Roles of PI3-kinase and ARAP2 in regulating glucose metabolism

Aditi Chaudhari

Department of Molecular and Clinical Medicine
Institute of Medicine
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Cover illustration: Infinite Opportunities…

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adit.chaudhari@wlab.gu.se

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Dedicated to Aai, my grandmother

and my parents…

"*The important thing is not to stop questioning; curiosity has its own reason for existing*"

-Albert Einstein

ABSTRACT

Insulin signaling is mediated by a complex, highly integrated network which functions to control multiple metabolic and growth processes throughout the organism. A key enzyme in the insulin signaling network is phosphatidylinositol 3-kinase (PI3-kinase). PI3-kinase catalyzes the production of the lipid second messenger, phosphatidylinositol 3, 4, 5- triphosphate (PIP3), which is involved in various cellular functions such as cell growth, survival and apoptosis. In this thesis, we have investigated the impact of oncogenic mutations of PI3-kinase, as well as deletion of its key subunit isoforms on glucose metabolism. We also identified a PH-domain containing protein ARAP2, and investigated its role in lipid droplet formation.

In Paper I, we investigated the effect of combined hepatic deletion of the PI3-kinase subunits p110α and p85α (L-DKO) on insulin signaling and glucose homeostasis. L-DKO mice developed impaired glucose-tolerance, but surprisingly displayed intact IRS1-associated lipid kinase activity. The mice exhibited decreased body weight, but similar adipose tissue weight, hepatic glucose production as well as normal insulin tolerance, demonstrating a paradoxical milder phenotype compared to mice having only p110α deleted in the liver.

In Paper II, we investigated the effects of the hot spot mutations E545K and H1047R of p110α on hepatic and whole body glucose homeostasis. The expression of these mutations resulted in a reprogrammed cellular metabolism with marked accumulation of lipids and glycogen in the liver. Wild-type (wt) p110α expression did not result in hepatic lipid or glycogen accumulation despite having similarly increased expression of glycolytic and lipogenic genes. Furthermore, there was no difference in the kinase activity between the wt and mutant-expressing mice, which suggest that the metabolic effects exhibited by the p110α mutants are linked to kinase-independent function(s) of the oncogenic p110α.

In Paper III, we identified ARAP2 as a PH-domain containing protein in the lipid droplet proteome. We show that knockdown of ARAP2 leads to diminished lipid droplet formation by decreasing the rate of triglyceride synthesis. The lower triglyceride synthesis rate resulted from decreased basal glucose uptake through lower expression of GLUT1, as well as reduced GLUT1 levels in the plasma membrane and lipid micro-domains. The effect on GLUT1 was mediated by increased glucosylceramide synthesis.

Keywords: Type 2 diabetes, phosphatidylinositol 3-kinase, metabolism, lipid droplets, ARAP2

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SAMMANFATTNING PÅ SVENSKA

Insulinsignalering medieras genom ett komplext sammanflätat nätverk som kontrollerar metabola processer och cell-tillväxt. Phosphatidylinositol 3-kinase (PI3-kinase) är ett centralt enzym i detta nätverk och spelar en betydande roll i ett flertal cellulära funtioner så som upptag av glukos, syntes av fetter och proteiner och cellens överlevnad. I den här doktorsavhandlingen har vi undersökt effekten av olika mutationer i genen som kodar för PI3-kinas, som tidigare visat sig vara vanligt förekommande i cancersjukdomar. Samtidigt har vi modifierat olika viktiga komponenter av enzymet för att studera dess påverkan på glukosmetabolism. Vi har även identifierat ett nytt protein, så kallad ARAP2 och undersökt dess roll i en av cellens grundläggande processer för inlagring av fett.

Delarbete I: Här har vi använt genetiskt modifierade möss som saknar två viktiga komponenter (p110α och p85α) av PI3-kinas i levern för att studera deras påverkan på insulinsignalering och glukosmetabolism. Mössen utvecklar försämrad förmåga att upprätthålla en normal glukoshomeostas och kan inte svara normalt på insulin, men blir inte diabetiska och uppvisar normal kroppsvikt, fettvävnad och intakt glukosproduktion i levern.

Delarbete II: Vi har studerat effekterna av två vanligt förekommande mutationer av PI3-kinas, som kallas E545K och H1047R. Vi undersökte effekterna på lever och helkropps glukosmetabolism och upptäckte att mutationerna resulterade i massiv fett-och glykogen-ansamling i levern. Vi fann att denna ansamling av fett och glykogen inte orsakades av en ökad aktivitet av PI3-kinas utan skedde via helt nya och tidigare okända signalvägar för enzymet.

Delarbete III: Här har vi identifierat ett nytt protein, så kallad ARAP2, som sitter på cellulära fettdroppar. Vi visar att sänkta nivåer av ARAP2 ger upphov till minskad fettproduktion vilket leder till minskad bildning av fettdroppar. Den minskade fettproduktionen beror på minskat glukosupptag, vilket i sin tur beror på minskat uttryck av en glukostransportör som kallas GLUT1.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Hepatic deletion of p110α and p85α results in insulin resistance despite sustained IRS1-associated lipid kinase activity**
Aditi Chaudhari, Katarina Ejeskär, Yvonne Wettergren, C. Ronald Kahn, and Victoria Rotter Sopasakis.
Manuscript

II. **p110α hot spot mutations in E545K and H1047R exert metabolic reprogramming independently of p110α kinase activity**
Aditi Chaudhari*, Daniel Krumlinde*, Annika Lundqvist, Levent Akyürek, Sashidhar Bandaru, Kristina Skålén, Marcus Ståhlman, Jan Borén, Yvonne Wettergren, Katarina Ejeskär, Victoria Rotter Sopasakis.
*Equal contribution
Mol Cell Biol (2015): 3258-73

III. **ARAP2, a novel regulator of sphingolipid metabolism affects GLUT1 mediated basal glucose uptake**
Aditi Chaudhari, Liliana Håversen, Reza Mobini, Linda Andersson, Marcus Ståhlman, Emma Lu, Mikael Rutberg, Per Fogelstrand, Kim Ekroos, Adil Mardinoglu, Malin Levin, and Jan Borén.
Submitted

CONTENT

ABBREVIATIONS

T2D	Type 2 diabetes
PI3-kinase	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 3, 4-biphosphate
PIP_3	Phosphatidylinositol 3, 4, 5-triphosphate
PH-domains	Pleckstrin homology domains
Akt/PKB	Protein kinase B
PDK1	Phosphoinositide-dependent kinase 1
PLD	Phospholipase D
LD	Lipid droplet
L-DKO	Liver-specific deletion of both p110α and p85α
IRS	Insulin receptor substrates
MAPK	Mitogen activated protein kinase
ERK	Extracellular signal-regulated kinase
SREBP-1c	Sterol regulatory element binding protein-1c
GAP	GTPase-activating protein
mTORC	Mechanistic target of rapamycin complex
SHIP	Phosphatidylinositol-3, 4, 5-triphosphate
PTEN	Phosphate and tensin homolog
DNL	*De novo* lipogenesis
KO	Knock-out
CPT1	Carnitine palmitoyltransferase I
G1P	Glucose-1-phosphate
TG	Triglycerides
FFA	Free fatty acid
PEPCK	phosphoenolpyruvate carboxykinase

GK	glucokinase
G6P	Glucose-6-phosphate
TCA	Tricarboxylic acid
ChREBP	Carbohydrate response element binding protein
GS	Glycogen synthase
FAS	Fatty acid synthase
GSK3	Glycogen synthase kinase 3
AMPK	AMP-activated protein kinase
VLDL	Very-low-density-lipoprotein
ACC	Acetyl CoA carboxylase
GA3P	Glyceraldehyde 3-phosphate
DHAP	Dihydroxyacetone phosphate
GCS	Glucosylceramide synthase
G3P	Glycerol 3-phoshpate
DGAT	Diacylglycerol acyltransferase
DG	1, 2- diacylglycerol
ER	Endoplasmic reticulum
Arf	ADP-ribosylation factor
FAT/ CD36	Fatty acid translocase
FATP	Fatty acid transfer protein
apoB	Apolipoprotein B
Mttp	Microsomal triglyceride transfer protein
ARAP2	Arf GAP with RhoGAP domain, Ank repeat and PH-domain 2
Cre	Cyclic recombinase
L-p110α KO	Liver-specific p110α knockout
HPLC	High performance liquid chromatography
UPLC	Ultra-performance liquid chromatography

MS	Mass spectrometry
siRNA	Small interfering RNA
GTT	Glucose tolerance test
ITT	Insulin tolerance test
PTT	Pyruvate tolerance test
H&E	Hematoxylin and eosin
SHC	Src homologous and collagen-like
PI	phosphoinositide
ACL	ATP citrate lyase
D-PDMP	D-thero-1-phenyl-2-decanoylamino-3-morpholino-1-propanol
DL-PPMP	DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol

1 INTRODUCTION

Type 2 diabetes (T2D) is the most common form of diabetes mellitus. Complications in diabetes result in various diseases such as cardiovascular diseases, renal failure, retinopathy and peripheral vascular diseases. T2D has emerged as a worldwide epidemic (1). In 2014, global prevalence of diabetes was approximately 9% (382 million) among adults and is estimated to rise to 592 million by the year 2035, with majority occurring in the low- and middle-income countries (2). While diabetes can be managed effectively, cost of diabetes on the healthcare system presents an economic burden on the societies worldwide. In 2010, the cost of diabetes on the Swedish healthcare system was estimated to be 4000 USD per person and the global expenditure is expected to rise >30% by 2030 (3). Diabetes is considered as one of the leading causes of death and hence prevention efforts are warranted.

T2D is a result from the interaction of genetic (~5%) and environmental factors. However, high calorie food intake and sedentary lifestyle is a major contributing factor to the development of metabolic diseases, obesity and type 2 diabetes (T2D) (4).

1.1 T2D and insulin resistance

Eating stimulates the secretion of insulin from pancreatic β-cells. The postprandial rise in glucose triggers the secretion of insulin from β-cells to allow the body to control the plasma glucose levels. This control is governed by the balance between glucose absorption, production by the liver, uptake and metabolism by peripheral tissues.

T2D is a disease state involving various metabolic perturbations, particularly insulin resistance. Insulin resistance is a decreased ability of insulin to inhibit glucose production in the liver and promote glucose uptake/utilization in tissues such as muscle and adipose tissue (5). In T2D, the resistant cells do not respond to insulin produced by pancreatic β-cells, and therefore, leads to increased levels of insulin (hyperinsulinemia) as a result of over-secretion of the hormone to compensate for the increased glucose levels (hyperglycemia). After an initial over-secretion of insulin, β-cells cannot keep pace to the

increasing demand of insulin and at later stages fail to produce and secrete insulin leading to the development of T2D (6, 7).

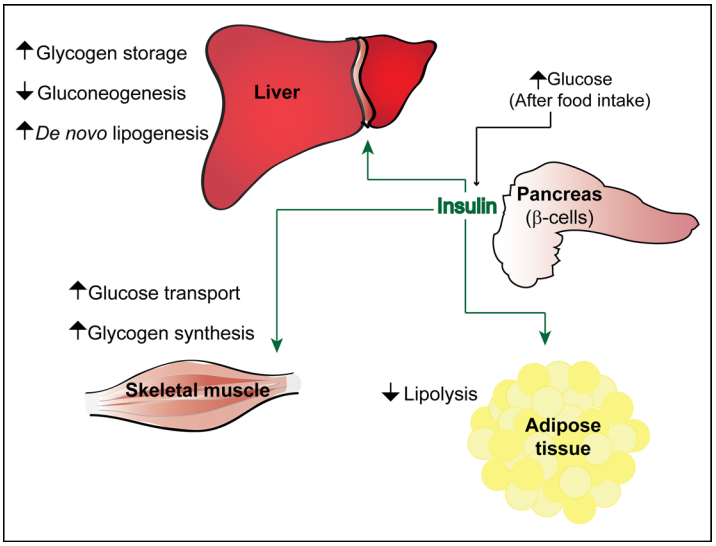

Figure 1. Actions of insulin in the control of whole-body metabolism

Insulin is a peptide hormone that plays a central role in the regulation of glucose homeostasis. Insulin triggers highly diverse physiological effects such as inhibiting hepatic glucose production and increasing glucose uptake in muscle and fat, serving as the primary regulator of blood glucose levels. It also promotes the storage of excess glucose in the form of glycogen in liver and muscle. In addition, to maintain glucose homeostasis, insulin also induces fat storage. In adipose tissue, insulin stimulates lipogenesis while inhibiting lipolysis; and it induces fatty acid uptake from the blood stream (8, 9) (Figure 1). Thus, impaired insulin action plays a major role in development of metabolic diseases.

1.2 Insulin signaling pathway

Insulin signaling is mediated by a complex, highly integrated network which functions to control multiple metabolic and growth processes throughout the organism (10). Insulin binds to the insulin receptor and leads to tyrosine phosphorylation of the insulin receptor itself and several other docking proteins such as insulin receptor substrates (IRS1 and IRS2) and src-homologous and collagen like protein (SHC) that then become phosphorylated by the receptor. These phosphorylated docking proteins then activate two major signaling

pathways, the Ras-mitogen-activated protein (MAP) kinase pathway and the phosphatidylinositol 3-kinase (PI3-kinase) – protein kinase B (Akt/PKB) pathway (Figure 2).

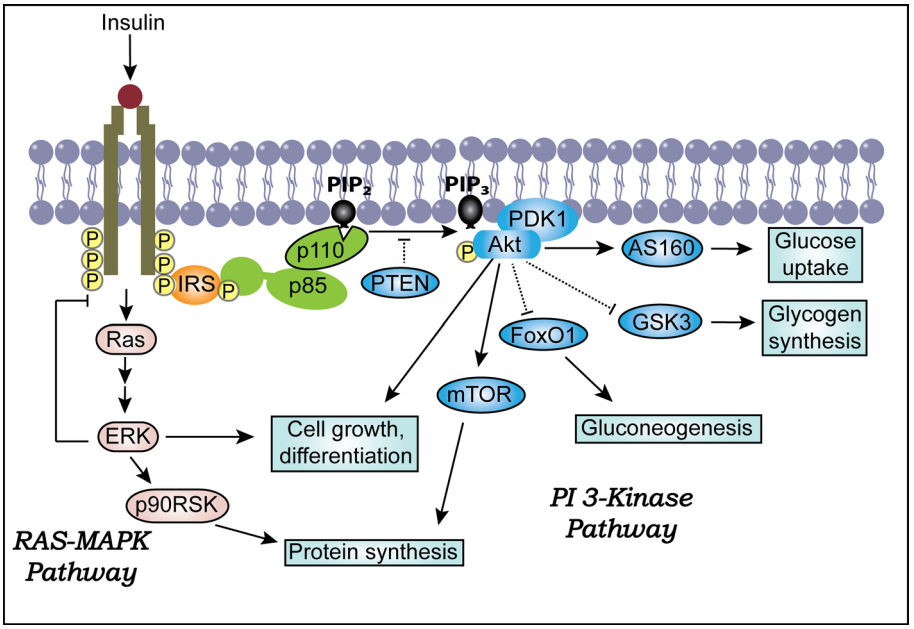

Figure 2. Schematic representation of pathways activated by insulin, PI3-kinase and MAP kinase. IRS, Insulin receptor substrate, p110-p85, subunits of PI3-kinase; PIP_2, phosphatidylinositol 4,5-biphosphate; PIP_3, phosphatidylinositol 3,4,5- triphosphate; PDK1, 3-phosphoinositide-dependent protein kinase 1; Akt, protein kinase B; PTEN, phosphatase and tensin homolog; AS160,Akt substrate of 160 kDa; GSK3, glycogen synthase kinase 3; FoxO1, Forkhead box protein O1; mTOR, mechanistic target of rapamycin; Ras, small GTPase; ERK, extracellular signal-regulated kinase; p90RSK, ribosomal S6 kinase

The MAP kinase pathway involves the tyrosine phosphorylation of the IRS proteins and/ or SHC, which in turn triggers a kinase cascade that phosphorylates and activates MAP kinase pathway and leads to the activation of extracellular signal-regulated kinase (ERK). Activation of ERK stimulates protein synthesis and cell proliferation and differentiation (11). In addition, hepatic ERK activation has been reported to enhance the transactivation of sterol regulatory element binding protein-1c (SREBP) target genes (12-14), to decrease energy expenditure and the expression of genes involved in fatty acid oxidation (15).

After phosphorylation, the docking proteins IRS (IRS1 or IRS2) activate the PI3-kinase pathway that regulates the metabolic actions of insulin. PI3-kinase then catalyzes the phosphorylation of the phosphatidylinositol (4,5)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)- trisphosphate (PIP_3), an important lipid second messenger that propagates metabolic signaling (16, 17). Inhibitors of PI3-kinase block almost all metabolic actions of insulin, which highlights the pivotal role of PI3-kinase in metabolic actions of insulin (18). PIP_3 controls a wide range of cellular processes *via* the downstream proteins such as 3-phosphoinositide-dependent protein kinase 1 (PDK1), Akt/PKB and nucleotide-exchange factors or GTPase-activating proteins (GAPs) for GTPases of the Rho, Ras and Arf families. PIP_3 is a recognition site for proteins containing pleckstrin homology (PH) domains, and is therefore involved in recruiting PH-domain containing proteins to the plasma membrane for activation. PIP_3 recruits PDK1 and Akt to the plasma membrane where PDK1 phosphorylates and activates Akt/PKB. Full activation of Akt involves phosphorylation at two specific sites – threonine (Thr) 308 and serine (Ser) 473, as well as PH-domain mediated lipid binding. PDK1 phosphorylates Akt at Thr308 (19). PIP_3 can also bind to mechanistic target of rapamycin complex 2 (mTORC2) (20), thus phosphorylating Akt at Ser473 (21). PIP_3 can be dephosphorylation at 5´- and 3´- positions by lipid phosphatases, phosphatidylinositol (3,4,5)-trisphosphate 5-phosphatase (SHIP) (22) and phosphatase and tensin homolog (PTEN) (23) respectively. A number of known downstream targets of Akt have been described, including glycogen synthase kinase 3 (GSK3) (glycogen synthesis), FoxO (apoptosis) and p70 S6 kinase (protein synthesis). Phosphorylation and activation of Akt triggers immediate downstream pathways such as *de novo* lipogenesis (DNL), gluconeogenesis, lipolysis, cellular uptake of glucose and protein synthesis (10).

The signaling mechanisms of PI3-kinase in various biological responses downstream of insulin have proven to be elusive. This thesis provides evidence of a novel way for PI3-kinase to transmit signals that play an important role in regulation of glucose and lipid metabolism.

1.3 PI-3 kinase and its role in metabolism

PI3-kinase is particular important for the insulin signal transduction and serves as a critical node in the insulin signaling pathway. PI3-kinase generates lipids

that regulate a variety of intracellular processes including glucose uptake, protein synthesis and glycogen synthesis (10). There are three main classes of PI3-kinase (24), of which class IA has been shown to play a pivotal role in insulin signaling. Class II PI3-kinases can be activated by tyrosine kinases, cytokine receptors and integrins, and are mainly known to regulate membrane trafficking and receptor internalization. The third class of PI3-kinase is thought to be involved in the mTOR mediated regulation of autophagy which is integrated with the insulin signaling pathway. In this thesis, we have studied the PI3-kinase class IA type (25).

Table 1. Three main classes of PI3-kinase.

PI3-kinase	Isoforms of PI3-kinase	Main substrate	Second messenger	Domain attracted to second messenger
Class IA	PI3Kα, PI3Kβ, PI3Kδ	PIP_2	PIP_3	Pleckstrin homology (PH) domain
Class IB	PI3Kγ			
Class II	PI3K-C2α, PI3K-C2β, PI3K-C2γ	PI, PIP	PIP, PIP_2	
Class III	Vps34	PI	PIP	FYVE domain

1.3.1 PI3-kinase class IA

PI3-kinase is a heterodimeric lipid kinase consisting of a regulatory subunit, p85, and a catalytic subunit, p110. Both subunits, however, exist as several isoforms. The catalytic subunit isoforms p110α, p110β, p110δ are encoded by the *Pik3ca*, *Pik3cb* and *Pik3cd* genes respectively (25). p37δ is a splice variant of p110δ and lacks the kinase domain completely (26). p110α and p110β are ubiquitously expressed whereas p110δ is mainly found in hematopoietic cells (27, 28).

The regulatory subunit isoforms of PI3-kinase are derived from three distinct genes *Pik3r1*, *Pik3r2* and *Pik3r3*. *Pik3r1* encodes p85α, along with the splice isoforms p55α and p50α (70-80% of the total), while *Pik3r2* and *Pik3r3* encodes for p85β and p55γ respectively. p85α is ubiquitously expressed and accounts for majority of the regulatory subunits in the cell whereas p55α and

p50α are expressed primarily in skeletal muscles and liver, respectively (10, 25) (Figure 3).

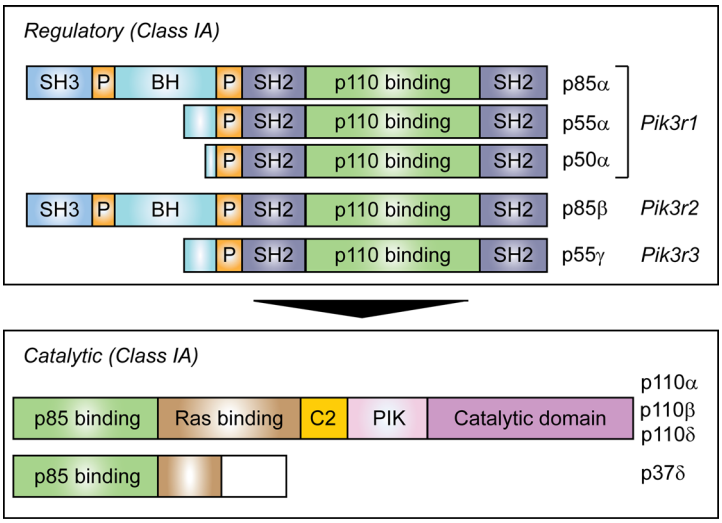

Figure 3. Class IA PI3-kinase, regulatory and catalytic subunit composition and domain structure. SH3, src homology 3; BH, breakpoint cluster region homology; SH2,src homology 2

The regulatory subunits have the ability to recruit the catalytic subunits, p110, to proteins containing tyrosine phosphorylated motifs (Figure 4). In an unstimulated state, the p85 stabilizes the thermally unstable catalytic subunit p110 and conformationally inhibits its lipid kinase activity (29). During insulin stimulation, the p85 subunit recruits the p110 subunit in close proximity of its lipid substrates and mediates the interaction between p110 and IRS1 by its SH2 domains (30, 31). This association between p85 and the IRS1 relieves the inhibition of p85 on p110 allowing the latter to generate PIP_3 (29, 32).

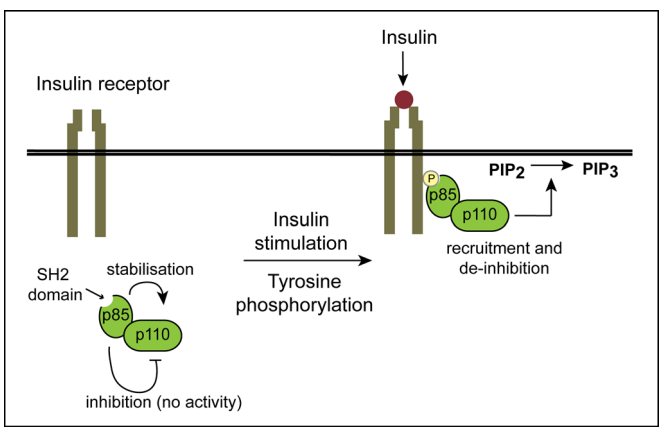

Figure 4. The role of p85 regulatory subunit in the regulation of PI3-kinase activity. SH2 domain, Src homology 2; PIP_2, phosphatidylinositol (4,5)-biphosphate;PIP_3, phosphatidylinositol (3,4,5)-triphosphate.

Regulation of the p85-p110 PI3-kinase is complex, and protein expression of p110 and p85 subunits is often interlinked. Deletion of p85α leads to a severe reduction in the expression of the most abundant p110 isoforms (p110α, p110β and p110δ). However, deletion of p85β, which is expressed at lower levels than the p85α, does not affect the expression of other catalytic subunits (33). This is in line with earlier studies indicating that the regulatory subunit stabilizes the p110 catalytic subunit (29).

In addition to the effects of the regulatory subunit on p110, targeting *Pik3r1* alters the expression of other regulatory subunits. For example, mice with homozygous or heterozygous knockout (KO) of *Pik3r1* have upregulated p85β expression (33, 34). No such alterations are found when *Pik3r2* gene is deleted. Thus, the upregulated regulatory subunits in *Pik3r1* KO mice might have distinct biological and signaling functions. KO of one type would facilitate recruitment of the remaining regulatory subunits for regulating signaling pathways. Studies have shown that the truncated form of p85α, p50α can compensate for the loss of full length p85α by being hyper-responsive to insulin (35). Given the important role of p85 in stabilizing p110, it is interesting that mice lacking all p85 isoforms show enhanced insulin sensitivity (36). Studies have shown that free p85 subunits act as a dominant negative regulator to inhibit PI3-kinase signaling by binding to tyrosine kinases, thus preventing the recruitment of catalytically competent p85-p110 heterodimers to the receptors (29, 37). Also, free p85 is able to sequester activated IRS1 in the cytoplasm, preventing the interaction between IRS1 and PI3-kinase (38). Thus, in addition to its role as a positive regulator, the regulatory subunit in its monomeric form is recognized as a negative regulator of PI3-kinase and insulin signaling.

Several knockout and transgenic mice studies have determined the role of catalytic subunits of PI3-kinase in mediating insulin signaling *in vivo*. Mice lacking either p110α or p110β die early in embryonic development (39, 40), indicating specific roles for each isoform during embryogenesis. However, mice with either p110α or p110β heterozygous deletion were viable and had no effect on insulin signaling, whereas mice doubly heterozygous of both isoforms, exhibit glucose intolerance (41), suggesting that both isoforms contribute to insulin signaling. Interestingly, mice with a kinase-dead knock-in form of p110β (p110β $^{K805R/wt}$) are viable, exhibiting mild late onset insulin resistance and only partially impaired Akt activation (42). In contrast, mice

with heterozygous knock-in of a kinase-dead allele of p110α (p110α $^{D933A/wt}$) showed a robust reduction in insulin-stimulated PI3-kinase activity, indicating that p110α plays a critical role in insulin signaling (43). Additionally, we have shown that liver-specific ablation of p110α results in impaired insulin action and glucose homeostasis. Overexpression of hepatic p110β did not improve the phenotype of these mice, suggesting that p110α plays a specific and critical role in the metabolic actions of insulin (44). Taken together, it is evident that p110α is the predominant catalytic isoform signaling downstream of tyrosine kinases.

1.3.2 PI3-kinase and cancer

Growing evidence suggests that T2D and insulin resistance are independent risk factors for development of several types of cancers (45-49). Increases in insulin and glucose levels might affect tumor growth by affecting cellular energy metabolism, ER stress, and dysfunctional autophagy or by increased levels of bio-active IGF-1 induced by insulin. In a recent study, hyperinsulinemia in postmenopausal women correlates with increased risk of breast cancer (50). Other studies have reported association between diabetes and endometrial, colorectal, pancreatic and liver cancer (51). A link between impaired PI3-kinase signaling and pancreatic cancer have also been reported (52). It is evident that insulin resistance is strongly associated with certain types of cancers and it is particularly interesting that PI3-kinase is a critical node in the insulin signaling pathway, controlling both metabolism and cell growth.

PI3-kinase is important for maintaining an intact metabolic state and is frequently mutated in various cancers (53-55). Several somatic mutations in the gene encoding p110α have been identified in various human cancers. Among them, E542K, E545K and H1047R account for more than 80% of all mutations in tumor cells and are often referred to as the hot spot mutations (56). The E542 and E545 mutations are located in the helical domain of p110α whereas the H1047 mutation resides in the kinase domain (Figure 5). The crystal structure analysis of the PI3-kinase complex showed that many of these mutations occur at the interfaces between p110α and p85α, or between the kinase domain of p110α and other domains within the catalytic subunit, thus affecting the regulation of the kinase activity of p85α or the catalytic activity of PI3-kinase (56, 57). H1047R is thought to constitutively activate the enzyme,

whereas mutation E545K might block the inhibitory effects of the regulatory subunit p85 on p110α (58, 59).

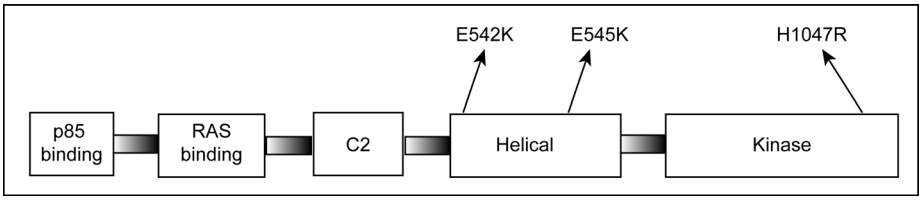

Figure 5. Schematic figure of the catalytic subunit of PI3-kinase and its functional domains showing hot spot mutations of p110α

These mutants have been shown *in vitro* to promote cellular proliferation and invasion by hyperactivating the downstream target Akt/PKB through increased lipid kinase activity (60, 61). However, virtually nothing is known about how p110α and its mutants affect cell- and whole-body metabolism. As these mutations exert gain of function effects, there is a large interest to create p110α kinase inhibitors as cancer therapy. Therefore, it is important to delineate whether there is a specific connection between PI3-kinase and cancer. Initially, we pondered on the potential link between increased insulin levels and oncogenic p110α mutations. We hypothesized that increased insulin levels would enhance the detrimental effects of p110α mutations E545K and H1047R. This thesis work provides evidence that the p110α hot spot mutations induce cellular metabolic reprogramming that would create particularly beneficial conditions for tumor growth and survival.

1.4 Glucose metabolism

Blood glucose levels in healthy individuals are normally maintained at ~90 mg/dl. This is a result of an intricate balance regulated by hormonal or nutritional signal between glucose utilization, production and removal of glucose from the blood stream. The liver plays a major role in whole-body glucose homeostasis by maintaining this balance between glucose production and glucose storage in the form of glycogen.

The liver produces glucose by breaking down glycogen (glycogenolysis) and by *de novo* synthesis of glucose (gluconeogenesis). Gluconeogenesis and glycogenolysis are two pathways that are interrelated to each other; a decrease

in gluconeogenesis generally is accompanied by an increase in glycogenolysis and *vice versa*. Hepatic gluconeogenesis is initiated by the induction of pyruvate carboxylase in the abundance of acetyl CoA. Inhibition of hepatic carnitine palmitoyltransferase I (CPT1), a mitochondrial fatty acid transporter leads to decreased fatty acid oxidation which represses hepatic gluconeogenesis (62).

1.4.1 Glycogenolysis and gluconeogenesis

Under fasting conditions, the liver provides energy to the body by breaking down glycogen and during prolonged starvation by gluconeogenesis (63). Glycogen phosphorylase is a major enzyme involved in glycogenolysis, which cleaves the glucose from the glycogen chain and produces glucose-1-phosphate (G1P) (64). G1P can be converted to G6P by phosphoglucomutase, which can then be incorporated into glycolysis, depending on the energy status of the cell.

Hepatic gluconeogenesis is initiated intramitochondrially when pyruvate carboxylase (PC) is induced in abundance of acetyl CoA, to form oxaloacetate. Oxaloacetate is eventually converted to glucose via several enzymatic processes (65, 66). Gluconeogenesis is regulated via the transcriptional activation of phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase and fructose-1,6-biphosphatase *via* the PI3-kinase pathway.

Overexpression of PEPCK in mice promotes insulin resistance (67). In contrast, PEPCK KO mice show decreased gluconeogenesis but importantly, they show a decreased removal of TCA anions that cause hepatic triglyceride (TG) accumulation and steatosis (68).

1.4.2 Glycolysis and glycogen synthesis

In the postprandial state, hepatic uptake of glucose from the bloodstream is mediated by glucose transporter GLUT2, a membrane bound transporter with high capacity and low affinity to glucose. GLUT2 functions in both taking up glucose from the bloodstream and releasing it to maintain glucose homeostasis (69). Another glucose transporter, GLUT1 is expressed in most cells and is responsible for basal glucose uptake (70). The expression and activity of both these glucose transporters is independent of insulin signaling. The regulation of GLUT1 is assumed to play an important role in insulin resistance (71).

Once taken up from the bloodstream, glucose is phosphorylated to glucose-6-phosphate (G6P) by the liver glucokinase (L-GK), a rate limiting enzyme for hepatic glucose utilization. In its phosphorylated form glucose is retained in the hepatocytes and cannot be exported back into the circulation. Depending on the metabolic state of the body, G6P is further processed by glycolysis or utilized for glycogen synthesis. Glycolysis (Figure 6) is a ten-step process that metabolizes glucose to produce pyruvate. The first committed step in glycolysis is the conversion of fructose-6-phosphate to fructose-1, 6-biphosphate (fbp1), catalysed by phosphofructokinase 1. The final step in glycolysis is the conversion of phosphoenolpyruvate to pyruvate, catalysed by pyruvate kinase. Pyruvate is further decarboxylized to acetyl CoA and then enters the tricarboxylic acid (TCA) cycle or utilized for *de novo* lipogenesis. Moreover, glycolysis is transcriptionally regulated by two major transcription factors SREBP-1c and carbohydrate response element binding protein (ChREBP). An alternative way to degrade glucose in hepatocytes is through the pentose phosphate pathway, which provides the cells with NADPH.

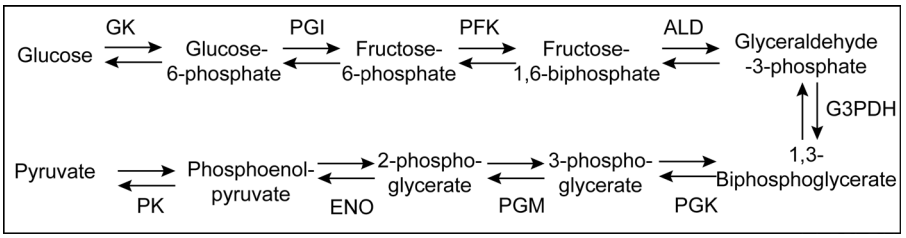

Figure 6. Schematic overview of key enzymes and metabolites involved in glycolysis. GK, glucokinase;PGI, phosphoglucoseisomerase;PGK, phosphofructokinase;ALD, aldolase; G3PDH, glyceraldehyde-3-phosphatase; PGK, phosphoglycerate kinase; PGM, phosphophoglycerate mutase; ENO, enolase; PK, pyruvate kinase

Glycogen synthase (GS) is a major enzyme that catalyses the formation of UDP-glucose from G6P (72). GS is activated by the allosteric activator, G6P and is inactive in its phosphorylated state. GS is inactivated by phosphorylation by glycogen synthase kinase 3 (GSK3), a downstream target of Akt/PKB. Glycogen synthesis is activated via the insulin-Akt-mediated inactivation of GSK3, thus resulting in an activation of GS and increased glycogen stores. Glycogen synthesis is also regulated by the protein phosphatase 1, which is activated by insulin. PP1 dephosphorylates and activates GS while inhibiting glycogenolysis by dephosphorylating glycogen phosphorylase. In addition, GS

can also be phosphorylated by AMP-activated protein kinase (AMPK) and protein kinase A.

Two prominent transcription factors SREBP-1c and ChREBP regulate the activities of the enzymes involved in glycolysis. ChREBP is activated by glucokinase, which requires the enhanced glucose uptake (73, 74) while SREBP-1c transcription is induced by the activation of the PI3-kinase pathway. In addition, ChREBP and SREBP-1c have also been shown to regulate lipogenesis through the activation of lipogenic genes (75, 76) and the Akt regulated very-low-density lipoprotein (VLDL) production (77, 78). Liver-specific SREBP-1c KO mice (79) and liver-specific ChREBP KO mice (80) exhibit impaired activation of lipogenic genes thus, confirming the roles of these transcription factors in regulation of hepatic glycolysis and fatty acid synthesis. This shows the close interaction between glucose and lipid metabolism (summarized in Figure 7).

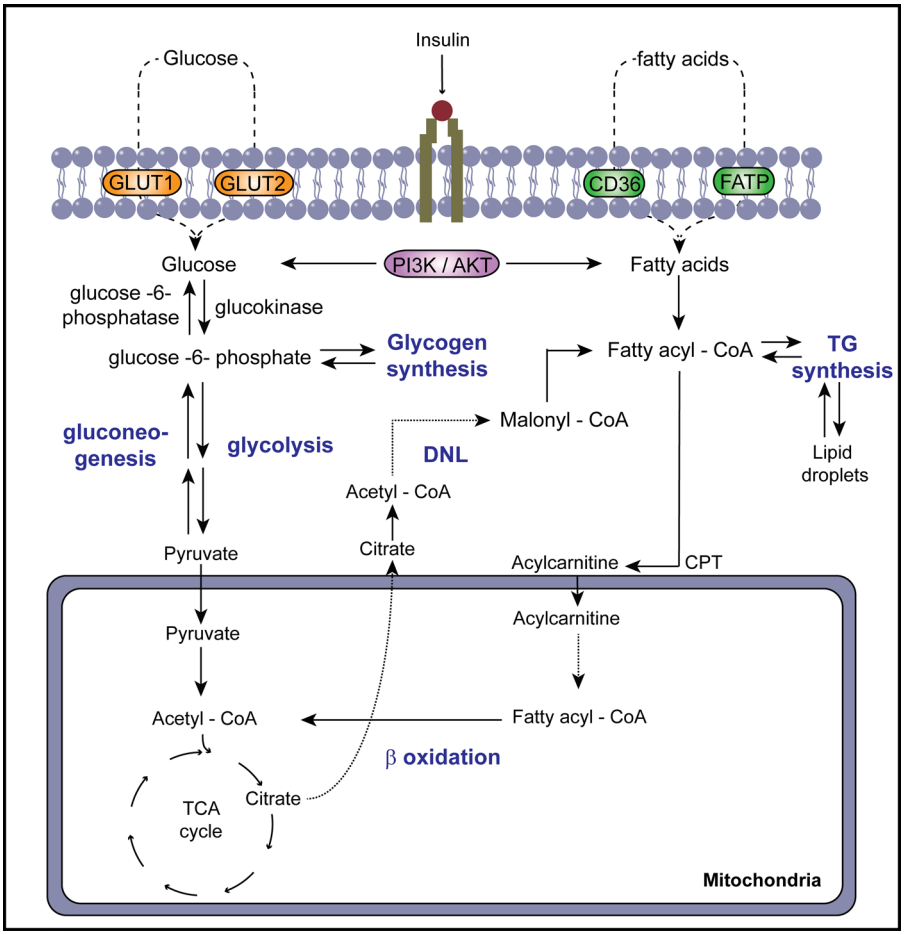

Figure 7. Schematic overview of hepatic lipid and glucose metabolism. GLUT, glucose transporter; CD36, cluster of differentiation 36; FATP, fatty acid transport protein; CPT, carnitine palmitoyltransferase; PI3K, phosphatidylinositol 3- kinase; Akt, protein kinase B; *DNL, de novo lipogenesis; TG, triglyceride; Fatty* acyl CoA, acyl CoA synthethase

1.5 Hepatic lipid metabolism

Lipids play a crucial role in different biological processes of the living cells such as storing energy, signal transduction and membrane structure. Lipids are a broad group of molecules which among others include fatty acids (FAs), glycerolipids and sphingolipids. FAs are comprised of an even number of carbon atoms with one carboxyl group. This carboxyl group serves as an important building block for the formation of more complex lipids. Glycerolipids comprise of essentially all glycerol-containing lipids among which include mono-, di- and tri-glycerols, in which FAs are esterified to form a glycerol backbone. Phospholipids that are part of the glycerolipid group and are comprised of two FAs esterified to glycerol and a hydrophilic (attracted to water) head group, making phospholipids hydrophilic in nature. Sphingolipids are a complex family of molecules that are structurally similar to phospholipids, but are comprised of a sphingoid backbone and FAs. Phospholipids and sphingolipids are major constituents of the cell membrane.

1.5.1 Hepatic *de novo* lipogenesis

Lipids are synthesized by two major processes, *de novo* lipogenesis (DNL) or esterification of free fatty acids (FFA) by an active uptake from the bloodstream into hepatocytes (Figure 8). DNL involves generation of FA from acetyl CoA or malonyl CoA and further processed into TG, to meet the needs of various cellular functions such as cellular membranes and signal transduction. FAs are either processed into TG and stored or rapidly metabolized *via* β-oxidation, depending on the cellular metabolic state. Lipids are synthesized endogenously from dietary sources such as carbohydrates or from endogenously stored energy depots.

Dietary carbohydrates are broken down to six carbon monosaccharides such as glucose that are subsequently metabolized to produce glyceraldehyde 3-phosphate (GA3P) and dihydroxyacetone phosphate (DHAP). These intermediates are then converted to pyruvate. Pyruvate then enters the TCA cycle in the mitochondria, for energy production, to yield citrate (81). Citrate is converted to acetyl CoA by the action of adenosine triphosphate citrate lyase (ACL), which is the first step in endogenous FA synthesis.

Fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) are responsible for carrying out the steps involved in FA synthesis. FAS and ACC expression

is stimulated by insulin *via* the PI3-kinase pathway (82) and mainly mediated by SREBP-1c and ChREBP (83).

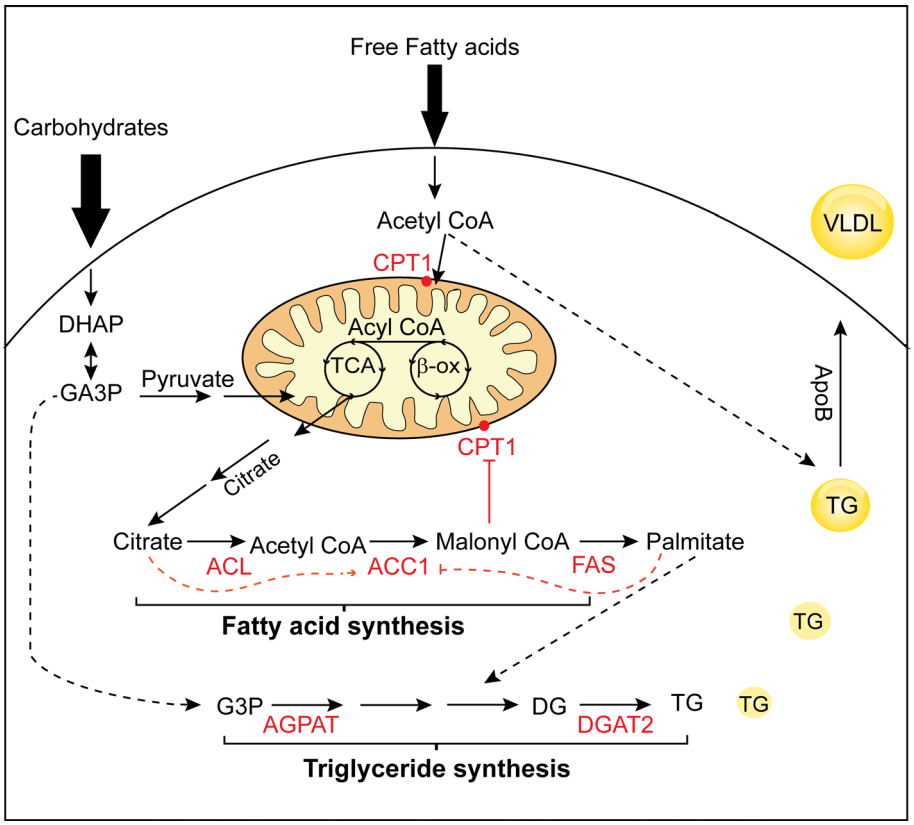

Figure 8. Hepatic de novo lipogenesis. DHAP, dihydroyacetone phosphate (DHAP); GA3P, glyceraldehyde 3-phosphate; ACL, ATP citrate lysate; ACC, acetyl carboxylase; FAS, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; G3P, glycerol-3 phosphate; DAG, 1,2-diacylglycerol; DGAT, diacylglycerol acyltransferase; TG, triglycerides; VLDL, very-low-density lipoprotein;CPT, carnitine palmitoyltransferase 1 [adapted from (84)]

DNL is initiated when acetyl CoA is carboxylated by ACC isoforms to form malonyl CoA, which is an intermediate that serves as a primary substrate for FA synthesis (85, 86). ACC isoforms can be regulated by phosphorylation of AMPK and phosphorylation of insulin-dependent Akt (87-89). The reaction catalysed by ACC is reversed by an enzyme malonyl CoA decarboxylase (MCD), which is another target of AMPK (90). Importantly, accumulation of malonyl CoA as a result of increased DNL, leads to the inhibition of the mitochondrial fatty acid transporter CPT1, a rate limiting enzyme for β-

oxidation in the mitochondria (91-94). Malonyl CoA further undergoes elongation by FAS to form a long chain fatty acid, palmitate (95). Palmitate is the major product of lipogenesis and can be further desaturated by stearoyl-CoA desaturase-1 (SCD-1) to form palmitoleic acid and oleic acid, major substrates for TG synthesis. Palmitate or AMPK is able to allosterically inhibit ACC (96).

Palmitate together with serine is able to induce endogenous *de novo* synthesis of ceramides. Alternatively, generation of ceramides is triggered by the action of sphingomyelinases, which hydrolyze sphingomyelin to yield ceramides. Once formed ceramides act as a central hub in sphingolipid metabolism. Ceramides are thought to activate PP2A and thus subsequently result in dephosphorylation and inactivation of Akt (97). Ceramides can then be converted to sphingomyelin by the action of sphingomyelin synthases (SMS1 and SMS2) or to glucosylceramide *via* glycosylation by glucosylceramide synthase (GCS). Glucosylceramide is subsequently converted to lactosylceramide which is later converted to complex glycosphingolipids such as GM3, GM2, GM1 and GD1 (Figure 9).

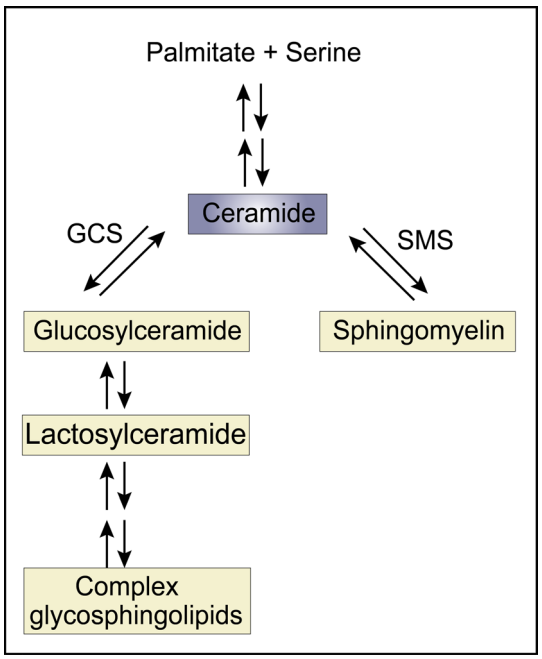

Figure 9. Summary of pathway leading to the formation of complex sphingolipids. GCS, glucosylceramide synthase; SMS, Sphingomyelin synthase

1.5.2 Triglyceride synthesis

FFA concentrations within the cells are maintained by constant incorporation/ storage into TGs or *via* oxidation to produce energy. Most TGs are formed through re-esterification of pre-existing FAs or by *de novo* incorporation of glycerol 3-phosphate (G3P) and sequential esterification of two fatty-acyl-CoA substrates by sn-1-glycerol-3-phosphate acyltransferase (GPAT). TG synthesis is catalysed by sn-1-acyl-glycerol-3-phosphate acyltransferase (AGPAT) and sn-1, 2-diacylglycerol acyltransferase (DGAT) (98) (Figure 8). DGAT exists in two isoforms DGAT-1 and DGAT-2 that catalyse the final esterification step of converting 1,2-diacylglycerol (DG) into TG (99). Both proteins are found in the ER. Studies showed that overexpression of either DGAT1 or DGAT2 increases TG synthesis while their absence decreases TG synthesis (100, 101). DGAT2 overexpression in cells results in larger accumulation of TG (102).

Hepatic accumulation of DG or phosphatidic acid is associated with impaired insulin signalling (103, 104) while accumulation of triglycerides, which are relatively inert may protect against lipotoxicity-induced insulin resistance (105). Therefore, incorporation of fatty acids into TG plays an essential role in preventing the accumulation of intracellular lipids in the liver that can cause liver dysfunction (106). TG synthesis is an integral part of the hepatic lipid metabolism that maintains the whole body lipid balance and dysregulation in this pathway can have detrimental effects on hepatic metabolism.

1.5.3 Lipid droplets

TGs are a major source of dietary energy and are crucial for both cellular and physiological energy homeostasis. Once formed, the majority of TGs are stored as neutral lipids in organelles known as lipid droplets (LDs). TGs are believed to be synthesized mainly in the ER (107, 108); although enzymes responsible for TG synthesis are also present on LDs (109). Numerous mechanisms have been proposed (110), but the precise mechanism for the formation and maturation of lipid droplets is poorly understood. LDs are thought to form *de novo* (111, 112) or could be derived from existing LDs by fusion (113).

One model suggests that neutral lipids synthesized in the endoplasmic reticulum (ER), accumulate in the lipid bilayer of the ER and as the concentration increases the lipids accumulate and form a lens which then develops into the core of the lipid droplet. The matured lipid droplet then buds

from the ER membrane to form an independent organelle with a phospholipid monolayer. Thus, the core of the LD is composed of neutral lipids which are bounded by a phospholipid monolayer studded with a unique set of proteins (114, 115) (Figure 10). In addition to storage of lipids, lipid droplets are linked to several cellular functions such as protein degradation, response to ER stress and protein glycosylation (116).

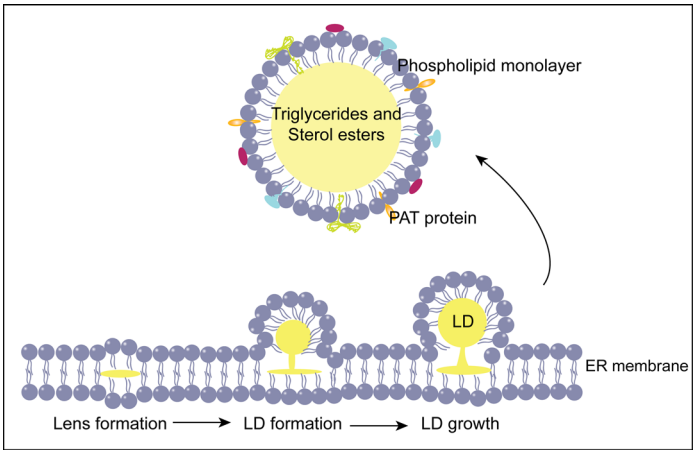

Figure 10. Overview of lipid droplet formation. Lens formation, initial accumulation of TG; LD, lipid droplet; ER, endoplasmic reticulum

LDs contain members of the proteins including perilipins (PLINs), adipose differentiation-related protein (ADRP/ also named as Plin 2) and the tail interacting protein 47 kDa (TIP47/ also named as Plin 3) (117-119). LD-associated proteins play an important structural role in regulating lipolysis or interacting with other proteins. Plin2 KO in the liver leads to reduced hepatic TG levels and improves insulin sensitivity, however the reduction in both Plin2 and Plin3 causes insulin resistance (120, 121). This suggests the importance of lipid droplet protein in insulin signalling. Further determination of the additional/ new LD-associated proteins holds the key to resolve the functional regulations of these cellular organelles. Proteomic analyses revealed one group of proteins, ADP-ribosylation factor (Arf) proteins (122), which are small GTPases that regulate intracellular traffic (123). Arf1-COPI proteins have been shown to regulate lipid droplet morphology and lipid utilization (124). Understanding the lipid droplet formation, accumulation and turnover allows us to gain more knowledge about how organs regulate circulating lipids.

1.5.4 Hepatosteatosis

Hepatosteatosis, also known as non-alcoholic steatohepatitis (NASH), is characterized by excessive lipid deposition within the LD in liver; and develops when fatty acid availability and *de novo* synthesis exceed the hepatic fatty acid disposal by oxidation and triglyceride export. It is well known that obesity, diabetes and insulin resistance are major risk factors involved in the development of hepatosteatosis (125). Accumulation of lipids in the liver activates various inflammatory cascades and fibrogenesis leading to chronic hepatic inflammation (126). In steatosis, FFA delivery to the liver is increased and accumulation of certain lipid species within cells cause cellular toxicity leading to impaired insulin signalling and mediate hepatocyte inflammation (127). Thus further development of steatosis into NASH is highly dependent on the exposure of the liver to lipotoxic species. It is well documented that accumulation of TG protects hepatocytes from cytotoxic effects of FFA-induced damage (128). The potential lipotoxic molecules include cholesterol (129-131) as well as diacylglycerol (132) and ceramides (133). These are often considered as products of impaired mitochondrial oxidative metabolism. Triglyceride accumulation is not hepatotoxic and could represent a defensive mechanism to balance excess FFAs (128, 134).

1.5.5 Fatty acid uptake

FFA derived from lipolysis (hydrolysis of TG) can be taken up by hepatocytes directly from the blood stream by passive absorption or facilitated by transport proteins. During passive absorption, the FFA passes through the membrane via flip flop and diffusion (135). However, the majority of the FFA uptake is mediated *via* a family of transporters: fatty acid translocase (FAT/ CD36), and fatty acid transport proteins (FATP). Mice lacking FATP2 or FATP5 in the liver exhibit decreased hepatic FA uptake (136, 137). CD36 is the most extensively studied and key to the FA transporter in the liver. *In vivo* studies show that overexpression of CD36 in the liver increases hepatic FA uptake (138), while CD36 deficient mice have elevated circulating FFA and TG levels and develop insulin sensitivity along with decreased circulating glucose (139, 140). Additionally, in HFD-fed mice, liver-specific deletion of CD36 reduces fatty liver and improves insulin sensitivity, whereas hepatic overexpression of CD36 exacerbated fatty liver (141).

FAs are disposed by the liver either by oxidation or by secretion as VLDL. FA oxidation is largely influenced by the FA influx, whereas VLDL secretion is more dependent on the FA influx and hormonal changes.

1.5.6 Fatty acid oxidation

In the liver, fatty acids are catabolized through the β-oxidation pathway in the mitochondria. FA oxidation and expression of FA transport proteins is closely regulated by various nuclear receptors such as peroxisome proliferator-activated receptors (PPARα and PPARγ) (142, 143). PPARα knockout supresses the expression of genes involved in FA uptake and oxidation resulting in a decreased basal-state hepatic FA uptake and oxidation (144).

Short-, medium-, and long-chain FAs are activated on the outer mitochondrial membrane by acyl CoA synthethase and converted to acyl CoA in the cytosol. Long chain FAs are unable to pass through the mitochondrial membrane, therefore they are converted to long-chain acylcartitine by CPT1. Malonyl CoA, an intermediate of DNL that accumulates after insulin receptor activation, is an allosteric inhibitor of CPT1 that regulates the entry of FAs into the mitochondria. Acylcartitine is subsequently shuttled through the mitochondrial membrane by caritine-acylcartinine translocase (CAT), followed by the conversion back to acyl CoA by CPT2.

In the mitochondrial matrix, β-oxidation of FAs is catalysed to form acetyl CoA, by cleaving two carbons from the long chain FA. Acetyl CoA then enters the TCA cycle for complete oxidation thus releasing NADH and $FADH_2$ leading to ATP synthesis. ATP is ultimately utilized by hepatocytes to provide energy for various cellular processes.

1.6 ARAP2 – an Arf-GAP protein containing PH-domains

ArfGAPs proteins are GTPases that control the function of Arf proteins by converting the active Arf-GTP form to the inactive Arf-GDP form. By regulating the Arfs proteins, ArfGAPs have been showed to play a role in actin remodelling, membrane trafficking (123) and cell signalling (145), suggesting a critical role of these proteins in various biological processes, such as secretion, endocytosis and phagocytosis. Thirty-one genes encoding the Arf-

GAP catalytic domains have been identified in humans and consist of four subfamilies among which include ASAP, ACAP, AGAP and ARAP (146). Shome and colleagues have shown that Arf proteins are stimulated by insulin and play a key role in insulin-mediated regulation of PLD1 (147), a mediator of lipid droplet formation, localization and growth of lipid droplets (148-150). Interestingly, PLD is activated downstream of PI3-kinase and plays a critical role in stimulation of glucose metabolism (151).

ARAP2 (Arf GAP with RhoGAP domain, Ank repeat and PH-domain 2) is a protein encoded by the *ARAP2* gene and is composed of a sterile α-motif (SAM), an ArfGAP domain, an inactive RhoGAP domain, Ankyrin repeat domain, RAS-associating (RA) domain and five PH-domains (Figure 11). ARAP2 lacks the Rho-GAP activity, and localises to the cell periphery on focal adhesions. ARAP2 has been shown to promote growth of focal adhesions and stress fibre (152). Chen and his colleagues have shown that ARAP2 is also involved in trafficking of integrin to endosomes (153). In addition, findings indicate that ARAP2 also functions downstream of PI3-kinase and plays a critical role in bacterial entry into the cells (154). In this thesis, we have focused on the ARAP2 protein and its role in lipid droplets formation as it was identified in the lipid droplet proteome.

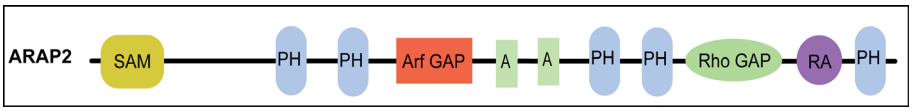

Figure 11. Schematic representation of ARAP2. SAM, sterile a-motif; PH, pleckstrin homology domain, ArfGAP, ArfGTPase- activating protein; RhoGAP, RhoGTPase-activating protein; A, Ankyrin repeat; RA, Ras-associating domain

2 METHODOLOGICAL CONSIDERATIONS

2.1 *In vivo* studies

2.1.1 Animals

In this thesis we have used mice with liver-specific deletion by utilizing the conditional inactivation of the target gene. The animals were kept in a temperature controlled environment on a 12 hour light cycle and fed a standard rodent chow and water *ad libitum*. All animals were fasted 12 hours before experiments, but water was available *ad libitum* during the whole procedure. All animal experiments were approved by the Animal Ethical Committee in Gothenburg. Our experimental design considered in detail the principles of use of animals in research studies; replace, refine and reduce (3Rs).

2.1.2 Deriving transgenic mice (Cre-loxP system)

The mouse, *Mus Musculus*, is the most commonly used model in medical research to study the human physiology and disease. There are powerful advantages of using mice as model organisms, as their genome and physiology have been studied extensively and several *in vivo* experimental protocols are available. The mouse and human share many similarities including their anatomy, physiology and most of the genome, thus making mouse genetic research applicable to humans (155). However, one has to be careful when extrapolating findings in mice to humans. Pragmatically, mice are also a convenient choice because they are small, have a shorter life span and reproduce quickly, thus making them appropriate models to execute large-scale experiments (156). The mouse genome is easy to manipulate (by adding or removing a gene), thus creating a transgenic mice, that makes it a powerful tool for modeling genetic disorders and evaluating therapeutics. Transgenic mice are created by introducing foreign DNA or deleting a gene from the mouse genome, depending on the goal of the experiment.

The mouse models used in this thesis had been created using the Cre-lox technology for conditional deletion of target genes. Using Cre-Lox system to produce transgenic mice allows the controlled expression of the gene within a specific tissue or cell, thus allowing us to define gene function in development

and physiology. Cyclic-recombinase (Cre) is a site specific integrase isolated from the P1 bacteriophage and catalyzes the recombination of DNA between the two loxP sites. The lox P sites are of 34-base pairs (bp) in length that consists of two inverted repeats that are flanked by 8bp spacer sequences. Cre cleaves the DNA sequence that is flanked by the loxP site (floxed) in the same orientation, thus knocking-out the gene (157).

In **Paper I**, we used the Cre-loxP system to inactivate the *Pik3ca* and the *Pik3r1*gene in hepatocytes, thus creating mice with liver-specific deletion of the catalytic subunit, p110α, and the regulatory subunit, p85α (L-DKO). We crossed the p110α lox-lox mice (44) with p85α lox-lox mice hemizygous for the Albumin Cre recombinase transgene (158).

In **Paper II**, we have used mice with liver-specific deletion of p110α isoform of the catalytic subunit by conditional inactivation of *Pik3ca* gene as described previously (44, 159). Adenovirus expressing the wild-type p110α or the E545K or H1047R mutant were created and purchased from Vector Biolabs (Philadelphia, PA). Adenoviruses are viral vectors that can accommodate large transgenes and code for proteins without integrating into the host genome. They can be used to achieve gain-of-function by overexpression or alternatively express antisense molecules to achieve loss of function (160). These adenoviral constructs were injected in p110α flox and liver-specific p110α knock-out (L-p110α KO) mice *via* the retro-orbital vein under isoflurane anesthesia, in a volume of 200 µl containing $10x10^{10}$ viral particles.

Advantages of using the adenoviral vectors are that they have high transduction efficiency and have a transient expression that peaks between 5-7 days. The L-p110α KO mice were included in the study for two reasons: to avoid interference of endogenously expressed p110α, but more importantly, to directly assess the effects on glucose homeostasis, since the L-p110α KO mice are severely insulin resistant (44).

Seven days after the adenoviral injections, mice were fasted and anesthetized followed by 5U of insulin injections or saline *via* the inferior vena cava. The inferior vena cava is the largest vein in the body. It collects blood from other tissues that are inferior to the heart and carries it directly to the heart. Importantly, the hepatic vein also provides blood into the vena cava from the digestive organs after it has passed through the hepatic portal system in the

liver. Injecting insulin into the vena cava allows it to enter the circulation immediately and exert its effects on different tissues in the body.

2.1.3 Evaluating glucose homeostasis and insulin sensitivity

The most commonly used tests to evaluate whether a genetic manipulation *in vivo* alters glucose metabolism is the measurement of fed and fasting circulating glucose and insulin. One must consider the conditions under which these variables are measured as the glucose and insulin levels are affected by a number of physiological and environmental factors such as activity levels, stress and the time of the day. To characterize the metabolic phenotype further, glucose tolerance tests (GTTs) and/or insulin tolerance tests (ITTs) are performed. Using these tests, one measures the changes in blood levels after a bolus dose of glucose (for GTT) or insulin (for ITT) over a 1- to 2-hour interval.

GTTs assess the disposal of the glucose load (generally 1-2 g/kg) administrated by different routes such as oral dosing or intraperitoneally or intravenous. Oral route is more physiological with insulin response peaking at 15 minutes. However, glucose tolerance flowing the oral glucose loading is influenced by intestinally derived factors that can cause alterations in insulin secretion or action (161, 162). Intra-peritoneal (i.p.) injections do not address the intestinal phase of glucose absorption. Intra-peritoneal injections are most commonly used, technically simple and easy. I.p. injected glucose is known to get a peak insulin response at 30 minutes. Performing intravenous injections require technical expertise and skill; the rate of injections need to be kept slow and the solution should not get out of the vein. Fasting periods is required before GTT to provide stable baseline measurements. Fasting periods can be overnight (~12-16h) or a short fast (~4-6h) in the morning. Overnight fasting produces low, stable baseline glucose and insulin levels (163, 164).

ITTs monitor the glucose concentration over time, but in response to bolus dose of insulin (0.5-2 U/kg) administration rather than glucose giving an estimate of the insulin sensitivity of the animal. The fall of blood glucose in response to insulin is a reflection on whole-body insulin action (165-167). ITTs are similar to GTTs, thus many of the technical considerations required for GTTs apply for an ITT.

The pyruvate tolerance test (PTT) elicits the conversion of pyruvate to glucose that reflects on the hepatic gluconeogenesis. PTT is another variant of GTT, in which a bolus dose of pyruvate (1-2 g/kg) is injected instead of glucose and the response in blood glucose concentration is followed. The results of intraperitoneal PTT are highly dependent on the variables that also influence GTT, including insulin sensitivity.

The results for GTTs, ITTs and PTTs can be expressed as both a time course of blood measurements and area under curve. In **Paper I & II** of this thesis, GTT, and PTT were performed on mice fasted for 12 hours. ITTs were performed on non-fasted mice to avoid a big drop in glucose levels after insulin injections. Circulating insulin levels were measured by an enzyme-linked immunosorbent assay.

2.2 *In vitro* studies

2.2.1 NIH-3T3 cells

Cell culture is the process by which cells are grown under controlled conditions outside their natural environment. The process of cell culture has been extensively used since early 1900's. Cell lines are immortalized and have been widely used as a model system to study different cellular mechanisms. A major advantage of using cell culture is reproducibility of results that can be obtained from using a batch of clonal cells. To be able to study the knockdown effects using small interfering RNA (siRNA) of ARAP2 we established an efficient transfection model. Transfection studies are mainly performed to study the function of genes by enhancing or inhibiting specific gene expression in cells (168).

NIH-3T3 mouse fibroblast cell line was derived from a cell line from the NIH Swiss mouse embryo fibroblasts (169). NIH-3T3 cells have the ability to grow at low density levels with a fast doubling time of approximately 18-20 hours.

The cells were maintained in Dulbecco's Modified Eagle medium containing 25 mM glucose supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (FCS).

In **Paper III**, a lipid-mediated delivery system was used to transfect foreign DNA or RNA into cells to produce genetically modified cells. In this study, cells were transfected with either ARAP2 or scrambled control siRNA's at 30% confluency using RNAiMAX; and for overexpression studies cells were transfected with the FLAG-tagged ARAP2 or control plasmid at 80-90% confluency using Lipofectamine 2000 in medium without FCS, penicillin and streptomycin. After four hours of transfection complete medium was added to transfected cells and they were let to incubate for 48 hours before harvesting.

For experiments involving GCS inhibitors, cells were transfected for 24 hours and treated thereafter with D-thero-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) or DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (DL-PPMP), 24 hours before harvesting. PDMP and PPMP are homologous with shorter (PDMP: C_{10}) or longer (PPMP: C_{16}) fatty acyl chains. Longer chain homologs were much more effective in inhibiting glucosylceramide synthesis in mammals (170). Additionally, a literature review suggests that PDMP and PPMP are specific inhibitors of GCS, but differentially affect the production of glycosphingolipids. For example, in BG1 cancerous cell line, PPMP significantly reduces lactosylceramide production while PDMP did not affect its production (171). This suggests that these GCS inhibitors differentially affect other glycosphingolipid formation in addition to glucosylceramide. Blocking glycosphingolipid production by two inhibitors with differential targets in the pathway ensures that the effects observed in our experiments are due to the inhibition of GCS.

Histological analysis is recognizing structure of cells and tissues and understanding the relationship between these structures and their physiological functions. Special histological stains are used to enhance biological structures. For example, in **Paper II**, we use the hematoxylin and eosin (H&E) combined with light microscopy to study the morphological changes in the liver tissue. H&E staining allows us to study a broad range of features of the cells such as cytoplasmic, nuclear and extracellular matrix. Hematoxylin is a basic dye that stains the nucleus blue due to the affinity to nucleic acids, whereas eosin is an acidic dye that stains the basic part of the cell such as cytoplasm (172). In addition, Ki-67 (detects nuclear protein) staining was used as a proliferation marker and Period-acid Schiff (PAS) (detects polysaccharides) was used to detect glycogen accumulation.

There are many other compounds use to selectively stain other cellular components such as Oil Red O. Using Oil Red O, a fat-soluble diazo dye we were able to visualize neutral lipids such as triglycerides, thus detecting lipid accumulation in cells (**Paper I & Paper II**).

2.3 Lipidomics

Lipidomics can be defined as the system-level analysis and characterization of lipids and their interacting partners (180). Comparing the lipid species in diseased states may elucidate the underlying metabolic pathways associated with the specific diseases. Analysis of lipids is based on lipid sample extraction, analysis/ separation and data analysis. However, due to the diverse lipid classes and molecular species it is impossible to use a common extraction, chromatography and detection method to identify all classes of lipids.

Sample extraction aims to recover lipids of interest into a solvent, to get as many lipids as possible or to discriminate selected lipid classes. Different extraction methods are available depending on the sample and the experiment. Sample preparation is important as it determines the quality of the experiment (181). Most commonly used extraction protocols are by Bligh and Dyer (182) and Folch method (183). In these protocols, a phase separation method is employed where the lipids are dissolved in organic solvents and separated into hydrophobic or aqueous phase. We extracted lipids using the Folch method in Paper II & Paper III.

Several techniques particularly mass spectrometry (MS) and chromatography have emerged as valuable tools to identify individual lipid species. Chromatographic methods involve dissolving lipids in a solvent, which carries it through a stationary phase for example silica in thin layer chromatography or small packaging particles in liquid chromatography. Thus various metabolites in the lipid mixture travel at different speeds and separate closely related metabolites through a mobile phase. In HPLC, the metabolite/ sample is forced through a column packed with a stationary phase particles (3-10 μm) at a high pressure and are separated by interactions with the particles and subsequently detected by a detector.

HPLC can be combined with different detectors depending on the complexity of the experiments and the information required. When information about the

lipid species is required HPLC is most commonly combined with a mass spectrometer. MS is an analytical tool that is able to identify lipids based on their mass. MS identify lipids with high resolution and sensitivity. Separating metabolites by chromatography before helps to separate the isomers having the same mass that is not normally differentiated by a mass detector.

Further advances in the instrumentation and technology have improved the chromatographic performance. Ultra-performance liquid chromatography (UPLC) is a new analytical tool that employs the same principles as HPLC but employs smaller particles (2 μm) to achieve superior resolution, speed and sensitivity (184). We perform targeted analysis to identify metabolites of interest, combining chromatography techniques with mass spectrometry to separate isomers having the same mass that is not normally differentiate by a mass detector.

In Paper II, quantified lipids using high performance liquid chromatography (HPLC) coupled with an evaporative light-scattering (ELS) detector and mass spectrometry. While, in Paper III lipid analysis was performed using UPLC coupled to mass spectrometry.

2.4 Microarray

In Paper III, we used the BeadChip microarray technology to identify the biological pathways affected by ARAP2 knockdown in an effort to unravel the complex biological mechanisms behind the effects of ARAP2 on glucose metabolism. Microarray allows us to measure the whole genome expression. Gene expression varies in tissues depending on the cell types and its condition (disease state). The ability to measure the expression of genes at the same time increases our knowledge and understanding of gene functions. Bead-chip array employs 3-micron silica beads help in micro-wells on the surface of an array substrate and uses the hybridization-based procedure. The beads are covered with hundreds of thousands of copies of specific oligonucleotides that act as probe of known DNA sequences to capture sequences from the sample. The microarrays use the multi-sample format for high throughput and reduced sample variability. Thus, using microarray technology is a high-throughput, reproducible method that accelerates targeted or whole-genome studies.

2.5 Lipid kinase assay

Lipid kinases are assayed *in vitro* to characterize their enzymatic properties. The current gold standard to measure the PI3-kinase lipid kinase activity involves a transfer of a phosphate onto a phosphoinositide which is subsequently assayed by thin-layer chromatography (TLC). The PI3-kinase phosphorylates the lipid in presence of [γ-^{32}P] ATP; the lipids are then extracted and separated by TLC. PI3-kinases are able to phosphorylate phosphoinositides (PI), phosphatidylinositol 4-phosphate (PI-4-P), phosphatidylinositol 3-phosphate (PI-4-P) and PIP_2 to produce phosphatidylinositol 3,4-biphosphate (PI-3,4-P_2) and PIP_3.

PIP_2 are not most commonly used as substrates for performing *in vitro* lipid kinase activity assays, as they are known to form aggregates that interferes with the assay. Thus, PI is the most commonly used substrate for characterizing the lipid kinases activity. In **Paper I & II** we used PI as a substrate to perform the lipid kinase activity assays.

3 AIM

The specific aims of the three papers included in this thesis are:

Paper I. To elucidate the impact of a combined hepatic deletion of the p110α and p85α subunits of PI3-kinase on insulin signaling and whole body glucose homeostasis.

Paper II. To investigate the effects of the p110α hot spot mutations E545K and H1047R on hepatic and whole body glucose metabolism.

Paper III. To identify PH-domain containing proteins involved in lipid droplet biology and elucidate their role in lipid droplet formation.

4 RESULTS AND DISCUSSION

Paper I: Hepatic deletion of p110α and p85α results in insulin resistance despite sustained IRS1-associated lipid kinase activity

In this paper we used the L-DKO mice to investigate the effect of combined hepatic deletion of p110α and p85α on insulin signaling and whole-body glucose homeostasis. We have previously shown that hepatic deletion of only p110α results in severe insulin resistance and impaired glucose tolerance, signifying that p110α is crucial for mediating insulin signaling (44). Moreover, mice deficient in all p85 isoforms in either muscle or liver exhibit severely impaired insulin signaling in these tissues (173, 174). These studies show that both subunits of PI3-kinase are essential for mediating insulin action. Liver plays a crucial role in maintaining glucose homeostasis; we therefore expected that deleting both these isoforms would result in severe and overt diabetes.

We found that combined hepatic deletion of both p110α and p85α subunits of PI3-kinase leads to decreased phosphorylation of downstream targets of PI3-kinase, such as Akt and p70S6 kinase, indicating that L-DKO mice have blunted insulin signaling downstream of PI3-kinase. Interestingly, despite the blunted hepatic insulin signal, resulting in reduced liver weight and impaired glucose tolerance, the L-DKO mice are not diabetic. The glucose levels were normal throughout the study (24 weeks) (Paper I, Fig. 4D and E). Furthermore, L-DKO mice had decreased body weight and, similar white adipose tissue weight, normal hepatic glucose production as well as normal insulin tolerance as compared to the controls. In contrast, mice with only p110α hepatic deletion (L-p110α KO) have increased body weight compared to controls as well as increased white adipose tissue mass and impaired gluconeogenic regulation (44). The overall observed phenotype for the L-DKO mice is thus, paradoxically, considerably less severe compared to mice with only p110α hepatic deletion.

Considering the critical role of the two PI3-kinase subunits in mutually stabilizing each other, we hypothesized that other regulatory isoforms, such as p85β or p55γ, would stabilize p110β in absence of p85α. Protein expression of p55γ was unchanged in the L-DKO mice compared to the controls. Due to the lack of functioning antibodies of p85β in mice, protein expression of p85β

remains to be determined; however the gene expression of p85β was not different between the L-DKO and the control mice, and using pan-p85 antibodies (detecting both p85 isoforms), we found only very low levels of p85β in the L-DKO mice. Additionally, we found that the total protein expression of p110β was decreased in L-DKO mice compared to the controls, likely due to destabilization of p110β. Taken together, this leads us to speculate that the protein expression of p85β remains unchanged in the L-DKO mice.

We then investigated the effect of the double (p110α and p85α) KO on the p110α lipid kinase activity, and as expected, the p110α kinase activity was significantly decreased in basal- and insulin-stimulated states of the L-DKO mice as compared to controls. However, the IRS1-associated lipid kinase activity in response to insulin was intact (Paper I, Fig. 2C). The intact IRS1-associated lipid kinase activity was surprising and completely in contrast to what is seen in L-p110α KO mice, where insulin stimulated IRS1-associated kinase activity was entirely ablated (44).

Previous studies by us and others have shown that p110β is unable to compensate for the loss of p110α, for example in our study of the L-p110α KO mice (44). We observed similar amounts of IRS1-associated p110β in the L-DKO mice and controls despite overall total decreased levels of p110β in the liver, and therefore speculated that perhaps p110β activity was increased in response to insulin in the L-DKO mice compared to controls, which would explain the sustained IRS1-associated lipid kinase activity. We found no difference in the p110β activity between the controls and the L-DKO mice in the basal state; however, the p110β kinase activity was significantly decreased, rather than increased, in insulin-stimulated state of L-DKO mice. We thus conclude that the sustained IRS1-associated lipid kinase activity in the L-DKO mice is not due to increased activity of p110β.

The decrease in p110β lipid kinase activity in response to insulin could be due to a shift in the lipid substrate concentration. Although it remains to be demonstrated, it is possible that there are high intracellular levels of the substrate PIP_2 accumulated in the livers of L-DKO mice as the PI3-kinase signaling is diminished. p110α and p110β have been known to differ in their lipid kinase activities (175). Studies have shown that p110β have specific and maximal activity at lower lipid concentrations as compared to p110α (176) and accumulation of PIP_2 levels might therefore have an inhibitory effect on p110β

activity. This differential preference for lipid concentration is thus a plausible explanation for the decrease in insulin-stimulated p110β lipid kinase activity. Increased PIP_2 levels might be sustained by activation of other enzymes/ kinases (Figure 12B). For example, PIP_2 together with activated Arf acts as a positive regulator or phospholipase D, mediating the synthesis of phosphatidic acid. Phosphatidic acid, in turn, activates phosphatidylinositol 4-phosphate 5-kinase (PIP5 kinase) to further produce PIP_2, thus creating a positive feedback loop (177).

The lack of the major regulatory subunit in the L-DKO mice, accompanied by absence of compensatory expression of other regulatory class 1A subunits, suggests that presence of other classes of phosphatidylinositol kinases account for the intact IRS1-associated kinase activity by directly binding to IRS1. Another possible theory is that increased levels of PIP_2 or other phospholipids in the absence of p85α and p110α (as discussed above) attract other lipid kinases to IRS1 through secondary binding. IRS1 has been shown to bind phosphatidylinositol phosphates through its PH-domain in the rank order PI(3,4,5)P3 = PI(4,5)P2 > PI(3,4)P2 = PI(4)P > PI(3)P = PI (178). If levels of PIP_2 increase markedly, this might impel the interaction between PIP_2 and IRS1, shifting its cellular distribution from the plasma membrane to other intracellular compartments where the IRS1-PIP_2 complex would be in close proximity to other lipid kinases (Figure 12C).

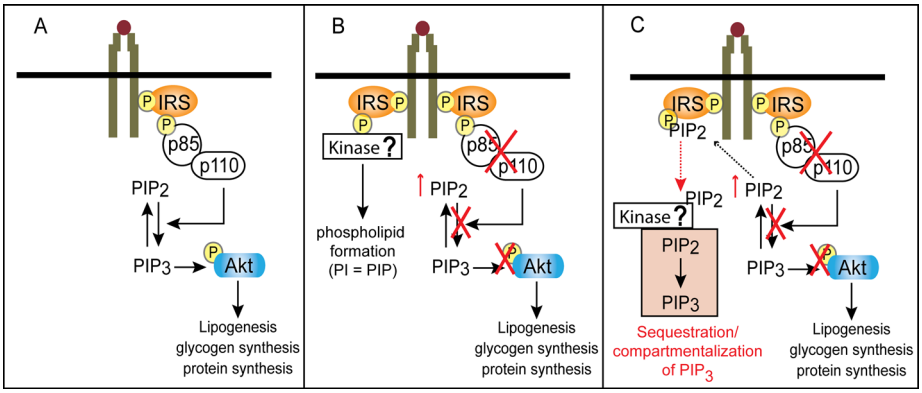

Figure 12. Proposed mechanisms for intact IRS1-associated lipid kinase activity in the L-DKO mice. (A) Normal signaling through p110α and p85α in floxed mice, Signaling in L-DKO mice in absence of p110α and p85α (B) other kinase/s bind to IRS1 and promote phosphorylation of phosphoinositides (C) PIP_2 accumulates and increased levels of PIP_2 binds to IRS1, which attracts other kinase/s that bind to PIP_2 and that are able to transfer a phosphate to the phosphoinositide substrate.

In summary, a combined deletion of hepatic p110α and p85α results in an impaired glucose homeostasis, but overall show a milder phenotype as compared to the mice with only p110α deletion in the liver. In addition, the L-DKO mice displayed an intact IRS1-associated lipid kinase activity. In order to gain more knowledge on the impact on, and regulation of, different classes of PI3-kinase as well as other lipid kinases in the L-DKO mice, and the status of phosphoinositide levels, more detailed molecular and signaling studies are necessary.

Paper II: p110α hot spot mutations E545K and H1047R exert metabolic reprogramming independently of p110α kinase activity

In this paper we investigated the effect of the p110α hot spot mutations E545K and H1047R on hepatic and whole body glucose metabolism. We hypothesized that effects of these mutations in p110α would exacerbate when combined with severe insulin resistance. We found that the expression of the hot spot mutations E545K and H1047R in p110α resulted in a rapid and marked increase in hepatic lipid and glycogen accumulation. This was observed within 7 days of virus injection. Surprisingly, the mice expressing the wild-type p110α did not develop hepatosteatosis. Development of hepatosteatosis is strongly associated with impaired glucose tolerance and insulin resistance (179, 180); therefore we investigated the effects of these mutants on glucose homeostasis.

As expected, both flox and L-p110α-KO mice showed improved glucose tolerance when injected with wild-type p110α, resulting in normalization of glucose tolerance in the insulin resistant L-p110α KO mice. Surprisingly, mice expressing the mutant p110α also showed improved glucose tolerance despite the rapid fatty liver development. Additionally, the development of hepatosteatosis in the mutant-expressing mice was not accompanied with impaired insulin tolerance. This suggests that glucose homeostasis and fatty liver development was uncoupled in these mice.

Hepatosteatosis is a result of lipid accumulation in the liver and effects on glucose metabolism, as a result of the hepatosteatosis, is dependent on the types of lipids accumulated in the hepatocytes (See section 1.5.4 above). Lipidomic analysis of the livers revealed that the accumulated lipids in the mutant livers were mainly triglycerides. There were no changes in other lipid species such as ceramides, sphingomyelin, phosphatidylcholine or cholesterol. Triglycerides are known as inert lipids and are considered non-toxic, mediating protection for the cells against FA-induced lipotoxicity (105). The absence of increased toxic species in the mutant-expressing mice might explain why they did not develop impaired glucose tolerance.

Lipid accumulation is often a result of increased lipogenesis; our results suggested that p110α mutations were potent in driving hepatic lipogenesis. We wanted to investigate if the increase in lipid synthesis could be attributed to an increase in the expression of key lipogenic genes. Unexpectedly, these

lipogenic genes were upregulated equally in the wild-type and mutant expressing livers. Similar levels of cleaved Srebp-1c were found in the nuclei of wild-type and mutant expressing mice. Furthermore, all key mediators of glycolysis and glycogen synthesis were significantly up-regulated in livers of mice expressing both mutant p110α-(E545K or H1047R) and wild-type p110α as compared to the p110α floxed controls. Nuclear levels of ChREBP, a key regulator of glucose-induced lipogenesis, were also similar between groups.

To elucidate the underlying mechanism, explaining why hepatic lipid accumulation was observed in mutant expressing mice but not wild-type expressing mice, we investigated if alterations in fatty acid β-oxidation, lipid secretion/uptake, and autophagic lipid degradation could explain the lipid accumulation in mutant expressing mice. Expression of the key mediators of β-oxidation, PPARα, AMPK and ACC were not changed between the wild-type and mutant expressing mice. There was no difference observed in the secretion or uptake of fatty acids as investigated by the fatty acid transporters CD36 or low-density lipoprotein receptor or expression of Mttp (unpublished data) or any differences in circulating triglycerides or FFA levels. Furthermore, the nuclear content of the transcription factor EB (TFEB), a key regulator for autophagy, was not changed in the mutant-expressing mice compared to the wild-type expressing mice (unpublished data). All these pathways were investigated, but failed to show any differences between mice expressing the wild-type p110α and the mutant p110α. Thus, we conclude that differential regulation of lipid metabolism was not the cause of the lipid accumulation seen in mice expressing mutant p110α.

The E545K and H1047R mutations of p110α have been shown *in vitro* to promote cellular proliferation and invasion, by hyperactivating the downstream target Akt/PKB (60, 61). We found that the phosphorylation of downstream target (pT-Akt and pS-Akt) was markedly increased in the basal state in L-p110α KO and flox mice expressing the E545K and H1047R mutants compared to the control mice. However, the mice expressing the wild-type p110α did not activate Akt in the basal state. As expected, key targets of Akt/PKB, such as GSK3β and ATP citrate lyase (ACL), in the mutant-expressing mice showed increased phosphorylation, in line with previous studies of the effects of p110α hot spot mutations (58, 60, 61).

Increased activation of ACL would logically generate a larger pool of cytosolic acetyl-CoA, which is crucial for lipogenesis. It is possible that increased levels of cytosolic acetyl-CoA, at least partially, can explain the lipid accumulation in the mutant-expressing mice, by driving lipogenesis. However, it seems unlikely that this would be the only mechanism involved as we cannot detect any differences of key lipogenic enzymes between controls and mutant-expressing mice. In addition to the increased basal activation of ACL, we also investigated enzymes important for acetyl-CoA secretion from hepatocytes via the urea cycle (arginase I, ornithine decarboxylase and diamine acetyltransferase 1) to explore the possibility of impaired secretion of acetyl-CoA in the mutant-expressing mice (unpublished data). Impaired acetyl-CoA secretion would contribute to further increasing intracellular acetyl-CoA levels in the mutant-expressing mice; however, we did not find differences in the activation of these enzymes.

Constitutive activation of the PI3-kinase-Akt pathway induces tumorigenesis, and tumor cells are known to activate the PI3-kinase Akt pathway in an insulin-independent manner (181). Maintaining this constitutive activation of Akt is crucial for glycolysis, lactate production and other downstream signals as a step to contribute to the coordination of tumor cell metabolism. Previous studies have reported the frequent occurrence of these hot spot mutations in several human cancers (53-55). Green *et al*., have shown that expression of p110α-H1047R expression alone failed to promote tumor formation, however, together with KRAS mutation enhanced tumorigenesis (182). Another study reported that a combined expression of p110α-H1047R mutants together with activated Her2/ Neu in mammary glands, augments its metastatic potential (183). These reports, taken together with our results, suggest that the p110α mutations E545K and H1047R might play a role in positively regulating metabolism in cells with high proliferation rate.

This raises the question, why do the p110α mutants reprogram metabolism? Hyper-activation of Akt is sufficient to induce a Warburg effect in either non-transformed or proliferating (cancerous) cells (184-186). Warburg suggested that cancer cells require increased glycolysis (187); to feed the elevated demand for energy. This is achieved by elevated glucose uptake by increasing the expression of specific glucose transporters such as GLUT1. GLUT1 has been found to be upregulated in wide variety of cancers such as endometrial, breast cancers and colorectal cancers, and its expression correlated with

metastatic potential (188, 189). Our unpublished data shows that the p110α mutant-expressing mice have increased GLUT1 protein levels in the plasma membrane fraction, suggesting that they have increased glucose uptake. Although the mutant-expressing mice do not show any cancer development for obvious reasons (the effects of the mutants were only studied over a period of seven days), our results also show an increased rate of glycolysis and pentose phosphate pathway. This increased glucose metabolism provides cells with intermediates required to control biosynthetic pathways and enables synthesis of proteins, lipids, and complex sugars at a high rate (190).

Apart from glucose, proliferating cells also require glutamine to ensure energetic precursors for amino acid and lipid synthesis (190, 191). Glutamine addiction is a major metabolic reprogramming that the proliferating cells undergo. Glutamine is converted to glutamate by glutaminase-1 (GLS1) after being taken up by the cell. Glutamate is then converted to α-ketoglutarate that then enters the TCA cycle in the mitochondria to provide ATP (192). In pancreatic cancer with KRAS mutations, glutamine is redirected to form aspartate that is converted to oxaloacetate (OAA), a part of the TCA cycle, which eventually produces pyruvate (193, 194). We speculate that the E545K and H1047R mutations of p110α reprogram metabolism *via* increased uptake of glucose and glutamine to supply cells with precursors for amino acid and lipid synthesis.

The increased activation of Akt/ PKB as a result of E545K and H1047R is generally thought to be due to increased lipid kinase activation of these mutants (60, 61). Our observation of increased activation of Akt, as well as the strong phenotype of the mutants displaying excessive lipid accumulation, we expected to see increased basal- and insulin-stimulated p110α kinase activity in the livers expressing the E545K and the H1047R mutants compared to the livers expressing the wild-type p110α. When assessing the lipid kinase activity, we found that the p110α kinase activity of the mutants was not different from that of the wild-type mice, suggesting a kinase-independent mechanism. Although no kinase-independent function of p110α has been described previously, a kinase-independent function for p110β has been reported (195). Furthermore, p37δ, a splice variant of p110δ, is able to activate the Akt/PKB pathway and promote proliferation despite lacking the kinase domain, thus indicating the importance of a kinase-independent signaling function of this PI3-kinase

isoform (26). It is plausible that the mutant p110α transmits signals through Akt in a similar kinase-independent fashion.

There are several possible scenarios for how the mechanism of a kinase-independent function of p110α mutants (E545K or H1047R) could occur:

1. Destabilizing of the regulatory subunit p85, as seen for p37δ (26), or by degrading or sequestering p85 through ubiquitination and/or confining p85 to signaling silent complexes (Figure 13A).

2. Decreased activation of the negative regulators of PI3-kinase, such as phosphatase PTEN, which dephosphorylates PIP_3 to PIP_2 (Figure 13B).

3. A mechanism that is completely independent of PI3-kinase activity and PIP3 production. The E545K or H1047R mutants of p110α might enable interactions with regulatory molecules that don't normally interact with p110α and thereby induce/ repress certain signaling mediators such as PDK1 or of the mTOR pathway, that are able to activate Akt independent of the PIP_3 generated (Figure 13C).

Alternatively, point 1 and 2 could be two steps in a common mechanism as seen for mice with hepatic p85α deletion. These mice show paradoxical increased insulin sensitivity, due to sustained PIP_3 levels over a longer period of time compared to controls. Overall PI3-kinase activity was decreased in the liver of these mice, but the PIP_3 levels were sustained because of decreased PTEN activity. The authors showed that p85α functioned as a negative regulator of insulin signaling by interacting with PTEN and increasing its activity (196).

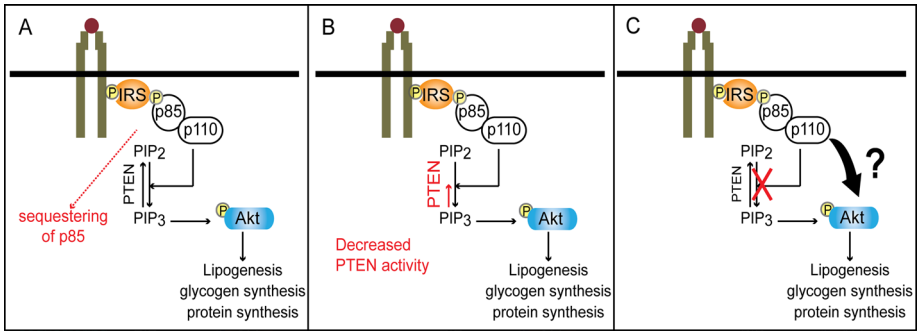

Figure 13. Proposed mechanisms of action in p110α mutant-expressing mice (A) the mutations destabilize p85α by degradation or sequesteing to different complexes, (B) decrease the PTEN activity which results in decreased phosphorylation of PIP_3 to PIP_2, (C) Other unknown mediators are able to phosphorylate Akt independent of PIP_3 generation.

In summary, we have shown that hepatic expression of p110α hot spot mutations E545K and H1047R results in rapid hepatosteatosis and glycogen accumulation paradoxically accompanied by improved glucose tolerance, whereas expression of wild-type p110α does not result in such a phenotype. Expression of p110α mutants resulted in hyper-activation of Akt /PKB in the basal state, versus the wild type p110α expressing livers. The p110α lipid kinase activity in mutant expressing livers was similar to the livers expressing the wild type p110α; a majority of the effects observed thus appear likely not to be due to an increase in the kinetic properties of p110α mutants, but possibly through a novel kinase-independent function.

One of our original hypotheses was that increased insulin levels, as seen for the markedly insulin resistant L-p110α KO mice, would enhance any detrimental effects of the E545K and H1047R mutants. Interestingly, we see no difference in the effects of these mutations on lipid and glycogen accumulation, glucose metabolism and their lipid kinase activity between the flox and L-p110α KO group. In addition, the effects that we do see occur in the basal state where no insulin is present. Thus, increased insulin levels do not enhance the effects on metabolism of these oncogenic mutations. Rather, the metabolic reprogramming is independent of insulin.

Paper III: ARAP2 promotes GLUT1-mediated basal glucose uptake through regulation of sphingolipid metabolism

The aim of this study was to identify PH-domain containing proteins and determine their role in lipid droplet formation. We used an *in vitro* model (NIH-3T3 cells) and identified a PH-domain containing protein, ARAP2 among others in the lipid droplet proteome. We further knocked down ARAP2 to determine its role in lipid droplet formation. Lipid droplet formation is mainly dependent on triglyceride synthesis from glucose and fatty acids (149, 197). We showed that ARAP2 knockdown decreases lipid droplet formation and triglyceride synthesis. To determine if the reduction in triglycerides is due to impaired fatty acid or glucose uptake we determined the cellular uptake of fatty acids and glucose after ARAP2 knockdown. The decrease in triglyceride synthesis after ARAP2 knockdown was not due to the decrease in fatty acid uptake, but rather due to decreased glucose uptake (Paper III, Fig 1D).

The regulation of basal glucose uptake in NIH-3T3 cells is highly dependent on the glucose transporter GLUT1 (198) which is expressed in virtually all tissues under normal conditions. ARAP2 knockdown decreased total GLUT1 levels. The localization of GLUT1 to specialized lipid micro-domains is essential for the activity of GLUT1 (199, 200). ARAP2 knockdown also decreased GLUT1 levels in the plasma membrane (Paper III, Fig 2D and E) and the lipid micro-domain fractions (Paper III, Fig 2F and G). To further understand how ARAP2 affects GLUT1 we analyzed global gene expression after ARAP2 knockdown. Microarray results showed that ARAP2 knockdown altered several pathways among which were steroid biosynthesis, fructose and mannose metabolism and sphingolipids metabolism.

Sphingolipids play important roles in maintaining membrane function and integrity. They are major constituents of the lipid micro-domains in various membranes primarily in the plasma membrane (201-204) and hence, ARAP2 knockdown altering the sphingolipids metabolism was in particularly interesting. Sphingolipids are derived from ceramide, a key lipid precursor formed by *de novo* synthesis or from hydrolysis of complex sphingolipids and sphingomyelin. Ceramide can be further converted to other bioactive lipids, such as sphingomyelin, glucosylceramide, lactosylceramide, GM3, GM2, GM1 and GD1 (Figure 7).

To further understand the link between ARAP2, sphingolipids and glucose

uptake we performed lipidomic analysis and we found that ARAP2 knockdown increases the glucosylceramide and lactosylceramide levels without affecting the ceramide levels. Therefore we speculated that ARAP2 might modulate glucosylceramide synthase (GCS), a rate-limiting enzyme in the glycosphingolipid synthesis. We found that the activity of GCS was increased (Paper III, Fig 3C) upon ARAP2 knockdown, while reduced upon ARAP2 overexpression (summarized in Figure 13).

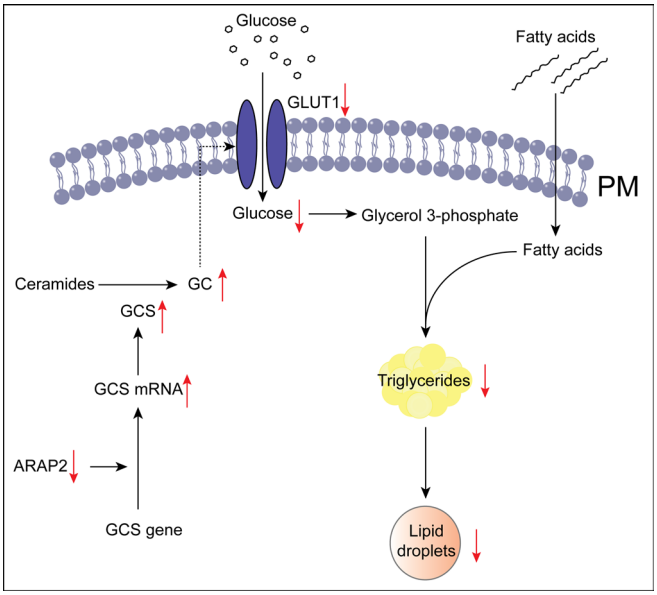

Figure 14. Summarizing the findings from paper III. The red arrows represent the effect of ARAP2 knockdown.

One explanation of how ARAP2 might directly or indirectly modify GCS could be that, ARAP2 affects the transcription factors that induce GCS expression. ARAP2 is a PH-domain containing protein that is cytosolic and associated with focal adhesions (152) and PH-domain containing proteins are recruited and activated at the plasma membrane by binding to PIP_3. These interactions of ARAP2 with PIP_3 might influence other signaling pathways that affect the transcription factors involved in GCS expression.

To determine if the effect on GLUT1 is mediated by altered GCS activity, we inhibited GCS using PPMP and PDMP inhibitors. We found that pharmacological inhibition of GCS reversed the effects of ARAP2 knockdown on basal glucose uptake (Paper III, Fig 4 A) and GLUT1 levels (Paper III, Fig 4 B and C) suggesting that ARAP2, may directly or indirectly, affect GLUT1 levels in the plasma membrane by modifying sphingolipids synthesis.

In addition to GLUT1, other receptors and proteins are localized to the cell surface in the glycosphingolipid-containing lipid micro-domains (205, 206). The lipid composition of these membrane micro-domains determines the characteristics and function of the proteins at the membrane (207). Thus, it remains to be determined if other receptors or lipid micro-domain proteins are also affected by ARAP2 knockdown. Accumulation of GM3, another type of glycosphingolipid localized to the caveolin enriched lipid micro-domains result in a marked reduction of the insulin receptor protein expression in the lipid micro-domains (208-211). Therefore, we cannot exclude the possibility that inactivation of ARAP2 might affect other complex glycosphingolipids and consequently affect the activity of other receptors.

Recent evidence has emerged suggesting sphingolipids to have an important role in the pathogenesis of various metabolic diseases associated with obesity such as cardiomyopathy, atherosclerosis and in particular diabetes (212). Sphingolipids have unique biophysical properties, due to their ability to alter membrane lipid composition they serve as signaling molecules. Several sphingolipids synthesis inhibitors show therapeutic potential as they influence broad spectrum of metabolic diseases such as T2D. To identify molecules such as ARAP2, involved in regulating intracellular signaling pathways, might prove beneficial in development of new therapeutic strategies.

In summary, we show that knockdown of ARAP2 decreases lipid droplet formation by decreasing the rate of triglyceride synthesis. This decrease is a result of decreased basal glucose uptake mediated by lower expression of GLUT1, as well as decreased GLUT1 levels in the plasma membrane and lipid micro-domains. Furthermore, we show that ARAP2 knockdown increases the activity and expression of GCS. The decrease in basal glucose uptake and GLUT1 levels in the lipid micro-domains were reversed by inhibition of GCS.

5 CONCLUSION

We conclude that:

Paper I

Combined hepatic deletion of the major subunits of PI3-kinase, p110α and p85α, does not result in diabetes, but rather a metabolic phenotype that is even less severe than when deleting p110α alone. The underlying mechanism for this phenomenon presumably involves signaling of other lipid kinases through IRS1.

Paper II

The oncogenic mutants, E545K and H1047R, of p110α, transmit metabolic signals that are independent of increased p110α kinase activity, resulting in major reprogrammed intracellular glucose- and lipid- metabolism.

Paper III

We identified a novel role for ARAP2, as a regulator of glucosylceramide synthesis and shown that it influences lipid droplet formation by regulating basal glucose uptake and triglyceride synthesis.

6 FUTURE PERSPECTIVES

Possible ways to continue on our findings from the papers included in this thesis:

For the L-DKO study, we will continue to investigate the effect of a combined liver-specific deletion of p110α and p85α on hepatic and glucose metabolism. We will investigate if there are any underlying processes that might compensate for the loss of p110α and p85α in the liver; or are there any other phosphoinositide phosphate/ kinase that are able to salvage glucose metabolism by maintaining normal blood glucose levels. We will also investigate the phospholipid status of the L-DKO mice to evaluate possible differences in the intracellular levels of PIP_2 and other phospholipids. This will help us narrow down possible kinase candidates that could contribute to the sustained IRS1-associated lipid kinase activity.

Our results show that the mice expressing p110α mutants E545K and H1047R reprogram metabolism to suit proliferating cells *via* a possible kinase-independent function of p110α. To further dissect the nature and mechanisms of a potential kinase-independent role of p110α, we will reconstitute the L-p110α knockout mice with a wild-type or a kinase-dead allele of p110α and/or p110α mutants and investigate its effects on hepatic and whole-body glucose metabolism. We will also include analysis of the PIP_3 levels to tease out if the underlying mechanism involves differential regulation of the PIP_3 pool or if the mechanism is completely independent of PIP_3 generation.

We hypothesize that altered glutamine metabolism contributes to the reprogrammed metabolism displayed by the p110α mutants as discussed above and will include these types of analyses as well in our search for the underlying process.

Furthermore, Straub and colleagues have shown that LD-associated PAT proteins have specific roles during maturation of LDs in normal or steatotic hepatocytes in fatty liver (117). The livers of the mice expressing the mutants accumulate lipid droplets; it would be interesting to investigate if the expression and localization of LD-associated proteins in p110α-E545K and the p110α-H1047R expressing mice liver play a role in the increased synthesis of

lipid droplets. Proliferating cells demand increased glucose uptake by increasing the expression of glucose transporter GLUT1 and our results in paper III show that ARAP2 affects the expression of GLUT1. Our preliminary data show that the livers of the mice expressing the p110α mutants have both increased GLUT1 content in the plasma membrane fraction as well as significantly increased ARAP2 gene expression, suggesting that ARAP2 and other regulators of lipid droplet formation and basal glucose transport could play an important role in the phenotype resulting from E545K and H1047R signaling.

ARAP2 is an Arf-GAP protein and previous studies have shown that the Arf-GAP activity of ARAP2 is regulated via its ArfGAP domain (154). We speculate that ARAP2 stimulates the conversion of Arf6-GTP to Arf6-GDP and thus knocking down ARAP2 might lead to accumulation of Arf6-GTP in the cells. We will test if ARAP2 regulates the Arf6 activity, by overexpressing a constitutively active and a dominant negative form of ARF6. We hypothesize that the expression of this constitutively active ARF6 will have the same effect as ARAP2 knockdown. To further validate these processes and to test if these processes remain consistent to other cell lines, we will investigate the effect of ARAP2 knockdown in cultured hepatocytes and also, to determine if ARAP2 influences glucose metabolism by affecting other glucose transporters.

Understanding the molecular mechanisms is necessary to get a complete comprehension of the underlying processes and interactions to further our knowledge for understanding the signaling in various disease states.

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"A book is almost always a collective effort, even if it has only a single author"

- Raghuram Rajan

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