N° d'ordre :

THÈSE

Présentée a

L'UNIVERSITÉ DE PAU ET DES PAYS DE L'ADOUR

ÉCOLE DOCTORALE 211 - SCIENCES EXACTES ET LEURS APPLICATIONS

par **Weiwei DAI**

Pour obtenir le grade de

DOCTEUR

SPÉCIALITÉ : **Sciences agronomiques, biotechnologies agro-alimentaires**

Amino acids regulate hepatic intermediary metabolism-related gene expression via mTORC1-dependent manner in rainbow trout (*Oncorhynchus mykiss*)

****

Les acides aminés régulent l'expression des gènes du métabolisme intermédiaire chez la truite par le biais de mTORC1 (*Oncorhynchus mykiss*)

Soutenue le: 27 October, 2015

Devant la commission d'examen formée de:

Mme. BONNIEU, Anne, Directrice de Recherche, INRA	Rapporteur
M. MAZURAIS, David, Ingénieur de Recherche HDR, Ifremer	Rapporteur
M. DAVAIL, Stéphane, Professeur, Université de Pau et des Pays de l'Adour	President
M. DUCHAMP, Claude, Professeur, Université de Lyon	Examinatrice
Mme. SKIBA-CASSY, Sandrine, Directrice de recherche, INRA	Directeur de thèse

I dedicate this thesis to INRA, NuMeA—where I grew and made progresses

----------*Weiwei DAI*

Table of Contents

LIST OF ABREVIATIONS

Abbreviations	Full name
Δ6/D6D	Δ6 fatty acyl desaturase
▵9/SCD1	Stearoyl-CoA desaturase-1
4EBP1	eukaryotic initiation factor 4E (eIF4E)-binding protein 1
6PF1K	6-phosphofructo-1-kinase
AACE	amino acid catabolizing enzymes
AAR	amino acid response
AAs	amino acids
ACC	Acetyl-CoA carboxylase
ACLY	ATP citrate lyase
AGC	protein kinases A, G, and C
ALAT	alanine transaminase
AMPK	AMP-activated protein kinase
AS160	Akt substrate of 160 kDa
ASAT	aspartate transaminase
ASNS	asparagine synthetase
ATF	activating transcription factor
Atg 4b	autophagy-related protein 4B
Atg12	autophagy-related protein 12
BCKD	branched-chain α-ketoacid dehydrogenase
BCKDK	branched-chain α-ketoacid dehydrogenase kinase
bHLH-LZ	basic helix-loop-helix–leucine zipper
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CARE	(C/EBP-ATF) response elements
CAT	cationic amino acid transporter 1
CBP	CREB-binding protein
CHO	carbohydrate
CHOP	C/EBP homology protein
ChREBP	carbohydrate responsive element-binding protein
CPT 1a	carnitine palmitoyltransferase 1 alpha
CPT 1b	carnitine palmitoyltransferase 1 beta
CREB	cAMP response element-binding protein
CREBH	ER-bound transcription factor
CRTC2	CREB regulated transcription coactivator
CRTCs	CREB regulated transcription coactivators
DGAT	diacylglycerol acyltransferase

DHA	docosahexaenoic acid
DNL	*de novo* lipogenesis
EAAs	essential amino acids
eEF2K	eukaryotic elongation factor 2 kinase
EF1	Eukaryotic translation elongation factor 1 alpha 1
EGF	epidermal growth factor
eIF2α	translation initiation factor 2α
Elovl2	elongation of very long chain fatty acids like-2
ElovL5	elongation of very long chain fatty acids like-5
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ERRα	estrogen-related receptor α
FA	fatty acids
FAD	fatty acid desaturases
FAS or FASN	fatty acid synthase
FBPase	fructose-1,6-bisphosphatase
FKBP12	12 kDa FK506-binding protein
FKHR	forkhead related protein
FM	fish meal
FO	fish oil
FOXO	Forkhead box protein O
G6P	glucose 6-phosphate
G6Pase I	glucose-6-phosphatase I
G6Pase II	glucose-6-phosphatase II
G6PDH	glucose-6-phosphate dehydrogenase
Gab1	Grb2-associated binder-1
Gabarapl1	gamma-aminobutyric acid
GAP	GTPase-activating protein
GCKR	glucokinase regulatory protein
GCN2	general control non-derepressible 2
GEF	guanine nucleotide exchange factor
GH	growth hormone
GK or GCK	glucokinase
GLS	glutaminase
Glu	glucose
GLUT	glucose transporter
GP	glycogen phosphorylase
GPCR	G protein-coupled receptor
GRB10	growth factor receptor-bound protein 10
GRB2	growth factor receptor-bound protein 2
GS	glycogen synthase
GSK3	glycogen synthase kinase 3

GTT	glucose tolerance test
HAA	high level of amino acids
HBP	hexosamine biosynthetic pathway
HERP	homocysteine-induced ER protein
HIF-1α	hypoxia-inducible factor 1-alpha
HK	hexokinase
HNF4α	hepatic nuclear factor-4-alpha
HNF6	hepatic nuclear factor 6
HOAD	3-hydroxyacyl-CoA dehydrogenase
HPHC	high protein high carbohydrate
HPLC	high protein low carbohydrate
IGF1	insulin-growth factor-1
IKK	IkappaB kinase
IP	intraperitoneal
IR	insulin receptor
IRS	insulin receptor substrates
JNK	c-Jun-N-terminal kinase
LAA	low level of amino acids
LATS	large tumour suppressor homologue
LC3B	light chain 3B
LCFA	long-chain fatty acids
LiRiKO	liver-specific rictor knockout
LPHC	low protein high carbohydrate
LXR	liver X receptor
MAP	mitogen-activated protein
MCD	Malonyl-CoA decarboxylase
ME	malic enzyme
mLST8	mammalian lethal with SEC thirteen 8
mPEPCK	metochonial phosphoenolpyruvate carboxykinase
MST1	mammalian sterile 20–like kinase-1
mTOR	mechanistic target of rapamycin
mTORC1	mechanistic target of rapamycin complex 1
mTORC2	mechanistic target of rapamycin complex 2
MUFA	monounsaturated fatty acid
NAFLD	nonalcoholic fatty liver disease
O-GlcNAc	*O*-linked β-N-acetylglucosamine
OGT	*O*-GlcNAc transferase
p38MAPK	mitogen-activated protein kinase p38

PBS	phosphate buffered saline
PDCD4	programmed cell death 4
PDGF	platelet-derived growth factor
PDK1	phosphoinositide dependent protein kinase 1
PEPCK tot	phosphoenolpyruvate carboxykinase total
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1α
PH	pleckstrin-homology
PI3K	phosphoinositide 3-kinase
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
PK	pyruvate kinase
PKA	protein kinase A
PKB or Akt	protein kinase B
PKC	protein kinase Ctheta
PKC-α	protein kinase C-α
PML	promyelocytic leukemia
Pol 1	Polymerase I
PP2A	protein phosphatase 2A
PPAR	peroxisome proliferator-activated receptor
PPP	pentose phosphate pathway
PRAS40	phosphorylation of proline-rich Akt substrate of 40 kDa
PRTE	pyrimidine-rich translational element
PTB	phosphotyrosine-binding
PtdIns3P	phosphatidylinositol-3-phosphate
p-βOx	peroxisomal β-oxidation
RAG	RAS-related GTP-binding protein
Rapa	rapamycin
RAPTOR	regulatory-associated protein of mTOR
REDD1	regulation of DNA damage response 1
RHEB	Ras homolog enriched in brain
RIA	homologous radioimmunoassay
RICTOR	rapamycin-insensitive companion of mTOR
ROK	Rho kinase
ROS	reactive oxygen species
rRNA	ribosomal RNA
RSK	p90 ribosomal S6 kinase
RTKs	receptor tyrosine kinases
S or Ser	serine residues
S6K1	p70 ribosomal S6 kinase 1
SCAP	SREBP cleavage-activating protein
SD	serine dehydratase
SGK1	protein kinase 1
SH2	Src-homology-2
SHC	Src-homology-2-containing protein
SIK2	salt-inducible serine/threonine kinase 2

SIN1	SAPK-interacting 1
SKAR	S6K1 aly/REF-like target
SLC	solute carrier
SNAT2	System A neutral amino acid transporter 2
SRE	sterol response element
SREBP1	sterol regulatory element binding protein 1-like
T2D	type 2 diabetes
TAG	triacylglycerol
TBC1D7	TBC1 (TRE2–BUB2–CDC16) domain family member 7
TCA	tricarboxylic acid cycle
TFEB	the transcription factor EB
TRB3	Tribbles 3
TSC	tuberous sclerosis complex
ULK1 or ATG1	UNC-51-like kinase 1
USF1	upstream transcription factor 1
VEGF	vascular endothelial growth factor
VLDLs	very low-density lipoproteins
VPS34	vacuolar protein sorting 34
Y or Thr	tyrosine residues
YAP	Yes-associated protein

LIST OF FIGURES

LIST OF TABLES

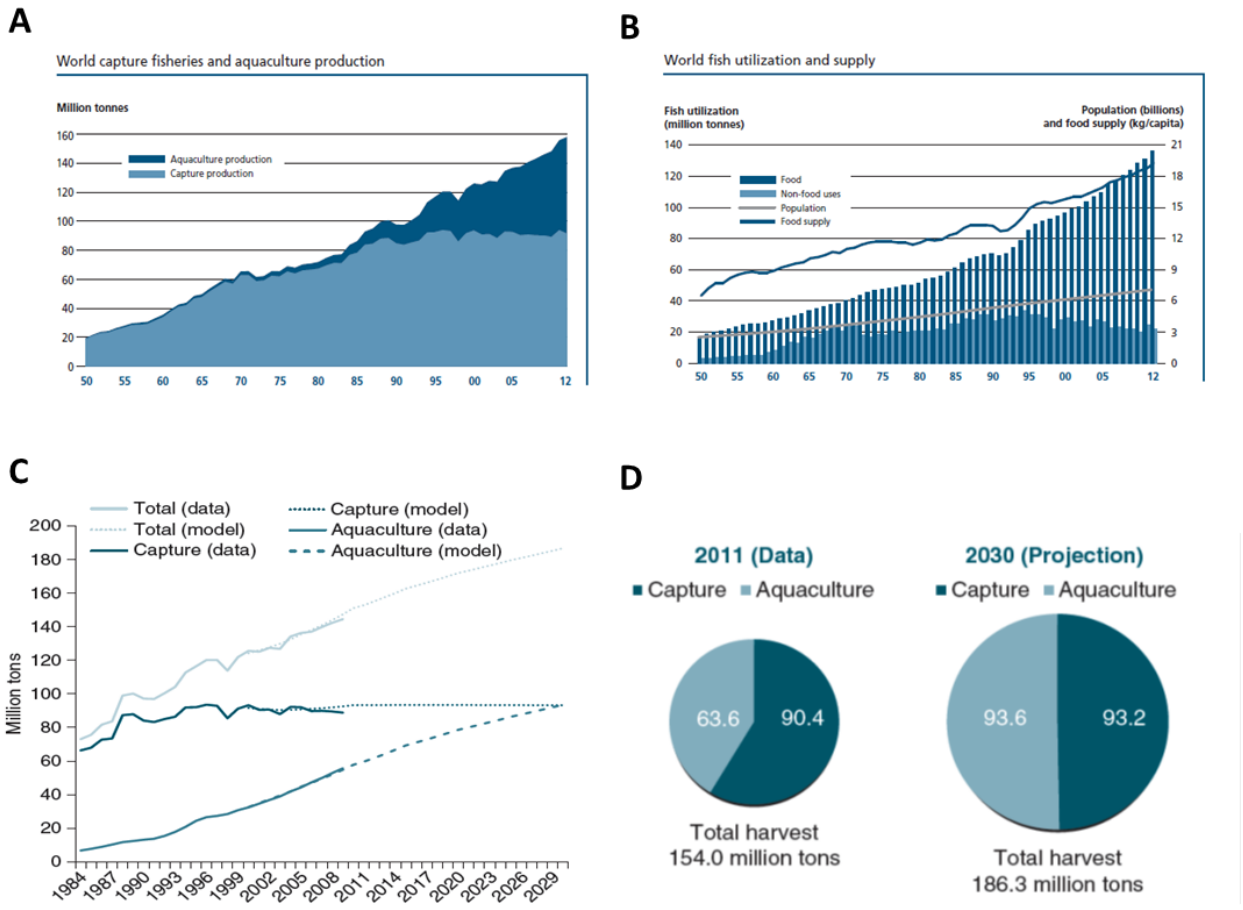
Chapter 1

INTRODUCTION

AND

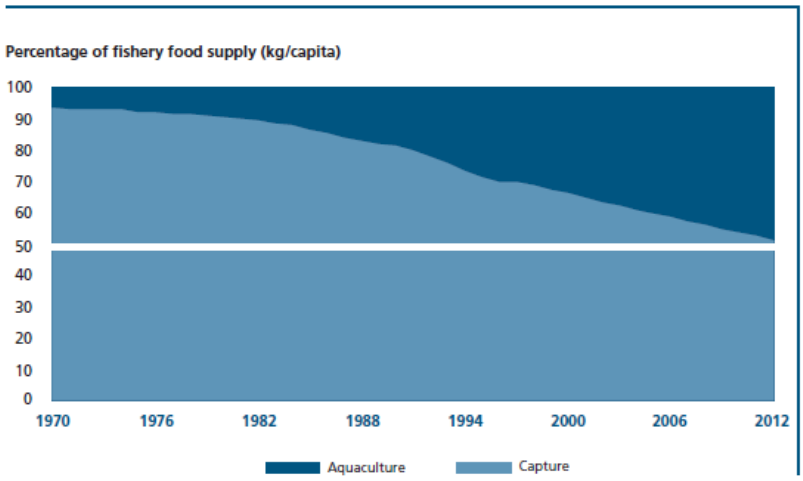
REVIEW OF LITERATURE

Figure 1. 1 Aquaculture developments: current status, trends and future prospects



A: World capture fisheries and aquaculture production (FAO, 2014); B: World fish utilization and supply (FAO, 2014); C: Global fish production: data and projections, 1984–2030 (The World Bank, 2013); D: Volume and share of capture and aquaculture production in global garvest (The World Bank, 2013).

Figure 1. 2 Relative contribution of aquaculture and capture ¬fisheries to food fish consumption



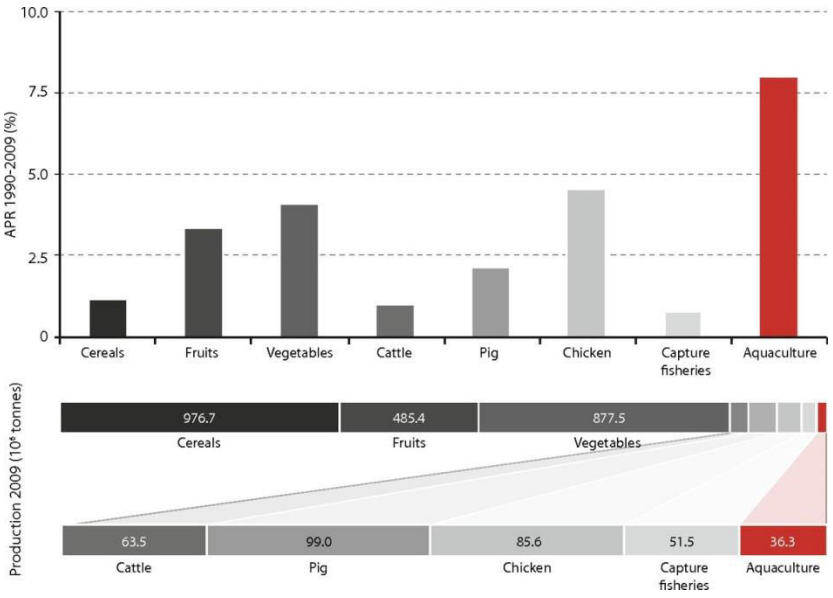
1.1 Aquaculture development: current status, trends and future prospects

1.1.1 Aquaculture's role in the global good system, trends and prospects

Fish is an important food commodity and currently accounts for 17% of animal-derived and 6.5% of total human protein consumption globally (FAO, 2014). Fish products comprise one of the most widely traded segments of the world food economy, valued at US$129 billion in 2012 (Troell et al., 2014). World per capita apparent fish consumption has increased dramatically during the past 50 years, from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 (FAO, 2014). Fish provided more than 2.9 billion people with almost 20 percent of their intake of animal protein and 4.3 billion people with about 15 percent of such protein (FAO, 2014). Fish is not only an excellent source of protein, but also a good source of highly unsaturated long-chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are important for optimal brain and neural system development in children, micronutrients (vitamins and minerals) and some less well-known nutrients such as taurine and choline. All these nutrient compositions make fish a truly important and unique food for human (FAO, 2014; Tacon and Metian, 2013).

Global fish production has grown steadily in the last five decades (Figure 1.1 A and B), with food fish supply increasing at an average annual rate of 3.2 percent, outpacing world population growth at 1.6 percent (FAO, 2014). Although capture fisheries provided most of the supply during the 1960s–1970s, aquaculture has contributed virtually all of the growth in per capita availabilities since the turn of the century (Troell et al., 2014). Aquaculture currently provides roughly half of the fish consumed worldwide (Figure 1.2), and its share is expected to increase in the future as wild fisheries reach or exceed their sustainable limits and meanwhile aquaculture technology and management continue to improve (Troell et al., 2014). Capture fisheries landings as a whole have plateaued at around 85–95 Mt (million metric tons, or megatonnes) per year. Moreover, there has been a gradual shift in wild fish capture from large and valuable carnivorous species to smaller, less valuable species that feed at lower trophic levels. Although catch rates for some species have not declined during the 1990s, most ocean fisheries stocks are now recognized as over or fully exploited (Naylor et al., 2009). Aquaculture production, meanwhile, has surged, particularly during the past 20–30 years (Naylor et al., 2000). In 2012, aquaculture industry set an all-time record, providing 67 million tons fish products, which accounts for about 49 percent of the fishery output for human consumption (FAO, 2014). In fact, aquaculture is the fastest growing food sector and continues to expand alongside terrestrial crop and livestock production (Figure 1.3) (Troell et al., 2014). In addition to rapid growth in volume and value, the sector is characterized by substantial investment in many regions of the world and rapid innovation in the breeding of cultured species, feed practices, and rearing systems (Naylor et al., 2000).

Figure 1. 3 Comparison of growth of aquaculture and main food commodities/groups



Comparison of growth of aquaculture and main food commodities/groups (Troell et al., 2014): Growth expressed as annual percentage for the period 1990–2009 and production for 2009. Data on meat from animals obtained from Tacon and Metian (Tacon and Metian, 2013) and FAO/FAOSTAT (FAO, 2013b). Similar sources (FAO, 2013a; FAO, 2013b) for data on production of cereals, fruits, and vegetables. The cultivation of fish and shellfish ("fish") in terrestrial freshwater and marine systems grew at an annual rate of 7.8% worldwide between 1990 and 2010; a rate that substantially exceeded that of poultry (4.6%), pork (2.2%), dairy (1.4%), beef (1.0%), and grains (1.4%) over the same period.

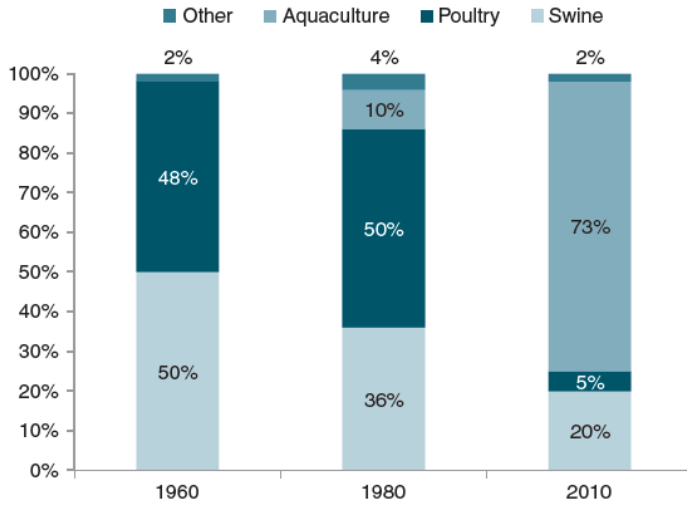
Based on the prediction of "Fish to 2030" (The World Bank, 2013), the projected capture production remains fairly stable over the 2010–2030 period (Figure 1.1 C). In contrast, the global aquaculture projection maintains its steady rise from historical levels, reaching the point where it equals global capture production by 2030 (Figure 1.1C and D). Global fish supply is projected to rise to 187 million tons by 2030 (OECD-FAO, 2012; The World Bank, 2013). As shown in Figure 1.1 D, while the share of capture fisheries is nearly 60 percent of global production in 2011, it is expected to fall to exactly half by 2030, after growing only by 2.8 million tons. Aquaculture is expected to grow by 30 million tons over this same period. In terms of food fish production, it predicts that aquaculture will contribute 62 percent of the global supply by 2030.

1.1.2 Towards sustainable aquaculture

Historically and nutritionally fish meal (FM) and fish oil (FO) are dominant ingredients in compound feeds for carnivorous finfish and marine shrimp (Naylor et al., 2000). These two ingredients respectively supply essential amino acids (such as lysine and methionine) that are deficient in plant proteins and highly unsaturated fatty acids (EPA and DHA) not found in vegetable oils (De Silva and Anderson, 1994).

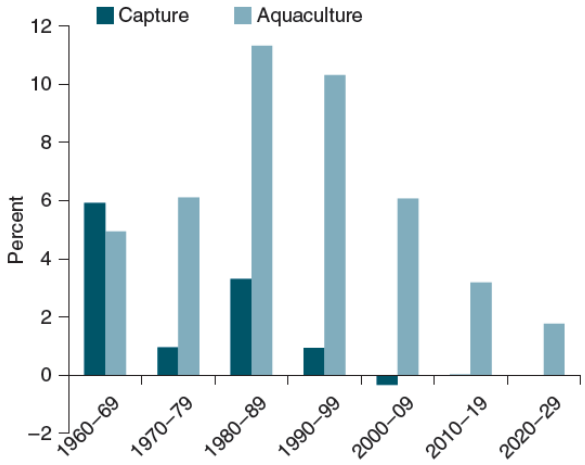
However, FM and FO production relies on wild forage fish stocks, which are now under a sustainable management to preserve this resource on the long term, and to ensure a constant availability of marine raw materials each year (FAO, 2012). Globally, 20 to 30 million metric tons (mmt) of reduction fish (1/4 to 1/3 of the global fish catch) are currently used for FM and FO production (Naylor et al., 2009), and the proportion is expected to remain unchanged as the capture production is projected to maintain nearly zero growth from 2010 to 2030 (The World Bank, 2013). With the production of farmed fish eclipsing that of wild fish, one major transition is already underway: aquaculture's share of global FM and FO consumption more than doubled over the past decade to 73% (Figure 1.4) and 88%, respectively (Naylor et al., 2009). As world aquaculture will keep growing in the coming decades (Figure 1.5), the demands for aquafeeds will certainly keep increasing as well, thus it is clear that the stagnant availability of wild-caught fish cannot go on feeding the growing aquaculture industry in the future, and the feed manufacturers must decrease their reliance on finite marine raw materials if they want to maintain their growth and profits. From the nutritional aspect, reducing aquaculture's dependence on marine resources highly depends on improving feed efficiencies and substituting away forage fisheries with suitable and sustainable nonfish-based alternatives, despite impressive gains have been already achieved in the past few decades (FEAP, 2011; Naylor et al., 2009).

Figure 1. 4 Global fishmeal utilization



The utilization of global fishmeal has evolved over the past half century. The importance of aquaculture as user of fishmeal has grown substantially as a result of the industry's rapid growth since the 1980s. From this observation, it is likely that fishmeal-intensive segments of the aquaculture industry (for example, salmon and shrimp) increasingly affect the dynamics of fishmeal and fish oil markets. In this context, modeling of demand for fishmeal and for fish oil as a function of aquaculture production seems essential (FAO, 2014).

Figure 1. 5 Average annual growth rates of capture and aquaculture production, 1960–2029



These trends are consistent with the overview given in the introductory chapter and reinforce the importance of aquaculture in augmenting global fish supply. However, the growth of aquaculture is expected to further decelerate. The projected annual growth rates for the projection periods of 2010–19 and 2020–29 are shown above. For these two periods, the projected growth rate of aquaculture production is below the level in the 1960s. Nearly zero growth is projected for capture production (The World Bank, 2013).

1.1.3 The importance of protein/amino acids research for aquaculture

Dietary intake of proteins is essential for animal growth and development (Tremblay et al., 2007). The ingested proteins are digested or hydrolyzed to release tri- or di-peptides and free amino acids (AAs), which are absorbed from the intestinal tract and distributed by the blood to the organs and tissues. These AAs are primarily used by the various tissues to synthesize new protein and polypeptides. Furthermore, AAs serve as precursors of non-protein nitrogenous high molecular weight compounds (such as nucleic acid, porphyrine and creatine), and substrates for energy. Additionally, AAs are key precursors for syntheses of hormones and low-molecular weight nitrogenous substances with each having enormous biological importance and also regulate key metabolic processes and pathways. Moreover, there is growing recognition that AAs also act as cell signaling molecules and regulators of gene expression and protein phosphorylation cascades (Wu, 2009; Wu et al., 2014).

Fish is a unique model for studying protein/AAs metabolism

Despite the well-studied roles of protein/AAs in modulating metabolic pathways and associated regulatory processes in mammals, relatively little is known about fish (Kaushik and Seiliez, 2010). Nevertheless, fish is an important and unique research model for studying protein/AAs metabolism as plenty of physiological and metabolic differences in protein/AAs metabolism have been identified between fish and terrestrial animals. For instance, the great majority of fish excrete 80% of their nitrogen from nitrogenous catabolism in the form of ammonia, a minimal cost for discharging

nitrogenous waste, while mammals and birds excrete urea and uric acid, respectively, after energy-expensive syntheses (Guillaume et al., 2001). Due to this unique manner of nitrogen excretion and the lack of urea cycle, arginine becomes one of the essential AAs (EAAs) for fish, while in uricotelic animals it can at least partly be provided by the urea cycle. Furthermore, fish possess relatively high dietary protein/AAs requirement, with AAs, rather than glucose, are preferentially used as energy substrates (Guillaume et al., 2001; Halver and Halver, 2002). Moreover, cysteine and tyrosine are also identified as two semi-EAAs since they can be only synthesized from the "coupling" of serine-methionine and phenylalanine respectively, and proline and glutamine are termed "semi-indispensable" due to their slow synthesis (Guillaume et al., 2001). Recent studies also revealed the essentiality of dietary taurine for many commercially relevant species, especially marine teleosts as they have low or negligible ability of taurine synthesis due to the absence or low cysteinsulphinate decarboxylase activity (Pinto et al., 2013), similar with domestic cat, a mammalian carnivore (Macdonald et al., 1984).

The importance for studying AA metabolism in fish

Based on traditional approaches (e.g., digestibility trials, nitrogen balance, assessments of growth, feed utilization and reproductive performance, isotope tracer techniques, as well as northern and western blots), significant developments have been already made for studying gross protein requirements, qualitative AA requirements, AA utilization and deposition, substitution of fish meal with alternative protein sources in the last half century (Guillaume et al., 2001; Halver and Halver, 2002; Kaushik and Seiliez, 2010; Wilson and Halver, 1986). However, due to the complexities and handicaps in studying fish nutrition, i.e. difficulties in study linked to the environment and the nutritional characteristics of fish, the large number of fish species and currently poorly applied advanced techniques in fish nutrition studies, our knowledge regarding the roles of AAs in regulating gene expression, cellular signaling pathways and intermediary metabolism remains limited (Kaushik and Seiliez, 2010). Therefore, it would be desirable to go on exploring the physiological and metabolic importance of protein/AAs at molecular and mechanism levels so as to expand our basic knowledge of AA biochemistry and nutrition in fish. Furthermore, the emergence of advanced bio-molecular method applied to fish (e.g., high-throughput functional genomics, microarray, metabolomics and proteomics) offers us new opportunities to do so. From the practical aspect, how to effectively improve the utilization of protein/AAs, especially the protein from non-fishmeal sources in fish is the key to go on improving feed efficiency, reducing the inclusion rates of FM in aquafeeds and cutting the rising costs for the feed enterprises. Thus, understanding the mechanisms underlying the regulation of intermediary metabolism, gene expression and cellular signaling pathways by protein/AAs may have both scientific and practical significance.

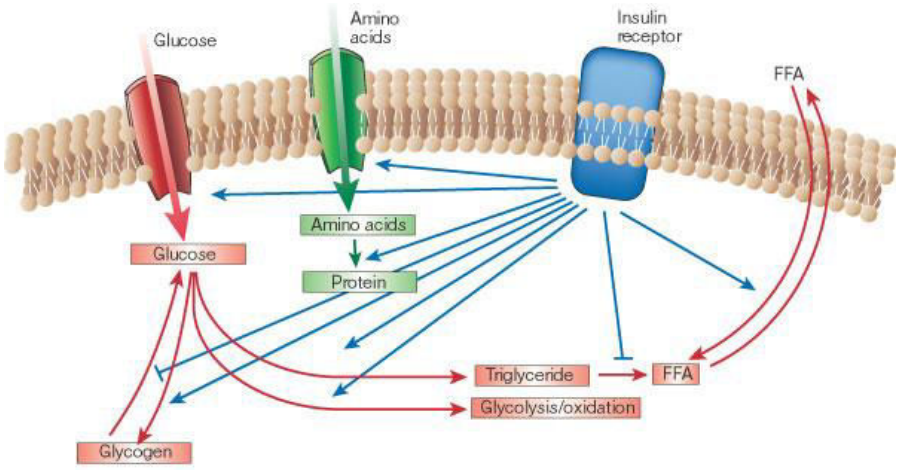
1.2 Cellular signaling

In this section, we briefly introduce two cellular signaling cascades, insulin signaling pathway and amino acid signaling pathway, including mechanistic target of rapamycin (mTOR) and amino acid response (AAR) pathways. The efforts mainly focus on the regulation of some crucial nodes, i.e. insulin receptor substrates (IRS), Akt, the mechanistic target of rapamycin complex 1 and 2 (mTORC1 and mTORC2), and the mechanisms by which they regulate intermediate metabolism (mainly in liver).

1.2.1 Insulin signaling in mammals

Insulin is a hormone released by pancreatic beta cells in response to elevated levels of nutrients in the blood, which serves as the primary regulator of blood glucose concentration. Insulin is the most potent anabolic hormone responsible for the control of cellular uptake, utilization and storage of glucose, amino acids and fatty acids by stimulating lipogenesis, glycogen and protein synthesis, respectively, while inhibiting the breakdown of glycogen, protein and fat (Figure 1.6). Insulin resistance or deficiency results in profound dysregulation of these processes, and produces elevations in fasting and postprandial glucose and lipid levels (Saltiel and Kahn, 2001; Wallis et al., 1985).

Figure 1. 6 The regulation of metabolism by insulin



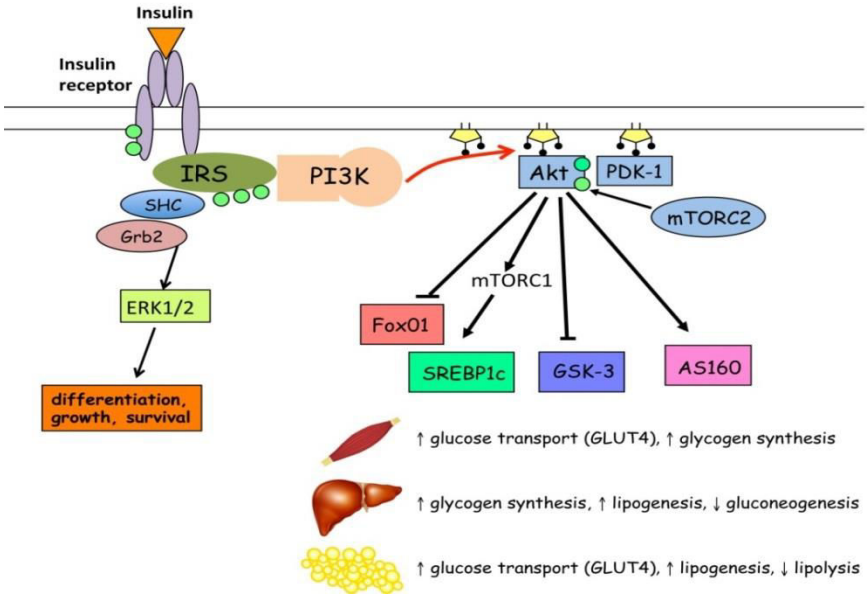
Insulin is the most potent anabolic hormone known, and promotes the synthesis and storage of carbohydrates, lipids and proteins, while inhibiting their degradation and release into the circulation. Insulin stimulates the uptake of glucose, amino acids and fatty acids into cells, and increases the expression or activity of enzymes that catalyze glycogen, lipid and protein synthesis, while inhibiting the activity or expression of those that catalyze degradation (Saltiel and Kahn, 2001).

1.2.1.1 Overview of insulin signaling pathway and some critical nodes

Growth factors such as insulin activate the insulin receptor (IR) tyrosine kinase, which phosphorylates and recruits different substrate adaptors such as the IRS family of proteins (Siddle, 2011). Tyrosine

phosphorylated insulin receptor substrates (IRS) then displays binding sites for numerous signaling partners (Taniguchi et al., 2006). Among them, phosphoinositide 3-kinase (PI3K) has a major role in insulin function, mainly via the activation of the Akt cascades (Manning and Cantley, 2007). Activated Akt induces glycogen synthesis through inhibition of glycogen synthase kinase 3 (GSK-3), protein synthesis via the activation of the mechanistic target of rapamycin (mTOR) pathway, and cell survival through inhibition of several pro-apoptotic agents (Forkhead box protein O (FoxO), mammalian sterile 20–like kinase-1(MST1) and GSK-3) (Siddle, 2011). Akt phosphorylates FoxO and directly inhibits transcription factors, which regulates metabolism and autophagy (Figure 1.7). Insulin stimulates glucose uptake in muscle and adipocytes via translocation of glucose transporter 4 (GLUT4) vesicles to the plasma membrane (Rowland et al., 2011). In addition, insulin signaling inhibits gluconeogenesis in the liver, through disruption of CREB/CBP/mTORC2 binding (Manning and Cantley, 2007). Insulin signaling induces fatty acid and cholesterol synthesis via the regulation of SREBP transcription factor (Wong and Sul, 2010). Insulin signaling also promotes fatty acid synthesis through activation of upstream transcription factor 1 (USF1) and liver X receptor (LXR) (Shao and Espenshade, 2012).

Figure 1. 7 Simplified insulin signaling pathway



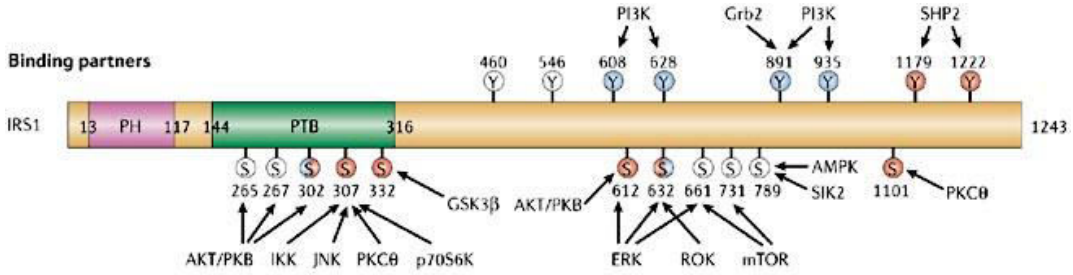
Binding of insulin to the insulin receptor initiates a signaling cascade that involves multiple phosphorylation events (green circles) and regulates glucose and lipid metabolism. In skeletal muscle, insulin promotes the translocation of the glucose transporter GLUT4 to the plasma membrane to increase glucose uptake and also stimulates glycogen synthesis. In liver, the major hepatic actions of insulin are the promotion of glycogen and lipid synthesis and the suppression of gluconeogenesis. In adipose tissue, insulin stimulates GLUT4-mediated glucose uptake and lipid synthesis, and additionally represses lipolysis, leading to net lipid accumulation. IRS, insulin receptor substrate; SHC, Src Homology 2 domain; GRB2, growth factor receptor-bound protein 2; ERK, extracellular-signal-regulated kinases or classical MAP kinases; PI3K Phosphoinositide 3-kinase; PDK1, phosphoinositide dependent protein kinase 1; mTORC mammalian target of rapamycin complex; FoxO1 Forkhead box protein O1; SREBP1c sterol regulatory element binding protein 1c; GSK-3, glycogen synthase kinase 3; AS160, 160 kDa Akt substrate. Picture from (Turner, 2013).

Insulin receptor substrates

At least 11 intracellular substrates of the insulin/ insulin-growth factor-1 (IGF1) receptor kinases have been identified. Six of these belong to the family of IRS proteins, and have been termed IRS1–6. Other substrates of the IR/IGF1R include Grb2-associated binder-1 (Gab1), Cas-Br-M (murine) ecotropic retroviral transforming sequence homologue (Cbl) and the various isoforms of Src-homology-2-containing protein (Shc). IRS1 and IRS2 are widely distributed, whereas IRS3 is largely limited to the adipocytes and brain, and IRS4 is expressed primarily in embryonic tissues or cell lines. IRS5 and IRS6 seem to have limited tissue expression and function in signaling (Taniguchi et al., 2006).

The IRS proteins contain up to 20 potential tyrosine-phosphorylation sites that, after phosphorylation by the IR, bind to intracellular molecules that contain Src-homology-2 domains (SH2 domains), such as the regulatory subunit of PI3K, Nck, Fyn, Grb-2, and SHP2 (Taniguchi et al., 2006). The ability of the IRS proteins to mediate intracellular signaling is mainly regulated by the action of tyrosine phosphatases (Taniguchi et al., 2006). However, IRS proteins also undergo serine phosphorylation in response to insulin and other stimuli, including cytokines and free fatty acids (Figure 1.8) (Gual et al., 2005). There are over 70 potential serine phosphorylation sites in IRS1, and in general, hyper-Ser phosphorylation serves as a negative feedback to down-regulate IRS1 function and can eventually promote IRS1 degradation and insulin resistance (Boura-Halfon and Zick, 2009; Taniguchi et al., 2006). Ser phosphorylation of IRS1 at critical sites can block Tyr phosphorylation and prevent IRS1 binding to the IR (Liu et al., 2004).

Figure 1. 8 Structure and interacting partners of the insulin receptor substrate 1



Insulin-receptor substrate 1 (IRS1) has a pleckstrin-homology (PH) domain (magenta), a phosphotyrosine-binding (PTB) domain (dark green) and several sites of phosphorylation on tyrosine and serine residues. The positions of the tyrosine residues (Y) that are phosphorylated by the IR and the downstream-signalling proteins that bind to these sites are shown. The positions of the serine residues (S) and the kinases responsible for their phosphorylation are also shown. Blue circles represent sites of positive regulation, whereas red circles represent sites of negative regulation. A combination of both colours shows sites in which the regulation has been reported to be either positive or negative under various conditions. White circles represent sites in which the effect of phosphorylation is currently unknown. Several proteins bind to or phosphorylate IRS, including phosphatidylinositol 3-kinase (PI3K), growth-factor-receptor-bound protein-2 (Grb2), Src-homology-2 (SH2) domain-containing tyrosine phosphatase-2 (SHP2), AKT/protein kinase B (PKB), IkappaB kinase (IKK), c-Jun-N-terminal kinase (JNK), protein kinase Ctheta (PKCtheta), p70 ribosomal protein S6 kinase (p70S6K), glycogen synthase kinase-3 beta (GSK3β), extracellular signal-regulated kinase (ERK), Rho kinase (ROK), target of rapamycin (TOR), AMP-activated protein kinase (AMPK), salt-inducible serine/threonine kinase 2 (SIK2)(Taniguchi et al., 2006).

Akt is a critical node

Akt, also known as protein kinase B (PKB), is a crucial signaling node in insulin signaling pathway, which is a direct downstream target of PI3K protein (Manning and Cantley, 2007). Once recruited to the plasma membrane, Akt is phosphorylated at two sites, one within the T-loop of the catalytic domain (Thr308) by the phosphoinositide-dependent kinase 1 (PDK1) and within the carboxyl terminal hydrophobic domain (Ser473) by the mechanistic target of rapamycin complex 2 (mTORC2) (Alessi et al., 1997; Shimobayashi and Hall, 2014). Current data in aggregate suggest that mTORC2-dependent phosphorylation of Akt is required for the full range of PI3K-dependent signaling to mTORC1, namely Akt S473 phosphorylation is required to fully activate Akt activity (Dibble and Cantley, 2015). However, loss of phosphorylation at Ser473 site affects only some Akt substrates, such as FoxO transcription factors, but not tuberous sclerosis complex-2 (TSC2), in response to growth factor signaling (Chiarini et al., 2015). Akt kinases control an array of diverse functions including cell proliferation and survival, cell size and response to nutrient availability, cell growth and metabolism, tissue invasion and angiogenesis (Altomare and Testa, 2005; Manning and Cantley, 2007; Testa and Tsichlis, 2005).

The Akt kinase family is comprised of three highly homologous isoforms: Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ) (Gonzalez and McGraw, 2009). The specificity of action of different Akt isoforms results from differences in their tissue distribution, subcellular localization and downstream-signaling targets. The relative ratios of expression of the three isoforms vary considerably among tissues. Akt3 is predominantly expressed in the nervous system and testis, whereas Akt1 and Akt2 are widely distributed, with Akt2 particularly enriched in insulin-sensitive tissues, such as liver and fat (Taniguchi et al., 2006). Demonstrations using knockout mice and siRNA indicate that the isoforms are involved in the regulation of different biological processes. Deletion of Akt1 results in growth retardation and reduced lifespan, but no metabolic abnormalities. By contrast, Akt2 knockout mice develop a type 2 diabetes-like phenotype, and cells derived from those mice show impaired glucose utilization, suggesting a central role for Akt2 in the maintenance of glucose homeostasis (Gonzalez and McGraw, 2009). Akt3 does not seem to have a role in glucose homeostasis, but has significant effects on neural development as Akt3 knockout mice display impaired brain development (Taniguchi et al., 2006).

1.2.1.2 Insulin regulates glucose metabolism

Insulin, secreted by the β-cells of the pancreas, is directly infused via the portal vein to the liver, where it exerts profound metabolic effects. One of the most important physiological functions of Akt is to modulate glucose metabolism, including stimulation of glucose uptake and glycolysis, promotion of glycogen synthesis and inhibition of gluconeogenesis (Figure 1.7) (Saltiel and Kahn, 2001).

Insulin stimulates glucose uptake

Among all the 13 glucose transporter proteins (GLUT1-GLUT12, and HMIT), glucose transporter 4 (GLUT4) displays the unique characteristic of a mostly intracellular disposition in the unstimulated state that is acutely redistributed to the plasma membrane in response to insulin and other stimuli. Thus, it represents a key determinant of glucose homeostasis (Huang and Czech, 2007). Insulin increases glucose uptake in cells by stimulating the translocation of the glucose transporter GLUT4 from intracellular sites to the cell surface, where it facilitates the reduction of plasma glucose levels (Bryant et al., 2002). Once activated by insulin, Akt stimulates Glut4 translocation through phosphorylation and inhibition of the Rab-GTPase-activating protein, AS160 (Akt substrate of 160 kDa), which triggers the activation of Rab small GTPases that are involved in the cytoskeletal re-organization that is required for the translocation of the GLUT4 to the plasma membrane (Taniguchi et al., 2006). Akt also phosphorylates and inhibits tuberin (also known as TSC2), which in turn stimulates glucose uptake through an mTOR dependent manner (the detailed mechanisms are explained in the TOR signaling pathway section) (Harris and Lawrence, 2003). Although insulin does not stimulate glucose uptake in liver (GLUT2), it blocks glycogenolysis and gluconeogenesis, and stimulates glycogen synthesis, thus regulating fasting glucose levels (Saltiel and Kahn, 2001).

Insulin stimulates glycogen synthesis

Insulin stimulates glycogen accumulation through a coordinated increase in glucose transport and glycogen synthesis. The hormone activates glycogen synthase by promoting its dephosphorylation, through the inhibition of kinases such as glycogen synthase kinase-3 (GSK3). Upon its activation downstream of PI3K, Akt phosphorylates and inactivates GSK3, decreasing the rate of phosphorylation of glycogen synthase, thus increasing its activity towards glycogen synthase. GSK3 can also phosphorylate several other substrates and is involved in many processes besides the regulation of glycogen synthesis (Saltiel and Kahn, 2001).

Insulin inhibits hepatic gluconeogenesis

Hepatic gluconeogenesis is essential for maintenance of normal blood glucose concentrations and insulin is the most important hormone to inhibit gluconeogenesis (Barthel and Schmoll, 2003; Saltiel and Kahn, 2001). Insulin suppresses gluconeogenesis at the transcriptional level by the transcription factors Forkhead box O1 (FoxO1) (Accili and Arden, 2004), peroxisome proliferator-activated receptor γ coactivator 1α (PGC)-1α (Li et al., 2007; Zhou et al., 2004), cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) and CREB regulated transcription coactivators (CRTCs) (Han et al., 2015). The phosphorylation of FoxO1 and PGC-1α by Akt reduces its entry into the nucleus, the

expression of key gluconeogenic genes, such as phosphoenolpyruvate carboxykinase (PEPCK), and the net glucose output from the liver (Calnan and Brunet, 2008). Furthermore, insulin directly regulates many cAMP signaling pathways at the transcriptional level by controlling the recruitment of ((cAMP response element–binding) CREB-binding protein) CBP and p300, and subsequently suppresses gluconeogenesis (Zhou et al., 2004). Notably, stimulation of the insulin receptor also results in the activation of the mitogen-activated protein (MAP) kinase cascade (Barthel and Schmoll, 2003). It is reported that insulin is able to stimulate the Raf/MEK/ERK1/2 pathway, which is sufficient to mediate the suppression of PEPCK and glucose 6-phosphatase (G6Pase) gene transcription (Hall et al., 2000; Schmoll et al., 2001). In line with these observations, recent work also shown that hepatic p38α regulates gluconeogenesis by suppressing AMP-activated protein kinase (AMPK) (Jing et al., 2015). However, the physiological significance of MAPK pathway in the context of hepatic glucose production is largely unclear.

1.2.1.3 Insulin stimulates lipid synthesis but inhibits lipid degradation

Insulin signaling also plays an important role in the regulation of fatty acids (FAs) metabolism, underscoring the close relation between lipid and glucose metabolism (Bechmann et al., 2012). Insulin stimulates fatty acids and triglyceride synthesis mainly by increasing the mRNA and the processed nuclear form of sterol regulatory element-binding protein-1c (SREBP-1c) (Jeon and Osborne, 2012; Shao and Espenshade, 2012), a transcription factor that activates all the genes needed to produce fatty acids and triglycerides in liver (Horton et al., 2002). Furthermore, insulin positively regulates Akt-mediated production of very low-density lipoproteins (VLDLs) (Savage and Semple, 2010). Moreover, insulin, along with other mediators (e.g., calpain-1), represses autophagy and thus the associated lipophagy within the hepatocyte, and thus induces lipogenesis and represses lipid degradation in the fed state as well (Rautou et al., 2010).

In adipocytes, glucose is stored primarily as lipid, owing to increased uptake of glucose and activation of lipid synthetic enzymes, including pyruvate dehydrogenase, fatty acid synthase and acetyl-CoA carboxylase (Saltiel and Kahn, 2001). Insulin also profoundly inhibits lipolysis in adipocytes, primarily through inhibition of the enzyme hormone-sensitive lipase (Anthonsen et al., 1998). This enzyme is acutely regulated by control of its phosphorylation state, which is activated by protein kinase A (PKA)-dependent phosphorylation, and inhibited as a result of a combination of kinase inhibition and phosphatase activation. Insulin inhibits the activity of the lipase primarily through reductions in cAMP levels, owing to the activation of a cAMP-specific phosphodiesterase in fat cells (Kitamura et al., 1999).

Insulin stimulates SREBP-1c at multiple levels

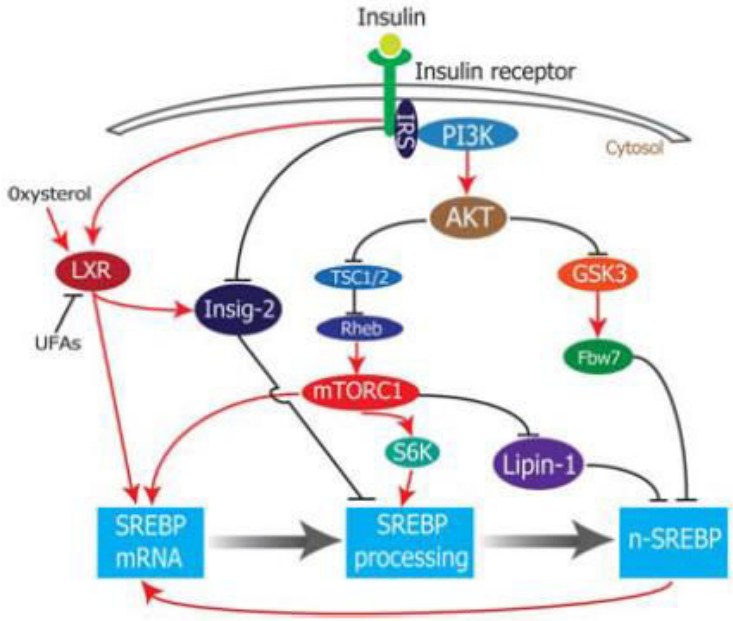
SREBPs comprise a subclass of basic helix-loop-helix–leucine zipper (bHLH-LZ) transcription factors that play a central role in cell metabolism by controlling synthesis of fatty acids, triglycerides, and cholesterol (Goldstein et al., 2006). In mammals, there are three mammalian SREBP isoforms (SREBP-1a, SREBP-1c, and SREBP-2), which are encoded by two genes, SREBF1 and SREBF2, and have distinct but overlapping functions (Shao and Espenshade, 2012). SREBP-1a activates fatty acid and cholesterol synthesis, SREBP-1c fatty acid synthesis, and SREBP-2 is the main regulator of cholesterol metabolism (Jeon and Osborne, 2012). Among the three SREBP-1 isoforms, SREBP-1c is the one whose expression is highest in liver and is the only one primarily controlled by insulin (Owen et al., 2012).

SREBP-1c, like the other two SREBP isoforms, is synthesized as a membrane-bound protein embedded in endoplasmic reticulum (ER) membranes. Immediately after synthesis, SREBP forms a complex with SREBP cleavage-activating protein (SCAP), a polytopic membrane protein that facilitates incorporation of the SREBP into COPII-coated vesicles that bud from the ER and fuse with the Golgi apparatus. There SREBP is processed by two proteases to liberate a soluble fragment that travels to the nucleus and activates transcription. Movement of the SCAP/SREBP complex is inhibited by Insig proteins, intrinsic ER proteins that bind the SCAP/SREBP complex and prevent its movement to the Golgi. The liver produces two Insig isoforms, Insig-1 and Insig-2, both of which retard the movement of SCAP/SREBP complexes (Owen et al., 2012).

SREBPs can be regulated at both transcriptional and posttranslational levels, and by different signaling pathways, which has been comprehensively and profoundly reviewed (Jeon and Osborne, 2012; Owen et al., 2012; Shao and Espenshade, 2012; Xiao and Song, 2013). Generally, insulin regulates SREBPs at multiple levels, including transcription of SREBPs mRNA, proteolytic processing of SREBPs, and stability and abundance of SREBPs (Figure 1.9) (Xiao and Song, 2013). Yecies et al. demonstrated that Akt activates sterol regulatory element binding protein 1-like (SREBP1c) via inhibition of expression of Insig2, an anchor protein that retains SREBP1c on the ER membrane (Yecies et al., 2011). Another proven mechanism by which Akt activates SREBP1c is via Akt-mediated phosphorylation and inhibition of GSK3. GSK3 phosphorylates mature SREBP1 and thereby promotes its degradation by the ubiquitin-proteasome pathway (Bengoechea-Alonso and Ericsson, 2009; Krycer et al., 2010). Furthermore, SREBP processing and activation can be regulated by mTORC1 through Ribosomal S6 kinase (S6K) and lipin 1 leading to the transcriptional induction of the SREBF1 and SREBF2 genes, encoding SREBP1 and SREBP2, respectively (Ricoult and Manning, 2013). Insulin-dependent SREBP-1c transcriptional regulation also requires liver X receptor (LXR) activation (Baranowski et al., 2014; Shao and Espenshade, 2012). Recent work also shown that activated mTOR also promotes SREBP1 maturation through the transcription

factor CREB regulated transcription coactivator 2 (CRTC2), which involves in regulating COPII-dependent SREBP1 transport from ER to Golgi (Han et al., 2015).

Figure 1. 9 The effects of insulin on SREBP pathway



From (Xiao and Song, 2013).

1.2.1.4 Insulin signaling regulates mitochondrial metabolism

Mitochondria are the major functional components of cellular fuel oxidation and ATP production (Cheng et al., 2010). Under normal conditions, mitochondria generate moderately reactive oxygen species (ROS) and enhance insulin sensitivity upon redox regulation of protein tyrosine phosphatase and insulin receptor, while chronic exposure to high ROS levels could alter mitochondrial function and thereby cause insulin resistance (Bonnard et al., 2008; Yu et al., 2006). Studies also suggest that insulin signaling underpins mitochondrial electron transport chain integrity and activity by suppressing FoxO1/HMOX1 and maintaining the NAD+/NADH ratio, the mediator of the SIRT1/PGC1α pathway for mitochondrial biogenesis and function (Cheng et al., 2010; Daitoku et al., 2003).

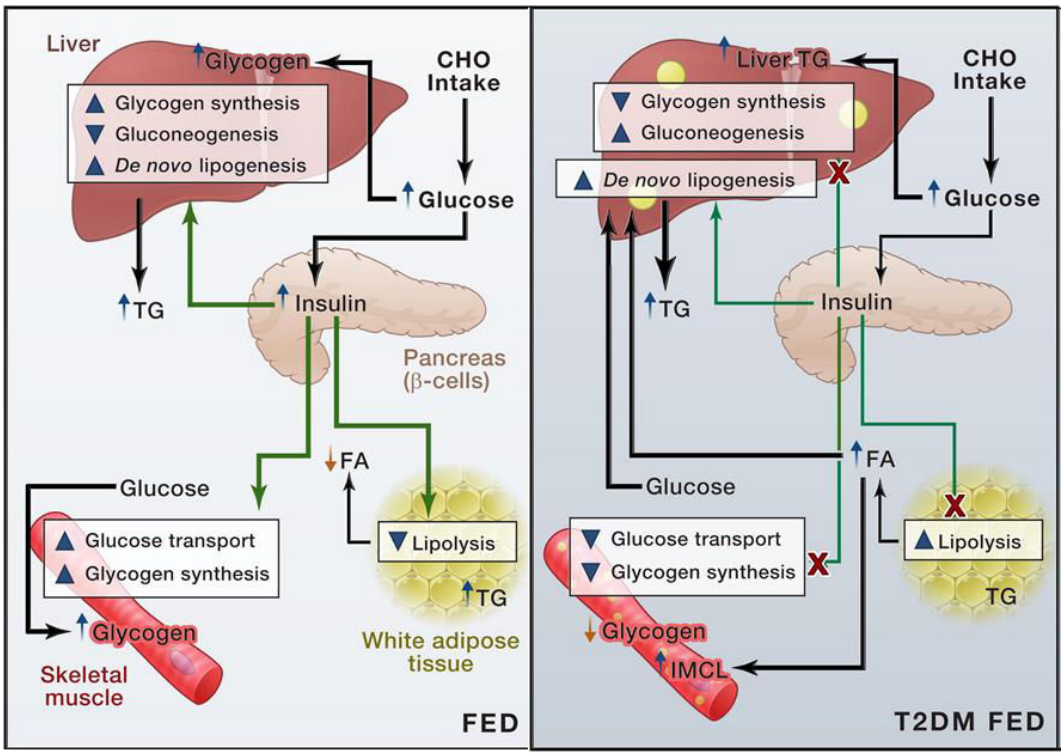
Other downstream metabolic pathways

In addition to the anabolic pathways described above, insulin also controls some other cellular pathways, such as protein and nucleotide synthesis, mitochondrial metabolism and biogenesis, and autophagy, which have been comprehensively and profoundly reviewed previously (Cheng et al., 2010; Laplante and Sabatini, 2012; Manning and Cantley, 2007).

1.2.1.5 Impaired insulin action and insulin resistance

Defective insulin secretion or impaired insulin action may lead to multiple metabolic abnormalities (Muoio and Newgard, 2008). Insulin resistance, which is defined as the inability of insulin to promote efficient glucose uptake by peripheral tissues, is a metabolic condition associated with obesity, type 2 diabetes (T2D), dyslipidemia, and cardiovascular diseases (Laplante and Sabatini, 2010). Under insulin-resistant status, impaired insulin signaling leads to hyperglycaemia due to impaired insulin-stimulated glucose uptake in the skeletal muscle, uncontrolled hepatic glucose production in the liver and hypertriglyceridaemia due to misdelivered glucose, enhanced lipogenesis in the liver, and increased lipolysis in the adipose tissue (Figure 1.10) (Muoio and Newgard, 2008; Samuel and Shulman, 2012). Chronically, these increases in circulating glucose and lipid levels can further impair insulin secretion and action, and cause other forms of tissue damage, thereby inducing or worsening obesity, T2D and nonalcoholic fatty liver disease (NAFLD) (Lynch and Adams, 2014; Newgard, 2012; Zoncu et al., 2011).

Figure 1. 10 Normal and impaired insulin action



(Left) Under normal fed state, dietary carbohydrate (CHO) increases plasma glucose and promotes insulin secretion from the pancreatic β cells. Insulin has numerous actions to promote storage of dietary calories, but only some are illustrated here. In the skeletal muscle, insulin increases glucose transport, permitting glucose entry and glycogen synthesis. In the liver, insulin promotes glycogen synthesis and *de novo* lipogenesis while also inhibiting gluconeogenesis. In the adipose tissue, insulin suppresses lipolysis and promotes lipogenesis.

(Right) In type 2 diabetes, insulin signaling is impaired (as depicted by the red "x"). With accumulation of intramyocellular lipid, insulin-mediated skeletal muscle glucose uptake is impaired. As a result, glucose is diverted to the liver. In the liver, increased liver lipid also impairs the ability of insulin to regulate gluconeogenesis and activate glycogen synthesis. In contrast, lipogenesis remains unaffected and, together with the increased delivery of dietary glucose, leads to increased lipogenesis and worsening nonalcoholic fatty liver disease (NAFLD). Impaired insulin action in the adipose tissue allows for increased lipolysis, which will promote re-esterification of lipids in other tissues (such as liver) and further exacerbates insulin resistance. Coupled with a decline in pancreatic β cells (depicted by the smaller lines emanating from the pancreas), hyperglycemia develops. FA, fatty acids; TAG, triacylglycerol (Samuel and Shulman, 2012).

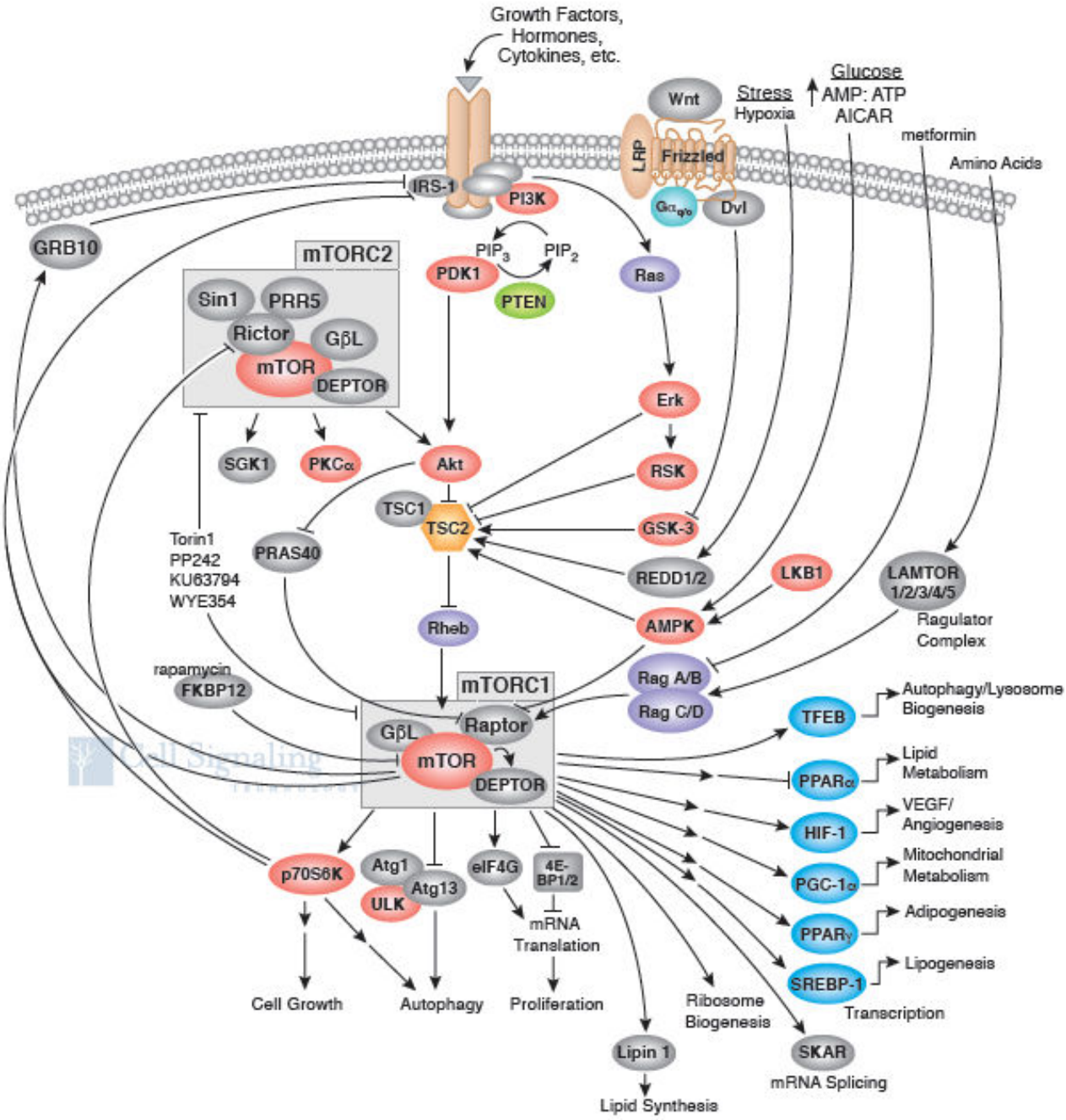
1.2.2 AA signaling pathways: mTOR & AAR pathway in mammals

Amino acids are versatile molecules used as substrates for synthesis of various components, and one of the most abundant macromolecules in cells. The accurate sensing of amino acid levels is the key for the efficient regulation of protein and amino acid synthesis and catabolism, as well as for the control of food intake. Cells sense extracellular and intracellular amino acids mainly through two distinct nutrient sensing cascades: mechanistic target of rapamycin (mTOR) and amino acid response (AAR) pathways, both of which involve in the regulation of cellular growth and metabolism (Efeyan et al., 2015).

1.2.2.1 Overview of mTOR signaling pathway in mammals

mTOR signaling pathway senses and integrates a variety of intracellular and extracellular cues to regulate organismal growth and homeostasis (Figure 1.11) (Laplante and Sabatini, 2012; Wullschleger et al., 2006). This pathway regulates many major cellular processes and is implicated in an increasing number of pathological conditions, including cancer, obesity, type 2 diabetes, neurodegeneration, and aging (Laplante and Sabatini, 2012). mTOR is an atypical serine/threonine kinase that is present in two structurally and functionally distinct complexes termed mTORC1 and mTORC2 (Figure 1.11). mTORC1, the first complex, is composed of mTOR, Raptor, GβL, and DEPTOR and is pharmacologically inhibited by rapamycin. It is a master growth regulator that senses and integrates diverse nutritional and environmental cues, including growth factors, energy levels, cellular stress, and amino acids. It couples these signals to the promotion of cellular growth by phosphorylating substrates that potentiate anabolic processes such as mRNA translation and lipid synthesis, or limit catabolic processes such as autophagy. The small GTPase Rheb (Ras homolog enriched in brain), in its GTP-bound state, is a necessary and potent stimulator of mTORC1 kinase activity, which is negatively regulated by its GAP (GTPase-activating protein), the tuberous sclerosis heterodimer TSC1/2. Most upstream inputs are funneled through Akt and TSC1/2 to regulate the nucleotide-loading state of Rheb. In contrast, amino acids signal to mTORC1 independently of the PI3K/Akt axis to promote the translocation of mTORC1 to the lysosomal surface where it can become activated upon contact with Rheb. This process is mediated by the coordinated actions of multiple complexes, notably the v-ATPase, Ragulator, the Rag GTPases, and GATOR1/2. The second complex, mTORC2, is composed of mTOR, Rictor, GβL, Sin1, PRR5/Protor-1, and DEPTOR. mTORC2 promotes cellular survival by activating Akt, regulates cytoskeletal dynamics by activating PKCα, and controls ion transport and growth via SGK1 phosphorylation. Aberrant mTOR signaling is involved in many disease states including cancer, cardiovascular disease, and diabetes (Cell Signaling Technology, 2014; Laplante and Sabatini, 2009; Zoncu et al., 2011).

Figure 1. 11 Overview of mTOR signaling pathway



From Cell Signaling Technology (Cell Signaling Technology, 2014).

1.2.2.2 mTORC1 signaling pathway in mammals

Overview of mTORC1 signaling

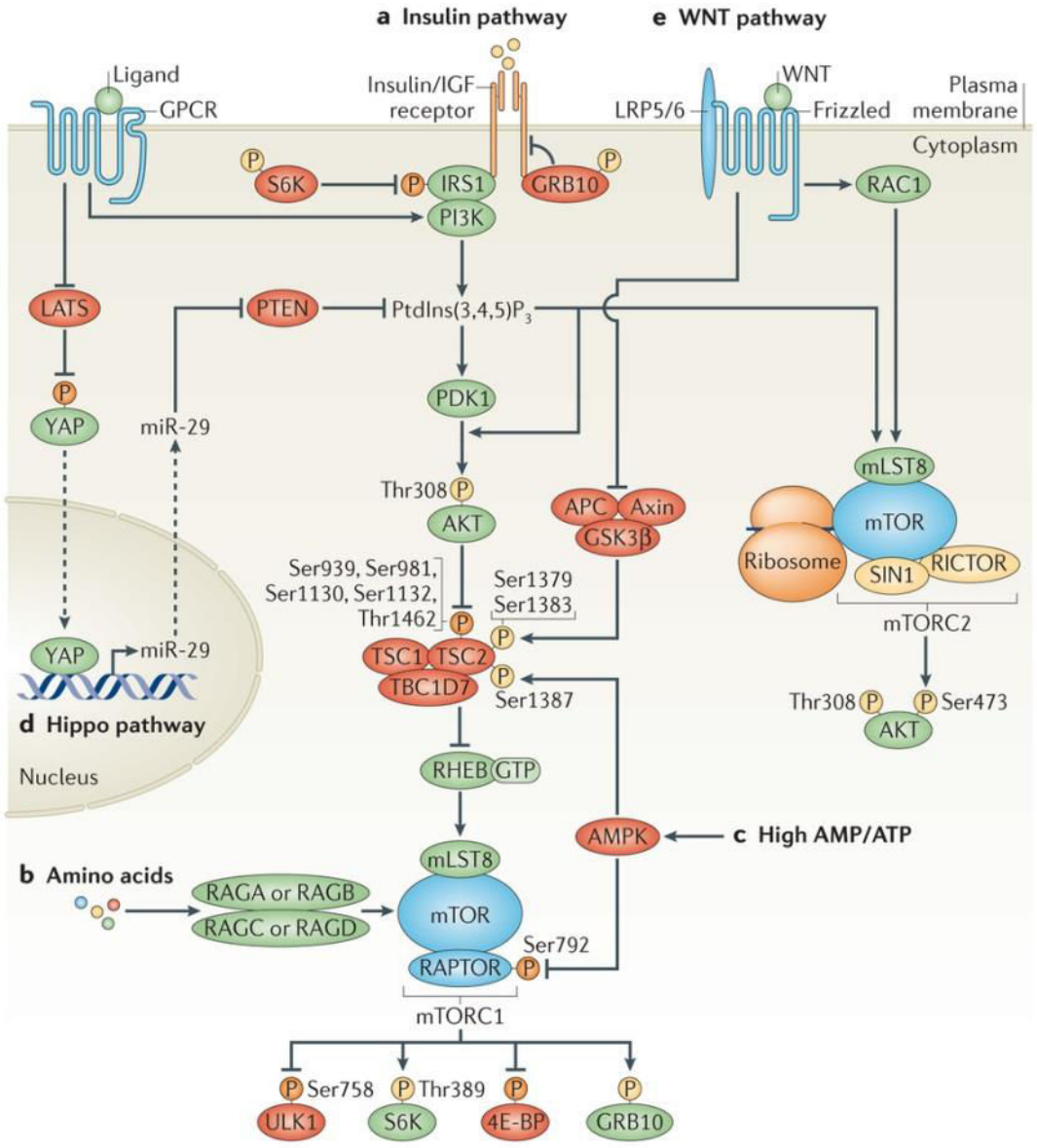
Of the two mTOR complexes, mTORC1 is by far the best characterized one (Laplante and Sabatini, 2013). mTORC1 integrates inputs from at least five major intracellular and extracellular cues—growth factors, stress, energy status, oxygen, and amino acids—to control many major processes, including protein and lipid synthesis and autophagy (Wullschleger et al., 2006). Ribosomal S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E) binding protein (4EBP) are the two best-characterized downstream proteins of mTORC1, which promote protein synthesis (Jewell and Guan, 2013). mTORC1 triggers anabolic cell growth and proliferation by stimulating mRNA translation, glycolysis, lipid synthesis, the pentose phosphate pathway and *de novo* pyrimidine synthesis and by reducing autophagy (Dibble and Manning, 2013). Deregulation of the mTOR signaling pathway has now been frequently linked to aging and several human diseases, such as type 2 diabetes, cancer, cardiovascular disease, obesity and neurodegeneration, which also highlights its importance in the maintenance of cellular homeostasis (Laplante and Sabatini, 2012).

Upsteam of mTORC1 signaling network

mTORC1 is activated by the small GTPase RAS homologue enriched in brain (Rheb) which resides at the lysosomal surface where it functions as a potent stimulator of the mTORC1 kinase activity (Bar-Peled and Sabatini, 2014; Dibble and Manning, 2013). Rheb in turn is negatively controlled by the trimeric tuberous sclerosis complex (TSC) whose TSC2 component harbors GTPase-activating protein (GAP) activity towards Rheb–converting it from the active GTP-bound state to the inactive GDP-bound state. The TSC complex, whose loss underlies a hamartomatous syndrome of the same name, serves as a central hub for numerous extracellular and intracellular inputs including mitogen and growth factor signaling, energy levels, oxygen availability, and genotoxic stress (Figure 1.12), which collectively exert their effects on the mTORC1 pathway by modulating the activity of the TSC complex (Bar-Peled and Sabatini, 2014; Laplante and Sabatini, 2009).

Growth factors Growth factors, hormones and cytokines (collectively referred to as growth factors here) stimulate mTORC1 through two major signaling cascades: the PI3K-Akt axis and Ras–Erk pathway (Dibble and Manning, 2013). Through binding to receptor tyrosine kinases (RTKs) and scaffolding adaptor proteins, a variety of secreted growth factors (insulin, IGF1, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF)) stimulate the recruitment and activation of PI3K. PI3K activity generates phosphatidylinositol-3,4,5-trisphosphate (PIP_3), which recruits PDK1 to the plasma membrane and phosphorylates Akt on Thr^{308}

Figure 1. 12 Upstream regulators of mTORC1



a | Growth factors such as insulin stimulate PI3K to generate phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P3), which promotes the phosphorylation (P) of AKT at Thr308 by phosphoinositide-dependent kinase 1 (PDK1). AKT phosphorylates tuberous sclerosis complex 2 (TSC2) on multiple sites to inhibit its GTPase-activating protein (GAP) activity for the small GTPase RAS homologue enriched in brain (RHEB). GTP-loaded RHEB then activates mammalian TOR complex 1 (mTORC1). Growth factors also stimulate mTORC2 by promoting its association with ribosomes in a PI3K-dependent manner. b | Amino acids stimulate mTORC1 by promoting the conversion of RAS-related GTP-binding protein (RAG) heterodimers to the active conformation, in which RAGA or RAGB is loaded with GTP and RAGC or RAGD is loaded with GDP. The active RAG heterodimer recruits mTORC1 to the surface of the lysosome where mTORC1 binds RHEB (Box 1). c | In response to low energy (high AMP/ATP ratio), AMP-activated protein kinase (AMPK) phosphorylates regulatory-associated protein of mTOR (RAPTOR) at Ser792 and TSC2 at Ser1387, leading to the inhibition of mTORC1. d | During the inhibition of the Hippo pathway component large tumour suppressor homologue (LATS) kinase, hypophosphorylated Yes-associated protein (YAP) translocates to the nucleus and promotes the expression of the microRNA miR-29. miR-29 targets PTEN mRNA and

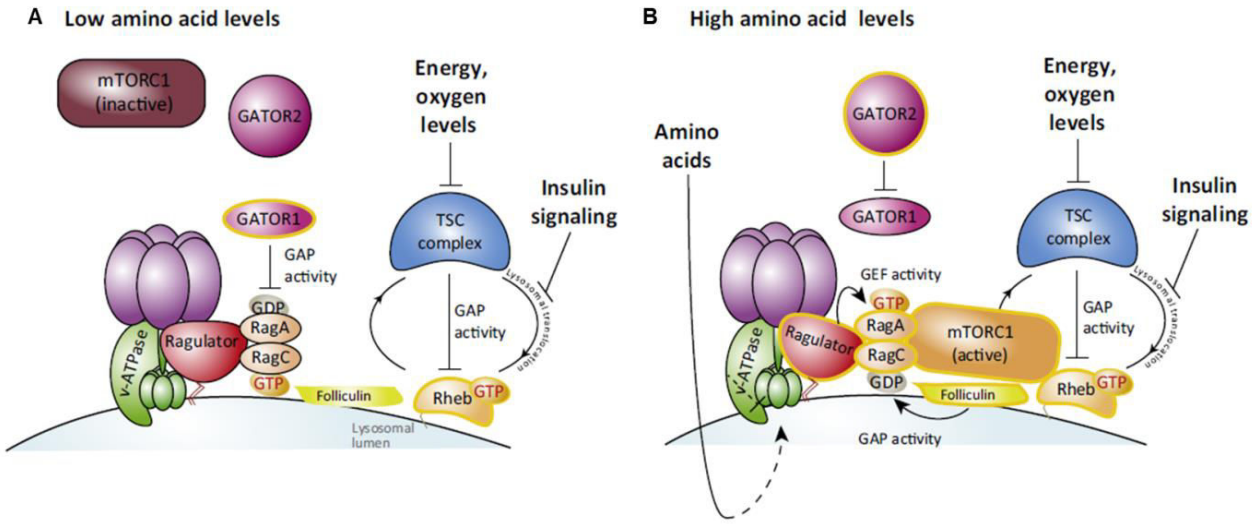
inhibits PTEN translation, which leads to increased levels of PtdIns(3,4,5)P3 and the activation of both mTORC1 and mTORC2. Dashed arrows represent translocation of the molecule. e | Glycogen synthase kinase 3β (GSK3β) activates the TSC complex by phosphorylating TSC2 at Ser1379 and Ser1383. Phosphorylation of these two residues requires priming by AMPK-dependent phosphorylation of Ser1387. WNT signalling inhibits GSK3β and the TSC complex, and thus activates mTORC1. mTORC2 is activated by WNT in a manner dependent on the small GTPase RAC1. Proteins shown in green promote mTOR activity or their activity is promoted by mTOR. Proteins shown in red inhibit mTOR activity or their activity is inhibited by mTOR. Phosphorylation depicted in yellow is an activation signal and phosphorylation depicted in orange is an inhibitory signal. 4E-BP, eIF4E-binding protein; APC, adenomatous polyposis coli; GPCR, G protein-coupled receptor; GRB10, growth factor receptor-bound protein 10; IGF, insulin-like growth factor 1;IRS1, insulin receptor substrate 1; LRP, low-density lipoprotein receptor-related protein; mLST8, mammalian lethal with SEC thirteen 8; RICTOR, rapamycin-insensitive companion of mTOR; S6K, ribosomal S6 kinase; SIN1, SAPK-interacting 1; TBC1D7, TBC1 (TRE2–BUB2–CDC16) domain family member 7; ULK1, UNC-51-like kinase 1. From Shimobayashi and Hall (Shimobayashi and Hall, 2014).

(Wullschleger et al., 2006). In turn, Akt stimulates mTORC1 activity through at least two distinct mechanisms, the phosphorylation of TSC-2 that blocks the inhibitory function of the TSC1–TSC2 complex and allows the activation of TORC1 by the Rheb GTPase (Porstmann et al., 2009; Sarbassov et al., 2005a) and the phosphorylation of proline-rich Akt substrate of 40 kDa (PRAS40), which results in dissociation of this inhibitory component from mTORC1 (Laplante and Sabatini, 2012; Sarbassov et al., 2005a). Ras is also activated downstream of RTKs and stimulates a kinase cascade leading to the activation of extracellular signal regulated kinase (Erk) and p90 ribosomal S6 kinase (Rsk). In response to growth factors, the Erk and Rsk protein kinases also phosphorylate specific residues on TSC2 within the TSC complex, thereby negatively regulating the ability of this complex to act as a GAP for Rheb. Consequently, GTP-bound Rheb accumulates and activates mTORC1. Thus, PI3K-Akt and Ras–Erk pathways represent two parallel inputs into mTORC1, with Akt phosphorylating PRAS40 and Erk and Rsk both phosphorylating residues on Raptor (Dibble and Manning, 2013).

Amino acids Amino acids are essential signals for mTORC1 activation (Kim and Guan, 2011). Amino acids signal to mTORC1 through the RAS-related GTP-binding protein (RAG) family of small GTPases (Kim et al., 2008; Sancak et al., 2008). The RAGs form heterodimers of either RAGA or RAGB with either RAGC or RAGD (Kim et al., 2008). Amino acids first enter the lysosome and triggers a conformational change within the v-ATPase, which in turn stimulates the conversion of RAG heterodimers from the inactive conformation ($RagA/B^{GTP}$–$RagC/D^{GDP}$) to the active conformation ($RagA/B^{GDP}$–$RagC/D^{GTP}$) (Figure 1.13) (Shimobayashi and Hall, 2014). The active RAG heterodimer recruits mTORC1 to the surface of the lysosome where Rheb is localized, thereby allowing the activation of mTORC1 by Rheb (Bar-Peled and Sabatini, 2014; Kim and Guan, 2011; Laplante and Sabatini, 2012; Yang et al., 2012a).

mTORC1 is not equally sensitive to all amino acids, for example, leucine, arginine and glutamine is particularly important for its activation (Dibble and Manning, 2013; Jewell and Guan, 2013). However, it is unclear whether mTORC1 truly senses individual amino acids, the total amino acid pool, or an amino acid byproduct (Bar-Peled and Sabatini, 2014; Dibble and Manning, 2013). In spite of the fact that

Figure 1. 13 The mTORC1 amino acid sensing pathway



The mechanistic target of rapamycin complex I (mTORC1) amino acid sensing pathway. (A) Under low amino acid conditions Ragulator is found in an inhibitory state with the v-ATPase, and GATOR1 [GAP activity towards Rags (Ras-related GTPases)] exerts its GTPase-activating protein (GAP) activity towards RagA, keeping this GTPase in the inactive GDP-bound state that is not sufficient to recruit mTORC1. Insulin signaling inhibits tuberous sclerosis complex (TSC) translocation to the lysosomal surface where it functions as a GAP for Rheb (Ras homolog enriched in brain), inactivating this G protein. (B) Upon amino acid stimulation, GATOR1 may be inhibited by GATOR2, and Ragulator and v-ATPase undergo a conformational change, unleashing the guanine nucleotide exchange factor (GEF) activity of Ragulator towards RagA, while the folliculin complex promotes RagC GTP hydrolysis. The now active heterodimer, consisting of GTP-bound RagA and GDP-loaded RagC, recruits mTORC1 to the lysosomal surface where it interacts with and is activated by Rheb. From Bar-Peled and Sabatini (Bar-Peled and Sabatini, 2014).

mTORC1 activity is highly responsive to changes in amino acid levels, it is not an amino acid sensor (Efeyan et al., 2015). Current research efforts are focusing on identifying membrane protein that links luminal amino acids to the mTORC1–activating machinery on the lysosomal membrane, such as the solute carrier (SLC) family of proteins (SLC38A9) as candidate amino acid sensor (Abraham, 2015; Jewell et al., 2015; Wang et al., 2015). Furthermore, it appears that the main purpose of the amino acid signal is to colocalize mTORC1 with its activator, Rheb (Bar-Peled and Sabatini, 2014). When cells are deprived of amino acids, mTORC1 is diffuse throughout the cytoplasm. However, upon addition of amino acids, mTORC1 rapidly translocates to the lysosomal surface where it is presumed to interact with the small GTPase Rheb (Sancak et al., 2008). Because mTORC1 kinase activation by Rheb occurs at the outer lysosomal surface, it is only possible following Rag GTPase-dependent recruitment of mTORC1. Hence, amino acid abundance and the consequent recruitment of mTORC1 is a prerequisite for the activation of mTORC1 by growth factors (Efeyan et al., 2015; Laplante and Sabatini, 2013).

Energy status The energy status of the cell is signaled to mTORC1 through AMP-activated protein kinase (AMPK), a master sensor of intracellular energy status (Jewell and Guan, 2013). In response to energy depletion (low ATP/ADP ratio), AMPK is activated and phosphorylates TSC2 at Ser1387, which increases the GAP activity of TSC2 towards Rheb and reduces mTORC1 activation (Laplante and Sabatini, 2013). Additionally, AMPK blocks mTORC1 activation in response to nutrient starvation conditions by directly phosphorylating Raptor at Ser792 (Shimobayashi and Hall, 2014).

Oxygen levels Under conditions of mild hypoxia, the reduction in ATP levels activates AMPK, which promotes TSC1/2 activation and inhibits mTORC1 signaling (Jewell and Guan, 2013). Hypoxia can also activate TSC1/2 through transcriptional regulation of DNA damage response 1 (REDD1), which blocks mTORC1 signaling by releasing TSC2 from its growth factor induced association with 14-3-3 proteins (DeYoung et al., 2008). Furthermore, promyelocytic leukemia (PML) tumor suppressor and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) reduce mTORC1 signaling during hypoxia by disrupting the interaction between mTOR and its positive regulator Rheb (Laplante and Sabatini, 2009).

WNT pathway WNT signaling inhibits glycogen synthase kinase 3β (GSK3β) and the TSC complex, and thus activates mTORC1 (Inoki et al., 2006). GSK3β activates the TSC complex by phosphorylating TSC2 at Ser1379 and Ser1383. Phosphorylation of these two residues requires priming by AMPK-dependent phosphorylation of Ser1387 (Zoncu et al., 2011).

Hippo pathway During the inhibition of the Hippo pathway component large tumour suppressor homologue (LATS) kinase, hypophosphorylated Yes-associated protein (YAP) translocates to the nucleus and promotes the expression of the microRNA miR-29. miR-29 targets PTEN mRNA and inhibits PTEN translation, which leads to increased levels of PtdIns(3,4,5)P3 and the activation of both mTORC1 and mTORC2 (Shimobayashi and Hall, 2014).

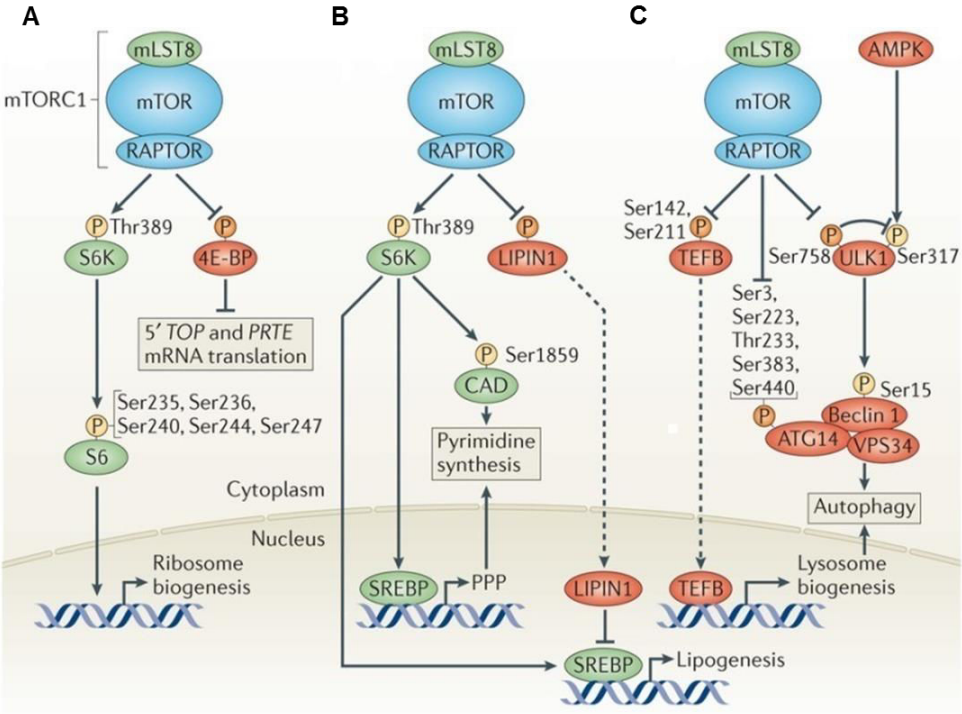
Other cellular conditions and signals

In addition to the key signals described above, other cellular conditions and signals, such as inflammation, p53, p38MAPK (mitogen-activated protein kinase p38) and Ras/Raf/MEK/ERK pathways, have all been shown to regulate mTORC1 signaling, which has been well reviewed previously (Dibble and Manning, 2013; Jewell and Guan, 2013; Laplante and Sabatini, 2009; Laplante and Sabatini, 2012).

Promotion of anabolic metabolism downstream of mTORC1

When active, mTORC1 stimulates cell growth and proliferation by promoting many anabolic processes, including biosynthesis of proteins, lipids and organelles, and by limiting catabolic processes such as autophagy (Figure 1.14) (Laplante and Sabatini, 2009).

Figure 1. 14 mTORC1 controls metabolism



Mammalian TOR complex 1 (mTORC1) promotes anabolic processes, such as the biosynthesis of proteins, nucleotides and lipids, and inhibits catabolic processes such as autophagy. A | mTORC1 phosphorylates (P) the hydrophobic motif (Thr389) in ribosomal S6 kinase (S6K), thereby activating it to subsequently phosphorylate ribosomal protein S6 at the sites indicated to promote ribosome biogenesis. mTORC1 also phosphorylates eIF4E-binding protein (4E-BP) at multiple sites to inhibit it. Inhibition of 4E-BP stimulates translation initiation, especially of 5′ oligopyrimidine tract (termed a 5′ TOP) and pyrimidine-rich translational element (PRTE) containing mRNAs. B | mTORC1 stimulates nucleotide and lipid synthesis. mTORC1 promotes the gene expression of key enzymes in the pentose phosphate pathway (PPP), at least in part by activating sterol regulatory element-binding proteins (SREBPs). mTORC1 also stimulates CAD (Gln-dependent carbamoyl-phosphate synthase, Asp carbamoyltransferase, dihydroorotase) by S6K-mediated phosphorylation at Ser1859, which leads to CAD activation and the stimulation of de novo pyrimidine synthesis. Furthermore, mTORC1 promotes lipogenic gene expression by activating S6K or by inhibiting the nuclear translocation of LIPIN1, both of which activate the transcription factor SREBP. C | mTORC1 inhibits autophagy by phosphorylating UNC-51-like kinase 1 (ULK1) at Ser758 and ATG14 at multiple sites. During mTORC1 inhibition, AMPK phosphorylates ULK1 at Ser317, and thereby activates ULK1, which phosphorylates Beclin 1 in the vacuolar protein sorting 34 (VPS34)–Beclin 1–ATG14 complex to initiate autophagy. mTORC1 also inhibits autophagy indirectly by blocking lysosome biogenesis, by phosphorylating and inhibiting the nuclear translocation of transcription factor EB (TFEB). The activity of proteins shown in green is promoted by mTOR. The activity of proteins shown in red is inhibited by mTOR. Phosphorylation depicted in yellow is an activation signal and phosphorylation depicted in red is an inhibitory signal. Dashed arrows represent translocation of the protein. mLST8, mammalian lethal with SEC thirteen 8; PDK, phosphoinositide-dependent kinase 1; RAPTOR, regulatory-associated protein of mTOR; RICTOR, rapamycin-insensitive companion of mTOR; SIN1, SAPK-interacting 1. From Shimobayashi and Hall (Shimobayashi and Hall, 2014).

mTORC1 controls protein synthesis

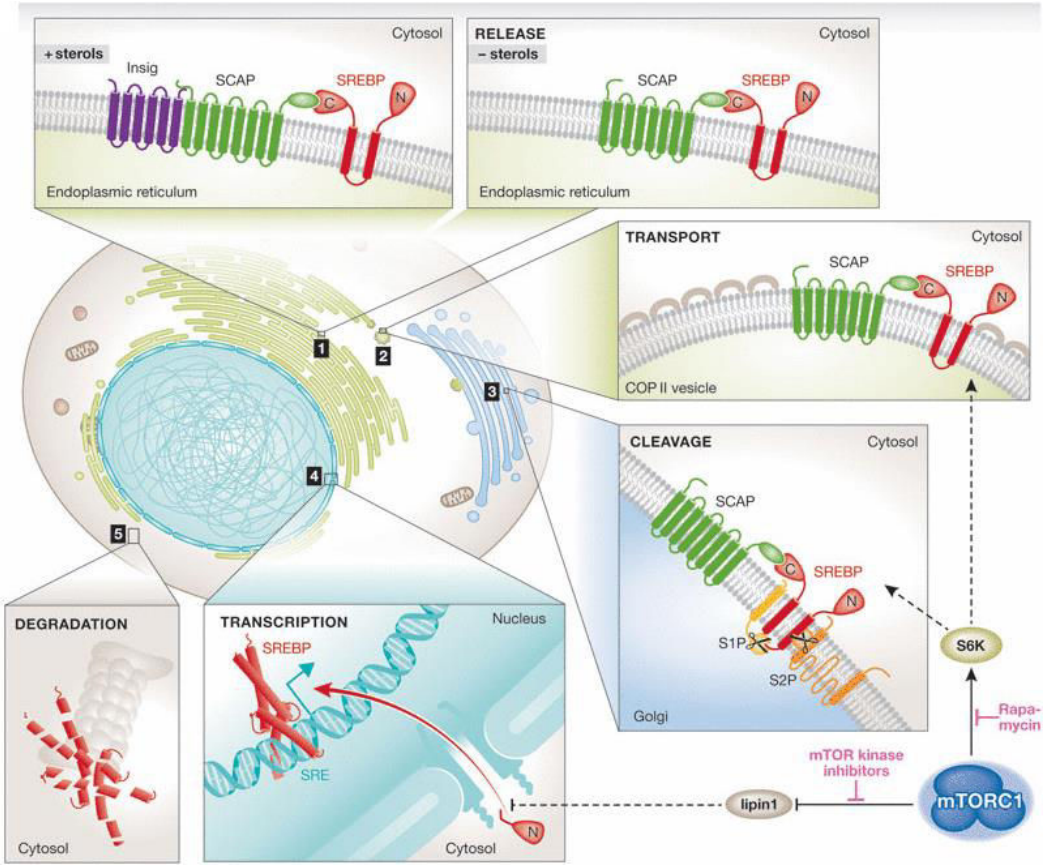
Protein synthesis is by far the best-characterized process controlled by mTORC1 (Laplante and Sabatini, 2012; Shimobayashi and Hall, 2014). When activated, mTORC1 stimulates both an acute increase in the translation of specific mRNAs and a broader increase in the protein synthetic capacity of the cell (Dibble and Manning, 2013). mTORC1 promotes protein synthesis by phosphorylating the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and the p70 ribosomal S6 kinase 1 (S6K1) (Figure 1.14 A) (Laplante and Sabatini, 2009). The phosphorylation of 4E-BP1 prevents its binding to eIF4E, enabling eIF4E at the 5'-cap of mRNAs and blocking assembly of the translation initiation complex to promote cap-dependent translation (Richter and Sonenberg, 2005). Activated S6K1 by mTORC1 increases in mRNA biogenesis, cap-dependent translation and elongation, and the translation of ribosomal proteins via regulating the activity of many proteins, such as S6K1 aly/REF-like target (SKAR), programmed cell death 4 (PDCD4), eukaryotic elongation factor 2 kinase (eEF2K) and ribosomal protein S6 (Ma and Blenis, 2009). Additionally, the activation of mTORC1 also can promote ribosome biogenesis by stimulating the transcription of ribosomal RNA through a process involving the protein phosphatase 2A (PP2A) and the transcription initiation factor IA (TIF-IA), which promotes its interaction with RNA Polymerase I (Pol I) and the expression of ribosomal RNA (rRNA) (Mayer et al., 2004). Therefore, the acute translational control over this class of mRNAs allows mTORC1 signaling to globally enhance cellular protein synthesis.

mTORC1 controls lipid metabolism

mTORC1 plays a central role in the control of lipid metabolism (Caron et al., 2015; Lamming and Sabatini, 2013; Ricoult and Manning, 2013). Activated mTORC1 can stimulate lipid synthesis, as well as promote glucose uptake, glycolysis and NADPH production to support this anabolic process (Dibble and Manning, 2013). mTORC1 promotes lipogenesis by activating SREBPs through multiple manners (Figure 1.14 B and 1.15) (Caron et al., 2015; Han et al., 2015). Using a small molecule inhibitor of S6K, Owen et al. (Owen et al., 2012) find that the transcriptional regulation of SREBP1c by insulin is not dependent on S6K, whereas posttranscriptional processing of SREBP1c is S6K dependent. mTORC1 also regulates SREBP by controlling the nuclear entry of lipin 1, a phosphatidic acid phosphatase that down-regulates SREBP activity (Peterson et al., 2011). Phosphorylation of lipin1 by mTORC1 regulates its subcellular localization, with phosphorylated lipin1 residing in the cytoplasm and dephosphorylated lipin1 accumulating in the nucleus. Nuclear lipin1 represses SREBP-dependent gene transcription by reducing the nuclear SREBP protein levels (Peterson et al., 2011). Moreover, latest study demonstrated that activated mTORC1 promotes the COPII-dependent SREBP1 maturation through the transcriptional coactivator CREB regulated transcription coactivator 2 (CRTC2) (Han et al., 2015). Han et al. (Han et al.,

2015) demonstrated that CRTC2 competes with Sec23A, a subunit of the COPII complex, to interact with Sec31A, another COPII subunit, thus disrupting SREBP1 transport from ER to the Golgi (Figure 1.15 B). During feeding, mTOR phosphorylates CRTC2 and attenuates its inhibitory effect on COPII-dependent SREBP1 maturation. Therefore, CRTC2 functions as a mediator of mTOR signaling to modulate COPII-dependent SREBP1 processing.

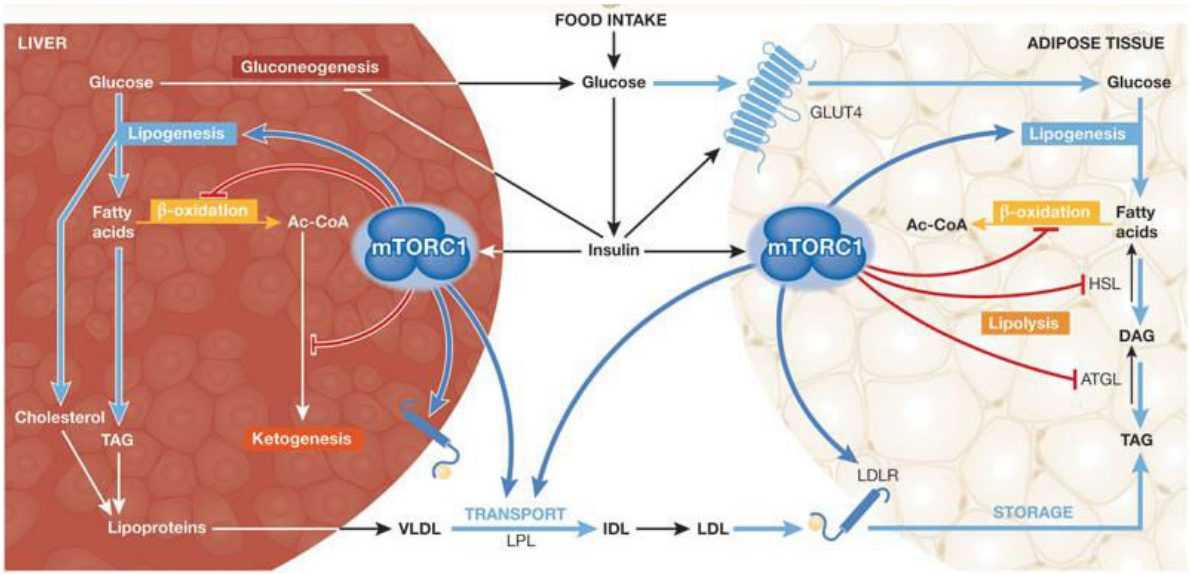
Figure 1. 15 The complex steps leading to SREBP activation and input from mTORC1 signaling



In the presence of sterols, SREBP resides in the endoplasmic reticulum bound to SCAP and the Insig proteins. When sterols become scarce SCAP undergoes a conformational change, which releases the SCAP–SREBP complex from the Insig, allowing its transport from the endoplasmic reticulum to the Golgi apparatus through COPII vesicles. Once in the Golgi, SREBP comes into contact with two site - specific proteases S1P and S2P. S1P cleaves the luminal loop of SREBP and S2P cleaves the amino - terminal transmembrane region of SREBP, which releases the N - terminal region of SREBP containing the DNA - binding and - transactivating domains. The NLS - containing processed form of SREBP enters the nucleus to activate transcription of genes containing SREs in their promoters. Finally, the processed form of SREBP is unstable and subject to proteasome - mediated degradation. In some settings, SREBP processing has been found to require S6K1 downstream from mTORC1 and is therefore sensitive to rapamycin. However, the nuclear shuttling of SREBP has been found to require lipin 1 downstream from mTORC1, the phosphorylation of which is largely resistant to rapamycin but sensitive to mTOR kinase domain inhibitors (Sidebar A). The precise molecular mechanisms by which either of these two mTORC1 targets regulates SREBP activation are unknown. COPII, coatamer protein II; Insig, insulin - induced gene; lipin 1, phosphatidate phosphatase LPIN1; mTORC1, mechanistic target of rapamycin complex 1; NLS, nuclear localization signal; S1/2P, site 1/2 protease; S6K1, ribosomal S6 kinase 1; SCAP, SREBP cleavage - activating protein; SRE, sterol response element; SREBP1/2, sterol regulatory element binding protein 1/2 (Ricoult and Manning, 2013).

Apart from lipid synthesis, mTORC1 also regulates some other aspects of lipid metabolism, including promotion of transport and storage, suppression of lipid oxidation and lipolysis, as well as adipocyte differentiation and function through controlling the master regulators like peroxisome proliferator-activated receptor γ (PPAR-γ), PPARα and so on (Figure 1.16) (Ricoult and Manning, 2013). mTORC1 also positively regulates the translation of hypoxia-inducible factor 1α (HIF1α), which induces the expression of glucose transporters and glycolytic enzymes and promotes a switch from mitochondrial oxidative metabolism to glycolysis (Duvel et al., 2010; Laplante and Sabatini, 2013). Furthermore, mTORC1 signaling also stimulates the expression of key enzymes related to pentose phosphate pathway (PPP) and metabolic flux specific to the oxidative, NADPH-producing branch of the PPP (Duvel et al., 2010). For example, mTORC1 regulates the expression of glucose-6-phosphate dehydrogenase (G6PD), a rate-limiting enzyme in the oxidative PPP, through a SREBP1-dependent manner (Dibble and Manning, 2013). Given that lipid synthesis is one of the most NADPH-demanding metabolic pathways, its co-regulation with the oxidative PPP by mTORC1 and SREBP is likely to help satisfy this requirement.

Figure 1. 16 The multifaceted role of mTORC1 in the control of lipid metabolism



The increase in insulin levels after a meal alters hepatic and adipose lipid metabolism, at least in part, through mTORC1 signaling. In the liver, mTORC1 promotes lipid synthesis through SREBP1c activation. In addition, mTORC1 signaling blocks lipid catabolism by blocking β oxidation and ketogenesis in the liver. Consequently, mTORC1 activation in the liver promotes the synthesis of TAGs and perhaps cholesterol, which are incorporated into VLDL for transport to peripheral tissues. Evidence suggests that mTORC1 signaling positively influences LPL activity, which promotes lipid delivery to peripheral tissues by hydrolyzing VLDL to IDL, which is then converted to LDL. Lipoprotein - bound TAGs are taken up by tissues, including adipocytes, through the LDLR. Both the expression and stability of LDLR, at least in the liver, are probably promoted by mTORC1 activation. In response to insulin, mTORC1 inhibits lipolysis in adipocytes by downregulating ATGL and HSL. Therefore, the systemic effects of postprandial mTORC1 activation are to promote the flux of carbon from glucose towards TAG storage in adipose tissue. See text for details regarding the evidence underlying this model. Ac-COA, acetyl-CoA; ATGL, adipose triglyceride lipase; DAG, diacylglycerol; GLUT4, glucose transporter type 4; HSL, hormone sensitive lipase; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LDLR, LDL receptor; LPL, lipoprotein lipase; mTORC1, mechanistic target of rapamycin complex 1; SREBP1c, sterol regulatory element binding protein 1c; TAG, triacylglycerol; VLDL, very low density lipoprotein (Ricoult and Manning, 2013).

mTORC1 controls autophagy

Autophagy (or macroautophagy) is a multi-stage cellular recycling and quality control process in which membranous structures called autophagosomes engulf cytosolic organelles and macromolecules and, through fusion with lysosomes, target their constituents for degradation into nutrient building blocks (Galluzzi et al., 2014; Rabinowitz and White, 2010). mTORC1 potently inhibits autophagy at multiple steps (Figure 1.14 C) (Dibble and Manning, 2013). The best-characterized mechanism is through the direct control of UNC-51-like kinase 1 (ULK1 or ATG1), a protein kinase that regulates the initiation of autophagosome formation (Rabinowitz and White, 2010). Under nutrient-rich conditions, mTORC1 phosphorylates ULK1 at Ser758 to inhibit its interaction with AMPK and thus prevent autophagy. During glucose starvation, AMPK inhibits mTORC1 and hence the phosphorylation of ULK1 at Ser758, which enables ULK1 to interact with AMPK. AMPK in turn phosphorylates ULK1 at Ser317 to activate it. Activated ULK1 is recruited to the vacuolar protein sorting 34 (VPS34)–Beclin 1–ATG14 (also known as Barkor) complex, where it phosphorylates Beclin 1 at Ser15. This phosphorylation activates the lipid kinase VPS34 to produce phosphatidylinositol-3-phosphate (PtdIns3P), which is essential for autophagosome formation. Therefore, mTORC1 phosphorylates the pro-autophagic kinase ULK1 to prevent its activation by AMPK and thereby inhibits autophagosome formation and autophagy (Shimobayashi and Hall, 2014). Furthermore, mTORC1 also directly phosphorylates the transcription factor EB (TFEB), a master regulator of lysosomal and autophagy genes, thereby exerting an inhibitory input that is likely to attenuate autophagy (Dibble and Manning, 2013). Activated mTORC1 phosphorylates TFEB at the lysosome surface, which promotes the binding of TFEB to 14-3-3 proteins and inhibits its transport into the nucleus, while inhibited mTORC1 reduces TFEB phosphorylation and its binding to 14-3-3 proteins, and so rapidly increase the accumulation of TFEB in the nucleus, where it orchestrates the expansion of lysosomal and autophagic compartments (Laplante and Sabatini, 2013). Moreover, mTORC1 directly phosphorylates ATG13 and ATG14 on multiple sites, which inhibits PtdIns3P production by the associated kinase VPS34 (Shimobayashi and Hall, 2014). Finally, loss of mTORC1-mediated phosphorylation of death-associated protein 1 (DAP1) at Ser3 and Ser51 during mTORC1 inhibition prevents excessive autophagy (Koren et al., 2010).

Other downstream metabolic pathways

In addition to the anabolic pathways described above, mTORC1 also controls some other cellular pathways, such as nucleotide synthesis and mitochondrial metabolism and biogenesis, which have been comprehensively and profoundly reviewed previously (Laplante and Sabatini, 2009; Laplante and Sabatini, 2013; Shimobayashi and Hall, 2014).

mTORC1/S6K1-induced negative feedback loop

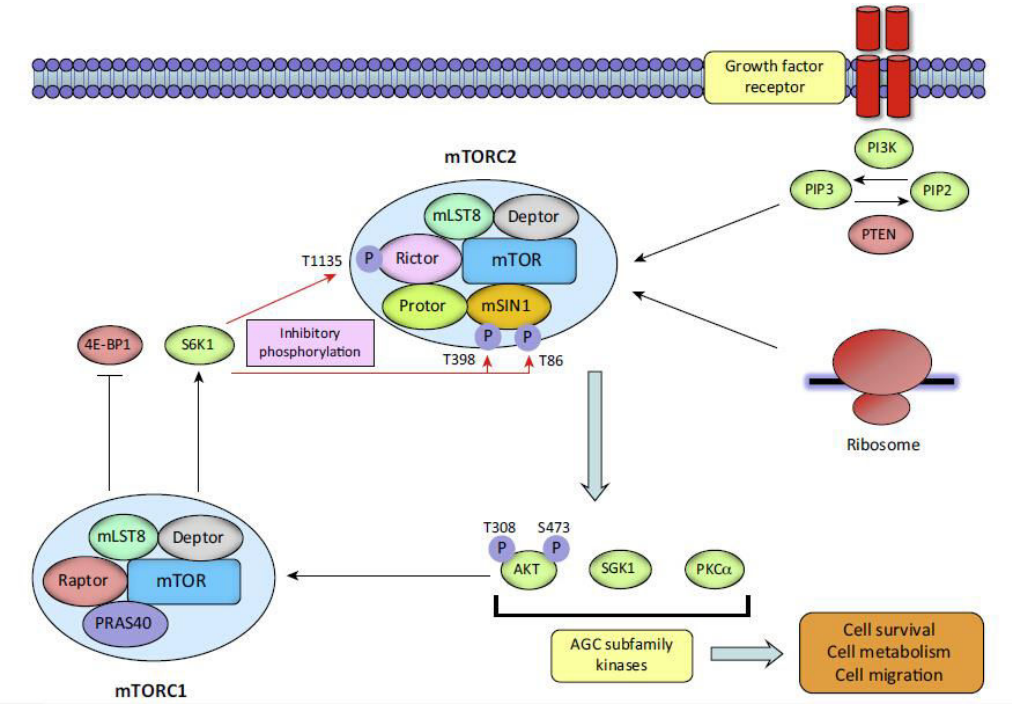
Active mTORC1 phosphorylates components of the protein synthesis machinery, including S6K1 and 4E-BP1 (Shimobayashi and Hall, 2014). S6K1 has several substrates including IRS1. S6K1 mainly stimulates IRS1 phosphorylation on serine residues (i.e. Ser^{307}, Ser^{302}). Importantly, this phosphorylation disrupts its interaction with IR, leads to proteasomal degradation and thereby hampers the ability of growth factors (insulin, insulin-like growth factor-1) to signal downstream of RTKs (Chiarini et al., 2015; Dann et al., 2007). This regulation results in inhibition of PI3K/Akt activation. As IRS1 is an upstream protein of mTORC1, S6K1-induced negative feedback loop indeed has an important role in the regulation of mTORC1 activity (Chiarini et al., 2015).

1.2.2.3 mTORC2 signaling pathway in mammals

Compare to mTORC1, the regulation of mTORC2 is poorly understood (Shimobayashi and Hall, 2014). Only growth factors stimulate mTORC2 kinase activity, by promoting its association with ribosomes in a PI3K-dependent manner (Zinzalla et al., 2011). Amino acids are required for the activation of mTORC1, but not mTORC2 (Laplante and Sabatini, 2013). Active mTORC2 allosterically activates the protein kinases A, G, and C (AGC) family members including Akt, serum- and glucocorticoid-induced protein kinase 1 (SGK1), and protein kinase C-α (PKC-α) (Figure 1.17) (Laplante and Sabatini, 2012). Through these downstream effectors, mTORC2 plays key roles in various biological processes, including cell survival, metabolism, proliferation and cytoskeleton organization (Laplante and Sabatini, 2009; Oh and Jacinto, 2011). Recruitment of Akt to the plasma membrane upon activation of PI3K results in a conformational change and phosphorylation of Thr308 and Ser473 (Scheid and Woodgett, 2003). Maximal Akt activity is dependent on the phosphorylation status of both residues (Alessi et al., 1996). Akt can be phosphorylated by PDK1 at Thr308, while mTORC2 directly activates Akt by phosphorylating its hydrophobic motif (Ser473) (Sarbassov et al., 2005b). Although the initial phosphorylation of Thr308 does not require prior Ser473 phosphorylation and the activity of Akt phosphorylated at Thr308 alone may be sufficient for a subset of its physiological roles (Guertin et al., 2006; Jacinto et al., 2006), numerous complimentary studies support a role for Akt Ser473 phosphorylation in increasing or maintaining Thr308 phosphorylation, Akt activity and mTORC1 signaling. Thus, mTORC2-dependent Akt phosphorylation on Ser473 is required for the full range of PI3K-dependent signaling to mTORC1 (Dibble and Cantley, 2015). However, loss of phosphorylation at Ser473 site, affects only some Akt substrates, such as FoxO transcription factors, but not TSC2, in response to growth factor signaling (Chiarini et al., 2015). Thus, it is not surprising that in some mTORC2 deficient conditions, Akt Thr308 phosphorylation and mTORC1 signaling can be even maintained at near-normal levels (Guertin et al., 2006; Hagiwara et al., 2012; Jacinto et al., 2006). mTORC2 also associates with actively translating

ribosomes to cotranslationally phosphorylate Akt (at Thr450), which prevents ubiquitinylation and degradation of Akt (Chiarini et al., 2015).

Figure 1. 17 The structure and signaling of mTORC2



Growth factor receptor-dependent activation of phosphoinositide 3-kinase (PI3K) promotes mTORC2 binding to ribosomes, and this activates mTORC2 in a fashion that is still incompletely understood. mTORC2 phosphorylates conserved motifs in AGC kinases to promote their allosteric activation. mTORC2 is a crucial node in growth factor receptor–PI3K signaling, phosphorylating Akt on Ser473 to promote its maximal activation. mTORC2 is negatively regulated by mTORC1. S6K1 downstream of mTORC1 phosphorylates Rictor on Thr1135 and mSIN1 on Thr86 and Thr398, inhibiting mTORC2-dependent phosphorylation of Akt. From (Masui et al., 2014).

mTORC2 regulates metabolism

Similar with mTORC1, mTORC2 also plays a crucial role for regulating lipid metabolism (Lamming and Sabatini, 2013; Masui et al., 2014), including regulation of hepatic lipogenesis (Hagiwara et al., 2012; Lee et al., 2008; Yuan et al., 2012), lipolysis (Cybulski et al., 2009) and adipogenesis (Yao et al., 2013). Using liver-specific rictor knockout (LiRiKO) mice, Hagiwara et al. (Hagiwara et al., 2012) observed that fed LiRiKO mice displayed loss of Akt Ser473 phosphorylation and reduced SREBP1c and PPARγ gene expression in the liver, leading to impaired hepatic glycolysis and lipogenesis. Yuan et al. (Yuan et al., 2012) also observed that, in the absence of mTORC2, hepatic expression of many key lipid synthesis genes, including acetyl-CoA carboxylase and fatty acid synthase, was reduced by 50% or more.

mTORC2 regulates glycolytic pathway mainly through mTORC2-mediated Akt phosphorylates on Ser473, which in turn increases the expression of glucose transporters (GLUT), phosphorylates and activates hexokinase 2 (HK2) or glucokinase, and the rate-limiting enzymes of glycolysis (6-phosphofructo-1-kinase (6PF1K) and pyruvate kinase (PK)) (Deprez et al., 1997; Gottlob et al., 2001; Hagiwara et al., 2012; Kohn et al., 1996). mTORC2 can also potentially suppress the transcription of gluconeogenic genes via a FoxO-dependent manner (Masui et al., 2014). In line with this, Lamming et al. (Lamming et al., 2012) observed that chronic administration of rapamycin disrupted mTORC2, which in turn attenuates insulin-mediated suppression of hepatic gluconeogenesis, thereby impairing glucose tolerance and insulin sensitivity. Moreover, mTORC2 can regulate glutaminolysis by controlling glutamine uptake and glutaminase (GLS) levels (Masui et al., 2014).

Hyper-activation of mTORC1 suppresses mTORC2

Of note, mTORC2 activity can be attenuated in response to growth factor signaling (Masui et al., 2014). Persistent mTORC1 activity can suppress mTORC2 through different post-translational modifications (Figure 1.17) (Julien et al., 2010; Liu et al., 2013; Zoncu et al., 2011). For instance, activated S6K1 phosphorylates Rictor on Thr1135, thereby inhibiting mTORC2-dependent Akt phosphorylation (Julien et al., 2010). S6K1 also phosphorylates mSIN1 on Thr86 and Thr398, suppressing mTORC2-dependent Akt phosphorylation via dissociating mSIN1 from the mTORC2 (Liu et al., 2013). Through these above mechanisms, hyperactive mTORC1 may result in dampened Akt activation, which translates into reduced glucose uptake and glycogen synthesis in liver and muscle, and increased gluconeogenesis and glucose release by the liver. Subsequently, these effects may lead to a worsening of the hyperglycaemia and hyperinsulinaemia, thereby inducing insulin resistance (Zoncu et al., 2011).

mTOR inhibition and inhibitors

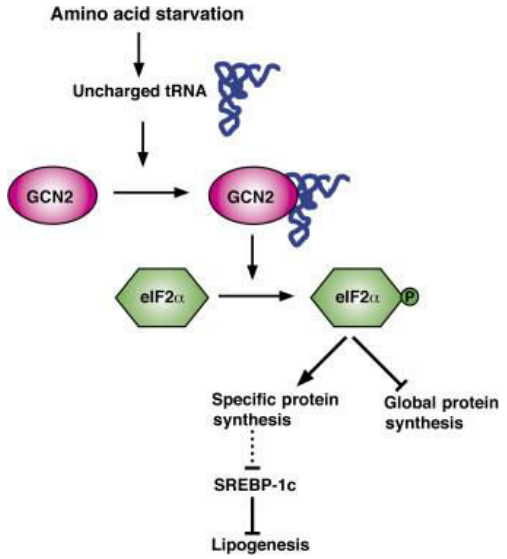
Rapamycin is an allosteric inhibitor of mTOR (Benjamin et al., 2011). In complex with the immunophilin FKBP12 (12 kDa FK506-binding protein), rapamycin binds and inhibits TOR exclusively in TORC1, whereas FKBP12–rapamycin cannot bind TOR in TORC2, making this complex insensitive to direct inhibition by rapamycin at least in a short time period (Jacinto et al., 2004; Laplante and Sabatini, 2012; Sarbassov et al., 2004). Yet the mechanism by which FKBP12–rapamycin inhibits mTORC1 is incompletely understood (Yang et al., 2013). Notably, although rapamycin does not exert acute effects on mTORC2, in certain cell types (Lamming et al., 2012; Sarbassov et al., 2005b; Ye et al., 2012), prolonged rapamycin treatment also results in a decrease of mTORC2 function and insulin sensitivity, perhaps as a result of depletion of the mTORC2 complex (Sarbassov et al., 2006).

Up to now, rapamycin and rapalogs are the most well-studied mTOR inhibitors (Ballou and Lin, 2008). However, studies also exposed some of rapamycin's limitations (Benjamin et al., 2011; Chiarini et al., 2015). First, rapamycin not only inhibits mTORC1 activation but also suppresses the mTORC1/S6K1-induced feedback loop, which might lead to reactivation of RTK, PI3K/Akt and Ras/Raf/MEK/ERK signaling. These unexpected results are big concerns. Second, rapamycin and its rapalogues do not target all mTORC1 outputs. For example, eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) is rephosphorylated and is refractory to long-term rapamycin treatment, even though S6K1 phosphorylation remains permanently inhibited under these conditions. Recent work conducted in Sabatini's laboratory has highlighted how the Thr37/46 residues of 4E-BP1 are good substrates for mTORC1 and, as such, are resistant to rapamycin. By contrast, the Ser65 residue is a poor substrate for mTORC1 and is dephosphorylated in response to rapamycin treatment (Chiarini et al., 2015). Finally, rapamycin is largely ineffective in inhibiting mTORC2 activity, which is a big difficulty for studying mTORC2 function with this drug. Due to these shortcomings and low specificity, scientists are developing a new generation of ATP-competitive inhibitors (i.e. Torin1 and PP242), which specifically target the catalytic site of mTOR, thus acting on both mTORC1 and mTORC2 but not on PI3K (Chiarini et al., 2015). The utilization of these inhibitors led to the observation that the ability of mTORC1 to phosphorylate 4E-BP and ULK1 is rapamycin insensitive (Benjamin et al., 2011; Shimobayashi and Hall, 2014). Furthermore, rapamycin has a biphasic effect on insulin sensitivity (Lamming et al., 2012; Ye et al., 2012). Rapamycin improves insulin sensitivity in acute studies *in vitro* and *in vivo* by disrupting a negative feedback loop mediated by S6K1, whereas chronic rapamycin treatment induces insulin resistance in rodents, at least in part due to disruption of mTORC2 (Ye et al., 2012).

1.2.2.4 AAR pathway in mammals

Deficiency of dietary protein or an imbalance of essential amino acids activates an amino acid response (AAR) signal transduction pathway, consisting of detection of uncharged tRNA by the general control non-derepressible 2 (GCN2) kinase (Kilberg et al., 2009). GCN2 is a sensor of amino acid deprivation that triggers a repression of global protein synthesis while simultaneously inducing translation of specific proteins (Kilberg et al., 2012). Uncharged tRNAs stimulate GCN2 which subsequently phosphorylates its substrate, the translation initiation factor 2α (eIF2α), leading to reduced global protein synthesis and simultaneously to increased translation of specific mRNAs, e.g. those coding for GCN4 in yeast and activating transcription factor 4 (ATF4) in mammals (Castilho et al., 2014). ATF4 is a transcription factor that regulates the expression of genes involved in amino acid metabolism, redox homeostasis and ER stress responses (Ye et al., 2010) and triggers increased transcription by binding to CCAAT-enhancer binding protein-activating transcription factor (C/EBP-ATF) response elements (CARE). These CARE-containing genes, including the system A sodium-dependent neutral amino acid transporter 2 (SNAT2), eukaryotic initiation factor 4E binding protein 1 (4E-BP1), asparagine synthetase (ASNS), ATF3, cationic amino acid transporter 1 (Cat-1), C/EBP homology protein (CHOP), homocysteine-induced ER protein (HERP), Tribbles 3 (TRB3), in turn modulate a wide spectrum of cellular events designed to adapt to dietary stress (Kilberg et al., 2009). In addition to regulating amino acid metabolism, recent work from Guo and Cavener (Guo and Cavener, 2007) also shown a broader role for GCN2 in controlling lipid homeostasis in response to amino acid deprivation (Figure 1.18). They demonstrated that GCN2 regulated expression of lipogenic genes through controlling SREBP-1c gene expression during leucine deprivation. Furthermore, B'chir et al. (B'chir et al., 2013) demonstrated that the eIF2α/ATF4 pathway is also essential for stress-induced autophagy gene expression.

Figure 1. 18 Schematic depicting the GCN2 pathway and its influence on lipogenesis



Model of GCN2-dependent regulation of lipid metabolism in liver. Leucine deprivation results in activation of GCN2 and phosphorylation of eIF2α. This is followed by repression of SREBP-1c and its downstream lipogenic genes and, in parallel, activation of ATF4 and C/EBPβ, as well as their downstream amino acid regulatory genes. Lines ending in arrows indicate processes that are activated; lines ending in bars indicate processes that are repressed. The dotted line indicates a process for which the molecular mechanism is unknown. From Guo and Cavener (Guo and Cavener, 2007).

1.2.3 AAs & insulin secretion, insulin action and insulin resistance in mammals

Dietary proteins and AAs have a biphasic effect on the regulation of insulin signaling pathway (Lynch and Adams, 2014; Newsholme et al., 2005; Tremblay et al., 2007).

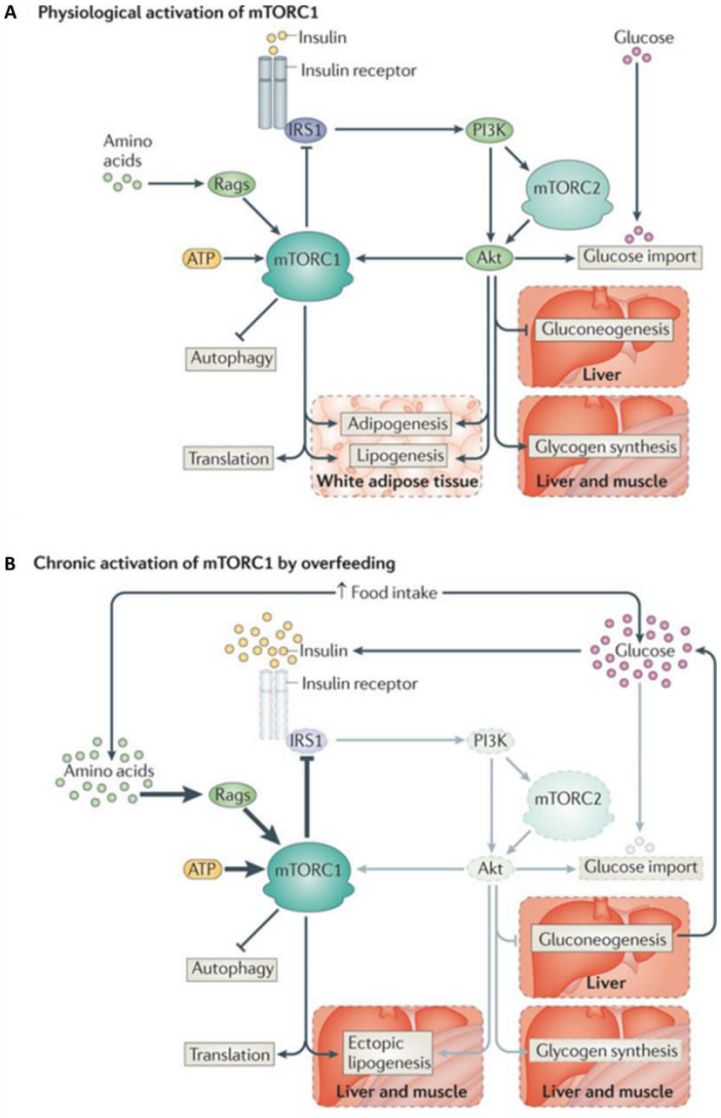
Physiological level of AAs promote insulin signaling and insulin action

At physiological levels, AAs stimulate insulin signaling and insulin action through two major manners. First, under appropriate conditions, amino acids pool or specific amino acids acutely and chronically stimulate insulin secretion from pancreatic β-cells *in vivo* and *in vitro* (Newsholme et al., 2005), which in turn activates IRS-PI3K-Akt cascades and subsequently promotes the GTP charge of Rheb (Shimobayashi and Hall, 2014). Four amino acids are particularly important for stimulating insulin secretion, which are leucine, isoleucine, alanine, and arginine (Newsholme et al., 2005). Second, AAs abundance can be sensed by mTORC1 (Bar-Peled and Sabatini, 2014), which in turn promotes *de novo* lipogenesis and glycolysis, through mTORC1-mediated activation of SREBP-1c and HIF1a, respectively (Laplante and Sabatini, 2013; Shimobayashi and Hall, 2014). AAs (particular leucine, arginine and glutamine) trigger the recruitment of mTORC1 to the outer lysosomal surface which is a prerequisite for the activation of mTORC1 by growth factors (Efeyan et al., 2015; Laplante and Sabatini, 2013). Through these manners, AAs can effectively contribute to the activation of insulin and mTORC1 signaling pathways, which in turn phosphorylate or activate their downstream effectors, thereby promoting insulin action (Figure 1.19 A).

Excessive AAs induce attenuated insulin action or insulin resistance

AAs are known to attenuate insulin sensitivity and action through at least two separated mechanisms. First, hyper-activation of mTORC1 by excessive AAs attenuates insulin action via triggering an mTORC1/S6K1- induced feedback loop, which enhances the phosphorylation of IRS1 on serine residues, dampening the function of IRS1 and preventing the IRS1 mediated recruitment of key downstream effectors to IR, thereby leading to insulin desensitization (Dann et al., 2007; Efeyan and Sabatini, 2010; Tremblay et al., 2007; Um et al., 2006). This results in dampened PI3K/Akt pathway as well as diminished mTORC2 activation (Masui et al., 2014; Shimobayashi and Hall, 2014; Zoncu et al., 2011), which in turn leads to reduced glucose uptake and glycogen synthesis in liver and muscle, and increased gluconeogenesis and glucose release by the liver (Figure 1.19 B) (Zoncu et al., 2011), yet overactivated mTORC1 retained its ability to promote lipogenesis (Laplante and Sabatini, 2010). Insulin resistance is a condition in which normal concentrations of insulin produce a subnormal biological response. It is common in many physiological and pathological states, including obesity, T2D, metabolic syndrome, polycystic ovarian disease, pregnancy and puberty (Feng et al., 2014; Samuel and Shulman, 2012).

Figure 1. 19 mTORC1 activation and insulin action



A | Mammalian target of rapamycin (mTOR) links nutrient abundance with growth and the accumulation of energy stores in anticipation of future nutrient shortage. Feeding raises nutrient (amino acids and glucose) and insulin levels in the bloodstream. These converge to activate mTOR complex 1 (mTORC1) and mTORC2. mTORC1 activates translation, cell mass increase (especially in skeletal muscle) and lipogenesis (which leads to lipid accumulation) in white adipose tissue, while inhibiting autophagy. mTORC2 promotes glucose import in most tissues and promotes glycogen synthesis, but inhibits gluconeogenesis, in the liver.

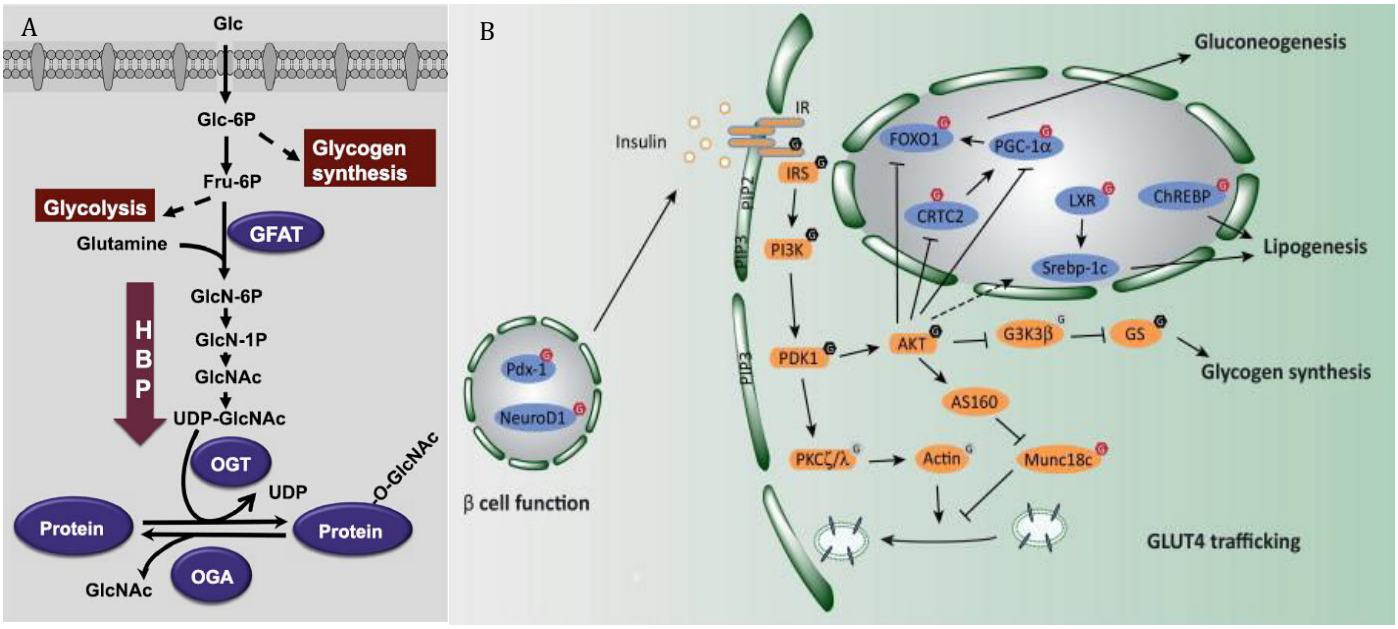
B | An overabundance of nutrients leads to chronic mTORC1 activation, which disrupts energetic homeostasis in different tissues. During chronic hyperinsulinaemia, as occurs in overfeeding states, mTORC1 activity towards S6K1 inhibits insulin receptor signalling at the cellular membrane, in particular in liver and muscle, contributing to the onset of the diabetic state. In an insulin-resistance state, phosphoinositide 3-kinase (PI3K) and Akt are not activated, leading to decreased cellular glucose uptake and to increased hepatic gluconeogenesis, which worsens the hyperglycaemic condition. Despite decreased insulin signalling and an absence of Akt activation, mTORC1 remains active, maintaining the negative feedback loop at work. The hyperactive amino acid input to mTORC1 may explain sustained mTORC1 activity in the context of insulin resistance and may drive the ectopic accumulation of lipids that occurs in muscle and liver. Inactivated pathways are faded out in the figure. From Zoncu et al. (Zoncu et al., 2011).

Persistent activation of mTORC1 may contribute to insulin resistance through (i) mediating excess fat deposition in WAT, liver and muscle; in turn, ectopic fat deposition has a role in the insurgence of insulin resistance (Zoncu et al., 2011); (ii) inducing hyper-activation of mTORC1/S6K1-induced negative feedback loop, which attenuates insulin action and leads to a worsening of the hyperglycaemia, hypertriglyceridaemia and hyperinsulinaemia. Chronically, these effects may lead to obesity or T2D (Lynch and Adams, 2014; Newgard, 2012; Zoncu et al., 2011).

Second, excessive AAs also attenuate insulin action by stimulating hexosamine biosynthetic pathway (HBP)/*O*-linked β-N-acetylglucosamine (*O*-GlcNAc) modification (Issad et al., 2010; Ruan et al., 2013;

Slawson et al., 2010). *O*-GlcNAcylation is a posttranslational modification by *O*-linked β-N-acetylglucosamine (*O*-GlcNAc) moiety at serine or threonine residues of proteins (Singh et al., 2015; Slawson et al., 2010). Similar to other posttranslational modifications such as phosphorylation and acetylation, *O*-GlcNAc can modify a wide spectrum of intracellular proteins, including signaling proteins, transcription factors, metabolic enzymes, and histones, through which it regulates crucial cellular processes, such as signal transduction, transcription, translation, and protein degradation (Issad et al., 2010; Slawson et al., 2010). Cytoplasmic and nuclear proteins can be dynamically modified by *O*-GlcNAc by the enzyme *O*-GlcNAc transferase (OGT) using UDP-GlcNAc as substrate, which is synthesized from glucose, glutamine, and UTP via the HBP (Figure 1.20 A) (Slawson et al., 2010). Excessive AAs (particular glutamine) can induce overexpression of OGT in muscle, fat, or liver, which in turn dampens insulin signaling via *O*-GlcNAc regulation of insulin signaling proteins, including IR, IRS1 and IRS2, PI3K, PDK1, Akt, GSK3 β and transcription factors, such as Foxo1, PGC-1α, liver X receptor (LXR), cAMP response element-binding protein (CREB) regulated transcription coactivator 2 (CRTC2) and carbohydrate responsive element-binding protein (ChREBP) (Figure 1.20 B) (Filhoulaud et al., 2013; Ruan et al., 2013; Slawson et al., 2010), as well as affecting β cell function (Hanover et al., 1999; Liu et al., 2000; Ruan et al., 2013). There is also evidence that high levels of nutrient flux bring about insulin resistance, a hallmark of T2D, via the HBP (Teo et al., 2010).

Figure 1. 20 O-GlcNAc regulation of insulin signaling



A| Hexosamine biosynthetic pathway targets protein O-GlcNAc modification. Glucose (Glc) uptaken by cells is mainly used in glycogen synthesis and glycolysis pathways. 2–5% of glucose fluxes into hexosamine pathway through the conversion of fructose-6-phosphate (Fru-6P) to glucosamine-6-phosphate (GlcN-6P) by a rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT). Subsequent acetylation and uridylation of GlcN-6P produce UDP-GlcNAc as a substrate for protein glycosylation. *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) catalyze the addition and removal of *O*-GlcNAc on proteins, respectively. B| On binding to insulin, the auto-phosphorylated IR catalyzes tyrosine phosphorylation of IRS proteins, which results in the docking and activation of phosphatidylinositol-3-OH kinase (PI3K). PI3K produces phosphatidylinositol 3,4,5-triphosphate (PIP3), which recruits PDK1 and AKT to the plasma membrane. AKT activated by PDK1 phosphorylates numerous substrates to mediate physiological functions. Subsequently, PIP3-binding OGT attenuates insulin signaling by *O*-GlcNAcylating IR, IRS, PDK1, and AKT. AKT phosphorylates AS160, a Rab GAP, to mediate the translocation of the glucose transporter GLUT4 to the membrane. Insulin also activates PKCζ/λ to stimulate the trafficking of GLUT4 vesicles by actin remodeling. *O*-GlcNAcylation suppresses GLUT4 trafficking by inhibiting Munc18c and possibly regulating PKCζ/λ and actin. *O*-GlcNAcylation antagonizes insulin suppression of gluconeogenesis by activating transcription factor and cofactors such as FOXO1, PGC-1α, and CRTC2. SREBP-1c and ChREBP are two key transcription factors that induce expression of lipogenic genes. *O*-GlcNAcylation regulates lipogenesis by directly stabilizing ChREBP and promoting Srebp-1c transcription through LXR activation. *O*-GlcNAcylation of GS suppresses glycogen synthesis. The possible role of *O*-GlcNAcylated GSK3β in glycogen storage has not been explored. In pancreatic β cells, *O*-GlcNAcylated Pdx-1 and NeuroD1 promote transcription of the insulin gene. Red and black hexagons containing the letter G indicate positive and negative regulation of the proteins by *O*-GlcNAcylation, respectively. Grey hexagons indicate that the role of *O*-GlcNAcylation is not known. From Singh et al. (Singh et al., 2015) and Ruan et al. (Ruan et al., 2013).

1.2.4 AAs and insulin/mTOR signaling pathways in fish

Despite the well-studied nature of insulin/mTOR signaling pathway in human, mammals or rodents, relatively less is known in fish, particularly for their roles in the regulation of metabolism.

Piscine insulin and its secretion

Piscine insulins are structurally close to other vertebrate insulins, with alpha and beta chains, linked through critical disulfide bridges (Mommsen and Plisetskaya, 1991; Navarro et al., 2002). The main insulin secretagogues recognized in fish are generally the same as in mammals (Navarro et al., 2002). Although glucose-stimulated insulin release has been reported *in vivo* and *in vitro* in many fish species, amino acids (especially arginine, lysine and leucine) play more potent role in stimulating insulin secretion in most fish species, especially in carnivorous ones (Mommsen and Plisetskaya, 1991). Plasma insulin levels are in the range of 0.2–5 nM, which tends to be surprisingly higher than those found in mammals (Mommsen and Plisetskaya, 1991). However, as noted by Plisetskaya (Plisetskaya, 1998), these values may also include pro-insulin due to the method used for measuring piscine plasma insulin (homologous radioimmunoassay, RIA) and it is unclear whether this precursor peptide has any specific physiological role in fish (Moon, 2001). Thus, until the time that pro-insulin can be precisely measured or more specific measure methods are adapted to fish, our knowledge about piscine insulin secretion will remain obscure.

Insulin signaling pathway in fish

Irrespective of the constraints in measuring piscine insulin, the existence of insulin sensitivity and intact functional mechanisms in fish has been demonstrated by the administration of exogenous insulin (like bovine insulin) in several *in vivo* and *in vitro* studies (Jin et al., 2014b; Navarro et al., 2006; Plagnes-Juan et al., 2008; Polakof et al., 2012a). As in mammals, insulin treatment effectively activates Akt phosphorylation (Jin et al., 2014b; Lansard et al., 2010; Plagnes-Juan et al., 2008). Probably through activating insulin/Akt signaling pathway, insulin was also found to reduce plasma glucose levels, stimulate glucose uptake in the peripheral tissues, up-regulate the gene expression and enzyme activity of glycolytic pathway, enhance glycogenesis and lipogenesis, and suppress gluconeogenesis and fatty acid oxidation potential (Jin et al., 2014b; Kamalam, 2013; Polakof et al., 2010b). However, controversial results exist, especially in the studies performed with insulin administration *in vivo* (Navarro et al., 2006; Polakof et al., 2012a) where insulin only displayed minor effects in stimulating the expression of genes related to glycolysis, lipogenesis and glycogen synthesis in liver and muscle, or in inhibiting the expression of gluconeogenesis and β-Oxidation in rainbow trout. Beyond this, several other hormones such as insulin-like growth factor (IGF) and growth hormone (GH) are also shown to effectively activate

insulin/mTOR signaling pathway (Castillo et al., 2006; Pozios et al., 2001; Velez et al., 2014), promote muscle growth, inhibit protein degradation and atrophy via PI3K-Akt-Foxo signaling pathway (Breves et al., 2014; Fuentes et al., 2013a; Reinecke et al., 2005) and regulate glucose and lipid metabolism (Hemre et al., 2002; Lu et al., 2015). Despite these identified roles of insulin in regulating intermediary metabolism, the underlying mechanisms still need to be defined in fish. Furthermore, it is worth to mention that some studies investigated the effects of insulin *in vivo* with the method of intraperitoneal (IP) administration of insulin or chronic insulin infusion (Jin et al., 2014b; Polakof et al., 2010a; Polakof et al., 2009b) but the results are not completely convincing, especially for the studies performed with physiological doses of exogenous insulin, as endogenous growth factor (i.e. insulin, IGF), hormone (i.e. GH, glucagon, leptin) and cytokine levels were undetermined. Studies in human reported that infusion of IGF-I first suppressed endogenous GH concentrations, while a rebound increase in GH concentrations occurred a few hours after the end of infusion (Chapman et al., 1998; Hartman et al., 1993). Presumably, exogenous insulin might have inevitably decreased the endogenous secretion of insulin, IGF and GH, and enhanced the release of glucagon and leptin, as in mammals (Sandhu et al., 2002), which may partially or totally counteract the exogenous bovine insulin effect at their measured time. Thus, some of the observed effects in these studies might not only attribute to exogenous insulin administration as expected or as previously concluded.

mTOR signaling pathway in fish

Seiliez et al. (Seiliez et al., 2008) first revealed both *in vivo* and *in vitro* the existence of several major kinases involved in mTOR signaling pathway (mTOR, S6K1 and 4EBP1) in fish species, and their responses to nutritional status (feeding). Following this effort, the existence of this pathway has been newly identified in many other fish species, including zebrafish (Seiliez et al., 2012), fine flounder (Fuentes et al., 2013a), Atlantic halibut (Fuentes et al., 2013a), gilthead sea bream (Velez et al., 2014), grass carp (Lu et al., 2015) and Jian carp (Feng et al., 2013; Hu et al., 2015). Progresses have also been made for identifying the physiological and metabolic roles of mTOR pathway in the regulation of growth and pathogenesis in fish. Similar with the other vertebrates, mTOR signaling pathway is significantly activated by refeeding (Fuentes et al., 2015; Fuentes et al., 2013a; Seiliez et al., 2008), association of insulin or IGF-1 (or GH) with amino acids (Fuentes et al., 2013b; Lansard et al., 2011; Lansard et al., 2010), while amino acids alone (without insulin or insulin homologs) fail to stimulate mTOR activation in primary cell culture of trout hepatocytes (Lansard et al., 2010). Moreover, acute administration of rapamycin effectively inhibits the activation of mTOR by AAs and insulin in primary trout hepatocytes (Lansard et al., 2010; Seiliez et al., 2008). Dietary compositions are also observed to mediate mTOR activation with a high protein low carbohydrate diet (HPLC) stimulated the activation of mTOR signaling while a low protein high carbohydrate diet (LPHC) failed to activate mTOR (Seiliez et al., 2011a).

Regarding the effect of individual amino acid, L-Leucine (Lansard et al., 2011; Xu et al., 2013a) and glutamine (Hu et al., 2015) partially activated mTOR activation, while L-Methionine and L-Lysine shown no or only minor effects in stimulating mTOR activation (Belghit et al., 2014; Lansard et al., 2011). Activated mTOR was observed to promote skeletal muscle growth (Fuentes et al., 2015; Fuentes et al., 2013b; Yogev et al., 2013), up-regulate the expression of genes related to glucose uptake (Glut4) (Seiliez et al., 2011a), glycolysis (glucokinase (GK), 6-phosphofructo-1-kinase (6PF1K) and pyruvate kinase (PK)) (Lansard et al., 2011; Lansard et al., 2010), lipogenesis (fatty acid synthase (FAS), ATP citrate lyase (ACLY) and SREBP1) (Lansard et al., 2010; Seiliez et al., 2011a) and AA catabolism (serine dehydratase (SD) and branched-chain α-ketoacid dehydrogenase (BCKD E2)) in liver, muscle and trout hepatocytes (Lansard et al., 2011; Lansard et al., 2010; Seiliez et al., 2011a), down-regulate the expression of genes related to *β*-Oxidation (carnitine palmitoyltransferase 1 alpha (CPT1a), carnitine palmitoyltransferase 1 beta (CPT1b) and 3-hydroxyacyl-CoA dehydrogenase (HOAD)) (Seiliez et al., 2011a), and inhibit autophagy by suppressing autophagy-related gene expression in muscle (light chain 3B (LC3B), gamma-aminobutyric acid (Gabarpl1), autophagy-related protein 12 (Atg12l), atrogin1 and Murf) and in trout myoblasts (Seiliez et al., 2012; Seiliez et al., 2011a), as well as regulate autophagy-related protein phosphorylation (LC3 and p62) (Heijnen et al., 2014). Interestingly, fructose treatment in zebrafish leads to hepatic lipid accumulation, mitochondrial abnormalities, and ER defects, while these effects was reversed by mTOR inhibition, implicating its important role in the regulation of lipid homeostasis (Sapp et al., 2014). Furthermore, using zebrafish models, researchers have explored the contributions of mTOR in the pathogenesis of many diseases, including cardiomyopathy (Ding et al., 2012a; Ding et al., 2012b), NAFLD (Sapp et al., 2014), Roberts Syndrome (Xu et al., 2013b) and colorectal cancer pathogenesis (Valvezan et al., 2014). In brief, it seems that the mTOR pathway is evolutionarily conserved in by far examined fish species and its responses to investigated nutritional stimuli are very similar to those occurring in mammals and other vertebrates.

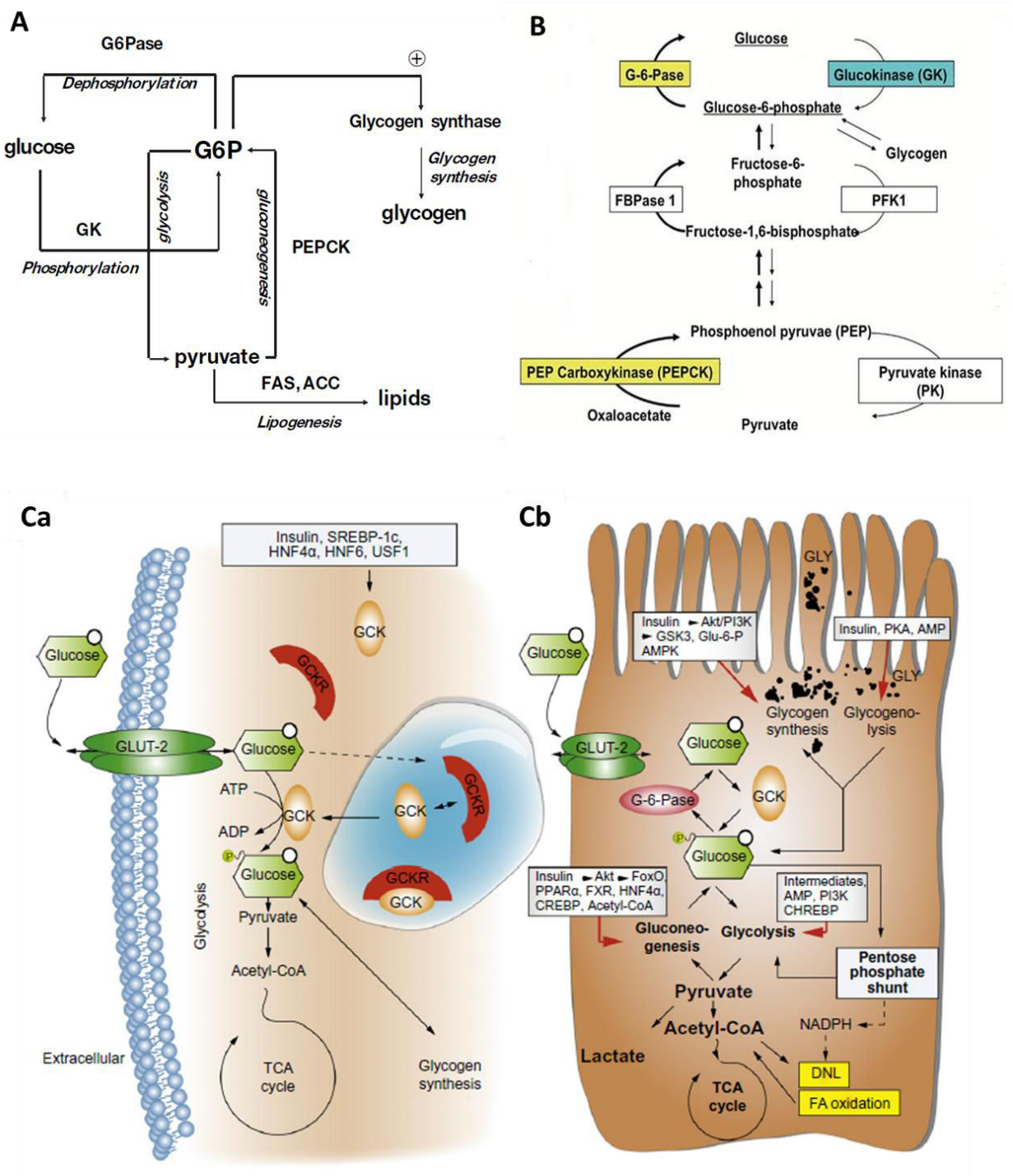
1.3 The regulation of hepatic intermediary metabolism in mammals and fish

In fish, as in mammals, the liver plays a central role in metabolic homeostasis and is a major site for synthesis, metabolism, storage and redistribution of carbohydrates, proteins and lipids (Bechmann et al., 2012; Enes et al., 2009). Generally, the metabolic machinery of intermediary metabolism in fish is much the same as that in mammals (Dabrowski and Guderley, 2002; Walton and Cowey, 1982). The main functional differences between fish and mammals lie in the means by which control is exercised, in the sensitivity of metabolic demand to biotic and abiotic factors, and in the exact roles of tissues and organs (Dabrowski and Guderley, 2002). Among those abiotic factors, temperature plays a crucial role on the regulation of intermediary metabolism in fish as the majority of fish are poikilotherm thus in thermal equilibrium with their ambient temperature (Dabrowski and Guderley, 2002; Guillaume et al., 2001). It pervasive affects enzyme activity and reaction rates of some pathways (Somero, 1997). Being poikilothermic also dramatically reduces energy expenditure and energy requirement in fish whereas mammals and other homeotherms expend much energy to maintain their body temperature (Guillaume et al., 2001). As fish live in water, their nitrogenous wastes are directly excreted in the environment. Thus fish eliminate most of their excess nitrogen as ammonia from the gills, thereby simplifying their intermediary metabolism (Dabrowski and Guderley, 2002). Furthermore, in contrast to the situation in mammals where carbohydrates and lipids are important energy sources while protein catabolism is of little significance in supplying energy, in most fish species dietary carbohydrates are not the principal source of energy or carbon, while proteins together with lipids serve as the major energy sources (Enes et al., 2009; Guillaume et al., 2001). Finally, many aspects of muscle metabolism are better understood in fish than in mammals as fish muscles possess separated fiber types, continuous muscle recruitment throughout much of the life cycle and other unique metabolic features (Guderley, 2004; Johnston, 1999; Seiliez et al., 2012; Shewfelt, 1981).

1.3.1 The regulation of hepatic glucose metabolism

Under hormonal control, liver responds to either feeding or fasting conditions to maintain glucose homeostasis as follows (Figure 1.21 A and B): in the postprandial period, blood glucose levels are elevated, which in turn stimulates insulin secretion. Following the release of insulin, liver takes up part of glucose to promote glucose disposal (glycolysis) and replenish glycogen stores (glycogenesis). Besides, when glucose stores are full, the liver promotes *de novo* lipid synthesize (lipogenesis) for long-term energy storage. Lipids are packaged in very low-density lipoprotein (VLDL) particles and then transported to the adipose tissue. Conversely during the starvation state, glycaemia falls and glucagon increases, the liver produces glucose to maintain circulating glucose levels by breaking down glycogen reserves (glycogenolysis) or by stimulating *de novo* glucose synthesis (gluconeogenesis)

Figure 1. 21 Hepatic glucose metabolic pathways and their regulation



A| Major glucose metabolic pathways in the liver. Glucose 6-phosphate (G6P) is a central intermediate in the hepatic glucose metabolism, which is shared by several pathways. Hepatic glucokinase (GK or GCK) allows for rapid and efficient phosphorylation of glucose in G6P. In hepatocytes, G6P concentrations are determined by the balance between glucose 6-phosphatase (G6Pase) and GK activities. G6P is an allosteric activator of glycogen synthase (GS), but is also a key metabolite than can enter glycolysis. In addition, since the major function of glycolysis in liver is to provide carbons from glucose for *de novo* lipid synthesis (lipogenesis), G6P is also considered as determinant for this pathway and for the induction of fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC). B| Rate-limiting enzymes of glucose phosphorylation, dephosphorylation (G6Pase), glycolysis (phosphofructokinase-1 (PFK1) and pyruvate kinase (PK)) and gluconeogenesis (phosphoenolpyruvate carboxykinase (PEPCK) and (Fructose 1,6-bisphosphatase) FBPase) pathway. C| Regulation of hepatic glucose metabolism. (a) After intestinal absorption, glucose (Glu) reaches the hepatocyte

via the portal vein. The insulin-independent glucose transporter 2 (GLUT2) shuttles Glu across the membrane. Abundance of glucose induces conformational changes of the glucokinase regulatory protein (GCKR), which binds to glucokinase (GCK or GK) and keeps it in the nucleus in the fasting state. GCK is then released into the cytosol and phosphorylates Glu to glucose-6-phosphate (G6P); depending on the nutritional state, it serves as a substrate for glycolysis or glycogen synthesis, respectively. GCK is transcriptionally regulated by insulin and nuclear receptor signaling. (b) G6P is a central intermediate in the hepatic glucose metabolism. It is degraded during glycolysis, which provides energy in the form of two ATP and two NADH molecules per glucose molecule. The product pyruvate is further decarboxylized to acetyl-CoA, which enters the intramitochondrial tricarboxylic acid cycle (TCA). Alternatively, G6P is degraded in the pentose-phosphate shunt, which provides NADPH, a co-substrate for lipogenesis. Acetyl-CoA is an important product of the TCA, linking glucose and lipid metabolism, as it is the substrate for lipogenesis. Gluconeogenesis and glycogenolysis provide G6P as a substrate for glucose synthesis in the fasting state. Glucogenolysis is catalyzed by glycogen phosphorylase, activated by AMP, and repressed by insulin. The key enzyme in gluconeogenesis is PEPCK, which is repressed by insulin signaling via Akt-mediated FoxO phosphorylation and activated by PPARα (Adapted from Postic et al. (Postic et al., 2004), Shoji et al. (Shoji et al., 2011) and Bechmann et al. (Bechmann et al., 2012)).

(Klover and Mooney, 2004; Polakof et al., 2012a; Postic et al., 2004; Vidal-Puig and O'Rahilly, 2001). Both transcriptional regulation of rate-limiting enzymes and modulation of enzyme activity through phosphorylation and allosteric regulation are involved in these processes (Figure 1.21 C) (Klover and Mooney, 2004).

1.3.1.1 Hepatocyte glucose uptake and phosphorylation

In the postprandial state, blood glucose passes the portal vein into liver cells via the glucose transporter type 2 (GLUT2), a membrane-bound transporter with high capacity and low affinity for glucose. In contrast to GLUT4, which is highly expressed in adipose tissue and skeletal muscle, and finely controlled by insulin (Bryant et al., 2002; Huang and Czech, 2007), the expression and activity of GLUT2 is independent of insulin signaling and the rate of entry into the hepatocyte is proportional to the extracellular glucose concentration (Leturque et al., 2009). Once taken up by the hepatocyte, glucose is rapidly phosphorylated to glucose-6-phosphate (G6P) by glucokinase (GK) (Figure 1.21 B), the first and rate-limiting enzyme for hepatic glucose utilization (Choi et al., 2013). Its function constitutes a simple, direct intracellular nutrient-sensing mechanism that controls systemic glucose homeostasis (Efeyan et al., 2015). In contrast to other hexokinases, GK (hexokinase IV) has significantly low affinity for glucose and is only active when glucose levels are relatively high (around 120 mg dl^{-1}, or 7 mM, or greater). Furthermore, since GK is not inhibited by G6P at physiological concentrations (Matschinsky, 2009), it allows for enhancing hepatic glycogen synthesis in hepatocyte (Choi et al., 2013). Moreover, as it is inactive under conditions of glucose limitation, it permits export of unphosphorylated glucose from the liver in order to supply the energetic demands of the brain and muscles (Efeyan et al., 2015). When hepatic glucose levels are high, GK-mediated conversion of glucose to the metabolic intermediate G6P allows glucose to be shunted into glycolysis (for energy production) or glycogen synthesis (Printz et al., 1993).

In rodents, GK enzyme is highly regulated in the liver, at both transcriptional and post-transcriptional levels (Iynedjian, 2009; Panserat et al., 2014; Vidal-Alabró et al., 2012). At the transcriptional level, hepatic GK is tightly controlled by insulin (positive) and glucagon (negative) (Iynedjian, 2009; Matschinsky, 2009; Postic et al., 1999). Insulin-mediated induction of GK gene

expression is triggered by the PI3K-Akt signaling pathway through the coordination of transcriptional factors, SREBP-1c, HIF1α, hepatic nuclear factor-4-alpha (HNF4α), hepatic nuclear factor 6 (HNF6), FoxO1 and upstream stimulatory factor 1 (USF1) (Bechmann et al., 2012; Duvel et al., 2010; Laplante and Sabatini, 2013; Vidal-Alabró et al., 2012). For instance, in cultured rat hepatocytes, the effect of insulin as inducer of GK was shown to be primarily at a transcriptional level, taking place in glucose free as well as glucose containing medium without any synergistic effect of glucose addition (Iynedjian, 1993). GK is also regulated by glucokinase regulatory protein (GKRP), which allosterically regulates the activity and subcellular localization of GK (Figure 1.21 Ca) (Choi et al., 2013). In the fasting state, GK is inactive and sequestered by GCKR within the nucleus. Post-prandial glucose abundance and insulin action synergistically cause rapid dissociation of GK from GCKR. Released GK returns to cytosol where it phosphorylates glucose to G6P (Beck and Miller, 2013).

In fish, the regulation of hepatic GK gene expression and activity seems to be highly dependent on blood glucose level changes induced by dietary carbohydrate modifications (Enes et al., 2009; Kamalam et al., 2012; Panserat et al., 2001b; Panserat et al., 2000; Panserat et al., 2014; Polakof et al., 2011b; Polakof et al., 2012a). The response of hepatic GK was repeatedly observed to increase proportionately with dietary starch content even in carnivorous rainbow trout, sea bass and sea bream (Capilla et al., 2003; Enes et al., 2008b; Kamalam et al., 2012). Recently, three independent studies reported that elevated GK expression and/or activity are associated with the activation of mTOR signaling pathway (Kamalam et al., 2012; Lansard et al., 2011; Lansard et al., 2010). Kamalam et al. (Kamalam et al., 2012) demonstrated that a diet rich in carbohydrate induced hyperglycemia, elevated hepatic GK gene expression, suppressed AMPK phosphorylation and enhanced S6 phosphorylation (mTOR downstream effector) in both fat line and lean line of rainbow trout. Using trout primary hepatocytes, Lansard et al. (Lansard et al., 2011; Lansard et al., 2010) demonstrated that the association of insulin with amino acids up-regulated the expression of GK through an mTOR-dependent manner. Due to the constraints in measuring piscine insulin *in vivo* (Plisetskaya, 1998), it is still unclear whether dietary carbohydrate modification- or plasma glucose level-induced GK responses are through insulin or relevant cellular signaling pathways (i.e. insulin or AMPK pathway). However, it is clear that mTOR is involved in the transcriptional regulation of GK in rainbow trout (at least *in vitro*), as demonstrated in mammals (Dibble and Manning, 2013). Recently, GKRP has been identified in rainbow trout, carp and goldfish, with biochemical and functional characteristics similar to that of mammals (Polakof et al., 2009a). Surprisingly, trout GKRP was the most potent among the analyzed fish species, but functionally less active than the mammalian isoform. However, it is unclear whether this protein has any role in "glucose intolerance" phenotype of carnivorous fish. In brief, it is rather clear that unlike cat, barn owl and some other mammalian carnivorous models, where hepatic GK expression and activity are lacking (Koizumi et al., 2005; Schermerhorn, 2013; Washizu et al., 1999), carnivorous fish has inducible hepatic GK (Panserat et al., 2000; Panserat et al.,

2014). Although some controversial results exist where IP administration of insulin fails to stimulate GK or HK expression in liver and muscle, respectively (Cowey et al., 1977; Jin et al., 2014b; Polakof et al., 2010a; Polakof et al., 2009b), it seems that the responses of GK to feeding status (Enes et al., 2009; Panserat et al., 2014), dietary carbohydrate inclusion (Kamalam et al., 2012; Seiliez et al., 2011a), insulin stimulations (*in vitro*) and mTOR activation (Lansard et al., 2011; Lansard et al., 2010; Plagnes-Juan et al., 2008) are quite decent and logical compared to what is known in mammals. However, whether the regulations of hepatic GK by dietary nutrients and hormone are fine, potent and timely enough to cope with those continuously dietary nutrient inputs in carnivorous fish remain unclear and need to be defined. Indeed, this is probably an issue related to insulin secretion and action. Given its pivotal role in glucose sensing and in the regulation of glucose homeostasis (Efeyan et al., 2015; Matschinsky, 2005; Postic et al., 1999), the physiological and functional role of this enzyme still need to be closely monitored.

1.3.1.2 Glycolysis and glycogen synthesis

Depending on the systemic metabolic state, G6P is either utilized for glycolysis or shunted into glycogen synthesis (Bechmann et al., 2012). Through ten step processes, glycolysis metabolizes glucose to pyruvate with a net gain of two ATP and two NADH molecules per glucose molecule (Lunt and Vander Heiden, 2011). Glycolysis is regulated by three key enzymes: GK, which provides G6P, 6-phosphofructo-1-kinase (6PF1K), which is inhibited by its product fructose-1,6-bisphosphate and pyruvate kinase (PK), the final step of glycolysis (Figure 1.21 B) (Pilkis and Granner, 1992). PK is activated by its substrate and inhibited by ATP abundance. Insulin, epinephrine, and glucagon modulate PK via the PI3K/Akt pathway (Bechmann et al., 2012) and ChREBP induces transcription of PK in response to glucose (Filhoulaud et al., 2013; Postic et al., 2007). In mammals, hepatic glycolytic gene expression is regulated by the availability of carbohydrate in the diet (Foufelle and Ferre, 2002) and insulin via transcriptional factors of SREBP1c, the forkhead related protein (FKHR) and HIF1a (Dibble and Manning, 2013; Duvel et al., 2010; Foufelle and Ferre, 2002). Pyruvate is further decarboxylized to acetyl-CoA and then processed in the TCA or utilized for *de novo* lipogenesis (DNL). The pentose phosphate pathway (PPP) is an alternative way for degradation of G6P in hepatocytes. It provides the cell with NADPH, an important antioxidant and co-substrate for DNL and cholesterol synthesis (Patra and Hay, 2014). G6P also can be converted to glucose-1 phosphate and glycogen is synthesized by the enzyme of glycogen synthase (GS). Liver glycogen serves as a glucose buffer, storing glucose when blood concentrations are high and releasing it when glucose is required in other parts of the body. Glucose is stored as glycogen in both liver and muscle. However, since muscle lacks the enzyme of glucose-6-phosphatase (G6Pase), an enzyme that converts G6P to glucose, muscle glycogen is unable to contribute to blood glucose although the muscle stores are large (Campbell, 2006). GS is regulated by the allosteric activator G6P and is inactive in the phosphorylated state. Furthermore, GS is regulated by insulin through GSK3 (Figure 1.21 Cb), which phosphorylates GS and is a direct downstream target of Akt. Other protein kinases

that phosphorylate GS are AMPK and protein kinase A (PKA). Insulin activates glycogen synthesis via repression of PKA (Bechmann et al., 2012).

Generally, *in vivo* studies in fish shown that, compared to GK, the expression and/or enzyme activity of 6PF1K and PK are less responsive to nutritional status, dietary composition, feeding regime or intraperitoneal (IP) administration of insulin (reviewed by (Enes et al., 2009)). Though starvation results in decreased 6PF1K and PK activity, the expression of 6PF1K or PK is barely affected by feeding status or dietary modifications (Enes et al., 2009; Kirchner et al., 2003; Panserat et al., 2001d). However, Cowey and Walton (Cowey and Walton, 1989) summarized that hepatic levels of glycolytic enzymes (6PF1K, PK and lactate dehydrogenase) are decreased when fish are fed a high protein/low carbohydrate (HP/LC) diet compared to a low protein/high carbohydrate diet (LP/HC). Although no effect was observed for the expression/activity of hepatic 6PF1K or PK in response to IP injection of insulin (Cowey et al., 1977; Jin et al., 2014b; Plagnes-Juan et al., 2008), recent *in vitro* studies with trout primary hepatocytes clearly demonstrated that insulin together with amino acids up-regulated the expression of 6PF1K and PK through a rapamycin-sensitive manner, indicating that insulin stimulates hepatic glycolytic pathway via Akt/mTOR signaling pathway (Lansard et al., 2011; Lansard et al., 2010; Plagnes-Juan et al., 2008). This observation is consistent with the known mechanisms in mammals (Dibble and Manning, 2013; Duvel et al., 2010). In line with these observations, glucagon decreases the affinity of PK for its substrate (phosphoenolpyruvate) and increases its sensitivity to ATP inhibition in both rainbow trout and sea raven hepatocytes (Enes et al., 2009).

As in vertebrates, hepatic glycogen content in fish serves as a universal reserve of glucose which can be used under conditions of food deprivation or low carbohydrate intake (Kamalam, 2013). A linear relationship between carbohydrate intake and hepatic glycogen content has been reported in many studies. For instance, glycogenesis originating from dietary starch was found to contribute up to 69% of the liver glycogen pools in gilthead sea bream, using 13C isotope-labelled starch (Ekmann et al., 2013). An *in vivo* study in rainbow trout hepatocytes indicated that both the glycogen synthetic and breakdown pathways are active concurrently and any subtle changes in the glycogen phosphorylase (GP) to synthase ratio may determine the hepatic glycogen content (Pereira et al., 1995). Nutritional status, carbohydrate intake, glucagon and insulin treatment are known to influence GS and GP activity (Kamalam, 2013). While feed deprivation and glucagon induces GP activity, dietary intake of carbohydrates and insulin decreases it. Thus, the regulation of hepatic glycogen synthesis in fish seems similar to higher vertebrates (Kamalam, 2013).

1.3.1.3 Glycogenolysis and gluconeogenesis

During periods of systemic glucose scarcity, the liver maintains glycaemia through gluconeogenesis and glycogen breakdown (Yoon et al., 2001). Glycogen is broken down through the action of the glycogen phosphorylase (GP). G6P is reformed and glucose released into the circulation via the action of glucose-6-phosphatase (G6Pase) (Nordlie et al., 1999). GP is regulated through allosteric activation by AMP and via phosphorylation by PKA, which is inhibited by insulin and stimulated by glucagon (Bechmann et al., 2012).

Hepatic gluconeogenesis occurs during prolonged fasting situations and begins intramitochondrially by the induction of pyruvate carboxylase in abundance of acetyl-CoA (Bechmann et al., 2012). The major gluconeogenic precursors are lactate, pyruvate, glycerol and gluconeogenic amino acids, principally alanine (Barthel and Schmoll, 2003; Nordlie et al., 1999). Release of gluconeogenic precursors from peripheral tissues occurs in situations such as starvation (amino acids from muscle) and severe exercise (lactate from muscle glycogen) (Nordlie et al., 1999). The rate of hepatic gluconeogenesis is controlled by the substrate availability, as well as by the direct effects of hormones (particularly insulin, glucagon and glucorticoids), which potently controls gluconeogenic enzymes at the transcriptional level (Bechmann et al., 2012; Yoon et al., 2001). Gluconeogenesis is principally controlled by three unidirectional enzymes: phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase) and G6Pase) (Figure 1.21 B) (Nordlie et al., 1999). The transcription of these enzymes is suppressed by insulin and stimulated by glucagon via FoxO1 (Puigserver et al., 2003), PPARγ coactivator-1α (PGC-1α) (Puigserver et al., 2003; Yoon et al., 2001), cAMP response element-binding protein (CREB) (He et al., 2009), CREB regulated transcription coactivators (CRTCs) (Barthel and Schmoll, 2003) and ER-bound transcription factor (CREBH) (Lee et al., 2010). In the fasted state, insulin levels drop while glucagon secretion goes up and catecholamines and glucocorticoids remain stable, resulting in increased glycogenolysis and gluconeogenesis. In the fed state, increased insulin secretion suppresses glycogenolysis and hepatic gluconeogenesis (Yoon et al., 2001).

Despite the poor utilization of glucose, fish still presumably require glucose since glucose represents the preferred fuel for brain and nervous tissue, red blood cells and gonads (Walton and Cowey, 1982). Furthermore, in general carnivores, glucose needs to be readily available for catching prey and a high rate of gluconeogenesis from amino acid catabolism is responsible for providing it (Robert and Backus, 2008). Hence in fish, especially in carnivorous fish, gluconeogenesis is likely to be important. Concerning the nutritional regulation, in omnivorous fish such as common carp, the responses of hepatic gluconeogenic enzymes (PEPCK, FBPase and G6Pase) are very much similar to higher vertebrates (Panserat et al., 2002; Shikata et al., 1994; Sugita et al., 1999; Sugita et al., 2001). However, in carnivorous fish species such as rainbow trout, sea bass and sea bream, these enzymes are barely affected by nutritional status or dietary carbohydrate inclusion (Caseras et al., 2002;

Fernández et al., 2007; Moreira et al., 2008; Panserat et al., 2001a; Panserat et al., 2001c) though minor changes at the expression level may have been observed in a few *in vivo* investigations (Kamalam et al., 2012; Panserat et al., 2001b; Seiliez et al., 2011a). Despite these poor regulation by dietary carbohydrate *in vivo*, studies performed with insulin stimulation of primary cell culture of hepatocyte or IP administration of insulin shown that insulin can effectively suppress the expression of hepatic gluconeogenic enzymes both *in vivo* and *in vitro* (Lansard et al., 2011; Lansard et al., 2010; Plagnes-Juan et al., 2008). Furthermore, glucagon incubation was also observed to successfully increased PEPCK activity in sea raven hepatocytes (Foster and Moon, 1990). In healthy human or mammals, refeeding or a diet rich in carbohydrate elevates blood glucose level, which stimulates insulin release. Elevated insulin subsequently promotes hepatic glycolysis and suppresses glucose production, including hepatic gluconeogenesis, thereby preventing hyperglycemia (Klover and Mooney, 2004). Thus, it is reasonable to presume that the impaired post-prandial down-regulation of hepatic gluconeogenesis in carnivorous fish is due to inadequate insulin effect, namely insulin secretion and/or action. Unlike human or omnivorous mammals but similar to what is observed in cat and other carnivorous vertebrates (Schermerhorn, 2013), carnivorous fish is more responsive to dietary protein modifications than dietary carbohydrate (Cowey et al., 1977; Enes et al., 2006; Enes et al., 2009; Hilton and Atkinson, 1982; Kamalam, 2013; Kamalam et al., 2012; Kirchner et al., 2003; Walton, 1986). For instance, Kirchner et al. (Kirchner et al., 2003) observed that low protein intake significantly reduced hepatic gluconeogenic enzyme activities and gene expression (FBPase and G6Pase) in rainbow trout. In line with this observation, other investigations in trout and sea bass also reported that higher hepatic gluconeogenic enzyme activities and/or mRNA levels were associated with high dietary protein diet/intake compared to low protein diet/intake (Cowey et al., 1977; Enes et al., 2006; Hilton and Atkinson, 1982; Kamalam et al., 2012; Kirchner et al., 2003; Walton, 1986). Furthermore, our previous *in vitro* study also demonstrated that increased AAs levels enhanced gluconeogenic mRNA levels (G6Pase and mPEPCK) independently of the insulin/TOR signaling pathway (Lansard et al., 2010).

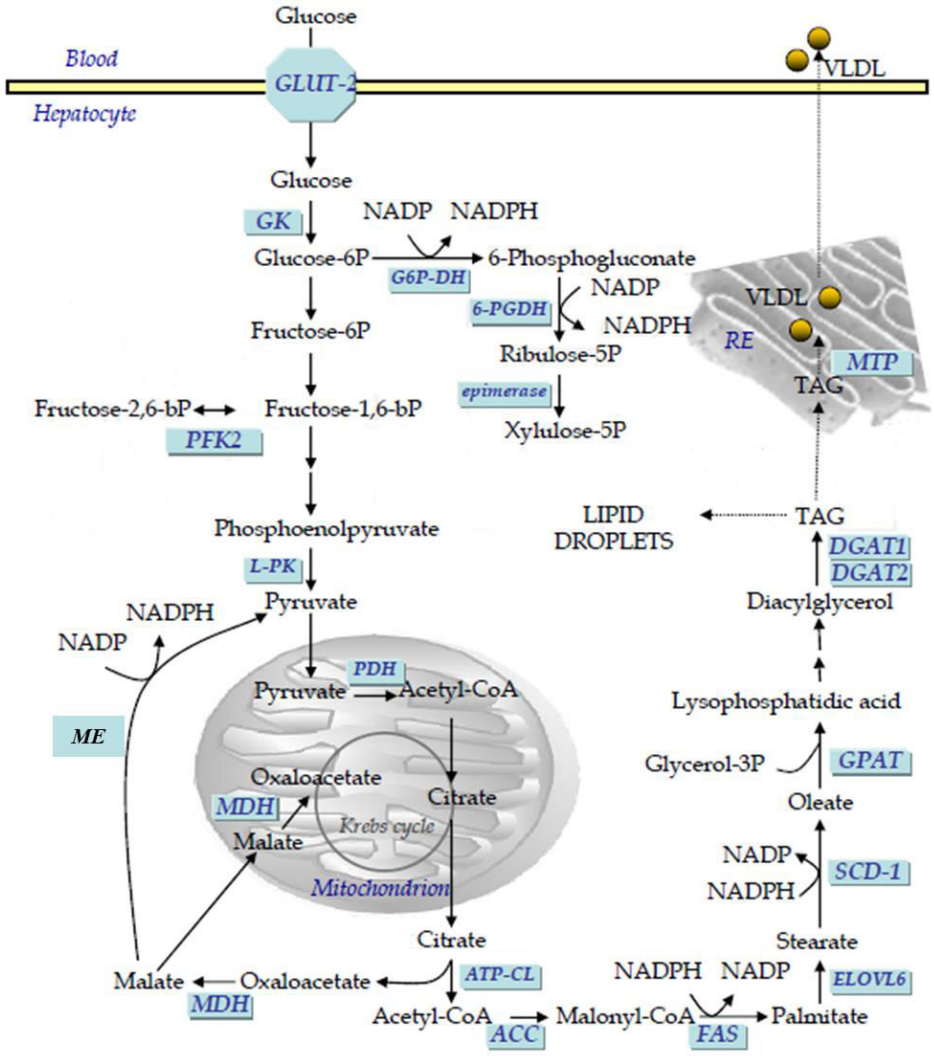
1.3.2 The regulation of hepatic lipid metabolism in mammals and fish

In mammals, the liver is crucial for maintaining overall energy homeostasis and for the conversion of carbohydrate into fat (Postic et al., 2007). When glucose is delivered into the portal vein in large quantities and hepatic glycogen concentrations are restored, glucose is converted in the liver into lipids through *de novo* lipogenesis (Figure 1.22). Then lipids are exported as very-low-density lipoprotein (VLDL) and ultimately stored as triglycerides (TGs) in adipose tissue, which, when necessary, provide energy through *β*-oxidation (Menendez and Lupu, 2007; Postic et al., 2007).

1.3.2.1 Hepatic lipogenesis

De novo lipogenesis (DNL) is the metabolic pathway that synthesizes fatty acids (FA) from excess carbon-donors (mainly acetyl-CoA and malonyl-CoA). These fatty acids can then be converted into triglycerides, the major energy storage form in vertebrates (Lee et al., 2008; Strable and Ntambi, 2010; Tocher, 2003). DNL is mainly catalyzed by ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Figure 1.22) (Towle et al., 1997). Flux through the lipogenic pathway depends on the availability of both lipogenic substrates and cofactors such as NADPH produced by the pentose phosphate pathway (Girard et al., 1997). In mammals, hepatic lipogenesis is very responsive to dietary modifications (Kersten, 2001). Consumption of a diet rich in carbohydrates stimulates the lipogenic pathway, whereas consumption of a diet rich in lipids and poor in carbohydrates, or rich in polyunsaturated fatty acids decreases this pathway (Kersten, 2001; Towle et al., 1997). High carbohydrate diet can induce hyperglycemia, thereby stimulating lipogenesis via several mechanisms (Kersten, 2001). First, by being glycolytically converted to acetyl-CoA, glucose renders substrate for fatty acid synthesis. Secondly, glucose positively stimulates glycolytic and lipogenic gene expression through transcription factor carbohydrate response element-binding protein (ChREBP) (Filhoulaud et al., 2013). Finally, glucose stimulates the release of insulin from the pancreas, thereby activating PI3K/Akt signaling pathway (Kersten, 2001; Saltiel and Kahn, 2001), which stimulates hepatic lipogenesis via the transcription factor of sterol regulatory element binding protein-1c (SREBP-1c) (Horton et al., 2002). Regarding dietary protein, data in rodents, chicken and humans revealed that the metabolic adaptation to a high protein diet included a down-regulation of lipogenesis at both gene expression and enzymatic levels (Abu-Elheiga et al., 2012; Allee et al., 1971; Pichon et al., 2006; Rietman et al., 2014a; Uebanso et al., 2009). These effects are partly mediated by hormones, which inhibit (growth hormone, leptin) or stimulate (insulin) hepatic lipogenesis (Kersten, 2001; Saltiel and Kahn, 2001). At the molecular level, hepatic lipogenesis is regulated in a combinatorial manner by transcription factors including peroxisome proliferator-activated receptor gamma (PPARγ), LXR (Li et al., 2011), ChREBP (Filhoulaud et al., 2013), SREBP-1c (Jeon and Osborne, 2012; Shao and Espenshade, 2012), XBP1(Lee et al., 2008), nuclear sterol-activated receptor LXR and FXR (Calkin and Tontonoz, 2012), as well as microRNA (miRNAs) (Dumortier et al., 2013; Fernandez-Hernando et al., 2011). Concerning cellular signaling

Figure 1. 22 Metabolic pathways leading to the synthesis of triglycerides from glucose in liver



Once inside the hepatocyte, glucose is metabolized on one hand through glycolysis to pyruvate (GK means glucokinase; PFK-2, 6, phosphofructo-2-kinase/fructose-2,6-bisphosphatase; L-PK, liverpyruvate kinase). On the other hand, glucose is oxidized through pentose phosphate pathway to obtain NADPH (G6P-DH means glucose-6-phosphate dehydrogenase; 6-PGDH, 6-phosphogluconate dehydrogenase). Pyruvate enters the mitochondrion to obtain citrate (PDH means, pyruvate dehydrogenase; MDH, malate dehydrogenase and EM, malic enzyme). De novo synthesis of fatty acids starts with citrate (ATP-CL means ATP citrate lyase; ACC, acetyl-CoA carboxylase) and after suffering elongation and desaturation reactions (ELOVL6 means elongase that catalyzes the conversion of palmitate to stearate; SCD-1, stearoyl-coenzyme A desaturase), fatty acids are converted to triglyceride (TAG) (GPAT means glycerol-3-phosphate acyltransferase; DGAT, diacylglycerol acyltransferase). Triglyceride can be stored in the liver but are mostly packaged into VLDL (very low-density lipoprotein) and secreted to bloodstream (MTP means microsomal triglyceride transfer protein). Adapted from (Vidal-Alabró et al., 2012).

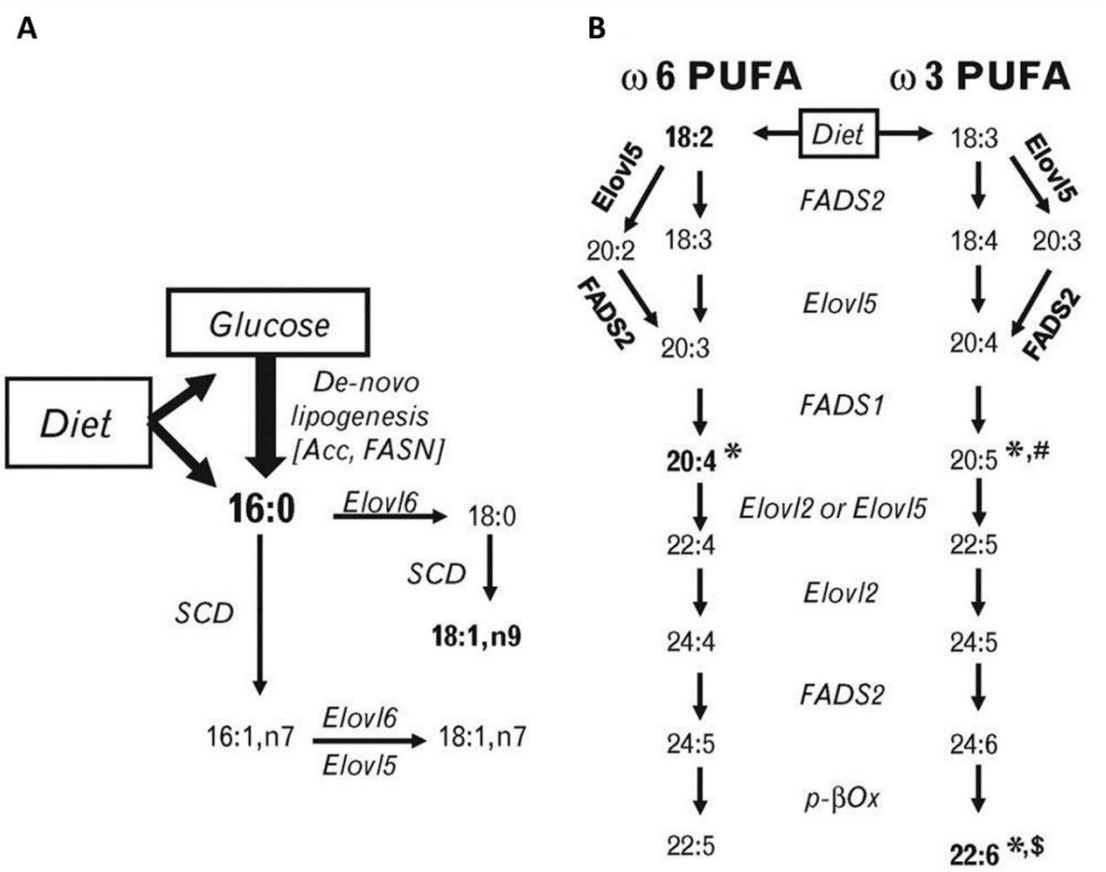
pathways, hepatic lipogenesis is regulated by insulin/mTOR (Ricoult and Manning, 2013), AMPK (Li et al., 2011), autophagy (Yang et al., 2010), p38MAPK (Xiong et al., 2007a) and GCN2-ATF4 (Guo and Cavener, 2007) pathways.

In fish, whereas numerous long-term feeding studies have shown that carbohydrate rich diets elevated the expression/activities of lipogenic enzymes and/or NADPH yielding as in mammals (Barroso et al., 2001; Dias et al., 1998; Enes et al., 2008a; Fynnaikins et al., 1992; Kamalam et al., 2012; Likimani and Wilson, 1982; Shiau and Chen, 1993; Shiau and Lin, 1993), studies in short-term

(i.e. one single meal, IP injection of glucose or feeding trial shorter than two weeks), particularly in carnivorous species, clearly unveiled a different landscape. Recent *in vivo* studies have demonstrated that high carbohydrate diets or glucose failed or only had very limited effects in stimulating lipogenic gene expression or enzyme activities (Hemre and Kahrs, 1997; Jin et al., 2014b; Seiliez et al., 2011a). For instance, Seiliez *et al*. (Seiliez et al., 2011a) reported that a diet rich in carbohydrate and low in protein failed to elevated the expression of gene related to lipogenesis (FAS, ACLY, glucose-6-phosphate dehydrogenase (G6PDH) and SREBP1). Jin *et al.* (Jin et al., 2014b) showed that intraperitoneal glucose administration only induced minor changes of the gene expression of ACLY, without influencing the expression of the other lipogenic genes (FAS, ACLY and SREBP1). Following radiolabelled $^{14}C_1$-glucose course, Hemre and Kars (Hemre and Kahrs, 1997) clearly showed that hepatic lipogenesis converted only a very small proportion of injected glucose into lipids in Atlantic cod. By using metformin, a common anti-diabetic drug, Panserat et al. (Panserat et al., 2009) observed that metformin effectively reduced postprandial hyperglycemia in trout fed high dietary carbohydrates, possibly through stimulating hepatic lipogenic mRNA levels and activities, indicating that biotransformation of glucose to fatty acids in trout may occur under non-physiological situations. In contrast, enhanced lipogenic gene expression and/or enzymatic activities was repeatedly observed in rainbow trout and blackspot seabream fed diets rich in protein and low in carbohydrate (Figueiredo-Silva et al., 2009; Seiliez et al., 2011a), implying that high protein diets appear to play a predominant role in stimulating lipogenic pathway in carnivorous fish. It is also worthy to note that most of these studies investigating high carbohydrate effects were associated with the expense of dietary protein/lipids modifications (Dias et al., 1998; Kamalam et al., 2012; Likimani and Wilson, 1982; Seiliez et al., 2011a; Suarez et al., 1995; Wilson and Likimani, 1981), hence it is difficult to distinguish which nutrient induced those metabolic changes. Moreover, some studies concluded that high carbohydrate diets/D-glucose enhanced hepatic lipogenesis mainly based on the results of NADPH yield, namely the gene expression and/or enzyme activities of G6PDH, 6PGD or ME (Enes et al., 2008a; Fynnaikins et al., 1992; Suarez et al., 1995), instead of *de novo* lipogenic gene expression or enzymatic activities. Therefore, the link between dietary carbohydrate/protein and DNL remains to be clearly defined in carnivorous fish. As many studies observed that dietary carbohydrate of glucose failed to stimulate hepatic lipogenesis but elevated the expression and/or activity related to NADPH production in fish (Hemre and Kahrs, 1997; Jin et al., 2014a; Seiliez et al., 2011a), it was suggested that the role of carbohydrates in DNL is more related to the production of cytosolic reducing equivalents (NADPH) than to the delivery of carbon backbones (acetyl-CoA) for DNL (Hemre et al., 2002). Regarding the regulation by dietary protein, previous *in vivo* studies tend to indicate that dietary protein play a potent role in regulating lipogenic and glycolytic pathways in fish (Dias et al., 1998; Figueiredo-Silva et al., 2009; Seiliez et al., 2011a). In line with these observations, the rate of FA synthesis from alanine was considerably greater than that from glucose in trout liver, implicating that AAs are the preferred carbon source in this fish species (Tocher et al., 2002). Furthermore, consistent with higher vertebrates, dietary lipid and n-3

HUFA suppress hepatic lipogenesis (Tocher et al., 2002), while insulin stimulates it both *in vivo* (Cowley and Sheridan, 1993; Polakof et al., 2011a) and *in vitro* (Lansard et al., 2010; Plagnes-Juan et al., 2008). Moreover, in rainbow trout liver, SREBP-1c expression was induced by insulin (Lansard et al., 2010; Polakof et al., 2010) and re-feeding (Skiba-Cassy et al., 2009), which was associated with elevated expression of lipogenic enzymes.

Figure 1. 23 Pathways for DNL, monounsaturated and polyunsaturated fatty acid synthesis



(a) *De-novo* lipogenesis (DNL) and monounsaturated fatty acid (MUFA) synthesis. The pathway illustrates the conversion of dietary glucose and palmitate to 16-carbon and 18-carbon saturated and monounsaturated fatty acids by acetyl CoA carboxylase (ACC), fatty acid synthase (FASN), fatty acid elongase-5 and fatty acid elongase-6 (Elovl5 and Elovl6), and stearoyl CoA desaturase (SCD). (b) Polyunsaturated fatty acid (PUFA) synthesis. The pathway illustrates the conversion of dietary essential fatty acids, C18 : 2, ω6 and C18 : 3, ω3 to C20–22 PUFAs by fatty acid desaturases (FADS1, FADS2), fatty acid elongases (Elovl2, Elovl5) and peroxisomal *β*-oxidation. Fatty acids in bold are the predominant PUFAs found in liver, that is, 18 : 2, ω6, 20 : 4, ω6, 22 : 6, ω3. *, precursors to bioactive eicosanoids and docosanoids; #, robust activator of PPARα; $, robust suppressor of nuclear content of SREBP-1. From (Jump, 2011).

Fatty acid bioconversion The liver also modifies fatty acid structure through metabolic pathways that include desaturation, elongation, mono-oxidation, and peroxisomal *β*-oxidation (chain shortening) (Wang et al., 2006). Palmitate (16:0) is a product of DNL but is also derived from the diet. Palmitate is subsequently elongated [fatty acid elongase (Elov)l5 and Elovl6] and desaturated [stearoyl CoA desaturase (SCD)] to form $C_{16\text{-}18}$ saturated and monounsaturated (ω7 and ω9) fatty acids. The essential fatty acids, linoleic acid (18:2, ω6) and α-linolenic acid (18:3, ω3), are derived from the diet. These fatty acids are desaturated [fatty acid desaturases (FADS)1 and FADS2] and

elongated (Elovl2 and Elovl5) to form the major C_{20-22} polyunsaturated fatty acids (PUFAs) appearing in cells, i.e., arachidonic acid (ARA; 20:4, ω6) and docosahexaenoic acid (DHA; 22:6, ω3). DHA production requires peroxisomal *β*-oxidation. C_{22} PUFAs are retroconverted to C_{20} PUFAs by p-βOx (Figure 1.23) (Jump et al., 2013). Fatty acid elongases and desaturases play an important role in hepatic and whole body lipid composition, and also require NADPH as a reducing reagent (Jump, 2011). Expression of the enzymes involved in DNL (ACC1, FASN) and monounsaturated fatty acids (MUFA) synthesis (Stearoyl-CoA desaturase-1 (SCD1) and Elovl6) synthesis are coordinately controlled during fasting and refeeding, and by insulin, dietary carbohydrate and dietary fat. This coordinate regulation is due, at least in part, to the hormonal and nutrient control of key transcription factors regulating the expression of these enzymes. These transcription factors include PPARα, SREBP-1 and the ChREBP/MLX, LXR/RXR and PPARα/RXR heterodimers (Jump, 2011; Wang et al., 2006).

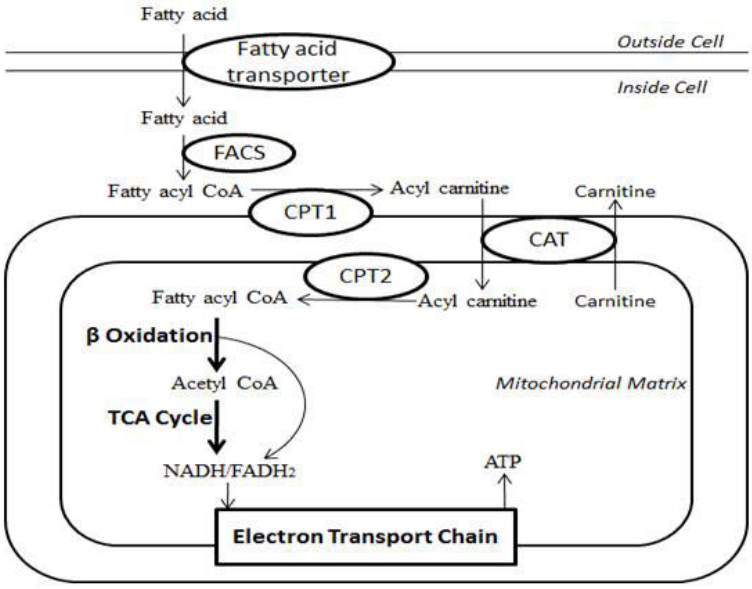
In fish, carbohydrate intake was found to increase the expression of Δ6 fatty acyl desaturase (D6D) in rainbow trout (Seiliez et al., 2001). Recently, Kamalam et al. (Kamalam et al., 2013a) demonstrated that low n-3 LC-PUFA diet enhanced the transcription of key desaturase (D6D) and elongase (Elovl5 and Elovl2) enzymes involved in LC-PUFA synthesis, in both fat and lean lines of rainbow trout. These up-regulations were associated with the activation of PPARγ, providing evidence for the first time that piscine PPARγ plays a role similar to their mammalian counterpart.

1.3.2.2 FA catabolism/*β*-Oxidation

Fatty acid *β*-oxidation is a major metabolic pathway responsible for mitochondrial breakdown of long chain acyl-CoA to acetyl-CoA (Fillmore et al., 2011). Indeed, *β*-oxidation is the predominant pathway providing energy during the fasting state in mammals (Bechmann et al., 2012) and by far in many fish species (Tocher et al., 2002). Oxidation of FAs occurs within mitochondria, peroxisomes and the ER, and facilitates degradation of activated FAs to acetyl-CoA, which is a rapid and effective way of energy allocation as the oxidation of one molecule of palmitate produces up to 129 ATP equivalents (Bechmann et al., 2012). FAs are activated by acyl-CoA-synthetase to acyl-CoA in the cytosol. This process is indispensable for enabling FAs to cross membranes and enter organelles. While short- and medium-chain FAs pass the mitochondrial membrane without activation, activated long-chain fatty acids (LCFAs) are shuttled across the membrane via carnitine palmitoyltransferase-1 (CPT1), the rate-limiting enzyme of fatty acid *β*-oxidation (Lu et al., 2014; Tocher, 2003). CPT1 conversion of the long-chain acyl-CoA to long-chain acylcarnitine allows the fatty acid moiety to be transported across the inner mitochondrial membrane via carnitine translocase (CAT), which exchanges long-chain acylcarnitines for carnitine. An inner mitochondrial membrane CPT2 then converts the long-chain acylcarnitine back to long-chain acyl-CoA. The long-chain acyl-CoA enters the fatty acid *β*-oxidation pathway, which results in the production of one acetyl-CoA from each cycle of fatty acid *β*-oxidation. Malonyl-CoA, an early intermediate of DNL that accumulates upon insulin

receptor activation, is an allosteric inhibitor of CPT1 (McGarry and Brown, 1997). Thus, in the fed state, FA oxidation is inhibited and DNL is promoted, allowing for storage and distribution of lipids. In general, short-, medium- and LCFAs are oxidized within mitochondria, while toxic, very-long-chain FAs are oxidized within peroxisomes. During the process of *β*-oxidation, electrons are indirectly donated to the electron transport chain to drive ATP synthesis (Figure 1.24). Acetyl-CoA can be further processed via the tricarboxylic acid cycle (TCA) or, in the case of FA abundance, be converted into ketone bodies. Many steps of *β*-oxidation are regulated at the transcriptional and post-transcriptional level. Transcriptional regulation are mainly mediated by transcription factors of PPARs, SREBP1, PGC-1α and estrogen-related receptor α (ERRα), while the post-transcriptional level mainly involves allosteric control of fatty acid *β*–oxidation (by the ratio of NADH/NAD+ and acetyl-CoA/CoA level), as well as ACC, Malonyl-CoA decarboxylase (MCD) and CPT1 regulation. Both mechanisms work in harmony to ensure a continual supply of long chain acyl-CoA for *β*-oxidation, and products of *β*-oxidation for mitochondrial energy production (Fillmore et al., 2011). Furthermore, insulin signaling is again involved in the regulation of FA oxidation and the formation of ketone bodies via transcriptional regulation of mitochondrial HMG-CoA synthase (Bechmann et al., 2012), as well as suppressing the *β*-oxidation via mTORC1 (Ricoult and Manning, 2013). The processes of mitochondrial *β*-oxidation and ketone body formation have been established in fish. Also it is demonstrated that fasting and low fat diet stimulate hepatic *β*-oxidation in teleost fish and elasmobranchs (Lu et al., 2014; Tocher, 2003).

Figure 1. 24 Fatty acid oxidation overview

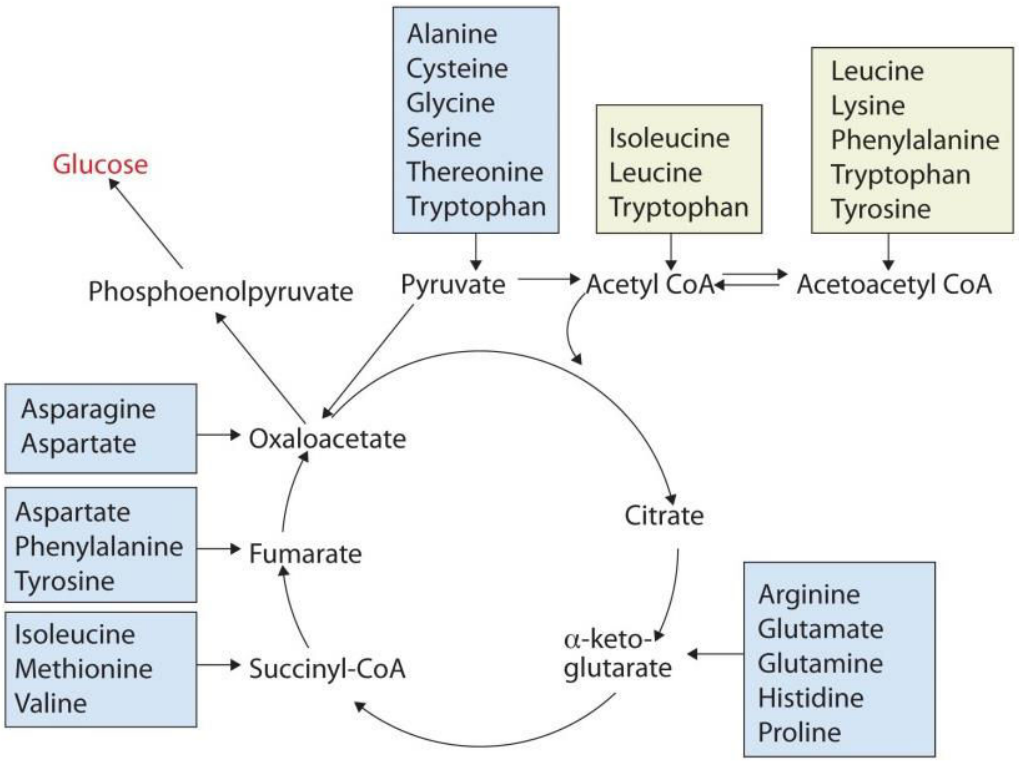


Fatty acids primarily enter a cell via fatty acid protein transporters on the cell surface. Once inside, fatty acyl-CoA synthase (FACS) adds a CoA group to the fatty acid. CPT1 then converts the long chain acyl-CoA to long chain acylcarnitine. The fatty acid moiety in transported by CAT across the inner mitochondrial membrane. CPT2 then converts the long chain acylcarnitine back to long chain acyl-CoA. The long chain acyl-CoA can then enter the fatty acid *β*-oxidation pathway, resulting in the production of one acetyl-CoA from each cycle of *β*-oxidation. This acetyl-CoA then enters the TCA cycle. The NADH and FADH2 produced by both *β*-oxidation and the TCA cycle are used by the electron transport chain to produce ATP. From (Fillmore et al., 2011).

1.3.3 The regulation of hepatic amino acid catabolism in mammals and fish

AA catabolism is an important energy source for mammalian organisms when other energy substrates are low, particularly circulating glucose and FA (Young and Marchini, 1990). During prolonged starvation or other energy demanding states, the protein breakdown in skeletal muscle is augmented, releasing to the circulation increasing amounts of AAs, which are taken up by the liver and catabolized by specific amino acid catabolizing enzymes (AACE) (Contreras et al., 2015). This catabolism contributes to the generation of metabolic energy and provides substrates for hepatic gluconeogenesis to maintain blood glucose concentration (Ruderman, 1975) as gluconeogenic amino acids can be converted to glucose via gluconeogenesis (Figure 1.25). On the other hand, a diet rich in protein induces high AA supply into the liver. It is well-known that when dietary protein intake does not exceed the amino acid requirements, AA catabolism barely occurs. However, when protein intake excesses the requirement, the excess of AA is catabolized by the AACE since the excess of dietary AA cannot be stored. Thus if excessive protein intake is not used efficiently by the body, this may impose a metabolic burden on the bones, kidneys and liver, and generate adverse effects, such as hypercalciuria and hyperuricosuria. Therefore, generally, the AACE activity increases when the supply of circulating AAs augments by fasting or after the consumption of an excess of dietary protein (Contreras et al., 2015). Recent studies shown that glucagon stimulates hepatic amino acid catabolism and decreases serum amino acid levels in mouse (Solloway et al., 2015). At the transcriptional level, investigators demonstrated that the expression of AACE is down-regulated through PPARα by attenuating HNF4α transcriptional activity, implicating an important role of PPARα in the regulation of protein metabolic homeostasis (Contreras et al., 2015). Interestingly, studies in trout shown that elevated hepatic AA catabolic gene expression was associated with enhanced mTOR activation both *in vivo* and *in vitro*, and these elevations diminished when mTOR activation was inhibited by acute rapamycin administrations (Lansard et al., 2011; Lansard et al., 2010; Seiliez et al., 2011a), implicating that hepatic AA catabolic gene expression is controlled by mTORC1. For instance, Seiliez et al. (Seiliez et al., 2011a) demonstrated that a high protein low carbohydrate diet upregulated the expression of serine dehydratase (SD) and branched-chain α-keto acid dehydrogenase 2 (BCKD E2) in liver and the expression of BCKD E2 in muscle. Consistently, insulin together with amino acids up-regulate SD gene expression in trout hepatocytes via an mTOR-dependent manner (Lansard et al., 2011; Lansard et al., 2010). Notably, investigations *in vivo* shown that AA catabolic enzyme is barely affected by dietary protein content similarly as in cat (Cowey and Walton, 1989; Macdonald et al., 1984).

Figure 1. 25 Catabolism of proteinogenic amino acids



Those amino acids that can form any of the intermediates of carbohydrate metabolism can subsequently be converted to glucose via a metabolic pathway known as gluconeogenesis. These amino acids are called glucogenic amino acids. Amino acids that are converted to acetoacetyl-CoA or acetyl-CoA, which can be used for the synthesis of ketone bodies but not glucose, are called ketogenic amino acids. Some amino acids fall into both categories. Leucine and lysine are the only amino acids that are exclusively ketogenic. From Ferrier et al. (Ferrier et al., 2011).

Table 1. 1 Protein requirement (% of diet) for adult and growing animals of several species

Species	Young	Adult
Man	8	5
Rat	12	4.2
Dog	12	4
Cat	29	19
Cat[b]	17	10
Mink	31	20
Fox	24	16
Rainbow trout	42-48	35-40

[a]Ideal protein, i.e. meeting all the known essential amino acid requirements.

[b]Values expressed as percentage of dietary energy.

(Adapted from (Hardy, 2002; Macdonald et al., 1984).

1.4 Rainbow trout as a model for nutrition and nutrient metabolism studies

The rainbow trout (*Oncorhynchus mykiss* Walbaum; family: Salmonidae) is a carnivorous (piscivorous) species that shows many particularities with respect to nutrition. The rainbow trout is characterized as a poor user of dietary carbohydrates illustrated by a clear glucose intolerance phenotype after carbohydrate intake (Hemre et al., 2002; Moon, 2001). Moreover, rainbow trout have relatively high dietary protein/AAs requirement (Table 1), with AAs being largely used for energy production by catabolism or for the production of fatty acids and glucose (Panserat et al., 2013; Walton and Cowey, 1982). Finally, trout possesses relatively low energy requirement compared to terrestrial vertebrates, which is intimately related to their poikilothermic lifestyle as for most aquatic animals (Guillaume et al., 2001). These unusual metabolic features may allow us to gain a better understanding of the nutritional regulation. Furthermore, it is also probably one of the most deeply studied fish species with a long research history performed in physiology, nutrition, ecology, genetics, pathology, carcinogenesis and toxicology. Additionally, its relatively large size compared with model fish such as zebrafish and medaka, makes rainbow trout a particularly well-suited alternative model to carry out biochemical and molecular studies on specific tissues or cells that are impossible to decipher in small fish models (Klionsky et al., 2012).

Rainbow trout represents a well-studied carnivorous model

Salmonids (including Atlantic salmon and rainbow trout) are predatory fish in nature and are placed at a highest trophic level of the food chain of the aquatic environment. These fish species are classified as carnivorous animals. In this context, they represent a very interesting model of animal nutrition because, up to now, few strict carnivorous animals have been extensively studied with the exception of cats, mink, raptors such as owls and the piscivorous fish species (Panserat et al., 2013). Furthermore, similar with domestic cat and dolphin, trout exhibits relatively high dietary protein/AAs requirement, poor utilization of dietary carbohydrates and glucose intolerant phenotype, thus it also represents a relevant carnivorous model organism for the investigation of protein and glucose metabolism (Hemre et al., 2002; Schermerhorn, 2013).

Trout is a relevant diabetic model

Glucose intolerance is a term that refers to the inability of an organism to rapidly deal with a glucose load. The consequences are persistent hyperglycemia and in many cases, reduced growth. Glucose intolerance is a clinical term used in the diagnosis of insulin-dependent diabetes mellitus and is assessed by the use of a glucose tolerance test (GTT). A GTT involves administering a bolus of glucose either orally or intravenously, and if plasma glucose values do not return to baseline within 12 h, the subject

(=human) is considered to have impaired glucose tolerance. The GTT has been used in many fish studies to test glucose tolerance and in most cases, the data consistently show that teleost fishes show persistent hyperglycemia after a glucose load. Thus, teleost fishes are generally considered to be glucose intolerant (Moon, 2001). Given that healthy carnivorous fish exhibit metabolic changes after fasting or a carbohydrate rich diet that are similar to those observed in humans with diabetes, trout can be used as a comparative natural model for the study of biological mechanisms associated with type 2 diabetes (Schermerhorn, 2013). As a relevant diabetic research model, carnivores have two main advantages. First, these features are presented in healthy fish, which do not have to be induced, representing a major advantage compared to other mammalian models. Second, these animals are long lived, easy to maintain in a controlled environment, cooperative and accessible for study, which permits long-term studies of individual animals. Thus, understanding how nutrients regulate intermediary metabolism in trout may have multi-significance.

1.5 Hypothesis and objectives of the thesis

In current thesis, we set to address four major questions below:

Question I: Does mTOR signaling pathway regulate metabolism-related gene expression in vivo?

Previous studies from the laboratory have demonstrated that TOR signaling pathway is involved in the control of lipogenic and glycolytic gene expression in primary cell culture of rainbow trout hepatocytes (Lansard et al., 2011; Lansard et al., 2010). We hypothesize that mTOR signaling pathway is also involved in the regulation of intermediary metabolism-related gene expression *in vivo*. Therefore, we assessed the potential involvement of mTORC1 signaling in the regulation of postprandial hepatic lipid and glucose metabolism-related gene expression in rainbow trout liver. We used re-feeding to activate mTOR signaling pathway and employed IP administration of rapamycin, a pharmacological inhibitor of mTOR, to achieve an acute inhibition of this pathway.

Question II: What are the respective role of protein and carbohydrate in the regulation of lipogenic gene expression, and what is the role of mTOR in these regulations?

De novo lipogenesis (DNL) is the metabolic pathway that synthesizes fatty acids from excess carbon-donors; these fatty acids can then be converted into triglycerides, the major energy storage form in vertebrates (Lee et al., 2008; Strable and Ntambi, 2010; Tocher, 2003). In mammals, hepatic lipogenesis is mainly stimulated by high dietary carbohydrates (Kersten, 2001; Towle et al., 1997), whereas suppressed by high dietary protein (Rietman et al., 2014a). However, the nutritional regulation of DNL in carnivorous fish seems to be relatively different, with limited effect of dietary carbohydrates or glucose on the stimulation of lipogenesis (Hemre and Kahrs, 1997; Jin et al., 2014b; Seiliez et al., 2011a), whereas a stimulatory role has been conferred to dietary protein (Figueiredo-Silva et al., 2009; Seiliez et al., 2011a). Therefore, we hypothesize that unlike omnivorous mammals, dietary proteins/AAs instead of dietary carbohydrate/glucose are the main regulators of hepatic lipogenesis in carnivorous fish. We thus evaluated and compared the respective role of dietary carbohydrate/glucose and protein/amino acids on the regulation of lipogenesis in rainbow trout via both *in vivo* and *in vitro* approaches.

Question III: Do high levels of amino acids negatively influence insulin signaling and action in fish?

As protein raw materials represent the highest expense, accounting for about 50 % of the total cost in aquafeeds (Rolland, 2014), one potential economic strategy is to maximize the inclusion of digestible carbohydrates (the cheapest and most abundant dietary energy source for all feed stock) and minimize the inclusion of protein used for energy fuel part in the diets (Kamalam, 2013; Stone, 2003; Wilson,

1994). However, fish, particularly carnivorous species, are well recognized for their low efficiency in using digestible carbohydrates and are classified as "glucose intolerant" (Falkmer, 1961; Mommsen and Plisetskaya, 1991; Moon, 2001), yet the underlying mechanisms for causing these characters still remain largely unclear (Kamalam, 2013; Polakof et al., 2012a). In mammals, excessive AAs can induce over-activation of mTORC1 pathway, which elicits a negative feedback signal that hyperphosphorylates IRS1 at serine residues, leading to reduced IRS1 function and impaired activation of the PI3K/Akt pathway, thereby diminishing insulin action (Boura-Halfon and Zick, 2009; Tremblay et al., 2007). Moreover, persistent activation of mTORC1 signaling pathway may lead to insulin resistance via the transcription factors FoxO1 and FoxO3 (Boura-Halfon and Zick, 2009; Codogno and Meijer, 2010; Lynch and Adams, 2014). Given that diets of carnivorous fish generally possess relatively high protein/AAs levels (two to four times higher) compared to those of humans, rodents or other terrestrial domestic animals (Schermerhorn, 2013; Walton and Cowey, 1982), we hypothesize that this nutritional feature may account for, or at least partially account for the persistent hyperglycemia and absence of postprandial down-regulation of gluconeogenesis, probably through attenuating insulin signaling pathway and insulin action via hyper-activation of mTORC1 signaling pathway. We thus investigated this hypothesis using trout primary hepatocytes.

Question IV: What mechanism governs glucose intolerance in fish? Role of mTOR signaling pathway

It is well-known that in fish hepatic GK expression and activity strongly respond to changes of blood glucose levels induced by different dietary carbohydrate inclusion (Enes et al., 2009; Panserat et al., 2014; Polakof et al., 2011b; Polakof et al., 2012a). Given that our previous study *in vitro* demonstrated that mTOR inhibition by rapamycin also repressed GK gene expression, we hypothesize that dietary carbodydrate regulates hepatic GK gene expression and activity via an mTOR-dependent manner. Furthermore, fish displays prolonged hyperglycemia after consuming a carbohydrate rich meal or after a glucose tolerance test (Moon, 2001), but it is unclear whether the persistent hyperglycemia in fish is due to poor glucose uptake (glucokinase or glycolysis), low utilization of glucose by the peripheral tissues and/or lack of suppression of endogeneous production (hepatic gluconeogenesis). We hypothesize that the poor inhibition of postprandial hepatic gluconeogenesis is the major responsible factor and have tried to address this question as well.

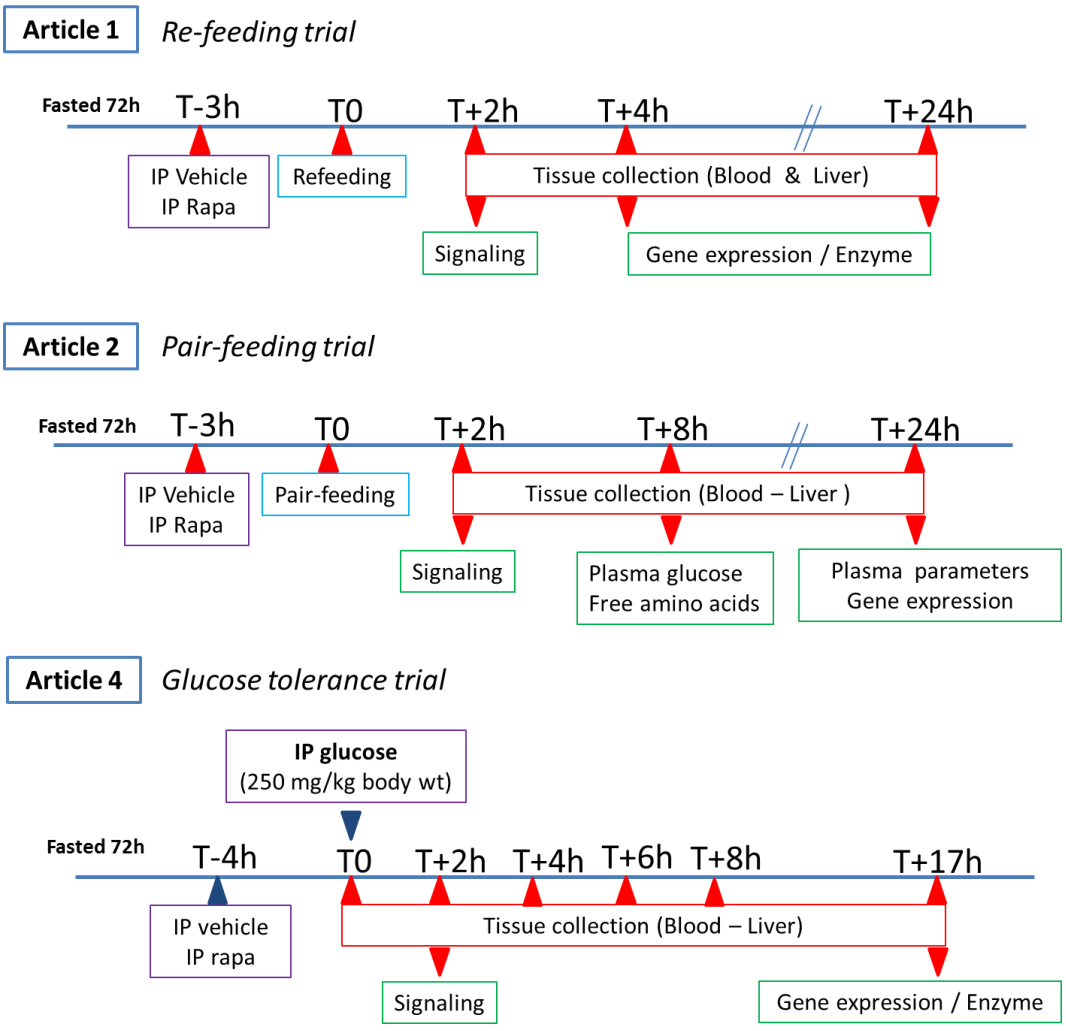
The present thesis brings forward our understandings about the regulatory roles of protein/AAs in the regulation of hepatic intermediary metabolism (glucose metabolism, fatty acid biosynthesis and amino acid catabolism), and determines the potential involvements of insulin/Akt and mTORC1 signaling pathways during these regulations. The most important results are recapitulated in chapter 3, followed by the general conclusion and future perspectives in chapter 4 and chapter 5.

Chapter 2

MATERIAL AND METHODS

Figure 2. 1 The experimental flow through rearing system setup used for conducting feeding trials at the INRA experimental farm, Donzacq

Figure 2. 2 Overview of experimental procedures of the in vivo experiments



2.1 Experimental trials

2.1.1 Ethics statement

All experiments were carried out in accordance with the clear boundaries of EU legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e. Directive 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decret no. 2001-464, May 29th, 2001). The investigators carrying out the experiment had ‘‘level 1’’ or ‘‘level 2’’ certification, bestowed by the Direction Départementale des Services Vétérinaires (French vetinary services) to carry out animal experiments (INRA 2002-36, April 14th, 2002).

2.1.2 *In vivo* experimental trial

2.1.2.1 Experimental and sampling procedures

Three experimental trials were conducted in the INRA experimental fish facilities of Donzacq (Landes, France; Figure 2.1). Juvenile rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were reared at a constant water temperature of 17.5±0.5°C, under natural photoperiod. They were fed a standard trout commercial diet (T-3P classic, Skretting, Fontaine-les-Vervins, France) during the acclimatization period. Prior to each trial, fish were food deprived for 72 h—the time required to ensure the complete emptying of the digestive tract. After a recovery period of 3 or 4 hours, trout were sedated with benzocaine (10mg/L) and treated with a single intraperitoneal (IP) injection of rapamycin (0.5 mg/kg) or vehicle (75% DMSO and 25% saline solution NaCl 0.9%) with 100 µL/100 g body mass. Fish were then fasted or refeed (article 1), controlled-fed (article 2) or IP injected with D-glucose (article 4) according to the procedures described below (Figure 2.2).

Refeeding trial (article 1): Two tanks per treatment (vehicle or rapamycin) were refed with the commercial diet (T-3P classic, Skretting, Fontaine-les-Vervins, France) to apparent satiation 3 h after the injection of rapamycin while the other two tanks remained fasted. Four fish per tank (two tanks per condition) were randomly sampled 2, 4 and 24 h after refeeding (*N*=8).

Controlled-feeding trial (article 2): 3 h after the IP injection of rapamycin, three tanks per treatment (vehicle or rapamycin) were assigned to one of the three experimental diets (Table 2.1), which were manufactured at the INRA facility of Donzacq (France) using a twinscrew extruder (Clextral, France). Three diets, high protein low carbohydrate (HPLC), high protein high carbohydrate (HPHC) and low protein high carbohydrate (LPHC) diets were formulated to contain different levels of dietary protein and carbohydrate as shown in Table 2.1. A controlled-feeding method was employed to supply fixed amount of dietary protein or carbohydrate while maintaining the other nutrient intake almost equivalent. HPLC, HPHC and LPHC diets were fed following the feeding ratios of 0.94%, 1.23% and

Table 2. 1 Diets composition and nutrients intake

Ingredients (%)	Diets		
	HPLC	HPHC	LPHC
Fish meal[a]	79.1	54.0	35.7
Fish oil[b]	8.7	5.9	11.3
Dextrin[c]	11.1	38.5	51.0
Vitamin premix[d]	1.1	0.8	1.0
Mineral premix[e]	1.1	0.8	1.0
Proximate analysis			
Crude protein (%DM)	57.5	40.1	26.2
Crude fat (%DM)	19.8	14.3	16.3
NFE (%DM)	10.6	36.1	48.2
Dry matter (%)	91.2	91.3	91.3
Feeding level (BW)	0.86%	1.23%	0.94%
Protein intake (g/10 kg BW)	**45.1**	**45.0**	**22.4**
NFE intake (g/10 kg BW)	**8.3**	**40.5**	**41.4**
Lipids intake (g/10 kg BW)	15.5	16.1	14.0

HPLC, high protein low carbohydrate; HPHC, high protein high carbohydrate; LPHC, low protein high carbohydrate; DM, dry matter; NFE, nitrogen-free extract/carbohydrate; BW, body weight.

[a]Fish meal (Sopropeche, Boulogne-sur-Mer, France).

[b]Fish oil (North sea fish oil; Sopropeche, Boulogne-sur-Mer, France).

[c]Dextrin (Lestrem 62, Roquette, France).

[d]Vitamin premix: 60 IU DL-a tocopherol acetate, 5 mg kg^{-1} diet sodium menadione bisulphate, 15,000 IU retinyl acetate, 3000 IU DL-cholecalciferol, 15 mg kg^{-1} diet thiamin, 30 mg kg^{-1} diet riboflavin, 15 mg kg^{-1} diet pyridoxine, 0.05 mg kg^{-1} diet B12, 175 mg kg^{-1} diet nicotinic acid, 500 mg kg^{-1} diet folic acid, 1000 mg kg^{-1} diet inositol, 2.5 mg kg^{-1} diet biotin, 50 mg kg^{-1} diet calcium panthotenate, 2000 mg kg^{-1} diet choline chloride (UPAE, Jouy, Inra, France).

[e]Mineral premix: 2.15 g calcium carbonate (40% Ca), 1.24 g magnesium oxide (60% Mg), 0.2 g ferric citrate, 0.4 mg kg^{-1} diet potassium iodide (75% I), 0.4 g zinc sulphate (36% Zn), 0.3 g copper sulphate (25% Cu), 0.3 g manganese sulphate (33% Mib), 5 g dibasic calcium phosphate (20% Ca, 18% P), 2 mg kg^{-1} diet cobalt sulphate, 3 mg kg^{-1} diet sodium selenite (30% Se), 0.9 g KCl, 0.4 g NaCl (UPAE, Jouy, INRA, France).

0.86% body weight, respectively. The remaining tanks remained fasted and used as controls. Eight fish per tank were randomly sampled 2, 8 and 24 h after refeeding (*N*=8).

Glucose tolerance trial (article 4): Four hours after rapamycin or vehicle administration, six fish per treatment (vehicle or rapamycin, two fish per tank) were sampled. Based on previous studies in fish (Moon, 2001), all remaining fish were sedated and subjected to IP administration with 250 mg D-glucose/kg body weight. Two fish per tank (three tanks per treatment) were randomly sampled 2, 4, 6, 8 and 17 h after glucose injection (*N*=6).

2.1.2.2 Sampling procedure

For each treatment, trout were anaesthetised with benzocaine (30mg/L) and killed by a sharp blow to the head. Blood was removed from the caudal vein into heparinized syringes and centrifuged (3000*g*, 5 min); the recovered plasma was immediately frozen and kept at –20°C. The stomach content of each fish was checked to confirm that the fish had effectively ingested the diet. Livers were dissected and immediately frozen in liquid nitrogen and kept at –80°C.

2.1.3 *In vitro* experiments

2.1.3.1 Animals

Sexually immature rainbow trout (240 to 300g) were obtained from the INRA experimental fish farm facilities (INRA, Donzacq, France). Fish were maintained in tanks kept in open circuits at 18°C with well-aerated water under natural photoperiod conditions. Trout were fed to satiety every two days with a commercial diet (T-3P classic, Trouw, France).

2.1.3.2 Primary cell culture of rainbow trout hepatocytes

Isolated liver cells were prepared from 3 day-fasted rainbow trout. Trout were left unfed for 3 days in order to empty the digestive tract and then facilitate the liver in situ perfusion. At the time of experiments, fish were anaesthetized by placing them in water containing 60 mg/L aminobenzoic acid, and hepatocytes were isolated by the in situ perfusion method described by Mommsen et al (Mommsen et al., 1994). Livers were excised and minced with a razor blade in modified Hanks' medium (136.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 5 mM $NaHCO_3$ and 10 mM HEPES) (Table 2.2) supplemented with 1mM EGTA. After filtration and centrifugation (120 g, 2 minutes), the resulting cell pellet was resuspended three successive times in modified Hanks' medium (1.5 mM $CaCl_2$ and 1.5% defatted bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA)). Cells were finally taken up in modified Hanks' medium supplemented with 1.5 mM $CaCl_2$, 1% defatted BSA, 3 mM glucose, MEM essential amino acids (1X) (Invitrogen Carlsbad,

Figure 2. 3 General procedures for trout primary hepatocytes harvest and stimulation

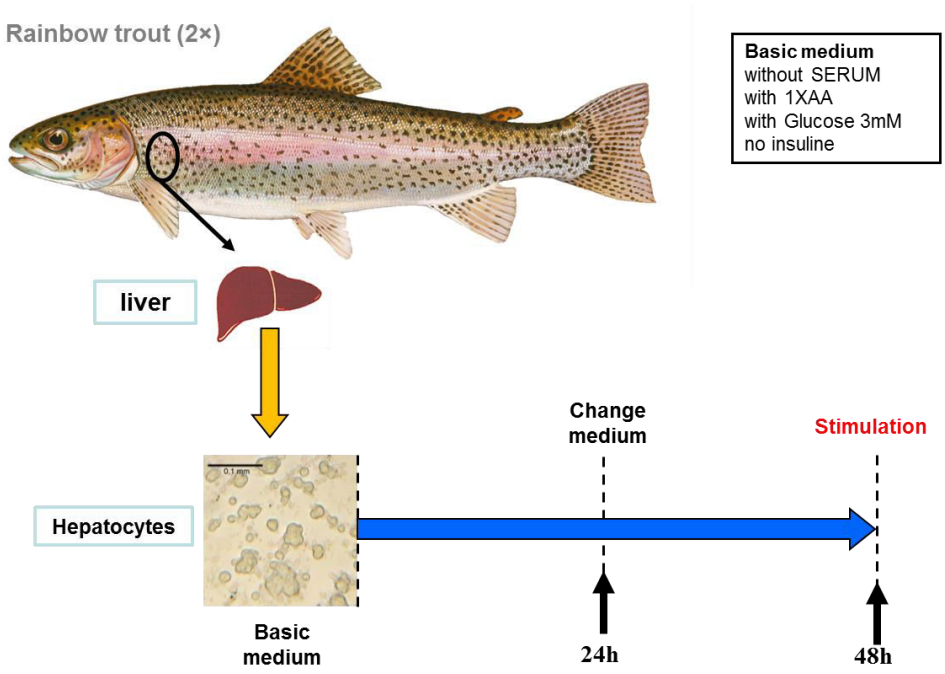
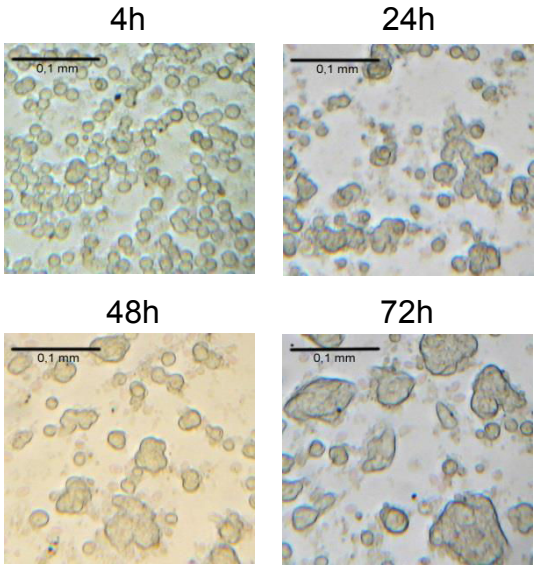


Figure 2. 4 Trout hepatocytes photos after 4, 24, 48 and 72 h culture



California, USA), MEM non-essential amino acids (1X) (Invitrogen Carlsbad, California, USA) and antibiotic antimycotic solution (1X) (Sigma, St. Louis, MO, USA) as the basic culture medium. Compositions of one fold MEM essential and non-essential amino acids are shown in Table 2.3. Cell viability (>98%) was assessed using the trypan blue exclusion method (0.04% in 0.15 M NaCl) and cells were counted using a haemocytometer. The hepatocyte cell suspension was plated in a six well Primaria culture dish (BD, USA) at a density of 3×10^6 cells/well and incubated at 18°C. The culture medium was changed every 24 h over the 48 h of primary cell culture as shown in Figure 2.3. Microscopic examination ensured that hepatocytes progressively re-associated throughout culture to form two-dimensional aggregates (Figure 2.4), consistent with earlier reports (Ferraris et al., 2002; Segner, 1998).

2.1.3.3 Stimulations and cell recovery

Article 2: For the cell culture involving different glucose and amino acid levels, 48 h-cultured cells were stimulated with 4×10^{-9} mol l^{-1} of bovine insulin (Sigma, St. Louis, MO, USA) corresponding to the post-prandial level of insulin (del sol Novoa et al., 2004; Mommsen and Plisetskaya, 1991), low or high level of glucose (3 mM/LGlu or 20 mM/HGlu) and low or high level of amino acids [one-fold (LAA) or four-fold (HAA) concentrated amino acids]. In the cell culture involving different levels of insulin, 48 h-cultured hepatocytes were stimulated with consistent glucose concentration (3 mM), low or high level of insulin (1×10^{-9} mol l^{-1}/low insulin or 4×10^{-9} mol l^{-1}/high insulin; corresponding to the two weeks fasted or post-prandial insulin levels, respectively (Mommsen and Plisetskaya, 1991)) and low or high level of amino acids (LAA or HAA). Cells were harvested 15 min after stimulation for western blot analysis or resuspended in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) 24 h after stimulation and stored at -80°C for subsequent mRNA extraction. The time intervals were chosen based on the phosphorylation peaks of Akt/TOR signaling pathway or relevant metabolic gene expression peaks, respectively.

Article 3: For the cell culture involving different amino acid levels, 48 h-cultured cells were stimulated with the mediums containing 4×10^{-9} mol l^{-1} of bovine insulin (Sigma, St. Louis, MO, USA) and one fold (I*1AA), two fold (I*2AA), or four fold concentrated MEM essential and non- essential amino acids mixture (I*4AA), respectively. Control cells were maintained the basic culture medium (1AA without insulin). In the second set of cell culture, 48 h-cultured hepatocytes were pre-incubated for 30 min with or without 100 nM rapamycin (Rapa) in order to inhibit TOR activation. The mediums were then replaced by a fresh one containing 4×10^{-9} mol l^{-1} insulin and four fold concentrated MEM essential and non-essential amino acids mixture (I*4AA). Cells were harvested 45 min after stimulation for western blot analysis or resuspended in TRIzol® Reagent 24 h after stimulation and stored at -80°C for subsequent mRNA extraction. The time intervals were chosen based on the phosphorylation peaks of TOR signaling pathway or relevant metabolic gene expression, respectively.

Table 2. 2 Composition of modified Hanks' medium

Composition	Concentration (mM)
NaCl	139,9
KCl	5,4
$MgS0_4 \times 7H_20$	0,8
$Na_2HP0_4 \times 7H_20$	0,33
KH_2P0_4	0,44
NNaHCO3	5
HEPES	5
HEPES×Na	5

Table 2. 3 Amino acid composition in the culture medium (1X)

Composition	Concentration (µM)
MEM essential	
L-arginine	600
L-cystine	100
L-histidine	200
L-isoleucine	400
L-leucine	400
L-lysine	400
L-methionine	100
L-phenylalanine	200
L-threonine	400
L-tryptophane	50
L-tyrosine	200
L-valine	400
MEM non essential	
Glycine	100
L-alanine	100
L-asparagine	100
L-acide aspartique	100
L-acide glutamique	100
L-proline	100
L-serine	100

2.2 Analytical methods

2.2.1 Plasma metabolites analysis

Plasma glucose (Glucose RTU, bioMérieux, Marcy l'Etoile, France), triglycerides (PAP 150, bioMérieux) and free fatty acid (NEFA C kit, Wako Chemicals, Neuss, Germany) levels were determined using commercial kits adapted to a microplate format, according to the recommendations of the manufacturer. Total plasma free amino acid levels were determined by the ninhydrin reaction (Moore, 1968), with glycine as standard.

2.2.2 Western blot analysis

Western blot analyses were performed with proteins extracted from both liver and hepatocytes. Frozen livers (200 mg; *N*=6) were homogenised on ice with an ULTRA-TURRAX® homogenizer (IKA®-WERKE, Germany) in 2 mL of buffer containing 150 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EGTA, 1 mmol/L EDTA (pH 7.4), 100 mmol/L NaF, 4 mmol/L sodium pyrophosphate, 2 mmol/L sodium orthovanadate, 1% Triton X-100, 0·5% NP-40-Igepal and a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Homogenates were centrifuged at 1500*g* for 15 min at 4°C and supernatant fractions were then centrifuged at 20,000*g* at 4°C for 30 min. The resulting supernatant fractions were recovered and stored at -80°C.

For cell culture samples, cells were carefully washed with two times 1ml of cold phosphate buffered saline (PBS) at the end of the stimulation period. Then 300µl of cell lysis buffer were added before being stored the culture dish at -80°C overnight. Then cells were scraped, collected and lysed on ice for 30 min. Lysates were centrifuged at 12.000g for 30 min at 4°C. The resulting supernatant fractions were recovered and stored at -80°C.

Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with BSA as standard. Lysates were subjected to SDS-PAGE and Western blotting using the appropriate antibody (Table 2.4 and 2.5). Anti-FoxO1 (no. 1874-1) and anti-p38 MAPK (no. sc-535) was purchased from Epitomics (California, United States), and the other antibodies were purchased from Cell Signaling Technologies (Ozyme, Saint Quentin Yvelines, France). All of these antibodies (except anti-phospho-Akt (Thr^{308}), anti-phospho-GSK 157 3α/β ($Ser^{21/9}$), anti-GSK 3α, anti-phospho-IRS-1 (Ser^{302}), anti-phospho-p38 MAPK and anti-p38 MAPK) successfully cross-reacted with rainbow trout (Kamalam et al., 2012; Seiliez et al., 2008; Seiliez et al., 2011b). For anti-phospho-Akt (Thr^{308}), anti-phospho-GSK 3α/β ($Ser^{21/9}$), anti-GSK 3α, anti-phospho-IRS-1 (Ser^{302}), anti-phospho-p38 MAPK and anti-p38 MAPK antibody, the molecular weight and amino acid sequences were monitored in the SIGENAE database (SIGENAE) to check for a good conservation of the antigen sequence. Conditions used for the *in vivo* and *in vitro* WB analysis were displayed in Table 2.4 and 2.5, respectively. After washing, membranes were incubated with an

Table 2. 4 General conditions used in the *in vivo* WB experiments (migration, transferring, reference of antibody and its conditions for incubation)

		Akt pathway				TOR pathway				
	Protein	**Akt S473**	**Akt T308**	**FoxO**	**GSK 3**	**TOR**	**S6K1**	**S6**	**4E-BP1**	**β-Tubulin**
Migration	**Molecular Weight (kDa)**	60	60	78-82, 95	49, 51	289	70.85	32	14-20	55
	Gel concentration (%)	10	10	10	10	7.5	10	10	15	10
	Loading quantity (μg)	10	10	10	10	20	20	10	10	10
	Migration (V)	80-100	80-100	80-100	80-100	45V, overnight	80-100	80-100	80-100	80-100
Transferring	**Transfering (V, h)**	100V, 2h	100V, 2h	100V, 2h	100V, 2h	45V, 4h	100V, 2h	100V, 2h	100V, 2h	100V, 2h
Phosph-Ab	**Antibody reference**	#9271	#9275	#9464	#9331	#2971	#9205	#4856	#9459	
	Phosphorylation site	Ser473	Thr308	T24/T32	S21/9	Ser2448	Thr389	S235/236	Thr37/46	
	Ab source	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	
	Ab concentration	1/1000	1/1000	1/1000	1/1000	1/1000	1/500	1/1000	1/1000	
	Time for incubation (d)	Overnight	Overnight	2 days	Overnight	2 days	2 days	Overnight	Overnight	
Total-Ab	**Antibody reference**	#9272	#9272	#1874-1	#9338	#2972	#9202	#2217	#9452	#2146
	Ab source	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit
	Ab concentration	1/1000	1/1000	1/1000	1/1000	1/1000	1/500	1/1000	1/1000	1/1000
	Time for incubation (d)	Overnight	Overnight	Overnight	Overnight	2 days	2 days	Overnight	Overnight	Overnight

Table 2. 5 General conditions used in the *in vitro* WB experiments (migration, transferring, reference of antibody and its conditions for incubation)

		Akt pathway			TOR pathway					
	Protein	**Akt S473**	**Akt T308**	**IRS 1**	**TOR**	**S6K1**	**S6**	**4E-BP1**	**p38 MAPK**	**β-Tubulin**
Migration	**Molecular Weight (kDa)**	60	60	180	289	70.85	32	14-20	43	55
	Gel concentration (%)	10	10	7.5	7.5	10	10	15	10	10
	Loading quantity (μg)	2	2	17	7	7	2	2	7	2
	Migration (V)	80-100	80-100	45V, overnight	45V, overnight	80-100	80-100	80-100	80-100	80-100
Transfering	**Transfering (V, time)**	100V, 2h	100V, 2h	45V, 4h	45V, 4h	100V,2h	100V, 2h	100V, 2h	100V, 2h	100V, 2h
Phosph-Ab	**Antibody reference**	#9271	#9275	#2384	#2971	#9205	#4856	#9459	#9211	
	Phosphorylation site	Ser473	Thr308	Ser302	Ser2448	Thr389	S235/236	Thr37/46	Thr180/Tyr182	
	Ab source	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	
	Ab concentration	1/1000	1/1000	1/1000	1/1000	1/500	1/1000	1/1000	1/1000	
	Time for incubation	Overnight	Overnight	2 days	2 days	2 days	Overnight	Overnight	Overnight	
Total-Ab	**Antibody reference**	#9272	#9272		#2972	#9202	#2217	#9452	SC-535	#2146
	Ab source	Rabbit	Rabbit		Rabbit	Rabbit	Rabbit	Rabbit	Rabbit	Rabbit
	Ab concentration	1/1000	1/1000		1/1000	1/500	1/1000	1/1000	1/200	1/1000
	Time for incubation	Overnight	Overnight		2 days	2 days	Overnight	Overnight	Overnight	Overnight

IRDye Infrared secondary antibody (LI-COR Biosciences, Lincoln, NE, USA). Bands were visualized by Infrared Fluorescence using the Odyssey Imaging System (LI-COR Biosciences) and quantified by Odyssey Infrared imaging system software (Version 3.0, LI-COR Biosciences).

2.2.3 Gene expression analysis: real time RT-PCR

Gene expression analyses were performed with total RNA extracted from both liver and hepatocytes. Total RNA was extracted from -80°C frozen livers or hepatocytes using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Total RNA was quantified by spectrophotometry (absorbance at 260 nm) and integrity was assessed using Agarose Gel Electrophoresis. One microgram of the resulting total RNA was reverse transcribed into cDNA using the SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and random primers (Promega, Charbonniéres, France) according to the instructions of each manufacturer. Target gene expression abundance was determined by quantitative real-time (q) RT-PCR, using specific primers (Cruz-Garcia et al., 2009; Kamalam et al., 2013b; Polakof et al., 2009b; Seiliez et al., 2011a; Skiba-Cassy et al., 2009). New primers were designed using Primer3 software. To confirm specificity, amplicons were purified and sequenced (Beckman Coulter Genomics, UK).

Quantitative RT-PCR was carried out on a LightCycler® 480 II (Roche Diagnostics, Neuilly sur Seine, France) using LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany). Quantitative RT-PCR analyses were focused on several key enzymes related to glycolysis, gluconeogenesis, lipogenesis, NADPH production, fatty acid bioconversion and AA catabolism (Table 2.6). When different isoforms of a gene were known in rainbow trout (as for CPT1 and G6Pase), gene expression analysis was performed on each isoform. Elongation factor-1alpha (EF1α) was employed as a non-regulated reference gene, as previously used in rainbow trout and it was stably expressed in our investigations.

PCR was performed using 2 µL of the diluted cDNA (76 times diluted) mixed with 0.24 µL of each primer (10 µM), 3 µL LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and 0.52 µL DNase/RNase/Protease-free water (5 prime GmbH, Hamburg, Germany) in a total volume of 6 µL. The PCR protocol was initiated at 95°C for 10 min for initial denaturation of the cDNA and hot-start Taq-polymerase activation, followed by 45 cycles of a three-step amplification program (15 s at 95°C; 10 s at Tm (59-65°C); 4.8 s at 72°C), according to the primer set used. Melting curves were systematically monitored (5 s at 95°C; 1 min at 65°C; temperature slope at 0.11°C/s from 65 to 97°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included quadruplicates for each sample (duplicates of reverse transcription and PCR amplification, respectively) and also negative controls (reverse transcriptase and RNA free samples). Relative quantification of target gene expression was determined using the E-Method of the LightCycler® 480 software (Version SW 1.5, Roche

Table 2. 6 Gene list used in RT-PCR analysis

Metabolic pathway	Abbreviations	Full name	Reference
Glucose dephosphorylation	G6Pase I	glucose-6-phosphatase I	EC 3.1.3.9
	G6Pase II	glucose-6-phosphatase II	EC 3.1.3.9
Gluconeogenesis	FBPase	fructose-1,6-bisphosphatase	EC 3.1.3.11
	mPEPCK	metochonial phosphoenolpyruvate carboxykinase	EC 4.1.1.32
	PEPCK tot	phosphoenolpyruvate carboxykinase total	EC 4.1.1.32
Glucose phosphorylation	GK	glucokinase	EC 2.7.1.2
Glycolysis	6PF1K	6-phosphofructo-1-kinase	EC 2.7.1.11
	PK	pyruvate kinase	EC 2.7.1.40
Transcriptional factor	HIF-1α	hypoxia-inducible factor 1-alpha	
Glucose transport	Glut 2	glucose transporter 2	
Lipogenesis	FAS	fatty acid synthase	EC 2.3.1.85
	ACC	Acetyl-CoA carboxylase	EC 6.4.1.2
	ACLY	ATP citrate lyase	EC 2.3.3.8
Transcriptional factor	SREBP1	sterol regulatory element binding protein 1-like	
	LXR	liver X receptor	
NADPH production	G6PDH	glucose-6-phosphate dehydrogenase	EC 1.1.1.49
	ME	malic enzyme	EC 1.1.1.40
Fatty acid bioconversion	Δ6/D6D	Δ6 fatty acyl desaturase	EC 1.14.19.3
	∆9/SCD1	Stearoyl-CoA desaturase-1	EC 1.14.19.1
	Elovl2	elongation of very long chain fatty acids like-2	EC 2.3.1.199
	ElovL5	elongation of very long chain fatty acids like-5	EC 2.3.1.199
Fatty acid β-oxidation	CPT 1a	carnitine palmitoyltransferase 1 alpha	EC 2.3.1.21
	CPT 1b	carnitine palmitoyltransferase 1 beta	EC 2.3.1.21
	Hoad	3-hydroxyacyl-CoA dehydrogenase	EC 1.1.1.35
Amino acid catabolism	SD	serine dehydratase	EC 4.3.1.17
	ALAT	alanine transaminase	EC 2.6.1.2
	ASAT	aspartate transaminase	EC 2.6.1.1
	BCKD	branched-chain α-ketoacid dehydrogenase	EC 1.2.4.4
	BCKDK	branched-chain α-ketoacid dehydrogenase kinase	EC 2.7.11.4
GCN2-ATF4 pathway	SNAT2	System A neutral amino acid transporter 2	EC 6.3.5.4
	CAT	cationic amino acid transporter 1	
	CHOP	C/EBP homology protein	
	ASNS	asparagine synthetase	
Autophagy	Atg 4b	autophagy related 4B	
	Atg12	autophagy-related protein 12	
	LC3B	light chain 3B	
	Gabarapl1	gamma-aminobutyric acid	
Reference Gene	EF1	elongation factor 1 alpha 1	

Diagnostics). PCR efficiency, which was measured by the slope of a standard curve using serial dilutions of cDNA, ranged between 1.85 and 2.

2.2.4 Enzyme activity analysis

Liver samples for GK enzyme activities were homogenized in ten volumes of ice-cold buffer (80 mmol/L Tris, 5 mmol/L EDTA, 2 mmol/L DTT, 1 mmol/L benzamidine, 1 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pH 7.6) and centrifuged at 900*g* at 4°C for 10 min. GK activities were assayed immediately in the supernatant as described by Figueiredo-Silva et al. (Figueiredo-Silva et al., 2010). Liver samples for FAS enzyme activities were homogenized in four volumes of ice-cold buffer (0.02 mol/L Tris-HCl, 0.25 mol/L sucrose, 2 mmol/L EDTA, 0.1 mol/L NaF, 0.5 mmol/L PMSF, 0.01 mol/L β-mercaptoethanol, pH 7.4), centrifuged at 24,000*g* at 4°C for 20 min and the supernatant were assayed immediately for FAS enzyme activity in pre-established conditions (Figueiredo-Silva et al., 2010). Livers for G6Pase enzyme activities were homogenized in eight volumes of ice-cold buffer (as described for GK), then centrifuged and the supernatant were assayed following the protocol of Alegre et al. (Alegre et al., 1988). Samples for FBPase enzyme activities were homogenized in seven volumes of ice-cold buffer (as described for GK), centrifuged at 900 *g* at 4°C for 10 min and the supernatant were assayed immediately for FBPase enzyme activity in pre-established conditions (Tranulis et al., 1996). Enzyme activity is defined as μmoles of substrate converted to product, per minute, at 37°C and is expressed per mg of liver protein. All measurements were performed in duplicate.

2.2.5 Statistical analysis

The effect of each condition and interactions were analyzed using two-way ANOVA ($P<0.05$), followed by a Tukey test when the interaction was significant. In cases where data were nonparametric or not homoscedastic, data transformations, such as logarithms, square roots and reciprocals, were used to meet ANOVA criteria. Normality was assessed using the Shaprio–Wilk test, while homoscedasticity was determined using Levene's test.

When hepatocyte primary cell cultures were repeated in different fish, results were analyzed using linear mixed-effects models (MIXED) procedure with fish ID as subject effect (SPSS 17.0). When interaction was significant, results were further analyzed using the EMMEANS subcommand. For all statistical analyses, the level of significance was set at $P<0.05$.

Chapter 3

RESULTS

The data generated from *in vivo* and *in vitro* experiments performed under the framework of this thesis, have been the subject of four peer reviewed publications. Therefore in this section, we present the original scientific articles in the order of the objectives of the thesis.

PUBLICATIONS DURING PhD:

(1) **Dai, W.**, Panserat, S., Mennigen, J. A., Terrier, F., Dias, K., Seiliez, I. and Skiba-Cassy, S. (2013). Post-prandial regulation of hepatic glucokinase and lipogenesis requires the activation of TORC1 signalling in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **216**, 4483-92.

(2) **Dai, W.**, Panserat, S., Kaushik, S., Terrier, F., Plagnes-Juan, E., Corraze, G., Seiliez, I. and Skiba-Cassy, S. (2015). Hepatic lipid biosynthesis is more responsive to protein than carbohydrate in rainbow trout during acute stimulations. *Am. J. Physiol. Regul. Integr. Comp. Physiol. (DOI: 10.1152/ajpregu.00281.2015).*

(3) **Dai W,** Panserat S, Plagnes-Juan E, Seiliez I, and Skiba-Cassy S. (2015). Amino acids attenuate insulin action on gluconeogenesis and promote fatty acid biosynthesis via mTORC1 signaling pathway in trout hepatocytes. *Cell Physiol Biochem* **36**: 1084-1100.

(4) **Dai, W.**, Panserat, S., Terrier, F., Seiliez, I. and Skiba-Cassy, S. (2014). Acute rapamycin treatment improved glucose tolerance through inhibition of hepatic gluconeogenesis in rainbow trout (*Oncorhynchus mykiss*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **307**, R1231-8.

PUBLICATION - 1

Does mTOR signaling pathway regulate metabolism-related gene expression *in vivo*?

Figure 3. 1 Experimental design, procedures and parameters from the first article

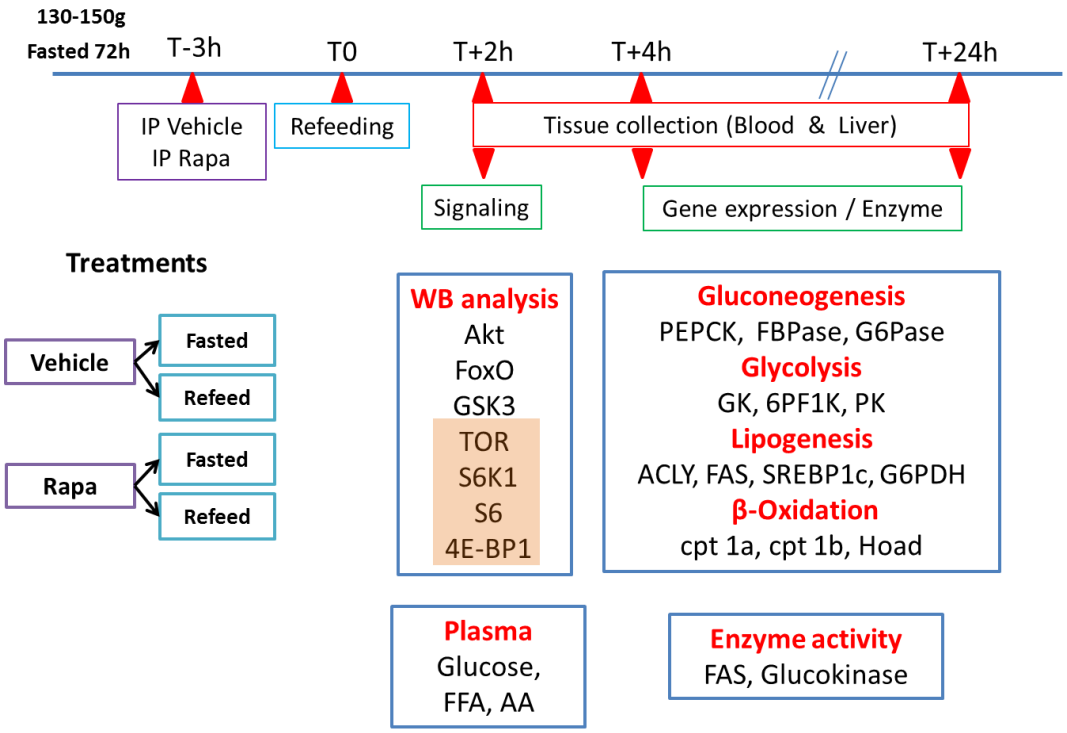
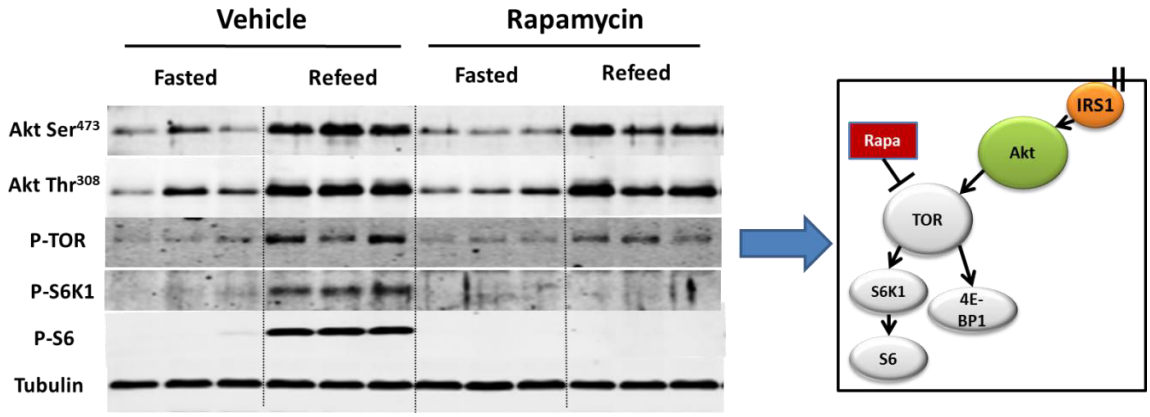


Figure 3. 2 Result highlights from the first article – Phosphorylation of representational proteins on Akt/TOR signaling pathway in trout liver



Rapamycin significantly inhibited the phosphorylation of TOR and its downstream effectors S6K1 and S6, without affecting Akt on Ser^{473} or Thr^{308}.

Presentation of the article

Objective

Despite the well-studied roles of TOR (target of rapamycin) in the regulation of lipid metabolism and glucose homeostasis in mammals, its role in the metabolic response to feeding status or dietary modifications in fish remains to be fully explored. In rainbow trout, mTOR signaling pathway has been characterized and the role of amino acids in the activation of this pathway has also been established. Moreover, previous studies from the laboratory have suggested that TOR signaling pathway is involved in the control of lipogenic and glycolytic gene expression. However, all these demonstrations were based on *in vitro* experiments using primary cell culture of rainbow trout hepatocytes. Our aim was to assess the potential involvement of mTOR signaling pathway in the regulation of postprandial hepatic lipid and glucose metabolism-related gene expression in rainbow trout. For that purpose, an experimental trial was implemented to inhibit the activation of TOR *in vivo* and analyze the consequences on the post-prandial regulation of hepatic gene expression.

Experiment and Analyses

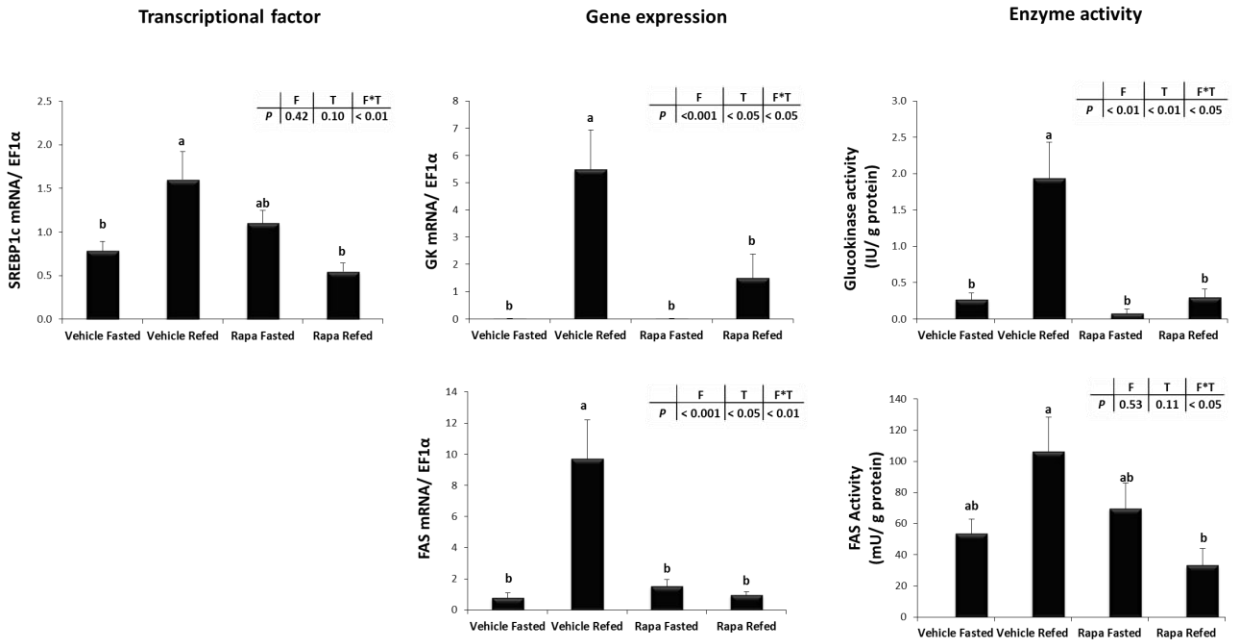
In order to abolish the potential activation of the TOR signaling pathway, fasted juvenile rainbow trout were subjected to a single intraperitoneal (IP) injection of rapamycin (0.5 mg/kg) or vehicle (75% DMSO and 25% saline solution NaCl 0.9%) with 100 µL/100 g body mass. Half of the fish were then refed with a commercial diet to apparent satiation 3 h after the IP injection of rapamycin while the other half remained fasted. Liver and blood samples were randomly collected 2, 4 and 24 h after refeeding (N=8). Post-prandial plasma metabolites, phosphorylation of proteins of the Akt/TOR signaling pathway, expression of genes related to glycolysis, gluconeogenesis, lipogenesis and fatty acid oxidation and activity of some selected enzymes were measured (Figure 3.1).

Results and Conclusion

As expected, refeeding resulted in enhanced phosphorylation of proteins of the Akt/TOR signaling pathway in vehicle treatment, confirming that refeeding effectively activated Akt/TOR signaling pathway *in vivo*. Furthermore, rapamycin significantly inhibited the postprandial phosphorylation of TOR and its downstream effectors S6K1, S6 and 4E-BP1, without affecting the phosphorylation of Akt, FoxO1/FoxO3 or GSK 3α/β, indicating that the activation of mTORC1 pathway in trout liver was successfully blocked by rapamycin (Figure 3.2).

Rapamycin administration also suppressed postprandial induction of FAS and GK at both gene expression and enzymatic activity levels, suggesting that GK and FAS activity are mainly controlled at the transcriptional level through an mTORC1-dependent manner. Rapamycin administration not only blunted the induction of FAS gene expression and activity but also limited the expression of SREBP1c, a transcription factor that activates all the genes needed to produce fatty acids and triglycerides in liver (Figure 3.3).

Figure 3. 3 Result highlights from the first article – Gene expression and enzyme activities of markers of hepatic glucose phosphorylation and lipogenesis



In conclusion, we demonstrated for the first time in fish that postprandial regulation of hepatic lipogenesis and glucokinase requires the activation of mTORC1 signaling in rainbow trout, confirming previous observations *in vitro*. We also identified the transcription factor SREBP1c as a potential mediator of the action of TOR signaling pathway on the regulation of lipogenic gene expression as previously demonstrated in mammals.

The Journal of Experimental Biology 216, 4483-4492

doi:10.1242/jeb.091157

RESEARCH ARTICLE

Post-prandial regulation of hepatic glucokinase and lipogenesis requires the activation of TORC1 signalling in rainbow trout (*Oncorhynchus mykiss*)

Weiwei Dai, Stéphane Panserat, Jan A. Mennigen, Frédéric Terrier, Karine Dias, Iban Seiliez and Sandrine Skiba-Cassy*

INRA, UR 1067 Nutrition, Metabolism, Aquaculture, Aquapôle, CD 918, F-64310 Saint-Pée-sur-Nivelle, France

*Author for correspondence (skiba@st-pee.inra.fr)

SUMMARY

To assess the potential involvement of TORC1 (target of rapamycin complex 1) signalling in the regulation of post-prandial hepatic lipid and glucose metabolism-related gene expression in trout, we employed intraperitoneal administration of rapamycin to achieve an acute inhibition of the TOR pathway. Our results reveal that rapamycin inhibits the phosphorylation of TORC1 and its downstream effectors (S6K1, S6 and 4E-BP1), without affecting Akt and the Akt substrates Forkhead-box Class O1 (FoxO1) and glycogen synthase kinase 3α/β (GSK 3α/β). These results indicate that acute administration of rapamycin in trout leads to the inhibition of TORC1 activation. No effect is observed on the expression of genes involved in gluconeogenesis, glycolysis and fatty acid oxidation, but hepatic TORC1 inhibition results in decreased sterol regulatory element binding protein 1c (SREBP1c) gene expression and suppressed fatty acid synthase (FAS) and glucokinase (GK) at gene expression and activity levels, indicating that FAS and GK activity is controlled at a transcriptional level in a TORC1-dependent manner. This study demonstrates for the first time in fish that post-prandial regulation of hepatic lipogenesis and glucokinase in rainbow trout requires the activation of TORC1 signalling.

Key words: TORC1, rapamycin, fatty acid synthesis, glycolysis, gene expression, rainbow trout.

Received 14 May 2013; Accepted 27 August 2013

INTRODUCTION

The target of rapamycin (TOR) is a central controller of the growth and metabolism of cells in response to nutrients, growth factors and cellular energy status in mammals (Wullschleger et al., 2006). TOR exists in two structurally and functionally distinct complexes, the rapamycin- and nutrient-sensitive TORC1 and the rapamycin- and nutrient-insensitive TORC2 (Kim and Guan, 2011). TORC1 integrates inputs from at least five major intracellular and extracellular cues – growth factors, stress, energy status, oxygen and amino acids – to control many major processes, including protein and lipid synthesis and autophagy (Wullschleger et al., 2006). The mechanism leading to TORC1 activation has already been established in mammals and fish (Kim and Guan, 2011; Lansard et al., 2010; Seiliez et al., 2008). In complex with the intracellular 12 kDa FK506-binding protein (FKBP12), rapamycin binds and inhibits TOR exclusively in TORC1, whereas FKBP12–rapamycin cannot bind TOR in TORC2, making this complex insensitive to direct inhibition by rapamycin at least over short periods (Laplante and Sabatini, 2012; Sarbassov et al., 2004). However, in many cell types (Sarbassov et al., 2005) and *in vivo* studies (Lamming et al., 2012), prolonged rapamycin treatment results in a decrease of TORC2 function, perhaps as a result of depletion of the TORC2 complex (Sarbassov et al., 2006).

It is well known in mammals that TORC1 plays an important role in the control of many aspects of cellular metabolism including lipid metabolism and glucose homeostasis (Wullschleger et al., 2006). TORC1 enhances *de novo* lipogenesis by regulating the expression and processing of sterol regulatory element-binding protein 1c (SREBP1c) (Wan et al., 2011; Yecies et al., 2011), a master regulator of sterol and lipogenic gene transcription (Krycer et al., 2010). Furthermore, TORC1 increases glycolytic flux by activating the transcription and the translation of hypoxia inducible factor 1a (HIF1a) (Düvel et al., 2010), a positive regulator of many glycolytic genes (Gordan et al., 2007).

In contrast, the role of TORC1 in the metabolic response to feeding status or dietary composition in fish has been less well investigated. Rainbow trout have relatively high dietary protein and essential amino acid requirements and poor utilization of dietary carbohydrates compared with humans, rodents and other terrestrial domestic animals (Walton and Cowey, 1982; Wilson, 1994); thus, it represents a suitable model to investigate the involvement of TORC1 in the nutritional control of metabolic gene expression. Most components of the TOR system [TOR, ribosomal protein S6 kinase (S6K1), ribosomal protein S6 (S6) and 4E-binding protein 1 (4E-BP1)] are evolutionarily conserved and have already been characterized in rainbow trout liver and muscle (Lansard et al., 2010; Seiliez et al., 2008; Seiliez et al., 2011a), and viscera and muscle from zebrafish (Seiliez et al., 2013). In fish, activation of the TOR pathway is controlled by re-feeding (Seiliez et al., 2008; Seiliez et al., 2013; Skiba-Cassy et al., 2009) as well as *in vivo* administration of insulin (Plagnes-Juan et al., 2008). As demonstrated using primary culture of rainbow trout hepatocytes, the joint action of insulin and amino acids (particularly leucine) seems to be essential to activate the TOR pathway whereas insulin alone is sufficient to activate protein kinase B (Akt) phosphorylation (Lansard et al., 2011; Lansard et al., 2010).

In rainbow trout, macronutrient composition of the diet affects the TOR signalling pathway and metabolism-related gene

expression. Increasing the dietary proportion of carbohydrates at the expense of proteins impairs activation of the TOR pathway and modifies the expression of several genes related to metabolism (Seiliez et al., 2011a; Skiba-Cassy et al., 2013). Furthermore, an *in vitro* investigation with rainbow trout hepatocytes demonstrated that insulin and amino acids together upregulate lipogenic and glycolytic gene expression in a TOR-dependent manner (Lansard et al., 2010) as observed in mammals (Porstmann et al., 2008). Thus, we put forward the hypothesis that TOR is involved in the post-prandial regulation of hepatic lipid and glucose metabolism-related gene expression in rainbow trout.

To determine the potential involvement of TORC1 signalling in the regulation of post-prandial hepatic metabolism-related gene expression in rainbow trout, we performed intraperitoneal (i.p.) administration of rapamycin. Our goal was to achieve an acute inhibition of the TORC1 signalling pathway *in vivo* and analyse the consequences of this TORC1 inhibition on the expression of several genes related to glucose utilization, gluconeogenesis, lipogenesis and fatty acid oxidation.

MATERIALS AND METHODS

Experimental and sampling procedure

Juvenile rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were reared in the INRA experimental facilities at Donzacq (Landes, France) at a constant water temperature of 17.5±0.5°C, under natural photoperiod. They were fed a standard trout commercial diet (T-3P classic, Skretting, Fontaine-les-Vervins, France) during the acclimatization period. Fish (mean ± s.e.m. body mass 140±10 g) were distributed into four tanks per treatment (15 fish per 70 l tank). Prior to the feeding trials, fish were food deprived for 72 h – the time required to ensure the complete emptying of the digestive tract. After this period, trout were sedated with benzocaine (10 mg l^{-1}) and treated with a single i.p. injection of rapamycin (0.5 mg kg^{-1}) or vehicle (75% DMSO and 25% saline solution, NaCl 0.9%) at 100 µl 100 g^{-1} body mass. Two tanks per treatment (vehicle or rapamycin) were re-fed with the commercial diet (T-3P classic) to apparent satiation 3 h after the injection while the other two tanks remained fasted. Four fish per tank (two tanks per condition) were randomly sampled at 2, 4 and 24 h after re-feeding (N=8). Trout were anaesthetized with benzocaine (30 mg l^{-1}) and killed by a sharp blow to the head. Blood was removed from the caudal vein into heparinized syringes and centrifuged (3000 ***g***, 5 min); the recovered plasma was immediately frozen and kept at –20°C. The stomach contents of each fish were checked to confirm that the fish had effectively ingested the diet. Livers were dissected and immediately frozen in liquid nitrogen and kept at –80°C. The experiments were carried out in accordance with the clear boundaries of EU legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e. Directive 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decree no. 2001-464, 29 May 2001). The investigators carrying out the experiment had ‘level 1’ or ‘level 2’ certification, bestowed by the Direction Départementale des Services Vétérinaires (French veterinary services) to carry out animal experiments (INRA 2002-36, 14 April 2002).

Plasma metabolite analysis

Plasma glucose (Glucose RTU, bioMérieux, Marcy l’Etoile, France), triglycerides (PAP 150, bioMérieux) and free fatty acid (NEFA C kit, Wako Chemicals, Neuss, Germany) levels were determined using commercial kits adapted to a microplate format, according to the recommendations of the manufacturer. Total plasma free amino acid levels were determined by the ninhydrin reaction (Moore, 1968), with glycine as standard.

Western blot analysis

Frozen livers (N=6; 200 mg) from 2 h re-fed trout were homogenized on ice with an ULTRA-TURRAX homogenizer (IKA-WERKE, Staufen, Germany) in 2 ml of buffer containing 150 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris, 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA (pH 7.4), 100 mmol l^{-1} NaF, 4 mmol l^{-1} sodium pyrophosphate, 2 mmol l^{-1} sodium orthovanadate, 1% Triton X-100, 0.5% NP-40-Igepal and a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Homogenates were centrifuged at 1500 ***g*** for 15 min at 4°C and supernatant fractions were then centrifuged at 20,000 ***g*** at 4°C for 30 min. The resulting supernatant fractions were recovered and stored at −80°C. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with BSA as standard. Lysates (10 µg of total protein for Akt/TOR/S6/4E-BP1 and 20 µg for S6K1/FoxO1) were subjected to SDS-PAGE and western blotting using the appropriate antibody. Anti-phospho-Akt (Ser473) (no. 9271), anti-phospho-Akt (Thr308) (no. 9275), anti-carboxyl terminal Akt (no. 9272), anti-phospho-TOR (Ser2448) (no. 2971), anti-TOR (no. 2972), anti-phospho-S6 protein kinase 1 (Thr389) (no. 9205), anti-S6 protein kinase 1 (no. 9202), anti-phospho-S6 (Ser235/236) (no. 4856), anti-S6 (no. 2217), anti-phospho-4E-BP1 (Thr37/46) (no. 9459), anti-4E-BP1 (no. 9452), anti-phospho-FoxO1 (Thr24)/FoxO3a (Thr32) (no. 9464), anti-phospho-glycogen synthase kinase 3α/β (GSK 3α/β; Ser21/9) (no. 9331), anti-GSK 3α (no. 9338) and anti-β-tubulin (no. 2146) were purchased from Cell Signaling Technologies (Ozyme, Saint Quentin Yvelines, France). Anti-FoxO1 (no. 1874-1) was purchased from Epitomics (Burlingame, CA, USA). All of these antibodies [except anti-phospho-Akt (Thr308), anti-phospho-GSK 157 3α/β (Ser21/9) and anti-GSK 3α] successfully cross-reacted with rainbow trout proteins (Kamalam et al., 2012; Seiliez et al., 2008; Seiliez et al., 2011b). For anti-phospho-Akt (Thr308), anti-phospho-GSK 3α/β (Ser21/9) and anti-GSK 3α antibody, the molecular weight and amino acid sequences were monitored in the SIGENAE database [information system of the Analysis of Breeding Animals’ Genome (AGENAE); http://www.sigenae.org/] to check for a good conservation of the antigen sequence. Membranes were washed then incubated with an IRDye Infrared secondary antibody (LI-COR Biosciences, Lincoln, NE, USA). Bands were visualized by Infrared Fluorescence using the Odyssey Imaging System (LI-COR Biosciences) and quantified by Odyssey Infrared imaging system software (v.3.0, LI-COR Biosciences).

Gene expression analysis: real-time PCR

Liver samples (N=6) for gene expression were collected from fasted fish and 4 and 24 h re-fed fish, the time interval being based on the post-prandial hepatic gene expression peak of rainbow trout (Mennigen et al., 2012). Total RNA was extracted from −80°C frozen livers using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s recommendations, quantified by spectrophotometry (absorbance at 260 nm) and its integrity assessed using agarose gel electrophoresis. A 1 µg sample of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III Reverse Transcriptase kit (Invitrogen) and random primers (Promega, Charbonniéres, France) according to the manufacturers’ instructions. Target gene expression levels were determined by quantitative real-time (q)RT-PCR, using specific primers (Skiba-Cassy et al., 2009).

qRT-PCR was carried out on a LightCycler 480 II (Roche Diagnostics) using LightCycler 480 SYBR Green I Master (Roche Diagnostics). qRT-PCR analyses were focused on several key enzymes of hepatic metabolism: glucokinase (GK; EC 2.7.1.2) for glucose phosphorylation; 6-phosphofructo-1-kinase (6PF1K; EC 2.7.1.11) and pyruvate kinase (PK; EC 2.7.1.40) for glycolysis; glucose-6-phosphatase (G6Pase; EC 3.1.3.9) for glucose dephosphorylation; fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) and phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) for gluconeogenesis; glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) for pentose phosphate shunt; ATP citrate lyase (ACLY; EC 2.3.3.8), fatty acid synthase (FAS; EC 2.3.1.85) and the transcription factor SREBP1c for lipogenesis; and carnitine palmitoyltransferase 1 (CPT1; EC 2.3.1.21) and 3-hydroxyacyl-CoA dehydrogenase (HOAD; EC 1.1.1.35) for fatty acid oxidation. When different isoforms of a gene were known in rainbow trout (as for CPT1), gene expression analysis was performed on each isoform. Elongation factor-1alpha (EF1α) was employed as a non-regulated reference gene, as previously used in rainbow trout, and it was stably expressed in our investigations (data not shown).

PCR was performed using 2 µl of the diluted cDNA (76 times diluted) mixed with 0.24 µl of each primer (10 µmol l^{-1}), 3 µl LightCycler 480 SYBR Green I Master (Roche Diagnostics) and 0.52 µl DNase/RNase/protease-free water (5 prime GmbH, Hamburg, Germany) in a total volume of 6 µl. The PCR protocol was initiated at 95°C for 10 min for initial denaturation of the cDNA and hot-start Taq polymerase activation, followed by 45 cycles of a three-step amplification program (15 s at 95°C; 10 s at melting temperature T_m (59–65°C); 4.8 s at 72°C), according to the primer set used. Melting curves were systematically monitored (5 s at 95°C; 1 min at 65°C; temperature slope at 0.11°C s^{-1} from 65 to 97°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included quadruplicates for each sample (duplicates of reverse transcription and PCR amplification, respectively) and also negative controls (reverse transcriptase- and RNA-free samples). Relative quantification of target gene expression was determined using the ΔΔCT method by the LightCycler 480 software (v.SW 1.5, Roche Diagnostics). PCR efficiency, which was measured by the slope of a standard curve using serial dilutions of cDNA, ranged between 1.85 and 2.

Enzyme activity analysis

Enzyme activity was measured from liver samples (N=6) collected from 24 h re-fed trout. Liver samples for GK enzyme activity were homogenized in 10 volumes of ice-cold buffer [80 mmol l^{-1} Tris, 5 mmol l^{-1} EDTA, 2 mmol l^{-1} DTT, 1 mmol l^{-1} benzamidine, 1 mmol l^{-1} 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pH 7.6] and centrifuged at 900 ***g*** at 4°C for 10 min. GK activity was assayed immediately in the supernatant as described elsewhere (Figueiredo-Silva et al., 2010). Liver samples for FAS enzyme activity were homogenized in four volumes of ice-cold buffer (0.02 mol l^{-1} Tris-HCl, 0.25 mol l^{-1} sucrose, 2 mmol l^{-1} EDTA, 0.1 mol l^{-1} NaF, 0.5 mmol l^{-1} PMSF, 0.01 mol l^{-1} β-mercaptoethanol, pH 7.4), centrifuged at 24,000 ***g*** at 4°C for 20 min and the supernatant assayed immediately for FAS enzyme activity in pre-established conditions (Figueiredo-Silva et al., 2010). Enzyme activity, defined as micromoles of substrate converted to product per minute, at 37°C, was expressed per gram of liver tissue. All measurements were performed in duplicate.

Statistical analysis

The data for post-prandial plasma metabolites are expressed as means ± s.e.m. (N=8). The results of western blot, gene expression and enzyme activity are expressed as means + s.e.m. (N=6). The effect of re-feeding, i.p. administration of rapamycin or vehicle, and their interaction were analysed using two-way ANOVA (P<0.05). When the interaction was significant, the results were further analysed using one-way ANOVA and a Tukey test. For all statistical analyses, the level of significance was set at P<0.05.

RESULTS

Plasma metabolite levels

Re-feeding significantly increased glucose levels at 4 h (P<0.01) and 24 h (P<0.001) and free amino acid levels at 24 h (P<0.001), while re-feeding markedly decreased free fatty acid at 4 h (P<0.001) (Table 1). There was no significant difference in the effects of vehicle and rapamycin on the plasma glucose, free fatty acid and free amino acid levels (P>0.05).

Akt/TOR signalling pathway

To validate an inhibitory effect on TOR signalling, we investigated the Akt/TOR signalling pathway in rainbow trout livers using western blot analyses. As illustrated in Fig. 1, re-feeding resulted in enhanced phosphorylation of Akt on Ser473 (P<0.001) and Thr308 (P<0.001), FoxO1 on Thr24 or FoxO3a on Thr32 (P=0.02), GSK 3α/β on Ser21/9 (P<0.01), TOR on Ser2448 (P<0.001), S6K1 on Thr389 (P<0.01), S6 on Ser235/236 (P<0.01) and 4E-BP1 on Thr37/46 (P<0.001) proteins. The levels of these total proteins were not modified by feeding or treatment, except for GSK 3α, which was significantly increased by re-feeding (P<0.01, Fig. 1E), whereas β-tubulin levels were not affected by re-feeding or treatment. Activation of Akt on Ser473 (P=0.52, Fig. 1A) and Thr308 (P=0.34,

Table 1. Post-prandial plasma metabolite levels in rainbow trout subjected to intraperitoneal administration of vehicle or rapamycin, 4 and 24 h after re-feeding

Plasma metabolite (mmol l^{-1})	Time (h)	Vehicle		Rapamycin		*P*-value		
		Fasted	Re-fed	Fasted	Re-fed	Feeding	Treatment	F×T
Glucose	4	3.77±0.12	4.44±0.21	3.75±0.13	4.08±0.20	<0.01	0.27	0.32
	24	3.84±0.11	4.92±0.17	3.81±0.30	5.21±0.38	<0.001	0.61	0.53
Free fatty acids	4	0.29±0.03	0.15±0.02	0.27±0.01	0.18±0.01	<0.001	0.75	0.17
	24	0.29±0.01	0.25±0.02	0.24±0.03	0.25±0.04	0.52	0.29	0.34
Free amino acids	4	3.64±0.22	3.77±0.21	3.47±0.15	4.00±0.21	0.11	0.89	0.33
	24	3.51±0.09	4.84±0.23	3.35±0.16	5.23±0.33	<0.001	0.63	0.20

Data are means ± s.e.m. (N=8). At each post-prandial time (row-wise), the effects of re-feeding (F), intraperitoneal (i.p.) administration of vehicle or rapamycin (T) and their interaction were analysed using two-way ANOVA (P<0.05), followed by a Tukey test when the interaction was significant.
F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction of F and T.

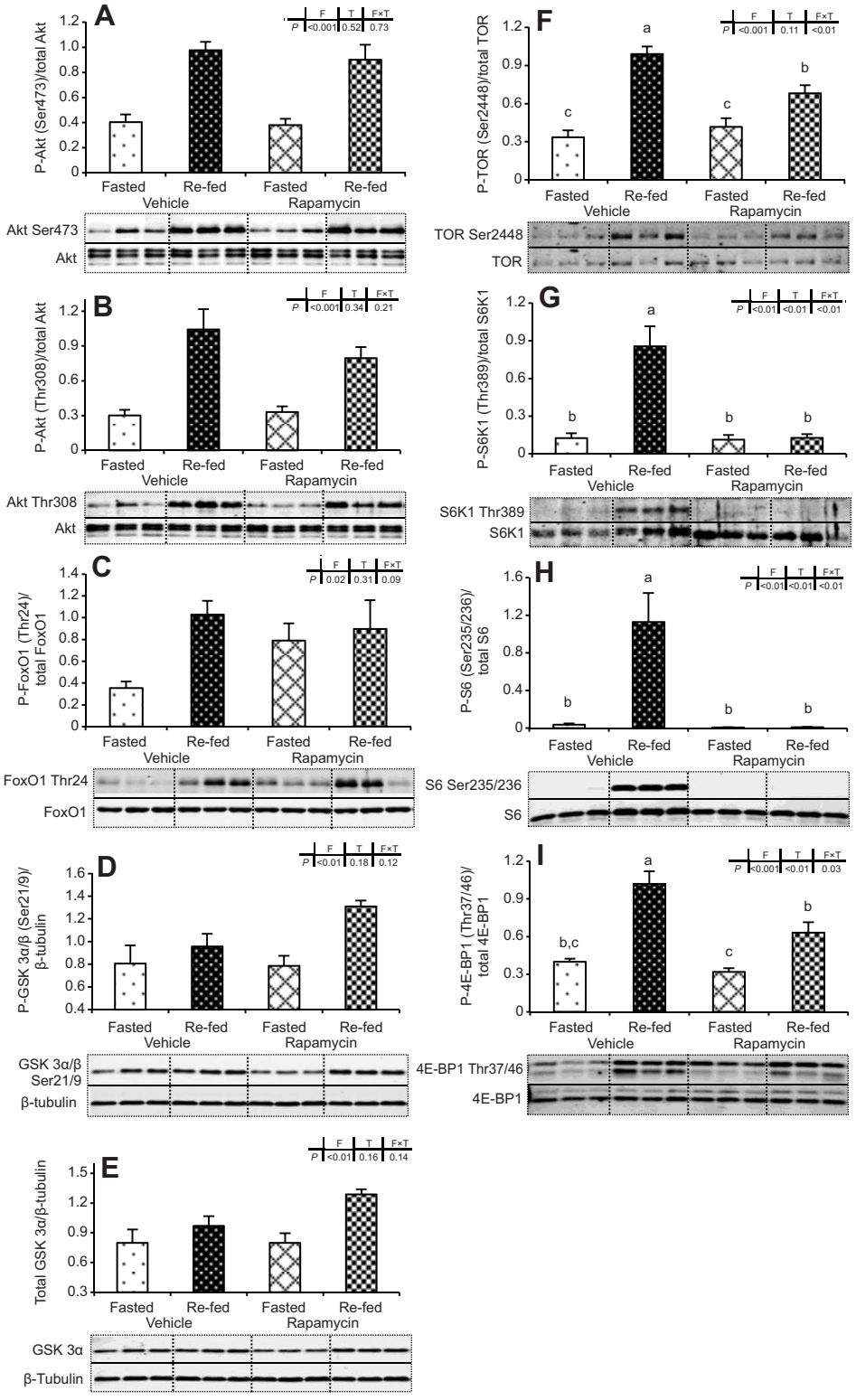


Fig. 1. Western blot analysis of hepatic (A) protein kinase B (Akt; Ser473), (B) Akt (Thr308), (C) forkhead-box class O1 (FoxO1; Thr24), (D,E) glycogen synthase kinase 3α/β (GSK 3), (F) target of rapamycin (TOR), (G) ribosomal protein S6 kinase (S6K1), (H) ribosomal protein S6 (S6) and (I) 4E-binding protein 1 (4E-BP1) protein phosphorylation in rainbow trout subjected to intraperitoneal (i.p.) administration of vehicle or rapamycin, 2 h after re-feeding. Gels were loaded with 10 μg of total protein per lane for Akt/GSK 3/TOR/S6/4E-BP1 and 20 μg for S6K1/FoxO1. A representative blot is shown. Graphs represent the ratio between the phosphorylated protein and the total amount of the target protein. Results are means + s.e.m. (N=6) and were analysed using two-way ANOVA (P<0.05), followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at P<0.05. F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction between F and T.

Fig. 1B) and the Akt substrates FoxO1 (P=0.31, Fig. 1C) and GSK 3 (P=0.18, Fig. 1D) was not affected by rapamycin, while the phosphorylation of TOR downstream effectors, including S6K1 (P<0.01, Fig. 1G), S6 (P<0.01, Fig. 1H) and 4E-BP1 (P<0.01, Fig. 1I) was totally or partially inhibited by rapamycin. The phosphorylation of S6K1 and S6 in vehicle re-fed fish increased 11- and 31-fold, respectively, compared with vehicle fasted fish, while the phosphorylation of these two proteins was totally abolished in rapamycin-treated fish, irrespective of the feeding status. Re-feeding induced a 2.0-fold increase in 4E-BP1 phosphorylation in rapamycin-treated fish, but this increase was significantly lower compared with the induction observed in vehicle controls. An interaction between feeding and treatment was observed for TOR protein phosphorylation (P<0.01, Fig. 1F), with TOR phosphorylation in vehicle re-fed fish being markedly higher than that in rapamycin re-fed fish.

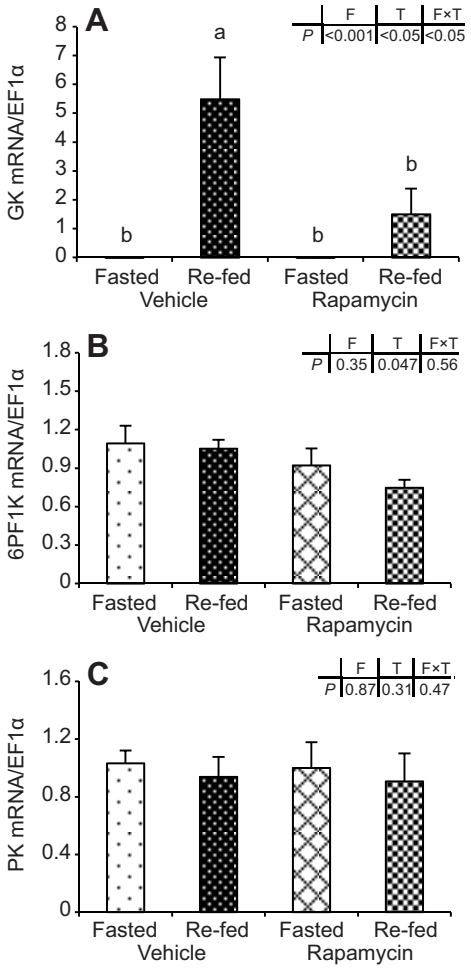


Fig. 2. Gene expression of selected glycolytic enzymes in the liver of rainbow trout subjected to i.p. administration of vehicle or rapamycin. (A) Glucokinase (GK), (B) 6-phosphofructo-1-kinase (6PF1K) and (C) pyruvate kinase (PK) mRNA levels were measured using quantitative real-time (q)RT-PCR, 24 h after re-feeding. Expression values are normalized with elongation factor-1 alpha (EF1α)-expressed transcripts. Results are means + s.e.m. (N=6) and were analysed using two-way ANOVA (P<0.05), followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at P<0.05. F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction between F and T.

mRNA levels of target genes

To determine the potential involvement of the TOR signalling pathway in the regulation of hepatic intermediary metabolism-related gene expression, we performed qRT-PCR on genes related to glucose and lipid metabolism. Samples collected at 4 h after re-feeding were unaffected by feeding or treatment (data not shown); the results summarized in Figs 2–5 correspond to samples collected 24 h after re-feeding. The results regarding glycolytic enzyme genes are presented in Fig. 2. GK mRNA levels were upregulated several hundredfold by re-feeding in the vehicle treatment, while this upregulation was significantly suppressed in the rapamycin treatment. Gene expression of 6PF1K was only slightly affected by the treatment (P=0.047, Fig. 2B) and that of PK remained stable irrespective of feeding (P=0.87, Fig. 2C) or treatment (P=0.31, Fig. 2C). As illustrated in Fig. 3A, re-feeding significantly increased (12-fold) FAS gene expression in the vehicle treatment, whereas this induction was totally inhibited by rapamycin. A similar pattern was also observed for SREBP1c mRNA levels (P<0.01, Fig. 3B), with a decrease in rapamycin re-fed fish compared with vehicle re-fed fish. Regardless of feeding or treatment, no significant variation was observed for G6PDH or ACLY mRNA levels. Regarding the mRNA expression of CPT1, both isoforms, CPT1a (Fig. 4A) and CPT1b (Fig. 4B), were positively affected by rapamycin. Nonetheless, re-feeding downregulated the expression of CPT1a in rapamycin-treated fish and that of CPT1b in both vehicle and rapamycin treatments. HOAD mRNA levels were also modulated by the treatment (P<0.01, Fig. 4C), with lower HOAD gene expression in the rapamycin treatment compared with the vehicle treatment irrespective of feeding status. In terms of gluconeogenesis (Fig. 5), mRNA levels of PEPCK (P<0.05), FBPase (P<0.05) and G6Pase (P<0.001) were downregulated by re-feeding irrespective of the treatment.

Enzyme activity analysis

To substantiate the results from gene expression analysis, two hepatic enzyme activities, GK and FAS, were determined 24 h after re-feeding (Fig. 6). Re-feeding enhanced GK enzyme activity 7.2-fold in vehicle-treated fish, but this increase was totally abolished by rapamycin treatment. An interaction between feeding and treatment was recorded for FAS enzyme activity (P<0.05, Fig. 6B), showing that rapamycin significantly decreased FAS enzyme activity in rapamycin re-fed fish compared with vehicle re-fed fish.

DISCUSSION

The regulation of metabolism-related gene expression by the TOR pathway has been investigated *in vitro* using several cellular models including primary culture of rainbow trout hepatocytes (Lansard et al., 2010). *In vivo*, most studies have focused on the chronic effects of rapamycin administration in mammals (Fang et al., 2013; Houde et al., 2010; Lamming et al., 2012; Sarbassov et al., 2006; Yang et al., 2012). In these experiments, modification of intermediary metabolism-related gene expression was not the only target of inhibition. For instance, plasma metabolites (Blum, 2002; Cunningham et al., 2007; Hagiwara et al., 2012), glucose intolerance (Chang et al., 2009; Houde et al., 2010; Lamming et al., 2012), insulin resistance (Blum, 2002; Hagiwara et al., 2012), insulin sensitivity (Wullschleger et al., 2006; Yang et al., 2012), islet mass (Yang et al., 2012) and other metabolic aspects have been modified and consequently influenced the expression of target genes. Here, we performed single i.p. administration of rapamycin to achieve an acute inhibition of the TOR signalling pathway in order to determine the potential direct involvement of this pathway in the regulation of post-prandial hepatic lipid and glucose metabolism-related gene expression in rainbow trout.

Akt/TOR signalling pathway

Investigation of the Akt/TOR signalling pathway revealed that administration of rapamycin significantly inhibits the post-prandial phosphorylation of TOR and its downstream effectors S6K1, S6 and 4E-BP1, without affecting Akt, FoxO1/FoxO3 and GSK 3α/β. These results indicate that the activation of the TOR pathway *in vivo* can be successfully blocked by a single injection of rapamycin, which is consistent with our previous finding in primary cultures of rainbow trout hepatocytes (Lansard et al., 2010) and other investigations in mammals (Owen et al., 2012a; Yang et al., 2012). Loss of function of TORC2 in mice through rictor or mLST8 ablation inhibits the phosphorylation of Akt on Ser473 (Hagiwara et al., 2012; Hresko and Mueckler, 2005), and its substrates FoxO1 and GSK 3α/β, while keeping the phosphorylation of TORC1 and

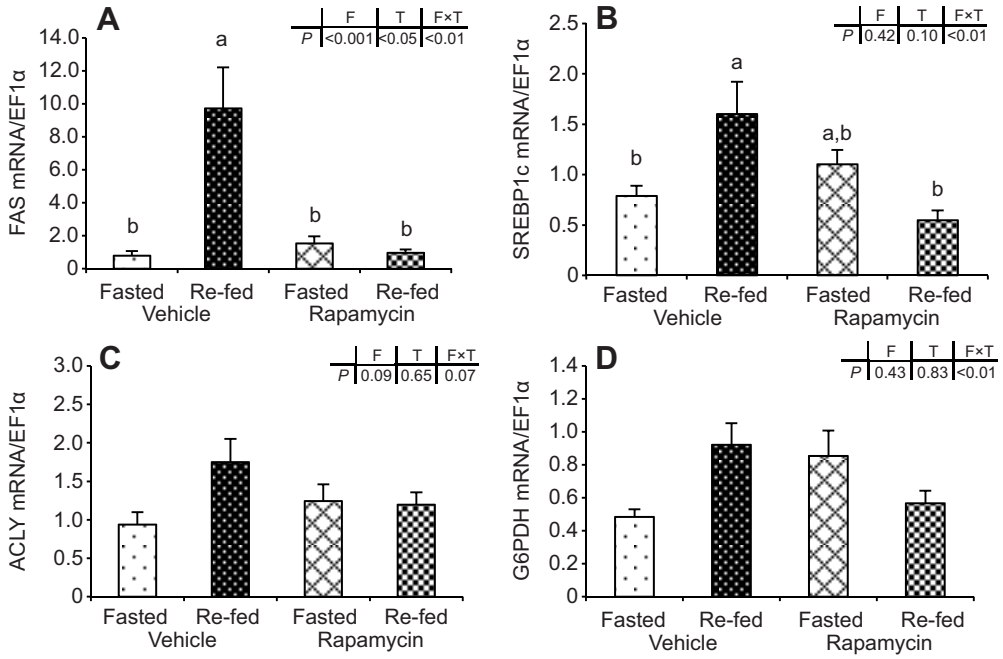


Fig. 3. Gene expression of selected enzymes and transcription factors involved in NADPH generation and lipogenesis in the liver of rainbow trout subjected to i.p. administration of vehicle or rapamycin, 24 h after re-feeding. (A) Fatty acid synthase (FAS), (B) sterol regulatory element binding protein 1-like (SREBP1c), (C) ATP citrate lyase (ACLY) and (D) glucose 6-phosphate dehydrogenase (G6PDH) mRNA levels were measured using qRT-PCR. Expression values are normalized with EF1α-expressed transcripts. Results are means + s.e.m. (N=6) and were analysed using two-way ANOVA (P<0.05), followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at P<0.05. F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction between F and T.

its downstream targets intact (Hagiwara et al., 2012; Sarbassov et al., 2005). In the present study, post-prandial phosphorylation of Akt Ser473, FoxO1/FoxO3 and GSK 3α/β was not affected by the administration of rapamycin, suggesting that acute administration of rapamycin in trout only inhibits the phosphorylation of TORC1, without affecting TORC2. Administration of rapamycin in trout reduces but does not totally abolish 4E-BP1 phosphorylation. This lower effect of rapamycin on 4E-BP1 could be explained by a partial recovery of 4E-BP1 phosphorylation during the post-prandial period. Indeed, rapamycin has been shown to differentially affect S6K1 and 4E-BP1 phosphorylation *in vitro* with a transient inhibitory effect on 4E-BP1 phosphorylation *versus* a long-term inhibition of S6K1 (Choo et al., 2008).

Lipid metabolism

The liver is an important site of lipid synthesis and export in fish (Tocher, 2003). We observed that TORC1 inhibition impairs post-prandial induction of both FAS gene expression and activity, suggesting that TORC1 controls the enzyme activity of FAS mainly at the transcriptional level. FAS is a downstream target gene of the transcription factor SREBP1c in mammals (Horton et al., 2002), but this link has not been unequivocally established in fish, despite reports in several studies performed in rainbow trout that FAS gene expression is associated with increased expression of SREBP1c (Lansard et al., 2010; Seiliez et al., 2011a; Skiba-Cassy et al., 2009). Our present findings confirm the relationship between SREBP1c and FAS gene expressions in trout and clearly demonstrate *in vivo* that post-prandial regulation of hepatic SREBP1c and FAS gene expression depends on the activation of TORC1.

In mammals, Akt acts at multiple regulatory steps, in both a TORC1-dependent and -independent manner, to control SREBP1c gene expression. A recent study revealed a TORC1-independent pathway by which Akt negatively regulates the expression of Insig-2a mRNA to promote SREBP1c expression (Yecies et al., 2011). Another potential mechanism involves Akt-mediated phosphorylation and inhibition of GSK 3, which induces the reduction of SREBP1c mRNA levels (Bengoechea-Alonso and Ericsson, 2009; Krycer et al., 2010). The present data indicate that the phosphorylation of Akt and GSK 3 is not modified after acute rapamycin administration, suggesting that the regulation of SREBP1c gene expression and lipogenesis in rainbow trout liver occurs at least in part independently of TORC2 and GSK 3 activation.

SREBP1c gene expression is induced by re-feeding and this induction is inhibited by rapamycin administration, suggesting that activation of TORC1 in trout liver is both necessary and sufficient to stimulate SREBP1c expression and its downstream targets. In mammals, TORC1 regulates SREBP1c mRNA induction, probably through the control of lipin 1 localization (Peterson et al., 2011). Peterson and colleagues (Peterson et al., 2011) demonstrated that TORC1 directly phosphorylates lipin 1, blocking lipin 1 nuclear entry, thereby promoting nuclear remodelling and mediating the effects of TORC1 on the SREBP target gene. Another potential mechanism by which TORC1 mediates SREBP1c maturation is *via* S6K1 (Cornu et al., 2013; Li et al., 2010; Owen et al., 2012b; Wang et al., 2011). Although acute administration of rapamycin in trout significantly impairs TORC1 activation and inhibits re-feeding induction of SREBP1c and FAS gene expression in trout, further explorations are needed to implicate lipin 1 or S6K1 as the crucial links in the TORC1 control of lipogenesis.

CPT1 is considered to be a rate-limiting enzyme of fatty acid β-oxidation whose expression is enhanced in fasted situations then rapidly reduced after re-feeding in mammals, birds and rainbow trout (Ryu et al., 2005; Seiliez et al., 2011a; Skiba-Cassy et al., 2007). The present study indicates that inhibition of CPT1 gene expression after re-feeding in trout is associated with the activation of Akt irrespective of TORC1 inhibition, suggesting that the regulation of β-oxidation might be dependent on Akt activation and at least partly independent of TORC1 phosphorylation. It has been proposed that Akt phosphorylates and inhibits the forkhead family transcription factor FoxA2, thereby controlling lipid export, fatty acid oxidation and accumulation of triglycerides in the liver (Wolfrum et al., 2004; Wolfrum and Stoffel, 2006). Administration of rapamycin significantly enhances CPT1 gene expression in both fasted and re-fed trout, while negatively affecting the expression of HOAD. The ability of rapamycin to affect gene expression has already been established *in vitro* (Deval et al., 2009). Further investigations are

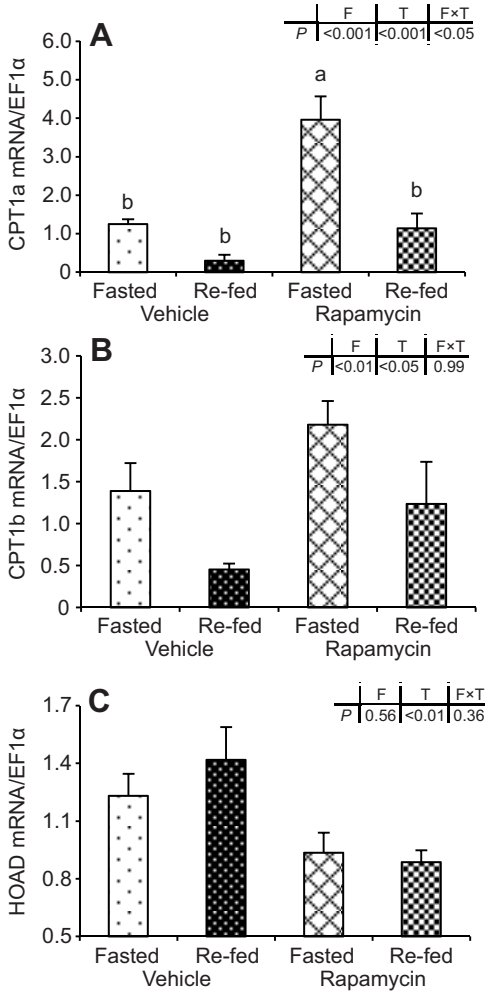


Fig. 4. Gene expression of selected fatty acid oxidation enzymes in the liver of rainbow trout subjected to i.p. administration of vehicle or rapamycin, 24 h after re-feeding. (A) Carnitine palmitoyl transferase isoforms a (CPT1a) and (B) b (CPT1b) and (C) 3-hydroxyacyl-CoA dehydrogenase (HOAD) mRNA levels were measured using qRT-PCR. Expression values are normalized with EF1α-expressed transcripts. Results are means + s.e.m. (*N*=6) and were analysed using two-way ANOVA (*P*<0.05), followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at *P*<0.05. F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction between F and T.

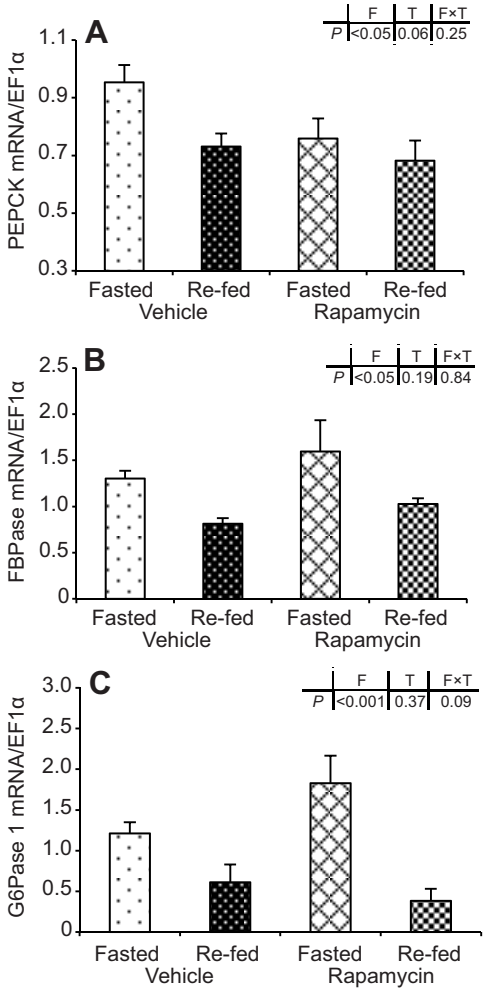


Fig. 5. Gene expression of selected gluconeogenic enzymes in the liver of rainbow trout subjected to i.p. administration of vehicle or rapamycin, 24 h after re-feeding. (A) Phosphoenolpyruvate carboxykinase (PEPCK), (B) fructose 1,6-bisphosphatase (FBPase) and (C) glucose-6-phosphatase isoform 1 (G6Pase1) mRNA levels were measured using qRT-PCR. Expression values are normalized with EF1α-expressed transcripts. Results are means + s.e.m. (*N*=6) and were analysed using two-way ANOVA (*P*<0.05), followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at *P*<0.05. F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction between F and T.

needed to determine the TORC1-independent mechanisms by which rapamycin controls the expression of some genes.

Glucose metabolism

GK, the rate-limiting enzyme of glycolysis in the liver, stimulates glycolysis and lipogenesis by enhancing glucose flux, including production of acetyl-CoA for lipid synthesis (Foufelle and Ferré, 2002). Unlike mammals, in which GK gene expression is mainly controlled by insulin (Iynedjian et al., 1989), expression of GK in rainbow trout is highly and predominantly controlled by dietary carbohydrates and glucose (Panserat et al., 2001a; Seiliez et al., 2011a). The present study provides the first evidence that rapamycin inhibits the post-prandial induction of GK enzyme at molecular and activity levels in trout liver, suggesting that GK and FAS activity may be controlled at a transcriptional level in a TORC1-dependent manner. This is consistent with our previous finding in rainbow trout hepatocytes (Lansard et al., 2010) and investigations in mammals (Düvel et al., 2010; Gosmain et al., 2005). How TORC1 regulates GK is quite complex: SREBP1c gene expression is involved in the regulation of lipid metabolism and also controls the expression of glucose metabolism-related genes, such as glucokinase and hexokinase-2 (HKII) in different tissues and cellular models (Ferré and Foufelle, 2007; Foufelle and Ferré, 2002; Gosmain et al., 2005). Other investigations demonstrate that TORC1 increases glycolysis by activating the transcription and translation of HIF1a (Düvel et al., 2010; Majumder et al., 2004), a positive regulator of many glycolytic genes. Furthermore, Düvel and colleagues (Düvel et al., 2010) revealed that HIF1a is stimulated by TORC1-mediated inhibition of 4E-BP1 and phosphorylation of S6K1. Although phosphorylation of 4E-BP1 and S6K1 as well as SREBP1c and GK gene expression are decreased in the liver of rapamycin re-

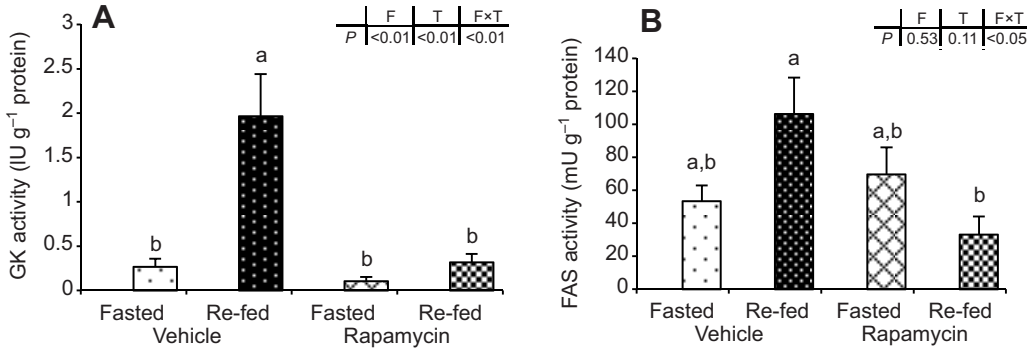


Fig. 6. Enzyme activity of (A) GK and (B) FAS in the liver of rainbow trout subjected to i.p. administration of vehicle or rapamycin, 24 h after re-feeding. Enzyme activity is defined as micromoles of substrate converted to product, per minute, at 37°C and is expressed per gram of liver tissue. Results are means + s.e.m. (N=6) and were analysed using two-way ANOVA (P<0.05), followed by a Tukey test when the interaction was significant. Values with different superscript letters are significantly different at P<0.05. F, fasted or re-fed; T, i.p. administration of vehicle or rapamycin; F×T, interaction between F and T.

fed trout, further investigations are required to determine the molecular mechanisms by which TORC1 regulates GK gene expression.

Despite a significant induction of GK gene expression, expression of 6PF1K and PK is poorly regulated by re-feeding or i.p. rapamycin administration, which is in agreement with a recent study showing that expression of 6PF1K in trout liver, muscle or adipose tissue is not much modified by dietary composition (Kamalam et al., 2012). In mammals, PK gene expression is subject to dietary and hormonal regulation mainly through carbohydrates, insulin and glucagon. Such regulation occurs at the transcriptional level (Hagiwara et al., 2012; Yamada and Noguchi, 1999) and also through post-transcriptional modification of the protein including phosphorylation and dephosphorylation (Assimacopoulos-Jeannet and Jeanrenaud, 1990). In fish, PK activity seems to be mainly controlled by re-feeding through post-translational mechanisms (Panserat et al., 2001b; Skiba-Cassy et al., 2009), explaining the absence of modification of PK gene expression after re-feeding in the present study.

In contrast with our previous observations showing that rapamycin inhibits insulin downregulation of G6Pase expression *in vitro* (Lansard et al., 2010), our present data indicate that rapamycin fails to interfere in the post-prandial downregulation of gluconeogenic gene expression, suggesting a weak contribution of TORC1 in the inhibition of gluconeogenesis in re-fed trout or implicating other endocrine or nutritional factors with counteracting effects. In rats, rapamycin was even able to stimulate G6Pase and PEPCK gene expression (Houde et al., 2010). This discrepancy between trout and rat may be attributed to the length of the rapamycin treatment. Unlike trout that received a single injection of rapamycin and exhibited poor modification of plasma metabolites, rats were submitted to a 2 week chronic rapamycin treatment and presented severe glucose intolerance and insulin resistance associated with impaired β-cell function that could have significantly influenced the expression of G6Pase and PEPCK.

Perspective and significance

The present study demonstrates the functional importance of hepatic TORC1 in the regulation of mRNA levels related to glucose utilization and lipogenesis in rainbow trout. This constitutes the first *in vivo* demonstration of the role of TORC1 in the metabolic response of the liver to nutritional status in fish. Hepatic TORC1 inhibition results in decreased SREBP1c gene expression and suppressed FAS and GK at gene expression and activity levels. Our data support a direct effect of TORC1 on SREBP1c, FAS and GK gene expression inasmuch as fish were subjected to a single injection of rapamycin and did not exhibit drastic changes in post-prandial metabolic parameters. Previous studies have reported that nutritional regulation of the TORC1 signalling pathway and glucose- and lipid-related gene expression are highly influenced by the dietary protein supply in rainbow trout (Seiliez et al., 2011a; Skiba-Cassy et al., 2013). In this carnivorous fish, a strict reduction of dietary protein supply impaired post-prandial activation of the TORC1 pathway and also strongly inhibited the expression of genes related to lipogenesis. Together with these studies, our current findings strongly suggest the TORC1 pathway is crucial for dietary protein to control post-prandial lipogenesis in rainbow trout. Further investigations are needed to confirm the role of TORC1 in the metabolic response of the liver to macronutrients, and identify the mechanisms by which TORC1 regulates SREBP1c expression as well as FAS and GK gene expression and enzyme activity. Understanding how amino acids influence metabolism-related gene expression in various physiological situations or metabolic disorders will be of great influence for limiting the development of metabolic pathologies or optimizing growth and development of livestock animals.

LIST OF ABBREVIATIONS

4E-BP1	4E-binding protein 1
6PF1K	6-phosphofructo-1-kinase
ACLY	ATP citrate lyase
Akt	protein kinase B
CPT1	carnitine palmitoyltransferase 1
EF1α	elongation factor-1 alpha
FAS	fatty acid synthase
FBPase	fructose 1,6-bisphosphatase
FKBP12	12-kDa FK506-binding protein
FoxO1	forkhead-box class O1
G6Pase	glucose 6-phosphatase
G6PDH	glucose 6-phosphate dehydrogenase
GK	glucokinase
GSK 3	glycogen synthase kinase 3α/β
HIF1a	hypoxia inducible factor 1a
HK	hexokinase
HOAD	3-hydroxyacyl-CoA dehydrogenase
i.p.	intraperitoneal
PEPCK	phosphoenolpyruvate carboxykinase
PK	pyruvate kinase
S6	ribosomal protein S6
S6K1	ribosomal protein S6 kinase

SREBP1c	sterol regulatory element binding protein 1c
TOR	target of rapamycin
TORC	target of rapamycin complex

ACKNOWLEDGEMENTS

We thank our colleague B. S. Kamalam for help with manuscript correction. We thank M. J. Borthaire, L. Larroquet, A. Herman, E. Plagnes-Juan, A. Surget and M. Cluzeaud for technical assistance in the laboratory. We also acknowledge the technical staff of the INRA experimental fish farm at Donzacq (Y. Hontang and F. Sandres) for fish rearing.

AUTHOR CONTRIBUTIONS

W.D. performed data acquisition, data analysis and wrote the manuscript. K.D. contributed to western blot and plasma amino acid analysis. F.T. participated in i.p. administration of rapamycin and fish rearing. J.M. supplied RT-PCR technical assistance. S.S.-C. and S.P. developed the study design. S.S.-C., S.P. and I.S. contributed to manuscript correction. S.S.-C. is the guarantor of this study and takes full responsibility for the data, analysis, statistical analysis and manuscript submission.

COMPETING INTERESTS

No competing interests declared.

FUNDING

W.D. gratefully acknowledges the financial assistance provided by the China Scholarship Council (CSC, File No. 2011633111) for his doctoral fellowship.

REFERENCES

Assimacopoulos-Jeannet, F. and Jeanrenaud, B. (1990). Insulin activates 6-phosphofructo-2-kinase and pyruvate kinase in the liver. Indirect evidence for an action via a phosphatase. *J. Biol. Chem.* **265**, 7202-7206.

Bengoechea-Alonso, M. T. and Ericsson, J. (2009). A phosphorylation cascade controls the degradation of active SREBP1. *J. Biol. Chem.* **284**, 5885-5895.

Blum, C. B. (2002). Effects of sirolimus on lipids in renal allograft recipients: an analysis using the Framingham risk model. *Am. J. Transplant.* **2**, 551-559.

Chang, G. R., Wu, Y. Y., Chiu, Y. S., Chen, W. Y., Liao, J. W., Hsu, H. M., Chao, T. H., Hung, S. W. and Mao, F. C. (2009). Long-term administration of rapamycin reduces adiposity, but impairs glucose tolerance in high-fat diet-fed KK/HlJ mice. *Basic Clin. Pharmacol. Toxicol.* **105**, 188-198.

Choo, A. Y., Yoon, S. O., Kim, S. G., Roux, P. P. and Blenis, J. (2008). Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. *Proc. Natl. Acad. Sci. USA* **105**, 17414-17419.

Cornu, M., Albert, V. and Hall, M. N. (2013). mTOR in aging, metabolism, and cancer. *Curr. Opin. Genet. Dev.* **23**, 53-62.

Cunningham, J. T., Rodgers, J. T., Arlow, D. H., Vazquez, F., Mootha, V. K. and Puigserver, P. (2007). mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature* **450**, 736-740.

Deval, C., Chaveroux, C., Maurin, A. C., Cherasse, Y., Parry, L., Carraro, V., Milenkovic, D., Ferrara, M., Bruhat, A., Jousse, C. et al. (2009). Amino acid limitation regulates the expression of genes involved in several specific biological processes through GCN2-dependent and GCN2-independent pathways. *FEBS J.* **276**, 707-718.

Düvel, K., Yecies, J. L., Menon, S., Raman, P., Lipovsky, A. I., Souza, A. L., Triantafellow, E., Ma, Q., Gorski, R., Cleaver, S. et al. (2010). Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol. Cell* **39**, 171-183.

Fang, Y., Westbrook, R., Hill, C., Boparai, R. K., Arum, O., Spong, A., Wang, F., Javors, M. A., Chen, J., Sun, L. Y. et al. (2013). Duration of rapamycin treatment has differential effects on metabolism in mice. *Cell Metab.* **17**, 456-462.

Ferré, P. and Foufelle, F. (2007). SREBP-1c transcription factor and lipid homeostasis: clinical perspective. *Horm. Res.* **68**, 72-82.

Figueiredo-Silva, A. C., Corraze, G., Kaushik, S., Peleteiro, J. B. and Valente, L. M. (2010). Modulation of blackspot seabream (*Pagellus bogaraveo*) intermediary metabolic pathways by dispensable amino acids. *Amino Acids* **39**, 1401-1416.

Foufelle, F. and Ferré, P. (2002). New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. *Biochem. J.* **366**, 377-391.

Gordan, J. D., Thompson, C. B. and Simon, M. C. (2007). HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. *Cancer Cell* **12**, 108-113.

Gosmain, Y., Dif, N., Berbe, V., Loizon, E., Rieusset, J., Vidal, H. and Lefai, E. (2005). Regulation of SREBP-1 expression and transcriptional action on HKII and FAS genes during fasting and refeeding in rat tissues. *J. Lipid Res.* **46**, 697-705.

Hagiwara, A., Cornu, M., Cybulski, N., Polak, P., Betz, C., Trapani, F., Terracciano, L., Heim, M. H., Rüegg, M. A. and Hall, M. N. (2012). Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c. *Cell Metab.* **15**, 725-738.

Horton, J. D., Goldstein, J. L. and Brown, M. S. (2002). SREBPs: transcriptional mediators of lipid homeostasis. *Cold Spring Harb. Symp. Quant. Biol.* **67**, 491-498.

Houde, V. P., Brûlé, S., Festuccia, W. T., Blanchard, P. G., Bellmann, K., Deshaies, Y. and Marette, A. (2010). Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue. *Diabetes* **59**, 1338-1348.

Hresko, R. C. and Mueckler, M. (2005). mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. *J. Biol. Chem.* **280**, 40406-40416.

Iynedjian, P. B., Jotterand, D., Nouspikel, T., Asfari, M. and Pilot, P. R. (1989). Transcriptional induction of glucokinase gene by insulin in cultured liver cells and its repression by the glucagon-cAMP system. *J. Biol. Chem.* **264**, 21824-21829.

Kamalam, B. S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S. and Panserat, S. (2012). Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. *J. Exp. Biol.* **215**, 2567-2578.

Kim, J. and Guan, K. L. (2011). Amino acid signaling in TOR activation. *Annu. Rev. Biochem.* **80**, 1001-1032.

Krycer, J. R., Sharpe, L. J., Luu, W. and Brown, A. J. (2010). The Akt-SREBP nexus: cell signaling meets lipid metabolism. *Trends Endocrinol. Metab.* **21**, 268-276.

Lamming, D. W., Ye, L., Katajisto, P., Goncalves, M. D., Saitoh, M., Stevens, D. M., Davis, J. G., Salmon, A. B., Richardson, A., Ahima, R. S. et al. (2012). Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. *Science* **335**, 1638-1643.

Lansard, M., Panserat, S., Plagnes-Juan, E., Seiliez, I. and Skiba-Cassy, S. (2010). Integration of insulin and amino acid signals that regulate hepatic metabolism-related gene expression in rainbow trout: role of TOR. *Amino Acids* **39**, 801-810.

Lansard, M., Panserat, S., Plagnes-Juan, E., Dias, K., Seiliez, I. and Skiba-Cassy, S. (2011). L-leucine, L-methionine, and L-lysine are involved in the regulation of intermediary metabolism-related gene expression in rainbow trout hepatocytes. *J. Nutr.* **141**, 75-80.

Laplante, M. and Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell* **149**, 274-293.

Li, S., Brown, M. S. and Goldstein, J. L. (2010). Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis. *Proc. Natl. Acad. Sci. USA* **107**, 3441-3446.

Majumder, P. K., Febbo, P. G., Bikoff, R., Berger, R., Xue, Q., McMahon, L. M., Manola, J., Brugarolas, J., McDonnell, T. J., Golub, T. R. et al. (2004). mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. *Nat. Med.* **10**, 594-601.

Mennigen, J. A., Panserat, S., Larquier, M., Plagnes-Juan, E., Medale, F., Seiliez, I. and Skiba-Cassy, S. (2012). Postprandial regulation of hepatic microRNAs predicted to target the insulin pathway in rainbow trout. *PLoS ONE* **7**, e38604.

Moore, S. (1968). Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. *J. Biol. Chem.* **243**, 6281-6283.

Owen, J. L., Zhang, Y., Bae, S. H., Farooqi, M. S., Liang, G., Hammer, R. E., Goldstein, J. L. and Brown, M. S. (2012a). Insulin stimulation of SREBP-1c processing in transgenic rat hepatocytes requires p70 S6-kinase. *Proc. Natl. Acad. Sci. USA* **109**, 16184-16189.

Owen, J. L., Zhang, Y., Bae, S. H., Farooqi, M. S., Liang, G., Hammer, R. E., Goldstein, J. L. and Brown, M. S. (2012b). Insulin stimulation of SREBP-1c processing in transgenic rat hepatocytes requires p70 S6-kinase. *Proc. Natl. Acad. Sci. USA* **109**, 16184-16189.

Panserat, S., Capilla, E., Gutierrez, J., Frappart, P. O., Vachot, C., Plagnes-Juan, E., Aguirre, P., Brèque, J. and Kaushik, S. (2001a). Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (*Oncorhynchus mykiss*) by a single meal with glucose. *Comp. Biochem. Physiol.* **128B**, 275-283.

Panserat, S., Plagnes-Juan, E. and Kaushik, S. (2001b). Nutritional regulation and tissue specificity of gene expression for proteins involved in hepatic glucose metabolism in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **204**, 2351-2360.

Peterson, T. R., Sengupta, S. S., Harris, T. E., Carmack, A. E., Kang, S. A., Balderas, E., Guertin, D. A., Madden, K. L., Carpenter, A. E., Finck, B. N. et al. (2011). mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway. *Cell* **146**, 408-420.

Plagnes-Juan, E., Lansard, M., Seiliez, I., Médale, F., Corraze, G., Kaushik, S., Panserat, S. and Skiba-Cassy, S. (2008). Insulin regulates the expression of several metabolism-related genes in the liver and primary hepatocytes of rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **211**, 2510-2518.

Porstmann, T., Santos, C. R., Griffiths, B., Cully, M., Wu, M., Leevers, S., Griffiths, J. R., Chung, Y. L. and Schulze, A. (2008). SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. *Cell Metab.* **8**, 224-236.

Ryu, M. H., Daily, J. W., 3rd and Cha, Y. S. (2005). Effect of starvation on hepatic acyl-CoA synthetase, carnitine palmitoyltransferase-I, and acetyl-CoA carboxylase mRNA levels in rats. *Nutrition* **21**, 537-542.

Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P. and Sabatini, D. M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol.* **14**, 1296-1302.

Sarbassov, D. D., Guertin, D. A., Ali, S. M. and Sabatini, D. M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098-1101.

Sarbassov, D. D., Ali, S. M., Sengupta, S., Sheen, J. H., Hsu, P. P., Bagley, A. F., Markhard, A. L. and Sabatini, D. M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol. Cell* **22**, 159-168.

Seiliez, I., Gabillard, J. C., Skiba-Cassy, S., Garcia-Serrana, D., Gutiérrez, J., Kaushik, S., Panserat, S. and Tesseraud, S. (2008). An *in vivo* and *in vitro* assessment of TOR signaling cascade in rainbow trout (*Oncorhynchus mykiss*). *Am. J. Physiol.* **295**, R329-R335.

Seiliez, I., Panserat, S., Lansard, M., Polakof, S., Plagnes-Juan, E., Surget, A., Dias, K., Larquier, M., Kaushik, S. and Skiba-Cassy, S. (2011a). Dietary carbohydrate-to-protein ratio affects TOR signaling and metabolism-related gene expression in the liver and muscle of rainbow trout after a single meal. *Am. J. Physiol.* **300**, R733-R743.

Seiliez, I., Sabin, N. and Gabillard, J. C. (2011b). FoxO1 is not a key transcription factor in the regulation of myostatin (mstn-1a and mstn-1b) gene expression in trout myotubes. *Am. J. Physiol.* **301**, R97-R104.

Seiliez, I., Médale, F., Aguirre, P., Larquier, M., Lanneretonne, L., Alami-Durante, H., Panserat, S. and Skiba-Cassy, S. (2013). Postprandial regulation of growth- and metabolism-related factors in zebrafish. *Zebrafish* **10**, 237-248.

Skiba-Cassy, S., Collin, A., Chartrin, P., Médale, F., Simon, J., Duclos, M. J. and Tesseraud, S. (2007). Chicken liver and muscle carnitine palmitoyltransferase 1: nutritional regulation of messengers. *Comp. Biochem. Physiol.* **147**, 278-287.

Skiba-Cassy, S., Lansard, M., Panserat, S. and Médale, F. (2009). Rainbow trout genetically selected for greater muscle fat content display increased activation of liver TOR signaling and lipogenic gene expression. *Am. J. Physiol.* **297**, R1421-R1429.

Skiba-Cassy, S., Panserat, S., Larquier, M., Dias, K., Surget, A., Plagnes-Juan, E., Kaushik, S. and Seiliez, I. (2013). Apparent low ability of liver and muscle to adapt to variation of dietary carbohydrate:protein ratio in rainbow trout (*Oncorhynchus mykiss*). *Br. J. Nutr.* **109**, 1359-1372.

Tocher, D. R. (2003). Metabolism and functions of lipids and fatty acids in teleost fish. *Rev. Fish. Sci.* **11**, 107-184.

Walton, M. J. and Cowey, C. B. (1982). Aspects of intermediary metabolism in salmonid fish. *Comp. Biochem. Physiol.* **73B**, 59-79.

Wan, M., Leavens, K. F., Saleh, D., Easton, R. M., Guertin, D. A., Peterson, T. R., Kaestner, K. H., Sabatini, D. M. and Birnbaum, M. J. (2011). Postprandial hepatic lipid metabolism requires signaling through Akt2 independent of the transcription factors FoxA2, FoxO1, and SREBP1c. *Cell Metab.* **14**, 516-527.

Wang, B. T., Ducker, G. S., Barczak, A. J., Barbeau, R., Erle, D. J. and Shokat, K. M. (2011). The mammalian target of rapamycin regulates cholesterol biosynthetic gene expression and exhibits a rapamycin-resistant transcriptional profile. *Proc. Natl. Acad. Sci. USA* **108**, 15201-15206.

Wilson, R. P. (1994). Utilization of dietary carbohydrate by fish. *Aquaculture* **124**, 67-80.

Wolfrum, C. and Stoffel, M. (2006). Coactivation of Foxa2 through Pgc-1beta promotes liver fatty acid oxidation and triglyceride/VLDL secretion. *Cell Metab.* **3**, 99-110.

Wolfrum, C., Asilmaz, E., Luca, E., Friedman, J. M. and Stoffel, M. (2004). Foxa2 regulates lipid metabolism and ketogenesis in the liver during fasting and in diabetes. *Nature* **432**, 1027-1032.

Wullschleger, S., Loewith, R. and Hall, M. N. (2006). TOR signaling in growth and metabolism. *Cell* **124**, 471-484.

Yamada, K. and Noguchi, T. (1999). Nutrient and hormonal regulation of pyruvate kinase gene expression. *Biochem. J.* **337**, 1-11.

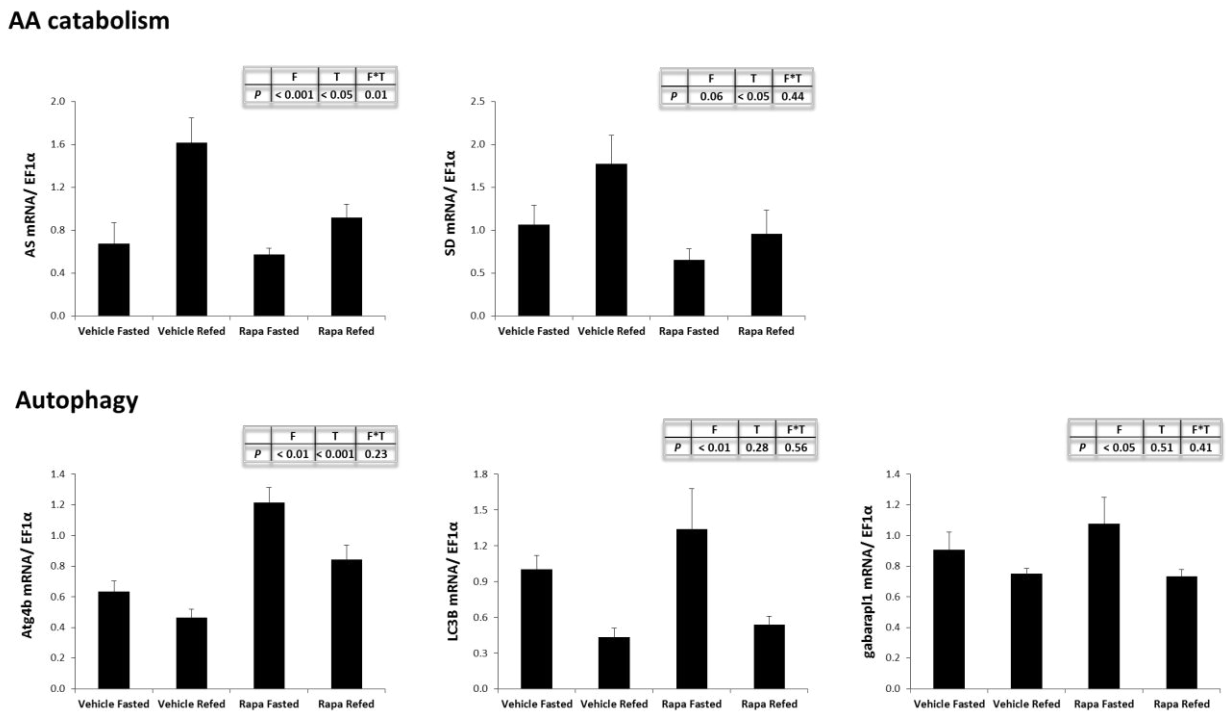
Yang, S. B., Lee, H. Y., Young, D. M., Tien, A. C., Rowson-Baldwin, A., Shu, Y. Y., Jan, Y. N. and Jan, L. Y. (2012). Rapamycin induces glucose intolerance in mice by reducing islet mass, insulin content, and insulin sensitivity. *J. Mol. Med.* **90**, 575-585.

Yecies, J. L., Zhang, H. H., Menon, S., Liu, S., Yecies, D., Lipovsky, A. I., Gorgun, C., Kwiatkowski, D. J., Hotamisligil, G. S., Lee, C. H. et al. (2011). Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. *Cell Metab.* **14**, 21-32.

Supplementary data

As mTOR signaling is involved in a broad range of action, we also examined the expression of genes related to AA catabolism (AS and SD) and autophagy (autophagy-related protein 4B (Atg4b), LC3b and gabarapl1), beside genes related to lipid and glucose metabolism.

Figure S 1 Result highlights from the first experiment – Gene expression of markers of hepatic amino acid catabolism and autophagy



Re-feeding significantly up-regulated the expression of AS without affecting SD mRNA level. mTORC1 inhibition significantly suppressed the expression of AS and SD, implicating that the regulation of amino acid catabolic gene expression maybe mTORC1-dependent.

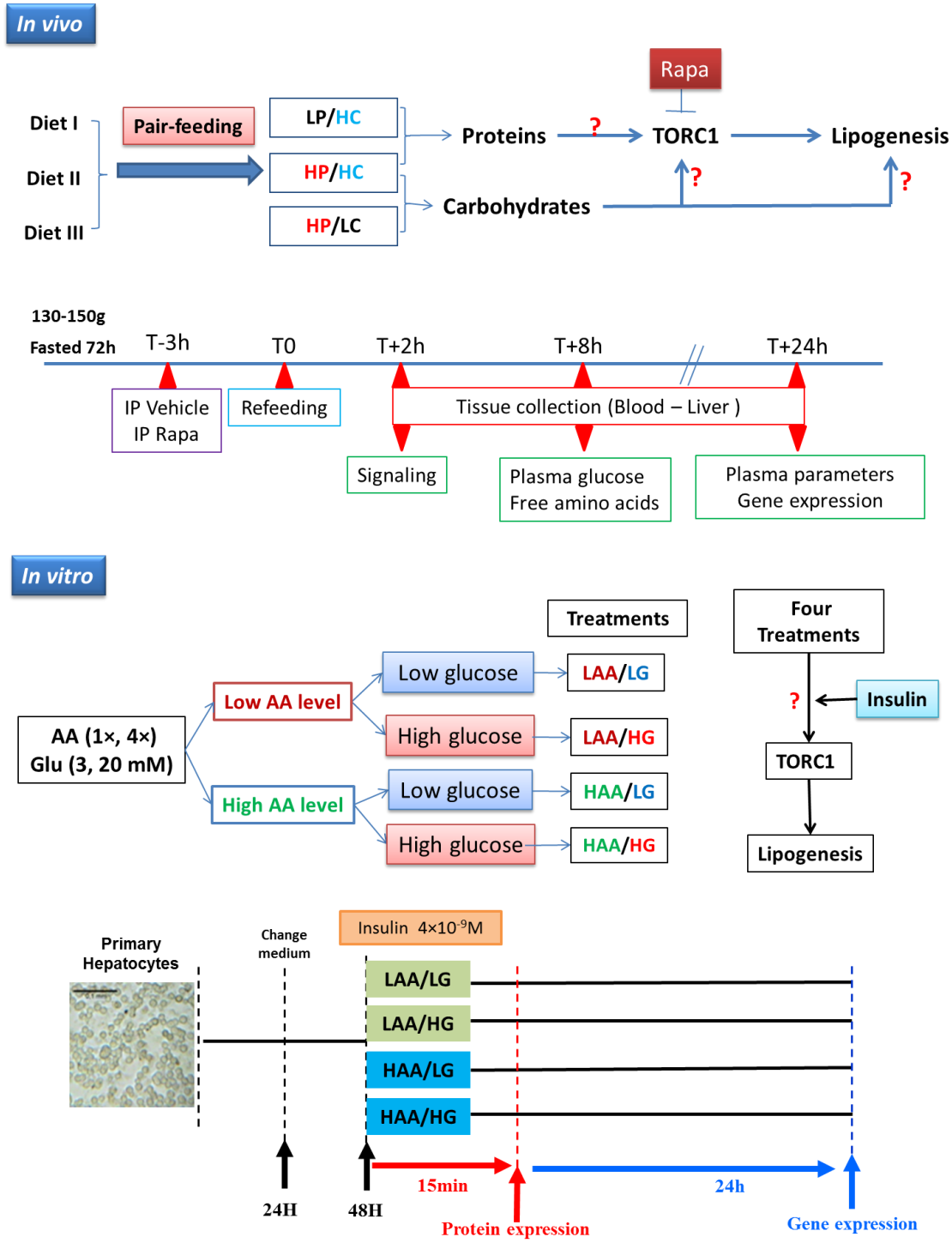
Concerning autophagy-related gene expression, re-feeding significantly down-regulated the expression of Atg4b, LC3b and gabarapl1. Despite the expression of LC3b and gabarapl1 was not affected by rapamycin, Atg4b gene expression was markedly up-regulated, indicating that Atg4b gene expression is negatively regulated by mTORC1.

***All the supplementary data are discussed in "Chapter 4 General discussion".*

PUBLICATION - 2

What are the respective role of protein and carbohydrate in the regulation of lipogenic gene expression, and what is the role of mTOR in these regulations?

Figure 3. 4 Experimental designs and procedures from the second article



Presentation of the article

Objective

De novo lipogenesis (DNL) is the metabolic pathway that synthesizes fatty acids from excess carbon-donors; these fatty acids can then be converted into triglycerides, the major energy storage form in vertebrates. In mammals, hepatic lipogenesis is mainly stimulated by high dietary carbohydrates, whereas suppressed by high dietary protein. The nutritional regulation of DNL seems to be relatively different in carnivorous fish with limited effect of dietary carbohydrates or glucose on the stimulation of lipogenesis and a stimulatory role proposed for dietary protein. Here we evaluated and compared the respective role of dietary carbohydrate/glucose and protein/amino acids on the regulation of lipogenesis in rainbow trout via both *in vivo* and *in vitro* approaches. A controlled-feeding method was employed *in vivo* in order to control the protein and carbohydrate supply to the fish allowing us to compare the metabolic consequences in fish with similar carbohydrate but different protein intakes, and fish with similar protein but different carbohydrate intakes. *In vitro*, primary cell culture of hepatocytes was stimulated with different levels of insulin, amino acids and glucose (Figure 3.4).

Experiment and Analyses

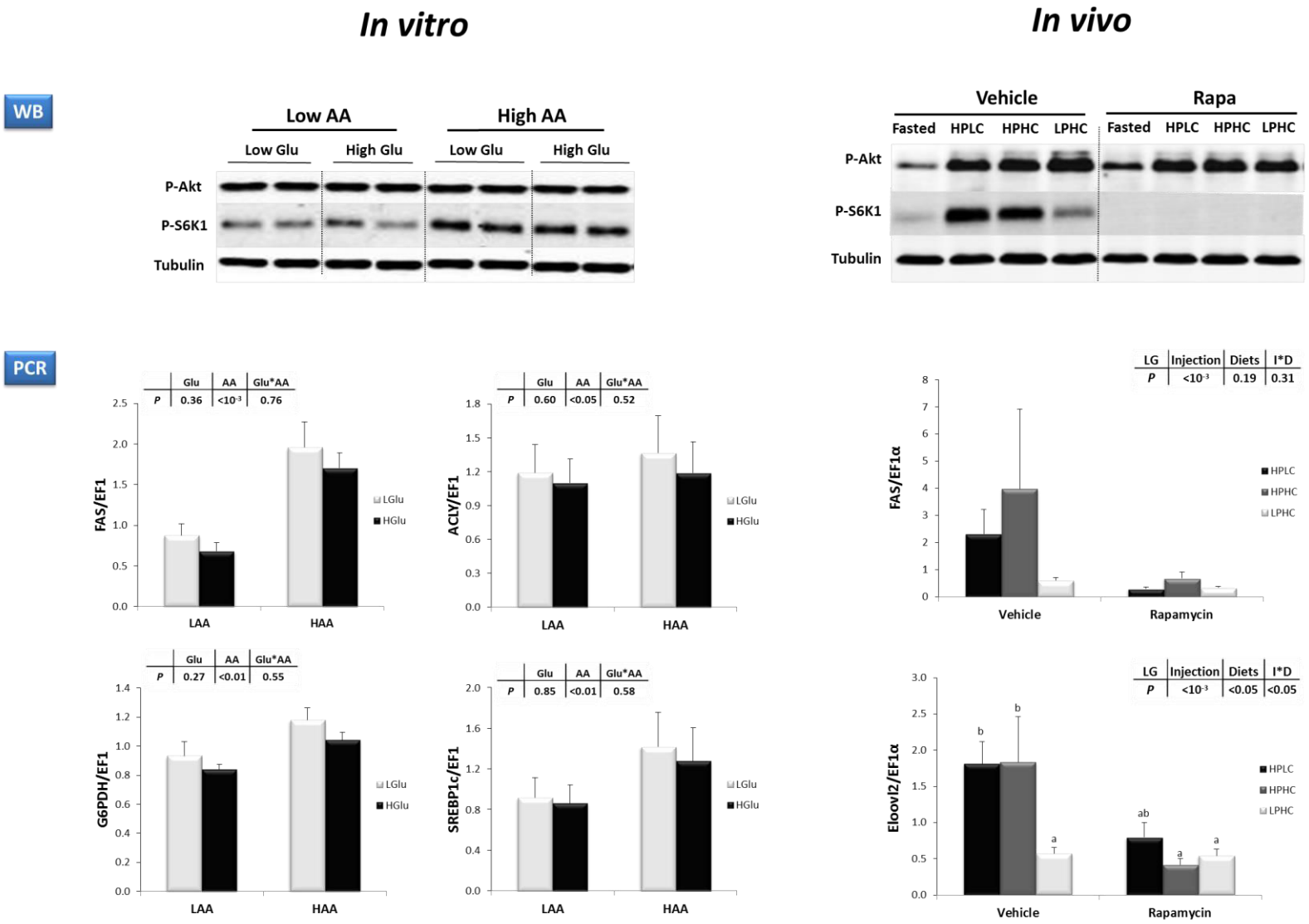
For the *in vivo* trial, trout were treated with a single intraperitoneal (IP) injection of rapamycin (0.5 mg/kg) or vehicle (75% DMSO and 25% saline solution NaCl 0.9%) with 100 µL/100 g body mass. Three hours after the IP administration, three tanks per treatment (vehicle or rapamycin) were assigned with the diets of HPLC, HPHC and LPHC, following the feeding ratios of 0.94%, 1.23% and 0.86% body weight, respectively. Blood and liver samples were randomly collected 2, 8 and 24 h after refeeding. Plasma metabolites, phosphorylation of proteins of the Akt/TOR signaling pathway and expression of genes related to lipogenesis were measured (Figure 3.4, *in vivo*).

For the *in vitro* trial, primary hepatocytes were stimulated with low or high level of glucose (3 mM or 20 mM) and low or high level of AAs (one-fold or four-fold concentrated AAs). Cells were harvested 15 min after stimulation for western blot analysis or resuspended in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) 24 h after stimulation and stored at -80°C for subsequent mRNA extraction. Phosphorylation of proteins on Akt/TOR signaling pathway and expression of genes related to lipogenesis, lipid bioconversion and NADPH production were measured (Figure 3.4, *in vitro*).

Results and Conclusion

In vitro data showed that, irrespective of glucose levels, high level of AAs enhanced S6K1 and S6 phosphorylation and up-regulated the expression of enzymes involved in DNL (FAS and ACLY), lipid bioconversion (Elovl5, Elovl2, D6D and SCD1), NADPH production (G6PDH and ME), and transcriptional factor SREBP1c. In line with these observations, *in vivo* data also showed that high dietary protein intake induced higher mTOR, S6K1, S6 and 4EBP1 phosphorylation and lipogenic

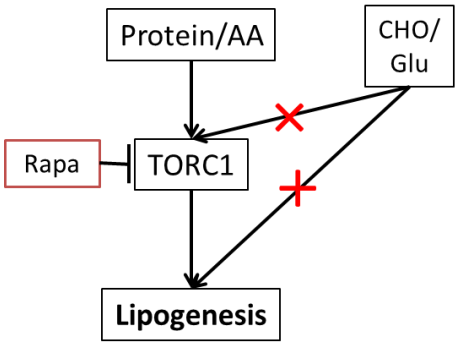
Figure 3. 5 Result highlights from the second article – Representational WB and RT-PCR results in trout liver and hepatocytes



gene expression (Elovl2) regardless of dietary carbohydrate intake, while these enhancements were markedly suppressed by rapamycin (Figure 3.5). Furthermore, despite the absence of statistical significance (probably due to different individual feed intake), the expression of FAS, ACLY and D6D exhibited higher mean mRNA levels in high protein intake groups.

Overall, we demonstrated that, unlike rodents or humans, hepatic lipid biosynthetic gene expression in rainbow trout is more responsive to dietary protein intake/AAs than dietary carbohydrate intake/glucose during acute stimulations. Results confirmed the involvement of the TOR pathway in the regulation of lipogenic gene expression and the stimulatory role of proteins in the regulation of this pathway (Figure 3.6). The poor induction of hepatic lipid biosynthetic gene expression by dietary carbohydrate intake/glucose provides one reasonable explanation for the poor utilization of dietary carbohydrates in rainbow trout, which may be applicable for general carnivores.

Figure 3. 6 Main conclusions from the second article



Hepatic fatty acid biosynthesis is more responsive to protein than carbohydrate in rainbow trout during acute stimulations

Weiwei Dai, Stéphane Panserat, Sadasivam Kaushik, Frédéric Terrier, Elisabeth Plagnes-Juan, Iban Seiliez and Sandrine Skiba-Cassy

INRA, UR 1067 Nutrition Métabolisme, Aquaculture, Pole d'hydrobiologie, CD 918, F-64310 Saint-Pée-sur-Nivelle, France.

ABSTRACT

The link between dietary carbohydrate/protein and *de novo* lipogenesis (DNL) remains debatable in carnivorous fish. We aimed to evaluate and compare the response of hepatic lipogenic gene expression to dietary carbohydrate intake/glucose and dietary protein intake/amino acids (AAs) during acute stimulations using both *in vivo* and *in vitro* approaches. For the *in vivo* trial, three different diets and a controlled-feeding method were employed to supply fixed amount of dietary protein or carbohydrate in a single meal; for the *in vitro* trial, primary hepatocytes were stimulated with low/high level of glucose (3 mM or 20 mM) and low/high level of AAs (one-fold or four-fold concentrated AAs). *In vitro* data showed that high level of AAs up-regulated the expression of enzymes involved in DNL (fatty acid synthase (FAS) and ATP citrate lyase (ACLY)), lipid bioconversion (elongation of very long chain fatty acids like-5 (Elovl5), Elovl2, Δ6 fatty acyl desaturase (D6D) and Stearoyl-CoA desaturase-1 (SCD1)), NADPH production (glucose-6-phosphate dehydrogenase (G6PDH) and malic enzyme (ME)), and transcriptional factor sterol regulatory element binding protein 1-like (SREBP1c), while high level of glucose only elevated the expression of ME. Data in trout liver also showed that high dietary protein intake induced higher lipogenic gene expression (FAS, ACLY and Elovl2) regardless of dietary carbohydrate intake, while high carbohydrate intake markedly suppressed the expression of Acetyl-CoA Carboxylase (ACC) and Elovl5. Overall, we conclude that, unlike rodents or humans, hepatic fatty acid biosynthetic gene expression in rainbow trout is more responsive to dietary protein intake/AAs than dietary carbohydrate intake/glucose during acute stimulations. This discrepancy probably represents one important physiological and metabolic difference between carnivores and omnivores.

Key words: TOR, fatty acid biosynthesis, lipogenesis, protein, carbohydrate, rainbow trout

1 INTRODUCTION

De novo lipogenesis (DNL) is the metabolic pathway that synthesizes fatty acids from excess carbon-donors; these fatty acids can then be converted into triglycerides, the major energy storage form in vertebrates (43, 83, 85). In mammals, hepatic lipogenesis is very responsive to dietary modifications (40). Consumption of a diet rich in carbohydrates stimulates the lipogenic pathway, whereas consumption of a diet rich in lipids and poor in carbohydrates, or rich in polyunsaturated fatty acids decreases this metabolic pathway (40, 86). High carbohydrate diet can induce hyperglycemia, thereby stimulating lipogenesis via several mechanisms (40). First, by being glycolytically converted to acetyl-CoA, glucose provides substrate for fatty acid synthesis. Secondly, glucose stimulates glycolytic and lipogenic gene expression (24). Finally, glucose stimulates the release of insulin from the pancreas, thereby activating insulin/Akt signaling pathway (40, 69), which stimulates hepatic lipogenesis via the transcription factor sterol regulatory element binding protein-1c (SREBP-1c) (32). Regarding dietary protein, rodent data showed that the metabolic adaptation to a high protein diet included a down-regulation of lipogenesis at both gene expression and enzymatic levels (64). Notably, the activity of lipogenic pathway also depends on the availability of cofactors such as NADPH produced by the pentose phosphate pathway (27), and DNL may serve as a key regulator in tandem with elongation and desaturation (10, 13, 36).

In fish, whereas numerous long-term feeding studies have shown that carbohydrate rich diets elevated the expression/activities of lipogenic enzymes and/or NADPH yielding as in mammals (4, 18, 20, 26, 38, 45, 78, 79), studies in short-term, particularly in carnivorous species, clearly unveiled a different landscape. Recent *in vivo* studies have demonstrated that high carbohydrate diets/ glucose failed or only had very limited effects in stimulating lipogenic gene expression/enzyme activities (30, 35, 75). For instance, Seiliez *et al.* (75) reported that a diet rich in carbohydrate and low in protein failed to elevated the expression of gene related to lipogenesis (FAS, ACLY, G6PDH and SREBP1) compared to fasted control. Jin *et al.* (35) also showed that intraperitoneal (IP) glucose

administration only induced minor changes for the gene expression of ATP citrate lyase (ACLY), without affecting the expression of other lipogenic genes (FAS and ACLY) or the transcription factor SREBP1. Following radiolabelled $^{14}C_1$-glucose course, Hermre and Kars (30) clearly showed that hepatic lipogenesis converted only a very small proportion of injected glucose into lipids in Atlantic cod. By using metformin, a common anti-diabetic drug, Panserat et al. (55) observed that metformin effectively reduced postprandial hyperglycemia in trout fed high dietary carbohydrates, possibly through stimulating hepatic lipogenic mRNA levels and activities, indicating the poor biotransformation of glucose to fatty acids in trout. In contrast, enhanced lipogenic gene expression and/or enzymatic activities was repeatedly observed in rainbow trout and blackspot seabream when fed diets rich in protein and low in carbohydrate (23, 75), implying that high protein diets appear to play a predominant role in stimulating lipogenic pathway in carnivorous fish. It is also worthy to note that most of these studies investigating high carbohydrate effects were associated with the expense of dietary protein/lipids modifications (18, 38, 45, 75, 84, 91), hence it is difficult to distinguish which nutrient induced those metabolic changes. Moreover, some studies concluded that high carbohydrate diets/D-glucose enhanced hepatic lipogenesis mainly based on the results of NADPH yield, namely the gene expression and/or enzyme activities of G6PDH, 6PGD or ME (20, 26, 84), instead of *de novo* lipogenic gene expression or enzymatic activities. Therefore, the link between dietary carbohydrate/protein and DNL remains debatable in carnivorous fish.

The aim of the current studies was to evaluate and compare the response of hepatic lipogenic gene expression to dietary carbohydrate intake and dietary protein intake *in vivo*, and also compare the response to glucose and amino acids (AAs) *in vitro* during short-term stimulations. Rainbow trout, a relevant carnivorous fish model, which possesses relatively high dietary protein/AAs requirements, poor utilization of dietary carbohydrates and "glucose intolerant" phenotype (16), was used as current research model. To prevent the other nutrient intake changes (except dietary carbohydrate or protein intake), we employed a controlled-feeding method for *in vivo* studies. As our recent work demonstrated that hepatic lipogenesis requires the activation of mechanistic target of rapamycin complex 1 (mTORC1) in rainbow trout (14, 15, 42) as in mammals (9, 41, 63), and elevated amino acid levels enhanced hepatic fatty acid biosynthetic gene expression through an mTORC1-dependent manner (15), we also investigated the potential involvement of mTORC1 signaling pathway in the present study. Acute administration of rapamycin, a pharmacological inhibitor of mTOR, was employed to achieve an inhibition of mTORC1 and its downstream effectors, including p70 ribosomal S6 kinase 1 (S6K1), S6 and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (14-16, 42, 74).

2 MATERIAL AND METHODS

2.1 Ethics statement

The experiments were carried out in accordance with legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e. Directive 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decret no. 2001-464, May 29th, 2001). The investigators carrying out the experiment had ''level 1'' or ''level 2'' certification, bestowed by the Direction Départementale des Services Vétérinaires (French veterinary services) to carry out animal experiments (INRA 2002-36, April 14th, 2002).

2.2 Experimental diets and feeding method

Diets were manufactured at the INRA facility of Donzacq (France) using a twinscrew extruder (Clextral, France). Three diets, high protein low carbohydrate (HPLC), high protein high carbohydrate (HPHC) and low protein high carbohydrate (LPHC), were formulated to contain different levels of dietary protein and carbohydrate as shown in Table 1. A controlled-feeding method, in which both diet compositions and feeding levels are strictly controlled for each treatment/tank, was employed to supply the fixed amount of dietary protein or carbohydrate intake as designed while the other nutrient intakes were almost equivalent (Table 1).

2.3 Experimental and sampling procedure

Juvenile rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were reared in the INRA experimental facilities at Donzacq (Landes, France) at a constant water temperature of 17.5±0.5°C, under natural photoperiod. Fish were fed a commercial trout diet (T-3P classic, Skretting, Fontaine-les-Vervins, France) during the acclimatization period. Fish (mean body mass 138.5±15 g) were randomly distributed into eight tanks (24 fish per 130 L tank). Prior to the feeding trials, fish were feed deprived for 72 h—the time

required to ensure the complete emptying of the digestive tract. After this period, trout were sedated with benzocaine (10mg/L) and treated with a single intraperitoneal (IP) injection of rapamycin (0.5 mg/kg) or vehicle (75% DMSO and 25% saline solution NaCl 0.9%) with 100 µL/100 g body mass. 3 h after the IP injection, three tanks per treatment (vehicle or rapamycin) were assigned with the diets, HPLC, HPHC and LPHC, following the feeding ratios of 0.94%, 1.23% and 0.86% body weight, respectively. Eight fish per tank were randomly sampled at 2, 8 and 24 h after the single meal. Trout were anaesthetized with benzocaine (30mg/L) and killed by a sharp blow to the head. Blood was removed from the caudal vein into heparinized syringes and centrifuged (3000*g*, 5 min); the recovered plasma was immediately frozen and kept at –20°C. The stomach content of each fish was checked to confirm that the fish had effectively ingested the diet. Livers were dissected and immediately frozen in liquid nitrogen and kept at –80°C.

Table 1 Diet composition and nutrients intake

Ingredients (%)	Diets		
	HPLC	HPHC	LPHC
Fish meal[a]	79.1	54.0	35.7
Fish oil[b]	8.7	5.9	11.3
Dextrin[c]	11.1	38.5	51.0
Vitamin premix[d]	1.1	0.8	1.0
Mineral premix[e]	1.1	0.8	1.0
Proximate analysis			
Crude protein (%DM)	57.5	40.1	26.2
Crude fat (%DM)	19.8	14.3	16.3
NFE (%DM)	10.6	36.1	48.2
Dry matter (%)	91.2	91.3	91.3
Feeding level (BW)	0.86%	1.23%	0.94%
Protein intake (g/10 kg BW)	**45.1**	**45.0**	**22.4**
NFE intake (g/10 kg BW)	**8.3**	**40.5**	**41.4**
Lipids intake (g/10 kg BW)	15.5	16.1	14.0

HPLC, high protein low carbohydrate; HPHC, high protein high carbohydrate; LPHC, low protein high carbohydrate; DM, dry matter; NFE, nitrogen-free extract/carbohydrate; BW, body weight.

[a]Fish meal (Sopropeche, Boulogne-sur-Mer, France).

[b]Fish oil (North sea fish oil; Sopropeche, Boulogne-sur-Mer, France).

[c]Dextrin (Lestrem 62, Roquette, France).

[d]Vitamin premix: 60 IU DL-a tocopherol acetate, 5 mg kg^{-1} diet sodium menadione bisulphate, 15,000 IU retinyl acetate, 3000 IU DL-cholecalciferol, 15 mg kg^{-1} diet thiamin, 30 mg kg^{-1} diet riboflavin, 15 mg kg^{-1} diet pyridoxine, 0.05 mg kg^{-1} diet B12, 175 mg kg^{-1} diet nicotinic acid, 500 mg kg^{-1} diet folic acid, 1000 mg kg^{-1} diet inositol, 2.5 mg kg^{-1} diet biotin, 50 mg kg^{-1} diet calcium panthotenate, 2000 mg kg^{-1} diet choline chloride (UPAE, Jouy, Inra, France).

[e]Mineral premix: 2.15 g calcium carbonate (40% Ca), 1.24 g magnesium oxide (60% Mg), 0.2 g ferric citrate, 0.4 mg kg^{-1} diet potassium iodide (75% I), 0.4 g zinc sulphate (36% Zn), 0.3 g copper sulphate (25% Cu), 0.3 g manganese sulphate (33% Mib), 5 g dibasic calcium phosphate (20% Ca, 18% P), 2 mg kg^{-1} diet cobalt sulphate, 3 mg kg^{-1} diet sodium selenite (30% Se), 0.9 g KCl, 0.4 g NaCl (UPAE, Jouy, INRA, France).

2.4 Primary culture of hepatocytes

For cell culture, trout (240 to 300g) were obtained from the INRA experimental fish farm facilities (Donzacq, France), then maintained in tanks kept in a flow-through system at 18°C with well-aerated water under natural photoperiod conditions. Fish were fed to satiety every two days with a commercial diet (T-3P classic, Trouw, France). Prior to the liver cells isolation, trout were left unfed for 72 h in order to empty the digestive tract and then facilitate the liver in situ perfusion. At the time of experiments, fish were anaesthetized by placing them in water containing 60 mg/L aminobenzoic acid, and hepatocytes were isolated from 3 different individuals by in situ perfusion method described by Mommsen et al (50). Livers were excised and minced with a razor blade in modified Hanks' medium (136.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 5 mM $NaHCO_3$ and 10 mM HEPES) supplemented with 1mM EGTA. After filtration and centrifugation (120 g, 2 minutes), the resulting cell pellet was resuspended three successive times in modified Hanks' medium (1.5 mM $CaCl_2$ and 1.5% defatted bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA)). Cells were finally taken up in modified Hanks' medium supplemented with 1.5 mM $CaCl_2$, 1% defatted BSA, 3 mM glucose, MEM essential amino acids (1X) (Invitrogen Carlsbad, California, USA), MEM non-essential amino acids (1X) (Invitrogen Carlsbad, California, USA) and antibiotic antimycotic solution (1X) (Sigma, St. Louis, MO, USA) as the basic culture medium. Cell viability (>98%) was assessed using the trypan blue exclusion method (0.04% in 0.15 M NaCl) and cells were counted using a haemocytometer. The hepatocyte cell suspension was plated in a six well Primaria culture dish (BD, USA) at a density of 3×10^6 cells/ well and incubated at 18°C. The culture medium was changed every 24 h over the 48 h of primary cell culture. Microscopic examination ensured that hepatocytes progressively re-associated throughout culture to form two-dimensional aggregates, in agreement with earlier reports (22, 73). For the first experiment involving different glucose and amino acid levels, 48 h-cultured cells were stimulated with 4×10^{-9} mol l^{-1} of bovine insulin (Sigma, St. Louis, MO, USA) corresponding to the post-prandial level of insulin (17, 51), low/high level of glucose (3 mM/Low Glu or 20 mM/High Glu) (57, 75) and low/high level of amino acids (one-fold (Low AA) or four-fold (High AA) concentrated amino acids). In the second *in vitro*

experiment, 48 h-cultured hepatocytes were stimulated with glucose (3 mM), low/high level of insulin (1×10^{-9} mol l^{-1}/Low Insulin or 4×10^{-9} mol l^{-1}/High Insulin; corresponding to the two weeks fasted or post-prandial insulin levels (51, 59)) and low/high level of amino acids (Low AA or High AA). Cells were harvested at 15 min for western blot analysis or re-suspended in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) 24 h after stimulation and stored at -80°C for subsequent mRNA extraction. The time intervals were chosen based on the phosphorylation peaks of Akt/TOR signaling pathway or relevant metabolic gene expression peaks, respectively (data not shown).

2.5 Plasma metabolites analysis

Plasma glucose (Glucose RTU, BioMérieux, Marcy l'Etoile, France) levels were determined using commercial kits adapted to a microplate format, according to the recommendations of the manufacturer. Total plasma free amino acid levels were determined by the ninhydrin reaction (52), with glycine as standard.

2.6 Protein extraction and Western blotting

Frozen livers (200 mg) from 2 h refed trout were homogenized on ice with an ULTRA-TURRAX® homogenizer (IKA®-WERKE, Germany) in 2 mL of lysis buffer (150 mM NaCl, 10 mM Tris , 1 mM EGTA, 1 mM EDTA (pH 7.4), 100 mM sodium fluoride, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1% Triton X-100, 0.5% NP-40-IGEPAL and a protease inhibitor cocktail (Roche, Basel, Switzerland)). Homogenates were centrifuged at 1500*g* for 15 min at 4°C and supernatant fractions were then centrifuged at 20,000*g* at 4°C for 30 min. The resulting supernatant fractions were recovered and stored at -80°C. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with BSA as standard. Lysates (10 µg of total protein for TOR/S6/4E-BP1 and 20 µg for S6K1) were subjected to SDS-PAGE.

For hepatocyte culture, cells were carefully washed with two times 1ml of cold phosphate buffered saline (PBS). Then 300µl of cell lysis buffer were added before being stored at -80°C overnight. Then cells were scraped, collected, and lysed on ice for 30 min. Lysates were centrifuged at 12.000g for 30 min at 4°C. The resulting supernatant fractions were recovered and stored at -80°C. Protein concentrations were measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with BSA as standard. Lysates (2 µg of total protein per lane for S6, 7 µg for S6K1, respectively) were subjected to SDS-PAGE.

All antibodies used were obtained from Cell Signaling Technologies (Ozyme, Saint Quentin Yvelines, France). Anti-phospho-TOR (Ser^{2448}) (no. 2971), anti-TOR (no. 2972), antiphospho-S6 protein kinase 1 (Thr^{389}) (no. 9205), anti-S6 protein kinase 1 (no. 9202), anti-phospho-S6 ($Ser^{235/236}$) (no. 4856), anti-S6 (no. 2217), anti-phospho-4E-BP1 ($Thr^{37/46}$) (no. 9459), anti-4E-BP1 (no. 9452) and anti-β-Tubulin (no. 2146) were purchased from Cell Signaling Technologies (Ozyme, Saint Quentin Yvelines, France). We have confirmed that all of these antibodies successfully cross-reacted with rainbow trout (14). After washing, membranes were incubated with an IRDye Infrared secondary antibody (LI-COR Biosciences, Lincoln, NE, USA). Bands were visualized by Infrared Fluorescence using the Odyssey Imaging System (LI-COR Biosciences) and quantified by Odyssey Infrared imaging system software (Version 3.0, LI-COR Biosciences).

2.7 Gene expression analysis

Total RNA samples were extracted from hepatocytes using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations, quantified by spectrophotometry (absorbance at 260 nm) and its integrity was assessed using Agarose Gel Electrophoresis. A 1 µg sample of the resulting total RNA was reverse transcribed into cDNA using the SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and random primers (Promega, Charbonniéres, France) according to the manufacturers' instructions. Target gene expression abundance was determined by quantitative real-time (q)RT-PCR, using specific primers (35, 39, 81). Primers targeting malic enzyme (forward: TACGTGCGGTGTGTGTGACG; reverse: GTGCCCACATCCAGCATGAC), were newly designed using Primer3 software. To confirm specificity, amplicons were purified and sequenced (Beckman Coulter Genomics, UK).

qRT-PCR was carried out on a LightCycler® 480 II (Roche Diagnostics, Neuilly sur Seine, France) using LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany). Quantitative RT-PCR analyses focused on several key enzymes of hepatic metabolism: ATP citrate lyase (ACLY; EC 2.3.3.8), Acetyl-CoA Carboxylase (ACC; EC 6.4.1.2) and fatty acid synthase (FAS; EC 2.3.1.85) for lipogenesis; glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) and malic enzyme (ME, EC 1.1.1.40) for pentose phosphate shunt; Δ6 fatty acyl desaturase (D6D; EC 1.14.19.3), Stearoyl-CoA desaturase-1 (SCD1 or D9D; EC 1.14.19.1), elongation of very long chain fatty acids like-5 (Elovl5; EC 2.3.1.199) and elongation of very long chain fatty acids like-2 (Elovl2; EC 2.3.1.199) for fatty acid bioconversion, and a relevant transcription factor sterol regulatory element binding protein 1-like (SREBP1c). Elongation factor-1alpha (EF1α) was employed as a non-regulated reference gene, as previously used in rainbow trout and it was

stably expressed in our investigations (data not shown).

PCR was performed using 2 µL of the diluted cDNA (76 times diluted) mixed with 0.24 µL of each primer (10 µM), 3 µL LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and 0.52 µL DNase/RNase/Protease-free water (5 prime GmbH, Hamburg, Germany) in a total volume of 6 µL. The PCR protocol was initiated at 95°C for 10 min for initial denaturation of the cDNA and hot-start Taq-polymerase activation, followed by 45 cycles of a three-step amplification program (15 s at 95°C, 10 s at melting temperature Tm (59-65°C), 4.8 s at 72°C), according to the primer set used. Melting curves were systematically monitored (5 s at 95°C, 1 min at 65°C, temperature slope at 0.11°C/s from 65 to 97°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included quadruplicates for each sample (duplicates of reverse transcription and PCR amplification, respectively) and also negative controls (reverse transcriptase and RNA free samples). Relative quantification of target gene expression was determined using the E-Method by the software of LightCycler® 480 (Version SW 1.5, Roche Diagnostics). PCR efficiency, assessed by the slope of a standard curve using serial dilutions of cDNA, ranged between 1.85 and 2.

2.8 Statistical analysis

The results of post-prandial plasma metabolites are expressed as means ± SEM (N=8). The results of western blot and gene expression are expressed as means + SEM (N=6). The effects of diets, IP administration of rapamycin or vehicle and interaction were analyzed using two-way ANOVA in SPSS 17.0. In cases where data were nonparametric or not homoscedastic, data transformations, such as logarithms, square roots and reciprocals, were used to meet ANOVA criteria. Normality was assessed using the Shaprio–Wilk test, while homoscedasticity was determined using Levene's test. For all statistical analyses, the level of significance was set at P<0.05.

For cell culture experiments, the results of western blot (N=6) and gene expression (N=9) are expressed as means + s.e.m. and were analyzed using linear mixed-effects models (MIXED) procedure with fish ID as subject effect (SPSS 17.0). When interaction was significant, results were further analyzed using the EMMEANS subcommand. For all statistical analyses, the level of significance was set at P<0.05.

3 RESULTS

3.1 High carbohydrate intake induced hyperglycemia irrespective of the protein intake

To check the effect of controlled-feeding, we investigated post-prandial plasma glucose and total plasma free amino acids (FAA) levels (Fig. 1). Compared to low carbohydrate intake treatment (HPLC), high carbohydrate intake treatments (HPHC and LPHC) induced higher plasma glucose levels 8 and 24 h after controlled-feeding irrespective of the administration of vehicle or rapamycin, and decreased FAA levels 24 h after controlled-feeding. Protein and carbohydrate intake also affected FAA levels 8 h after controlled-feeding (P<0.05), with HPLC treatment inducing higher FAA levels than LPHC treatment. Notably, rapamycin significantly decreased plasma glucose levels but elevated FAA levels 8 h after controlled-feeding compared to vehicle-treated groups.

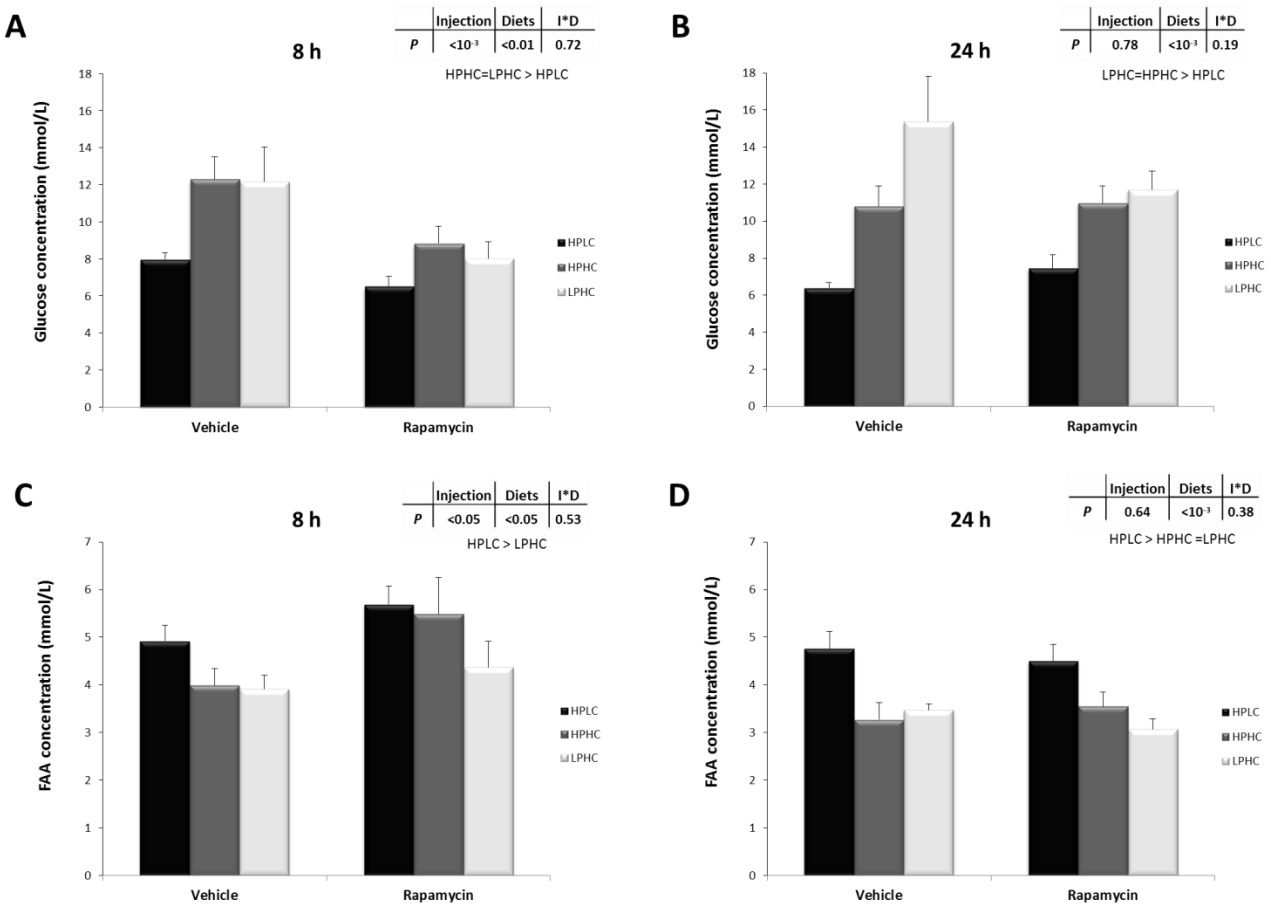


Fig. 1 Post-prandial plasma glucose (A and B) and total free amino acids (FAA, C and D) levels in rainbow trout subjected to intraperitoneal (IP) administration of vehicle or rapamycin, 8 and 24 h after controlled-feeding. Data are means + s.e.m. (*N*=8). The effects of IP administration of vehicle or rapamycin (Injection), diets and interaction (I*D) were analyzed using two-way ANOVA ($P<0.05$) followed by a Tukey multiple comparison test. HPLC, high protein low carbohydrate; HPHC, high protein high carbohydrate; LPHC, low protein high carbohydrate.

3.2 High protein intake induced enhanced mTORC1 activation

We further investigated the effects of administration of vehicle or rapamycin and different nutrients intake on the regulation of mTORC1 signaling pathway in trout liver (Fig. 2). Protein phosphorylation of TOR, S6K1, S6 and 4EBP1 was markedly inhibited by rapamycin irrespective of the nutrients intake. High protein intake (HPLC and HPHC) induced higher phosphorylation of TOR and 4EBP1 compared to low protein intake (LPHC), while no statistical difference was observed for the phosphorylation of S6K1 and S6.

3.3 Rapamycin inhibited the expression of hepatic fatty acid biosynthetic genes

We further measured the expression of selected enzymes involved in fatty acid biosynthesis in the liver (Fig. 3). Compared to vehicle-treated groups, rapamycin significantly repressed the mRNA levels of FAS, ACLY, ACC, Elovl2, Elovl5, D6D and G6PDH without affecting ME or SREBP1c. Nutrient intake significantly affected the expression of ACC and Elovl5 (Fig. 3C and 3E; $P<0.05$), with high protein/low carbohydrate intake groups (HPLC) obtaining higher ACC gene expression compared to high protein/high carbohydrate intake groups (HPHC), and high carbohydrate intake (HPHC and LPHC) suppressing the expression of Elovl5 compared to low carbohydrate intake groups (HPLC). An interaction between injection and diets was recorded for Elovl2 (Fig. 3D; $P<0.05$), showing that high protein intake (HPLC and HPHC) induced higher Elovl2 gene expression compared with low protein intake (LPHC) in vehicle-treated groups while no modifications for rapamycin-treated

groups. The mRNA levels of FAS, ACLY, D6D, G6PDH, ME or SREBP1c were not affected by protein or carbohydrate intake.

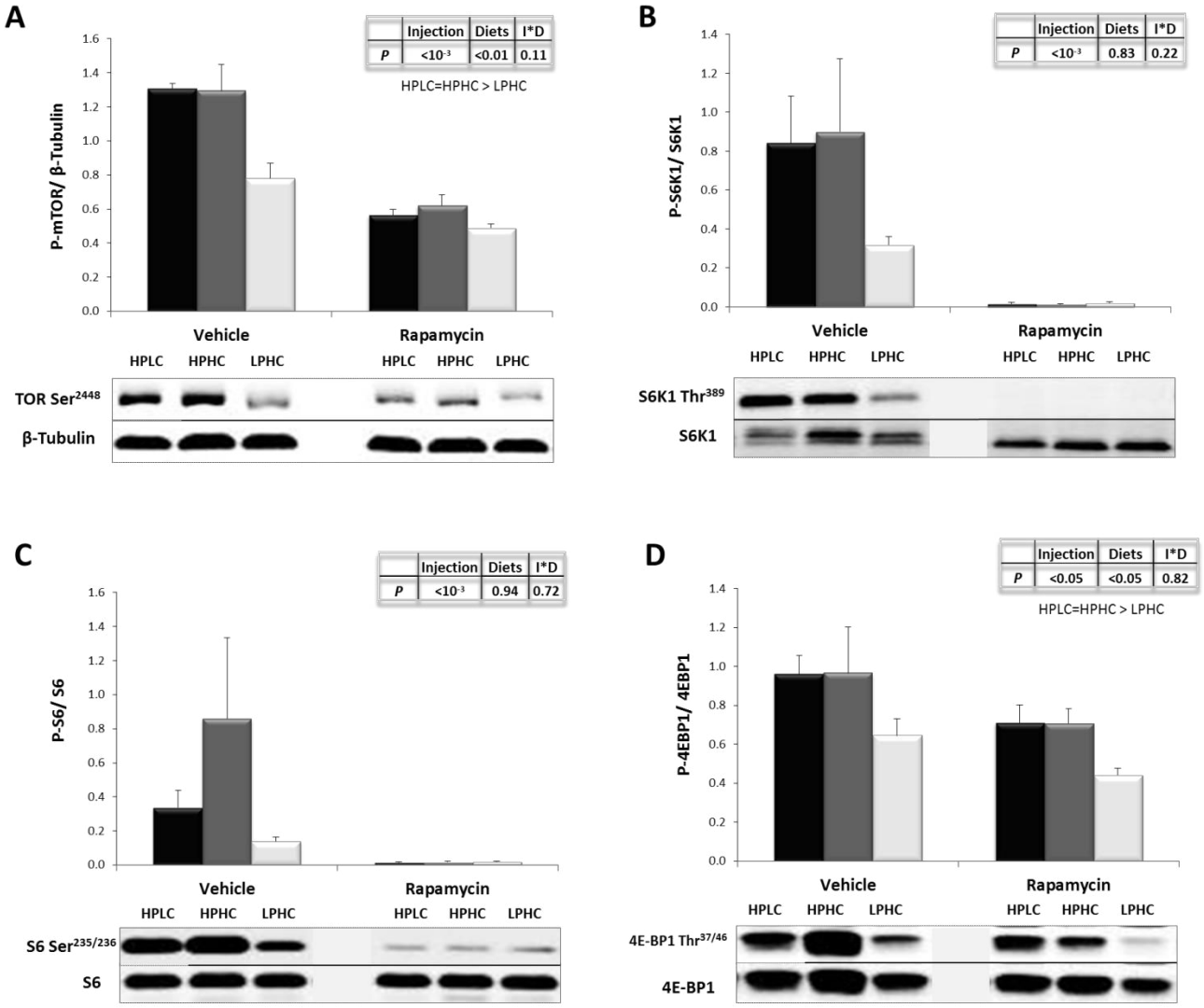


Fig. 2 Western blot analysis of hepatic (A) TOR, (B) S6K1, (C) S6 and (D) 4E-BP1 protein phosphorylation in rainbow trout subjected to IP administration of vehicle or rapamycin, 2 h after controlled-feeding. Gels were loaded with 10 µg of total protein per lane for S6/4E-BP1 and 20 µg for TOR/S6K1, respectively. A representative blot is shown. Results are means + s.e.m. (N=6) and were analyzed using two-way ANOVA (P<0.05) followed by a Tukey multiple comparison test. HPLC, high protein low carbohydrate; HPHC, high protein high carbohydrate; LPHC, low protein high carbohydrate. Injection=IP administration of vehicle or rapapamycin; Diets=HPLC, HPHC or LPHC; I*D=interaction between Injection and Diets.

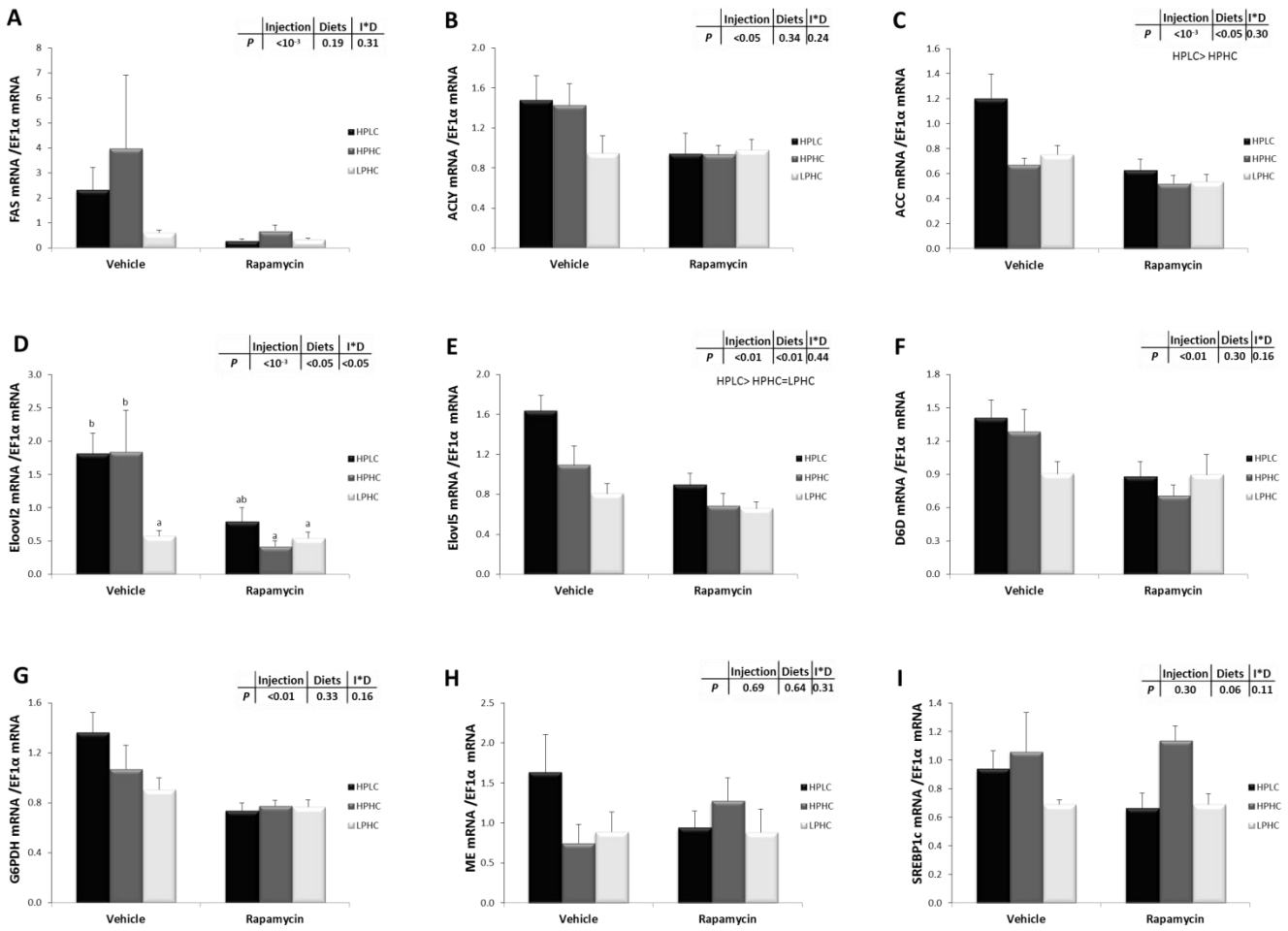


Fig. 3 Gene expression of selected enzymes involved in fatty acid biosynthesis in the liver of rainbow trout subjected to IP administration of vehicle or rapamycin, 24 h after controlled-feeding. mRNA levels of genes involved in de novo lipogenesis including (A) FAS, (B) ACLY and (C) ACC, fatty acid bioconversion including (D) Elovl2, (E) Elovl5 and (F) D6D, NADPH production including (G) G6PDH and (H) ME and transcriptional factor (I) SREBP1c were measured using real-time RT-PCR. Expression values are normalized with elongation factor-1 alpha (EF1α)-expressed transcripts. Results are means + s.e.m. (N=6) and were analyzed using two-way ANOVA ($P<0.05$) followed by a Tukey multiple comparison test. HPLC, high protein low carbohydrate; HPHC, high protein high carbohydrate; LPHC, low protein high carbohydrate. Injection=IP administration of vehicle or rapapamycin; Diets=HPLC, HPHC or LPHC; I*D=interaction between Injection and Diets.

3.4 High level of AAs enhanced mTORC1 activation irrespective of glucose levels

To overcome the complexity *in vivo*, especially the metabolite exchange between organs and hormonal effects, we used cell culture approach to investigate the effects of different glucose/amino acid levels on the regulation of mTORC1 signaling pathway in trout hepatocytes (Fig. 4). The phosphorylation of S6K1 and S6 was markedly enhanced by elevated AA levels while no glucose effect or interaction was observed.

3.5 High level of AAs predominantly up-regulated fatty acid biosynthetic gene expression

We further investigated the effects of different glucose/AA levels on the regulation of fatty acid biosynthetic gene expression in trout hepatocytes (Fig. 5). We observed that mRNA levels of the key enzymes involved in *de novo* lipogenesis (FAS and ACLY), fatty acid bioconversion (Elovl5, Elovl2, D6D and SCD1), NADPH production (G6PDH and ME), and transcriptional factor SREBP1c were significantly up-regulated by high AA levels, while glucose only increased the expression of ME. The expression of ACC in trout hepatocytes was too low to be measured.

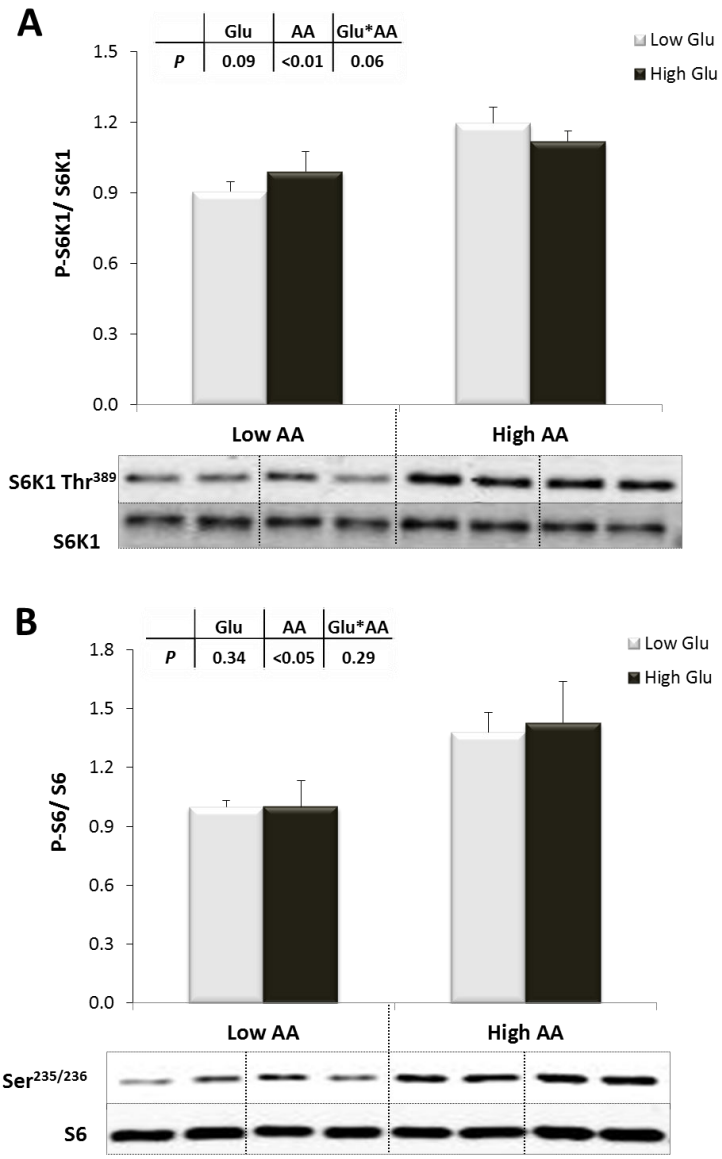


Fig. 4 Effects of different glucose (Glu) and amino acid (AA) levels on the phosphorylation of (A) S6K1 and (B) S6 protein in trout hepatocytes. The experiment was conducted in 48 h-cultured trout hepatocytes. Cells were then stimulated for 15 min with low/high level of glucose (3 mM/Low Glu or 20 mM/High Glu) and low/high level of amino acids (one-fold (Low AA) or four-fold (High AA) concentrated amino acids). Gels were loaded with 2 µg of total protein per lane for S6, 7 µg for S6K1, respectively. A representative blot is shown. Graphs represent the ratio between the phosphorylated protein and the total amount of the target protein. Results are means + s.e.m. (N=6) and were analyzed using linear mixed-effects models (MIXED) procedure (P<0.05). Glu=Low or High glucose; AA=Low or High level of amino acids; Glu*AA=interaction between Glu and AA.

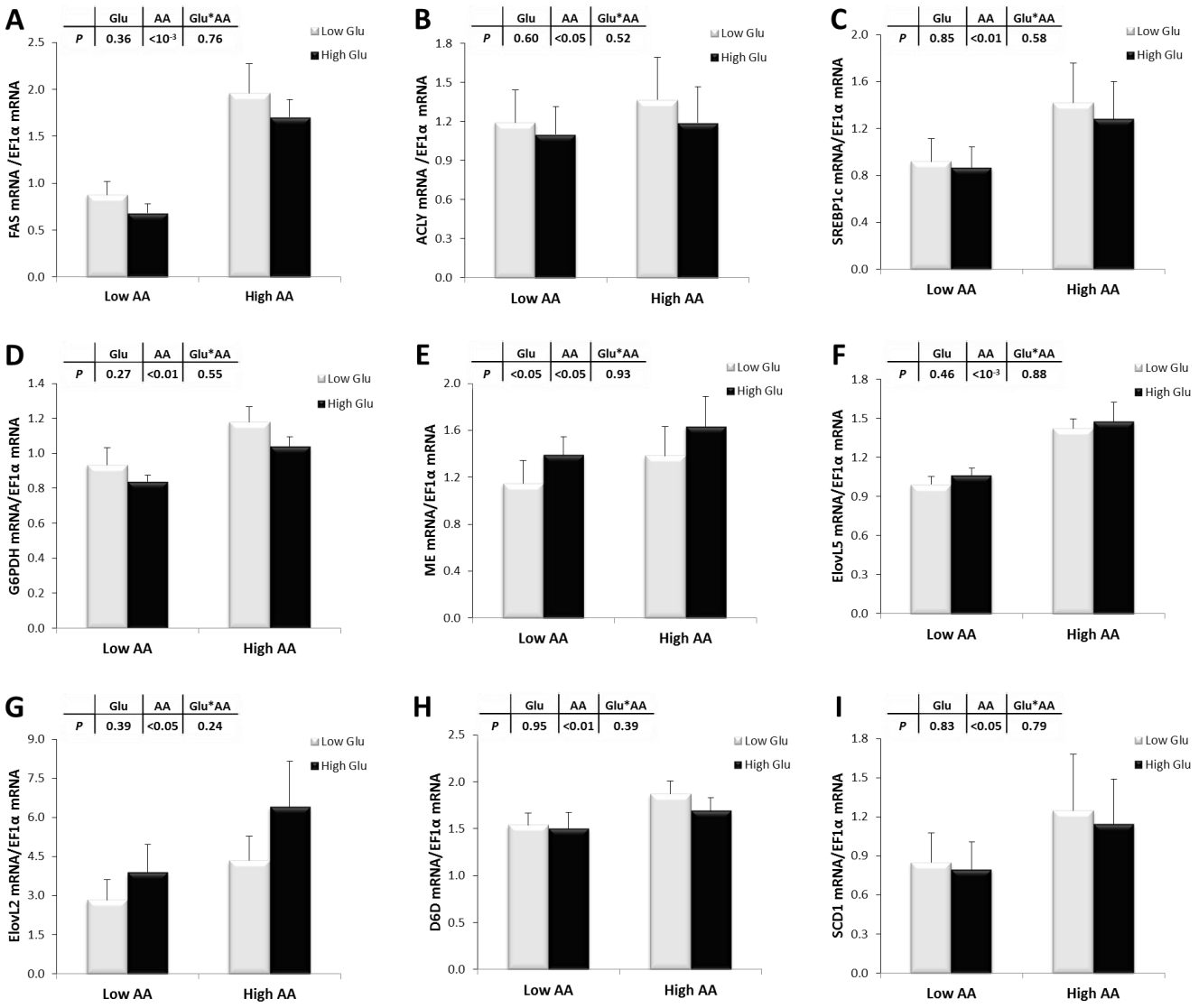


Fig. 5 Effects of different glucose (Glu) and amino acid (AA) levels on the regulation of fatty acid biosynthetic gene expression in trout hepatocytes. The experiment was conducted in 48 h-cultured trout hepatocytes. Cells were then stimulated with low/high level of glucose (3 mM/Low Glu or 20 mM/High Glu) and low/high level of amino acids (one-fold (Low AA) or four-fold (High AA) concentrated amino acids). mRNA levels of genes involved in *de novo* lipogenesis including (A) FAS and (B) ACLY, transcriptional factor (C) SREBP1c, NADPH production including (D) G6PDH and (E) ME, and fatty acid bioconversion including (F) Elovl5, (G) Elovl2, (H) D6D and (I) SCD1 in the primary hepatocytes of rainbow trout were measured 24 h after the stimulations. Expression values are normalized with elongation factor-1 alpha (EF1α)-expressed transcripts. Results are means + s.e.m. (N=9) and were analyzed using linear mixed-effects models (MIXED) procedure (P<0.05). Low or High glucose; AA=Low or High level of amino acids; Glu*AA=interaction between Glu and AA.

3.6 Fatty acid biosynthetic gene expression were also up-regulated by insulin levels

Considering that high dietary protein intake *in vivo* can induce not only elevated plasma AA levels but also higher insulin secretion (25, 51), we further stimulated trout hepatocytes with different insulin and AA levels to determine which one is the main factor on the regulation of fatty acid biosynthetic gene expression (Fig. 6). We observed that the mRNA levels of FAS, Elovl5 and Elovl2 were up-regulated by both elevated insulin and AA levels, while the gene expression of ACLY, G6PDH, ME, D6D and SCD1 were only up-regulated by increased AA levels. No change was observed for SREBP1c in current trial.

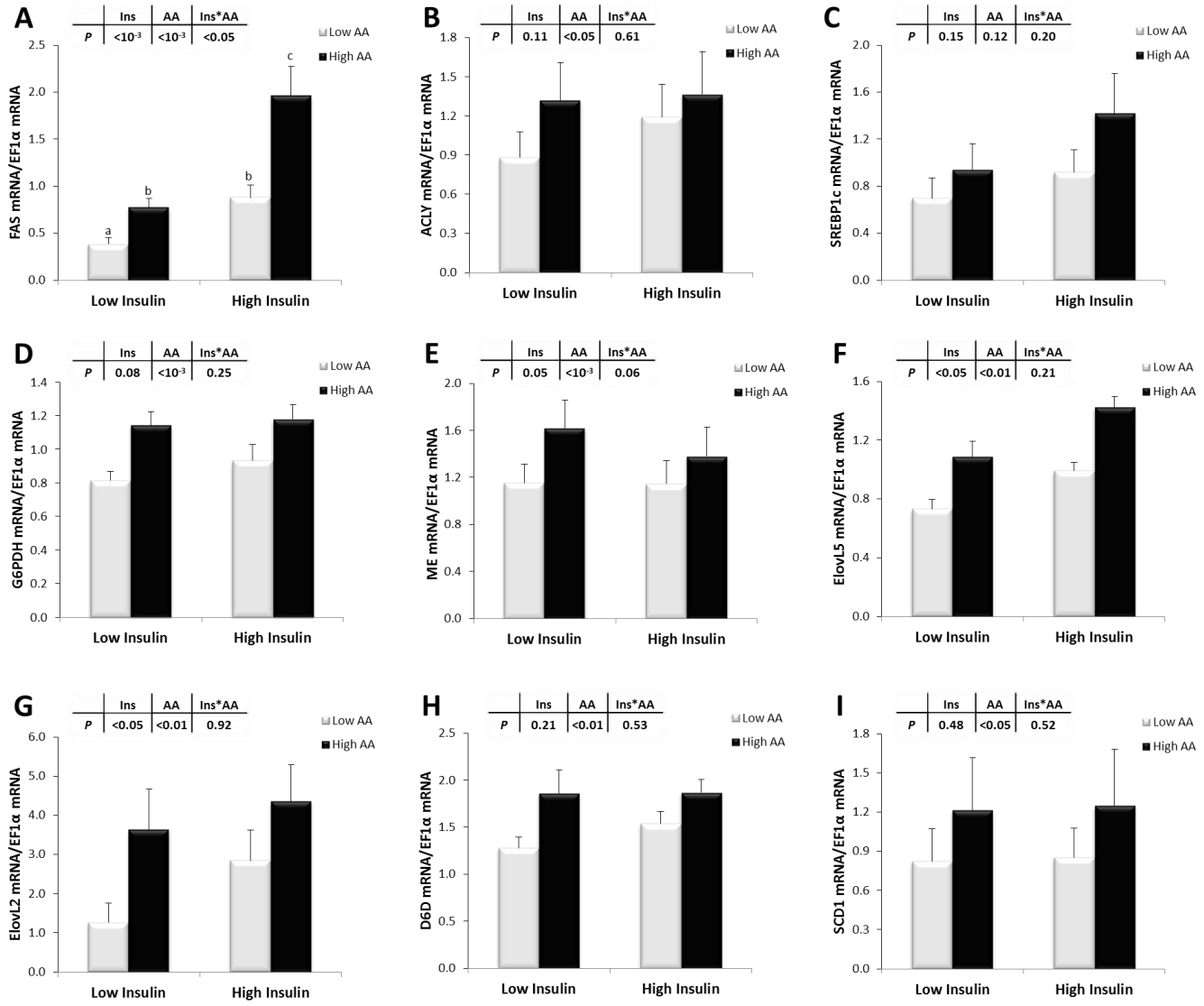


Fig. 6 Effects of different insulin (Ins) and amino acid (AA) levels on the regulation of fatty acid biosynthetic gene expression in trout hepatocytes. The experiment was conducted in 48 h-cultured trout hepatocytes. Cells were then stimulated with low/high level of insulin (1×10^{-9} mol l^{-1}/Low Insulin or 4×10^{-9} mol l^{-1}/High Insulin) and low/high level of amino acids (Low AA or High AA). mRNA levels of genes involved in *de novo* lipogenesis including (A) FAS and (B) ACLY, transcriptional factor (C) SREBP1c, NADPH production including (D) G6PDH and (E) ME, and fatty acid bioconversion including (F) Elovl5, (G) Elovl2, (H) D6D and (I) SCD1 in the primary hepatocytes of rainbow trout were measured 24 h after the stimulations. Expression values are normalized with elongation factor-1 alpha (EF1α)-expressed transcripts. Results are means + s.e.m. (N=9) and were analyzed using linear mixed-effects models (MIXED) procedure (P<0.05). When interaction was significant, results were further analyzed using the EMMEANS subcommand. Ins=Low or High Insulin; AA=Low or High level of amino acids; Ins*AA=interaction between Ins and AA.

4 DISCUSSION

Protein metabolism in rainbow trout is intimately linked to hepatic glucose metabolism since protein/amino acid catabolism provides substrates that channel into gluconeogenic pathways and excessive AAs attenuate insulin action on hepatic gluconeogenesis via mTORC1/S6K1-induced feedback loop (15, 42). However, our latest data also showed that high levels of AAs promote hepatic fatty acid biosynthesis through up-regulating the expression of genes related to DNL, NADPH production and fatty acid bioconversion, and by elevating amino acid catabolic pathway in trout hepatocytes (15). In this study, we further evaluated and compared the capacity of dietary protein intake/AAs and dietary carbohydrate intake/glucose in stimulating hepatic lipogenic gene expression in rainbow trout liver and primary hepatocytes during short-term stimulations. Unlike earlier studies in rodents, chickens or humans (1, 2, 56, 64, 87), we provide both *in vivo* and *in vitro* evidence that hepatic fatty acid biosynthetic gene expression in rainbow trout is more responsive to dietary protein intake/AAs than dietary carbohydrate intake/glucose during acute stimulations; dietary protein intake/AAs stimulates fatty acid biosynthetic gene expression via an mTORC1-dependent manner.

4.1 High carbohydrate intake induced hyperglycemia irrespective of the protein intake

High carbohydrate intake induced higher plasma glucose levels 8 and 24 h after controlled-feeding, and markedly decreased free amino acid (FAA) levels 24 h after controlled-feeding. These results are consistent with previous demonstrations in rainbow trout (60, 61, 75, 82), showing that high carbohydrate diets induced hyperglycemia and reduced plasma AA levels. These plasma parameters clearly confirmed the efficiency of controlled-feeding method. However, despite these confirmations, one limit of this method still needs to be considered. With controlled-feeding method, the amount of nutrient intake for each tank/treatment had been well controlled, however, whether each individual had ingested the same amount of feed was not guaranteed, which may account for the high individual variations as observed for the phosphorylation of S6K1 and S6, and mRNA levels of FAS and ACLY in the *in vivo* trail.

It is worthy to note that rapamycin markedly decreased plasma glucose levels 8 h after controlled-feeding and suppressed the expression of G6Pase I and G6Pase II (data not shown), in line with our previous finding showing that acute rapamycin administration reduced the hyperglycemia induced by IP glucose injection via inhibiting hepatic gluconeogenesis at both gene expression and enzyme activity levels in rainbow trout (16). Interestingly, rapamycin also enhanced plasma FAA levels 8 h after controlled-feeding. This elevated FAA level was negatively associated with the reduced glycemia, which is consistent with previous observations in trout (60, 61, 75, 82). Given that AAs, especially gluconeogenic AAs, can promote glucose production through hepatic gluconeogenic pathway (5, 15), we presume that both the elevated FAA levels and the reduced glycemia by rapamycin may be related to the suppressed hepatic gluconeogenesis as previously demonstrated (16), implicating the importance of hepatic gluconeogenesis in modulating glucose homeostasis. Notably, consistent with this deduction, recent work using stable tracer method by Viegas et al. (89) revealed that supplementation of digestible starch resulted in a significant reduction of gluconeogenic contributions to systemic glucose appearance in seabass.

4.2 High protein intake/AAs levels enhanced mTORC1 activation regardless of carbohydrate intake/glucose levels

mTOR is an evolutionarily conserved serine/threonine kinase that senses both nutrients and insulin signaling to control a myriad of cellular processes (80). Here we observed that high protein intake induced higher phosphorylation of TOR and 4EBP1 compared to low protein intake treatment regardless of dietary carbohydrate intake. Despite no statistical difference, the phosphorylation of S6K1 and S6 also showed elevated mean values in trout under high protein intake treatments compared to low protein intake treatment, similar as TOR and 4EBP1 results. *In vitro* data further confirmed the *in vivo* observations showing that high level of AAs enhanced S6K1 and S6 phosphorylation irrespective of glucose levels.

Therefore, our results suggest that high protein intake/AA levels enhanced mTORC1 activation regardless of carbohydrate intake/glucose levels in rainbow trout. This finding is in agreement with previous *in vivo* findings in trout, reporting that diet rich in protein and low in carbohydrates induced higher S6 phosphorylation in liver and elevated S6K1, S6 and 4EBP1 phosphorylation in muscle compared to high carbohydrates low protein diet (75). It is also consistent with studies in rat demonstrating that high protein intake/AAs elevated the phosphorylation of mTOR and 4EBP1 compared with low protein intake/AAs (11).

4.3 Hepatic fatty acid biosynthesis was more responsive to dietary protein intake/AAs than dietary carbohydrate intake/glucose

In vitro data clearly showed that the expression of genes related to hepatic DNL (FAS and ACLY), fatty acid bioconversion (Elovl5, Elovl2, D6D and SCD1), NADPH production (G6PDH and ME), and SREBP1c, a transcription factor that activates all the genes needed to produce fatty acids and triglycerides in liver (32), were coordinately up-regulated by high level of AAs, while high level of glucose only elevated the expression of ME, indicating that in rainbow trout hepatocytes, fatty acid biosynthetic gene expression is more responsive to AAs than glucose. In line with these observations, *in vivo* data also showed that high protein intake induced higher Elovl2 gene expression regardless of carbohydrate intake, while high carbohydrate intake suppressed the expression of ACC and Elovl5. Notably, despite the absence of statistical significance for the expression of FAS, ACLY and D6D by nutrient intake, they exhibited higher mean mRNA levels in high protein intake groups. Therefore, we conclude that hepatic fatty acid biosynthetic gene expression in rainbow trout is more responsive to dietary protein intake/AAs than dietary carbohydrate intake/glucose during acute stimulations.

The finding that dietary protein/AAs is a more potent stimulator for hepatic fatty acid biosynthesis compared to dietary carbohydrate/glucose is in agreement with previous *in vitro* observations demonstrating that increased AA levels up-regulated fatty acid biosynthetic gene expression in trout (15) and *in vivo* observations reporting that high protein low carbohydrate diets stimulated lipogenic gene expression (FAS and SREBP1) and enzyme activity (FAS, G6PDH and ME) compared to low protein high carbohydrate diets in rainbow trout and blackspot seabream, respectively (23, 75). However, these results are different from the demonstrations in rodents, chickens or humans reporting that high protein (HP) diets induce down-regulations of fatty acid biosynthesis in liver at both gene expression and enzymatic levels (1, 2, 7, 56, 64, 67, 87, 90). Data in mouse have shown that an increased flux of AAs reaching the hepato-portal area in HP diet conditions promotes AA catabolism and acetyl-CoA synthesis, however, it was suggested that the synthesized acetyl-CoA is either channeled into TCA cycle or used for β-hydroxybutyrate production, but not converted to fatty acids through unknown reasons (64, 72, 92). Therefore, DNL rates might be lower or even absent after an HP diet (72). Based on our results, we reason that, unlike rodents or humans, protein/AAs is more potent in stimulating hepatic lipogenesis compared to carbohydrate/glucose in rainbow trout, confirming the surmise of Tocher (85), who suggested that AAs are the preferred carbon source for lipogenesis in trout. Future work with tracer method is needed to further confirm this conclusion at nutrient flux level. It is of interest to note that current observation in rainbow trout is similar as in domestic cat, a terrestrial carnivorous animal, in which acetate is the predominant carbon source for DNL in both liver and adipose tissue instead of glucose which is the major substrate for DNL in human and rodents (62, 66, 88). However, unlike trout, human or rodents where liver is the primary site for DNL (6, 29, 85), in cats, adipose tissue serves this function, followed by liver, mammary glands and muscle (66, 88).

The results that high carbohydrate intake/glucose failed to stimulate hepatic lipogenic gene expression but up-regulated ME (*in vitro*) are consistent with previous studies reporting that high carbohydrate diets/glucose failed or only had limited effects in stimulating lipogenic gene expression and enzyme activities in rainbow trout (30, 35, 75). Notably, similar demonstrations were reported in cat and mink (68, 88), showing that fatty acids are not synthetized from glucose, probably due to the absence of

glucokinase in feline liver (48, 71). The results also confirmed the presumption of Hemre et al. (31) that carbohydrate is needed to stimulate lipid biosynthesis not so much through delivery of carbon backbones, but rather via increased availability of cytosolic reducing equivalents, and are consistent with some other demonstrations in carnivorous fish (4, 20, 26, 84). However, this is different from which is known in mammals that diets high in simple carbohydrates led to elevated expression of enzymes involved in DNL, MUFA synthesis (SCD1) and PUFA synthesis (Elovl2, Elovl5 and D6D) (10, 27, 36, 40, 86). The poor induction of hepatic lipogenic/fatty acid biosynthetic gene expression to dietary carbohydrate intake/glucose could be one of the contributors to the poor dietary carbohydrate utilization in rainbow trout. As trout represents a relevant carnivorous model (54, 71), we presume this demonstration may be applicable for general carnivores, including piscivorous fish and terrestrial carnivores.

4.4 Dietary protein/AAs stimulate fatty acid biosynthesis via mTORC1-dependent manner

In vitro data showed that elevated fatty acid biosynthetic gene expression by high level of AAs was associated with the enhanced phosphorylation of S6K1 and S6, which is totally consistent with our previous investigation (15). Furthermore, we also reported that elevated fatty acid biosynthetic gene expression was markedly suppressed by mTORC1 inhibition (15). *In vivo* data showed that high protein intake not only induced higher TOR and 4EBP1 phosphorylation but also induced higher Elovl2 gene expression regardless of carbohydrate intake. Although not statistically significant, the expression of FAS, ACLY and D6D exhibited similar patterns as the phosphorylation of S6K1 and S6, showing that high protein intake induced higher mean mRNA levels. Moreover, mTORC1 inhibition suppressed the expression of FAS, ACLY, ACC, Elovl2, Elovl5, D6D and G6PDH, indicating that the regulation of hepatic fatty acid biosynthetic gene expression is mTORC1 dependent, which is consistent with our latest *in vitro* demonstrations in trout (15). Therefore, our data clearly demonstrate that dietary protein intake/AAs stimulate fatty acid biosynthetic gene expression through an mTORC1-dependent manner. Consistent with this observation, recent investigation in zebrafish also shown that mTORC1 activation is required for hepatic lipid accumulation as rapamycin reverses hepatic lipid accumulation and associated gene expression changes (lipogenic, inflammatory, oxidative stress and ER stress genes) (70).

Indeed, the regulation of fatty acid biosynthesis, especially the regulation of lipogenesis is very complex. Mammalian studies have demonstrated that several cellular signaling pathways, including insulin/mTOR (63), AMPK (AMP-activated protein kinase) (44), autophagy (94), p38MAPK (mitogen-activated protein kinase p38) (93) and AAR (amino acid response) (28), and transcription factors including peroxisome proliferator-activated receptor gamma (PPARγ), LXR (44), ChREBP (24), SREBP1c (34, 77), XBP1 (43), nuclear sterol-activated receptor LXR and FXR (8), as well as microRNA (miRNAs) (19, 21) are involving in the regulation of hepatic lipogenesis. Thus, we assume that mTORC1 signaling pathway is probably not the only signaling pathway that involving in the nutrients-mediated control of fatty acid biosynthesis in rainbow trout. Future studies are merited to verify the potential involvement of other signaling pathways or transcription factors in fish.

4.5 Dietary protein intake stimulated hepatic fatty acid biosynthesis probably mainly through AAs themselves rather than insulin

It is well known that, compared to carbohydrates, AAs are more potent insulin secretagogues in most fish species (51). Therefore, one potential explanation for the elevated lipogenic gene expression in high dietary protein diet conditions can be due to the higher insulin secretion induced by the HP diets as insulin has been proven a potent mediator for stimulating mTOR signaling and anabolic pathways, including glycolysis and lipid synthesis (15, 57, 69). Given that currently the method for precisely measuring trout plasma insulin concentration is no longer available (58), we tested this hypothesis *in vitro*. Using trout primary hepatocytes, we observed that increased insulin levels markedly up-regulated the gene expression of FAS, Elovl5 and Elovl2, while increased AA levels significantly up-regulated the expression of enzymes involved in DNL (FAS and ACLY), lipid bioconversion (Elovl5,

Elovl2, D6D and SCD1) and NADPH production (G6PDH and ME). The results that insulin stimulated the expression of FAS, Elovl5 and Elovl2 are consistent with our previous *in vitro* demonstrations (15) and also in agreement with data from a previous study showing that addition of insulin elevated FAS gene expression (42). Regarding AAs effect, it is totally consistent with our previous *in vitro* observations (15). Therefore, we propose that dietary protein intake stimulates hepatic fatty acid biosynthesis mainly through AAs themselves; HP diet/AAs induced release of insulin may also contribute to the elevated lipogenic gene expression, however, its stimulation effect is less potent compared to AAs themselves.

In summary, we provided both *in vivo* and *in vitro* evidence to demonstrate that hepatic fatty acid biosynthetic gene expression in rainbow trout is more responsive to dietary protein intake/AAs than dietary carbohydrate intake/glucose during acute stimulations; dietary protein intake/AAs stimulates fatty acid biosynthetic gene expression via an mTORC1-dependent manner. We also propose that dietary protein intake stimulates hepatic fatty acid biosynthetic gene expression mainly through AAs themselves instead of stimulating the release of insulin.

4.6 Perspectives and Significance

Unlike rodents, chickens or humans (1, 2, 56, 64, 87), we demonstrated that hepatic fatty acid biosynthesis is more responsive to protein than carbohydrate in rainbow trout. This demonstration is supported by previous investigations or presumptions in fish (23, 31, 54, 75, 85), and similar as cat and mink (68, 88). Thus, the feature we revealed here probably represents one important physiological and metabolic difference between carnivores and omnivores. In human and other mammalian species, consumption of high dietary protein can induce hyper-TOR activation, which may in turn induce suppressed autophagy, thereby promoting endoplasmic reticulum (ER) stress and, chronically, causing mitochondrial dysfunction and insulin resistance (3, 46, 76, 94). Furthermore, high dietary protein stimulates insulin hypersecretion and overactivation of mTORC1 that can blunt insulin sensitivity via mTORC1/S6K1-induced negative feedback loop, which will further increase demand of insulin secretion. The increased demand for insulin biosynthesis in turn increases demand in the ER, gradually leading to ER stress, increased protein misfolding, activation of unfolded protein response (UPR) and dysregulated mitochondria function. Collectively, these adverse events may eventually cause insulin resistance (12, 33, 37, 47, 65, 76, 94). Considering that rainbow trout possesses high dietary protein consumption and acute stimulation with excessive AAs induces overactivation of mTORC1, which in turn attenuates insulin-mediated repression of gluconeogenesis via mTORC1/S6K1-induced negative feedback loop (15), we presume that the poor carbohydrate utilization in rainbow trout is possibly due to a combination of mitochondrial dysfunction, ER stress, hypo-autophagy, accumulation of misfolded proteins and altered UPR as in mammals (53, 95). Interestingly, in line with this presumption, recent investigation in zebrafish reported that fructose treatment of larval zebrafish induced hepatic lipid accumulation, which was associated with mTORC1 activation, inflammation and oxidative stress (70). Therefore, future work are needed to investigate the parameters related to inflammatory stresses and mitochondrial functions as they may account for the low utilization efficiency of dietary carbohydrate/glucose and poorly inhibited hepatic gluconeogenesis in rainbow trout as in mammals. Notably, recent work by Marandel et al., (49) concluded that maintenance of ohnologous g6pcb2 pair and futile glucose/ glucose-6-phosphate cycling may contribute in a significant way to the glucose-intolerant phenotype of trout and may partially explain its poor use of dietary carbohydrates. To some extent, these two deductions are consistent with each other and may coordinately exist in rainbow trout. Furthermore, as we reason that protein or AAs are more potent in the stimulation of DNL than carbohydrate or glucose in rainbow trout and AAs are probably used as substrates for DNL, future work with tracer method is needed to measure the true contributions of AAs and AA catabolism to fat synthesis at nutrient flux level.

ACKNOWLEDGMENTS

We thank K. Dias, V. Véron, A. Herman and M. Cluzeaud for technical assistance in the laboratory. We also acknowledge the technical

staff of the INRA experimental fish farm at Saint Pee sur Nivelle (P. Aguirre and Y. Mercier) for fish rearing.

FUNDING

W. Dai gratefully acknowledges the financial assistance provided by the China Scholarship Council (CSC, File No. 2011633111) for his doctoral fellowship.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

W. D. performed sampling, primary cell culture, data acquisition, data analysis and wrote the manuscript. E. P.-J. contributed in the primary cell culture. F. T. contributed in the intraperitoneal administration of rapamycin and fish rearing. S. S.-C. and S. K. developed the study design. S. S.-C., S. P., S. K. and I. S. contributed to the manuscript correcting. S. S.-C. is the guarantor of this study and take full responsibility for the data, analysis, statistical analysis and manuscript submission.

AUTHOR CONTRIBUTIONS

W. D. performed sampling, primary cell culture, data acquisition, data analysis and wrote the manuscript. E. P.-J. contributed in the primary cell culture. F. T. contributed in the intraperitoneal administration of rapamycin and fish rearing. S. S.-C. and S. K. developed the study design. S. S.-C., S. P., S. K. and I. S. contributed to the manuscript correcting. S. S.-C. is the guarantor of this study and take full responsibility for the data, analysis, statistical analysis and manuscript submission.

REFERENCES

1. **Abu-Elheiga L, Wu H, Gu Z, Bressler R, and Wakil SJ.** Acetyl-CoA carboxylase 2-/- mutant mice are protected against fatty liver under high-fat, high-carbohydrate dietary and de novo lipogenic conditions. *J Biol Chem* 287: 12578-12588, 2012.

2. **Allee GL, O'Hea EK, Leveille GA, and Baker DH.** Influenza of dietary protein and fat on lipogenesis and enzymatic activity in pig adipose tissue. *J Nutr* 101: 869-878, 1971.

3. **Arruda AP, Pers BM, Parlakgul G, Guney E, Inouye K, and Hotamisligil GS.** Chronic enrichment of hepatic endoplasmic reticulum-mitochondria contact leads to mitochondrial dysfunction in obesity. *Nat Med* 20: 1427-1435, 2014.

4. **Barroso JB, Peragon J, Garcia-Salguero L, de la Higuera M, and Lupianez JA.** Carbohydrate deprivation reduces NADPH-production in fish liver but not in adipose tissue. *Int J Biochem Cell Biol* 33: 785-796, 2001.

5. **Berg JM, Tymoczko JL, and Stryer L.** *Biochemistry*. New York: W H Freeman, 2010.

6. **Bergen WG and Mersmann HJ.** Comparative aspects of lipid metabolism: Impact on contemporary research and use of animal models. *J Nutr* 135: 2499-2502, 2005.

7. **Blouet C, Mariotti F, Azzout-Marniche D, Bos C, Mathe V, Tome D, and Huneau JF.** The reduced energy intake of rats fed a high-protein low-carbohydrate diet explains the lower fat deposition, but macronutrient substitution accounts for the improved glycemic control. *J Nutr* 136: 1849-1854, 2006.

8. **Calkin AC and Tontonoz P.** Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR. *Nat Rev Mol Cell Biol* 13: 213-224, 2012.

9. **Caron A, Richard D, and Laplante M.** The Roles of mTOR Complexes in Lipid Metabolism. *Annu Rev Nutr* 35: 321-348, 2015.

10. **Chong MF, Hodson L, Bickerton AS, Roberts R, Neville M, Karpe F, Frayn KN, and Fielding BA.** Parallel activation of de novo lipogenesis and stearoyl-CoA desaturase activity after 3 d of high-carbohydrate feeding. *Am J Clin Nutr* 87: 817-823, 2008.

11. **Chotechuang N, Azzout-Marniche D, Bos C, Chaumontet C, Gausseres N, Steiler T, Gaudichon C, and Tome D.** mTOR, AMPK, and GCN2 coordinate the adaptation of hepatic energy metabolic pathways in response to protein intake in the rat. *Am J Physiol Endocrinol Metab* 297: E1313-1323, 2009.

12. **Codogno P and Meijer AJ.** Autophagy: A Potential Link between Obesity and Insulin Resistance. *Cell metabolism* 11: 449-451, 2010.

13. **Collins JM, Neville MJ, Hoppa MB, and Frayn KN.** De novo lipogenesis and stearoyl-CoA desaturase are coordinately regulated in the human adipocyte and protect against palmitate-induced cell injury. *J Biol Chem* 285: 6044-6052, 2010.

14. **Dai W, Panserat S, Mennigen JA, Terrier F, Dias K, Seiliez I, and Skiba-Cassy S.** Post-prandial regulation of hepatic glucokinase and lipogenesis requires the activation of TORC1 signalling in rainbow trout (Oncorhynchus mykiss). *J Exp Biol* 216: 4483-4492, 2013.

15. **Dai W, Panserat S, Plagnes-Juan E, Seiliez I, and Skiba-Cassy S.** Amino acids attenuate insulin action on gluconeogenesis and promote fatty acid biosynthesis via mTORC1 signaling pathway in trout hepatocytes. *Cell Physiol Biochem* 36: 1084-1100, 2015.

16. **Dai WW, Panserat S, Terrier F, Seiliez I, and Skiba-Cassy S.** Acute rapamycin treatment improved glucose tolerance through inhibition of hepatic gluconeogenesis in rainbow trout (Oncorhynchus mykiss). *Am J Physiol-Reg I* 307: R1231-R1238, 2014.

17. **del sol Novoa M, Capilla E, Rojas P, Baro J, Gutierrez J, and Navarro I.** Glucagon and insulin response to dietary carbohydrate in rainbow trout (Oncorhynchus mykiss). *Gen Comp Endocrinol* 139: 48-54, 2004.

18. **Dias J, Alvarez MJ, Diez A, Arzel J, Corraze G, Bautista JM, and Kaushik SJ.** Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European seabass (Dicentrarchus labrax). *Aquaculture* 161: 169-186, 1998.

19. **Dumortier O, Hinault C, and Van Obberghen E.** MicroRNAs and metabolism crosstalk in energy homeostasis. *Cell metabolism* 18: 312-324, 2013.

20. **Enes P, Panserat S, Kaushik S, and Oliva-Teles A.** Growth performance and metabolic utilization of diets with native and waxy maize starch by gilthead sea bream (Sparus aurata) juveniles. *Aquaculture* 274: 101-108, 2008.

21. **Fernandez-Hernando C, Suarez Y, Rayner KJ, and Moore KJ.** MicroRNAs in lipid metabolism. *Curr Opin Lipidol* 22: 86-92, 2011.

22. **Ferraris M, Radice S, Catalani P, Francolini M, Marabini L, and Chiesara E.** Early oxidative damage in primary cultured trout hepatocytes: a time course study. *Aquatic Toxicology* 59: 283-296, 2002.

23. **Figueiredo-Silva AC, Corraze G, Rema P, Sanchez-Gurmaches J, Gutierrez J, and Valente LMP.** Blackspot seabream (Pagellus bogaraveo) lipogenic and glycolytic pathways appear to be more related to dietary protein level than dietary starch type. *Aquaculture* 291: 101-110, 2009.

24. **Filhoulaud G, Guilmeau S, Dentin R, Girard J, and Postic C.** Novel insights into ChREBP regulation and function. *Trends Endocrinol Metab* 24: 257-268, 2013.

25. **Floyd JC, Jr., Fajans SS, Conn JW, Knopf RF, and Rull J.** Stimulation of insulin secretion by amino acids. *J Clin Invest* 45: 1487-1502, 1966.

26. **Fynnaikins K, Hung SSO, Liu W, and Li HB.** Growth, lipogenesis and liver composition of juvenile white sturgeon fed different levels of D-glucose. *Aquaculture* 105: 61-72, 1992.

27. **Girard J, Ferre P, and Foufelle F.** Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. *Annu Rev Nutr* 17: 325-352, 1997.

28. **Guo F and Cavener DR.** The GCN2 eIF2alpha kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid. *Cell metabolism* 5: 103-114, 2007.

29. **Hellerstein MK, Christiansen M, Kaempfer S, Kletke C, Wu K, Reid JS, Mulligan K, Hellerstein NS, and Shackleton CHL.** Measurement of Denovo Hepatic Lipogenesis in Humans Using Stable Isotopes. *Clin Res* 39: A332-A332, 1991.

30. **Hemre GI and Kahrs F.** C-14-glucose injection in Atlantic cod, Gadus morhua, metabolic responses and excretion via the gill membrane. *Aquacult Nutr* 3: 3-8, 1997.

31. **Hemre GI, Mommsen TP, and Krogdahl A.** Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. *Aquacult Nutr* 8: 175-194, 2002.

32. **Horton JD, Goldstein JL, and Brown MS.** SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 109: 1125-1131, 2002.

33. **Hummasti S and Hotamisligil GS.** Endoplasmic Reticulum Stress and Inflammation in Obesity and Diabetes. *Circ Res* 107: 579-591, 2010.

34. **Jeon TI and Osborne TF.** SREBPs: metabolic integrators in physiology and metabolism. *Trends Endocrinol Metab* 23: 65-72, 2012.

35. **Jin J, Medale F, Kamalam BS, Aguirre P, Veron V, and Panserat S.** Comparison of glucose and lipid metabolic gene expressions between fat and lean lines of rainbow trout after a glucose load. *PLoS One* 9: e105548, 2014.

36. **Jump DB.** Fatty acid regulation of hepatic lipid metabolism. *Curr Opin Clin Nutr Metab Care* 14: 115-120, 2011.

37. **Jung HS and Lee MS.** Role of autophagy in diabetes and mitochondria. *Ann Ny Acad Sci* 1201: 79-83, 2010.

38. **Kamalam BS, Medale F, Kaushik S, Polakof S, Skiba-Cassy S, and Panserat S.** Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. *J Exp Biol* 215: 2567-2578, 2012.

39. **Kamalam BS, Panserat S, Aguirre P, Geurden I, Fontagne-Dicharry S, and Medale F.** Selection for high muscle fat in rainbow trout induces potentially higher chylomicron synthesis and PUFA biosynthesis in the intestine. *Comp Biochem Physiol A Mol Integr Physiol* 164: 417-427, 2013.

40. **Kersten S.** Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO reports* 2: 282-286, 2001.

41. **Lamming DW and Sabatini DM.** A Central role for mTOR in lipid homeostasis. *Cell metabolism* 18: 465-469, 2013.

42. **Lansard M, Panserat S, Plagnes-Juan E, Seiliez I, and Skiba-Cassy S.** Integration of insulin and amino acid signals that regulate hepatic

metabolism-related gene expression in rainbow trout: role of TOR. *Amino Acids* 39: 801-810, 2010.

43. **Lee AH, Scapa EF, Cohen DE, and Glimcher LH.** Regulation of hepatic lipogenesis by the transcription factor XBP1. *Science* 320: 1492-1496, 2008.

44. **Li Y, Xu S, Mihaylova MM, Zheng B, Hou X, Jiang B, Park O, Luo Z, Lefai E, Shyy JY, Gao B, Wierzbicki M, Verbeuren TJ, Shaw RJ, Cohen RA, and Zang M.** AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. *Cell metabolism* 13: 376-388, 2011.

45. **Likimani TA and Wilson RP.** Effects of Diet on Lipogenic Enzyme-Activities in Channel Catfish Hepatic and Adipose-Tissue. *J Nutr* 112: 112-117, 1982.

46. **Lowell BB and Shulman GI.** Mitochondrial dysfunction and type 2 diabetes. *Science* 307: 384-387, 2005.

47. **Lynch CJ and Adams SH.** Branched-chain amino acids in metabolic signalling and insulin resistance. *Nature Reviews Endocrinology* 10: 723-736, 2014.

48. **Macdonald ML, Rogers QR, and Morris JG.** Nutrition of the Domestic Cat, a Mammalian Carnivore. *Annu Rev Nutr* 4: 521-562, 1984.

49. **Marandel L, Seiliez I, Veron V, Skiba-Cassy S, and Panserat S.** New insights into the nutritional regulation of gluconeogenesis in carnivorous rainbow trout (Oncorhynchus mykiss): a gene duplication trail. *Physiological genomics* 47: 253-263, 2015.

50. **Mommsen TP, Moon TW, and Walsh TJ.** Hepatocytes: isolation, maintenance and utilization. In: *Biochemistry and Molecular Biology of Fishes*, edited by Hochachka PW and Mommsen TP. Amsterdam: Elsevier Science B.V., 1994.

51. **Mommsen TP and Plisetskaya EM.** Insulin in Fishes and Agnathans - History, Structure, and Metabolic-Regulation. *Rev Aquat Sci* 4: 225-259, 1991.

52. **Moore S.** Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. *J Biol Chem* 243: 6281-6283, 1968.

53. **Muoio DM and Newgard CB.** Mechanisms of disease:Molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat Rev Mol Cell Biol* 9: 193-205, 2008.

54. **Panserat S, Kaushik S, and Médale F.** Rainbow trout as a model for nutrition and nutrient metabolism studies. In: *Trout : from physiology to conservation*, edited by Polakof S and Moon TW. Hauppauge, USA: Nova Science Publishers, 2013, p. 452 p.

55. **Panserat S, Skiba-Cassy S, Seiliez I, Lansard M, Plagnes-Juan E, Vachot C, Aguirre P, Larroquet L, Chavernac G, Medale F, Corraze G, Kaushik S, and Moon TW.** Metformin improves postprandial glucose homeostasis in rainbow trout fed dietary carbohydrates: a link with the induction of hepatic lipogenic capacities? *Am J Physiol Regul Integr Comp Physiol* 297: R707-715, 2009.

56. **Pichon L, Huneau JF, Fromentin G, and Tome D.** A high-protein, high-fat, carbohydrate-free diet reduces energy intake, hepatic lipogenesis, and adiposity in rats. *J Nutr* 136: 1256-1260, 2006.

57. **Plagnes-Juan E, Lansard M, Seiliez I, Medale F, Corraze G, Kaushik S, Panserat S, and Skiba-Cassy S.** Insulin regulates the expression of several metabolism-related genes in the liver and primary hepatocytes of rainbow trout (Oncorhynchus mykiss). *J Exp Biol* 211: 2510-2518, 2008.

58. **Plisetskaya EM.** Some of my not so favorite things about insulin and insulin-like growth factors in fish. *Comp Biochem Physiol B Biochem Mol Biol* 121: 3-11, 1998.

59. **Plisetskaya EM and Sullivan CV.** Pancreatic and thyroid hormones in rainbow trout (Salmo gairdneri): what concentration does the liver see? *Gen Comp Endocrinol* 75: 310-315, 1989.

60. **Polakof S, Panserat S, Plagnes-Juan E, and Soengas JL.** Altered dietary carbohydrates significantly affect gene expression of the major glucosensing components in Brockmann bodies and hypothalamus of rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 295: R1077-1088, 2008.

61. **Polakof S, Skiba-Cassy S, Kaushik S, Seiliez I, Soengas JL, and Panserat S.** Glucose and lipid metabolism in the pancreas of rainbow trout is regulated at the molecular level by nutritional status and carbohydrate intake. *J Comp Physiol B* 182: 507-516, 2012.

62. **Richard MJ, Holck JT, and Beitz DC.** Lipogenesis in liver and adipose tissue of the domestic cat (Felis domestica). *Comp Biochem Physiol B* 93: 561-564, 1989.

63. **Ricoult SJH and Manning BD.** The multifaceted role of mTORC1 in the control of lipid metabolism. *EMBO reports* 14: 242-251, 2013.

64. **Rietman A, Schwarz J, Blokker BA, Siebelink E, Kok FJ, Afman LA, Tome D, and Mensink M.** Increasing protein intake modulates lipid metabolism in healthy young men and women consuming a high-fat hypercaloric diet. *J Nutr* 144: 1174-1180, 2014.

65. **Rietman A, Schwarz J, Tome D, Kok FJ, and Mensink M.** High dietary protein intake, reducing or eliciting insulin resistance? *Eur J Clin Nutr* 68: 973-979, 2014.

66. **Robert C and Backus RC.** Advances in knowledge about feline metabolism. *Continuing Education for Veterinarians* 30, 2008.

67. **Rosebrough RW, Poch SM, Russell BA, and Richards MP.** Dietary protein regulates in vitro lipogenesis and lipogenic gene expression in

broilers. *Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology* 132: 423-431, 2002.

68. **Rouvinen-Watt K, Harris L, Dick M, Pal C, Lei S, Mustonen AM, and Nieminen P.** Role of hepatic de novo lipogenesis in the development of fasting-induced fatty liver in the American mink (Neovison vison). *Br J Nutr* 108: 1360-1370, 2012.

69. **Saltiel AR and Kahn CR.** Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414: 799-806, 2001.

70. **Sapp V, Gaffney L, EauClaire SF, and Matthews RP.** Fructose Leads to Hepatic Steatosis in Zebrafish That Is Reversed by Mechanistic Target of Rapamycin (mTOR) Inhibition. *Hepatology* 60: 1581-1592, 2014.

71. **Schermerhorn T.** Normal glucose metabolism in carnivores overlaps with diabetes pathology in non-carnivores. *Front Endocrinol (Lausanne)* 4: 188, 2013.

72. **Schwarz J, Tome D, Baars A, Hooiveld GJ, and Muller M.** Dietary protein affects gene expression and prevents lipid accumulation in the liver in mice. *PLoS One* 7: e47303, 2012.

73. **Segner H.** Isolation and primary culture of teleost hepatocytes. *Comparative Biochemistry and Physiology A: Comparative Physiology* 120: 71-81, 1998.

74. **Seiliez I, Gabillard JC, Skiba-Cassy S, Garcia-Serrana D, Gutierrez J, Kaushik S, Panserat S, and Tesseraud S.** An in vivo and in vitro assessment of TOR signaling cascade in rainbow trout (Oncorhynchus mykiss). *Am J Physiol-Reg I* 295: R329-R335, 2008.

75. **Seiliez I, Panserat S, Lansard M, Polakof S, Plagnes-Juan E, Surget A, Dias K, Larquier M, Kaushik S, and Skiba-Cassy S.** Dietary carbohydrate-to-protein ratio affects TOR signaling and metabolism-related gene expression in the liver and muscle of rainbow trout after a single meal. *Am J Physiol Regul Integr Comp Physiol* 300: R733-743, 2011.

76. **Senft D and Ronai ZA.** UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. *Trends Biochem Sci* 40: 141-148, 2015.

77. **Shao W and Espenshade PJ.** Expanding roles for SREBP in metabolism. *Cell metabolism* 16: 414-419, 2012.

78. **Shiau SY and Chen MJ.** Carbohydrate Utilization by Tilapia (Oreochromis-Niloticus X Oreochromis-Aureus) as Influenced by Different Chromium Sources. *J Nutr* 123: 1747-1753, 1993.

79. **Shiau SY and Lin SF.** Effect of Supplemental Dietary Chromium and Vanadium on the Utilization of Different Carbohydrates in Tilapia, Oreochromis-Niloticusxo-Aureus. *Aquaculture* 110: 321-330, 1993.

80. **Shimobayashi M and Hall MN.** Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nat Rev Mol Cell Biol* 15: 155-162, 2014.

81. **Skiba-Cassy S, Lansard M, Panserat S, and Medale F.** Rainbow trout genetically selected for greater muscle fat content display increased activation of liver TOR signaling and lipogenic gene expression. *Am J Physiol-Reg I* 297: R1421-R1429, 2009.

82. **Skiba-Cassy S, Panserat S, Larquier M, Dias K, Surget A, Plagnes-Juan E, Kaushik S, and Seiliez I.** Apparent low ability of liver and muscle to adapt to variation of dietary carbohydrate:protein ratio in rainbow trout (Oncorhynchus mykiss). *Br J Nutr* 109: 1359-1372, 2013.

83. **Strable MS and Ntambi JM.** Genetic control of de novo lipogenesis: role in diet-induced obesity. *Crit Rev Biochem Mol Biol* 45: 199-214, 2010.

84. **Suarez MD, Hidalgo MC, Gallego MG, Sanz A, and Delahiguera M.** Influence of the Relative Proportions of Energy Yielding Nutrients on Liver Intermediary Metabolism of the European Eel. *Comparative Biochemistry and Physiology a-Physiology* 111: 421-428, 1995.

85. **Tocher DR.** Metabolism and functions of lipids and fatty acids in teleost fish. *Rev Fish Sci* 11: 107-184, 2003.

86. **Towle HC, Kaytor EN, and Shih HM.** Regulation of the expression of lipogenic enzyme genes by carbohydrate. *Annu Rev Nutr* 17: 405-433, 1997.

87. **Uebanso T, Taketani Y, Fukaya M, Sato K, Takei Y, Sato T, Sawada N, Amo K, Harada N, Arai H, Yamamoto H, and Takeda E.** Hypocaloric high-protein diet improves fatty liver and hypertriglyceridemia in sucrose-fed obese rats via two pathways. *Am J Physiol Endocrinol Metab* 297: E76-84, 2009.

88. **Verbrugghe A and Bakovic M.** Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis. *Nutrients* 5: 2811-2835, 2013.

89. **Viegas I, Rito J, Jarak I, Leston S, Caballero-Solares A, Meton I, Pardal MA, Baanante IV, and Jones JG.** Contribution of dietary starch to hepatic and systemic carbohydrate fluxes in European seabass (Dicentrarchus labrax L.). *Br J Nutr* 113: 1345-1354, 2015.

90. **Wang Y, Botolin D, Xu J, Christian B, Mitchell E, Jayaprakasam B, Nair MG, Peters JM, Busik JV, Olson LK, and Jump DB.** Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity. *J Lipid Res* 47: 2028-2041, 2006.

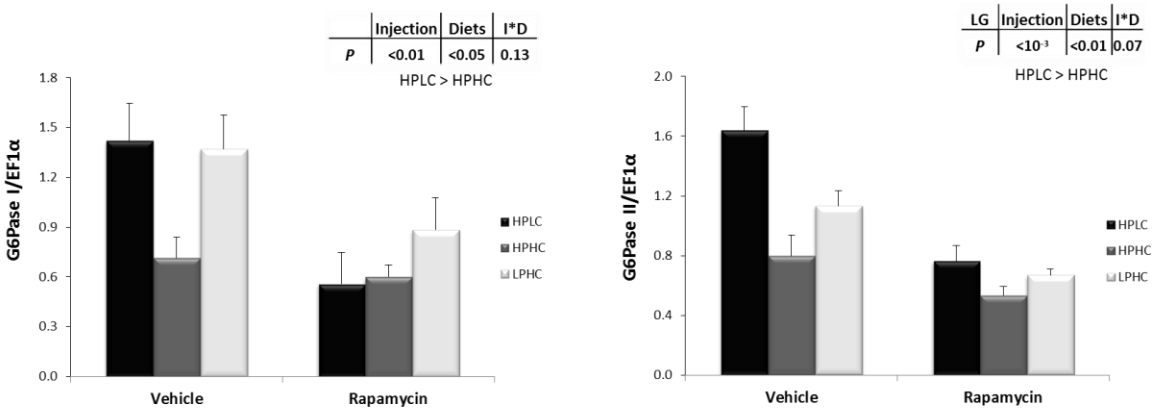
91. **Wilson RP and Likimani TA.** Effects of Diet on Lipogenic Enzyme-Activities in Channel Catfish Hepatic and Adipose-Tissue. *Fed Proc* 40: 882-882, 1981.

92. **Wu G.** Amino acids: metabolism, functions, and nutrition. *Amino Acids* 37: 1-17, 2009.
93. **Xiong Y, Collins QF, An J, Lupo E, Jr., Liu HY, Liu D, Robidoux J, Liu Z, and Cao W.** p38 mitogen-activated protein kinase plays an inhibitory role in hepatic lipogenesis. *J Biol Chem* 282: 4975-4982, 2007.
94. **Yang L, Li P, Fu SN, Calay ES, and Hotamisligil GS.** Defective Hepatic Autophagy in Obesity Promotes ER Stress and Causes Insulin Resistance. *Cell metabolism* 11: 467-478, 2010.
95. **Zoncu R, Efeyan A, and Sabatini DM.** mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Bio* 12: 21-35, 2011.

Supplementary data

Similar as in article four (Dai et al., 2014), we observed that rapamycin significantly decreased plasma glucose levels 8 h after controlled-feeding. We also demonstrated that acute rapamycin administration reduced the hyperglycemia induced by IP glucose injection probably via inhibiting hepatic gluconeogenesis (Dai et al., 2014). Thus, we further analyzed the expression of G6Pase, the last rate-limiting enzyme of gluconeogenesis, which is involved in glucose dephosphorylation (Nordlie et al., 1999).

Figure S 2 Gene expression of markers of hepatic gluconeogenesis



Gene expression of G6Pase in the liver of rainbow trout subjected to IP administration of vehicle or rapamycin, 24 h after controlled-feeding. Expression values are normalized with elongation factor-1 alpha (EF1α)-expressed transcripts. Results are means + s.e.m. (*N*=6) and were analyzed using two-way ANOVA (*P*<0.05) followed by a Tukey multiple comparison test. "LG" indicates data were transformed and statistical analyzed with log transforms. HPLC, high protein low carbohydrate; HPHC, high protein high carbohydrate; LPHC, low protein high carbohydrate. Injection=IP administration of vehicle or rapapamycin; Diets=HPLC, HPHC or LPHC; I*D=interaction between Injection and Diets.

Gene expression of G6Pase I and G6Pase II in high protein low carbohydrate intake treatment was markedly higher compared to high protein high carbohydrate intake treatment.

Rapamycin significantly reduced the expression of G6Pase I and G6Pase II, which is consistent with the observations in article 4 (Dai et al., 2014). This reduction probably accounted for the reduced glycemia and was related to the enhanced plasma free amino acid levels.

PUBLICATION - 3

Do high levels of amino acids negatively influence insulin signaling and action in fish?

Figure 3. 7 Experimental design and procedures from the third article

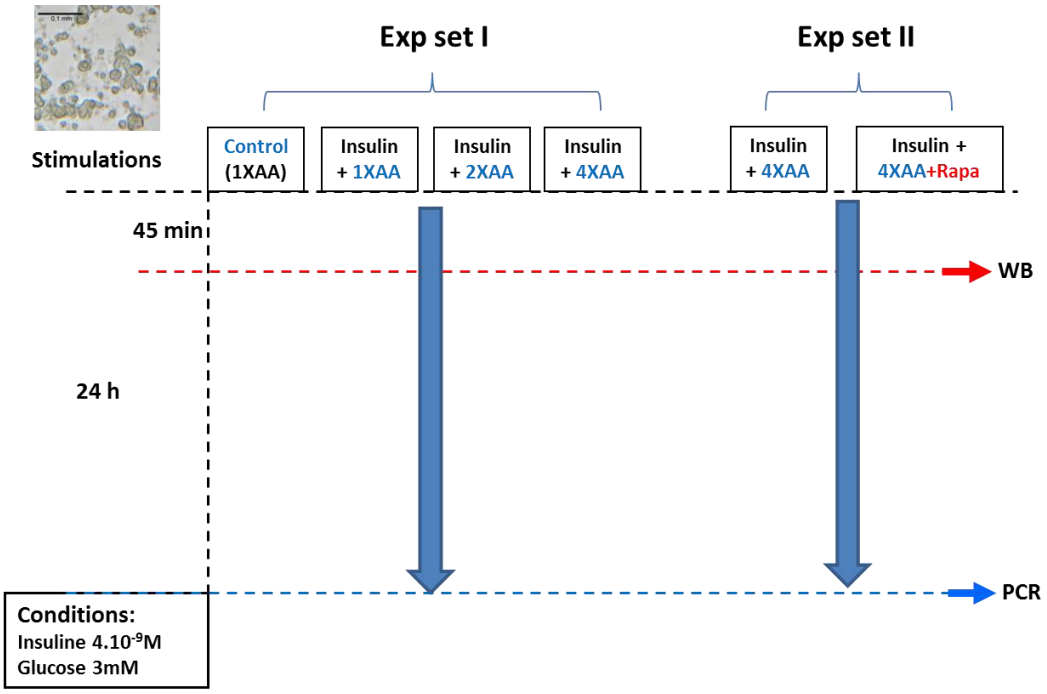
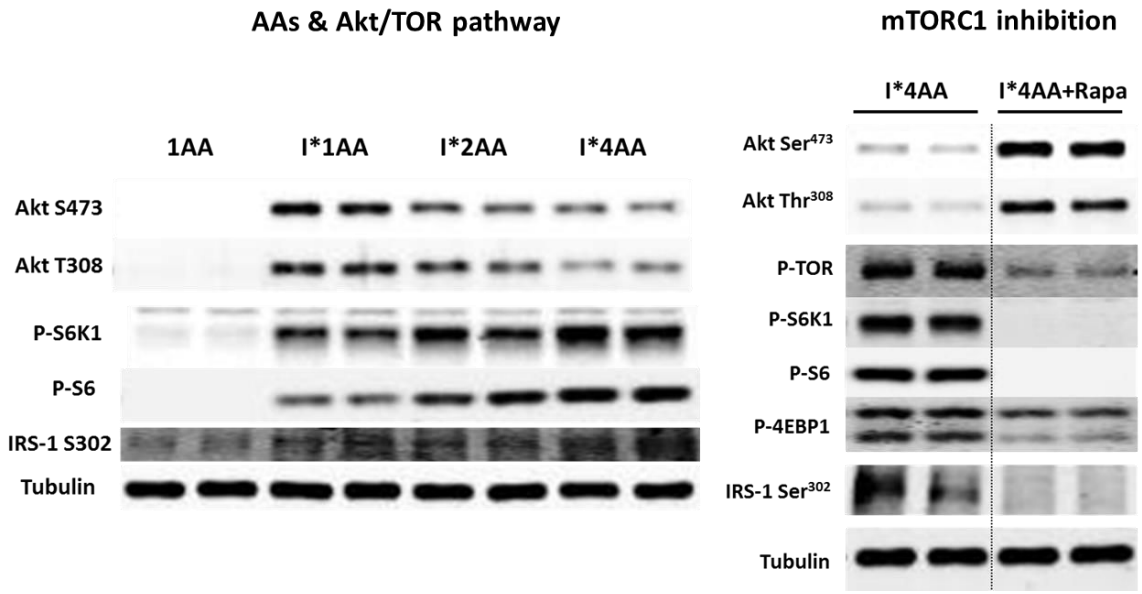


Figure 3. 8 Result highlights from the third article – Phosphorylation of representational proteins on Akt/TOR signaling pathway in trout hepatocytes



Increased AA levels not only elevated the phosphorylation of mTORC1 downstream effectors, S6K1 and S6, but also enhanced the phosphorylation of IRS-1 on Ser^{302} and decreased the phosphorylation of Akt on both Ser^{473} and Thr^{308}. Rapamycin not only inhibited the proteins on mTORC1 signaling pathway but also inhibited IRS-1 Ser^{302} phosphorylation and restored Akt phosphorylation.

Presentation of the article

Objective

Rainbow trout, like the other carnivorous animals consumes diets with relatively high protein content compared to humans, rodents and other terrestrial domestic animals. In fish as in other vertebrates, amino acids not only serve as building blocks for protein synthesis but also modulate signaling pathways, regulating not only protein metabolism but also glucose and lipid metabolism. In mammals, the over-activation of mTOR signaling pathway induced by excessive amino acids can result in a negative feedback loop on the early events of insulin signaling pathway that may lead to insulin resistance (Zoncu et al., 2011). This feedback involves phosphorylation of IRS1 on serine residues (Boura-Halfon and Zick, 2009; Taniguchi et al., 2006). Due to the high dietary protein content in trout (more than 40%), we proposed to verify the occurrence of such mechanism in trout and analyze its potential consequences on metabolism-related gene expression. *In vitro* studies using freshly isolated trout hepatocytes were designed to evaluate the effects of increasing levels of AAs on the regulation of hepatic fatty acid biosynthetic, glycolytic, gluconeogenic and amino acid catabolic gene expression and also assess the potential involvements of Akt/mTORC1 and p38 MAPK signaling pathway in these regulations.

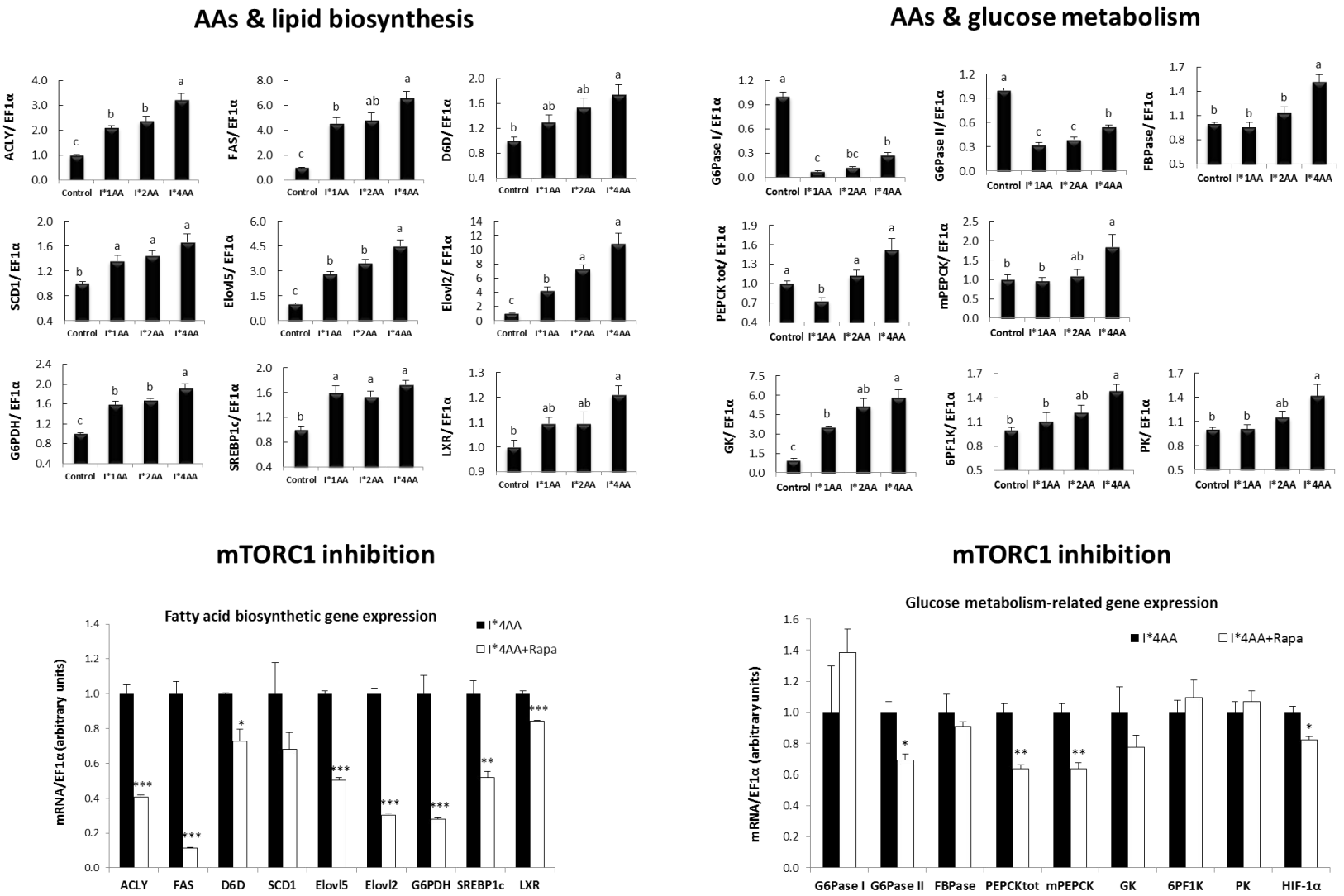
Experiment and Analyses

For the first experiment involving different amino acid levels (Figure 3.7 Exp set I), 48 h-cultured cells were stimulated with the mediums containing 4×10^{-9} mol l^{-1} of bovine insulin (Sigma, St. Louis, MO, USA) corresponding to the post-prandial level of insulin irrespective of dietary carbohydrate level (del sol Novoa et al., 2004), and one fold (I*1AA), two fold (I*2AA), or four fold concentrated MEM essential and non-essential amino acids mixture (I*4AA), respectively. Hepatocytes maintained in the basic culture medium (1AA without insulin) were used as control cells. In the second set of experiments (Figure 3.7 Exp set II), 48 h-cultured hepatocytes were pre-incubated for 30 min with or without 100 nM rapamycin (Rapa), a pharmacological TOR inhibitor. The mediums were then replaced by the medium containing 4×10^{-9} mol l^{-1} insulin and four fold concentrated essential and non-essential amino acids mixture (I*4AA). Cells were harvested 45 min after stimulation for western blot analysis or resuspended in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) 24 h after stimulation and stored at -80°C for subsequent mRNA extraction (Figure 3.7). Phosphorylation of proteins of the Akt/TOR signaling pathway and expression of genes related to fatty acid biosynthesis, glucose metabolism and AA catabolism were measured.

Results and Conclusion

Increased AA levels enhanced the phosphorylation of S6K1, S6, and IRS-1 on Ser^{302} but suppressed Akt and p38 MAPK phosphorylation. High levels of AAs also up-regulated the expression of genes related to gluconeogenesis and fatty acid biosynthesis. mTORC1 inhibition not only inhibited the

Figure 3. 9 Result highlights from the third article – Gene expression of markers of hepatic lipid biosynthesis and glucose metabolism

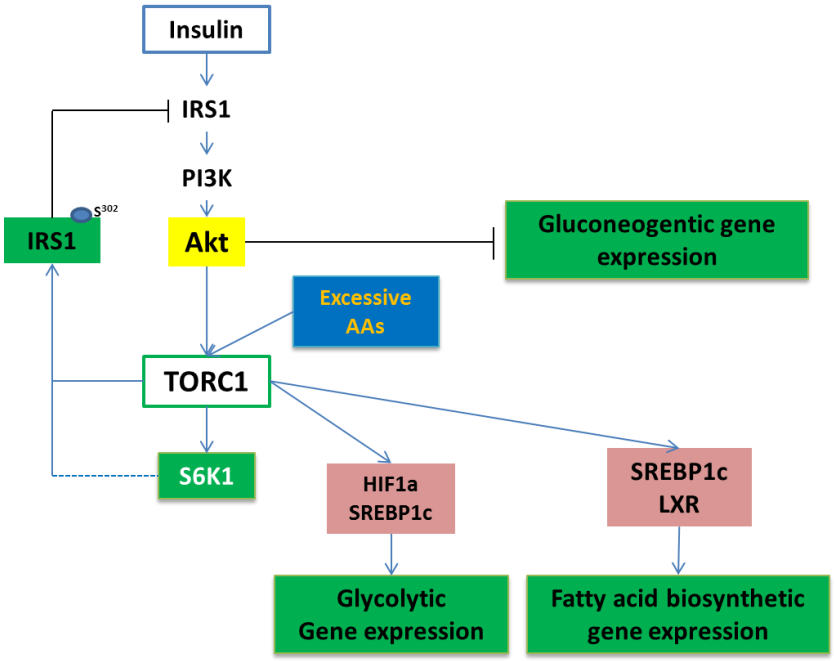


phosphorylation of mTORC1 downstream targets, but also blunted IRS-1 phosphorylation on Ser^{302} and restored excessive AAs-suppressed Akt phosphorylation (Figure 3.8 right). Rapamycin also inhibited fatty acid biosynthetic and gluconeogenic gene expression (Figure 3.9 lower panel).

Moreover, increased AA levels also markedly elevated the expression of genes related to AA catabolism, suggesting that elevated AA levels promoted hepatic fatty acid biosynthesis and glucose metabolism not only through modulating relevant signaling pathways, but also by providing gluconeogenic and lipogenic substrates.

In conclusion, we demonstrated that, in trout primary hepatocytes, increased AA levels (i) up-regulate fatty acid biosynthetic gene expression through an mTORC1-dependent manner, (ii) attenuate insulin-mediated repression on hepatic gluconeogenesis through elevated IRS-1 phosphorylation on Ser^{302}, which in turn impairs the activation of Akt pathway and weakens insulin action (Figure 3.10) and (iii) elevate AA catabolic gene expression. The present study highlights the key role of amino acids and mTORC1 signaling in this negative feedback that might explain the absence of postprandial inhibition of hepatic gluconeogenesis and the glucose intolerance phenotype in rainbow trout.

Figure 3. 10 Main conclusions from the third article



Cellular Physiology and Biochemistry

Cell Physiol Biochem 2015;36:1084-1100
DOI: 10.1159/000430281
Published online: June 25, 2015
Accepted: April 20, 2015

1421-9778/15/0363-1084$39.50/0

Original Paper

Amino Acids Attenuate Insulin Action on Gluconeogenesis and Promote Fatty Acid Biosynthesis via mTORC1 Signaling Pathway in trout Hepatocytes

Weiwei Dai Stéphane Panserat Elisabeth Plagnes-Juan Iban Seiliez
Sandrine Skiba-Cassy

INRA, UR 1067 Nutrition, Métabolisme, Aquaculture, Pole d'hydrobiologie, CD 918, Saint-Pée-sur-Nivelle, France

Key Words
Amino acids • mTORC1 • Insulin action • Fatty acid biosynthesis • Gluconeogenesis • Rainbow trout

Abstract
Background/Aims: Carnivores exhibit poor utilization of dietary carbohydrates and glucose intolerant phenotypes, yet it remains unclear what are the causal factors and underlying mechanisms. We aimed to evaluate excessive amino acids (AAs)-induced effects on insulin signaling, fatty acid biosynthesis and glucose metabolism in rainbow trout and determine the potential involvement of mTORC1 and p38 MAPK pathway. ***Methods:*** We stimulated trout primary hepatocytes with different AA levels and employed acute administration of rapamycin to inhibit mTORC1 activation. ***Results:*** Increased AA levels enhanced the phosphorylation of ribosomal protein S6 kinase (S6K1), S6, and insulin receptor substrate 1 (IRS-1) on Ser^{302} but suppressed Akt and p38 phosphorylation; up-regulated the expression of genes related to gluconeogenesis and fatty acid biosynthesis. mTORC1 inhibition not only inhibited the phosphorylation of mTORC1 downstream targets, but also blunted IRS-1 Ser^{302} phosphorylation and restored excessive AAs-suppressed Akt phosphorylation. Rapamycin also inhibited fatty acid biosynthetic and gluconeogenic gene expression. ***Conclusion:*** High levels of AAs up-regulate hepatic fatty acid biosynthetic gene expression through an mTORC1-dependent manner, while attenuate insulin-mediated repression of gluconeogenesis through elevating IRS-1 Ser^{302} phosphorylation, which in turn impairs Akt activation and thereby weakening insulin action. We propose that p38 MAPK probably also involves in these AAs-induced metabolic changes.

Sandrine Skiba-Cassy

INRA, UR1067 Nutrition, Métabolisme, Aquaculture
F-64310 Saint-Pée-sur-Nivelle (France)
Tel: +33 5 59 51 59 93, Fax +33 5 59 54 51 52, E-Mail skiba@st-pee.inra.fr

Introduction

The liver is a key organ in the systemic response to insulin, controlling both glucose and lipid metabolism [1]. In hepatocytes, binding of insulin to their receptors leads to recruitment and phosphorylation of the insulin receptor substrate 1 (IRS-1) on tyrosine residues, which in turn recruits phosphoinositide 3-kinase (PI3K) and activates Akt, a critical node of the insulin signaling pathway leading to at least the regulation of glucose uptake, glycogen synthesis, glycolysis and gluconeogenesis [2]. The activation of Akt inhibits glucose production through direct phosphorylation of transcription factors Forkhead-box Class O1 (FoxO1) [3] and peroxisome proliferator-activated receptor γ coactivator 1α (PGC)-1α [4]. Another important anabolic role of insulin is to activate fatty acid synthesis. Insulin stimulates hepatic lipogenesis by increasing the mRNA and the processed nuclear form of sterol regulatory element-binding protein-1c (SREBP-1c), a transcription factor that activates all the genes needed to produce fatty acids and triglycerides in liver [5]. Insulin-dependent SREBP-1c transcriptional regulation also requires liver X receptor (LXR) activation [6, 7].

Dietary proteins and amino acids (AAs) have emerged as potent modulators of insulin signaling, thereby regulating glucose and lipid metabolism. Under appropriate conditions, AAs (particularly leucine, isoleucine, alanine, and arginine) can enhance pancreatic insulin secretion and repress hepatic gluconeogenesis through the IRS-Akt-Foxo pathway [8, 9]. Furthermore, AAs abundance can be sensed by mechanistic target of rapamycin complex 1 (mTORC1) [10], a crucial signaling node controlling cell growth and proliferation, which promotes *de novo* lipogenesis and glycolysis, through mTORC1-mediated activation of SREBP-1c and hypoxia inducible factor 1a (HIF1a), respectively [11, 12]. Moreover, excessive AAs can induce elevated activation of the mTORC1/S6K1 pathway, which elicits a negative feedback signal that hyperphosphorylates IRS-1 at serine residues, leading to reduced IRS-1 function and impaired activation of the PI3K/Akt pathway, thereby diminishing insulin action [13, 14]. Persistent activation of mTORC1 signaling pathway may lead to insulin resistance [14, 15], which is defined as the inability of insulin to promote efficient glucose uptake by peripheral tissues, and commonly associated with obesity, human type 2 diabetes and nonalcoholic fatty liver disease [16, 17].

The p38 mitogen-activated protein kinase (MAPK) is a major kinase in the MAPK family and plays a pivotal role in regulating many essential cellular processes, such as proliferation, inflammation, differentiation, survival and migration [18, 19]. Recent studies show that p38 MAPK also responds to key metabolic factors, thereby controlling glucose and lipid metabolism [20]. Cao et al. [21] demonstrated that activation of p38 by fasting in liver and glucagon in primary hepatocytes stimulates hepatic gluconeogenesis via regulating the phosphorylation of cAMP-response element-binding protein (CREB) and the expression of PGC1α. Furthermore, p38 plays an inhibitory role on hepatic lipogenesis, probably through the inhibtion of the transcription of central lipogenic genes, such as SREBP-1c and PGC-1β [22]. However, whether nutrients regulate p38 MAPK activation, thereby regulating intermediary metabolism remains largely unknown.

Rainbow trout, like domestic cat and dolphin, exhibits relatively high dietary protein/AAs requirement, poor utilization of dietary carbohydrates and glucose intolerant phenotype, thus it represents a relevant carnivorous model organism for the investigation of protein and glucose metabolism [23, 24]. Furthermore, given that healthy carnivores exhibit metabolic changes after fasting or a carbohydrate rich diet that are similar to those observed in humans with diabetes, trout can be used as a comparative natural model for the study of common pathologies associated with type 2 diabetes [23]. Therefore, understanding how nutrients regulate intermediary metabolism in trout may have dual significance.

Despite the well-studied roles of protein/AAs in modulating insulin action and intermediary metabolism in mammals, relatively little is known in trout. Diets of rainbow trout generally contain more than 40% protein, which has long been suspected as a causal factor for the persistent hyperglycemia and absence of postprandial down-regulation of

gluconeogenesis [25, 26]. In line with this hypothesis, Kirchner et al. [26] observed that low protein intake reduced hepatic gluconeogenic enzyme activities and gene expression (FBPase and G6Pase). Furthermore, in a previous study, we also demonstrated that AAs increased gluconeogenic mRNA levels (G6Pase and mPEPCK) independently of the insulin/TOR signaling pathway [27]. However, whether excessive AAs elevate hepatic gluconeogenesis via affecting insulin signaling has not been investigated in fish thus far. As for the regulation of lipogenesis, previous *in vivo* studies tend to indicate that dietary protein play a potent role in regulating mTOR signaling pathway [28], and lipogenic and glycolytic pathways in fish [28-30]. Moreover, our *in vitro* studies demonstrated that AAs (particularly leucine) together with insulin successfully stimulated the activation of mTOR signaling pathway [27, 31], which was proven as an upstream activator for hepatic lipogenesis in trout liver and hepatocytes [27, 32]. However, whether different AA levels affect mTORC1 activation and thereby regulating hepatic lipogenesis in fish is still unknown.

The aim of this work was to (1) study whether excessive amino acids (AAs) affect the regulation of hepatic metabolism-related gene expression and (2) determine the potential involvement of mTORC1 and p38 MAPK pathways in these regulations.

Material and Methods

Animals

Sexually immature rainbow trout (240 to 300g) were obtained from the INRA experimental fish farm facilities (INRA, Donzacq, France). Fish were maintained in tanks kept in open circuits at 18°C with well-aerated water under natural photoperiod conditions. Trout were fed to satiety every two days with a commercial diet (T-3P classic, Trouw, France). The experiments were carried out in accordance with the clear boundaries of EU legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e. Directive 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decree no. 2001-464, 29 May, 2001). The investigators carrying out the experiment had ''level 1'' or ''level 2'' certification, bestowed by the Direction Départementale des Services Vétérinaires (French veterinary services) to carry out animal experiments (INRA 2002-36, 14 April, 2002).

Hepatocyte Cell Culture

Isolated liver cells were prepared as previously described [25, 27]. Cells were finally taken up in modified Hanks' medium supplemented with 1.5 mM $CaCl_2$, 1% defatted BSA, 3 mM glucose, MEM essential amino acids (1X) (Invitrogen Carlsbad, California, USA), MEM non-essential amino acids (1X) (Invitrogen Carlsbad, California, USA) and antibiotic antimycotic solution (1X) (Sigma, St. Louis, MO, USA) as the basic culture medium. Cell viability (>98%) was assessed using the trypan blue exclusion method (0.04% in 0.15 M NaCl) and cells were counted using a haemocytometer. The hepatocyte cell suspension was plated in a six well Primaria culture dish (BD, USA) at a density of 3×10^6 cells/ well and incubated at 18°C. The culture medium was changed every 24 h over the 48 h of primary cell culture. Microscopic examination ensured that hepatocytes progressively re-associated throughout culture to form two-dimensional aggregates, in agreement with earlier reports [33, 34]. For the first experiment involving different amino acid levels, 48 h-cultured cells were stimulated with the mediums containing 4×10^{-9} mol l^{-1} of bovine insulin (Sigma, St. Louis, MO, USA) corresponding to the post-prandial level of insulin irrespective of dietary carbohydrate level [35], and one fold (I*1AA), two fold (I*2AA), or four fold concentrated MEM essential and non-essential amino acids mixture (I*4AA), respectively, while the control cells maintained the basic culture medium (1AA but no insulin). In the second experiment, 48 h-cultured hepatocytes were pre-incubated for 30 min with or without 100 nM rapamycin (Rapa), a pharmacological TOR inhibitor. The mediums were then replaced by the medium containing 4×10^{-9} mol l^{-1} insulin and four fold concentrated MEM essential and non-essential amino acids mixture (I*4AA). Cells were harvested at 45 min for western blot analysis or resuspended in TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) 24 h after stimulation and stored at -80°C for subsequent mRNA extraction. The time intervals were chosen based on the phosphorylation peaks of TOR signaling pathway or relevant metabolic gene expression peaks, respectively (data not shown).

Protein extraction and Western blotting

At the end of the stimulation period, cells were carefully washed with two times 1ml of cold phosphate buffered saline (PBS). Then 300µl of cell lysis buffer [36] were added before being stored the culture dish at -80°C overnight. Then cells were scraped, collected, and lysed on ice for 30 min. Lysates were centrifuged at 12.000g for 30 min at 4°C. The resulting supernatant fractions were recovered and stored at -80°C. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with BSA as standard. Lysates (2 µg of total protein per lane for Akt/S6/4EBP1, 7 µg for TOR/S6K1/p38 MAPK and 17 µg for IRS-1, respectively) were subjected to SDS-PAGE and Western blotting using the appropriate antibodies. Anti-phospho-Akt (Ser^{473}) (no. 9271), anti-phospho-Akt (Thr^{308}) (no. 9275), anti-carboxyl terminal Akt (no. 9272), anti-phospho-TOR (Ser^{2448}) (no. 2971), anti-TOR (no. 2972), anti-phospho-S6 protein kinase 1 (Thr^{389}) (no. 9205), anti-S6 protein kinase 1 (no. 9202), anti-phospho-S6 ($Ser^{235/236}$) (no. 4856), anti-S6 (no. 2217), anti-phospho-4E-BP1 ($Thr^{37/46}$) (no. 9459), anti-4E-BP1 (no. 9452), anti-phospho-IRS-1 (Ser^{302}) (no. 2384), anti-phospho-p38 MAPK (no. 9211) and anti-β-Tubulin (no. 2146) were purchased from Cell Signaling Technologies (Ozyme, Saint Quentin Yvelines, France). Anti-p38 MAPK (no. sc-535) was purchased from Santa Cruz Biotechnology **(**Dallas, Texas, USA**).** All of these antibodies (except anti-phospho-IRS-1 (Ser^{302}), anti-phospho-p38 MAPK and anti-p38 MAPK) were successfully cross-reacted with rainbow trout [32]. For anti-phospho-IRS-1 (Ser^{302}), anti-phospho-p38 MAPK and anti-p38 MAPK antibody, the molecular weight and amino acid sequences were monitored in the SIGENAE database [37] to check for a good conservation of the antigen sequence. After washing, membranes were incubated with an IRDye Infrared secondary antibody (LI-COR Biosciences, Lincoln, NE, USA). Bands were visualized by Infrared Fluorescence using the Odyssey Imaging System (LI-COR Biosciences) and quantified by Odyssey Infrared imaging system software (Version 3.0, LI-COR Biosciences).

Gene expression analysis

Total RNA samples were extracted from hepatocytes using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations, quantified by spectrophotometry (absorbance at 260 nm) and its integrity was assessed using Agarose Gel Electrophoresis. A 1 µg sample of the resulting total RNA was reverse transcribed into cDNA using the SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and random primers (Promega, Charbonniéres, France) according to the manufacturers' instructions. Target gene expression abundance was determined by quantitative real-time (q) RT-PCR, using specific primers [38-42]. Primers targeting alanine transaminase 2 (forward: TGGGTGCGTACAGTGCCAGT; reverse: GACGCACCCTCACCACACAC; Sigenae AU081029.s.om.10), aspartate transaminase 1 (forward: TCAAGAGTGGCAGGAACATCA; reverse: AGCGTCTCTGAAGATGGGTGT; Sigenae CA359859.s.om.10), aspartate transaminase 2 (forward: TCTGTGCCCAGTCCTTCTC; reverse: GGAGGGTTGGACCAGGT; Sigenae CA344854.s.om.10.) and branched-chain α-ketoacid dehydrogenase α subunit (forward: TCGCTGTGTACAACGCTACC; reverse: GCCTCCATCACCATCTTGC) were newly designed using Primer3 software. To confirm specificity, amplicons were purified and sequenced (Beckman Coulter Genomics, UK).

qRT-PCR was performed as previously described [36]. Quantitative RT-PCR analyses were focused on several key enzymes of hepatic metabolism, which were glucokinase (GK; EC 2.7.1.2), 6-phosphofructo-1-kinase (6PF1K; EC 2.7.1.11), pyruvate kinase (PK; EC 2.7.1.40) and transcription factor hypoxia-inducible factor 1-alpha (HIF-1α) for glycolysis; glucose transporter 2 (GLUT2) for glucose transport ; glucose-6-phosphatase (G6Pase; EC 3.1.3.9) for glucose dephosphorylation; fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) and phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) for gluconeogenesis; ATP citrate lyase (ACLY; EC 2.3.3.8) and fatty acid synthase (FAS; EC 2.3.1.85) for lipogenesis; glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) for pentose phosphate shunt; Δ6 fatty acyl desaturase (D6D; EC 1.14.19.3), Stearoyl-CoA desaturase-1 (SCD1 or D9D; EC 1.14.19.1), elongation of very long chain fatty acids like-5 (Elovl5; EC 2.3.1.199) and elongation of very long chain fatty acids like-2 (Elovl2; EC 2.3.1.199) for fatty acid bioconversion, and two relevant transcription factors sterol regulatory element binding protein 1-like (SREBP1c) and liver X receptor (LXR); serine dehydratase (SD; EC:4.3.1.17), alanine transaminase (ALAT; EC 2.6.1.2), aspartate transaminase (ASAT; EC 2.6.1.1), branched-chain α-ketoacid dehydrogenase (BCKD; EC 1.2.4.4) and branched-chain α-ketoacid dehydrogenase kinase (BCKDK; EC 2.7.11.4) for amino acid catabolism. When different isoforms of a gene were known in rainbow trout (as for G6Pase), gene expression analysis was performed on each isoform. Elongation factor-1alpha (EF1α) was employed as

a non-regulated reference gene, as previously used in rainbow trout and it was stably expressed in our investigations (data not shown).

Statistical analysis

The results of western blot (N=4) and gene expression (N=6) are expressed as means + s.e.m. and were analyzed using one-way ANOVA, followed by a Tukey test. In cases where data were nonparametric or not homoscedastic, data transformations, such as logarithms, square roots and reciprocals, were used to meet ANOVA criteria. Normality was assessed using the Shaprio–Wilk test, while homoscedasticity was determined using Levene's test. For all statistical analyses, the level of significance was set at P<0.05.

Results

Amino acid levels affected the phosphorylation of Akt/TOR signaling pathway

First, we investigated whether the phosphorylation of Akt/TOR signaling pathway was affected by different amino acid levels (Fig. 1).

As expected, I*1AA treated cells showed higher Akt (on Thr^{308} and Ser^{473}), S6K1 and S6 phosphorylation levels compared to control cells, indicating that insulin effectively triggered

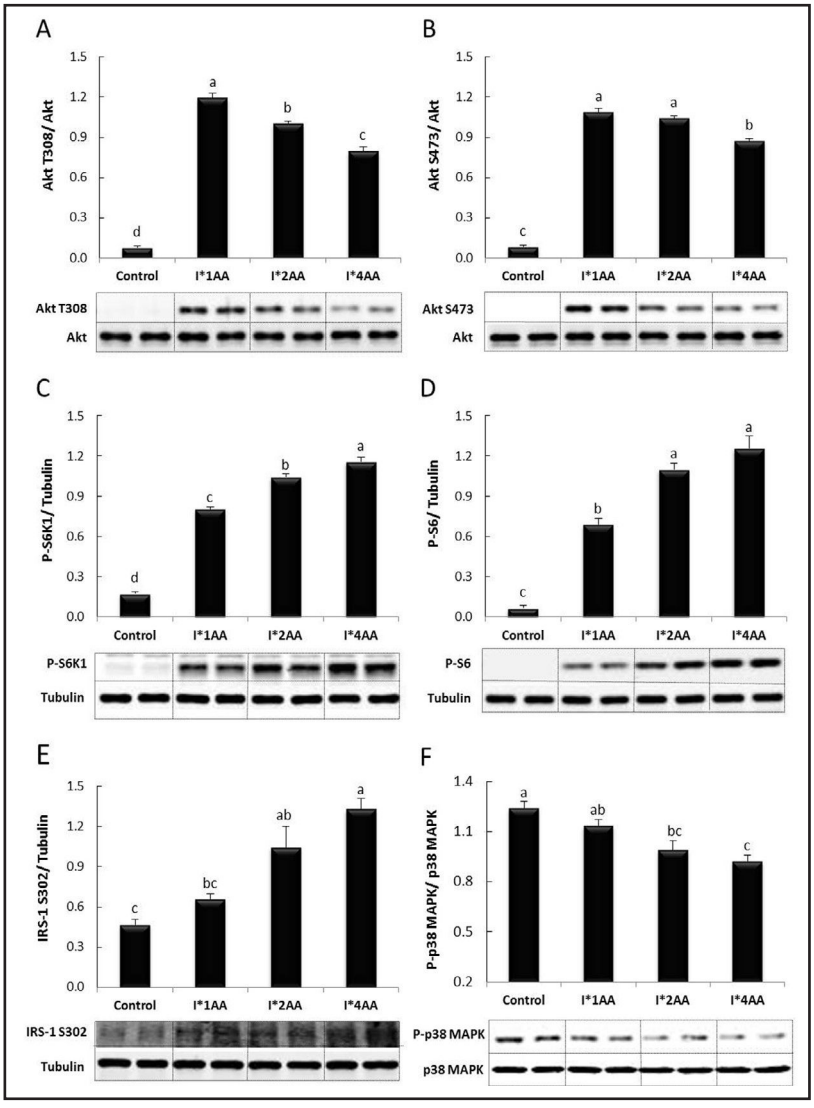


Fig. 1. Effects of different amino acid levels on the phosphorylation of (A) Akt Ser^{473}, (B) Akt Thr^{308}, (C) S6K1, (D) S6, (E) IRS-1 Ser^{302} and (F) p38 MAPK protein in trout hepatocytes. The experiment was conducted in 48 h-cultured trout hepatocytes. Cells were then stimulated for 45 min with the mediums containing 4×10^{-9} mol l^{-1} insulin (I), and one fold (I*1AA), two fold (I*2AA), or four fold concentrated amino acids (I*4AA), respectively, while the control cells maintained the basic culture medium (1AA but no insulin). Gels were loaded with 2 μg of total protein per lane for Akt/S6, 7 μg for S6K1/p38 MAPK and 17 μg for IRS-1, respectively. A representative blot is shown. Results are means + s.e.m. (N=4) and were analyzed using one-way ANOVA, followed by a Tukey test. Values with different superscript letters are significantly different at P<0.05.

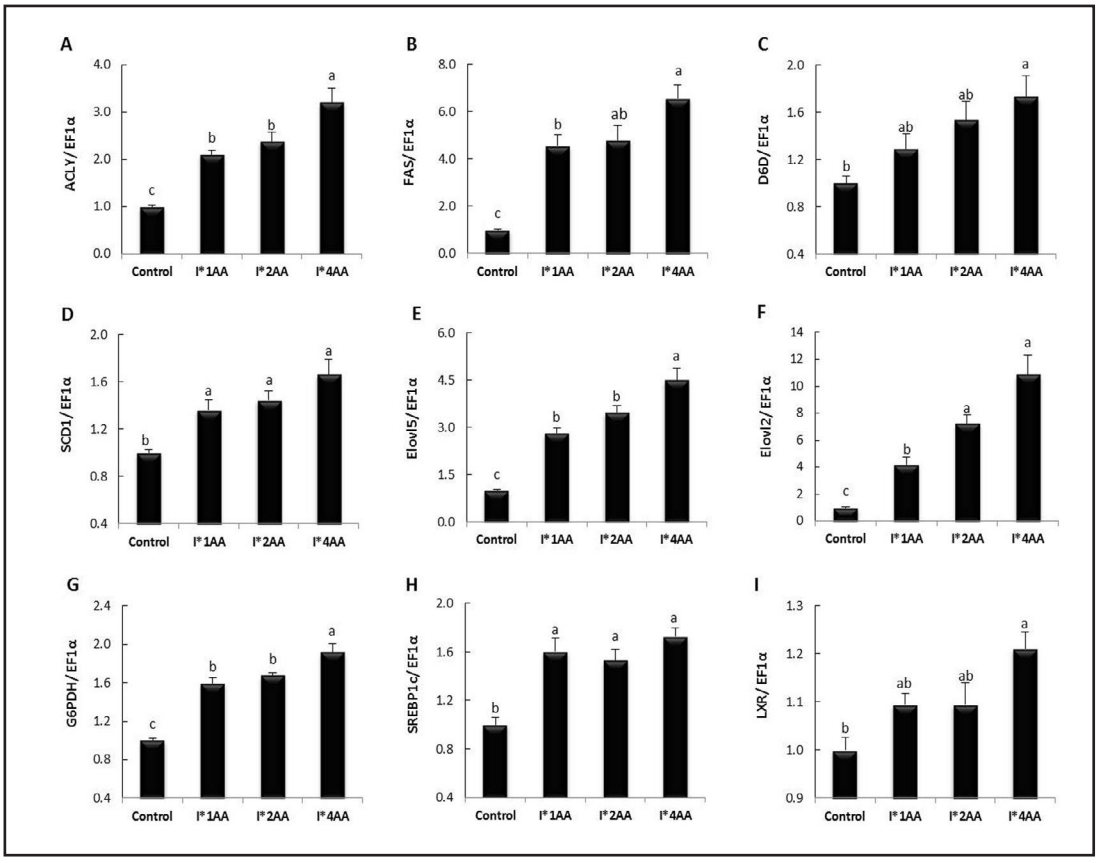


Fig. 2. Effects of different amino acid levels on the regulation of fatty acid biosynthetic gene expression in trout hepatocytes. mRNA levels of genes involved in *de novo* lipogenesis including (A) ACLY and (B) FAS, fatty acid bioconversion including (C) D6D, (D) SCD1, (E) Elovl5 and (F) Elovl2, NADPH production including (G) G6PDH, and relevant transcriptional factors (H) SREBP1c and (I) LXR in the primary hepatocytes of rainbow trout after 24 h stimulation. The experiment was conducted in 48 h-cultured trout hepatocytes. Cells were then stimulated with the mediums containing 4×10^{-9} mol l^{-1} insulin (I), and one fold (I*1AA), two fold (I*2AA), or four fold concentrated amino acids (I*4AA), respectively, while the control cells maintained the basic culture medium (1AA but no insulin). Expression values are first normalized with elongation factor-1 alpha (EF1α)-expressed transcripts then further normalized to the levels of control hepatocytes. Results are means + s.e.m. (N=6) and were analyzed using one-way ANOVA, followed by a Tukey test. Values with different superscript letters are significantly different at P<0.05.

the activation of Akt/TOR signaling pathway as previously demonstrated [27]. Of note, the phosphorylation of IRS-1 on Ser302 and p38 MAPK was not affected by the addition of insulin in 1AA treated cells.

Increased amino acid levels gradually enhanced S6K1 phosphorylation (Fig. 1C). Furthermore, phosphorylated S6 in I*2AA and I*4AA treated cells was also higher compared to I*1AA treated cells (Fig. 1D). In contrast, increased amino acid levels gradually decreased Akt phosphorylation on Thr^{308} (Fig. 1A) and the phosphorylation of Akt on Ser^{473} in I*4AA treated cells was significantly lower than in I*1AA or I*2AA treated cells (Fig. 1B). Because the phosphorylation of IRS-1 on serine residues is known as a potential link between TOR/S6K1 and Akt protein phosphorylation in mammals, we further studied the phosphorylation of IRS-1 on Ser^{302} and found that phosphorylated IRS-1 on Ser^{302} in I*4AA treated cells was significantly higher than in I*1AA treated cells (Fig. 1E). Moreover, the phosphorylation of p38 MAPK in I*4AA treated cells was markedly lower compared to I*1AA treated cells (Fig. 1F).

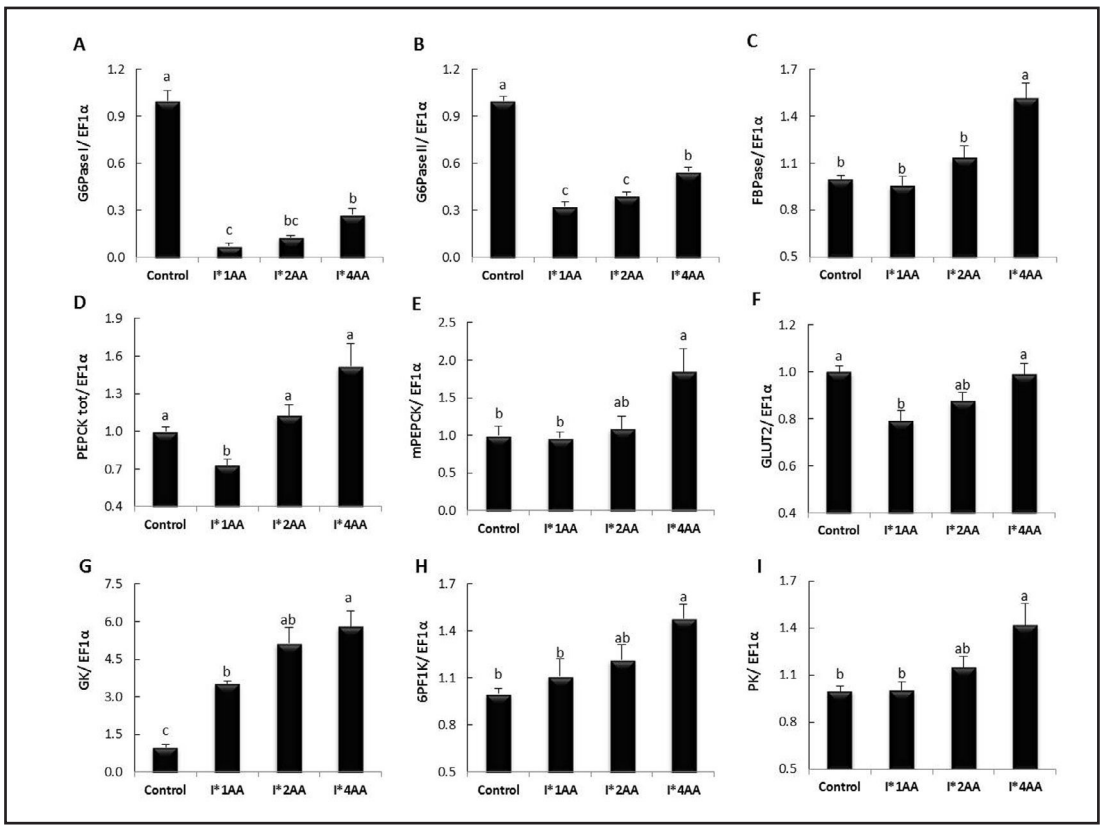


Fig. 3. Effects of different amino acid levels on the regulation of glucose metabolism-related gene expression in trout hepatocytes. mRNA levels of genes involved in gluconeogenesis including (A) G6Pase I, (B) G6Pase II, (C) FBPase, (D) PEPCKtot and (E) mPEPCK, glucose transport including (F) GLUT2, and glycolysis including (G) GK, (H) 6PF1K and (I) PK in the primary hepatocytes of rainbow trout after 24 h stimulation. The experiment was conducted in 48 h-cultured trout hepatocytes. Cells were then stimulated with the mediums containing 4×10^{-9} mol l^{-1} insulin (I), and one fold (I*1AA), two fold (I*2AA), or four fold concentrated amino acids (I*4AA), respectively, while the control cells maintained the basic culture medium (1AA but no insulin). Expression values are first normalized with elongation factor-1 alpha (EF1α)-expressed transcripts then further normalized to the levels of control hepatocytes. Results are means + s.e.m. (N=6) and were analyzed using one-way ANOVA, followed by a Tukey test. Values with different superscript letters are significantly different at P<0.05.

Fatty acid biosynthetic gene expression was up-regulated by high level of AAs

We further investigated the effects of different amino acid levels on the regulation of fatty acid biosynthetic gene expression in trout hepatocytes (Fig. 2).

Except D6D (Fig. 2C) and LXR (Fig. 2I), all the examined mRNA levels of the key enzymes involved in *de novo* lipid synthesis including ACLY, FAS, SCD1, Elovl5, Elovl2 and G6PDH, and SREBP1c, a transcription factor that activates *de novo* lipogenesis, were all significantly up-regulated in I*1AA treated cells compared to control cells, indicating that insulin significantly up-regulated fatty acid biosynthetic gene expression in trout hepatocytes, which is consistent with previous demonstrations in trout [27, 43] and mammals [44].

Moreover, mRNA levels of the key enzymes involved in *de novo* lipogenesis, including ACLY (Fig. 2A) and FAS (Fig. 2B), fatty acid bioconversion including Elovl5 (Fig. 2E) and Elovl2 (Fig. 2F), and NADPH production including G6PDH (Fig. 2G) were all significantly higher in I*4AA treated cells than in I*1AA treated cells. Furthermore, similar enhancements were also observed between I*2AA and I*4AA treated cells for the mRNA levels of ACLY (Fig. 2A), Elovl5 (Fig. 2E) and G6PDH (Fig. 2G). Of note, despite no significant difference was observed

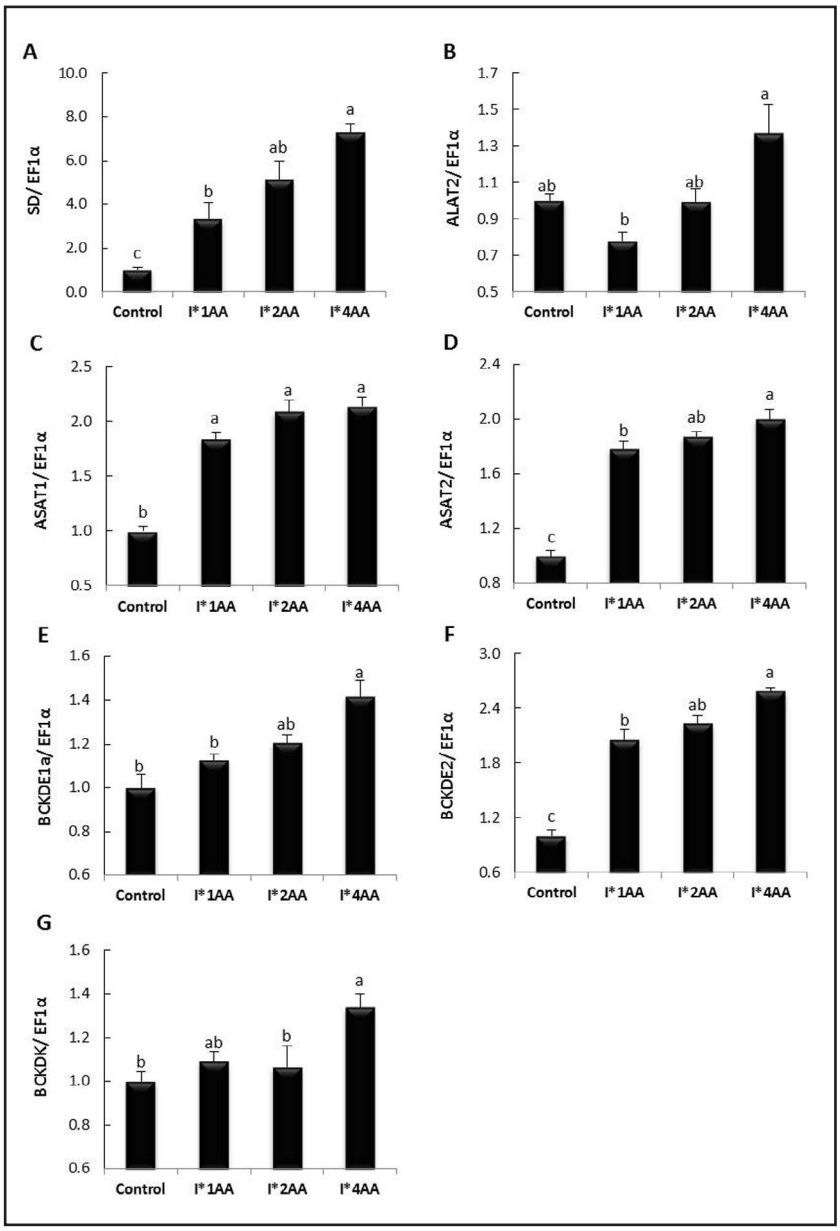


Fig. 4. Effects of different amino acid levels on the regulation of amino acid catabolic gene expression in trout hepatocytes. mRNA levels of genes involved in amino acid catabolism including (A) SD, (B) ALAT2, (C) ASAT1, (D) ASAT2, (E) BCKDE1α, (F) BCKDE2 and (G) BCKDK in the primary hepatocytes of rainbow trout after 24 h stimulation. The experiment was conducted in 48 h-cultured trout hepatocytes. Cells were then stimulated with the mediums containing 4×10^{-9} mol l^{-1} insulin (I), and one fold (I*1AA), two fold (I*2AA), or four fold concentrated amino acids (I*4AA), respectively, while the control cells maintained the basic culture medium (1AA but no insulin). Expression values are first normalized with elongation factor-1 alpha (EF1α)-expressed transcripts then further normalized to the levels of control hepatocytes. Results are means + s.e.m. (N=6) and were analyzed using one-way ANOVA, followed by a Tukey test. Values with different superscript letters are significantly different at P<0.05.

for the mRNA levels of D6D (Fig. 2C), SCD1 (Fig. 2D) or LXR (Fig. 2I), they all exhibited similar patterns as the other examined fatty acid biosynthetic genes, which were positively regulated by increased amino acid levels.

High level of AAs up-regulated gluconeogenic and glycolytic gene expression

We also investigated the effects of different amino acid levels on the regulation of gluconeogenic and glycolytic gene expression in trout hepatocytes (Fig. 3). The addition of insulin significantly decreased the mRNA levels of G6Pase I (Fig. 3A), G6Pase II (Fig. 3B), PEPCKtot (Fig. 3D) and GLUT2 (Fig. 3F), and significantly increased GK mRNA level (Fig. 3G), while no significant variations was observed for the mRNA levels of FBPase (Fig. 3C), mPEPCK (Fig. 3E), 6PF1K (Fig. 3H) and PK (Fig. 3I). Messenger RNA levels of the key enzymes involved in gluconeogenesis (G6Pase I, G6Pase II, FBPase, PEPCKtot and mPEPCK),

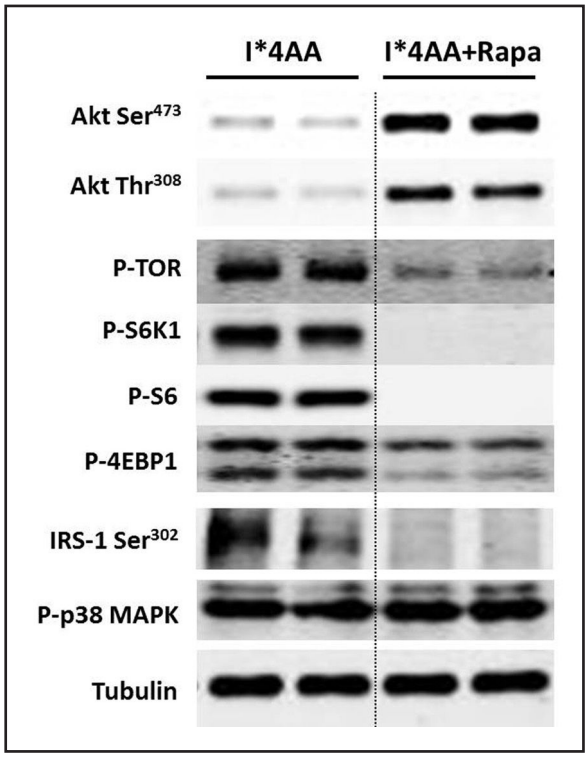


Fig. 5. Effects of mTORC1 overactivation (I*4AA) and inhibition (I*4AA+Rapa) on the phosphorylation of Akt Ser^{473}, Akt Thr^{308}, TOR, S6K1, S6, 4E-BP1, IRS-1 Ser^{302} and p38 MAPK protein in trout hepatocytes. Cells were pre-incubated for 30 min with or without 100 nM rapamycin (Rapa), a TOR inhibitor. The culture medium was then replaced for 45 min by the medium containing 4×10^{-9} mol l^{-1} insulin (I) and four fold concentrated amino acids (I*4AA). Gels were loaded with 2 μg of total protein per lane for Akt/S6/4EBP1, 7 μg for TOR/S6K1/p38 MAPK and 17 μg for IRS-1, respectively. Western blots were performed on four individual samples and similar results were obtained. This figure includes representative blots.

glucose transporter (GLUT2), and glycolysis (GK, 6PF1K and PK) were all significantly higher in I*4AA treated cells than in I*1AA treated cells, indicating that high level AAs up-regulated gluconeogenic and glycolytic gene expression.

High level of AAs up-regulated AA catabolic gene expression

As shown in Fig.4, the addition of insulin upregulated the mRNA levels of SD (Fig. 4A), ASAT1 (Fig. 4C), ASAT2 (Fig. 4D) and BCKDE2 (Fig. 4F), while no changes for the mRNA levels of ALAT2 (Fig. 4B), BCKDE1α (Fig. 4E) and BCKDK (Fig. 4G). Despite no modulation for ASAT1, messenger RNA levels of SD, ALAT2, ASAT2, BCKDE1α and BCKDE2 were all significantly higher in I*4AA treated cells compared to I*1AA treated cells, and BCKDK increased in I*4AA treated cells compared to I*2AA treated cells, indicating that high level AAs up-regulated AA catabolic gene expression.

Excessive AAs decreased Akt phosphorylation through mTORC1-dependent manner

In order to investigate whether excessive AAs reduced Akt phosphorylation through mTORC1/S6K1-induced negative feedback loop involving the phosphorylation of IRS-1 on serine residues, we further treated trout hepatocytes with I*4AA in the presence or absence of rapamycin.

As expected, rapamycin partially inhibited the phosphorylation of TOR and 4E-BP1 and totally abolished the phosphorylation of S6K1 and S6 as previously observed [27, 32]. More importantly, we observed that rapamycin significantly inhibited the phosphorylation of IRS-1 on Ser^{302} and strongly increased the Akt phosphorylation (on Thr^{308} and Ser^{473}) (Fig. 5). p38 phosphorylation was not affected by rapamycin, indicating that the phosphorylation of p38 MAPK is mTORC1-independent, which is compatible with previous demonstration [45].

Inhibition of mTORC1 activation resulted in suppressed fatty acid biosynthetic and gluconeogenic gene expression

We further examined the effects of mTORC1 overactivation and inhibition on the regulation of fatty acid biosynthetic, gluconeogenic, glycolytic and AA catabolic gene expression in trout hepatocytes. Messenger RNA levels of the key enzymes involved in *de*

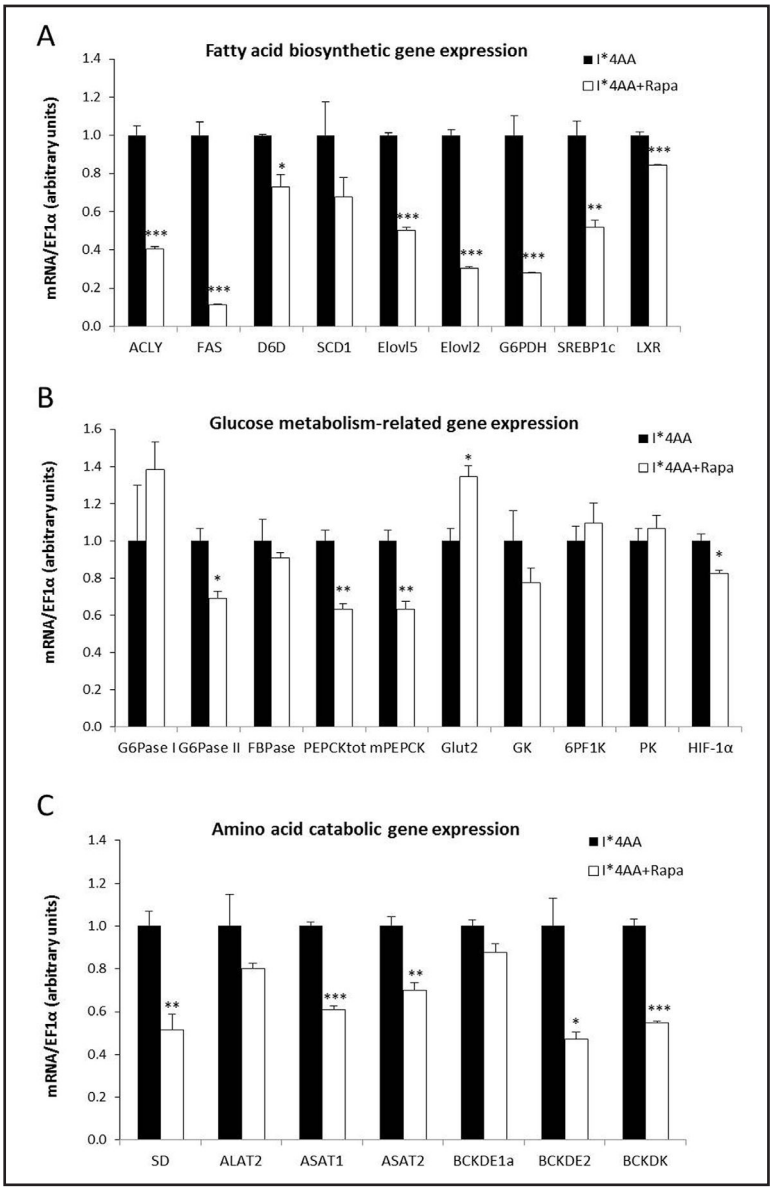


Fig. 6. Effects of mTORC1 overactivation (I*4AA) and inhibition (I*4AA+Rapa) on the regulation of fatty acid biosynthetic, gluconeogenic and glycolytic gene expression in trout hepatocytes. (A) mRNA levels of genes involved in *de novo* lipid synthesis including ACLY, FAS, D6D, SCD1, Elovl5, Elovl2, G6PDH, SREBP1c and LXR; (B) mRNA levels of genes involved in gluconeogenesis including G6Pase I, G6Pase II, FBPase, PEPCKtot and mPEPCK, glucose transport GLUT2, and glycolysis including GK, 6PF1K, PK and HIF1-α; (C) mRNA levels of genes involved in amino acid catabolism including SD, ALAT2, ASAT1, ASAT2, BCKDE1α, BCKDE2 and BCKDK, in the primary hepatocytes of rainbow trout after 24 h stimulation. Cells were pre-incubated for 30 min with or without 100 nM rapamycin (Rapa), then stimulated with the medium containing 4×10^{-9} mol l^{-1} insulin (I) and four fold concentrated amino acids (I*4AA) for 24 h. Expression values are first normalized with elongation factor-1 alpha (EF1α)-expressed transcripts then further normalized to the levels of I*4AA hepatocytes (N=6). "*" indicates significant difference at P<0.05, "**"indicates P<0.01, "***"indicates P<0.001.

novo lipid synthesis (ACLY, FAS, D6D, Elovl5, Elovl2 and G6PDH), gluconeogenesis (G6Pase II, PEPCKtot and mPEPCK) and amino acid catabolism (SD, ASAT1, ASAT2, BCKDE2 and BCKDK) were all significantly reduced by rapamycin (Fig. 6A, 6B and 6C). In addition, three transcription factors, SREBP1c and LXR for activating *de novo* lipid synthesis, and HIF-1α for regulating glycolysis, were significantly reduced by rapamycin (Fig. 6A and 6B). Messenger RNA levels of SCD1, G6Pase I, FBPase, GK, 6PF1K, PK, ALAT2 and BCKDE1α were not significantly affected by rapamycin.

Discussion

Rainbow trout, like the other carnivorous animals consumes diets with relatively high protein content compared to humans, rodents and other terrestrial domestic animals

[23, 46]. Presumably, ingested protein/AAs not only regulate protein metabolism, but also modulate glucose and lipid metabolism. The current studies were designed to evaluate the effects of different AA levels on the regulation of hepatic fatty acid biosynthetic, glucose metabolism-related and amino acid catabolic gene expression, and also to assess the potential involvements of Akt/mTORC1 and p38 MAPK signaling pathway in these regulation processes using freshly isolated trout hepatocytes. We demonstrated that high levels of AAs up-regulate hepatic fatty acid biosynthetic gene expression through an mTORC1-dependent manner, while attenuating insulin-mediated down-regulation of gluconeogenesis through elevated IRS-1 phosphorylation on Ser^{302}, which in turn impairs the activation of Akt pathway and insulin action.

AAs modulated insulin signaling and gluconeogenesis

Our first effort revealed that high levels of AAs attenuated insulin/Akt signaling through mTORC1/S6K1-induced feedback loop in rainbow trout hepatocytes. Increased AA levels not only elevated the phosphorylation of mTORC1 downstream effectors, S6K1 and S6, but also enhanced the phosphorylation of IRS-1 on Ser^{302} and decreased the phosphorylation of Akt on both Ser^{473} and Thr^{308}. Given that IRS-1 Ser^{302} phosphorylation is known as a direct target of mTORC1 and S6K1 in mammals [14, 47], which disrupts the ability of the phosphotyrosine-binding domain to interact with activated insulin receptor, thereby decreasing insulin signaling [48], we first inferred that high levels of AAs enhanced IRS-1 Ser^{302} phosphorylation and repressed Akt phosphorylation through mTORC1/S6K1-induced feedback loop. By using rapamycin, we further demonstrated that mTORC1 inhibition blunted IRS-1 Ser^{302} phosphorylation and restored Akt phosphorylation in excessive (four fold concentrated) AAs-treated hepatocytes, confirming the existence of mTORC1/S6K1-operated negative feedback loop decreasing insulin sensitivity under AAs excess conditions in trout. Therefore, we indeed demonstrated that increased AA levels promoted mTORC1/S6K1 activation, which in turn operated a negative feedback loop toward Akt signaling pathway through, at least in part, enhanced IRS-1 phosphorylation on Ser^{302}. This demonstration is in agreement with previous *in vitro* [49-51] and *in vivo* [52, 53] mammalian investigations.

An intriguing finding of the present study is that high levels of AAs elevated hepatic gluconeogenic gene expression through attenuating insulin signaling. Consistent with previous demonstrations in trout [25, 27], insulin effectively suppressed the gene expression of two key gluconeogenic enzymes, G6Pase and PEPCK. However, increased AA levels attenuated these suppressions and increased the gene expression of FBPase, suggesting that insulin-mediated down-regulation of hepatic gluconeogenesis was attenuated by increased AA sufficiency. This finding is in agreement with previous *in vivo* findings in trout and sea bass, which reported that higher hepatic gluconeogenic enzyme activities and/or mRNA levels were associated with high dietary protein diet/intake compared to low protein diet/intake [26, 54-58]. Furthermore, the negative relation between Akt phosphorylation and gene expression of G6Pase and PEPCK was consistently observed in all the insulin-, different AA levels- and rapamycin-mediated modulations, indicating that hepatic gluconeogenesis was negatively controlled by Akt phosphorylation in trout hepatocytes, as in mammals [2, 59]. Therefore, we concluded that high levels of AAs attenuated insulin-mediated down-regulation of hepatic gluconeogenic gene expression through mTORC1/S6K1-induced feedback loop toward Akt pathway. Similar inhibitory effects on insulin action as a consequence of increased AA supply have also been reported in mammalian hepatocytes [50, 60], muscle cells [49] and adipocytes [51, 61, 62], as well as *in vivo* studies [53, 63, 64]. The present work together with previous study [27] provides strong evidences that increased AA sufficiency up-regulates hepatic gluconeogenesis through insulin/TOR-independent manner(s) and as well as impairing insulin action. However further studies are needed to test whether AAs (particularly glutamine) also promote glucose production through stimulating hexosamine biosynthetic pathway/O-GlcNAc signaling [65]. Furthermore, considering that persistent activation of mTORC1 and S6K1 may promote insulin resistance and glucose intolerance [13, 15], our finding, that high levels of AAs up-regulated hepatic

gluconeogenic gene expression through attenuating insulin signaling, indeed highlighted the potential role of AAs as an inducer for insulin resistance and impaired glucose homeostasis in carnivorous species such as salmonids. However, it is noteworthy that AAs elevation *in vivo* can also stimulate higher insulin secretion [66, 67], which may counteract the effects of AAs on glucose production [53]. Thus, whether high protein diet/intake modulates insulin action and glucose production *in vivo* still needs to be investigated in fish.

AAs regulated fatty acid biosynthesis and glycolysis

Impaired insulin signaling by high levels of AAs partially lost its ability to inhibit hepatic gluconeogenesis, yet it retained its ability to promote fatty acid biosynthesis. mTORC1 inhibition by rapamycin markedly suppressed the expression of genes involved in *de novo* lipogenesis (ACLY and FAS), fatty acid bioconversion (D6D, Elovl5 and Elovl2), NADPH production (G6PDH), and transcriptional factors SREBP1c and LXR, confirming that mTORC1 signaling is essential to stimulate specific metabolic pathways, including *de novo* lipogenesis, fatty acid bioconversion and the oxidative arm of the pentose phosphate pathway (PPP), which is consistent with previous demonstration in mammals [68]. Furthermore, we observed that enhanced gene expression of ACLY, FAS, Elovl5, Elovl2 and G6PDH by high levels of AAs, was also associated with enhanced mTORC1 activation. Therefore, we concluded that increased AA levels up-regulated fatty acid biosynthetic gene expression through an mTORC1-dependent manner. This conclusion is consistent with previous findings in trout [27, 32, 39], which showed that mTOR activation is positively related to elevated lipogenic mRNA levels and/or enzyme activities. The results are also consistent with the findings in mammals [69, 70] and in fish [28-30], showing that high protein diets induced higher fatty acid synthetic gene expression and/or enzyme activities compared to low protein diets. In contrast, all of these results differ from the *in vitro* findings of Allee et al. [71] and Rosebrough et al. [72], who observed that lipogenesis and malic enzyme activity were inversely related to dietary protein levels. Part of these discrepancies may be ascribed to species/tissue specificity or varied protein levels. mTORC1 activation regulates fatty acid biosynthetic gene expression probably through the transcription factors, SREBP1c and LXR [6]. Whereas insulin and amino acids effectively enhanced the expression of SREBP1c and LXR, we failed to demonstrate their AAs-induced changes at the transcriptional level. Given that both SREBP1c and LXR can be regulated transcriptionally and posttranscriptionally [73, 74], we presume that in trout hepatocytes high levels of AAs contribute to the posttranscriptional regulation of SREBP1c and LXR, probably through posttranslational modifications including phosphorylation, ubiquitinylation, and acetylation.

Increased AA availability also elevated glycolytic gene expression. GK, also known as hexokinase IV, plays a pivotal role in blood glucose homeostasis by catalyzing the phosphorylation of glucose, providing metabolites for glycolysis, glycogenesis and pentose phosphate pathway in hepatocytes [75]. We observed that insulin significantly up-regulated GK mRNA level in trout hepatocytes, which is consistent with the previous demonstrations in fish [25, 27, 76] and mammals [77-79] but differs from other studies based on intraperitoneal administration of insulin in trout [80, 81]. GK and the other two glycolytic genes, 6PF1K and PK, were all up-regulated by increased AA levels. This result is in line with the foresaid elevated fatty acids biosynthetic gene expression, as elevated glycolysis can render more available substrates to support the enhanced fatty acid biosynthesis and increased NADPH-demanding. Given that glycolysis is known to be positively regulated by the transcription factors HIF-1α and SREBP1c [68, 82], and both HIF-1α and SREBP1c mRNA levels were inhibited by rapamycin in the current study, we thus suggest that high levels of AAs up-regulated hepatic glycolysis through an mTORC1-dependent manner as well. This deduction is consistent with the observation of Lansard et al. [27], who observed that insulin combined with AAs activated TOR signaling pathway and up-regulated the expression of GK, 6PF1K and PK. As for GLUT2 gene expression, despite it was significantly decreased by insulin and up-regulated by rapamycin and elevated AAs, all the changes were quite minor, confirming that GLUT2, which has low affinity for glucose, is poorly regulated by metabolic factors [83].

AAs modulated AA catabolism and p38 MAPK phosphorylation

AAs are not only signaling molecules, but are also glucose and fatty acids precursors, which can supply carbon skeleton for hepatic gluconeogenesis and lipogenesis via AA catabolism [84]. As expected, increased AA levels markedly elevated the expression of genes related to AA catabolism (SD, ALAT2, ASAT2, BCKDE1α, BCKDE2 and BCKDK), indicating that elevated AA levels promoted hepatic fatty acid biosynthesis and glucose metabolism not only through modulating relevant signaling pathways, but also serving as gluconeogenic and lipogenic substrates by promoting AA catabolism. The present study also demonstrated that several AA catabolic genes were controlled by the mTOR pathway. Further studies are needed to explore the mechanisms linking these signaling pathways to AA catabolic gene expression.

p38 MAPK phosphorylation was not affected by insulin in hepatocytes, which is consistent with previous demonstrations showing that insulin-mediated p38 MAPK activation mainly occur in adipocytes and skeletal muscle [85]. p38 phosphorylation was insensitive to rapamycin, indicating that high levels of AAs decreased p38 phosphorylation through an mTORC1-independent manner. The AA-induced decrease of p38 phosphorylation was associated with the up-regulation of hepatic lipogenic, G6PDH and gluconeogenic gene expression. These results are compatible with previous reports indicating that p38 MAPK negatively regulates hepatic lipogentic and G6PDH mRNA levels [86, 87]. However, they contrast with the other studies reporting that p38 plays a stimulatory role in hepatic gluconeogenic gene expression [88, 89]. Nevertheless, we uncovered a new player involved in AA signaling, which confirmed previous deductions by Casas-Terradellas et al. [90] and may provide new insights into the understanding of nutrients-mediated intermediary metabolism.

Conclusions and prospects

Taken together, we demonstrated that, in primary trout hepatocytes, increased AA levels could (i) up-regulate fatty acid biosynthetic gene expression through an mTORC1-dependent manner, (ii) attenuate insulin-mediated repression on hepatic gluconeogenesis through elevated IRS-1 phosphorylation on Ser^{302}, which in turn impairs the activation of Akt pathway and weakens insulin action, (iii) elevate AA catabolic gene expression, and (iiii) partly suppress p38 MAPK phosphorylation. The results that, under excessive AAs conditions, insulin partially lost its ability to suppress gluconeogenesis but continued to promote fatty acid biosynthetic gene expression, resemble the pathological features observed in the insulin-resistant state [91, 92], which further highlights the potential of carnivorous fish to be used as a natural model for the study of common mammalian pathologies. Future studies are needed to determine which AA truly contributes to mTORC1 activation, and gene expression regulation, and how dietary AA profiles may influence these regulations.

Acknowledgments

W. Dai gratefully acknowledges the financial assistance provided by the China Scholarship Council (CSC, File No. 2011633111) for his doctoral fellowship.

We thank K. Dias, V. Véron, A. Herman and M. Cluzeaud for technical assistance in the laboratory. We also acknowledge the technical staff of the INRA experimental fish farm at Saint Pee sur Nivelle (P. Aguirre and Y. Mercier) for fish rearing.

Disclosures Statement

No conflicts of interest, financial or otherwise, are declared by the authors.

References

1 Yecies JL, Zhang HH, Menon S, Liu S, Yecies D, Lipovsky AI, Gorgun C, Kwiatkowski DJ, Hotamisligil GS, Lee CH, Manning BD: Akt stimulates hepatic srebp1c and lipogenesis through parallel mtorc1-dependent and independent pathways. Cell Metabol 2011;14:21-32.
2 Manning BD, Cantley LC: Akt/pkb signaling: Navigating downstream. Cell 2007;129:1261-1274.
3 Accili D, Arden KC: Foxos at the crossroads of cellular metabolism, differentiation, and transformation. Cell 2004;117:421-426.
4 Li X, Monks B, Ge Q, Birnbaum MJ: Akt/pkb regulates hepatic metabolism by directly inhibiting pgc-1alpha transcription coactivator. Nature 2007;447:1012-1016.
5 Horton JD, Goldstein JL, Brown MS: Srebps: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 2002;109:1125-1131.
6 Shao W, Espenshade PJ: Expanding roles for srebp in metabolism. Cell metabolism 2012;16:414-419.
7 Baranowski M, Zabielski P, Blachnio-Zabielska AU, Harasim E, Chabowski A, Gorski J: Insulin-sensitizing effect of lxr agonist t0901317 in high-fat fed rats is associated with restored muscle glut4 expression and insulin-stimulated as160 phosphorylation. Cell Physiol Biochem 2014;33:1047-1057.
8 Newsholme P, Brennan L, Rubi B, Maechler P: New insights into amino acid metabolism, beta-cell function and diabetes. Clin Sci (Lond) 2005;108:185-194.
9 Cheng Z, Tseng Y, White MF: Insulin signaling meets mitochondria in metabolism. Trends Endocrinol Metab 2010;21:589-598.
10 Bar-Peled L, Sabatini DM: Regulation of mtorc1 by amino acids. Trends Cell Biol 2014;24:400-406.
11 Shimobayashi M, Hall MN: Making new contacts: The mtor network in metabolism and signalling crosstalk. Nat Rev Mol Cell Biol 2014;15:155-162.
12 Laplante M, Sabatini DM: Regulation of mtorc1 and its impact on gene expression at a glance. J Cell Sci 2013;126:1713-1719.
13 Tremblay F, Lavigne C, Jacques H, Marette A: Role of dietary proteins and amino acids in the pathogenesis of insulin resistance. Annu Rev Nutr 2007;27:293-310.
14 Boura-Halfon S, Zick Y: Phosphorylation of irs proteins, insulin action, and insulin resistance. Am J Physiol-Endoc M 2009;296:E581-E591.
15 Lynch CJ, Adams SH: Branched-chain amino acids in metabolic signalling and insulin resistance. Nature Rev Endocrinol 2014;10:723-736.
16 Um SH, D'Alessio D, Thomas G: Nutrient overload, insulin resistance, and ribosomal protein s6 kinase 1, s6k1. Cell Metabol 2006;3:393-402.
17 Feng YY, Xu XQ, Ji CB, Shi CM, Guo XR, Fu JF: Aberrant hepatic microrna expression in nonalcoholic fatty liver disease. Cell Physiol Biochem 2014;34:1983-1997.
18 Wagner EF, Nebreda AR: Signal integration by jnk and p38 mapk pathways in cancer development. Nat Rev Cancer 2009;9:537-549.
19 Ono K, Han J: The p38 signal transduction pathway: Activation and function. Cell Signal 2000;12:1-13.
20 Gehart H, Kumpf S, Ittner A, Ricci R: Mapk signalling in cellular metabolism: Stress or wellness? EMBO reports 2010;11:834-840.
21 Cao W, Collins QF, Becker TC, Robidoux J, Lupo EG Jr, Xiong Y, Daniel KW, Floering L, Collins S: P38 mitogen-activated protein kinase plays a stimulatory role in hepatic gluconeogenesis. J Biol Chem 2005;280:42731-42737.
22 Xiong Y, Collins QF, An J, Lupo E, Jr., Liu HY, Liu D, Robidoux J, Liu Z, Cao W: P38 mitogen-activated protein kinase plays an inhibitory role in hepatic lipogenesis. J Biol Chem 2007;282:4975-4982.
23 Schermerhorn T: Normal glucose metabolism in carnivores overlaps with diabetes pathology in non-carnivores. Front Endocrinol (Lausanne) 2013;4:188.
24 Hemre GI, Mommsen TP, Krogdahl A: Carbohydrates in fish nutrition: Effects on growth, glucose metabolism and hepatic enzymes. Aquacult Nutr 2002;8:175-194.
25 Plagnes-Juan E, Lansard M, Seiliez I, Medale F, Corraze G, Kaushik S, Panserat S, Skiba-Cassy S: Insulin regulates the expression of several metabolism-related genes in the liver and primary hepatocytes of rainbow trout (oncorhynchus mykiss). J Exp Biol 2008;211:2510-2518.
26 Kirchner S, Kaushik S, Panserat S: Low protein intake is associated with reduced hepatic gluconeogenic enzyme expression in rainbow trout (oncorhynchus mykiss). J Nutr 2003;133:2561-2564.

27 Lansard M, Panserat S, Plagnes-Juan E, Seiliez I, Skiba-Cassy S: Integration of insulin and amino acid signals that regulate hepatic metabolism-related gene expression in rainbow trout: Role of tor. Amino Acids 2010;39:801-810.
28 Seiliez I, Panserat S, Lansard M, Polakof S, Plagnes-Juan E, Surget A, Dias K, Larquier M, Kaushik S, Skiba-Cassy S: Dietary carbohydrate-to-protein ratio affects tor signaling and metabolism-related gene expression in the liver and muscle of rainbow trout after a single meal. Am J Physiol-Reg I 2011;300:R733-R743.
29 Dias J, Alvarez MJ, Diez A, Arzel J, Corraze G, Bautista JM, Kaushik SJ: Regulation of hepatic lipogenesis by dietary protein/energy in juvenile european seabass (dicentrarchus labrax). Aquaculture 1998;161:169-186.
30 Figueiredo-Silva AC, Corraze G, Rema P, Sanchez-Gurmaches J, Gutierrez J, Valente LMP: Blackspot seabream (pagellus bogaraveo) lipogenic and glycolytic pathways appear to be more related to dietary protein level than dietary starch type. Aquaculture 2009;291:101-110.
31 Lansard M, Panserat S, Plagnes-Juan E, Dias K, Seiliez I, Skiba-Cassy S: L-leucine, l-methionine, and l-lysine are involved in the regulation of intermediary metabolism-related gene expression in rainbow trout hepatocytes. J Nutr 2011;141:75-80.
32 Dai W, Panserat S, Mennigen JA, Terrier F, Dias K, Seiliez I, Skiba-Cassy S: Post-prandial regulation of hepatic glucokinase and lipogenesis requires the activation of torc1 signalling in rainbow trout (oncorhynchus mykiss). J Exp Biol 2013;216:4483-4492.
33 Segner H: Isolation and primary culture of teleost hepatocytes. Comp Biochem Physiol A: Comp Physiol 1998;120:71-81.
34 Ferraris M, Radice S, Catalani P, Francolini M, Marabini L, Chiesara E: Early oxidative damage in primary cultured trout hepatocytes: A time course study. Aquatic Toxicology 2002;59:283-296.
35 del sol Novoa M, Capilla E, Rojas P, Baro J, Gutierrez J, Navarro I: Glucagon and insulin response to dietary carbohydrate in rainbow trout (oncorhynchus mykiss). Gen Comp Endocrinol 2004;139:48-54.
36 Dai WW, Panserat S, Terrier F, Seiliez I, Skiba-Cassy S: Acute rapamycin treatment improved glucose tolerance through inhibition of hepatic gluconeogenesis in rainbow trout (oncorhynchus mykiss). Am J Physiol-Reg I 2014;307:R1231-R1238.
37 SIGENAE: Information system of breeding animals' genome. Http://www.Sigenae. Org/.
38 Kamalam BS, Panserat S, Aguirre P, Geurden I, Fontagne-Dicharry S, Medale F: Selection for high muscle fat in rainbow trout induces potentially higher chylomicron synthesis and pufa biosynthesis in the intestine. Comp Biochem Physiol A Mol Integr Physiol 2013;164:417-427.
39 Skiba-Cassy S, Lansard M, Panserat S, Medale F: Rainbow trout genetically selected for greater muscle fat content display increased activation of liver tor signaling and lipogenic gene expression. Am J Physiol-Reg I 2009;297:R1421-R1429.
40 Polakof S, Skiba-Cassy S, Panserat S: Glucose homeostasis is impaired by a paradoxical interaction between metformin and insulin in carnivorous rainbow trout. Am J Physiol Regul Integr Comp Physiol 2009;297:R1769-1776.
41 Cruz-Garcia L, Minghetti M, Navarro I, Tocher DR: Molecular cloning, tissue expression and regulation of liver x receptor (lxr) transcription factors of atlantic salmon (salmo salar) and rainbow trout (oncorhynchus mykiss). Comp Biochem Physiol B Biochem Mol Biol 2009;153:81-88.
42 Seiliez I, Panserat S, Lansard M, Polakof S, Plagnes-Juan E, Surget A, Dias K, Larquier M, Kaushik S, Skiba-Cassy S: Dietary carbohydrate-to-protein ratio affects tor signaling and metabolism-related gene expression in the liver and muscle of rainbow trout after a single meal. Am J Physiol Regul Integr Comp Physiol 2011;300:R733-743.
43 Cowley DJ, Sheridan MA: Insulin stimulates hepatic lipogenesis in rainbow-trout, oncorhynchus-mykiss. Fish Physiol Biochem 1993;11:421-428.
44 Saltiel AR, Kahn CR: Insulin signalling and the regulation of glucose and lipid metabolism. Nature 2001;414:799-806.
45 Casas-Terradellas E, Tato I, Bartrons R, Ventura F, Rosa JL: Erk and p38 pathways regulate amino acid signalling. Biochim Biophys Acta 2008;1783:2241-2254.
46 Walton MJ, Cowey CB: Aspects of intermediary metabolism in salmonid fish. Comp Biochem Phys B 1982;73:59-79.

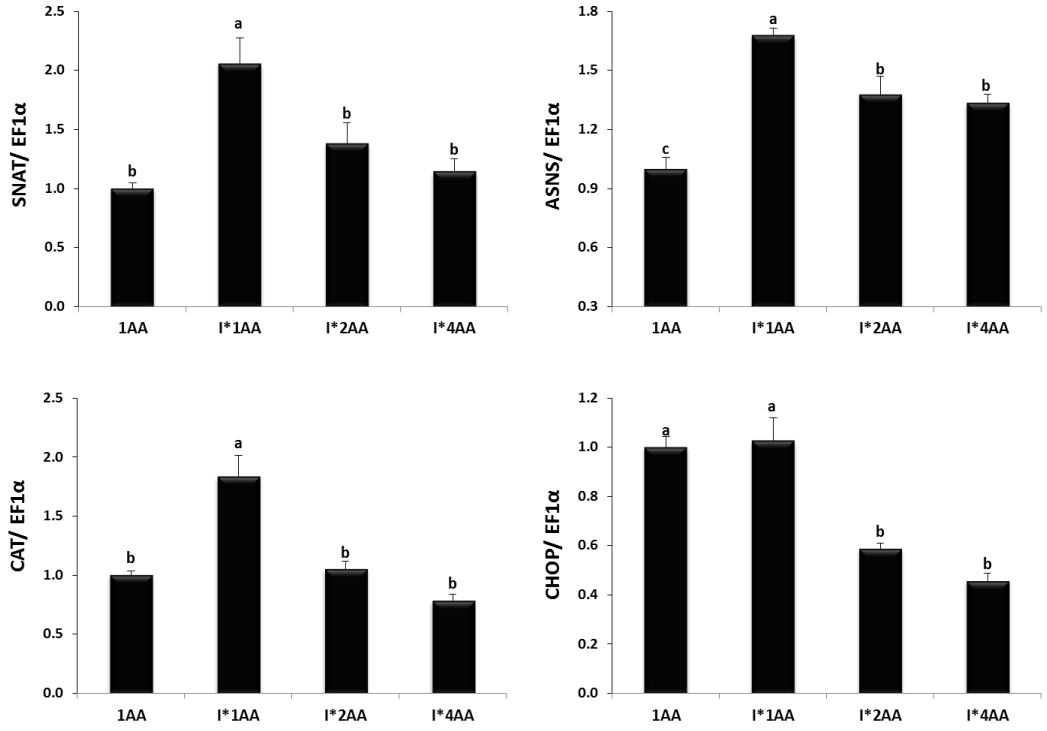
47 Dann SG, Selvaraj A, Thomas G: Mtor complex1-s6k1 signaling: At the crossroads of obesity, diabetes and cancer. Trends Mol Med 2007;13:252-259.
48 Harrington LS, Findlay GM, Gray A, Tolkacheva T, Wigfield S, Rebholz H, Barnett J, Leslie NR, Cheng S, Shepherd PR, Gout I, Downes CP, Lamb RE: The tsc1-2 tumor suppressor controls insulin-pi3k signaling via regulation of irs proteins. J Cell Biol 2004;166:213-223.
49 Tremblay F, Marette A: Amino acid and insulin signaling via the mtor/p70 s6 kinase pathway - a negative feedback mechanism leading to insulin resistance in skeletal muscle cells. J Biol Chem 2001;276:38052-38060.
50 Patti ME, Brambilla E, Luzi L, Landaker EJ, Kahn CR: Bidirectional modulation of insulin action by amino acids. J Clin Invest 1998;101:1519-1529.
51 Takano T, Usui I, Haruta T, Kawahara J, Uno T, Iwata M, Kobayashi M: Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. Mol Cell Biol 2001;21:5050-5062.
52 Layman DK, Shiue H, Sather C, Erickson DJ, Baum J: Increased dietary protein modifies glucose and insulin homeostasis in adult women during weight loss. J Nutr 2003;133:405-410.
53 Tremblay F, Krebs M, Dombrowski L, Brehm A, Bernroider E, Roth E, Nowotny P, Waldhausl W, Marette A, Roden M: Overactivation of s6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. Diabetes 2005;54:2674-2684.
54 Cowey CB, Knox D, Walton MJ, Adron JW: Regulation of gluconeogenesis by diet and insulin in rainbow-trout (salmo-gairdneri). Br J Nutr 1977;38:463-470.
55 Hilton JW, Atkinson JL: Response of rainbow-trout (salmo-gairdneri) to increased levels of available carbohydrate in practical trout diets. Br J Nutr 1982;47:597-607.
56 Walton MJ: Metabolic effects of feeding a high protein-low carbohydrate-diet as compared to a low protein-high carbohydrate-diet to rainbow-trout salmo-gairdneri. Fish Physiol Biochem 1986;1:7-15.
57 Enes P, Panserat S, Kaushik S, Oliva-Teles A: Effect of normal and waxy maize starch on growth, food utilization and hepatic glucose metabolism in european sea bass (dicentrarchus labrax) juveniles. Comp Biochem Phys A 2006;143:89-96.
58 Kamalam BS, Medale F, Kaushik S, Polakof S, Skiba-Cassy S, Panserat S: Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. J Exp Biol 2012;215:2567-2578.
59 Cheng ZY, White MF: The aktion in non-canonical insulin signaling. Nat Med 2012;18:351-353.
60 Khamzina L, Veilleux A, Bergeron S, Marette A: Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: Possible involvement in obesity-linked insulin resistance. Endocrinology 2005;146:1473-1481.
61 Haruta T, Uno T, Kawahara J, Takano A, Egawa K, Sharma PM, Olefsky JM, Kobayashi M: A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. Mol Endocrinol 2000;14:783-794.
62 Tremblay F, Gagnon A, Veilleux A, Sorisky A, Marette A: Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to akt and glucose transport in 3t3-l1 and human adipocytes. Endocrinology 2005;146:1328-1337.
63 Linn T, Santosa B, Gronemeyer D, Aygen S, Scholz N, Busch M, Bretzel RG: Effect of long-term dietary protein intake on glucose metabolism in humans. Diabetologia 2000;43:1257-1265.
64 Rossetti L, Rothman DL, DeFronzo RA, Shulman GI: Effect of dietary protein on in vivo insulin action and liver glycogen repletion. Am J Physiol 1989;257:E212-219.
65 Slawson C, Copeland RJ, Hart GW: O-gicnac signaling: A metabolic link between diabetes and cancer? Trends Biochem Sci 2010;35:547-555.
66 Mommsen TP, Plisetskaya EM: Insulin in fishes and agnathans - history, structure, and metabolic-regulation. Rev Aquat Sci 1991;4:225-259.
67 Floyd JC, Jr., Fajans SS, Conn JW, Knopf RF, Rull J: Stimulation of insulin secretion by amino acids. J Clin Invest 1966;45:1487-1502.
68 Duvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, Triantafellow E, Ma QC, Gorski R, Cleaver S, Heiden MGV, MacKeigan JP, Finan PM, Clish CB, Murphy LO, Manning BD: Activation of a metabolic gene regulatory network downstream of mtor complex 1. Mol Cell 2010;39:171-183.

69 Herzberg GR, Rogerson M: The role of dietary-protein in hepatic lipogenesis in the young-rat. Br J Nutr 1981;45:529-538.
70 Iritani N, Fukuda H, Tada K: Nutritional regulation of lipogenic enzyme gene expression in rat epididymal adipose tissue. J Biochem 1996;120:242-248.
71 Allee GL, O'Hea EK, Leveille GA, Baker DH: Influenza of dietary protein and fat on lipogenesis and enzymatic activity in pig adipose tissue. J Nutr 1971;101:869-878.
72 Rosebrough RW, Poch SM, Russell BA, Richards MP: Dietary protein regulates in vitro lipogenesis and lipogenic gene expression in broilers. Comp Biochem Physiol-Mol Int Physiol 2002;132:423-431.
73 Laurencikiene J, Ryden M: Liver x receptors and fat cell metabolism. Int J Obes (Lond) 2012;36:1494-1502.
74 Jeon TI, Osborne TF: Srebps: Metabolic integrators in physiology and metabolism. Trends Endocrinol Metab 2012;23:65-72.
75 Zhang W, Patil S, Chauhan B, Guo S, Powell DR, Le J, Klotsas A, Matika R, Xiao X, Franks R, Heidenreich KA, Sajan MP, Farese RV, Stolz DB, Tso P, Koo SH, Montminy M, Unterman TG: Foxo1 regulates multiple metabolic pathways in the liver: Effects on gluconeogenic, glycolytic, and lipogenic gene expression. J Biol Chem 2006;281:10105-10117.
76 Egea M, Meton I, Baanante IV: Sp1 and sp3 regulate glucokinase gene transcription in the liver of gilthead sea bream (sparus aurata). J Mol Endocrinol 2007;38:481-492.
77 Iynedjian PB: Molecular physiology of mammalian glucokinase. Cell Mol Life Sci 2009;66:27-42.
78 Matschinsky FM: Assessing the potential of glucokinase activators in diabetes therapy. Nat Rev Drug Discov 2009;8:399-416.
79 Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, Shelton KD, Lindner J, Cherrington AD, Magnuson MA: Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using cre recombinase. J Biol Chem 1999;274:305-315.
80 Jin JY, Panserat S, Kamalam BS, Aguirre P, Veron V, Medale F: Insulin regulates lipid and glucose metabolism similarly in two lines of rainbow trout divergently selected for muscle fat content. Gen Comp Endocrinol 2014;204:49-59.
81 Polakof S, Skiba-Cassy S, Choubert G, Panserat S: Insulin-induced hypoglycaemia is co-ordinately regulated by liver and muscle during acute and chronic insulin stimulation in rainbow trout (oncorhynchus mykiss). J Exp Biol 2010;213:1443-1452.
82 Massa ML, Gagliardino JJ, Francini F: Liver glucokinase: An overview on the regulatory mechanisms of its activity. Iubmb Life 2011;63:1-6.
83 Mueckler M, Thorens B: The slc2 (glut) family of membrane transporters. Mol Aspects Med 2013;34:121-138.
84 Berg JM, Tymoczko JL, Stryer L: Biochemistry, ed 7th edition. New York, W H Freeman, 2010.
85 Antonescu CN, Huang C, Niu W, Liu Z, Eyers PA, Heidenreich KA, Bilan PJ, Klip A: Reduction of insulin-stimulated glucose uptake in l6 myotubes by the protein kinase inhibitor sb203580 is independent of p38mapk activity. Endocrinology 2005;146:3773-3781.
86 Xiong Y, Collins QF, An J, Lupo E, Liu HY, Liu DL, Robidoux J, Liu ZQ, Cao WH: P38 mitogen-activated protein kinase plays an inhibitory role in hepatic lipogenesis. J Biol Chem 2007;282:4975-4982.
87 Talukdar I, Szeszel-Fedorowicz W, Salati LM: Arachidonic acid inhibits the insulin induction of glucose-6-phosphate dehydrogenase via p38 map kinase. J Biol Chem 2005;280:40660-40667.
88 Cao WH, Collins QF, Becker TC, Robidoux J, Lupo EG, Xiong Y, Daniel KW, Floering L, Collins S: P38 mitogen-activated protein kinase plays a stimulatory role in hepatic gluconeogenesis. J Biol Chem 2005;280:42731-42737.
89 Qiao LP, MacDougald OA, Shao JH: Ccaat/enhancer-binding protein alpha mediates induction of hepatic phosphoenolpyruvate carboxykinase by p38 mitogen-activated protein kinase. J Biol Chem 2006;281:24390-24397.
90 Casas-Terradellas E, Tato I, Bartrons R, Ventura F, Rosa JL: Erk and p38 pathways regulate amino acid signalling. Bba-Mol Cell Res 2008;1783:2241-2254.
91 Samuel VT, Shulman GI: Mechanisms for insulin resistance: Common threads and missing links. Cell 2012;148:852-871.
92 Kahn SE, Hull RL, Utzschneider KM: Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006;444:840-846.

Supplementary data

As amino acid response (AAR) pathway is another important pathway involving in AA sensing and also related to the regulation of hepatic lipogenesis (Efeyan et al., 2015; Guo and Cavener, 2007), we measured the expression of its downstream target genes (SNAT, ASNS, CAT and CHOP) to determine the potential roles of AAs in regulating AAR pathway and determine the potential involvement of AAR pathway in mediating hepatic lipogenesis.

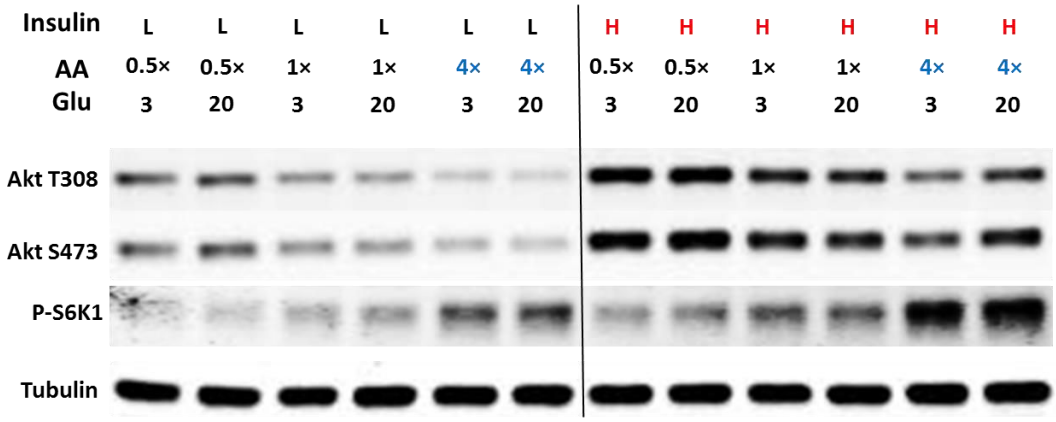
Figure S 3 Gene expression of markers of ATF4 downstream target genes



Compared to one fold concentrated AA (1AA), higher AA levels (2AA or 4AA) reduced the expression of SNAT, ASNS, CAT and CHOP, indicating that increased AA levels significantly down-regulated the expression of ATF4 downstream target genes.

Apart from the data we presented in the article, we actually tested greater range of amino acid concentrations, different glucose and insulin levels on the regulation of Akt and S6K1 phosphorylation in rainbow trout hepatocytes. Some highlighted results are shown below.

Figure S 4 Effects of different insulin, glucose (Glu) and amino acid (AA) levels on the phosphorylation of Akt and S6K1 in trout hepatocytes



The experiment was conducted in 48 h-cultured trout hepatocytes. Cells were then stimulated for 45 min with low/high level of insulin (1×10^{-9}M or 4×10^{-9}M), low/high level of glucose (3 mM or 20 mM) and different levels of amino acids (nought point five-fold, one-fold or four-fold concentrated amino acids). Gels were loaded with 10 μg of total protein per lane for Akt/Tubulin and 20 μg for TOR, respectively. A representative blot is shown (*N*=6). AA, amino acid; Glu, glucose; L, low Insulin (1×10^{-9}M); H, (high Insuline 4×10^{-9}M); 0.5×, 1× and 4× represent naught point five, one and four-fold concentrated amino acids, respectively; 3 and 20 represent low (3 mM) and high (20 mM) level of glucose.

Elevated insulin level promoted both Akt and S6K1 phosphorylation irrespective of glucose or AA levels; elevated AA levels enhanced S6K1 phosphorylation but suppressed Akt phosphorylation whatever insulin levels.

PUBLICATION - 4

What mechanism governs glucose intolerance in fish?

Role of mTOR signaling pathway

Figure 3. 11 Experimental design, procedures and parameters from the fourth article

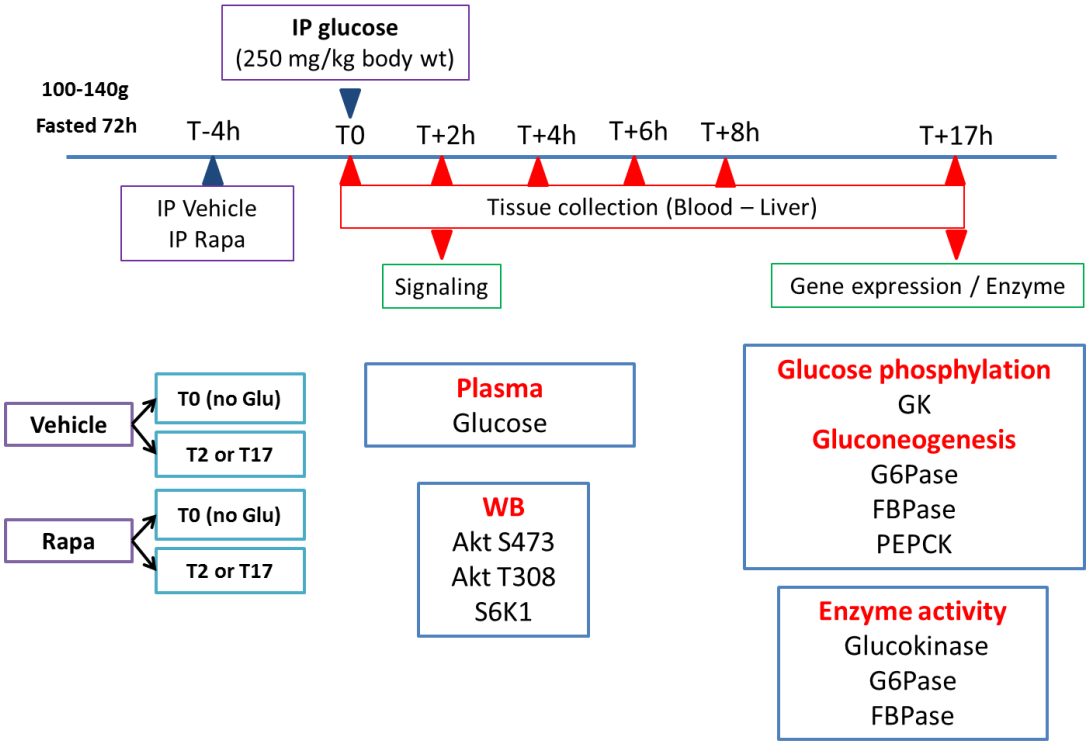
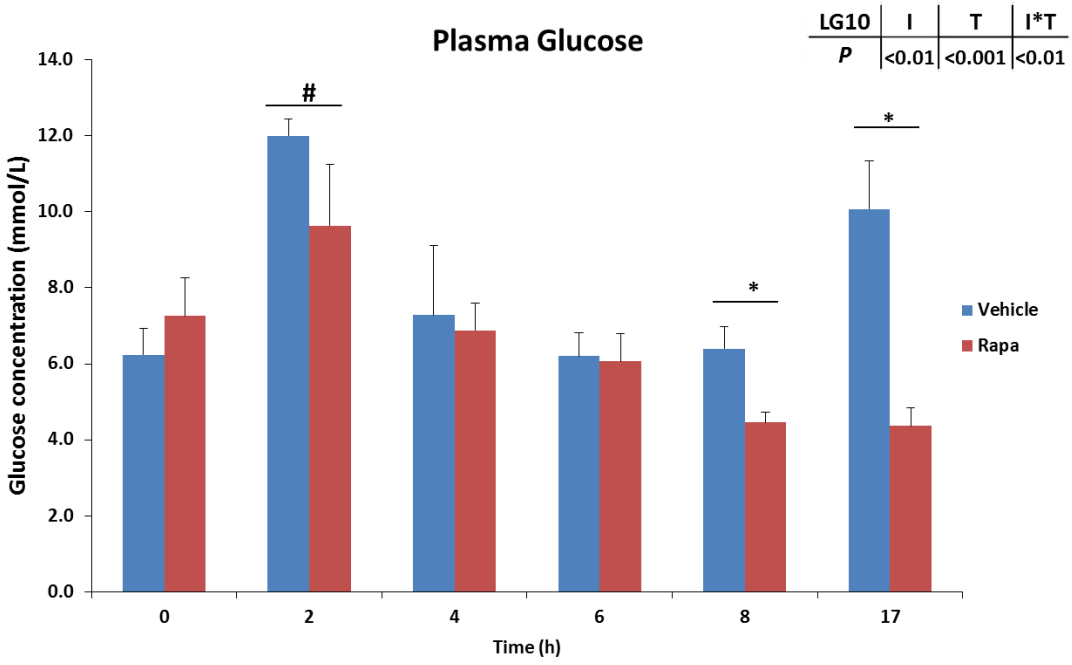


Figure 3. 12 Plasma glucose levels from the fourth article – 0, 2, 4, 6, 8 and 17 h after IP injection of 250mg D-glucose/kg body weight



*# Shows significant difference between different times. * Indicates significant difference between the injection of vehicle and rapamycin at a given time (P<0.05). I, IP administration of vehicle or rapamycin; T, different times; I*T, interaction between I and T.*

Presentation of the article

Objective

In mammals, hepatic GK expression is strictly controlled by insulin through PI3-kinase/Akt pathway. However, in fish, the regulation of GK gene expression seems to be clearly dependent on carbohydrate supply. Nevertheless, our recent data indicated that hepatic GK expression and activity require the activation of mTORC1 signaling in trout liver and hepatocytes. In order to better understand the mechanism behind fish glucose intolerance and the potential implication of TOR signaling pathway, IP administration of rapamycin was performed and followed by an IP injection of glucose to investigate the consequences on plasma glucose levels and expression of main genes involved in hepatic glucose phosphorylation and production.

Experiment and Analyses

72 h fasted juvenile rainbow trout were sedated and treated with a single IP injection of rapamycin (0.5 mg/kg) or vehicle (75% DMSO and 25% saline solution, NaCl 0.9%) at 100 µL/100 g body mass. Four hours after the first injection, six fish per treatment (vehicle or rapamycin, two fish per tank) were sampled, while all remaining fish were sedated and subjected to the second IP administration with 250 mg D-glucose/kg body weight. Liver and blood samples were randomly collected 2, 4, 6, 8 and 17 h after glucose injection (N=6). Post-prandial plasma glucose, phosphorylation of proteins of the Akt/TOR signaling pathway, expression of genes related to glucose phosphorylation and gluconeogenesis, and activity of some selected enzymes were measured (Figure 3.11).

Results and Conclusion

IP administration of glucose induced hyperglymia for both vehicle and rapamycin treatments, which peaked at 2 h. Plasma glucose level in vehicle-treated fish was significantly higher than in rapamycin-treated fish at 8 and 17 h where it returned to the basal level in rapamycin-treated fish (Figure 3.12).

Glucose administration significantly enhanced the phosphorylation of Akt and S6K1 in vehicle treated fish, while rapamycin completely abolished the activation of S6K1 in rapamycin-treated fish, without inhibiting the phosphorylation of Akt on Thr^{308} or Ser^{473} (Figure 3.13).

Despite no change for mPEPCK gene expression, mRNA levels of GK, G6Pase I and II, and FBPase was reduced by rapamycin 17 h after glucose administration. The inhibitory effect of rapamycin on GK and FBPase gene expression was further substantiated at the activity level (Figure 3.14).

Figure 3. 13 Result highlights from the fourth article – Phosphorylation of representational proteins on Akt/TOR signaling pathway in trout liver

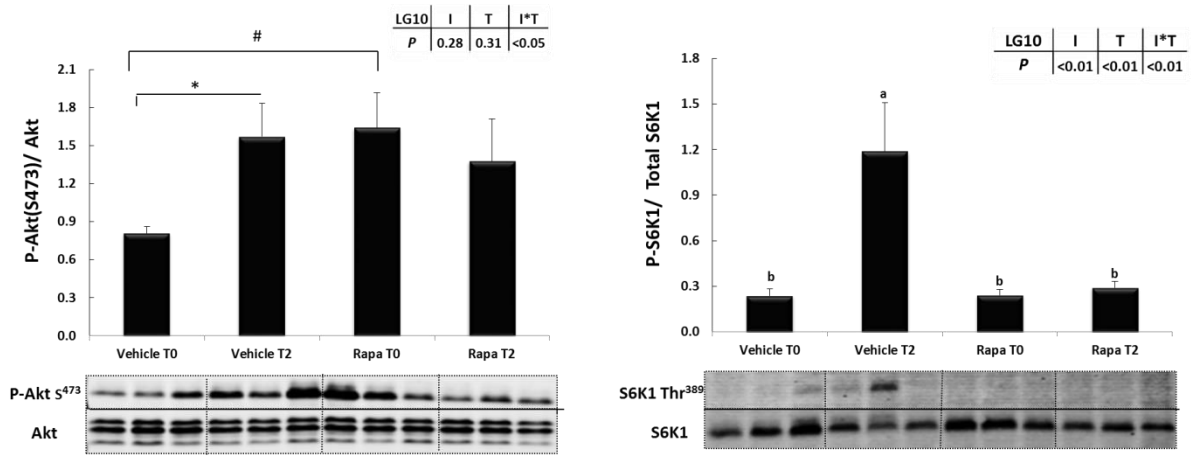
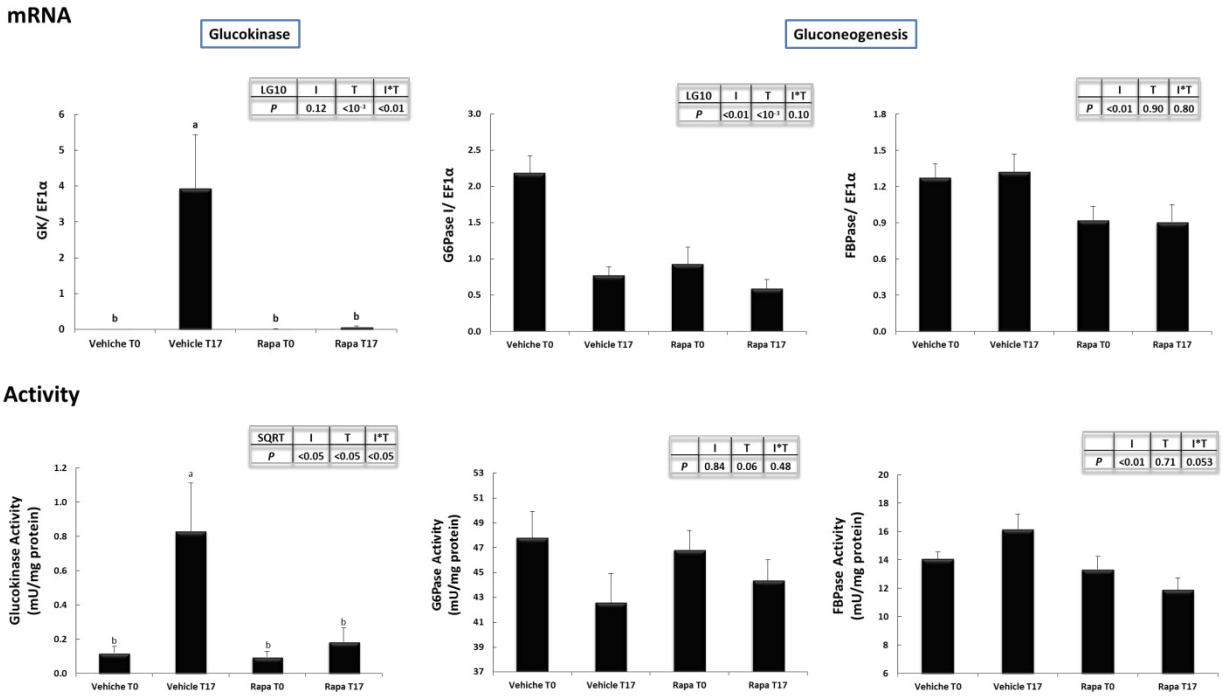


Figure 3. 14 Result highlights from the fourth article – Gene expression and enzyme activities of markers of hepatic glucose utilization and gluconeogenesis



Unlike mammals, we observed that acute rapamycin treatment improved glucose tolerance through the inhibition of hepatic gluconeogenesis in rainbow trout. The result highlighted the crucial role of hepatic gluconeogenesis in regulating plasma glucose homeostasis. The suppression of GK gene expression and activity by rapamycin provided the first *in vivo* evidence in fish that glucose regulates hepatic GK gene expression and activity through a mTORC1-dependent manner.

Am J Physiol Regul Integr Comp Physiol 307: R1231–R1238, 2014.
First published August 27, 2014; doi:10.1152/ajpregu.00166.2014.

Acute rapamycin treatment improved glucose tolerance through inhibition of hepatic gluconeogenesis in rainbow trout (*Oncorhynchus mykiss*)

Weiwei Dai, Stéphane Panserat, Frédéric Terrier, Iban Seiliez, and Sandrine Skiba-Cassy

Institut National de la Recherche Agronomique, UR 1067 Nutrition Métabolisme, Aquaculture, Pole d'Hydrobiologie, CD 918, Saint-Pée-sur-Nivelle, France

Submitted 24 April 2014; accepted in final form 25 August 2014

Dai W, Panserat S, Terrier F, Seiliez I, Skiba-Cassy S. Acute rapamycin treatment improved glucose tolerance through inhibition of hepatic gluconeogenesis in rainbow trout (*Oncorhynchus mykiss*). *Am J Physiol Regul Integr Comp Physiol* 307: R1231–R1238, 2014. First published August 27, 2014; doi:10.1152/ajpregu.00166.2014.—Our aim was to investigate the potential role of TOR (target of rapamycin) signaling pathway in the regulation of hepatic glucose metabolism in rainbow trout. Fasted fish were first treated with a single intraperitoneal injection of rapamycin or vehicle and then submitted to a second intraperitoneal administration of glucose 4 h later. Our results revealed that intraperitoneal administration of glucose induced hyperglycemia for both vehicle and rapamycin treatments, which peaked at 2 h. Plasma glucose level in vehicle-treated fish was significantly higher than in rapamycin-treated fish at 8 and 17 h, whereas it remained at the basal level in rapamycin-treated fish. Glucose administration significantly enhanced the phosphorylation of Akt and ribosomal protein S6 kinase (S6K1) in vehicle-treated fish, while rapamycin completely abolished the activation of S6K1 in rapamycin-treated fish, without inhibiting the phosphorylation of Akt on Thr-308 or Ser-473. Despite the lack of significant variation in phosphoenolpyruvate carboxykinase mRNA abundance, mRNA abundance for glucokinase (GK), glucose 6-phosphatase (G6Pase) I and II, and fructose 1,6-bisphosphatase (FBPase) was reduced by rapamycin 17 h after glucose administration. The inhibition effect of rapamycin on GK and FBPase was further substantiated at the activity level. The suppression of GK gene expression and activity by rapamycin provided the first in vivo evidence in fish that glucose regulates hepatic GK gene expression and activity through a TORC1-dependent manner. Unlike in mammals, we observed that acute rapamycin treatment improved glucose tolerance through the inhibition of hepatic gluconeogenesis in rainbow trout.

target of rapamycin; rapamycin; gluconeogenesis; glucokinase; glucose homeostasis; rainbow trout

PLASMA GLUCOSE LEVEL IS THE result of glucose intake, utilization, and endogeneous production. Regarding the study of glucose homeostasis, carnivorous animals like salmonids are a useful model. In their natural habitat, carnivorous animals such as the cat, mink, trout, salmon, sea bass, and sea bream consume prey high in protein but low in carbohydrates and are, therefore, metabolically adapted to lower glucose but higher protein utilization (4, 26, 73).

The presence of high levels of carbohydrates in fish diet is highly problematic since carnivorous fish are recognized for their low efficiency in using digestible carbohydrates and are typically classified as "glucose-intolerant" (11, 39, 40). Oral, intravenous or intraperitoneal administration of glucose or a carbohydrate-rich diet result in persistent hyperglycemia in various fish species, including rainbow trout (3, 32, 40, 41). Several parameters may contribute to this phenotype. Glucokinase (GK), also known as hexokinase IV, plays a pivotal role in blood glucose homeostasis by catalyzing the phosphorylation of glucose, providing metabolites for glycolysis, glycogenesis, and pentose phosphate pathway in hepatocytes (1, 5) and sensing glucose for insulin secretion in pancreatic β-cells (35). The rate of gluconeogenesis is mainly controlled by the activities of unidirectional enzymes, including phosphoenolpyruvate carboxykinase (mPEPCK), fructose-1, 6-bisphosphatase (FBPase), and glucose-6-phosphatase (G6Pase). G6Pase catalyzes the last step, which is shared by gluconeogenesis and glycogenolysis. Unlike the cat, a mammalian carnivorous model, where hepatic GK expression and activity are lacking (26, 73) and hepatic de novo lipogenesis does not use carbon backbones from glucose as substrate (71), carnivorous fish have inducible GK (43) and can use exogenous glucose for de novo lipogenesis (4). In trout, hepatic GK gene expression and activity are highly induced by dietary carbohydrates (24, 43) or glucose (54) instead of insulin; the persistent hyperglycemia is often associated with low utilization of glucose by the peripheral tissues and impaired postprandial downregulation of gluconeogenesis in liver (50, 52), similar to those typical features in human Type 2 diabetes (56).

Recently, the TOR (target of rapamycin) signaling pathway has emerged as a potential mediator of the regulation of glucose homeostasis (6). The TOR kinase exists as two physically and functionally distinct protein complexes, TORC1 and TORC2, which differ in their regulation, downstream targets, and sensitivity to the allosteric TOR inhibitor rapamycin (16). TORC1 integrates signals from growth factors, amino acids, and cellular energy status, while TORC2 is activated by growth factors only (6). TORC1 controls cell growth and protein translation through the subsequent activation of the p70 ribosomal S6 protein kinase 1 (S6K1) and the ribosomal protein S6 (74). TORC1 is also involved in the regulation of metabolism-related gene expression. It enhances lipogenic gene expression by regulating the expression and processing of sterol regulatory element-binding protein 1c (SREBP1c) (72, 77) and promotes the expression of genes that regulate glucose transport and glycolysis (8, 31). TORC2 responds to the presence of growth factors such as insulin by phosphorylating several members of the AGC kinase subfamily such as Akt (30). In the liver, TORC2 also controls glucose homeostasis via activation of glycolysis and inhibition of gluconeogenesis (17). TORC2 stimulates glycolysis through activation of GK (17, 27).

Despite the well-studied nature of TOR in mammals, the physiological role(s) of TOR in the regulation of glucose utilization and gluconeogenesis in fish remains to be fully explored. Acute inhibition of TORC1 activity by rapamycin in trout liver and primary hepatocytes has shown that GK expres-

Address for reprint requests and other correspondence: Sandrine SKIBA-CASSY, INRA, UR1067 Nutrition, Métabolisme, Aquaculture, F-64310 Saint-Pée-sur-Nivelle, France (e-mail: skiba@st-pee.inra.fr).

sion and activity were controlled by the TOR pathway (7, 29). However, contradictory results were obtained for genes involved in gluconeogenesis. Experiments performed in primary cell culture of hepatocytes tend to indicate that the regulation of expression of G6Pase is dependent upon the TOR pathway (7, 29), while acute administration of rapamycin in vivo failed to modify the postprandial expression of G6Pase, FBPase, or mPEPCK in trout liver (7) despite effective inhibition of TORC1 activity. Moreover, abundant investigations in fish indicated that hepatic GK expression and activity are mainly induced by dietary carbohydrate or glucose (10, 42, 43, 55), whereas in mammalian liver, GK gene expression is strictly controlled by insulin (22, 23, 36, 57). Thus, we hypothesized that dietary carbohydrate/glucose regulates hepatic GK activity through a TORC1-dependent manner. To test this hypothesis, we pretreated trout with intraperitoneal administration of rapamycin or not to achieve an acute inhibition of the TORC1 signaling pathway (7) followed by an intraperitoneal injection of glucose to investigate the consequences on plasma glucose levels, as well as on the regulation of expression of main genes involved in hepatic glucose utilization and production, which were glucokinase (GK) for glucose phosphorylation, glucose-6-phosphatase (G6Pase) for glucose dephosphorylation, fructose-1,6-bisphosphatase (FBPase), and phosphoenolpyruvate carboxykinase (mPEPCK) for gluconeogenesis.

MATERIALS AND METHODS

Experimental and sampling procedure. Juvenile rainbow trout (*Oncorhynchus mykiss*) were reared in the Institut National de la Recherche Agronomique (INRA) experimental facilities at Donzacq (Landes, France) at a constant water temperature of 17.5 ± 0.5°C, under a natural photoperiod. They were fed a standard trout commercial diet (T-3P classic, Skretting, Fontaine-les-Vervins, France) during the acclimatization period. Fish (mean body mass 140 g) were distributed into six tanks (12 fish per 70-liter tank). Prior to the intraperitoneal administration, fish were food-deprived for 72 h (time required to ensure the complete emptying of the digestive tract). After this period, trout were sedated with benzocaine (10 mg/l) and treated with a single intraperitoneal injection of rapamycin (0.5 mg/kg) or vehicle (75% DMSO and 25% saline solution, NaCl 0.9%) at 100 μl/100 g body mass. Four hours after the first injection, six fish per treatment (vehicle or rapamycin, two fish per tank) were sampled, while all remaining fish were sedated and subjected to the second intraperitoneal administration with 250 mg D-glucose/kg body wt based on previous studies in fish (40). Two fish per tank (three tanks per treatment) were randomly sampled at 2, 4, 6, 8, and 17 h after glucose injection ($n = 6$). Trout were anesthetized with benzocaine (30 mg/l) and killed by a sharp blow to the head. Blood was removed from the caudal vein into heparinized syringes and centrifuged (3,000 *g*, 5 min); the recovered plasma was immediately frozen and kept at −20°C. Livers were dissected and immediately frozen in liquid nitrogen and kept at −80°C. The experiments were carried out in accordance with the clear boundaries of European Union legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e., Directive 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decree no. 2001–464, May 29, 2001). The investigators carrying out the experiment had "'level 1"' or "'level 2"' certification, bestowed by the Direction Départementale des Services Vétérinaires (French veterinary services) to carry out animal experiments (INRA 2002–36, 14 April, 2002).

Plasma metabolites analysis. Plasma glucose (Glucose RTU, bioMérieux, Marcy l'Etoile, France) levels were determined using commercial kits adapted to a microplate format, according to the recommendations of the manufacturer.

Western blot analysis. Frozen livers ($n = 6$; 200 mg) from fasted fish (T0) and 2 h glucose-injected fish (T2) were homogenized on ice with an ULTRA-TURRAX homogenizer (IKA-WERKE, Staufen, Germany) in 2 ml of buffer containing 150 mmol/l NaCl, 10 mmol/l Tris, 1 mmol/l EGTA, 1 mmol/l EDTA (pH 7.4), 100 mmol/l NaF, 4 mmol/l sodium pyrophosphate, 2 mmol/l sodium orthovanadate, 1% Triton X-100, 0·5% NP-40-Igepal and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Homogenates were centrifuged at 1,500 *g* for 15 min at 4°C, and supernatant fractions were then centrifuged at 20,000 *g* at 4°C for 30 min. The resulting supernatant fractions were recovered and stored at −80°. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany) with BSA as standard. Lysates (10 μg of total protein for Akt and 20 μg for S6K1) were subjected to SDS-PAGE and Western blot analysis using the appropriate antibody. Anti-phospho-Akt (Ser-473) (no. 9271), anti-phospho-Akt (Thr-308) (no. 9275), anti-carboxyl terminal Akt (no. 9272), antiphospho-S6 protein kinase 1 (Thr-389) (no. 9205), and anti-S6 protein kinase 1 (no. 9202) were purchased from Cell Signaling Technologies (Ozyme, Saint Quentin Yvelines, France). All of these antibodies were successfully cross-reacted with rainbow trout proteins (7, 60, 62). Membranes were washed and then incubated with an IRDye Infrared secondary antibody (LI-COR Biosciences, Lincoln, NE). Bands were visualized by infrared fluorescence using the Odyssey imaging system (LI-COR Biosciences) and were quantified by Odyssey Infrared imaging system software (version 3.0; LI-COR Biosciences).

Gene expression analysis: real-time PCR. Liver samples ($n = 6$) for gene expression were collected from fasted fish (T0) and 17-h glucose-injected fish (T17), which was chosen on the basis of the hepatic gene expression peak or trough of rainbow trout (38). Total RNA was extracted from −80°C frozen livers using TRIzol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendations, quantified by spectrophotometry (absorbance at 260 nm), and its integrity was assessed using agarose gel electrophoresis. A 1-μg sample of the resulting total RNA was reverse transcribed into cDNA using the SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA) and random primers (Promega, Charbonniéres, France), according to the manufacturers' instructions. Target gene expression abundance was determined by quantitative real-time (q)RT-PCR, using specific primers (63).

qRT-PCR was carried out on a LightCycler 480 II (Roche Diagnostics, Neuilly sur Seine, France) using LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany). We used qRT-PCR analyses to focus on several key enzymes of hepatic metabolism, which were glucokinase (GK; EC 2.7.1.2) for glucose phosphorylation, glucose-6-phosphatase (G6Pase; EC 3.1.3.9) for glucose dephosphorylation, fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11), and phosphoenolpyruvate carboxykinase (mPEPCK; EC 4.1.1.32) for gluconeogenesis. When different isoforms of a gene were known in rainbow trout (as for G6Pase), gene expression analysis was performed on each isoform. Elongation factor-1α (EF1α) was employed as a nonregulated reference gene, as previously used in rainbow trout, and it was stably expressed in our investigations (data not shown).

PCR was performed using 2 μl of the diluted cDNA (76 times diluted) mixed with 0.24 μl of each primer (10 μM), 3 μl LightCycler 480 SYBR Green I Master (Roche Diagnostics) and 0.52 μl DNase/RNase/Protease-free water (5 prime, Hamburg, Germany) in a total volume of 6 μl. The PCR protocol was initiated at 95°C for 10 min for initial denaturation of the cDNA and hot-start Taq-polymerase activation, followed by 45 cycles of a three-step amplification program [15 s at 95°C, 10 s at melting temperature T_m (59–65°C), 4.8 s at 72°C], according to the primer set used. Melting curves were systematically monitored (5 s at 95°C, 1 min at 65°C, temperature slope at

0.11°C/s from 65 to 97°C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included quadruplicates for each sample (duplicates of reverse transcription and PCR amplification, respectively) and also negative controls (reverse transcriptase and RNA free samples). Relative quantification of target gene expression was determined using the ΔΔCT method by the software of LightCycler 480 (version SW 1.5; Roche Diagnostics). PCR efficiency, which was assessed by the slope of a standard curve using serial dilutions of cDNA, ranged between 1.85 and 2.

Enzyme activity analysis. Enzyme activity was measured from liver samples ($n = 6$) collected from fasted (T0) and 17-h glucose-injected trout (T17). Liver samples for GK enzyme activities were homogenized in four volumes of ice-cold buffer [50 mmol/l Tris, 5 mmol/l EDTA, 2 mmol/l DTT and a protease inhibitor cocktail (Sigma, St. Louis, MO; P2714), pH 7.4] and centrifuged at 900 *g* at 4°C for 10 min. GK activities were assayed immediately in the supernatant, as described elsewhere (13, 43). Livers for G6Pase enzyme activities were homogenized in eight volumes of ice-cold buffer (as described for GK), then centrifuged, and the supernatant was assayed following the protocol of Alegre et al. (2). Samples for FBPase enzyme activities were homogenized in seven volumes of ice-cold buffer (as described for GK), centrifuged at 900 *g* at 4°C for 10 min, and the supernatant was assayed immediately for FBPase enzyme activity in pre-established conditions (65). Enzyme activity is defined as micromoles of substrate converted to product, per minute, at 37°C and is expressed per milligram of liver protein.

Statistical analysis. The data for plasma glucose, Western blot, gene expression, and enzyme activity are expressed as means ± SE ($n = 6$). The effects of time (T), intraperitoneal administration of vehicle or rapamycin (I), and their interaction were analyzed using two-way ANOVA, followed by a Tukey test when the interaction was significant. In cases where data were nonparametric or not homoscedastic, data transformations, such as logarithms, square roots, and reciprocals, were used to meet ANOVA criteria. Normality was assessed using the Shaprio-Wilk test, while homoscedasticity was determined using Levene's test. For all statistical analyses, the level of significance was set at $P<0.05$.

RESULTS

Plasma glucose levels. Plasma glucose levels peaked at 2 h after the glucose administration and then returned to their initial level from 4 h until 8 h for both vehicle and rapamycin treatments (Fig. 1). In vehicle-treated fish, plasma glucose level measured at 17 h in vehicle treatment was not significantly different compared with its peak level, while it stayed lower than its peak level in rapamycin treatment. As indicated by the statistical interaction between time and intraperitoneal administration of vehicle or rapamycin ($P < 0.01$), plasma glucose level in vehicle-treated fish was significantly higher than in rapamycin-treated fish at 8 h and 17 h after glucose administration ($P < 0.01$).

Akt/TOR signaling pathway. Two-way ANOVA statistical analysis showed that the phosphorylation of Akt on Thr-308 and Ser-473 was not affected by glucose administration ($P > 0.05$) or intraperitoneal injection of vehicle or rapamycin ($P > 0.05$) (Fig. 2, *A* and *B*). On the basis of the significant interaction between glucose administration and injection of vehicle or rapamycin ($P < 0.05$), we further analyzed the data and found that glucose administration significantly induced the phosphorylation of Akt on Ser-473 in vehicle-treated fish ($P < 0.05$) but not in rapamycin-treated fish. Moreover, rapamycin significantly increased Akt Ser-473 phosphorylation level before glucose injection ($P < 0.05$). Phosphorylation of S6K1 was significantly enhanced by glucose administration in vehicle treatment, while this enhancement was totally abolished by rapamycin treatment ($P < 0.01$; Fig. 2*C*).

Messenger RNA levels of target genes. To determine the potential involvement of the TOR signaling pathway in the regulation of hepatic glucose metabolism-related gene expression, we performed qRT-PCR analysis (Fig. 3). GK mRNA abundance was significantly upregulated by glucose administration in vehicle-treated fish, while rapamycin treatment inhibited this upregulation (Fig. 3*A*). As regards gluconeogenesis, both glucose administration and rapamycin treatment inhibited mRNA abundance of G6Pase I ($P < 0.01$) (Fig. 3*B*) and G6Pase II ($P < 0.01$) (Fig. 3*C*), whereas only rapamycin treatment downregulated FBPase gene expression ($P < 0.01$) (Fig. 3*D*). No significant variation was observed for mPEPCK mRNA abundance (Fig. 3*E*).

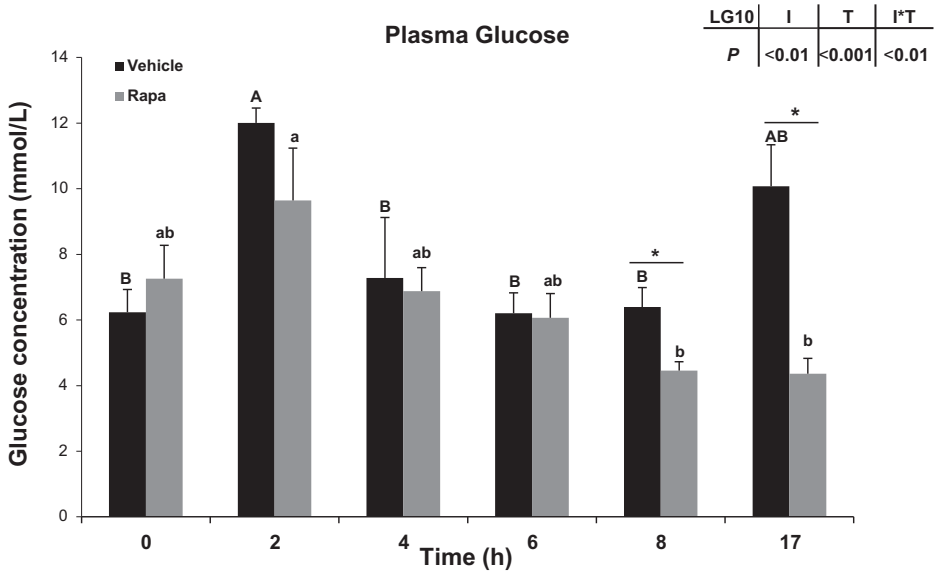


Fig. 1. Plasma glucose concentrations in rainbow trout subjected to intraperitoneal administration of vehicle or rapamycin (Rapa), 0, 2, 4, 6, 8, and 17 h after intraperitoneal injection of 250 mg D-glucose/kg body wt (0 corresponds to fasted fish or non-glucose-injected fish). Data are expressed as means ± SE. ($n = 6$). The effects of time (T), intraperitoneal administration of vehicle or rapamycin (I) and their interaction were analyzed using two-way ANOVA (shown in the upper right table, $P < 0.05$), followed by a Tukey test when the interaction was significant. Letters A and B or a and b indicate significant differences between each time point for vehicle or Rapa treatment, respectively. *Significant difference between the injection of vehicle and rapamycin at a given time ($P < 0.05$). LG10 indicates data were transformed and statistically analyzed with log transforms. "I" represents intraperitoneal administration of vehicle or rapamycin, "T" represents different times, and "I*T" represents interaction between I and T.

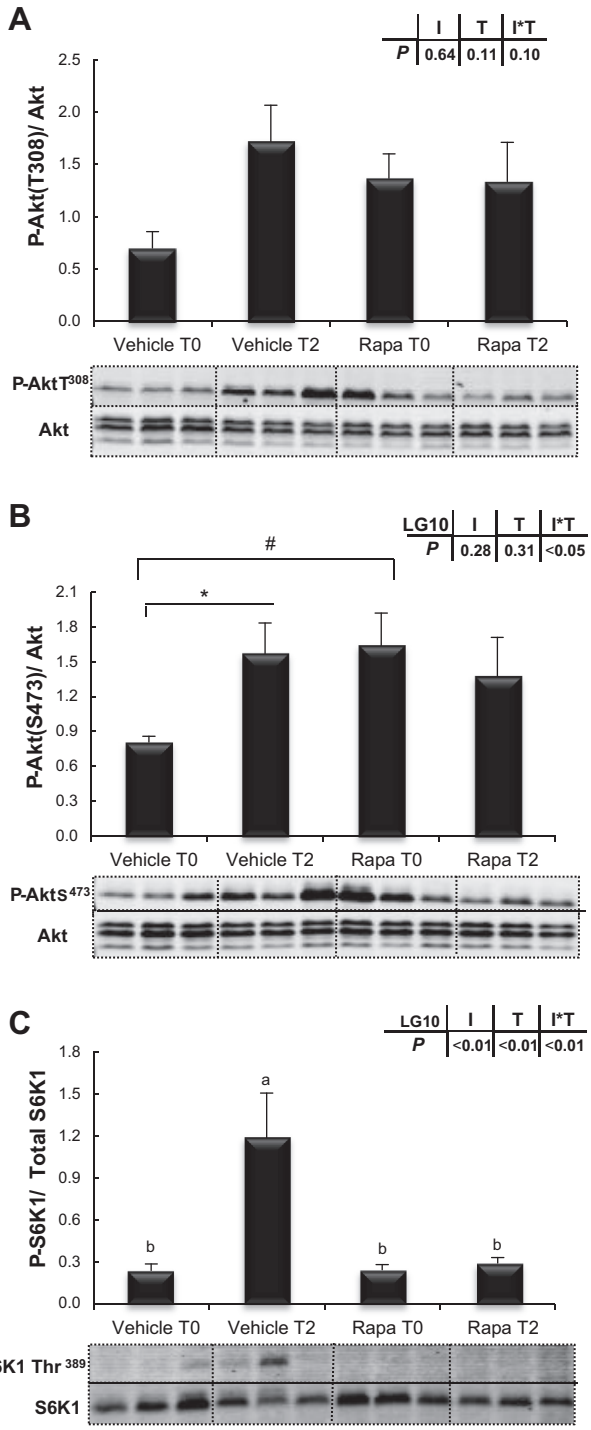


Fig. 2. Western blot analysis of hepatic protein kinase B (Akt Thr-308) (*A*), Akt (Ser-473) (*B*), and ribosomal protein S6 kinase (S6K1) protein phosphorylation (*C*) in rainbow trout subjected to intraperitoneal administration of vehicle or rapamycin (Rapa), 0 and 2 h after IP injection of 250 mg D-glucose/kg body wt (0 corresponds to fasted fish or non-glucose-injected fish). Gels were loaded with 10 μg of total protein per lane for Akt Thr-308/Akt Ser-473 and 20 μg for S6K1, respectively. A representative blot is shown. Graphs represent the ratio between the phosphorylated protein and the total amount of the target protein. Results are expressed as means ± SE. ($n = 6$) and were analyzed using two-way ANOVA, followed by a Tukey test when the interaction was significant. *Significant effect of glucose injection in vehicle fish. #Significant effect of rapamycin administration before glucose injection. Values with different superscript letters (a, b) are significantly different at $P < 0.05$. See Fig. 1 legend for details.

Enzyme activity analysis. To substantiate the results from gene expression analysis, hepatic enzyme activities of GK, G6Pase, and FBPase were determined before and 17 h after glucose injection (Fig. 4). Glucose injection significantly increased GK enzyme activity in vehicle-treated fish, while this increase was blocked in rapamycin-treated fish (Fig. 4*A*). G6Pase enzyme activity remained unchanged regardless of time or IP administration of vehicle or rapamycin (Fig. 4*B*), whereas FBPase enzyme activity was significantly decreased by intraperitoneal administration of rapamycin ($P < 0.01$, Fig. 4*C*).

DISCUSSION

In the present study, we investigated the role of TOR signaling pathway in the regulation of glycemia in rainbow trout. For that purpose, we performed intraperitoneal administration of rapamycin, followed by intraperitoneal administration of glucose 4 h after the first injection. Glucose administration has been reported to cause hyperglycemia in a number of teleost species (18, 20, 25, 40). In the present experiment, plasma glucose levels exhibited a biphasic response. Consistent with previous trout observations (18, 32), the first phase consisted of a peak of plasma glucose occurring 2 h after glucose injection with a recovery of the basal level (0 h) at least 4 h after glucose injection in both vehicle and rapamycin-treated fish. It is worthwhile to note that in the second phase, plasma glucose level in vehicle-treated fish was significantly higher than in rapamycin-treated fish between 8 and 17 h and displayed an increasing tendency, whereas it remained at the basal level in rapamycin-treated fish, indicating that, unlike mammals (19, 28, 75), acute rapamycin treatment improved glucose homeostasis in rainbow trout.

Investigation of TOR signaling pathway reveals that glucose administration significantly enhanced the phosphorylation of S6K1 in vehicle-treated fish, while rapamycin completely abolished this activation, confirming the efficiency of rapamycin in inhibiting TORC1 (7). Considering Akt Ser-473, glucose administration significantly activates Akt signaling pathway, suggesting efficient induction of insulin secretion (39, 52). However, because of the high level of Akt phosphorylation in rapamycin-treated fish before glucose administration, no further enhancement of Akt phosphorylation was recorded after glucose administration. This absence of significant difference could be due to acute induction of insulin release by rapamycin treatment (14) or rapamycin inhibition of the S6K1-induced negative feedback loop on early events of insulin signaling (19, 66, 68–70).

The regulation of GK expression in healthy mammals is dual: in the pancreatic β-cells, it is controlled by the blood glucose level, while in the liver, it is strictly controlled by insulin (22, 36, 57) through PI3-kinase/Akt pathway (34). In cultured rat hepatocytes, the effect of insulin as an inducer of GK was shown to be primarily at a transcriptional level, taking place in glucose-free medium, as well as glucose containing medium without any synergistic effect of glucose supplement (21). In fish, the regulation of GK gene expression seems to be clearly dependent on carbohydrate supplement (10, 42, 43, 49, 55). The present study clearly confirms this feature at both gene expression and activity levels. The suppression of GK gene expression and activity by rapamycin provided the first in

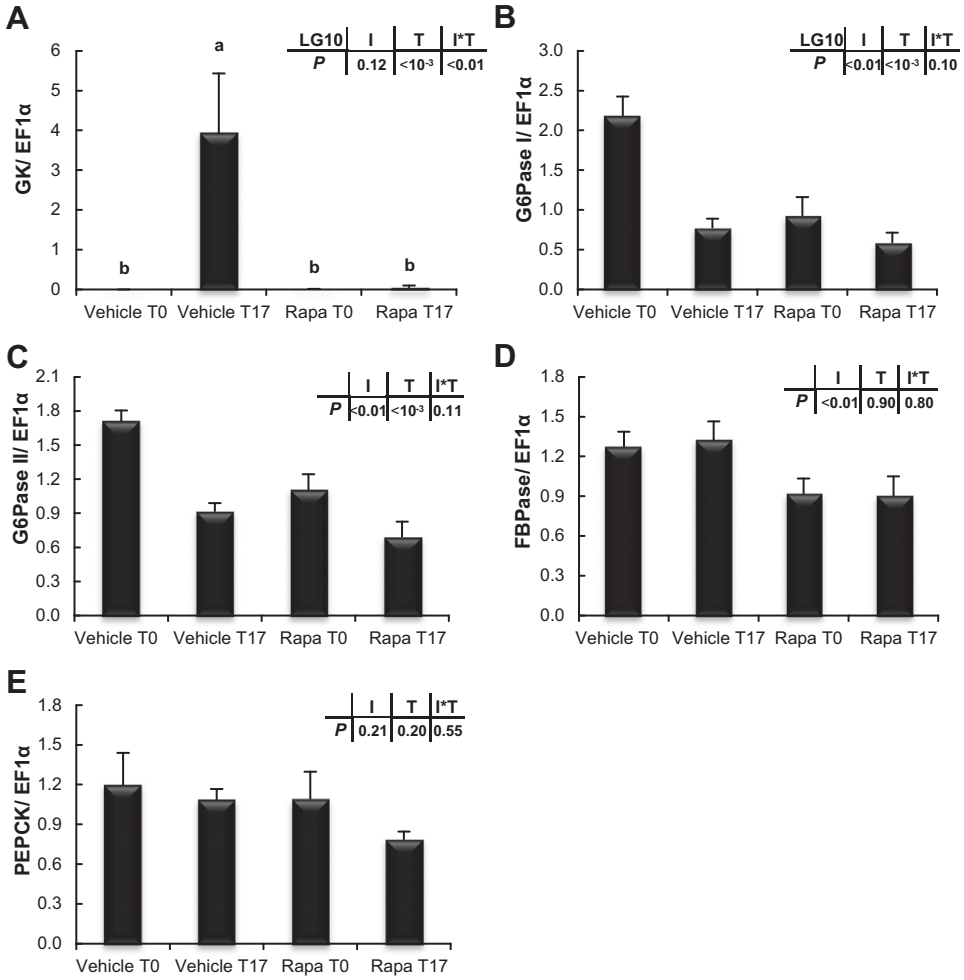


Fig. 3. Gene expression of selected glycolytic and gluconeogenic enzymes in the liver of rainbow trout subjected to intraperitoneal administration of vehicle or rapamycin (Rapa), 0 and 17 h after IP injection of 250 mg D-glucose/kg body wt (0 corresponds to fasted fish or non-glucose injected fish). Glucokinase (GK) (*A*), glucose-6-phosphatase isoform 1 (G6Pase I) (*B*), G6Pase II (*C*), fructose 1,6-bisphosphatase (FBPase) (*D*), and phosphoenolpyruvate carboxykinase (mPEPCK) (*E*) mRNA abundance were assessed using quantitative real-time (q)RT-PCR. Expression values are normalized with elongation factor-1α (EF1α)-expressed transcripts. Results are expressed as means ± SE. ($n = 6$) and were analyzed using two-way ANOVA, followed by a Tukey test when the interaction was significant. Values with different superscript letters (a, b) are significantly different at $P < 0.05$. See Fig. 1 legend for details.

vivo evidence in fish that glucose regulates hepatic GK gene expression and activity through a TORC1-dependent manner, confirming previous data, indicating that GK gene expression may be inhibited by rapamycin in rainbow trout hepatocytes (29). Given the key role of GKRP (glucokinase regulatory protein) in the allosterical regulation of GK activity and subcellular localization (48), further investigations are needed to explore its potential involvement in the nutritional regulation in fish.

Hepatic endogenous production of glucose is driven by the availability of gluconeogenic substrates and the activity of three key gluconeogenic enzymes: mPEPCK, FBPase, and G6Pase (10, 15). Among these three enzymes, only G6Pase gene expression (G6Pase I and II) was reduced by glucose injection in both rapamycin and vehicle-treated fish, whereas mPEPCK and FBPase mRNA abundance remained stable. Moreover, neither G6Pase nor FBPase activities were controlled by glucose. This phenotype is in agreement with the well-known glucose-intolerant phenotype of carnivorous fish (42, 44, 45, 64), in which carbohydrate-rich diets do not affect the activity or gene expression of the three hepatic key enzymes of gluconeogenesis. While G6Pase is poorly regulated at the activity level in fish, G6Pase gene expression showed a more sensitive response to feeding (7, 42), macronutrients composition of the diets (61), and in vitro insulin treatment (29, 52), even though its expression is poorly regulated by dietary carbohydrate levels (46, 47). Thus, it is consistent to record an inhibitory effect by glucose on hepatic G6Pase at gene expression level but no regulation at its activity level. This discrepancy between gene expression and enzyme activity may be attributed to posttranscriptional regulation. MicroRNAs are small regulatory molecules, which posttranscriptionally regulate mRNA stability and translation. They represent one mechanism that may finely regulate enzyme activities. Indeed, recent findings indicate that miR-29a-c can negatively regulate hepatic gluconeogenesis in mice through direct targeting of G6Pase mRNA (33). In trout, the hepatic expression of miR29a has been shown to be regulated by feeding (37); however, further experiments are needed to investigate the functional interaction between miR29 and G6Pase mRNA.

In terms of rapamycin effect, only FBPase was reduced at both gene expression and activity levels, while G6Pase I and G6Pase II merely decreased at the expression level and mPEPCK remained unaffected. Thus, we concluded that acute administration of rapamycin inhibited hepatic gluconeogenesis at the level of FBPase by limiting both its gene expression and activity. This inhibition of gluconeogenesis by rapamycin may finally explain why plasma glucose level remains at the basal level in rapamycin-treated fish between 8 and 17 h after glucose administration. Therefore, the absence of inhibition of hepatic gluconeogenesis in vehicle-treated fish might lead to the higher glycemia between 8 and 17 h after glucose admin-

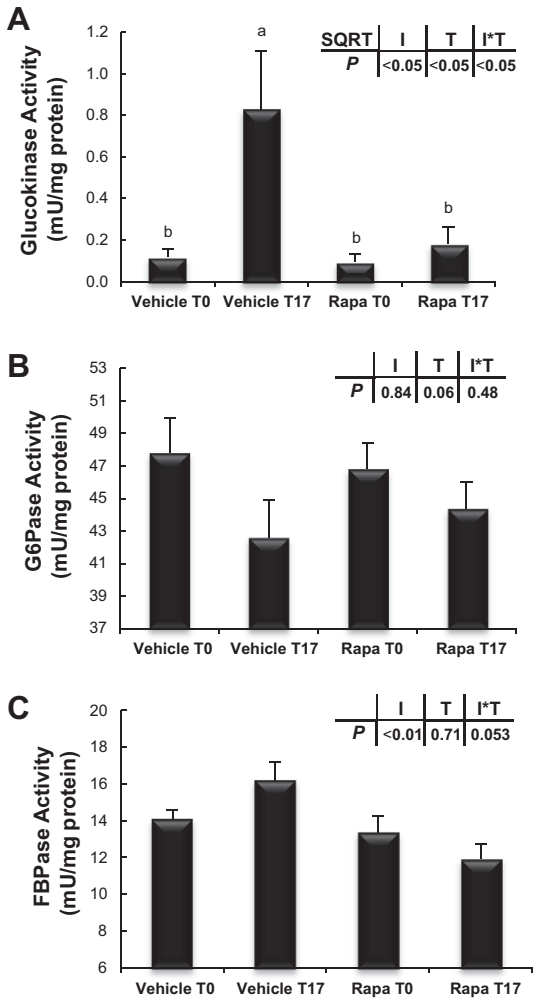


Fig. 4. Enzyme activity of GK (*A*), G6Pase (*B*), and FBPase (*C*) in the liver of rainbow trout subjected to intraperitoneal administration of vehicle or rapamycin (Rapa), 0 and 17 h after intraperitoneal injection of 250 mg D-glucose/kg body wt (0 corresponds to fasted fish or non-glucose-injected fish). Enzyme activity is defined as micromoles of substrate converted to product, per minute, at 37°C and is expressed per milligram of liver protein. Results are expressed as means ± SE ($n = 6$) and were analyzed using two-way ANOVA, followed by a Tukey test when the interaction was significant. Values with different superscript letters (a, b) are significantly different at $P < 0.05$. SQRT indicates data were transformed and statistically analyzed with square roots. See Fig. 1 legend for details.

istration despite a significant induction of hepatic GK in gene expression and activity. However, GK activity measured at 17 h after glucose administration was relatively low compared with the levels that could be reached after glucose administration in trout (24, 42), suggesting that gluconeogenesis may be predominant upon glucose utilization at this time point. Interestingly, the effects caused by acute rapamycin treatment in fish are opposite to the effects caused by chronic rapamycin treatment in mice (12, 19, 28, 75). While acute rapamycin treatment in trout improved glucose intolerance through the downregulation of FBPase, a rate-limiting enzyme of gluconeogenesis, chronic administration of rapamycin in mice resulted in glucose intolerance and hyperlipidemia as a result of insulin resistance and enhanced hepatic gluconeogenesis (19, 28, 75).

Since TORC1 inhibition is the main consequence of acute rapamycin administration (59), our results support the idea that TORC1 activity positively contributes to G6Pase gene expression, which is in line with our previous demonstration in primary cell culture of hepatocytes (29). The mechanism linking rapamycin to the downregulation of FBPase gene expression and activity is less clear than for G6Pase, since rapamycin does not regulate FBPase gene expression in hepatocytes (29). Notably, rapamycin not only suppressed gluconeogenesis but also slightly enhanced Akt phosphorylation, these associated effects tend to indicate that hepatic gluconeogenesis and FBPase may be negatively controlled by TORC2 in rainbow trout, through enhanced phosphorylation of Akt on Ser^{473}, in agreement with previous in vivo demonstrations in mice (28).

Perspectives and Significance

The present study highlighted specific features of rainbow trout glucose homeostasis, including TORC1-dependent regulation of GK regulation and the predominant role of gluconeogenesis in the control of glucose homeostasis. In mammals, TORC1 activation is sufficient to stimulate glucose uptake and glycolysis through the transcriptional factor HIF1a (9, 76). Further investigations are now needed to explore the involvement of HIF1a in the TORC1-dependent control of GK in fish. Furthermore, F-2,6-bisphosphate is known as a potent allosteric activator of 6PF1K and a competitive inhibitor of FBPase (51, 58). F-2,6-bisphosphatase expression and activity are warranted to further explore its role as a potential regulator of FBPase. Moreover, current hyperglycemia and impaired downregulation of gluconeogenesis in rainbow trout may be attributed to defective insulin actions (52, 53). A potential inducer for insulin resistance is amino acid (AA), which is thought to play a significant role in the pathogenesis of insulin resistance by modulating the endocrine function of the pancreas, acting as gluconeogenic precursors, stimulating hexosamine biosynthesis, or activating the S6K1-induced feedback loop toward insulin receptor substrate 1 (67). It would be interesting to perform an in vivo investigation with a pair-feeding method and two diets containing high-protein/low-carbohydrate (HPLC) or high-protein/high-carbohydrate (HPHC), or an in vitro experiment with high amino acid levels and graded levels of glucose to test this hypothesis in the future.

ACKNOWLEDGMENTS

We thank K. Dias, V. Véron, A. Herman, E. Plagnes-Juan, A. Surget, and M. Cluzeaud for technical assistance in the laboratory. We also acknowledge the technical staff of the Institut National de la Recherche Agronomique experimental fish farm at Donzacq (Y. Hontang, and F. Sandres) for fish rearing.

GRANTS

W. Dai gratefully acknowledges the financial assistance provided by the China Scholarship Council (File no. 2011633111) for his doctoral fellowship.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: W.D. and F.T. performed experiments; W.D. and S.S.-C. analyzed data; W.D., S.P., I.S., and S.S.-C. interpreted results of experiments; W.D. prepared figures; W.D. drafted manuscript; W.D., S.P.,

F.T., I.S., and S.S.-C. approved final version of manuscript; S.P., F.T., I.S., and S.S.-C. conception and design of research; S.P., I.S., and S.S.-C. edited and revised manuscript.

REFERENCES

1. **Agius L.** Glucokinase and molecular aspects of liver glycogen metabolism. *Biochem J* 414: 1–18, 2008.
2. **Alegre M, Ciudad CJ, Fillat C, Guinovart JJ.** Determination of glucose-6-phosphatase activity using the glucose dehydrogenase-coupled reaction. *Anal Biochem* 173: 185–189, 1988.
3. **Bergot F.** Specific problems related to carbohydrate incorporation in rainbow trout diets. *Ann Nutrit Aliment* 33: 247–257, 1979.
4. **Brauge C, Corraze G, Medale F.** Effect of dietary levels of lipid and carbohydrate on growth performance, body composition, nitrogen excretion and plasma glucose levels in rainbow trout reared at 8 or 18°C. *Reprod Nutr Dev* 35: 277–290, 1995.
5. **Choi JM, Seo MH, Kyeong HH, Kim E, Kim HS.** Molecular basis for the role of glucokinase regulatory protein as the allosteric switch for glucokinase. *Proc Natl Acad Sci USA* 110: 10171–10176, 2013.
6. **Cornu M, Albert V, Hall MN.** mTOR in aging, metabolism, and cancer. *Curr Opin Genet Dev* 23: 53–62, 2013.
7. **Dai W, Panserat S, Mennigen JA, Terrier F, Dias K, Seiliez I, Skiba-Cassy S.** Post-prandial regulation of hepatic glucokinase and lipogenesis requires the activation of TORC1 signalling in rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 216: 4483–4492, 2013.
8. **Dibble CC, Manning BD.** Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nat Cell Biol* 15: 555–564, 2013.
9. **Duvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, Triantafellow E, Ma Q, Gorski R, Cleaver S, Vander Heiden MG, MacKeigan JP, Finan PM, Clish CB, Murphy LO, Manning BD.** Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol Cell* 39: 171–183, 2010.
10. **Enes P, Panserat S, Kaushik S, Oliva-Teles A.** Nutritional regulation of hepatic glucose metabolism in fish. *Fish Physiol Biochem* 35: 519–539, 2009.
11. **Falkmer S.** Experimental diabetes research in fish. *Acta Endocrinol Suppl* (*Copenh*) 37 *Suppl* 59: 1–122, 1961.
12. **Festuccia WT, Blanchard PG, Belchior T, Chimin P, Paschoal VA, Magdalon J, Hirabara SM, Simoes D, St-Pierre P, Carpinelli AR, Marette A, Deshaies Y.** PPARγ activation attenuates glucose intolerance induced by mTOR inhibition with rapamycin in rats. *Am J Physiol Endocrinol Metab* 306: E1046–E1054, 2014.
13. **Figueiredo-Silva AC, Corraze G, Kaushik S, Peleteiro JB, Valente LM.** Modulation of blackspot seabream (Pagellus bogaraveo) intermediary metabolic pathways by dispensable amino acids. *Amino Acids* 39: 1401–1416, 2010.
14. **Fuhrer DK, Kobayashi M, Jiang H.** Insulin release and suppression by tacrolimus, rapamycin and cyclosporin A are through regulation of the ATP-sensitive potassium channel. *Diabetes Obes Metab* 3: 393–402, 2001.
15. **Gross DN, van den Heuvel AP, Birnbaum MJ.** The role of FoxO in the regulation of metabolism. *Oncogene* 27: 2320–2336, 2008.
16. **Guertin DA, Sabatini DM.** The pharmacology of mTOR inhibition. *Sci Signal* 2: pe24, 2009.
17. **Hagiwara A, Cornu M, Cybulski N, Polak P, Betz C, Trapani F, Terracciano L, Heim MH, Ruegg MA, Hall MN.** Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c. *Cell Metab* 15: 725–738, 2012.
18. **Harmon JS, Eilertson CD, Sheridan MA, Plisetskaya EM.** Insulin suppression is associated with hypersomatostatinemia and hyperglucagonemia in glucose-injected rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 261: R609–R613, 1991.
19. **Houde VP, Brule S, Festuccia WT, Blanchard PG, Bellmann K, Deshaies Y, Marette A.** Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue. *Diabetes* 59: 1338–1348, 2010.
20. **Ince BW, Thorpe A.** Effects of insulin and of metabolite loading on blood metabolites in the European silver eel, (*Anguilla anguilla* L.). *Gen Comp Endocrinol* 23: 460–471, 1974.
21. **Iynedjian PB.** Mammalian glucokinase and its gene. *Biochem J* 293: 1–13, 1993.
22. **Iynedjian PB.** Molecular physiology of mammalian glucokinase. *Cell Mol Life Sci* 66: 27–42, 2009.
23. **Iynedjian PB, Pilot PR, Nouspikel T, Milburn JL, Quaade C, Hughes S, Ucla C, Newgard CB.** Differential expression and regulation of the glucokinase gene in liver and islets of Langerhans. *Proc Natl Acad Sci USA* 86: 7838–7842, 1989.
24. **Kamalam BS, Medale F, Kaushik S, Polakof S, Skiba-Cassy S, Panserat S.** Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. *J Exp Biol* 215: 2567–2578, 2012.
25. **Khanna SS, Rekhari K.** Effect of glucose loading on the blood sugar and the histology of the pancreatic islets in a freshwater teleost, Heteropneustes fossilis. *Acta Anat (Basel)* 82: 126–137, 1972.
26. **Koizumi N, Kondoh M, Mizuguchi H, Nakanishi T, Masuyama A, Ida F, Fujii M, Hayakawa T, Nakashima E, Tanaka K, Watanabe Y.** Comparison of transgene expression mediated by several fiber-modified adenovirus vectors in trophoblast cells. *Placenta* 26: 729–734, 2005.
27. **Lamming DW, Demirkan G, Boylan JM, Mihaylova MM, Peng T, Ferreira J, Neretti N, Salomon A, Sabatini DM, Gruppuso PA.** Hepatic signaling by the mechanistic target of rapamycin complex 2 (mTORC2). *FASEB J* 28: 300–315, 2014.
28. **Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, Davis JG, Salmon AB, Richardson A, Ahima RS, Guertin DA, Sabatini DM, Baur JA.** Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. *Science* 335: 1638–1643, 2012.
29. **Lansard M, Panserat S, Plagnes-Juan E, Seiliez I, and Skiba-Cassy S.** Integration of insulin and amino acid signals that regulate hepatic metabolism-related gene expression in rainbow trout: role of TOR. *Amino Acids* 39: 801–810, 2010.
30. **Laplante M, Sabatini DM.** mTOR signaling in growth control and disease. *Cell* 149: 274–293, 2012.
31. **Laplante M, Sabatini DM.** Regulation of mTORC1 and its impact on gene expression at a glance. *J Cell Sci* 126: 1713–1719, 2013.
32. **Legate NJ, Bonen A, Moon TW.** Glucose tolerance and peripheral glucose utilization in rainbow trout (*Oncorhynchus mykiss*), American eel (*Anguilla rostrata*), and black bullhead catfish (*Ameiurus melas*). *Gen Comp Endocrinol* 122: 48–59, 2001.
33. **Liang J, Liu C, Qiao A, Cui Y, Zhang H, Cui A, Zhang S, Yang Y, Xiao X, Chen Y, Fang F, Chang Y.** MicroRNA-29a-c decrease fasting blood glucose levels by negatively regulating hepatic gluconeogenesis. *J Hepatol* 58: 535–542, 2013.
34. **Massa ML, Gagliardino JJ, Francini F.** Liver glucokinase: An overview on the regulatory mechanisms of its activity. *IUBMB Life* 63: 1–6, 2011.
35. **Matschinsky F, Liang Y, Kesavan P, Wang LQ, Froguel P, Velho G, Cohen D, Permutt MA, Tanizawa Y, Jetton TL, Niswender K, Magnuson MA.** Glucokinase as pancreatic β-cell glucose sensor and diabetes gene. *J Clin Invest* 92: 2092–2098, 1993.
36. **Matschinsky FM.** Assessing the potential of glucokinase activators in diabetes therapy. *Nat Rev Drug Discov* 8: 399–416, 2009.
37. **Mennigen JA, Panserat S, Larquier M, Plagnes-Juan E, Medale F, Seiliez I, Skiba-Cassy S.** Postprandial regulation of hepatic microRNAs predicted to target the insulin pathway in rainbow trout. *PLoS One* 7: e38604, 2012.
38. **Mennigen JA, Panserat S, Larquier M, Plagnes-Juan E, Medale F, Seiliez I, Skiba-Cassy S.** Postprandial regulation of hepatic microRNAs predicted to target the insulin pathway in rainbow trout. *PLoS One* 7: e38604, 2012.
39. **Mommsen TP, Plisetskaya EM.** Insulin in fishes and Agnathans: history, structure, and metabolic-regulation. *Rev Aquat Sci* 4: 225–259, 1991.
40. **Moon TW.** Glucose intolerance in teleost fish: fact or fiction? *Comp Biochem Physiol B Biochem Mol Biol* 129: 243–249, 2001.
41. **Novoa MD, Capilla E, Rojas P, Baro J, Gutierrez J, Navarro I.** Glucagon and insulin response to dietary carbohydrate in rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol* 139: 48–54, 2004.
42. **Panserat S, Capilla E, Gutierrez J, Frappart PO, Vachot C, Plagnes-Juan E, Aguirre P, Breque J, Kaushik S.** Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (*Oncorhynchus mykiss*) by a single meal with glucose. *Comp Biochem Physiol B Biochem Mol Biol* 128: 275–283, 2001.
43. **Panserat S, Medale F, Blin C, Breque J, Vachot C, Plagnes-Juan E, Gomes E, Krishnamoorthy R, Kaushik S.** Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. *Am J Physiol Regul Integr Comp Physiol* 278: R1164–R1170, 2000.

44. **Panserat S, Medale F, Breque J, Plagnes-Juan E, Kaushik S.** Lack of significant long-term effect of dietary carbohydrates on hepatic glucose-6-phosphatase expression in rainbow trout (*Oncorhynchus mykiss*). *J Nutr Biochem* 11: 22–29, 2000.
45. **Panserat S, Plagnes-Juan E, Breque J, Kaushik S.** Hepatic phosphoenolpyruvate carboxykinase gene expression is not repressed by dietary carbohydrates in rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 204: 359–365, 2001.
46. **Panserat S, Plagnes-Juan E, Kaushik S.** Gluconeogenic enzyme gene expression is decreased by dietary carbohydrates in common carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*). *Biochim Biophys Acta* 1579: 35–42, 2002.
47. **Panserat S, Plagnes-Juan E, Kaushik S.** Nutritional regulation and tissue specificity of gene expression for proteins involved in hepatic glucose metabolism in rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 204: 2351–2360, 2001.
48. **Panserat S, Rideau N, Polakof S.** Nutritional regulation of glucokinase: a cross-species story. *Nutr Res Rev:* 27: 1–27, 2014.
49. **Panserat S, Rideau N, Polakof S.** Nutritional regulation of glucokinase: a cross species story. *Nutrit Res Rev* 27: 21–47, 2014.
50. **Panserat S, Skiba-Cassy S, Seiliez I, Lansard M, Plagnes-Juan E, Vachot C, Aguirre P, Larroquet L, Chavernac G, Medale F, Corraze G, Kaushik S, Moon TW.** Metformin improves postprandial glucose homeostasis in rainbow trout fed dietary carbohydrates: a link with the induction of hepatic lipogenic capacities? *Am J Physiol Regul Integr Comp Physiol* 297: R707–R715, 2009.
51. **Pilkis SJ, Claus TH.** Hepatic gluconeogenesis/glycolysis: regulation and structure/function relationships of substrate cycle enzymes. *Annu Rev Nutr* 11: 465–515, 1991.
52. **Plagnes-Juan E, Lansard M, Seiliez I, Medale F, Corraze G, Kaushik S, Panserat S, Skiba-Cassy S.** Insulin regulates the expression of several metabolism-related genes in the liver and primary hepatocytes of rainbow trout *(Oncorhynchus mykiss). J Exp Biol* 211: 2510–2518, 2008.
53. **Polakof S, Moon TW, Aguirre P, Skiba-Cassy S, Panserat S.** Effects of insulin infusion on glucose homeostasis and glucose metabolism in rainbow trout fed a high-carbohydrate diet. *J Exp Biol* 213: 4151–4157, 2010.
54. **Polakof S, Moon TW, Aguirre P, Skiba-Cassy S, Panserat S.** Glucose homeostasis in rainbow trout fed a high-carbohydrate diet: metformin and insulin interact in a tissue-dependent manner. *Am J Physiol Regul Integr Comp Physiol* 300: R166–R174, 2011.
55. **Polakof S, Panserat S, Soengas JL, Moon TW.** Glucose metabolism in fish: a review. *J Comp Physiol B* 182: 1015–1045, 2012.
56. **Polakof S, Skiba-Cassy S, Panserat S.** Glucose homeostasis is impaired by a paradoxical interaction between metformin and insulin in carnivorous rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 297: R1769–R1776, 2009.
57. **Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, Shelton KD, Lindner J, Cherrington AD, Magnuson MA.** Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic β cell-specific gene knock-outs using Cre recombinase. *J Biol Chem* 274: 305–315, 1999.
58. **Rider MH, Bertrand L, Vertommen D, Michels PA, Rousseau GG, Hue L.** 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis. *Biochem J* 381: 561–579, 2004.
59. **Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM.** Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* 22: 159–168, 2006.
60. **Seiliez I, Gabillard JC, Skiba-Cassy S, Garcia-Serrana D, Gutierrez J, Kaushik S, Panserat S, Tesseraud S.** An in vivo and in vitro assessment of TOR signaling cascade in rainbow trout (*Oncorhynchus mykiss*). *Am J Physiol Regul Integr Comp Physiol* 295: R329–R335, 2008.
61. **Seiliez I, Panserat S, Lansard M, Polakof S, Plagnes-Juan E, Surget A, Dias K, Larquier M, Kaushik S, and Skiba-Cassy S.** Dietary carbohydrate-to-protein ratio affects TOR signaling and metabolism-related gene expression in the liver and muscle of rainbow trout after a single meal. *Am J Physiol Regul Integr Comp Physiol* 300: R733–R743, 2011.
62. **Seiliez I, Sabin N, Gabillard JC.** FoxO1 is not a key transcription factor in the regulation of myostatin (mstn-1a and mstn-1b) gene expression in trout myotubes. *Am J Physiol Regul Integr Comp Physiol* 301: R97–R104, 2011.
63. **Skiba-Cassy S, Lansard M, Panserat S, Medale F.** Rainbow trout genetically selected for greater muscle fat content display increased activation of liver TOR signaling and lipogenic gene expression. *Am J Physiol Regul Integr Comp Physiol* 297: R1421–R1429, 2009.
64. **Skiba-Cassy S, Panserat S, Larquier M, Dias K, Surget A, Plagnes-Juan E, Kaushik S, Seiliez I.** Apparent low ability of liver and muscle to adapt to variation of dietary carbohydrate:protein ratio in rainbow trout (*Oncorhynchus mykiss*). *Br J Nutr* 109: 1359–1372, 2013.
65. **Tranulis MA, Dregni O, Christophersen B, Krogdahl A, Borrebaek B.** A glucokinase-like-enzyme in the liver of Atlantic salmon (*Salmo salar*). *Comp Biochem Physiol B Biochem Mol Biol* 114: 35–39, 1996.
66. **Tremblay F, Gagnon A, Veilleux A, Sorisky A, Marette A.** Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3–L1 and human adipocytes. *Endocrinology* 146: 1328–1337, 2005.
67. **Tremblay F, Lavigne C, Jacques H, Marette A.** Role of dietary proteins and amino acids in the pathogenesis of insulin resistance. *Annu Rev Nutr* 27: 293–310, 2007.
68. **Tremblay F, Marette A.** Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. *J Biol Chem* 276: 38,052–38,060, 2001.
69. **Tzatsos A, Kandror KV.** Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent mTOR-mediated insulin receptor substrate 1 phosphorylation. *Mol Cell Biol* 26: 63–76, 2006.
70. **Um SH, D'Alessio D, Thomas G.** Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. *Cell Metab* 3: 393–402, 2006.
71. **Verbrugghe A, Bakovic M.** Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis. *Nutrients* 5: 2811–2835, 2013.
72. **Wan M, Leavens KF, Saleh D, Easton RM, Guertin DA, Peterson TR, Kaestner KH, Sabatini DM, Birnbaum MJ.** Postprandial hepatic lipid metabolism requires signaling through Akt2 independent of the transcription factors FoxA2, FoxO1, and SREBP1c. *Cell Metab* 14: 516–527, 2011.
73. **Washizu T, Tanaka A, Sako T, Washizu M, Arai T.** Comparison of the activities of enzymes related to glycolysis and gluconeogenesis in the liver of dogs and cats. *Res Vet Sci* 67: 205–206, 1999.
74. **Wullschleger S, Loewith R, Hall MN.** TOR signaling in growth and metabolism. *Cell* 124: 471–484, 2006.
75. **Yang SB, Lee HY, Young DM, Tien AC, Rowson-Baldwin A, Shu YY, Jan YN, Jan LY.** Rapamycin induces glucose intolerance in mice by reducing islet mass, insulin content, and insulin sensitivity. *J Mol Med* 90: 575–585, 2012.
76. **Yecies JL, Manning BD.** Transcriptional control of cellular metabolism by mTOR signaling. *Cancer Res* 71: 2815–2820, 2011.
77. **Yecies JL, Zhang HH, Menon S, Liu S, Yecies D, Lipovsky AI, Gorgun C, Kwiatkowski DJ, Hotamisligil GS, Lee CH, Manning BD.** Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. *Cell Metab* 14: 21–32, 2011.

Chapter 4

GENERAL DISCUSSION

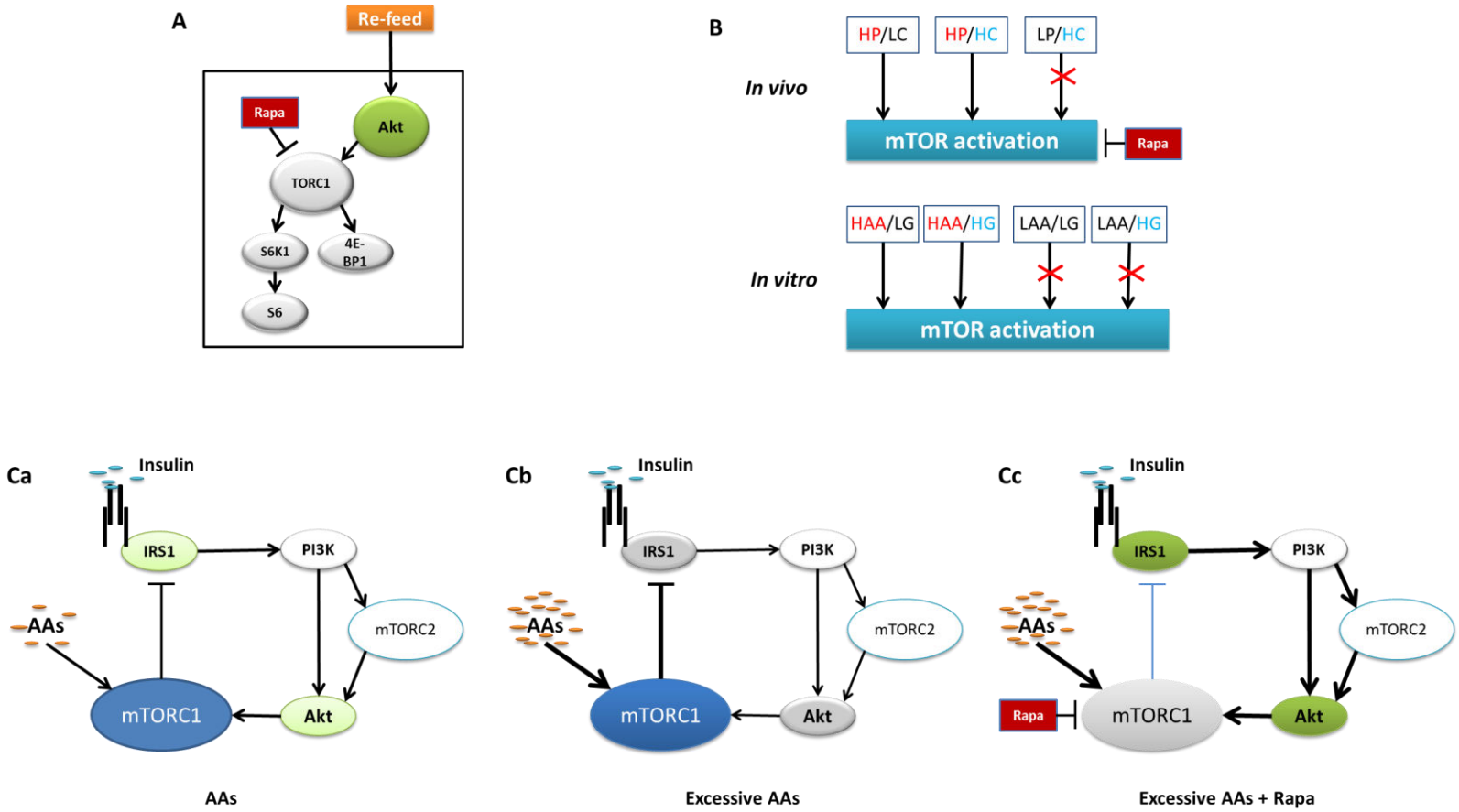
This thesis investigated the roles of nutrients, especially dietary protein/amino acids (AAs) on the regulation of hepatic intermediary metabolism and further determined the potential involvement of relevant signaling pathways (e.g. insulin/Akt, mTORC1 and p38 MAPK) in these regulation processes in rainbow trout (*Oncorhynchus mykiss*), a carnivorous fish and relevant diabetic research model. To achieve this goal, we first identified mTORC1 downstream target genes with RT-PCR method in rainbow trout liver and primary hepatocytes (Publication 1 and 3). Secondly, we evaluated and compared the effects of dietary protein intake/AAs and dietary carbohydrate intake/glucose on the stimulation of hepatic lipogenic gene expression during short-term stimulations using both *in vivo* and *in vitro* approaches (Publication 2). Thirdly, we evaluated the effects of different AA levels on the regulation of expression of genes related to hepatic fatty acid biosynthesis, glucose metabolism and AA catabolism, and also assessed the potential involvements of Akt/mTORC1 and p38 MAPK signaling pathway in these regulations using freshly isolated trout hepatocytes (Publication 3). Finally, we investigated the role of mTOR signaling pathway in the regulation of glucose homeostasis (*in vivo*) (Publication 4). The results presented in the previous chapters are developed into a comprehensive discussion as follows:

4.1 mTOR activation, overactivation and inhibition

Re-feeding significantly stimulated mTORC1 activation and the phosphorylation of its downstream effectors (S6K1, S6 and 4EBP1) in trout liver, while acute inhibition of rapamycin effectively suppressed these up-regulations (Figure 4.1 A).

Investigation on Akt/TOR signaling pathway reveals that administration of rapamycin significantly inhibited the postprandial phosphorylation of TOR and its downstream effectors S6K1, S6 and 4E-BP1, without affecting the phosphorylation of Akt, FoxO1/FoxO3 or GSK 3α/β. These results indicate that the activation of mTOR pathway induced by refeeding can be successfully blocked by a single injection of rapamycin, which is consistent with our previous finding in primary culture of trout hepatocytes (Lansard et al., 2010) and other investigations in mammals (Owen et al., 2012; Yang et al., 2012b). Unlike the Thr^{308} residue of Akt, which is phosphorylated by 3-phosphoinositide dependent protein kinase 1 (PDK1), Akt Ser^{473} is directly phosphorylated by mTORC2 (Dibble and Cantley, 2015; Shimobayashi and Hall, 2014). Given that postprandial hepatic phosphorylation of Akt Ser^{473} and its downstream effectors FoxO1/FoxO3 and GSK 3α/β are not affected by rapamycin, we conclude that acute administration of rapamycin in rainbow trout only inhibited the phosphorylation of TORC1, without affecting TORC2. It is also worth to note that administration of rapamycin in trout reduced but did not totally abolish 4E-BP1 phosphorylation even though S6K1 and S6 phosphorylation were fully inhibited, implicating that rapamycin only incompletely inhibits mTORC1 target phosphorylation as previously demonstrated in mammals (Benjamin et al., 2011). Recent work conducted in Sabatini's

Figure 4. 1 mTOR activation, overactivation and inhibition by rapamycin



(A) Re-feeding significantly stimulated mTORC1 activation and the phosphorylation of its downstream effectors (S6K1, S6 and 4EBP1) in trout liver, while acute inhibition of rapamycin effectively suppressed these up-regulations (article 1). (B) In trout liver, high protein intake (HP) induced higher TOR activation compared to low protein intake treatment (LP) regardless of carbohydrate intake; similarly, in trout hepatocytes, high level of AAs (HAA) induced enhanced mTOR activation compared to low level of AAs (LAA) irrespective of glucose levels (article 2). (C) a| Insulin together with normal AAs led to mTORC1 activation; b| Excessive AAs induced mTORC1 overactivation and attenuated insulin signaling through mTORC1-induced feedback loop; c| Under excessive AAs conditions, rapamycin effectively inhibited mTORC1 activation and its inhibition effect on insulin signaling, leading to restored or enhanced insulin-PI3K-Akt signaling (article 3). Rapa, rapamycin; HP/LC, high protein/low carbohydrate; HPHC, high protein/high carbohydrate; LPHC, low protein/low carbohydrate; HAA, high AAs; LAA, low AAs; HG, high glucose; LG, low glucose; AAs, amino acids.

laboratory has highlighted how the Thr37/46 residues of 4E-BP1 are good substrates for mTORC1 but are resistant to rapamycin (Chiarini et al., 2015), which probably explains the poorly inhibited 4EBP1 phosphorylation observed in the current thesis.

High protein intake/AAs levels induced enhanced mTORC1 activation regardless of carbohydrate intake/glucose levels

We observed that high protein intake induced higher phosphorylation of TOR and 4EBP1 compared to low protein intake regardless of dietary carbohydrate intake. Despite no statistical difference for the phosphorylation of S6K1 and S6, it also showed elevated mean values in high protein intake treatments compared to low protein intake treatment, similar as TOR and 4EBP1 results. *In vitro* data further confirmed the *in vivo* observations showing that high level of AAs induced enhanced S6K1 and S6 phosphorylation compared to low level of AAs irrespective of glucose levels. Therefore, we conclude that high protein intake/AAs levels enhanced mTORC1 activation regardless of carbohydrate intake/glucose levels in rainbow trout (Figure 4.1 B). This finding is in agreement with previous *in vivo* findings in trout, which reported that diet rich in protein and low in carbohydrates induced higher S6 phosphorylation in liver and elevated S6K1, S6 and 4EBP1 phosphorylation in muscle compared to high carbohydrates low protein diet (Seiliez et al., 2011a). It is also consistent with studies in rat demonstrating that high protein intake/AAs elevated the phosphorylation of mTOR and 4EBP1 compared with low protein intake/AAs (Chotechuang et al., 2009).

Although IP administration of glucose activated the mTOR pathway *in vivo* (Dai et al., 2014), we assume that this was due to an indirect instead of a direct effect of glucose. Glucose administration has been reported to cause hyperglycemia in a number of teleost species (Harmon et al., 1991; Ince and Thorpe, 1974; Khanna and Rekhari, 1972; Moon, 2001), and hyperglycemia can, in turn, induce hyperinsulinemia through stimulating insulin release (Mommsen and Plisetskaya, 1991). Furthermore, under low nutrient conditions, autophagy-mediated protein catabolism results in the production of free amino acids that provide building blocks for protein synthesis and serve to maintain intracellular amino acid pools (Kaur and Debnath, 2015). Given that we recorded increased glycemia and enhanced Akt phosphorylation, which is mainly stimulated by insulin, we deduce that IP administration of glucose activated mTOR activation through a combination effect of hyperglycemia-induced insulin release and autophagy-produced AAs. However, this deduction needs further verification.

Excessive AAs induced overactivation of mTORC1 and attenuated insulin/Akt signaling through enhancing mTORC1/S6K1-induced feedback loop

Using trout primary hepatocytes, we further demonstrated that increased AA levels not only elevated the phosphorylation of mTORC1 downstream effectors, S6K1 and S6, but also enhanced the phosphorylation of IRS-1 on Ser^{302} and decreased the phosphorylation of Akt on both Ser^{473} and Thr^{308}. One potential mechanism that can link these observed results is the mTORC1/S6K1-induced feedback loop. Namely, enhanced mTOR and S6K1 phosphorylation probably suppressed Akt phosphorylation via enhancing mTORC1/S6K1-mediated phosphorylation of IRS-1 on serine residues, which dampens the function of IRS-1 and leads to insulin desensitization (Boura-Halfon and Zick, 2009; Dann et al., 2007; Efeyan and Sabatini, 2010). By using rapamycin, we further observed that mTORC1 inhibition blunted IRS-1 Ser^{302} phosphorylation and restored Akt phosphorylation in excessive (four fold concentrated) AAs-treated hepatocytes, confirming the existence of mTORC1/S6K1-operated negative feedback loop, which decreases insulin sensitivity under AAs excess conditions in rainbow trout hepatocytes. Therefore, we indeed demonstrated that increased AA levels promoted mTORC1/S6K1 activation, which in turn operated a negative feedback loop toward Akt signaling pathway through, at least in part, enhanced IRS-1 phosphorylation on Ser^{302} (Figure 4.1 C). This demonstration is in agreement with previous *in vitro* (Patti et al., 1998; Takano et al., 2001; Tremblay and Marette, 2001) and *in vivo* (Layman et al., 2003; Tremblay et al., 2005b) mammalian investigations. Considering that Akt phosphorylation on Ser^{473} is known to be mainly regulated by mTORC2 (Dibble and Cantley, 2015; Masui et al., 2014), which is stimulated by insulin via its association with ribosomes in a PI3K-dependent manner (Shimobayashi and Hall, 2014), we presume that mTORC2 phosphorylation was also repressed by mTORC1 overactivation-enhanced feedback loop as in mammals (Masui et al., 2014; Zoncu et al., 2011), probably via inhibiting the phosphorylation of Rictor on Thr1135 and mSIN1 on Thr86 and Thr398 (Masui et al., 2014).

Akt phosphorylation was enhanced by increased insulin level but suppressed by elevated AA level

As shown in Figure 1.19, both Akt phosphorylation and mTORC1 activation can be regulated by insulin and amino acids. Insulin can positively regulate Akt phosphorylation through IRS-PI3K and mTORC2 dependent manners, while elevated AA availability attenuates insulin signaling and decreases insulin-mediated Akt phosphorylation via enhancing mTORC1/S6K1-induced feedback loop on IRS1 serine residues (Manning and Cantley, 2007; Masui et al., 2014; Zoncu et al., 2011). Consistent with mammals, we observed in rainbow trout that Akt phosphorylation was significantly enhanced by higher insulin concentration irrespective of glucose or AA levels, while it was markedly suppressed by elevated AA levels via enhanced mTORC1 activation-induced feedback inhibition effect (Figure S4, unpublished data). To our knowledge, this is the first demonstration in carnivorous fish showing that increased insulin level positively regulates Akt phosphorylation. Therefore, we conclude that elevated insulin level promotes both Akt phosphorylation and mTORC1 activation, while elevated AA levels enhance mTORC1 activation but repress the activation of insulin signaling and Akt phosphorylation.

4.2 mTORC1 activation regulates hepatic intermediary metabolism

The work performed in current thesis allows us to identify some mTORC1- and Akt-controlled downstream target genes related to several metabolic pathways, including lipid and glucose metabolism, and AA catabolism.

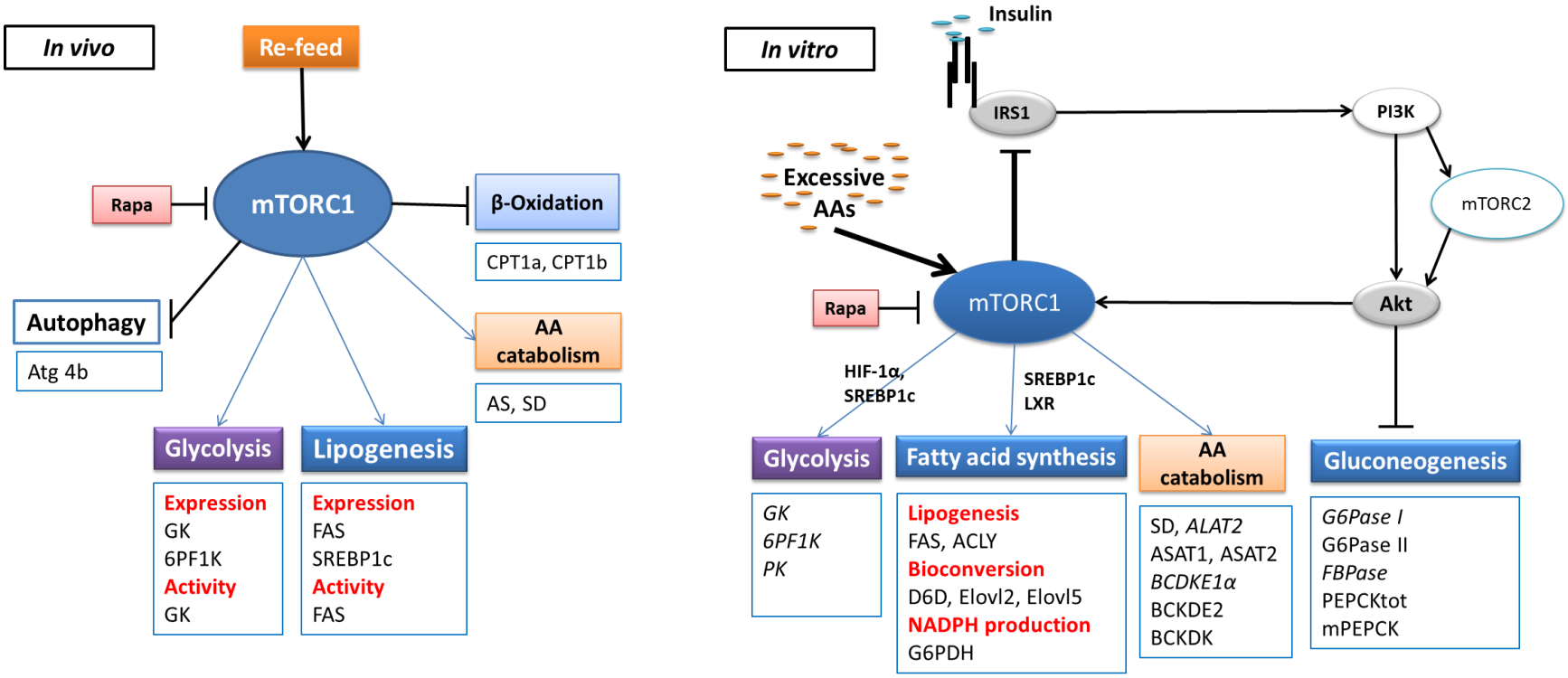
mTORC1 activation stimulates hepatic fatty acid biosynthesis

Our *in vivo* results demonstrated that mTORC1 inhibition impairs postprandial induction of FAS gene expression and enzyme activity, as well as the expression of SREBP1c (Dai et al., 2013), a transcription factor that activates all the genes needed to produce fatty acids and triglycerides in liver (Horton et al., 2002). Similarly, *in vitro* data also shown that mTORC1 inhibition suppressed the expression of FAS, ACLY and SREBP1c (Dai et al., 2015b), which is totally consistent with our previous demonstration in trout hepatocytes (Lansard et al., 2010). Thus, we conclude that mTORC1 activation promotes hepatic *de novo* lipogenesis in rainbow trout, in agreement with previous demonstrations in mammals (Duvel et al., 2010). Furthermore, mTORC1 inhibition also markedly suppressed the expression of genes involved in fatty acid bioconversion (D6D, Elovl5 and Elovl2), NADPH production (G6PDH) and transcriptional factor LXR in trout hepatocytes, indicating that mTORC1 signaling is essential in stimulating fatty acid bioconversion and the oxidative arm of the pentose phosphate pathway (PPP), which is consistent with previous demonstration in mammals (Duvel et al., 2010). Given that lipid synthesis is one of the most NADPH-demanding metabolic pathways, its co-regulation with the oxidative PPP by mTORC1 and SREBP1c is likely to help satisfy this requirement. LXR is another important transcriptional factor involves in the control of lipogenesis in the liver (Schultz et al., 2000), and is required by insulin-dependent SREBP1c transcriptional regulation (Shao and Espenshade, 2012). Based on our demonstration, we therefore conclude that the expression of SREBP1c and LXR is controlled by the mTOR pathway and mTORC1 promotes fatty acid biosynthesis through the transcription factor of SREBP1c- and LXR-dependent manner.

mTORC1 activation promotes glycolysis

Lansard et al. (Lansard et al., 2010) first revealed the link between mTOR signaling and glycolytic gene expression in carnivorous fish using cultured liver cells, showing that mTOR inhibition by rapamycin markedly suppressed the expression of genes involved in glycolysis (GK, 6PF1K and PK). We further confirmed *in vivo* that mTORC1 inhibition suppressed GK at both gene expression and enzyme activity levels (Dai et al., 2013; Dai et al., 2014), as well as the expression of 6PF1K (Dai et al., 2013). Moreover, our latest investigation recorded that mTORC1 inhibition also suppressed the expression of HIF-1α and SREBP1c (Dai et al., 2015b), two transcriptional factors known to positively regulate the expression of

Figure 4. 2 mTORC1-mediated downstream target genes



Gene name with italic format represents its expression is either affected by mTORC1 inhibition or altered mTORC1 activation.

glucose transporters and glycolytic enzymes and promote a switch from mitochondrial oxidative metabolism to glycolysis (Duvel et al., 2010; Laplante and Sabatini, 2013). Thus, our data strongly suggest that mTORC1 promotes hepatic glycolysis through HIF-1α and SREBP1c-dependent manner.

mTORC1 activation stimulates AA catabolism

Additionally, we observed that SD and AS expression were intimately regulated by mTORC1 activation *in vivo* (Figure S1). Furthermore, studies using trout hepatocytes demonstrated that acute inhibition of rapamycin not only inhibited SD expression (Dai et al., 2015b; Lansard et al., 2010) but also markedly suppressed the expression of other AA catabolic genes, including ASAT1, ASAT2, BCKDE2 and BCKDK (Dai et al., 2015b). Taken together, these results indicate that mTORC1 positively regulates hepatic amino acid catabolic gene expression in rainbow trout. It is well known that AAs are not only signaling molecules, but are also glucose and fatty acids precursors, which can supply carbon skeleton for hepatic gluconeogenesis and lipogenesis via AA catabolism (Berg et al., 2010). Thus, this observation is in line with foresaid demonstrations as elevated AA catabolism by mTORC1 activation can render more available substrates to support the other enhanced anabolic pathways, including fatty acid biosynthesis and NADPH-demanding. Moreover, this regulation also seems logical and reasonable when we take the poor glucose utilization in carnivorous fish into consideration. In mammals, dietary carbohydrate/ glucose provide substrates and NADPH to feed the demanding of the anabolic pathways but its efficiency in carnivorous fish is relatively poor compared to terrestrial mammals.

***mTORC1 activation negatively regulates β*-oxidation**

CPT1 is considered as a rate-limiting enzyme of fatty acid *β*-oxidation (Lu et al., 2014; Tocher, 2003). We observed that mTORC1 inhibition markedly up-regulated the expression of CPT1a and CPT1b, indicating that mTORC1 may negatively regulate hepatic *β*-oxidation, consistent with previous demonstrations in mammals (Ricoult and Manning, 2013). This demonstration can well-explain our previous observations showing that the expression of CPT1a, CPT1b (in liver and muscle) and HOAD (in muscle) was negatively associated with mTOR activation in rainbow trout liver and muscle (Seiliez et al., 2011a). This is also consistent with our previous *in vivo* observation that insulin markedly suppressed the expression of CPT1a and CPT1b as insulin together with AAs can effectively stimulates mTOR signaling in trout (Lansard et al., 2011; Lansard et al., 2010; Plagnes-Juan et al., 2008).

mTORC1 negatively controls hepatic autophagy

It is well-known in mammals that mTORC1 potently inhibits autophagy via multiple steps (Dibble and Manning, 2013; Shimobayashi and Hall, 2014). In line with these demonstrations, we observed that mTORC1 inhibition induced elevated expression of Atg4b in trout liver. Thus, we presume that mTORC1

negatively controls hepatic autophagy in rainbow trout as in mammals. Consistent with this deduction, we observed that refeeding markedly down-regulated the expression of Atg4b, LC3B and gabarapl1, which was associated with enhanced mTORC1 activation (Figure S1). Furthermore, Belghit et al. (Belghit et al., 2013) demonstrated that refeeding not only enhanced the phosphorylation of S6K1 but also suppressed the phosphorylation of LC3II (lapidated form of LC3b) in trout muscle. Moreover, Seiliez et al. (Seiliez et al., 2011a) reported that high protein/low carbohydrate diet induced higher S6K1, S6 and 4EBP1 phosphorylation in trout muscle, as well as lower LC3II phosphorylation (Belghit et al., 2013) compared to a low protein/high carbohydrate diet. These results are all in line with current deduction.

Phosphorylated Akt negatively regulates hepatic gluconeogenesis

Acute administration of rapamycin effectively inhibited excessive AAs-induced mTORC1 overactivation (Dai et al., 2015b). Associated with this inhibition, the expression of genes related to gluconeogenesis (G6Pase II, PEPCKtot and mPEPCK) was markedly reduced, indicating that mTORC1 positively regulates hepatic gluconeogenesis. It is well-known in mammals that gluconeogenesis is negatively regulated by insulin mainly through Akt-dependent manner (Accili and Arden, 2004; Li et al., 2007), and mTORC1 overactivation can attenuate this insulin/Akt-mediated regulation on gluconeogenesis through mTORC1/S6K1-induced feedback loop (Figure 1.19 B) (Manning and Cantley, 2007; Masui et al., 2014; Zoncu et al., 2011). Furthermore, as shown in Figure 4.1 C, we indeed recorded mTORC1 overactivation- and inhibition-induced down-regulation and up-regulation of Akt phosphorylation, respectively. Therefore, we presume that in rainbow trout as in mammals mTORC1 positively regulates hepatic gluconeogenesis through weakening Akt phosphorylation. Consistent with this deduction, our *in vivo* study also shown that elevated Akt phosphorylation on Ser^{473} by rapamycin (probably through inhibiting mTORC1/S6K1-induced feedback loop or stimulating insulin secretion) was associated with suppressed expression of G6Pase I, G6Pase II and FBPase, and enzyme activity of FBPase (Dai et al., 2014). Furthermore, in another *in vivo* study, hepatic gluconeogenic gene expression (G6Pase 1, FBPase and PEPCK) remained unaffected when Akt phosphorylation was unchanged although rapamycin effectively inhibited refeeding-induced mTORC1 activation (Dai et al., 2013). Moreover, the negative association between Akt phosphorylation and gluconeogenic gene expression was repeatedly observed in the insulin- and different AA levels-mediated modulations in rainbow trout both *in vivo* and *in vitro* (Dai et al., 2015b; Plagnes-Juan et al., 2008). Therefore, we conclude that Akt negatively regulates hepatic gluconeogenesis in rainbow trout.

In summary, mTORC1 activation stimulates hepatic fatty acid biosynthetic, glycolytic and amino acid catabolic gene expression, but inhibits the expression of genes related to β-oxidation and autophagy. Akt negatively regulates hepatic gluconeogenic gene expression (Figure 4.2).

4.3 Nutritional regulation of hepatic fatty acid biosynthesis in rainbow trout

Hepatic fatty acid biosynthesis is more responsive to dietary protein intake/AAs than dietary carbohydrate intake/glucose in rainbow trout

Our *in vitro* data (Dai et al., 2015a) clearly showed that the expression of genes related to hepatic DNL (FAS and ACLY), fatty acid bioconversion (Elovl5, Elovl2, D6D and SCD1), NADPH production (G6PDH and ME) and transcription factor SREBP1c were coordinately up-regulated by high level of AAs, while high level of glucose only elevated the expression of ME, indicating that in rainbow trout hepatocytes, fatty acid biosynthetic gene expression is more responsive to AAs than glucose. In line with these observations, *in vivo* data also showed that high protein intake induced higher Elovl2 gene expression regardless of carbohydrate intake, while high carbohydrate intake suppressed the expression of ACC and Elovl5. Notably, despite the absence of statistical significance, the expression of FAS, ACLY and D6D exhibited higher mean mRNA levels in high protein intake groups. Therefore, we conclude that hepatic fatty acid biosynthetic gene expression in rainbow trout is more responsive to dietary protein intake/AAs than dietary carbohydrate intake/glucose during acute stimulations.

The finding that dietary protein/AAs is a more potent stimulator for hepatic fatty acid biosynthesis compared to dietary carbohydrate/glucose is in agreement with previous *in vitro* observations demonstrating that increased AA levels up-regulated fatty acid biosynthetic gene expression (Dai et al., 2015b) and *in vivo* observations reporting that high protein low carbohydrate diets stimulated lipogenic gene expression (FAS and SREBP1) and enzyme activity (FAS, G6PDH and ME) compared to low protein high carbohydrate diets in rainbow trout and blackspot seabream, respectively (Figueiredo-Silva et al., 2009; Seiliez et al., 2011a). However, these results are in contrast with the demonstrations in rodents, chickens or humans reporting that high protein (HP) diets induce down-regulations of fatty acid biosynthesis in liver at both gene expression and enzymatic levels (Abu-Elheiga et al., 2012; Allee et al., 1971; Pichon et al., 2006; Rietman et al., 2014a; Uebanso et al., 2009). Data in mouse have shown that an increased flux of AAs reaching the hepato-portal area in HP diet conditions promotes AA catabolism and acetyl-CoA synthesis, however, it was suggested that the synthesized acetyl-CoA is either channeled into TCA cycle or used for β-hydroxybutyrate (BHB) production, but not converted to fatty acids through unknown reasons (Rietman et al., 2014a; Schwarz et al., 2012; Wu, 2009). Therefore, DNL rates might be lower or even absent after an HP diet (Schwarz et al., 2012). Based on our results, we reason that, unlike rodents or humans, protein/AAs is a more potent mediator for stimulating hepatic lipogenesis compared to carbohydrate/glucose in rainbow trout, confirming the surmise of Tocher (Tocher, 2003), who suggested that AAs are the preferred carbon source for lipogenesis in trout. Future work with tracer method is needed to further confirm this conclusion at nutrient flux level. It is of interest to note

that current observation in rainbow trout is similar as in domestic cat, a terrestrial carnivorous animal, in which acetate is the predominant carbon source for DNL in both liver and adipose tissue instead of glucose which is the main carbon source for DNL in human and rodents (Richard et al., 1989; Robert and Backus, 2008; Verbrugghe and Bakovic, 2013). However, unlike trout, human or rodents where liver is the primary site for DNL (Bergen and Mersmann, 2005; Tocher, 2003), in cats, adipose tissue serves this function, followed by liver, mammary glands and muscle (Robert and Backus, 2008; Verbrugghe and Bakovic, 2013).

The results that high carbohydrate intake/glucose failed to stimulate hepatic lipogenic gene expression but up-regulated ME (*in vitro*) are consistent with previous studies reporting that high carbohydrate diets/glucose failed or only had limited effects in stimulating lipogenic gene expression and enzyme activities in rainbow trout (Hemre and Kahrs, 1997; Jin et al., 2014a; Seiliez et al., 2011a). Notably, similar demonstrations were reported in cat and mink (Rouvinen-Watt et al., 2012; Verbrugghe and Bakovic, 2013), showing that fatty acids are not synthetized from glucose, probably due to the absence of glucokinase in feline liver (Macdonald et al., 1984; Schermerhorn, 2013). The results also confirmed the presumption of Hemre et al. (Hemre et al., 2002) that carbohydrate is needed to stimulate lipid biosynthesis not so much through delivery of carbon backbones, but rather via increased availability of cytosolic reducing equivalents, and are consistent with some other demonstrations in carnivorous fish (Barroso et al., 2001; Enes et al., 2008a; Fynnaikins et al., 1992; Suarez et al., 1995). However, this is in contrast with mammals where diets high in simple carbohydrates led to elevated expression of enzymes involved in DNL, MUFA synthesis (SCD1) and PUFA synthesis (Elovl2, Elovl5 and D6D) (Chong et al., 2008; Girard et al., 1997; Jump, 2011; Kersten, 2001; Towle et al., 1997). The poor induction of hepatic lipogenic/fatty acid biosynthetic gene expression by dietary carbohydrate intake/glucose during acute stimulations provides one clear and reasonable explanation for the poor dietary carbohydrate utilization in rainbow trout confirming the presumption of Panserat et al. (Panserat et al., 2013).

In human and other mammalian species, consumption of high dietary protein can induce hyper-TOR activation, which may in turn induce suppressed autophagy, thereby promoting ER stress and, chronically, causing mitochondrial dysfunction and insulin resistance (Arruda et al., 2014; Lowell and Shulman, 2005; Senft and Ronai, 2015; Yang et al., 2010). Furthermore, high dietary protein stimulates insulin hypersecretion and overactivation of mTORC1 that can blunt insulin sensitivity via mTORC1/S6K1-induced negative feedback loop, which will further increase demand of insulin secretion. The increased demand for insulin biosynthesis in turn increases demand in the ER, gradually leading to ER stress, increased protein misfolding, activation of unfolded protein response (UPR) and dysregulated mitochondria function. Collectively, these adverse events may eventually cause insulin resistance (Codogno and Meijer, 2010; Hummasti and Hotamisligil, 2010; Jung and Lee, 2010; Lynch and Adams,

2014; Rietman et al., 2014b; Senft and Ronai, 2015; Yang et al., 2010). Considering that rainbow trout possesses high dietary protein consumption and acute stimulation with excessive AAs induces overactivation of mTORC1, which in turn attenuates insulin-mediated repression of gluconeogenesis via mTORC1/S6K1-induced negative feedback loop (Dai et al., 2015b), we presume that the poor carbohydrate utilization in rainbow trout is possibly due to a combination of mitochondrial dysfunction, ER stress, hypo-autophagy, accumulation of misfolded proteins and altered UPR as in mammals (Muoio and Newgard, 2008; Zoncu et al., 2011). Interestingly, in line with this presumption, recent investigation in zebrafish reported that fructose treatment of larval zebrafish induced hepatic lipid accumulation, which was associated with mTORC1 activation, inflammation and oxidative stress (Sapp et al., 2014). Therefore, future work are needed to investigate the parameters related to inflammatory stresses and mitochondrial functions as they may account for the low utilization efficiency of dietary carbohydrate/glucose and poorly inhibited hepatic gluconeogenesis in rainbow trout as in mammals.

Dietary protein/AAs stimulates hepatic fatty acid biosynthesis through mTORC1 dependent manner

In vitro data showed that elevated fatty acid biosynthetic gene expression by high level of AAs was associated with the enhanced phosphorylation of S6K1 and S6 (Dai et al., 2015a; Dai et al., 2015b), and the elevated fatty acid biosynthetic gene expression was markedly suppressed by mTORC1 inhibition (Dai et al., 2015b). *In vivo* data also showed that high protein intake not only induced higher TOR and 4EBP1 phosphorylation but also induced higher Elovl2 gene expression regardless of carbohydrate intake. Although not statistically significant, the expression of FAS, ACLY and D6D exhibited similar patterns as the phosphorylation of S6K1 and S6, showing that high protein intake induced higher mean mRNA levels. Furthermore, mTORC1 inhibition suppressed the expression of FAS, ACLY, ACC, Elovl2, Elovl5, D6D and G6PDH, indicating that the regulation of hepatic fatty acid biosynthetic gene expression is mTORC1-dependent, which is consistent with our latest demonstrations in trout hepatocytes (Dai et al., 2015b). Therefore, our data clearly demonstrate that dietary protein intake/AAs stimulate fatty acid biosynthetic gene expression through an mTORC1-dependent manner. Consistent with this observation, recent investigation in zebrafish also shown that mTORC1 activation is required for hepatic lipid accumulation as rapamycin reverses hepatic lipid accumulation and associated gene expression changes (lipogenic, inflammatory, oxidative stress and ER stress genes) (Sapp et al., 2014).

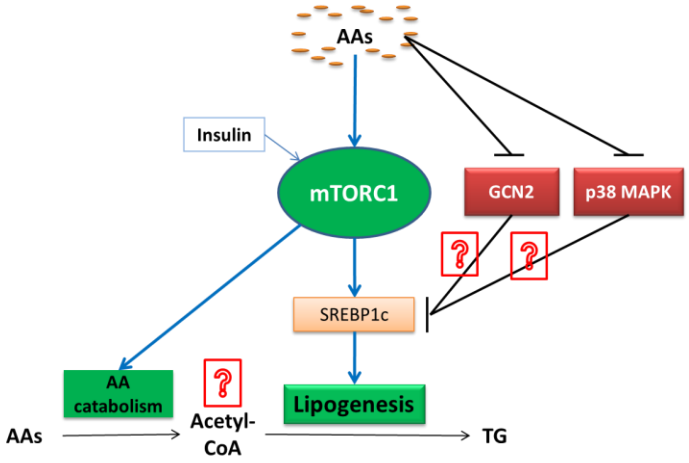
GCN2 and p38 MAPK pathway probably also involve in the AAs-mediated regulation of hepatic fatty acid biosynthesis

The mTOR signaling pathway probably is not the only cellular signaling pathway that regulates hepatic lipogenesis in rainbow trout. It is of interest to note that AA-induced decrease of p38 phosphorylation was associated with the up-regulation of hepatic lipogenic (ACLY and FAS) and G6PDH gene expression,

which are compatible with previous reports in mammals indicating that p38 MAPK negatively regulates mRNA levels of hepatic lipogenic genes and G6PDH (Talukdar et al., 2005; Xiong et al., 2007b). Furthermore, as shown in Figure S3, increased AA levels significantly down-regulated the expression of ATF4 downstream target genes, including SNAT, ASNS, CAT and CHOP, implicating that GCN2-ATF4 pathway was down-regulated by elevated AA levels. Given that GCN2 can also regulate the expression of lipogenic genes through controlling SREBP-1c gene expression (Guo and Cavener, 2007), we presume that, apart from mTORC1 signaling pathway, both GCN2 and p38 MAPK pathway participate in the AAs-mediated regulation of hepatic fatty acid biosynthesis. Further experiments are needed to confirm it in the future.

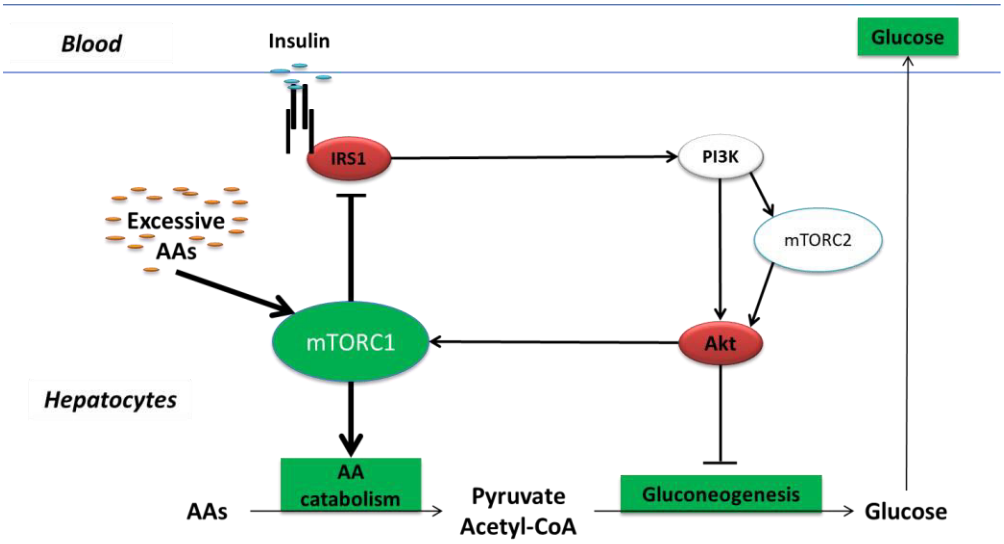
In summary, AAs promote hepatic lipogenesis via at least three manners: 1. enhancing mTORC1 activation, thereby up-regulating hepatic lipogenic gene expression; 2. promoting AA catabolic gene expression via mTORC1-dependent manner; 3. down-regulating GCN2 and p38 MAPK pathway, thereby stimulating hepatic lipogenesis (need to be confirmed) (Figure 4.3).

Figure 4. 3 Amino acids promote hepatic lipogenesis via multiple manners



Amino acids (AAs) promote hepatic lipogenesis via at least three manners: 1. Enhancing mTORC1 activation, thereby up-regulating hepatic lipogenesis through the transcriptional factor SREBP1c; 2. Promoting AA catabolism via mTORC1-dependent manner; 3. Down-regulating GCN2 and p38 MAPK pathway, thereby stimulating hepatic lipogenesis (need to be confirmed). Green colour means protein function or relevant pathway is enhanced; red colour means relevant pathways are suppressed; question mark represents the regulation needs to be confirmed in the future.

Figure 4. 4 Excessive amino acids up-regulate hepatic gluconeogenesis via multiple manners



Excessive amino acids (AAs) induce mTORC1 overactivation, which in turn promotes hepatic gluconeogenesis via: (1) attenuating insulin-mediated repression on hepatic gluconeogenesis through elevated IRS-1 phosphorylation on Ser^{302}, which in turn impairs the activation of Akt pathway and weakens insulin action; (2) elevating AA catabolic gene expression. Elevated hepatic gluconeogenesis, in turn, increases blood glucose levels. Green colour means protein function or relevant pathways that are enhanced; red colour means protein activation that is significantly suppressed.

4.4 The regulation of hepatic gluconeogenesis

AAs attenuate insulin-mediated repression of gluconeogenesis through elevating IRS-1 Ser^{302} phosphorylation, which in turn impairs Akt phosphorylation and weakens insulin action

An intriguing finding of the current thesis is that high levels of AAs elevated hepatic gluconeogenic gene expression through attenuating insulin signaling. Consistent with previous demonstrations in trout (Lansard et al., 2010; Plagnes-Juan et al., 2008), insulin effectively suppressed the gene expression of two key gluconeogenic enzymes, G6Pase and PEPCK. However, increased AA levels attenuated these suppressions and increased the gene expression of FBPase, suggesting that insulin-mediated down-regulation of hepatic gluconeogenesis was attenuated by increased AA sufficiency. This finding is in agreement with previous *in vivo* findings in trout and sea bass, which reported that higher hepatic gluconeogenic enzyme activities and/or mRNA levels were associated with high dietary protein diet/intake compared to low protein diet/intake (Cowey et al., 1977; Enes et al., 2006; Hilton and Atkinson, 1982; Kamalam et al., 2012; Kirchner et al., 2003; Walton, 1986). Furthermore, the negative relation between Akt phosphorylation and gene expression of G6Pase and PEPCK was consistently observed in all insulin-, AA levels- and rapamycin-mediated modulations, confirming that hepatic gluconeogenesis was negatively controlled by Akt phosphorylation in trout hepatocytes, as in mammals (Cheng and White, 2012; Manning and Cantley, 2007). Therefore, we concluded that high levels of AAs attenuated insulin-mediated down-regulation of hepatic gluconeogenic gene expression through mTORC1/S6K1-induced feedback loop. Similar inhibitory effects on insulin action as a consequence of increased AA supply have also been reported in mammalian hepatocytes (Khamzina et al., 2005; Patti et al., 1998), muscle cells (Tremblay and Marette, 2001) and adipocytes (Haruta et al., 2000; Takano et al., 2001; Tremblay et al., 2005a), as well as *in vivo* studies (Linn et al., 2000; Rossetti et al., 1989; Tremblay et al., 2005b).

The present work together with previous study (Lansard et al., 2010) provides strong evidences that increased AA sufficiency up-regulates hepatic gluconeogenesis through mTOR-dependent and independent manners, respectively. However future studies are needed to test whether AAs (particularly glutamine) also dampen insulin signaling and promote glucose production through stimulating hexosamine biosynthetic pathway/O-GlcNAc signaling (Slawson et al., 2010). Furthermore, considering that persistent activation of mTORC1 and S6K1 may promote insulin resistance and glucose intolerance in mammals (Lynch and Adams, 2014; Tremblay et al., 2007), our finding that high levels of AAs up-regulated hepatic gluconeogenic gene expression through attenuating insulin signaling, indeed highlighted the potential role of AAs as casual factor for poorly inhibited postprandial gluconeogenesis and "glucose intolerant" phenotype in carnivorous species such as salmonids. However, it is noteworthy

that AAs elevation *in vivo* can also stimulate higher insulin secretion (Floyd et al., 1966; Mommsen and Plisetskaya, 1991), which may counteract the effects of AAs on glucose production (Tremblay et al., 2005b). Thus, whether high protein diet/intake impairs insulin action and induces elevated glucose production *in vivo* still needs to be investigated in fish through prolonged *in vivo* studies in the future. Moreover, given that insulin signaling is required for mitochondrial function and metabolism (Cheng et al., 2010), we reason that impaired insulin action may have dysregulated mitochondrial functions as well, which may account for the unstrained hyperglycemia through reducing insulin responsiveness in skeletal muscle and liver and decreasing glucose-stimulated insulin secretion by pancreatic β cells (Cheng et al., 2010; Lowell and Shulmanz, 2005; Senft and Ronai, 2015; Szendroedi et al., 2012). As proposed by Lowell and Shulman (Lowell and Shulman, 2005), a decrease in mitochondrial fatty acid oxidation, caused by mitochondrial dysfunction and/or reduced mitochondrial content, produces increased levels of intracellular fatty acyl CoA and diacylglycerol. These molecules activate novel protein kinase C, which in turn activates a serine kinase cascade [possibly involving inhibitor of nuclear factor κB kinase (IKK) and c-Jun N-terminal kinase–1], leading to increased serine phosphorylation of IRS-1. Increased serine phosphorylation of IRS-1 on critical sites (e.g., IRS-1 Ser^{307}) blocks IRS-1 tyrosine phosphorylation by the insulin receptor, which in turn inhibits the activity of PI3K. This inhibition results in suppression of insulin-stimulated glucose transport, thereby inducing insulin resistance in skeletal muscle and hyperglycemia. Furthermore, mitochondrial dysfunction may also disrupt insulin secretion from pancreatic β cells. These potential mechanisms merit future investigations in fish.

AAs provide substrates for gluconeogenesis through elevating AA catabolism

AAs are not only signaling molecules, but are also glucose and fatty acids precursors, which can supply carbon skeleton for hepatic gluconeogenesis and lipogenesis via AA catabolism (Berg et al., 2010; Walton and Cowey, 1982). As we observed that increased AA levels markedly elevated the expression of genes related to AA catabolism (SD, ALAT2, ASAT2, BCKDE1α, BCKDE2 and BCKDK), we presume that elevated AA levels promote hepatic fatty acid biosynthesis and glucose metabolism not only through modulating relevant signaling pathways, but also by serving as gluconeogenic and lipogenic substrates through promoting AA catabolism (Figure 4.3 and 4.4).

AAs either from dietary sources or from catabolism of body proteins are believed to be an important source of gluconeogenic substrates in rainbow trout (Walton and Cowey, 1982), but their true contribution remains unclear as the proportion of protein/AAs diverted into gluconeogenesis has not been measured. In healthy human or rats, it was reported that the amount of AA-derived carbon skeletons used for gluconeogenesis is very limited and this pathway cannot cope with the excess of incoming AA even under optimal gluconeogenic conditions (Fromentin et al., 2013; Schwarz et al., 2012).

However, unlike human or omnivorous animals where maximal gluconeogenesis only occurs in the post-absorptive state or during prolonged fasting stimulations, gluconeogenesis is more or less permanently "switched on" in carnivorous animals, including cat and rainbow trout (Legate et al., 2001; Macdonald et al., 1984). Furthermore, in carnivores, their natural diet contains little carbohydrate; therefore, a significant proportion of protein must be diverted into gluconeogenesis to supply the needs of the brain and the other glucose-requiring tissues (Eisert, 2011; Robert and Backus, 2008). Thus, we presume that the persistent high rate of gluconeogenesis in these glucose-requiring tissues is supplied by AAs, and the amount of AA-derived carbon skeletons used for gluconeogenesis in rainbow trout should be higher than in herbivores and omnivores. Further investigations are needed to assess this in the future.

Poorly inhibited hepatic gluconeogenesis maybe account for the prolonged hyperglycemia

It is also worthy to note that rapamycin significantly decreased plasma glucose levels 8 h after controlled-feeding and suppressed the expression of G6Pase I and G6Pase II (Figure S2), which is in line with our previous finding showing that acute rapamycin administration reduced the hyperglycemia induced by IP glucose injection via inhibiting hepatic gluconeogenesis at both gene expression and enzyme activity levels in rainbow trout (Dai et al., 2014). Interestingly, rapamycin also significantly enhanced plasma free amino acid levels 8 h after controlled-feeding. This elevated free AA level was negatively associated with the reduced glycemia, which is consistent with previous observations in trout (Polakof et al., 2008; Polakof et al., 2012b; Seiliez et al., 2011a; Skiba-Cassy et al., 2013). Given that AAs, especially gluconeogenic AAs, can promote glucose production through hepatic gluconeogenic pathway (Berg et al., 2010; Dai et al., 2015b), we presume that both the elevated free AA levels and the reduced glycemia by rapamycin may be related to the suppressed hepatic gluconeogenesis as we previously demonstrated (Dai et al., 2014), implicating the important role of hepatic gluconeogenesis in modulating glucose homeostasis. Notably, consistent with this deduction, recent work using stable tracer method by Viegas et al. (Viegas et al., 2015) revealed that supplementation of digestible starch resulted in a significant reduction of gluconeogenic contributions to systemic glucose appearance in seabass. Based on our demonstrations, it is attractive to postulate that poorly inhibited hepatic gluconeogenesis is account for the prolonged hyperglycemia and glucose intolerance phenotype in rainbow trout.

In summary, excessive AAs induce mTORC1 overactivation, which in turn promotes hepatic gluconeogenesis via (1) attenuating insulin-mediated repression on hepatic gluconeogenesis through elevated IRS-1 phosphorylation on Ser^{302}, which in turn impairs the activation of Akt pathway and weakens insulin action; (2) elevating AA catabolic gene expression (Figure 4.4).

4.5 The regulation of AA catabolism

AAs promote AA catabolic gene expression via mTORC1-dependent manner

In vitro results demonstrated that increased AA levels markedly elevated the expression of genes related to AA catabolism (SD, ALAT2, ASAT2, BCKDE1α, BCKDE2 and BCKDK) as well as mTORC1 activation. Acute administration of rapamycin not only inhibited mTORC1 activation but also suppressed the elevated hepatic AA catabolic gene expression (Dai et al., 2015b). Therefore, we conclude that AAs promote AA catabolic gene expression through mTORC1-dependent manner. Indeed the positive association between mTOR activation and hepatic AA catabolic gene expression has been previously reported (Dai et al., 2013; Lansard et al., 2011; Lansard et al., 2010; Seiliez et al., 2011a; Skiba-Cassy et al., 2009), supporting our current conclusion. For instance, insulin together with AAs up-regulate SD gene expression in trout hepatocytes through an mTOR-dependent manner (Lansard et al., 2011; Lansard et al., 2010). Though refeeding failed to stimulate SD mRNA level *in vivo*, mTORC1 inhibition significantly suppressed its expression (Figure S1). Furthermore, a diet rich in protein and low in carbohydrate not only induced higher mTOR activation but also upregulated the expression of SD and BCKD E2 in liver and the expression of BCKD E2 in muscle (Seiliez et al., 2011a). Thus, it is not surprising to see that insulin also up-regulated AA catabolic gene expression (SD, ASAT1, ASAT2, BCKDE2, GDH1 and GDH3 (GDH1 and GDH3 data are not presented in the paper)) as the addition of insulin stimulated mTORC1 activation (Dai et al., 2015b).

Current observation that mTORC1 activation positively regulates hepatic AA catabolic gene expression in rainbow trout merits our attention. In human and rats, after feeding a high protein diet, the activities of the AA catabolic enzymes in the liver and kidney are increased, facilitating disposal of excess nitrogen. The opposite is true; if a lower than normal level of protein is fed, the amino acid catabolism decreases, enabling the animal to preserve amino acids (Verbrugghe and Bakovic, 2013). Normally, AA catabolism increases when the supply of circulating AAs augments by fasting or after the consumption of an excess of dietary protein (Contreras et al., 2015), which is promoted by glucagon (Solloway et al., 2015). However, different with these demonstrations, we observed that AA catabolic gene expression was stimulated by mTORC1 activation and insulin in rainbow trout. This discrepancy may be due to the metabolic differences between carnivorous and omnivorous animals. Indeed, previous investigations *in vivo* had demonstrated that when fish are fed a high protein/low carbohydrate (HP/LC) diet compared to a low protein/high carbohydrate (LP/HC) diet, the enzymes which initiate amino acid catabolism are barely affected (Cowey and Walton, 1989; Kirchner et al., 2003), as in cats where hepatic AA catabolism is poorly inhibited even when fed a low protein diet (Macdonald et al., 1984). Considering that refeeding, even with a low protein diet may induce mTORC1 activation and, in turn, stimulate AA catabolism, our

observation, to same extend, is in line with these demonstrations (mTOR activation has not been analyzed in these studies). This observation is also consistent with the foresaid elevated fatty acids biosynthetic and gluconeogenic gene expression as elevated AA catabolism can render more available substrates to support these enhanced anabolic pathways. It is worthy to point out that if this conclusion is true it would possess significant importance as it may account for the high protein requirement (Macdonald et al., 1984; Robert and Backus, 2008), poor inhibition of postprandial hepatic gluconeogenesis (Robert and Backus, 2008), poor glucose utilization in peripheral tissues, as well as some other metabolic particularities in rainbow trout or general carnivores, which demands further explorations. However, to our knowledge, no equivalent counterpart has been reported yet, even in mammals or cat. Thus, we still need to be cautious about this conclusion and future studies are needful to further confirm current conclusion.

Limitations of the thesis

1. Due to the constraints in measuring piscine insulin, plasma insulin levels have not been measured in the in vivo experiments (publication 2 & 4)

In publication 2, we observed that hepatic fatty acid biosynthesis was more responsive to dietary protein intake compared to dietary carbohydrate intake. In rainbow trout, insulin has been proven a potent mediator for stimulating mTOR signaling and anabolic pathways, including glycolysis and lipid synthesis (Dai et al., 2015b; Plagnes-Juan et al., 2008; Saltiel and Kahn, 2001). Given that AAs are more potent insulin secretagogues compared to carbohydrates in most fish species (Mommsen and Plisetskaya, 1991), one potential explanation for the elevated lipogenic gene expression in high dietary protein diet conditions can be due to the higher insulin secretion induced by the HP diets. However, due to the constraints in measuring piscine insulin (Plisetskaya, 1998), we were unable to check this hypothesis *in vivo*.

Similarly, in publication 4, the elevated phosphorylation of Akt on Ser^{473} could be due to elevated insulin release induced by acute administration of rapamycin (Fuhrer et al., 2001) or rapamycin inhibition improved insulin sensitivity as rapamycin can interrupt mTORC1/S6K1-induced negative feedback loop on insulin signaling (Houde et al., 2010; Tremblay et al., 2005a; Tremblay and Marette, 2001; Tzatsos and Kandror, 2006; Um et al., 2006). Since we cannot measure plasma insulin levels, we were unable to verify these potential underlying mechanisms.

2. Controlled-feeding method probably induced high variations for the in vivo molecular parameters

With controlled-feeding method, the amount of nutrient intake for each tank/treatment can be well controlled. However, whether each individual fish ingests the average amount of feed is not guaranteed, which may induce high variations and affect the statistical significance. In our experiment, despite the clear confirmations from plasma parameters, statistical analysis shown that some more sensitive molecular parameters, including the phosphorylation of S6K1 and S6, gene expression of FAS, ACLY, D6D and G6PDH, were not affected by different nutrient intake. This lack of statistical significance was probably due to the uneven nutrient intake between fish, which may have caused high variations and ruined the statistical significance. Due to this limit, we were not able to give a very solid conclusion as we expected. Indeed, a controlled-feeding method associated with force-feeding might be considered to overcome the above limits induced by controlled-feeding method alone. However, this would also bring other limits linked to stress whose effect on glycemia has been already demonstrated in fish (Plagnes-Juan et al., 2008).

3. In experiment four, no muscle samples

It is well known that fish displays prolonged hyperglycemia after consuming a carbohydrate rich meal or after a glucose tolerance test (Moon, 2001), but it is unclear whether the persistent hyperglycemia in fish is due to poor glucose uptake (glucokinase or glycolysis), low utilization of glucose by the peripheral tissues or lacking suppression of endogeneous production of glucose or a combinations of all. We suspected that the poor inhibition of postprandial hepatic gluconeogenesis could be the major responsible factor and thus tested this hypothesis *in vivo* (publication 4). As expected, we observed that decreased plasma glucose levels were associated with suppressed hepatic gluconeogenesis at both gene expression (G6Pase I, II and FBPase) and enzyme activity (FBPase) levels, and inhibited hepatic glucose utilization (GK was inhibited at both expression and activity levels) (Dai et al., 2014). Presumably, glucose uptake in muscle might have also been suppressed as rapamycin can inhibit Glut4 through mTORC1-dependent manner (Duvel et al., 2010; Laplante and Sabatini, 2013). Thus, presumably, suppressed hepatic gluconeogenesis was the major factor that accounted for the decreased glucose levels, however, without muscle samples and relevant data on the contribution of glucose utilization in muscle, we were unable to give a certain conclusion although our results were quite promising.

4. The setting of 1AA as low AA level and 4×10^{-9} mol l^{-1} insulin as normal insulin level in the in vitro trials may be a little too high

To keep consistent conditions with previous published cell culture experiments in the lab, we used one fold concentrated AAs and 4×10^{-9} mol l^{-1} as low AA level and insulin level, respectively. That was a safe choice and we also successfully obtained the results as we hypothesized/expected. However, our later tests (Figure S4, unpublished data) indicated that setting 0.3 or 0.5 fold concentrated AAs as low AA level and 1×10^{-9} mol l^{-1} as normal insulin level should be adequate, and would have provided more clear and significant results than that we presented in current thesis. This is not a strict "limitation" for current thesis, but it should be considered in future studies.

Chapter 5

CONCLUSIONS AND PERSPECTIVES

The present study permits the following conclusions:

1. Re-feeding stimulates mTORC1 activation and the phosphorylation of its downstream effectors (S6K1, S6 and 4EBP1) in trout liver, while acute inhibition of rapamycin suppresses these up-regulations;

2. High protein intake/AAs levels induce enhanced mTORC1 activation regardless of carbohydrate intake/glucose levels;

3. Excessive AAs induce overactivation of mTORC1 and attenuate insulin/Akt signaling through enhancing mTORC1/S6K1-induced feedback loop;

4. Akt phosphorylation is enhanced by increased insulin but suppressed by elevated AA levels;

5. mTORC1 activation promotes hepatic fatty acid biosynthesis, glycolysis and amino acid catabolism, but inhibits β-oxidation and autophagy; Akt negatively regulates hepatic gluconeogenesis;

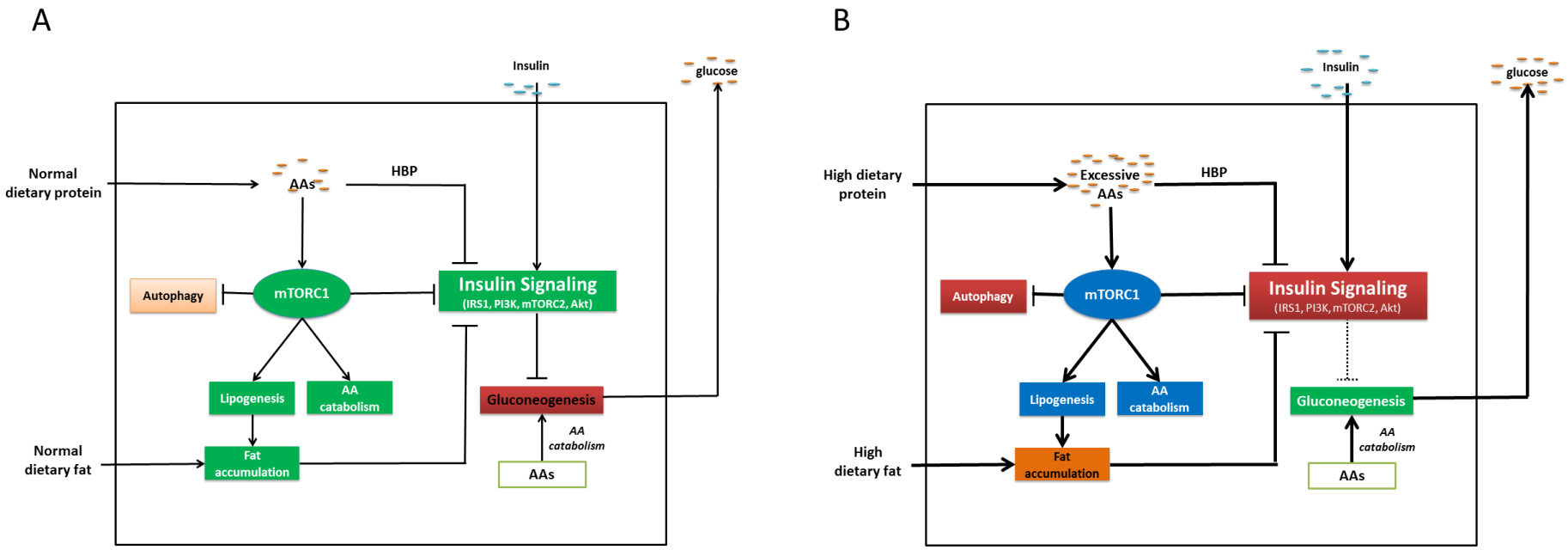
6. Hepatic fatty acid biosynthesis is more responsive to dietary protein intake/AAs than dietary carbohydrate intake/glucose in rainbow trout;

7. AAs promote hepatic fatty acid biosynthesis via at least two manners: (1) enhancing mTORC1 activation, thereby up-regulating fatty acid biosynthetic gene expression; (2) promoting AA catabolism via mTORC1-dependent manner;

8. AAs promote hepatic gluconeogenesis via: (1) attenuating insulin-mediated repression on hepatic gluconeogenesis through elevated IRS-1 phosphorylation on Ser^{302}, which in turn impairs the activation of insulin/Akt pathway; (2) providing substrates for gluconeogenesis through elevating AA catabolism;

9. Hepatic gluconeogenesis probably plays a major role in controlling glucose homeostasis, which maybe account for the prolonged hyperglycemia and glucose intolerance phenotype of carnivorous fish.

Figure 5. 1 Hypothetical mechanisms linking chronic feeding of high protein/high fat diet and hyperglycemia in rainbow trout



(A) In healthy mammals, feeding a diet with normal level of protein and lipids raises nutrient concentration (amino acids (AAs) and glucose), which, in turn, elevates insulin levels in the bloodstream. Released insulin through activating insulin signaling inhibits gluconeogenesis in liver. Ingested AAs induce normal mTORC1 activation, thereby stimulating hepatic lipogenesis and AA catabolism, whereas suppressing autophagy directly and attenuating insulin signaling through mTORC/S6K1-induced feedback loop.

(B) A hypothetical model suggesting how chronic feeding of high protein/high fat diet induces hyperglycemia in rainbow trout. In carnivorous fish, like rainbow trout, chronic feeding of high protein/high fat diet leads to enhanced mTORC1 activation, which in turn promotes elevated lipogenesis and AA catabolism, suppresses autophagy, and enhance mTORC1/S6K1-induced feedback loop, thereby impairing insulin signaling. Furthermore, excessive AAs, especially glutamine stimulate hexosamine biosynthetic pathway (HBP)/*O*-linked *β*-N-acetylglucosamine (*O*-GlcNAc), which attenuates insulin action. Moreover, chronic mTORC1 activation promotes fat accumulation through elevated hepatic lipogenesis; in turn, ectopic fat deposition also has a role in dampening insulin signaling. Collectively, these effects may lead to a worsening of the hyperglycaemia and hyperinsulinaemia. Presumably, mTORC1 promoted or poorly regulated AA catabolism provides substrates for the elevated hepatic gluconeogenesis. Other factors not shown here may also play a role in attenuating insulin action or inducing elevated glycemia, including mitochondrial dysfunction, ER stress, hypo-autophagy, accumulation of misfolded proteins and altered unfolded protein response (UPR).

Remaining questions and perspectives

1. Does high dietary protein and high protein diet-induced hyper-TOR activation account for the persistent hyperglycemia and poor glucose utilization in rainbow trout?

It is well-known that the impairment of PI3K-Akt signaling in the liver promotes gluconeogenesis and contributes to the hyperglycemia and hyperinsulinemia observed in insulin resistance and type 2 diabetes (Laplante and Sabatini, 2012). Given we have demonstrated *in vitro* that acute stimulation with excessive AAs induces overactivation of mTORC1, which in turn, promotes hepatic lipogenesis and attenuates insulin-mediated repression of gluconeogenesis through elevating IRS-1 Ser^{302} phosphorylation, thereby weakening Akt activation and insulin action (Dai et al., 2015b), we presume that chronic feeding of high protein diet maybe account for the postprandial persistent hyperglycemia in rainbow trout as it may cause insulin resistance, attenuate insulin action or induce elevated glycemia via at least five potential manners below (Figure 5.1):

Hyper-mTORC1 activation-induced feedback loop

Chronic feeding of high protein diet may lead to sustained activation of mTORC1 and enhance mTORC1/S6K1-induced feedback loop, which elevates the phosphorylation of IRS-1 on serine residues, dampening the function of IRS-1 and preventing the IRS-1 mediated recruitment of key downstream effectors to IR, thereby leading to insulin desensitization and impairment of PI3K-Akt signaling (Dann et al., 2007; Efeyan and Sabatini, 2010; Tremblay et al., 2007; Um et al., 2006). Notably, this may also result in diminished mTORC2 activation (Masui et al., 2014; Shimobayashi and Hall, 2014; Zoncu et al., 2011), which can also lead to increased gluconeogenesis and glucose release by the liver (Zoncu et al., 2011). Chronically, high blood levels of AAs will keep mTORC1 at work, driving the S6K1-IRS1 loop and, consequently, insulin resistance and hyperglycemia. Although it has been well-demonstrated *in vitro* in rainbow trout (Dai et al., 2015b), future studies are needed to demonstrated this cellular mechanism *in vivo* using long-term feeding trials.

Activate hexosamine biosynthetic pathway (HBP)

The HBP combines various metabolic inputs to ultimately serve in the synthesis of UDP-GlcNAc, the donor substrate for the enzyme of *O*-GlcNAc transferase (OGT). Approximately 2–3% of cellular glucose is funneled into the HBP (Slawson et al., 2010). There are evidences that high levels of circulating glucose and free fatty acids bring about insulin resistance, a hallmark of type 2 diabetes (T2D), via the HBP (Ruan et al., 2013; Teo et al., 2010). Furthermore, excessive AAs, especially glutamine also attenuate insulin action by stimulating HBP pathway (Figure 1.20) (Issad et al., 2010; Ruan et al., 2013;

Slawson et al., 2010). Moreover, increased acetyl-CoA can be funneled into the HBP causing an increase in HBP flux and consequently increasing O-GlcNAcylation as well (Slawson et al., 2010). Given that persistent hyperglycemia, high availability of AAs and acetyl-CoA all occur in rainbow trout, it is attractive to postulate that hyper- or overexpression of OGT-dampened insulin signaling exists in rainbow trout.

Ectopic fat deposition

Chronic mTORC1 activation mediates excess fat deposition in WAT, liver and muscle and ectopic fat deposition has a role in the insurgence of insulin resistance (Perry et al., 2014; Zoncu et al., 2011). Hepatic triglyceride synthesis is the sum of two main processes: (i) DNL of fatty acids from acetyl CoA and (ii) esterification of fatty acids from all sources (DNL, fatty acids from adipose lipolysis or chylomicron lipolysis, and re-esterification of intrahepatic lipid) (Vatner et al., 2015). For the first component, whereas insulin fails to suppress hepatic glucose production, it continues to stimulate DNL, promoting the synthesis of TAGs and perhaps cholesterol, which are incorporated into VLDL for transport to peripheral tissues (Brown and Goldstein, 2008). The second component is primarily dependent on substrate delivery and largely independent of hepatic insulin action (Vatner et al., 2015). Given that marine fish and rainbow trout not only consume high dietary protein but also high content of lipids, and are capable of efficiently digest, assimilate and deposit large quantities of oil in their body tissues (Tocher, 2003), we assume that ectopic fat deposition, if any, contributes to the potential insulin resistance and hyperglycemia. In line with this hypothesis, Figueiredo-Silva et al. (Figueiredo-Silva et al., 2012) demonstrated that high levels of dietary fat impair glucose homeostasis in rainbow trout.

ER stress and mitochondria dysfunction

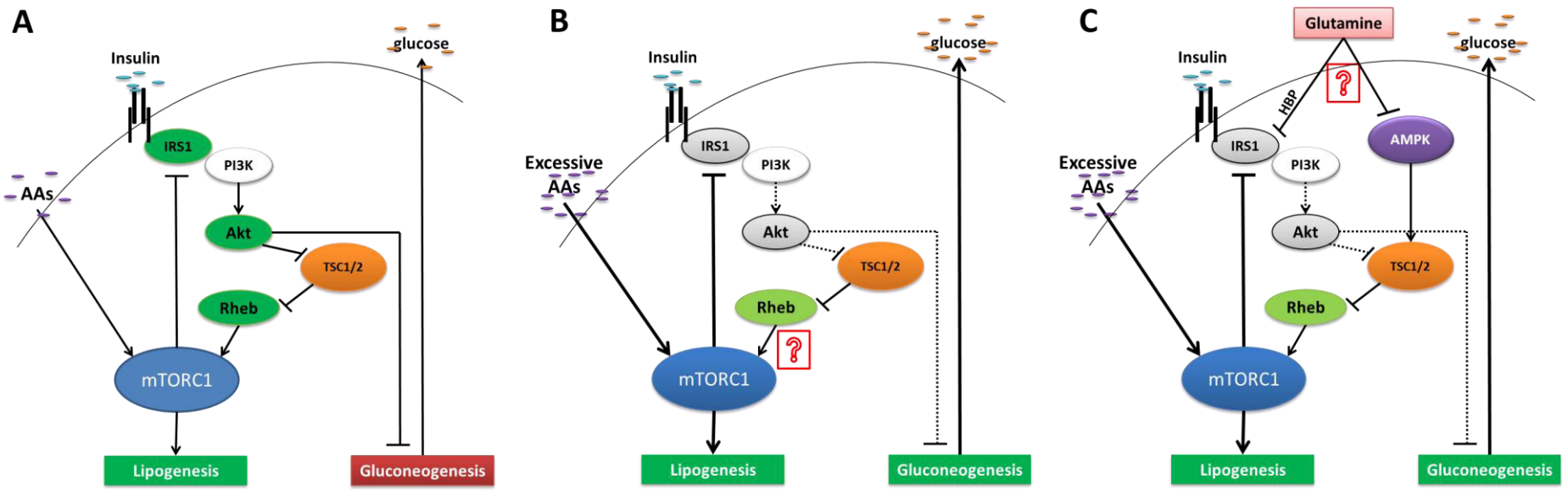
As we previously discussed in 4.3, we reason that the poor carbohydrate utilization in rainbow trout is possibly due to a combination of mitochondrial dysfunction, ER stress, hypo-autophagy, accumulation of misfolded proteins and altered unfolded protein response (UPR) as in obesity or diabetes (Codogno and Meijer, 2010; Hotamisligil, 2010; Jung and Lee, 2010; Lynch and Adams, 2014; Rietman et al., 2014b; Senft and Ronai, 2015), which can be possibly caused by chronic feeding of high protein/high fat diet, sustained activation of mTORC1 and/or ectopic lipid accumulation. Interestingly, consistent with this presumption, recent investigation in zebrafish reported that fructose treatment of larval zebrafish induced hepatic lipid accumulation, which was associated with mTORC1 activation, inflammation and oxidative stress (Sapp et al., 2014). Thus, it is worth to investigate the parameters related to inflammatory stresses and mitochondria functions as they may be the key factors for understanding the low efficiency of glucose utilization and poorly inhibited hepatic gluconeogenesis in trout as in mammals.

mTORC1 activated AA catabolism

As demonstrated in current thesis, AA catabolism is positively controlled by mTORC1 activation in trout hepatocytes (Dai et al., 2015b). It is reasonable to postulate that sustained activation of mTORC1 by chronic feeding high protein diets will keep AA catabolism at work, thereby providing steady substrates for poorly inhibited hepatic gluconeogenesis. The above four manners probably cause poor inhibition of hepatic gluconeogenesis, while AAs seem to provide substrates to satisfy the unstrained gluconeogenic pathway through elevating AA catabolism, thereby inducing hyperglycemia *in vivo*. Future work is needed to measure the contribution of AAs for glucose production through *in vivo* investigations.

Collectively, these above potential factors induced by chronic feeding of high protein/high fat diets may account for low efficiency of glucose utilization and poor inhibition of hepatic gluconeogenesis in rainbow trout. Therefore, future work is needed to investigate these aspects *in vitro* and *in vivo.* Prolonged feeding trials and other feeding methods, like nutrient restriction (NR) should be considered.

Figure 5. 2 The potential roles of glutamine in stimulating mTORC1 activation and inhibiting insulin signaling pathway in rainbow trout



(A) Under normal amino acid (AA) levels, insulin inhibits hepatic gluconeogenesis through insulin/Akt signaling pathway; insulin together with AAs activate lipogenesis (a model in healthy mammals);

(B) Under excessive AAs conditions, overactivated mTORC1 attenuates insulin action on hepatic gluconeogenesis, thereby up-regulating gluconeogenesis; however, through unknown reasons, insulin remains its ability to enhance lipogenesis despite insulin partially losses its ability to inhibit gluconeogenesis (a potential model in carnivorous fish);

(C) A hypothetical model suggesting how mTORC1 activation could drive both lipogenesis and gluconeogenesis under excessive AAs conditions. Different from the other AAs, glutamine probably can directly inhibit AMPK, which in turn promote TSC complex, thereby negatively controlling Rheb to activate mTORC1. Furthermore, high level of glutamine probably also attenuate insulin action by stimulating hexosamine biosynthetic pathway (HBP).

2. The potential roles of glutamine in stimulating mTORC1 activation and attenuating insulin signaling via HBP

In mammals, glutamine is well-known as a functional AA, playing important roles in regulating key metabolic pathways that are necessary for maintenance, growth, reproduction and immunity (Wu, 2009), but its roles are still poorly explored in fish. Our intense interests towards glutamine mainly come from three aspects described below:

Glutamine stimulates mTORC1 activation

In mammals, leucine, arginine and glutamine are three particularly important AAs in stimulating mTORC1 activation (Dibble and Manning, 2013; Jewell and Guan, 2013). In fish, we demonstrated that unlike lysine or methionine, leucine combined with insulin partially stimulated mTOR activation in primary cell culture of rainbow trout hepatocytes (Lansard et al., 2011). However, it remains unclear whether arginine and glutamine can contribute to mTORC1 activation in trout as in mammals, which requires further investigations. Furthermore, one big remaining question following current thesis is how excessive AAs kept mTORC1 and hepatic lipogenesis active under the condition where early events of insulin signaling was impaired by mTORC1/S6K1-induced feedback loop. Indeed, this question is very much similar to the long-standing paradoxical phenomenon of selective hepatic insulin resistance in T2D, in which impaired insulin signaling partially lost its ability to inhibit hepatic gluconeogenesis yet it retained its ability to promote hepatic lipogenesis (Laplante and Sabatini, 2010; Vatner et al., 2015). Recent study in yeast shown that glutamine, the preferred nitrogen source, activates mTORC1 independently of Gtr/Rag (Stracka et al., 2014), which may explain the paradox and the above question. Moreover, it is known that excessive AAs down-regulate AMPK, thereby activating mTORC1 and in turn may induce insulin resistance (Saha et al., 2013). This can be another potential explanation that needs to be explored. We presumed that excessive glutamine contributes to this AMPK down-regulation, and our preliminary results seem consistent with this presumption (unpublished data). Therefore, due to these three aspects, it is worth to investigate the role of glutamine in stimulating mTORC1 in rainbow trout. Metformin, an antidiabetic drug, which activates AMPK (Hardie et al., 2012; Ouyang et al., 2011), should be considered for performing this potential investigation in the future.

Glutamine activates HBP pathway

As discussed previously, excessive glutamine may also attenuate insulin action by stimulating HBP pathway (Issad et al., 2010; Ruan et al., 2013; Slawson et al., 2010). And, to our knowledge, this pathway remains unexplored in any fish species yet.

Glutamine abundance

Generally, glutamate and glutamine represent 20% of AA in animal proteins. Concerning fish, glutamine is synthesized from glutamate by ATP-dependent glutamine synthetase, which is abundant in brain, intestine, liver, red muscle, gill/kidney, white muscle and heart (Li et al., 2009). Glutamate is transaminated by many amino acids and the liver is the main site for such a transformation (Guillaume et al., 2001). Furthermore, carnivorous fish consume high dietary protein and possess consistently high rate of AA catabolism (Walton and Cowey, 1982). Thus, not surprisingly, glutamine is one of the most abundant free α-AA in fish plasma and muscle (Li et al., 2009). Presumably, abundant glutamine plays important functional and physiological roles in regulating metabolic pathways in trout.

Due to these three major reasons above, we propose that it is worthy to explore the role(s) of glutamine in rainbow trout in the future. However, due to the complexity *in vivo*, especially the metabolite exchange between organs and hormonal effects, we suggest that it is better to carry out the potential experiments *in vitro*.

3. The control of AA catabolism needs further investigations

The observation that mTORC1 activation promotes hepatic AA catabolic gene expression would possess significant importance as it may account for the high protein requirement (Macdonald et al., 1984; Robert and Backus, 2008), poor inhibition of postprandial hepatic gluconeogenesis (Robert and Backus, 2008), poor glucose utilization in peripheral tissues, as well as some other metabolic particularities in rainbow trout or general carnivores. Future work is highly required to investigate the mechanism(s) by which mTORC1 regulates AA catabolic gene expression/enzymes in rainbow trout. Furthermore, as no equivalent counterpart has been reported yet (even in mammals or cat) and rapamycin indeed can also affect some other proteins (like Akt, PI3K) through inhibition the mTORC1/S6K1-induced feedback loop (Benjamin et al., 2011), future work with other more specific mTOR inhibitors (i.e. Torin1 and PP242) or other manners (i.e. diets with different protein levels or using controlled-feeding method to provide varied protein intake) are needful to further confirm current conclusion.

4. Measure the contribution of AA and AA catabolism to hepatic fatty acid biosynthesis and gluconeogenesis using tracer method

Different from the demonstrations in rodents, chickens or humans (Abu-Elheiga et al., 2012; Allee et al., 1971; Pichon et al., 2006; Rietman et al., 2014a; Uebanso et al., 2009), we observed that hepatic fatty acid biosynthetic gene expression in rainbow trout is more responsive to dietary protein intake/AAs than dietary carbohydrate intake/glucose during acute stimulations (Dai et al., 2015b). This

demonstration is supported by previous investigations in fish (Figueiredo-Silva et al., 2009; Hemre et al., 2002; Panserat et al., 2013; Seiliez et al., 2011a; Tocher, 2003). Therefore, we reason that, unlike rodents or humans, protein/AAs is a more potent mediator for stimulating hepatic lipogenesis compared to carbohydrate/glucose in rainbow trout, and AAs are probably used as substrates for hepatic lipogenesis. Furthermore, as we previously discussed in chapter 4.4, we presume that the persistent high rate of gluconeogenesis in rainbow trout is fed by AAs, and the amount of AA-derived carbon skeletons used for gluconeogenesis should be higher in trout than in herbivores and omnivores. Therefore, to further confirm the above two presumptions at nutrient flux level, future work with tracer method is needed to measure the true contributions of AAs and AA catabolism to fat and glucose synthesis.

5. Explore more mTOR downstream target genes in rainbow trout using RNA-sequencing method

We have demonstrated in current thesis that mTORC1 activation positively regulates AA catabolism gene expression (Dai et al., 2015b), which receives no equivalent reports in current available demonstrations in mammals. This discrepancy may due to the differences between mammals and fish or the differences between omnivores and carnivores. Hence, we propose to use RNA-sequencing or microarray method to go on exploring more mTOR downstream target genes in rainbow trout in the future in order to futher explore the role of mTOR in the regulation of fish physiology and perhaps identify parameters that may explain the metabolic features of carnivores (RNA-seq experiments have been performed on primary cell culture of hepatocytes, but data processing needs to be completed).

BIBLIOGRAPHY

Abraham, R. T. (2015). Making sense of amino acid sensing. *Science* **347**, 128-129.
Abu-Elheiga, L., Wu, H., Gu, Z., Bressler, R. and Wakil, S. J. (2012). Acetyl-CoA carboxylase 2-/- mutant mice are protected against fatty liver under high-fat, high-carbohydrate dietary and de novo lipogenic conditions. *J. Biol. Chem.* **287**, 12578-88.
Accili, D. and Arden, K. C. (2004). FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell* **117**, 421-426.
Alegre, M., Ciudad, C. J., Fillat, C. and Guinovart, J. J. (1988). Determination of glucose-6-phosphatase activity using the glucose dehydrogenase-coupled reaction. *Anal. Biochem.* **173**, 185-9.
Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B. A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* **15**, 6541-51.
Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R. J., Reese, C. B. and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B alpha. *Curr. Biol.* **7**, 261-269.
Allee, G. L., O'Hea, E. K., Leveille, G. A. and Baker, D. H. (1971). Influenza of dietary protein and fat on lipogenesis and enzymatic activity in pig adipose tissue. *J. Nutr.* **101**, 869-78.
Altomare, D. A. and Testa, J. R. (2005). Perturbations of the AKT signaling pathway in human cancer. *Oncogene* **24**, 7455-7464.
Anthonsen, M. W., Ronnstrand, L., Wernstedt, C., Degerman, E. and Holm, C. (1998). Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro. *J. Biol. Chem.* **273**, 215-221.
Arruda, A. P., Pers, B. M., Parlakgul, G., Guney, E., Inouye, K. and Hotamisligil, G. S. (2014). Chronic enrichment of hepatic endoplasmic reticulum-mitochondria contact leads to mitochondrial dysfunction in obesity. *Nat. Med.* **20**, 1427-35.
B'chir, W., Maurin, A. C., Carraro, V., Averous, J., Jousse, C., Muranishi, Y., Parry, L., Stepien, G., Fafournoux, P. and Bruhat, A. (2013). The eIF2 alpha/ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic Acids Res.* **41**, 7683-7699.
Ballou, L. M. and Lin, R. Z. (2008). Rapamycin and mTOR kinase inhibitors. *J. Chem. Biol.* **1**, 27-36.
Bar-Peled, L. and Sabatini, D. M. (2014). Regulation of mTORC1 by amino acids. *Trends Cell Biol.* **24**, 400-406.
Baranowski, M., Zabielski, P., Blachnio-Zabielska, A. U., Harasim, E., Chabowski, A. and Gorski, J. (2014). Insulin-sensitizing effect of LXR agonist T0901317 in high-fat fed rats is associated with restored muscle GLUT4 expression and insulin-stimulated AS160 phosphorylation. *Cell. Physiol. Biochem.* **33**, 1047-57.
Barroso, J. B., Peragon, J., Garcia-Salguero, L., de la Higuera, M. and Lupianez, J. A. (2001). Carbohydrate deprivation reduces NADPH-production in fish liver but not in adipose tissue. *Int. J. Biochem. Cell Biol.* **33**, 785-96.
Barthel, A. and Schmoll, D. (2003). Novel concepts in insulin regulation of hepatic gluconeogenesis. *Am J Physiol-Endoc M* **285**, E685-E692.
Bechmann, L. P., Hannivoort, R. A., Gerken, G., Hotamisligil, G. S., Trauner, M. and Canbay, A. (2012). The interaction of hepatic lipid and glucose metabolism in liver diseases. *J. Hepatol.* **56**, 952-964.
Beck, T. and Miller, B. G. (2013). Structural basis for regulation of human glucokinase by glucokinase regulatory protein. *Biochemistry* **52**, 6232-9.
Belghit, I., Panserat, S., Sadoul, B., Dias, K., Skiba-Cassy, S. and Seiliez, I. (2013). Macronutrient composition of the diet affects the feeding-mediated down regulation of autophagy in muscle of rainbow trout (O. mykiss). *PLoS One* **8**, e74308.
Belghit, I., Skiba-Cassy, S., Geurden, I., Dias, K., Surget, A., Kaushik, S., Panserat, S. and Seiliez, I. (2014). Dietary methionine availability affects the main factors involved in muscle protein turnover in rainbow trout (Oncorhynchus mykiss). *Br. J. Nutr.* **112**, 493-503.
Bengoechea-Alonso, M. T. and Ericsson, J. (2009). A phosphorylation cascade controls the degradation of active SREBP1. *J. Biol. Chem.* **284**, 5885-95.
Benjamin, D., Colombi, M., Moroni, C. and Hall, M. N. (2011). Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nature reviews. Drug discovery* **10**, 868-80.
Berg, J. M., Tymoczko, J. L. and Stryer, L. (2010). Biochemistry. New York: W H Freeman.
Bergen, W. G. and Mersmann, H. J. (2005). Comparative aspects of lipid metabolism: Impact on contemporary research and use of animal models. *J. Nutr.* **135**, 2499-2502.

Bonnard, C., Durand, A., Peyrol, S., Chanseaume, E., Chauvin, M. A., Morio, B., Vidal, H. and Rieusset, J. (2008). Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J. Clin. Invest.* **118**, 789-800.
Boura-Halfon, S. and Zick, Y. (2009). Phosphorylation of IRS proteins, insulin action, and insulin resistance. *Am J Physiol-Endoc M* **296**, E581-E591.
Breves, J. P., Tipsmark, C. K., Stough, B. A., Seale, A. P., Flack, B. R., Moorman, B. P., Lerner, D. T. and Grau, E. G. (2014). Nutritional status and growth hormone regulate insulin-like growth factor binding protein (igfbp) transcripts in Mozambique tilapia. *Gen. Comp. Endocrinol.* **207**, 66-73.
Brown, M. S. and Goldstein, J. L. (2008). Selective versus total insulin resistance: a pathogenic paradox. *Cell metabolism* **7**, 95-6.
Bryant, N. J., Govers, R. and James, D. E. (2002). Regulated transport of the glucose transporter GLUT4. *Nat. Rev. Mol. Cell Biol.* **3**, 267-77.
Calkin, A. C. and Tontonoz, P. (2012). Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR. *Nat. Rev. Mol. Cell Biol.* **13**, 213-24.
Calnan, D. R. and Brunet, A. (2008). The FoxO code. *Oncogene* **27**, 2276-88.
Campbell, L. (2006). Liver: metabolic functions. *Physiology*.
Capilla, E., Medale, F., Navarro, I., Panserat, S., Vachot, C., Kaushik, S. and Gutierrez, J. (2003). Muscle insulin binding and plasma levels in relation to liver glucokinase activity, glucose metabolism and dietary carbohydrates in rainbow trout. *Regul. Pept.* **110**, 123-32.
Caron, A., Richard, D. and Laplante, M. (2015). The Roles of mTOR Complexes in Lipid Metabolism. *Annu. Rev. Nutr.* **35**, 321-48.
Caseras, A., Metón, I., Vives, C., Egea, M., Fernández, F. and Baanante, I. (2002). Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (Sparus aurata). *British Journal of Nutrition* **88**, 607-614.
Castilho, B. A., Shanmugam, R., Silva, R. C., Ramesh, R., Himme, B. M. and Sattlegger, E. (2014). Keeping the eIF2 alpha kinase Gcn2 in check. *Biochim. Biophys. Acta* **1843**, 1948-68.
Castillo, J., Ammendrup-Johnsen, I., Codina, M., Navarro, I. and Gutierrez, J. (2006). IGF-I and insulin receptor signal transduction in trout muscle cells. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **290**, R1683-90.
Cell Signaling Technology. (2014). mTOR Signaling Pathway. Massachusetts, USA: Cell Signaling Technology, Inc.
Chapman, I. M., Hartman, M. L., Pieper, K. S., Skiles, E. H., Pezzoli, S. S., Hintz, R. L. and Thorner, M. O. (1998). Recovery of growth hormone release from suppression by exogenous insulin-like growth factor I (IGF-I): evidence for a suppressive action of free rather than bound IGF-I. *J. Clin. Endocrinol. Metab.* **83**, 2836-42.
Cheng, Z., Tseng, Y. and White, M. F. (2010). Insulin signaling meets mitochondria in metabolism. *Trends Endocrinol. Metab.* **21**, 589-98.
Cheng, Z. Y. and White, M. F. (2012). The AKTion in non-canonical insulin signaling. *Nat. Med.* **18**, 351-353.
Chiarini, F., Evangelisti, C., McCubrey, J. A. and Martelli, A. M. (2015). Current treatment strategies for inhibiting mTOR in cancer. *Trends Pharmacol. Sci.* **36**, 124-35.
Choi, J. M., Seo, M. H., Kyeong, H. H., Kim, E. and Kim, H. S. (2013). Molecular basis for the role of glucokinase regulatory protein as the allosteric switch for glucokinase. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 10171-10176.
Chong, M. F., Hodson, L., Bickerton, A. S., Roberts, R., Neville, M., Karpe, F., Frayn, K. N. and Fielding, B. A. (2008). Parallel activation of de novo lipogenesis and stearoyl-CoA desaturase activity after 3 d of high-carbohydrate feeding. *Am. J. Clin. Nutr.* **87**, 817-23.
Chotechuang, N., Azzout-Marniche, D., Bos, C., Chaumontet, C., Gausseres, N., Steiler, T., Gaudichon, C. and Tome, D. (2009). mTOR, AMPK, and GCN2 coordinate the adaptation of hepatic energy metabolic pathways in response to protein intake in the rat. *Am. J. Physiol. Endocrinol. Metab.* **297**, E1313-23.
Codogno, P. and Meijer, A. J. (2010). Autophagy: A Potential Link between Obesity and Insulin Resistance. *Cell metabolism* **11**, 449-451.
Contreras, A. V., Rangel-Escareno, C., Torres, N., Aleman-Escondrillas, G., Ortiz, V., Noriega, L. G., Torre-Villalvazo, I., Granados, O., Velazquez-Villegas, L. A., Tobon-Cornejo, S. et al. (2015). PPARalpha via HNF4alpha regulates the expression of genes encoding hepatic amino acid catabolizing enzymes to maintain metabolic homeostasis. *Genes & nutrition* **10**, 452.

Cowey, C. B., Knox, D., Walton, M. J. and Adron, J. W. (1977). Regulation of Gluconeogenesis by Diet and Insulin in Rainbow-Trout (Salmo-Gairdneri). *Br. J. Nutr.* **38**, 463-470.
Cowey, C. B. and Walton, M. J. (1989). Intermediary metabolism. In *Fish nutrition*, (ed. J. E. Halver), pp. 259-329. New York, NY: Academic press.
Cowley, D. J. and Sheridan, M. A. (1993). Insulin stimulates hepatic lipogenesis in rainbow trout, Oncorhynchus mykiss. *Fish Physiol. Biochem.* **11**, 421-8.
Cruz-Garcia, L., Minghetti, M., Navarro, I. and Tocher, D. R. (2009). Molecular cloning, tissue expression and regulation of liver X receptor (LXR) transcription factors of Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **153**, 81-8.
Cybulski, N., Polak, P., Auwerx, J., Ruegg, M. A. and Hall, M. N. (2009). mTOR complex 2 in adipose tissue negatively controls whole-body growth. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 9902-9907.
Dabrowski, K. and Guderley, H. (2002). Intermediary Metabolism. In *Fish Nutrition*, eds. J. E. Halver and R. Hardy), pp. 500: Academic Press.
Dai, W., Panserat, S., Kaushik, S., Terrier, F., Plagnes-Juan, E., Seiliez, I. and Skiba-Cassy, S. (2015a). Hepatic lipid biosynthesis is more responsive to protein than carbohydrate in rainbow trout during acute stimulations. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*
Dai, W., Panserat, S., Mennigen, J. A., Terrier, F., Dias, K., Seiliez, I. and Skiba-Cassy, S. (2013). Post-prandial regulation of hepatic glucokinase and lipogenesis requires the activation of TORC1 signalling in rainbow trout (Oncorhynchus mykiss). *J. Exp. Biol.* **216**, 4483-4492.
Dai, W. W., Panserat, S., Plagnes-Juan, E., Seiliez, I. and Skiba-Cassy, S. (2015b). Amino Acids Attenuate Insulin Action on Gluconeogenesis and Promote Fatty Acid Biosynthesis via mTORC1 Signaling Pathway in trout Hepatocytes. *Cell. Physiol. Biochem.* **36**, 1084-1100.
Dai, W. W., Panserat, S., Terrier, F., Seiliez, I. and Skiba-Cassy, S. (2014). Acute rapamycin treatment improved glucose tolerance through inhibition of hepatic gluconeogenesis in rainbow trout (Oncorhynchus mykiss). *Am J Physiol-Reg I* **307**, R1231-R1238.
Daitoku, H., Yamagata, K., Matsuzaki, H., Hatta, M. and Fukamizu, A. (2003). Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. *Diabetes* **52**, 642-9.
Dann, S. G., Selvaraj, A. and Thomas, G. (2007). mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. *Trends Mol. Med.* **13**, 252-259.
De Silva, S. S. and Anderson, T. A. (1994). Fish Nutrition in Aquaculture. London: Springer.
del sol Novoa, M., Capilla, E., Rojas, P., Baro, J., Gutierrez, J. and Navarro, I. (2004). Glucagon and insulin response to dietary carbohydrate in rainbow trout (Oncorhynchus mykiss). *Gen. Comp. Endocrinol.* **139**, 48-54.
Deprez, J., Vertommen, D., Alessi, D. R., Hue, L. and Rider, M. H. (1997). Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. *J. Biol. Chem.* **272**, 17269-17275.
DeYoung, M. P., Horak, P., Sofer, A., Sgroi, D. and Ellisen, L. W. (2008). Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes Dev.* **22**, 239-51.
Dias, J., Alvarez, M. J., Diez, A., Arzel, J., Corraze, G., Bautista, J. M. and Kaushik, S. J. (1998). Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European seabass (Dicentrarchus labrax). *Aquaculture* **161**, 169-186.
Dibble, C. C. and Cantley, L. C. (2015). Regulation of mTORC1 by PI3K signaling. *Trends Cell Biol.*
Dibble, C. C. and Manning, B. D. (2013). Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nat. Cell Biol.* **15**, 555-564.
Ding, Y. H., Sun, X. J., Redfield, M., Kushwaha, S. and Xu, X. L. (2012a). Target of rapamcyin (TOR)-based therapeutics for cardiomyopathy Insights from zebrafish genetics. *Cell Cycle* **11**, 428-429.
Ding, Y. H., Sun, X. J. and Xu, X. L. (2012b). TOR-autophagy signaling in adult zebrafish models of cardiomyopathy. *Autophagy* **8**, 142-143.
Dumortier, O., Hinault, C. and Van Obberghen, E. (2013). MicroRNAs and metabolism crosstalk in energy homeostasis. *Cell metabolism* **18**, 312-24.
Duvel, K., Yecies, J. L., Menon, S., Raman, P., Lipovsky, A. I., Souza, A. L., Triantafellow, E., Ma, Q. C., Gorski, R., Cleaver, S. et al. (2010). Activation of a Metabolic Gene Regulatory Network Downstream of mTOR Complex 1. *Mol. Cell* **39**, 171-183.
Efeyan, A., Comb, W. C. and Sabatini, D. M. (2015). Nutrient-sensing mechanisms and pathways. *Nature* **517**, 302-10.

Efeyan, A. and Sabatini, D. M. (2010). mTOR and cancer: many loops in one pathway. *Curr. Opin. Cell Biol.* **22**, 169-176.
Eisert, R. (2011). Hypercarnivory and the brain: protein requirements of cats reconsidered. *J. Comp. Physiol. B* **181**, 1-17.
Ekmann, K. S., Dalsgaard, J., Holm, J., Campbell, P. J. and Skov, P. V. (2013). Effects of dietary energy density and digestible protein:energy ratio on de novo lipid synthesis from dietary protein in gilthead sea bream (Sparus aurata) quantified with stable isotopes. *Br. J. Nutr.* **110**, 1771-81.
Enes, P., Panserat, S., Kaushik, S. and Oliva-Teles, A. (2006). Effect of normal and waxy maize starch on growth, food utilization and hepatic glucose metabolism in European sea bass (Dicentrarchus labrax) juveniles. *Comp Biochem Phys A* **143**, 89-96.
Enes, P., Panserat, S., Kaushik, S. and Oliva-Teles, A. (2008a). Growth performance and metabolic utilization of diets with native and waxy maize starch by gilthead sea bream (Sparus aurata) juveniles. *Aquaculture* **274**, 101-108.
Enes, P., Panserat, S., Kaushik, S. and Oliva-Teles, A. (2008b). Hepatic glucokinase and glucose-6-phosphatase responses to dietary glucose and starch in gilthead sea bream (Sparus aurata) juveniles reared at two temperatures. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **149**, 80-6.
Enes, P., Panserat, S., Kaushik, S. and Oliva-Teles, A. (2009). Nutritional regulation of hepatic glucose metabolism in fish. *Fish Physiol. Biochem.* **35**, 519-39.
Falkmer, S. (1961). Experimental diabetes research in fish. *Acta Endocrinol. Suppl. (Copenh.)* **37(Suppl 59)**, 1-122.
FAO. (2013a). FAOSTAT. Rome: Food and Agriculture Organization.
FAO. (2013b). FISHSTAT Plus: Universal Software for Fishery Statistical Time Series.
FAO. (2014). The State of World Fisheries and Aquaculture 2014, pp. 223pp. Rome: FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS.
FEAP. (2011). FEAP Annual Report 2011. FEAP Secretariat rue de Paris 9 B-4020 Liege Belgium.
Feng, L., Peng, Y., Wu, P., Hu, K., Jiang, W. D., Liu, Y., Jiang, J., Li, S. H. and Zhou, X. Q. (2013). Threonine affects intestinal function, protein synthesis and gene expression of TOR in Jian carp (Cyprinus carpio var. Jian). *PLoS One* **8**, e69974.
Feng, Y. Y., Xu, X. Q., Ji, C. B., Shi, C. M., Guo, X. R. and Fu, J. F. (2014). Aberrant hepatic microRNA expression in nonalcoholic fatty liver disease. *Cell. Physiol. Biochem.* **34**, 1983-97.
Fernandez-Hernando, C., Suarez, Y., Rayner, K. J. and Moore, K. J. (2011). MicroRNAs in lipid metabolism. *Curr. Opin. Lipidol.* **22**, 86-92.
Fernández, F., Miquel, A. G., Córdoba, M., Varas, M., Metón, I., Caseras, A. and Baanante, I. V. (2007). Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead sea bream (< i> Sparus aurata</i>, L.) fingerlings. *J. Exp. Mar. Biol. Ecol.* **343**, 1-10.
Ferraris, M., Radice, S., Catalani, P., Francolini, M., Marabini, L. and Chiesara, E. (2002). Early oxidative damage in primary cultured trout hepatocytes: a time course study. *Aquatic Toxicology* **59**, 283-96.
Ferrier, D. R., Champe, P. C. and Harvey, R. A. (2011). Amino Acid Degradation and Synthesis. In *Biochemistry*, eds. M. D. Hagerstwon W. Lippincott and Wilkins).
Figueiredo-Silva, A. C., Corraze, G., Kaushik, S., Peleteiro, J. B. and Valente, L. M. (2010). Modulation of blackspot seabream (Pagellus bogaraveo) intermediary metabolic pathways by dispensable amino acids. *Amino Acids* **39**, 1401-16.
Figueiredo-Silva, A. C., Corraze, G., Rema, P., Sanchez-Gurmaches, J., Gutierrez, J. and Valente, L. M. P. (2009). Blackspot seabream (Pagellus bogaraveo) lipogenic and glycolytic pathways appear to be more related to dietary protein level than dietary starch type. *Aquaculture* **291**, 101-110.
Figueiredo-Silva, A. C., Panserat, S., Kaushik, S., Geurden, I. and Polakof, S. (2012). High levels of dietary fat impair glucose homeostasis in rainbow trout. *J. Exp. Biol.* **215**, 169-78.
Filhoulaud, G., Guilmeau, S., Dentin, R., Girard, J. and Postic, C. (2013). Novel insights into ChREBP regulation and function. *Trends Endocrinol. Metab.* **24**, 257-268.
Fillmore, N., Alrob, O. A. and Lopaschuk, G. D. (2011). FATTY ACID β-OXIDATION: Cardiovascular Research Centre, Mazankowski Alberta Heart Institute University of Alberta, Edmonton, Canada.
Floyd, J. C., Jr., Fajans, S. S., Conn, J. W., Knopf, R. F. and Rull, J. (1966). Stimulation of insulin secretion by amino acids. *J. Clin. Invest.* **45**, 1487-502.

Foster, G. D. and Moon, T. W. (1990). The role of glycogen phosphorylase in the regulation of glycogenolysis by insulin and glucagon in isolated eel (Anguilla rostrata) hepatocytes. *Fish Physiol. Biochem.* **8**, 299-309.
Foufelle, F. and Ferre, P. (2002). New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. *Biochem. J.* **366**, 377-391.
Fromentin, C., Tome, D., Nau, F., Flet, L., Luengo, C., Azzout-Marniche, D., Sanders, P., Fromentin, G. and Gaudichon, C. (2013). Dietary proteins contribute little to glucose production, even under optimal gluconeogenic conditions in healthy humans. *Diabetes* **62**, 1435-42.
Fuentes, E. N., Einarsdottir, I. E., Paredes, R., Hidalgo, C., Valdes, J. A., Bjornsson, B. T. and Molina, A. (2015). The TORC1/P70S6K and TORC1/4EBP1 signaling pathways have a stronger contribution on skeletal muscle growth than MAPK/ERK in an early vertebrate: Differential involvement of the IGF system and atrogenes. *Gen. Comp. Endocrinol.* **210**, 96-106.
Fuentes, E. N., Safian, D., Einarsdottir, I. E., Valdes, J. A., Elorza, A. A., Molina, A. and Bjornsson, B. T. (2013a). Nutritional status modulates plasma leptin, AMPK and TOR activation, and mitochondrial biogenesis: Implications for cell metabolism and growth in skeletal muscle of the fine flounder. *Gen. Comp. Endocrinol.* **186**, 172-80.
Fuentes, E. N., Valdes, J. A., Molina, A. and Bjornsson, B. T. (2013b). Regulation of skeletal muscle growth in fish by the growth hormone--insulin-like growth factor system. *Gen. Comp. Endocrinol.* **192**, 136-48.
Fuhrer, D. K., Kobayashi, M. and Jiang, H. (2001). Insulin release and suppression by tacrolimus, rapamycin and cyclosporin A are through regulation of the ATP-sensitive potassium channel. *Diabetes Obesity & Metabolism* **3**, 393-402.
Fynnaikins, K., Hung, S. S. O., Liu, W. and Li, H. B. (1992). Growth, lipogenesis and liver composition of juvenile white sturgeon fed different levels of D-glucose. *Aquaculture* **105**, 61-72.
Galluzzi, L., Pietrocola, F., Levine, B. and Kroemer, G. (2014). Metabolic control of autophagy. *Cell* **159**, 1263-76.
Girard, J., Ferre, P. and Foufelle, F. (1997). Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. *Annu. Rev. Nutr.* **17**, 325-352.
Goldstein, J. L., DeBose-Boyd, R. A. and Brown, M. S. (2006). Protein sensors for membrane sterols. *Cell* **124**, 35-46.
Gonzalez, E. and McGraw, T. E. (2009). The Akt kinases Isoform specificity in metabolism and cancer. *Cell Cycle* **8**, 2502-2508.
Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R. B. and Hay, N. (2001). Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev.* **15**, 1406-1418.
Gual, P., Le Marchand-Brustel, Y. and Tanti, J. F. (2005). Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. *Biochimie* **87**, 99-109.
Guderley, H. (2004). Metabolic responses to low temperature in fish muscle. *Biol Rev* **79**, 409-427.
Guertin, D. A., Stevens, D. M., Thoreen, C. C., Burds, A. A., Kalaany, N. Y., Moffat, J., Brown, M., Fitzgerald, K. J. and Sabatini, D. M. (2006). Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKC alpha but not S6K1. *Dev. Cell* **11**, 859-871.
Guillaume, J., Kaushik, S., Bergot, P. and Métailler, R. (2001). Nutrition and Feeding of Fish and Crustaceans. London: Springer.
Guo, F. and Cavener, D. R. (2007). The GCN2 eIF2alpha kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid. *Cell metabolism* **5**, 103-14.
Hagiwara, A., Cornu, M., Cybulski, N., Polak, P., Betz, C., Trapani, F., Terracciano, L., Heim, M. H., Ruegg, M. A. and Hall, M. N. (2012). Hepatic mTORC2 Activates Glycolysis and Lipogenesis through Akt, Glucokinase, and SREBP1c. *Cell metabolism* **15**, 725-738.
Hall, R. K., Yamasaki, T., Kucera, T., Waltner-Law, M., O'Brien, R. and Granner, D. K. (2000). Regulation of phosphoenolpyruvate carboxykinase and insulin-like growth factor-binding protein-1 gene expression by insulin - The role of winged helix/forkhead proteins. *J. Biol. Chem.* **275**, 30169-30175.
Halver, J. E. and Halver, R. W. (2002). Fish Nutrition: Academic Press.
Han, J., Li, E., Chen, L., Zhang, Y., Wei, F., Liu, J., Deng, H. and Wang, Y. (2015). The CREB coactivator CRTC2 controls hepatic lipid metabolism by regulating SREBP1. *Nature*.
Hanover, J. A., Lai, Z., Lee, G., Lubas, W. A. and Sato, S. M. (1999). Elevated O-linked N-acetylglucosamine metabolism in pancreatic beta-cells. *Arch. Biochem. Biophys.* **362**, 38-45.

Hardie, D. G., Ross, F. A. and Hawley, S. A. (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Bio* **13**, 251-262.
Hardy, R. W. (2002). Rainbow trout, Oncorhynchus mykiss. In *Nutrient requirements and feeding of finfish for aquaculture*, eds. C. D. Webster and C. Lim), pp. 184. Hagerman Fish Culture Experiment Station, University of Idaho, 3059F National Fish Hatchery Road, Hagerman, ID 83332, USA.
Harmon, J. S., Eilertson, C. D., Sheridan, M. A. and Plisetskaya, E. M. (1991). Insulin suppression is associated with hypersomatostatinemia and hyperglucagonemia in glucose-injected rainbow trout. *Am. J. Physiol.* **261**, R609-13.
Harris, T. E. and Lawrence, J. C., Jr. (2003). TOR signaling. *Science's STKE : signal transduction knowledge environment* **2003**, re15.
Hartman, M. L., Clayton, P. E., Johnson, M. L., Celniker, A., Perlman, A. J., Alberti, K. G. and Thorner, M. O. (1993). A low dose euglycemic infusion of recombinant human insulin-like growth factor I rapidly suppresses fasting-enhanced pulsatile growth hormone secretion in humans. *J. Clin. Invest.* **91**, 2453-62.
Haruta, T., Uno, T., Kawahara, J., Takano, A., Egawa, K., Sharma, P. M., Olefsky, J. M. and Kobayashi, M. (2000). A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. *Mol. Endocrinol.* **14**, 783-94.
He, L., Sabet, A., Djedjos, S., Miller, R., Sun, X. J., Hussain, M. A., Radovick, S. and Wondisford, F. E. (2009). Metformin and Insulin Suppress Hepatic Gluconeogenesis through Phosphorylation of CREB Binding Protein. *Cell* **137**, 635-646.
Heijnen, H. F., van Wijk, R., Pereboom, T. C., Goos, Y. J., Seinen, C. W., van Oirschot, B. A., van Dooren, R., Gastou, M., Giles, R. H., van Solinge, W. et al. (2014). Ribosomal protein mutations induce autophagy through S6 kinase inhibition of the insulin pathway. *PLoS genetics* **10**, e1004371.
Hemre, G. I. and Kahrs, F. (1997). C-14-glucose injection in Atlantic cod, Gadus morhua, metabolic responses and excretion via the gill membrane. *Aquacult Nutr* **3**, 3-8.
Hemre, G. I., Mommsen, T. P. and Krogdahl, A. (2002). Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. *Aquacult Nutr* **8**, 175-194.
Hilton, J. W. and Atkinson, J. L. (1982). Response of Rainbow-Trout (Salmo-Gairdneri) to Increased Levels of Available Carbohydrate in Practical Trout Diets. *Br. J. Nutr.* **47**, 597-607.
Horton, J. D., Goldstein, J. L. and Brown, M. S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* **109**, 1125-1131.
Hotamisligil, G. S. (2010). Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell* **140**, 900-17.
Houde, V. P., Brule, S., Festuccia, W. T., Blanchard, P. G., Bellmann, K., Deshaies, Y. and Marette, A. (2010). Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue. *Diabetes* **59**, 1338-48.
Hu, K., Zhang, J. X., Feng, L., Jiang, W. D., Wu, P., Liu, Y., Jiang, J. and Zhou, X. Q. (2015). Effect of dietary glutamine on growth performance, non-specific immunity, expression of cytokine genes, phosphorylation of target of rapamycin (TOR), and anti-oxidative system in spleen and head kidney of Jian carp (Cyprinus carpio var. Jian). *Fish Physiol. Biochem.* **41**, 635-49.
Huang, S. and Czech, M. P. (2007). The GLUT4 glucose transporter. *Cell metabolism* **5**, 237-52.
Hummasti, S. and Hotamisligil, G. S. (2010). Endoplasmic Reticulum Stress and Inflammation in Obesity and Diabetes. *Circ. Res.* **107**, 579-591.
Ince, B. W. and Thorpe, A. (1974). Effects of insulin and of metabolite loading on blood metabolites in the European silver eel, (Anguilla anguilla L.). *Gen. Comp. Endocrinol.* **23**, 460-71.
Inoki, K., Ouyang, H., Zhu, T. Q., Lindvall, C., Wang, Y., Zhang, X. J., Yang, Q., Bennett, C., Harada, Y., Stankunas, K. et al. (2006). TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* **126**, 955-968.
Issad, T., Masson, E. and Pagesy, P. (2010). O-GlcNAc modification, insulin signaling and diabetic complications. *Diabetes Metab.* **36**, 423-435.
Iynedjian, P. B. (1993). Mammalian glucokinase and its gene. *Biochem. J.* **293 (Pt 1)**, 1-13.
Iynedjian, P. B. (2009). Molecular physiology of mammalian glucokinase. *Cell. Mol. Life Sci.* **66**, 27-42.
Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S. Y., Huang, Q., Qin, J. and Su, B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* **127**, 125-37.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M. A., Hall, A. and Hall, M. N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nature cell biology* **6**, 1122-8.
Jeon, T. I. and Osborne, T. F. (2012). SREBPs: metabolic integrators in physiology and metabolism. *Trends Endocrinol. Metab.* **23**, 65-72.
Jewell, J. L. and Guan, K. L. (2013). Nutrient signaling to mTOR and cell growth. *Trends Biochem. Sci.* **38**, 233-242.
Jewell, J. L., Kim, Y. C., Russell, R. C., Yu, F. X., Park, H. W., Plouffe, S. W., Tagliabracci, V. S. and Guan, K. L. (2015). Differential regulation of mTORC1 by leucine and glutamine. *Science* **347**, 194-198.
Jin, J., Medale, F., Kamalam, B. S., Aguirre, P., Veron, V. and Panserat, S. (2014a). Comparison of glucose and lipid metabolic gene expressions between fat and lean lines of rainbow trout after a glucose load. *PLoS One* **9**, e105548.
Jin, J. Y., Panserat, S., Kamalam, B. S., Aguirre, P., Veron, V. and Medale, F. (2014b). Insulin regulates lipid and glucose metabolism similarly in two lines of rainbow trout divergently selected for muscle fat content. *Gen. Comp. Endocrinol.* **204**, 49-59.
Jing, Y., Liu, W., Cao, H., Zhang, D., Yao, X., Zhang, S., Xia, H., Li, D., Wang, Y. C., Yan, J. et al. (2015). Hepatic p38alpha regulates gluconeogenesis by suppressing AMPK. *J. Hepatol.* **62**, 1319-27.
Johnston, I. A. (1999). Muscle development and growth: potential implications for flesh quality in fish. *Aquaculture* **177**, 99-115.
Julien, L. A., Carriere, A., Moreau, J. and Roux, P. P. (2010). mTORC1-Activated S6K1 Phosphorylates Rictor on Threonine 1135 and Regulates mTORC2 Signaling. *Mol. Cell. Biol.* **30**, 908-921.
Jump, D. B. (2011). Fatty acid regulation of hepatic lipid metabolism. *Curr. Opin. Clin. Nutr. Metab. Care* **14**, 115-20.
Jump, D. B., Tripathy, S. and Depner, C. M. (2013). Fatty acid-regulated transcription factors in the liver. *Annu. Rev. Nutr.* **33**, 249-69.
Jung, H. S. and Lee, M. S. (2010). Role of autophagy in diabetes and mitochondria. *Ann Ny Acad Sci* **1201**, 79-83.
Kamalam, B. S. (2013). Metabolic utilization of dietary carbohydrates in lean and fat lines of rainbow trout (Oncorhynchus mykiss), vol. Doctor, pp. 225: L'UNIVERSITÉ DE PAU ET DES PAYS DE L'ADOUR.
Kamalam, B. S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S. and Panserat, S. (2012). Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. *J. Exp. Biol.* **215**, 2567-2578.
Kamalam, B. S., Medale, F., Larroquet, L., Corraze, G. and Panserat, S. (2013a). Metabolism and fatty acid profile in fat and lean rainbow trout lines fed with vegetable oil: effect of carbohydrates. *PLoS One* **8**, e76570.
Kamalam, B. S., Panserat, S., Aguirre, P., Geurden, I., Fontagne-Dicharry, S. and Medale, F. (2013b). Selection for high muscle fat in rainbow trout induces potentially higher chylomicron synthesis and PUFA biosynthesis in the intestine. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **164**, 417-27.
Kaur, J. and Debnath, J. (2015). Autophagy at the crossroads of catabolism and anabolism. *Nat Rev Mol Cell Bio* **16**, 461-472.
Kaushik, S. J. and Seiliez, I. (2010). Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. *Aquac Res* **41**, 322-332.
Kersten, S. (2001). Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO reports* **2**, 282-6.
Khamzina, L., Veilleux, A., Bergeron, S. and Marette, A. (2005). Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. *Endocrinology* **146**, 1473-81.
Khanna, S. S. and Rekhari, K. (1972). Effect of glucose loading on the blood sugar and the histology of the pancreatic islets in a freshwater teleost, Heteropneustes fossilis. *Acta Anat. (Basel)* **82**, 126-37.
Kilberg, M. S., Balasubramanian, M., Fu, L. and Shan, J. (2012). The transcription factor network associated with the amino acid response in mammalian cells. *Advances in nutrition* **3**, 295-306.
Kilberg, M. S., Shan, J. and Su, N. (2009). ATF4-dependent transcription mediates signaling of amino acid limitation. *Trends Endocrinol. Metab.* **20**, 436-43.
Kim, E., Goraksha-Hicks, P., Li, L., Neufeld, T. P. and Guan, K. L. (2008). Regulation of TORC1 by Rag GTPases in nutrient response. *Nat. Cell Biol.* **10**, 935-45.
Kim, J. and Guan, K. L. (2011). Amino acid signaling in TOR activation. *Annu. Rev. Biochem.* **80**, 1001-32.
Kirchner, S., Kaushik, S. and Panserat, S. (2003). Low protein intake is associated with reduced hepatic gluconeogenic enzyme expression in rainbow trout (Oncorhynchus mykiss). *J. Nutr.* **133**, 2561-2564.

Kitamura, T., Kitamura, Y., Kuroda, S., Hino, Y., Ando, M., Kotani, K., Konishi, H., Matsuzaki, H., Kikkawa, U., Ogawa, W. et al. (1999). Insulin-induced phosphorylation and activation of cyclic nucleotide phosphodiesterase 3B by the serine-threonine kinase Akt. *Mol. Cell. Biol.* **19**, 6286-6296.
Klionsky, D. J. Abdalla, F. C. Abeliovich, H. Abraham, R. T. Acevedo-Arozena, A. Adeli, K. Agholme, L. Agnello, M. Agostinis, P. Aguirre-Ghiso, J. A. et al. (2012). Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* **8**, 445-544.
Klover, P. J. and Mooney, R. A. (2004). Hepatocytes: critical for glucose homeostasis. *Int. J. Biochem. Cell Biol.* **36**, 753-8.
Kohn, A. D., Summers, S. A., Birnbaum, M. J. and Roth, R. A. (1996). Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J. Biol. Chem.* **271**, 31372-31378.
Koizumi, N., Kondoh, M., Mizuguchi, H., Nakanishi, T., Masuyama, A., Ida, F., Fujii, M., Hayakawa, T., Nakashima, E., Tanaka, K. et al. (2005). Comparison of transgene expression mediated by several Fiber-modified adenovirus vectors in trophoblast cells. *Placenta* **26**, 729-34.
Koren, I., Reem, E. and Kimchi, A. (2010). DAP1, a Novel Substrate of mTOR, Negatively Regulates Autophagy. *Curr. Biol.* **20**, 1093-1098.
Krycer, J. R., Sharpe, L. J., Luu, W. and Brown, A. J. (2010). The Akt-SREBP nexus: cell signaling meets lipid metabolism. *Trends Endocrinol. Metab.* **21**, 268-76.
Lamming, D. W. and Sabatini, D. M. (2013). A Central role for mTOR in lipid homeostasis. *Cell metabolism* **18**, 465-9.
Lamming, D. W., Ye, L., Katajisto, P., Goncalves, M. D., Saitoh, M., Stevens, D. M., Davis, J. G., Salmon, A. B., Richardson, A., Ahima, R. S. et al. (2012). Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. *Science* **335**, 1638-43.
Lansard, M., Panserat, S., Plagnes-Juan, E., Dias, K., Seiliez, I. and Skiba-Cassy, S. (2011). L-Leucine, L-Methionine, and L-Lysine Are Involved in the Regulation of Intermediary Metabolism-Related Gene Expression in Rainbow Trout Hepatocytes. *J. Nutr.* **141**, 75-80.
Lansard, M., Panserat, S., Plagnes-Juan, E., Seiliez, I. and Skiba-Cassy, S. (2010). Integration of insulin and amino acid signals that regulate hepatic metabolism-related gene expression in rainbow trout: role of TOR. *Amino Acids* **39**, 801-810.
Laplante, M. and Sabatini, D. M. (2009). mTOR signaling at a glance. *J. Cell Sci.* **122**, 3589-3594.
Laplante, M. and Sabatini, D. M. (2010). mTORC1 activates SREBP-1c and uncouples lipogenesis from gluconeogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 3281-2.
Laplante, M. and Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell* **149**, 274-293.
Laplante, M. and Sabatini, D. M. (2013). Regulation of mTORC1 and its impact on gene expression at a glance. *J. Cell Sci.* **126**, 1713-1719.
Layman, D. K., Shiue, H., Sather, C., Erickson, D. J. and Baum, J. (2003). Increased dietary protein modifies glucose and insulin homeostasis in adult women during weight loss. *J. Nutr.* **133**, 405-10.
Lee, A. H., Scapa, E. F., Cohen, D. E. and Glimcher, L. H. (2008). Regulation of hepatic lipogenesis by the transcription factor XBP1. *Science* **320**, 1492-6.
Lee, M. W., Chanda, D., Yang, J. Q., Oh, H., Kim, S. S., Yoon, Y. S., Hong, S., Park, K. G., Lee, I. K., Choi, C. S. et al. (2010). Regulation of Hepatic Gluconeogenesis by an ER-Bound Transcription Factor, CREBH. *Cell metabolism* **11**, 331-339.
Legate, N. J., Bonen, A. and Moon, T. W. (2001). Glucose tolerance and peripheral glucose utilization in rainbow trout (Oncorhynchus mykiss), American eel (Anguilla rostrata), and black bullhead catfish (Ameiurus melas). *Gen. Comp. Endocrinol.* **122**, 48-59.
Leturque, A., Brot-Laroche, E. and Le Gall, M. (2009). GLUT2 mutations, translocation, and receptor function in diet sugar managing. *Am. J. Physiol. Endocrinol. Metab.* **296**, E985-92.
Li, P., Mai, K., Trushenski, J. and Wu, G. (2009). New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. *Amino Acids* **37**, 43-53.
Li, X., Monks, B., Ge, Q. and Birnbaum, M. J. (2007). Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1alpha transcription coactivator. *Nature* **447**, 1012-6.
Li, Y., Xu, S., Mihaylova, M. M., Zheng, B., Hou, X., Jiang, B., Park, O., Luo, Z., Lefai, E., Shyy, J. Y. et al. (2011). AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. *Cell metabolism* **13**, 376-88.

Likimani, T. A. and Wilson, R. P. (1982). Effects of Diet on Lipogenic Enzyme-Activities in Channel Catfish Hepatic and Adipose-Tissue. *J. Nutr.* **112**, 112-117.
Linn, T., Santosa, B., Gronemeyer, D., Aygen, S., Scholz, N., Busch, M. and Bretzel, R. G. (2000). Effect of long-term dietary protein intake on glucose metabolism in humans. *Diabetologia* **43**, 1257-1265.
Liu, K., Paterson, A. J., Chin, E. and Kudlow, J. E. (2000). Glucose stimulates protein modification by O-linked GlcNAc in pancreatic beta cells: linkage of O-linked GlcNAc to beta cell death. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 2820-5.
Liu, P., Gan, W., Inuzuka, H., Lazorchak, A. S., Gao, D., Arojo, O., Liu, D., Wan, L., Zhai, B., Yu, Y. et al. (2013). Sin1 phosphorylation impairs mTORC2 complex integrity and inhibits downstream Akt signalling to suppress tumorigenesis. *Nat. Cell Biol.* **15**, 1340-50.
Liu, Y. F., Herschkovitz, A., Boura-Halfon, S., Ronen, D., Paz, K., LeRoith, D. and Zick, Y. (2004). Serine phosphorylation proximal to its phosphotyrosine binding domain inhibits insulin receptor substrate 1 function and promotes insulin resistance. *Mol. Cell. Biol.* **24**, 9668-9681.
Lowell, B. B. and Shulman, G. I. (2005). Mitochondrial dysfunction and type 2 diabetes. *Science* **307**, 384-387.
Lowell, B. B. and Shulmanz, G. I. (2005). Mitochondrial dysfunction and type 2 diabetes. *Science* **307**, 384-387.
Lu, K. L., Xu, W. N., Wang, L. N., Zhang, D. D., Zhang, C. N. and Liu, W. B. (2014). Hepatic beta-oxidation and regulation of carnitine palmitoyltransferase (CPT) I in blunt snout bream Megalobrama amblycephala fed a high fat diet. *PLoS One* **9**, e93135.
Lu, R. H., Zhou, Y., Yuan, X. C., Liang, X. F., Fang, L., Bai, X. L., Wang, M. and Zhao, Y. H. (2015). Effects of glucose, insulin and triiodothyroxine on leptin and leptin receptor expression and the effects of leptin on activities of enzymes related to glucose metabolism in grass carp (Ctenopharyngodon idella) hepatocytes. *Fish Physiol. Biochem.* **41**, 981-9.
Lunt, S. Y. and Vander Heiden, M. G. (2011). Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu. Rev. Cell Dev. Biol.* **27**, 441-64.
Lynch, C. J. and Adams, S. H. (2014). Branched-chain amino acids in metabolic signalling and insulin resistance. *Nature Reviews Endocrinology* **10**, 723-736.
Ma, X. M. and Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control. *Nat. Rev. Mol. Cell Biol.* **10**, 307-18.
Macdonald, M. L., Rogers, Q. R. and Morris, J. G. (1984). Nutrition of the Domestic Cat, a Mammalian Carnivore. *Annu. Rev. Nutr.* **4**, 521-562.
Manning, B. D. and Cantley, L. C. (2007). AKT/PKB signaling: navigating downstream. *Cell* **129**, 1261-1274.
Masui, K., Cavenee, W. K. and Mischel, P. S. (2014). mTORC2 in the center of cancer metabolic reprogramming. *Trends Endocrinol. Metab.* **25**, 364-373.
Matschinsky, F. M. (2005). Glucokinase, glucose homeostasis, and diabetes mellitus. *Current diabetes reports* **5**, 171-6.
Matschinsky, F. M. (2009). Assessing the potential of glucokinase activators in diabetes therapy. *Nature reviews. Drug discovery* **8**, 399-416.
Mayer, C., Zhao, J., Yuan, X. and Grummt, I. (2004). mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev.* **18**, 423-34.
McGarry, J. D. and Brown, N. F. (1997). The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur. J. Biochem.* **244**, 1-14.
Menendez, J. A. and Lupu, R. (2007). Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat. Rev. Cancer* **7**, 763-77.
Mommsen, T. P., Moon, T. W. and Walsh, T. J. (1994). Hepatocytes: isolation, maintenance and utilization. In *Biochemistry and Molecular Biology of Fishes*, vol. 3 eds. P. W. Hochachka and T. P. Mommsen). Amsterdam: Elsevier Science B.V.
Mommsen, T. P. and Plisetskaya, E. M. (1991). Insulin in Fishes and Agnathans - History, Structure, and Metabolic-Regulation. *Rev Aquat Sci* **4**, 225-259.
Moon, T. W. (2001). Glucose intolerance in teleost fish: fact or fiction? *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **129**, 243-9.
Moore, S. (1968). Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. *J. Biol. Chem.* **243**, 6281-3.

Moreira, I., Peres, H., Couto, A., Enes, P. and Oliva-Teles, A. (2008). Temperature and dietary carbohydrate level effects on performance and metabolic utilisation of diets in European sea bass (< i> Dicentrarchus labrax</i>) juveniles. *Aquaculture* **274**, 153-160.
Muoio, D. M. and Newgard, C. B. (2008). Mechanisms of disease:Molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat. Rev. Mol. Cell Biol.* **9**, 193-205.
Navarro, E., Capilla, J., Castillo, A., Albalat, M., Díaz, M. A., Gallardo, J., Blasco, J. V., Planas, J. and Gutiérrez, M. (2006). Insulin metabolic effects in fish tissues. *Fish Endocrinol.*, 15–48.
Navarro, I., Rojas, P., Capilla, E., Albalat, A., Castillo, J., Montserrat, N., Codina, M. and Gutierrez, J. (2002). Insights into insulin and glucagon responses in fish. *Fish Physiol. Biochem.* **27**, 205-216.
Naylor, R. L., Goldburg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C., Clay, J., Folke, C., Lubchenco, J., Mooney, H. and Troell, M. (2000). Effect of aquaculture on world fish supplies. *Nature* **405**, 1017-24.
Naylor, R. L., Hardy, R. W., Bureau, D. P., Chiu, A., Elliott, M., Farrell, A. P., Forster, I., Gatlin, D. M., Goldburg, R. J., Hua, K. et al. (2009). Feeding aquaculture in an era of finite resources. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 15103-10.
Newgard, C. B. (2012). Interplay between Lipids and Branched-Chain Amino Acids in Development of Insulin Resistance. *Cell metabolism* **15**, 606-614.
Newsholme, P., Brennan, L., Rubi, B. and Maechler, P. (2005). New insights into amino acid metabolism, beta-cell function and diabetes. *Clin. Sci. (Lond.)* **108**, 185-94.
Nordlie, R. C., Foster, J. D. and Lange, A. J. (1999). Regulation of glucose production by the liver. *Annu. Rev. Nutr.* **19**, 379-406.
OECD-FAO. (2012). World Agricultural Outlook 2012–2021. Rome: FAO; Paris: OECD.
Oh, W. J. and Jacinto, E. (2011). mTOR complex 2 signaling and functions. *Cell Cycle* **10**, 2305-16.
Ouyang, J. Y., Parakhia, R. A. and Ochs, R. S. (2011). Metformin Activates AMP Kinase through Inhibition of AMP Deaminase. *J. Biol. Chem.* **286**, 1-11.
Owen, J. L., Zhang, Y., Bae, S. H., Farooqi, M. S., Liang, G., Hammer, R. E., Goldstein, J. L. and Brown, M. S. (2012). Insulin stimulation of SREBP-1c processing in transgenic rat hepatocytes requires p70 S6-kinase. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 16184-9.
Panserat, S., Capilla, E., Gutierrez, J., Frappart, P., Vachot, C., Plagnes-Juan, E., Aguirre, P., Brèque, J. and Kaushik, S. (2001a). Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (Oncorhynchus mykiss) by a single meal with glucose. *Comp Biochem Physiol B Biochem Mol Biol* **128**, 275-83.
Panserat, S., Capilla, E., Gutierrez, J., Frappart, P. O., Vachot, C., Plagnes-Juan, E., Aguirre, P., Breque, J. and Kaushik, S. (2001b). Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (Oncorhynchus mykiss) by a single meal with glucose. *Comp Biochem Phys B* **128**, 275-283.
Panserat, S., Kaushik, S. and Médale, F. (2013). Rainbow trout as a model for nutrition and nutrient metabolism studies. In *Trout : from physiology to conservation*, eds. S. Polakof and T. W. Moon), pp. 452 p. Hauppauge, USA: Nova Science Publishers.
Panserat, S., Medale, F., Blin, C., Breque, J., Vachot, C., Plagnes-Juan, E., Gomes, E., Krishnamoorthy, R. and Kaushik, S. (2000). Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **278**, R1164-70.
Panserat, S., Plagnes-Juan, E., Brèque, J. and Kaushik, S. (2001c). Hepatic phosphoenolpyruvate carboxykinase gene expression is not repressed by dietary carbohydrates in rainbow trout (Oncorhynchus mykiss). *J Exp Biol* **204**, 359-65.
Panserat, S., Plagnes-Juan, E. and Kaushik, S. (2001d). Nutritional regulation and tissue specificity of gene expression for proteins involved in hepatic glucose metabolism in rainbow trout (Oncorhynchus mykiss). *J. Exp. Biol.* **204**, 2351-60.
Panserat, S., Plagnes-Juan, E. and Kaushik, S. (2002). Gluconeogenic enzyme gene expression is decreased by dietary carbohydrates in common carp (Cyprinus carpio) and gilthead seabream (Sparus aurata). *Biochim Biophys Acta* **1579**, 35-42.
Panserat, S., Rideau, N. and Polakof, S. (2014). Nutritional regulation of glucokinase: a cross species story. *Nutrition Research Reviews* **In press**.
Panserat, S., Skiba-Cassy, S., Seiliez, I., Lansard, M., Plagnes-Juan, E., Vachot, C., Aguirre, P., Larroquet, L., Chavernac, G., Medale, F. et al. (2009). Metformin improves postprandial glucose homeostasis in rainbow trout fed dietary carbohydrates: a link with the induction of hepatic lipogenic capacities? *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297**, R707-15.

Patra, K. C. and Hay, N. (2014). The pentose phosphate pathway and cancer. *Trends Biochem. Sci.* **39**, 347-54.
Patti, M. E., Brambilla, E., Luzi, L., Landaker, E. J. and Kahn, C. R. (1998). Bidirectional modulation of insulin action by amino acids. *J. Clin. Invest.* **101**, 1519-29.
Pereira, C., Vijayan, M. M., Storey, K. B., Jones, R. A. and Moon, T. W. (1995). Role of Glucose and Insulin in Regulating Glycogen-Synthase and Phosphorylase Activities in Rainbow-Trout Hepatocytes. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology* **165**, 62-70.
Perry, R. J., Samuel, V. T., Petersen, K. F. and Shulman, G. I. (2014). The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. *Nature* **510**, 84-91.
Peterson, T. R., Sengupta, S. S., Harris, T. E., Carmack, A. E., Kang, S. A., Balderas, E., Guertin, D. A., Madden, K. L., Carpenter, A. E., Finck, B. N. et al. (2011). mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway. *Cell* **146**, 408-20.
Pichon, L., Huneau, J. F., Fromentin, G. and Tome, D. (2006). A high-protein, high-fat, carbohydrate-free diet reduces energy intake, hepatic lipogenesis, and adiposity in rats. *J. Nutr.* **136**, 1256-60.
Pilkis, S. J. and Granner, D. K. (1992). Molecular Physiology of the Regulation of Hepatic Gluconeogenesis and Glycolysis. *Annu. Rev. Physiol.* **54**, 885-909.
Pinto, W., Figueira, L., Santos, A., Barr, Y., Helland, S., Dinis, M. T. and Aragao, C. (2013). Is dietary taurine supplementation beneficial for gilthead seabream (Sparus aurata) larvae? *Aquaculture* **384**, 1-5.
Plagnes-Juan, E., Lansard, M., Seiliez, I., Medale, F., Corraze, G., Kaushik, S., Panserat, S. and Skiba-Cassy, S. (2008). Insulin regulates the expression of several metabolism-related genes in the liver and primary hepatocytes of rainbow trout (Oncorhynchus mykiss). *J. Exp. Biol.* **211**, 2510-2518.
Plisetskaya, E. M. (1998). Some of my not so favorite things about insulin and insulin-like growth factors in fish. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **121**, 3-11.
Polakof, S., Medale, F., Larroquet, L., Vachot, C., Corraze, G. and Panserat, S. (2011a). Insulin stimulates lipogenesis and attenuates Beta-oxidation in white adipose tissue of fed rainbow trout. *Lipids* **46**, 189-99.
Polakof, S., Miguez, J. M. and Soengas, J. L. (2009a). A hepatic protein modulates glucokinase activity in fish and avian liver: a comparative study. *J. Comp. Physiol. B* **179**, 643-52.
Polakof, S., Moon, T. W., Aguirre, P., Skiba-Cassy, S. and Panserat, S. (2010a). Effects of insulin infusion on glucose homeostasis and glucose metabolism in rainbow trout fed a high-carbohydrate diet. *J. Exp. Biol.* **213**, 4151-4157.
Polakof, S., Moon, T. W., Aguirre, P., Skiba-Cassy, S. and Panserat, S. (2011b). Glucose homeostasis in rainbow trout fed a high-carbohydrate diet: metformin and insulin interact in a tissue-dependent manner. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **300**, R166-74.
Polakof, S., Panserat, S., Plagnes-Juan, E. and Soengas, J. L. (2008). Altered dietary carbohydrates significantly affect gene expression of the major glucosensing components in Brockmann bodies and hypothalamus of rainbow trout. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R1077-88.
Polakof, S., Panserat, S., Soengas, J. L. and Moon, T. W. (2012a). Glucose metabolism in fish: a review. *J. Comp. Physiol. B* **182**, 1015-45.
Polakof, S., Skiba-Cassy, S., Choubert, G. and Panserat, S. (2010b). Insulin-induced hypoglycaemia is co-ordinately regulated by liver and muscle during acute and chronic insulin stimulation in rainbow trout (Oncorhynchus mykiss). *J. Exp. Biol.* **213**, 1443-1452.
Polakof, S., Skiba-Cassy, S., Kaushik, S., Seiliez, I., Soengas, J. L. and Panserat, S. (2012b). Glucose and lipid metabolism in the pancreas of rainbow trout is regulated at the molecular level by nutritional status and carbohydrate intake. *J. Comp. Physiol. B* **182**, 507-16.
Polakof, S., Skiba-Cassy, S. and Panserat, S. (2009b). Glucose homeostasis is impaired by a paradoxical interaction between metformin and insulin in carnivorous rainbow trout. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297**, R1769-76.
Porstmann, T., Santos, C. R., Lewis, C., Griffiths, B. and Schulze, A. (2009). A new player in the orchestra of cell growth: SREBP activity is regulated by mTORC1 and contributes to the regulation of cell and organ size. *Biochemical Society transactions* **37**, 278-83.
Postic, C., Dentin, R., Denechaud, P. D. and Girard, J. (2007). ChREBP, a transcriptional regulator of glucose and lipid metabolism. *Annu. Rev. Nutr.* **27**, 179-192.
Postic, C., Dentin, R. and Girard, J. (2004). Role of the liver in the control of carbohydrate and lipid homeostasis. *Diabetes Metab.* **30**, 398-408.
Postic, C., Shiota, M., Niswender, K. D., Jetton, T. L., Chen, Y., Moates, J. M., Shelton, K. D., Lindner, J., Cherrington, A. D. and Magnuson, M. A. (1999). Dual roles for glucokinase in glucose homeostasis as

determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J. Biol. Chem.* **274**, 305-15.
Pozios, K. C., Ding, J., Degger, B., Upton, Z. and Duan, C. (2001). IGFs stimulate zebrafish cell proliferation by activating MAP kinase and PI3-kinase-signaling pathways. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **280**, R1230-9.
Printz, R. L., Magnuson, M. A. and Granner, D. K. (1993). Mammalian glucokinase. *Annu. Rev. Nutr.* **13**, 463-96.
Puigserver, P., Rhee, J., Donovan, J., Walkey, C. J., Yoon, J. C., Oriente, F., Kitamura, Y., Altomonte, J., Dong, H. J., Accili, D. et al. (2003). Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1 alpha interaction. *Nature* **423**, 550-555.
Rabinowitz, J. D. and White, E. (2010). Autophagy and metabolism. *Science* **330**, 1344-8.
Rautou, P. E., Mansouri, A., Lebrec, D., Durand, F., Valla, D. and Moreau, R. (2010). Autophagy in liver diseases. *J. Hepatol.* **53**, 1123-1134.
Reinecke, M., Bjornsson, B. T., Dickhoff, W. W., McCormick, S. D., Navarro, I., Power, D. M. and Gutierrez, J. (2005). Growth hormone and insulin-like growth factors in fish: where we are and where to go. *Gen. Comp. Endocrinol.* **142**, 20-4.
Richard, M. J., Holck, J. T. and Beitz, D. C. (1989). Lipogenesis in liver and adipose tissue of the domestic cat (Felis domestica). *Comp. Biochem. Physiol. B* **93**, 561-4.
Richter, J. D. and Sonenberg, N. (2005). Regulation of cap-dependent translation by eIF4E inhibitory proteins. *Nature* **433**, 477-80.
Ricoult, S. J. H. and Manning, B. D. (2013). The multifaceted role of mTORC1 in the control of lipid metabolism. *EMBO reports* **14**, 242-251.
Rietman, A., Schwarz, J., Blokker, B. A., Siebelink, E., Kok, F. J., Afman, L. A., Tome, D. and Mensink, M. (2014a). Increasing protein intake modulates lipid metabolism in healthy young men and women consuming a high-fat hypercaloric diet. *J. Nutr.* **144**, 1174-80.
Rietman, A., Schwarz, J., Tome, D., Kok, F. J. and Mensink, M. (2014b). High dietary protein intake, reducing or eliciting insulin resistance? *Eur. J. Clin. Nutr.* **68**, 973-9.
Robert, C. and Backus, R. C. (2008). Advances in knowledge about feline metabolism. *Continuing Education for Veterinarians* **30**.
Rolland, M. (2014). Effects of dietary methionine on feed utilization, plasma amino acid profiles and gene expression in rainbow trout (Oncorhynchus mykiss). In *DTU Aqua, National Institute of Aquatic Resources*, vol. Doctor, pp. 173: Technical University of Denmark.
Rossetti, L., Rothman, D. L., DeFronzo, R. A. and Shulman, G. I. (1989). Effect of dietary protein on in vivo insulin action and liver glycogen repletion. *Am. J. Physiol.* **257**, E212-9.
Rouvinen-Watt, K., Harris, L., Dick, M., Pal, C., Lei, S., Mustonen, A. M. and Nieminen, P. (2012). Role of hepatic de novo lipogenesis in the development of fasting-induced fatty liver in the American mink (Neovison vison). *Br. J. Nutr.* **108**, 1360-70.
Rowland, A. F., Fazakerley, D. J. and James, D. E. (2011). Mapping Insulin/GLUT4 Circuitry. *Traffic* **12**, 672-681.
Ruan, H. B., Singh, J. P., Li, M. D., Wu, J. and Yang, X. (2013). Cracking the O-GlcNAc code in metabolism. *Trends Endocrinol. Metab.* **24**, 301-9.
Ruderman, N. B. (1975). Muscle amino acid metabolism and gluconeogenesis. *Annu. Rev. Med.* **26**, 245-58.
Saha, A. K., Balon, T. W., Coughlan, K. A. and Ruderman, N. B. (2013). Insulin Resistance due to Nutrient Excess: Mechanisms of AMPK Downregulation. *FASEB J.* **27**.
Saltiel, A. R. and Kahn, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414**, 799-806.
Samuel, V. T. and Shulman, G. I. (2012). Mechanisms for insulin resistance: common threads and missing links. *Cell* **148**, 852-71.
Sancak, Y., Peterson, T. R., Shaul, Y. D., Lindquist, R. A., Thoreen, C. C., Bar-Peled, L. and Sabatini, D. M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* **320**, 1496-501.
Sandhu, M. S., Dunger, D. B. and Giovannucci, E. L. (2002). Insulin, insulin-like growth factor-I (IGF-I), IGF binding proteins, their biologic interactions, and colorectal cancer. *J. Natl. Cancer Inst.* **94**, 972-80.
Sapp, V., Gaffney, L., EauClaire, S. F. and Matthews, R. P. (2014). Fructose Leads to Hepatic Steatosis in Zebrafish That Is Reversed by Mechanistic Target of Rapamycin (mTOR) Inhibition. *Hepatology* **60**, 1581-1592.

Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P. and Sabatini, D. M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol.* **14**, 1296-302.
Sarbassov, D. D., Ali, S. M. and Sabatini, D. M. (2005a). Growing roles for the mTOR pathway. *Current opinion in cell biology* **17**, 596-603.
Sarbassov, D. D., Ali, S. M., Sengupta, S., Sheen, J. H., Hsu, P. P., Bagley, A. F., Markhard, A. L. and Sabatini, D. M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol. Cell* **22**, 159-68.
Sarbassov, D. D., Guertin, D. A., Ali, S. M. and Sabatini, D. M. (2005b). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098-1101.
Savage, D. B. and Semple, R. K. (2010). Recent insights into fatty liver, metabolic dyslipidaemia and their links to insulin resistance. *Curr. Opin. Lipidol.* **21**, 329-336.
Scheid, M. P. and Woodgett, J. R. (2003). Unravelling the activation mechanisms of protein kinase B/Akt. *FEBS Lett.* **546**, 108-12.
Schermerhorn, T. (2013). Normal glucose metabolism in carnivores overlaps with diabetes pathology in non-carnivores. *Front. Endocrinol. (Lausanne)* **4**, 188.
Schmoll, D., Grempler, R., Barthel, A., Joost, H. G. and Walther, R. (2001). Phorbol ester-induced activation of mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase and extracellular-signal-regulated protein kinase decreases glucose-6-phosphatase gene expression. *Biochem. J.* **357**, 867-873.
Schultz, J. R., Tu, H., Luk, A., Repa, J. J., Medina, J. C., Li, L. P., Schwendner, S., Wang, S., Thoolen, M., Mangelsdorf, D. J. et al. (2000). Role of LXRs in control of lipogenesis. *Genes Dev.* **14**, 2831-2838.
Schwarz, J., Tome, D., Baars, A., Hooiveld, G. J. and Muller, M. (2012). Dietary protein affects gene expression and prevents lipid accumulation in the liver in mice. *PLoS One* **7**, e47303.
Segner, H. (1998). Isolation and primary culture of teleost hepatocytes. *Comparative Biochemistry and Physiology. A: Comparative Physiology* **120**, 71-81.
Seiliez, I., Gabillard, J. C., Riflade, M., Sadoul, B., Dias, K., Averous, J., Tesseraud, S., Skiba, S. and Panserat, S. (2012). Amino acids downregulate the expression of several autophagy-related genes in rainbow trout myoblasts. *Autophagy* **8**, 364-375.
Seiliez, I., Gabillard, J. C., Skiba-Cassy, S., Garcia-Serrana, D., Gutierrez, J., Kaushik, S., Panserat, S. and Tesseraud, S. (2008). An in vivo and in vitro assessment of TOR signaling cascade in rainbow trout (Oncorhynchus mykiss). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R329-35.
Seiliez, I., Panserat, S., Kaushik, S. and Bergot, P. (2001). Cloning, tissue distribution and nutritional regulation of a Δ6-desaturase-like enzyme in rainbow trout. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **130**, 83-93.
Seiliez, I., Panserat, S., Lansard, M., Polakof, S., Plagnes-Juan, E., Surget, A., Dias, K., Larquier, M., Kaushik, S. and Skiba-Cassy, S. (2011a). Dietary carbohydrate-to-protein ratio affects TOR signaling and metabolism-related gene expression in the liver and muscle of rainbow trout after a single meal. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **300**, R733-43.
Seiliez, I., Sabin, N. and Gabillard, J. C. (2011b). FoxO1 is not a key transcription factor in the regulation of myostatin (mstn-1a and mstn-1b) gene expression in trout myotubes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **301**, R97-104.
Senft, D. and Ronai, Z. A. (2015). UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. *Trends Biochem. Sci.* **40**, 141-8.
Shao, W. and Espenshade, P. J. (2012). Expanding roles for SREBP in metabolism. *Cell metabolism* **16**, 414-419.
Shewfelt, R. L. (1981). Fish Muscle Lipolysis - a Review. *J. Food Biochem.* **5**, 79-100.
Shiau, S. Y. and Chen, M. J. (1993). Carbohydrate Utilization by Tilapia (Oreochromis-Niloticus X Oreochromis-Aureus) as Influenced by Different Chromium Sources. *J. Nutr.* **123**, 1747-1753.
Shiau, S. Y. and Lin, S. F. (1993). Effect of Supplemental Dietary Chromium and Vanadium on the Utilization of Different Carbohydrates in Tilapia, Oreochromis-Niloticusxo-Aureus. *Aquaculture* **110**, 321-330.
Shikata, T., Iwanaga, S. and Shimeno, S. (1994). Effects of dietary glucose, fructose, and galactose on hepatopancreatic enzyme activities and body composition in carp. *Fish. Sci.* **60**.
Shimobayashi, M. and Hall, M. N. (2014). Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nat Rev Mol Cell Bio* **15**, 155-162.
Shoji, I., Deng, L. and Hotta, H. (2011). Molecular mechanism of hepatitis C virus-induced glucose metabolic disorders. *Frontiers in microbiology* **2**, 278.

Siddle, K. (2011). Signalling by insulin and IGF receptors: supporting acts and new players. *J. Mol. Endocrinol.* **47**, R1-R10.
SIGENAE. Information System of Breeding Animals' Genome. http://www.sigenae. org/.
Singh, J. P., Zhang, K., Wu, J. and Yang, X. (2015). O-GlcNAc signaling in cancer metabolism and epigenetics. *Cancer Lett.* **356**, 244-50.
Skiba-Cassy, S., Lansard, M., Panserat, S. and Medale, F. (2009). Rainbow trout genetically selected for greater muscle fat content display increased activation of liver TOR signaling and lipogenic gene expression. *Am J Physiol-Reg I* **297**, R1421-R1429.
Skiba-Cassy, S., Panserat, S., Larquier, M., Dias, K., Surget, A., Plagnes-Juan, E., Kaushik, S. and Seiliez, I. (2013). Apparent low ability of liver and muscle to adapt to variation of dietary carbohydrate:protein ratio in rainbow trout (Oncorhynchus mykiss). *Br. J. Nutr.* **109**, 1359-1372.
Slawson, C., Copeland, R. J. and Hart, G. W. (2010). O-GIcNAc signaling: a metabolic link between diabetes and cancer? *Trends Biochem. Sci.* **35**, 547-555.
Solloway, M. J., Madjidi, A., Gu, C., Eastham-Anderson, J., Clarke, H. J., Kljavin, N., Zavala-Solorio, J., Kates, L., Friedman, B., Brauer, M. et al. (2015). Glucagon Couples Hepatic Amino Acid Catabolism to mTOR-Dependent Regulation of alpha-Cell Mass. *Cell reports* **12**, 495-510.
Somero, G. N. (1997). Handbook of Physiology.
Stone, D. A. J. (2003). Dietary carbohydrate utilization by fish. *Rev Fish Sci* **11**, 337-369.
Strable, M. S. and Ntambi, J. M. (2010). Genetic control of de novo lipogenesis: role in diet-induced obesity. *Crit. Rev. Biochem. Mol. Biol.* **45**, 199-214.
Stracka, D., Jozefczuk, S., Rudroff, F., Sauer, U. and Hall, M. N. (2014). Nitrogen source activates TOR (target of rapamycin) complex 1 via glutamine and independently of Gtr/Rag proteins. *J. Biol. Chem.* **289**, 25010-20.
Suarez, M. D., Hidalgo, M. C., Gallego, M. G., Sanz, A. and Delahiguera, M. (1995). Influence of the Relative Proportions of Energy Yielding Nutrients on Liver Intermediary Metabolism of the European Eel. *Comparative Biochemistry and Physiology a-Physiology* **111**, 421-428.
Sugita, T., Shimeno, S., Hosokawa, H. and Masumoto, T. (1999). Response of hepatopancreatic enzyme activities and metabolic intermediate concentrations to bovine insulin and glucose administration in carp Cyprinus carpio. *Nippon Suisan Gakkaishi* **65**, 896-900.
Sugita, T., Shimeno, S., Ohkubo, Y., Hosokawa, H. and Masumoto, T. (2001). Response of enzyme activities and metabolic intermediate concentrations to glucagon administration in hepatopancreas and muscle of carp†. *Fish. Sci.* **67**, 157-162.
Szendroedi, J., Phielix, E. and Roden, M. (2012). The role of mitochondria in insulin resistance and type 2 diabetes mellitus. *Nat. Rev. Endocrinol.* **8**, 92-103.
Tacon, A. G. J. and Metian, M. (2013). Fish Matters: Importance of Aquatic Foods in Human Nutrition and Global Food Supply. *Rev Fish Sci* **21**, 22-38.
Takano, T., Usui, I., Haruta, T., Kawahara, J., Uno, T., Iwata, M. and Kobayashi, M. (2001). Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. *Mol. Cell. Biol.* **21**, 5050-5062.
Talukdar, I., Szeszel-Fedorowicz, W. and Salati, L. M. (2005). Arachidonic acid inhibits the insulin induction of glucose-6-phosphate dehydrogenase via p38 MAP kinase. *J. Biol. Chem.* **280**, 40660-40667.
Taniguchi, C. M., Emanuelli, B. and Kahn, C. R. (2006). Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Bio* **7**, 85-96.
Teo, C. F., Wollaston-Hayden, E. E. and Wells, L. (2010). Hexosamine flux, the O-GlcNAc modification, and the development of insulin resistance in adipocytes. *Mol. Cell. Endocrinol.* **318**, 44-53.
Testa, J. R. and Tsichlis, P. N. (2005). AKT signaling in normal and malignant cells. *Oncogene* **24**, 7391-7393.
The World Bank. (2013). Fish to 2030: prospects for fisheries and aquaculture. In *WORLD BANK REPORT NUMBER 83177-GLB*, pp. 80pp. Washington, DC: Agriculture and Environmental Services.
Tocher, D. R. (2003). Metabolism and functions of lipids and fatty acids in teleost fish. *Rev Fish Sci* **11**, 107-184.
Tocher, D. R., Fonseca-Madrigal, J., Bell, J. G., Dick, J. R., Henderson, R. J. and Sargent, J. R. (2002). Effects of diets containing linseed oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes in Atlantic salmon (Salmo salar). *Fish Physiol. Biochem.* **26**, 157-170.
Towle, H. C., Kaytor, E. N. and Shih, H. M. (1997). Regulation of the expression of lipogenic enzyme genes by carbohydrate. *Annu. Rev. Nutr.* **17**, 405-33.

Tranulis, M. A., Dregni, O., Christophersen, B., Krogdahl, A. and Borrebaek, B. (1996). A glucokinase-like-enzyme in the liver of Atlantic salmon (Salmo salar). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **114**, 35-9.
Tremblay, F., Gagnon, A., Veilleux, A., Sorisky, A. and Marette, A. (2005a). Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. *Endocrinology* **146**, 1328-1337.
Tremblay, F., Krebs, M., Dombrowski, L., Brehm, A., Bernroider, E., Roth, E., Nowotny, P., Waldhausl, W., Marette, A. and Roden, M. (2005b). Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. *Diabetes* **54**, 2674-2684.
Tremblay, F., Lavigne, C., Jacques, H. and Marette, A. (2007). Role of dietary proteins and amino acids in the pathogenesis of insulin resistance. *Annu. Rev. Nutr.* **27**, 293-310.
Tremblay, F. and Marette, A. (2001). Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway - A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. *J. Biol. Chem.* **276**, 38052-38060.
Troell, M., Naylor, R. L., Metian, M., Beveridge, M., Tyedmers, P. H., Folke, C., Arrow, K. J., Barrett, S., Crepin, A. S., Ehrlich, P. R. et al. (2014). Does aquaculture add resilience to the global food system? *Proc. Natl. Acad. Sci. U. S. A.* **111**, 13257-13263.
Turner, N. (2013). Mitochondrial Metabolism and Insulin Action. In *Type 2 Diabetes*, (ed. K. Masuo), pp. 71-114. license: InTech.
Tzatsos, A. and Kandror, K. V. (2006). Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent mTOR-mediated insulin receptor substrate 1 phosphorylation. *Mol. Cell. Biol.* **26**, 63-76.
Uebanso, T., Taketani, Y., Fukaya, M., Sato, K., Takei, Y., Sato, T., Sawada, N., Amo, K., Harada, N., Arai, H. et al. (2009). Hypocaloric high-protein diet improves fatty liver and hypertriglyceridemia in sucrose-fed obese rats via two pathways. *Am. J. Physiol. Endocrinol. Metab.* **297**, E76-84.
Um, S. H., D'Alessio, D. and Thomas, G. (2006). Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. *Cell metabolism* **3**, 393-402.
Valvezan, A. J., Huang, J., Lengner, C. J., Pack, M. and Klein, P. S. (2014). Oncogenic mutations in adenomatous polyposis coli (Apc) activate mechanistic target of rapamycin complex 1 (mTORC1) in mice and zebrafish. *Dis. Model. Mech.* **7**, 63-71.
Vatner, D. F., Majumdar, S. K., Kumashiro, N., Petersen, M. C., Rahimi, Y., Gattu, A. K., Bears, M., Camporez, J. P., Cline, G. W., Jurczak, M. J. et al. (2015). Insulin-independent regulation of hepatic triglyceride synthesis by fatty acids. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 1143-8.
Velez, E. J., Lutfi, E., Jimenez-Amilburu, V., Riera-Codina, M., Capilla, E., Navarro, I. and Gutierrez, J. (2014). IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes. *Gen. Comp. Endocrinol.* **205**, 296-304.
Verbrugghe, A. and Bakovic, M. (2013). Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis. *Nutrients* **5**, 2811-35.
Vidal-Alabró, A., Méndez-Lucas, A., Semakova, J., Gómez-Valadés, A. G. and Perales, J. C. (2012). Liver Glucokinase and Lipid Metabolism. *InTech*.
Vidal-Puig, A. and O'Rahilly, S. (2001). Metabolism. Controlling the glucose factory. *Nature* **413**, 125-6.
Viegas, I., Rito, J., Jarak, I., Leston, S., Caballero-Solares, A., Meton, I., Pardal, M. A., Baanante, I. V. and Jones, J. G. (2015). Contribution of dietary starch to hepatic and systemic carbohydrate fluxes in European seabass (Dicentrarchus labrax L.). *Br. J. Nutr.* **113**, 1345-54.
Wallis, M., Howell, S. L. and Taylor, K. W. (1985). The Biochemistry of the Polypeptide Hormones. New York: John Wiley & Sons.
Walton, M. J. (1986). Metabolic Effects of Feeding a High Protein-Low Carbohydrate-Diet as Compared to a Low Protein-High Carbohydrate-Diet to Rainbow-Trout Salmo-Gairdneri. *Fish Physiol. Biochem.* **1**, 7-15.
Walton, M. J. and Cowey, C. B. (1982). Aspects of Intermediary Metabolism in Salmonid Fish. *Comp Biochem Phys B* **73**, 59-79.
Wang, S. Y., Tsun, Z. Y., Wolfson, R. L., Shen, K., Wyant, G. A., Plovanich, M. E., Yuan, E. D., Jones, T. D., Chantranupong, L., Comb, W. et al. (2015). Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. *Science* **347**, 188-194.
Wang, Y., Botolin, D., Xu, J., Christian, B., Mitchell, E., Jayaprakasam, B., Nair, M. G., Peters, J. M., Busik, J. V., Olson, L. K. et al. (2006). Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity. *J. Lipid Res.* **47**, 2028-41.

Washizu, T., Tanaka, A., Sako, T., Washizu, M. and Arai, T. (1999). Comparison of the activities of enzymes related to glycolysis and gluconeogenesis in the liver of dogs and cats. *Res. Vet. Sci.* **67**, 205-6.
Wilson, R. P. (1994). Utilization of Dietary Carbohydrate by Fish. *Aquaculture* **124**, 67-80.
Wilson, R. P. and Halver, J. E. (1986). Protein and Amino-Acid-Requirements of Fishes. *Annu. Rev. Nutr.* **6**, 225-244.
Wilson, R. P. and Likimani, T. A. (1981). Effects of Diet on Lipogenic Enzyme-Activities in Channel Catfish Hepatic and Adipose-Tissue. *Fed. Proc.* **40**, 882-882.
Wong, R. H. F. and Sul, H. S. (2010). Insulin signaling in fatty acid and fat synthesis: a transcriptional perspective. *Curr. Opin. Pharmacol.* **10**, 684-691.
Wu, G. (2009). Amino acids: metabolism, functions, and nutrition. *Amino Acids* **37**, 1-17.
Wu, G. Y., Bazer, F. W., Dai, Z. L., Li, D. F., Wang, J. J. and Wu, Z. L. (2014). Amino Acid Nutrition in Animals: Protein Synthesis and Beyond. *Annu Rev Anim Biosci* **2**, 387-417.
Wullschleger, S., Loewith, R. and Hall, M. N. (2006). TOR signaling in growth and metabolism. *Cell* **124**, 471-84.
Xiao, X. and Song, B. L. (2013). SREBP: a novel therapeutic target. *Acta biochimica et biophysica Sinica* **45**, 2-10.
Xiong, Y., Collins, Q. F., An, J., Lupo, E., Jr., Liu, H. Y., Liu, D., Robidoux, J., Liu, Z. and Cao, W. (2007a). p38 mitogen-activated protein kinase plays an inhibitory role in hepatic lipogenesis. *J. Biol. Chem.* **282**, 4975-4982.
Xiong, Y., Collins, Q. F., An, J., Lupo, E., Liu, H. Y., Liu, D. L., Robidoux, J., Liu, Z. Q. and Cao, W. H. (2007b). p38 mitogen-activated protein kinase plays an inhibitory role in hepatic lipogenesis. *J. Biol. Chem.* **282**, 4975-4982.
Xu, B., Lee, K. K., Zhang, L. and Gerton, J. L. (2013a). Stimulation of mTORC1 with L-leucine rescues defects associated with Roberts syndrome. *PLoS genetics* **9**, e1003857.
Xu, B. S., Lee, K. K., Zhang, L. and Gerton, J. L. (2013b). Stimulation of mTORC1 with L-leucine Rescues Defects Associated with Roberts Syndrome. *PLoS genetics* **9**.
Yang, H., Gong, R. and Xu, Y. (2012a). Control of cell growth: Rag GTPases in activation of TORC1. *Cellular and molecular life sciences : CMLS*.
Yang, H., Rudge, D. G., Koos, J. D., Vaidialingam, B., Yang, H. J. and Pavletich, N. P. (2013). mTOR kinase structure, mechanism and regulation. *Nature* **497**, 217-23.
Yang, L., Li, P., Fu, S. N., Calay, E. S. and Hotamisligil, G. S. (2010). Defective Hepatic Autophagy in Obesity Promotes ER Stress and Causes Insulin Resistance. *Cell metabolism* **11**, 467-478.
Yang, S. B., Lee, H. Y., Young, D. M., Tien, A. C., Rowson-Baldwin, A., Shu, Y. Y., Jan, Y. N. and Jan, L. Y. (2012b). Rapamycin induces glucose intolerance in mice by reducing islet mass, insulin content, and insulin sensitivity. *J. Mol. Med. (Berl.)* **90**, 575-85.
Yao, Y. X., Suraokar, M., Darnay, B. G., Hollier, B. G., Shaiken, T. E., Asano, T., Chen, C. H., Chang, B. H. J., Lu, Y. L., Mills, G. B. et al. (2013). BSTA Promotes mTORC2-Mediated Phosphorylation of Akt1 to Suppress Expression of FoxC2 and Stimulate Adipocyte Differentiation. *Sci Signal* **6**.
Ye, J., Kumanova, M., Hart, L. S., Sloane, K., Zhang, H., De Panis, D. N., Bobrovnikova-Marjon, E., Diehl, J. A., Ron, D. and Koumenis, C. (2010). The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation. *EMBO J.* **29**, 2082-96.
Ye, L., Varamini, B., Lamming, D. W., Sabatini, D. M. and Baur, J. A. (2012). Rapamycin has a biphasic effect on insulin sensitivity in C2C12 myotubes due to sequential disruption of mTORC1 and mTORC2. *Frontiers in genetics* **3**, 177.
Yecies, J. L., Zhang, H. H., Menon, S., Liu, S., Yecies, D., Lipovsky, A. I., Gorgun, C., Kwiatkowski, D. J., Hotamisligil, G. S., Lee, C. H. et al. (2011). Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. *Cell metabolism* **14**, 21-32.
Yogev, O., Williams, V. C., Hinits, Y. and Hughes, S. M. (2013). eIF4EBP3L acts as a gatekeeper of TORC1 in activity-dependent muscle growth by specifically regulating Mef2ca translational initiation. *PLoS Biol.* **11**, e1001679.
Yoon, J. C., Puigserver, P., Chen, G. X., Donovan, J., Wu, Z. D., Rhee, J., Adelmant, G., Stafford, J., Kahn, C. R., Granner, D. K. et al. (2001). Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* **413**, 131-138.
Young, V. R. and Marchini, J. S. (1990). Mechanisms and Nutritional Significance of Metabolic Responses to Altered Intakes of Protein and Amino-Acids, with Reference to Nutritional Adaptation in Humans. *Am. J. Clin. Nutr.* **51**, 270-289.

Yu, T., Robotham, J. L. and Yoon, Y. (2006). Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 2653-8.
Yuan, M. S., Pino, E., Wu, L. F., Kacergis, M. and Soukas, A. A. (2012). Identification of Akt-independent Regulation of Hepatic Lipogenesis by Mammalian Target of Rapamycin (mTOR) Complex 2. *J. Biol. Chem.* **287**, 29579-29588.
Zhou, X. Y., Shibusawa, N., Naik, K., Porras, D., Temple, K., Ou, H. S., Kaihara, K., Roe, M. W., Brady, M. J. and Wondisford, F. E. (2004). Insulin regulation of hepatic gluconeogenesis through phosphorylation of CREB-binding protein. *Nat. Med.* **10**, 633-637.
Zinzalla, V., Stracka, D., Oppliger, W. and Hall, M. N. (2011). Activation of mTORC2 by Association with the Ribosome. *Cell* **144**, 757-768.
Zoncu, R., Efeyan, A. and Sabatini, D. M. (2011). mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Bio* **12**, 21-35.

ACKOWLEDGEMENT

Looking back to my four years pleasant journey in France, I am grateful to numerous scientists, technicians and friends, who have contributed towards shaping this thesis.

First and foremost, I would like to express my deepest appreciation to my supervisor Dr. Sandrine SKIBA-CASSY for her supervision and guidance during my doctoral research endeavor for the past four years! As my supervisor, she has constantly guided me to remain focused on achieving my goals. Her observations and comments helped me to establish the overall direction of the research and to move forward with investigation in depth. I thank her for all her tremendous trust and the freedom that she gave me, which fully mobilized my inner enthusiasm and potentiality. Due to her patient and proper guidance, the "stubborn" me and her, we formed an "efficient team" and eventually accomplished the achievements we made in the current thesis, which I am pretty proud of.

I would like to thank Dr. Stéphane Panserat ("Panpan") for being our perfect researcher model, my ideal office mate, an intimate friend and my "first supporter"! Being his office mate, giving me great opportunity to know him closely and learn from this "scientific giant" both scientificly and personally. Scientificly, he is such a great scientist with pure research spirit, broad and solid knowledge, and enormous curiosity; personally, he is very easygoing, humorous and always very supportive. I am very grateful for his tremendous help, support and encouragement, and looking forward to go on learning from him in the future.

I am greatly indebted to Dr. Sadasivam Kaushik (Sachi) for creating the opportunity for me to come to INRA Saint Pée and I am also grateful for his comprehensive and meticulous care, and myriad help! As a senior scientist, his enormous contribution for world aquaculture nutrition field and his research spirit will keep inspiring me throughout my research career.

I also want to thank Dr. Iban Seilez for all his enormous help during my entire PhD study. As the leading scientist of our "amino acid research group", his profound knowledge about molecular biology and sharp mind give me a lot of inspiration and help.

It was a delight to be closely associated with Dr. F. Medale, Dr. Genevieve, Dr. Inge, Dr. Stephanie, Dr. H . Alami-Durante, Dr. C. Burel, Dr. Marandel and Dr. S. Nahon for parts of my work or scientific discussion. I thank them for their amity, intellectual inputs and knowledge sharing.

I would like to express my sincere thanks to Mme. Karine, Mme. Elisabeth, Mme. Marianne, Mr. Vincent, Mr. Alexander, Mme. Anne, Mme. Laurence and Mme. Marie-Jo for imparting cheerful and diligent technical support, training and trouble-shooting in the lab. In the same vein, I also thank Mr. Fred, Mr. Franck, Mr. Peyo, Mr. Yvan, Mr. Didier, Mr. Hontang and Mr. Maunas for their meticulous and complete assistance in conducting experimental trials and for conserving fish lines.

The atmosphere in the lab and my French sojourn was always superlative and conducive to work, thanks to the amazing camaraderie of my mates Karine, Biju, Antony, Viviana, Ikram, Alexander, Yujie, Tengfei, Junyan, Haokun, Vincent, Pedro, Filipa, Jan, Mukundh, Daniel, Jingwei, Jeanne and many others. I am especially grateful to my best friends Karine, Biju, Antony, Yujie and Alex, and my kind landlords Mme/Mr. Daguerre for making me feel at home with their kindness after lab hours, and offer us continuously delicious desserts.

I am so grateful to Mme. Elissalde for her kind and untiring help in all the official administrative formalities in France. I also thank Mme. Maryse, Celine and Pascale for their cheerful help in the NUMEA unit and library. A special thank for Dr. Olivier Lepais (UMR, Ecobiop) for all his kind and patient help for training and helping a "blank" to complete the entire RNA-seq experiments and data analysis; I am also equally grateful for having him as my good friend.

I would like to dedicate this thesis to my family and my love, Dandan Xu for their unselfish and sacrificial love, patience, and understanding—they allowed me to spend most of the time on this thesis. Indeed, I have been selfishly chasing my own dream and goal for many years, while they always unconditionally trust and support their stubborn, self-righteous son/husband. No matter how far I can go, how high I can fly in the future, they will be always my spiritual pillar and roots.

Last, but not least, I gratefully acknowledge the financial support that I received from the China Scholarship Council (CSC, File No. 2011633111) for providing me four years scholarship, which enabled me to come to INRA Saint Pée and pursue my dreams in the last four years and in the future. Without their support, this fantastic journey was simply impossible to occur.

Weiwei DAI

October, 28th, 2015, St Pée sur Nivelle, France

LIST OF PUBLICATIONS

PUBLICATIONS DURING PhD:

(1) **Dai, W.**, Panserat, S., Mennigen, J. A., Terrier, F., Dias, K., Seiliez, I. and Skiba-Cassy, S. (2013). Post-prandial regulation of hepatic glucokinase and lipogenesis requires the activation of TORC1 signalling in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **216**, 4483-92.

(2) **Dai, W.**, Panserat, S., Kaushik, S., Terrier, F., Plagnes-Juan, E., Corraze, G., Seiliez, I. and Skiba-Cassy, S. (2015). Hepatic lipid biosynthesis is more responsive to protein than carbohydrate in rainbow trout during acute stimulations. *Am. J. Physiol. Regul. Integr. Comp. Physiol. (DOI: 10.1152/ajpregu.00281.2015).*

(3) **Dai W,** Panserat S, Plagnes-Juan E, Seiliez I, and Skiba-Cassy S. (2015). Amino acids attenuate insulin action on gluconeogenesis and promote fatty acid biosynthesis via mTORC1 signaling pathway in trout hepatocytes. *Cell Physiol Biochem* **36**: 1084-1100.

(4) **Dai, W.**, Panserat, S., Terrier, F., Seiliez, I. and Skiba-Cassy, S. (2014). Acute rapamycin treatment improved glucose tolerance through inhibition of hepatic gluconeogenesis in rainbow trout (*Oncorhynchus mykiss*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **307**, R1231-8.

COMMUNICATIONS IN CONGRESSES/SYMPOSIA:

(1) **Dai, W.** and Skiba-Cassy, S. (2012). The role of protein/amino acids on hepatic metabolism related gene expression in rainbow trout (*Oncorhynchus mykiss*). *Doctoriales transfrontalières* UPPA-UPV/EHU. 22-26, October, Anglet, France. **Oral presentation**.

(2) **Dai, W.** and Skiba-Cassy, S. (2013). The role of protein/amino acids on the regulation of hepatic metabolism-related gene expression in rainbow trout. *Journées de l'Ecole Doctorale sciences exactes et leurs applications*, 4-5, July, Pau, France. **Poster**.

(3) **Dai, W.** (2014). Thesis assessment. PhD third year. *Journées de l'Ecole Doctorale sciences exactes et leurs applications*, 5-6 June, Pau, France. **Oral presentation**.

(4) **Dai, W.**, Panserat, S., Kaushik, S., Terrier, F., Plagnes-Juan, E., Seiliez, I. and Skiba-Cassy, S. (2014). Lipogenesis is mainly regulated by protein through TORC1 dependent manner in rainbow trout liver and primary cell culture of hepatocytes. *Aquaculture Europe 2014***,** 15-17, October, San Sebastián, Spain. **Oral presentation**.

Amino acids regulate hepatic intermediary metabolism-related gene expression via mTORC1-dependent manner in rainbow trout (*Oncorhynchus mykiss*)

Abstract: During my doctoral study, we used rainbow trout, a representative carnivorous fish and relevant diabetic model, to study the mechanisms underlying the regulation of hepatic intermediary metabolism by nutrients (amino acids (AAs) and glucose), and determine the potential involvement of insulin/Akt and mTORC1 signaling pathways in these regulations. Using acute administration of rapamycin, a pharmacological inhibitor of TOR, we first identified that mTORC1 activation promotes the expression of genes related to fatty acid biosynthesis, glycolysis and amino acid catabolism, while Akt negatively regulates gluconeogenic gene expression in rainbow trout liver and primary hepatocytes. Furthermore, we demonstrated hepatic fatty acid biosynthetic gene expression is more responsive to dietary protein intake/AAs than dietary carbohydrate intake/glucose during acute stimulations *in vivo* and *in vitro*. Moreover, we further showed that high levels of AAs up-regulate hepatic fatty acid biosynthetic gene expression through an mTORC1-dependent manner, while excessive AAs attenuate insulin-mediated repression of gluconeogenesis through elevating IRS-1 Ser^{302} phosphorylation, which in turn impairs Akt phosphorylation and dampens insulin action. Finally, using glucose tolerance test and acute inhibition of rapamycin, we concluded that hepatic gluconeogenesis probably plays a major role in controlling glucose homeostasis, which maybe account for the prolonged hyperglycemia and glucose intolerance phenotype of carnivorous fish. The present thesis brings forward our understandings about the roles of protein/AAs in the regulation of hepatic intermediary metabolism in trout and identifies relevant cellular signaling pathways mediating the action of amino acids on metabolism. It also clarifies some nutritional characteristics of the trout.

Key words: amino acids, insulin, lipogenesis, gluconeogenesis, glycolysis, amino acid catabolism, mTOR, Akt, liver, rainbow trout

Les acides aminés régulent l'expression des gènes du métabolisme intermédiaire chez la truite par le biais de mTORC1 (*Oncorhynchus mykiss*)

Résumé: Au cours de ma thèse, nous avons utilisé la truite arc-en-ciel, un poisson carnivore et modèle potentiellement pertinent du diabète, pour étudier des mécanismes de régulation du métabolisme intermédiaire hépatique par les nutriments (acides aminés (AA) et le glucose). Nous nous sommes plus particulièrement intéressés aux voies de signalisation de l'insuline et des acides aminés (Akt et mTORC1). Grâce à l'utilisation de rapamycine, un inhibiteur pharmacologique de mTORC1, nous avons montré que l'activation de mTORC1 stimule l'expression de gènes de la lipogenèse, de la glycolyse et du catabolisme des acides aminés, tandis que la voie de signalisation Akt inhibe celle des gènes impliqués dans la néoglucogenèse. Ces études ont été conduites dans le foie de truite ou en culture primaire d'hépatocytes de truite arc-en-ciel. En outre, nous avons démontré lors de stimulations à court terme *in vivo* et *in vitro* que l'expression hépatique des gènes de la lipogenèse est plus sensible à l'apport de protéines alimentaires ou d'AA qu'à l'apport de glucides ou de glucose. De plus, nous avons observé que des taux élevés d'AA conduisent, par le biais de l'activation de la voie de signalisation mTORC1, à une augmentation de l'expression des gènes lipogéniques mais surtout à une répression de l'inhibition de l'expression des gènes de la néoglucogenèse induite par l'insuline. Cet effet s'accompagne d'une augmentation de la phosphorylation de IRS-1 sur le résidu Ser^{302} qui pourrait être responsable de la baisse de phosphorylation d'Akt et par conséquent d'une inhibition de l'action de l'insuline. Enfin, en réalisant un test de tolérance au glucose chez des truites préalablement traitées avec de la rapamycine, nous avons conclu que la néoglucogenèse hépatique joue un rôle probablement majeur dans le contrôle de l'homéostasie glucidique chez la truite. Ainsi, une absence d'inhibition de la néoglucogenèse pourrait contribuer au maintien de l'hyperglycémie prolongée et au phénotype d'intolérance au glucose caractéristique des poissons carnivores. Cette thèse met en avant le rôle des protéines/AA dans la régulation du métabolisme intermédiaire de la truite et identifie certaines voies de signalisation cellulaire sollicités par les acides aminés pour réguler le métabolisme. Elle permet ainsi d'éclaircir certaines particularités nutritionnelles de la truite.

Mots clés : acides aminés, insuline, lipogenèse, néoglucogenèse, glycolyse, catabolisme des acides aminés, mTOR, Akt, foie, truite arc-en-ciel