insulin resistance, a close relationship between excessive nutrient stress and aberrations in mediators of immunity and inflammation have been uncovered. This led to the development of the concept of “metainflammation” describing the low-grade chronic inflammatory response to obesity\(^{46-56}\). In general terms, inflammation is a coordinated response to harmful stimuli to remove pathogens and restore homeostasis. Adipose tissue
inflammation involves many components of classically activated inflammatory responses, such as increased circulation of inflammatory cytokines, recruitment and activation of immune cells into the impaired tissue, and production of reparative responses\textsuperscript{46-56}. However, AT inflammation/metainflammation in response to obesity is unique compared with canonical inflammation, such as infection or autoimmune diseases.

Obesity induces both a persistent low-grade activation of immune cells that over time can negatively affect metabolic homeostasis as well as acute attacks of nutrition-related immune cell activation induced by excessive nutrient availability and associated stress signals\textsuperscript{46-56}. Obesity-induced chronic inflammation leads to increased pro-inflammatory changes in not just AT but also multiple organs including liver, muscle, pancreas and the brain\textsuperscript{46-56}. This multi-organ inflammation associated with obesity presents a unique disease state and creates a difficult challenge to tease out direct mechanisms in the involvement of adipose tissue dysfunction and inflammation in the onset of insulin resistance.

\textbf{Obesity driven AT inflammation}

A long standing debate has been how obesity and inflammation can synergistically and separately drive insulin resistance. The discovery that obesity directly induces polarization of AT macrophages suggested that obesity can itself drive pro-inflammatory responses in the tissue leading to metabolic dysregulation\textsuperscript{59,60}. Adipose tissue insulin resistance and dysfunctional lipid storage are important events that can cause proinflammatory responses (\textbf{Figure 1.4}). A reduced ability for AT to store excessive lipids, resulting in lipid spillover has been proposed as a potential factor\textsuperscript{46-56}. 
Lipid spillover can induce macrophage infiltration into the AT. Direct and paracrine signals from pro-inflammatory macrophages can in turn impair insulin signaling and adipogenesis in adipose tissue\textsuperscript{46-56}. Additionally, FFAs from improper lipid storage can activate inflammatory pathways and serve as ligands for TLR2/4 to induce JNK and NF\kappa B to stimulate pro-inflammatory gene transcription and impair insulin signaling\textsuperscript{46-56}.

WAT also acts as an endocrine organ and releases cytokines, including IL6, IL1\beta, TNF\alpha, and MCP1. These stress signals are typically activated when excessive hypertrophy results in cell death. Increased activation of NF\kappa B transcriptional programs results in increased production of proinflammatory cytokines such as TNF\alpha, IL6, IL1\beta, and MCP1 to further activate pro-inflammatory gene transcription in a feed forward mechanism\textsuperscript{46-56}. These cytokines also help to recruit immune cells into AT and contribute to pro-inflammatory signaling. Immune cells can then polarize to create a pro-inflammatory environment in the adipose tissue and feed back to the adipocyte to cause decreased IRS-1 and AKT phosphorylation as well as GLUT4 translocation. This cascade is thought to continuously signal to drive chronic adipose tissue inflammation\textsuperscript{46-56}.

Obesity can furthermore cause increased adipocyte cell death which contributes to immune cell infiltration into the tissue to clean up the cellular debris\textsuperscript{46-56}. Due to increased adipocyte hypertrophy and lack of sufficient blood supply to reach the over-expanded tissue, hypoxia has also been implicated to propagate inflammation associated with obesity\textsuperscript{46-56}. Hypoxia has been shown to activate HIF1\alpha which can activate NF\kappa B signaling\textsuperscript{46-56}. ER stress has been implicated in causing inflammation in obesity as well. It has been shown that overnutrition can cause activation of the unfolded protein response
Obesity and dysregulated inflammatory signaling coordinately result in AT dysfunction. This causes macrophage infiltration, polarization of immune cells to pro-inflammatory phenotypes and increased production of pro-inflammatory signals in AT. This figure is adapted from: Osborn, O. & Olefsky, J. M. The cellular and signaling networks linking the immune system and metabolism in disease. Nat Med 18, 363-374, doi:10.1038/nm.2627 (2012).
and ER stress, which can activate JNK and cause defective insulin signaling\textsuperscript{46-56}. Lipid intermediates such as DAGs, ceramides and sphingolipids can also induce adipocyte cell stress and pro-inflammatory signaling cascades\textsuperscript{46-56}. Lastly, oxidative stress and mitochondrial dysfunction have been suggested as cell-intrinsic mechanisms to drive metabolic tissue inflammation.

\textit{AT inflammation driving insulin resistance}

As much as AT dysfunction can drive pro-inflammatory signaling activation, increased inflammation can also drive adipose tissue and systemic metabolic dysfunction, creating a pathophysiologic feedforward loop to drive insulin resistance (Figure 1.4). Many theories have developed as to how inflammation contributes to obesity-induced insulin resistance. In seminal work by Hotamisligil and Spiegelman, TNF\(\alpha\) was shown to inhibit insulin signaling in adipocytes and contribute to obesity-induced insulin resistance, which was the first evidence that adipose tissue inflammation was pertinent in metabolic dysfunction\textsuperscript{62-65}. TNF\(\alpha\) has been found to be upregulated in obese insulin resistant patients and TNF\(\alpha\) stimulation leads to the activation of the stress kinase, JNK, which was then found to directly serine phosphorylate IRS1 to inhibit its ability to mediate the insulin signaling pathway. Once activated, JNK is also able to translocate to the nucleus and activate transcription factors such as C-Jun and ATF2 to induce pro-inflammatory gene expression\textsuperscript{46-56}. Since these initial observations, many other mechanisms of inflammation have been implicated in the development of insulin resistance.
Multiple inflammatory signals contribute to adipose tissue dysfunction, including increases in circulating cytokines, decreases in protective, insulin sensitizing factors, such as adiponectin, and communication between inflammatory cells and adipocytes. Obesity has been shown to activate stress signals such as increased FFAs, ROS, hyperglycemia and increased cytokine production which can activate TNFα, TLR and NLR, NOD like receptor, signaling cascades\textsuperscript{46-56}. These cellular stress signaling pathways typically converge on the activation of JNK as previously mentioned, as well as IKK and NFκB. Briefly, the IKK complex is recruited and activated which then phosphorylates IκBα, the inhibitory protein that normally binds to NFκB and inhibits its translocation, causing IκBa degradation and allows NFκB to enter the nucleus and mediate transcription of an inflammatory gene program and production of pro-inflammatory cytokines, such as MCP1, TNFα, IL6, IL1β and more. This can lead to increased ROS production and hypoxia in the adipose tissue. Overall, cytokine production and increased lipid species leads to hyperactivation of inflammatory pathways exacerbating their negative impact on adipocytes. Inflammatory cytokines produced by dysfunctional adipose tissue can also increase SOC3 expression which can also interfere with insulin receptor activity, similarly how JNK can impair IRS activity\textsuperscript{46-56}. Overall, adipose tissue inflammation can impair glucose transport, adipocyte function and insulin sensitivity, yet more in depth understanding of how inflammation directly inhibits these processes is necessary.
Macrophage infiltration into adipose tissue and involvement in remodeling

Immune system cells involved in these inflammatory responses include macrophages, dendritic cells, mast cells, neutrophils, T cells, B cells, and NK cells. Particularly, obesity has been shown to lead to increased infiltration of macrophages into the adipose tissue\textsuperscript{47-50}. Macrophages are derived from monocytes and act phagocytically to engulf and digest unwanted substances such as cellular debris or foreign species. Other immune cells are also found in adipose tissue depots however macrophages are the most dominant, representative of approximately 40-60\% of cells in the adipose tissue, and functionally central\textsuperscript{47-50}. AT macrophages can have different phenotypes, described as M1 classically activated or M2 alternatively activated. M2 macrophages are uniformly dispersed throughout the adipose tissue, specifically express ARG1, CD206 and CD301, and have been shown to assist remodeling of the tissue. These macrophages are less pro-inflammatory than M1 macrophages and produce IL10, an anti-inflammatory cytokine that helps to maintain and potentiate insulin signaling in adipocytes\textsuperscript{47-50}.

M1 classically activated macrophages have a pro-inflammatory phenotype, expressing NOS2, TNFα, CD11C, and are typically observed in a setting of obesity\textsuperscript{47-50}. Often, these macrophages are found in crown-like structures around dying adipocytes, where they are able to phagocytose lipids released from the necrotic cells to help reduce lipotoxic species release into circulation. Fatty acids have also been shown to promote M1 macrophage recruitment into adipose tissue. M1 macrophages secrete pro-inflammatory cytokines that can exacerbate adipose tissue inflammation\textsuperscript{47-50,66}. Although these two phenotypes for macrophages have been described as very distinct populations,
adipose tissue macrophages exhibit plasticity and can encompass a wide spectrum of activation phenotypes that cannot solely be defined as M1 or M2.

Macrophages are mainly recruited into adipose tissue depots through the production of MCP1 from adipocytes which is the main chemoattractant for macrophage infiltration into tissues\textsuperscript{67}. Interestingly, macrophage recruitment has been shown more recently to have a positive function in the adipose tissue. In an interesting study, macrophage infiltration into adipose tissue has been shown to be required for healthy expansion of the adipose tissue\textsuperscript{31}. In general, resident macrophages help to remodel the adipose tissue to allow for expansion and response to metabolic cues\textsuperscript{57,68}. Further expansion of these studies can help clarify the role for polarized macrophages in obesity and how they may be targeted to treat metabolic syndromes.

**Ubiquitination as a mechanism of post translational modifications in signaling pathways**

**Overall mechanism of ubiquitination**

Ubiquitination, also known as ubiquitylation, is a reversible posttranslational modification involved in nearly all cellular processes. Ubiquitin is a small, 76 amino acid and lysine rich, regulatory protein. It is universally expressed in eukaryotes and beyond its essential function for protein degradation processes, it is also involved in a myriad of other biological events, resembling other posttranslational modifications such as phosphorylation. Ubiquitination is the enzymatic process by which one single ubiquitin protein or a chain of ubiquitin molecules is added to a substrate protein. This
modification often marks the target protein for proteasomal degradation, however non-degradative ubiquitin marks also exist$^{69-75}$.

The process of ubiquitination is regulated by three main classes of enzymes. These include ubiquitin-activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3). The diversity of these enzymes allows for profound specificity of substrate recognition. There are two E1s identified, approximately 50 E2 enzymes, and hundreds of E3 enzymes which control the three stages of ubiquitination known as activation, conjugation, and ligation$^{69-75}$. The first step involves the ATP-dependent activation of the ubiquitin molecule by the E1 enzyme which forms a thioester bond with ubiquitin. Subsequently, the ubiquitin conjugating enzyme (E2) is necessary to transfer the ubiquitin from the E1 to the active cysteine site of the E2 via an additional thioester bond. The E2 enzyme has a specific structure that allows it to bind the ubiquitin and E1 at the same time to allow this step to occur. Finally, the ubiquitin ligase (E3) is required to recognize and bind the target substrate and covalently attach the ubiquitin molecule to it. Two distinct E3 families of enzymes exist based on the presence of two domains, known as the homologous to E6-AP carboxyl terminus (HECT) domain or really interesting new gene (RING) domain. For HECT domain E3s, ubiquitin is transferred to the HECT domain of the enzyme followed by its transfer to the substrate. Evidence from studies on RING domain E3s suggest that ubiquitin can be transferred directly from the E2 to the substrate. In both cases, the reaction generates an isopeptide bond between ubiquitin and a lysine residue on the substrate protein$^{69-75}$. If the enzymatic process is repeated multiple times, several ubiquitin molecules can be added to form a ubiquitin chain in which each
Figure 1.5 – The ubiquitination pathway.

Ubiquitination is a multiple step enzymatic process that involves three classes of enzymes to ultimately add ubiquitin to a target substrate. This figure was adapted from: Weissman, A. M. Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* 2, 169-178, doi:10.1038/35056563 (2001).
ubiquitin is conjugated to a lysine residue on the previous one (Figure 1.5). Removal of ubiquitin chains, or deubiquitination, is mediated by deubiquitinating enzymes, DUBs, which act as thiol proteases to specifically cleave this posttranslational modification76.

**Differential ubiquitin chain formations**

The way in which ubiquitin molecules are linked to each other to form chains and how they are linked to protein substrates provides extreme flexibility to this posttranslational modification strategy. Because ubiquitin itself has seven conserved lysine residues, which are all potential sites of isopeptide linkage, the choice of lysine position to build a multi-ubiquitin chain can result in a multitude of protein substrate fates69-75.

The most notable and studied type of chain has been K48 ubiquitin chains, which are involved in tagging proteins for proteosomal degradation. These specific chains are recognized by the 26S subunit of the proteasome. K11 chains have also been shown to be involved in targeting substrate proteins for proteasomal degradation, specifically in the context of cell cycle regulation during mitosis. Monoubiquitination or the addition of multi monoubiquitin marks have been implicated in mediating protein interactions, localization, and protein activity. K63 ubiquitination has been shown to be involved in activation of kinase signaling cascades in the context of inflammatory pathways, DNA repair, general trafficking, endocytosis, and protein-protein interactions. K6, K27, K29 and K33 linked chains are also under investigation and have been implicated in protein degradation and DNA repair. Additionally, ubiquitin chains of mixed topology are also being identified and their functions are under intense investigation69-75. In conclusion,
different types of ubiquitin chains can be formed to function for specific applications and this ubiquitin code is still being unraveled.

**Role of K63 ubiquitination in various signal transduction pathways**

K63 ubiquitination is a non-proteolytic posttranslational mark involved in the regulation of various cellular processes and signaling pathways. Currently, Ubc13 is the only identified E2 ubiquitin conjugating enzyme responsible for K63 ubiquitin chain formation. It works in concert with the cofactor, Uev1A, to promote full activation to catalyze K63 chains. Uev1A has a similar structure to Ubc13, however lacks the catalytic domain critical for E2 function. Ubc13/Uev1A cooperate with various E3 ubiquitin ligases to confer substrate specificity and differential pathway regulation.

This posttranslational modification has an important function in the nucleus. It has been shown to be essential in mediating the recruitment of DNA damage repair machinery. Specifically, nuclear Ubc13 has been shown to act with the E3 ubiquitin ligase, RNF8, to recruit repair factors involved in homologous recombination to properly regulate DNA repair upon double strand breaks. Additionally, Ubc13 works with other nuclear E3s to regulate DNA replication stress. More recently, Ubc13-mediated K63 ubiquitination has been found to recruit chromatin remodeling machinery as well. Our lab has demonstrated the functions of Ubc13/RNF8 to help remodel chromatin landscapes necessary to allow PPARγ binding and activation of a subset of PPARγ target genes. K63 ubiquitination clearly plays an important nuclear role which should be studied in further detail to elucidate other mechanisms.
K63 ubiquitination is also an important posttranslational modification used to mediate inflammation and immune response pathways\textsuperscript{37,77,78}. Ubc13-mediated K63 ubiquitin chains have been found to be involved in multiple stages downstream TNF\(\alpha\), TLR ligand, and IL1\(\beta\) stimulation to activate NF\(\kappa\)B and MAPK signaling. For example, Ubc13 has been shown to interact with the RING domain of TRAF2 in the TNF\(\alpha\) receptor signaling pathway to mediate K63 ubiquitination of RIP1 to induce JNK activation. Additionally, Ubc13 has been found to act with TRAF6 to mediate non-proteolytic ubiquitination of protein substrates, such as TRAF6 itself and TAK1, involved in activating the IKK complex in TLR signaling cascades\textsuperscript{37,77,78}. Many additional examples of K63 ubiquitination in these pathways exist and interestingly, this regulation of signaling has been expanded and implicated in IGF and EGF growth factor mediated pathways\textsuperscript{37}.

The addition and removal of K63 ubiquitin marks are under tight control. The cleavage of K63 ubiquitin chains is achieved in part by the DUB, OTUB1, which was found to bind and inhibit the activity of Ubc13\textsuperscript{67-69,75,76}. Various mouse models have been developed in order to study the regulation of K63 ubiquitin as well. Interestingly, whereas Ubc13 knockout mice show significantly impaired K63 ubiquitination, the fact that this posttranslational modification is not completely abolished leaves open the possibility that more E2 conjugating enzymes that can make K63 chains may exist\textsuperscript{67-69,75,76}. Expanding the studies of K63 ubiquitination to other signaling cascades can help shed light on additional mechanisms of regulation in a multitude of pathways.
G-Protein Pathway Suppressor 2 (GPS2)

Overview and initial discovery

G-protein pathway suppressor 2 (GPS2) is a 37 kDa, relatively unstructured and ubiquitously expressed protein. It is found localized in the cytoplasm, nucleus, and, more recently, in mitochondria. Total body knockout of GPS2 is embryonic lethal, with mice only surviving through embryonic development days E9.5- E10.5\(^{80}\). These data suggests that GPS2 is an extremely important protein warranting further studies to understand its full function.

GPS2 was originally discovered in 1996 in a screen interrogating suppressors of signal transduction in the yeast pheromone response pathway\(^{81-83}\). In this work, human GPS2 was identified as a potent suppressor of RAS- and MAPK-mediated signaling pathways, resulting in compromised JNK activity\(^{81}\). Through an additional independent yeast- two hybrid screen, GPS2 was identified to interact with Human T lymphotropic virus type I Tax oncoprotein and was found as a suppressor of TAX- mediated TNF\(\alpha\) signaling. In this study, overexpression of GPS2 was again found to potently suppress JNK activation\(^{83}\). These studies were the first to suggest that GPS2 could play an important role in signal transduction.

Follow up studies identifying GPS2 nuclear localization instead focused on GPS2 function in regulating gene transcription and other nuclear functions\(^{79,84-86}\). Work from various laboratories, for example, indicate that nuclear GPS2 acts as a transcriptional coactivator for several nuclear receptors and transcription factors\(^{79,87-93}\). This function of GPS2 has been implicated in regulating cell proliferation as well as DNA damage
GPS2 transcriptional activating function was also found to play a role in viral transcription and replication. Additionally, the discovery of GPS2 as a fundamental subunit of NCoR/SMRT corepressor complexes suggested that nuclear GPS2 could function as a corepressor as well\textsuperscript{93}.

GPS2 cytoplasmic function has been further elucidated since the original work uncovering GPS2 potential role in signal transduction. Our lab has reported a number of distinct, but coordinated functions for GPS2 in the cytosol as a regulator of pro-inflammatory response pathways, that appear to be conserved in adipose tissue, macrophages and B cells\textsuperscript{84-86}. Notably, our work suggests that both nuclear and cytosolic functions are based on GPS2-mediated inhibition of K63 ubiquitination events\textsuperscript{79,84-86}.

Overall, the cumulative, initial studies of GPS2 imply it is an exceedingly vital protein in regulating both transcriptional activation and repression as well as mediating important signal transduction pathways.

**GPS2 role in mediating transcriptional repression**

GPS2 has been identified as an fundamental component of corepressor transcriptional complexes, suggesting it plays a role in transcriptional repression. A report was published in 2002 first showing that GPS2 was an integral member of the NCoR corepressor complex\textsuperscript{93}. GPS2 was identified by mass spectrometry as a stoichiometric subunit of the NCoR-HDAC3 complex via interaction with the repressor domain (RD1) of NCoR, as well as interactions with TBL1 and TBLR1\textsuperscript{93}. This work showed that promoter bound GPS2 was able to repress transcription by its association with the NCoR complex and suggested that GPS2-mediated inhibition of JNK activation
was a result of GPS2-NCoR transcriptional repression\textsuperscript{93}. Just as GPS2 was found to be an integral component of the NCoR corepressor complex, it was also shown that GPS2 was a fundamental subunit of the SMRT corepressor complex, which functions very similarly to NCoR through recruitment of HDACs\textsuperscript{90}. In this work GPS2 was shown to be an important component of this complex and functionally involved in ER\textalpha-mediated transcriptional repression\textsuperscript{90}.

The structural interaction of the NCoR/SMRT transcriptional repressor complex was investigated revealing how GPS2 how binds in this complex. SMRT and GPS2 form an anti-parallel coiled coil in which amino acids 167-207 are sufficient to interact with amino acids 53-90 of GPS2\textsuperscript{96}. In turn, TBL1 crystal structure revealed that TBL1 N terminal domain dimerized and amino acids 1-71 of each TBL1 protein could interact with amino acids 227-297 of SMRT and amino acids 1-60 of GPS2, each containing a short helical sequence\textsuperscript{96}.

In support of the role of GPS2 in mediating transcriptional repression, recent studies indicate that downregulation of GPS2 with the associated corepressor SMRT in adipose tissue of obese individuals plays a critical role in the upregulation of a pro-inflammatory gene program\textsuperscript{97}. Additionally, it was suggested that GPS2 may play a role in transrepression of PPAR\textgamma and LXR but more mechanistic data is required to identify GPS2 exact role in repression in these model systems\textsuperscript{80,91}. This evidence along with data showing GPS2 direct interaction within the NCoR/SMRT complex supports the idea that GPS2 is involved with transcriptional repression although direct evidence for GPS2 alone acting to control repression remains to be further elucidated.
GPS2 role in mediating transcriptional activation

Despite data showing that GPS2 may play a role in transcriptional repression, more evidence has suggested that GPS2 plays a role in transcriptional activation with various nuclear receptors and transcription factors\(^{82,87,88,91,95,98-104}\). These initial studies led the groundwork for mechanistic studies indeed showing GPS2 plays a crucial role in transcriptional activation of FXR, LXR, and PPAR\(_\gamma\)^{79,89,105}.

The first evidence for the role of GPS2 as a transcriptional coactivator was suggested in greater mechanistic detail in studies of bile acid biosynthesis in liver cells\(^{105}\). It was found that GPS2 is a cofactor for SHP, a known physiological repressor of bile acid biosynthesis. By interacting with SHP and additional binding partners, LRH-1, HNF\(\alpha\), and FXR, GPS2 was found differentially regulating the transcription of the genes, CYP7A1 and CYP8B1, key enzymes involved in hepatic bile acid synthesis\(^{105}\). Specifically, GPS2 was found to modulate SHP repression by enhancing interactions with NCoR-HDAC3 repressor complex. GPS2 was shown to differentially regulate CYP7A1 and CYP8B1, which was found to be a result of GPS2 repressor of the nuclear receptor LRH-1 on CYP7A1 and GPS2 enhancement of HNF4\(\alpha\) on CYP8B1. It was observed that GPS2 interacted with FXR and potentiated FXR-mediated transcription of the CYP8B1 promoter. It was implied that CYP8B1 transcriptional induction is ligand-dependent and requires GPS2 to induce enhancer-promoter communication\(^{105}\). This report was the first to succinctly explore the differential gene-specific repressor versus activator functions of GPS2.
Because GPS2 was shown to function in cholesterol homeostasis in liver by regulating bile acid biosynthesis via target gene specific repressor as well as activator roles, an additional report then investigated the function of GPS2 in regulating LXR-mediated cholesterol efflux in liver and macrophages. This work showed that GPS2 is selectively required for LXR activation of the cholesterol efflux gene, ABCG1, while not affecting other LXR-dependent cholesterol transport genes. Whereas in the case of the gene ABCA1, GPS2 was present on the promoter in the unliganded state and was dismissed upon ligand binding; in the case of ABCG1, GPS2 recruitment to the promoter was dependent on ligand binding suggesting that GPS2 functions as a coactivator for LXR on this specific gene. In this case, GPS2 was found to bind the heterodimer LXR/RXR on the ABCG1 promoter. As was suggested in the case of CYP8B1, GPS2 was proposed to mediate ABCG1 promoter enhancer looping and communication upon LXR ligand activation of the gene. Lastly, this work showed that GPS2 was required to initiate H3K9 demethylation of the ABCG1 gene.

GPS2 has been shown to be involved in the regulation of the nuclear receptor, PPARγ, whereby the NCoR/SMRT corepressor complex was shown to repress PPARγ gene expression and 3T3-L1 cells devoid of NCoR/SMRT had an increase in adipogenic differentiation. As expected since GPS2 plays a key role in the regulation of PPARγ, GPS2 has been implicated in various studies in the regulation of adipogenesis. Interestingly, however, our lab discovered a novel mechanism of GPS2 regulation of PPARγ, distinct from its role with the NCoR/SMRT corepressor complex. GPS2 was shown to act as a pioneering and coactivating factor for PPARγ recruitment on a subset of
Figure 1.6 – Model of GPS2-mediated regulation of PPARγ recruitment in adipocytes.

GPS2 inhibition of Ubc13/RNF8 results in chromatin remodeling events to allow the binding of PPARγ on promoters to regulate a subset of target genes. This figure was adapted from: Cardamone, M. D. et al. GPS2/KDM4A pioneering activity regulates promoter-specific recruitment of PPARgamma. Cell reports 8, 163-176, doi:10.1016/j.celrep.2014.05.041 (2014).
target genes in which PPARγ binding is necessary on promoters, rather than on enhancers\textsuperscript{79}. In this work it was revealed that GPS2 can stabilize KDM4A, an H3K9 histone demethylase, by inhibiting Ubc13 and RNF8 ubiquitination. This priming of the chromatin environment was required for broad PPARγ promoter-specific binding\textsuperscript{79}. This regulatory strategy was found to be required for the regulation of a select PPARγ transcriptional program that included the rate-determining enzymes of lipolysis, ATGL and HSL (\textbf{Figure 1.6})\textsuperscript{79}.

In agreement with this mechanistic study dissecting how GPS2 regulates PPARγ recruitment and adipocyte function, GPS2 has been shown to play an important role in the regulation of lipid metabolism \textit{in vivo} as well. Transgenic mice overexpressing GPS2 in AT were found to have increased expression of ATGL and HSL, both at the mRNA and protein level\textsuperscript{79}. This in turn led to increased phosphorylation and activation of HSL, as a marker of lipolysis. The white adipose tissue also exhibited an enhanced response to isoproterenol induced lipolysis\textsuperscript{79}. This mechanism led to smaller adipocytes in the adipose tissue depots from TG mice. These data suggest that GPS2 plays an important role in regulating lipid metabolism in adipose tissue however the complete understanding of this role \textit{in vivo} remains to be elucidated.

\textit{The role of cytoplasmic GPS2 in regulating inflammation}

In the initial discovery of GPS2, it was noted that GPS2 could potently suppress the activity of JNK, an important mediator of downstream inflammatory signaling cascades\textsuperscript{81,83}. Accordingly, subsequent studies have discovered numerous roles of GPS2
in regulating inflammation in various cell types including adipose tissue, macrophages, and B cells.84,91,97,108.

In particular, work by our lab uncovered a specific role for GPS2 outside the nucleus in the regulation of inflammatory signal transduction. GPS2 can interact with TRAF2/Ubc13 K63 ubiquitination machinery to affect the activation of the TNFα signaling pathway (Figure 1.7A).84 GPS2 was found to regulate the activation of TNFR1 in response to TNFα via the inhibition of JNK activation84. This mechanism led to hyperactivation of a proinflammatory gene transcription program in the absence of GPS284. Interestingly, this mechanism was found to regulate adipogenesis as well. Removal of GPS2 in 3T3L1 cells prior to inducing differentiation resulted in decreased expression of PPARγ and these cells were unable to induce expression of mature adipogenic genes and fully undergo differentiation84. Downregulation of TRAF2 or treatment of GPS2 knockdown cells with a JNK inhibitor was able to rescue this phenotype, suggesting that unregulated inflammation, as a result of lack of GPS2, could negatively impact the early stages of adipocyte differentiation84.

In continuation of this work by our lab, we studied the effect of GPS2 overexpression in adipose tissue on inflammation in vivo. In this work addressing that GPS2 acts as a negative regulator of the TNFα signaling pathway, aP2-driven overexpression of GPS2 resulted in mice that were protected from hyperinflammatory signaling in the adipose tissue84. These mice on high fat diet exhibited decreased phosphorylation of JNK and decreased expression of inflammatory gene markers, resulting in improved adipose tissue insulin signaling84. Fitting with these results, it was
Figure 1.7 – Model of GPS2 anti-inflammatory action.

found in human patients there was an inverse correlation between GPS2 and SMRT levels and inflammatory status in obese adipose tissue and this effect was found to be reversible with bariatric surgery. Lack of GPS2 and SMRT in adipocytes led to increased pro-inflammatory gene expression and secretion of inflammatory cytokines. Surprisingly, the GPS2 TG mice with decreased inflammation did not have improved systemic metabolic parameters, which was thought to result from an increase in circulating resistin and increased lipid deposition in the liver.

In the case of macrophages, an inverse correlation exists between GPS2 levels in AT macrophages and markers of inflammation with hyperglycemia in adipose tissue. Macrophage specific GPS2 knockout mice exhibited a pro-inflammatory gene signature which was exacerbated when treated with TLR4 agonists. This was found in part to be mediated by GPS2 interaction with AP-1 (c-Jun) however the cytoplasmic function of GPS2 was not explored in this context. Upon high fat diet feeding, mice with GPS2 deficiency in macrophages were found to have increased AT and systemic inflammation, increased macrophage infiltration in adipose tissue with a pro-inflammatory phenotype. This resulted in increased liver lipid deposition, impaired glucose and insulin tolerance, and decreased phosphorylation and activation of AKT as a marker of insulin signaling. Conversely, overexpression of GPS2 in macrophages reversed this phenotype. Collectively these data point to GPS2 as a key mediator of macrophage inflammatory function, specifically in the context of diet-induced insulin resistance.

Most recently, we reported that GPS2 plays an important anti-inflammatory role in B cells as well. In this work, using B cell specific knockout of GPS2, it was found that
these mice had hyperactive TLR and BCR signaling pathways through the cytoplasmic function of GPS2 to inhibit Ubc13 activity (Figure 1.7B)\textsuperscript{85}. Loss of GPS2 regulation of these pathways resulted in significantly increased pro-inflammatory gene expression\textsuperscript{85}. GPS2 regulation of these pathways affected various stages of B cell development in which these pathways are critical in the development of various mature functional B cell populations\textsuperscript{85}. Overall, the combination of these data in various tissues clearly show that GPS2 has a fundamental role in the regulation of signal transduction pathways that are central to inflammatory processes.

\textit{GPS2 mechanism of action and regulation}

While studied in various signaling pathways, cellular locations and numerous processes, our lab has identified that there is one unifying function of GPS2 in all these various applications. GPS2 is able to endogenously inhibit the enzymatic activity of the E2 ubiquitin conjugating enzyme, Ubc13\textsuperscript{85}. Although it does not appear that GPS2 blocks ubiquitin charging nor mono- or di-ubiquitin formation, it seems as though GPS2 blocks the formation of polyubiquitin chains. This has been addressed by showing the addition of GPS2 blocks ubiquitin chain formation on many substrates in various immunoprecipitation and \textit{in vitro} ubiquitination assays\textsuperscript{84,85}. More thorough structural studies are necessary to understand the exact mechanism of how GPS2 can inhibit Ubc13.

Further studies in trying to understand how GPS2 itself is regulated have revealed that GPS2 activity is mediated by posttranslational modifications, protein interactions, localization and a balance between stabilization and proteasomal degradation. It was found that the N terminal domain of GPS2 was necessary for its targeting into the
Although an identified nuclear localization sequence of GPS2 was not found to interfere with nuclear import, this region of GPS2 was found to interact with the N terminus of TBL1. GPS2 interaction with TBL1 was found necessary for its nuclear localization as well as stabilization. Furthermore, it was found that GPS2 undergoes sumoylation at two distinct amino terminal sites, K45 and K71, by the small-ubiquitin-like modifier, SUMO-1, and this modification could be reversed by SENP1. Certain evidence suggested that sumoylation was necessary for GPS2 and TBL1 binding. However, more thorough studies found the mutation of these two sumoylation sites interrupted GPS2 and TBL1 binding not because of sumoylation per se but because these mutation affected the proper folding of GPS2. This was proven in studies in which knocking down the SUMO-conjugating enzyme, Ubc9, did not impair GPS2 and TBL1 binding.

The stabilization of GPS2 through its interaction with TBL1 significantly protected GPS2 from proteasomal degradation mediated by Siah. GPS2 was found to undergo polyubiquitination in its C terminus. Interestingly, the C terminal domain of GPS2 and the WD40 domain in the C terminal domain of TBL1 were found to interact. Various screening studies suggested that arginine residues of GPS2 underwent methylation and it was later discovered that these arginine residues flanked the sites of ubiquitination on GPS2. Specifically, it was observed that PRMT6 regulated methylation of GPS2 on arginine residues 312 and 323. This methylation ensured C terminal GPS2 and TBL1 binding, preventing proteasomal degradation of GPS2 by Siah. These data suggest that various posttranslational modifications of GPS2...
regulate protein activity, localization and stability, however more extensive studies are required to reveal the full program responsible for the modulation of GPS2.

**Significance and research goals**

Obesity has emerged as a worldwide health epidemic, particularly as a serious risk factor in the development of various metabolic syndromes, including T2D. More specifically, obesity-associated inflammation is widely recognized as a critical factor in the development of insulin resistance leading to T2D. However, anti-inflammatory drug therapies have proven unsuccessful in treating these metabolic syndromes. In addition, recent reports have shown that healthy AT expansion, which results in adequate lipid storage in this tissue and not in other peripheral organs, can abrogate the development of insulin resistance. Together, these observations raise the fundamental question of whether inhibiting inflammation is an appropriate approach in the attempt to develop therapeutics and treat insulin resistance or whether the focus for clinical therapies should concentration on hijacking mechanisms of healthy AT expansion for the prevention of this disease.

The research goal of this project was to understand the intersection as well as delineation between lipid metabolism and inflammation in AT under homeostatic and dietary stress conditions and how they may be modulated to alleviate insulin resistance. Since GPS2 has recently emerged as an critical, novel regulator in metabolic organs, we characterized the *in vivo* function of GPS2 using the adipocyte-specific GPS2 knockout mouse model to explore the critical role of GPS2 in regulating lipid metabolism and obesity-induced inflammation in AT. Based on previous data, we hypothesized that the
imbalance in the flux between fatty acid mobilization and utilization along with proper remodeling and expansion of AT, potentially through assistance from infiltrated immune cells, can result in an improved metabolic status of GPS2-AKO mice. Importantly, this hypothesis implicates an unexpected uncoupling between lipid metabolism and inflammation within AT under conditions of excessive nutrient load, thus suggesting that inhibiting inflammation in the context of obesity may not be the ideal strategy to ameliorate metabolic dysfunction. This project facilitates a strong approach to gain a better understanding of the casual relationship between obesity, inflammation, and insulin resistance.

In order to test our hypothesis, we first characterized the effect of GPS2 on the insulin signaling pathway and lipid storage capacity of white adipocytes. The goal of this part of the project was to elucidate the mechanisms underlying GPS2 regulation of lipid flux in adipocytes. We discovered that modulating GPS2 can affect the lipid storage capacity of adipocytes by modulating AKT activation affecting pathways involved in lipid storage and mobilization. Additionally, we illustrated the effects of GPS2 knockout on mitochondrial function and brown fat activity. Secondly, we aimed to understand the contribution of AT inflammation to the overall metabolic profile of GPS2 AKO mice. We revealed that despite increased obesity and AT inflammation, GPS2-AKO mice remained metabolically healthy. These data suggest that an uncoupling of inflammation with whole-body metabolic status may exist if continued healthy expansion to extend the maximum storage capacity of the adipose tissue can be accomplished.
This thesis project represents an innovative means to comprehensively investigate the complex relationship between insulin signaling regulated lipid flux and AT inflammation in the development of insulin resistance, with important implications for the discovery and design of effective therapeutic approaches against metabolic diseases, especially T2D.
CHAPTER TWO – MATERIALS AND METHODS

Animal studies

Fat-specific GPS2 knockout mice (GPS2-AKO) were generated using a cre/lox approach and maintained on a mixed 129sv/C57BL6J background. Conditional Gps2 floxed mice were generated by inGenious Targeting Laboratory. The 9.52 kb region used to construct the targeting vector was first sub cloned from a positively identified C57BL/6 (RP23: 91G16) BAC clone into a ~2.4kb backbone vector (pSP72, Promega) containing an ampicillin selection cassette. The total size of the targeting construct (including vector backbone and Neo cassette) is ~13.62kb. The region was designed such that the short homology arm (SA) extends about 2.55kb 3’ to exon 6. The long homology arm (LA) ends 5’ to exon 3 and is 6.07kb long. A pGK-gb2 loxP/FRT Neo cassette is inserted on the 3’ side of exon 6 and the single loxP site is inserted 5’ of exon 3. The target region is 0.90 kb and includes exon 3-6. The targeting vector was linearized by NotI and then transfected by electroporation of BA1 (C57BL/6x129/SvEv) hybrid embryonic stem cells. After selection with G418 antibiotic, surviving clones were expanded for PCR analysis to identify recombinant ES clones and control for retention of the third LoxP site. Secondary confirmation of positive clones was performed by Southern Blotting analysis prior to microinjection into C57BL/6 blastocysts. Resulting chimeras with a high percentage agouti coat color were mated to wild-type C57BL/6J mice to generate F1 heterozygous offspring. The Neo cassette was excised by crossing with FLP mice (Jackson Laboratories) to generate F2 heterozygous mice with Neo deletion in somatic cells and F3 heterozygous mice with Neo deletion in germ cells.
Adipose tissue specific deletion was achieved by crossing Gps2\(^{\text{flox/flox}}\) mice with heterozygous Adipoq-Cre C57BL/6J transgenic mice expressing Cre recombinase under control of the adiponectin promoter\(^{113}\). Male mice and littermate controls were used for all experiments. Mice were maintained on standard laboratory chow diet in temperature controlled facility on a 12-hour light/dark cycle.

Homozygous Gps2\(^{\text{flox/flox}}\) mice were then used for crossing with heterozygous 129sv CD19-cre mice\(^{114}\) (Jackson Laboratories) to create B cell-specific GPS2 knockout mice. The CD19 promoter specifically directs expression at the earliest stages and throughout B-lymphocyte development and differentiation\(^{114}\). Because the Cre cassette is inserted into exon 2 of CD19 gene, functionally disrupting the gene, homozygous CD19-cre mice are CD19-deficient. However, heterozygous mice are phenotypically normal and can be used for specific deletion of floxed targets in B-lymphocytes\(^{114}\). Wild-type mice used as control for all analyses presented here were littermates Gps2\(^{\text{flox/flox}}\)/CD19Cre\(^{-}\). All experiments are done with mice 10 to 16 wks old.

Genotypes of mice were confirmed by PCR for the two floxed GPS2 alleles and for the presence of CRE using the primers listed in Table 3.

Mice used for HFD studies were placed on 45% HFD (Research Diet) at 6-8 weeks of age. Body weights were measured weekly for the 16-week diet regiment or under chow conditions. Weight measurements were also taken after an overnight fast in chow mice 4-6 months of age and at time of sacrifice for all mice.
All animal studies were approved by the Boston University Institutional Animal Care and Use Committee (IACUC) and performed in strict accordance of NIH guidelines for animal care.

**Tissue fractionation, primary cell culturing and differentiation**

Tissue fractionation of primary mature adipocytes and the stromal vascular fraction (SVF) were isolated from adipose tissue depots using collagenase type I/dispase II (Fisher) digestion in 4% BSA (Sigma) Kreb-Ringer HEPES (KRH) (1.3M NaCl, 4.7 mM KCl, 1.24 mM MgSO4, 3.3 mM CaCl2-2H2O, with 1X HEPES/PHOS) buffer for 45 minutes, then filtered and adipocytes were separated by floating and washed 3 times in 1% BSA KRH buffer prior to resuspension in lysis buffer for protein extraction or Trizol for RNA analysis.

The SVF was spun down at 900 rpm for 10 minutes. Cells were cultured in high glucose DMEM with 10% fetal bovine serum (Hyclone) and 1X pen/strep until confluent. Two days after confluence, *in vitro* adipogenic differentiation was induced using a standard insulin (Sigma), IBMX (Sigma), and DEX (Sigma) cocktail for WAT and the same cocktail supplemented with 10 uM T3 for BAT depots for 12 additional days. Maintenance media was replenished every two days.

**Body composition analysis and metabolic testing**

Mice body composition was assessed by non-invasive MRI scanning on an EchoMRI 700 (BUSM Metabolic Phenotyping core). Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT) were performed according to established protocols. Mice were starved overnight or 4-6 hours for GTT and ITT, respectively. Blood glucose levels
were measured, at the described time points after glucose (Sigma) (1.5mg/g body weight) or insulin (Humulin R, Lilly) (0.5U/kg body weight) i.p. injection with an insulin syringe (BD), from tail nicking using a OneTouch Ultra meter. For HFD studies, GTT’s and ITT’s were performed at 14 weeks and 15 weeks of diet treatment, respectively.

**Plasma collection and assays and ELISAs**

Mice were fasted overnight and terminally anesthetized using 2-bromo-2-chloro-1,1,1-trifluoroethane (Sigma). 26 gauge syringes were coated with 0.1M EDTA and plasma was collected by cardiac puncture into tubes with 100 uL 0.1M EDTA. Samples were kept on ice then spun at maximum speed at RT for 5 minutes. Plasma fraction was removed and used for assays and ELISAs. MCP1, Adiponectin, Leptin, and Insulin were profiled using Milliplex Multiplex Assays (Millipore, BUSM Analytical Core). Additional plasma Insulin levels were measured using the Rat/Mouse Insulin ELISA kit (Millipore) according to manufacturer’s instructions. Plasma glycerol levels were measured using the Free Glycerol Determination Kit (Sigma) and Plasma FFA levels were measured using Free Fatty Acid Fluorometric Assay Kit (Cayman Chemical) following manufacturer’s instructions.

**Lipolysis assays**

*In vitro* differentiated cells were washed with 1X PBS (137 mM NaCl, 5.4 mM KCl, 16.2 mM Na2HPO4, 2.9 mM KH2PO4) then incubated with high glucose DMEM +2% BSA without serum for 2-3 hours. Cells were washed with phenol-free DMEM without serum then incubated in serum free phenol free DMEM +2%FFA- free BSA (Sigma) with or without 10uM isoproterenol (Sigma) for 1 hour. Media was collected for
measuring the released glycerol using the free glycerol reagent (Sigma). Glycerol concentrations were calculated from a standard curve and values normalized to protein concentration per well assessed by Bradford assay (Bio-Rad) as described above. Results shown include data points from three independent experiments, each time the assay was performed with technical triplicates.

**Staining**

Upon harvesting, adipose tissue depot and liver tissues were incubated at 4°C in Z-fix solution (Anatech LTD) overnight. Tissues were then transferred to 70% ethanol, paraffin embedded, sectioned, and stained with hematoxylin & eosin (Tuft Pathology Core/BNORC Adipose Biology Core). Imaging of adipocyte cell size was performed as described 116,117.

*In vitro* differentiated brown adipocytes were fixed in 10% formalin at room temperature for 30 minutes. Oil Red O (ORO) stock solution was prepared by adding 0.3G ORO powder (Alfa Aesar) in 100 mL isopropanol. Cells were washed 2X with 1XPBS then incubated in Oil Red O (ORO) working solution (3 parts ORO stock with 2 parts water and filtered through .2 uM filter) for 5 minutes. Stain was removed by washing with 60% isopropanol then with distilled water until excess stain was removed and visualized immediately by microscopy.

Immunofluorescence for macrophage staining was performed on fixed tissue sections. Sections were deparaffinized by washing 3X 5 minutes in xylene in a glass container. Slides were then washed as follows: 2X 5 minutes in 100% ethanol, 2X 5 minutes in 95% ethanol, 1X 5 minutes in 80% ethanol, 1X 5 minutes in 70% ethanol 1X
5 minutes in 50% ethanol, and 2X 5 minutes in dH2O. Slides were placed into a wash container with antigen retrieval buffer (10 mM sodium citrate, 0.05% Tween 20, pH6) and heated on full power in a microwave until boil began. Power was slowly reduced in microwave for 9 minutes and slides were cooled for 20 minutes at room temperature then washed 3X 5 minutes in water. Slides were drained and a hydrophobic barrier was created around each tissue section using an ImmEdge pen. Slides were washed with 1X PBS for 5 minutes then placed into a wash container with permeabilization buffer (0.2% Triton X-100 in PBS) for 45 minutes at room temperature. Slides were again washed with 1X PBS and blocked in blocking buffer (0.5% BSA, 1:1000 donkey serum in PBS) for 1 hour at room temperature in a humidified chamber. Slides were washed 3X for 5 minutes in 1X PBS and primary conjugated antibody (Alexa Fluor 647 anti-mouse Mac-2, Biolegend) was added and incubated overnight in a humidified chamber at 4C. Slides were washed 3X 5 minutes in 1X PBS then mounted with coverslips using mounting media containing DAPI. Slides were stored at 4C in the dark and then imaged by fluorescent microscopy.

**Cell culture and isolation**

3T3-L1 preadipocytes (American Type Culture Collection) were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) (Corning) with 10% fetal calf serum (Hyclone) under 5% CO2. Cell lines were trypsinized using 0.25% Trypsin (Corning) and split twice weekly prior to reaching confluence. Cells were serum starved overnight prior to 100nM insulin (Life Technologies) treatment for described time points.
**Transient Transfection**

Cells were transfected using Lipofectamine2000 (Invitrogen) and Opti-MEM reduced serum media (Thermo Fisher Scientific) according to each manufacturer’s instructions. The following siRNA’s were used: MISSION siRNA universal negative control (Sigma), siGPS2 (#s80309, 20nM, Ambion), siUBC13 (#s123784, 20nM, Ambion) with more detailed information found in Table 1.

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**Table 1. siRNA sequences.**

**Site-directed mutagenesis and AKT cell lines**

AKT plasmid constructs were kindly provided from the Guertin Lab and site-specific mutations were created using the QuikChange II XL Site-directed Mutagenesis Kit (Agilent) then shipped back to the Guertin Lab for reexpression in AKT null cell lines.

AKT null cells are UBC-Cre ERT2; AKT1 fl/fl, AKT2 fl/fl in an AKT3 KO background. To generate this line, primary brown adipocyte precursors (bAPC) cells were isolated from P1 neonates and immortalized with pBabe-SV40 Large T antigen. To induce AKT1 and AKT2 deletion UBC-Cre ERT2; AKT1 fl/fl, AKT2 fl/fl cells were treated on 2 consecutive days with 1uM 4-hydroxy tamoxifen and on the 3rd day the media was changed to regular media to allow protein turnover. On the 4th day, cells were plated during the morning at 80% confluence, serum removed later in the day for overnight starvation prior to 15’ insulin treatment (150nM) and protein extraction.
**Protein isolation, immunoprecipitation and western blot analysis**

Whole cell extracts were prepared from cultured cells by homogenization in lysis buffer (50 mM Tris HCl pH 8, 250 mM NaCl, 5 mM EDTA, 0.5% NP-40) supplemented with 0.1mM PMSF, 10mM NEM (Thermo fisher scientific), 1X protease inhibitors (Pierce) and 1X phosphatase inhibitors (Pierce). Cells were resuspended in lysis buffer for 20 minutes on ice then spun at maximum speed for 10 minutes at 4C. Supernatant was then removed. WCE from tissue were homogenized using 1 mm ZrOx beads in lysis buffer (50mM Tris-HCl pH8, 300 mM NaCl, 5 mM EDTA, 0.1mM PMSF, 10 mM NEM, 1X protease inhibitor cocktail, 1X phosphatase inhibitor cocktail) in the Bullet Blender (Next Advance, Inc) at setting 9 for 5 minutes. Homogenates were then spun 2X 5 minutes at 1000 RPM at 4C to remove lipids then rotated for 10 minutes in lysis buffer supplemented with 2% Triton. Samples were spun at maximum speed for 10 minutes at 4C and the supernatant was collected. All protein concentrations were analyzed using the Bradford assay (Bio-Rad) to a BSA standard curve and normalized for protein loading.

Immunoprecipitation were performed on whole cell lysates upon incubation of indicated antibodies overnight followed by collection of immune complexes with 50 uL Protein A-sepharose 4B conjugate (Thermo fisher scientific). Beads were precipitated by centrifugation at 1000 RPM for 1 minute and washed 3X with IPH buffer (50 mM Tris (pH 7.4), 300 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) and eluted in NuPAGE LDS sample buffer (Life Technologies) substituted with 100 mM DTT for analysis.

Extracts were boiled in NuPAGE LDS sample buffer for 10 minutes and loaded in 10% Mini-PROTEAN TGX gels (Bio-Rad) in SDS-PAGE running buffer (25 mM Tris,
192 mM glycine, 0.1% SDS (Boston Bioproducts) at 180 V for 1 hour. Proteins were immobilized onto PVDF membranes (Millipore) by transferring in 1X transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol) at 100 V for 45 minutes. Membranes were then blocked in 5% BSA (Fisher) diluted in PBS-T (137 mM NaCl, 5.4 mM KCl, 16.2 mM Na2HPO4, 2.9 mM KH2PO4, 0.1% Tween-20) for 1 hour at room temperature. Membranes were incubated in primary antibody overnight at 4°C. The following day membranes were washed 3 times 10 minutes in PBS-T. Membranes in unconjugated primary antibodies were incubated in secondary antibody for 1 hour at room temperature, washed again 3 times 10 minutes in TBS-T and immediately visualized. All blots were visualized using Clarity Western ECL Substrate (Bio-Rad), exposed for 1 to 500 seconds, and captured on Chemi DocTM XRS+ imaging station (Bio-Rad) using Image Lab software (Bio-Rad). Expressed proteins were compared to Precision Plus Protein Standard protein ladder (Bio-Rad) to determine size. All antibodies are outlined in Table 2.

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Anti-GPS2-N antibody was generated in rabbit against a specific peptide representing aa 1-11.

**RNA isolation and gene expression analysis**

Total RNA was extracted from flash frozen tissue. Tissue was homogenized in 1 mL Trizol reagent (Sigma) with RNase free 1 mm zirconium oxide beads using a Bullet Blender. Samples were spun at 12000 X g for 10 minutes at 4C. Supernatant was added to a fresh tube and 200 uL chloroform was added. Samples were shaken by hand for 15 seconds then centrifuged at 12000 X g for 15 minutes at 4C. The aqueous phase was removed in then total RNA was purified using RNeasy plus mini columns (Qiagen).

Total RNA extracted from cultured cells or primary adipocytes was purified using RNeasy plus mini columns (Qiagen). 1ug of RNA was reverse transcribed using the
iScript cDNA Synthesis System (Bio-Rad) according to manufacturer’s instructions.

Quantitative PCR was performed using Fast SYBR Master Mix (Applied Biosystems) and measured on the ViiA 7 real time PCR system (Applied Biosystems). Transcript levels were analyzed using ΔΔCT method and normalized to the housekeeping gene, Cyclophilin A. Primer sequences are outlined in Table 3. All experiments were performed in triplicate and statistical significance was calculated by two-tailed Student’s t test.

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TFAM | Mouse | RT-qPCR | CTGCACTCTGCCCATCCAAA | CTGAGCATTCGCAGGCCTTT

Table 3. Primer sequences.

Chromatin Immunoprecipitations

All cells were cross-linked in 1% formaldehyde then washed with ice cold 1X PBS containing 1X protease inhibitor cocktail. Hypotonic lysis was performed by adding 500uL hypotonic lysis buffer ((10mM HEPES, 1.5 mM MgCl2, 10mM KCl, .5 mM DTT, .2mM PMSF, and 1X protease inhibitor cocktail), incubating on ice for 20 minutes and spun at maximum speed for 10 minutes. The supernatant was discarded. Cell pellet was incubated in 100uL per IP of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCL pH 8, 1X protease inhibitor cocktail) for minutes on ice then sonicated 2X 10 minutes at maximum power in a Biorupter (Diagenode) sonicator. Debris was removed by centrifugation for 10 minutes at 13,000 RPM at 4C in special thin plastic tubes. The supernatant was diluted 10-fold in ChIP dilution buffer (.01% SDS, 1.1% Triton, 1.2 mM EDTA, 16.7 mM Tris-HCL pH8, 167 mM NaCl, 1X protease inhibitor cocktail). 50 uL of chromatin solution was set aside as input. Antibodies as described in Table was added to 1 mL of chromatin solution and incubated overnight at 4C with rotation. 1 mL of chromatin solution without antibody was used for IgG control. Immune complexes were collected using 50 uL of Protein A agarose slurry for one hour at 4C with rotation. Beads were pelleted for 1 minute at 1000 RPM at 4C then washed in Centrifugal Filters (Millipore) for 3 minutes at 5000 RPM with 500 uL of each subsequent listed buffer: Low salt wash buffer (.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris-HCl pH8, 150 mM NaCl), High salt wash buffer ((.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris-HCl
pH8, 500 mM NaCl), Lithium Chloride buffer (.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-Hcl pH8) and TE buffer (1 mM EDTA, 10 mM Tris-HCl pH8). Immune complexes were eluted by adding 300 uL of elution buffer (1% SDS, 0.1 M NaHCO3) to pelleted beads then put on the shaker and incubated at RT for 30 minutes. Beads were spun down and the supernatant was kept. Cross-links were reversed at 65C overnight. 250 uL elution buffer was added to inputs and cross-links were reversed at 65C overnight. DNA was recovered with phenol/chloroform extraction by adding 300 uL phenol:chloroform: IAA vortexed for 10 seconds, then spun at 4C at maximum speed for 15 minutes. Supernatant was removed and 3 uL of glycogen was added and incubated for 1 minute. 30 uL Na acetate (3M) was added and incubated for 1 minute. 750 mL of cold 100% ethanol was added and samples were placed in -80C for 2 hours. Samples were spun down at maximum speed for 20 minutes, washed with 1 mL 70% ethanol and pellets were dried overnight at RT on the bench. DNA pellets were then resuspended in water and used for qPCR reactions.

**Genomic DNA isolation, mitochondria content and PCR**

To perform mouse genotyping, genomic DNA was isolated from cut tails by adding 1 mL 50 mM Sodium hydroxide (NaOH). This was heated for 10 minutes at 100C then neutralized with 80 uL 1mM Tris-HCL, pH 8. 2uL was added to the PCR reaction. DNA was extracted from BAT following manufacturer’s instructions of the BuccalAmp DNA Extraction Kit (Epicentre). DNA amplification of the mitochondrial-encoded NADH dehydrogenase 1 (mt-ND1) relative to nuclear TFAM was used to determine mitochondrial DNA copy numbers using primers listed in Table 3.
PCR reactions were performed using Fermentas DreamTaq Green PCR Master Mix (Thermo Scientific) according to manufacturer’s guidelines on Veriti thermos cycler (ABI) machine. Reactions were run on 1.5% agarose (Bio-Rad) gels in 1X TBE buffer (0.9 M Tris pH 8, 28 mM Na2EDTA, 1.12 M Boric acid) containing 1X GreenGlo Safe DNA Dye (Denville), visualized on Chemi DocTM XRS+ imaging station (Bio-Rad) using Image Lab software (Bio-Rad) and products were compared to exACTGene 1kb DNA ladder (Fisher) ladder to determine size.

**Mitochondria isolation, content and Seahorse**

Brown Adipose Tissue was isolated from GPS2-WT and KO mouse and weighed. Isolated BAT was rinsed and minced in ice-cold PBS then homogenized in a glass-teflon dounce homogenizer containing SHE pH=7.2 (250 mM Sucrose, 5mM HEPES, 2 mM EGTA, BSA 2%) + BSA buffer. After 9-10 strokes through the teflon pestle the homogenate was centrifuged at 900xg for 10 min at 4°C. The resulting supernatant was then centrifuged at 9000xg for 10 min at 4°C and the pellet was washed once and then re-suspended in SHE without BSA. Protein content was measured by BCA assay (Bio-Rad).

For mitochondrial activity, 4µg of mitochondrial protein fractions were loaded per well for complex I driven respiration (pyruvate+malate) and 2µg for complex II-driven respiration (succinate+rotenone) in 25 µl of Mitochondrial Assay Solution pH 7.2 (MAS: 100mM KCl, 10mM KH2PO4, 2mM MgCl2, 5mM HEPES, 1mM EGTA, 0.1% BSA and 1mM GDP) per Seahorse XF96 well. The plate was centrifuged at 4°C, 5 min at 3400 rpm. Then, 110 µl of MAS with the respective fuels were carefully added per well. Plate was warmed at 37°C for 4 min then oxygen consumption was measured at 37°C.
using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA). Mitochondrial stress test compounds (10 µM oligomycin, 2.5 µM FCCP and 10 µM antimycin A) were injected through ports A, B, and C, respectively, to measure mitochondrial respiration linked to ATP synthesis, leak, and maximal respiratory capacity according to the manufacturer’s instructions. Pyruvate was used at 5mM, malate 5mM, succinate 5mM and rotenone 2µM. ADP was injected at port A (3.5mM), Oligomycin A at port B (3.5µM), FCCP at port C (4µM) and Antimycin A at port D (4µM).

**Isolation of B cells**

Bone marrow cells were flushed from femoral bone by flush, peritoneal cavity cells were isolated by lavage of the peritoneal cavity and splenic B cells were isolated from single cells suspension of total splenocytes prepared according to standard protocols. B cell purification was achieved by magnetic depletion using the MACS Pan B cells Isolation Kit (Miltenyi Biotech). Purity of the B cells populations was verified by flow cytometric analysis with CD19, CD11b and CD3 labeling.

**FACS analysis**

SVF cells were purified as described above. Flow cytometry analyses were performed using the following anti-mouse antibodies: PerCP anti CD11b (Biolegend), FITC antiF4/80 (BioRAD); Single cell suspensions were stained with Aqua Zombie dye, washed, pre-blocked with mouse FcBlock (Biolegend), and stained with antibody cocktails in the presence of Brilliant Violet buffer (BD Biosciences). Ultracomp beads (eBioscience) stained with abovementioned antibodies and ArC beads (Life Technologies) stained with Aqua Zombie dye were used for compensation in
FACSDIVA. All data were acquired on a SORP LSRII (BD Biosciences). At least 20,000 events were collected from each sample. All manual analysis was performed by a blinded investigator using FlowJo 10. Results presented are from three independent experiments, with statistical significance calculated by two-tailed Student’s t test.
CHAPTER THREE – SYSTEMIC INSULIN SENSITIVITY IS REGULATED BY
GPS2 THROUGH INHIBITION OF AKT UBIQUITINATION AND
ACTIVATION IN ADIPOSE TISSUE

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This chapter is also adapted from: Lentucci, C., Belkina, A.*, Cederquist, C.T.*, et al. (2017). Inhibition of Ubc13-mediated ubiquitination by GPS2 regulates multiple stages of B cell development. J Biol Chem, online Dec 30. 85

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Abstract
Insulin signaling plays a unique role in the regulation of energy homeostasis and the impairment of insulin action is associated with altered lipid metabolism, obesity, and Type 2 Diabetes. The main aim of this study was to provide further insight into the regulatory mechanisms governing the insulin signaling pathway by investigating the role of non-proteolytic ubiquitination in insulin-mediated activation of AKT. The molecular mechanism of AKT regulation through ubiquitination is first dissected in vitro in 3T3-L1 preadipocytes and then validated in vivo using mice with adipo-specific deletion of GPS2, an endogenous inhibitor of Ubc13 activity (GPS2-AKO mice). Our results indicate that K63 ubiquitination is a critical component of AKT activation in the insulin signaling pathway and that counter-regulation of this step occurs by GPS2 preventing AKT ubiquitination through inhibition of Ubc13 enzymatic activity. Removal of this
negative checkpoint, through GPS2 downregulation or genetic deletion, results in sustained activation of insulin signaling both in vitro and in vivo. As a result, the balance between lipid accumulation and utilization is shifted toward storage in the adipose tissue and GPS2-AKO mice become obese under a normal laboratory chow diet. However, the adipose tissue of GPS2-AKO mice is not inflamed, the levels of circulating adiponectin are elevated and systemic insulin sensitivity is overall improved. My findings characterize a novel layer of regulation of the insulin signaling pathway based on non-proteolytic ubiquitination of AKT and define GPS2 as a previously unrecognized component of the insulin signaling cascade. In accordance with this role, we have shown that GPS2 presence in the adipocytes modulates systemic metabolism by restricting the activation of insulin signaling during the fasted state, whereas in absence of GPS2, the adipose tissue is more efficient at storing lipids and obesity becomes uncoupled from inflammation and insulin resistance.

Introduction

The adipose tissue is an extremely flexible organ that plays a critical role in the regulation of energy homeostasis through both triglyceride storage and adipokine secretion\(^9,11^9\). The health of the white adipose tissue, and thus of the whole body, largely depends on the existence of a proper balance between lipid storage and utilization, which is challenged under conditions of excess food intake. Major pathological consequences of overnutrition and obesity result from an increase in lipid flux to non-adipose organs and the development of insulin resistance\(^57,12^0\). During the normal cycles of daily fasting and feeding, adipose tissue metabolism is tightly regulated to respond to the energetic
demands of the organism. Insulin signaling plays a key role in this process by promoting nutrient storage during the fed state via enhanced glucose uptake and triglyceride synthesis, and by inhibiting triglyceride breakdown and fatty acid release through lipolysis \(^{121,122}\). The AKT/PKB kinase is an obligatory mediator of the insulin signaling pathway downstream of phosphatidylinositol 3-kinase (PI3K) \(^{24,123}\). Full AKT activation is promoted by dual phosphorylation by PDK1, a PI3K-dependent kinase, and the mTOR-rictor complex. The key rate-limiting event for AKT activation is not the phosphorylation per se, but rather its translocation to the plasma membrane. Accordingly, full signaling capacity is achieved by tethering AKT to the plasma membrane \(^{124-127}\).

Intriguingly, recent evidence indicates that AKT recruitment to the membrane, and thus activation, upon stimulation with the growth factors EGF and IGF is unexpectedly regulated through K63 ubiquitination \(^{36,40,128}\). However, it is currently unknown whether AKT ubiquitination similarly contributes to the regulation of metabolic homeostasis through modulation of insulin signaling.

Ubiquitination is a reversible modification that is achieved via the sequential actions of several classes of enzymes, including an ubiquitin (Ub)-activating enzyme (E1), an Ub-conjugating enzyme (E2), and an Ub ligase (E3) \(^{129,130}\). Poly-ubiquitination of target proteins with chains of different topology can promote either protein degradation or serve, as in the case of other post-translational modifications, to influence protein function and interactions \(^{73,131}\). The key E2 enzyme for promoting the formation of non-proteolytic, K63 ubiquitin chains is Ubc13, which catalyzes the synthesis of ubiquitin chains in complex with the non-catalytic subunits Mms2/Uev1A and specific
E3 ligases \(^{132-134}\). Consistent with the flexibility of ubiquitination as a tight regulatory switch for signaling pathways is the existence of multiple strategies to rapidly reverse the active mark. Among them, best characterized is the removal of ubiquitin modifications by chain specific deubiquitinases \(^{129,135-137}\), as reported in the case of AKT deubiquitination by CYLD \(^{42,138}\). However, an equally, if not more effective strategy is restricting the deposition of the ubiquitin chains through inhibition of the ubiquitination machinery. Examples of inhibitors behaving in this manner are the ubiquitin thioesterase OTUB1 in the DNA damage response pathway and G-protein Pathway Suppressor 2 (GPS2) in the TNFR1 signaling pathway \(^{84,139}\).

GPS2 is a small multifunctional protein that was originally identified while screening for suppressors of Ras activation in the yeast pheromone response pathway \(^{81}\). Recent studies by our lab and others indicate that GPS2 plays an important anti-inflammatory role in adipose tissue and macrophages and is required for the expression of genes regulating cholesterol and triglyceride metabolism \(^{79,84,89,91,97}\). Due to multiple functional interactions existing between GPS2 and various transcriptional regulators, GPS2 activity has been mainly studied in the context of its nuclear functions, including both transcriptional repression and activation \(^{79,84,87-90,92,93,95,97,98,105}\). However, GPS2 also plays an important non-transcriptional role in the cytosol by regulating JNK activation downstream of TNFR1 \(^{84}\). Intriguingly, our findings reveal that GPS2 activity in different cellular compartments relies on a conserved regulatory strategy based on the inhibition of ubiquitin conjugating complexes that are responsible for the formation of K63 ubiquitin chains (TRAF2/Ubc13 in the cytosol and RNF8/Ubc13 in the nucleus) \(^{79,84}\). Furthermore,
recent data indicate that GPS2 directly inhibits Ubc13 enzymatic activity, suggesting that GPS2-mediated regulation might extend to other signaling pathways relying on Ubc13-mediated ubiquitination events. Here, we have investigated the hypothesis that GPS2 is required for restricting the activation of the insulin signaling pathway through inhibition of Ubc13-mediated ubiquitination of AKT.

**Results**

*AKT activation in the insulin signaling cascade is regulated by ubiquitination*

Non-proteolytic ubiquitination of AKT has been recently described as a key step in the activation cascades downstream of EGF and IGF receptor stimulation. Despite some differences in the enzymatic machineries that are enlisted in the two different pathways, in both cases, K63 ubiquitination plays a critical role in promoting AKT recruitment to the plasma membrane. Membrane recruitment of AKT through anchoring to phosphoinositol-3,4,5-triphosphate (PIP3) is also a limiting step in the insulin signaling pathway. However, it is currently unknown whether ubiquitination is required for the activation of AKT upon insulin stimulation. To address this question, we first asked whether AKT is post-translationally modified by the addition of K63 ubiquitin chains upon insulin stimulation of 3T3-L1 preadipocytes. As shown in Figure 3.1A, we observed that AKT undergoes K63 ubiquitination within minutes of insulin stimulation. The peak of ubiquitination slightly precedes AKT maximal phosphorylation by the phosphoinositide-dependent protein kinase 1 (PDK1) (Figure 3.1A), in accord with the proposed function. Next, we addressed whether ubiquitination occurs on the same sites described in the context of EGF/IGF signaling, and whether this
Figure 3.1 – AKT K63 ubiquitination and activation is regulated by the ubiquitin conjugating enzyme Ubc13.

(A) Detection of K63 ubiquitination by IP/WB of AKT in 3T3-L1 preadipocytes serum starved overnight prior to stimulation with 100 nM insulin. Phosphorylation of AKT on Thr308 is measured in parallel on whole cell extracts from the same cells and normalized to total AKT levels. (B) Inducible AKT1 and AKT2 deletion upon 4-hydroxy tamoxifen treatment (4OH-Tam) is rescued by transient overexpression of AKT2 WT or ubiquitination mutants. Protein expression levels and insulin mediated activation of AKT2 and downstream effectors PRAS40 and ribosomal protein S6 are assayed by WB in whole cell extracts. (C) WB analysis of phospho-AKT/total AKT on whole cell extracts from siCTRL versus siUBC13 transiently transfected 3T3-L1 preadipocytes. Cells were stimulated with insulin (5’) after overnight serum starvation. All western blots are representative images of at least three independent experiments.
modification is required for activation of the insulin pathway. To answer these questions, we reconstituted AKT1/2/3 null cells, generated from primary brown adipocyte precursors, with either wild type HA-AKT2 or HA-AKT2 mutants in which we had disrupted either Lys8 (AKT2-K8R), Lys14 (AKT2-K14R), or both (AKT2-K8R/K14R) by targeted mutagenesis. Their overexpression in AKT null cells revealed that the single mutants were still partially phosphorylated upon insulin stimulation, whereas removal of both ubiquitination sites resulted in a complete loss of AKT phosphorylation (Figure 3.1B). The activation of downstream AKT targets, such as PRAS40 and ribosomal protein S6, was also inhibited in cells reconstituted with the double mutant (Figure 3.1B). Together, these results indicate that AKT ubiquitination is a required step for full activation of the insulin pathway. To further confirm this conclusion and explore which ubiquitination machinery mediates AKT ubiquitination within the insulin pathway, we downregulated the E2 conjugating enzyme Ubc13 responsible for the synthesis of K63 ubiquitin chains by transient siRNA transfection. Ubc13, in concert with specific E3 ligases, was previously found responsible for mediating the ubiquitination, and thus activation, of AKT upon stimulation of IGF, EGF and ErbB receptors. In accord with these results, we observed that basal activation of AKT, albeit already very low, is reduced upon Ubc13 downregulation. In addition, our results indicate that disrupting AKT ubiquitination through downregulation of Ubc13 significantly impaired insulin-independent phosphorylation of AKT (Figure 3.1C Figure 3.3A). Together our data indicate that AKT is K63 ubiquitinated upon insulin stimulation and that Ubc13-mediated K63 ubiquitination is required for full activation of AKT and its downstream targets,
Figure 3.2 – AKT K63 ubiquitination and activation is regulated by GPS2, the negative regulator of the ubiquitin conjugating enzyme Ubc13.

(A) WB analysis of phospho-AKT/total AKT and phospho-GSK3B/total GSK3B in cytosolic cell extracts from proliferating 3T3-L1 preadipocytes transiently transfected with siCTRL (WT) or siGPS2 (GPS2-KD). (B) WB analysis of phospho-AKT/total AKT in whole cell extracts from 293T cells. (C) WB analysis of phospho-AKT/total AKT in whole cell extracts from WT and GPS2-KD 3T3-L1 preadipocytes starved overnight prior to insulin stimulation (3’). All western blots are representative images of at least three independent experiments.
hence raising the interesting question of whether AKT ubiquitination can be modulated to regulate insulin signaling.

Previous studies from our lab show that GPS2 negatively regulates different cellular functions through inhibition of K63 ubiquitination events. Recent work has revealed that this effect is achieved through direct inhibition of Ubc13 enzymatic activity. Because our data indicate that Ubc13-mediated ubiquitination is required for AKT phosphorylation and activation, we then asked whether GPS2 negatively regulated the activation of the insulin pathway. To investigate this hypothesis, we monitored AKT activation in 3T3-L1 preadipocytes upon modulation of GPS2 expression. First, we downregulated GPS2 by transient siRNA transfection in 3T3-L1 preadipocytes (GPS2-KD). A significant increase in the basal level of AKT phosphorylation was observed upon GPS2 downregulation, indicating that GPS2 is required for restricting the constitutive activation of AKT in proliferating cells under basal conditions. Similar results were observed in human 293T cells, suggesting that GPS2-mediated regulation of AKT is conserved among different cell types. Next, to specifically investigate the relevance of GPS2-mediated inhibition in the context of insulin signaling, we starved GPS2 KD cells and subjected them to insulin stimulation. Again, AKT activation was enhanced in GPS2-KD cells.

Together, these results reveal that Ubc13-mediated K63 ubiquitination of AKT represents a novel, key regulatory node of the insulin signaling pathway. GPS2, by virtue of its ability to inhibit Ubc13 enzymatic activity, emerges as a novel regulator of the
Figure 3.3 – Efficient knockdown of Ubc13 and GPS2 is achieved in cell lines.

(A) siRNA validation by RT-qPCR of transiently transfected 3t3-L1 cells comparing siCTRL versus siUBC13 treated cells. Results are expressed as mean-/+SEM from 3 independent experiments and statistical significance was calculated comparing the 3 independent experiments. (B) siRNA validation by RT-qPCR of transiently transfected 3t3-L1 cells comparing siCTRL versus siGPS2 treated cells. Results are expressed as mean-/+SEM from 3 independent experiments and statistical significance was calculated comparing the 3 independent experiments. Statistical significance was calculated by two-tailed Student’s t test; * represents p value <0.05 and ** p value < 0.01.
insulin pathway required for restricting the activation of AKT and downstream effectors both in presence and absence of stimulation.

**Constitutive AKT ubiquitination and activation in GPS2-deficient adipocytes**

Properly functioning insulin signaling is especially important in adipocytes to maintain cell homeostasis, and allow for appropriate responses to metabolic cues. Based on our findings of GPS2 acting as a negative regulator of AKT activation, we hypothesized that GPS2 would play an important physiologic role in adipose tissue. To investigate the physiologic relevance of GPS2 in regulating insulin signaling in the adipose tissue, we generated an adipo-specific knockout mouse model (GPS2-AKO) by crossing GPS2^{fl/fl} mice, carrying LoxP sites flanking exons 3-6, with Adipo-Cre mice (Jackson laboratories). The efficiency of this deletion strategy was confirmed by genomic PCR, western blotting and gene expression analysis (Figure 3.4A-D). Together, these experiments confirmed that GPS2 deletion is specific to mature adipocytes within the adipose tissue. Then, we investigated in *in vitro* differentiated adipocytes from the stromal vascular fraction (SVF) of either wild type (WT) or mutant (GPS2-AKO) mice the hypothesis that loss of GPS2 results in increased ubiquitination and activation of AKT. The efficiency of differentiation was similar for cells of both genotype (Figure 3.5). However, when assaying the level of AKT ubiquitination by IP/WB using an antibody specific against K63 ubiquitin chains for detection, we found a striking increase in AKT-associated ubiquitin chains in GPS2-deficient adipocytes compared to their wildtype littermates (Figure 3.6A). The increase in ubiquitination was associated with enhanced basal phosphorylation on both Ser473 and Thr308 both in absence of
Figure 3.4 – Adipocyte-specific GPS2 Knockout mice show specific deletion for GPS2 only in adipose tissue.

(A) Confirmation of GPS2 deletion in the adipose tissue of GPS2-AKO mice by genomic DNA analysis. (B) RT-qPCR analysis of GPS2 mRNA expression in white epididymal (EPI), subcutaneous (SC), and brown (BAT) adipose tissue depots and liver (LIV) from AKO versus WT mice, n=6-7. (C) GPS2 mRNA expression in primary adipocytes isolated from white fat depots, n=3-4. (D) Confirmation of protein knockout by WB in extracts from WAT, BAT, and LIV tissues. Results are expressed as mean +/-SEM. Statistical significance was calculated by two-tailed Student’s t test; * represents p value < 0.05 and **p value < 0.01. All western blots are representative images of at least three independent experiments.
Figure 3.5 – No differences are observed in differentiation capacity in WT vs GPS2-AKO.

RT-qPCR analysis of gene markers of adipogenesis in in vitro differentiated adipocytes from WT versus AKO mice at specified time points. Results are expressed as mean +/-SEM. Statistical significance was calculated by two-tailed Student’s t test; * represents p value <0.05 and ** p value < 0.01.
Figure 3.6 – In vitro differentiated adipocytes from GPS2-AKO mice display hyperubiquitination and increased phosphorylation of AKT.

(A) IP/WB analysis of AKT ubiquitination with K63 ubiquitin chains in in vitro differentiated adipocytes from AKO versus WT mice. (B) WB analysis of phospho-AKT/AKT in whole cell extracts from in vitro differentiated adipocytes upon insulin stimulation (3’) after overnight serum starvation. All western blots are representative images of at least three independent experiments.
stimulation and upon short-term insulin treatment (Figure 3.6B), confirming that AKT ubiquitination and activation are strongly enhanced in cultured adipocytes depleted of GPS2.

Next, we confirmed that insulin-dependent stimulation of AKT is affected by GPS2 deletion in vivo by monitoring AKT activation in adipose tissue under fasted and insulin stimulated conditions. Strikingly, we observed a significant increase in basal phosphorylation of AKT in both the whole subcutaneous adipose tissue depot and in mature adipocytes isolated from the epididymal adipose tissue depot of GPS2-AKO mice following overnight fasting (Figure 3.7A). In the same conditions, removal of GPS2-mediated inhibition of Ubc13 activity in adipocytes also led to increased activation of JNK (Figure 3.7B), as expected based on previous findings. In addition, we observed a significant increase in the level of AKT phosphorylation and activation in the subcutaneous adipose tissue when mice were injected with insulin for a short time prior to sacrifice (Figure 3.7C). Enhanced activation of insulin signaling pathways downstream of AKT in the adipose tissue from GPS2-AKO mice was confirmed by augmented phosphorylation of AKT target substrate GSK3β (Figure 3.7C). Therefore, our results together indicate that GPS2 deletion promotes sustained basal and insulin-stimulated phosphorylation and activation of AKT, and downstream effectors of the insulin signaling pathway, in vivo in the adipose tissue.
Altered regulation of lipolysis and lipogenesis in GPS2-AKO mice cause adipocyte hypertrophy and excessive body adiposity

A tight regulation of insulin action in adipose tissue is required for the proper modulation of nutrient storage and mobilization throughout the daily cycles of fasting and feeding \(^{24,142,143}\). Our in vitro and in vivo results combined suggest that removal of GPS2-mediated regulation is sufficient to promote aberrant activation of the insulin signaling pathway through constitutive phosphorylation and activation of AKT. Thus, we asked whether the fat-specific deletion of GPS2 affects nutrient storage, lipid fluxes and whole body metabolism in vivo. First, we observed that GPS2-AKO mice were healthy and fertile, but could be easily distinguished from their wild type (WT) littermates based on their larger size (Figure 3.8A). The difference in body weight was not apparent at birth but increased gradually with time (Figure 3.8B) and became significant once mice reached adulthood (Figure 3.8C). Echo-MRI scans of these mice confirmed that the increase in body mass was attributed to an increase in fat mass, without significant changes in lean mass (Figure 3.9A). Gross appearance revealed that all adipose tissue depots appeared larger when comparing GPS2-AKO to WT littermates (Figure 3.9B). Weights of individual white fat depots were significantly higher for GPS2-AKO mice (Figure 3.9C). The interscapular brown adipose tissue (BAT) also tended to be larger (Figure 3.9C). And, the enhanced adiposity correlated with a substantial increase in the size of adipocytes (hypertrophy) within both the SC and EPI depots (Figure 3.9D). In conclusion, these data indicate that GPS2-depleted adipocytes expand more than their
Figure 3.7 – Primary adipocytes and adipose tissue from GPS2-AKO mice show increased phosphorylation of AKT under various conditions and increased phosphorylation of JNK is observed in GPS2-AKO adipocytes.

(A) WB analysis of phospho-AKT/total AKT on whole cell extracts from WAT tissue and primary adipocytes isolated from the EPI tissue after overnight fast. (B) WB analysis of phospho-JNK/total JNK on whole cell extracts from primary adipocytes. (C) WB analysis of phospho-AKT/total AKT and phospho-GSK3B/total GSK3B in whole cell extracts from WAT after i.p. insulin injection (0.5 U/kg body weight). All western blots are representative images of at least three independent experiments.
Figure 3.8 – GPS2-AKO mice have increased total body weight compared to WT mice.

(A) Representative image of whole body morphology of adult WT and GPS2-AKO mice. (B) Progressive body weight gain in AKO versus WT mice, WT n=5-9, KO n=7-11. (C) Total body weight of 4-6 months old mice, n=5. Results are expressed as mean +/-SEM. Statistical significance was calculated by two-tailed Student’s t test; *represents p value < 0.05 and **p value < 0.01.
wild type counterparts, resulting in elevated adipose tissue mass and larger body weight, even when mice are fed a normal chow diet.

Adipocytes are the primary site of energy storage. The balance between lipid storage in the form of triglycerides and mobilization as released free fatty acids is directly regulated by insulin through AKT-dependent as well as AKT-independent pathways. In this context, a major physiological function of insulin in the postprandial, anabolic state is to support the accumulation of lipid storage by promoting de novo lipogenesis while restraining lipid mobilization via lipolysis. Based on the enhanced AKT activation we observed in the adipose tissue of both fasted and insulin-stimulated GPS2-AKO mice, we asked whether an impairment of the regulated cycles of lipogenesis and lipolysis was underlying the increased adipocyte cell size and body adiposity of these mice.

Our previous work showed that GPS2 overexpression in aP2-GPS2 transgenic mice promoted a strong increase in the phosphorylation, and thus activation, of hormone sensitive lipase, HSL. Here, we found that GPS2 deletion promoted the opposite phenotype. In isolated adipocytes from fasted GPS2-AKO mice, HSL phosphorylation was completely blunted in contrast to the strong signal observed in adipocytes from WT littermates (Figure 3.10A). Similar results were observed upon isoproterenol stimulation of in vitro differentiated adipocytes from WT or GPS2-AKO mice, with the induced HSL phosphorylation being severely reduced in absence of GPS2 (Figure 3.10B). In agreement with GPS2 being required for the priming of HSL and ATGL promoters only during the early stages of adipocyte differentiation prior to the expression of
Figure 3.9 – Increased fat mass and adipocyte cell size is observed in GPS2-AKO mice.

(A) Body composition analysis of total fat versus lean mass measured by EchoMRI scanning of WT and AKO mice at 4-6 months old, n=5. (B) Representative images of gross appearance of EPI, SC, and BAT depots in AKO versus WT mice at 4-6 months old. (C) Individual adipose tissue depot and liver weights in AKO versus WT mice at 4-6 months old, n =9-11. (D) Representative H&E staining of paraffin-embedded EPI and SC sections imaged at 10X magnification. Results are expressed as mean +/-SEM. Statistical significance was calculated by two-tailed Student’s t test; *represents p value < 0.05 and **p value < 0.01.
Figure 3.10 – Adipocytes from GPS2-AKO mice have impaired phosphorylation of HSL, as a marker of lipolysis, despite trends of increased ATGL and HSL.

(A) WB analysis of phospho-HSL/total HSL in whole cell extracts of primary adipocytes isolated from the EPI adipose tissue depot of AKO versus WT mice after overnight fasting. (B) WB analysis of phospho-HSL/total HSL protein levels in whole cell extracts from in vitro differentiated adipocytes in fasted or isoproterenol (10 μM) stimulated conditions. (C) RT-qPCR analysis of ATGL and HSL, lipolysis markers, in WAT of AKO versus WT mice, n= 4-5. Results are expressed as mean+/−SEM. (C) Western blot analysis of ATGL and HSL normalized to β-tubulin in whole cell extracts from WAT from AKO versus WT mice in fasting conditions. Statistical significance was calculated by two-tailed Student’s t test; * represents p value <0.05 and ** p value < 0.01. All western blots are representative images of at least three independent experiments.
adiponectin\textsuperscript{79}, we did not observe any significant change in the expression of HSL upon GPS2 deletion in adiponectin-expressing, mature adipocytes. However, we unexpectedly observed a trend for increased ATGL mRNA and protein levels, which could reflect an attempt at compensating for the reduced lipolysis (Figure 3.10B-D). Upstream regulation of the lipolysis pathway was also impaired as shown by reduced phosphorylation of PKA and PKA substrates in subcutaneous white adipose tissue homogenates (Figure 3.11A). To further confirm that lipid mobilization from the adipose tissue is impaired in GPS2-AKO mice, we confirmed that lipolysis is impaired in absence of GPS2 \textit{ex vivo} and \textit{in vivo}. Ex \textit{vivo}, the rate of lipolysis, as assessed by measuring glycerol release from \textit{in vitro} differentiated adipocytes, was severely reduced in GPS2-deficient cells compared to wild type counterparts (Figure 3.11B). \textit{In vivo}, GPS2-AKO mice lost significantly less body weight after an overnight fast than their WT littermates, in accordance with their impaired ability to promote lipid release from triglyceride stores (Figure 3.11C). Thus, our current and previous work combined indicates that GPS2 is indeed required for lipid mobilization through lipolysis. Interestingly, it appears that GPS2 plays complementary genomic and non-genomic roles in regulating different steps of the lipolysis pathway in differentiating pre-adipocytes and mature adipocytes.

Next, we investigated the effect of enhanced insulin signaling on the regulation of lipogenesis. In both adipose tissue and liver, acute stimulation of de novo lipogenesis (DNL) is mainly regulated by insulin and glucose availability through the transcriptional activities of SREBP1, ChREBP and LXR\textsubscript{s}\textsuperscript{121,146,147}. Gene expression analysis by RT-qPCR showed a significant increase in the mRNA levels of
Figure 3.11 – Improved lipid storage in the white adipose tissue of GPS2-AKO mice is a result of impaired lipolysis.

(A) WB analysis for markers of active lipolysis in whole cell extracts from the WAT of fasted mice. (B) Basal and isoproterenol induced lipolysis assay on in vitro differentiated adipocytes. Statistical significance was calculated comparing 3 independent experiments. (C) Percentage of total body weight loss after prolonged overnight fast, n = 7. Results are expressed as mean ±SEM. Statistical significance was calculated by two-tailed Student’s t test; *represents p value < 0.05 and **p value < 0.01. All western blots are representative images of at least three independent experiments.
Figure 3.12 – Improved lipid storage capacity in GPS2-AKO mice is a result of increased lipogenesis.

(A) qPCR analysis of lipogenic markers in WAT of AKO versus WT mice, n =5-7. (B) WB analysis of phospho-ACC/total ACC and phospho-AMPK/total AMPK in whole cell extracts from the WAT of fasted mice. Results are expressed as mean +/-SEM. Statistical significance was calculated by two-tailed Student’s t test; *represents p value < 0.05 and **p value < 0.01. All western blots are representative images of at least three independent experiments.
the lipogenic genes *Fasn, Dgat1, Dgat2, AGPAT2* and *Acaca* in the subcutaneous WAT of GPS2-AKO mice compared to WT littermates (**Figure 3.12A**). In addition to the transcriptional regulation of enzyme availability, insulin-mediated activation of AKT impinges upon DNL through AMPK inhibition, resulting in dephosphorylation and activation of the Acetyl-CoA carboxylase (ACC)\(^{148}\). In agreement with AKT being constitutively activated in GPS2-AKO mice, we observed both a significant decrease in active AMPK and decreased phosphorylation of ACC in the white adipose tissue of GPS2-AKO mice (**Figure 3.12B**). Together, these results are consistent with the hypothesis that both in the fed and fasted state lipogenesis is enhanced while lipid mobilization is impaired in GPS2-AKO mice, markedly shifting the balance toward fat storage.

**Fat-specific deletion of GPS2 results in improved systemic insulin sensitivity**

Sustained insulin signaling, improved lipid storing capacity of the adipose tissue, impaired adipose tissue lipolysis and enhanced DNL have all been associated with improved systemic insulin sensitivity\(^ {142,149-152}\), suggesting that adipo-specific GPS2 deletion might be beneficial for the organism. However, obesity is generally associated with chronic inflammation and with the development of insulin resistance\(^ {50,153,154}\). To investigate how the removal of GPS2 and the corresponding enhanced insulin signaling in the adipose tissue affect the maintenance of systemic homeostasis, we first assessed the level of local inflammation in the adipose tissue by RT-qPCR and FACS analysis. Surprisingly, given GPS2 previously reported anti-inflammatory role and the obesity that characterize GPS2-AKO mice, no significant differences were observed in either the
Figure 3.13 – GPS2-AKO mice do not display adipose tissue inflammation despite their obesity.

(A) RT-qPCR analysis of pro-inflammatory genes and macrophage markers in the WAT. Results are expressed as mean +/- SEM. Statistical significance was calculated by two-tailed Student’s t test; *represents p value < 0.05 and **p value < 0.01. (B) Macrophage infiltration in the WAT as measured by FACS analysis with quantification of f4/80 and CD11b positive cells. FACS statistical significance was calculated comparing 3 independent experiments.
expression of gene markers of inflammation or in the amount of macrophages present within the adipose tissue (Figures 3.13A-B).

Next, we sampled plasma from fasted WT and GPS2-AKO littermates for the presence of secreted adipokines. Plasma levels of the anti-inflammatory and insulin-sensitizing hormone adiponectin\textsuperscript{155,156} were found significantly elevated in GPS2-AKO mice despite the reported increase in body adiposity (Figure 3.14A), whereas no significant differences were observed in the amount of circulating leptin (Figure 3.14A). Consistent with an increase in circulating adiponectin and with adiponectin playing an important role in promoting the “healthy expansion” of the adipose tissue\textsuperscript{157,158}, we did not observe any evidence of hepatic steatosis in the liver of GPS2-AKO mice despite their increase in total body fat (Figure 3.14B). Accordingly, the expression of both ChREBP and SREBP1c, key markers of DNL, were found downregulated in the liver of GPS2-AKO mice (Figure 3.14C). The expression of key gluconeogenic enzymes, such as G6Pase, FBP1 and PEPCK was also reduced in the liver of GPS2-deficient mice (Figure 3.14C). Furthermore, we sampled circulating free fatty acids (FFA) from GPS2-AKO and WT littermates and found a slight trend to decrease but no significant differences. While this result could initially appear surprising in face of the decreased lipolysis, it has to be taken in consideration that in a context of increased obesity circulating FFA are usually elevated, thus maintenance of the baseline level actually reflects a decrease compared to the expected phenotype (Figure 3.14D). In conclusion, these results indicate that despite the increase in adiposity, which typically correlates with metabolic dysfunction and peripheral tissue lipid deposition, GPS2 deletion in the
Figure 3.14 – GPS2-AKO mice have improved adipokine and circulating markers and improved liver function.

(A) Plasma adiponectin and leptin levels after an overnight fast, n = 7. (B) Representative H&E staining of paraffin-embedded liver sections imaged at 10X magnification. (C) Hepatic expression of gluconeogenic and de novo lipogenesis genes by RT-qPCR analysis. (D) Plasma non esterified free fatty acid levels in AKO versus WT mice after overnight fast, n=3. Results are expressed as mean +/- SEM. Statistical significance was calculated by two-tailed Student’s t test; *represents p value < 0.05 and **p value < 0.01.
adipose tissue appears to have a positive effect on whole body lipid metabolism with reduced spillover to non-adipose tissues such as the liver.

Improved lipid storing capacity of the adipose tissue can contribute to improved systemic insulin sensitivity \(^{57,120,149,150}\). Blood glucose levels after overnight food deprivation were comparable between WT and GPS2-AKO matched littermate mice (Figure 3.15A), and glucose tolerance tests (GTT) were also normal (Figure 3.15B). However, plasma insulin levels under the same experimental fasting conditions were significantly reduced in GPS2-AKO mice (Figure 3.15C), consistent with our previous data that AKT is activated \textit{in vitro} in basal conditions and \textit{in vivo} under food deprivation upon GPS2 deletion/downregulation. To directly test the insulin sensitivity of GPS2-AKO mice, we injected matching cohorts of WT and GPS2-AKO littermates with a suboptimal dose of insulin after a short morning fast. Baseline insulin levels of GPS2-AKO mice trended to be lower under these experimental conditions as well (Figure 3.15D), and insulin-induced glucose clearance was greater in GPS2-AKO mice than WT littermates (Figure 3.15E), confirming the predicted improvement in insulin sensitivity.

In conclusion, our results together indicate that enhancing insulin signaling in the adipose tissue, through GPS2 deletion and modulation of AKT ubiquitination status, has a positive effect on whole body insulin sensitivity despite the increased adiposity.

\textit{GPS2 regulation of AKT is conserved in other cell types}

While investigating the role of GPS2 in B cells using CD19-cre driven B-cell specific GPS2 knockout mice, results reveal that the development of BKO cells is impaired at the pre-BCR stage of the B cell maturation process in the bone marrow.
Figure 3.15 – GPS2-AKO mice have improved systemic insulin sensitivity.

(A) Fasting blood glucose levels in AKO versus WT mice after overnight fast, n =8. (B) Glucose tolerance test following glucose i.p. injection (1.5 mg/g of body weight) after an overnight fast, n =4. (C) Plasma insulin levels in AKO versus WT mice after overnight fast, n =5-7. Statistical significance was calculated comparing 3 independent experiments. (D) Plasma insulin levels in AKO versus WT mice after a 6 hour fast, n=3-6. (E) Insulin tolerance test after a 0.5 U/kg body weight insulin i.p. injection in AKO versus WT mice following a 4 h fast, n =7-8. Results are expressed as mean +/- SEM. Statistical significance was calculated by two-tailed Student’s t test; *represents p value < 0.05 and **p value < 0.01.
Interestingly, a similar phenotype is promoted by the deletion of FOXO1 in pre-B/immature cells. CD19-cre driven deletion of FOXO1 is associated with a later impairment at the pre-B cell stage, defective light chains rearrangement and a lower number of recirculating mature cells\(^\text{159}\). Among genes found significantly altered in splenic GPS2-BKO cells were the pre-BCR adaptor protein BLNK/SLP-65, the MAPK-activated kinase MK5 and the recombination activating gene RAG, all known to play key roles in FOXO1-mediated regulation of the rearrangement of immunoglobulin chains\(^\text{160}\).

These data, along with our results showing that GPS2 can regulate AKT activation in adipocytes, prompted us to investigate the possibility that lack of GPS2 in B cells affects the early stages of B cell differentiation through increased AKT activation and defective transcriptional regulation of key FOXO1 target genes. We first confirmed GPS2 knockout efficiency in bone marrow B cells by RT-qPCR analysis was comparable to GPS2-BKO plenic B cells (Figure 3.16A). As previously described that GPS2 deletion in adipocytes leads to constitutive AKT activation, we asked whether GPS2-mediated regulation of AKT is conserved in B cells. Indeed, as shown in Figure 3.16B, AKT phosphorylation was found increased in GPS2-BKO B cells. As expected in the presence of enhanced AKT activation, we observed a striking upregulation of FOXO1 phosphorylation (Figure 3.16B). It is well known that FOXO factors are negatively regulated by the PI3K/AKT pathway via phosphorylation and nuclear exclusion in order to regulate FOXO1-target gene expression\(^\text{160}\). Consistent with the increased FOXO1 phosphorylation, we observed a significant decrease in the expression of FOXO1 target genes \textit{RAG1}, \textit{RAG2} and \textit{BLNK} (Figure 3.16C). To investigate the nuclear exclusion of
Figure 3.16 – Defective B cell development in the bone marrow of GPS2-BKO mice results from GPS2 regulation of AKT activation and direct transcriptional control.

(A) GPS2 mRNA expression in BM B cells from GPS2-BKO and control littermates measured by RT-qPCR. (B) Western blotting analysis for P-AKT (Ser 473) and P-FOXO1 in splenic B cells. (C) Down regulation of BLNK1, RAG1, RAG2, and FOXO1 in BM from GPS2-BKO and control littermates by RT-qPCR. Bar graphs represents the sample mean of three independent experiments +/- SD. (D) Chromatin immunoprecipitation (ChIP) analysis to measure GPS2 occupancy on the promoters of putative target genes in B cells purified from BM of WT mice. (E) ChIP for GPS2 and FOXO1 in B cells purified from BM of either WT or GPS2-BKO mice. For ChIPs, data are representative of three independent experiments. Bar graphs represents the sample mean of technical replicates +/- SD; * indicate p-value<0.05; ** indicate p-value<0.01.)
FOXO1 in GPS2-BKO B cells, we found that FOXO1 binding on target gene promoters was drastically reduced in GPS2-BKO cells (Figure 3.16E). Interestingly, the expression of FOXO1 gene itself was also found significantly downregulated in GPS2-depleted cells (Figure 3.16C). Thus, we asked whether GPS2 regulated the transcription of these genes directly as well. Results of our chromatin immunoprecipitation analysis revealed significant GPS2 binding to the promoters of BLNK, RAG1, RAG2 and FOXO1 genes in WT B cells isolated from the bone marrow (Figure 3.16D). In turn, we observe significantly less GPS2 binding on these promoters thus suggesting a direct role for GPS2 in the transcriptional forward loop that regulate FOXO1 and some of its target genes in developing B cells (Figure 3.16E). These results strongly confirm that the role of GPS2 in regulating AKT phosphorylation and activation is a conserved mechanism in various cell types yet also suggests that GPS2 direct transcriptional control of FOXO1 could have a function in GPS2-AKO adipocytes as well (Figure 3.17).

**Discussion**

Insulin action is critically required for promoting cell growth, regulating tissue development, and controlling whole body metabolism through modulation of glucose homeostasis and lipid metabolism. It’s disruption or reduced functionality leads to the development of insulin resistance, a hallmark of Type 2 Diabetes and other metabolic disorders. The signaling mechanisms that mediate the physiological responses to insulin have been thoroughly studied with the identification of multiple transduction pathways, including both positive and negative regulators. Major players in these pathways are kinases, which are usually counter-regulated by opposing phosphatases (i.e. PI3K and
PTEN). Our work has further extended this signaling network by revealing the existence of an additional level of regulation based on the opposing actions of an ubiquitin conjugating enzyme, Ubc13, and its endogenous inhibitor, GPS2. Insulin-mediated ubiquitination of AKT, via Ubc13-mediated synthesis of K63 ubiquitin chains, is an unexpected, critical step for the activation of downstream signaling events (Figure 3.17A). Non-proteolytic ubiquitination of AKT had been previously reported for growth factor-mediated pathways, and a cancer-associated mutation that affects the ubiquitination of AKT1 (E17K) had been linked to AKT hyperactivation in breast and colon cancer. In this context, the gain of an additional lysine in the PH domain was found to promote enhanced AKT ubiquitination, constitutive recruitment to the membrane and activation. Interestingly, exome sequencing of patients with severe hypoglycemia but undetectable plasma insulin has recently led to the identification of a homologous activating mutation in AKT2 that might be similarly promoting enhanced ubiquitination. Overexpression of the E17K mutant in 3T3-L1 preadipocytes promoted insulin-independent localization of both AKT and GLUT4 to the plasma membrane and nuclear export of FoxO1, indicating constitutive activation of the AKT signaling pathway. These observations support our findings that regulation of AKT activation by ubiquitination is a conserved feature among the metabolic and growth signaling pathways mediated by different AKT isoforms. They also indicate that the non-proteolytic ubiquitination of AKT represents a key regulatory node for both adipose tissue and systemic homeostasis and metabolism.
Figure 3.17 – Model of GPS2 regulation of AKT.

(A) Model of GPS2 regulation of the insulin signaling pathway in adipocytes. (B) Model of GPS2 regulation of AKT in B cells.
In addition to revealing a role for K63 ubiquitination in the insulin pathway, our work has identified GPS2 as an endogenous inhibitor of this regulatory step. We previously characterized GPS2 as an inhibitor of Ubc13/TRAF2-6 activity in the context of pro-inflammatory pathways downstream of TNFα and TLR receptors \(^84\). Here, we confirm that a significant increase in JNK activation in primary adipocytes is observed upon GPS2 deletion. We also report the novel finding that GPS2 is a critical player in the insulin transduction pathway. Our data, in particular, reveal that physiological levels of GPS2 in adipocytes are required for preventing the constitutive ubiquitination of AKT, and therefore restricting its activation, as well as the activation of downstream signaling events, when insulin levels are low. In accord with these conclusions, we found that GPS2 deletion in mice adipose tissue results in constitutive ubiquitination and activation of AKT, leading to disrupted lipid metabolism and increased adiposity, but also elevated adiponectin levels. Interestingly, overexpression studies in mice indicate that increasing the level of secreted adiponectin contributes to mimicking a state of constant feeding and results in improved insulin sensitivity despite the substantial expansion of the adipose tissue \(^9,142,158\). Similarly, deletion of PTEN, a well-known inhibitor of AKT, promotes sustained insulin signaling and improved insulin sensitivity despite a significant increase in body weight \(^142\). Our results indicate that the absence of GPS2 similarly favors the retention of triglycerides in the adipose tissue, and thus improvement of insulin sensitivity in mice that become obese when fed a regular laboratory chow diet.

Interestingly, our previous work showed GPS2 playing an important anti-inflammatory role in the regulation of pro-inflammatory signaling pathways. GPS2 anti-
inflammatory role was then confirmed by macrophage-specific and B-cell-specific deletion studies, and by GPS2 overexpression in the adipose tissue (both adipocytes and macrophages) of DIO obese mice. In contrast, we report here that GPS2 adipo-specific deletion has a positive effect on adipocyte insulin signaling and systemic insulin sensitivity in chow-fed mice, and that, under these conditions, inflammation of the adipose tissue is undetectable in GPS2-AKO mice despite their increase in body fat. These observations are in agreement with a recent report that hyperinsulinemia can drive adipose tissue inflammation whereas a reduction in circulating insulin levels causes a decrease in the expression of pro-inflammatory markers and macrophage expansion in the adipose tissue. Thus, our findings reveal that GPS2 plays an intriguing role at the intersection between inflammatory and metabolic pathways, and suggest that sustained AKT activation through enhanced ubiquitination is likely to overcome the negative effect that the elevated levels of P-JNK might have on insulin signaling via IRS1 phosphorylation. Finally, it is important to note that there are layers to the phenotype associated with the loss of GPS2 function that might depend on the genetic background and the experimental conditions. For example, while the GPS2-AKO mice described here become obese even when fed a chow diet, similar mice generated by Fan and colleagues were not obese at baseline and did not appear to gain extra weight upon HFD feeding. Thus, further studies that explore the interplay between increased activation of pro-inflammatory signaling pathways and enhanced insulin signaling in GPS2-AKO mice will be critical for a comprehensive understanding of the crosstalk between these pathways in condition of diet- or genetic-induced obesity. Interestingly, our results
indicate that the synergistic roles played by GPS2 in the regulation of JNK activity and insulin signaling are mediated through a conserved mechanism based on the inhibition of Ubc13 enzymatic activity. This raises the interesting question of whether the ubiquitination machinery responsible for the synthesis of non-proteolytic K63 ubiquitin chains represents a key regulatory node between inflammation and lipid metabolism at the onset of obesity.

Lastly, it is worth noting that there is a large body of work describing GPS2 as a transcriptional cofactor, involved in mediating both gene repression, as part of the NCoR/SMRT complex, and activation, through direct interaction with nuclear receptors and other TFs. For example, in the adipose tissue, downregulation of GPS2 was associated with the derepression of inflammatory target genes in human obese patients, and with the activation of key enzymes for triglyceride breakdown, HSL and ATGL, in murine differentiating adipocytes. Here, we have confirmed a critical role for GPS2 in the regulation of adipocyte triglyceride turnover. However, the expression of HSL and ATGL was not impaired in GPS2-null primary adipocytes, indicating that lipolysis is likely suppressed due to the sustained activation of insulin signaling even under baseline or fasted conditions, rather than direct transcriptional effects. Thus, our previous and current findings, together, indicate that while GPS2 is essential for the early development of preadipocytes, its deletion at later stages does not affect differentiation but GPS2 remains essential for maintaining metabolic homeostasis in mature adipocytes. Intriguingly, increased adiposity and altered lipid mobilization have been previously reported also in the case of mice lacking a binding partner of GPS2, the transcriptional
corepressor TBLR1. The phenotype observed upon TBLR1 deletion appears to similarly extend beyond the transcriptional regulation of HSL and ATGL, with TBLR1 being described as required for controlling multiple steps of the lipolytic cascade, including both changes in gene expression and a decrease in the phosphorylation of HSL and other PKA substrates. This suggests that multiple components of the NCoR/SMRT corepressor complex might play complementary genomic and non-genomic functions towards the coordinated regulation of cell homeostasis.
CHAPTER FOUR – GPS2 REGULATES MITOCHONDRIA AND BROWN ADIPOSE TISSUE FUNCTION

This chapter is adapted from Cardamone, M.D., Cederquist, C.T.*, Tanasa, B.*, Huang, J., Mahdaviani, K., Li, W. Rosenfeld, M.G., Lisesa, M., and Perissi, V. GPS2 regulates mitochondria biogenesis via mitochondria retrograde signaling and chromatin remodeling of nuclear-encoded mitochondrial genes. Under review.

*= These authors contributed equally to this work.

Abstract

Brown adipose tissue (BAT) is a specialized type of adipose tissue (AT) containing multiple small lipid droplets and an extremely high density of mitochondria. This depot is characterized by high expression of the uncoupling protein (UCP1) which allows BAT to burn fatty acids for the generation of heat rather than ATP production. These features allow BAT to be specifically involved in energy control and thermoregulation. Dysfunctionality of this tissue has been associated with metabolic diseases and, in turn, therapeutics to increase BAT activity are under investigation to target Type 2 diabetes and metabolic syndrome.

Mitochondrial biogenesis and homeostasis are critically important for the function of BAT. Because most mitochondrial proteins are encoded in the nuclear genome, these functions are directly dependent on the regulation of gene transcription in the nucleus. Studies performed in vitro in 3T3-L1 cells have revealed that GPS2 is required for the transcriptional activation of a vast nuclear-encoded mitochondrial gene program. This suggested that GPS2 transcriptional functions in vivo will be particularly relevant in the
context of BAT. In addition, our previous work has shown that GPS2 plays an essential role in the balance of lipid storage capacity by regulating lipolysis and lipogenesis in WAT, suggesting a similar phenotype may be observed in BAT as well.

Indeed, in our phenotypic analysis, we observe that the regulatory strategies in which GPS2 is essential for supporting mitochondrial biogenesis and lipid storage are conserved in BAT from GPS2-AKO mice. Specifically, we found that loss of GPS2 in these mice led to impaired mitochondria biogenesis, which in concordance with impaired lipolysis, resulted in whitening of this AT depot. Together, our study shows that modulation of GPS2 in BAT represents a critical node for the coordinated regulation of metabolic pathways modulating the balance between lipid storage and energy expenditure.

**Introduction**

Brown adipose tissue (BAT) is a distinct, specialized type of adipose tissue that is found to play a critical role in energy homeostasis. The tissue derives its name and color due to high mitochondrial content and extensive vascularization. It was initially thought to be abundant only in newborns or small mammals, naturally susceptible to loss of heat, as a mechanism to adequately maintain body temperature. However, it was recently found that adult humans also have metabolically active BAT, found distributed in various regions of the body such as cervical, supraclavicular, axillary, paravertebral, and upper abdominal locations\textsuperscript{166,167}. The discovery of adult BAT has led to more extensive research on the development and function of this specialized AT depot.
Classic brown fat is derived during embryogenesis from mesenchymal precursors of a Pax7+ and Myf5+ lineage, similar to skeletal muscle, unlike classic WAT which arises from a Pax7- and Myf5- lineage\textsuperscript{168-171}. Through these studies, another developmentally distinct type of brown fat was uncovered, known as beige/brite adipose tissue\textsuperscript{172-175}. This type of BAT is recruited postnatally through either a mechanism of transdifferentiation of mature white adipocytes into a brown adipocyte-like phenotype or by induction of brown-like specific progenitors, which is still under investigation\textsuperscript{176-179}. Despite differences in developmental origin, BAT also acts as an endocrine organ and can release various factors such as IGF1, IL6, and FGF21 similarly to the endocrine capabilities of WAT. Both AT depots are therefore critical for the regulation of systemic metabolism.

The differences in function of BAT versus WAT have been broadly studied as well. Whereas white adipocytes comprised of a large, unilocular lipid droplet are predominantly used for energy storage, brown adipocytes contain small, multilocular lipid droplets, densely packed mitochondria and specifically express uncoupling protein 1, UCP1. BAT also has a distinct gene expression signature from WAT that includes high expression of PGC1a, PRDM16, PPARα, CIDEA, ELOVL3 and DIO2. This gene expression program also includes enhanced expression of genes involved in mitochondrial biogenesis and function\textsuperscript{170,175,180-182}. These characteristics are required for the primary function of this depot to regulate thermogenesis and energy expenditure.

Proper functionality and response of BAT to external and internal metabolic signals consequently relies heavily on appropriate mitochondria function and plasticity of
mitochondrial biogenesis. Mitochondria are highly dynamic, cytoplasmic organelles with distinct compartments: outer membrane, inner membrane, intermembrane space, cristal membranes, intracristal space and a matrix$^{183,184}$. This organelle is involved in almost all metabolic pathways, however the most distinctive, crucial role is to synthesize a majority of cellular ATP used as energy currency. This process requires the oxidation of nutrients into Acetyl-CoA which enter the citric acid (TCA) cycle producing high energy electrons that feed into the various complexes of the electron transport chain (ETC). As these reactions proceed, energy released causes protons to get pumped out of the mitochondria matrix space and create a pH gradient which helps to drive ATP synthase activity and the production of ATP from ADP via phosphorylation$^{183,184}$. Additionally, mitochondria contain their own small genome, however most mitochondrial proteins are encoded by nuclear DNA, synthesized in the cytoplasm and imported into mitochondria$^{183,184}$. This system relies heavily on exquisite communication between these organelles because in response to various metabolic conditions, mitochondria can increase or decrease their number or undergo remodeling via fission and fusion to adapt to the energy demands of a cell$^{170,175,180-182}$. For example, the proper expansion of mitochondrial mass and increased functionality of mitochondria-specific metabolic pathways has been shown to be important for the process of adipogenesis.

The multilocular lipid droplets in BAT allow for the rapid release of free fatty acids (FFAs) from triglyceride stores to be burned by mitochondria that uniquely contain UCP1. UCP1 acts as a proton channel that causes dissipation of the proton gradient formed in the inner mitochondrial membrane from the products of oxidative
phosphorylation of nutrient stores. This causes a proton leak allowing UCP1-rich mitochondria to generate heat rather than ATP, the complete process known as non-shivering thermogenesis\textsuperscript{170,175,180-182}. BAT can become even more active in response to various stimuli, which include B adrenergic agonist stimulation or cold exposure. These stimuli induce the release of norepinephrine from the sympathetic nervous system (SNS) to significantly increase UCP1 and other brown fat specific and mitochondria genes ultimately triggering enhanced BAT activation. Additional stimuli such as cAMP analogs, thyroid hormone, insulin, thiazolidinediones, and retinoic acid can also contribute to BAT activation\textsuperscript{170,175,180-182}. Overall, UCP1 uncoupling activity and mitochondrial function have been shown to be essential for BAT-mediated maintenance of thermogenesis as well as the induction of browning in WAT depots.

Mitochondria also clearly play a central role in regulating metabolism of both AT depots via documented roles in affecting lipolysis and lipogenesis. Lipolysis allows for the breakdown of free fatty acids (FFAs) that can diffuse or travel through carnitine shuttles into the mitochondrial matrix whereby the FFAs are converted to Acetyl-CoA via beta oxidation to be further used in the TCA cycle and ETC. Mitochondria, in turn, have the ability to provide key intermediates, such as glycerol 3-phosphate, Acetyl-CoA, and pyruvate, for the processes of de novo lipogenesis and triglyceride synthesis\textsuperscript{13,183-185}.

Mitochondria dysfunction in both brown and white adipocytes has been recently associated with metabolic dysfunction. Mitochondrial dysfunction resulting in impaired oxidation of fuels and lipid accumulation are associated with obesity and insulin resistance however the direct role of AT mitochondria in the development of these
pathologies is still under stringent investigation\textsuperscript{186-188}. One example shows that defects in the electron transport chain can result in increased reactive oxygen species, which can interfere with proper adipocyte function in the maintenance of glucose homeostasis and adipokine secretion\textsuperscript{189,190}. Due to the significant capacity of BAT to dissipate energy which requires the catabolism of triglycerides and glucose, brown fat is thought to be a potential, potent target for the treatment of obesity-associated disorders\textsuperscript{191,192}. Therefore, an increase in the fundamental knowledge about the regulation of BAT formation and its activity is necessary to determine its full therapeutic potential.

We have shown that GPS2 is a multi-functional protein that recently emerged as an important regulator of inflammation and lipid metabolism, all processes tightly linked to mitochondrial functions\textsuperscript{79,84,89,91,97}. In addition, we have also identified a direct mechanism in which GPS2 controls mitochondria, independently of regulation of signal transduction of metabolic pathways. Although GPS2 was previously reported in both the nuclear and cytosolic compartments\textsuperscript{84}, we have revealed that GPS2 is a previously unrecognized mitochondrial protein. In these studies, we have shown that after induction of mitochondrial stress inducing mitochondrial biogenesis, GPS2, via de-sumoylation by SENP1, was able to translocate from the outer mitochondrial membrane into the nucleus\textsuperscript{94,193-196}. GPS2 was recruited specifically on nuclear-encoded mitochondrial gene promoters and affected the processivity of core transcription machinery, including Pol2 processivity (\textbf{Figure 4.1}). In agreement with these findings, GPS2 downregulation (GPS2-KD) in 3T3-L1 preadipocytes was associated with a significant reduction in
Figure 4.1 – Model of GPS2 regulation of mitochondrial retrograde signaling in 3T3-L1 adipocytes.

Upon mitochondrial stress, GPS2 is de-sumoylated on the outer mitochondrial membrane and translocates into the nucleus where it can regulate core transcription machinery on mitochondria gene promoters.
mitochondrial mass and mitochondrial gene expression, thereby affecting the respiration capacity of GPS2-KD cells.

Together, these results confirm that GPS2 plays a major role as a mediator of mitochondria retrograde signaling which is important in promoting biogenesis of new mitochondria through the activation of an extensive mitochondrial gene program. This data all raised the question of how GPS2 affects mitochondrial function and biogenesis in vivo. Therefore, we sought to investigate the role of GPS2 in regulating BAT, a mitochondria-rich tissue, using GPS2-AKO mice. Our data in this model suggests that this newly identified role of GPS2 is physiologically relevant and loss of GPS2 results in dysfunctional brown adipose tissue.

Results

**GPS2-AKO mice display decreased mitochondrial function in brown adipose tissue**

To complete our investigation of the GPS2-AKO mice, we next decided to investigate the specific phenotype of BAT. We first confirmed that GPS2 was absent in this AT depot (Figure 4.2A). In WAT of GPS2-AKO mice, we showed that adipocytes had significantly impaired lipolysis. We find this mechanism of lipid utilization is also impaired in BAT by measuring the activation of HSL (Figure 4.2B). We also showed that the sumoylated form of GPS2, which controls its translocation to the nucleus is expressed in BAT and as expected this form is absent in GPS2-AKO BAT (Figure 4.2A). The removal of GPS2 from BAT led to a significant decrease in nuclear-encoded mitochondria gene expression (Figure 4.2C). These results suggest the mechanisms of
Figure 4.2 – GPS2-AKO mice have impaired lipolysis and mitochondrial gene expression in the brown adipose tissue.

(A) WB analysis of GPS2 using two independent antibodies in WT versus GPS2-AKO mice normalized to B tubulin expression. (B) WB analysis of phospho-HSL/total HSL in whole cell extracts of brown adipose tissue in GPS2-AKO versus WT mice after overnight fasting. (C) RT-qPCR analysis of mitochondrial and brown fat specific gene markers, in BAT of GPS2-AKO versus WT mice, n= 4-5. Results are expressed as mean+/SEM. Statistical significance was calculated by two-tailed Student’s t test; * represents p value <0.05 and ** p value < 0.01. All western blots are representative images of at least three independent experiments.
GPS2 in regulating lipid storage capacity and in mediating mitochondria retrograde signaling are preserved in BAT.

The mechanism described *in vitro* in 3T3-L1 cells showed that knockdown of GPS2 resulted in impaired mitochondria biogenesis. To determine whether this phenotype was conserved *in vivo*, we first measured the number of mitochondria in the tissue and found mitochondria content was significantly decreased in GPS2-deficient mice (*Figure 4.3A*). In agreement with these results, it was also observed that the whole tissue had decreased levels of various subunits of each complex of the oxidative phosphorylation pathway (*Figure 4.3B*). On the contrary, respiration measured in mitochondria isolated from BAT was unaffected by the loss of GPS2 (*Figure 4.3C*). Together, these data indicate that mitochondria biogenesis was defective BAT of GPS2-AKO mice however the functionality of the remaining mitochondria in the tissue were not impaired per se. These results confirmed that GPS2 regulates nuclear-encoded mitochondria gene expression and mitochondria biogenesis in BAT.

**GPS2-AKO mice display increased lipid accumulation in brown adipose tissue and in in vitro differentiated brown adipocytes**

Mitochondria dysfunction and impaired biogenesis as well as impaired lipolysis have been associated with whitening of BAT. As described above, GPS2 deletion in WAT has been recently reported to promote obesity associated with increased deposition of lipids in the WAT and improved systemic insulin sensitivity. In agreement with the impaired lipolysis and compromised mitochondria biogenesis observed in BAT from GPS2-AKO mice, histological inspection revealed that GPS2 deletion promotes the
Figure 4.3 – GPS2-AKO mice have impaired mitochondria biogenesis in the brown adipose tissue.

(A) Mitochondrial mass calculated by mtDNA/gDNA content, n=4-5. Results are expressed as mean +/- SEM. (B) WB analysis of various complexes in the electron transport chain in whole cell extracts of brown adipose tissue from GPS2-AKO versus WT mice after fasting normalized to HSP70. (C) Quantification of oxygen consumption rates (OCR) in BAT isolated mitochondria from control and GPS2-AKO mice in chow diet under the different respiratory states. State 2 quantifies respiration driven by proton leak, state 3 quantifies respiration linked to maximal ATP synthesis and maximal represents maximal electron transport chain activity induced by FCCP. Bar graphs represent average +/- SEM for complex I-driven respiration (pyruvate-malate, n=6 mice per group), complex II-driven respiration (succinate-rotenone, n=6 mice per group). Statistical significance was calculated by two-tailed Student’s t test; * represents p value <0.05 and ** p value < 0.01. All western blots are representative images of at least three independent experiments.
whitening of this depot (Figure 4.4A). Confirming these results, adipocytes arising from
differentiation of SVF from BAT of GPS2-AKO mice contain larger lipid droplets than
adipocytes derived from WT SVF (Figure 4.4B). Together, these results suggest the role
of GPS2 in regulating mitochondria biogenesis through mediating mitochondrial
retrograde signaling to control nuclear-encoded mitochondria gene expression is
physiologically relevant. Furthermore, GPS2 control of mitochondria biogenesis and
lipolysis led to a whitening of GPS2-AKO mice BAT.

**Discussion**

In conclusion, we have characterized the GPS2-AKO mouse by dissecting how
the physiology of BAT is affected by loss of GPS2. Particularly, GPS2-AKO mice have a
dysfunctional BAT depot with reduced mitochondria biogenesis, decreased lipolysis, and
a whitened phenotype. We have confirmed that the role of GPS2 in regulating lipid
storage capacity in WAT is also conserved in brown fat. Moreover, our results together
confirm the *in vivo* relevance of GPS2 in regulating the transcriptional activity of
nuclear-encoded mitochondrial gene expression for the proper regulation of
mitochondrial biogenesis.

These *in vivo* data indicate that GPS2 plays a critical role in regulating lipid
metabolism and energy expenditure in BAT. More investigation into the mechanisms
leading to mitochondrial dysfunction in WAT depots should be undertaken since such
dysfunction is linked to obesity-associated insulin resistance. It will be important to
determine whether the mechanisms by which GPS2 regulates mitochondrial homeostasis
contribute to the increased lipid storage capacity in WAT depots and additionally whether
Figure 4.4 – GPS2 deficiency in brown adipose tissue results in a whitening phenotype in the tissue depot and \textit{in vitro} differentiated brown adipocytes compared to WT mice.

(A) Representative H&E staining of BAT of WT and GPS2-AKO mice showing increased lipid deposition in the GPS2-AKO mice compared to WT mice under 10X magnification. (B) Representative Oil Red O staining of \textit{in vitro} differentiated SVF from brown adipose tissue of WT versus GPS2-AKO mice.
they help to drive the decreased lipolysis and increased lipogenesis observed in the GPS2-AKO mice.

In addition, the phenotype observed in the BAT of GPS2-AKO mice raises the question of whether GPS2 could affect the browning process in WAT depots. Browning is the process by which certain white adipose tissue depots can take on a brown fat phenotype when subjected with certain stimuli\textsuperscript{174,175,199-201}. These stimuli include chronic cold exposure, B-adrenergic stimulation, or pharmacological treatments such as PPAR\gamma agonists. These effectors induce white adipocytes to either adopt a brown fat gene expression program or turn on this BAT gene expression program in newly recruited and differentiated beige/brite adipocytes. Once activated, these beige/brite cells possess many characteristics of classical brown fat including the presence of multiple mitochondria and multiple lipid droplets. UCP1 levels significantly increase and genes related to mitochondrial biogenesis are also increased which allow the cells to participate in adaptive thermogenesis and energy expenditure\textsuperscript{174,175,199-201}. Because GPS2 is required for normal mitochondrial function in BAT, one would expect that its loss in beige/brite adipocytes of WAT would prevent these cells from participating in thermogenesis and energy expenditure. Additionally, the absence of GPS2 would also lead to defective lipolysis in these cells and consequently a reduction in fatty acids that fuel thermogenesis.

Intriguingly, mitochondria dysfunction has been shown to contribute to insulin resistance in many ways\textsuperscript{202,203}. A decreased number of mitochondria, decreased mitochondrial gene expression, abnormal morphology and impaired functions in oxidative phosphorylation are commonly found aberrations in insulin-resistant metabolic
tissues, including adipose tissue, skeletal muscle and liver. These mitochondria abnormalities are associated with intracellular lipid accumulation, insulin resistance, and the overall pathophysiology of T2D\textsuperscript{204-206}. Despite these data, this phenomenon is not observed in GPS2-AKO mice on chow or HFD. In fact, GPS2-AKO mice have improved glucose and insulin tolerance. This observation raises many interesting questions. The first being whether mitochondrial function is directly related to the progression of insulin resistance or is a secondary effect of improper energy partitioning. If it is a primary determinant in disease progression, how exactly do dysfunctional mitochondria contribute to insulin resistance and can the dysfunction be by-passed by maintain the correct balance of lipid storage versus lipolysis. Further investigation is necessary to answer these questions and shed light on whether modulating mitochondrial function could help improve metabolic diseases.

Lastly, it is worth noting that both mitochondrial function and BAT activity decline with age. Specifically, humans and rodents lose their ability to regulate body temperature\textsuperscript{13}. This decline in thermoregulation is often related to BAT atrophy and a loss of UCP1 activity\textsuperscript{180,207}. In an investigation of the effect of aging on BAT activity in lean and obese mice, it was found that old obese animals exhibited decreased expression of UCP1, BAT thermogenic capacity and overall BAT activity\textsuperscript{208}. Our novel findings showing that mitochondrial biogenesis is regulated by GPS2 raises the possibility that it is a target for development of therapeutics to treat age-associated diseases, most notably those affecting mitochondrial-rich tissues such as the brain.
CHAPTER FIVE – GPS2 MODULATES THE INTERPLAY BETWEEN
ADIPOSE TISSUE INFLAMMATION AND SYSTEMIC METABOLIC HEALTH

STATUS

Abstract

Obesity-associated inflammation is widely recognized as a critical factor in the development of insulin resistance leading to Type 2 diabetes (T2D) and other metabolic disorders. In response to obesity, adipose tissue (AT) becomes dysfunctional and increases the production of pro-inflammatory cytokines. This can drive infiltration of immune cells into AT and the development of systemic, low-grade, chronic inflammation associated with insulin resistance. However, surprisingly, treatment with anti-inflammatory drugs has not proven successful in treating these metabolic syndromes and the critical question of whether inhibiting inflammation is a good approach in the attempt to treat insulin resistance remains unanswered.

While characterizing the GPS2-AKO mice, we found that adipocytes had increased cell size, resulting in increased fat mass and total body weight. Typically, obesity is associated with increased inflammation and metabolic dysfunction, however these phenotypes were not observed in GPS2-AKO mice on chow diet, which instead showed improved insulin signaling. This suggested that obesity could be uncoupled from inflammation and whole body metabolism in the presence of sustained AT insulin signaling. However, these observations are in contrast with previous reports that GPS2 plays an important anti-inflammatory role in various metabolic organs. GPS2 is found to inhibit signal transduction of TNFα and TLR pathways as well as act as a cofactor
regulating gene expression by inhibiting transcription of pro-inflammatory targets. Because of the anti-inflammatory role of GPS2, we hypothesize that a lack of GPS2 in AT under excessive nutrient stress could result in increased inflammation leading to a decline in systemic metabolism.

In order to further our characterization of GPS2-AKO mice, we exposed the mice to a high fat diet (HFD) regimen to dissect the critical role of GPS2 in regulating obesity-induced inflammation and lipid metabolism in vivo. Unexpectedly, we observe that the GPS2-AKO mice become more obese and inflamed than WT mice during HFD feeding yet are protected from developing insulin resistance. Our data suggests that increased AT lipid storage capacity and a reduction of peripheral lipid deposition has a positive, protective role against the development of insulin resistance, despite increased stimulation of inflammatory responses in AT. We hypothesize the role of GPS2 in controlling macrophage infiltration could potentially lead to healthy tissue remodeling and expansion. The GPS2-AKO mice represent a unique model to improve our comprehension of the interplays between inflammation and adipose tissue functionality in the development of insulin resistance and this work contributes to understanding whether inhibiting inflammation in the context of obesity is a viable strategy to ameliorate metabolic functionality.

**Introduction**

Adipose tissue is a highly active organ involved in numerous metabolic and immune processes. AT function and secretory abilities are able to act locally as well as influence other organs systems that play crucial roles in the regulation of whole body
homeostasis. Obesity-associated insulin resistance can drive a phenotype of low-grade chronic inflammation. Specifically, dysfunctional AT propagates the secretion of chemoattractants such as MCP-1 and pro-inflammatory cytokines such as IL-6, TNFα, and IL1B, which increase immune cell infiltration into AT, most notably macrophages. Macrophages with either an M1 or M2 phenotype can elicit either pro-inflammatory or anti-inflammatory responses respectively\textsuperscript{209}, and M1 pro-inflammatory macrophages are predominately recruited under conditions of obesity\textsuperscript{210}. Moreover, dysfunctional AT lipid metabolism leads to increased circulating FFAs which further initiates pro-inflammatory signaling cascades in recruited immune cells. It has been found that these cumulative signaling events cause a feedback loop of pro-inflammatory cytokines exacerbating the AT pathological state by continuously disrupting the insulin signaling cascade in adipocytes. AT dysfunction and induction of inflammation can further cause defects in hepatic and skeletal muscle glucose homeostasis, resulting in systemic insulin resistance and the development of T2D. A plethora of reports have shown that obesity- associated inflammation strongly correlates with increased insulin resistance however anti-inflammatory drug therapies have proven unsuccessful in treating metabolic diseases\textsuperscript{51,58,211-218}.

Thus, an open question remains whether the recruitment of the immune system in the context of obesity is solely a pathological process. In this regard, it has been proposed that the presence of immune cells in metabolic organs, such as AT, help to support the physiologic function of these metabolic tissues. In support of this idea, recent reports have shown that macrophage infiltration in AT is required for proper tissue expansion
and remodeling. Specifically, local inflammation has been shown to act as an adaptive response for AT expansion to permit storage of excess nutrients. Immune cell infiltration, in the context of obesity, also allows macrophages to play an important role in lipid trafficking and uptake to reduce lipotoxicity. Local inflammatory responses have been shown to be required to recruit progenitors as well as allow for enhanced adipogenesis in order to deal with increased lipid load as well. Therefore, it is clear that inflammation is important for the homeostasis of AT, yet becomes a pathophysiologic signal when lipid load and inflammatory signaling become uncontrollable.

GPS2 plays an important anti-inflammatory role in metabolic organs, specifically adipose tissue. Reports in human studies show an inverse correlation between GPS2 and adiposity and markers of AT inflammation, specifically IL6. This was attributed to the function of GPS2 to repress pro-inflammatory genes as part of the NCoR/SMRT corepressor complex. Additionally, our lab has shown that cytoplasmic GPS2 acts to inhibit both the TNFα and TLR signaling pathways by inhibiting ubiquitination events required for downstream signaling thereby blocking JNK activation. The in vivo relevance of this GPS2 function was first demonstrated in aP2 driven GPS2-TG mice by showing these mice are protected from HFD-induced increases in AT inflammation, providing additional proof that GPS2 has an anti-inflammatory function in adipocytes. TG mice showed decreased phosphorylation of JNK and decreased expression of pro-inflammatory markers in AT. Very surprisingly however, these mice did not have improvements in systemic metabolism as measured by glucose and insulin tolerance tests.
Figure 5.1 – GPS2-AKO mice gain significantly more total body weight on high fat diet.

(A) Body weight gain of WT versus GPS2-AKO mice after 16 weeks of 45% HFD feeding regimen. (B) Body weight gain curve measured weekly during 16 weeks of HFD in WT versus GPS2-AKO mice, n=3. Results are expressed as mean +/- SEM. Statistical significance was calculated by two-tailed Student’s t test; *represents p value < 0.05 and **p value < 0.01.
These data suggest that there is an uncoupling between lipid metabolism and inflammation during the onset of insulin resistance. Therefore, there is still a great deal to be learned about the possible beneficial effects of inflammation in protecting from systemic metabolic dysfunction.

In order to further elucidate the relationship between lipid metabolism and inflammation on metabolic flexibility, the characterization of GPS2-AKO mice was expanded to understand their phenotype on HFD. This is an interesting model to use because GPS2-AKO mice become obese even under conditions of chow diet yet remain metabolically healthy, due to enhanced AT insulin signaling controlling triglyceride handling. However, removal of GPS2, which has been shown to control key inflammatory signaling cascades, presumably would result in increased AT inflammation. Our initial results show that GPS2-AKO mice on HFD become more obese and inflamed than WT mice, however continue to show improved systemic metabolism while WT mice develop insulin resistance. By further studying GPS2-AKO mice on HFD we hope to dissect the relevance and interplay among inflammation and lipid storage in the development of insulin resistance.

**Results**

*GPS2-AKO mice display increased adiposity without signs of enhanced peripheral lipid deposition on high fat diet*

During the 16 weeks on a 45% HFD, both WT and GPS2-AKO mice gained a significant amount of weight, however, GPS2-AKO mice gained significantly more body weight than WT mice (Figure 5.1A). Similar to observations of GPS2-AKO mice on
Figure 5.2 – Increased adipose tissue size and mass are observed in GPS2-AKO mice on HFD compared to WT mice.

(A) Body composition analysis of total fat versus lean mass measured by EchoMRI scanning of WT and GPS2-AKO mice on HFD, n=2. (B) Individual adipose tissue depot and liver weights in GPS2-AKO versus WT mice after 16 weeks HFD, n=3. (C) Representative H&E staining of paraffin-embedded EPI sections from WT versus GPS2-AKO mice after 16 weeks HFD imaged at 10X magnification. Results are expressed as mean +/- SEM. Statistical significance was calculated by two-tailed Student’s t test; *represents p value < 0.05 and **p value < 0.01.
chow diet, these mice became more obese than WT counterparts upon completion of the HFD regiment (Figure 5.1B). The increase in body weight observed in GPS2-AKO mice on HFD was a result of increased fat mass, whereas no changes in mass of non-adipose tissues were observed (Figure 5.2A-B). H&E staining of AT depots, specifically the epididymal (EPI) AT, suggested that the increase in AT mass in GPS2-AKO mice was in part due to adipocyte hypertrophy as observed by the significant increase in adipocyte cell size (Figure 5.2C). Interestingly, GPS2-AKO mice showed signs of reduced lipid deposition in the liver, suggesting that the AT depots had an expanded capacity to store lipids (Figure 5.3).

The imbalance in lipid storage in GPS2-AKO mice on a chow diet could be explained by the observed changes in lipolysis and lipogenesis. It was also observed that GPS2-AKO mice had impaired lipolysis on HFD. This was determined by measuring ATGL and HSL gene expression in WAT depots and in isolated adipocytes from the EPI depot which were reduced in GPS2-AKO mice on HFD (Figure 5.4A-B). Additionally, there was a significant reduction in phosphorylated and activated HSL in WAT depots of these HFD fed mice (Figure 5.4C). Furthermore, GPS2-AKO mice on HFD had decreased circulating levels of free fatty acids and free glycerol (Figure 5.5A-B). These data suggest that GPS2-AKO mice on HFD exhibit an increased adiposity phenotype as a result of impaired lipolysis.
Figure 5.3 – Less lipid accumulation in liver of GPS2-AKO mice compared to WT mice on HFD.

(A) Representative H&E staining of paraffin-embedded liver sections from WT versus GPS2-AKO mice after 16 weeks HFD, imaged at 10X magnification.
Figure 5.4 – GPS2-AKO mice have impaired lipolysis on HFD.

(A) RT-qPCR analysis of ATGL and HSL, lipolysis markers, in EPI WAT of GPS2-AKO versus WT mice after 16 weeks HFD, n=2. (B) RT-qPCR analysis of ATGL and HSL, lipolysis markers, in EPI isolated adipocytes from GPS2-AKO versus WT mice after 16 weeks HFD, n=2. (C) WB analysis of phospho-HSL/total HSL in whole cell extracts of the EPI adipose tissue depot of GPS2-AKO versus WT mice after 16 weeks HFD.
The increased AT mass observed in GPS2-AKO mice on HFD was not only attributed to impaired lipolysis but also a significant increase in de novo lipogenesis based on the increased expression of select lipogenic genes (Figure 5.6A). In an attempt to explain how GPS2 regulates lipogenic gene expression, we reanalyzed ChIP-sequencing data available in the lab. In these data sets, we observed that GPS2 was present with the NCoR/SMRT corepressor complex on promoters of many essential genes involved in regulating lipogenesis in 3T3-L1 cells. In preliminary ChIP experiments, it was confirmed that GPS2 was present on the promoters of DGAT1 and FASN, which was lost in GPS2-AKO adipocytes (Figure 5.6B-C). These data present a potential mechanism of how GPS2 could coordinately regulate lipogenesis gene expression.

Overall, these data show that GPS2-AKO mice on HFD have decreased lipolysis and increased lipogenesis. The imbalance of these pathways can thus contribute to the increased lipid storage observed in adipocytes resulting in increased AT mass and body weight.

**Removal of GPS2 results in increased macrophage infiltration and inflammation in adipose tissue**

Although no changes in inflammatory gene expression were observed under chow diet in GPS2-AKO mice, this result was very surprising considering a clear anti-inflammatory role of GPS2 has been observed in multiple cell types. We postulated that without a nutrient challenge mice would not elicit a strong induction of inflammation and that HFD would be a better condition to investigate AT inflammation
Figure 5.5 – GPS2-AKO mice show trends for decreased circulating free fatty acids and glycerol as markers of lipolysis.

(A) Measurements of levels of circulating free fatty acids in WT versus GPS2-AKO mice after 16 weeks of HFD, n=2. (B). Measurements of levels of circulating glycerol in WT versus GPS2-AKO mice after 16 weeks of HFD, n=2.
in GPS2-AKO mice compared to WT mice. Therefore, we subjected mice to 16 weeks of 45% HFD feeding. In agreement with previous reports, we found that GPS2-AKO mice had increased expression of inflammatory genes under HFD. We observed an upregulation in genes for general macrophages, M1 phenotype macrophages, as well as M2 macrophages in EPI AT of GPS2-AKO mice, suggesting there was not a distinct polarized phenotype of macrophages (Figure 5.7A-C). To understand the cell type within the AT depot where the expression of inflammation was most pronounced, we digested EPI AT and separated mature adipocytes from the SVF, where immune cells and precursors have been shown to reside. Through fractionation, we observed that the majority of the increased inflammatory gene expression came from the SVF (Figure 5.8A). These data suggested there could be an increase in inflammatory gene expression in cell types residing in the SVF or it could represent an increase in the number of immune cells per se. We observed a significant increase in circulating levels of MCP1, the main chemoattractant responsible for macrophage infiltration into various tissues, including AT (Figure 5.8B). This observation suggested the increase in inflammatory gene expression is due to an overall increase in the macrophage population of the EPI WAT. This notion was further supported by an increased staining of the EPI depot with Mac2, a marker for macrophages (Figure 5.8C). Consistent with previous reports about GPS2 anti-inflammatory function, we have added to this body of work by reporting that GPS2-AKO mice have increased AT inflammation when challenged with HFD.
Figure 5.6 – GPS2-AKO mice have increased lipogenic gene expression potentially via loss of direct transcriptional control.

(A) RT-qPCR analysis of lipogenic gene markers in WT versus GPS2-AKO mice after 16 weeks of HFD, n=2. (B) ChIP analysis of GPS2 binding on the DGAT1 promoter in isolated adipocytes from WT and GPS2-AKO mice under chow diet. (C) ChIP analysis of GPS2 binding on the FASN promoter in isolated adipocytes from WT and GPS2-AKO mice under chow diet.
Figure 5.7 – GPS2 deficiency results in increased inflammatory gene expression in adipose tissue.

(A) RT-qPCR analysis of general macrophage markers in EPI WAT from WT versus GPS2-AKO mice after 16 weeks HFD, n=2. (B) RT-qPCR analysis of M2 phenotype macrophage markers in EPI WAT from WT versus GPS2-AKO mice after 16 weeks HFD, n=2. (C) RT-qPCR analysis of M1 phenotype macrophage markers in EPI WAT from WT versus GPS2-AKO mice after 16 weeks HFD, n=2.
*Fat-specific deletion of GPS2 results in improved systemic metabolism on high fat diet*

Since obesity and inflammation are associated with insulin resistance, we wanted to understand how the severely increased adiposity and increased inflammation in GPS2-AKO mice affected their total body metabolic parameters. Surprisingly, analysis of glucose tolerance tests in GPS2-AKO mice after 14 weeks on a HFD showed improved glucose tolerance compared to WT mice ([Figure 5.9A](#)). Comparably, fasting glucose levels were reduced in GPS2-AKO mice after both short and overnight fasts ([Figure 5.9B-C](#)). Similarly, after 15 weeks of HFD, GPS2-AKO mice showed improved insulin tolerance ([Figure 5.10A](#)), in concert, with a reduction in fasting insulin levels in GPS2-AKO mice compared to WT mice ([Figure 5.10B](#)). Additionally, GPS2-AKO mice had increased circulating levels of adiponectin, the insulin sensitizing adipokine ([Figure 5.11A](#)), however, no changes in leptin levels were observed ([Figure 5.11B](#)). These data combined corroborate that removal of GPS2 in AT is protective of systemic glucose and insulin sensitivity, despite an increase in adiposity and AT inflammation under HFD conditions.
Figure 5.8 – GPS2-AKO mice have increased macrophage infiltration in white adipose tissue.

(A) Levels of circulating MCP1 in WT versus GPS2-AKO mice after 16 weeks HFD, n=2. (B) RT-qPCR expression of F4/80 after digestion of mature adipocytes from SVF in WT versus GPS2-AKO mice after 16 weeks HFD, n=2. (C) Representative images of MAC2 staining for macrophage infiltration into EPI WAT in WT versus GPS2-AKO mice after 16 weeks HFD. DAPI staining was used for normalization.
Figure 5.9 – GPS2-AKO mice have improved glucose tolerance on high fat diet compared to WT mice.

(A) Glucose tolerance test following glucose i.p. injection (1.5 mg/g of body weight) after an overnight fast in WT versus GPS2-AKO mice after 14 weeks HFD, n=3. (B) Fasting blood glucose levels in AKO versus WT mice after 4 hour fast at 15 weeks HFD GPS2-, n=3. Results are expressed as mean +/- SEM. (C) Fasting blood glucose levels in GPS2-AKO versus WT mice after overnight fast after 16 weeks HFD, n=2.
Discussion

Although our studies of GPS2-AKO mice on HFD are preliminary and require increased cohort numbers to confirm statistical significance of many of these experiments, they have revealed an intriguing disconnection between obesity and AT inflammation in the development of insulin resistance. Specifically, we have shown that an increase in AT inflammation does not necessarily drive an insulin resistant state in the context of obesity. Intriguingly, similar reports have begun to emerge, pointing out that inflammation is not the sole causative factor in the progression of insulin resistance. In fact, inflammation has been shown to have a positive function in AT in certain contexts, most predominantly in the modulation of proper expansion and remodeling of the tissue\textsuperscript{66,219,220}. In concert with our data, these results suggest that there is a disconnection between inflammation and adiposity in the development of insulin resistance. Further studies to characterize the inflammatory status of GPS2-AKO mice as well as other models can help shed light on the progression of when AT inflammation turns from a healthy response to tissue expansion into a negative contributor to insulin resistance.

Evolutionarily, inflammation has developed as an adaptive mechanism to fight off invading pathogens. Thus inflammatory mechanisms in AT appear to have adapted as a response to excessive nutrient intake and circulating lipids to avoid systemic lipotoxicity\textsuperscript{46}. It has been shown that during obesity the role of macrophages is to clean up lipid spillover and promote lipid trafficking in states of excessive nutrient storage\textsuperscript{221}. These data suggest that insulin resistance develops when AT inflammation reaches a critical level. It would be interesting to perform a stringent profiling of macrophages in
Figure 5.10 – GPS2-AKO mice have improved insulin tolerance compared to WT mice on high fat diet

(A) Insulin tolerance test after a 0.5 U/kg body weight insulin i.p. injection in GPS2-AKO versus WT mice following a 4 h fast after 15 weeks HFD, n=3. Results are expressed as mean +/- SEM. (B) Plasma insulin levels in GPS2-AKO versus WT mice after overnight fast at the end of 16 weeks HFD treatment, n=2.
GPS2-AKO mice on HFD to understand their function and address if they are synergistically adding to the enhanced insulin signaling observed in GPS2-AKO mice, overall resulting in a more metabolically healthy mouse.

In addition, recent reports have suggested that macrophages contribute to the expansion of AT by aiding in the recruitment of adipogenic progenitors\textsuperscript{222,223}. It would be interesting to determine whether there is an increase in adipocyte progenitors of GPS2-AKO mice following macrophage infiltration. Although we observed significant adipocyte hypertrophy in GPS2-AKO mice on HFD, it would be interesting and important to determine whether there is a corresponding increase in hyperplasia. An attractive hypothesis for future experimentation is that the influx of macrophages due to HFD initiates recruitment of progenitors with subsequent adipogenesis, which then leads to a healthy, insulin sensitive AT in the GPS2-AKO mice.

Data showing that improper lipid storage, not necessarily inflammation, causes insulin resistance has led to the adipose tissue expandability hypothesis\textsuperscript{120,225}. This hypothesis states that AT has a defined limit of expansion during storage of dietary (calories) lipid and once this limit is reached the excess lipid is deposited in other tissues including skeletal muscle and liver. Such ectopic lipid deposition then leads to lipotoxicity in those tissues that results in insulin resistance which contributes to overall glucose intolerance. This hypothesis has been supported by various mouse models in which enhanced AT expansion results in increased obesity yet the mice remain metabolically healthy\textsuperscript{57}. Most notably, knockout of collagen 6, a highly enriched ECM component of adipose tissue, resulted in uninhibited, stress-free expansion of adipose
Figure 5.11 – GPS2-AKO mice have a trend towards an improved adipokine profile compared to WT mice on HFD.

(A) Plasma adiponectin levels in WT versus GPS2-AKO mice after 16 weeks HFD, n=3.
(B) Plasma leptin levels in WT versus GPS2-AKO mice after 16 weeks HFD, n=3. Results are expressed as mean ± SEM.
tissue depots on either HFD or an ob/ob background\textsuperscript{226}. This expansion was not accompanied by increased inflammation or worsening of the whole body metabolic profile of these mice, supporting the idea that healthy adipose tissue expansion can counteract insulin resistance induced by obesity\textsuperscript{57,226}. Using adiponectin overexpressing mice, it was also found that forced expansion of adipose tissue prevents metabolic disease despite excessive obesity\textsuperscript{158}. We would like to speculate that GPS2-AKO mice represent a similar model system mimicking metabolically healthy obesity. Although further investigation into the inflammatory profiling of GPS2-AKO mice must be done, the initial description of their phenotype on HFD has led to many exciting observations that change several paradigms in the field of obesity, metabolism and inflammation.
CHAPTER SIX – FUTURE DIRECTIONS, GENERAL DISCUSSION, AND FINAL CONCLUSIONS

Summary of key findings

In summary, this work has identified a novel mechanism of regulation of AKT in the insulin signaling pathway. Specifically, AKT undergoes Ubc13-mediated K63 ubiquitination which is required for its activation upon insulin stimulation. This work has also identified GPS2 as a regulator of this process via its ability to negatively regulate Ubc13. GPS2-AKO mice display a phenotype of having constitutively active insulin signaling in WAT depots, which results in decreased lipolysis and increased lipogenesis. Imbalance of these pathways results in hypertrophic adipocytes, increased fat mass and body weight. Decreased lipolysis was also confirmed in BAT, which along with GPS2 effect on mitochondrial biogenesis, results in whitening of this specific fat depot. Unexpectedly, GPS2-AKO mice remain insulin sensitive despite becoming obese on both chow and HFD, which we postulate is a function of GPS2-AKO mice having increased lipid storage capacity in AT and a reduction of peripheral tissue lipid deposition. Our work validates that GPS2 also plays an anti-inflammatory role in AT under dietary stressed conditions. Preliminary work from GPS2-AKO mice exposed to HFD demonstrates they have increased AT inflammation and macrophage infiltration, however this does not influence the metabolic flexibility of these mice. Thus, this work suggests that enhanced insulin signaling outmaneuvers effects of increased inflammatory signaling events in adipocytes, pointing to a shift in the paradigm of thinking for treating T2D and metabolic diseases.
Future directions: The role of GPS2 in regulating AKT in the insulin signaling cascade

In this work, we have identified a novel mechanism of regulation in the insulin signaling cascade in which AKT undergoes GPS2 and Ubc13 regulated K63 ubiquitination for proper phosphorylation and activation. An unanswered question of this work is mechanistically why K63 ubiquitination is necessary for AKT full activation in insulin signaling. We hypothesize that it helps drive AKT to the plasma membrane, as previously suggested in the case of IGF and EGF-mediated AKT activation. In order to address this question, it would be useful to perform fractionation experiments to see if modulation of GPS2 and Ubc13 could affect AKT membrane translocation upon insulin stimulation. Recent evidence has suggested that p62, a known K63 ubiquitin binding protein, helps bridge interactions between IRS-1 and AKT at the membrane upon insulin stimulation. This finding could help explain why this specific post translational modification of AKT is important for the activation of the insulin signaling cascade by allowing interactions with key components at the plasma membrane. Future experiments modulating p62 could help answer these pressing questions and further dissect the mechanism of AKT activation.

Prior to the studies reported in this thesis, AKT K63 ubiquitination has been studied in the context of cancer models. Mutations that add an additional lysine residue in the PH domain of AKT exhibit hyperactive AKT signaling in this context. These data suggest that K63 ubiquitination is an important post translational modification that can govern AKT signaling in cancer. Therefore, it would be interesting to see if...
defects in GPS2-mediated regulation of AKT activation are involved in tumors, especially with dysfunctional AKT/PI3K signaling. Evidence of this has been suggested in work showing that GPS2 acts as a tumor suppressor in liposarcomas. Therefore, studying the role of GPS2 in cancer progression could prove to be useful and advantageous in the search for novel targets for cancer treatment.

Lastly, K63 ubiquitination has emerged as an increasingly common posttranslational modification in signal transduction pathways. It would be useful to expand the studies of GPS2 and Ubc13 regulation to see if their modulation of kinase activation is conserved with different growth factor stimulation and extends to additional signaling pathways involved in metabolic homeostasis. This method of regulation could help further explain how metabolic pathways are coordinated within a cell.

**Future directions:** GPS2 regulation of adipose tissue lipid storage capacity, mitochondrial biogenesis and whole body metabolism

In this thesis, we have shown how GPS2 regulates various pathways involved in metabolic regulation of an adipocyte. Lipolysis, lipogenesis, and mitochondrial function are tightly linked in the coordinated response of a cell to fasting and feeding. Data showing that GPS2 can regulate all these processes leads us to speculate that it may be involved in managing communication within a cell to regulate metabolic responses from the level of signal transduction to transcriptional input as a metabolic integrator. In this context, it will be worth exploring the mechanism by which GPS2 can directly regulate lipogenic gene expression, and whether this could be a function of GPS2 with the NCoR/SMRT corepressor complex, as our data suggests GPS2 binding on lipogenic
gene promoters. In addition, our work indicates that GPS2 is localized in the cytoplasm, nucleus and mitochondria. In future directions, it would be extremely interesting to understand if GPS2 compartmentalization could lead to coordinated activation and inactivation of energy storage, utilization and oxidative phosphorylation during fasting and feeding cycles. More extensive studies in understanding GPS2 localization during fasting or growth factor stimulation can help answer some of these questions. We envision a network whereby, in fasting conditions, endogenous GPS2 is present in the cytoplasm to inhibit AKT activation and mitochondrial GPS2 translocates into the nucleus to positively and negatively regulate gene expression of mitochondrial and lipogenic genes, respectively. Under feeding conditions, we speculate that GPS2 is sequestered out of the cytoplasm, to allow for AKT activation, possibly by retaining it in the mitochondria whereby it is not permissive to enter the nucleus. Much greater detailed exploration of these mechanisms is required to prove these hypotheses but they open the possibility that GPS2 plays a key role in the coordinated regulation of metabolic homeostasis.

On a systems level, we have shown that enhancing mechanisms involved in positively regulating AT lipid storage concurs with improvements in systemic metabolism. These data suggest that these pathways could be the most successful targets to efficaciously treat metabolic diseases. Interestingly, a study parallel to ours showed that GPS2-AKO mice were not protected from insulin resistance under 60% HFD\textsuperscript{108} whereas our work revealed that GPS2-AKO mice fed either chow or 45% HFD diet remain insulin sensitive, despite being obese\textsuperscript{86}. The discrepancies in these data could
suggest that there are inherent differences as to how these models were generated or, more interestingly, that there is a threshold for the level of protection from insulin resistance with a loss of GPS2. Further characterization of the metabolic health of GPS2-AKO mice during the progression of either 45% or 60% HFD would be useful to undertake. Using comparisons between these diet treatments could help answer the question of whether there is indeed a threshold of GPS2 protection and, if so, whether it is uncontrolled lipid spillover on 60% HFD that causes insulin resistance rather than increased AT inflammation, which is observed under both diet regimens.

**Future directions: GPS2 effects on inflammation and macrophage infiltration**

Our current work in characterizing GPS2-AKO mice on HFD shows that these mice are protected from systemic metabolic dysfunction despite severe obesity and increased AT inflammation, however more work in characterizing the local and systemic inflammation in this mouse model is required. This work supports various data suggesting AT inflammation is not the sole insulin resistance driver and treating T2D with anti-inflammatory drugs may not be the most appropriate choice. For the future directions in understanding how GPS2 regulates inflammation, we plan to continue the characterization of the inflammatory profile of GPS2-AKO mice on HFD. Evidence suggests that these mice have increased macrophage infiltration however we are currently unclear about the function of these macrophages. It would be useful to answer the question of whether these macrophages display polarization of a specific M1 vs M2 phenotype to clarify why increased AT inflammation is not driving insulin resistance in this model under dietary stress. We hypothesize that these macrophages might be helping...
to remodel AT to allow for enhanced expansion. We propose this is in part based on
reports showing macrophages help to recruit progenitors\textsuperscript{223}. To verify these hypotheses,
future directions will also include experiments to sort and profile macrophages and
progenitor cells within AT depots of GPS2-AKO mice.

Furthermore, data showing that GPS2 and Ubc13 regulate both the insulin
signaling pathway and TNFα/TLR pro-inflammatory signaling cascades, suggest that
there is significantly more integration between these pathways than previously described
especially in regards to how these pathways can influence each other upon metabolic
disease progression\textsuperscript{84-86}. Interestingly, in cells lacking TRAF6, TNFα stimulation led to
prolonged hyperphosphorylation of AKT, which coincided with the activation of
upstream PI3K, suggesting these pathways are tightly intertwined\textsuperscript{231}. These studies again
point to GPS2 as a mediator of Futures studies in understanding the interplay between
these pathways and how/when they are both regulated by GPS2 and Ubc13 will provide
insight into how impaired adipocyte insulin signaling and increased AT inflammation
instigate each other in the development of insulin resistance.

\textbf{Concluding remarks}

In conclusion, this project highlights the importance of gaining a better
understanding of the casual relationship between lipid storage and obesity, inflammation,
and insulin resistance. The studies performed in this thesis display extensive evidence
that GPS2 acts as a key metabolic integrator within AT with great implications in
systemic coordination of metabolism as well. Our recent studies, along with the previous
studies of GPS2 in liver and immune cells, from seemingly diverse research areas, all
suggest that regulation of lipid metabolism and inflammation through GPS2 is fundamental to many physiologic and pathophysiologic metabolic processes. GPS2 appears to act to coordinately modulate metabolic pathways within a cell to regulate responses to nutritional cues in a synchronized manner. The coordinated actions of GPS2 allow full integration of signal transduction cascades between various cellular compartments and organelles, ultimately transmitting these signals into transcriptional program regulation. Furthermore, these studies uncover the important role of non-proteolytic ubiquitination as a novel, unappreciated regulatory mechanism in AT and immune cell metabolic pathways. This thesis represents an innovative means to comprehensively investigate the complex relationship between lipid flux and inflammation in the development of insulin resistance with important implications for the design of effective therapeutic approaches against T2D. This project is significant because understanding the role of GPS2 in AT has the potential of providing insightful information for the development of new treatments against obesity-associated metabolic disorders.
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