

**Phosphatase PPP2R5C Couples Hepatic
Glucose and Lipid Homeostasis**

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PhD Thesis

**Phosphatase PPP2R5C Couples Hepatic
Glucose and Lipid Homeostasis**

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Yong-Sheng Cheng
Abgabedatum: January 20, 2015

Zusammenfassung

Das Verständnis des Entstehungsmechanismus von Krebs und Stoffwechselkrankheiten ist von großer Wichtigkeit um neue Behandlungen für diese Krankheiten zu entwickeln. Unter den verschiedenen Signalwegen, welche in diesen Krankheiten involviert sind, spielt Insulinsignalisierung eine bedeutende Rolle in der Modulierung von Zellwachstum und der Stoffwechselrate. Die bisherigen Forschungsergebnisse unseres Labors [1] zeigen dass die *Drosophila* PP2A regulatorische Untereinheit B' ein negativer Regulator von S6K ist; eine bedeutende downstream Komponente der Insulin und mTOR Regulierung. Um die Funktion dieser regulatorischen Untereinheit und ihrer Auswirkung auf Translationale Medizin besser zu verstehen, werden Mausmodelle und Zellkulturen zur Charakterisierung der Funktion von Säugetier Homolog PPP2R5C in Mäusen verwendet—welches sich als molekular Checkpoint zur Regulierung der Balance zwischen Glukose und Lipid Homöostase in Mausleber erwies. Knockdown von PPP2R5C in Hepa 1-6 und Primären Maus-Hepatozyten zeigt dass PPP2R5C ein negativer Regulator für Triglyceridespeicherung und Glykolyse sein könnte. Knockdown von PPP2R5C in mehreren Maus Zelllinien resultiert in erhöhter Glukoseaufnahme und Glykolyserate. Knockdown von PPP2R5C, insbesondere in Mausleber, verändert den Maus Metabolismus auf dramatische Weise. Leber Triglycerid und Glykogen werden gesteigert und Leber Cholesterin vermindert. Trotz keiner Veränderung im Blutglukosespiegel haben die Knockdown Mäuse besser Insulinsensitivität und Glukosetoleranz. Zwischen fasten und füttern haben die Knockdown Mäuse auch erhöhte VLDL Aussonderungen der Leber. Microarray und qPCR Analyse zeigt auch dass mehrere Gene; die in Glykolyse und Lipogenese involviert sind, nach PPP2R5C Knockdown hochgeregelt werden. Den meisten dieser Gene könnte eine Steigerung von HIF1 α und SREBP-1 Aktivität zugeschrieben werden. PPP2R5C Substrat-Trapping identifiziert mehrere Hauptregulatoren des Stoffwechselvorgangs, wie etwa AMPK, HIF1 α und STAT3. *In vitro* Knockdown von PPP2R5C zeigt auch erhöhte AMPK Aktivität und erhöhte HIF1 α Phosphorylierung auf. Interessanterweise ist Maus PPP2R5C in *db/db* Mausleber hochgeregelt, welches ein Mausmodell von Typ 2 Diabetes ist. Des Weiteren ist menschliche PPP2R5C in der Leber von Diabetes Patienten auch erhöht.

Abstract

Understanding the mechanism of how cancer and metabolic disorders arise is important for finding new treatment for these diseases. Among different signaling pathways involved in these diseases, insulin signaling holds a special role in modulating cell growth and metabolic rate. Our lab's previous finding [1] shows that *Drosophila* PP2A regulatory subunit B' is a negative regulator of S6K, a major downstream component in insulin and mTOR signaling. In order to understand better the function of this regulatory subunit and its implication in translational medicine, mouse model and cell culture are employed to characterize the function of mammalian homolog PPP2R5C in mouse, which is found to be the molecular checkpoint regulating the balance between glucose and lipid homeostasis in mouse liver. Knockdown of PPP2R5C in Hepa 1-6 and mouse primary hepatocytes shows that PPP2R5C could be a negative regulator for triglyceride storage and glycolysis. Knocking down of PPP2R5C in several mouse cell lines results in increased glucose uptake and glycolysis rate. Knockdown of PPP2R5C specifically in mouse liver changes mouse metabolism dramatically. Liver triglyceride and glycogen are increased and liver cholesterol is decreased. Despite no change in blood glucose level, the knockdown mice have better insulin sensitivity and glucose tolerance. During fasting and refed, the knockdown mice have also increased VLDL secretion from liver. Microarray and qPCR analysis also reveal multiple genes involved in glycolysis and lipogenesis are up-regulated upon PPP2R5C knockdown, and most of these genes could be attributed to increase in HIF1 α and SREBP-1 activity. Substrate trapping for PPP2R5C identifies several master regulators in metabolic process, such as AMPK, HIF1 α and STAT3. *In vitro* knockdown of PPP2R5C also show increased AMPK activity and increased HIF1 α phosphorylation. Interestingly, mouse PPP2R5C is up-regulated in *db/db* mouse liver, which is a mouse model of type 2 diabetes. In addition, human PPP2R5C is also elevated in liver from diabetic patients.

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Contents

List of Figures	ix
List of Tables	xii
1. Background	1
1.1. Cancer metabolism	1
1.1.1. Metabolism switch in quiescent vs proliferative cell	2
1.1.2. Signaling in metabolism reprogramming	3
1.2. Liver metabolism	3
1.2.1. Liver glucose regulation	4
1.2.2. Liver lipid metabolism	7
1.3. PP2A and its biology	9
1.3.1. PP2A structure	10
1.3.2. PP2A in signaling	13
1.3.3. PP2A in cancer	15
2. Results	17
2.1. Transcriptional response of PPP2R5C in pathophysiology	19
2.2. PPP2R5C negatively regulates glycolysis and lipogenesis	23
2.2.1. Selecting shRNA/miRNA for efficient knockdown of PPP2R5C .	23
2.2.2. Generating Guinea Pig antibody for endogenous PPP2R5C detection	25
2.2.3. PPP2R5C KD in Hepa 1-6 increases glycolysis	27
2.2.4. PPP2R5C KD promotes glucose uptake rate in Hepa 1-6	29
2.2.5. PPP2R5C deficiency promotes <i>de novo</i> lipogenesis	33
2.3. PPP2R5C <i>in vivo</i> knockdown promotes glucose uptake, triglyceride synthesis	36
2.3.1. PPP2R5C knockdown has no impact on animal health	36
2.3.2. PPP2R5C KD promotes glucose uptake <i>in vivo</i> with better insulin sensitivity	40

Contents

2.3.3. PPP2R5C KD promotes anabolic changes in liver	42
2.4. PPP2R5C negatively regulates VLDL secretion in liver	51
2.5. PPP2R5C regulates metabolism via AMPK and HIF1 α	56
2.5.1. PPP2R5C's substrates include multiple metabolic regulators	56
2.5.2. AMPK is PPP2R5C's substrate involved in glucose uptake	57
2.5.3. HIF1 α is PPP2R5C's substrate involved in glycolysis and lipogenesis	59
2.5.4. SREBP-1 is involved in lipogenesis phenotype	60
2.5.5. Microarray analysis of PPP2R5C KD in mouse liver	63
2.6. Human PPP2R5C in Type 2 Diabetes	65
2.6.1. PPP2R5C misregulation in human liver	65
2.6.2. PPP2R5C misregulation in human adipose tissues	69
3. Discussions	71
3.1. PPP2R5C in liver metabolism	71
3.2. PPP2R5C substrates	73
3.3. PPP2R5C's metabolic control in cancer cells	75
3.4. PPP2R5C in human metabolic diseases	75
4. Methodology	77
4.1. Molecular Biology	77
4.1.1. DNA digestion by restriction enzymes	77
4.1.2. Polymerase chain reaction(PCR)	77
4.1.3. Agarose gel electrophoresis	79
4.1.4. Agarose gel purification	79
4.1.5. DNA ligation	80
4.1.6. Transformation of <i>E. coli</i>	80
4.1.7. Bacteria cultivation and plasmid purification	80
4.1.8. Cloning and selection of shRNA or miRNA candidates	81
4.1.9. Cloning miR30-based shRNA for inducible piggyBac shRNA system	81
4.2. Gene expression analysis	81
4.2.1. Tissue pulverization	81
4.2.2. RNA isolation from tissue sample	81
4.2.3. RNA isolation from cell sample	82
4.2.4. cDNA synthesis	82
4.2.5. Quantitative PCR (qPCR) analysis	83
4.2.6. Microarray analysis of mouse tissue and cell sample	83

Contents

4.3.	Cell biology	84
4.3.1.	Cell culture for Hepa 1-6, HEK293T, HEK293A, Hela Cells	84
4.3.2.	Transfection assay	84
4.3.3.	Mouse primary hepatocyte cultivation	84
4.3.4.	PP2A substrate trapping in Hepa 1-6	85
4.3.5.	Luciferase assay	85
4.3.6.	Inducible shRNA stable cell line generation	86
4.3.7.	FACS analysis of 2NBDG uptake	86
4.4.	Virus production for mouse in vivo knock-down	86
4.4.1.	shRNA packaging Adenovirus construction and production	86
4.4.2.	miRNA packaging Adeno-Associated Virus construction and pro- duction	89
4.5.	Metabolite measurement in cellular and tissue samples	91
4.5.1.	Glucose consumption in Hepa 1-6 and primary hepatocytes	91
4.5.2.	Lactate production	91
4.5.3.	Free fatty acid measurement	92
4.5.4.	Triglyceride measurement	93
4.5.5.	Cholesterol measurement	93
4.5.6.	Glycogen content determination	93
4.5.7.	Ketone body measurement	94
4.5.8.	ATP measurement	94
4.5.9.	Intraperitoneal glucose tolerance test (IPGTT)	94
4.5.10.	Serum insulin measurement	94
4.5.11.	Serum lipoprotein analysis	95
4.5.12.	Serum ALT measurement	95
4.5.13.	Seahorse analysis of glycolysis in hepa 1-6	95
4.6.	Biochemical methods	96
4.6.1.	Protein expression in bacteria	96
4.6.2.	Antibody production and purification	96
4.6.3.	SDS-PAGE and western blot	96
4.6.4.	Phos-tag® analysis of phosphorylated proteins	97
4.6.5.	Immunoprecipitation	97
4.7.	Animal experiments	97
4.8.	Data analysis and plotting	97
A. Materials		99
A.1.	Chemicals and kits	99

Contents

A.2. Antibodies	103
A.3. Instruments	104
A.4. Solutions and buffers	105
A.5. Primer list for quantitative RT-PCR	106
A.6. shRNA/miRNA target sequences	107
A.7. Software list	108
B. Supplementary Table	109
B.1. Enriched TFs in mouse cell lines upon PPP2R5C KD	109
B.2. Enriched TFs in mouse liver upon PPP2R5C KD	110
B.2.1. Enriched TFs in mouse liver during fasting	110
B.2.2. Enriched TFs in mouse liver during <i>ad libitum</i> feeding	111
Bibliography	112
Glossary	132

List of Figures

1.1. Glucose redistribution in whole body	5
1.2. Lipid redistribution in whole body	8
1.3. PP2A in signaling	14
2.1. Mouse PPP2R5C gene structure	18
2.2. PPP2R5C expression in liver	20
2.3. PPP2R5C expression in adipose tissue	21
2.4. PPP2R5C expression in muscle	22
2.5. PPP2R5C mRNA levels in Type I&II Diabetes or Hepatosteatosis	23
2.6. shRNA/miRNA KD efficiency on PPP2R5C luciferase reporter	24
2.7. shRNA/miRNA KD efficiency on PPP2R5C protein level	25
2.8. Recombinant Variant 3 expression and purification	26
2.9. Specificity of GP antibody for PPP2R5C	27
2.10. Knockdown profile of endogenous PPP2R5C in Hepa 1-6	28
2.11. PPP2R5C KD increases glycolysis in Hepa 1-6	28
2.12. PPP2R5C KD promotes glycolysis by seahorse measurement	29
2.13. FACS filtering for single live Hepa 1-6 cell	30
2.14. Density plot of 2NBDG+ Hepa 1-6	31
2.15. Relative quantification of 2NBDG uptake upon PPP2R5C KD	32
2.16. 2NBDG uptake in primary hepatocytes	32
2.17. Triglyceride in primary hepatocytes upon PPP2R5C KD	33
2.18. PPP2R5C KD has no effect on ATP and Glycogen in 1° Hepatocytes	34
2.19. Triglyceride increases in Hepa 1-6 upon PPP2R5C KD	35
2.20. Free Fatty Acid uptake is not responsible for increased triglyceride	35
2.21. KD efficiency for miR12 <i>in vivo</i>	37
2.22. Serum ALT after PPP2R5C KD	38
2.23. Body weight change after PPP2R5C KD	38
2.24. Body fat content profile after PPP2R5C KD	39
2.25. Body lean mass profile after PPP2R5C KD	39

List of Figures

2.26. Abdominal fat after PPP2R5C KD	40
2.27. Blood glucose after PPP2R5C KD	41
2.28. Serum insulin drops after PPP2R5C KD	41
2.29. ISI index increases after PPP2R5C KD	42
2.30. GTT shows better glucose tolerance in PPP2R5C KD	43
2.31. AUC analysis for GTT results	43
2.32. Serum insulin level in GTT	44
2.33. Liver weight increases after PPP2R5C KD	45
2.34. Liver glycogen increases after PPP2R5C KD	46
2.35. Liver triglyceride changes after PPP2R5C KD	46
2.36. Liver NEFA has no change after PPP2R5C KD	47
2.37. Liver triglyceride increases in pilot experiment	47
2.38. Serum total ketone body upon PPP2R5C KD	48
2.39. Serum hydroxybutyrate upon PPP2R5C KD	49
2.40. Serum HB to TKB ratio upon PPP2R5C KD	49
2.41. Serum triglyceride changes after PPP2R5C KD	50
2.42. Serum NEFA changes in refed	51
2.43. Serum FPLC profile	52
2.44. Triglyceride concentration in lipoprotein particles	53
2.45. VLDL peak analysis	53
2.46. Cholesterol concentration in lipoprotein particles	54
2.47. Liver cholesterol drops upon PPP2R5C KD	55
2.48. Serum cholesterol level remains no change	55
2.49. PPP2R5C substrate trapping scheme	57
2.50. Metabolic regulators as PPP2R5C's substrates	58
2.51. AMPK activity increases upon PPP2R5C knockdown	59
2.52. HIF1 α 's phosphorylation analysis by Phos-tag [®]	60
2.53. HIF1 α 's transcriptional activity increases upon PPP2R5C KD	61
2.54. SREBP-1 protein increases upon PPP2R5C KD	62
2.55. SREBP-1 activity increases upon PPP2R5C KD in primary hepatocytes	63
2.56. SREBP-1 activity increased in mouse liver	64
2.57. Human PPP2R5C mRNA levels in human liver	66
2.58. Human PPP2R5C levels correlates with obesity type	67
2.59. All covariates correlated with PPP2R5C levels	68
2.60. Correlation profile for FPG and IL6	69
2.61. PPP2R5C mRNA levels in human adipose tissue	70

List of Figures

3.1. Whole organismal model for PPP2R5C's metabolic control	72
3.2. Cell-autonomous model for PPP2R5C in metabolic control	74

List of Tables

1.1. PP2A gene superfamily	10
1.2. Kinase/phosphatase gene number	11
2.1. Activated transcription factors in HepKD mouse liver during refed.	65
A.1. Chemical and kit list	99
A.2. Antibody list	103
A.3. Instrument list	104
A.4. Buffers list	105
A.5. Q-PCR primer list	106
A.6. shRNA/miRNA target sequences	108
A.7. Software list	108
B.1. Activated TFs in mouse primary hepatocytes and Hepa 1-6.	109
B.2. Activated TFs in HepKD mouse liver during fasting.	110
B.3. Activated TFs in HepKD mouse liver during random fed.	111

1. Background

1.1. Cancer metabolism



Comparing with normal differentiated quiescent cells, proliferative tumor cells exhibit distinct metabolic profile, which is generating energy from aerobic glycolysis, a phenomenon called "Warburg Effect" initially described by Otto Warburg at last century [2, 3, 4]. In proliferative tissue or tumor, quickly dividing cells ferment major fraction of glucose into secreted lactate and inefficiently produce ATP from glycolysis (2 ATP molecules per glucose) instead of oxidative phosphorylation in mitochondria (~36 ATP molecules per glucose).

The preference over fermentation in glucose utilization is first discovered in yeast. Otto Warburg's findings in proliferative ascites tumor cells establish the glucose utilization into lactate secretion, even under hyperoxia condition. Warburg and his contemporaries postulated that the aerobic glycolysis is specific marker for cancer cells, and defective mitochondria is responsible for energy production switch to glycolysis. The "Warburg Effect" is generally true for majority of cancer cells, and extensively applied in clinical diagnosis for tumor detection in human by ^{18}F -deoxyglucose positron emission tomography (FDG-PET) [5].

Beside the extensive clinical application of warburg effect, recent studies in cancer cell show that most cancer cells are not defective in mitochondria function, including oxidative phosphorylation for efficient ATP generation [6, 7, 8]. These observations suggest the existence of alternative explanation for ATP production switch from oxidative phosphorylation to aerobic glycolysis, which is the altered metabolism in cancer cell for supporting anabolic growth requirements in proliferation, including fast ATP generation, massive biosynthesis of macromolecules and tight maintenance of cellular redox status [9]. It is now clear that metabolism change in cancer cell is driven by growth factor signaling transduction, and not secondary indirect consequences upon increasing demand from fast growth and dividing, but rather tightly regulated metabolism repro-

1. Background

gramming to increase nutrient uptake and flux under control of activated oncogenes or inactivated tumor suppressors [5]. This new understanding of cancer metabolism has become one of the hallmarks of cancer [10].

1.1.1. Metabolism switch in quiescent vs proliferative cell

Most non-proliferative cells in differentiated tissues are quiescent and producing ATP efficiently via oxidative phosphorylation in mitochondria. In presence of oxygen, these cells preferentially generate ATP from oxidation of glucose into CO₂ by oxidizing glycolytic product pyruvate in TCA cycle happening in mitochondria. Besides 2 net gain ATP generated from glycolysis, NADH, GTP and FADH₂ are produced during sequential oxidation of pyruvate in TCA cycle. These products from TCA cycle are fueling oxidative phosphorylation complexes I-V to generate ~36 ATP/glucose in total. Under anaerobic conditions, differentiated cells could produce large amount of lactate from glycolysis while oxidative phosphorylation is bypassed.

In contrast, cancer cells utilize 10% glucose for biosynthetic pathway upstream of pyruvate production, and the rest 90% glucose for pyruvate production [5]. Among this 90% glucose, 5% of them will be metabolized via oxidative phosphorylation and the rest 85% of them will be converted to lactate. This process could only generate ~4 ATP/glucose. One of the possible reasons for employing this low efficiency ATP production by cancer cells is that nutrient availability is not a issue for them. Cancer cells live in a environment having continuous supply of glucose and other nutrients. There are evidences that ATP production is never a limiting factor during cell division [11, 12]. Even highly stimulated to growth and dividing, cancer cells are still able to maintain high ATP/ADP and NADH/NAD⁺ ratios.

Beyond ATP requirement, cancer cells also need double its cellular content for cell division. There are huge needs for nucleotides, amino acids, and lipids for macromolecular biosynthesis and new membrane formation. Although ATP is indispensable for most biomass accumulation reactions, other intermediate metabolites are also needed. For example, the palmitate synthesis for reconstituting new cellular membrane, requires 8 molecules of acetyl-CoA as carbon source, 14 molecules of NADPH for reducing power, as well as 7 molecule of ATP. One molecule of glucose could generate up to 36 ATP, or 30 ATP plus 2 NADPH through phosphate pentose pathway, or just provide 6 carbon for macromolecular biosynthesis. Now back to palmitate synthesis, one molecule of glucose could provide ~5 times ATP needed for 16-carbon fatty acid synthesis, or 1/7 of the

1. Background

NADPH needed for it. There is 35-fold asymmetry between the need for ATP and that for NADPH, given that 3 additional glucose for making acetyl-CoA as carbon source for palmitate synthesis [13]. Thus, during cell proliferation, majority of glucose can not undergo oxidative phosphorylation to generate ATP, otherwise the resulting high ratio of ATP/ADP will negatively control the flux of glycolysis and compromise the production of acetyl-CoA and NADPH, which leads to impaired biomass accumulation. The "by-product" lactate could also be converted into glucose again in liver by cori cycle [13].

1.1.2. Signaling in metabolism reprogramming

Recently, there are increasing evidences support the hypothesis that metabolic reprogramming is a hallmarks of tumor development and the primary consequence from mutation in oncogenes and tumor suppressor genes [10, 5]. Cancer cell proliferation not only relies on large amount of energy consumption, but also needs other build blocks for cell growth, such as amino acids for protein synthesis and fatty acids for lipid bilayer formation. For these purposes, cell metabolism must undergo massive reprogramming to fulfill the increased anabolic demand for cell growth and division. Interestingly, cancer cell metabolic reprogramming and metabolic syndromes such as type 2 diabetes and hepatosteatosis (fatty liver), are sharing a broad range of signaling pathways. One of these is Insulin/PI3K/AKT/mTOR pathway. Activation of PI3K/AKT is probably the most prominent lesion in various types of cancer. mTOR, a downstream target of PI3K/AKT, is well-characterized for its role in enhancing protein synthesis, glycolysis and lipogenesis via S6K, HIF1 α and SREBP-1 respectively. Dissecting the signaling network behind the insulin signaling could potentially reveal more pharmaceutical targets for treating both cancer and metabolic syndromes.

1.2. Liver metabolism

Liver is the largest organ in the body, contributing about 2% total body weight in human and 5% in mouse. Liver is a lobular structure composed of many cylindrical *liver lobule* as basic function unit. Each unit containing 3 types of cell: hepatocytes, endothelial cell and Kupffer cell (local macrophage cell in liver). Liver performs many important functions in physiology: (1) filtration and storage of blood (2) metabolism of carbohydrates, lipids, proteins, hormones and foreign chemicals (3) bile acid synthesis (4) vitamin and iron storage (5) synthesis of serum proteins, such as albumin, coagulation factors .

1. Background

In this thesis project, liver glucose and lipid metabolism are the main focus. The liver is especially essential organ for maintaining blood glucose level. Liver can serve as a glucose buffer. In postprandial phase, large amount of blood glucose is transported into liver for glycogen synthesis and lipid *de novo* synthesis [14], which allow removal of excess blood glucose and returning into blood when glucose level drops below normal. Liver can also synthesize glucose via gluconeogenesis when blood glucose falls below normal. During this process, large amount of amino acid and glycerol released from triglyceride are converted into glucose and released into circulation to maintain normal level of blood glucose.

For lipid metabolism, liver is the major organ for certain aspect of fat metabolism, including: (1) fat oxidation (2) synthesis of lipoproteins, cholesterol and phospholipids (3) free fatty acid and triglyceride synthesis from carbohydrates and proteins . The major sites for *de novo* lipogenesis are liver and adipose tissue. In liver, the products from lipogenesis are either stored as lipid droplets in liver or secreted in the form of VLDL, which delivers the endogenous derived lipids to peripheral organs.

1.2.1. Liver glucose regulation

After digestion in alimentary tract, the final products of carbohydrate are glucose, galactose and fructose. Much of the fructose and all of galactose are interconverted into glucose in liver. Thus glucose becomes the dominant carbohydrate circulating in blood.

In postprandial phase, Liver plays a critical role in nutrient absorption and metabolism, since it is the first barrier to filter all the ingested nutrients through hepatic port vein. This process filters out micro-organisms absorbed together with nutrients, such as bacteria, fungi, viruses and parasites. As the first access to nutrients, liver is exposed to higher nutrient levels than peripheral organs. Large amount of glycogen is synthesized in liver in order to relieve the modest hyperglycemia after meal for normal individual. In addition, liver also produce glucose to maintain glucose homeostasis during fasting state. In diabetic person, liver is one of the major culprit for hyperglycemia for impaired balance between glucose uptake and production in liver [15].

1.2.1.1. Liver glucose uptake

In postprandial phase and high glucose load via oral or enteral delivery, liver shifts the balance toward more glucose uptake than endogenous production (NHGU shifts from

1. Background

modest to high, from 2.8-11.1 to 25-27.8 $\mu\text{mol}/\text{kg}/\text{min}$ in dog [16, 17].). The glucose uptake experiments in human and dog have shown that liver NHGU takes up 25-40% of the administered glucose, while muscle and adipose tissues take one third, and the noninsulin-sensitive glucose obligating tissues (brain, red blood cells, etc.) absorb the remaining one third (Figure 1.1). Actually, the liver NHGU underestimates the role of liver in glycemic control. The total capacity for liver in glucose disposal upon oral glucose load is about 60-65%, demonstrates the great importance of liver in glucose clearance and production [14]. Once taken up by liver, glucose is converted to glucose-6-phosphate by liver glucokinase (L-GCK). Unlike other hexokinases, L-GCK is not inhibited by glucose-6-phosphate. This allows actively glycogen storage in postprandial phase.

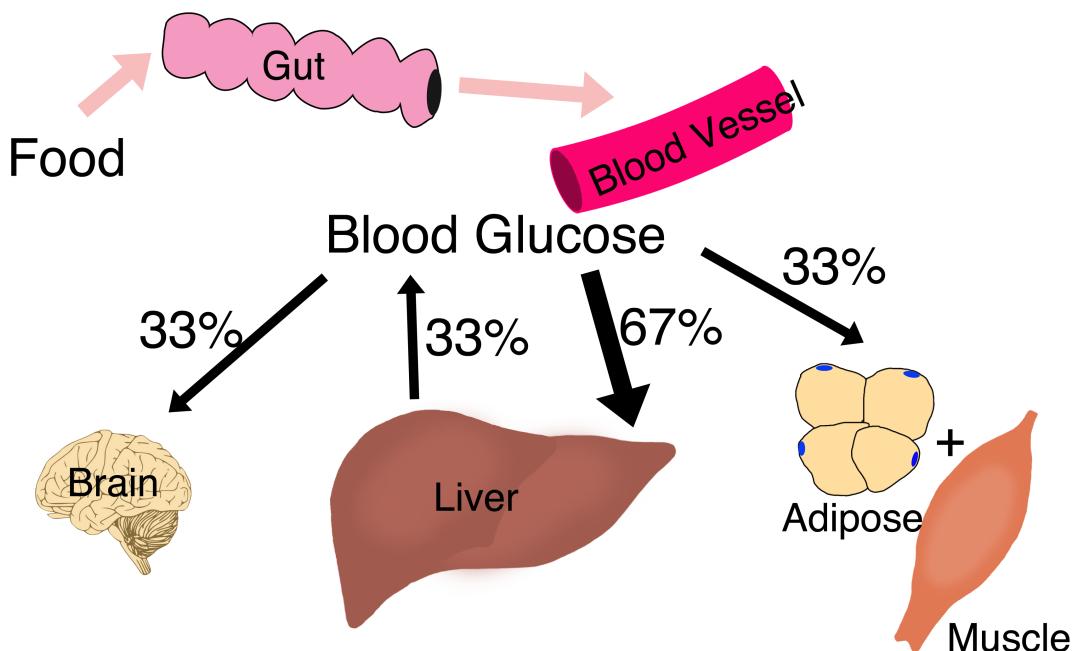


Figure 1.1.: Glucose absorption among metabolism related tissues. Adapted from Moore et al. [14].

1.2.1.2. Glycolysis for energy and metabolite intermediate production

Glucose-6-phosphate is further metabolized in glycolysis or stored in form of glycogen. Glycolysis converts glucose into pyruvate with 2 ATP and 2 NADH released from one glucose molecule. Pyruvate is further decarboxylated to acetyl-CoA and then submitted for TCA cycle or *de novo* lipogenesis. The pentose phosphate pathway (PPP) is another way of metabolizing glucose, which generates NADPH as antioxidant or reducing equivalent for *de novo* lipogenesis and cholesterol synthesis.

1. Background

1.2.1.3. Glycogen storage in liver

After glucose is absorbed into liver, it can be metabolized in glycolysis pathway to release energy and building block for protein and lipid synthesis, or it can be stored as glycogen for future use such as releasing glucose in fasting state. Liver can store as much as 5-8% of its weight as glycogen. The conversion from glucose to glycogen allows liver cell store large amount of carbohydrate without increasing intracellular osmotic pressure.

Glycogenesis for glycogen formation

The chemical process of glycogen synthesis is that glucose-6-phosphate is interconverted into glucose-1-phosphate; this is converted to uridine diphosphate glucose, which is finally synthesized into glycogen. There are several enzymes involved in this process: (1) Phosphoglucomutase for reversible interconversion between glucose-6-phosphate and glucose-1-phosphate; (2) UTP-glucose-1-phosphate uridylyltransferase for UDP-glucose synthesis from UTP and glucose-1-phosphate; (3) Glycogenin for initiating glycogen synthesis by catalyzing attachment of a glucose molecule to one of its own tyrosine residues; (4) Glycogen Synthase (GS) for catalyzing elongation of glycogen chains; .

GS can be regulated by several pathways. Glucose-6-phosphate can allosterically activate GS. Phosphorylation of GS can reduce its activity. Numerous kinases have been shown to regulate GS by phosphorylation [18]. Phosphorylation of GS occurs both in primary and secondary phosphorylation sites. Primary phosphorylation events are initiated by phosphorylase kinase, PKA, AMPK, PKC, CaMK-II, and CK-II. Secondary phosphorylation events are initiated by GSK-3 and CK-I.

Glycogenolysis for glycogen removal

In fasting state, liver produces glucose for whole body by breaking down glycogen into glucose in a process called glycogenolysis. This process is catalyzed by glycogen phosphorylase. Glycogen phosphorylase is regulated by allosteric activation of AMP and activated by phosphorylation via PKA. The product from glycogenolysis is glucose-1-phosphate, which will be further converted to glucose-6-phosphate by phosphoglucomutase. And the glucose 6-phosphatase remove the phosphate group from glucose-6-phosphate to produce glucose which will be transported out of liver.

1. Background

1.2.2. Liver lipid metabolism

1.2.2.1. Dietary lipid absorption

During digestion, triglycerides from food are split into monoglycerides and fatty acids, which are re-esterified in intestinal epithelial cell into triglycerides and released into lymphatic system as lipoprotein droplets called chylomicrons (Figure 1.2). Chylomicrons also contain cholesterol and phospholipids absorbed from food. The half-life of chylomicrons is less than 1 hour. Most of chylomicrons are cleared out in capillary of muscle, adipose and liver by the action of lipoprotein lipase (LPL). The remnants of chylomicron are absorbed by liver via LDL receptor, LDL receptor-related protein (LRP) and scavenger receptor B-1 mediated endocytosis. The engulfed chylomicrons in hepatocytes are digested in lysosome to release glycerol, fatty acids, cholesterol, which are recycled into VLDL.

1.2.2.2. Lipoprotein particle for lipid transportation and redistribution

Besides chylomicrons, there are four major types of lipoprotein particles circulating in plasma (Figure 1.2). Most of these lipoprotein particles are synthesized from liver and employed to redistribute triglycerides, cholesterol, and phospholipids among peripheral tissues. These lipoprotein particles are classified based on their density measured in ultracentrifugation: (1) VLDL is synthesized in liver and containing highest amount of triglycerides (~70%) and modest amount of cholesterol (~7.5%) and phospholipids [19]; (2) IDL is derived from VLDL, in which triglycerides are partially removed; (3) LDL is derived from IDL, in which almost all triglycerides are absorbed by peripheral tissues, left with very high amount of cholesterol (~45%) [19]; (4) HDL is synthesized in liver or intestine epithelium, containing high concentration of protein (~50%) and modest amount of cholesterol (~20%) and phospholipids [19]; .

In contrast to chylomicron for exogenous lipid transportation, the primary function of VLDL is transporting endogenous liver-derived triglycerides and cholesterol to peripheral tissues such as adipose tissue and muscle. As partially digested lipoprotein from VLDL, IDL is either taken up by liver or continually circulating in plasma to convert into LDL. LDL contains high amount of cholesterol, which is also called "bad cholesterol" in comparison to "good cholesterol" in HDL. Increasing blood cholesterol from LDL is a strong risk factor for causing atherosclerosis. HDL is responsible for delivery cholesterol to peripheral tissues and removal of excess cholesterol from plasma in a process called

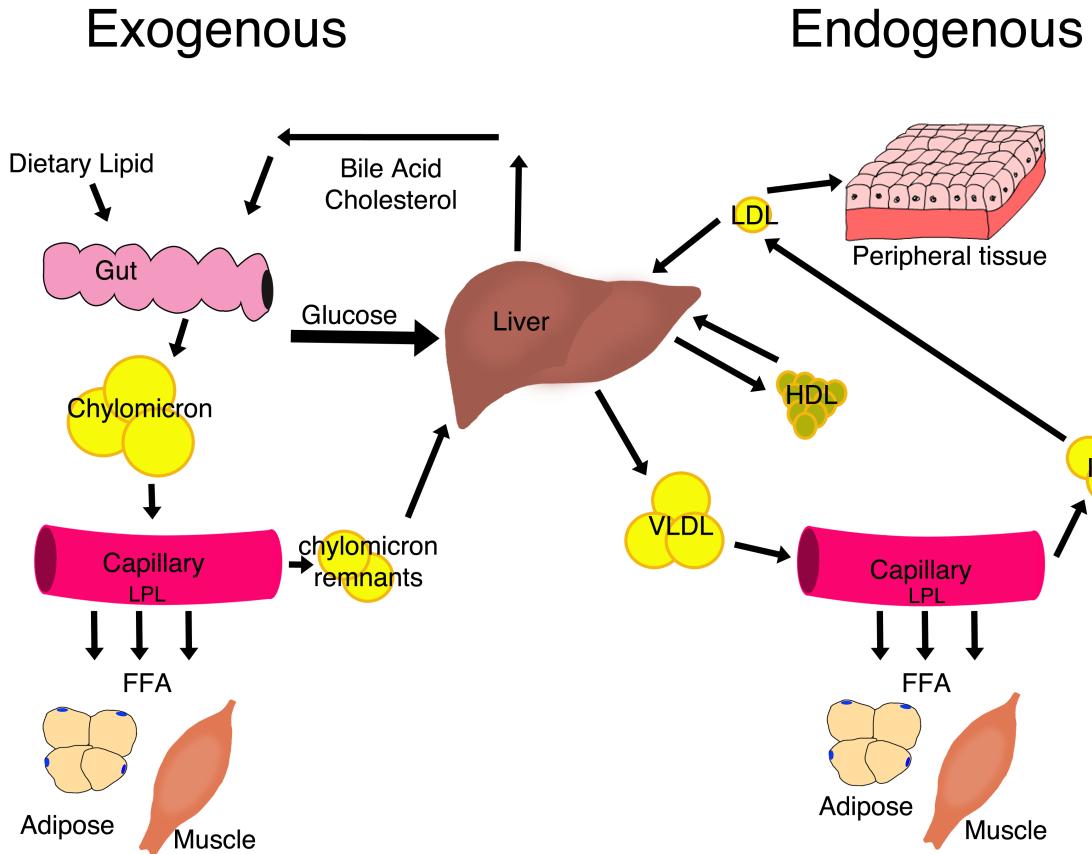


Figure 1.2.: Lipid absorption and lipoprotein particle redistribution among metabolism related tissues.

reverse cholesterol transport [20, 21].

1.2.2.3. *de novo* lipogenesis in liver

Whenever a greater amount of carbohydrate than it can be used immediately such as in glycolysis or stored in form of glycogen, the excess is quickly converted to triglycerides. Most of the triglyceride synthesis occurs in liver, while a small fraction also occurs in adipose tissue. Hepatic *de novo* lipogenesis include fatty acid synthesis from acetyl-CoA and malonyl-CoA, and further synthesis of triglycerides. Fatty acid synthesis is catalyzed by acetyl-CoA carboxylase for malonyl-CoA synthesis and fatty acid synthase for fatty acid elongation up to 16 carbon. Fatty acid and its metabolites are the major culprit for lipotoxicity. Thus fatty acids are quickly further stored as triglycerides, which are relatively inert and shown to have hepatic protective role. Triglyceride synthesis starts from glycerol-3-phosphate and fatty acid-CoA by glycerol-3-phosphate acyltransferase (GPAT) to form lysophosphatidic acid, then further adding fatty acid stepwise

1. Background

by acylglycerolphosphate acyltransferase (AGPAT), phosphatidic acid phosphohydro-lase (PAP) and diacylglycerol acyltransferase (DGAT) to have triglycerides. Finally, triglycerides are packaged into VLDL.

1.2.2.4. β -oxidation of fatty acids

Degradation and oxidation of fatty acids occurs in mitochondria, peroxisomes and ER [22]. For β -oxidation in mitochondria, fatty acids are first transported via help from carrier called carnitine. Then fatty acids in mitochondria is progressively processed to release acetyl-CoA and reducing equivalent such as FADH₂ and NADH. β -oxidation of fatty acid will release tremendous amount of energy from it. For example, one molecule of stearic acid will release net gain of 146 molecule of ATP after complete oxidation [23]. Acetyl-CoA can also be converted to ketone body in case of excess fatty acid, or further processed in TCA cycle. Two acetyl-CoA can be condensed into acetoacetic acid. Acetoacetic acid can also be converted to β -hydroxybutyric acid [24]. In ER, long-chain fatty acids can be degraded via ω -oxidation by cytochrome P450 [25]. PPAR α and insulin are positive and negative regulator for fatty acid oxidation [22].

1.3. PP2A and its biology

Reversible protein phosphorylation is a ~~one~~ of the most abundant post-translational modifications (PTMs), in which protein residue serine/theronine/tyrosine is phosphorylated by kinase and de-phosphorylated by phosphatase. Regulation of protein phosphorylation is considered to be one of the most common way of protein function regulation, which switch protein between active and inactive form, or between stabilization and degradation, or different cellular localizations [26, 27, 28]. While the previous basic research and pharmaceutical development is mainly focused on kinase activity modulation to affect protein phosphorylation, it is now also being recognized that protein phosphatase could also be important regulator in protein phosphorylation and provide new drug candidate to change protein phosphorylation pharmacologically [29, 30, 31, 32, 33].

1. Background

Table 1.1.: PP2A gene superfamily composition^a.

Subunit	Gene Name	Protein Name
PP2A-A	PPP2R1A	PR65 α
	PPP2R1B	PR65 β
PP2A-C	PPP2CA	PP2Ac α
	PPP2CB	PP2Ac β
PP2A-B	PPP2R2A	PR55 α
	PPP2R2B	PR55 β
	PPP2R2C	PR55 γ
	PPP2R2D	PR55 δ
PP2A-B'	PPP2R5A	PR56/61 α
	PPP2R5B	PR56/61 β
	PPP2R5C	PR56/61 γ
	PPP2R5D	PR56/61 δ
	PPP2R5E	PR56/61 ϵ
PP2A-B''	PPP2R3A	PR130, B'' α 1
	PPP2R3A	PR72, B'' α 2
	PPP2R3B	PR70, B'' β
	PPP2R3C	G5PR
	PPP2R3D	PR59, B'' δ
PP2A-B'''	STRN	Striatin/PR110
	STRN3	SG2NA/PR93
	PPP2R4	PR53

^a Adapted from Perrotti et al [34].



1.3.1. PP2A structure

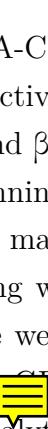
Protein phosphatase 2 (PP2A) is a heterotrimeric serine/threonine phosphatase with broad substrate specificity and diverse cellular functions. PP2A is composed of a dimeric core enzyme formed by scaffold A subunit and catalytic C subunit, and a regulatory B subunit for expanding PP2A's substrate specificity. While A and C subunit sequence have extraordinary sequence conservation throughout eukaryotes, the regulatory B subunits show more heterogeneous sequence evolution. Multicellular eukaryotes are believed to express four classes of regulatory subunits: B, B', B'', and B''' , with at least 13 members in these different subfamilies (Table 1.1).

Beyond this layer of complexity in gene family, each gene can also have various splicing isoforms. In total, different combination of certain isoform of certain gene subfamily member provide more than 200 possible variations of PP2A. This will also explain why phosphatases, even less represented in genome than kinases, can counteract phosphorylation events by many kinases. Table 1.2 shows the unbalanced number of genes for

1. Background

phosphatase and kinase in different genome, from Yeast to Human.

1.3.1.1. PP2A catalytic subunit

Mammalian PP2A catalytic subunit (PP2A-C) is okadaic acid sensitive metallophosphatase, which require normally Mg^{2+} in active center for its activity. PP2A-C is encoded by two gene subfamily member, α and β , which share 97% amino acid sequence identity with only minor difference at beginning N-terminal. PP2A-C α and β isoform also share 98-99% sequence similarity across mammalian species, and the differences are also located at N-terminal. Even comparing with other protein phosphatase, such as PP1, PP2B, and PP4-6, PP2A-C still share well-conserved residues in catalytic center with the phosphoesterase signature motif:  DxHG----(~25)----GDxVD----(~25)----GNHD/E [36]. In 3D structure, PP2A catalytic subunit share even higher structural similarity with PP1 and PP5 shown by 3D superimposition [37].

The C-terminal tail ($^{304}TPDYEL^{309}$) is extremely conserved between species. And the reason for this evolutional conservation can be explained from structural study of PP2A. The C-terminal Leucine can undergo reversible carboxymethylation by CMT (C-terminal leucine methyltransferase) and CME (PP2A-specific carboxymethyl esterase) [38]. Methylated C-terminal tail nestles into the pocket formed by scaffold subunit and regulator subunit of PP2A. And this arrangement will facilitate the assembly of PP2A holoenzyme.

The two sub-member of PP2A-C are differentially expressed. The PP2A-C α is about 10 times more efficiently transcribed than PP2A-C β [39], probably due to the higher expression capacity of PP2A-C α promoter [40]. Overexpression of PP2A-C in mammalian cell was not successful. And knockout mice of PP2A-C α is not viable and die at embryonic 6.5 day [41], which indicate the importance of PP2A-C α in mouse development and its non-redundancy in term of rescuing by PP2A-C β .

Table 1.2.: Kinase/phosphatase gene number imbalance in genome^a.

Gene Family	<i>S. cerevisiae</i>	<i>D. melanogaster</i>	<i>C. elegans</i>	<i>H. sapiens</i>
Total Gene Number	6122	13600	18988	25000
Total Protein Kinase Number	124	236	493	518
Total Protein Phosphatase Number	37	93	185	119 (21 Protein S/T Phosphatase)

^a Adapted from Seshacharyulu et al [35].

1. Background

1.3.1.2. PP2A scaffold subunit

The Scaffold A subunit is also encoded by two distinct isoforms, PP2A-A α and PP2A-A β , which share 86% sequence identity. A subunit is the structural subunit which provide scaffold for regulatory subunit and Catalytic C subunit association. Since the different regulatory subunit bind with the same or overlapping surface on A subunit, the association of regulatory subunit to holoenzyme is mutually exclusive [42, 43].

The structure of PP2A-A is quite unique, composed of 15 tandem repeats of rod-like HEAT (Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase TOR1) domain, and building C-shaped horse hoof like structure which facilitate the recruitment of regulatory subunit and other substrates [42].

Mice that are homozygous for PPP2R1A^{−/−} are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities. However, Oocyte-specific knock-out of PPP2R1A leads to severe defect in female meiosis and fertility in mice [44]. Mouse model of human cancer-associated mutations in PPP2R1A increases lung cancer incidence [45].

1.3.1.3. PP2A regulatory subunit

Regulatory subunits of PP2A are diverse (Table 1.1) and low in sequence similarity between these four gene families, even though they all bind to similar repeats in A subunit. From the crystal structure, it is postulated that regulatory subunit, together with catalytic subunit, establish the binding groove for substrates. The diversity of regulatory subunit can partially explain how PP2A counteract phosphorylation from multiple kinases by having diversified substrate recognition.

PP2A-B

PP2A-B gene family has four sub-members (Table 1.1), which are all tissue-specifically expressed [46]. PR55 α and PR55 δ are expressed in almost all tissues, while PR55 β and PR55 γ are highly expressed in brain. Structurally, PP2A-B family protein's common feature is existence of 5 degenerated WD40 repeats, which are believed to be involved in protein-protein interactions. It has been shown that PR55 α and PR55 β could interact with intracellular domain of TGF- β receptor via WD40 repeats [47].

1. Background

PP2A-B'

PP2A-B' gene family currently contains 5 member (Table 1.1). While PR61 α , PR61 β and PR61 ϵ mainly are localized in cytoplasm, PR61 γ and PR61 δ are localized both in cytoplasm and nucleus. For tissue expression pattern, PR61 α and PR61 γ are expressed in almost all tissues, especially high in heart and skeletal muscle. And PR61 β and PR61 δ are highly enriched in brain [48, 49, 50].

All PP2A-B' genes share a conserved central region (80% identical), with different C and N terminal. This indicates the central region is probably more involved in association with scaffold and catalytic subunit, while C and N terminal could be involved in regulating substrate diversity. The structure of PP2A holoenzyme shows PP2A-B' also have psuedo-HEAT repeat like structure [42]. The modular structure of PP2A-B' preserved the structural similarity for stability of holoenzyme, while the terminal sequence difference allows structural diversity at N and C terminal for substrate diversity.

PP2A-B''

Initially, PR72 and PR130 were discovered as founding members of PP2A-B'' [51]. The only sequence difference between them was located at N terminal which raises the possibility that PR72 and PR130 are originated from alternative splicing. Two other member PR48 and PR59 were discovered by yeast two-hybrid as interaction partners for retinoblastoma-related p107 protein and Cdc6 respectively [52, 53].

PP2A-B'''

PR93 and PR110 were discovered as members of PP2A-B''' family based on their sequence conservation with PP2A-B' family. These two protein are all calmodulin-binding protein and suggesting their associated PP2A holoenzyme are involved in calcium-dependent signaling [54].

1.3.2. PP2A in signaling

PP2A is essential to majority of signaling pathways, including cell cycle control, Wnt signaling, insulin signaling, apoptosis, cell adhesion and cytoskeleton dynamics, etc. (Figure 1.3).

1. Background

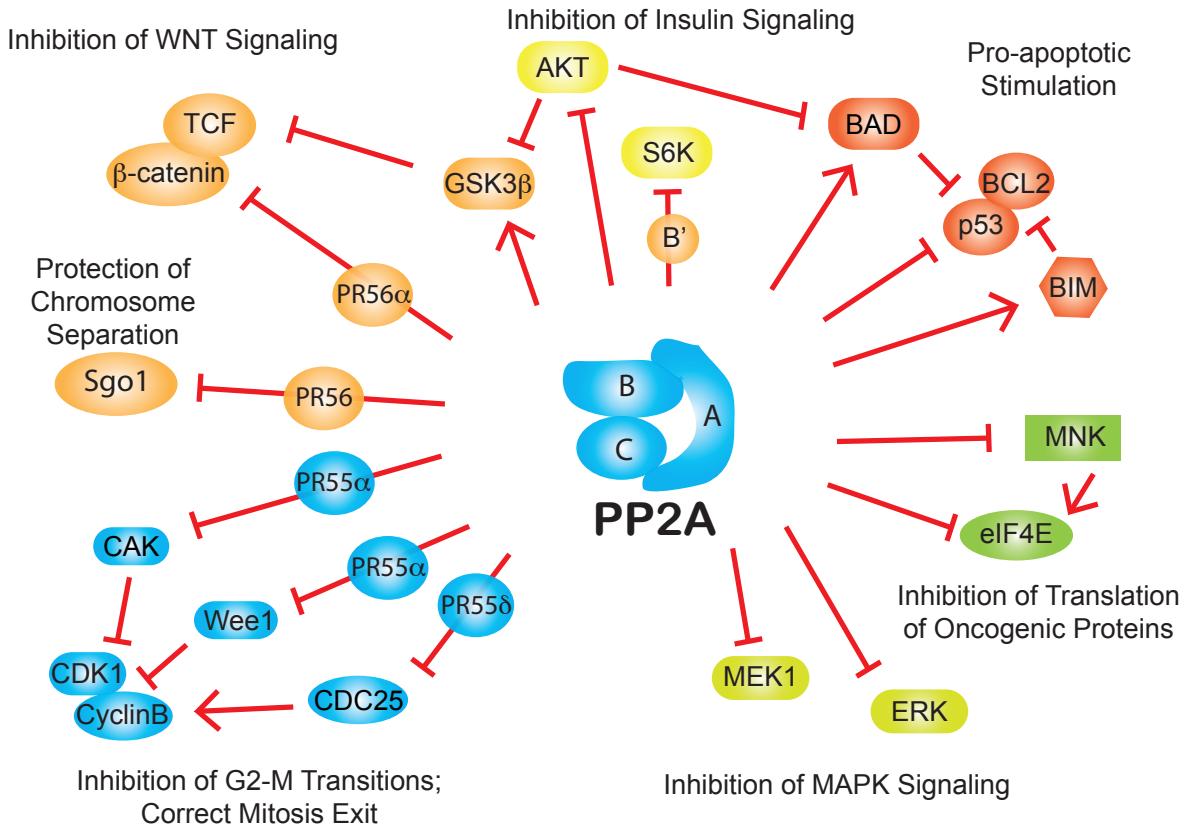


Figure 1.3.: PP2A is involved in many signaling pathways by counteracting phosphorylation events by many kinases. Adapted from Perrotti et al. [34].

Multiple PP2A B subunits have been demonstrated to be involved in regulating metabolism via Insulin/AKT/mTOR signaling pathways. In *Drosophila*, Hahn et al demonstrated that PP2A regulatory B' target S6K to modulate the phosphorylation of its activation site, and this regulation is conserved in HeLa cell line [1]. Another *Drosophila* B' subunit Widerborst modulates activated AKT via direct interaction and changes lipid droplet size and expression of lipid storage protein perilipin [55]. *C. elegans* B' subunit pptr-1 could also directly regulate AKT's phosphorylation *in vivo* and impact the life span, fat storage phenotype of worms [56]. In mammalian system, AKT was found to be associated with PP2A-B55 holoenzyme, and its phosphorylation at Thr-308 was compromised by overexpressing PR55 α subunit in both FL5.12 and NIH3T3 cells [57].

In Xenopus, B' subunit PR56 α was discovered to be the negative regulator for β -Catenin phosphorylation, which leads to degradation of β -Catenin via the ubiquitin/proteasome pathway [58]. However, recent evidences show PP2A has more complicated control in Wnt signaling [59]. Before Wnt ligand binding, β -Catenin is located in destruction

1. Background

complex including APC, AXIN, and GSK3 β . PP2A B subunits, PR61 α - δ , bind to either APC or AXIN to destabilizing β -Catenin. However the mechanism is still not clear. Upon Wnt ligand binding, Wnt downstream effector Naked, which causes a negative feedback on Wnt signaling, requires PR72 for its negative regulation, and is repressed by PR130 (alternative splicing form of PR72). Additionally, both PR61 ϵ and PR55 could enhance Wnt signaling by destabilizing inhibitory GSK3 β (Figure 1.3).

For MAPK kinase signaling cascades, PP2A has also both inhibitory and activating role [59]. Related to PP2A's activating role, PR55 α binds and dephosphorylates KSR1 and RAF upon RAS activation. And this leads to plasma membrane recruitment of RAF and subsequent enhanced binding between RAF and RAS, therefore stronger activation of MAPK signaling. On the other hand, PR55 γ interacts with c-SRC and inhibits c-SRC's positive regulation on RAF independently from RAS activation. Furthermore, PR61 β and PR61 γ directly de-phosphorylate ERK (Figure 1.3).

PP2A has also pro-apoptotic activity via its inhibitory effect on AKT, which inactivates anti-apoptotic protein BCL2 and activate pro-apoptotic factors like BAD and BIM [59, 60] (Figure 1.3). PP2A directly binds BCL2 and BIM and de-phosphorylates them. Also, PP2A directly binds to BH4 domain of BCL2 and removes the phosphorylation at Ser70 of BCL2, which causes enhanced interaction between p53 and BCL2 to inhibit BCL2's anti-apoptotic function. Additionally, inhibition of PP2A by okadaic acid or siRNA increases eIF4E phosphorylation via MNK kinase [61]. This finding is consistent with the tumor suppressor role of PP2A.

PP2A has fundamental role in controlling cell cycle. During G1-S transition, PR56 γ is translocated into nucleus and PP2A terminal methylation levels also change [59]. Also, PR55 α is inhibiting CDK1-Cyclin B complex via inhibition of CAK and Wee1 kinase. PR56 δ could also de-phosphorylate CDC25 and inactivate it. PR56 family could also regulate sister chromatid cohesion, which is important for proper chromosome segregation in mitosis and meiosis [44, 62]. In my own PhD thesis project, over-expression of mouse PP56 γ (PPP2R5C) in Hepa 1-6 has resulted in dense chromosome in DAPI staining (data not shown).

1.3.3. PP2A in cancer

PP2A has been demonstrated with multiple evidences for its tumor suppressor activity in human cell transformation [63, 64]. Viral antigen SV40 small T antigen or tumor-inducing toxins like okadaic acid and microcystin-LR have been shown to the viral or

1. Background

chemical inhibitor for PP2A activity [65, 66]. In addition, *in vivo* inhibitor for PP2A CIP2A, an endogenous interacting protein for PP2A, has been found to be the stabilizer for c-Myc and mediating PP2A's inhibition in human malnignancies [30, 67].

PP2A scaffold and regulatory subunits have been shown to be mutated or down-regulated in multiple cancers [35]. Knockdown of PR56 γ in HEK cell inhibits PP2A phosphatase activity similar to the extent achieved by SV40 small T antigen and induces anchorage-independent tumor growth [68]. PP2A PR56 γ containing holoenzyme has tumor growth suppression activity via de-phosphorylation on p53 at Thr55 [69, 70], and leads to growth arrest and inhibition of cell proliferation.

2. Results



Human PPP2R5C has been shown in regulating p53 tumor suppressor activity upon DNA damage[69, 70]. After DNA damage, PR56 γ containing PP2A holoenzyme binds p53 and de-phosphorylates Thr55 of p53, which leads to induction of downstream transcriptional target p21 and following inhibition of cell proliferation [69]. In addition, the interaction between PR56 γ and p53 upon DNA damage require ATM-dependent phosphorylation of Ser15 in p53 [70]. However, p53 protein sequence alignment between human and mouse shows that Thr55 in mouse is missing, and this deletion rules out the possibility of mouse PR56 γ 's ability to inhibit cell proliferation via p53. Indeed, PPP2R5C knockdown in mouse cell lines has mild effects on proliferation (*circa* 5% increase in total protein content, data not shown).



Human PPP2R5C also involves in TCR-induced NF- κ B activity[71]. NF- κ B is activated upon T cell stimulation by complex phosphorylation event cascade. PPP2R5C was found to be the negative regulator to fine-tune and terminate NF- κ B activation. PPP2R5C silencing in stimulated primary human T cell causes increased phosphorylation in IKK and I κ B α .

Knockout mice in PPP2R5C results in heart development defects, including formation of incomplete ventricular septum and a decrease in the number of ventricular cardiomyocytes [72]. In addition, PPP2R5C knockout mice have a decrease in locomotive coordination and gripping strength, which indicates that PPP2R5C is also required for efficient neuromuscular function. Finally, the knockout mice have also neonatal growth deficiency, but survived knockout mice develop ~~into~~ obesity after weaning and have 31% more body weight in age of 6 months. However, the exact mechanism or substrate for PPP2R5C knockout to cause obesity is still not clear.



Proteomic study on PPP2R5C interacting partners reveals several interesting candidates as PPP2R5C's phosphatase substrates [73, 74]. A GST tagged PPP2R5C is used to unravel its potential interaction partners by Mass Spectrometry approach. And several proteins, such as calcium pump SERCA2a, are involved in calcium homeostasis. Over-

2. Results

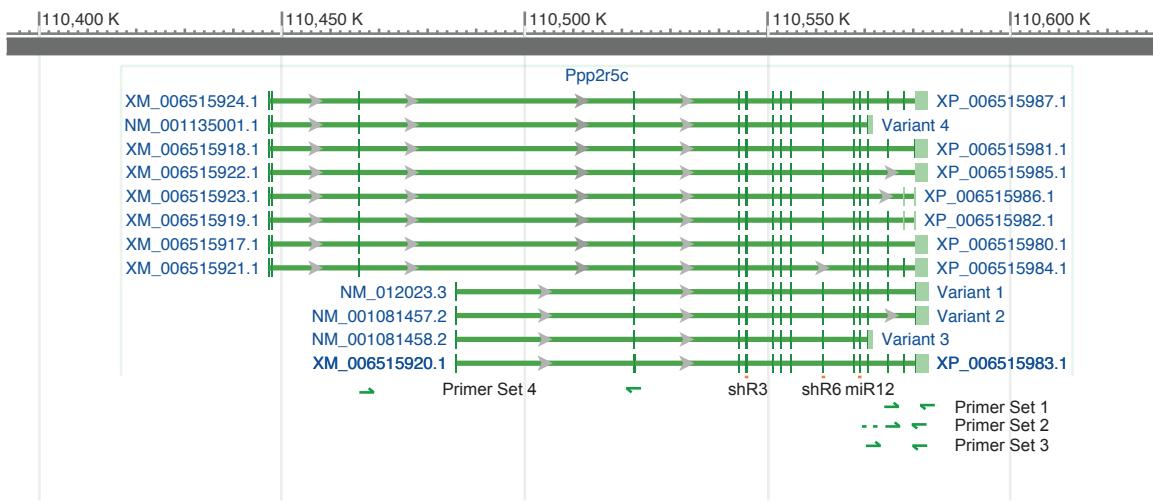


Figure 2.1.: Mouse PPP2R5C gene structure. Four splicing isoforms, Variant 1 to 4, are displayed with exons and introns according to NCBI genebank annotation. Primer set for Variant 1 to 4 are also shown in their corresponding positions. miRNAs/shRNAs targeting all isoforms, including shR3, shR6 and miR12, are also shown at their relative positions.

expression of PPP2R5C in cultured myocytes impairs the cell contractility [74]. Another TAP tagged PPP2R5C based proteomic strategy also finds Liprin α 1 is interacting with PPP2R5C independent of PP2A holoenzyme. Liprin α 1 is suggested to stabilize PPP2R5C and regulate focal adhesion [73].

In our lab, homolog of PPP2R5C in Drosophila, PP2A-B', has been previously shown that it regulates organismal metabolism [1] via directly de-phosphorylating S6K1 in *Drosophila*. In agreement with S6K activation phenotype, fly with PP2A-B' whole body knockout has increased insulin signaling phenotype, which is decreased life span and whole body triglyceride. The initial study in Hela cell also shows that human homolog of PP2A-B', PPP2R5C, also negatively regulate S6K phosphorylation. The naturally following question would be whether there is any link between PPP2R5C and metabolic status in more translational related context, such as in mice or human patients. In mice, PPP2R5C gene contains splicing isoforms according to NCBI's genbank annotation when the project was started. The gene structure is shown in Figure 2.1. Now with deeper annotation, there are 11 different isoforms for mouse PPP2R5C have been annotated in NCBI.

2.1. Transcriptional response of PPP2R5C in pathophysiology

Since genes for metabolic regulators are often present in regulatory transcriptional feedback loops [75], the relationship between PPP2R5C expression and organismal nutritional status was tested in various mice tissue sample. Indeed, obese mice lacking leptin receptor (*db/db*) have significantly elevated levels of various PPP2R5C mRNA isoforms in liver (Figure 2.2). By ANOVA analysis, at least in Variant 1, 2 and 4, there are significant increases in transcriptional level of PPP2R5C in *db/db* mice liver. Variant 3 has also shown similar trend through not significant. However, the total protein level of PPP2R5C, measured by home-made antibody for mouse PPP2R5C, did not show a significant increase in protein level. This discrepancy in mRNA and protein level of PPP2R5C further indicates the up-regulation in *db/db* is a transcriptional feedback.

Another interesting finding in liver PPP2R5C mRNA profiling is that at least Variant 4 in wildtype mice has a transcriptional increase in response to fasting. And this response is reversed after 6 hour re-feeding. Though not significant, Variant 2 shows similar trend. Additionally, there are also some significant changes in Variant 1 within wildtype or *db/db* mice between different nutritional statuses. However, these trends are not clear in other isoforms and could be isoform-specific features.

In mouse abdominal white adipose tissue, there is also a significant trend in which most of PPP2R5C variants have elevated mRNA levels in *db/db* mice, especially for Variant 4 (Figure 2.3). There is significant transcriptional increase in *db/db* mice in most nutritional statuses even with larger standard deviation compared with liver PPP2R5C. This phenomenon is also validated to be true in another independent qPCR analysis of mouse adipose sample, which was done by Katrin Straßburger in our lab (data not shown). The same transcriptional change pattern shared between liver and adipose tissue PPP2R5C indicates a similar function performed by PPP2R5C in liver and adipose tissue. Interestingly, this match between adipose tissue and liver had also been reiterated in liver and subcutaneous adipose tissue in healthy human control and type 2 diabetic patients (See details in Section 2.6). This further indicates the conserved function of PPP2R5C between mice and human, and raises the interest to study PPP2R5C's role in metabolic diseases, such as Type 2 Diabetes.

PPP2R5C expression is nutritionally regulated in other metabolic relevant tissues such as muscle, albeit in a complex way. In mouse gastrocnemius muscle, PPP2R5C expres-

2. Results

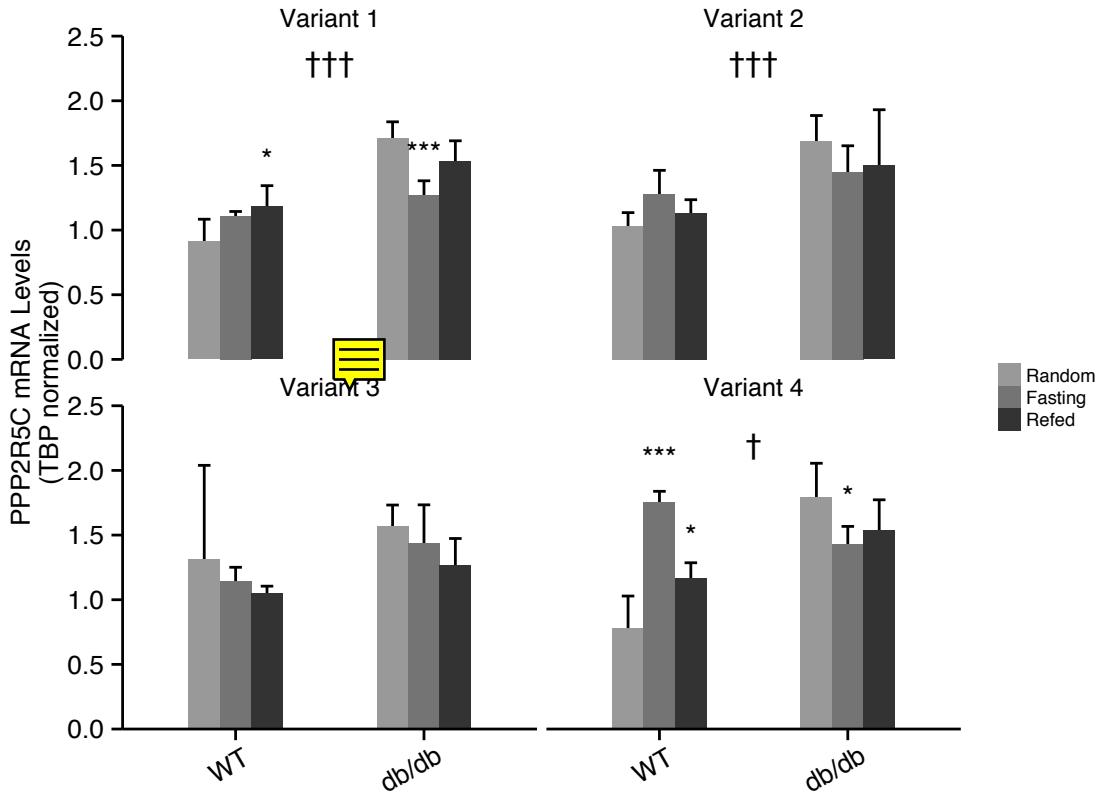


Figure 2.2.: PPP2R5C is regulated in mouse liver. Mouse PPP2R5C transcript variant 1–4 mRNA levels in liver from 8–12 week C57BL/6 wildtype (WT) or *db/db* male mice under different nutritional status is shown here. Mice were fed with normal chow diet without limit ("Random"). For "Fasting" and "Refed", mice were first starved for 16 hours and then allowed to normal chow diet access for 6 hours. Error bar: std. dev. (this is the same for all following figures). * and *** for p-value<0.05 and 0.001 by t-test within each mice genotype (WT or *db/db*) and feeding regime (Random, Fasting, and Refed) in R. p-value was adjusted by BenjaminiHochberg procedure in R. † and ††† for p-value<0.05 and 0.001 by ANOVA analysis with comparison between wt and *db/db* mice.

sion is increased upon refeeding (Figure 2.4). This is significant for all variants except Variant 1, though trend is still clear. However, this regulation is blunted in *db/db* mice compared to control mice in almost all isoforms but Variant 3. Variant 3 in both wt and *db/db* mice show increased expression upon refeeding. In addition, Variant 3 has also increased in *db/db* mice. In comparison with liver and adipose PPP2R5C, The opposite transcriptional response of PPP2R5C in muscle during switch from fasting to refed suggests distinctive role of PPP2R5C in metabolic relevant tissues under different nutrient conditions.

Finally, among various mouse model for different diseases such as Type I&II diabetes and hepatosteatosis, liver PPP2R5C shows significantly reduced total mRNA level compared

2. Results

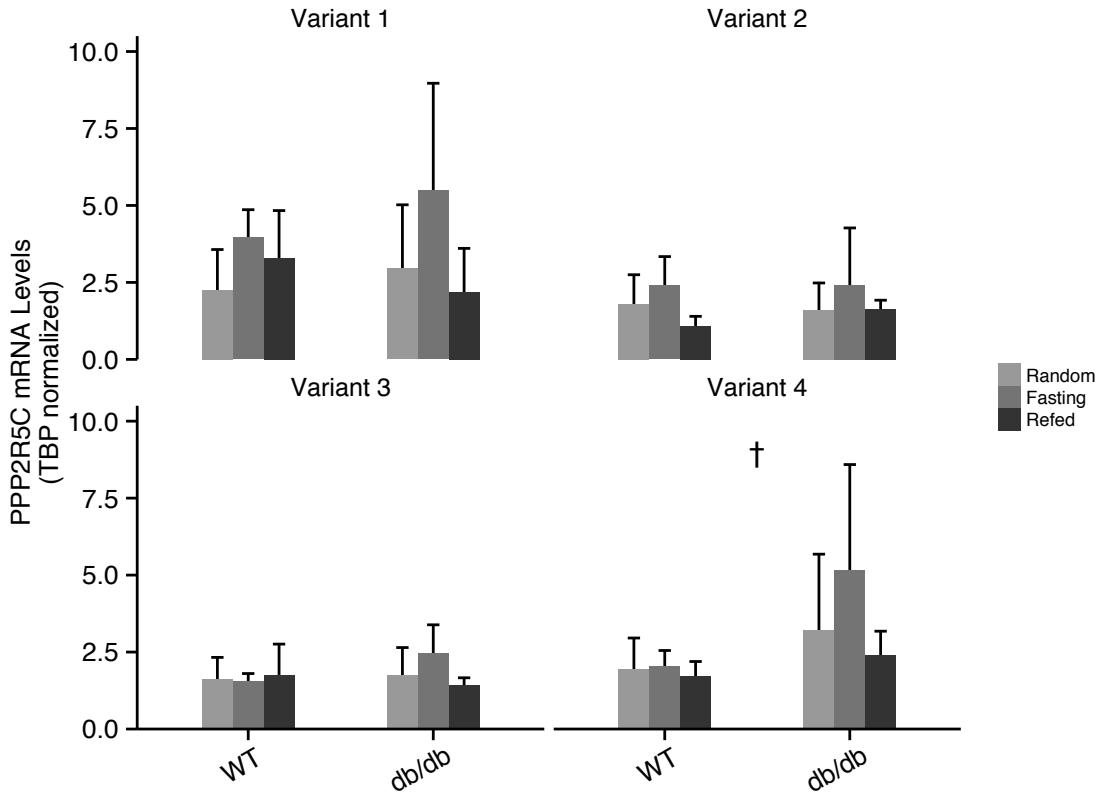


Figure 2.3.: PPP2R5C is regulated in mouse adipose tissue. Mouse PPP2R5C transcript variant 1–4 mRNA levels in adipose from 8–12 week C57BL/6 wildtype or *db/db* male mice under different nutritional status is shown here. Mice were fed with normal chow diet without limit ("Random"). For "Fasting" and "Refed", mice were first starved for 16 hours and then allowed to normal chow diet access for 6 hours. PPP2R5C Variant 1–4 are referenced according to NCBI. † for p-value<0.05 by ANOVA analysis with comparison between wt and *db/db* mice.

to control only in ~~in hepatosteatosis model~~ (Figure 2.5). However, in other disease models, there are no significant change in PPP2R5C mRNA level. In Dexamethasone induced mouse type 2 diabetes model, no strong increase in liver PPP2R5C mRNA levels shows a discrepancy with genetic model of type 2 diabetes mouse model (*db/db* in Figure 2.2). This difference could be due to different mechanisms for developing symptoms of type 2 diabetes in mouse [76, 77].

In sum, although I have no data suggesting that these transcriptional changes are of functional relevance, these results suggest there might be links between PPP2R5C and metabolic state or regulation. Although whole body knockout mouse model for PPP2R5C shown increased adiposity [72], distinct functions of PPP2R5C for metabolic homeostasis in a tissue-specific manner (in mammals) are widely unknown. A tissue-

2. Results

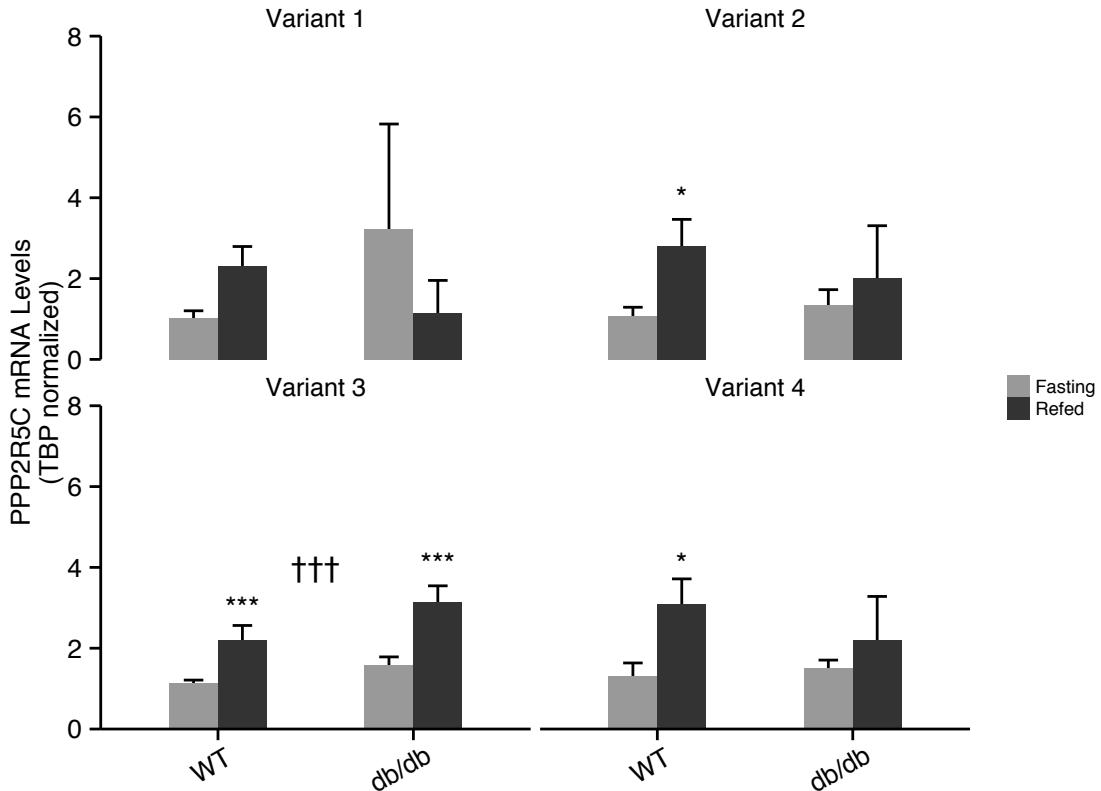


Figure 2.4.: PPP2R5C is regulated in mouse muscle. Mouse PPP2R5C transcript variant 1–4 mRNA levels in muscle from 8–12 week C57BL/6 wildtype or *db/db* male mice under different nutritional status is shown here. For "Fasting" and "Refed", mice were first starved for 16 hours and then allowed to normal chow diet access for 6 hours. * and *** for p-value<0.05 and 0.001 by t-test within each mice genotype (WT or *db/db*) and feeding regime (Random, Fasting, and Refed) in R. p-value was adjusted by BenjaminiHochberg procedure in R. ††† for p-value<0.05 by ANOVA analysis with comparison between wt and *db/db* mice.

specific knockout or knockdown of PPP2R5C would shed more light on its functions in metabolic control. Since PPP2R5C has more than four splicing isoforms when the project was started, the primer sets 1–4 used throughout the thesis are representing more than one transcript of PPP2R5C (Figure 2.1). RNA sequencing for these samples would give more information about the differential regulation of various splicing isoforms.

2. Results

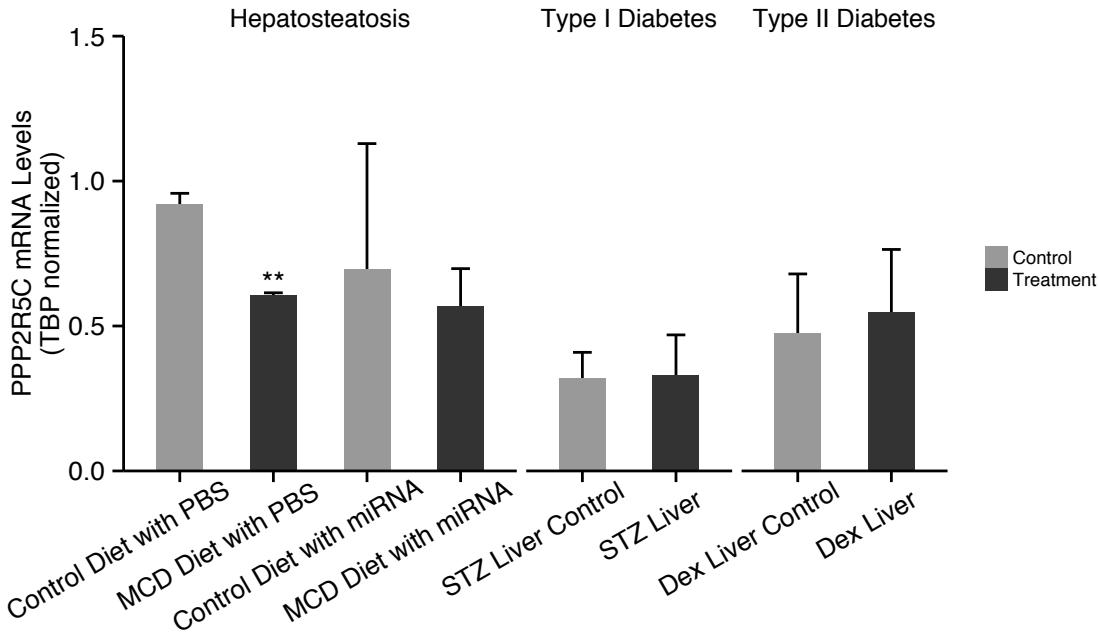


Figure 2.5.: PPP2R5C transcriptional change in different pathophysiological conditions. Liver PPP2R5C mRNA levels were evaluated by qPCR in control or treatment group for mouse model in Hepatosteatosis, Type I diabetes, and Type II diabetes. For Hepatosteatosis model, 16 week old male C57Bl6/J mice was fed with Methionine-Choline Deficient (MCD) diet or control diet for 4 weeks. In Type I diabetes model, mice was treated with Streptozotocin (STZ) or control. For Type II diabetes model, mice was treated with Dexamethasone (Dex) or control. ** for p-value<0.01 by t-test in R.

2.2. PPP2R5C negatively regulates glycolysis and lipogenesis

2.2.1. Selecting shRNA/miRNA for efficient knockdown of PPP2R5C

In order to characterize mammalian function of PPP2R5C, mouse model was chosen to decipher the molecular link for PPP2R5C in its potential role in metabolism. At first step, different shRNAs and miRNAs against common region of all PPP2R5C mRNA isoform was designed from online RNAi design website from Invitrogen [78] (final candidates used throughout the thesis are shown in Figure 2.1 and Table A.6). shRNAs were cloned into pENTR U6, and miRNAs were cloned into pcDNA 6.2 GW EmGFP, both were purchased from Invitrogen. To have a reporter for PPP2R5C protein level, PPP2R5C Variant 2 was cloned into 3' UTR of renilla luciferase reporter (pRL-CMV rel-

2. Results

lina) and co-transfected with expression vectors for shRNAs and miRNAs in HEK293T cell. Knockdown efficiency was monitored for shRNAs and miRNAs as firefly luciferase normalized activity (Figure 2.6). After comparing the dynamic efficiency with different shRNA or miRNA to reporter plasmid ratios, shR3 is discovered to be the shRNA with strongest knockdown, and miR2 is the current best miRNA candidate. Furthermore, knockdown efficiency was also cross-validated by western blot of HA-tagged PPP2R5C Variant 2 (Figure 2.7a). Again, shR3 is shown to be the best shRNA candidate with almost complete knockdown when co-expressed with Variant 2. Thus, shR3 was chosen to be shRNA for PPP2R5C knockdown in different models, from cell to mouse. However, miRNA candidates have different performance in protein level knockdown (Figure 2.7a). miR3 has better knockdown efficiency than miR2, but still has not reach the extent of shR3.

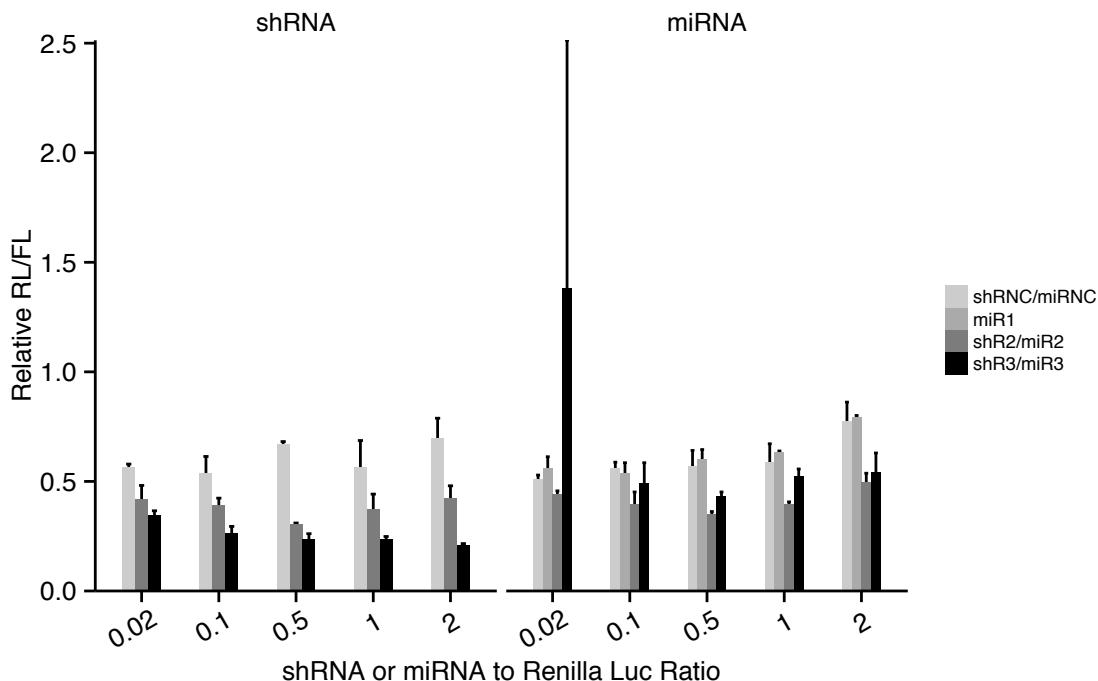


Figure 2.6.: KD efficiency for shRNAs/miRNAs are measured on PPP2R5C variant 2 luciferase reporter. PPP2R5C variant 2 was cloned into 3' UTR of firefly luciferase reporter (pRL-CMV rellina). RL-Var2 reporter was used to evaluated shRNA/miRNA KD efficiency via co-transfection with miRNA/shRNA expression construct at different ratios in HEK293T cell. Rellina luciferase (RL) activity was normalized against firefly luciferase (FL).

Due to lower performance in knockdown efficiency, a bigger scale of searching miRNA candidate was needed. 12 new miRNAs were designed and cloned as before. Similar western blot validation was performed across various miRNAs (Figure 2.7). In this experiment, miR12 was discovered to be the best miRNA against PPP2R5C so far

2. Results

(only 5% Variant 2 left comparing with control miRNA at protein level, Figure 2.7b), and chosen to be the miRNA for PPP2R5C knockdown in vivo.

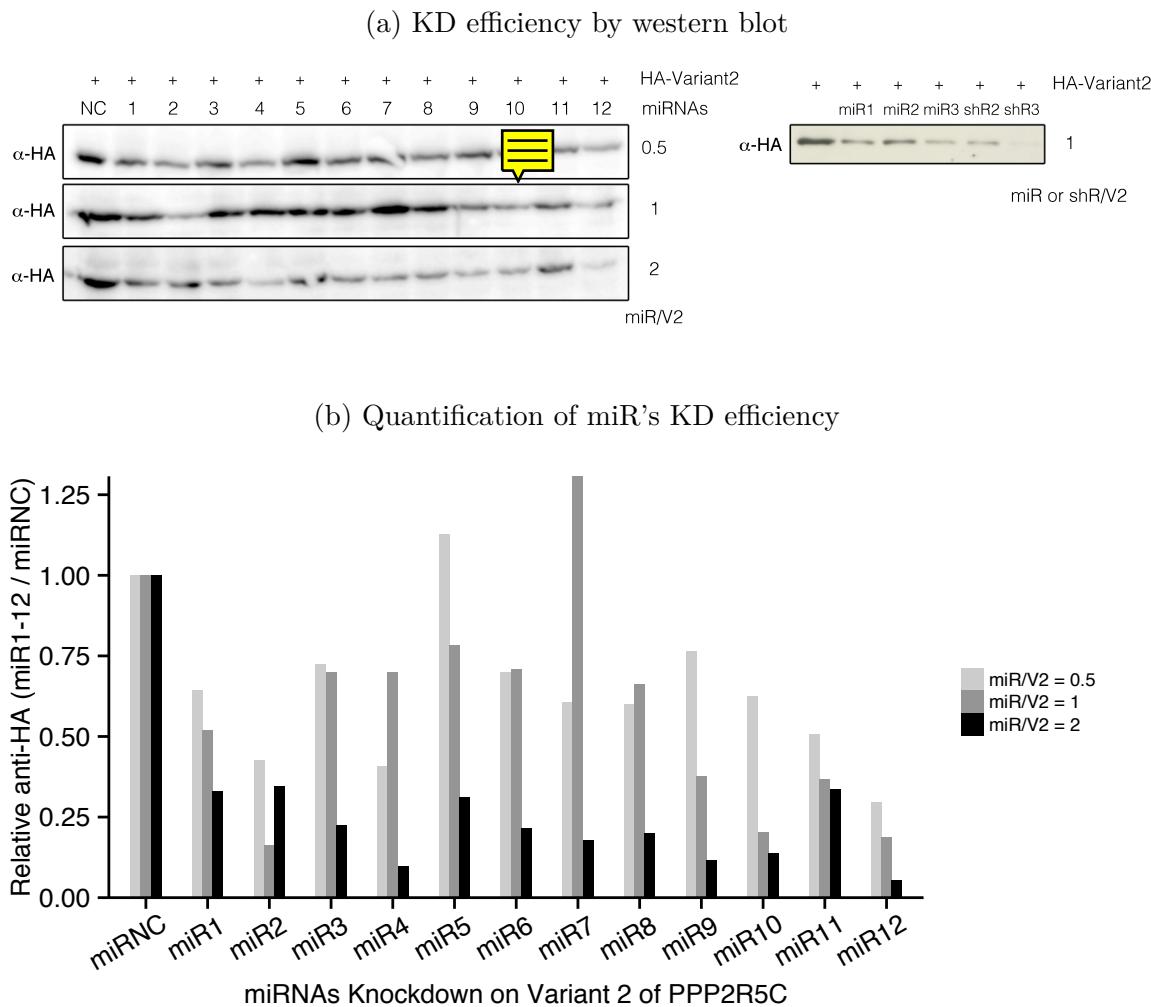


Figure 2.7.: Knockdown (KD) efficiency validated by western on PPP2R5C. (a) PPP2R5C KD efficiency by miR1–12 and shR2–3. miRNA or shRNA construct was co-transfected with HA tagged Variant 2 of PPP2R5C at different ratio in 293T cell. Total lysates after 3 day transfection was used to measure KD efficiency by blotting with α -HA antibody. (b) Quantification of miRNAs KD efficiency on PPP2R5C in Image J.

2.2.2. Generating Guinea Pig antibody for endogenous PPP2R5C detection

To monitor PPP2R5C's endogenous regulation as well as knockdown efficiency, a specific antibody for mouse PPP2R5C has to be generated. Although there were some commercial antibodies against mammalian PPP2R5C, most of them were failed in specificity

2. Results

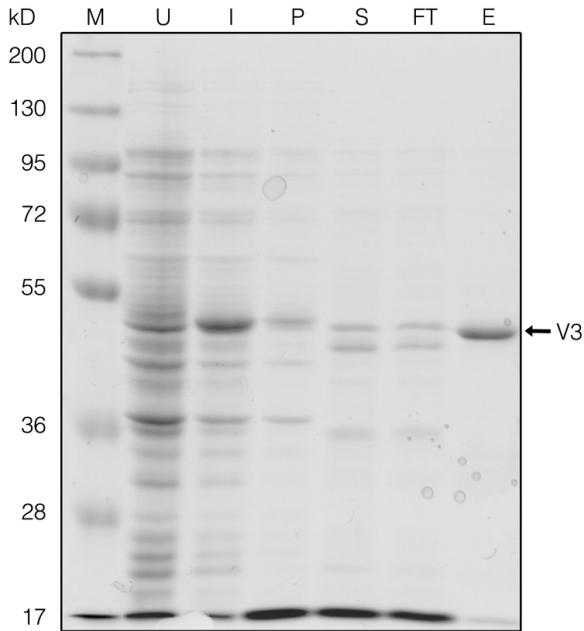


Figure 2.8: PPP2R5C Variant 3 (V3) was cloned into pETM-11 (EMBL protein expression/purification facility) to be expressed in *E. coli*. BL21-CodonPlus(DE3)-RIL strain. pETM-11 with V3 was electroporated into RIL and induced at 1 mM IPTG (Isopropyl β -D-1-thiogalactopyranoside) at 18 °C overnight. 1 mL bacteria before induction (U) and after induction (I) were taken to control the induction efficiency. Bacteria pellet after induction was lysed in lysis buffer (10mM MgCl₂, 150mM NaCl, 10mM Imidazole, 20mM Tris pH 7.5) with 1 mg/ml Lysozyme, and separated into insoluble (P) and soluble fraction (S). Soluble fraction was loaded onto Ni-NTA column, and the flow-through fraction (FT) was collected. Final elute was collected in lysis buffer with 500mM Imidazole (E). All fractions from purification were run in 12% SDS-PAGE gel with protein marker (M) and stained with GelCode coomassie stain.

test, in which Variants 1–4 were over-expressed in mouse Hepa 1-6 cells. Then I decided to make a polyclonal antibody against mouse PPP2R5C in the lab. Variant 3 was chosen to be the immunizing antigen in guinea pig due to its smaller size, and potentially better solubility in bacteria than other isoforms. Recombinant Variant 3 was successfully produced in bacteria with a 6×His tag. IMAC (Immobilized metal ion Affinity Chromatography) purification gave a good amount enough for immunization (~1 mg). And the purity of recombinant Variant 3 was also good enough to have high specificity during antibody production (*circa* 89% in coomassie brilliant blue staining, Figure 2.8).

Polyclonal antibody production in guinea pig was performed with Freund's adjuvant. After 4th boosting immunization, sera from guinea pig was collected by heart puncture under animal welfare regulation in Germany. Specificity of anti-sera for PPP2R5C was tested in both endogenous PPP2R5C knockdown (KD) and different PPP2R5C isoform over-expression (Figure 2.9). Even at 1:5000 dilution, PPP2R5C anti-sera could still detect various PPP2R5C isoform over-expression and endogenous knockdown of PPP2R5C by adenovirus-packaged shR3 in Hepa 1-6.

Interestingly, adenovirus-packaged shRNC, which is a non-targeting scramble shRNA, strongly decreases PPP2R5C protein level, especially for band size-matched with Variant

2. Results

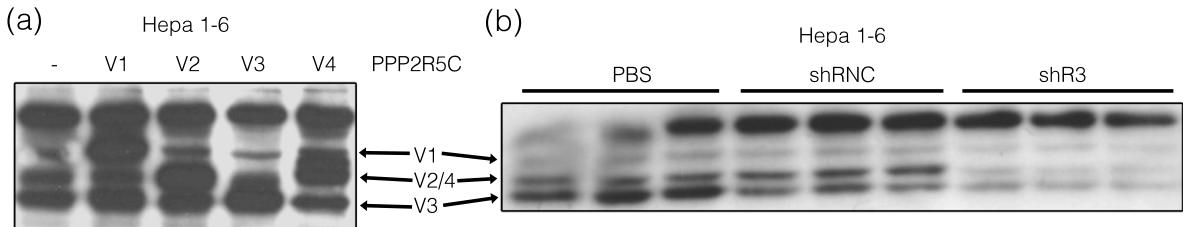


Figure 2.9.: Antibody specificity for PPP2R5C in Hepa 1-6 lysate. (a) Variant 1–4 (V1–4) were over-expressed in Hepa 1-6 for 3 day. Total lysate from control (no over-expression) or V1–4 were submitted for western blotting by guinea pig antibody against PPP2R5C. V1–4 were corresponding to 3 lower bands. The most upper band could be non-specific band or other protein with high PPP2R5C homology, such as PPP2R5D. (b) knockdown efficiency on endogenous PPP2R5C by adenovirus packaged with shR3. 3 lower bands is size matched to V1–4 and showed clear reduction in shR3. Band corresponding to V3 already showed reduction in shRNC comparing to PBS control.

3. And this is also true for transcriptional level of PPP2R5C (Figure 2.10). Specifically, Variant 3 mRNA level dropped to around 50% from low dosage to high dosage of adenovirus. This indicates non-specific knockdown effect on Variant 3. It is known the adenovirus has strong immunogenic effect [79]. Strong immunogenicity could initiate some inflammation response which then affect the expression of PPP2R5C Variant 3. Interestingly, PPP2R5C has recently been shown to be involved in NF- κ B mediated inflammation response [71]. PPP2R5C suppression upon adenovirus infection suggests a feedback loop of PPP2R5C transcription in immune response.

2.2.3. PPP2R5C KD in Hepa 1-6 increases glycolysis

With efficient *in vitro* knockdown tools for PPP2R5C, Hepa 1-6 was studied to decipher the functional role of PPP2R5C, especially in metabolism control. As shown in Figure 2.9, endogenous PPP2R5C could be successfully knocked down at efficiency of 80% by adenovirus-packaged shR3. After 3 day infection, mutant Hepa 1-6 cell with PPP2R5C knockdown shows clear increase in glycolysis, which is initially observed as deeper brownness of culture media in KD cells. Indeed, glucose consumption assay (Figure 2.11a) indicates a strong increase in glycolysis rate even in the last 24 hour of infection period. Accordingly, lactate production (Figure 2.11b) has similar extent of increase as in glucose consumption. These evidence demonstrate that PPP2R5C could be a negative regulator in glycolysis.

In addition, seahorse experiment also confirmed the increased glycolysis phenotype after PPP2R5C knockdown. When normalized to cell nuclei counting by DAPI staining, Hepa

2. Results

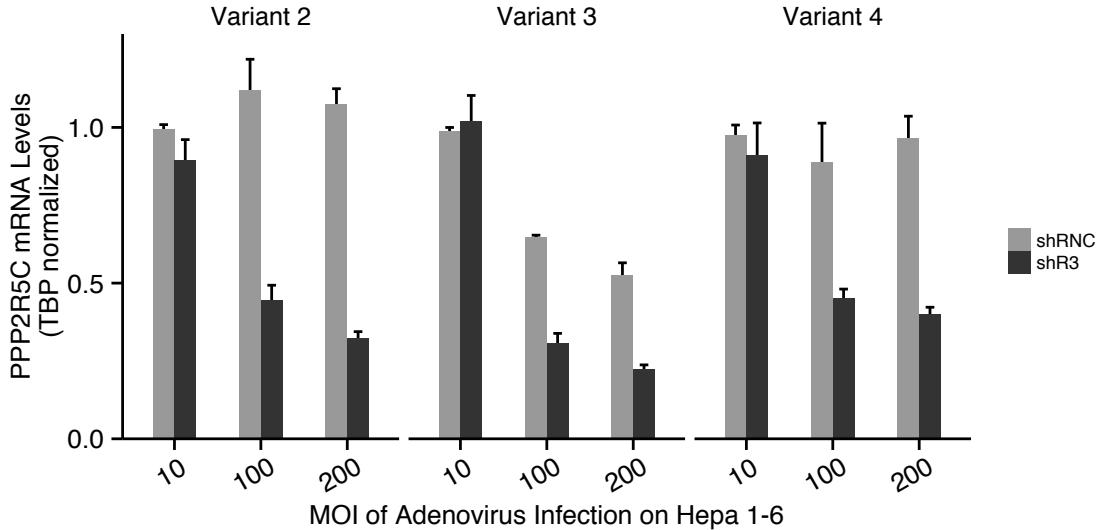


Figure 2.10.: Knockdown profile of endogenous PPP2R5C isoforms in Hepa 1-6. Adenovirus with different MOI (10, 100, 200) was diluted in PBS and infecting Hepa 1-6 for 3 day. Quantitative PCR analysis of PPP2R5C Variant 2/3/4 shows nice knockdown efficiency profile, which is dependent on MOI for all isoforms. MOI at 10 shows almost no reduction in mRNA level for all isoforms. MOI at 100 and 200 show similar level of significant knockdown. Variant 3 is sensitive to control Adenovirus (shRNC) and shows reduction in mRNA level at MOI of 100 or 200.

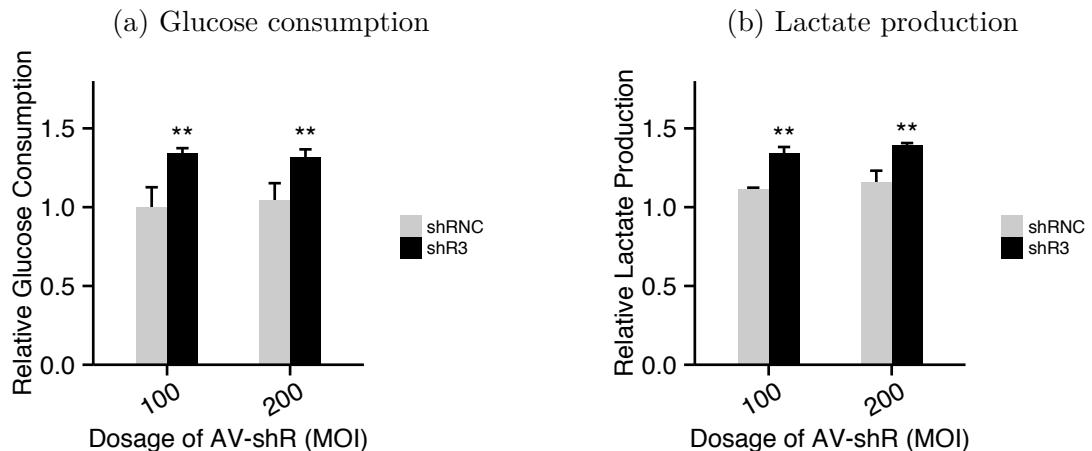


Figure 2.11.: PPP2R5C knockdown promote glucose consumption and lactate production in Hepa 1-6. Hepa 1-6 cell was infected with adenovirus packaged either with non-targeting scramble shRNA (shRNC) or PPP2R5C targeting (shR3) at MOI of 100 or 200 for 3 day knockdown. Infection was done in first 24 hours and then Hepa 1-6 cell was washed by fresh medium. Glucose consumption (a) and lactate production (b) were measured in last 24 hour window of knockdown. They were background-subtracted from glucose and lactate concentration in fresh medium and normalized to total protein. ** for p-value<0.01 by t-test within each MOI in R.

2. Results

1-6 knockdown cells have increased AUC (Area Under Curve) comparing with control adenovirus infected cells (Figure 2.12). The increase becomes statistically significant after oligomycin is added, which boosts the maximal glycolytic activity. Combining with data from glucose consumption and lactate production (Figure 2.11), it is clearly demonstrated that PPP2R5C knockdown promote glucose uptake and glycolysis in Hepa 1-6 cells.

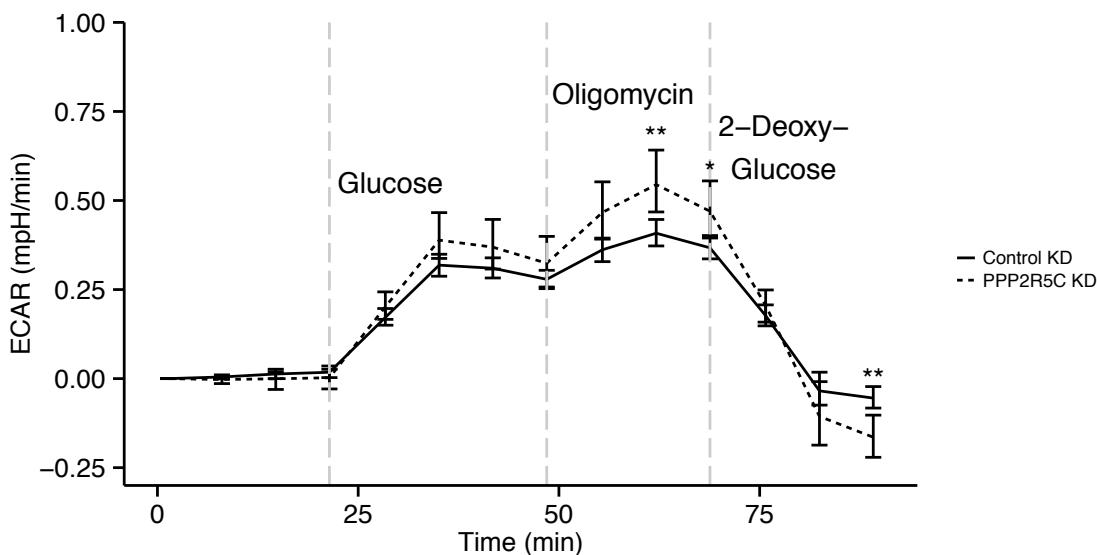


Figure 2.12.: PPP2R5C Knockdown promote glycolysis in seahorse experiment. Adenovirus with MOI=100 was diluted in PBS and infected Hepa 1-6 for 2 day. Then infected Hepa 1-6 was digested by trypsin from culture plate and re-plated in 96-well plate with balanced cell number between different groups (Control KD for shRNC, and PPP2R5C KD for shr3). Glycolysis activity was measured on seahorse instrument with glycolysis stress kit. Glucose, oligomycin and 2-Deoxy-Glucose were added at the time of vertical dashed line according to the protocol in the kit. The extracellular acidification rate (ECAR, mpH/min) was recorded as a indirect readout for intracellular glycolysis rate. * and ** for p-value<0.05 and 0.01 by wilcoxon signed-rank test.

2.2.4. PPP2R5C KD promotes glucose uptake rate in Hepa 1-6

Although PPP2R5C is shown to be a negative regulator for glycolysis, it is still not clear the increased glucose consumption is due to increased cell number with same glucose uptake rate or increased glucose uptake rate within the same number of cells. On one hand, the total protein content after PPP2R5C knockdown has not changed between control and PPP2R5C knockdown cells (data not shown), which indicates no strong increase in cell proliferation. On other hand, a short-term glucose uptake assay was employed to assess the glucose uptake activity of Hepa 1-6 by measuring 2NBDG uptake

2. Results

in 20 min via FACS (Figures 2.13 to 2.15). 2NBDG is a fluorescent 2-deoxyglucose analog which can be used to monitor glucose uptake rate [80, 81, 82, 83, 84].

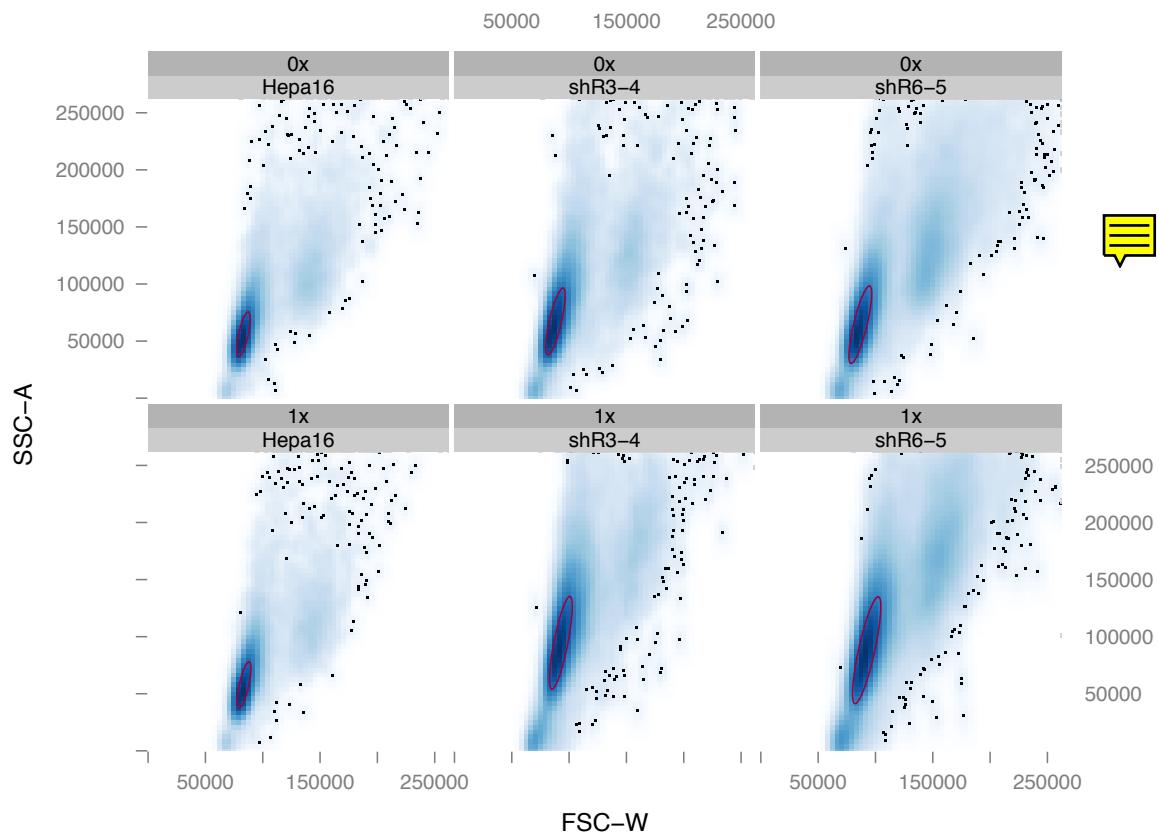


Figure 2.13.: Single live Hepa 1-6 cell filtering in FACS by R. X-coordinate is marker for cell size (FSC-W, Forward-Scattered Width), and y-coordinate is cell granularity (SSC-A, Side-Scattered Area). Hepa 1-6 with stable shRNAs (shR3 or shR6) or not were induced at 30 μ g/mL cumate (1x, 0x for DMSO control) for 3 day and then starved in serum-free DMEM overnight. shR3-4 and shR6-5 were single clone for shR3 and shR6. Then these cells were sensitized in KRPH (Krebs-Ringer-Phosphate-HEPES) buffer for 1 hour and followed by 20 min incubation with 100 μ M 2NBDG. Finally, Hepa 1-6 cells were digested with trypsin for 3 minutes and subjected to FACS analysis. Single live Hepa 1-6 cells were clustered in FSC-SSC scatter plot and filtered out in R by using package flowCore (indicated by red circle on scatter plot).

Additionally, 2 set of shRNAs (shR3 and shR6) were cloned into miR30-based inducible shRNA expression vector, and stable Hepa 1-6 cell lines harboring genome-integrated inducible shR3 or shR6 were generated after puromycin selection. The stable inducible cell lines were used as cross-validation for results from adenovirus-packaged shR3, and supposed to eliminate potential virus-mediated effects given the fact of high immunogenicity of adenovirus.

All the data from FACS were analyzed in flowCore [85], flowStats [86] and flowViz [87]

2. Results

Glucose Uptake Profiles

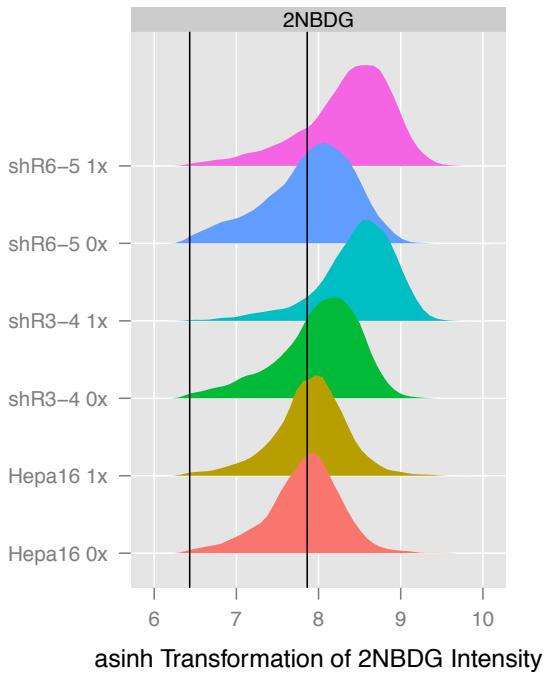


Figure 2.14: Density plot of Hepa 1-6 cell with positive 2NBDG uptake. Filtered single live Hepa 1-6 cells were compared for 2NBDG fluorescence intensity between Hepa 1-6 cell with 2NBDG incubation and the one without 2NBDG incubation to calculate threshold for 2NBDG uptake (left vertical line). Median intensity in Hepa 1-6 without cumate induction (right vertical line) was calculated for better comparison between different cell lines. shR3-4 and shR6-5 were single clone for shR3 and shR6. 0x and 1x were DMSO control and 30 μ g/mL cumate induction for 3 days.

package in R. There are mainly two population in all cell lines (from empty Hepa 1-6 to two stable inducible shRNA cell lines) (Figure 2.13). And these two populations have not changed after shRNA expression by induction with 30 μ g/ml cumate, which indicates no significant cell morphological change after PPP2R5C knockdown. Based on particle size (FSC), it is clear that the population at the left is single live cells, and the population on the right is doublet Hepa 1-6 cells from incomplete digestion by trypsin.

In the first step, single live Hepa 1-6 cells were filtered out based on size (red circled area in Figure 2.13) with 2D-normal distributed contour. Then, single live Hepa 1-6 cells were analyzed to calculate the proportion of 2NBDG positive staining with 2NBDG unstained Hepa 1-6 cell as negative staining control. From the asinh transformed density map of all 2NBDG positive staining, there is a obvious red-shift in density map after PPP2R5C knockdown (Figure 2.14), and almost all single live Hepa 1-6 cells have 2NBDG uptake. For empty Hepa 1-6 cells, there is no red-shift in density map after cumate induction. These data clearly show that PPP2R5C knockdown could increase glucose uptake even in short time period. The quantification of median fluorescence intensity (MFI) is normalized and showed in Figure 2.15. The same phenotype has also been observed in a third independent shRNA for PPP2R5C (shR8, data not shown).

In mouse primary hepatocytes, there is also nice cross-validation of glucose uptake phenotype (Figure 2.16). At two different nutritional statuses, either starved for serum

2. Results

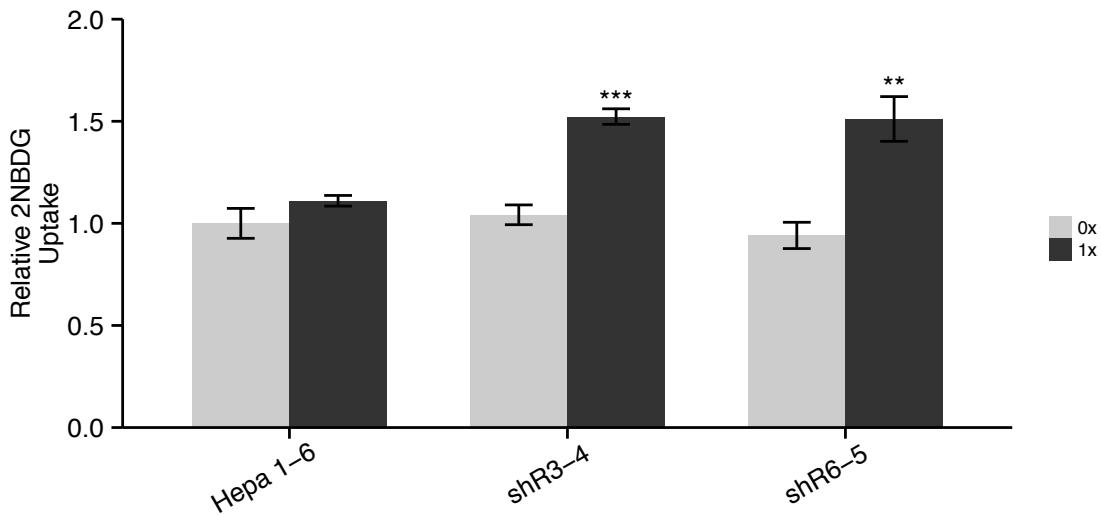


Figure 2.15.: 2NBDG uptake in Hepa 1-6 upon PPP2R5C knockdown. FACS data for 2NBDG uptake in empty Hepa 1-6, Hepa 1-6 with inducible shR3 and 6 (clone shR3-4 and shR6-5) were induced at DMSO (0x) or 30 µg/mL cumate (1x) and analyzed as described in Figure 2.14 and Figure 2.13. ** and *** for p-value<0.01 and 0.001 by t-test in R.

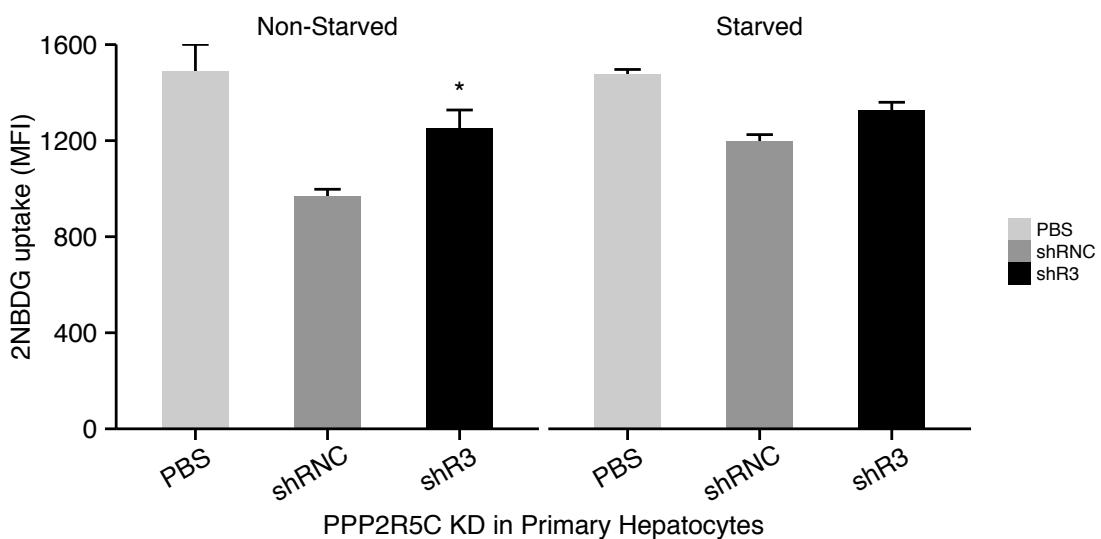


Figure 2.16.: 2NBDG uptake in mouse primary hepatocytes upon PPP2R5C knockdown by adenovirus with shRNC or shR3. FACS data were collected and analyzed as described in Figure 2.15. * for p-value<0.05 by t-test in R. n=3.

2. Results

or non-starved, glucose uptake, which is determined by FACS measurement of 2NBDG uptake as in Figure 2.15, shows clear trend of increase in PPP2R5C knockdown cells comparing with non-targeting shRNA controls. For non-starved condition, the increase is statistically significant. Again, the non-targeting shRNA control adenovirus still has some virus effect on glucose uptake comparing with PBS control.

2.2.5. PPP2R5C deficiency promotes *de novo* lipogenesis

Another interesting phenotype by PPP2R5C knockdown is increased lipid storage in cultured primary hepatocytes (Figure 2.17). With two difference sources of mouse primary hepatocytes, PPP2R5C knockdown via adenovirus-packaged shR3 shows significant increase (approximately 2-fold) in triglyceride storage comparing to non-targeting shRNC or PBS control. Although relative extent of lipid storage increase is proportionally augmented within duration of knockdown, prolonged *in vitro* cultivation of primary hepatocytes sometimes ended up with de-differentiation of hepatocyte. The de-differentiated primary hepatocytes usually shows distorted and shrunken cell shape. The lipid storage increase phenotype could not be observed under this condition, such as 3 day adenovirus infected hepatocytes from Prof. Herzig's Lab at DKFZ (Herzig 3 Day in Figure 2.17).

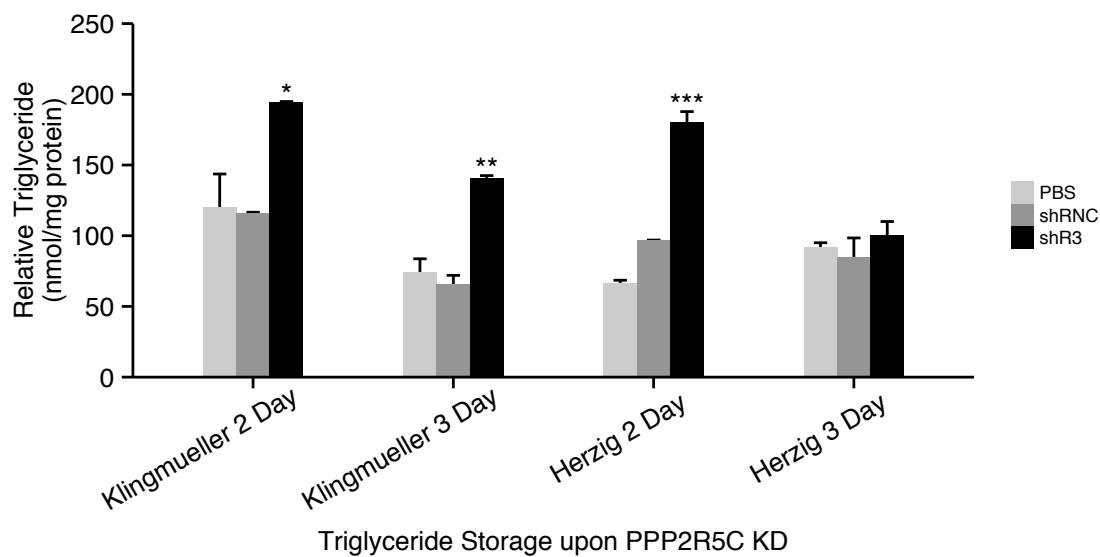


Figure 2.17.: PPP2R5C knockdown increases lipid storage in primary hepatocytes. Primary hepatocytes were infected with adenovirus at the same condition as in Figure 2.16. Lipid fraction was extracted by methanol-chloroform method [88] and measured for free glycerol released from lipase digestion. *, ** and *** for p-value<0.05, 0.01 and 0.001 by t-test in R, n=3.

In contrary to shRNC effect on glycolysis phenotype, non-targeting scramble shRNA

2. Results

showed no effect on lipid storage when that was comparing with PBS control. This discrepancy possibly implied complex and different mechanism in changing glycolysis and lipid storage after PPP2R5C knockdown.

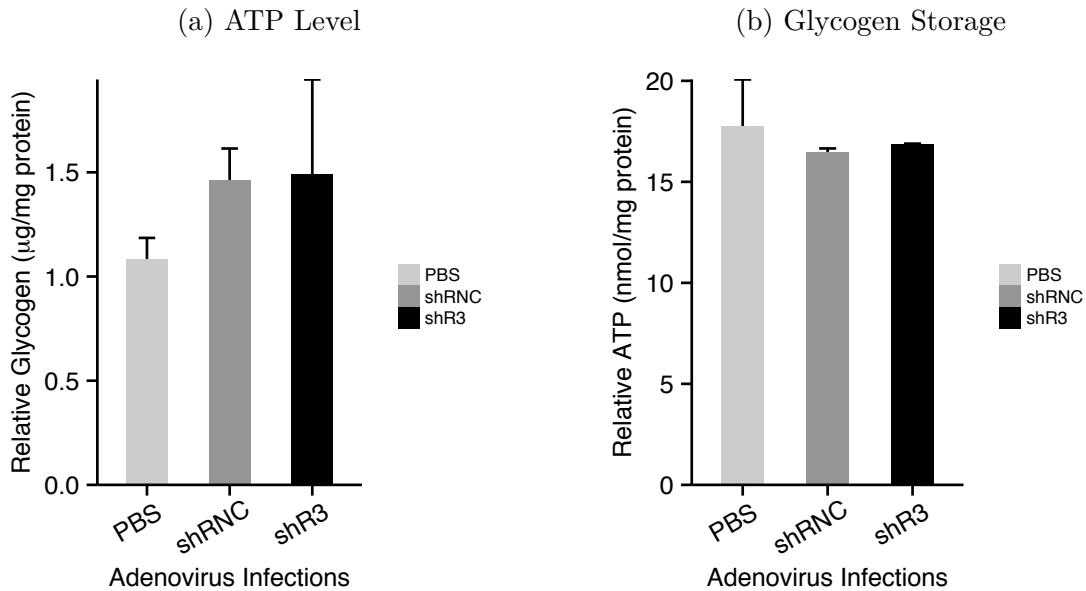


Figure 2.18.: PPP2R5C knockdown does not change ATP level (a) or glycogen storage (b) in mouse primary hepatocytes. Primary hepatocytes were infected with adenovirus at the same condition as in Figure 2.15. n=3.

In addition to lipid storage and glycolysis phenotype, PPP2R5C knockdown in primary hepatocyte has no effect on ATP level (Figure 2.18a) and glycogen level (Figure 2.18b). This indicates the excessive energy from increased glucose uptake is shunted into energy storage as triglyceride storage, but not as ATP or glycogen in cultivated primary hepatocytes.

Moreover, the lipid storage phenotype in PPP2R5C knockdown is also conserved in another cell type, Hepa 1-6 (Figure 2.19). Even treated with mTOR inhibitor rapamycin, the lipid storage phenotype is still present without compromising its increase. This evidence indicates the mechanism how PPP2R5C affect lipid storage is either at downstream of mTOR or in parallel with mTOR. It has been shown that mTORC1 activation leads to increase in glycolysis and lipogenesis via downstream activation of HIF1 α and SREBP-1 [89]. Increased glycolysis and lipogenesis in PPP2R5C knockdown is a phenocopy for mTORC1 activation.

In order to further clarify the lipid storage phenotype, free fatty acid uptake from culture media and intracellular free fatty acid were measured in Hepa 1-6 upon PPP2R5C

2. Results

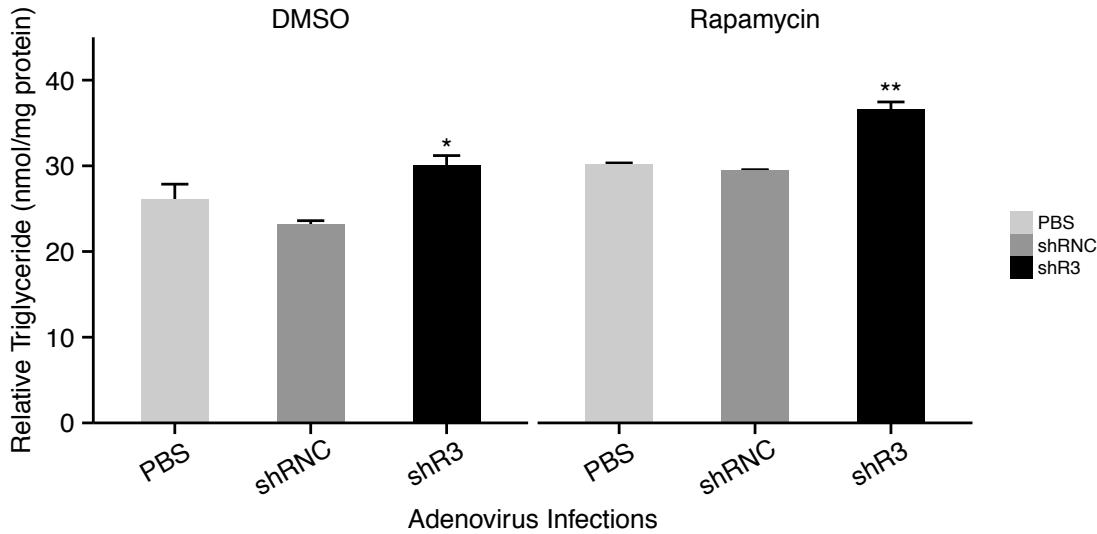


Figure 2.19.: PPP2R5C knockdown increases lipid storage in Hepa 1-6 independent of mTOR. Hepa 1-6 were infected with adenovirus at the same condition as in Figure 2.11. Lipid fraction was extracted by methanol-chloroform method and measured for free glycerol released from lipase digestion. *, ** for p-value<0.05, 0.01 by t-test in R, n=3.

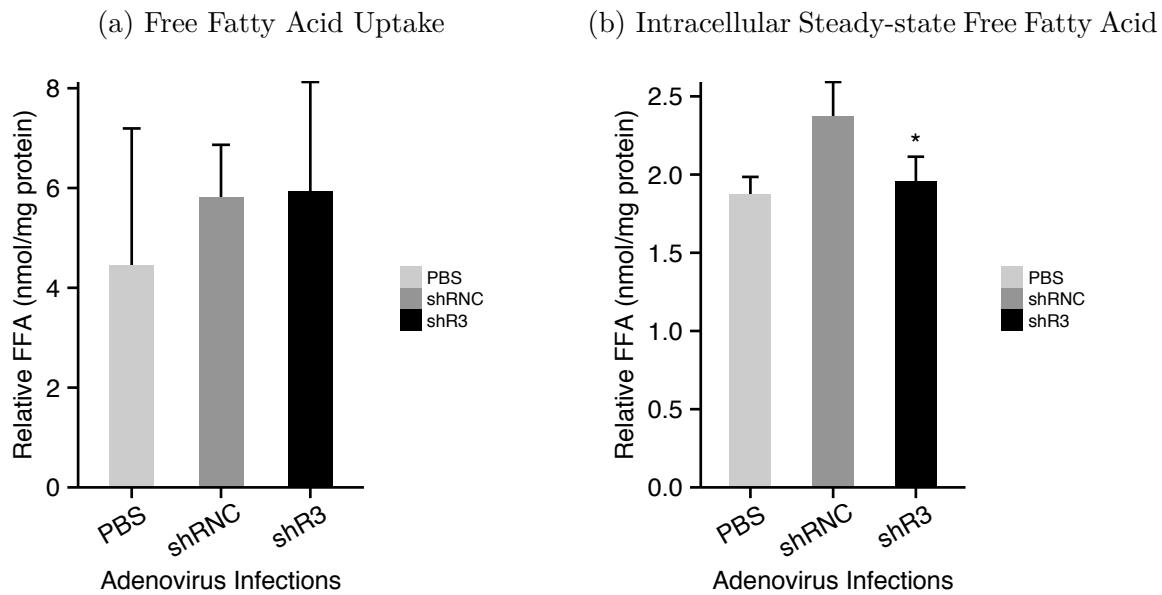


Figure 2.20.: Free fatty acid in culture medium is not responsible for increased triglyceride storage inside Hepa 1-6. (a) Free fatty acid from culture media was enriched by methanol-chloroform method [88] and uptake was calculated from the difference in free fatty acid between fresh medium and medium after 3 day culturing. (b) Intracellular free fatty acid level. Hepa 1-6 cells were infected with adenovirus at the same condition as in Figure 2.15. * for p-value<0.05 by t-test in R, n=3.

2. Results

knockdown (Figure 2.20). Neither of them are increased after PPP2R5C knockdown. No increase in free fatty acid uptake suggested accumulated lipid storage could come from *de novo* lipogenesis. If comparing the relative concentration of free fatty acid uptake during 3 day infection with that for fatty acid equivalent increment in triglyceride after PPP2R5C knockdown (approximately comparing 6 nmol/(mg protein) with 30 nmol/(mg protein), 1 molecule of triglyceride consists of 3 molecule of fatty acid), it is very unlikely triglyceride storage come from re-esterification of absorbed free fatty acid, but rather from *de novo* biosynthesis. Also, the slight decrease in intracellular free fatty acid concentration after PPP2R5C implies higher flux from intracellular free fatty acid toward triglyceride synthesis when comparing with shRNC.

2.3. PPP2R5C *in vivo* knockdown promotes glucose uptake, triglyceride synthesis

It was interesting to know the PPP2R5C's role in cellular model. Data from *in vitro* cellular model suggested PPP2R5C's potential role in metabolism is shunting the transported glucose into lipid storage in a cell-autonomous fashion. But it was always more physiological-relevant when investigating PPP2R5C's function in metabolism *in vivo*. To achieve this purpose, adeno-associated virus (AAV) was employed to specifically express miRNA against PPP2R5C (miR12, see Figure 2.1 and Table A.6) in mouse liver and perform long-term knockdown *in vivo* as described [90]. Previously, miR12 was selected as miRNA candidate based on its highest efficacy in knockdown tested in HEK293T cell. AAV packaged miR12 was produced from Vector Biolabs (Philadelphia, USA) due to insufficient in-house virus production yield. Pilot mouse experiment was performed to evaluate the knockdown efficiency *in vivo*. From qPCR analysis of total PPP2R5C mRNA (Figure 2.21a) and protein level (Figure 2.21b), there is a strong reduction in both transcription and protein level.

2.3.1. PPP2R5C knockdown has no impact on animal health

With 7 week of PPP2R5C knockdown in mouse liver, there is no severe side effect from AAV infection, which could be demonstrated by low level of serum ALT level and further no increase in knockdown mice (Figure 2.22). ALT is an enzyme mainly expressed in liver, much less expressed in kidney, heart, muscle. The serum ALT level is normally

2. Results

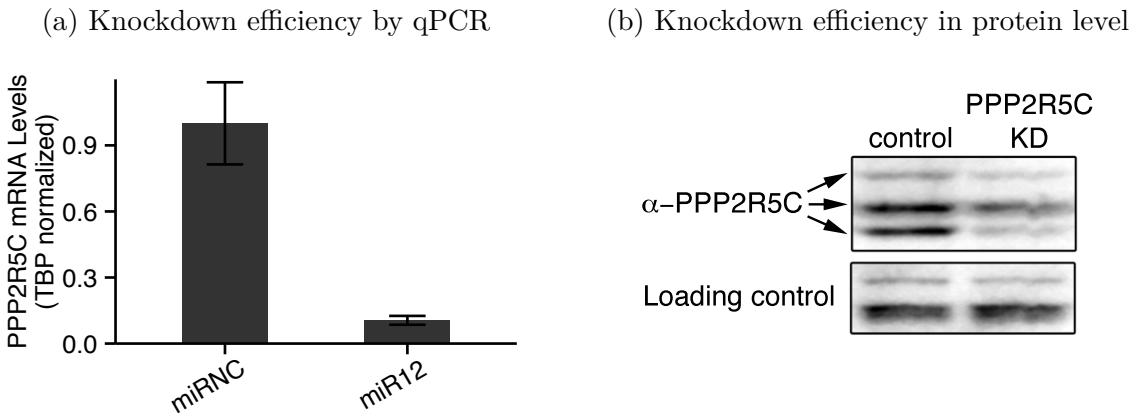


Figure 2.21.: PPP2R5C knockdown efficiency *in vivo*. (a) miR12's *in vivo* knockdown efficiency was compared with non-targeting control miRNC after 2 week AAV injection. qPCR was performed with probe set for total mRNA of PPP2R5C in liver. (b) Knockdown efficiency was evaluated at protein level. Liver endogenous PPP2R5C was detected by home-made guinea pig antibody against it. Non-specific binding band was showed at bottom as loading control. n=5 for qPCR analysis.

low, and only become high when liver is damaged or diseased (leakage across damaged hepatic cell membrane). Serum ALT level around 20 U/L was much lower than that from mice suffering liver injury (several hundreds to even thousands U/L [91]).

During AAV infection, mice from control and knockdown group (miRNC vs miR12) have no significant difference in body weight growing profile (Figure 2.23). Furthermore, body composition analysis was also performed on these mice every 2 or 3 weeks (Figures 2.24 and 2.25) by echoMRI measurement. There is no significant difference in fat and lean mass profile between control and knockdown group. Although mice would be subjected into fasting show some difference before virus injection, and the difference remained constant during knockdown. After sacrificing, mice were measured for abdominal white adipose tissue weight (Figure 2.26). There is also no change in abdominal white adipose tissue weight upon PPP2R5C KD. In summary, 7 week liver-specific PPP2R5C knockdown via AAV infection does not change mouse whole body metabolism significantly.

2. Results

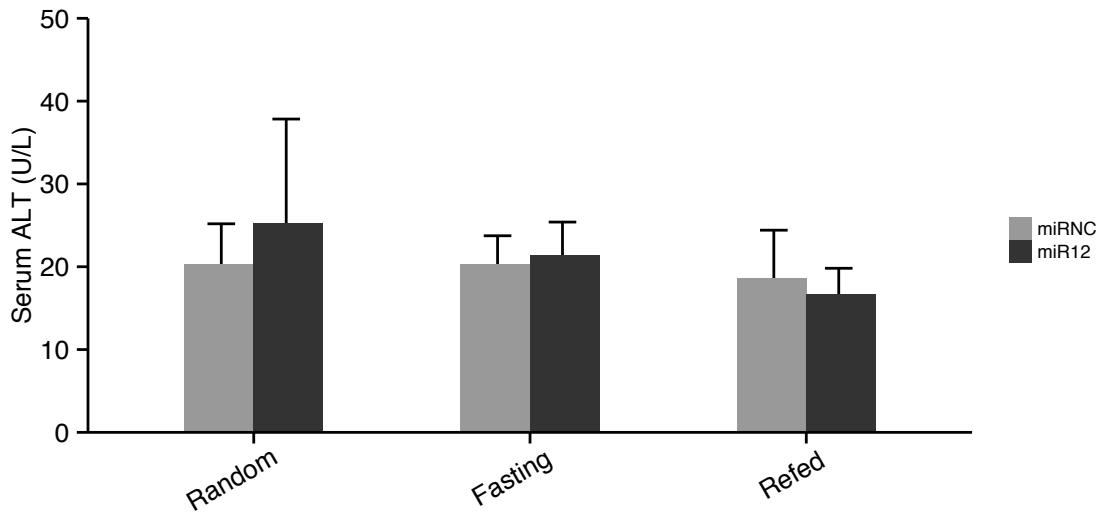


Figure 2.22.: Serum ALT (Alanine Aminotransferase) level indicates no liver injury. 8-10 week CL57BL/6 male mice were injected with adeno-associated virus packaged miRNA against mouse PPP2R5C (miR12) or scramble miRNA (miRNC) at 1×10^{11} viral particles/mouse via tail injection in 100 μ L PBS. And injected mice was sacrificed after 7 week knockdown. Before sacrificing, mice were divided into different groups for various treatment, including *ad libitum* fed (Random), 16 hour fasting (Fasting), and 16 hour fasting followed by 6 hour feeding (Refed). 5 μ L serum was used to measure ALT enzymatic activity. n=5 or 6.

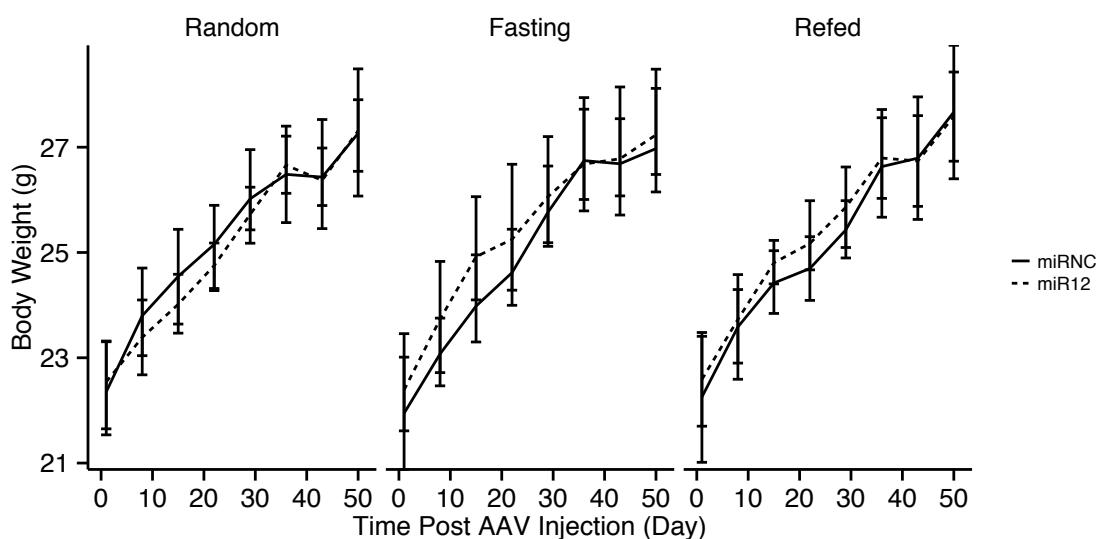


Figure 2.23.: Body weight profile has no change after PPP2R5C knockdown. Mice in Figure 2.22 were measured for body weight at each week. Time 0 was the body weight before virus injection. n=5 or 6.

2. Results

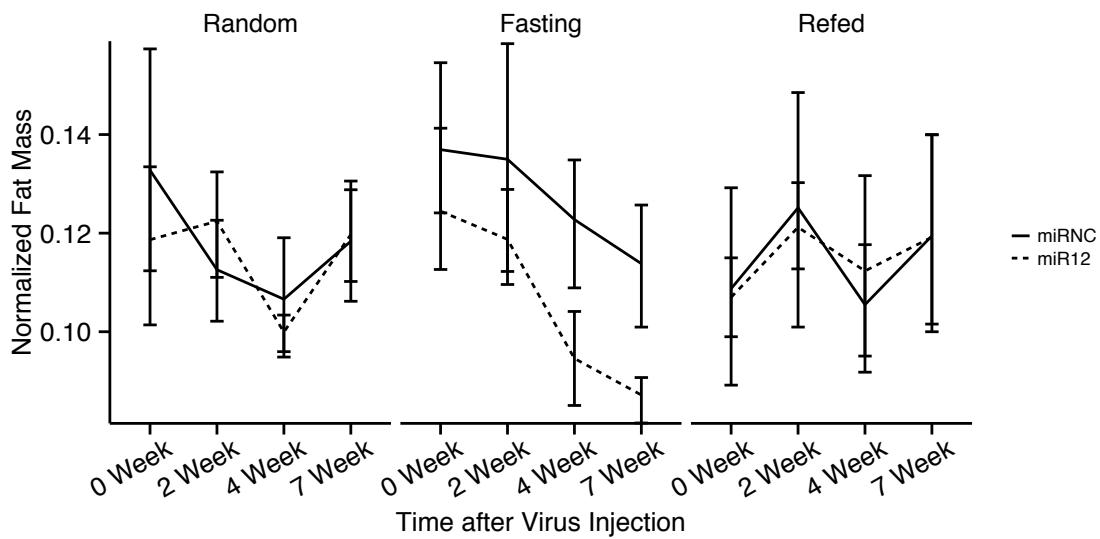


Figure 2.24.: Body fat content profile has no change after PPP2R5C knockdown. Mice were the same as Figure 2.22. Whole body fat composition was calculated from fat content normalized to body weight at each time point. Week 0 indicated fat composition before virus injection. n=5, or 6

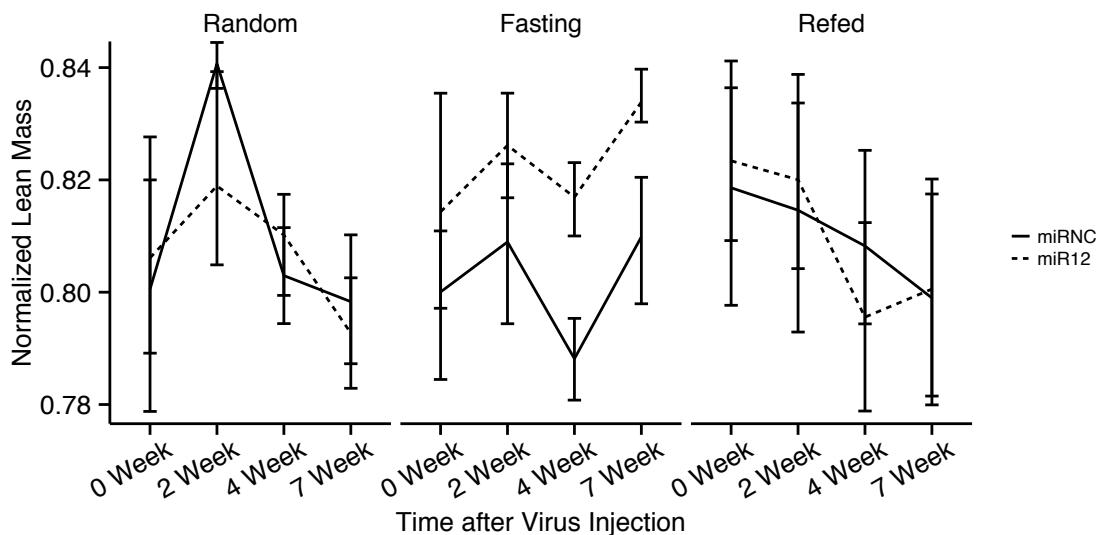


Figure 2.25.: Body lean mass profile has no change after PPP2R5C knockdown. Mice were the same as Figure 2.22. Whole body lean mass composition was calculated from lean mass content normalized to body weight at each time point. Week 0 indicated fat composition before virus injection. n=5, or 6

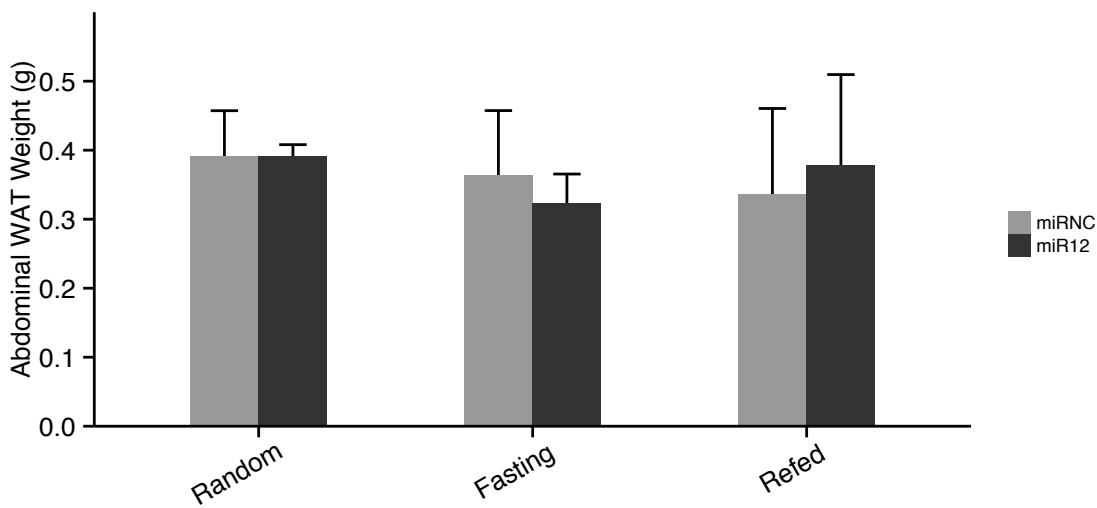


Figure 2.26.: Abdominal white adipose tissue (Abd.WAT) has no change after PPP2R5C knockdown. Mice were the same as Figure 2.22. After mice were treated for different nutritional status (Random, Fasting and Refed), Abd.WAT tissue was collected from each mouse and weighted before stored in -80°C. n=5, or 6

2.3.2. PPP2R5C KD promotes glucose uptake *in vivo* with better insulin sensitivity

Since liver is important for maintaining euglycemia, especially in postprandial phase [14], blood glucose level were measured for *ad libitum* feeding ("Random"), or after 16 hour fasting ("Fasting"), followed by 6 hour refeeding ("Refed") in control and knockdown mice (Figure 2.27). Surprisingly, there are no significant decrease in all three conditions, even given that PPP2R5C KD in cultivated hepatocytes increased glucose uptake (Figure 2.16).

Although there is no change in blood glucose level after PPP2R5C knockdown, the serum insulin concentrations drop almost 2-fold after PPP2R5C knockdown (Figure 2.28) when mice are fed *ad libitum*. In Fasting and Refed group, serum insulin levels are also decreased comparing with control mice. The decreased circulating insulin levels indicated increased insulin sensitivity. Indeed, the insulin sensitivity index [92] in random and refed group are also increased in PPP2R5C knockdown mice (Figure 2.29).

Besides the increased insulin sensitivity, glucose uptake capacity is so increased in 6 hour fasted mice after PPP2R5C knockdown, which is shown by better glucose tolerance in glucose tolerance test (Figure 2.30). This data is nicely correlated with increased glucose uptake and glycolysis in cellular models (Hepa 1-6 and mouse primary hepatocytes,

2. Results

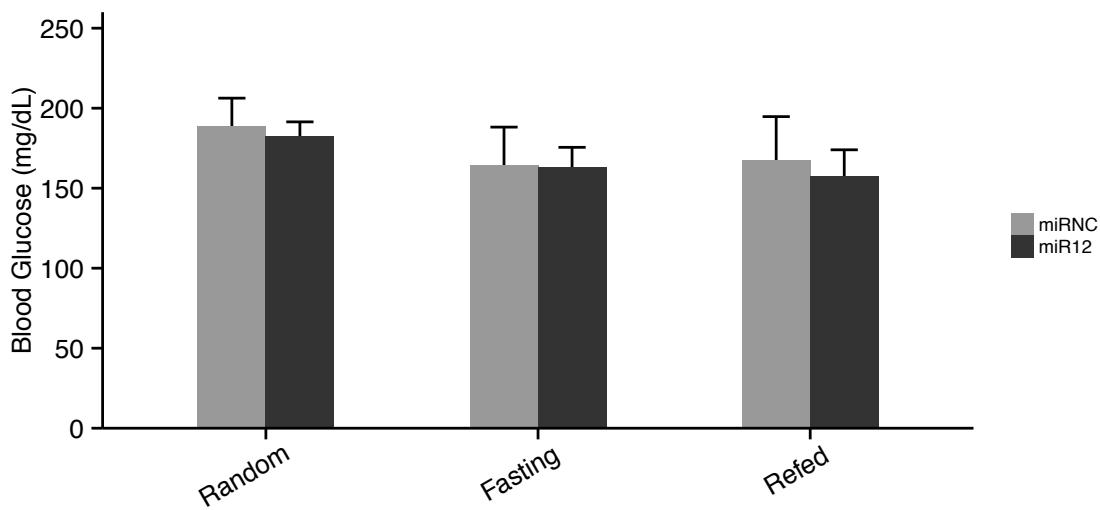


Figure 2.27.: Blood glucose level has no change after PPP2R5C knockdown. Mice were the same as Figure 2.22. Blood glucose level was immediately measured after sacrifice. n=5, or 6

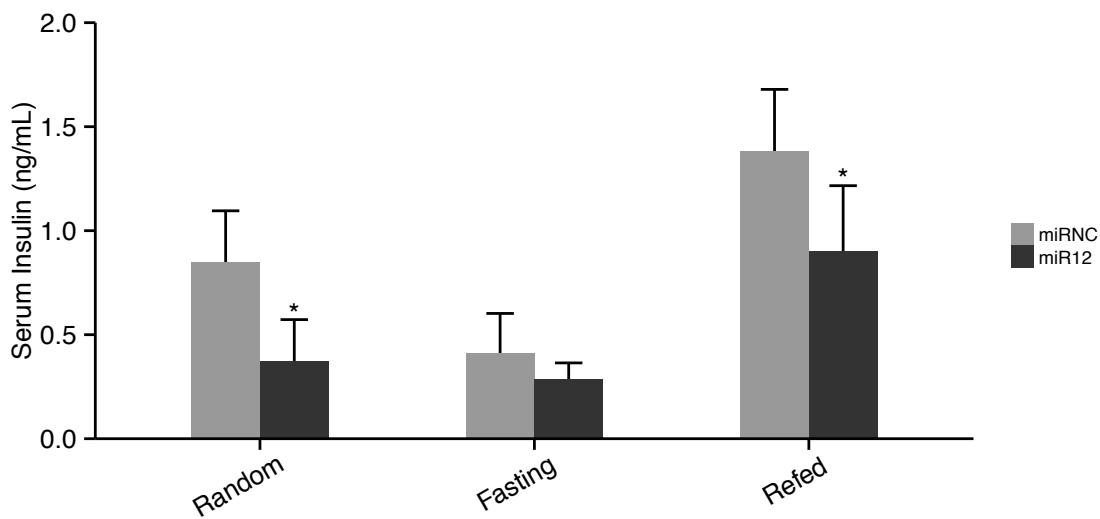


Figure 2.28.: Serum insulin drops after PPP2R5C knockdown in *ad libitum* fed (Random) or 6-hour Refeeding after 16-hour fasting. Serum insulin was measured using ELISA kit for mouse insulin, and standard curve for ELISA was fitted from serial diluted insulin standards (0.1-6.9 ng/mL) with 5-parameter logistic model in R. * for p-value<0.05 by t-test in R for comparing miR12 to miRNC for each nutrition group. n=5, or 6

2. Results

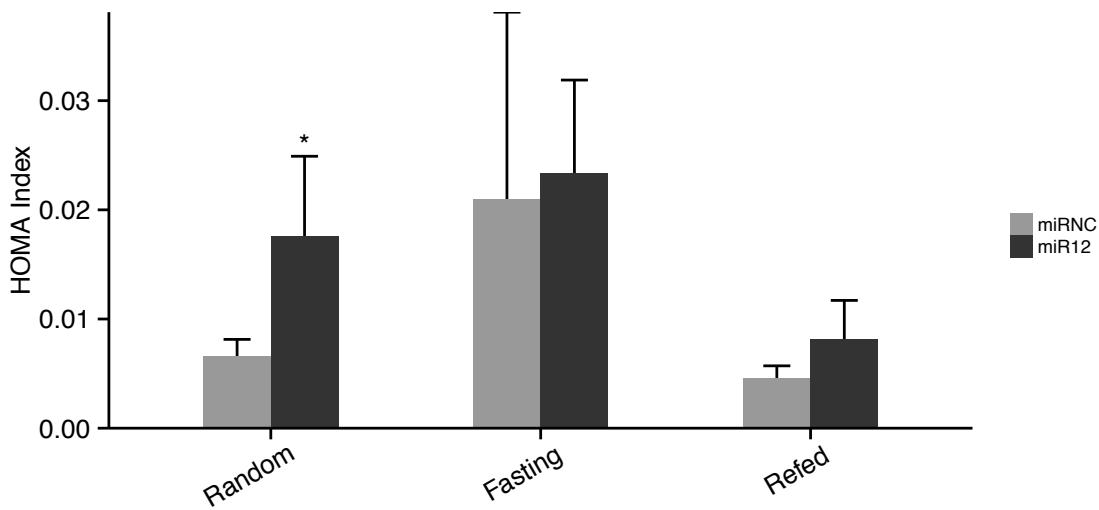


Figure 2.29.: Insulin sensitivity index (ISI) increases after PPP2R5C knockdown in *ad libitum* fed (Random) or 6 hour re-feeding after 16 hour fasting. ISI index was calculated from the inverse of product between blood glucose concentration and insulin concentration. * for p-value<0.05 by t-test in R for comparing miR12 to miRNC for each nutrition group. n=5, or 6

Figures 2.12, 2.15 and 2.16). AUC analysis for GTT data also demonstrates decreased AUC in PPP2R5C knockdown (Figure 2.31). Although the absolute serum insulin levels from the same mouse in GTT experiment could not be calculated due to their level are below the limit of detection, raw O.D. 450 is used to estimate relative serum insulin level (Figure 2.32). And the relative serum insulin levels in PPP2R5C knockdown mice are even lower than control mice, given that they still had quicker glucose clearance rate (Figure 2.30). In sum, these data indicate PPP2R5C liver-specific knockdown mice have both increased insulin sensitivity and glucose uptake.

2.3.3. PPP2R5C KD promotes anabolic changes in liver

The increased glucose uptake in liver after PPP2R5C KD indicates a more anabolic metabolism in hepatocytes in knockdown mice. In agreement with this, liver has significant weight gain upon PPP2R5C knockdown in all groups (Figure 2.33), instead of no systematic anabolic change in whole body metabolism (Figures 2.23 to 2.26). The increase in Fasting and Refed group are even stronger than that in random group. In postprandial phase, glucose is absorbed by hepatocytes to synthesize glycogen and lipids [93, 22]. The increase could be partially explained by increase in liver glycogen (Figure 2.34) and triglyceride (Figure 2.35), even all these data are normalized to liver

2. Results

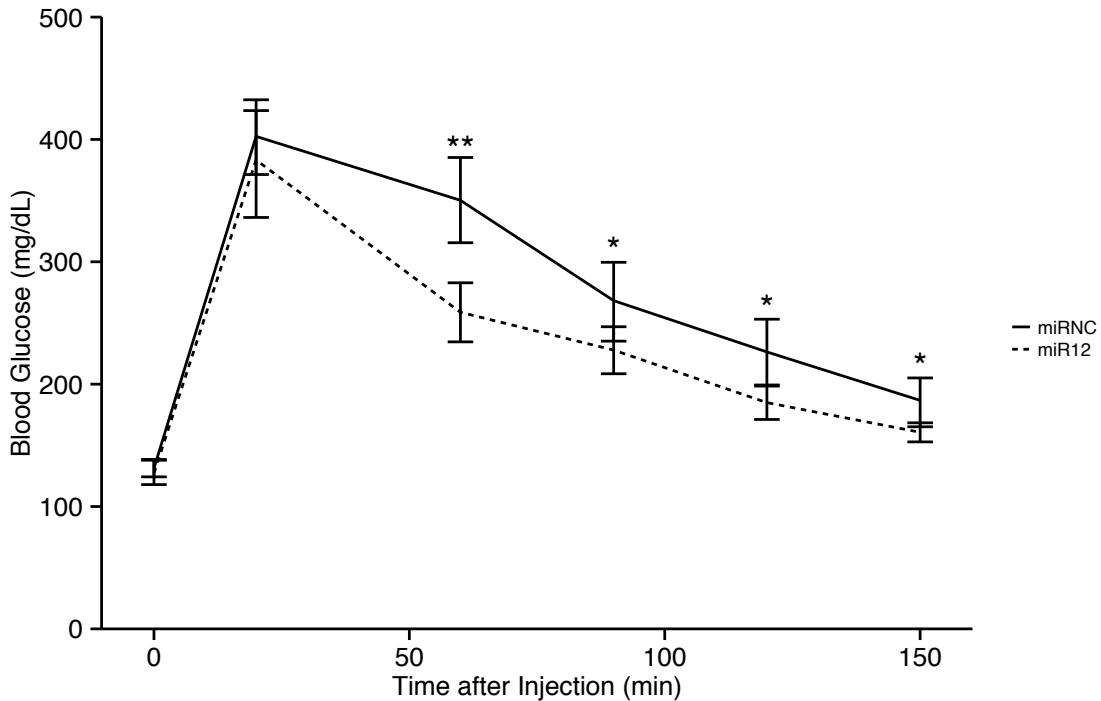


Figure 2.30.: Glucose tolerance test (GTT) shows better glucose tolerance in PPP2R5C KD. GTT was performed 4 weeks after virus injection at dosage of 2g glucose/kg body weight. Glucose was solubilized in PBS and injected intraperitoneally. Blood glucose before injection was recorded as it for time 0 min, and also measured at time 20, 60, 90, 120 and 150 min. * and ** for p-value<0.05 and 0.01 by wilcoxon signed-rank test in R for comparing miR12 to miRNC. n=12. Repeated Measures ANOVA in R showed significant difference in blood glucose changing profile with p-value of 0.0098.

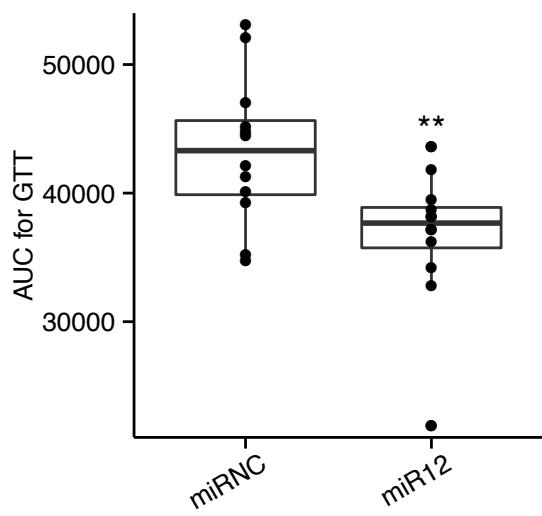


Figure 2.31: Area Under Curve (AUC) analysis for GTT blood glucose profile in Figure 2.30. ** for p-value<0.01 by t-test in R for comparing miR12 to miRNC. n=12.

2. Results

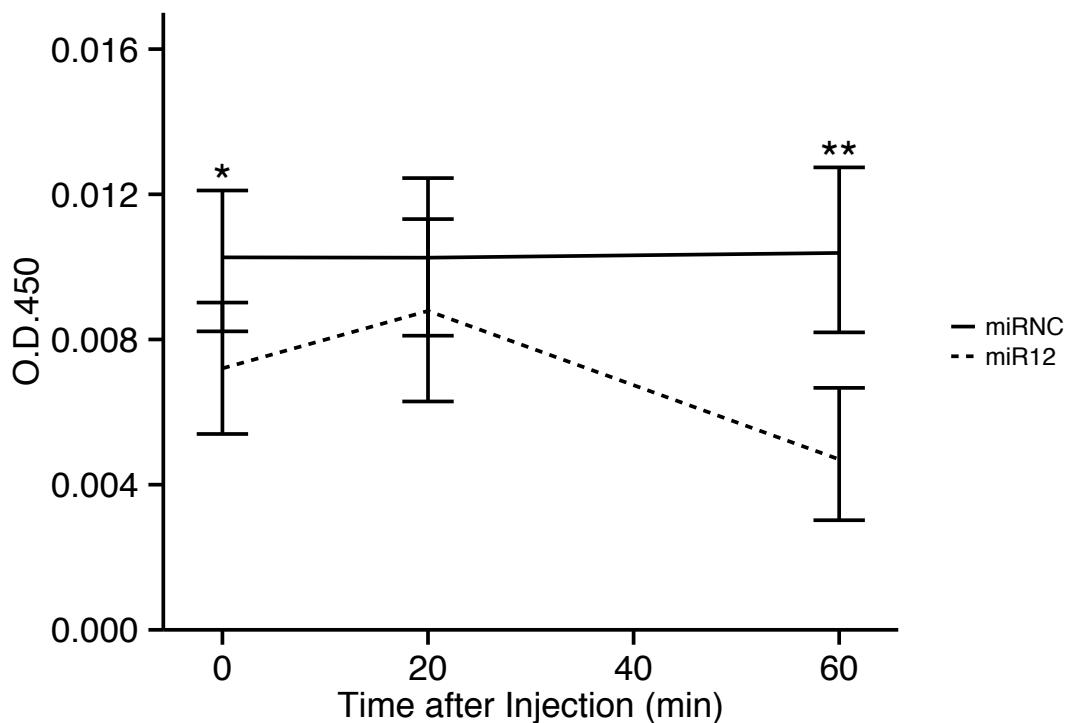


Figure 2.32.: Serum insulin relative level decreases in PPP2R5C. Due to O.D. 450 for some mice were even below the blank sample in standard curve, absolute serum insulin level could not be calculated properly. Instead, raw O.D. 450 was employed to show the relative serum insulin level in GTT samples. * and ** for p-value<0.05 and 0.01 by two sample t-test in R for comparing miR12 to miRNC. Repeated Measures ANOVA in R showed significant difference in serum insulin raw O.D. 450 profile with p-value of 0.0061. n=12.

2. Results

weight. Most strikingly, although glycogen levels drop in control livers upon fasting, as they enter a catabolic state to provide the rest of the organism with glucose, PPP2R5C knockdown livers displayed almost no drop in glycogen upon fasting (Figure 2.34).

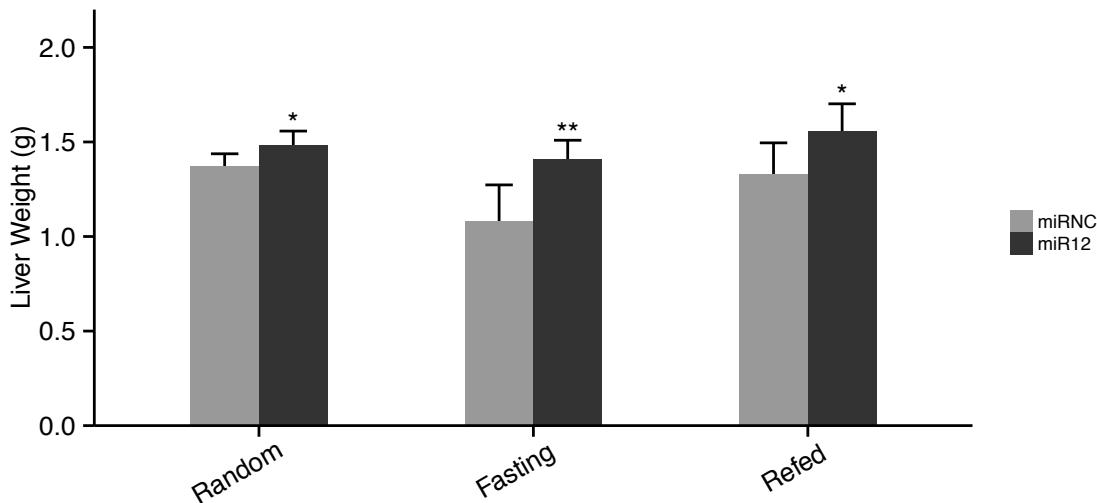


Figure 2.33.: Liver weight increases after PPP2R5C knockdown in all nutritional status. Mice were the same as in Figure 2.22. Dissected liver was weighted before aliquoting for cryosection, paraformaldehyde fixation and -80°C storage. n=5, or 6.

Glucose is also used by hepatocytes for lipid biosynthesis. If combining data from GTT experiment (Figure 2.30) and cell-autonomous increased glucose uptake lipogenesis in *in vitro* cultivated hepatocytes (Figures 2.16 and 2.17), it implies the increased glucose uptake in liver would also shunted into lipid synthesis. In agreement with this, mice with PPP2R5C KD have significantly elevated triglyceride levels in their livers in the random feeding state. Due to two outliers of liver NEFA (Figure 2.36) in control mice in random group, liver triglyceride levels in these two control mice are also higher. By ANCOVA analysis with liver NEFA as covariate, liver triglyceride in knockdown mice in random group is found to be significantly higher (p-value=0.04).

Although this effect is visible 7 weeks after PPP2R5C knockdown (Figure 2.35), it is even more pronounced 2 weeks after knockdown (Figure 2.37), possibly due to the reduced knockdown efficiency upon counter-regulation over time, or compensatory regulatory mechanism developing over time. However, Oil Red O staining on liver sample does not show visible change in lipid droplet, which indicates the increased triglyceride storage is mainly microvesicular lipid droplets which are not easily visible under microscope after Oil Red O staining.

One possible explanation for the increased liver triglyceride levels could be reduced liver

2. Results

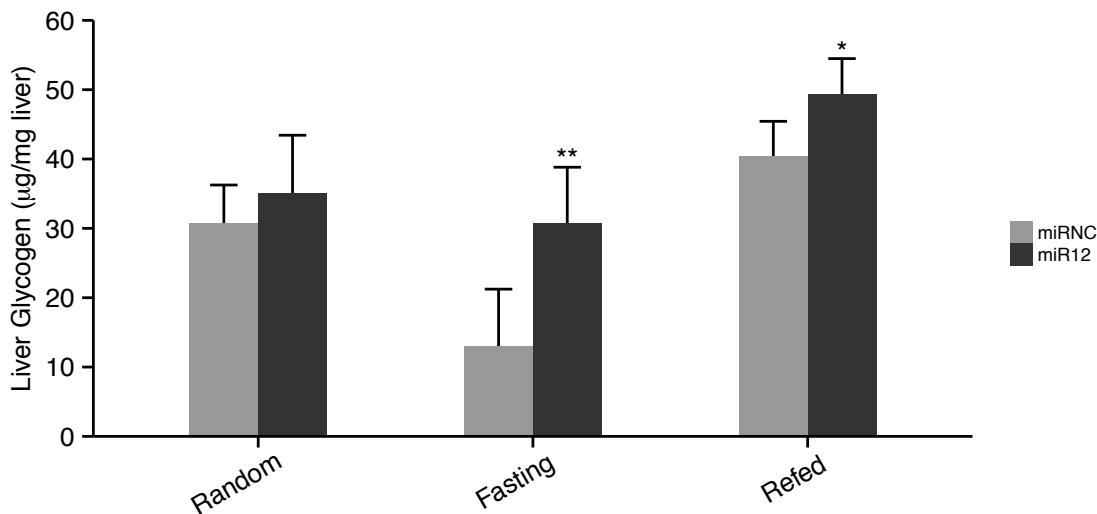


Figure 2.34.: Liver glycogen increases after PPP2R5C knockdown in fasting and refed. Mice were the same as in Figure 2.22. Frozen liver sample was pulverized in tissue homogenizer with pre-cooling in liquid nitrogen. Glycogen was extracted from ~150 mg liver powder and measured for glucose level after overnight amyloglucosidase digestion. * and ** for p-value<0.05 and 0.01 by t-test in R for comparing miR12 to miRNC for each nutrition group. n=5, or 6.

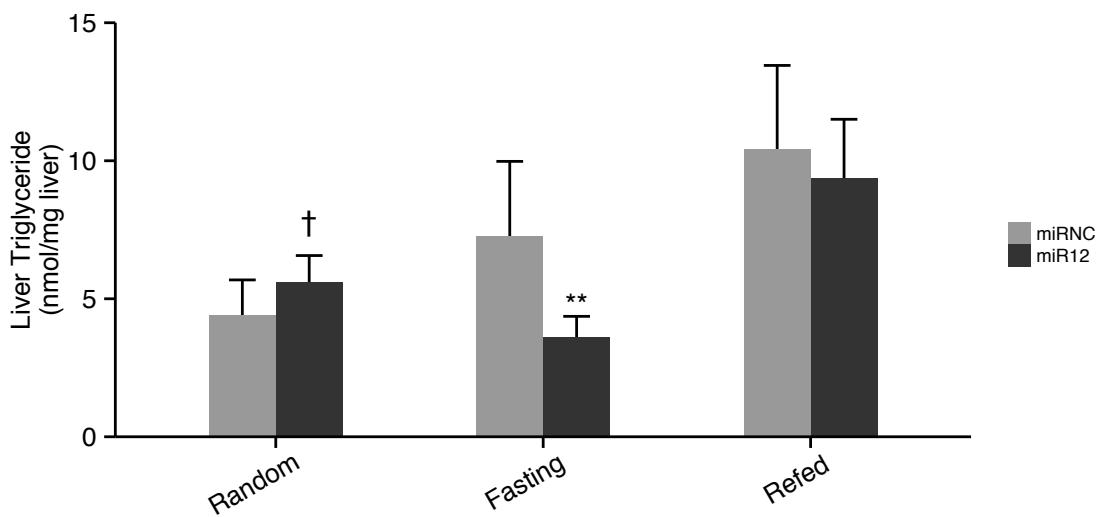


Figure 2.35.: Liver triglyceride increases after PPP2R5C knockdown in random but decreases in fasting. Mice were the same as in Figure 2.34. Lipid fraction was extracted from liver by methanol-chloroform and triglyceride was measured as free glycerol released from lipase digestion. † for p-value<0.05 for ANCOVA analysis for comparing liver triglyceride between miRNC and miR12 in random fed, in which liver non-esterified fatty acids (NEFA) (Figure 2.36) as co-variate. ** for p-value<0.01 by t-test in R. n=5, or 6.

2. Results

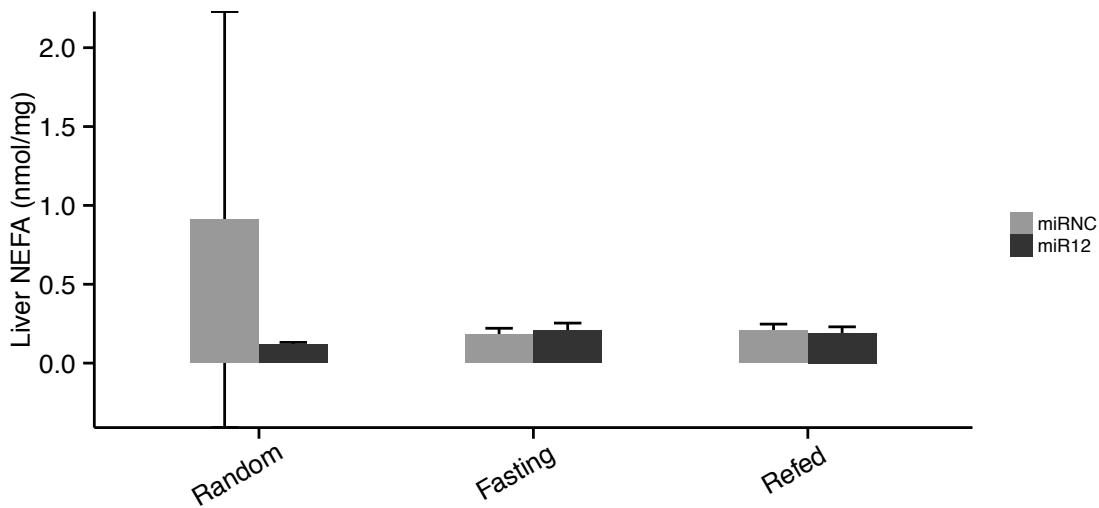


Figure 2.36.: Liver NEFA level has no significant change after PPP2R5C knockdown in all feeding regimes. Mice were the same as in Figure 2.34. Lipid fraction was extracted from liver by methanol-choloroform and NEFA was measured. n=5, or 6.

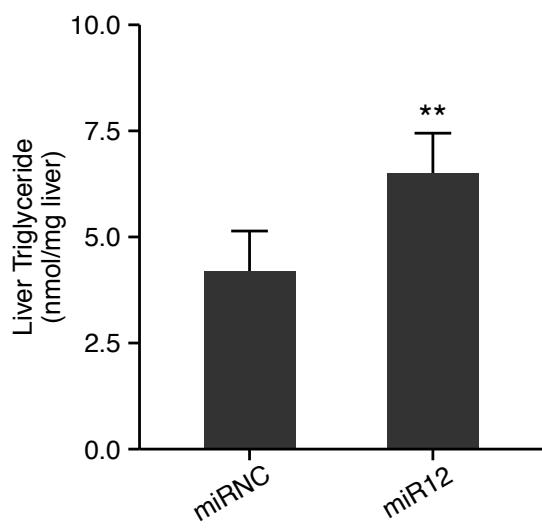


Figure 2.37: Liver triglyceride increases after PPP2R5C knockdown in random fed. 8-10 week CL57BL/6 male mice were injected with adeno-associated virus packaged miRNA against mouse PPP2R5C (miR12) or scramble miRNA (miRNC) at 0.5 or 1×10^{11} viral particles/mouse via tail injection in 100 μ L PBS. And Injected mice was sacrificed after 2 week knockdown. For liver triglyceride analysis, data from low and high dose virus injection were combined. ** for p-value<0.01 by t-test in R for comparing miR12 to miRNC. n=5.

2. Results

fatty acid β -oxidation. However, the serum ketone body concentration of total ketone body species or hydroxybutyrate are maintained at the same level after PPP2R5C (Figures 2.38 and 2.39). Total ketone body concentration are increased in fasting group and decreased after refeeding for both control and PPP2R5C mice, which is expected since starvation in mice would increase β -oxidation activity in liver. Additionally, hydroxybutyrate concentration normalized to total ketone body also did not show any relative change upon PPP2R5C KD (Figure 2.40). Circulating ketone body levels are not changed upon PPP2R5C KD is indicating that accumulation of hepatic triglyceride levels are not due to impaired utilization in β -oxidation but presumably due to increased *de novo* lipogenesis, which have been demonstrated in *in vitro* cell culture models (Figures 2.17 and 2.19).

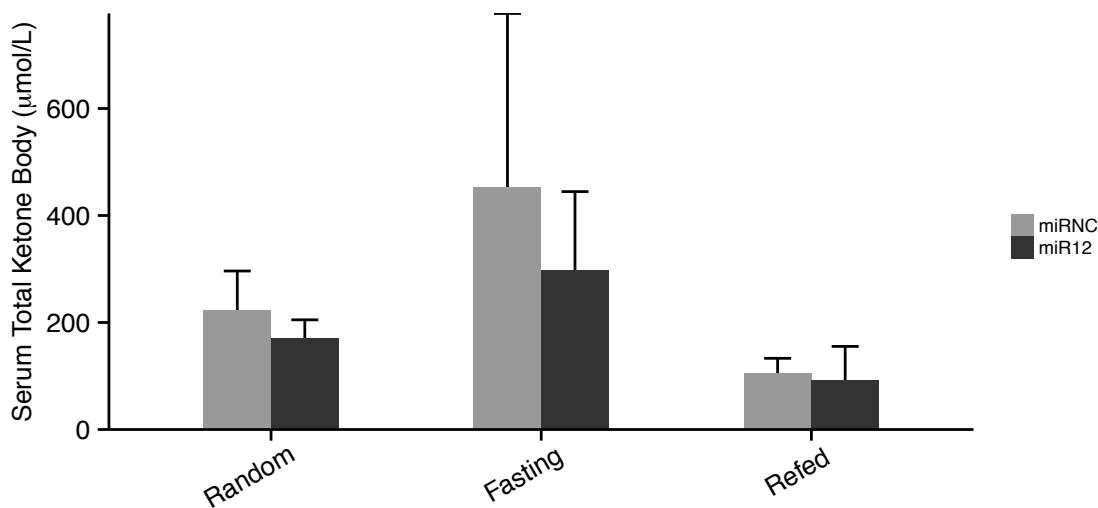


Figure 2.38.: Serum total ketone body (TKB) does not change upon PPP2R5C knockdown in all feeding regimes. Mice were the same as in Figure 2.34. n=5, or 6.

Surprisingly, *in vivo*, triglyceride levels drop significantly in PPP2R5C knockdown livers upon fasting (Figure 2.35). I have tested if this could be due to increased lipid secretion from liver. Indeed, Serum triglyceride in fasting and refed group after PPP2R5C KD are increased significantly (Figure 2.41). This leads to a drop not only in triglyceride but also cholesterol in PPP2R5C knockdown livers upon fasting and refed (Figure 2.47). Upon refeeding, PPP2R5C knockdown livers re-accumulated triglycerides very rapidly, reaching control levels within 6 hours of refeeding (Figure 2.35), consistent with elevated lipid biosynthesis rates in PPP2R5C knockdown livers. Taken together, PPP2R5C knockdown livers have more glucose uptake than control livers, thereby produce more triglycerides, and secrete elevated lipid amounts into circulation. The steady state levels of

2. Results

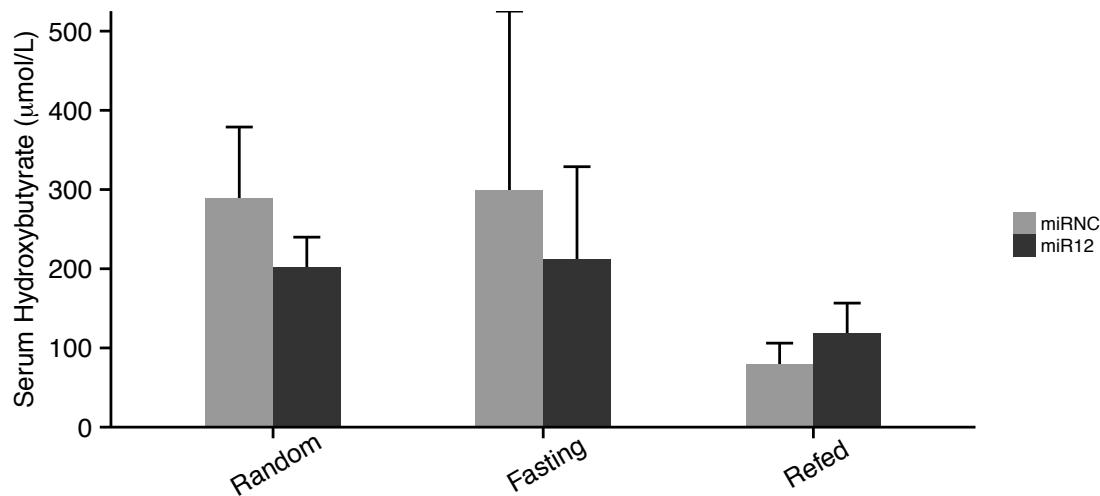


Figure 2.39.: Serum hydroxybutyrate (HB) concentration does not change upon PPP2R5C knockdown in all feeding regimes. Mice were the same as in Figure 2.34. n=5, or 6.

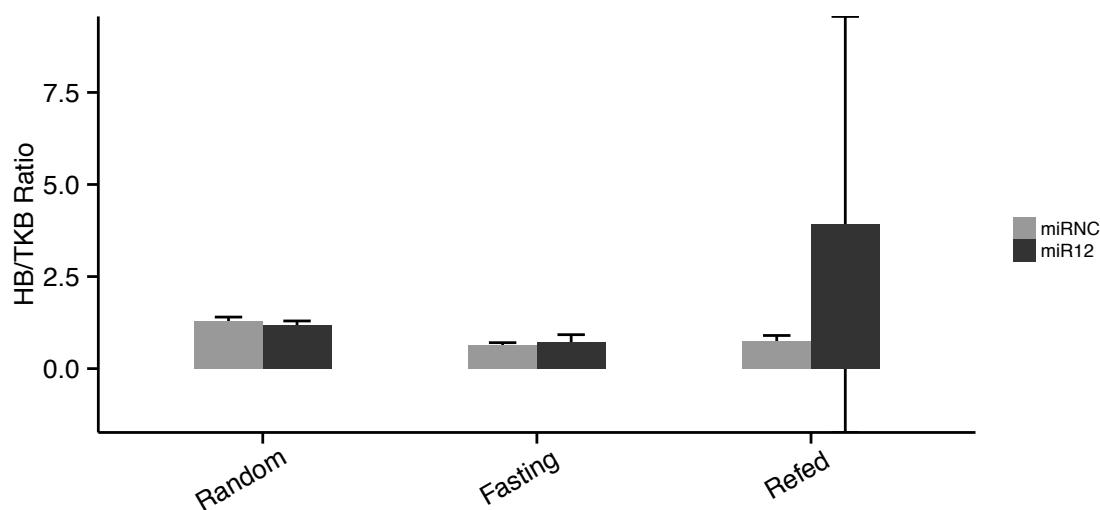


Figure 2.40.: Serum HB to TKB ratio does not change upon PPP2R5C knockdown in all feeding regimes. Mice were the same as in Figure 2.34. n=5, or 6.

2. Results

triglyceride in PPP2R5C knockdown livers likely reflecte this balance between increased biosynthesis and increased secretion, leading to a drop in liver triglyceride upon fasting when less dietary glucose is available for lipid biosynthesis. If serum triglyceride and liver triglyceride are considered together, the overall triglyceride at least in random and refed condition are increased in knockdown mice.

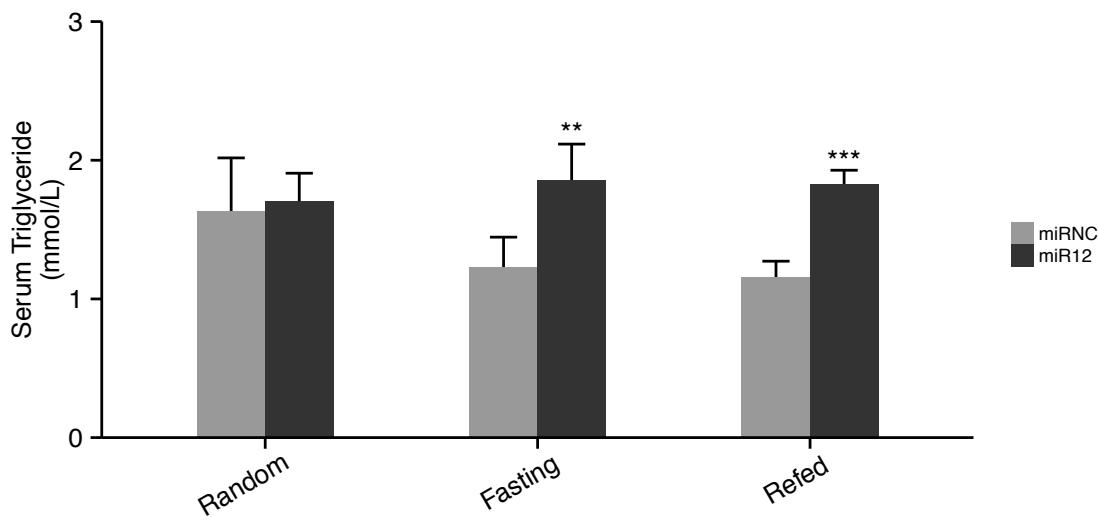


Figure 2.41.: Serum triglyceride increases after PPP2R5C knockdown in fasting and refed. Mice were the same as in Figure 2.34. 2 μ L serum from these mice was measured as free glycerol released from lipase digestion. ** and *** for p -value<0.01 and 0.001 by t-test in R. n=5, or 6.

Lipoactive hormones such as epinephrine, norepinephrine, glucagon, thyrotropin, and adrenocorticotropin release free fatty acids (Serum NEFA) into serum from lipolysis in adipose tissue. And liver can re-absorb almost 75% of the serum NEFA to re-esterify them into triglyceride and release into serum as VLDL particle. Upon PPP2R5C knockdown, the steady state level of serum NEFA in random and fasting feeding regime are not changed (Figure 2.42), which further indicates the increased lipid storage is rather sourced from *de novo* lipogenesis in liver. In refed, mice with PPP2R5C knockdown even have significant increased serum NEFA. During refed, serum NEFA could also be originated from food intake. And it is possible the liver lipid synthesis capacity has already reach its plateau and the NEFA is accumulated in serum to have a higher concentration in PPP2R5C knockdown mice than control. This postulation also fits with the fact that liver triglyceride in refed condition stays at very high level but there is still more triglyceride released into serum, which suggestes higher triglyceride synthesis and secretion in liver during refed.

2. Results

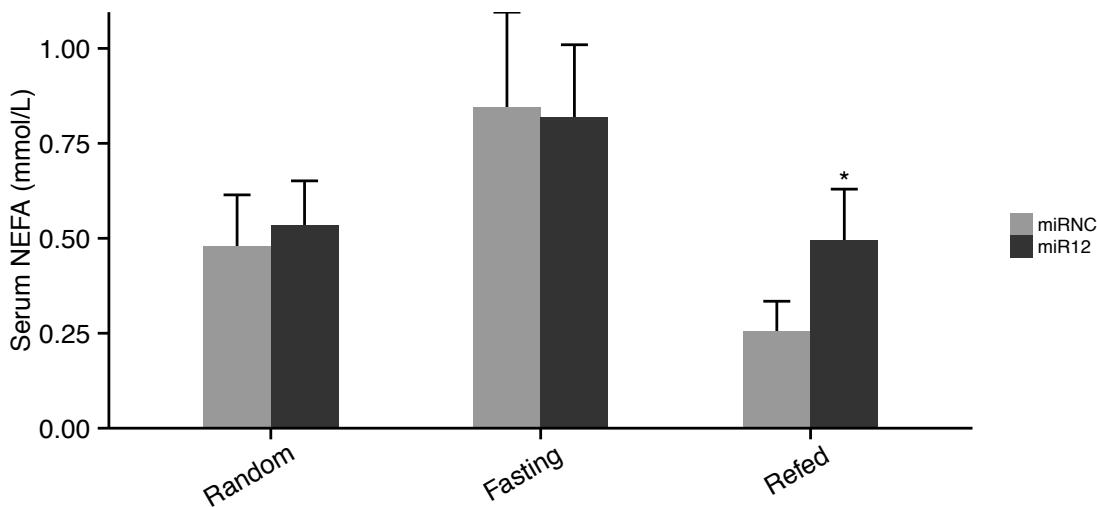


Figure 2.42.: Serum NEFA is decreased in refed. Mice were the same as in Figure 2.34. Lipid fraction was extracted from liver by methanol-chloroform and triglyceride was measured as free glycerol released from lipase digestion. 2 μ L serum from these mice was measured for NEFA. * for p-value<0.05 by t-test in R. n=5, or 6.

2.4. PPP2R5C negatively regulates VLDL secretion in liver

Given the fact that serum triglyceride was increased during fasting and refed, a more detailed analysis of serum lipid composition was performed by FPLC fractionation of serum, in order to find which lipoprotein particle was responsible for increased serum triglyceride. VLDL, IDL, LDL and HDL could be nicely separated on a high resolution size-exclusion chromatography column. In Random group, mice after PPP2R5C KD has no difference in serum lipoprotein particle profile (Total protein content (A₂₈₀) in lipoprotein fractions was shown in Figure 2.43). However, there are significant increase in VLDL fraction (approximately 5-fold increase) in Fasting and Refed group after PPP2R5C knockdown. Concordantly, triglyceride distribution profile in serum is also agreed with increased VLDL intensity (Figure 2.44). Quantification for VLDL peak apex and peak area is shown in Figure 2.45. VLDL particles are lipoprotein particles synthesized in liver and secreted into bloodstream for transporting endogenous triglyceride, cholesterol, phospholipids and cholesterol esters [94]. Comparing with other lipoprotein particles, VLDL has highest ratio of triglyceride, and could explain the significant increase in serum triglyceride in the form of VLDL. Increased VLDL secretion is also observed in human upon increased *de novo* lipogenesis [95].

2. Results

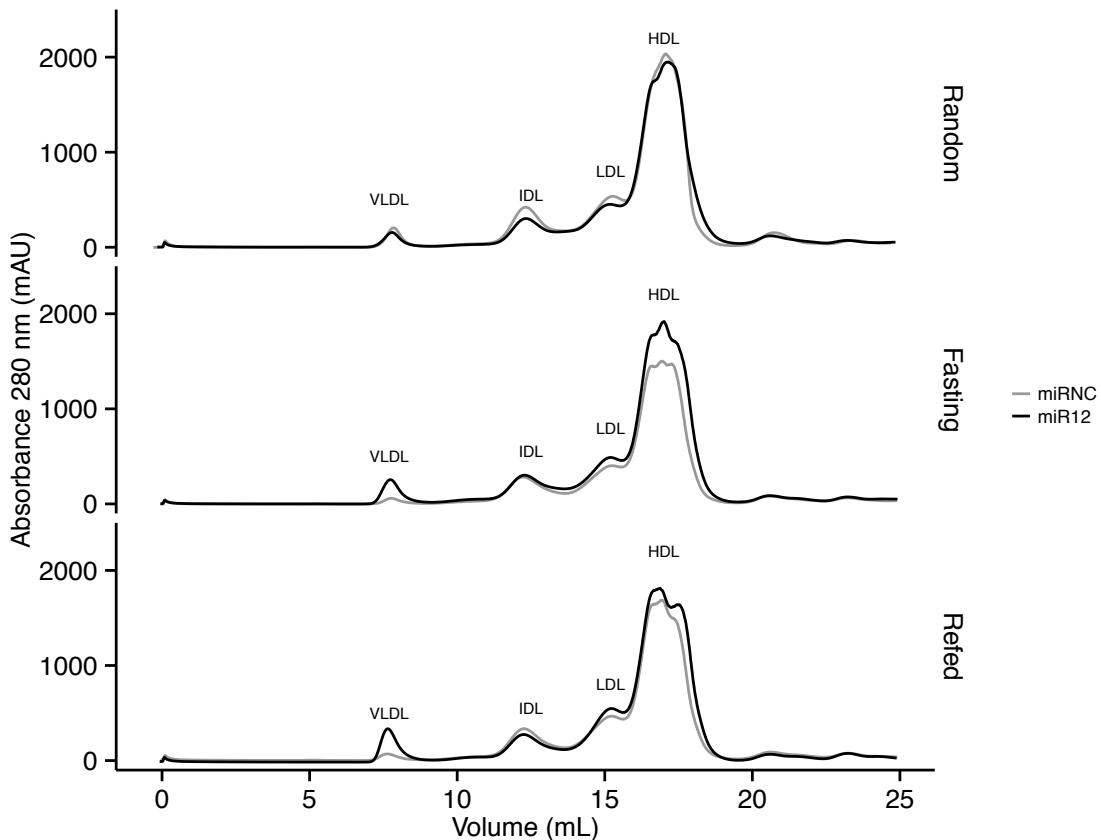


Figure 2.43.: Increased VLDL fraction after PPP2R5C KD. Serum lipoprotein particles was analyzed by FPLC. 200 μ L pooled serum for 5 or 6 mice in the same virus and feeding group was subjected on FPLC separation on high-resolution size-exclusion chromatography. FPLC profile was recorded as UV 280nm, which was the indicator for protein concentration. Separated serum was collected in fractions with 0.5 mL. Mice were the same as in Figure 2.34.

2. Results

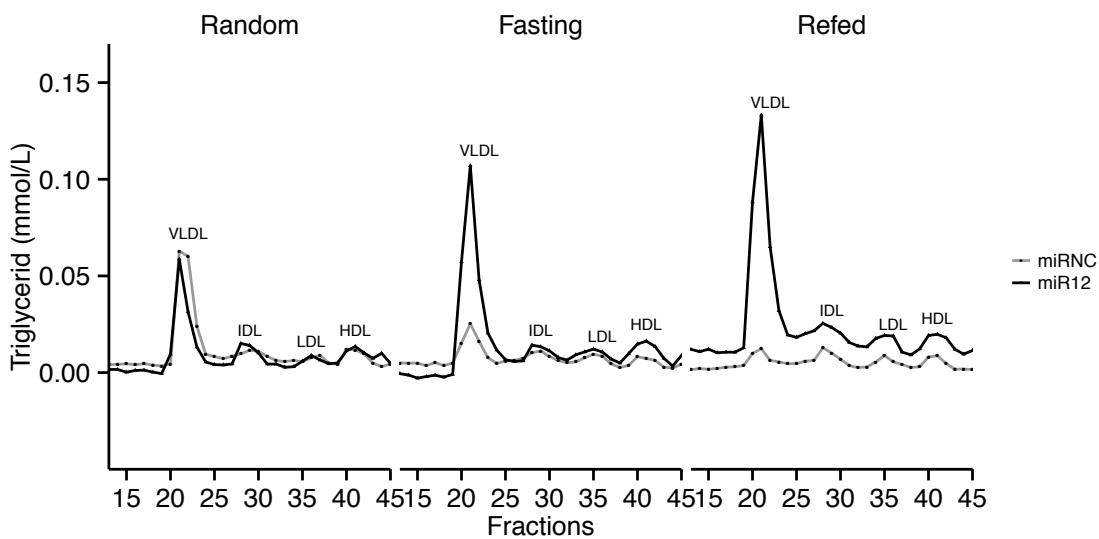


Figure 2.44.: Triglyceride concentration profile correlated with its FPLC profile. 160 μ L from each fraction was used to measure triglyceride concentration as free glycerol released from lipase digestion.

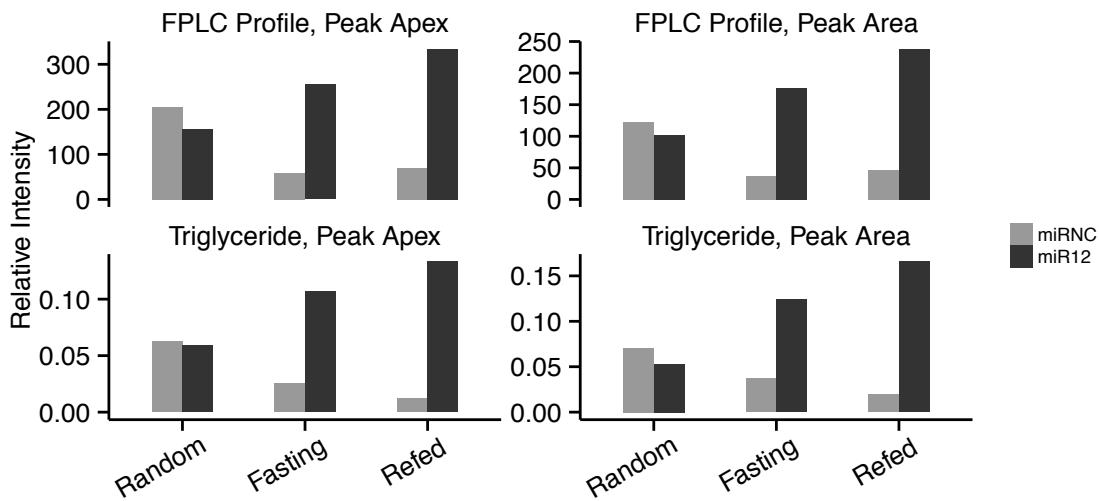


Figure 2.45.: Quantification of VLDL peak apex and area. Start and end point for each peak in Figure 2.43 were manually selected by examining the emerging and vanishing time point for each peak comparing with background. Peak Apex and Area were calculated in R by finding maximal intensity and trapezoid integration from start point to end point for VLDL peak.

2. Results

In agreement with increased serum VLDL fraction, cholesterol level in this fraction has also increased (Figure 2.46). This data further validates the hypothesis that increased glucose uptake in liver is shunted into lipogenesis and eventually released into serum in the form of VLDL after PPP2R5C knockdown. Indeed, liver cholesterol levels also drop in fasting and refed condition due to increased VLDL secretion (Figure 2.47). Liver cholesterol is probably passively packaged into VLDL particle together with triglyceride and released into serum, due to increased lipogenesis in liver upon PPP2R5C KD. However, the total serum cholesterol levels have not change (Figure 2.48) since cholesterol from VLDL is only a minor fraction comparing to cholesterol form IDL and HDL peaks (Figure 2.46), which are not changed dramatically upon PPP2R5C KD (Figure 2.43).

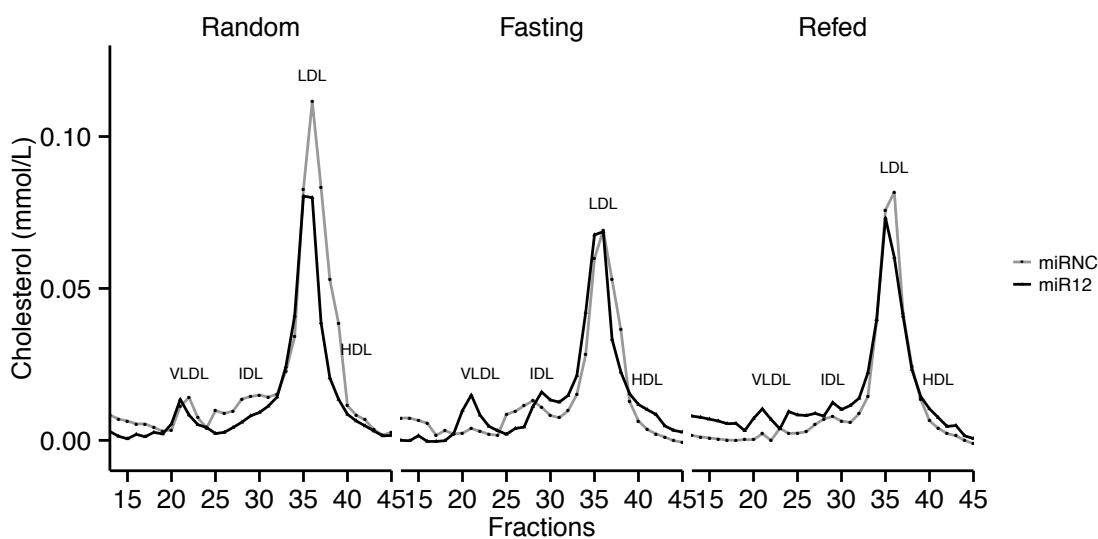


Figure 2.46.: Cholesterol concentration profile in various lipoprotein particles. $40 \mu\text{L}$ from each fraction was used to measure cholesterol concentration.

In summary, due to increased lipogenesis derived from increased glucose uptake in liver after PPP2R5C KD, VLDL secretion from liver is also increased and contributing to anabolically storage glucose in liver. The increased glucose clearance capacity of liver after PPP2R5C KD enable efficient euglycemic control with less insulin secretion from pancrea.

2. Results

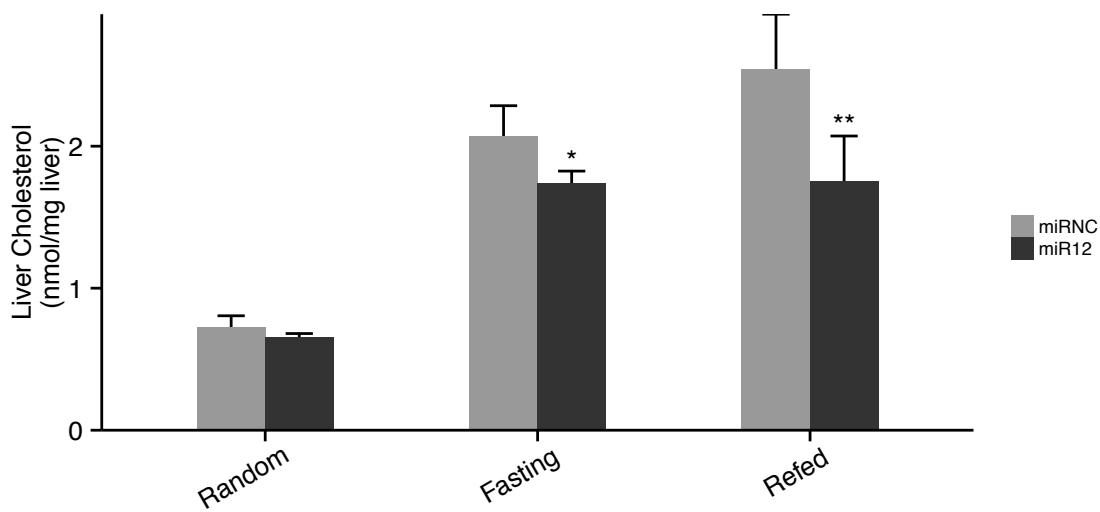


Figure 2.47.: Liver cholesterol is dropped in fasting and refed after PPP2R5C KD. Mice were the same as in Figure 2.34. Lipid fraction was extracted from liver by methanol-chloroform and cholesterol was directly measured. * and ** for p-value<0.05 and 0.01 by t-test in R for comparing miR12 to miRNC for each nutrition group. n=5, or 6.

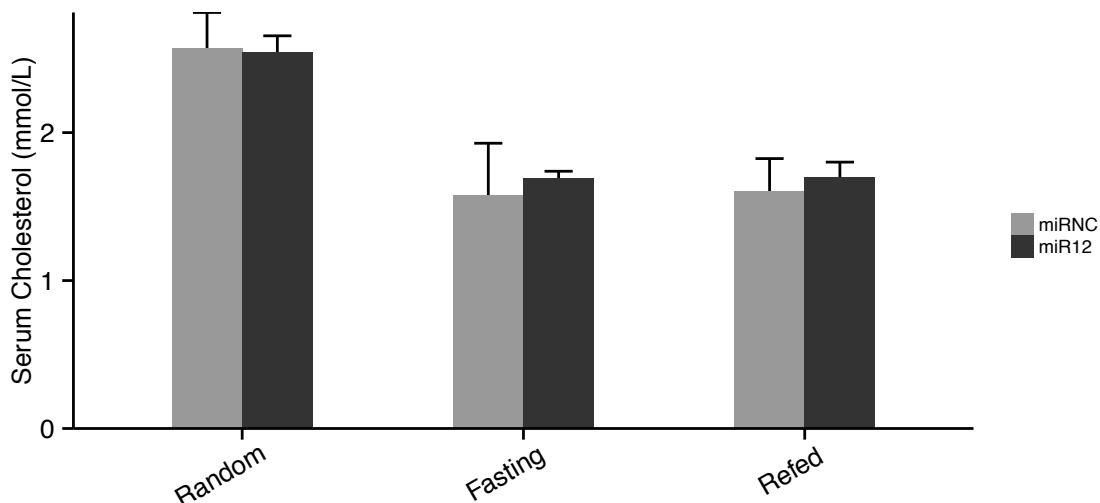


Figure 2.48.: Serum cholesterol has no change after PPP2R5C KD in all feeding regimes. Mice were the same as in Figure 2.34. 2 μ L serum was used to measure cholesterol directly. n=5, or 6.

2.5. PPP2R5C regulates metabolism via AMPK and HIF1 α

2.5.1. PPP2R5C's substrates include multiple metabolic regulators

PPP2R5C is a regulatory subunit of PP2A, thought to provide substrate specificity to the phosphatase holoenzyme. Therefore, a proteomic approach was needed to identify target substrates that bind PPP2R5C. And potential substrates involved in metabolism control would provide clue to elucidate the mechanism of PPP2R5C's negative regulation in glycolysis and lipogenesis. However, phosphatase substrate discovery is difficult. Beside occasionally successes in finding phosphatase interacting substrates, there are few reports regarding system-wide substrate identification for phosphatases, mostly protein tyrosine phosphatases [96, 97, 98]. In case for protein tyrosine phosphatases, a substrate trapping mutant competes with its endogenous counterpart for substrates. Due to its structural simplicity (single subunit and verified catalytic dead mutant, usually a cysteine to serine mutation in catalytic active center), protein tyrosine phosphatase is much easier for pulling down binding substrates. For Serine/Threonine kinases, MAP kinase phosphatase-1 is another success example of substrate trapping [99].

Since protein-protein interactions between phosphatases and substrates are notoriously transient and difficult to detect via conventional methods, such as co-immunoprecipitation strategies. Here in the thesis project, the BioID method [100] was employed to identify PPP2R5C interacting proteins, including its potential substrates. A fusion between PPP2R5C and the biotin ligase BirA mutant (BirA*) was expressed in Hepa 1-6. The mutant biotin ligase (P118G) will generate reactive biotin (biotin-5'-AMP) and ligate biotin onto proteins *in vitro* [101, 102]. With co-expression of substrate trapping mutant of PP2A catalytic subunit C, fusion protein from PPP2R5C and BirA* led to *in vivo* biotinylation of PPP2R5C interacting proteins, which can subsequently be isolated by collecting cellular lysates and streptavidin pulldown (Figure 2.49). By this way, these potential substrates with weak interactions with PPP2R5C would have higher chance to be identified by western blot or mass spectrometry. BirA* was fused to either the N-terminus or the C-terminus of PPP2R5C (Myc-BirA-PPP2R5C and PPP2R5C-BirA-HA respectively), and Myc or HA tagged BirA* alone was used as negative control. In addition, the catalytic dead mutation (D85N), which was known to eliminate phosphatase activity [36], was introduced into the catalytic subunit of PP2A (PP2CA). This design was the first attempt to perform a substrate trapping assay for

2. Results

PP2A, and expected to employ PP2A's substrate-trapping mutation might extend the duration of interaction between PP2A and its substrate proteins.

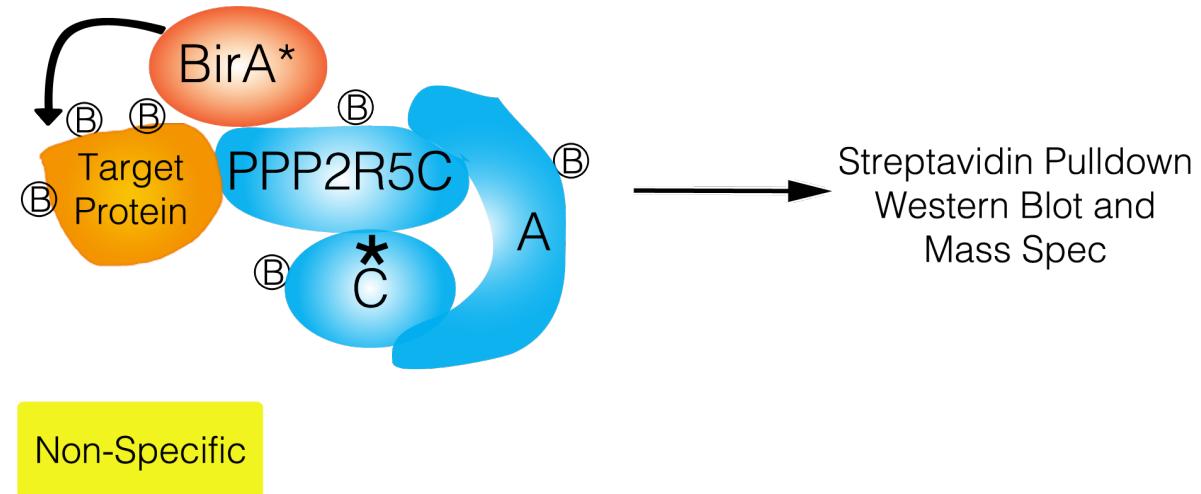


Figure 2.49.: PPP2R5C containing PP2A holozyme substrate trapping and *in vivo* biotinylation of its substrates.

In the BioID experiment, various known regulators of liver metabolism were found to be interacting with PPP2R5C, including AMPK $\beta 1$, HIF1 α , S6K1, STAT3 (Figure 2.50a). Comparing to either Myc-BirA* or BirA*-HA controls, HIF1 α and other potential substrate have increased biotinylation, which is shown as stronger band after streptoavidin pulldown and detection with total antibodies for individuals. When co-expressed with substrate trapping mutant of PP2A catalytic subunit, the interaction between PPP2R5C and its substrates is further increased. In contrast, no binding of PPP2R5C to SREBP-1, or a panel of negative control proteins are detected, including HSP90, YAP, TSC1 and Rpl26 (Figure 2.50b).

2.5.2. AMPK is PPP2R5C's substrate involved in glucose uptake

AMPK is the master regulator in energy homeostasis. It is a trimeric heterogenous complex composed of α for catalytic activity, β for regulatory function, and γ for sensing AMP/ATP ratio. Given the interaction between AMPK $\beta 1$ and PPP2R5C, there might also some functional link between PP2A and AMPK. Indeed, there are several reports showed the AMPK phosphorylation is negatively regulated by PP2A [103, 104, 105]. However, which regulatory subunit in PP2A is responsible for AMPK dephosphorylation is still unknown. With PPP2R5C knockdown, the PPP2R5C containing

2. Results

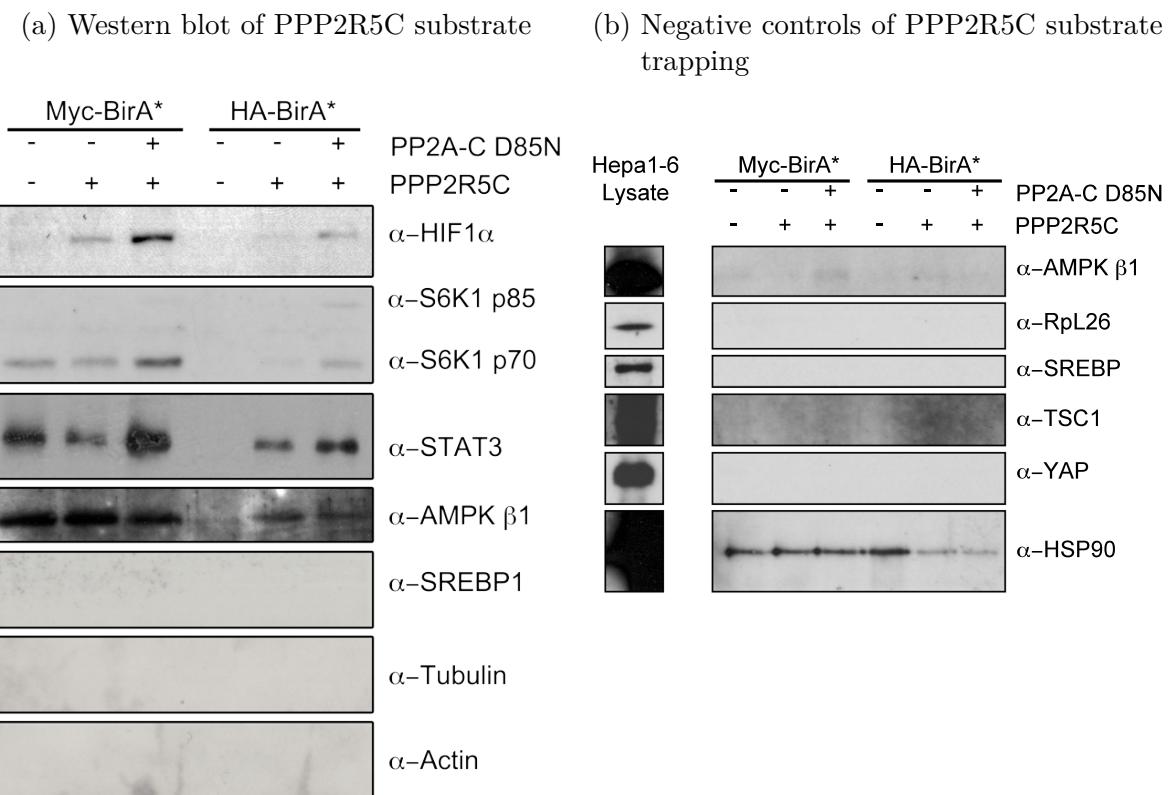


Figure 2.50.: PPP2R5C's substrates involved in metabolic control. (a) PPP2R5C interacting proteins were validated by western blot. SREBP-1, Tubulin or Actin were used as non-interacting controls. (b) AMPK β 1 subunit and several other negative controls were used to validate the specificity of PPP2R5C's substrate trapping in a independent repeat experiment.

2. Results

sub-pool of PP2A holoenzyme is reduced. And AMPK activity is markedly increased upon PPP2R5C knockdown with different methods, either adenovirus mediated knockdown (Figure 2.51a) or inducible shRNA stable cell line (Figure 2.51b). This data clearly shows that PPP2R5C is at least one of the regulatory subunit of PP2A involving AMPK inhibition. Accordingly, AMPK activity change was further validated for downstream effectors' activity such as ACC1 phosphorylation, TBC1D1 phosphorylation. It is known that TBC1D1, the mouse homolog for human AS160, is upstream regulator for Glut1 translocation and its phosphorylation at S700 promote glucose uptake [106, 107]. This raises the possibility of AMPK activation after PPP2R5C knockdown is mediating the increased glucose uptake phenotype (Figures 2.15 and 2.16).

- (a) AMPK activity increases upon PPP2R5C KD (b) Activity increase of AMPK and its downstream effectors

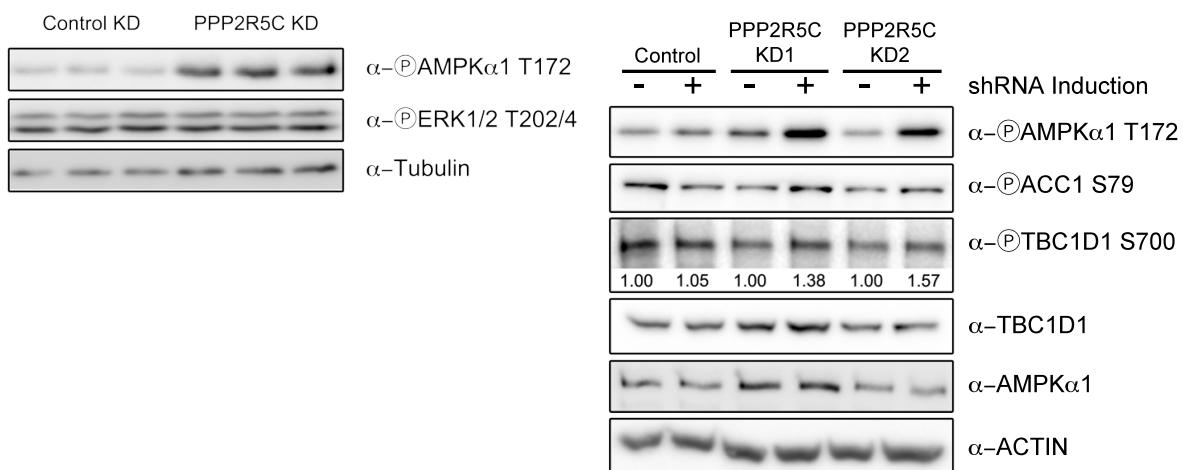


Figure 2.51.: AMPK activity is up-regulated upon PPP2R5C knockdown. (a) AMPK activity is increased after PPP2R5C knockdown by adenovirus packaged shR3 comparing to shRNC (PPP2R5C KD vs Control KD). (b) Inducible shR3 and shR6 (PPP2R5C KD1 and KD2) were used to examine the AMPK activity change in order to eliminate any virus mediated effect in (a).

2.5.3. HIF1 α is PPP2R5C's substrate involved in glycolysis and lipogenesis

HIF1 α is also known for controlling glycolysis and lipogenesis in liver [108, 109, 110, 111, 112]. The interaction between HIF1 α and PPP2R5C, which is demonstrated by substrate trapping in Figure 2.50, indicates that HIF1 α could also be one of PPP2R5C's

2. Results

potential substrates involved in metabolism control. However, there is no phospho-specific antibody commercially available for HIF1 α . Then the functional relevance of PPP2R5C knockdown on HIF1 α 's phosphorylation status was evaluated by Phos-tag® gel (Figure 2.52). Phos-tag® gel is made from an acrylamide analog containing chemical group for binding phosphorylated ions specifically [113, 114, 115, 116, 117]. When protein is phosphorylated, its mobility in Phos-tag® gel will be retarded. As a result, phosphorylated and non-phosphorylated proteins will be separated and protein phosphorylation with unknown site will be possibly examined without phospho-specific antibodies. The HIF1 α has increased phosphorylated form upon PPP2R5C knockdown, and this phosphorylation is also validated by CIP treatment (Figure 2.52).

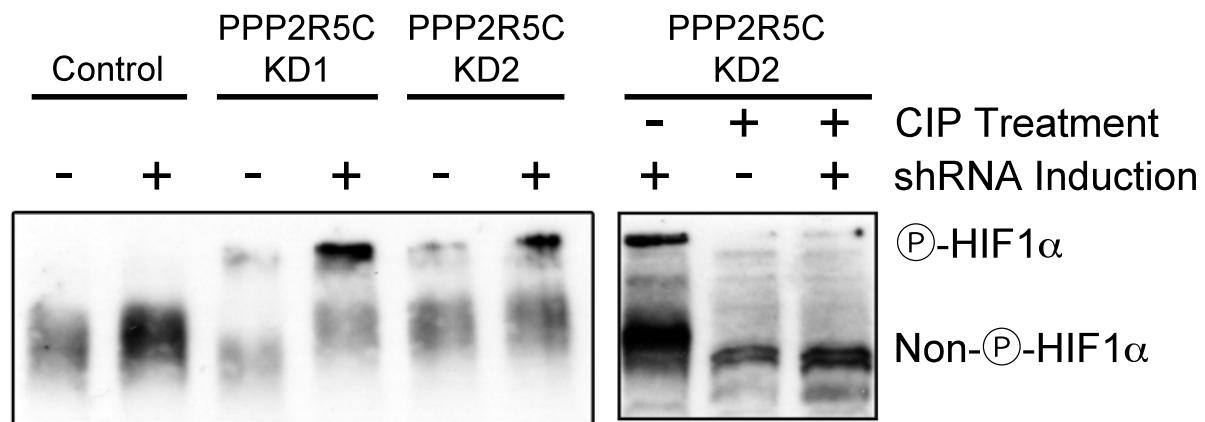


Figure 2.52.: HIF1 α 's phosphorylation is increased after PPP2R5C KD. HIF1 α 's phosphorylation status was evaluated in Phos-tag® gel after inducible shR3 and shR6 expression (PPP2R5C KD1 and KD2).

In agreement with phosphorylation increase upon PPP2R5C knockdown, HIF1 α 's transcriptional activity is also up-regulated after PPP2R5C knockdown (Figure 2.53) in primary hepatocytes. Four canonical down-stream targets of HIF1 α are all increased in mRNA level. Three of these four targets are involved in glycolysis, including LDHa, HK2, and PKM2, could help to explain the increased glycolysis after PPP2R5C knock-down.

2.5.4. SREBP-1 is involved in lipogenesis phenotype

Although SREBP-1 was not interacting with PPP2R5C in substrate trapping experiment, SREBP-1 could still be involved in lipogenesis phenotype after PPP2R5C in two possible ways. First, the decrease in liver cholesterol in fasting and refed group indicates that SREBP-1 activity increase could be the direct result from liver cholesterol decrease due to the fact that cholesterol and its derivatives are endogenous molecules have been

2. Results

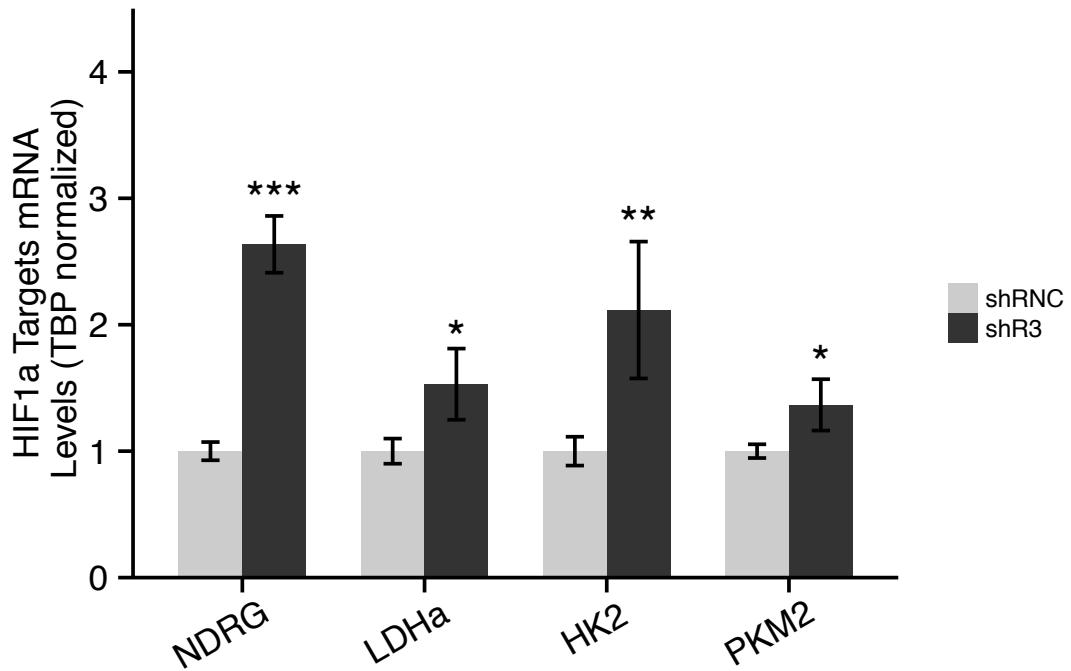


Figure 2.53.: HIF1 α 's transcriptional activity is increased after PPP2R5C KD. Four HIF1 α 's targets were examined for its total mRNA level in mouse primary hepatocytes after PPP2R5C KD.

2. Results

demonstrated to regulate SREBP-1 expression [118, 119]. Decreased cholesterol concentration will relieve the cholesterol's suppression on SREBP-1 activity. Secondly, SREBP-1 could act as downstream effector in HIF1 α mediated lipogenesis [110].

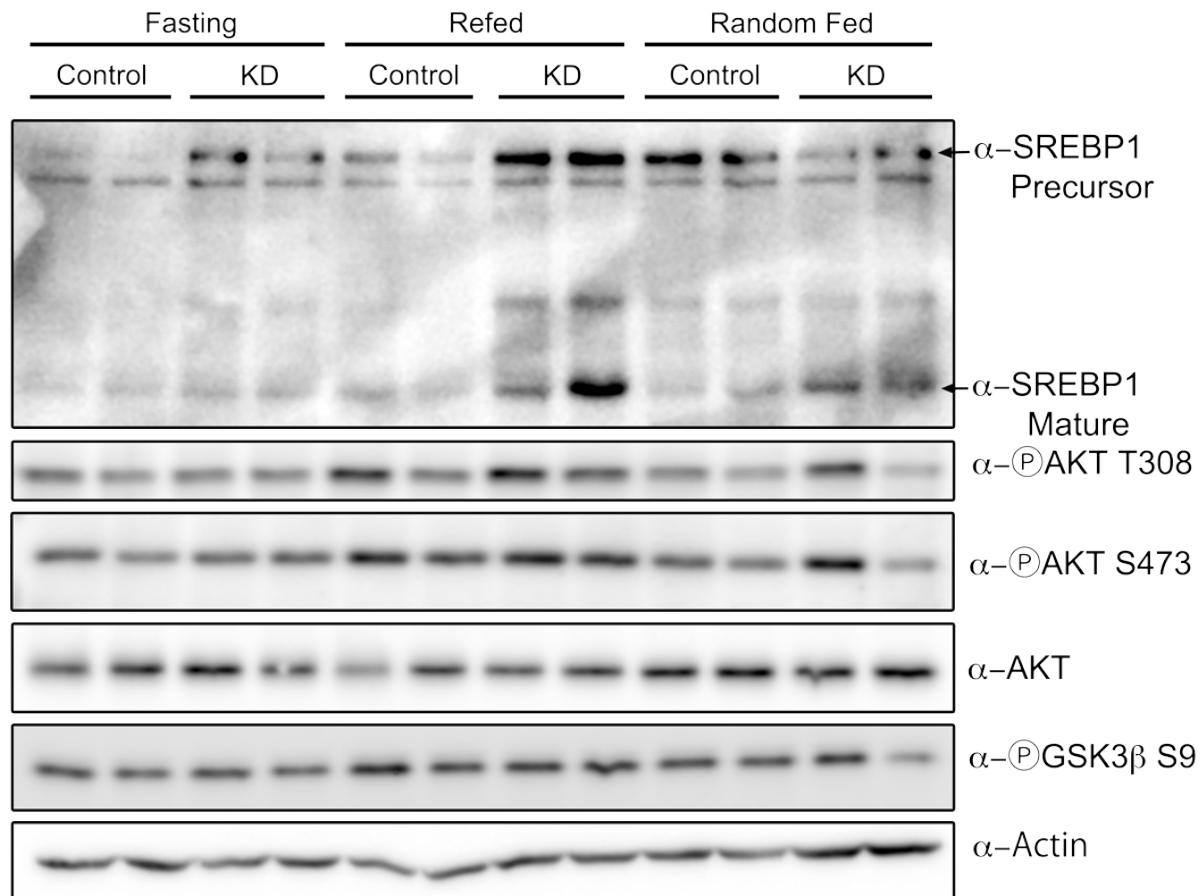


Figure 2.54.: SREBP-1 protein level is increased after PPP2R5C KD. Insulin signaling activity was monitored by AKT and GSK3 β phosphorylation.

Indeed, SREBP-1 activity is increased in mouse liver upon PPP2R5C knockdown when mice are subjected to fasting and refed (Figure 2.54). Together, the insulin signaling activity is remained the same. This rules out the possibility that the increased SREBP-1 activity is originated from insulin signaling, a positive regulator for SREBP-1 activity. In addition, the SREBP-1 transcriptional activity is also increased in mouse primary hepatocytes (Figure 2.55) and mouse liver (Figure 2.56) after PPP2R5C knockdown. Four canonical SREBP-1 targets involved in lipogenesis (DGAT2, GPAT1, ACLY, and SLC25A1) are all significantly up-regulated after PPP2R5C knockdown. Since SREBP-1 could auto-activate itself, increased SREBP-1 mRNA level is also observed in mouse liver after PPP2R5C knockdown (Figure 2.56). It is known that HIF1 α could still regulate lipogenesis in mouse liver [112], even the mice are not under hypoxia. Both HIF1 α activity increase and cholesterol lowering after PPP2R5C knockdown would potentially

2. Results

increase the SREBP-1 activity, which promotes lipogenesis.

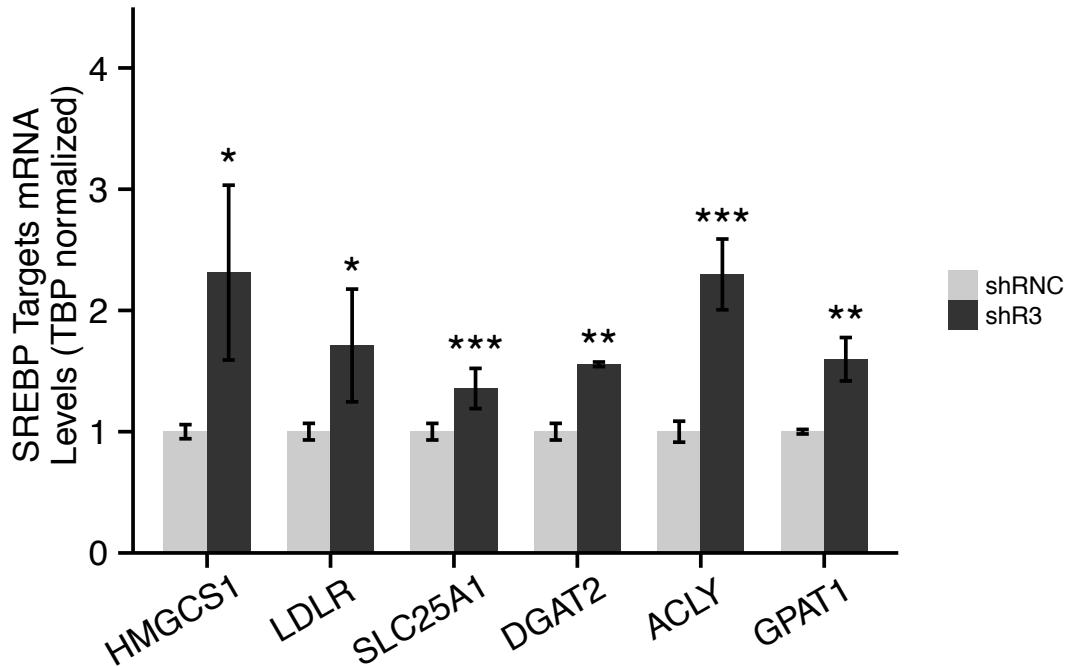


Figure 2.55.: SREBP-1 transcriptional activity is upregulated in PPP2R5C KD. Four SREBP-1's targets (DGAT2, GPAT1, ACLY, and SLC25A1 are all genes involved in lipogenesis) were examined for its total mRNA level in mouse primary hepatocytes after PPP2R5C KD.

2.5.5. Microarray analysis of PPP2R5C KD in mouse liver

Gene expression analysis in mouse liver with PPP2R5C KD has also shown that increased SREBP-1 activity based on transcription factor (TF) enrichment analysis from differentially expressed genes with 2-fold change cutoff. TF enrichment analysis was done with online web server of TFactS [120]. During refed, SREBP-1 and HIF1 α were enriched based on their potential target gene activation (Table 2.1). The activated TF lists upon PPP2R5C KD in fasting and random fed are shown in Supplementary Table Tables B.2 and B.3. The activated TF list upon PPP2R5C KD in mouse primary hepatocyte and Hepa 1-6 is shown in Supplementary Table B.1. All these lists have SREBP-1 (gene name SREBF1) in the activation TF list based on target gene expression.

2. Results

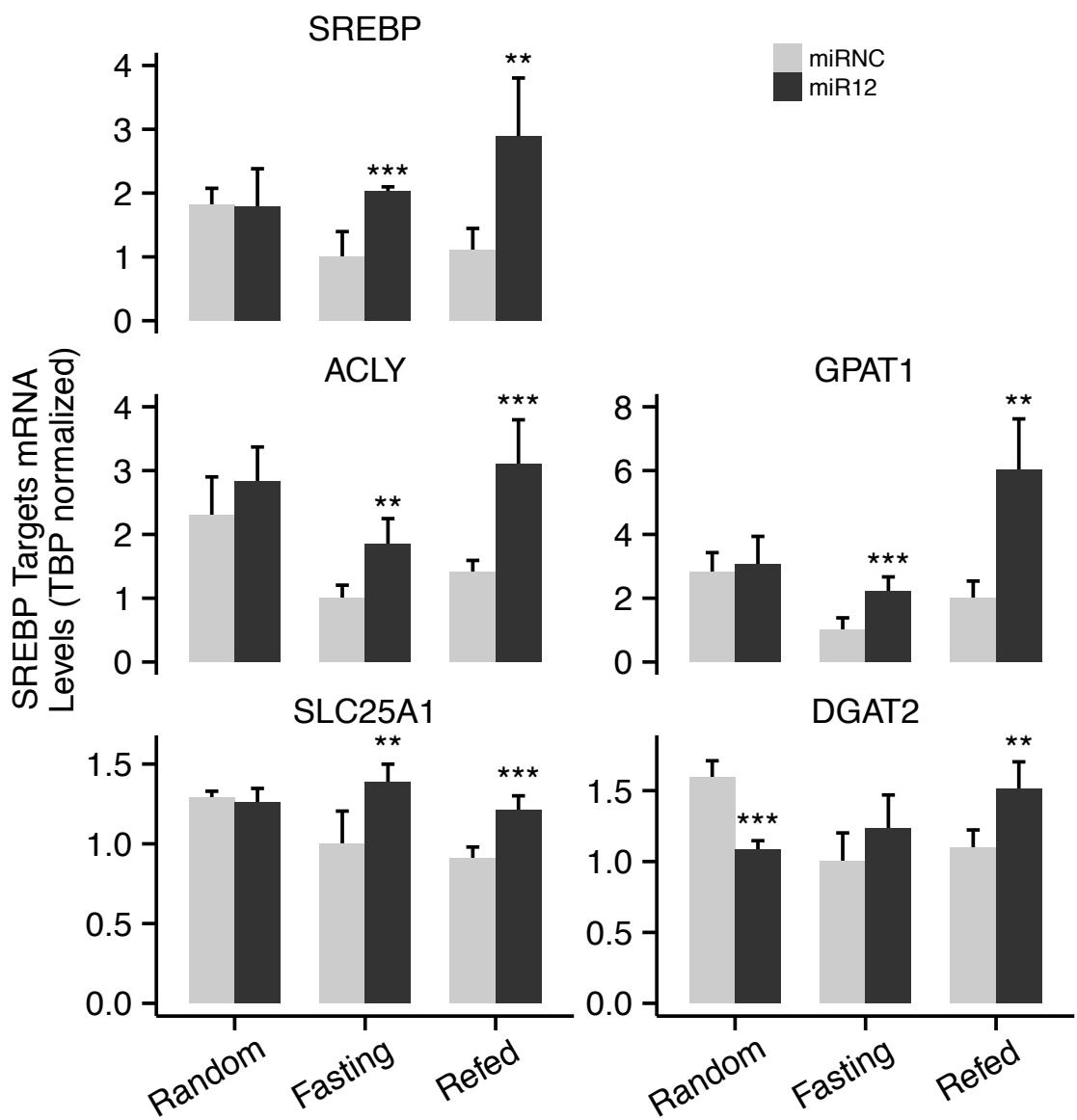


Figure 2.56.: SREBP-1 transcriptional activity is upregulated in mouse liver after PPP2R5C KD. Total mRNA of SREBP-1 and its downstream targets in lipogenesis were investigated in mouse liver.

2. Results

Table 2.1.: Activated transcription factors in HepKD mouse liver during refed.

Transcription Factor	P-value	FDR control (B-H) ^a
SREBF1	0.00038	0.001613
TP53	0.00266	0.003226
ID3	0.00921	0.004839
ID2	0.00921	0.006452
NOTCH2	0.01226	0.008065
ID1	0.01226	0.009677
PPARA	0.03340	0.011290
SMAD1	0.04233	0.012900
NR2F1	0.04825	0.014520
HIF1A	0.06579	0.016130
STAT1	0.07446	0.017740
E2F1	0.07917	0.019350
FOXO1	0.08369	0.020970
CEBPA	0.09721	0.022580
SREBF2	0.10000	0.024190

^a Adjusted p-value (false discovery rate) by Benjamini-Hochberg method [121].

2.6. Human PPP2R5C in Type 2 Diabetes

2.6.1. PPP2R5C misregulation in human liver

In a cohort study of 76 liver sample from human, including 40 healthy donors and 26 type 2 diabetic patients (performed by my collaborator Prof. Matthias Blüher in University of Leipzig), human PPP2R5C total mRNA levels was checked by quantitative PCR with 18S rRNA as normalization control. The human liver PPP2R5C mRNA is significantly increased in type 2 diabetes (Figure 2.57). The up-regulation of human PPP2R5C in type 2 diabetic patients is correlated with the findings that almost all variants of mouse PPP2R5C are increased in liver of *db/db* mice, which is a mouse model for type 2 diabetes (Figure 2.2). This up-regulation in human liver could be considered as negative feedback of PPP2R5C transcription in order to curtail the lipid synthesis capacity of liver, or a potential reason for insulin resistance since PPP2R5C KD could improve insulin sensitivity. In type 2 diabetic patient, majority of them will accumulate lipid in liver and develop fatty liver disease in addition to type 2 diabetes. PPP2R5C increase seems to have protective role for fatty liver development.

Another interesting finding in this cohort study is that human liver PPP2R5C mRNA levels negatively correlate with Clamp Glucose Infusion Rate (GIR), which is a mea-

2. Results

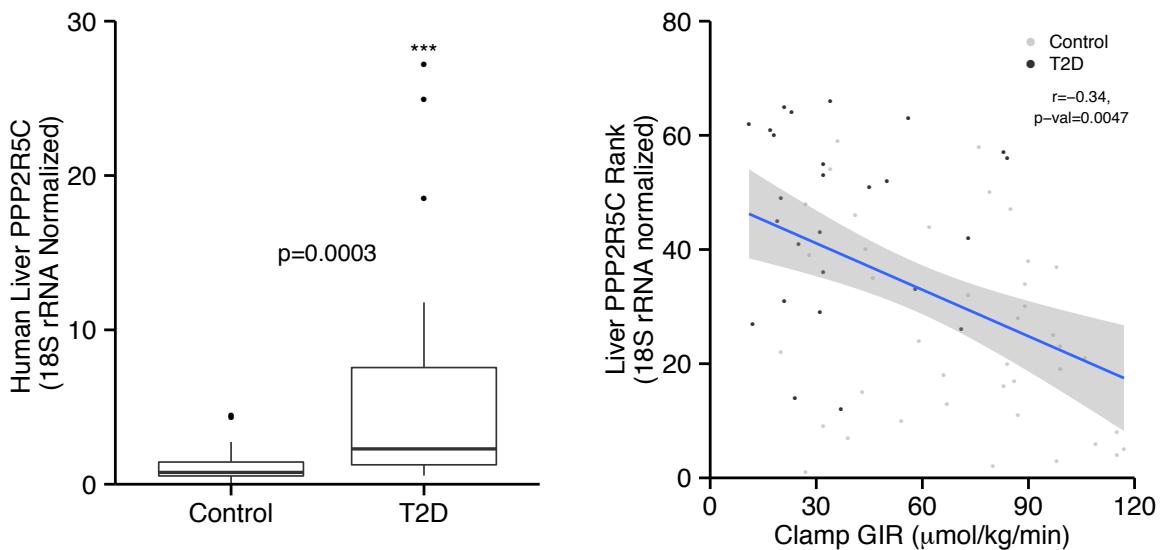


Figure 2.57.: Human liver PPP2R5C mRNA levels in healthy control (Control) and type 2 diabetic patients (T2D). PPP2R5C was normalized to 18S rRNA level in liver, and shown as box-and-whisker plot. *** for p -value <0.001 by t-test in R for comparing Control to T2D. $n=40$ and 26 for Control and T2D respectively. Cohort study and qPCR experiments were performed by Prof. Matthias Blüher, Nora Klöting and Arne Dietrich in University of Leipzig, and analyzed by Yong-Sheng Cheng.

surement for glucose uptake rate in human (Figure 2.57). This piece of data also agrees with the molecular function of PPP2R5C in mouse liver. Knockdown of PPP2R5C in mouse liver increases glucose uptake rate after 6 hour fasting in mice during GTT test, which is demonstrated by increased glucose tolerance after PPP2R5C KD (Figure 2.30). This negative correlation between PPP2R5C and Clamp GIR is independent of disease statuses. Both healthy donors and type 2 diabetic patients have the negative correlation, which is shown in different grey scale in scatter plot of Figure 2.57 and correlation graph for all covariates (Figure 2.59).

Obesity has been shown to be a strong risk factor for type 2 diabetes [122]. The association between obesity and type 2 diabetes is 30% of cases in those of Chinese and Japanese descent, 60-80% of cases in those of European and African descent, and 100% of cases in Pima Indians and Pacific Islanders [123]. Especially, visceral obesity is considered as major culprit in developing insulin resistance [124, 125]. Apart from Clamp GIR, human liver PPP2R5C mRNA levels also vary among different obesity groups. All healthy controls and type 2 diabetic patients can be separated into three groups based on the severity of obesity, from lean, to subcutaneous obesity (SC) or visceral obesity (VIS). Increasing PPP2R5C over severity of obesity also indicates a negative feedback control on PPP2R5C's mRNA level for reduce lipid synthesis and VLDL secretion from

2. Results

liver (Figure 2.58). Due to the lipid buffering function of liver, the secreted lipid in the form of VLDL will promote peripheral organs such as adipose tissue and muscle to deposit more fat content.

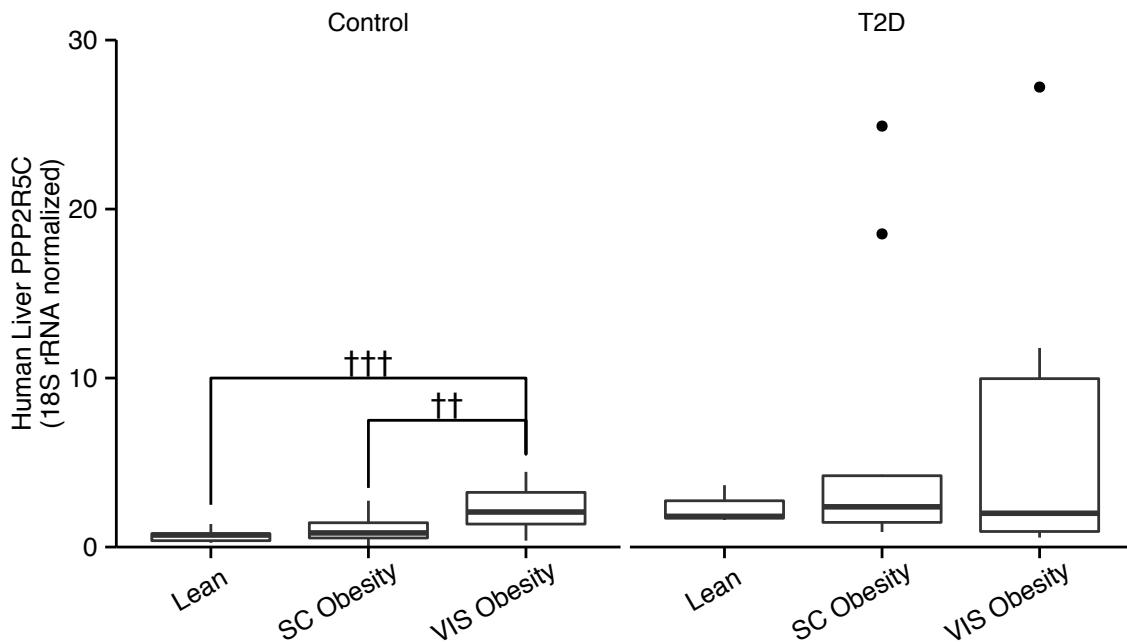


Figure 2.58.: Human liver PPP2R5C mRNA levels correlates with the type and severity of obesity. Healthy or type 2 diabetic person are divided into lean, subcutaneous obese and visceral obese. †† and † † † for p-value<0.01 and 0.001 by ANOVA analysis with comparison between obesity groups. Cohort study and qPCR experiments were performed by Prof. Matthias Blüher, Nora Klöting and Arne Dietrich in University of Leipzig, and analyzed by Yong-Sheng Cheng.

Despite correlation with Clamp GIR, PPP2R5C was also found to be correlated with other 9 covariates in human (Figure 2.59). Among these covariates, the correlation profile could be divided into three groups. The first group includes visceral adipose tissue area, glycated haemoglobin, and serum triglyceride. In this group, the positive correlations are present in all subjects (Total) and healthy sub-group (Control), and the correlations are lost in type 2 diabetic patients. Visceral adipose tissue area is another indicator for obese severity, and linearly correlates with the amount of visceral adipose tissue. Thus the correlation pattern for visceral adipose tissue is similar as the one for obesity group covariate (Figure 2.58). Glycated haemoglobin is measurement of average plasma glucose concentration over prolonged time window, and considered as a better marker for hyperglycemia. It is created by non-enzymatic glycation of haemoglobin while exposed to plasma glucose. This marker could represent 2-3 month average plasma

2. Results

glucose level before the time point of measurement. Since mouse liver PPP2R5C KD can improve insulin sensitivity and then potentially lower blood glucose in hyperglycemia, the positive correlation between human liver PPP2R5C and glycated haemoglobin could be explained by the negative regulation of insulin sensitivity of PPP2R5C in liver. Serum triglyceride has been shown the strongest risk factor for type 2 diabetes. And one of the major contribution for serum triglyceride is secreted VLDL from liver [126]. The positive correlation between liver PPP2R5C and serum triglyceride or glycated haemoglobin only in healthy control indicated liver PPP2R5C could be a negative feedback to control lipid synthesis in liver.

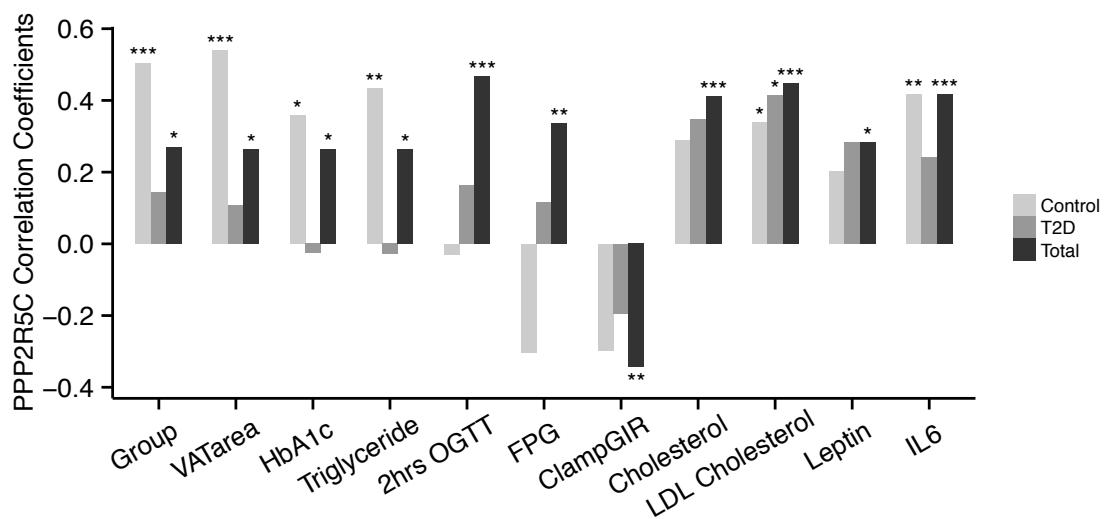


Figure 2.59.: All covariates statistically significantly correlated with human liver PPP2R5C mRNA level with Pearson method for correlation. The p-value cutoff is 0.05 for significance of correlation in all subjects in the cohort. Correlation analysis was also performed individually for healthy control and type 2 diabetic patient. All covariates are obesity group (Group, including lean, sc, vis), visceral adipose tissue area (VATarea, cm²), glycated haemoglobin (HbAc1, %), serum triglyceride (Triglyceride, mg/dL), blood glucose after 2 hour OGTT (2hrs OGTT, mmol/L), fasting plasma glucose (FPG, mmol/L), clamp glucose infusion rate (Clamp GIR, μmol/kg/min), serum cholesterol (Cholesterol, mg/dL), LDL cholesterol (mg/dL), leptin (ng/mL), and IL6 (pmol/L). *, ** and *** for p-value <0.05, 0.01 and 0.001 by correlation test in R by Pearson method. Cohort study and qPCR experiments were performed by Prof. Matthias Blüher, Nora Klöting and Arne Dietrich in University of Leipzig, and analyzed by Yong-Sheng Cheng.

The second group includes blood glucose after 2 hour OGTT and fasting plasma glucose. The positive correlations in this group come from the group difference between healthy control and type 2 diabetes. 2 hour OGTT glucose level above 7.8 mmol/L (140 mg/dL) indicates hyperglycemia [127]. Fasting plasma glucose is normal within the range of 4 to 5.5 mmol/L (70 to 99 mg/dL), while continual fasting levels of 5.5 to 7 mmol/L (101–125 mg/dL) indicates possible pre-diabetes, and FPG above 7 mmol/L (126 mg/dL)

2. Results

indicates high risk of diabetes [127]. The two positive correlations are originated from their higher levels in type 2 diabetic patients, which results a positive correlation (shown in Figure 2.60 FPG vs PPP2R5C scatter plot).

The last group includes serum cholesterol, LDL cholesterol, leptin, and IL6. The positive correlations in this group are shown in all subjects (Total) and all sub-groups (Control and T2D). Serum cholesterol and LDL cholesterol (so called "Bad" cholesterol) are normally higher in type 2 diabetic patients and will increase the risk for cardiovascular disease. Leptin and IL6 have been also shown to related to type 2 diabetes and insulin resistance [128, 129]. Positive correlation between liver PPP2R5C and these four covariates indicate PPP2R5C's role in negatively regulating insulin sensitivity. The individual scatter plot for PPP2R5C vs IL6 are shown in Figure 2.60 as an example for this group of covariates.

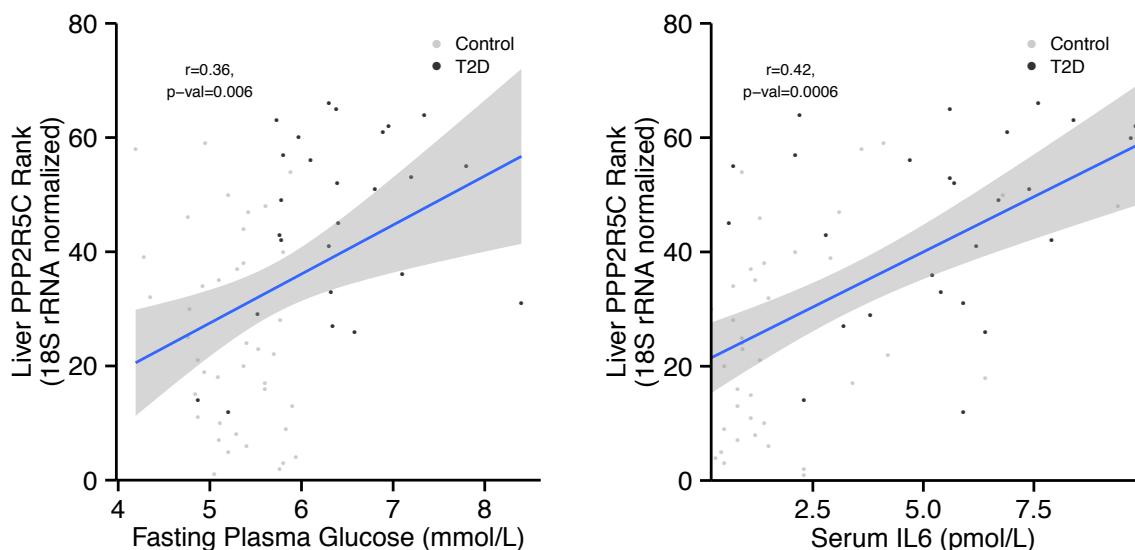


Figure 2.60.: Correlation profile discrepancy for fasting plasma glucose (FPG) and IL6. In FPG, the positive correlation is due to group difference in healthy control and type 2 diabetic patient. For IL6, the correlation is across disease groups. Cohort study and qPCR experiments were performed by Prof. Matthias Blüher, Nora Klöting and Arne Dietrich in University of Leipzig, and analyzed by Yong-Sheng Cheng.

2.6.2. PPP2R5C misregulation in human adipose tissues

With another cohort study in human adipose tissue, PPP2R5C is also found to be upregulated in subcutaneous adipose tissues, but not visceral adipose tissues from type 2 diabetic patients (Figure 2.61, coordinated by Joan J Vendrell in Hospital Universitari de Tarragona Joan XXIII, Spain). This cohort study is suggesting that similar metabolism

2. Results

control from PPP2R5C may also apply to other tissues besides liver. These data fit nicely with the expression data from mice adipose tissues (Figure 2.3), which shown increased mRNA levels of PPP2R5C in adipose tissue of *db/db* mice.

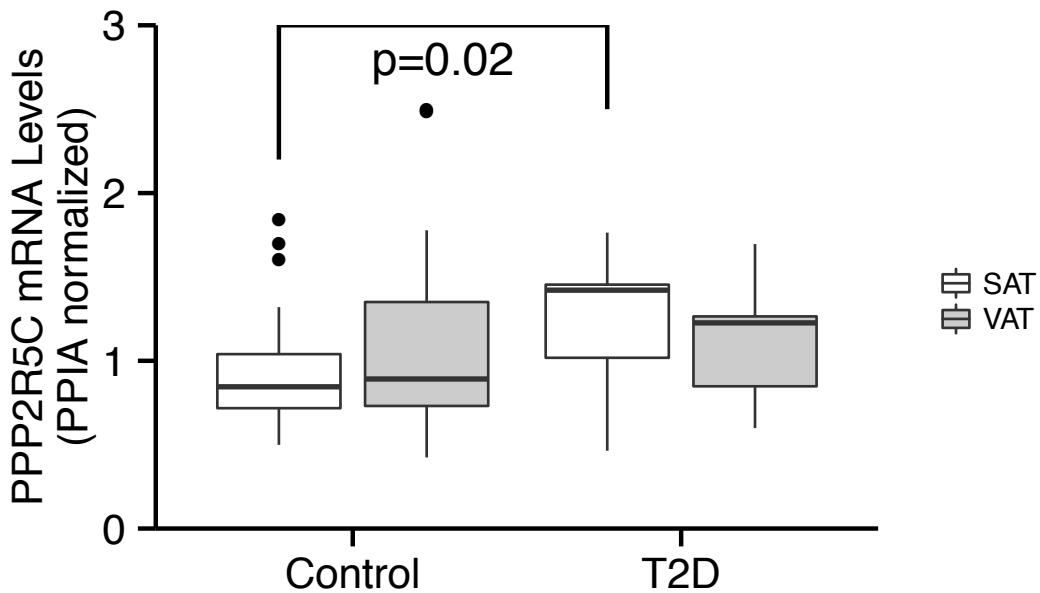


Figure 2.61.: PPP2R5C mRNA levels in human adipose tissue from healthy donors and T2D patients. Adipose PPP2R5C mRNA levels were analyzed by qPCR with cyclophilin 1A (PPIA) as normalization control. Cohort study and qPCR experiments were performed by Sonia Fernandez-Veledo (Hospital Universitari de Tarragona Joan XXIII) and Antonio Zorzano (IRB Barcelona), and analyzed by Yong-Sheng Cheng. t-test for Control and T2D was done in R.

3. Discussions

3.1. PPP2R5C in liver metabolism

In this thesis project, PPP2R5C, a PP2A regulatory subunit, is identified as a metabolism modulator in hepatocytes. Reduced PPP2R5C expression leads to increased glucose uptake and increased *de novo* lipogenesis in *in vitro* cultivated hepatocytes and mouse hepatoma cell line. These phenotypes are reiterated *in vivo* whereby liver-specific knockdown of PPP2R5C generated mice with improved glucose tolerance but elevated circulating VLDL levels (Figure 3.1). Hence PPP2R5C is fine-tuning the balance the liver needs to strike between preventing circulating glucose levels from becoming too elevated in postprandial phase (or high glucose load in GTT), and yet not flooding the circulatory system with lipids. Interestingly, PPP2R5C liver-specific knockdown mice have reduced levels of serum insulin (Figure 2.28) but normal levels of circulating glucose (Figure 2.27). This is probably due to the rheostat for euglycemia, the pancrea, is not affected in PPP2R5C KD mice. Hence, the pancrea is still working normally to maintain proper blood glucose level, reducing its insulin secretion to compensate for the elevated glucose clearance by the liver.

Due to increased VLDL secretion in fasting and refed condition, steady-state triglyceride level in liver shows a decrease in fasting and no further increase in refed, unlike the increased triglyceride during *ad libitum* fed. Given the continuous consumption of triglyceride from circulating VLDL by adipose tissue, muscle and other peripheral tissues during fasting, the overall triglyceride production in liver could still be increased. This hypothesis could be further resolved by *in vivo* tracer study to clarify. During refed, the combined triglyceride from liver and serum are increased upon PPP2R5C KD, and the liver triglyceride was increasing faster than control during re-accumulating triglyceride after fasting (Figure 3.1). These evidence demonstrated the general function of PPP2R5C in inhibiting lipogenesis during all feeding regimes (at least for *ad libitum* fed and refed). Another consequence after increased VLDL secretion is that liver cholesterol levels drops significantly during fasting and refed (Figure 2.47). The decreased

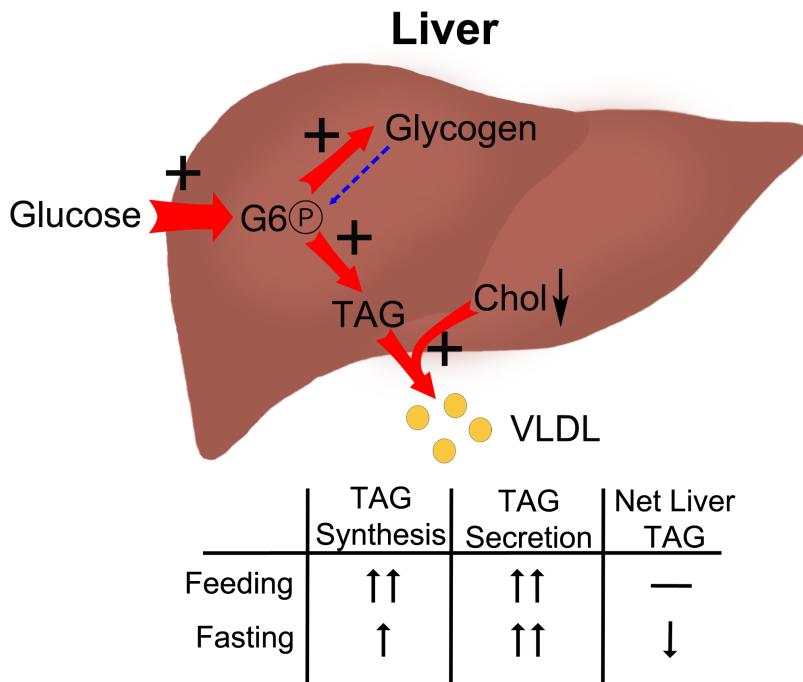


Figure 3.1.: Whole organismal control model for PPP2R5C in glucose and lipid homeostasis in liver. Upon PPP2R5C knockdown in liver, glucose uptake is increased in postprandial phase or high glucose load during GTT. Glucose in liver is quickly converted to glucose-6-phosphate in order to stay in liver. The absorbed glucose is further stored either as glycogen or triglyceride. Increased triglyceride storage triggers VLDL secretion and results in passively lowering liver cholesterol. During fasting, the higher rate of VLDL secretion could cause the decreased liver triglyceride, while increased triglyceride synthesis rate during refeeding balances triglyceride production and secretion from liver and results in no net change in liver steady-state triglyceride level.

level of cholesterol could also contribute the SREBP-1 activation in liver (Figures 2.54 and 2.56), since cholesterol is endogenous inhibitor for SREBP-1 activation [119].

The phenotypes reported here were obtained by targeting PPP2R5C with multiple different miRNA or shRNA sequences (See Table A.6). For instance, the increased glucose uptake was observed in cell culture using two independent shRNAs (Figure 2.15) and a third independent target sequence *in vivo* (Figure 2.30). This excludes the possibility that the phenotypes could arise from possible off-target effects.

3.2. PPP2R5C substrates



Although the phenotypes of PPP2R5C knockdown are quite specific both in cell culture and *in vivo* mouse experiment, they likely result from an effect of PPP2R5C on multiple downstream targets. Hence it will likely be difficult or impossible to identify a single downstream target as the main one mediating the effects of PPP2R5C. Here 4 protein complexes were identified as PPP2R5C interactors including AMPK, HIF1 α , STAT3 and S6K. I tested whether phosphorylation of these proteins increases upon PPP2R5C knockdown, as would be expected of a PPP2R5C target. For S6K, phosphorylation on Thr389 was checked by using phospho-specific antibody, and it did not elevate upon PPP2R5C knockdown. For this reason, S6K branch was not pursued further, although this does not exclude that S6K phosphorylation on another site could be regulated by PPP2R5C.

For STAT3, phospho-specific antibody for S727 of STAT3 was used to check phosphorylation change upon PPP2R5C KD, and there was no change in two independent inducible shRNA mediated KD (data not shown). Again, Phos-tag® gel was used to test its motility shift due to phosphorylation in an approach similar to what I did for HIF1 α (Figure 2.52). Although there was no obvious changes in STAT3's motility, an important caveat from my experience is that Phos-tag® gels only resolve phosphorylations on roughly half of the proteins I have tested and know to be phosphorylated. For these reasons, AMPK and HIF1 α were focused for deciphering PPP2R5C's role in metabolism control.

For AMPK and HIF1 α , both phosphorylation and activity of them were elevated upon PPP2R5C knockdown. Both AMPK and HIF1 α are known to increase glycolytic flux in response to stress conditions, either reduced energy supply or impaired mitochondrial function [130, 131]. Therefore the two likely act in concert to drive glucose uptake and glycolysis upon PPP2R5C knockdown (Figure 3.2). For PPP2R5C's control in glucose uptake and glycolysis branch, at least in Hepa 1-6 and primary hepatocytes, AMPK activity and its downstream effector TBC1D1 in glucose uptake are shown to be increased.

Indeed, the functional role of AMPK has also been carefully studied *in vivo* in mouse liver, with increased liver AMPK activity leading to decreased blood glucose and fatty liver [136] and reduced liver AMPK activity leading to glucose intolerance [137], in agreement with potential increased AMPK activity upon PPP2R5C KD. The functional relevance of HIF1 α is less clear, given that the mice I was studying were housed under

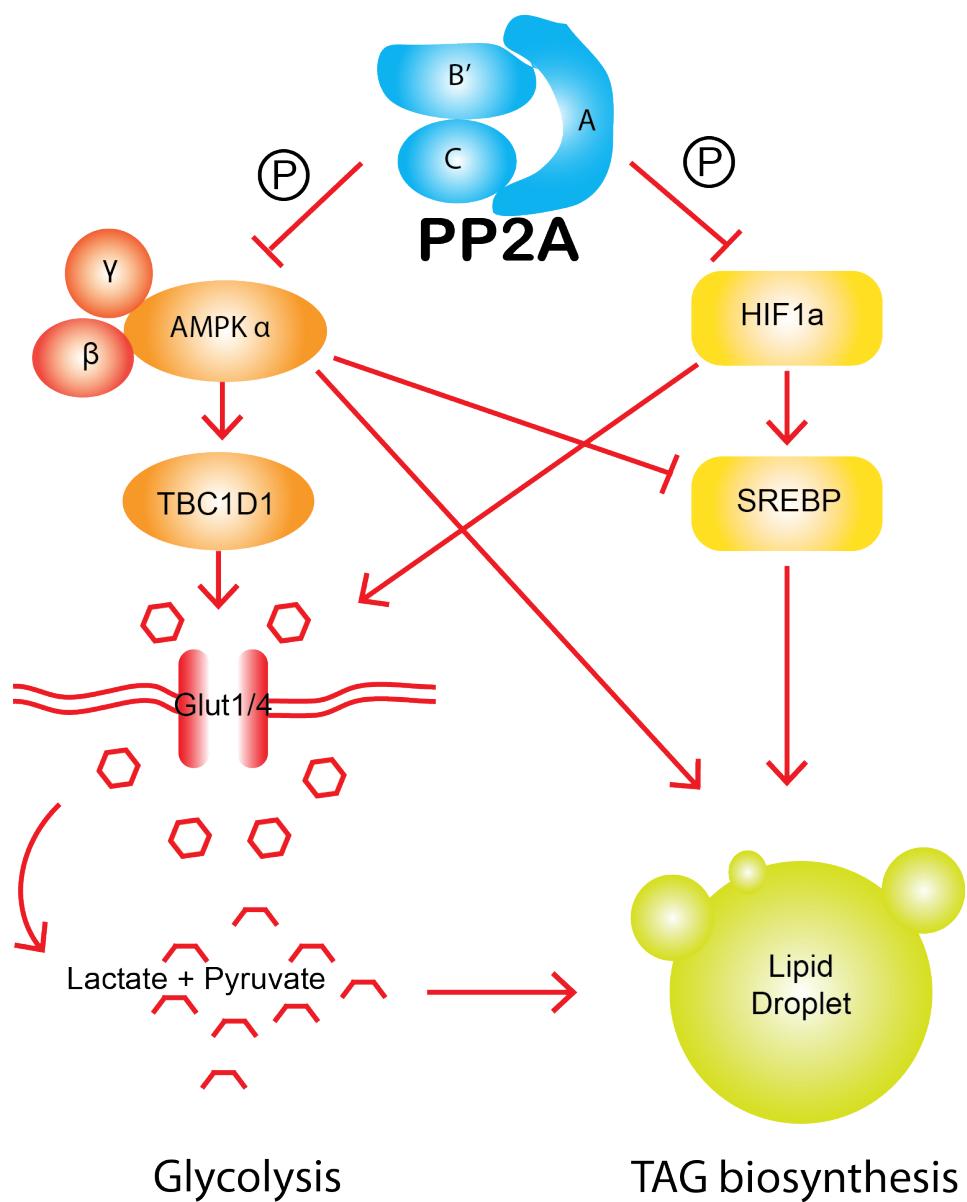


Figure 3.2.: Signaling model of PPP2R5C in glycolysis and lipogenesis in cell. PPP2R5C containing PP2A complex inhibits AMPK and HIF1 α activity via de-phosphorylation. Although AMPK has been shown to be negative regulator for SREBP-1 [132], SREBP-1 could still be activated by upstream HIF1 α activation [111] and potentially lower liver cholesterol in PPP2R5C knockdown at long term. Activated AMPK [107, 133, 106] and HIF1 α [108, 131] could both contribute to increased glucose uptake and glycolysis in a cell-autonomous way. Even increased glycolysis itself could also contribute to *de novo* lipogenesis [134, 135].

normoxia. To my knowledge, whether HIF1 α plays a role in mouse liver metabolism in such circumstances has not been studied. Mice with liver-specific ablation of HIF1 β develop diabetic phenotypes [112], however HIF1 β binds also other partners besides HIF1 α . In cell culture experiments with Hepa 1-6 or primary hepatocytes, also conducted under normoxia, HIF1 α is indeed functionally relevant because I can detect HIF1 α protein by

3. Discussions

western blot (Figures 2.50 and 2.52) and I see induction of HIF1 α target genes upon PPP2R5C knockdown (Figure 2.53). HIF1 α is known to be frequently up-regulated and functionally relevant in cancers, therefore HIF1 α is likely to be a relevant downstream PPP2R5C target in this pathological context. Finally, SREBP-1 activation was observed in PPP2R5C KD livers, which likely accounts for their increased lipogenesis. SREBP-1 can be activated downstream of HIF1 α but additional mechanisms are likely to link PPP2R5C to SREBP-1 activation [138].

3.3. PPP2R5C's metabolic control in cancer cells

PPP2R5C has been linked to cancer development. One mechanism appears to be via dephosphorylation of p53 on Thr55 [69, 70, 139]. The results described here suggest that upon PPP2R5C knockdown, cells also increase their glucose uptake, glycolytic rate, and lipid biosynthesis—all of which are metabolic hallmarks for cancer cells. This also fits with the tumor suppressor function of PPP2R5C, which is inhibiting the cell proliferation in cancer cells through reprogramming metabolism toward anabolic growth. Therefore, it will be interesting to study in the future whether these metabolic effects might be contributing towards the tumor suppressive properties of PPP2R5C. Additionally, with recent advances in genome editing technology, it is now possible to specifically activate PPP2R5C expression with the help from CRISPR/Cas9 mediated genome-loci specific targeting and gene activation [140, 141]. Local activation of PPP2R5C in tumor cells would be a new perspective in drug development for cancer, turning PP2A a tumor suppressor as new druggable target.

3.4. PPP2R5C in human metabolic diseases

From PPPR5C knockdown experiments, it has been clearly shown that PPP2R5C expression in liver inhibits glucose uptake and reduces insulin sensitivity. Astoundingly, PPP2R5C expression level in human liver also correlates with insulin resistance. Type-2 diabetic patients have significantly elevated PPP2R5C liver expression levels compared to controls (Figure 2.57). In fact, even within the control population PPP2R5C expression correlates with reduced insulin sensitivity (Figure 2.57), raising the hypothesis for future studies that increased PPP2R5C expression might play a causative role in insulin resistance.

3. Discussions

In type 1 or 2 diabetes, postprandial hyperglycemia and impaired hepatic glycogen storage are the two most prominent characteristics [14]. Targeting postprandial glucose (PPG) level has also become a major interest in drug development for diabetes [142]. In fact, 2-hour GTT is still considered to be the gold standard for diagnosis of diabetes. And PPG has also been shown as frequently the earliest abnormality of type 2 diabetes, and an independent risk for cardiovascular diseases [143]. In this thesis project, liver specific knockdown of PPP2R5C has been shown to induce profound metabolic changes, including increased insulin sensitivity without affect fasting blood glucose level, increased glucose uptake and consequently increased glycogen and triglyceride storage in liver. PPP2R5C liver-specific knockdown has provided a new drug target for controlling PPG via multiple possible pathways.

Either via liver-specific siRNA mediated knockdown or interrupting interactions between PPP2R5C and PP2A holoenzyme (A and C subunits), PPP2R5C's substrates can be specifically modulated in term of increasing their phosphorylation levels. Liver specific siRNA delivery has been quite successful in recent studies [144, 145]. In future, with more efficient and specific siRNA delivery system development, it is reasonable to test the clinical possibility of liver specific knockdown of PPP2R5C to improve postprandial hyperglycemia in diabetic patients. With recent development in phosphatase-based drug development [31], it is also plausible to develop activators or inhibitors for PP2A. Inhibitors, which could disrupt PPP2R5C's interaction with PP2A holoenzyme (A and C subunits) or specific substrates like AMPK or HIF1 α , can be employed to target liver and mimic the liver specific knockdown effect on metabolic benefits for diabetes.

4. Methodology

4.1. Molecular Biology

4.1.1. DNA digestion by restriction enzymes

All plasmids and PCR products were digested by restriction enzymes from Fermentas (Now part of Thermo Fisher Scientific). DNA was digested with restriction enzymes in 1×FastDigest® buffer for 2 hrs in 37°C.

4.1.2. Polymerase chain reaction(PCR)

For Cloning PCR, Taq polymerase from New England Biolabs was used and condition was as following.

Recipes:

Components	2.5×Master Mix(μL)
H ₂ O	42
Forward Primer (100μM)	0.2
Reverse Primer (100μM)	0.2
dNTP (10mM)	2
10×Standard Taq buffer	5
Taq DNA polymerase	1

For each reaction, bacterial clone was touched by pipette tip and washed in 10 μL sterile 1×PBS and then 1 μL was add into 20 μL Master Mix. Then the PCR was run with following condition.

PCR running condition:

4. Methodology

Step	Temperature	Time	Cycles
Denaturing	95°C	3'	1
Denaturing	95°C	30"	
Annealing	60°C	30"	30
Elongation	72°C	30"	
Final	72°C	5'	1

For cloning Genomic DNA fragment from mouse liver gDNA, Expand® Long Range DNA polymerase from Roche was used and condition was following:

Recipes:

Components	1× Master Mix(μL)
H ₂ O	fill up to 50 μL
Template (gDNA,10-500ng/μL)	0.5
Forward Primer (100μM)	0.3
Reverse Primer (100μM)	0.3
dNTP (10mM)	2.5
5× Expand long rang buffer	10
DMSO	0,1,2 or 3
Expand long range enzyme mix	0.7

PCR running condition:

Step	Temperature	Time	Cycles
Denaturing	92°C	2'	1
Denaturing	92°C	10"	
Annealing	65°C	15"	10
Elongation	68°C	60"/kb	
Denaturing	92°C	10"	
Annealing	65°C	15"	25
Elongation	68°C	60"/kb + 20"/cycle	
Final	68°C	7'	1

For cloning cDNA from mouse, Hotstart® polymerase from Qiagen was used and condition as following:

Recipes:

4. Methodology

Components	1×Master Mix(μL)
H ₂ O	fill up to 50 μL
Template (cDNA)	0.5
Forward Primer (100μM)	1
Reverse Primer (100μM)	1
dNTP (10mM)	2
10× Hotstart® Taq buffer	5
Hotstart® Taq DNA polymerase	0.5

PCR running condition:

Step	Temperature	Time	Cycles
Denaturing	95°C	14'	1
Denaturing	92°C	30''	
Annealing	60°C	30''	35
Elongation	72°C	60''/kb	
Final	72°C	5'	1

4.1.3. Agarose gel electrophoresis

Agarose gel electrophoresis was employed to separate and purify DNA products from PCR, plasmid digestion, etc.. Normally, 0.7–1.5% ultrapure agarose gel was prepared in 1×TAE by microwave mediated boiling. For separating ds/ss oligos, 4% agarose gel was prepared with occasional shaking and mixing during boiling to help dissolve in TAE. DNA samples were mixed with 1/10 volume of 10×GelRed dye stock solution. Running condition was 95–125V (constant voltage) depend on the size of electrophoresis chamber used. Running time was 45 min as standard, and varied depending on separation resolution needed (ie. separating size difference of 100-200 bp need more running time).

4.1.4. Agarose gel purification

The cut agarose gel was submitted to purification using NucleoSpin® Gel and PCR Clean-up kit from Macherey-Nagel. The purification procedure followed the manufacturer's instructions.

4. Methodology

4.1.5. DNA ligation

DNA ligation was done by mixing insert and linearized vector in 3:1 ratio (molarity ratio, 10:1 for blunt end) with following recipes.

Recipes:

Components	10 μ L reaction
H ₂ O	fill up to 10 μ L
Insert	varies for insert size
Vector	1
10× T4 DNA ligase buffer(NEB)	1
T4 DNA ligase(NEB)	0.5

Ligation was usually done within 20 min at room temperature. For ligation between dsDNA oligo and vector, ligation could also done at 16 or 4°C overnight for better ligation efficiency.

4.1.6. Transformation of *E. coli*

XL1 Blue competent cell was used for most gDNA, cDNA and oligo cloning. For cloning miRNA or shRNA, SURE 2 competent cell from Agilent Technologies and TOP10 from Life Technologies were used respectively. 1–4 μ L ligation product was used to transformation and mixed with 50–100 μ L competent cell suspension, then incubated on ice for 45 minute. Heat shock was done at 42°C for 30–45 seconds. Following incubation with 1 ml more LB medium at 37°C was only needed for kanamycin resistant plasmid and occasionally for ampicillin resistance plasmid if transformation efficiency is very low.

4.1.7. Bacteria cultivation and plasmid purification

Transformed bacteria with plasmid was incubated at 37°C and shake at 230 rpm overnight in 2–5 ml LB medium or other special medium (NZY+ medium for SURE 2). Plasmid was isolated and purified with NucleoSpin® Plasmid from Macherey-Nagel. All procedures were followed according to the manufacture's standard protocols. All purified plasmids were stored at -20°C. If sequencing is needed, All plasmids were send to GATC Biotech for sequencing.

4. Methodology

4.1.8. Cloning and selection of shRNA or miRNA candidates

shRNAs or miRNAs against common region in all PPP2R5C transcripts were designed on Invitrogen's BLOCK-iTTM RNAi designer website (<http://rnaidesigner.lifetechnologies.com/rnaiexpress/>). 3 independent shRNA and 14 miRNA were selected based on their top positions on rank list. Oligos for these shRNAs and miRNAs were synthesized from Sigma and cloned into Invitrogen's BLOCK-iTTM adenovirus and adeno-associated virus system especially according to manufacturer's instruction. Knockdown efficiency was evaluated by co-expression of miRNA/shRNA with HA-tagged Variant 2 of PPP2R5C and checked by western blot.

4.1.9. Cloning miR30-based shRNA for inducible piggyBac shRNA system

miR30-based shRNA was designed either using shRNA3 sequence designed at Invitrogen's RNAi website or new sequences predicted from Gregory Hannon's laboratory website for shRNA design (http://cancan.cshl.edu/RNAi_central/RNAi.cgi?type=shRNA). 3 independent shRNAs were synthesis and cloned into piggyBac transposase system with inducible shRNA expression (System Biosciences, PBQMSH812A-1).

4.2. Gene expression analysis

4.2.1. Tissue pulverization

Frozen tissue was transferred into liquid nitrogen pre-cooled adapter sets with steel beads. The tissue was pulverized by TissueLyser IITM(Qiagen) for 1 min and at a frequency of 30 Hz (repeat if tissue was not homogeneously powder). Transfer pulverized powder into original tubes for these tissue samples.

4.2.2. RNA isolation from tissue sample

~50 mg of frozen tissue were weighted and transferred into a 2 ml RNase/DNase/Protease-free reaction tube containing 1 ml of QiazolTM Lysis reagent and a stainless steel bead. The samples were homogenized using the TissueLyser IITM (Qiagen) for 1 min and at a

4. Methodology

frequency of 30 Hz. Lysate was transferred into a new 1.5 ml RNase/DNase free tube, and 200 μ L chloroform was added into each tube. The mixture was further vortexed for 15 seconds at room temperature and then incubated under the hood for 15 minutes. To separate the RNA containing water phase, sample was centrifuged at 14, 000 rpm for 15 minutes at 4°C. Then 400 μ L upper water phase was taken out and mixed with equal volume of isopropanol in a new tube, then another 14, 000 rpm 15 minute centrifugation at 4°C was applied to separate the RNA pellet. After washing with 70% ethanol twice, RNA pellet was dried at room temperature till no visible solution, and then re-solubilized in 50 μ L water. To increase solubility, the RNA solution was incubated at 60°C for 10 min. The samples were stored at -80°C until further use.

4.2.3. RNA isolation from cell sample

1 ml Trizol™ Lysis reagent was directly applied onto cell in 6-well plate after removal of medium. Plate was then shaken on head-to-tail rotator for 2 min to allow complete lysis of cell till no visible debris left. Then the lysate was transferred into 1.5 ml tube and 200 μ L chloroform was added and mixed by vortexing for 15 seconds. RNA containing water phase was separated by centrifugation at 14, 000 rpm for 15 minutes at 4°C. Then 400 μ L upper water phase was taken out and mixed with equal volume of isopropanol in a new tube, then another 14, 000 rpm 15 minute centrifugation at 4°C was applied to separate the RNA pellet. After washing with 70% ethanol twice, RNA pellet was dried at room temperature till no visible solution, and then re-solubilized in 50 μ L water. To increase solubility, the RNA solution was incubated at 60°C for 10 min. The samples were stored at -80°C until further use.

4.2.4. cDNA synthesis

2–5 μ L (depend on concentration, make sure about 2 μ g total RNA) RNA sample was used to synthesize cDNA from it. At first, RNA mix was prepared as following and heated at 65°C for 5 min:

Components	14.5 μ L in total
H ₂ O	10.5
Oligo dT ₂₀ (50 μ M)	1
dNTP mix(10mM)	1
RNA sample	2

4. Methodology

Then add 5.5 μ L RT mix from following recipes, and mix well.

Components	5.5 μ L in total
5 \times RT buffer	4
Ribolock	0.5
Reverse Transcriptase(RevertAid)	1

Then sample was put on PCR machine from Bio-Rad (DNAEngine) at 50°C for 50 minutes and then inactivate enzyme in reaction by heated sample up to 85°C for 5 minutes. After these steps, cDNA sample was stored at -20°C.

4.2.5. Quantitative PCR (qPCR) analysis

cDNA sample prepared as above was diluted in 1:60 in RNase/DNase/Protease-free water. Then 4 μ L of diluted cDNA as template for qPCR analysis. Working master mix for qPCR was made from 5 μ L 2 \times Maxima SYBR Green/ROX qPCR Master Mix (Fermentas), and 1 μ L oligo mix (2.5 μ M each primer). The PCR reaction mix was transferred to a MicroAmp™ Optical 96-well reaction plate (Applied Biosystems). All reactions were performed in technical duplicates. Quantitative PCR was performed using a StepOne Real Time PCR System (Applied Biosystems, now part of Thermo Fisher Scientific). Gene expression levels were calculated by ΔC_t method.

4.2.6. Microarray analysis of mouse tissue and cell sample

Expression profile analysis by microarray was done for RNA sample from Hepatoma cell line Hepa 1-6, primary mouse hepatocytes and mouse liver tissues with infection by adenovirus packaged with control shRNA or shRNA targeting common region of all mouse PPP2R5C splicing isoforms at MOI (Multiplicity of Infection) of 100. RNA was isolated as protocol in Section 4.2.3 and send to DKFZ's in-house Genomics & Proteomics Core Facility for microarray analysis. RNA sample was analyzed on Bioanalyzer (Agilent Technologies) for quantity control and then submitted for cDNA synthesis and microarray analysis using MouseWG-6 v2.0 Expression BeadChip Kit from Illumina Inc. The raw data from core facility was send back and further processed and analysed on DKFZ's in-house Chipster server [146]. Alternatively, raw data was processed in R and analyzed with limma package in R.

4.3. Cell biology

4.3.1. Cell culture for Hepa 1-6, HEK293T, HEK293A, Hela Cells

All cell lines were maintained and propagated in Dulbecco's Modified Eagle Medium with 4.5 g/L glucose (DMEM), 10% fetal calf serum (FCS) and 1×penicillin/streptomycin (100 IU and 100 µg/mL). HEK293A and HEK293T cells also required 1×Non-Essential Amino Acids (NEAA). Cell was split in 1:10 twice per week. Experiments involving eukaryotic cells were performed under sterile conditions. Media and reagents were pre-heated to 37°C prior to use. All cells were cultivated at 37°C, 5% CO₂ and 95% humidity in 96-well, 24-well, 12-well, 6-well, 10 cm or 15 cm cell culture dishes.

4.3.2. Transfection assay

For transfection plasmid into cells, Effectene® Tranfection Reagent from Qiagen was used according to standard protocols provided in kit's instruction. Medium with transfection reagent was exchanged with fresh medium after overnight incubation. Additional 1-2 day was needed for proper expression of exogenous genes.

4.3.3. Mouse primary hepatocyte cultivation

Mouse primary hepatocytes were isolated and *in vitro* cultivated as standard procedure in Prof. Herzig's lab [147]. Male 8–12 week old C57Bl/6J mice were housed for 1 week and then anesthetized by intra-peritoneal injection of 5 mg 10% ketamine hydrochloride/100 mg body weight and 1 mg 2% xylazine hydrochloride/100 mg body weight. When there was no response from pressing mouse foot, it was then allowed to open the abdominal cavity. The liver was then perfused with HANKS I buffer via the portal vein for 5 min at 37°C and subsequently with HANKS II buffer for 5–7 min until complete disruption of the liver structure is visible (color change from red to pale). Then the liver was cut out and the liver capsule was removed and washed gently in adhesion buffer (recipe in Table A.4) until no visible cell was left attached onto the capsule. Then the liver cell suspension was filtered through a 100 µm mesh fitted into 50 mL Falcon tube (BD Biosciences). Hepatocytes were washed twice and gently collected by centrifugation at 37.5 g at room temperature. Cell suspension from one mice was equally distributed in collagen I-coated 6-well plates (roughly 1 million cell for complete coverage) without

4. Methodology

checking the cell viability by trypan blue staining. Hepatocytes were infected with recombinant adenoviruses (MOI = 10, 100 or 200) 4 hours after seeding and harvested for gene expression analysis or submitted for Triglyceride, free fatty acid, glucose, and lactate measurement after 48 or 72 hours later.

4.3.4. PP2A substrate trapping in Hepa 1-6

Protein-protein interaction mapping by biotinylation [100] was adapted to discover new substrate of PP2A holoenzyme with PPP2R5C as regulatory B' subunit. In order to stabilize interaction between substrates and PP2A, a phosphatase dead mutant of C catalytic subunit of PP2A was also co-expressed together with promiscuous biotin ligase tagged PPP2R5C Variant 1. The mechanics behind this method design is called substrate trapping. It has been successfully used to find several protein phosphatases' substrates [98, 97, 96]. 2 μ g of each plasmid was transfected into Hepa 1-6 cell in 6-well plate with 50 μ M biotin in medium. After 24 hrs of expression, cell was washed twice in PBS and lysed in BioID lysis buffer 1. Then equal volume of BioID lysis buffer 2 was added and mixed. Clarified supernatant was collected and incubated with 100 μ L Dyna-Beads (MyOne Streptavidin C1 from Life Technologies) overnight. On the second day, beads were collected and washed twice with BioID wash buffer 1 on a magnetic separator (DynaMagTM-Spin Magnet). The washing was repeated once with BioID wash buffer 2, once with BioID wash buffer 3 and twice with BioID wash buffer 4 (all buffers used in substrate trapping are listed in Table A.4). Finally, protein was eluted from beads by BioID elution buffer. Protein sample was either submitted for western blot cross validation or mass spectrometry identification, which is performed at DKFZ's in-house proteomics core facility.

4.3.5. Luciferase assay

All promoter reporters used in this project were cloned into pGL3 promoter from Promega. Transfection of luciferase reporters into hepa 1-6 cell was titrated and optimized for 96-well or 24-well plate. Cell was lysed in either 50 μ L or 200 μ L passive lysis buffer from Promega's Duo-luciferase reporter assay system, and renilla luciferase was used as control.

4. Methodology

4.3.6. Inducible shRNA stable cell line generation

Hepa 1-6 cell was transfected with piggBac transposase expression plasmid and shRNA containing plasmid using Effectene® transfection reagents from Qiagen. After 6 hour post-transfection, cell was selected under 3 μg /mL puromycin until clones were formed under microscope check. shRNA integrated cell was either submitted for continuous selection for two week or picked as single clone for continuous selection for additional two week. Generated stable cell line was submitted for 1 week puromycin selection every month during culture.

4.3.7. FACS analysis of 2NBDG uptake

Stable cell line with inducible shRNA or empty hepa 1-6 cell was cultivated and induced for 3–4 days and then starved in serum-free DMEM overnight. Then these cells were sensitized in KRPH buffer (20 mM HEPES, 5 mM KH₂PO₄, 1 mM MgSO₄, 1 mM CaCl₂, 136 mM NaCl, 4.7 mM KCl, adjust pH to 7.4 (from pH 5.1 to 7.4)) for 1 hour and then mixed with 2NBDG up to 100 μM , 20 min to allow glucose analog uptake. Uptake was stopped by washing with PBS for 3 times and then digested in 0.25% trypsin for 3 min. Digestion was stopped by adding equal volume of FBS (fetal bovine serum from PAA). All the cells were suspended and washed in PBS with 2% FBS for 3 times before FACS measurement. 2NBDG intensity was recorded in the same channel for GFP on BD's FACSCanto™ II. FACS data was analysed either in FlowJo or R.

4.4. Virus production for mouse *in vivo* knock-down

4.4.1. shRNA packaging Adenovirus construction and production

4.4.1.1. Adenovirus with shRNA-NC/3 construction

The BLOCKiT™ Adenoviral RNAi System from Life Technologies was employed to clone and package control shRNA or shRNA3 (targeting common region of all splicing isoforms of *PPP2R5C*) from ds oligos. Oligonucleotide sequences were designed using Invitrogen's online RNAi Design server [78]. Two complementary ssDNA oligos against the target gene sequence were ordered from Sigma, and re-suspended as 200 μM in water. Then oligo mixes with 1×annealing buffer was denatured at 98°C and annealed

4. Methodology

from 98°C to 90°C, then hold for 5 minutes, and annealed again from 90°C to room temperature. The annealed ds oligo products was checked by 4% agarose gel and cloned into the pENTR™/U6 vector according to the manufacturers instructions. The sequence verified constructs were recombined with the pAd/BLOCK-iT™ DEST vector, which contains the adenovirus serotype 5 DNA but not the E1 and E3 genes that are required for viral replication. The viral vector containing the shRNA sequence was linearized by restriction digest using the enzyme PacI and transfected into HEK239A cells using Lipofectamine 2000 reagent according to the manufacturers instructions. HEK239A cells express the viral E1 and E3 genes necessary for viral lysis, which allows the virus to be propagated in culture medium. Viral plaques become visible from 6 to 10 days after transfection and cell monolayer started to form plaques. When ~70% of cells were round and detaching, it was time to harvest them.

4.4.1.2. Adenovirus harvesting

HEK239A cells containing adenovirus were harvested from the medium after complete detachment of all round infected cells. The medium was collected from up to 20×15 cm culture dishes and centrifuged at 2, 000 rpm, 4°C for 10 min. The supernatant was then discarded and the pellet was resuspended in 4 mL PBS-TOSH buffer inside 15 mL Falcon tube. The cell pellets in Falcon tube were frozen in liquid nitrogen and subsequently thawed in room temperature on vortex for 3 times for maximal cell lysis and adenovirus releasing. After 3 times lysis the cell suspension was centrifuged at 2, 000 rpm, 4°C for 10 minutes. The clarified supernatant was then stored at -80°C or directly submitted for a CsCl gradient purification.

4.4.1.3. Adenovirus purification by CsCl gradient

Virus lysates from Section 4.4.1.2 was filled with PBS-TOSH upto 20 mL final volume. CsCl gradients were prepared in ultracentrifuge tubes (Beckmann Polyallomer 25mm×89 mm) and were weight-balanced after addition of each solution. At first, ~9 ml 4 M CsCl was added, then ~9 ml of 2.2 M CsCl was added and finally the viral lysate was carefully added on top in one liquid droplet by one fashion. In the end, there should be 3 different visible gradient layers. These gradients were centrifuged at 24, 000 rpm, 4°C in ultracentrifuge XL-70 (Beckmann) with a SW28 swing bucket rotor for 2 hrs. After ultracentrifugation a visible white band representing the conentrated adenovirus fraction was formed between the 4 M and 2.2 M CsCl gradients. The band was collected

4. Methodology

by inserting into the tube with 25G needle connected with 5 ml syringe. The collected virus (~3 ml) fraction was then diluted with equal volume of saturated CsCl and changed into a 12 mL ultracentrifuge tube (Beckmann Polyallomer 14mm×89 mm). ~2 ml of 4 M CsCl and 2.2 M CsCl were utilized again to form gradients. Then a centrifugation at 35, 000 rpm, 4°C was applied in a SW41 Ti swing bucket rotor for 3 hrs. The viral fraction was visible again as a white band between the 4 M and 2.2 M CsCl gradients. The fraction (~700 μ L) was collected using needle and 1 ml syringe. Finally, the viral fractions were dialyzed (Spectra/Por® Biotech, MWCO 15,000, 10 mm diameter) in 1 L 1×PBS with 10% glycerol (v/v) for 2 times (1 and 24 hrs each) at 4°C. After dialysis, 50–200 μ L adenovirus in PBS were aliquoted and stored at -80°C before use.

4.4.1.4. Adenovirus titration

Adenovirus titer was determined by the Tissue Culture Infectious Dose 50 (TCID50) assay. For titer measurement, 10^4 HEK293A cells/well were cultivated in 100 μ L DMEM medium with 2% FCS (v/v), 1timesP/S and 1% NEAA in 96-well plate. In order to have more accurate TCID50 calculation, technical duplicates of 96-well plate were required for titer measurement. Cells were completely attached to plate after 4 hours seeding. During cell attachment, serial dilutions of the adenoviruses (1.65 ml for each dilution, from 10^{-6} – 10^{-13}) were prepared in the same medium as that for cultivation. 100 μ L of each virus dilution was added to ten wells and 100 μ L of medium without virus was added to the rest two well in the same row in 96-well plate as negative control wells. The infected cells were cultivated for 10 days for continuous monitoring plaque formation everyday. At day 10, the number of wells with at least one plaque was investigated under microscope for each dilution, and the titer was calculated with following formula:

$$Ta = \text{viruses per } 100 \mu\text{L} = 10^{1+(S-0.5)}$$

S = the sum of all positive wells starting from the 10^{-1} dilution, whereby 10 positive wells correspond to the value 1.

$$T = \text{viruses per } 1 \text{ ml} = 10 \times Ta$$

4. Methodology

4.4.2. miRNA packaging Adeno-Associated Virus construction and production

4.4.2.1. miRNA containing AAV construction

PPP2R5C-specific or non-targeting scramble control miRNA was cloned into Invitrogen's adeno-associated virus system for long-term knockdown of *PPP2R5C* *in vivo*. The Oligonucleotide sequences were designed using Invitrogen's online RNAi Design server [78], and listed in Table A.4. The oligos were then synthesized from Sigma, then annealed and cloned into the pcDNA6.2-GW/EmGFP-miR vector. Later these oligos were sub-cloned into pdsAAV-LP1-EGFPmut AAV vector [90] between the BglII and SalI sites. The pdsAAV plasmids with miRNAs were co-transfected into HEK293T cells for AAV production, together with the pDG Δ VP helper plasmid [148] and a mutated p5E18-VD2/8 expression vector [149] encoding AAV2 rep and a mutated AAV8 cap protein. For virus production, cells from 6 \times 15 cm culture dish with 90% confluence were scrapped and resuspended in 1100 ml DMEM medium (with 10% FCS, 1*times*P/S). 1000 ml of the cell suspension was transferred to a 10 \times cell-stack chamber for first round virus production. And the left 100 ml was transferred to a 1 \times cell stack chamber as cell source for second round virus production in 10 \times cell-stack (could also be used as control chamber for checking cell density). 2 days after plating, the cells in 10 \times cell-stack chamber were reaching 90% confluent and co-transfected with the plasmids encoding the viral genes using the PEI method in the amounts described below.

Plasmid	amount in μ g
AAV-miRNA expression vector	395
p5E18 VD2/8 helper plasmid	497
pDG Δ VP helper plasmid	1353

After 1 or 2 day transfection, cells were ready for harvesting and washed with 1 \times PBS once. Then 10 or 100 mL trypsin was added to 1 \times or 10 \times cell-stack for 5 minute digestion at 37°C respectively. 40 ml or 350 mL full DMEM medium with serum was added into for quenching trypsin digestion. And then the cells were transferred into a 50 mL falcon tube, or a 500 mL conical tube. For cells in 50 mL falcon tube, they were repopulated in used 10 \times cell-stack chamber for second round cell transfection and harvesting. The cells in 500 mL conical tube were spun down at 2000 rpm for 10 min. The supernatant was discarded and the cell pellets were resuspended in 8 mL lysis buffer (150 mM NaCl and 50 mM Tris-HCl, pH 8.5) and transferred in 15 Falcon tube, frozen in liquid nitrogen

4. Methodology

and stored at -80°C.

4.4.2.2. AAV crude lysate preparation

AAV lysates from step above were thawed at 37°C until half frozen and half suspension mixture formed. Then the votexing was employed to have complete thawing. The virus containing supernatant was collected by 10 minute centrifugation at 3500×g. The cell pellets were remixed in 4 mL lysis buffer and then snap-frozen in liquid nitrogen. The freezing-and-thaw cycle was repeated for 3 times to have maximal cell lysis. The final thawing step was employing 1 minute sonication before centrifugation for better cell lysis. Finally, suspensions from all steps were pooled and digested with benzonase (50 U/ml) for 30 min at 37°C to remove any transfected plasmid and naked DNA. This virus lysate was then centrifuged at 4°C and 3,500 g for 10 min to remove the pellet and then stored at -80°C until further use.

4.4.2.3. AAV iodixanol gradient purification

AAV crude lysates were further purified by two-step iodixanol gradient. 4 gradients with different concentration were prepared as in table as follow (quantity for 15 gradients):

Iodixanol Gradient	%15	%25	%40	%60
OptiPrep	17.5 mL	31.2 mL	40 mL	60 mL
PBS-MK-NaCl	52.5 mL			
PBS-MK		43.8 mL	20 mL	
0.5% Phenol Red		187.5 μ L		150 μ L
Total	70 mL	75 mL	60 mL	60 mL

Lysates were transferred into centrifugation tube via pasteur pipette. 4 different gradients were sequentially under-layered through pasteur pipette. Then centrifugation tube was sealed and ~1 mL air bubble was left inside. Gradients were centrifuged at 50, 000 rpm, 2.5 hours and 10°C in 50.2Ti rotor. Purified virus fraction was taken out by inserting 20G needle into 60% layer and collecting roughly 3.5 mL 40% gradient fraction. In second step gradient purification, only 25%, 40% and 60% gradient were used for purification. Other procedures were the same as in step 1. After two round gradient purification, virus fraction was dialyzed against PBS overnight with 3 changes of PBS. Then virus fraction was transferred into Vivaspin-6 tube and spun and resuspended at

4. Methodology

2000–4000 rpm for 3–5 min until the final volume is 1000-1500 μL . Finally, virus elute was aliquoted and stored at -80°C until further use.

4.4.2.4. AAV titration

5 μL virus solution was mixed with 5 μL H₂O and 10 μL NaOH and then incubated at 55°C to allow complete release of viral genome. Then this solution was neutralized by adding 10 μL HCl and diluted by adding 970 μL H₂O. Finally, 5 μL was submitted to qPCR analysis with probe for GFP sequence in AAV genome. For quantification, the standards were prepared by diluting AAV genome plasmid from 10¹³ copies/mL step-wise into 10².

4.5. Metabolite measurement in cellular and tissue samples

4.5.1. Glucose consumption in Hepa 1-6 and primary hepatocytes

Hepa 1-6 or mouse primary hepatocytes was cultured as condition described before. At day 0, adenovirus packaged with scramble shRNA or shRNA3 (targets all PPP2R5C transcripts) were incubated with cell for 3-day infection experiment at MOI of 10, 100, or 200. After 24 hours, media with virus was washed away by 3 time washing with fresh medium. Cell media was replaced everyday, and media for last 24 hour infection was collected for glucose consumption assay. Glucose concentrations from 2.5 μL of these media or control media (fresh media) were measured by Glucose HK assay kit from Sigma, and relative glucose consumption was calculated by subtracting glucose concentration from fresh medium.

4.5.2. Lactate production

The same media collected for glucose consumption assay was also used for lactate production assay. 2 μL of each medium was submitted for lactate concentration measurement by using a lactate kit from Roche (D-Lactic acid/L-Lactic acid, Cat. No. 11 112 821 035). And lactate production rate in 24 hour was calculated from relative lactate production compared with fresh medium.

4. Methodology

4.5.3. Free fatty acid measurement

Sample for free fatty acid measurement was prepared differently among various samples. For media sample, all the lipid content was enriched by Methanol-Chloroform method [88]. 2 volume of methanol:chloroform mix (2:1 in volume ratio) was mixed well with 1 volume of media (if media does not contain Triton X-100, add 10 μ L chloroform:Triton X-100 mix (1:1 in volume ratio)), and then was shaken at room temperature at 250 rpm to allow complete extraction of lipid fraction. Then these solutions were spun down at maximal speed on a desktop centrifuge for 5 min, room temperature to separate the water:methanol phase and chloroform phase. Then lipid fraction was collected from the lower chloroform phase, and mixed with 0.4 volume of 0.9% NaCl for cleaning. Final spin on this solution will separate the chloroform phase with water phase on the top. Final lipid fraction was collected and dried in a speed-vac for at least 3 hours until there was a Triton X-100 pellet formed. Finally, pellet was dissolved in water and submitted to fatty acid measurement using the kit from Cayman Chemical (Free Fatty Acid Fluorometric Assay Kit) under the instruction manual from manufacturer.

For cellular samples lysed by IP lysis buffer (150 mM NaCl, Tris pH 7.5, 1% Triton X-100), they could be submitted to fatty acid measurement directly if the fatty acid concentration was within the range of standard curve. If not, then these samples must be enriched as before. For tissue sample such as mouse liver, The sample is needed to be pulverized and weighted before suspending in methanol:chloroform mix (2:1 in volume ratio). For 100 mg liver tissue powder, 1.5 mL mix was added and shaken at room temperature for 20 min. The following procedure for collecting lipid fraction was similar with these for media samples.

For serum samples, 2 μ L of serum was subjected to fatty acid measurement by NEFA HR kit from Wako Chemicals. 2 μ L serum sample or different amount of standard (1 mM/L oleic acid, 0.5 - 5 μ L) was first mixed with 200 μ L R1 reagent, and incubated at 37 °C in Tecan microplate reader Infinite M200 for 2 min to read the absorbance 546 nm as blank. Then 100 μ L R2 reagent was added at 3 min, and absorbance at 7.5 min was recorded as final reading. Serum NEFA was calculated from back calculation from standard curve.

4. Methodology

4.5.4. Triglyceride measurement

Sample preparation procedure for triglyceride measurement were the same as the ones for free fatty acid measurement. After sample was lysed or enriched, normally 2–20 μL sample was mixed and incubated with 5 μL lipase solution (10 mg/mL) at 37 °C overnight to release the glycerol from triglyceride. Then free glycerol content was measured by free glycerol assay kit from Sigma. Triglyceride concentration was calculated and normalized to the total protein content in the very same sample.

4.5.5. Cholesterol measurement

Cholesterol was measured from the same sample for triglyceride or free fatty acid measurement. For serum sample, 2–4 μL serum was directly submitted for measurement. All cholesterol measurements were done using cholesterol (liquid) assay kit from Randox Laboratories (Cat. No. CH201) according to the standard protocol shipped with the kit. Cholesterol concentration was calculated from control with known concentration.

4.5.6. Glycogen content determination

For measuring glycogen from cellular lysate, such as Hepa 1-6 in 6 well plate, cell was first lysed in 500 μL IP buffer and 30 μL of the lysate was mixed with 1 μL amyloglucosidase solution (14 U/ μL) overnight at 37 °C. For tissue sample such as mouse liver, ~50 mg liver was weighted (recording the weight for normalization) and homogenized in 1 mL 30% KOH with Qiagen tissue lyser. Then the homogenate was incubated at 95 °C for 30 min and clarified by maximal centrifugation in a desktop centrifuge for 10 min. Supernatant was collected and mixed with 1.5 mL 95% ethanol to precipitate glycogen. Glycogen pellet was collected by spinning at 3000 $\times g$ for 20 min and then washed with 95% ethanol, dried at room temperature. Then pellet was dissolved in 500 μL water (in case there was solubility issue, heat to 37 °C for 30 min). 5 μL glycogen solution was digested using 295 μL amyloglucosidase solution (30 U/mL in 0.2 M NaAc pH 4.8) and then neutralized with 6 μL 30% KOH. 10 μL of the digested glycogen was submitted to glucose measurement using the same kit for glucose consumption assay.

4. Methodology

4.5.7. Ketone body measurement

Total ketone body and 3-hydroxybutyrate were measured using Autokit total ketone bodies and 3-HB kit from Wako Diagnostics. 2 μ L of each serum was taken for measurement. And procedures were the same according to the manufacturer's instruction. All the reading was done in 96 transparent well and recorded on Tecan infinite M200 at 405 nm.

4.5.8. ATP measurement

Intracellular ATP level was measured using ATPliteTM from Perkin Elmer. 50 μ L of Hepa 1-6 lysate (lysis in 500 μ L IP buffer for 10^7 cell) was mixed with equal volume of reagent and read for luminescence intensity within 20 min on Tecan infinite M200.

4.5.9. Intraperitoneal glucose tolerance test (IPGTT)

Mice were starved for 6 hours before injecting 2g glucose/kg body weight intraperitoneally. Blood glucose was collected from cutting at tail and measured right after injection. Then 50 μ L blood was also collected from the same cutting point for measuring insulin in the serum. At time point 20, 60, 90, 120, 150 min, blood glucose concentration was measured and extra 50 μ L blood was collected for later insulin measurement at time point of 20 and 60 minute. Blood was incubated at 4 °C to allow separation of serum and serum was collected by spinning at 5000 rpm for 30 min. All blood glucose measurement was done by using a glucose measurement kit from One Touch Glucose Monitor (Lifescan).

4.5.10. Serum insulin measurement

Mouse insulin in the serum was performed using mouse insulin ELISA kit from Alpco. 5 μ L serum was used for mouse serum from random fed mice. For GTT assay or serum from fasting mouse and refed mice, 25 μ L was taken for measurement. Insulin concentration was calculated from 5 parameter logistic regression of standard curve data (0.188–6.9 ng/mL) in R package drc. All the reading was done in 96 transparent well and recorded on Tecan infinite M200 at 450 nm.

4. Methodology

4.5.11. Serum lipoprotein analysis

200 μ L of pooled serum from 5 or 6 mice (40 or 33.3 μ L for each mouse) and 100 μ L 1× PBS were mixed and spun for 2 min at 10, 000×g, 4 °C to clarify. And then the 300 μ L mixed solution was subjected to FPLC separation on Superose™ 6 10/300 GL column (GE Healthcare) in 25 mL PBS at flow rate of 0.5 mL/min. Lipoprotein particle peaks were monitored with UV 280nm. VLDL, LDL and HDL eluted at 7-8 mL, 12-14 mL and 16-18 mL respectively. 0.5 mL/Fraction was used to collect each fraction, and then 160 and 40 μ L from each fraction was used to determine the triglyceride and cholesterol content per fraction respectively.

4.5.12. Serum ALT measurement

Serum ALT (Alanine Aminotransferase) activity was measured by the Infinity ALT/GPT Reagent (Thermo Scientific). 5 μ L serum or H₂O was added into 100 μ L ALT reagent on 96-well transparent plate (SARSTEDT). Then the microplate was incubated at 37 °C and recorded UV 355 nm on microplate reader (Infinite® M200, Tecan) at time of 1 min and 9 min. Then the ALT activity was calculated as following equation.

$$ALT = \frac{\Delta A_{1min-9min}}{8} \times 11029.4(\text{U/L})$$

4.5.13. Seahorse analysis of glycolysis in hepa 1-6

Pre-split hepa 1-6 cell was washed once with 180 μ L assay medium (DMEM (Sigma D5030) with 143 mM NaCl, 2 mM L-Glutamine, pH 7.35±0.05. Adjust pH at day of assay.) and incubated with 175 μ L assay medium at 37 °C for 1 hour in 96-well plate from XF96 glycolysis stress kit (Seahorse Bioscience). Then 25 μ L of each glucose (10 mM in assay medium), oligomycin (2.5 or 1 μ M in assay medium) and 2-deoxyglucose (100 mM in assay medium) was injected into plate reservoir and then glycolysis rate was recorded on XF96^e Extracellular Flux Analyzer from Seahorse Bioscience. All the data collection and analysis was done in built in software for XF96. Data normalization was done by counting average nuclei number in Cell Profiler from DAPI staining and normalization to average nuclei count.

4. Methodology

4.6. Biochemical methods

4.6.1. Protein expression in bacteria

Variant 3 of PPP2R5C was sub-cloned into 6 × His tag purification system. Expression plasmid was transformed into 4 different bacteria strains by electroporation in Bio-Rad's Gene Pulser Xcell™ Electroporation Systems, include Lucigen, Rossetta, RP and RIL. Strain with highest induction under IPTG was selected for protein production. 1 L bacteria culture was shake at 37 °C until O.D. 600 exceed 2 and then IPTG induction was done at 18 °C overnight at 1 mM. Protein purification on 6 × His tag resin from Qiagen were performed according to suppliers' instruction.

4.6.2. Antibody production and purification

Purified PPP2R5C was dialysed in PBS with 5% glycerol overnight and then concentrated by Vivaspin 2 with 3000 MWCO until protein concentration reached 1 mg/mL. Antigen was mixed with equal volume of Freund's adjuvant complete and injected 250 μL per guinea pig at each 3–4 week. Around 50 μL blood was collected at 1 week after injection. Serum was separated and tested for specificity for antigen at 1:200–1000 dilution in western blot.

4.6.3. SDS-PAGE and western blot

All protein samples, including lysates from cell, tissue or serum fractions, were denatured in 1×Laemmli buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and boiled at 95 °C for 5 min. SDS-PAGE gels were prepared at different percentage using Tris-Glycine gel system. Gel was running at 20 mA/gel with 200 V limit for 1 hour. And then gel was washed in transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol) shortly before assembled into wet transfer sandwich with whatman paper and 0.22 μM nitrocellulose membrane from GE Healthcare. Protein was transferred onto nitrocellulose membrane at 100 V for 1 hour at 4 °C. Afterwards, protein was visualized by Ponceau S staining (0.2% in 3% TCA) and blocked with 5% BSA or skim milk (Sigma) for 1 hour. Then membrane was washed with PBST (1×PBS, 0.1%Tween-20) for 3 times, 10 min each. Incubation with primary antibody was done at 4 °C overnight. Then the membrane was washed for 3

4. Methodology

times, 10 min each. Secondary antibody was diluted in 5% skim milk and incubated with membrane at room temperature for 1 hour. Finally, the membrane was washed 3 times, 10 min each, and developed with ECL reagents from Thermo Scientific.

4.6.4. Phos-tag[®] analysis of phosphorylated proteins

For protein separation using Phos-tag[®] analysis, all protein lysate samples were cleaned by Methanol-Chloroform precipitation [150] and re-solubilized in 1×Lamelli buffer. 25 μ M Phos-tag[®] was used to incorporate with 8% SDS-PAGE gel. Other procedures were performed under the instruction manual of Phos-tag[®].

4.6.5. Immunoprecipitation

Total protein lysates were prepared by lysing cell in IP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100) for 15 minutes on ice and then collecting supernatant after centrifugation of 14, 000 rpm at 4 °C for 15 minutes. Then lysates were incubated with 1 μ L antibody (0.5 mg/ml) for 3 hours at 4 °C, and 30 μ L Protein A Agarose slurry (Roche) for additional 0.5 hour. Agarose beads were washed in cold IP buffer for 3 times with centrifugation at 2000 rpm, 4 °C for 1 minute to discard the supernatant. Final IP fraction was eluted in 2×Lamelli buffer and boiled at 95 °C for 5 minutes.

4.7. Animal experiments

8–10 week C57BL/6J male mice was purchased from Charles River Laboratories and maintained with unlimited water and normal chow food at 12 hour light–dark cycle. After 1 week adaptation, mice were tail-injected 100 μ L control or knockdown AAV diluted in 1×PBS. After 7 week infection, mice were subjected to *ad libitum* feeding, 16 hour fasting or 16 hour fasting + 6 hour refeeding.

4.8. Data analysis and plotting

Data for mouse serum and liver metabolites were imported and analysed in R [151]. For liver TG in random fed mice, ANCOVA model was used to estimate the effect

4. Methodology

of PPP2R5C knockdown by AAV. Two variables liver NEFA and AAV (miRNC and miR12) were used as covariates to predict the liver TG. In GTT measurement, repeated measures ANOVA model was used to evaluated the effect of PPP2R5C knockdown on glucose clearance rate. For other metabolite measurements, pairwise.t.test function in R was employed to perform multiple comparisons between 3 different treatments (Random Fed, Fasting, Refed) and 2 different AAVs (miRNC, miR12). p-value form multiple testing was controlled by Benjamini & Hochberg method [121]. All the plotting was done either using R core graphics or ggplot2 [152].

A. Materials

A.1. Chemicals and kits

Table A.1.: Chemicals and kits used in all experiments

Chemical	Company	Ordering No.
N-Z-Amine A	Sigma	C0626
Ammonium persulfate	Sigma	A9164
starPure Agarose	Starlab	N3101
Bis-Tris	Sigma	B7535
Bromphenolblau	Roth	T1161
Baetopeptone	BD	211672
Cobal chlorid	Sigma	60818
EDTA disodium salt dihydrate	AppliChem	A1104
Glycine	Sigma	33226
Glycerol	Sigma	15523
β -Glycerophosphate disodium salt hydrate	Sigma	G6376
Guanidine hydrochloride	Sigma	G4505
HEPES	AppliChem	A37240500
Hematoxylin Solution	Sigma	MHS16
Imidazole	AppliChem	A10730100
Kanamycin sulfate	AppliChem	A14930025
Lithium chloride	Sigma	62478
MOPS buffer grade	AppliChem	A10761000
Magnesium sulfate heptahydrate	Sigma	13142

A. Materials

Chemical	Company	Ordering No.
Magnesium chloride hexahydrate	Merck	1058331000
Na ₂ HPO ₄	Sigma	S5136-100G
Nile Red	MP	180085405
Nonidet® P40	AppliChem	A16940250
Orange G	Fluka	75380
Oil Red O solution	Sigma	O1516
Oil Red O powder	MP	155984
pH-Pufferloesung pH 10	Roth	H910.1
pH-Pufferloesung pH 7	Roth	H908.1
pH-Pufferloesung pH 4	Roth	H906.1
Polyethylene glycerol	Sigma	P3640
Pierce 660nm Protein Assay	Thermo	22660
Ponceau S solution	SERVA	3342701
Potassium chloride	Roth	67811
Potassium hydroxide	Fluka	60375
Di-Potassium Hydrogenphosphate	Roth	P7491
Potassium Dihydrogenphosphate	Roth	39041
Potassium acetate	Roth	T8742
Di-Potassium Hydrogenphosphate Trihydrate	Roth	68781
Propylene Glycerol	MP	151957
Polyethylenimine	Polysciences Inc	24765
Sodium hydroxide pellets	Riedel	30620
Skim milk powder	Fluka	70166
Sodium phosphate dibasic dihydrate	Sigma	30412
Sodium hydrogencarbonate	AppliChem	A03841000
Sodium hydrogenphosphate monohydrate	Roth	K3002
Sodium carbonate	Roth	A1351

A. Materials

Chemical	Company	Ordering No.
SDS ultrapure	AppliChem	A11121000
Sodium bisulfite	Sigma	243973
Sodium deoxycholate	Sigma	D6750
Sodium fluorid	AppliChem	A39040100
Sodium azid	AppliChem	A14300100
Tween-20	AppliChem	A13891000
Trizma Base (Tris)	Sigma	T1503
Thiourea	Sigma	T8656
Triton® X-100	AppliChem	A4975
Tryptone/peptone	from Roth	8952.3
Casein		
Urea	SERVA	24524
Nuclease Protease Free	Acros organics	327390050
Water		
Yeast Extract	Fluka	70161
LB Broth	Sigma	L3022
Ampicillin sodium salt	Sigma	A9518
Biotin	Sigma	B4639
CHAPS	AppliChem	A1099
DTT	AppliChem	A11010025
L-(+)-Arabinose, 98%	Sigma	A91906
L-Glutathione reduced	Sigma	G6013
Glutathione Sepharose	Amersham	17075601
Ni-NTA agarose	Quiagen	1018244
NHS-activated Sepharose	GE Healthcare	17090601
Fast Flow		
monoclonal anti-HA	Sigma	A2095
Agarose		
Prot-G-Agarose	Roche	11719416001
Prot-A-Agarose	Roche	11134515001
Amyloglucosidase	Sigma	10115
BSA Albumin Fraction V	AppliChem	A1391
Free Glycerol Reagent	Sigma	F6428
GelCode Blue Stain	Thermo	24590
Reagent		

A. Materials

Chemical		Company	Ordering No.
Glucose reagent	(HK) assay	Sigma	G3293
Acrylamide 30% 4k	37.5:1	AppliChem	A16721000
PhosSTOP		Roche	04906837001
Protease inhibitor mix		Roche	11836145001
Protease inhibitor mix	EDTA-free	Roche	11836170001
Ultrasensitive mouse Insulin ELISA		Mercodia	10-1249-01
D-Lactic Acid / L-Lactic acid kit		Roche	11112821035
Freund's Adjuvant, Complete		Sigma	F5881
Freund's Adjuvant, Incomplete		Sigma	F5506
Streptavidin HRP		invitrogen	T20932
Lipofectamin 2000		Invitrogen	11668-027
Puromycin dihydrochloride		AppliChem	A2856
effectene reagent kit		Quiagen	301427
Opti-MEM reduced		Gibco	31985-062
Pen-Strep 100x		PAA	P11-010
L-Glutamine solution		Gibco	25030-024
Trypsin-EDTA solution		Gibco	25200-056
MEM non essential Amino Acids		Sigma	M7145
FBS Gold		PAA	A11-151
DMEM (4.5g/l Gluc; + L-Glut)		Gibco	41965-039
Glycoysis stress kit		Seahorse Bioscience, Massachusetts, USA	102194-100
BLOCK-iT TM U6 RNAi Entry Vector Kit		Invitrogen, Karlsruhe	K4945-00
BLOCK-iT TM Adenoviral RNAi Expression System		Invitrogen, Karlsruhe	K4941-00

A. Materials

Chemical	Company	Ordering No.
Cholesterol (liquid) assay	Randox, Crumlin, UK	CH201
Total Ketone Bodies	Wako, Neuss	415-73301&411-73401
HR Series NEFA-HR	Wako, Neuss	999-34691&991-34891

A.2. Antibodies

Table A.2.: Antibodies used in all experiments

Antibody	Suppliers
anti myc-Tag (71D10)	Cell Signaling 2278
anti Phospho-p70 S6K (Thr389)	Cell signalling 9205L
anti HA tag (clone 3F10)	Roche 11 867 423 001
anti AKT (total protein)	Cell Signaling 9272
anti FLAG tag M2	Sigma F 1804
anti phospho-ERK1/2(p44/42)	Cell Signaling 4370
anti AMPK β 1	Cell signaling 4148
anti AMPK β 2	Cell signaling 4178
S6 ribosomal protein (54D2)	Cell Signaling 2317
anti phospho-S6 ribosomal Protein (Ser235/236)	Cell signaling 4857
anti p70 S6 Kinase	Cell Signaling 9202
anti human PP2A catalytic	BD 610555
anti p-AKT(Thr308)	Cell Signaling 2965S
anti p-AKT(Ser473)	Cell Signaling 9271
anti Ribosomal Protein L26	Cell Signaling 2065
anti YAP	Cell Signaling 4912
anti p-GSK3-beta (Ser9)	Cell Signalling 5558
anti p-AMPK α (T172) (40H9)	Cell signaling 2535S
anti GFP	Teleman's production
anti AMPK α	Cell signaling 2532S
anti HSP90 (C45G5)	Cell signaling 4877
anti p-Acetyl-CoA Carboxylase (Ser79)	Cell Signaling 3661
anti TSC1	Cell Signaling 4906
anti SREBP1	Santa Cruz sc-8984

A. Materials

Antibody	Suppliers
anti SREBP-1	BD Biosciences (557036)
anti HIF1 α	Cell Signaling 3716
anti p-Stat3 (Ser727)	Cell Signalling 9134
anti STAT3	Cell Signaliing 9139
anti HIF1 α	GeneTex
anti GAPDH	Cell Signaling 2118
anti CHD4	Cell signaling 4245
anti VDUP1 (B-2)	Santa Cruz sc-166234
anti p-TBC1D1 (Ser660)	Cell signaling 6928
anti p-TBC1D1 (Ser700)	Cell signaling 6929
anti p-AMPK b1 S108	Cell signaling 4181
anti SREBP-2 (H-164)	Santa Cruz sc-5603
anti Glut1 (H-43)	Santa Cruz sc-7903
anti TBC1D1 (V796)	Cell signaling 4629S
anti Acetyl-CoA Carboxylase (C83B10)	Cell Signaling 3676

A.3. Instruments

Table A.3.: Instruments used in all experiment

Instruments	Suppliers
Peltier Thermal Cycler DNAEngine	Bio-Rad, Munich
Step One Plus Real-Time PCR System	Applied Biosystems, USA (Now part of Thermo Scientific)
MicroPulser	Bio-Rad, Munich
FlexCycler	Analytik jena, Germany
Uvsolo TS Imaging system	Biometra, Gottingen
SPECTROstar Omega	BMG LABTECH GmbH, Ortenberg
TriStar2 multimode reader	Berthold Technolog, Bad Wildbad
Avanti J-25	Beckmann, Munich
Duomax 1030 horizontal shaker	Heidolph, Kehlheim
Analytic Scales	Satorius, Gottingen
CERTOMAT BS-T	Satorius, Gottingen
Wet Transfer Blotting	Bio-Rad, Munich
Cell counter	Bio-Rad, Munich

A. Materials

Instruments	Suppliers
Centrifuge 5810R	Eppendorf, Hamburg
Centrifuge 5430	Eppendorf, Hamburg
Electrophoresis system	Bio-Rad, Munich
Film cassette	GE Healthcare, Salt Lake City, USA
Film Developer	Sanyo, Munich
AKTA purifier 10	GE Healthcare, Salt Lake City, USA
Odyssey® Fc	LI-COR Biotechnology GmbH, Bad Homberg
Infinite M200	Tecan Group Ltd., Switzerland
One Touch Glucose monitor	Lifescan, Neckargemund
pH-meter	Satorius, Gottingen
Nano-Drop 1000	Thermo Scientific, USA
XF96 analyser	Seahorse Bioscience, Massachusetts, USA
Tissue lyzer	Qiagen, Hilden
FACS Canto II	BD Biosciences, San Jose, USA
Benchtop Heater PCH-1	Grant Instruments, UK
Electrophoresis Power Supply	VWR international, Darmstadt
DNA gel electrophoresis chamber	Peqlab Biotechnology, Erlangen
Magnetic mixer MR 2002	Heidolph, Kehlheim
Compatible Control CC-1	Peter Huber Kaltemaschinenbau GmbH, Offenburg

A.4. Solutions and buffers

Table A.4.: Buffers used in all experiments

Buffers	Recipes
HANKS Buffer	2L containing 16.0 g NaCl, 7.1 g HEPES, 800 mg KCl, 120 mg Na ₂ HPO ₄ , 120 mg KH ₂ PO ₄ , pH 7.4
HANKS I	400 mL HANKS buffer, 152 mg EGTA (1 mM final), 4 mL 10% Glucose (0.1% final)
HANKS II	400 mL HANKS buffer, 100 mg Collagenase CLS II (0.3 mg/ml final), 389 mg CaCl ₂ •2H ₂ O (10 mM final), 4 mL 10% Glucose (0.1% final)

A. Materials

Buffers	Recipes
Adehesion Medium	10% FCS, 1% P/S, 2mM Glutamine, 0.01 mg/mL Insulin, 100 nM Dexamethsone in Williams E Medium
PBS-Tosh	30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na ₂ HPO ₄ , 1.46 mM KH ₂ PO ₄ , 10 mM MgCl ₂ , pH 7.2
IP lysis and washing buffer	150 mM NaCl, 50 mM Tris, 1% Triton X-100, pH 7.4
BioID lysis buffer 1	50 mM Tris, pH 7.4, 500 mM NaCl, 0.4% SDS, 5 mM EDTA, 1 mM DTT, and 1×Complete protease inhibitor from Roche
BioID lysis buffer 2	50 mM Tris, pH 7.4
BioID wash buffer 1	2% SDS
BioID wash buffer 2	0.1% deoxycholate, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA, and 50 mM Hepes, pH 7.5
BioID wash buffer 3	250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, and 10 mM Tris, pH 8.1
BioID wash buffer 4	50 mM Tris, pH 7.4, and 50 mM NaCl
BioID elution buffer	Biotin saturated 1×Laemmli sample buffer

A.5. Primer list for quantitative RT-PCR

Table A.5.: Primer list for quantitative RT-PCR

Oligo No.	Primer Sequence	Name
OAT1632	CGGTCGCAATGGAGACA	Foward primer for mPPP2R5C variant 1+4
OAT1633	GGGGACCGATCCTTCTTCA	Reverse primer for mPPP2R5C variant 1+4
OAT1634	GCGAATCCCCAGGCACA	Foward primer for mPPP2R5C variant 2
OAT1635	GTGTGGGGTCCTGAGG	Reverse primer for mPPP2R5C variant 2
OAT1636	GCACTCAGCAGTTCAAAGC	Foward primer for mPPP2R5C variant 3
OAT1637	CACGCAAAGCCTAACAC	Reverse primer for mPPP2R5C variant 3

A. Materials

Oligo No.	Primer Sequence	Name
OAT1638	CGTCGTCCCTGTGAAGAA	Foward primer for mPPP2R5C variant 4
OAT1639	GATCCGCAGGAGGAACAT	Reverse primer for mPPP2R5C variant 4
OYC175	AGTTTGTATTGCAGCTTCTA	Foward primer for mPPP2R5C
OYC176	TCCAGTAACTCCGCTATG	Reverse primer for mPPP2R5C
OYC124	AGGAGTATGGGCTTCATTGGGCA	Foward primer for mAcy
OYC125	TCCCAGGGTGACGATACAGCC	Reverse primer for mAcy
OYC185	CCTGTGCTACCTTCTCTCTA	Foward primer for mGPAT
OYC186	CTTCCTGGTCATCTGCTCT	Reverse primer for mGPAT
OYC197	CACACATCACTTAGCCAAC	Foward primer for mHMGCS1
OYC198	GTCCTTCTGTGTTTCATC	Reverse primer for mHMGCS1
OYC199	ATCTTCTCCCTATTGCACT	Foward primer for mLDR
OYC200	TGGGTTGTCAAAGTTATGC	Reverse primer for mLDR
OYC209	AGAAAGGTGGCAGGAGATCG	Foward primer for mDGAT2
OYC210	GTCAGCAGGTTGTGTGTCTT	Reverse primer for mDGAT2
OYC213	ATGACCAGACTTCCTCCAAC	Foward primer for mSlc25a1
OYC214	GTATGTTCCCTTAGCCCTT	Reverse primer for mSlc25a1
OYC233	AGTTCCATTGACAAGGCCAT	Foward primer for mSREBP1
OYC234	TACCGTGAGCTACCTGGACT	Reverse primer for mSREBP1
OYC395	CCATTCCACCATGATTAAGGGTCT	Foward primer for mLDA
OYC396	CGAGATTCCATTGTCCCAGGATA	Reverse primer for mLDA
OYC397	CAGATCTCTCAGCCCCCAA	Foward primer for mNDRG1
OYC398	GGCGAGTCATGCTGGCAGAA	Reverse primer for mNDRG1
OYC399	ACAAGCTTCATCCTCACTTGCC	Foward primer for mHK2
OYC400	GGAAGGACACGTCACATTCGGA	Reverse primer for mHK2
OYC110	ATGGATGTTGGCAAGGCCGA	Foward primer for mPKM2
OYC111	AGGCACTACACGCATGGTGTGG	Reverse primer for mPKM2

A.6. shRNA/miRNA target sequences

A. Materials

Table A.6.: shRNA/miRNA target sequences

shRNA/miRNAs	Target Sequence
shR3	CGTGCTTACATCAGGAAACA
shR6	TCAGAGTTGTGAAGATCATG
miR12	AGACAATAACACGGCTTGATAT

A.7. Software list

Table A.7.: List of software used

Software	Provider
Image J	NIH
R	R core team
Office Suite	Microsoft
Photoshop	Adobe
ApE	M. Wayne Davis
Oligo 7	Molecular Biology Insights
RStudio	RStudio
FlowJo	FLOWJO

B. Supplementary Table

B.1. Enriched TFs in mouse cell lines upon PPP2R5C KD

Table B.1.: Activated TFs in mouse primary hepatocytes and Hepa 1-6.

Transcription Factor	P-value	FDR control (B-H).
PPARA	0.00138	0.00263
SREBF1	0.02730	0.00526
CTNNB1	0.06480	0.00789
E2F2	0.15100	0.01050
SREBF2	0.16400	0.01320
E2F3	0.16900	0.01580
CREB1	0.24900	0.01840
TCF7L2	0.27600	0.02110
GLI2	0.42600	0.02370
GLI1	0.47900	0.02630
E2F1	0.56500	0.02890
MYC	0.65600	0.03160
SP3	1.00000	0.03420
EGR1	1.00000	0.03680
SP1	1.00000	0.03950
HBP1	1.00000	0.04210
FOXO1	1.00000	0.04470
TP53	1.00000	0.04740

B.2. Enriched TFs in mouse liver upon PPP2R5C KD

B.2.1. Enriched TFs in mouse liver during fasting

Table B.2.: Activated TFs in HepKD mouse liver during fasting.

Transcription Factor	P-value	FDR control (B-H).
FOXM1	0.00799	0.001724
CDX1	0.00799	0.003448
EBF1	0.00799	0.005172
SREBF1	0.01626	0.006897
USF1	0.02379	0.008621
PDX1	0.03161	0.010340
GATA2	0.04706	0.012070
SMAD1	0.05471	0.013790
NR2F1	0.06229	0.015520
TCF7	0.06606	0.017240
CEBPA	0.12450	0.018970
E2F1	0.12510	0.020690
SREBF2	0.12810	0.022410
SMAD4	0.13510	0.024140
HNF1A	0.14560	0.025860
ETS1	0.19940	0.027590
CTNNB1	0.34110	0.029310
STAT5A	1.00000	0.031030
STAT5B	1.00000	0.032760
STAT3	1.00000	0.034480
STAT1	1.00000	0.036210
RELA	1.00000	0.037930
TP53	1.00000	0.039660
CEBPB	1.00000	0.041380
NFKB1	1.00000	0.043100
GATA3	1.00000	0.044830
GLI1	1.00000	0.046550
E2F4	1.00000	0.048280
FOXO1	1.00000	0.050000

B.2.2. Enriched TFs in mouse liver during *ad libitum* feeding

Table B.3.: Activated TFs in HepKD mouse liver during random fed.

Transcription Factor	P-value	FDR control (B-H).
EBF1	0.00554	0.002
CDX1	0.00554	0.004
PPARA	0.03011	0.006
GATA2	0.03280	0.008
SMAD1	0.03818	0.010
TCF7	0.04619	0.012
HIF1A	0.05941	0.014
CEBPA	0.08792	0.016
SREBF2	0.09047	0.018
RELA	0.09302	0.020
SMAD4	0.09556	0.022
YY1	0.11320	0.024
STAT5A	0.11570	0.026
STAT5B	0.11570	0.028
SREBF1	0.13050	0.030
GLI2	0.25420	0.032
GLI1	0.29180	0.034
E2F1	0.35640	0.036
CTNNB1	0.58250	0.038
FOXO4	1.00000	0.040
ETS1	1.00000	0.042
STAT1	1.00000	0.044
STAT3	1.00000	0.046
E2F4	1.00000	0.048

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Glossary

2NBDG 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose. 29

AAV Adeno-Associated Virus. 36, 37, 89–91, 97, 98

ACLY ATP-citrate lyase. 62

AKT V-Akt Murine Thymoma Viral Oncogene Homolog, or Protein Kinase B. 14

ALT Alanine Aminotransferase, marker for liver injury. 36

AMPK 5'-AMP-Activated Protein Kinase. 6, 57, 59

ANCOVA Analysis of covariance. 45, 46

ANOVA Analysis of Variance. 19–22, 43, 44, 67

APC Adenomatous Polyposis Coli. 15

ATP Adenosine triphosphate. 34, 57

BioID proximity-dependent **biotin** identification. 85

BirA Bifunctional ligase/repressor BirA. 56, 57

BSA Bovine Serum Albumin. 96

CAK CDK-activating kinase. 15

CaMK-II Calcium/Calmodulin-Dependent Protein Kinase II. 6

cDNA Complementary DNA. 78, 80, 82, 83

CK-I Casein Kinase 1. 6

CK-II Casein Kinase 2. 6

Glossary

DAPI 4',6-diamidino-2-phenylindole. 15

DGAT2 Diacylglycerol O-Acyltransferase 2. 62

DMEM Dulbecco's Modified Eagle's Medium. 84, 86, 88, 95

DNA Deoxyribonucleic acid. 77–80, 87

dNTP Deoxyribonucleotide. 77–79, 82

ELISA Enzyme-linked immunosorbent assay. 94

FACS Fluorescence-activated cell sorting. 30

FCS Fetal Calf Serum. 84, 88, 106

FPLC Fast protein liquid chromatography. 51, 95

gDNA Genomic DNA. 78, 80

GPAT1 Glycerol-3-Phosphate Acyltransferase 1, Mitochondrial. 62

GSK-3 glycogen synthase kinase 3 β . 6

GST Glutathione S-transferase. 17

GTT Glucose tolerance test. 42, 45, 94, 98

HDL High-density lipoproteins. 7, 51, 95

HIF1 α Hypoxia Inducible Factor 1, Alpha Subunit. 57, 59, 60, 62

IDL Intermediate-density lipoproteins. 7, 51

I κ B α Nuclear Factor Of κ Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Alph. 17

IKK inhibitor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Kinase, upstream regulator of NF- κ B. 17

LB Luria-Bertani. 80

LDL Low-density lipoproteins. 7, 51, 95

Glossary

miRNA microRNA. 23, 24, 36, 38, 81, 89

MOI Multiplicity of Infection. 28

mTOR Mechanistic Target Of Rapamycin (Serine/Threonine Kinase). 14, 34

NEAA Non-essential amino acid. 84, 88

NEFA Non esterified fatty acid. 46, 50, 92, 98

NF-κB Nuclear Factor Of κ Light Polypeptide Gene Enhancer In B-Cells. 17, 27

NHGU Net Hepatic Glucose Uptake, $\mu\text{mol}/\text{kg}/\text{min}$. 4, 5

P/S Penicillin-Streptomycin (10,000 U/mL). 88, 89, 106

PCR Polymerase chain reaction. 77, 79, 83

PEI Polyethylenimine. 89

PKA Protein Kinase A, cAMP-Dependent. 6

PKC Protein Kinase C. 6

PP2A Protein Phosphatase 2A. 11, 18, 56, 57, 59

PPP2R5C Protein Phosphatase 2, Regulatory Subunit B', Gamma. 19–21, 23–27, 29, 31, 33, 34, 36, 40, 42, 45, 48, 50, 51, 54, 56, 57, 60, 62

qPCR Quantitative Real-Time PCR. ii, iii, 83, 91

S6K1 Ribosomal Protein S6 Kinase, 70kDa, Polypeptide 1. 18, 57

shRNA Short hairpin RNA. 23, 26, 30, 31, 33, 80, 81, 83, 86, 91

SLC25A1 Solute Carrier Family 25 (Mitochondrial Citrate Transporter), Member 1. 62

STAT3 Signal Transducer And Activator Of Transcription 3 (Acute-Phase Response Factor). 57

TAP Tandem Affinity Purification. 18

TCR T Cell Receptor. 17

Glossary

VLDL Very-low-density lipoproteins. 4, 7, 51, 54, 66, 71, 95

WD40 WD or beta-transducin repeats are short ~40 amino acid motifs with terminal Trp-Asp (W-D) dipeptide. 12