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# Nutritional regulation of the hepatokine FGF21 in the liver : interdependence of the transcription factors ChREBP and PPAR $\alpha$

Alison Iroz

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***École doctorale Bio Sorbonne Paris Cité 562***

*Institut Cochin / Signalisation de l'insuline et du glucose, et glucotoxicité*

**Nutritional regulation of the hepatokine FGF21 in the liver:  
interdependence of the transcription factors ChREBP and PPAR $\alpha$**

Alison Iroz

**Thèse de doctorat de Biologie Cellulaire et Moléculaire,  
Physiologie, Physiopathologie**

**Dirigée par Catherine Postic**

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**Titre :** Régulation nutritionnelle de l'hépatokine FGF21 dans le foie: interdépendance des facteurs de transcription ChREBP et PPAR $\alpha$  en réponse au glucose

**Résumé :** L'hépatokine FGF21 (Fibroblast Growth factor 21) joue un rôle primordial dans le contrôle de l'homéostasie énergétique. Des études chez l'Homme et l'animal mettent en évidence ses effets bénéfiques dans la lutte contre l'hyperglycémie, la dyslipidémie et l'obésité. Connue pour être induite en réponse au jeûne par le récepteur nucléaire PPAR $\alpha$  (Proliferator Activated Receptor  $\alpha$ ), des études récentes suggèrent l'implication du facteur de transcription ChREBP (Carbohydrate Responsive Element Binding Protein) dans la réponse nutritionnelle de FGF21. Dans ce contexte, les objectifs de thèse ont été : 1) d'obtenir une meilleure compréhension de la régulation de FGF21 dans le foie par le jeûne et le glucose via les acteurs moléculaires ChREBP et PPAR $\alpha$ ; 2) de déterminer la pertinence physiologique de l'axe ChREBP-PPAR $\alpha$ -FGF21 en réponse au glucose. Nos résultats mettent en évidence que l'expression hépatique de ChREBP est nécessaire à l'induction de FGF21 en réponse au glucose *in vitro* et *in vivo*. De manière inattendue, lorsque l'expression de PPAR $\alpha$  est spécifiquement invalidée dans le foie, la réponse au glucose de FGF21 est diminuée de manière significative car ChREBP ne peut se lier à son élément de réponse de type ChoRE, présent sur le promoteur de *fgf21*. La réponse synergique de ChREBP et de PPAR $\alpha$  sur FGF21 a été également mise en évidence dans des cultures primaires d'hépatocytes humains. Chez les souris invalidées pour PPAR $\alpha$  dans le foie, l'absence de FGF21 circulant entraîne une augmentation de la préférence au sucrose. Notre étude révèle l'existence d'un dialogue fonctionnel unique entre ChREBP et PPAR $\alpha$  pour la régulation de FGF21 en réponse au glucose.

**Mots clefs:** Hépatokines, FGF21, ChREBP, PPAR $\alpha$ ,

**Title:** Nutritional Regulation of the hepatokine FGF21 in the liver: interdependence of the transcription factors ChREBP and PPAR $\alpha$

**Abstract:** The hepatokine FGF21 (Fibroblast Growth factor 21) plays an important role in the control of energy homeostasis. Studies in humans and animals have established FGF21 as an important therapeutic target for its beneficial effects on hyperglycemia, dyslipidemia and obesity. Induced in response to fasting by the PPAR $\alpha$  nuclear receptor (Proliferator Activated Receptor  $\alpha$ ), recent studies suggest the involvement of ChREBP (Carbohydrate Responsive Element Binding) in the nutritional response of FGF21. In this context, the thesis objectives were: 1) to obtain a better understanding of the regulation of FGF21 in the liver by fasting and glucose via the molecular actors ChREBP and PPAR $\alpha$ ; 2) to determine the physiological relevance of the ChREBP-PPAR $\alpha$ -FGF21 axis in response to glucose. Our results demonstrate that hepatic expression of ChREBP is necessary for the induction of FGF21 in response to glucose *in vitro* and *in vivo*. Unexpectedly, when PPAR $\alpha$  expression is specifically invalidated in the liver, the glucose response of FGF21 is significantly decreased as ChREBP cannot bind to its ChoRE response element present on the *fgf21* promoter. The synergistic response of ChREBP and PPAR $\alpha$  to FGF21 was also demonstrated in primary cultures of human hepatocytes. In mice deficient for PPAR $\alpha$  in the liver, the absence of circulating FGF21 leads to an increase in their preference to sucrose. Our study reveals the existence of a unique functional dialogue between ChREBP and PPAR $\alpha$  for the regulation of FGF21 in response to glucose.

**Keywords:** Hepatokines, FGF21, ChREBP, PPAR $\alpha$ , glucose signal



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## Table of Abbreviations

### #)

-COOH :	Carboxylic acid groups
16:0/18:1-GPC :	1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine

### A)

ACC :	Acetyl-coa carboxylase
ACOX1 :	Acyl coa oxidase-1
ACP :	Acyl carrier protein
ACTH :	Adrenocorticotropic hormone
AF-1 :	Activating function-1
AKT :	Protein kinase B
ALT :	Alanine transaminase
AMPK :	AMP-activated protein kinase
Angptl8 :	Angiopoietin-like protein 8
AST :	Aspartate aminotransferase
ATF4 :	Activating transcription factor 4
ATG :	Transcriptional start sight
ATGL :	Adipose triglyceride lipase
ATP :	Adenosine triphosphate

### B,C)

BMI :	Body mass index
cAMP :	Cyclic AMP
ChIP :	Chromatin immunoprecipitation

CHOP :	CCAAT enhancer binding protein homologous protein
ChoRE :	Carbohydrate response element
ChREBP :	Carbohydrate responsive element binding protein
CNS :	Central nervous system
COUP-TFII :	Chicken ovalbumin upstream promoter transcription factor II
CPT-1 :	Carnitine acyl transferase I
CPT-II :	Carnitine acyl transferase II
CREB :	Camp-response element binding protein
CREBH :	Camp-responsive element binding protein
CRH :	Corticotropin-releasing hormone
CRTC2 :	CREB-regulated transcription co activator 2

#### **D,E)**

DBD :	DNA-binding C domain
Elovl :	Elongation of long-chain fatty acids
ER :	Endoplasmic reticulum
ERK :	Extracellular signal-regulated kinase

#### **F)**

FABP :	Fatty acid-binding protein
FAD :	Flavin adenine dinucleotide
FAS :	Fatty acid synthase
Fat :	Fatty acid translocase
Fatp :	Fatty acid transport protein
FFA :	Free fatty acids
FGF :	Fibroblast growth factor

FGF21 :	Fibroblast growth factor 21
FGFR :	FGF receptors
FoxO1 :	Forkhead O box 1
FRS2 :	FGF receptor substrate 2
FSP :	Fructose 6-phosphate
FXR :	Farnesoid x receptor

## **G)**

G3P :	Glycerol-3-phosphate
G6P :	Glucose-6-phosphate
G6Pase :	Glucose-6-phosphatase
GDF-15 :	Growth differentiation factor-15
GDP :	Guanosine diphosphate
GH :	Growth hormone
GK :	Glucokinase
GKRP :	Glucokinase regulatory protein
GLUT :	Glucose transporter
GRACE :	Glucose response conserved element
Grb14 :	Growth factor receptor bound protein 14
Grb2 :	Growth factor receptor-bound protein 2
GTP :	Guanosine triphosphate

## **H)**

HAT :	Histone acetyltransferase
HCC :	Hepatocellular carcinoma
HDAC3 :	Histone deacetylase 3

HDL :	High-density lipoproteins
HFD :	High fat diet
HMG-CoA :	3-hydroxy-3-methylglutaryl-coa
Hmgcs2 :	3-hydroxy-3-methylglutaryl-coa synthase 2
HNF4 :	Hepatocyte nuclear factor 4
HNF4 $\alpha$ :	Hepatocyte nuclear factor 4 $\alpha$
HNF6 :	Hepatic nuclear factor 6
HOMA-IR :	Homeostasis model assessment of insulin resistance
HSL :	Hormone sensitive lipase

## **I)**

IGF-1 :	Insulin-like growth factor
IL-1 $\beta$ :	Interleukin-1 $\beta$
IL-6 :	Interleukin-6
IRS :	Insulin receptor substrate

## **J,k)**

JAK :	Janus kinases
JNK :	C-Jun N-terminal kinases
KDa :	Kilodalton

## **L)**

L-PK :	Liver-pyruvate kinase
LBD :	Ligand-binding E domain
LDL :	Low-density lipoproteins
LHA :	Lateral hypothalamic area

LID :	Low glucose inhibitory domain
LIRKO :	Liver insulin receptor knockout
LPL :	Lipoprotein lipase
LXR :	Liver X receptor
LXR $\alpha$ :	Liver X receptor $\alpha$
LXRE	LXR response elements
Lys :	Lysine

## **M)**

MAPK :	Mitogen-activated protein kinase
MCD :	Methionine choline-deficient
MCR :	Mondo conserved regions
Mlx :	Max-like protein X
mTOR :	Mechanistic target of rapamycin
mTORC1 :	mtor protein complex
MUFA :	Monounsaturated fatty acids

## **N)**

NAD :	Nicotinamide adenine dinucleotide
NAFLD :	Non-alcoholic fatty liver disease
NASH :	Non-alcoholic steatohepatitis
NCoR1 :	Nuclear receptor co repressor
NES :	Nuclear export signals
NLS :	Nuclear localization signal

## O,P)

OGT	O-glcnac transferase
OGTT :	Oral glucose tolerance test
P13K :	Phosphatidyl-3-kinase
PC :	Pyruvate carboxylase
PDK1 :	Pyruvate dehydrogenase kinase 1
PEP :	Phosphoenolpyruvate
PEPCK :	Phosphoenolpyruvate carboxykinase
PFK :	Phosphofructokinase-1
PGC-1 $\alpha$ :	Peroxisome proliferator-activated receptor gamma co activator 1- $\alpha$
PIP2 :	Phosphatidylinositol 4,5-bisphosphate
PIP3 :	Phosphatidylinositol (3,4,5)-trisphosphate
PKA :	Protein kinase A
PKC :	Protein kinase C
PNPLA3 :	Patatin-like phospholipase domain-containing 3
PP2A :	Phosphoprotein phosphatase 2A
PPAR :	peroxisome proliferator-activated receptor
PPAR $\gamma$ :	Peroxisome proliferator-activated receptor $\gamma$
PPAR $\alpha$ :	Proliferator-activated receptor $\alpha$
PPRE :	PPAR response element
PRAS40 :	Proline-rich Akt substrate 40
PTEN :	Phosphatase and tensin homolog
PUFA :	Polyunsaturated fatty acids
PVN :	Periventricular hypothalamic nucleus

**R)**

R :	Hydrocarbon chains
Rev-ERB $\alpha$ :	Reverse erythroblastosis virus $\alpha$
ROR $\alpha$ :	Retinoic acid receptor-related orphan receptors $\alpha$
RXR :	Retinoid X receptor
RXR $\alpha$ :	Retinoid X receptor $\alpha$

**S)**

SCD1 :	Stearoyl-coa desaturase-1
SCOT :	Succinyl-coa-oxoacid transferase
Ser :	Serine
SFA :	Saturated fatty acids
SHC :	Src homologous and collagen protein
SIK2 :	Salt inducible kinase 2
SMRT :	Silencing mediator retinoid and thyroid hormone protein
SNP :	Single nucleotide polymorphism
SOS :	Son of sevenless
SPPARM $\alpha$	Selective PPAR $\alpha$ modulators
SRE :	Sterol response element
SREBP-1c :	Sterol regulatory element binding protein 1c
STAT :	Signal transducers and activators of transcription
STZ :	Streptozotocin

**T)**

T1D :	Type 1 diabetes
T2D :	Type 2 diabetes
TAG :	Triacylglycerols
TCA :	Tricarboxylic acid
TH :	Thyroid hormone
Thiolase :	Acetoacetyl-coa thiolase
Thr :	Threonine
TNF $\alpha$ :	Tumor necrosis factor $\alpha$
TSC2 :	Tuberous sclerosis complex 2
TZD :	Thiazolidinediones

**U,V,X,Z)**

UCP1 :	Uncoupling protein 1
VGLUT2 :	Vesicular glutamate transporter 2
VLDL :	Very low density lipoproteins
VMH :	Ventromedial
Xu5P :	Xylulose 5-phosphate
ZIP-like :	Leucine-zipper-like domain



## ***INTRODUCTION***



## ***Part 1: The role of the liver in conserving energy homeostasis***

### ***I-Introduction to the liver***

The liver, located in the abdominal cavity under the diaphragm, is the second largest organ in humans and has the ability to rapidly regenerate. It is the main deposit for glucose and energy storage and its primary function is to maintain normal plasma blood glucose concentrations, which are constantly fluctuating due to irregular feeding and fasting periods, 4 grams of glucose per liter is considered normal for healthy human adults (Wasserman, 2009). The liver has several other important functions such as removing toxins from the blood and producing bile acids necessary for the breakdown of fatty acids in the gut.

The predominant cell type found in the liver is the hepatocyte that makes up 80% of the total cell population (Taub, 2004). Other cell types that are less abundantly present but important for the anchoring of blood-borne immune cells are Kupffer and sinusoidal endothelial cells (Wohlleber and Knolle, 2016). Lymphocytes present in the liver are important for oval cell-mediated hepatic regeneration (Strick-Marchand et al., 2008). Because of its important role for blood cleaning and for the regulation of energy metabolism, the liver is highly oxygenated by the hepatic artery and the portal vein allowing the liver to pump between 800 and 1200 ml/min (Eipel et al., 2010). The portal vein is important because it brings blood from the gut and pancreas carrying with it nutrients and hormonal signals that regulate hepatic function. Of particular importance for metabolism, are the glucose regulating hormones insulin and glucagon from the  $\alpha$ -cells of the endocrine pancreas.

Insulin stimulates the uptake of glucose into the hepatocytes. Indeed, the liver is able to store glucose as glycogen by the gluconeogenic pathway or as esterified fatty acids in the form of triacylglycerols (TAG). A proportion of TAG is stored in the liver itself while the majority is exported in the form of very low density lipoproteins (VLDL) out of the liver for storage in adipose tissue. During fasting, to maintain glucose concentrations in the blood, glucagon induces the liver to export glucose into the plasma, via gluconeogenesis. Ketone bodies, from lipid  $\beta$ -oxidation, are also important energy sources for the brain and retina (Ruderman et al., 1974). More recently the liver has been credited for the production of secretory proteins, coined hepatokines, which are able to circulate to target tissues propagating signaling pathways important to energy homeostasis (Figure 1).

## ***II-Metabolic pathways involved in glucose and lipid homeostasis control***

### ***A/The insulin hormone and its receptor***

Insulin is a small peptide hormone that is produced by  $\beta$ -cells located in the pancreatic islets of Langerhans. It is the hormone responsible for the uptake of glucose in peripheral tissues following a meal rich in carbohydrates.

The insulin receptor is a tyrosine kinase receptor with enzymatic activity. The receptor is made up of two extracellular  $\alpha$  subunits and two  $\beta$  subunits that contain intracellular tyrosine kinase domains. The four insulin receptor subunits are held together by disulfide bonds (Van Obberghen et al., 1983). Only one insulin molecule is able to bind to one of the two active sites located on the extracellular  $\alpha$  subunits. Ligand binding brings together the  $\beta$  subunits leading to receptor autophosphorylation and conformational change of the tyrosine kinase domain activation loop which when inactive blocks the adenosine triphosphate (ATP) attachment site

**Figure 1: Physiological regulation of hepatic glucose and lipid metabolism**

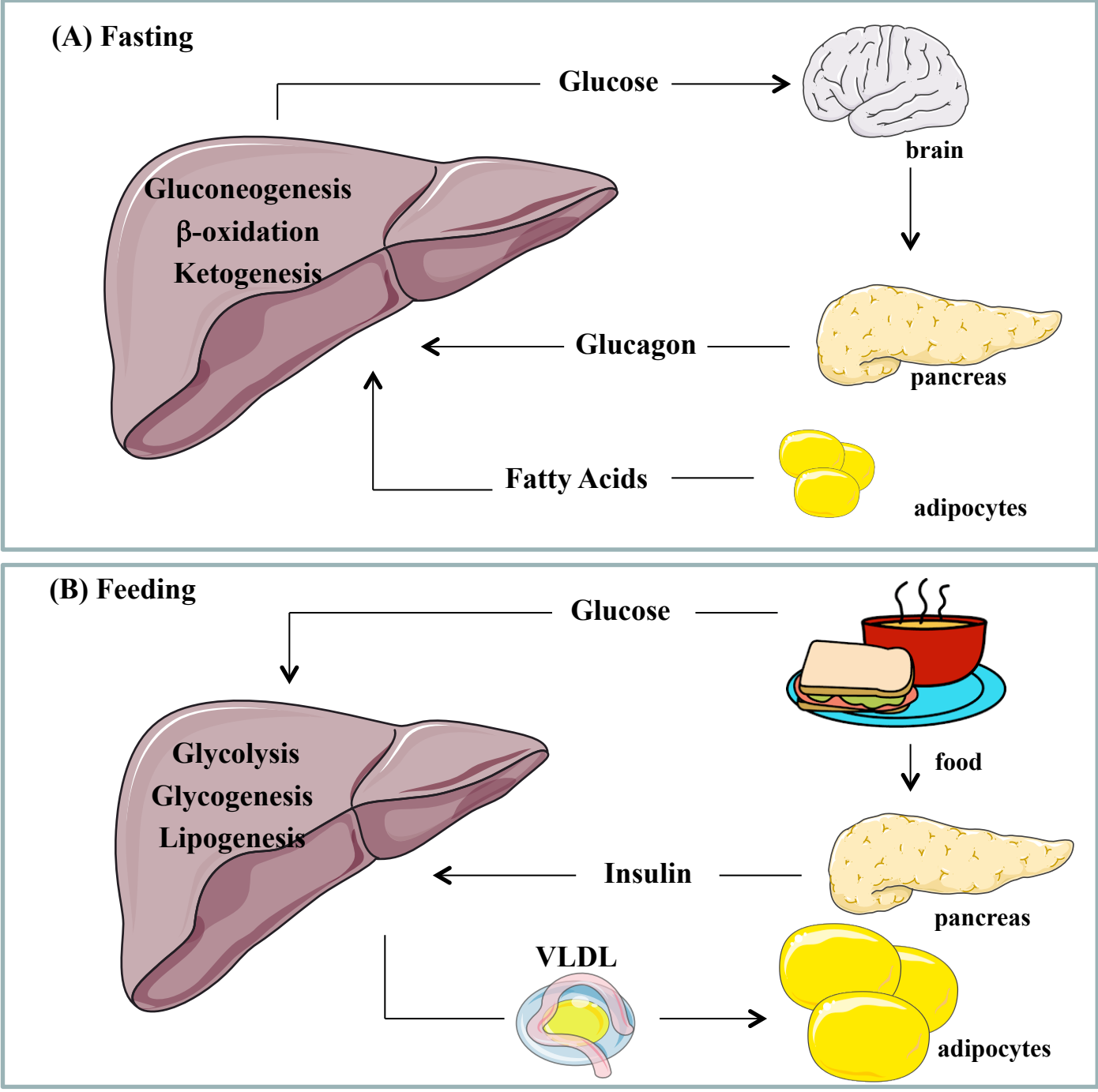


Figure 1: (A) During fasting secretion of glucagon from pancreatic  $\alpha$ -cells stimulates glycogenolysis and gluconeogenesis for the production of glucose primarily for the brain. Free fatty acids liberated from adipose tissue promote  $\beta$ -oxidation and ketogenesis of hepatic lipid stores to provide energy for peripheral tissue. (B) Following a meal glucose absorbed by the gut stimulates the release of insulin from pancreatic  $\beta$ -cells insulin and uptake of glucose by the liver. Glucose is converted into glycogen and lipids. Lipids packaged as very low density lipoproteins (VLDL) are shuttled to the adipose tissue for storage.

key to the receptors catalytic activity (De Meyts, 2000). Once active the receptor is quickly internalized and degraded by intracellular endosomes or recycled and returned to the plasma membrane in response to insulin (Goodner et al., 1988). In addition to cellular degradation, the insulin receptor and its mitogenic signaling pathway are negatively regulated by growth factor receptor bound protein 14 (Grb14) (Morzyglod et al., 2016).

#### A-1/The insulin receptor deletion models

Global knockout of the insulin receptor causes severe hyperglycemia and hyperinsulinemia leading to diabetic acidosis and death a few days after birth (Accili et al., 1996; Kitamura et al., 2003).

The liver insulin receptor knockout (LIRKO) mouse was developed to isolate the role of hepatic insulin resistance. LIRKO mice have highlighted the importance of hepatic insulin signaling to maintain normal glucose concentrations and regulate lipid homeostasis. Absence of the hepatic insulin receptor caused hyperglycemia despite hyperinsulinemia at 2 months of age and older mice (6 months old) showed markers of hepatic dysfunction as marked by increased levels of aspartate aminotransferase (AST) (Michael et al., 2000). The effect of hepatic insulin resistance was not limited to impaired glucose homeostasis. LIRKO mice displayed hypercholesterolemia and developed severe atherosclerosis following a 12 week diet rich in cholesterol while wild type mice did not display severe atherosclerosis (Biddinger et al., 2008). These experiments linked hepatic insulin resistance to atherosclerosis, a major precursor of heart attack and stroke.

## A-2/The insulin receptor substrate IRS1/2

Conformational change of the tyrosine kinase domain activation loop on the insulin receptor allows for the tyrosine-phosphorylation of adaptor proteins including, insulin receptor substrate (IRS) and Src homologous and collagen protein (Shc) (Figure. 2). Six isoforms of insulin receptor substrate family are documented but, in the liver, IRS-1 and IRS-2 are responsible for the transduction of the insulin signaling during feeding. The IRS-1/2 protein binds directly to the insulin receptor pocket liberated by the activation loop, via its phosphorylation binding site domain located on the N-terminus. Binding triggers phosphorylation of the IRS-1/2 protein and propagates its downstream effects via the phosphatidylinositol-3-kinases (PI3K) signaling cascade which regulates genes important for glucose metabolism (Shepherd et al., 1998; Taniguchi et al., 2005). The two adaptor proteins have both complementary and distinct roles. Knockdown of either IRS-1 or IRS-2 proteins inhibits activation of downstream target gene causing insulin resistance and glucose intolerance in both cases and  $\beta$ -cell failure in the case of IRS-2 deletion (Kubota et al., 2000; Valverde et al., 2003). IRS-1 knockdown increases hepatic gluconeogenesis while IRS-2 knockdown increases genes of liponeogenesis (Taniguchi et al., 2005).

The Shc protein binds in a similar fashion as IRS-1 and IRS-2 to the insulin receptor during refeeding in chicken livers (Dupont et al., 1998). Its activation enhances the mitogen-activated protein kinase (MAPK) signaling pathway important for growth factors as exogenous Shc stimulated the MAPK pathway but not the PI3K pathway (Giorgetti et al., 1994) (Figure 2). Because of its role in cardiovascular development Shc is leads to embryonic mortality (Mlih et al., 2015)

**Figure 2:**

**The major insulin signaling pathways**

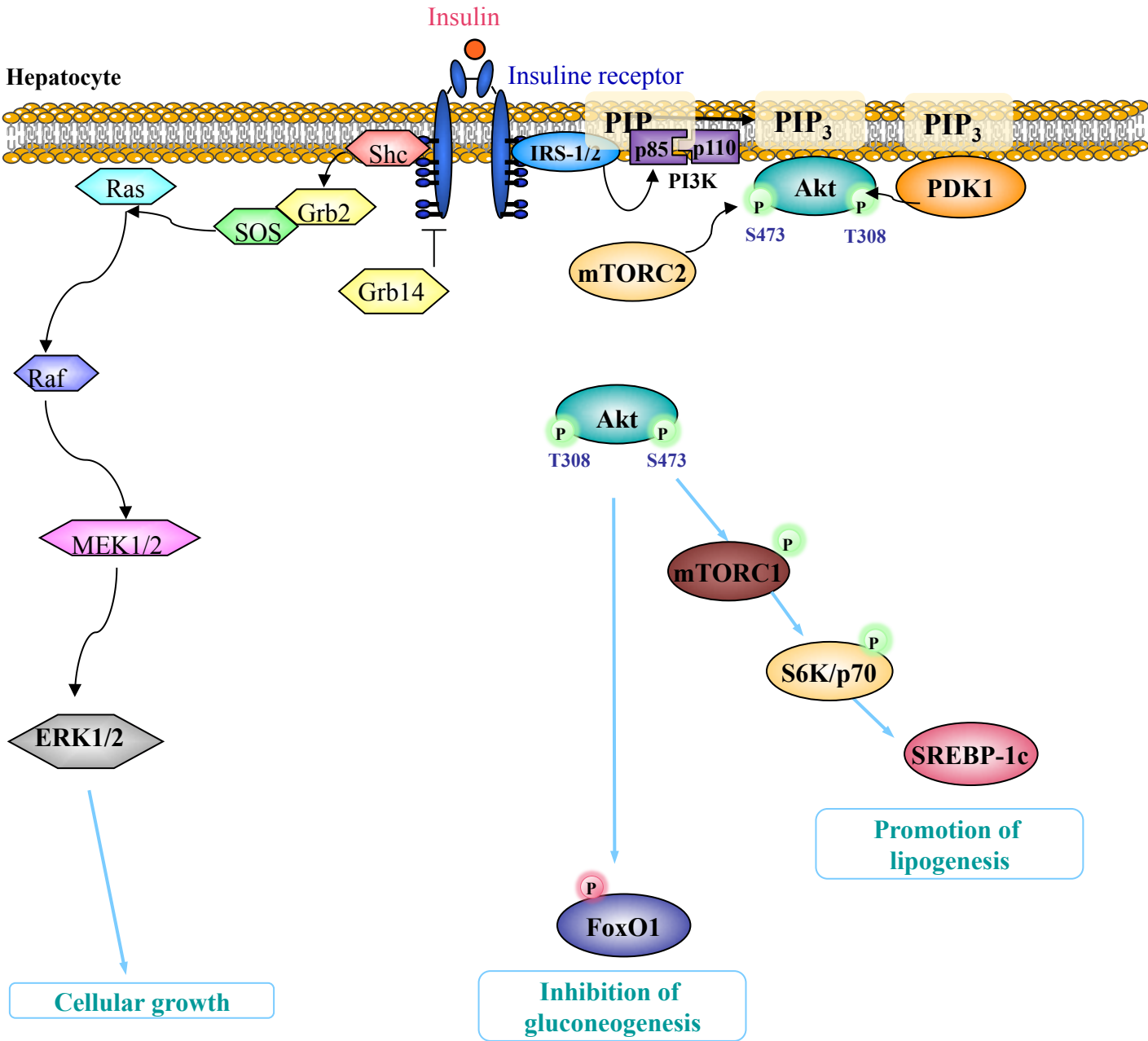


Figure 2: Insulin binding to the its receptor activates auto phosphorylation of the tyrosine subunits and recruitment of scaffolding proteins which propagates the insulin signaling pathway leading to activation of ERK and Akt. Downstream of these pathways insulin signaling is able to induce cell growth, inhibit gluconeogenesis and promote lipogenesis. Gab14 block the insulin receptor catalytic activity and prevent activation of Akt.



## ***B/The major insulin signaling pathways***

Insulin receptor activation propagates the signaling cascade of several different pathways involved in cell growth and survival namely, MAPK and PI3K (Figure 2).

### **B-1/The MAP-kinase Pathway**

The activated tyrosine domain of the insulin receptor initiates a sequential string of phosphorylation events in order to effect cellular metabolism. The first step is the binding of the tyrosine domain activation loop by the growth factor receptor-bound protein 2 (Grb2) and its associated kinase son of sevenless (SOS). In the next step, SOS converts a guanosine diphosphate (GDP), attached to a membrane bound protein called RAS, to guanosine triphosphate (GTP) (Gehart et al., 2010). Activated GTP bound RAS then activates another membrane bound protein B-Raf. Next MEK1/2 binds to the c-terminal catalytic domain of B-Raf. MEK1/2 is phosphorylated on both the threonine and tyrosine residues converting it to the MAP-kinase extracellular signal-regulated kinase (ERK) 1/2 (Boulton et al., 1991; Crews et al., 1992). Finally, phosphorylation of ERK1/2 causes a conformational change and promotes dimerization of ERK1/2 leading to its translocation from the cytoplasm to the nucleus where it activates transcription factors initiating transcription of target genes to control insulin-mediated metabolism. ERK1 is required in the early steps of adipogenesis (Bost et al., 2005). ERK1 knockout mice are protected from diet-induced obesity and insulin resistance (Jager et al., 2011). However these results are conflicting because, liver-specific activation of ERK1/2 has been shown to reverse insulin-deficiency in type 2 diabetes (T2D) by increasing  $\beta$ -cell mass via a central nervous system (CNS) neuronal-mediated pathway. This study supports evidence that the liver is able to regulate glucose homeostasis through efferent vagal signals (Imai et al., 2008).

The MAP-kinase pathway is also able to stimulate two other classes of MAP-kinases, the p38 MAP-kinase and c-Jun N-terminal kinases (Moxham et al., 1996). The p38 MAP-kinases is stimulated by insulin in adipocytes and skeletal muscle and is important for the production of pro-inflammatory cytokines (Antonescu et al., 2005). JNK activates cell death pathways and plays a critical role in T2D as JNK activation inhibits transduction of the insulin receptor while JNK deletion protects against diet induced insulin resistance (Hirosumi et al., 2002; Vernia et al., 2014).

## B-2/ The PI3K pathway

The PI3K pathway is essential to cell survival and growth through its activation of two major kinases (1) protein kinase B (Akt), responsible for insulin-stimulated glucose uptake and storage and (2) the mechanistic target of rapamycin (mTOR), which promotes the synthesis of fatty acids and proliferation. The signaling cascade begins with the phosphorylation of the insulin receptor and binding of IRS-1/2. IRS-1/2 activates P13K by provoking a conformational change in its p85/p110 complex promoting its catalytic activity (Taniguchi et al., 2006). This induces its translocation and phosphorylating activity. P13K phosphorylates a 6-carbon ring called inositol hanging from a phosphate group head in the plasma membrane. The inositol has two more phosphate groups on its fourth and fifth carbon and the entire complex is referred to as phosphatidylinositol 4,5-bisphosphate (PIP2) (Rameh et al., 1997). P13K catalyzes the addition of a phosphate group to the third carbon on the inositol ring, converting PIP2 to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). The conversion of PIP2 to PIP3 is reversible during periods of low nutrient availability by activation of the phosphatase and tensin homolog (PTEN). During insulin signaling PIP3 bound to the cell membrane is plentiful allowing for the binding of different enzymes important for the propagation of the PI3K pathway. One of those enzymes is pyruvate dehydrogenase kinase 1 (PDK1), which is activated by PIP3 binding

(Walker et al., 1998). Another enzyme is the kinase Akt that also binds to a PIP3 however Akt binding to PIP3 does not allow for its activation (James et al., 1996). The activated kinase PDK1 is necessary for activating Akt by the addition of a phosphate group on the Akt residues threonine (thr) 308 and serine (ser) 473 (Alessi et al., 1996; Stephens et al., 1998).

### B-3/Downstream insulin targets mTOR and SREBP-1c

Once activated, Akt detaches from the membrane bound PIP3 and targets key transcription factors promoting glycolysis, lipogenesis, and proliferation (Engelman et al., 2006). Activation of Akt is also important for activation of mTOR, which is situated in the center of a protein complex (mTORC1). Akt regulates mTOR by the addition of a phosphate group to the mTORC1 suppressor protein proline-rich Akt substrate 40 (PRAS40) and also by phosphorylating the GTPase, tuberous sclerosis complex 2 (TSC2) (Zhou and Huang, 2010). Inhibition of TSC2 allows for the accumulation of Rheb-GTP rather than Rheb-GDP further stimulating mTORC1 activity (Maiese, 2016; Tee et al., 2002).

Following intake of a high carbohydrate or fructose diet, insulin via the Akt pathway stimulates the transcription of sterol regulatory element binding protein (SREBP-1c) and induces its proteolytic cleavage to its mature and active form (Fleischmann and Iynedjian, 2000). SREBP-1c is an important transcription factor for the synthesis of fatty acids, TAG, and phospholipids (Ferré and Foufelle, 2007; Foretz et al., 1999). Once active, the transcription binds to the regulatory binding element (SRE) inducing glycolytic and lipogenic genes (Dentin et al., 2004; Foretz et al., 1999). It has been recently demonstrated that treatment with dexamethasone, a synthetic glucocorticoid, decreases SREBP-1c activity in livers of refed C57BL/6J or fed *ob/ob* mice, by activating fasting induced insulin induced gene 2, a negative

regulator of SREBP-1c maturation. This also leads to a significant decrease in hepatic TAG accumulation (Roqueta-Rivera et al., 2016).

### ***C/The regulation of glucose use and production in the liver***

Following a meal, glucose absorbed by the gut enters the blood stream and is taken up by hepatocytes via the glucose transporter Glut-2. Inside the cytosol, glucose undergoes glycogenesis for glycogen storage or glycolysis for the harvest of energy rich pyruvate. These steps will not be detailed in this introduction.

#### **C-1/Glycogen synthesis and breakdown**

During periods of feeding the body is able to store glucose for energy in the form of glycogen, a polysaccharide. This allows for short-term energy storage and glycogen is the first energy substrate used when glucose in the blood is in low supply. Glycogen is made by glycosidic linking of the glucose ring at the  $\alpha$ -1,4 carbon or  $\alpha$ -1,6 allowing glycogen to be highly branched. This allows glucose to be quickly broken off of glycogen at several ends. There are several steps in glycogen synthesis involving glucose activation, linkage and glycogen branching. These steps will not be detailed in this introduction.

## C-2/Glycolysis

Glycolysis is the process of converting glucose to ATP for cellular energy. The process takes place in the cytoplasm of most cell types and includes three major phases: a preparatory phase, where ATP is used, a rate-limiting phase and a harvest phase, which generates ATP, implementing 10 enzymes. The net gain of the reaction is 2 ATPs + 2 nicotinamide adenine dinucleotide (NAD)H + 2 pyruvate from one glucose molecule (Figure 3).

- The preparatory phase

Once passed the cellular membrane the preparatory phase begins and glucose must be converted to glucose-6-phosphate (G6P). The kinase responsible for G6P synthesis in liver is glucokinase (GK). GK is regulated at both the transcriptional and substrate level. *Gk* mRNA is expressed in low quantities during fasting, however following a meal, insulin signaling induces the transcription of *gk* through the transcription factor SREBP-1c which binds the SRE binding region of the *gk* promoter (Foretz et al., 1999; Kim et al., 2004). In addition, GK is regulated by substrate concentration level. When glucose concentrations are low, GK present in the cell is bound to the glucokinase regulatory protein (GKRP) (Van Schaftingen et al., 1994). GKRP sequesters GK in the nucleus rendering it inactive. Glucose present in the cell competes for GK binding and dissociates GK from GKRP allowing it to translocate from the nucleus to the cytoplasm (Brown et al., 1997). Once in the cytoplasm, GK uses one ATP to transfer a phosphate to glucose resulting in one G6P and one ADP. G6P is then converted to fructose 6-phosphate (F6P) by the phosphoglucose isomerase enzyme.

# Figure 3: Hepatic glycolysis

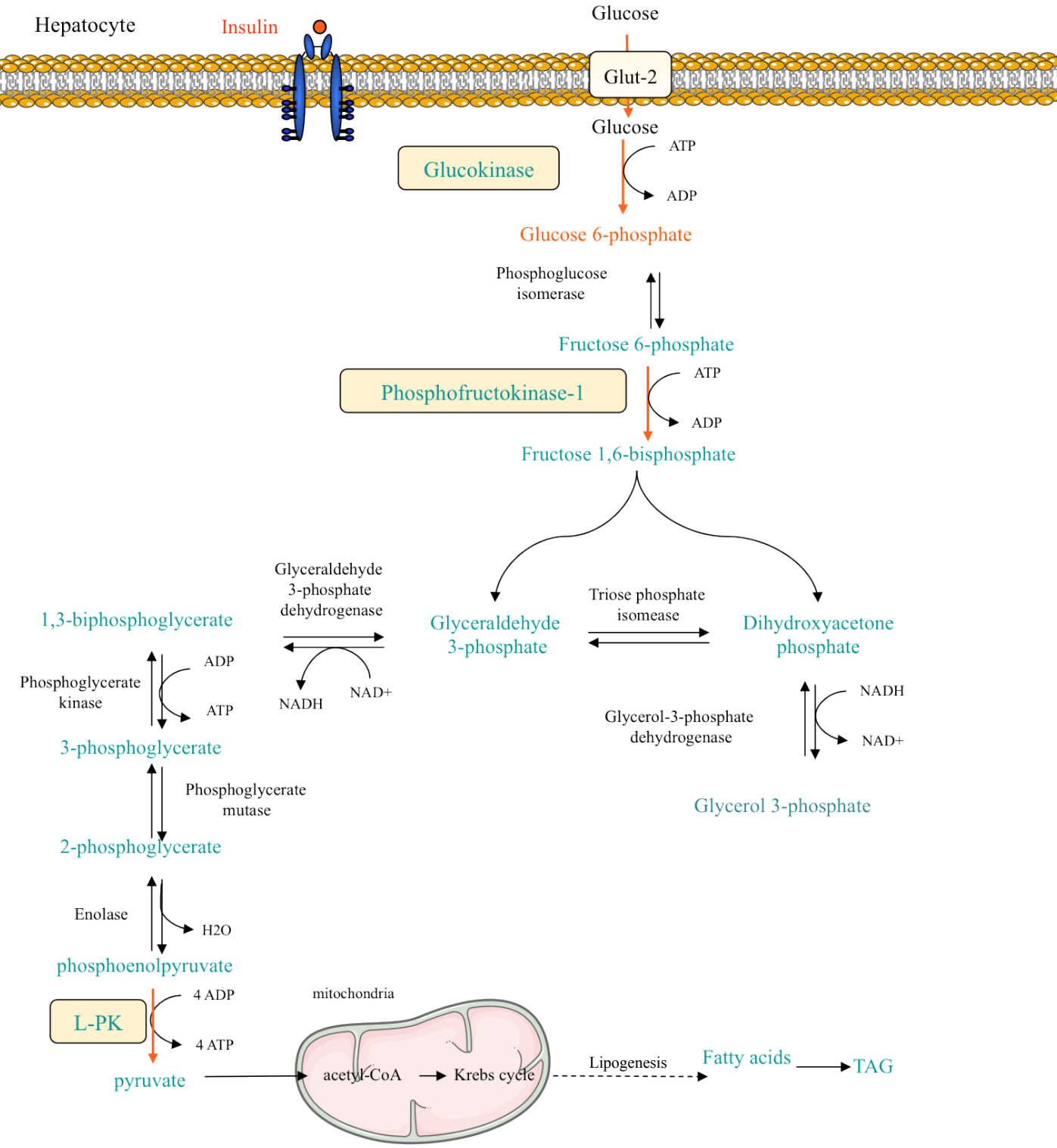


Figure 3: Glycolysis is the process of converting glucose to ATP for cellular energy. The process includes three major phases for the breakdown of 6-carbon glucose to 3-carbon pyruvate followed by the production of acetyl-CoA. Acetyl-CoA is then able to enter the Krebs cycle for the production of ATP and NADH. Excess acetyl-CoA can also enter lipogenesis for the production of fatty acids and TAG.

- The rate limiting phase

The next major rate-limiting step is the conversion of F6P to fructose-1,6-bisphosphate by phosphofructokinase-1 (PFK). Initial control of PFK begins with activation via transcription factors followed by refeeding of mice and is inhibited by the activation of the cyclic AMP (cAMP) pathway (Rongnoparut et al., 1991). Once translated, both an active site and an allosteric binding site regulate PFK activity allowing the same enzyme to promote glycolysis and gluconeogenesis. The active site has a higher ATP affinity allowing it to be active and promote glycolysis when energy substrates are low. The allosteric site has low affinity and only binds ATP when it is abundant promoting gluconeogenesis. In glycolysis this step consumes one ATP and is especially important because build-up of the PFK product, fructose 1,6-bisphosphate, positively regulates the last major kinase in the pay-off phase, liver-pyruvate kinase (L-PK) (Pilkis and Claus, 1991).

- The pay-off phase

L-PK is regulated both by covalent modification of phosphates and by allosteric regulation of ATP binding (El-Maghrabi et al., 2001). During fasting, L-PK is phosphorylated by the protein kinase A (PKA), rendering it inactive. When energy availability is high, L-PK is dephosphorylated by the phosphoprotein phosphatase 2A (PP2A) (Assimacopoulos-Jeannet and Jeanrenaud, 1990) rendering it active for the conversion of phosphoenolpyruvate (PEP) to pyruvate (Noguchi et al., 1985; Pilkis et al., 1988). These final steps in the conversion of PEP to pyruvate provide 4 ATP. The end product pyruvate is then transported into the mitochondria by mitochondrial pyruvate carrier 1 where it is converted to acetyl-CoA and enter the Krebs cycle for the generation of additional ATP (Bricker et al., 2012). L-PK, is up regulated by a high glucose

diet (Vaulont et al., 1986) which stimulates transcription factors essential to *L-pk* expression. During a glucose influx, the glucose sensitive transcription factor carbohydrate responsive element binding protein (ChREBP) binds to the carbohydrate response element (ChoRE) region of the *L-pk* promoter and activates its transcription (Kawaguchi et al., 2001). ChREBP creates a binding complex with hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) on the *L-pk* promoter (Diaz Guerra et al., 1993). While HNF4 $\alpha$  over expression increases L-PK, ChREBP null mice have a marked reduction in *L-pk* mRNA indicating that ChREBP is its primary transcriptional regulator (Ishii et al., 2004). The orphan receptor chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), negatively regulated by glucose and insulin blocks *L-pk* transcription by competing with the ChREBP binding region on the *L-pk* promoter (Diaz Guerra et al., 1993; Perilhou et al., 2008). A later section of this introduction will be dedicated to ChREBP.

### C-3/Gluconeogenesis

Not all tissues in the body are able to produce their own energy during fasting from the breakdown of amino acids and glycerol. Some tissue types rely solely on the availability of plasma glucose and depend on the liver to supply glucose during intermittent periods of feeding and fasting. This pathway is set in motion by increasing acetyl-CoA and citrate concentrations that build up during prolonged fasting once glycogen stores have been depleted. The process is similar to the reversal of glycolysis, but it begins with pyruvate and replaces three enzymes of glycolysis with four different enzymes specific to gluconeogenesis. In addition, the step from pyruvate to PEP is completed in a two-step process that is necessary to over-power the highly potentialized energy of L-PK towards pyruvate (Figure 4).



- Pyruvate conversion to phosphoenolpyruvate

Pyruvate must first be converted into PEP but, as mentioned above, because of the high-energy potential of the reaction towards pyruvate, the cell must use a two-step process implementing two gluconeogenic specific enzymes as well and the addition of an intermediate substrate. This first process takes place in the mitochondria and requires the enzyme pyruvate carboxylase (PC) for the conversion of pyruvate to oxaloacetate and uses one NADH. The second step in the process is converting oxaloacetate to PEP by the enzyme phosphoenolpyruvate carboxykinase (PEPCK) (Owen et al., 2002). The next seven steps of gluconeogenesis are similar to glycolysis in the reverse order.

- Formation of glucose

The last step of gluconeogenesis takes place in the endoplasmic reticulum (ER) by the enzyme glucose-6-phosphatase (G6Pase). This enzyme cleaves a phosphate from glucose allowing it then to be transported out of the cell and into circulation increasing plasmatic blood glucose levels.

### ***D/Fatty acids use and production in the liver***

Dietary fatty acids are an important source of energy, one 18 carbon fatty acid generates 180 ATPs while 3 glucose molecules, also containing a total of 18 carbons, provide only 96 ATPs (biochemistryquestions, 2008).

**Figure 4:**

# Hepatic gluconeogenesis

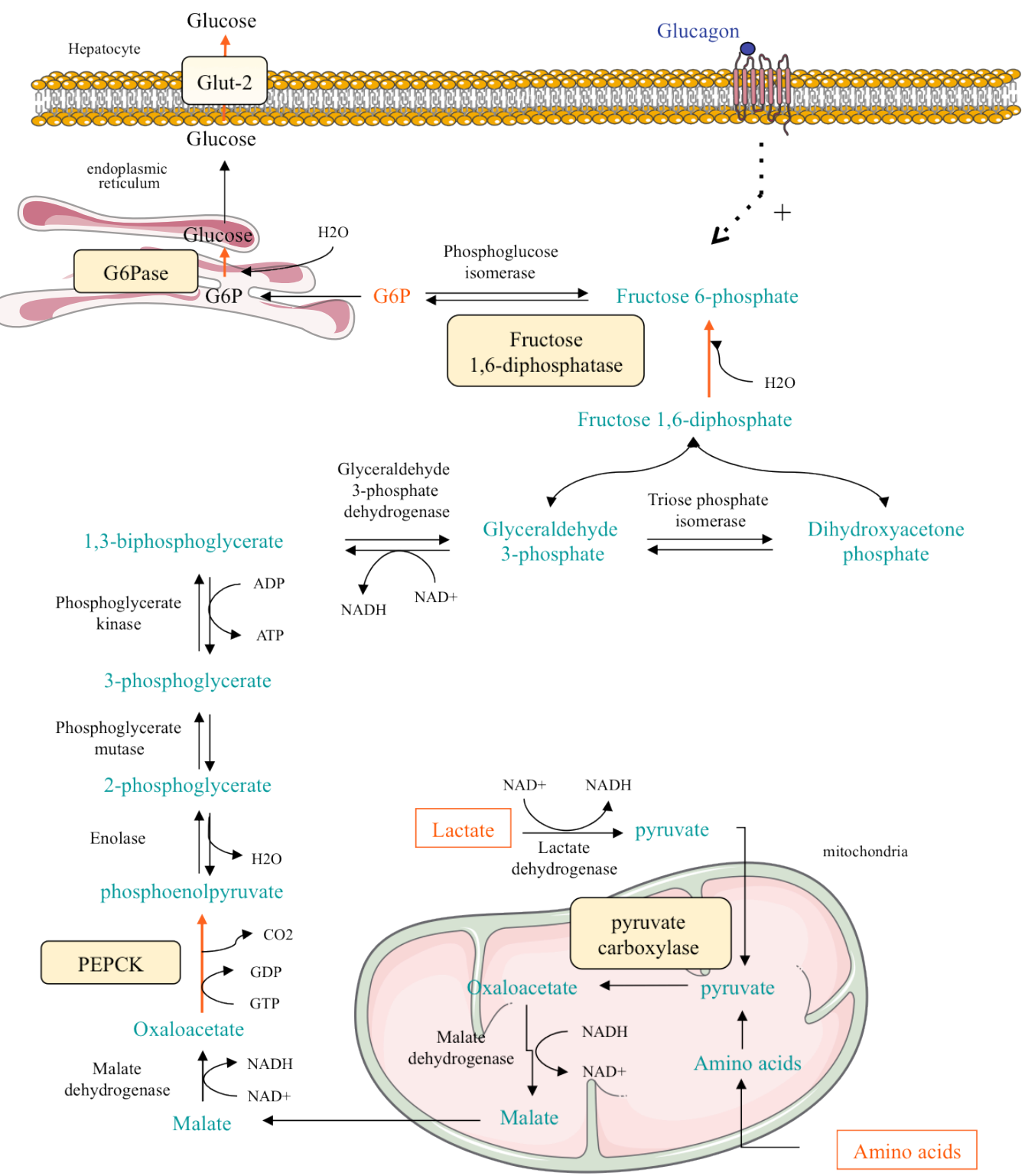


Figure 4: Gluconeogenesis by the liver is an essential source of energy for the brain and other peripheral tissues during fasting. The pathway is set in motion by glucagon released from the pancreatic  $\alpha$ -cells and also by increasing acetyl-CoA and citrate concentrations within the hepatocyte. Gluconeogenesis is regulated by the availability of rate limiting enzymes PEPCK for the second step in the conversion of pyruvate to phosphoenolpyruvate and the phosphate cleaving enzyme G6Pase, allowing glucose to be transported out of the hepatocyte and into the blood stream.

## D-1/ Fatty acid structure

Lipids are hydrophobic, water insoluble macromolecules. Lipids serve multiple functions such as energy storage, building blocks for cellular membranes and hormones used for signaling (Rustan and Drevon, 2001). Free fatty acids (FFA) are hydrocarbon chains (R) with carboxylic acid groups ( $-\text{COOH}$ ) that are used for energy because of their rich electron source. The R group of any given FFA refers to the hydrocarbon chain, which can be from 4 to 36 carbons long. The carbon located closest to the carboxylic acid group is considered carbon number one. FFA can be referred to as short ( $<8$ ) medium (8-16) or long ( $>16$ ) chain FFA. Their hydrocarbon chain is usually even (Figure 5).

There are two major classes of fatty acids, saturated fatty acids (SFA) (no carbon-carbon double bonds) and unsaturated fatty acid (at least one carbon-carbon double bond). Unsaturated FFA are classified by, whether their double bonds are in a cis or trans configuration, and also by the number and location of the double bonds. The cis double bond has an  $\text{H}^+$  on the same side causing the hydrocarbon chain to kink while trans double bonds have an  $\text{H}^+$  on opposite sides of the bond resulting in a straight hydrocarbon chain.

Unsaturated fatty acids containing only one double bond are called monounsaturated fatty acids (MUFA). Any unsaturated fatty acids with two or more double bonds are called polyunsaturated fatty acids (PUFA). In addition, the kinking of the cis configuration also interferes with the stacking ability of the FFA, lowering their melting point, and making them liquid at room temperature. MUFA with a single double bond, in the trans configuration, are able to stack and are solid at room temperature, with a higher melting point, they are harder to be broken apart for energy.

Figure 5:

## Structure and nomenclature of fatty acids

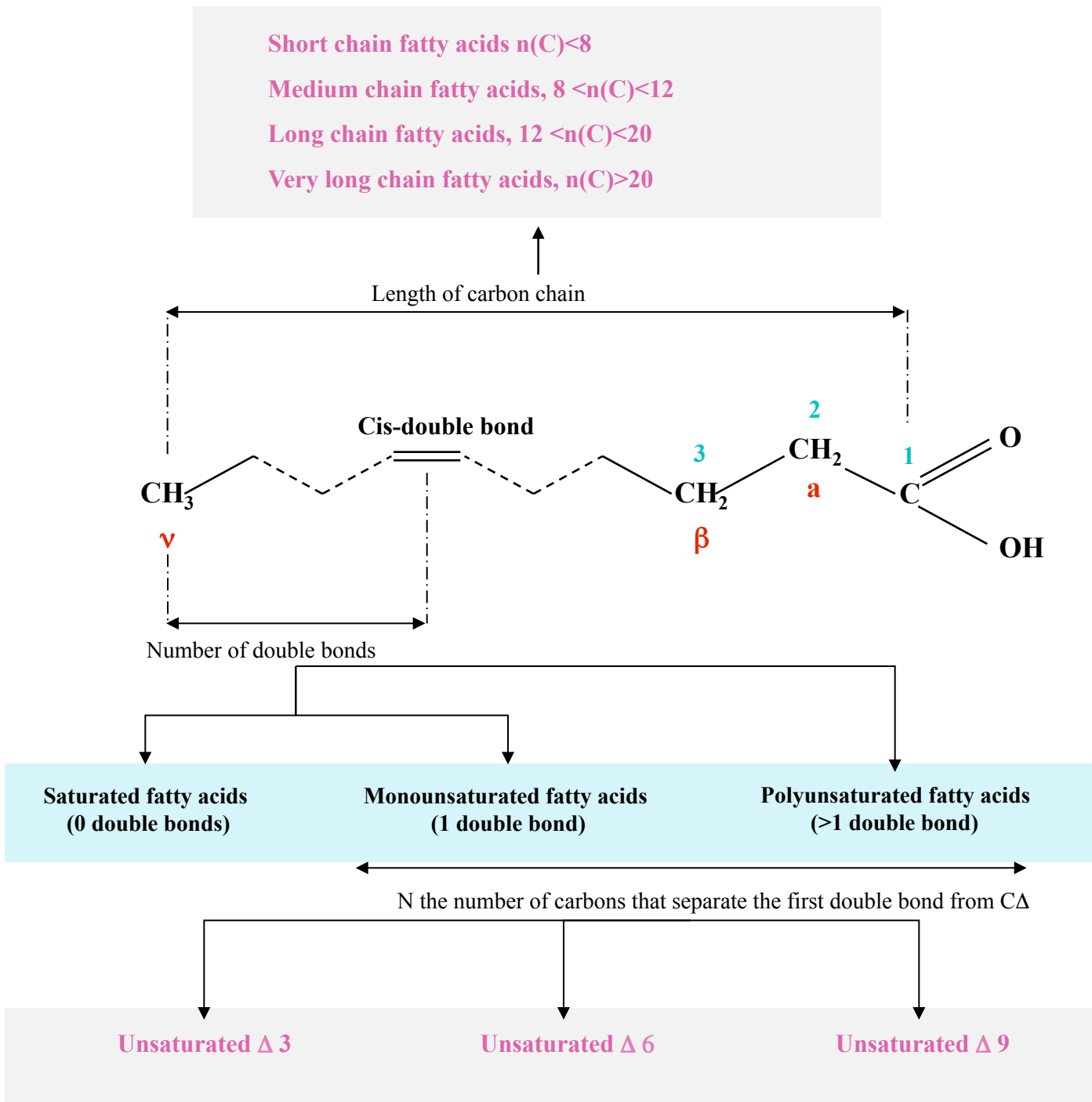


Figure 5: The fatty acids are aliphatic hydrocarbon chains having a carboxylic acid function at the carbon atom (C)  $n^{\circ}1$ . The C atoms which are located after the acid function are called a, b, ... and  $\omega$  for the carbon of the methyl terminal function. Four families of fatty acids are defined by the number of carbons, short chain, medium chain, long chain and very long chain fatty acids. Likewise, depending on the number of double bonds, they are further divided as saturated, monounsaturated and polyunsaturated fatty acids. Finally, the number of C atoms separating the CA from the first double bond makes it breaks the unsaturated fatty acids into 3 families with distinct functions,  $\Delta 3$   $\Delta 6$   $\Delta 9$  fatty acids.

Unsaturated fatty acids are also classified by the location of their first double bond located on the 3, 6 or 9<sup>th</sup> carbon closest to the terminal end carbon (omega carbon) of the fatty acid chain i.e. furthest from the carboxylic group. This allows for the classification of fatty acid families as omega 3, 6, or 9.

## D-2/ Fatty acid transport

Fatty acids are transported in the form of TAG, a 3-carbon glycerol backbone with three FFA tails. The molecules are bound together by ester bonds between the glycerol's oxygen and the fatty acid acyl groups. Because of their hydrophobic nature TAG groups together to form fatty droplets within the lumen of the small intestine. Bile secreted from the liver works as a detergent to emulsify the fatty droplets allowing access of lipase. Lipase cleaves the ester bond by adding a water, separating glycerol from the three fatty acids. The FFA's are then able to diffuse across the small intestine.

Once absorbed into the lumen FFA must be re-esterified back to TAG. They are packaged inside lipo-proteins called chylomicrons that have outer hydrophilic ring and inner TAG cores allowing TAG to enter into the large lacteals of the lymphatic system. Too large to enter the blood stream directly through capillary beds, chylomicrons use the lymphatic system to enter large blood vessels and then capillary beds. To become small enough to escape the capillaries and move into tissues, chylomicrons undergo delipidation by lipoprotein lipase (LPL), activated by insulin, to form chylomicron remnant. Like in the lumen of the intestine, LPL cleaves the ester group by hydrolysis of TAG liberating the glycerol and fatty acids. Almost all peripheral tissues except the brain and red blood cells can take up FFA for energy.

The liver is also an important site of FFA uptake and storage. Chylomicron remnants not used by peripheral tissues are taken up and packaged as TAG in VLDL. The liver is also able to take from the blood high-density lipoproteins (HDL) and low-density lipoproteins (LDL). Importantly, the liver is also able to synthesize its own fatty acids from glucose via *de novo* lipogenesis from the glycerol backbone.

### D-3/ Fatty acid synthesis from glucose : The lipogenic pathway

The role of hepatic lipogenesis is to convert excess glucose into fatty acids. Pyruvate generated through glycolysis enters the mitochondrial matrix to begin the tricarboxylic acid (TCA) cycle or be used to generate acetyl-CoA, the building blocks of fatty acids. In the mitochondrial matrix pyruvate first undergoes decarboxylation, by snipping of a carboxyl group, leaving a two-carbon molecule and releasing carbon dioxide, by the enzyme pyruvate decarboxylase. The two-carbon molecule is then oxidized converting NAD<sup>+</sup> to NADH. The two-carbon molecule is called an acetyl group. A CoA is then added with the help of ATP to produce acetyl-CoA (Figure 6).

### D-3-1/Acetyl-CoA transport

Synthesis of FFA takes place in the cellular cytosol requiring the transport of the generated acetyl CoAs from the mitochondrial matrix to the cytosol. The enzyme citrate synthase from the TCA cycle links two acetyl-CoA's, removing the CoA. Citrate is then free to move across the mitochondrial membrane into the cytosol. Once in the cytosol the enzyme ATP

citrate lyase cleaves 2 carbons off citrate generating a four-carbon molecule that is converted back to pyruvate generating a NADPH and a free acetyl-CoA. NADPH generated from acetyl-CoA transport and the pentose phosphate pathway will be the electron donor for the biosynthesis of lipids.

#### D-3-2/ Acetyl-CoA carboxylase

The first committed step in the building of fatty acids is carboxylating acetyl-CoA to a three-carbon malonyl-CoA (Brownsey et al., 2006). This step is catalyzed by acetyl-CoA carboxylase (ACC) and requires ATP. The CoA on both acetyl and malonyl must then be replaced by acyl carrier protein (ACP). Malonyl-CoA will be used as the two-carbon donor required for building the carbon chain of the fatty acid.

#### D-3-3/ The fatty acid synthase complex

Fatty acid synthase (FAS) is a large a 200 kDa (kilodalton) complex that consists of three enzymatic domains. The FAS complex dimerises and is able to synthesize up to 16-carbon long saturated fatty acids by transporting the fatty acid around its enzymatic domains, adding two carbons for each rotation of up to 7 rotations (Jensen-Urstad and Semenkovich, 2012). Each rotation requires 2 NADPH in addition to one acetyl-ACP and one carbon donating malonyl-ACP. The acetyl-malonyl attaches to a specialized domain of the FAS complex called the ACP. The ACP works as the transporter moving the fatty acid along to each domain of the FAS complex (Colli et al., 1969; Wakil, 1989).

Figure 6:

# The lipogenic pathway

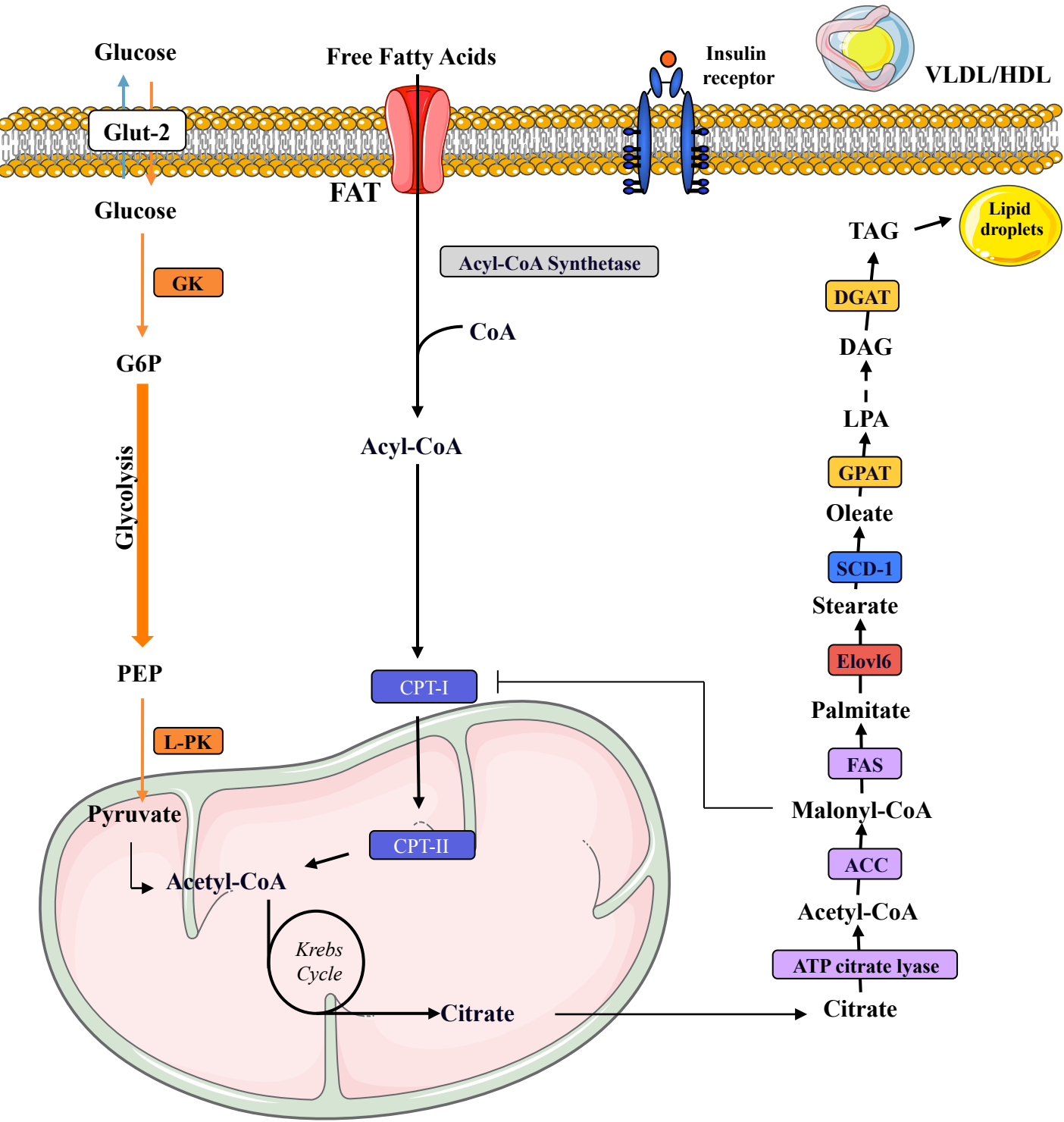


Figure 6: Lipogenesis provides for storage of excess acetyl-CoA generated from glycolysis and provides important signaling molecules for cell structure and survival. Insulin and glucose signaling lead to the upregulation of lipogenic enzymes for the elongation and desaturation of fatty acids. Build up of malonyl-CoA negatively regulates the lipogenic pathway by blocking the CPT-1 enzyme which transports acetyl-CoA into the mitochondria and Krebs cycle. FFA generated from lipogenesis are stored in lipid droplets as TAG or exported into the blood stream in lipoproteins including VLDL or HDL.



The first domain contains three enzymes and is responsible for condensing malonyl-ACP and acetyl-ACP and releasing carbon dioxide. The second domain includes four enzymes involved in the first reduction, the dehydration and second reduction step of fatty acid synthesis. The last domain contains a cleaving enzyme, thioesterase, which breaks the ester bond between the newly formed fatty acid such as palmitate, a 16-carbon saturated fatty acid, and the ACP group (Chakravarty et al., 2004). Experiments using liver specific FAS knockout mice suggested that FAS regulates lipid metabolism by generating endogenous activators of peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) pools in adult liver (Chakravarthy et al., 2005). Hepatic FAS knockout mice develop mild hepatic steatosis consistent with suppressed PPAR $\alpha$  activity. Indeed, a follow up paper by the same group identified the FAS dependent expression of an endogenous PPAR $\alpha$  ligand, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (16:0/18:1-GPC). Infusing the portal vein of C57BL/6 mice with 16:0/18:1-GPC increased hepatic PPAR $\alpha$  activity and expression of its target genes (Chakravarthy et al., 2009). The expression of FAS is nutritionally regulated and is induced in response to the increase of both insulin and glucose after a meal (Latasa et al., 2000). FAS expression is inhibited by glucagon when nutritional substrates are low (Wilson et al., 1986).

## ***E/Elongation and desaturation of fatty acids***

### **E-1/ Fatty acid elongation**

Elongation of fatty acids takes place mostly in the smooth ER and requires that the fatty acid be attached to a phospholipid. Like in fatty acid synthesis, malonyl-CoA is used as a carbon donor adding an acetyl group to the CoA end of the fatty acid. The process is similar to the FAS complex with exceptions to the enzymes used. To synthesize fatty acids longer than the 16

carbons added by the FAS complex a different family of enzymes, called the elongation of long-chain fatty acids (Elovl) is activated. There are 7 isoforms of Elovl. The isoforms 5 (Elovl5) and 6 (Elovl6) are expressed in the liver and have important roles in human health. Elovl5 elongates 20 carbon PUFA to 22 or 24 carbon PUFA and is regulated by the insulin sensitive transcription factor SREBP-1c. These long chain PUFA are reported to induce PPAR $\alpha$  activity (Bordoni et al., 2006) and inhibit the phosphorylation of Akt on ser473, increasing its activity and improving insulin sensitivity (Wang et al., 2008). Elovl6 is synergistically regulated by SREBP-1c and ChREBP (Bae et al., 2016). Elovl6 is a microsomal enzyme able to lengthen MUFAs. It is essential for the synthesis of oleic acid (18:1 *cis* 9) important for its role in reducing inflammatory cytokines including tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (Vassiliou et al., 2009). Its role in hepatic health and insulin sensitivity, however, is paradoxical as global deletion of Elovl6 protects mice from diet induced insulin resistance (Matsuzaka and Shimano, 2009). In contrary, deletion of Elovl6, in *ob/ob* mice, did not alter the development of obesity, fatty liver, hyperglycemia, or hyperinsulinemia (Moon et al., 2014). The LDL receptor recognizes lipoprotein in the blood and communicates peripheral fatty acid needs to the liver. LDL receptor deficient (LDLr<sup>-/-</sup>) mice are insensitive to circulating lipoprotein concentrations and therefore over compensate by exporting VLDL into the blood stream causing inflammation and atherosclerosis. In a separate study, LDLr<sup>-/-</sup> mice were crossed with Elovl6 knockout mice (Elovl6<sup>-/-</sup>Ldlr<sup>-/-</sup>). The double knockout Elovl6<sup>-/-</sup>Ldlr<sup>-/-</sup> displayed reduced hepatic inflammation, oxidative stress and liver injury in comparison to Elovl6<sup>+/+</sup>LDLr<sup>-/-</sup> mice (Kuba et al., 2015).

## E-2/ Fatty acid desaturation

Desaturated fatty acids include carbon double bonds. A saturated fatty acid is referred to as saturated because the carbons are bound to maximal number of hydrogen's. The double

bonds reduce the number of hydrogen's that can be bond to the fatty acid and are thus considered unsaturated. Unsaturated fatty acids containing omega-3 and omega-6 double bonds come strictly from the diet and are important hormone and signaling molecules (Zivkovic et al., 2011). Other unsaturated fatty acids can be synthesized by the body by a family of enzymes called fatty acyl-CoA desaturase, which removes hydrogens to generate carbon-9 double bonds. The enzyme stearoyl-CoA desaturase-1 (SCD1) catalyzes the biosynthesis of oleic acid (C18:1 cis-9) (Ntambi, 1992). The other three desaturases require the essential fatty acid linoleic acid to synthesis PUFA, like arachidonic acid (20:4  $\omega$ -6), through successive actions of elongation and desaturation. SCD1 has undergone significant research to chart its role in human health. Early research using SCD1 knockout mice reported that disruption of *Scd1* reduced body adiposity, increased insulin sensitivity, and reduced diet-induced weight gain in mice (Miyazaki et al., 2009; Ntambi et al., 2002). However, the production of MUFA may also be a defense mechanism allowing the liver to safely store excess energy as SCD1 deficient mice, insensitive to circulating TAG concentrations, show increased inflammation and atherosclerosis (Gillingham et al., 2011; MacDonald et al., 2009). Furthermore, SCD1 reduces inflammatory saturated fatty acid stearoyl (C18:0) and palmitoyl-CoA (C16:0) by converting them to MUFA's oleic (C18:1 cis-9) and palmitoleic acids (C16:1 cis-9) (Ntambi, 1999). The production of oleic acid by SCD1 has been shown to be essential in protecting the liver against dietary lipid-induced injury (Liu et al., 2016a). It has been reported that activation of SCD1 activity dissociates hepatic steatosis from insulin resistance by increasing the ratio of MUFA to saturated fatty acids (Benhamed et al., 2012; MacDonald et al., 2009).

## E-3/TAG formation and storage

The glycerolipid biosynthetic or Kennedy pathway, named for the work of Eugene Kennedy and his co-workers in the 1950's, is responsible for *de novo* synthesis of TAG and phospholipids for efficient storage of energy. TAG is formed by ester binding of a glycerol-3-phosphate (G3P) and three fatty acids through dehydration. The fatty acids can be saturated or unsaturated and do not need to be the same FFA species. TAG is hydrophobic and anhydrous, allowing the body to store them as a more condensed and lighter form of energy. TAG biosynthesis is broken up into four steps but will not be covered by this introduction. Following biosynthesis TAG is stored in lipid droplets and then packaged for secretion into VLDL. The VLDL is transported through the blood stream to be taken up by adipocytes. Over production of VLDL can cause build up in artery walls leading to a major health concern, atherosclerosis (Talayero and Sacks, 2011).

## ***F/Fatty acid oxidation***

### F-1/Fatty acid $\beta$ -oxidation

$\beta$ -oxidation of short and medium chain fatty acids takes place within the mitochondrial matrix. Long chain fatty acids  $\beta$ -oxidation starts in cellular organelles called peroxisomes. Once long chain fatty acids are reduced to medium chain fatty acids they are transported to the mitochondria to complete  $\beta$ -oxidation. The breakdown of fatty acid is a four step process, (1) *first oxidation*, (2) *hydration*, (3) *second oxidation* and (4) *cleavage* (Figure 7).

In the cellular cytosol fatty acids are activated by the addition of a CoA to the fatty acid acyl group. However the acyl-CoA cannot cross the mitochondrial membrane where  $\beta$ -oxidation takes place. So it uses carnitine and the carnitine acyl transferase I (CPT-I) enzyme to take off the CoA group, converting the acyl-CoA to acyl carnitine. Acyl carnitine enters the mitochondrial via translocase embedded in the outer mitochondrial membrane (Bressler and Katz, 1965). Once in the mitochondrial matrix the acyl carnitine is reconverted back to acyl CoA by removing the carnitine and adding a CoA group, again by carnitine acyl transferase (CPT-II). Once in the mitochondrial the fatty acid undergoes the following steps. *Step 1 (First oxidation)* :  $\beta$ -oxidation first begins with the oxidation of the second carbon, also know as the  $\beta$  carbon, resulting in a double trans bond between the second and third carbon. A double bond must be present in the trans configuration for a fatty acid to undergo  $\beta$ -oxidation. This oxidation step is catalyzed by the enzyme acyl-CoA dehydrogenase and requires the electron acceptor flavin adenine dinucleotide (FAD), which is reduced to FADH<sub>2</sub> (Ghisla et al., 1984; Wang et al., 2014). *Step 2 (Hydration)*: Water is used to add a hydroxyl group to carbon two by the enzyme enoyl-CoA hydratase generating a single bond between carbons two and three. *Step 3 (Second oxidation)*: the hydroxyl group on carbon two is oxidized by  $\beta$ -hydroxyacyl-CoA dehydrogenase using the electron acceptor NAD<sup>+</sup> reducing it to NADH (Gillingham et al., 1999). *Step 4 (Cleavage)*: the number one and two carbons are cleaved by acetoacetyl-CoA thiolase (thiolase) generating an acetyl-CoA that is used by the TCA cycle for the generation of ATP and a fatty acid that is 2 carbons shorter. The fatty acid re-enters the  $\beta$ -oxidation pathway, cleaving off two carbons at a time, always between the second and third carbon. In the rare case of an odd number carbon, the fatty acid undergoes normal  $\beta$ -oxidation until a 3-carbon structure called propionyl-CoA is left. Addition of a carbon and movement of a methyl group allows for the metabolism of propionyl-CoA to succinyl-CoA. Like acetyl-CoA, succinyl-CoA can enter into the TCA cycle. Section adapted from [Biochemistry Free for All](#) by Ahern and Rajagopal 2016.

Figure 7:

Fatty Acid β-Oxidation

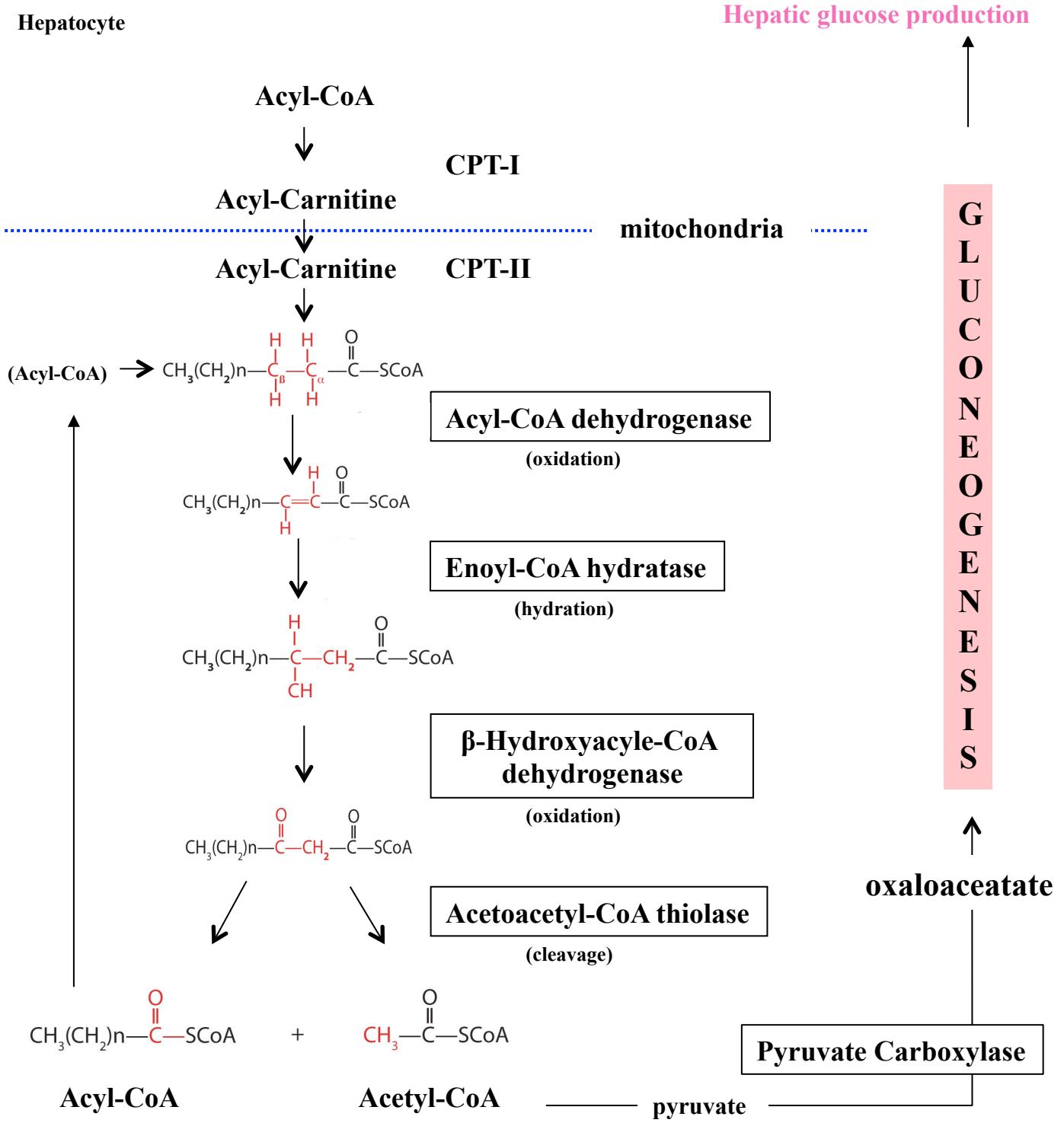


Figure 7: β-oxidation is the process of harvesting acetyl-CoA from stored lipid for gluconeogenesis. β-oxidation of short and medium chain fatty acids takes place within the mitochondrial matrix. Long chain fatty acids β-oxidation begins in cellular organelles called peroxisomes. Once long chain fatty acids are reduced to medium chain fatty acids they are transported to the mitochondria to complete β-oxidation. The breakdown of fatty acid is a four step process, (1) first oxidation, (2) hydration, (3) second oxidation and (4) cleavage

## F-2/Ketone Bodies

Ketone bodies provide a valuable water-soluble energy source that can quickly enter the blood stream and cross the blood-brain barrier when glucose has been depleted by the body. Produced in the mitochondria of hepatocytes, ketone bodies are especially critical for the brain during starvation as they can provide the brain with nearly 65% of its required energy. Up-regulation of ketogenesis also improves clearance of excessive hepatic TAG. However, in the case of T2D, over production of ketone bodies can lead to a life threatening condition called ketoacidosis.

### F-2-1/ Ketone body synthesis

Ketone body production is a four-step enzymatic pathway that begins with the last enzyme of  $\beta$ -oxidation, thiolase. *Step 1*: Thiolase II, uses two 2-carbon acetyl-CoAs to build a 4-carbon acetoacetyl-CoA. *Step 2*: The rate limiting enzyme, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase condenses the 4-carbon acetoacetyl-CoA, from *step 1*, with an additional acetyl-CoA to get a 6-carbon molecule HMG-CoA. The pathway at this point can either use HMG-CoA to synthesize cholesterol in the cytosol or continue to *step 3* of mitochondrial ketogenesis. *Step 3*: The enzyme hydroxymethylglutaryl-CoA lyase converts HMG-CoA, from *step 2*, to acetoacetate + acetyl-CoA. *Step 4*: 3-hydroxybutyrate dehydrogenase then uses acetoacetate, from *step 3*, and an NADH to synthesize  $\beta$ -hydroxybutyrate + NAD<sup>+</sup>.  $\beta$ -hydroxybutyrate can then exit the mitochondria and enter into the blood stream to be used as an energy substrate for peripheral tissues. Additionally, acetoacetate, like  $\beta$ -hydroxybutyrate, from *step 3* can be transported to other tissues for energy use or be decarboxylated non-enzymatically to a chemically volatile molecule called acetone that is not used by the body (Figure 8).

## F-2-2/Ketone body oxidation

$\beta$ -hydroxybutyrate is able to cross the blood-brain barrier and provides other extrahepatic tissues with fuel in times of depleted glucose sources. The first step is the oxidation of  $\beta$ -hydroxybutyrate to acetoacetate by the reduction of NAD<sup>+</sup> to NADH. For the second step the cell then uses an intermediate of the TCA cycle, succinyl-CoA, to activate acetoacetate by adding a CoA group and uses the enzyme succinyl-CoA-oxoacid transferase (SCOT) to convert acetoacetate into acetoacetyl-CoA. This is the rate-limiting step in the oxidation process. SCOT gene expression is induced by high intracellular concentrations of acetoacetyl-CoA (Cotter et al., 2013). To prevent a futile cycle of ketone synthesis and breakdown the liver does not express the SCOT gene enabling its ability to breakdown ketone bodies and sparing them for peripheral tissue use. The last step is the cleavage step, which uses the enzyme methylacetoacetyl-CoA thiolase to cleave two acetyl-CoA from acetoacetyl-CoA (Søvik, 1993). The harvested acetyl-CoA's then enter the TCA cycle for the production of energy substrates NADH, FADH<sub>2</sub>, which donates electrons to the electron transport chain for the generation of ATP.

## F-2-3/Ketone bodies and disease

In a disease states such as Type 1 diabetes (T1D) or T2D, the absence or resistance of insulin activates the hormone sensitive lipase (HSL) driving lipolysis and ketogenesis resulting in diabetic ketoacidosis. Ketone bodies have an acidic nature and if not used by peripheral tissues build up in the blood lowering arterial blood pH < 7.0 causing a syndrome know as diabetic ketoacidosis. Acidosis can be life threatening and is characterized by loss of breath, muscle seizures and dizziness leading to loss of consciousness (Konstantinov et al., 2015). In addition to diabetes, mutation in the gene OXCT1 causes SCOT deficiencies consequently leading to ketoacidotic attack in newborns and young children (Abdelkreem et al., 2016). On the flip side,



Figure 8:

### Ketone body synthesis

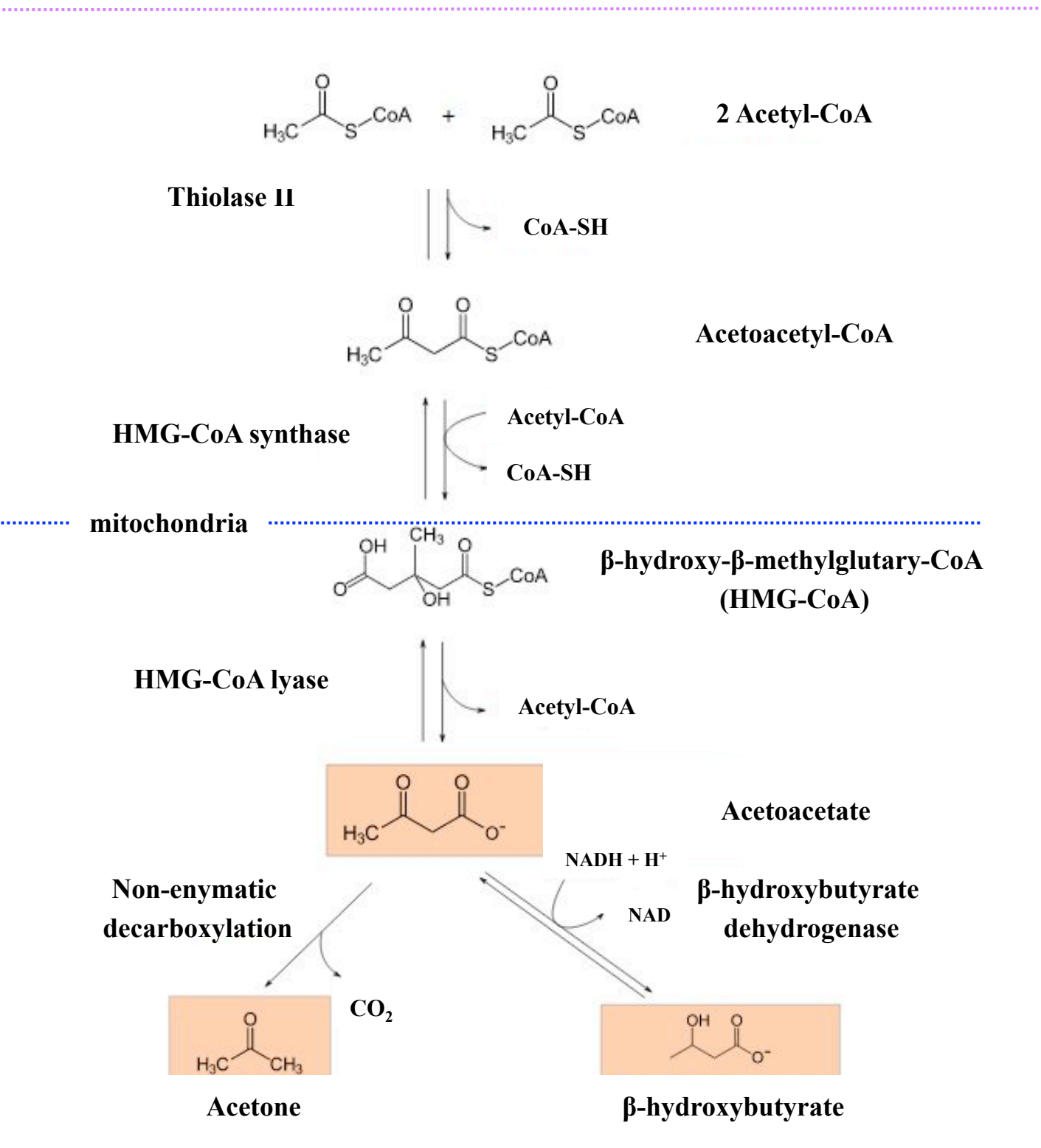


Figure 8: Under starvation, production of ketone bodies is turned on in hepatocyte mitochondria. In the ketogenesis process, 3 ketone derivatives of acetyl-CoA groups are made; acetoacetate, acetone, and  $\beta$ -hydroxybutyrate. Once produced the acetyl-CoA loaded ketones are sent to the brain for energy. Enzymes are displayed on the left (except for  $\beta$ -hydroxybutyrate dehydrogenase) and substrate names on the right.

ketogenic insufficiency, caused by obesity-induced hyperinsulinemia, has been reported to negatively impact hepatic glucose and lipid metabolism leading to fatty acid build up in the liver. Hypoketogenic mice lacking the rate-limiting ketogenic enzyme HMG-CoA synthase have a 7-fold increase in hepatic TAG concentration, increased hepatic inflammation and are hypoglycemic, highlighting the importance of ketogenesis for the clearance of hepatic TAG and for the maintenance of normal blood glucose concentrations (Cotter et al., 2014). The nuclear transcription factor PPAR $\alpha$  is critical for regulating HMG-CoA synthesis through direct promoter binding (Rodríguez et al., 1994). PPAR $\alpha$  knockout mice are hypoglycemic during fasting due to their inability to induce ketogenesis (Kersten et al., 1999).

As mentioned in the section “Fatty acid  $\beta$ -oxidation,” the CPT-I enzyme is essential for the transport of long-chain fatty acids into the mitochondria. CPT-I deficiency prevents long-chain fatty acid oxidation that causes hypoglycemia and is suggested to be a cause for sudden unexpected death in infancy (Fohner et al., 2017). CPT-II dysfunction is caused by polymorphisms in the F352C and V368I alleles and is more common in people of the north coast of Alaska, northeast Siberia and Japan (Yamamoto et al., 2014).

## **Conclusion of Part 1**

The liver plays a central role in regulating glucose and lipid metabolism. During feeding, insulin secreted from pancreatic  $\beta$ -cells stimulates the usage of glucose in liver and peripheral tissues. Glucose undergoes glycolysis to produce acetyl-CoA. Acetyl-CoA is used for the production of fatty acids through the lipogenic pathway. During fasting, glucagon secreted by the  $\alpha$ -cells of the endocrine pancreas stimulates mobilization of hepatic glycogen stores in the liver. Fatty acid  $\beta$ -oxidation provides acetyl-CoA and co-factors for the production of glucose

through gluconeogenesis and for the production of ketone bodies both important sources of peripheral tissue energy during fasting.

## ***Part 2: The pathology of type 2 diabetes and non-alcoholic fatty liver disease***

### ***I- The prevalence of type 2 diabetes, obesity and non-alcoholic fatty liver disease***

Nutrition is central to maintaining our health and most importantly, our quality of life. Western society has been inundated by the obesity and diabetic epidemic, which caused over 3.7 million deaths in 2012 and degrades the daily quality of life for over 422 million adults each day or about 8.5% of the population (WHO, 2016). In addition to reduced energy and mobility, uncontrolled hyperglycemia and obesity causes serious health risks including : heart attack, stroke, vision loss, foot amputation and kidney failure with many patients resorting to costly dialysis. The catastrophic expense of obesity and diabetes in wealthier countries is estimated at a GDP loss of 900 billion per year (WHO, 2011). This has led to a booming research focused on understanding how the body orchestrates whole body energy metabolism (Figure 9).

Obesity is associated with TAG accumulation in liver leading to hepatic steatosis onset. Without life style changes to limit obesity, hepatic steatosis progresses into non-alcoholic fatty liver disease (NAFLD) which encompasses severe syndromes. NAFLD estimated to be present in 20-40% of western population is highly associated with obesity, as 80% of NAFLD patients have an increased body mass index (BMI). In conjunction with inflammation hepatic steatosis can progress to non-alcoholic steatohepatitis (NASH). Of those individuals diagnosed with NASH, 5-25% of them will progress further to irreversible liver scarring called cirrhosis. Individuals with NASH in combination with cirrhosis are significantly more likely (12,7% over 3 years) to

develop hepatocellular carcinoma (HCC) (White et al., 2012). Genetic factors can also predispose NAFLD patients to the progression into cirrhosis such as single nucleotide polymorphism (SNP). Among them, a SNP in patatin-like phospholipase domain-containing 3 (PNPLA3) gene is associated with NAFLD (Romeo et al., 2008) as individuals carrying the PNPLA3 SNP allele are at a higher risk of developing liver cirrhosis (Krawczyk et al., 2011). A SNP in the gene coding for epidermal growth factor is also associated with an increased risk of HCC and may be a potential marker for liver disease (Shen et al., 2015).

### ***A/Mechanisms linking obesity to insulin resistance and type 2 diabetes***

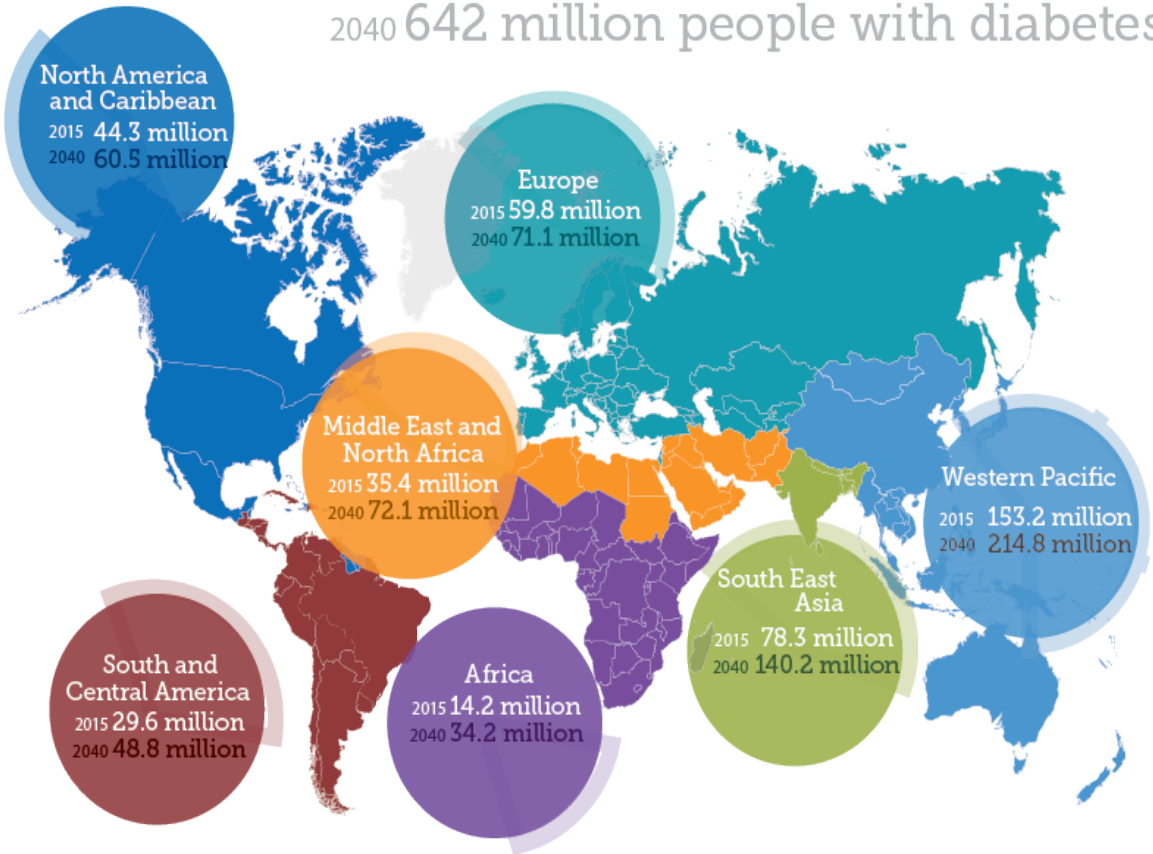
Obesity is commonly measured by a weight-for-height BMI. Individuals with a BMI over 25 are classified as overweight or obese (66.9% of the US population and 49.9% for France). Obesity developed through a chronic imbalance between increased energy intake and decreased energy expenditure (Figure 10). The obese phenotype is hallmarked by hyperglycemia, hyperinsulinemia and dyslipidemia. Increased output of insulin by pancreatic  $\beta$ -cells is initially able to maintain normal blood glucose concentration, however, over time the peripheral tissues response to insulin becomes impaired leading to the pre-diabetic condition of hyperinsulinemia and hyperglycemia. Insulin resistance is in part caused by inflammatory cytokines which block the insulin receptor's ability to signal downstream targets in skeletal muscle, adipocyte tissue and/or liver. In skeletal muscle, insulin resistance is associated with a decrease in PI3K activity (Rondinone et al., 1997) and in the adipose tissue with decreased IRS-1 expression both abnormalities leading to inhibition of glucose uptake (Kim et al., 1999). Diacylglycerols and SFA, sent to the liver by over-extended adipose tissue overloads the livers ability to store fatty acids safely, causing oxidative stress and mitochondrial malfunction and activation of cellular stress pathways such as the JNK pathway (Odegaard and Chawla, 2013). This leads to hepatic insulin resistance and uncouples insulin's affects on glucose and lipid metabolism as insulin signaling is

Figure 9:

# The Global Diabetes Epidemic

International Diabetes Federation, diabetes atlas - 7th edition 2015

Worldwide 2015 415 million people with diabetes  
2040 642 million people with diabetes



2015



One in 11 adults has diabetes

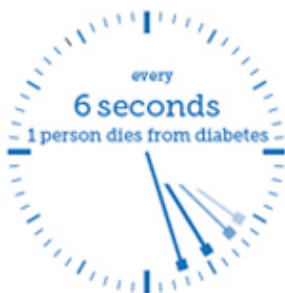
2040



One in 10 adults will have diabetes



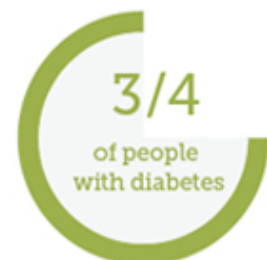
One in two adults with diabetes is undiagnosed



5.0 million deaths in 2015



12% of global health expenditure is spent on diabetes



no longer able to block gluconeogenesis, by suppression of PEPCK, however, insulin mediated-activation of mTORC1 remains intact, further stimulating lipogenesis (Li et al., 2010). This is known as selective insulin resistance. Indeed, It has been reported that the lipogenic transcription factor SREBP-1c can be activated by mTORC1 through an Akt independent pathway in obese rodents (Laplante and Sabatini, 2010), suggesting that in addition to Akt activation SREBP-1c activity is driven by a parallel and distinct pathway. However, later reports using transgenic mice with an active form of mTORC1 were unable to induce SREBP-1c independently of insulin (Yecies et al., 2011) (Figure 11). Another pathway that could explain sustained SREBP-1c activity in the context of insulin resistance is the ER stress pathway. Expression of a ER stress blocker in liver of *ob/ob* mice reduced the cleavage and the expression of SREBP-1c thereby reducing hepatic TAG concentrations and improving insulin sensitivity (Kammoun et al., 2009).

## ***B/Genetic models of obesity and type 2 diabetes***

One of the first breakthrough in understanding how the body controls energy intake came in 1969 when D. Coleman cleverly used parabiotic studies in genetically obese mice (*ob/ob*, *db/db*) to illustrate the role of a circulatory factor on satiety and suppression of food intake. To characterize the development of the *ob/ob* mutation in which mice display moderate glucose intolerance and islet hypertrophy as to the more severe phenotype of the *db/db* mutation characterized by a shortened life expectancy and islet atrophy. Pairing of the distinct phenotypes with wild type mice dissociated the cause of the mutation allowing for the distinction of a missing satiety hormone, in the case of the *ob/ob* model, who showed a significant food intake limiting effect on the *ob/ob* partner when paired with a wild type, and the complete loss of a functional satiety center in *db/db* which resisted the food intake limiting effect described in the *ob/ob* wild type pairing. Importantly, *db/db* wild type pairing induced severe





feeding apathy in the wild type leading to starvation and death (Coleman and Hummel, 1969). His work led to the identification of leptin.

### ***C/ Non Alcoholic fatty Liver Disease (NAFLD)***

Diagnosed as benign and reversible through simple life style changes such as weight loss, hepatic steatosis is caused by an accumulation of TAG in the liver (Cohen et al., 2011) (Figure 12). TAG arises from three distinct sources; dietary fat intake (15% of hepatic TAG), dysregulated lipolysis in adipose tissue (59% of hepatic TAG), and *de novo* lipogenesis (26% of hepatic TAG) (Donnelly et al., 2005). Over stimulation of *de novo* lipogenesis has been linked to atherosclerosis and the development of T2D (Lede et al., 2016).

NASH and its progressive stages are difficult to identify because patients display little symptoms. While plasmatic markers can give clues, liver biopsy is currently the accepted method of diagnosing stages of NASH. Following liver biopsy a NASH activity score is assigned to determine the stage of the disease; 0-2 no NASH; 3-4 pre-NASH; 5-8 NASH. Thiazolidinediones (TZD), known agonists of Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), are currently the most commonly used treatment to reduce the progression of NASH. However, a novel dual PPAR $\alpha$  and PPAR $\beta/\delta$  agonist was also recently describe as efficient for treatment of NASH (Oseini and Sanyal, 2017).

Figure 11:

## Selective hepatic insulin resistance

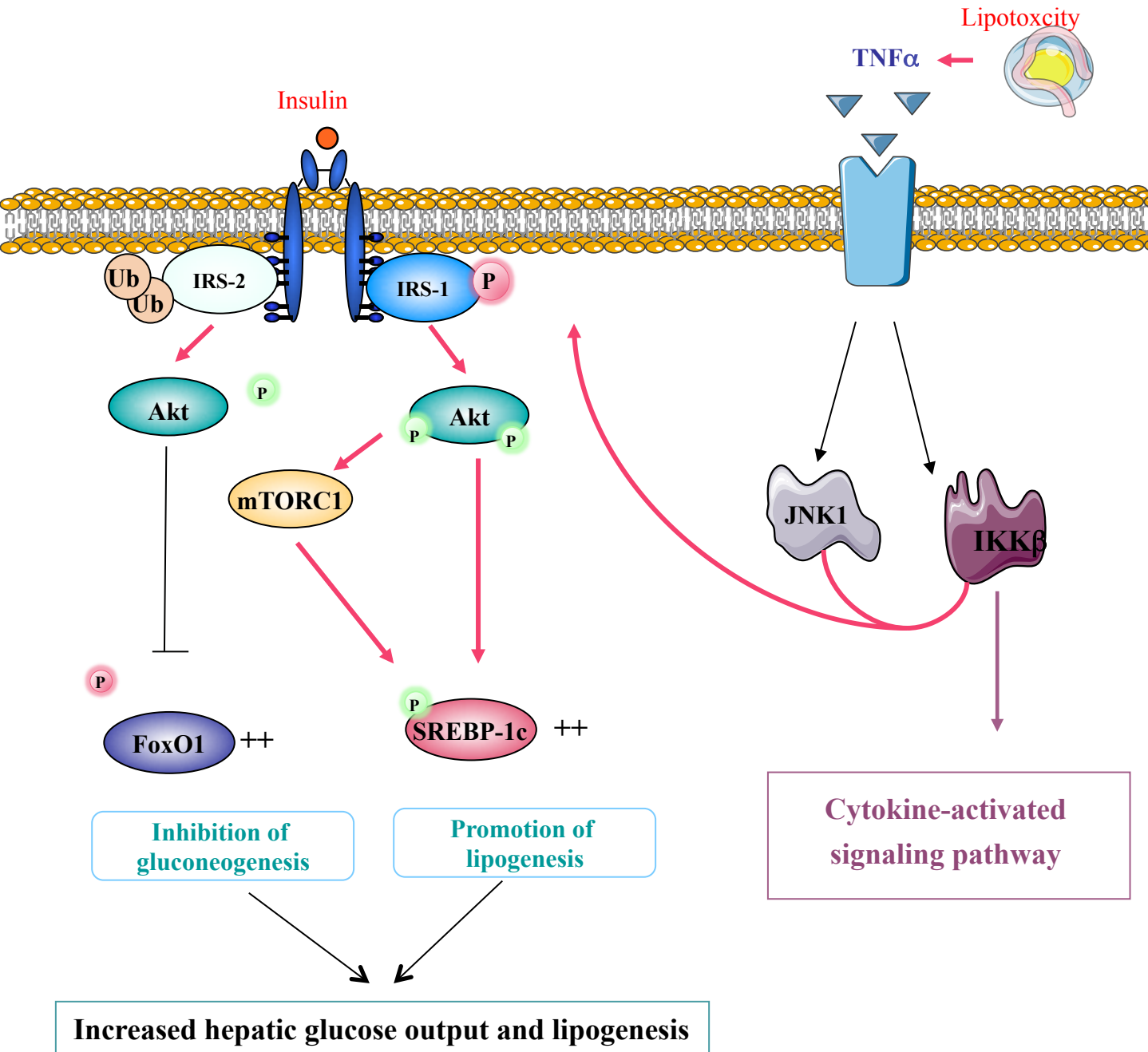


Figure 11: Inflammation and activated cytokines can induce insulin resistance, especially by acting on IRS-1 and 2. Inhibition of IRS-2 blocks Akt from suppressing Foxo1 resulting in up regulation of gluconeogenic genes. However a combination ER stress and mTORC1 activity induces SREBP-1c promoting the lipogenic program.

## ***D/The “two hit” hypothesis, progressing from simple steatosis to NASH***

A “two hit” hypothesis was described to explain the progression of hepatic steatosis to NASH (Day, 2002). *The first hit*: Hepatic accumulation of TAG in the liver. *The second hit*: Already under metabolic strain the liver develops irreversible hepatic damage in the form of lesions (Figure 13).

These lesions are caused by oxidative stress as a result of increased peroxisomal oxidation. Hepatic lipid flooding causes mitochondrial dysfunction and activation of the unfolded protein response further inhibiting insulin action, leading to hepatocyte apoptosis. Decreased mitochondrial function leads to increased reactive oxygen species (ROS) that impair cell function and initiate cell death signaling pathways such as JNK (Rocha et al., 2016). Inflammation is caused by a build up of hepatic FFA, which activate toll-like receptor and production of other cytokines, including, TNF $\alpha$ , interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6) (Shi et al., 2006). Activation of cell death signaling pathway leads to additional hepatic necrosis and scarring. The liver then secretes plasmatic markers of stress including alanine transaminase (ALT) and AST. Left untreated hepatic injury induces fibrosis progressing from NASH to hepatic cirrhosis (Tsochatzis et al., 2014).

However, the “two hit” hypothesis has been put under questions as research has led to a better understanding of the importance of lipid species and storage in insulin resistance and NASH. For example, hepatic over expression of the transcription factor ChREBP induces hepatic steatosis, however, mice do not develop inflammation or insulin resistance (Benhamed et al., 2012b). Suppression of histone deacetylase 3 (HDAC3) has also been associated with the remodeling of glucose and lipid metabolism. Liver specific HDAC3 deletion causes hepatic

**Figure 12:**

**The histological progression of NAFLD**

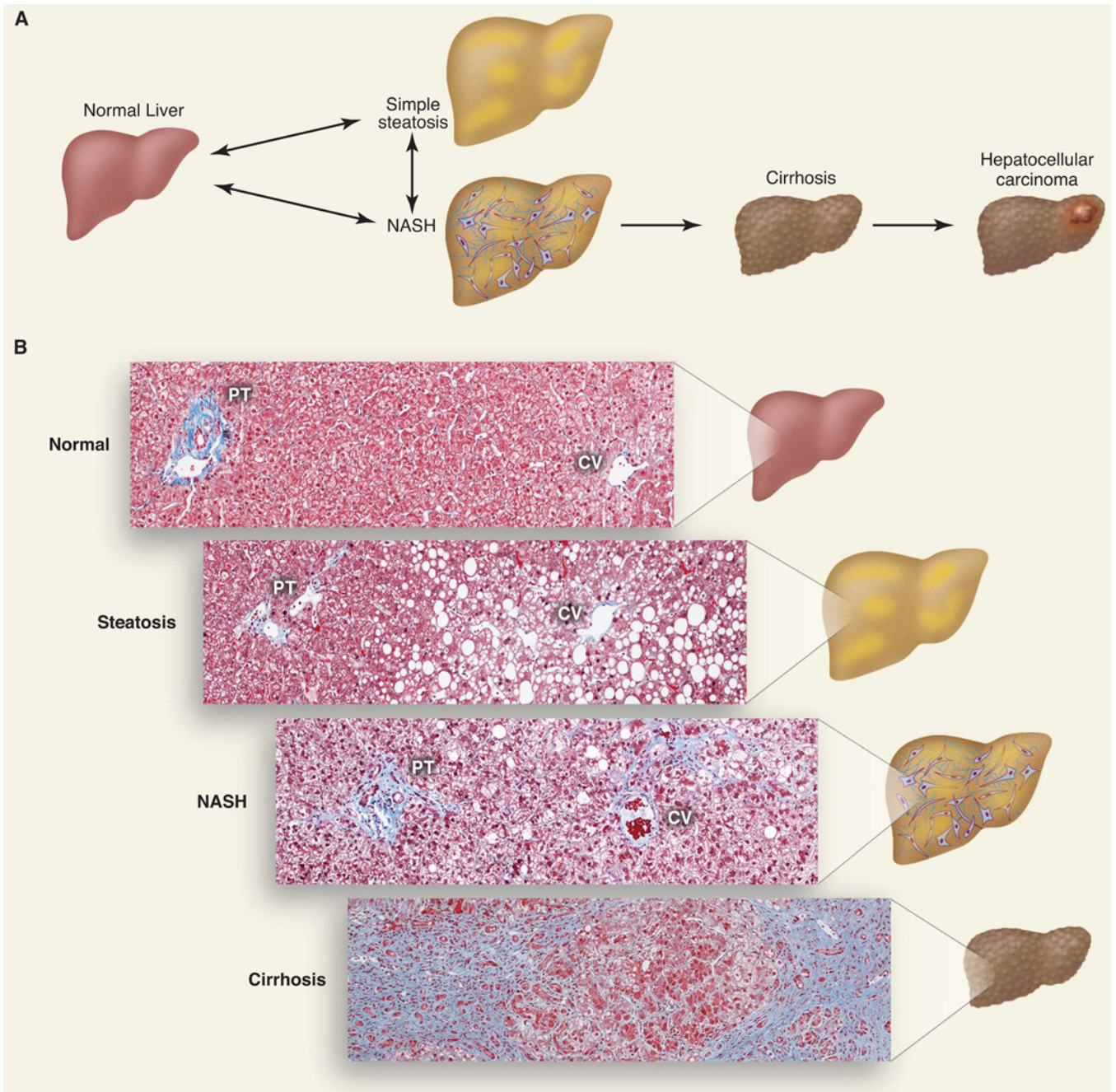


Figure 12: The disease spectrum of NAFLD. (A) Schematic of progression of NAFLD. The accumulation of TAG within lipid droplets in hepatocytes causes steatosis. Steatosis associated with inflammation, cell death, and fibrosis is referred to as NASH, which can progress to cirrhosis. Individuals with cirrhosis have an increased risk of hepatocellular carcinoma. (B) Histological sections illustrating normal liver, steatosis, NASH, and cirrhosis. Collagen fibers are stained blue with Masson's trichrome stain. The portal triad (PT), which consists of the hepatic artery, portal vein, and bile duct, and the central vein (CV) are shown. Adapted from Cohen et al. 2011 Science

steatosis but these mice remain insulin sensitive and were better able to store lipids (Sun et al., 2012). HDAC3 inhibition also increases the expression of anti-stress proteins such as fibroblast growth factor 21 (FGF21) (Archer et al., 2012; Rando et al., 2016), which improves mitochondrial function reducing oxidative stress and attenuates hepatic fibrosis (Gómez-Sámano et al., 2016; Xu et al., 2016).

## ***II-The role of hepatokines in energy metabolism***

Hepatokines have emerged as interesting pharmaceutical targets for the regulation of inflammation, dyslipidemia and may act as potential insulin sensitizers (Figure 14). The notion of a hepatokine that could improve  $\beta$ -cell proliferation was based on the findings of a parabiosis study in which LIRKO mice were paired with lean controls (El Ouaamari et al., 2013). Control partners of LIRKO mice showed increased plasma insulin concentrations associated with  $\beta$ -cell proliferation. Importantly, serum from LIRKO mice increased  $\beta$ -cell proliferation in cultured human islets, indicating that the secretome of an insulin-resistant liver is able to induce physiological changes in  $\beta$ -cell function/mass (El Ouaamari et al., 2013). However, despite extensive analysis of known circulating factors, a potential candidate was not identified by the paper.

### ***A/ Fetuin-A***

The first liver-derived factor suggested to regulate metabolic balance through integrated organ cross-talk was fetuin-A, a 64 kDa glycoprotein encoded by the Ahsg gene. Circulating levels of fetuin-A are increased in obesity and related disorders such as the metabolic syndrome and T2D. Fetuin-A concentrations are tightly correlated with impaired insulin sensitivity and

**Figure 13: The development of hepatic steatosis and the “two hit” hypothesis**

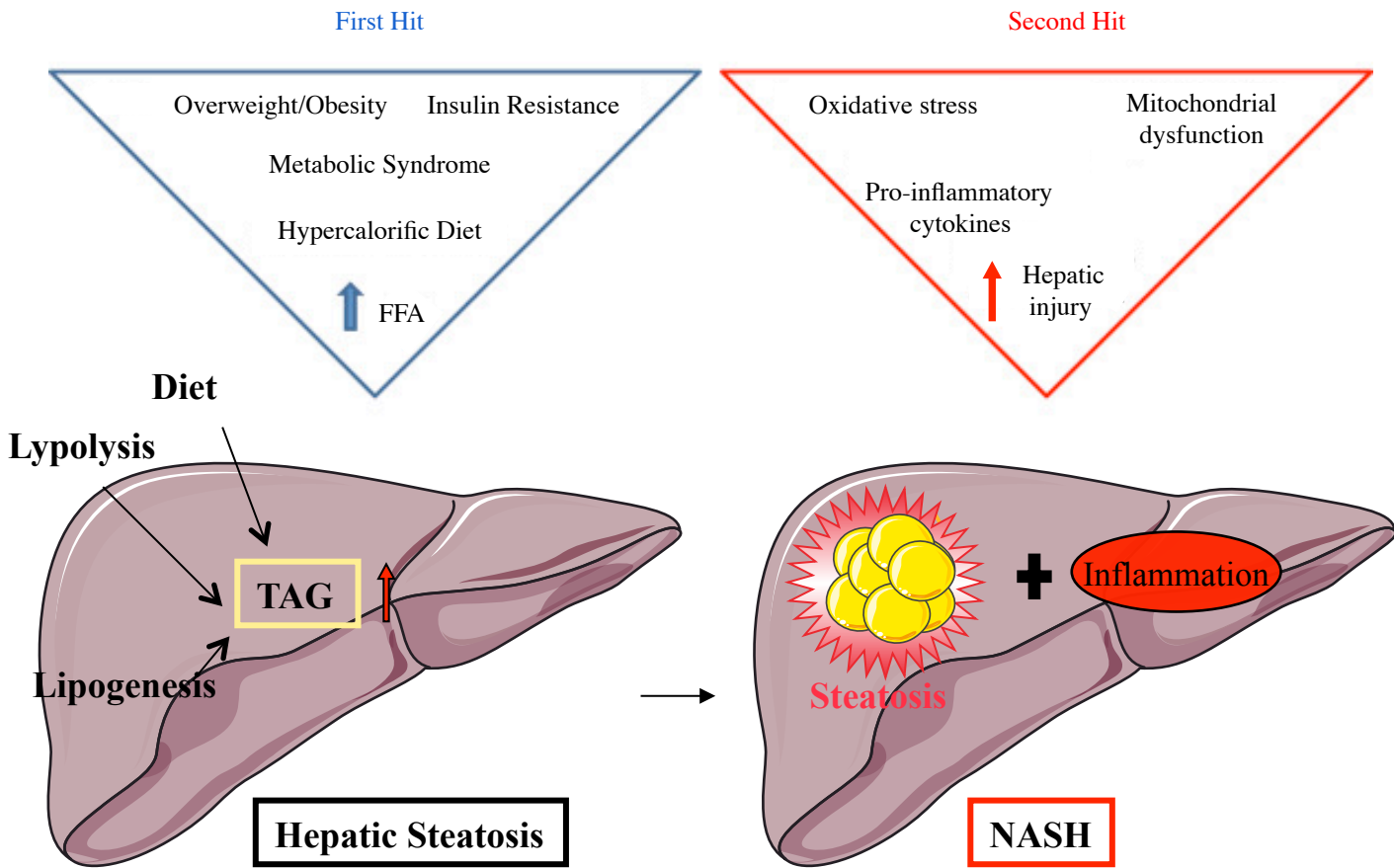


Figure 13: Progression of hepatic steatosis to NASH can be explained by the so called “two hit” hypothesis (1) Increased plasma FA from the diet and lipolysis of adipose tissue as well as increased synthesis of *de novo* fatty acids leads to increased concentrations of TAG in liver. (2) Over production of toxic lipids causes hepatic injuries through oxidative stress, mitochondrial dysfunction, and the release of pro-inflammatory cytokines. Adapted from Giorgio et al. 2013 BMC Pediatr.

glucose intolerance as it directly inhibits the downstream phosphorylation events of the insulin signaling cascade and the translocation of the glucose transporter Glut4 in insulin target tissues. Interestingly, this liver-derived protein is also associated with fatty liver and correlates with NAFLD in humans (Stefan and Häring, 2013). Identified as an adaptor protein for saturated fatty acid-mediated activation of toll-like receptor 4, this fetuin-A fatty acid synergy may induce inflammatory signaling and insulin resistance—two important driving forces behind T2D (Pal et al., 2012). Pioglitazone is a TZD that significantly reduces serum fetuin-A levels in patients with T2D. However, other insulin sensitising therapies, such as metformin and aerobic exercise, do not seem to affect fetuin-A levels (Ochi et al., 2014). Although its regulation is still largely unknown, fetuin-A still represents an attractive target for the development of T2D treatments.

## ***B/ Growth differentiation factor-15***

Currently under discussion is the clinical significance of growth differentiation factor-15 (GDF-15) as a biomarker for T2D progression. GDF-15 is a member of the transforming growth factor- $\beta$  superfamily (Bootcov et al., 1997), which has been identified as a key regulator in the promotion of liver fibrosis (Dooley and ten Dijke, 2012). High plasma GDF-15 has been linked to progression insulin resistance severity in obese patients and to early stages of T2D (Dostálová et al., 2009). GDF-15 is also reported to be independently associated with progression of cardiovascular disorders (Adela et al., 2016). Produced from various cell types, especially macrophages, GDF-15 is found in the heart, brain, liver and placenta. GDF-15 is increased in response to oxidative stress, and during cellular proliferation and is increased in the presence of inflammatory cytokines by the tumor suppressor protein p53 (Zimmers et al., 2006). GDF-15 blocks the JNK pathway and activates Akt as well as intracellular SMAD proteins, which activate the antihypertrophic pathway (Berezin, 2016). Transgenic mice over expressing GDF-15 in

macrophages were reported to have decreased food intake, body weight, and adiposity and improved glucose tolerance on both a normal and HFD diet suggesting that increased GDF-15 may have a protective effect against T2D or that T2D may be a GDF-15 resistant state (Macia et al., 2012). Other studies reported that the glucose-mediated induction of GDF-15 may protect endothelial cells from high glucose induced cellular injury. Indeed, siRNA-induced GDF-15 knockdown in human umbilical venous endothelial cells attenuated Akt signaling and promoted activation of JNK (Li et al., 2013). These studies suggest that GDF-15 may be a potential biomarker or therapeutic target for cardiovascular disease and T2D. However, its poor specificity for a particular disease may hinder its clinical application as a biomarker (Mueller et al., 2015). A recent publication has linked metformin use to increased plasma GDF-15 in T2D patients. However, it was not determined whether the therapeutic actions of metformin may in part be mediated by GDF-15 (Gerstein et al., 2017) (Figure 14).

### ***C/Angiopoietin-like protein 8***

Angiopoietin-like protein 8 (Angptl8), gained extensive popularity following a paper were authors credited Angptl8 for increased  $\beta$ -cell proliferation and mass following use of an insulin receptor antagonist S961, renaming the hepatokine betatrophin (Yi et al., 2013). However, this paper was highly challenged and later retracted by the authors (Yi et al., 2017). Indeed, Angptl8 knockout mice, while exhibiting profoundly reduced plasma TAG levels, show no abnormalities in terms of glucose homeostasis and/or in  $\beta$ -cell expansion in response to insulin resistance resulting from either HFD or from the administration of S961. In addition, over expression of Angptl8 in the liver of mice did not affect  $\beta$ -cell expansion (Cox et al., 2015; Gusarova et al., 2014).



**Figure 14: Hepatokines induced in response to obesity and Type 2 diabetes**

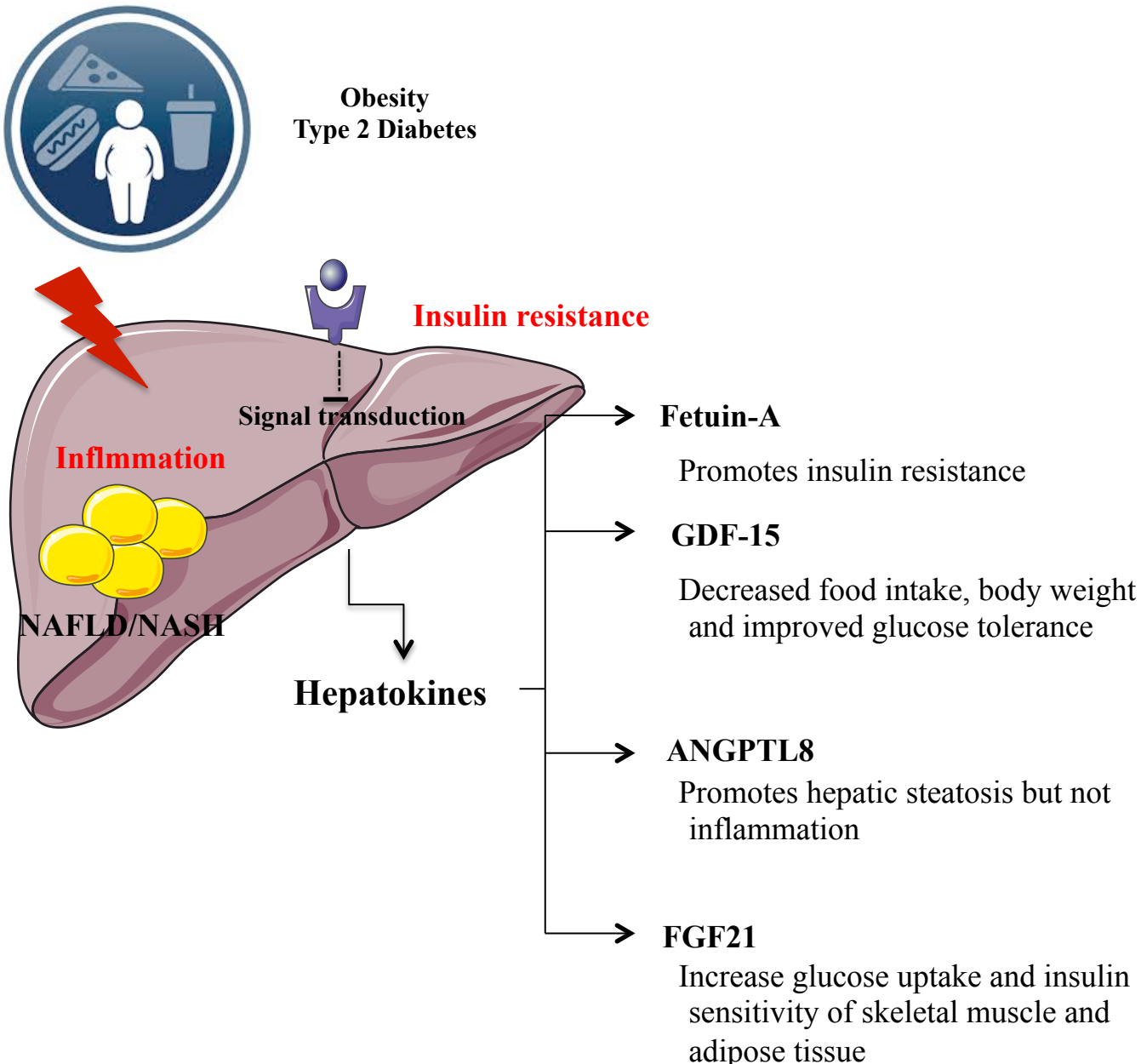


Figure 14: In response to hepatic inflammation and insulin resistance the liver releases proteins into the blood stream called hepatokines that are attractive targets for the development of novel type 2 diabetes treatments. Fetuin-A, GDF-15, ANGPTL8 and FGF21 are examples of metabolic relevant hepatokines.

Hepatic Angptl8 is stimulated by insulin possibly through liver X receptor (LXR) and SREBP-1c dependent pathway and is inhibited by AMPK (Lee et al., 2015). In hepatocytes Angptl8 is activated by the MAP-kinase pathway via ERK1/2 (Zhang et al., 2016). Knockdown of ATGL using siRNA results in TAG accumulation in hepatocytes (Ong et al., 2010). Over expression of Angptl8 in livers of mice promotes hepatic steatosis but does not promote inflammation or insulin resistance suggesting the Angptl8 activation could synthesize less toxic lipid species (Wu et al., 2011). Regardless, plasma TAG levels correlate with Angptl8 expression supporting the possibility that inhibiting Angptl8 may represent an efficient therapeutic strategy for the prevention of hypertriacylglycerolaemia (Abu-Farha et al., 2016; Gómez-Ambrosi et al., 2016) (Figure 14).

### ***D/Fibroblast growth factor 21***

The beneficial effects of FGF21 in the diabetic model were first described in 2005 when FGF21 protein was administered to *ob/ob* mice. The authors observed that mice administered FGF21 protein had significantly lower blood glucose and circulating insulin concentrations as well as reduced plasma triglycerides. In transgenic mice over expression of FGF21 resulted in resistance to diet induced obesity, significantly lower fat in the liver, and smaller adipocytes (Kharitonov et al., 2005). These findings were confirmed in diabetic monkeys (Coskun et al., 2008). The glucose lowering effect of FGF21 was shown to be mediated through FGF21's stimulation of Glut1 transporter mRNA as well as in an increase in Glut1 at the cell membrane by ERK 1/2 activation (Kharitonov et al., 2005). FGF21 had a limiting effect on circulating triglycerides and cholesterol contributing to overall weight loss. Further developments in mouse islets demonstrated that FGF21 could improve function and survival of pancreatic  $\beta$ -cells when an increase in insulin staining intensity as well as an increase in islet number per pancreas section was observed (Wente et al., 2006) (Figure 14). FGF21 is regulated by PPAR $\alpha$  during a

ketogenic state or fasting and by glucose via ChREBP (Badman et al., 2007; von Holstein-Rathlou et al., 2016). A part of this introduction will be dedicated to the transcriptional control of FGF21 by PPAR $\alpha$  and ChREBP.

## **Conclusion of Part 2**

Obesity-related insulin resistance from excessive energy intake aggravates hepatic TAG accumulation in the liver leading to a condition called NAFLD. Toxic lipid accumulation in the liver causes hepatic insulin resistance increasing hepatic glucose production and causing dyslipidemia. In response to energy overload, liver secretes hormone-like peptides called hepatokines to induce metabolic adaptations.

## ***Part 3: Transcriptional regulation by glucose, a central role for ChREBP***

### ***I-The ChREBP protein***

Highly enriched in the liver, ChREBP mediates the hepatic response to dietary carbohydrates. Glucose promotes translocation of ChREBP from the cytoplasm to the nucleus initiating ChREBP binding to the ChoRE region of genes essential for the induction of glycolysis (*L-pk*) (Ishii et al., 2004; Yamashita et al., 2001) and lipogenesis (*Acc, Fas, Scd1*) (Dentin et al., 2004). ChIP-seq analysis of genome wide ChREBP binding has enabled a broad identification of ChREBP target genes the majority of which are involved in metabolism, insulin signaling and tumorigenesis (Poungvarin et al., 2015). For the purpose of this manuscript we will focus on ChREBP in the liver.

### ***A/ChREBP and ChREBP $\beta$ protein structure***

#### **A-1/The ChREBP protein structure**

Weighing in at 94 kDa, ChREBP is a 864 amino acids member of the basic helix-loop-helix leucine zipper family. The ChREBP protein contains several functional domains. Located near the N-terminus is the nuclear localization signal (NLS) (residues 158-173; rat isoform) and two nuclear export signals (NES1/2) (residues 5-15 and 85-95; rat isoform) (Fukasawa et al., 2010). These regions make up the mono conserved region (MCR) (Li et al., 2008). At the C-

terminus a basic helix-loop-helix leucine zipper (residues 382-583; human isoform) as well as a leucine-zipper-like domain (ZIP-like) (residues 795-835; human isoform) can be found. ChREBP contains also a proline-rich region. The phosphorylation sites (ser196 and ser626) and (thr666 for mouse and thr665 for rat protein) are also present on the ChREBP protein and are important for its post-translational activation (Poupeau and Postic, 2011; Yamashita et al., 2001) (Figure 15A).

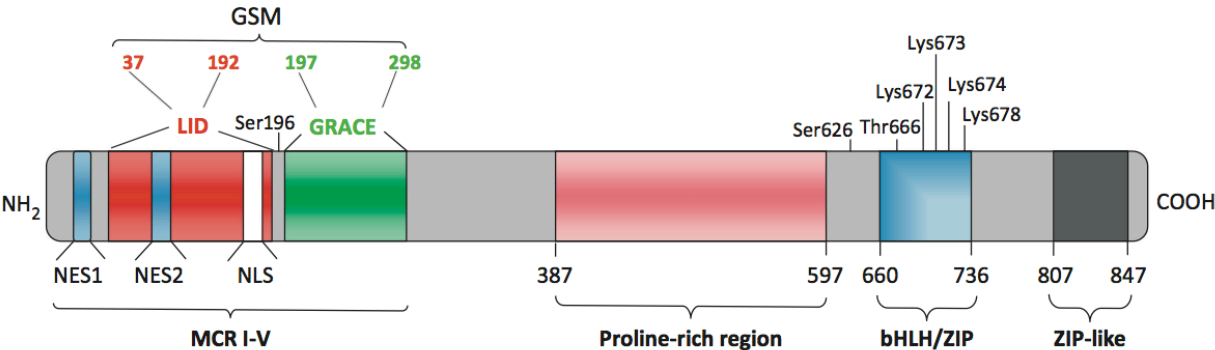
## A-2/The ChREBP $\beta$ protein structure

In 2012, Herman and colleagues reported a novel ChREBP isoform, coined ChREBP $\beta$ . After the discovery of ChREBP $\beta$ , the full length protein was renamed ChREBP $\alpha$  (but will still be referred as ChREBP for the purpose of this manuscript). ChREBP $\beta$  is suggested to originate from an alternative first exon promoter (Exon1b) resuming its transcription at a later transcriptional start sight (ATG). This results in a shorter ChREBP $\beta$  protein (687 amino acids) lacking the N-terminus domains including the NLS and the NES2. These two domains are referred as the low glucose inhibitory domain (LID) associated with ChREBP inhibition under low glucose concentrations (Li et al., 2008). The ChREBP $\beta$  protein does contain the glucose sensing region (GRACE; residues 197-298), which promotes transactivation in response to high glucose concentrations. ChREBP $\beta$  was reported to be highly active regardless of glucose concentrations (Herman et al., 2012). In white adipose tissue of C57BL/6J mice fasted overnight, *Chrebp $\beta$*  expression is significantly reduced while *Chrebp $\alpha$*  declines modestly. Three hours of refeeding induces a significant up-regulation in expression of both *Chrebp $\alpha$*  and *Chrebp $\beta$* . *Chrebp $\beta$*  is most drastically affected with a 4.3 fold increased suggesting that importance of ChREBP $\beta$  for the regulation of target genes in adipose tissue (Herman et al., 2012) (Figure 15B).

Figure 15:

Structure of the ChREBP $\alpha$  and ChREBP $\beta$  proteins

(A) ChREBP protein (ChREBP $\alpha$ )



(B) ChREBP variant (ChREBP $\beta$ )

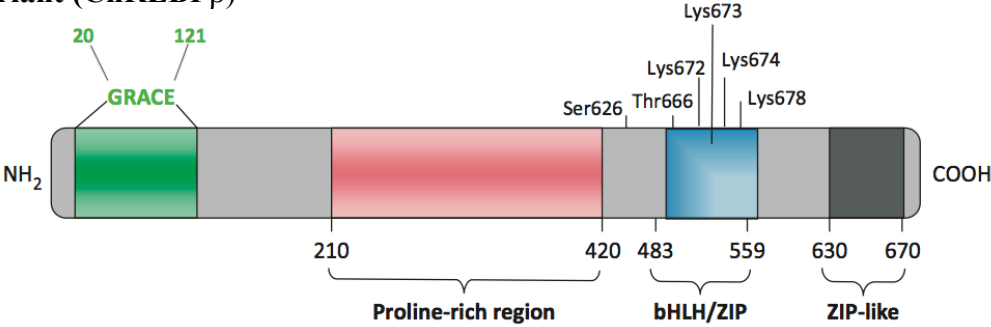


Figure 15: (A) ChREBP $\alpha$  protein structure. ChREBP $\alpha$  is a 864 amino acids in length and contains several domains including a nuclear localization signal (NLS), two nuclear export signals (NES1 and NES2) near the N terminus, polyproline domains, a bHLH/LZ domain, and a leucine-zipper-like (Zip-like) domain. ChREBP contains at least three phosphorylation target sites for PKA (ser196, ser626, and thr666). Structure/function analysis identified a glucose-sensing module (GSM), evolutionally conserved in Mondo proteins, which contains a low-glucose inhibitory domain, denoted LID, and a glucose-response conserved element, denoted GRACE. LID is able to inhibit the ChREBP transactivation activity conferred by GRACE, and this inhibition is lifted under high glucose conditions. (B) ChREBP $\beta$  protein structure. Although translation from the start-site in exon 1 a produces the full-length protein (ChREBP- $\alpha$ ), translation initiating at the next start-site located in exon 4 produces a shorter protein of 687 amino acids (ChREBP $\beta$ ). The ChREBP $\beta$  protein is missing the first 177 amino acids. Adapted from Filhoulaud et al, Trends 2013

## ***II- Post-translational and transcriptional regulation of ChREBP***

The activation mechanism of ChREBP remains controversial but it is agreed that ChREBP is inhibited by glucagon and activated by products of glucose metabolism (Kawaguchi et al., 2001).

### ***A/Post-transcriptional regulation of ChREBP***

ChREBP translocation from the cytosol to the nucleus was described to be driven through a dephosphorylation mechanism by xylulose 5-phosphate (Xu5P)(Kabashima et al., 2003; Kawaguchi et al., 2001b). Xu5P activates PP2A which initiates dephosphorylation of ChREBP on the ser196 residue leading to ChREBP translocation to the nucleus where it is again dephosphorylated on its thr666 residue by a PP2A-mediated mechanism (Dentin et al., 2012). Once dephosphorylated ChREBP undergoes the additional post transcriptional modification of O-GlcNAcylation, a glucose dependent reaction in which a high-energy donor substrate is added to a protein by O-GlcNAc transferase (OGT) in order to modify protein activity or synthesis (Hart and Akimoto, 2009). Immunoprecipitation experiments completed in hepatocytes of wild type mice (Glucose 25mM) demonstrated that ChREBP is highly O-GlcNAcylated, (Guinez et al., 2011). Over expression of OGT, is able to modify and increase transcriptional activity of ChREBP as observed by an increase of ChREBP lipogenic target genes *Acc* and *Fas* in refed mice. Further investigation by Guinez and colleagues reported that by limiting O-GlcNAcylation of ChREBP by O-GlcNAcase, the enzyme responsible for reversing the action of OGT, limits ChREBP activity and improves hepatic steatosis as well as the lipid profile of *db/db* mice (Figure 16).

## A-1/ChREBP regulation by the LID and Grace domain

Alternatively, a second mechanism described in the literature illustrates, control of ChREBP by an intra-molecular glucose-sensing region. The MCR of ChREBP contains a LID (residues 37-192) and a glucose response conserved element (GRACE, residues 197-298) (Li et al., 2008). It is suggested that glucose regulates ChREBP by direct binding of a glucose metabolite with the MCR and/or by recruiting co-regulatory proteins (McFerrin and Atchley, 2012). Under low glucose concentration, LID is deactivated by phosphorylation on several residues allowing for activation of GRACE and recruitment of co factors (Tsatsos et al., 2008). In line with these findings, a truncated form of ChREBP called ChREBP<sup>CA</sup> (constitutively active ChREBP), lacking the first 196 N-terminal amino acids of the LID domain, is constitutively active under low glucose concentrations ( Li et al., 2008).

## A-2/Regulation of ChREBP regulation by ketone bodies and AMP

During fasting, phosphorylation dependent cytoplasmic retention has been identified as the master inhibitory mechanism of ChREBP in response to glucagon-induced PKA (Kawaguchi et al., 2001a). However, ketone bodies can also act as low glucose sensing molecules. During periods of low energy substrate availability  $\beta$ -hydroxybutyrate and acetoacetate have been shown to restrict ChREBP localization to the cytoplasm of primary rat hepatocytes (Nakagawa et al., 2013). In addition, AMP (Blázquez et al., 1999), was shown to inhibit ChREBP activity. Stimulated by low ATP levels in the cell, AMP maintains energy homeostasis by activating catabolic pathways and inhibiting transcription factors essential to lipogenesis (Li et al., 2011). AMP has recently been shown to inhibit ChREBP activity through two different mechanisms: (a) activation of AMPK leading to the phosphorylation of ser568 near the basic helix-loop-helix/ZIP



**Figure 16: Post-translational regulation of ChREBP in response to glucose**

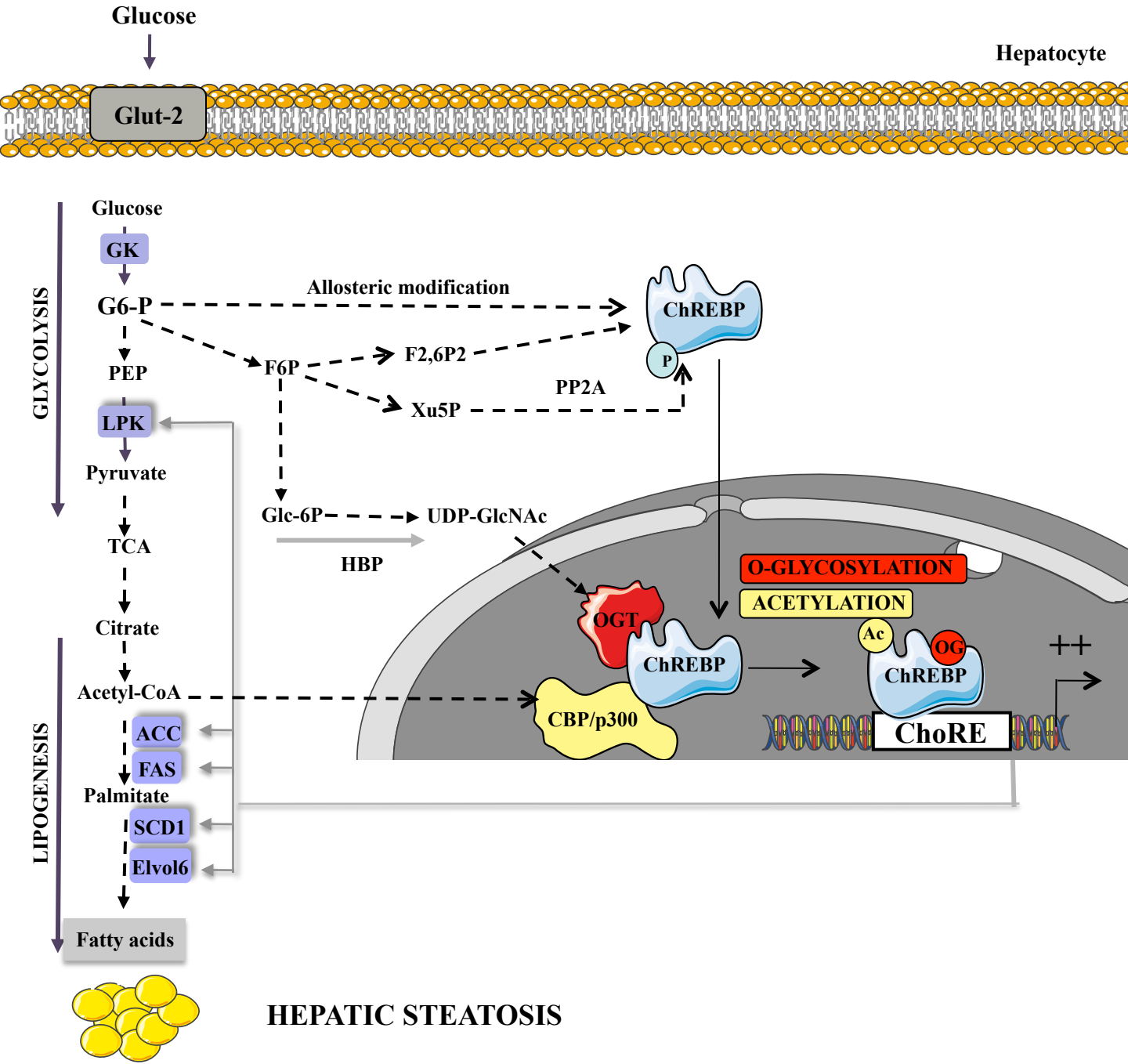


Figure 16: Key metabolites, including G6P or Xu-5P may regulate activation of ChREBP activity in response to glucose. Under high glucose concentrations, G6P or other metabolites may bind a specific recognition motif causing an allosteric conformational change leading to ChREBP activation. Within the nucleus, ChREBP undergoes two important post-translational modifications: acetylation through HAT coactivator activity of CBP/p300 and O-GlcNAcylation through O-GlcNAc transferase (OGT) activity, that both contribute to enhanced ChREBP transcriptional activity and DNA binding to ChoRE motifs present on glycolytic (*L-pk*) and lipogenic (*Acc*, *Fas*, *Scd1*, *Elov6*) target genes. Adapted from Filhoulaud et al, Trends 2013

in the C-terminal region of ChREBP, stabilizing ChREBP with translocation inhibitors and (b) AMP binding directly to ChREBP on the N-terminal region again sequestering ChREBP to the cytosol (Sato et al., 2016).

### A-3/ChREBP co activators

ChREBP binds with its transcription partner Max-like protein X (Mlx) creating a heterotetramer on the ChoRE (Stoeckman et al., 2004). ChREBP transcriptional activity is improved by recruitment of histone acetyltransferase (HAT) p300 which joins the ChREBP/Mlx binding complex acetylating ChREBP on lys (lysine) 672 (Bricambert et al., 2010). The participation of p300 was demonstrated to impact ChREBP potency as over expression of phosphorylated p300 at ser89, restricted HAT activity in mice blunting ChREBP transcriptional activity (Bricambert et al., 2010). ChREBP is able to form a binding complex with HNF4 $\alpha$  and its binding partner CBP (Burke et al., 2009). The importance of this complex could lie in the CBP binding partner, which is activated in response to glucose or cAMP signaling. Experiments confirmed that repression of CBP leads to the down regulation of *L-pk* despite over expression of ChREBP (Burke et al., 2009). Without the presence of CBP to hold together the ChREBP/HNF4 $\alpha$  complex, factors of repression can bind to the glucose-sensing region. COUP-TFII, a repressor of insulin target genes is able to compete for the HNF4 $\alpha$  binding site repressing *L-pk* transcription (Lou et al., 1999; Perilhou et al., 2008)

## ***B/Transcriptional regulation of ChREBP***

### **B-1/ChREBP auto-regulation**

While a ChoRE binding region has not yet been identified it has been suggested that ChREBP may auto-regulate through a feed-forward mechanism by binding near the alternative exon 1 on the *Chrebp* promoter inducing expression of the highly active *Chrebp* $\beta$ . In support of this theory, experiments using a dominant negative Mlx mutant, which inhibits ChREBP activity, reduced endogenous *Chrebp* $\beta$  expression (Herman et al., 2012) (Figure 17).

### **B-2/ChREBP activation by LXR $\alpha$**

Highly expressed in the liver, liver X receptor  $\alpha$  (LXR $\alpha$ ) controls cholesterol synthesis and promotes lipogenesis (Peet et al., 1998; Schultz et al., 2000). By creating a complex with its binding partner retinoid X receptor (RXR), LXR $\alpha$  binds to response LXR response elements (LXRE) 1 and 2 (Mitro et al., 2007). In 2007, two LXR $\alpha$  binding regions were identified on the promoter of ChREBP, LXRE1 and LXRE2 (-2566 bp to -2302 bp) (Cha and Repa, 2007). Indeed stimulation of fed male C57Bl/6 mice with T0901317 (50 mg/kg body weight), a LXR $\alpha$  agonist, increased ChREBP promoter occupancy by the RXR/LXR heterodimer and was associated with an increase of total *Chrebp* mRNA. This also paralleled with a 2.5 fold in expression of *L-pk* mRNA (Cha and Repa, 2007). However, these findings are controversial as LXR $\alpha$  knockout mice, fed a high carbohydrate diet (18 hours), had no significant difference in *L-pk* gene expression as compared to controls. This suggests that the activation of ChREBP by glucose occurs in an LXR-independent mechanism (Denechaud et al., 2008) (Figure 18).

**Figure 17: Physiological relationship between ChREBP $\alpha$  and ChREBP $\beta$**

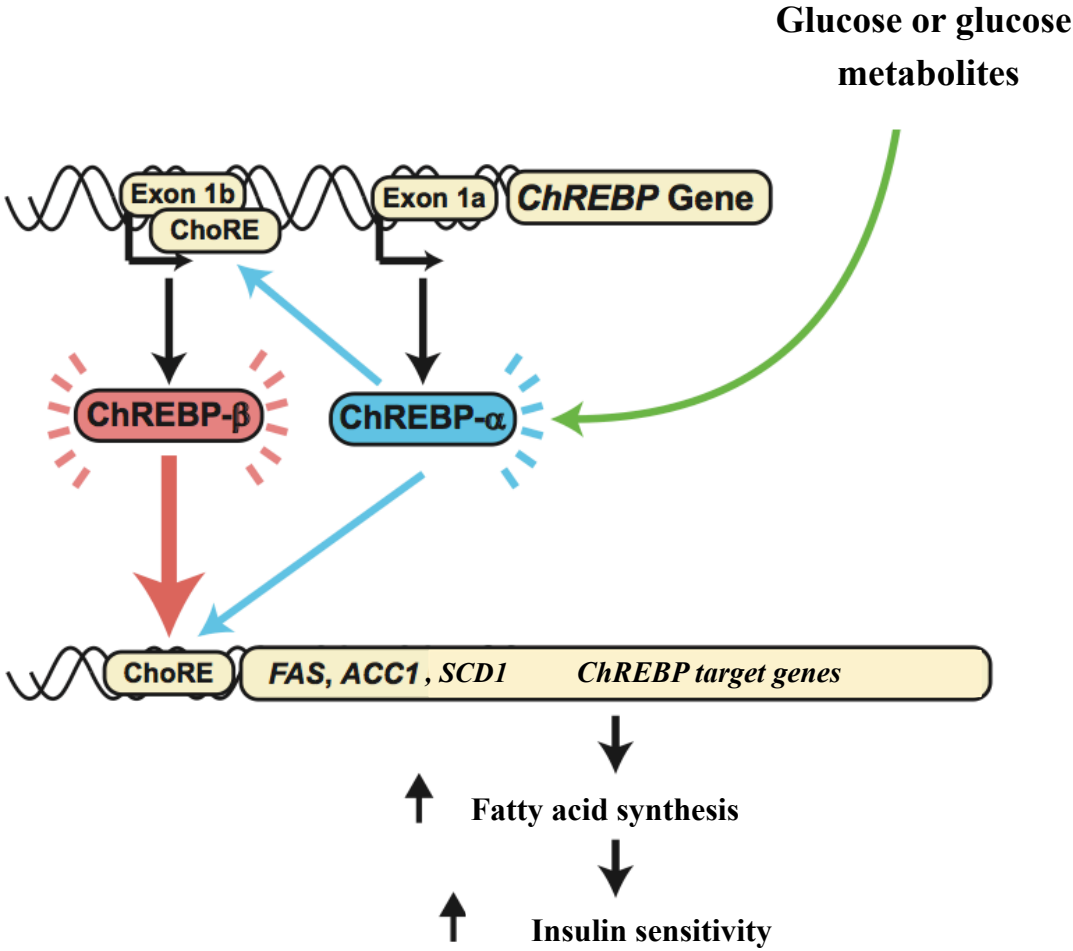


Figure 17: The isoform ChREBP $\alpha$  is activated by glucose or glucose metabolites. Once ChREBP $\alpha$  translocates to the nucleus its binds to its ChoRE region. Herman et al. 2012 suggested that ChREBP $\alpha$  is able to induce translation of the more potent ChREBP $\beta$  isoform through direct binding of the ChoRE located near the exon 1b. Adapted from Herman et al. 2012 Nature

### B-3/ChREBP activation by thyroid hormone

In conjunction with LXR $\alpha$ , thyroid hormone (TH) is known to up-regulate lipogenesis (Hashimoto et al., 2007). Luciferase assays confirmed that treatment with TH positively regulated *ChREBP* transcription and furthermore increased *ChREBP* mRNA and ChREBP protein in Hepal1-6 cells and in the liver of hypothyroid mice (Hashimoto et al., 2009). Specifically, the positive regulation of ChREBP by TH has been attributed to the isoform TR $\beta$ 1 of the TH receptor, more highly expressed in the liver than the TR $\alpha$ 1/2 isoform (White and Dauncey, 1999). In similarity to LXR $\alpha$ , TR $\beta$ 1 creates a binding complex with RXR, and can bind both LXRE binding regions, preferentially the LXRE2. However, in the current paper using TR $\beta$ 1 and LXR $\alpha$  knockout mice, ChREBP regulation by nutrition over-dominated the independent role of either TR $\beta$ 1 or LXR $\alpha$  (Gauthier et al., 2010) (Figure 18).

### B-4/ChREBP repression by the nuclear receptor farnesoid X receptor

It has now been demonstrated that the nuclear receptor farnesoid X receptor (FXR), involved in bile acid metabolism, is able to inhibit the transcription of *L-pk* (Caron et al., 2013). A relationship between FXR and ChREBP was first observed in 2005 when FXR knockout mice displayed an increase in ChREBP target genes expression *L-pk* and *Acc* in the liver (Duran-Sandoval et al., 2005). A more recent publication by the same team outlines the molecular mechanisms involved. In wild-type mice orally administered with INT-747, a synthetic FXR agonist mRNA of *L-pk* was significantly blunted during refeeding. In addition, studies conducted in human hepatocyte cell lines IHH cells reported that the down regulation of *L-pk* was specific to FXR. Chromatin immunoprecipitation (ChIP) experiments demonstrated that *in vivo* FXR directly interacts with the ChREBP binding complex. This was surprisingly maintained during high

Figure 18: Transcriptional regulation of ChREBP during feeding

## Feeding

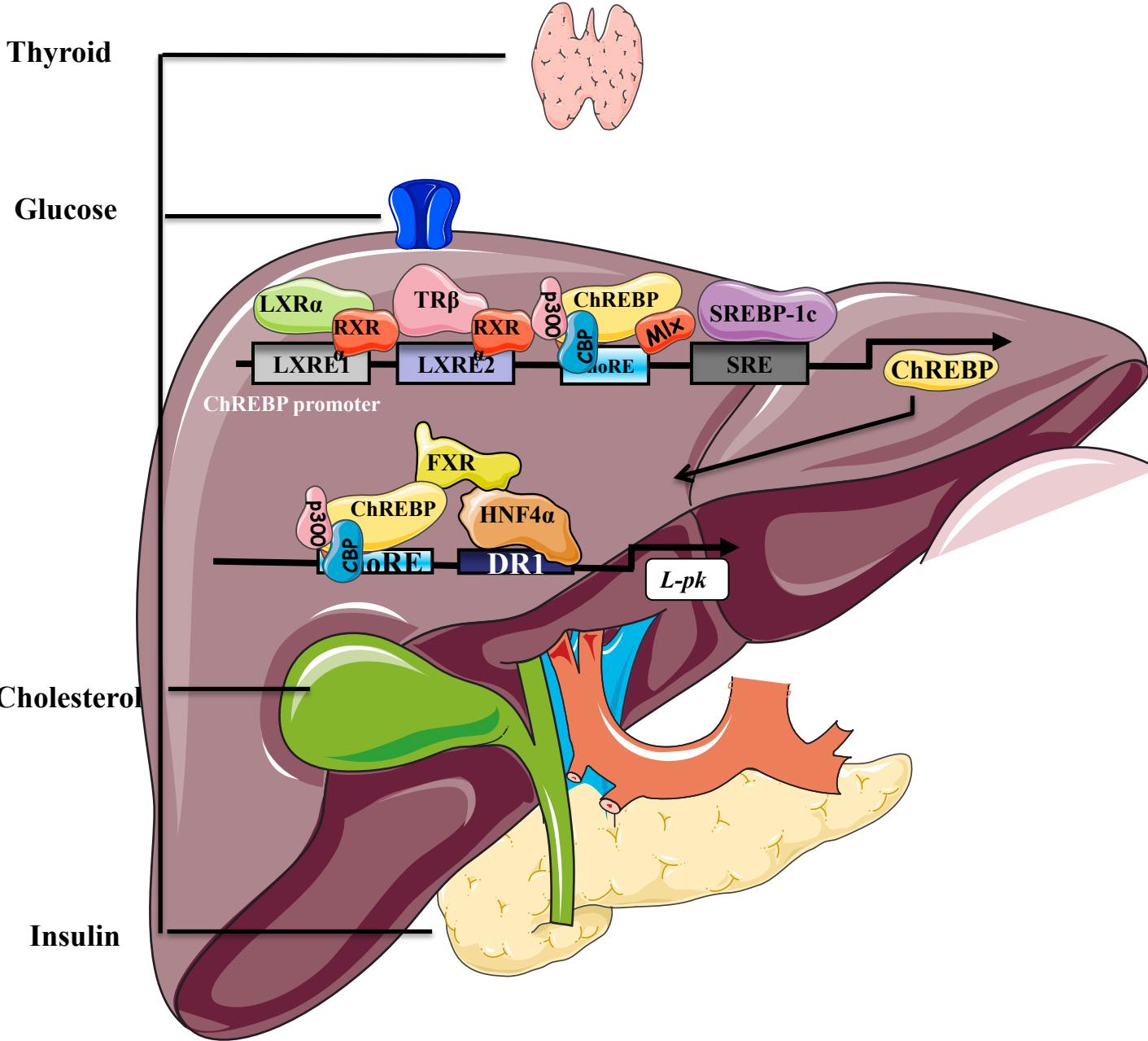


Figure 18: ChREBP transcription and activity is up regulated in response to feeding signals to promote energy storage. Regulated by glucose metabolites, ChREBP is also stimulated at the transcription level by the transcription factors LXR $\alpha$ , TR $\beta$  and SREBP-1c. To induce transcription of its target genes, ChREBP forms a dimer complex with CBP/p300 and its binding partner Mix and binds to the ChoRE region. This complex then attaches to the HNF4 $\alpha$ /FXR complex on the DR1 region of the *Lpk* promoter driving transcription.

glucose conditions (Figure 18). FXR suppression activity was linked to the recruitment of the co repressor SMRT. Indeed, siRNA mediated inhibition of SMRT abolished the FXR-dependent inhibitory effect on the *L-pk* promoter activity. The authors conclude that the deacetylation activity of SMRT led to release, of not only ChREBP on the ChoRE binding region, but also of its co activators CBP/p300 previously reported necessary for the glucose induced expression of *L-pk* (Caron et al., 2013) (Figure 19).

### ***III- Role of ChREBP in the regulation of energy homeostasis***

Since its discovery, in 2001, by the group of K. Uyeda, the transcription factor ChREBP has been established as a key regulator of glucose metabolism and lipid homeostasis. ChREBP protein is highly expressed in the liver, pancreatic  $\beta$ -cells, white adipose tissue, and skeletal muscle.

#### ***A/Lessons from ChREBP deficiency***

Early work conducted with the global ChREBP knockout mice (ChREBP<sup>-/-</sup> mice) reported that ChREBP deficiency leads to glucose and carbohydrate intolerance with a decline in plasma FFA levels and progressive hypothermia, culminating in death 1 week following a diet of 70% sucrose (Iizuka, 2004). ChREBP knockout mice also displayed glucose intolerance and inulin resistance when challenged with a high starch diet (Ishii et al., 2004).

However, when ChREBP<sup>-/-</sup> mice are crossed into a genetic background of obesity (*ob/ob*

Figure 19:

Transcriptional suppression of ChREBP during fasting

Fasting

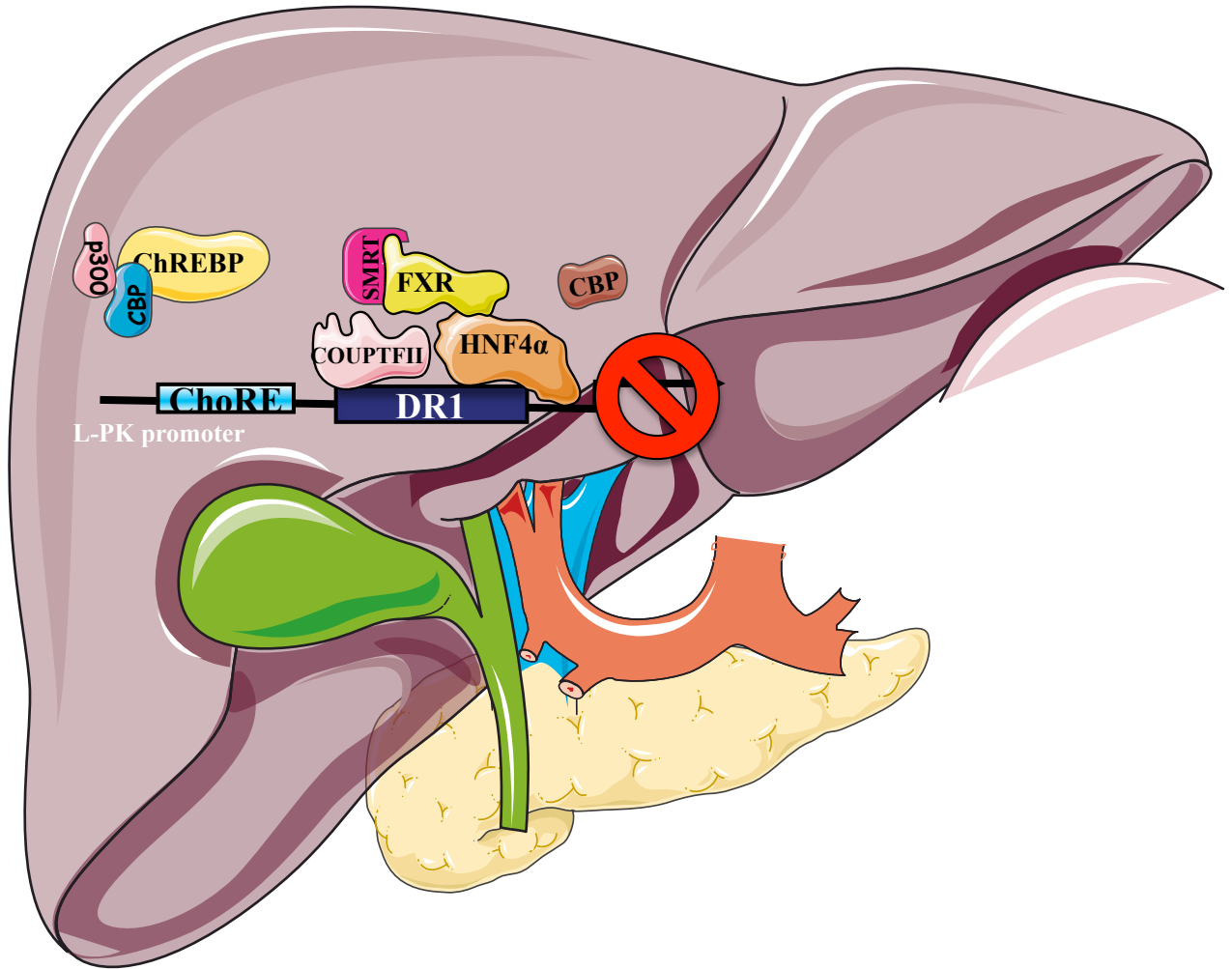


Figure 19: ChREBP activity is turned off during fasting to prevent storage of lipids and glucose. The receptor FXR is able to recruit the co-repressor SMRT which causes release of CBP/p300. When CBP/p300 is removed the binding complex between HNF4 $\alpha$  and ChREBP is released, inhibiting ChREBP activity. In addition, the repressor transcription factor COUP-TFII is then able to bind to the DR1 binding region preventing ChREBP's access to its target gene promoter.



mice), hepatic steatosis and insulin resistance were reduced (Iizuka et al., 2006). ChREBP deletion also reduced expression of the hypothalamic appetite-stimulating neuropeptide agouti-related protein resulting in decreased food intake and consequently reduced body weight (Iizuka et al., 2006). This suggests that ChREBP activity is able to signal through the CNS (Iizuka et al., 2006). When ChREBP was selectively silenced through a shRNA strategy in liver of *ob/ob* mice, a significant reduction of hepatic fatty acid synthesis rates was observed due to a decreased in ChREBP target genes *Acc*, *Fas* and *Scd1* mRNA. Recent studies have also linked ChREBP to fructose metabolism as glucose production from fructose was reduced 60% in ChREBP knockout mice (Kim et al., 2016).

### ***B/Specific role of ChREBP in liver***

The role of ChREBP in liver function is complex as it may depend on the nutritional status and/or the genetic background. Indeed, liver specific over expression of ChREBP through adenoviral gene transfer in C57BL/6 mice, displayed hepatic steatosis onset (from enhanced *de novo* lipogenic genes, *Fas*, *Scd1* and *Elovl6*). But despite increased hepatic steatosis, ChREBP overexpressing mice did not display insulin resistance. Indeed, Benhamed and colleagues demonstrated that in liver of mice overexpressing ChREBP fed a HFD (60% energy derived from fat) for 14 weeks, saturated fatty acids concentrations were reduced restoring in turn Akt phosphorylation. The observed benefits were largely attributed to ChREBP target gene *Scd1* a crucial enzyme in fatty acid metabolism converting stearate (C18:0) to oleate (C18:1) a monounsaturated fatty acid, known to improve insulin sensitivity and to decrease inflammation in cases of lipid overload. In vitro knockdown of *Scd1* blunted the beneficial effects of ChREBP on Akt phosphorylation even in the context of lipid overload (Benhamed et al., 2012).

## ***C/Relevance of ChREBP to human physiology and pathology***

In human liver, ChREBP expression is strongly correlated with *de novo* lipogenesis and metabolic risk markers of NAFLD and also positively correlated with insulin resistance (HOMA-IR) in some cohorts (Eissing et al., 2013; Hurtado del Pozo et al., 2011; Lambert et al., 2014). In particular, ChREBP $\beta$  activity was reported to mimic up regulation of ChREBP dependent genes (*Fas*, *Scd1*, *G6pc*) in human liver biopsy of obese patients and was suggested to be a main driver of NAFLD development (Eissing et al., 2013). Up regulation of *G6pc* by ChREBP $\beta$  was suggested to be a mechanism which likely contributes to the apparent paradox of selective hepatic insulin resistance in humans (Kim et al., 2016). However, others have found that while hepatic ChREBP expression did positively correlate to the degree of hepatic steatosis its expression inversely correlated to insulin resistance in patients with NASH (Benhamed et al., 2012a). The benefits and disadvantages of ChREBP expression in the human liver is still under investigation.

### **Conclusion of Part 3**

Glucose metabolites promote translocation of ChREBP from the cytosol to the nucleus initiating ChREBP binding to the ChoRE of genes essential for the induction of glycolysis and lipogenesis. Two isoforms of ChREBP have been identified ChREBP $\alpha$  and ChREBP $\beta$ . ChREBP $\beta$  lacks the glucose inhibitory domain (LID) allowing it to be highly active regardless of the glucose concentration. The role of ChREBP in the development of NASH and insulin resistance is paradoxical as hepatic ChREBP knockdown prevents hepatic steatosis, however, over expression of ChREBP in the liver dissociates hepatic steatosis from insulin resistance. Clinical and mouse studies strongly suggest that the role of ChREBP in liver function is complex as it may depend on the nutritional status and/or the genetic background.

## ***Part 4: The nuclear receptor PPAR $\alpha$ , a key regulator of lipid oxidation***

Hepatic PPAR $\alpha$  is essential in the adaptive fasting response as a key nuclear receptor that regulates transcription of rate limiting genes necessary for lipid trafficking,  $\beta$ -oxidation and ketogenesis. PPAR $\alpha$ -deficient mice exhibit impaired fatty acid oxidation and ketogenesis and are sensitive to diet and age induced hepatic steatosis (Kersten et al., 1999; Kroetz et al., 1998; Montagner et al., 2016). Down regulated in advance stages of NASH, PPAR $\alpha$  inducing-agonists are important anti-diabetic drug targets that normalize dyslipidemia and reduce inflammation.

### ***I-The peroxisome proliferator-activated receptor family***

The lipid sensitive nuclear receptor PPAR $\alpha$  is a member of the PPAR (peroxisome proliferator-activated receptor) family comprised of three distinct PPAR isoforms that are specific in their tissue distribution and binding ligands (Issemann and Green, 1990). This diversity allows PPAR to perform both overlapping and distinct functions in their respective tissues (Dreyer et al., 1993). PPAR $\beta/\delta$  is strongly expressed in muscle fibers and is important for fatty acid catabolism prevented lipid-induced ER stress (Salvadó et al., 2014). PPAR $\gamma$ , in white adipose tissue, is targeted by pharmaceuticals for its lipid storage and insulin sensitizing capacities. Like PPAR $\beta/\delta$  and PPAR $\gamma$ , PPAR $\alpha$  is also distributed throughout metabolically active tissues such as the kidney, muscle and liver and is especially characterized for its ability to control fatty acid metabolism in response to nutrient availability (Keller et al., 1993).

The *PPAR $\alpha$*  gene (NR1C1) is located on chromosome 22 and 15 in humans and mice respectively (Pawlak et al., 2015). Human and mouse *PPAR $\alpha$*  share a 91% homology in their 468 amino acid sequence (Gearing et al., 1994); however, comparative transcriptomic studies revealed only partial overlap of human and mouse *PPAR $\alpha$*  target genes which suggest that species specific discrepancies should be considered when investigating *PPAR $\alpha$*  action (Rakhshandehroo et al., 2009). Despite the variation in target genes the *PPAR $\alpha$*  structure remains highly conserved across species. The *PPAR $\alpha$*  protein consists of an N-terminal A/B domain containing a ligand-independent trans-activation function called activating function-1 (AF-1), a DNA-binding C domain (DBD), a D hinge region, located at the C terminus, and a large ligand-binding E domain (LBD) (Feige et al., 2006).

## ***II-Regulation of PPAR $\alpha$ activation***

Long chain FFA from the diet, liberated during fasting, are credited to be the preferred *PPAR $\alpha$* . Hydrolysis of hepatic TAG stores by ATGL can also activate *PPAR $\alpha$*  and induce its activity in a ligand independent fashion (Sapiro et al., 2009) (Figure 20).

*PPAR $\alpha$*  is also able to regulate in a feed forward loop generating endogenous ligands via its regulation of the FAS enzyme. The use of FAS knockout mice, deficient in *PPAR $\alpha$*  activity, allowed for the identification of a novel *PPAR $\alpha$*  ligand 16:0/18:1-GPC which is synthesized via *de novo* lipogenesis (Chakravarthy et al., 2009). During hepatic injury and inflammation oxidized phospholipids stimulate *PPAR $\alpha$*  (Delerive et al., 2000). In addition to exogenous and endogenous fatty acids *PPAR $\alpha$*  is the main target of the anti-diabetic drug fenofibrate that reduces hypertriglyceridemia and lowers the risk of cardio vascular disorder (Schoonjans et al., 1996;

Figure 20:

## Regulation of PPAR $\alpha$ in response to fasting

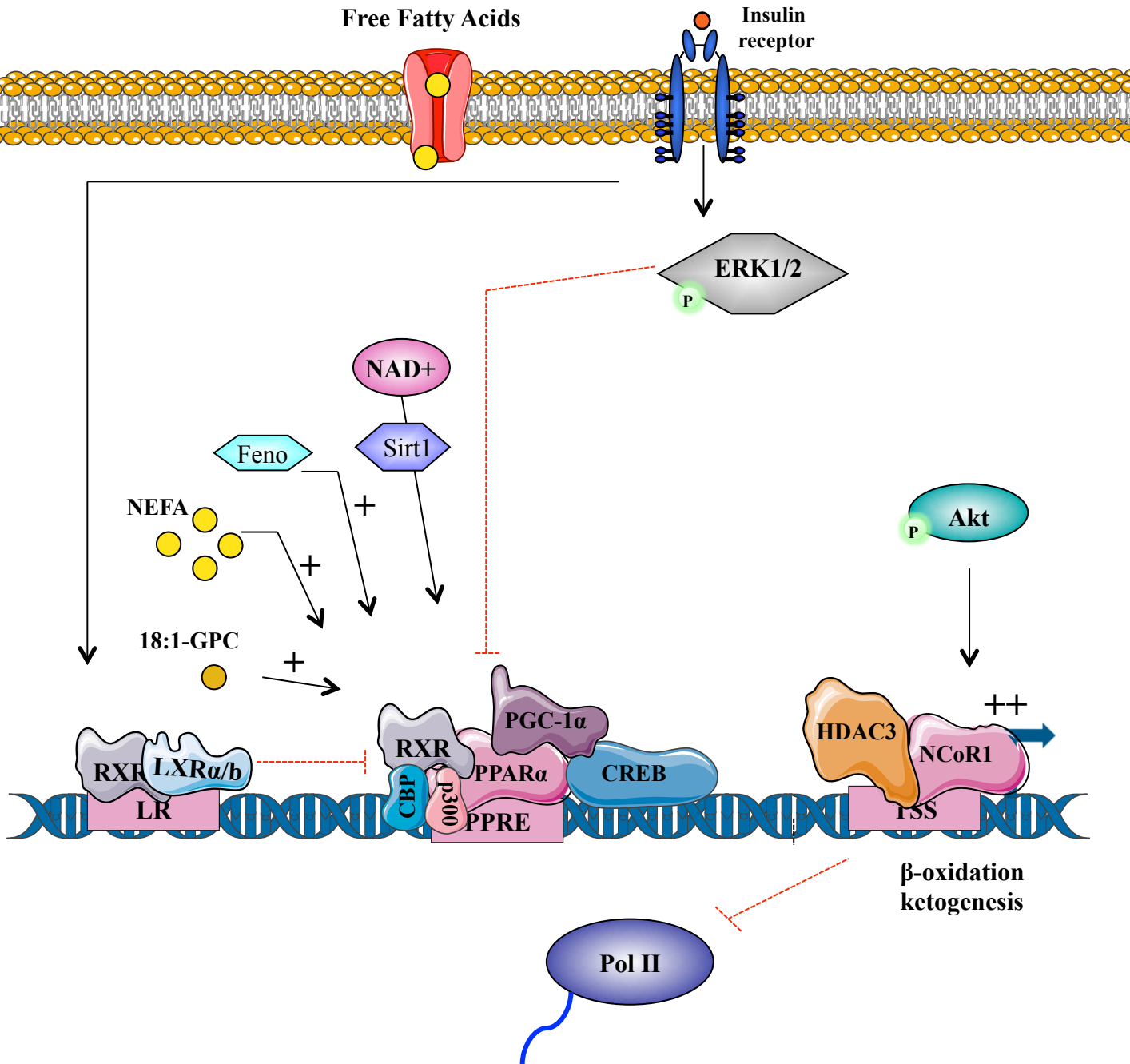


Figure 20: PPAR $\alpha$  is stimulated by free fatty acids, liberated from adipose tissue or from the diet, the drug fenofibrate and by build up of oxidative stress. Activation of PPAR $\alpha$  and its co activators induces  $\beta$ -oxidation and ketogenesis genes. Insulin signaling blocks PPAR $\alpha$  by the MAP-kinase/ERK pathway and through the NCoR1/HDAC3 recruitment to the TSS of the promoter which prevents the binding of essential transcriptional machinery including Pol II.

Staels et al., 1998).

Suppression of PPAR $\alpha$  is regulated by intracellular signaling cascades such as MAP kinase, stimulated by insulin, that mediates ligand independent phosphorylation of PPAR $\alpha$  via the AF-1 region (Juge-Aubry et al., 1999). The nuclear receptor LXR is also able to inhibit PPAR $\alpha$  activity by sequestering its obligatory co activator retinoid X receptor  $\alpha$  (RXR $\alpha$ ) (Ide et al., 2003)

### ***A/PPAR $\alpha$ co activators***

Transcriptional activity of PPAR $\alpha$  is dependent on its dimerize with its obligatory co activator RXR $\alpha$  but other co activators are also important for PPAR $\alpha$  activity (Gearing et al., 1994). Other co activator interactions are also induced by ligand binding to the LBD, which causes a conformational change of PPAR $\alpha$  allowing it to interact directly with co activators CREB, peroxisome proliferator-activated receptor gamma co activator 1- $\alpha$  (PGC-1 $\alpha$ ) and CBP/p300. The binding partner complex includes a HAT protein essential for chromatin accessibility and recruitment of transcriptional machinery (Feige et al., 2006). PGC-1 $\alpha$  is a key regulator of glucose lipid and energy metabolism through its co activity with nuclear receptors (Yang et al., 2009). In the liver PGC-1 $\alpha$  activity is dependent on post-translational modifications including deacetylation. Build up of NAD<sup>+</sup> during oxidative stress or low energy substrate availability stimulates the nicotinamide adenine dinucleotide-dependent deacetylase Sirt1. Deacetylation of PGC-1 $\alpha$  by Sirt1 allows PGC-1 $\alpha$  to generate the foundation of the PPAR $\alpha$  co activator complex (Rodgers et al., 2005; Sugden et al., 2010). This system works in a feed forward fashion, as PPAR $\alpha$  target gene, *Fgf21*, is then able to activate Sirt1/PGC-1 $\alpha$  in order to maintain PPAR $\alpha$  activity and the adaptive fasting response through mobilization and oxidation of lipids long after depletion of glycogen stores depleted by early glycogen activity. This is especially evident as PGC-1 $\alpha$  and Sirt1 knockout mice, even when treated with exogenous

FGF21, are unable to induce hepatic gluconeogenesis during prolonged fasting (Potthoff et al., 2009; Purushotham et al., 2009) (Figure 20).

### ***B/PPAR $\alpha$ co repressors***

The D hinge region is important for recruitment of co repressors such as nuclear receptor co repressor (NCoR1) and SMRT which together recruit HDAC3 (Chen and Evans, 1995; Dowell et al., 1999; Mottis et al., 2013). During periods of nutrient availability such as the case in obesity models, the mTOR signaling cascade promotes localization of NCoR1 to the nucleus (Sengupta et al., 2010). Interaction of PPAR $\alpha$  with NCoR1 is then promoted by SUMOylation of PPAR $\alpha$  on lys185 (Pourcet et al., 2010). HDAC3 recruitment is essential to NCoR1/SMRT-mediated suppression of its nuclear receptor targets (Ishizuka and Lazar, 2003). Ubiquitination of the PPAR $\alpha$  co repressor complex is triggered by PPAR $\alpha$  ligand binding (Perissi et al., 2010) (Figure 20).

### ***C/Reciprocal regulation of PPAR $\alpha$ and ChREBP***

Recent identification of a feed-back loop between ChREBP and PPAR $\alpha$ , for the regulation of lipogenesis in brown adipose tissue, first brought to thought a potential cross-talk. To understand the feed-back loop between ChREBP and PPAR $\alpha$ , investigators used Wy14643, a selective PPAR $\alpha$  agonist, to activate PPAR $\alpha$  *in vitro*. Studies confirmed that PPAR $\alpha$  activation partially suppressed glucose induction of *ChREBP $\beta$*  and *Fas* mRNA in the brown adipose tissue cell line HB 2 and reduced *ChREBP* luciferase activity. These results were reciprocal as during

fasting of male C57BL/6J mice, when PPAR $\alpha$  is normally up regulated, adenoviral transfection of a constitutively active ChREBP suppressed PPAR $\alpha$  induction of its key gene such as acyl coA oxidase-1 (*ACOX1*) (Iizuka et al., 2013). Other studies found similar interactions. In human islet cells, glucose stimulation (25mM) represses expression of PPAR $\alpha$ . Furthermore, the repression of PPAR $\alpha$  was shown to be ChREBP dependent as siRNA-mediated knockdown of ChREBP abrogates glucose repression of PPAR $\alpha$  (Boergesen et al., 2011). Identification of an endogenous PPAR $\alpha$  ligand, 16:0/18:1-GPC, dependent on the ChREBP target gene *Fas* (Chakravarthy et al., 2009), creates a new level of complexity to draw out a clear model of the ChREBP-PPAR $\alpha$  cross-talk. In the liver ChREBP and PPAR $\alpha$  share common target genes including *Fgf21* and *Scd1* (Benhamed et al., 2012a; Iizuka et al., 2009; Lundåsen et al., 2007; Miller and Ntambi, 1996). Experiments conducted in both ChREBP and PPAR $\alpha$  knockout mice would improve current understanding of their reciprocal role, and would address the relationship of their role in the liver which to date has not yet been explored.

### ***III-Role PPAR $\alpha$ in the control of energy homeostasis***

PPAR $\gamma$  activity in white adipose tissue is important for initiating, the hepatic catabolic program. FFA liberated from white adipose tissue stores by PPAR $\gamma$  via induction of ATGL into the plasma are taken up by the liver (Kershaw et al., 2007). Activation of PPAR $\alpha$  allows for long-chain fatty acid uptake into hepatocytes in part due to its control of fatty acid translocase and fatty acid transport protein (Motojima et al., 1998). In the cell, PPAR $\alpha$  target genes (*Cpt-I*, and *Cpt-II*) orchestrate lipid trafficking. These proteins shuttle fatty acids to cellular peroxisomes and mitochondria for  $\beta$ -oxidation. PPAR $\alpha$  is also essential for the induction of the  $\beta$ -oxidation program via up regulation of gene including the rate-limiting enzyme *Acox1* in peroxisomal fatty acid oxidation. In the mitochondria PPAR $\alpha$  generates energy not only through  $\beta$ -oxidation but



also through generation of ketone bodies from acetyl-CoA including  $\beta$ -hydroxybutyrate and acetoacetate by up regulation of 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*Hmgcs2*).

### ***A/Lessons from global and tissue-specific models of PPAR $\alpha$ deficiency***

The role of PPAR $\alpha$  on insulin sensitivity has been controversial as early studies demonstrated improved insulin sensitivity, as measured by glucose and insulin tolerance tests, in global PPAR $\alpha$  knockout mice fed a HFD as compared to controls (Guerre-Millo et al., 2001). However, long-term treatment of mice with a PPAR $\alpha$  activator, fenofibrate, not only corrects dyslipidemia but also improves insulin sensitivity. In humans, fenofibrate significantly lowers the risk associated with cardiovascular disease (Guerre-Millo et al., 2000; Keating, 2011; Scott et al., 2009). The disparity may be due to reduced hepatic glucose output observed in PPAR $\alpha$  knockout mice, which are unable to mobilize lipid stores for gluconeogenesis. PPAR $\alpha$  also promotes safer storage of TAG and cholesterol in the liver and white adipose tissue by reducing lipid shuttling to peripheral tissues, which causes lipotoxicity. Sensitive to nutritional challenges of diet induced obesity and hepatic stress, PPAR $\alpha$  knockout mice fed a HFD or methionine choline-deficient (MCD) diet developed severe fibrosis, steatosis and macrophage infiltration (Abdelmegeed et al., 2011; Ip et al., 2003). In addition, a prolonged inflammatory response was observed in PPAR $\alpha$  global knockout mice (Devchand et al., 1996).

Liver specific PPAR $\alpha$  knockout mice (PPAR $\alpha^{\text{hep}/-}$ ) are hypoglycemic and are unable to mobilize hepatic lipid stores for peripheral energy use during prolonged fasting (Montagner et al., 2016). Unable to mobilize hepatic TAG in livers of aged PPAR $\alpha^{\text{hep}/-}$  mice balloon turning white, engorged with TAG and cholesterol metabolites (Montagner et al., 2016) Reactivation of PPAR $\alpha$  exclusively in the liver reduced both hepatic and plasmatic pro-inflammatory cytokines

of the JNK pathway down regulating inflammatory cytokines IL-1, IL-6 and TNF $\alpha$  (Mansouri et al., 2008). It would be interesting to investigate if suppression of pro-inflammatory cytokines by hepatic PPAR $\alpha$  could also improve insulin sensitivity in these mice. Because of its beneficial effect on whole body lipid homeostasis, PPAR $\alpha$  is a major target of drug developers (Figure 21).

### ***B/Pharmaceutical activation of PPAR $\alpha$***

The PPAR $\alpha$  agonist fenofibrate has been used since 1998 to reduce TAG and improve the HDL to LDL ratio in patients with dyslipidemia. Clinical trials are cluttered with mixed results on their efficacy that may be due to genetic variability and low fenofibrate PPAR $\alpha$  affinity (Kraja et al., 2010; Pawlak et al., 2015). Drug side effects accompany the beneficial effects of fenofibrate treatment. The most commonly reported are gastrointestinal disorders and elevated homocysteine and creatinine concentrations, markers of vascular and renal dysfunction (Keating and Ormrod, 2002). Difference in species-specific action has also complicated research efforts. For example, fenofibrate treatment in diabetic mouse models lowers hyperglycemia (Carmona et al., 2005; Park et al., 2006), while no effect on blood glucose has been reported in clinical trials (Tsunoda et al., 2016).

Novel PPAR $\alpha$  agonist labeled selective PPAR $\alpha$  modulators (SPPARM $\alpha$ ) are under development (Hennuyer et al., 2016). The SPPARM $\alpha$ , K-877, is in clinical trial and has been effective in lowering TAG with less side effects than fenofibrate (Fruchart, 2009). Studies using human hepatocytes show that K-877 directly stimulates PPAR $\alpha$  leading to an up regulation of PPAR $\alpha$  target genes including apolipoprotein A I/II, important for the transport and distribution

**Figure 21:**

**Role of PPAR $\alpha$  in hepatic energy balance**

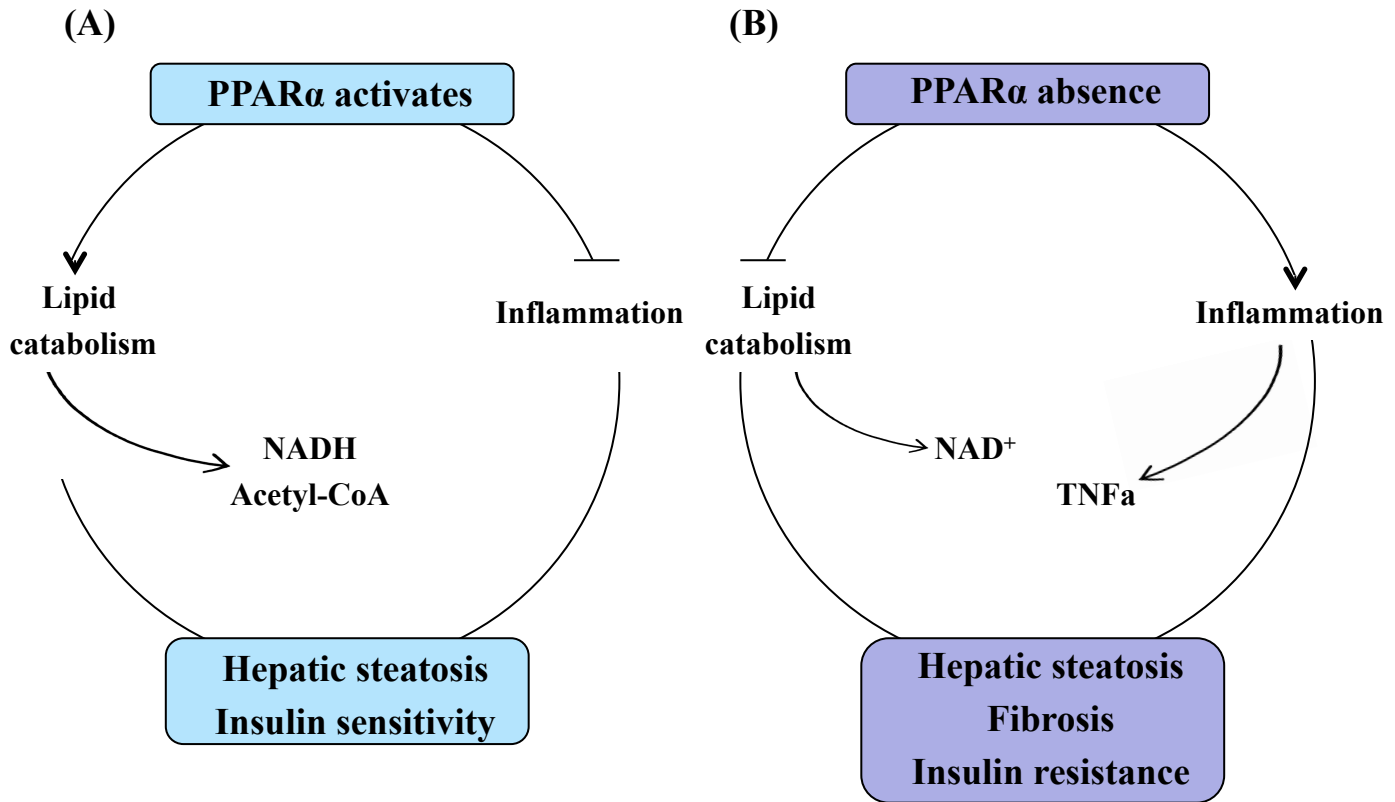


Figure 21: (A) PPAR $\alpha$  activation jump starts lipid catabolism for the production of NADH and acetyl-CoA generating ketone bodies for the use of peripheral tissue energy. Proper storage and turnover of fatty acids reduces inflammation and hepatic steatosis and improves insulin sensitivity (B) PPAR $\alpha$  deficient mice are hypoglycemic following a fast because they are unable to mobilize lipid stores. This causes a build up of fatty acids and markers of oxidative stress measured by an accumulation of NAD<sup>+</sup>. PPAR $\alpha$  knockout mice show also increased inflammation and inflammatory cytokines including TNF- $\alpha$ . Adapted from Rando et al. 2016 elife

of lipids through-out the body, and a decrease in inflammatory cytokines (Ishibashi et al., 2016; Raza-Iqbal et al., 2015). In phase II clinical trials, the SPPARM $\alpha$  GFT505 targets both PPAR $\alpha$  and PPAR $\beta/\delta$  and has been reported to reduced plasma TAG and improved insulin sensitivity without activation of PPAR $\gamma$  and its negative side effects including fluid retention and heart failure (Cariou et al., 2011, 2013; Hanf et al., 2014). Importantly, PPAR $\alpha$  activators increase mRNA and protein expression of the PPAR $\alpha$  target gene *Fgf21*. Primate and mouse studies have reported that PPAR $\alpha$ -dependent activation of FGF21 improves peripheral glucose utilization and induce secretion of the insulin sensitizing adipokine adiponectin and reduces body weight.

## Conclusion of Part 4

PPAR $\alpha$  is essential in the adaptive fasting response as a key nuclear receptor that regulates transcription of rate limiting genes necessary for lipid trafficking,  $\beta$ -oxidation and ketogenesis. PPAR $\alpha$  is regulated in a feed forward loop through the synthesis of endogenous ligands via the lipogenic enzyme FAS. In addition, its activity is repressed by HDAC3 recruitment to the promoter of its target genes. The anti-diabetic drug fenofibrate improves dyslipidemia in a PPAR $\alpha$  dependent manner. Selective PPAR $\alpha$  modulators are currently under clinical investigation for the development of novel T2D and NASH therapies.

## ***Part 5: FGF21, a major hepatokine of energy homeostasis***

Discovered in 2000 by the laboratory of Dr. Nobuyuki Itoh (Nishimura et al., 2000) the first breakthrough in understanding the physiological relevance of FGF21 was identified through the use of phenotypic screening. A phenotypic screen searches large libraries of chemical compounds and measures their effects on cell line characteristics. The adipocytes cell line 3T3-L1 was used in a glucose uptake assay to search for potential drug targets. Of those proteins FGF21 was found to improve glucose uptake in 3T3-L1 cells as well as in mouse and human primary adipocytes independently of insulin (Kharitononkov et al., 2005). In the same publication, it was reported that FGF21 up regulated expression of the *Glut1* glucose transporter via phosphorylation of the MAPK signaling pathway consequently improving glucose uptake. These findings propagated a tidal wave of research that classified FGF21 as a hepatokine able to act directly on the white adipose tissue, brown adipose tissue, CNS and liver to improve hepatic insulin sensitivity, reduce glucose output and correct dyslipidemia with few adverse side effects (Lin et al., 2013; Sarruf et al., 2010). In clinical trial today are several FGF21-like analogs, however, a better understanding of its physiological regulation would help pinpoint drug target discovery (Figure 22).

### ***I-The fibroblast growth factor family***

The fibroblast growth factor (FGF) gene family includes 22 peptides targeting a wide range of metabolic functions such as cell growth, cell survival and metabolism. These small peptides ranging from 17-35 kDa in size share a 120 amino acid conserved core region and are categorized into three distinct subfamilies determined by their ability to act as intracrine,

paracrine or endocrine signaling molecules. Intracrine FGFs (FGF 11/12/13/14) mainly function as intracellular signaling molecules in the CNS in a receptor independent fashion (Goldfarb et al., 2007). Paracrine FGFs (1-10; 16-18) include a heparan sulfate binding domain, which is essential for ligand receptor binding onto one of the four different cell surface tyrosine kinase FGF receptors (FGFR1-4). Ligand binding and tissue specificity is determined by alternative splicing of the FGFR binding domain (Itoh and Ornitz, 2004). Because of their important role in cellular development and angiogenesis, knockout of paracrine FGFs leads to embryonic mortality and overexpression is linked to carcinogenesis (Galzie et al., 1997; Sato et al., 2009). However, not all FGFs promote proliferation, endocrine FGFs are transported through the bloodstream to exert metabolic effects on peripheral tissues.

Endocrine FGFs, FGF15/19 (FGF15 is the mouse ortholog to the human FGF19), FGF21 and FGF23 act in a tissue-specific manner by binding either FGFR1c or FGFR4 and co-receptor  $\alpha/\beta$ Klotho present on the cell surface of target tissues (Ding et al., 2012; Kurosu et al., 2006; Suzuki et al., 2008). Produced by enterocytes in the small intestine, FGF15/19 expression is up regulated in response to bile acid accumulation in the gut. Once secreted, FGF15/19 enters the portal vein, repressing bile acid production and gluconeogenesis in the liver (Fernandes-Freitas and Owen, 2015). FGF23 is a bone-derived hormone that targets the kidney to suppress reabsorption of phosphate and regulate vitamin D metabolism (Erben and Andrukhova, 2016). Highly conserved across species, FGF21 in circulation is derived exclusively from the liver (Markan et al., 2014) and targets multiple tissues central to energy metabolism to coordinate the adaptive response to physiological stresses including starvation and carbohydrate overload (Berglund et al., 2009; Emanuelli et al., 2014; von Holstein-Rathlou et al., 2016; Markan et al., 2014).

**Figure 22:**

**Physiological roles of FGF21**

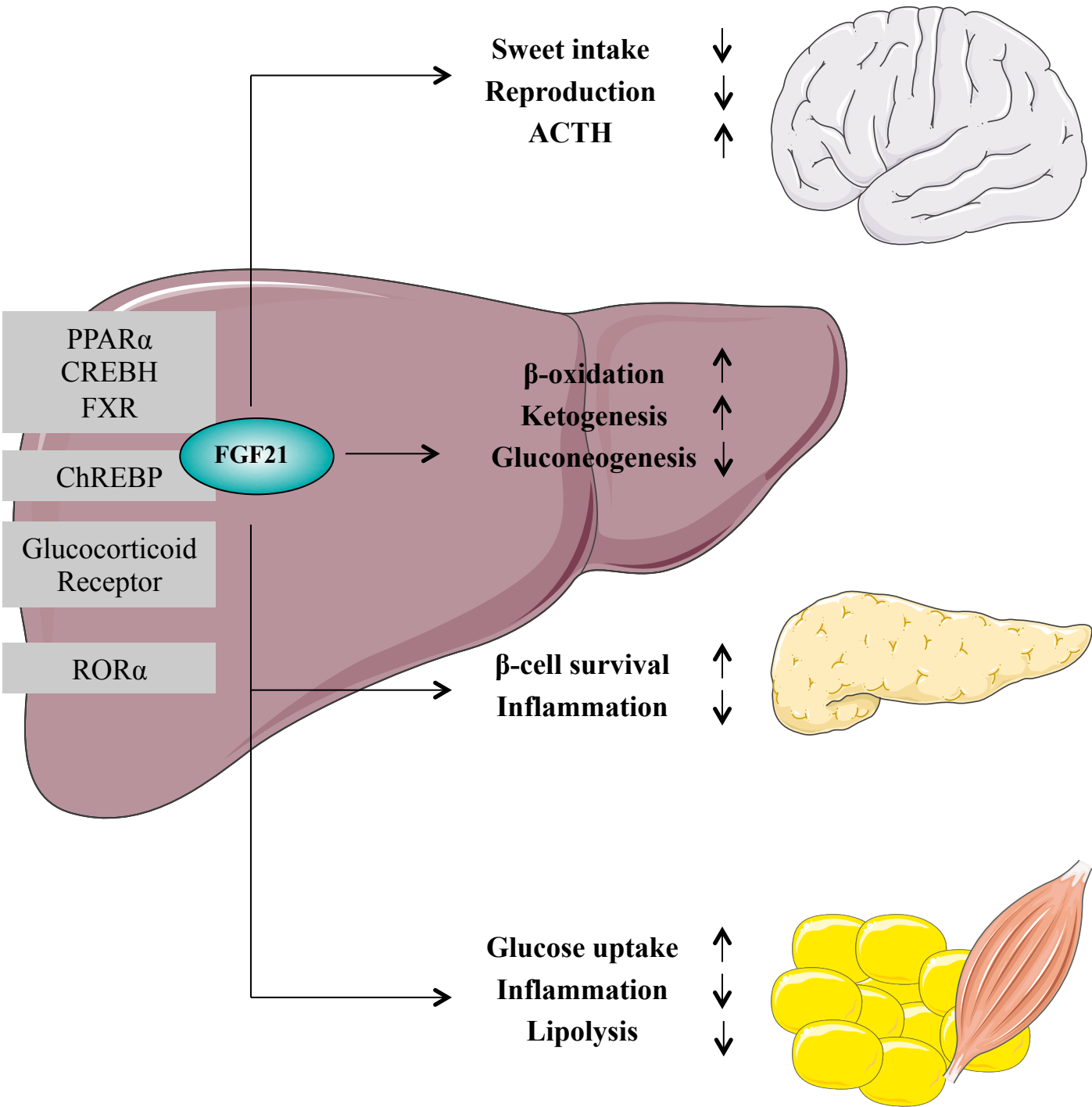


Figure 22: FGF21 is up regulated by PPAR $\alpha$ , CREBH, FXR, ChREBP, GR and ROR $\alpha$ . Secreted into the plasma from the liver, FGF21 can improve glucose homeostasis by acting on the brain, the liver itself, the pancreas, the skeletal muscle and adipose tissues (white and brown).

## ***II-FGF21 signaling and sites of expression***

FGF21 in circulation is derived exclusively from the liver (Markan et al., 2014a). Upon secretion, FGF21 binds the FGFR1c/ $\beta$ Klotho dimer and initiates phosphorylation of FGF receptor substrate 2 (FRS2) and subsequent downstream pathways including MAPK/ERK, Akt and AMPK to regulate targets (Itoh and Ornitz, 2004; Salminen et al., 2016). Dimerization of co-receptors FGFR1c and  $\beta$ Klotho is essential to FGF21 signaling as all FGF21 effects on metabolism are lost in whole-body  $\beta$ Klotho-knockout mice (Adams et al., 2012; Ding et al., 2012). FGFR1c/ $\beta$ Klotho activation initiates phosphorylation of FRS2 and subsequent downstream pathways including MAPK/ERK, Akt and mTORC1. Activation of these pathways improves insulin sensitivity and positively regulates FGF21 expression in adipocytes. The janus kinases (JAK) and signal transducers and activators of transcription (STAT) pathway, important for growth, is down regulated by FGF21 (Figure 23).

## ***III-FGF21 is important for energy homeostasis control***

### ***A/Lessons from global and tissue-specific models of FGF21 deficiency***

The generation of mouse models of FGF21 deficiency has been critical in determining its role in energy homeostasis. FGF21 whole body knockout mice display obesity and impaired glucose homeostasis and are unable to respond to long term fasting (Potthoff et al., 2009). Normally present in the plasma during fasting, FGF21 knockout causes intolerance to long term fasting or to a ketogenic diet suggesting its importance for the mobilization and oxidization of stored fatty acids (Badman et al., 2009). As would be expected following these observation, the



Figure 23:

FGF21 signaling pathway in adipose tissue

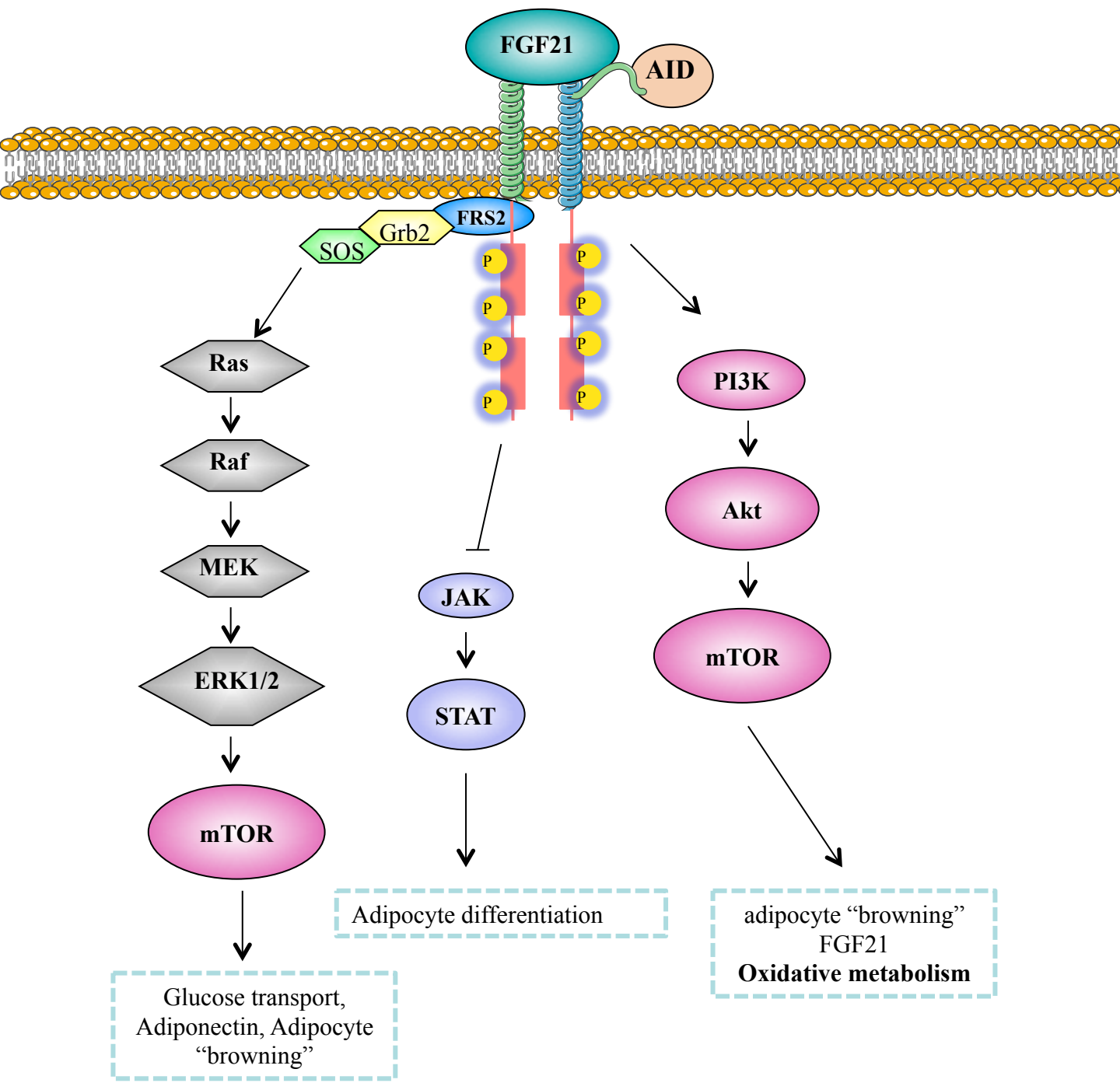


Figure 23: The FGF21 receptor FGFR1c has an auto inhibitory domain (AID) and requires interaction with a co-receptor,  $\beta$ -klotho. FGF21 binding allows for dimerization with  $\beta$ -klotho and autophosphorylation. The FGFR1c/ $\beta$ Klotho initiates phosphorylation of FGF receptor substrate 2 (FRS2) and subsequent downstream pathways including MAPK/ERK, Akt and mTORC1. Activation of these pathway improves insulin sensitivity and positively regulates FGF21 expression in adipocytes. The JAK/STAT pathway, important for growth, is down- regulated by FGF21.

expression of *Acox1* and *Hmgcs2*, two important genes for the ketogenic program, were reduced in liver of FGF21 knockout mice (Badman et al., 2009). In addition to long term fasting, FGF21 is elevated in mice challenged with a low protein diet suggesting that FGF21 responds to other types of nutrient deficiencies. Indeed, FGF21 is a target gene for activating transcription factor 4 (ATF4) and CCAAT enhancer binding protein homologous protein (CHOP), both important for adaptation to dietary and ER stress (De Sousa-Coelho et al., 2012, 2013; Wan et al., 2014). Its importance in ER stress and protein restriction is supported by mouse studies fed a MCD diet. FGF21 knockout mice are sensitive to MCD accumulating TAG in the liver leading to hepatic inflammation and fibrosis (Fisher et al., 2014). Additionally, whole body FGF21 knockout mice have hepatic insulin resistance and increased hepatic glucose production (Camporez et al., 2015). However, despite FGF21's importance for hepatic energy metabolism and its ability to induce MAP kinase signaling in the liver, its direct role on the liver has been debated and several studies have shown that FGF21 works indirectly on the liver via white adipose tissue and the CNS (Lin et al., 2013; Sarruf et al., 2010). The development of global  $\beta$ Klotho knockout mice with restored  $\beta$ Klotho expression in the liver would help better address this issue.

Undisputable, is the importance of the liver for secretion of FGF21. Using FGF21<sup>fl/fl</sup> and albumin-Cre transgenic mice, Markan et al. generated a hepatocyte-specific deletion of FGF21. In this line of mice, FGF21 expression was not only absent in the liver but also in circulation. Furthermore, this study demonstrates that hepatic FGF21 is necessary for the whole body insulin sensitizing and glucose lowering effects of FGF21 (Markan et al., 2014b). Hepatic specific deletion of FGF21 has also revealed a mechanism in which FGF21 improves hepatic insulin sensitivity. The JNK signaling pathway, activated by saturated fatty acid and cellular stress (Jaeschke and Davis, 2007), contributes to the direct development of insulin resistance by phosphorylating the insulin signaling adapter protein IRS-1 (Aguirre et al., 2000) and indirectly by promoting infiltration of inflammatory cytokines in peripheral tissues (Sabio et al., 2008) and

by the suppression of PPAR $\alpha$ . JNK knockout releases suppression on PPAR $\alpha$  and restores insulin sensitivity in a hepatic FGF21 dependent manner (Vernia et al., 2014, 2016).

### ***B/Role of FGF21 in white adipose tissue: importance of the adiponectin-FGF21 axis***

Adiponectin produced by white adipose tissue is a key regulator of whole body energy balance with an important anti-inflammatory role. Unlike other adipokines it is negatively correlated with obesity and diabetes (Hotta et al., 2000). Mice lacking adiponectin have severe insulin resistance and glucose intolerance when challenged with a HFD (Maeda et al., 2002). Transgenic mice over expressing adiponectin are refractory to obesity induced insulin resistance and have improved carbohydrate and lipid metabolism (Combs et al., 2004) (Figure 24). The role of the PPAR $\gamma$  agonist TZD on adiponectin is especially important as today TZD's are known to up regulate adiponectin in patients and is reported to be a critical mediator of the insulin sensitizing action of the anti-diabetic and anti-NASH drug (Polyzos and Mantzoros, 2016; Yu et al., 2002). Abundantly expressed in the liver the adiponectin receptor is required for its anti-diabetic effects including insulin sensitivity via increased *Irs-2* expression in obese mice (Awazawa et al., 2011; Berg et al., 2001). In addition to IRS-2, adiponectin reduces hepatic glucose production by signaling through the AMP-activated protein kinase (AMPK) which blocks G6Pase and PEPCK (Combs and Marliss, 2014; Liu et al., 2016b). To alleviate hepatic steatosis, adiponectin improves fatty acid turn over and disposal by activating the PPAR $\alpha$  pathway (Yamauchi et al., 2002). Exogenous administration of recombinant adiponectin to *ob/ob* mice alleviated hepatic steatosis and reduced inflammation by suppressing the production of the pro-inflammatory cytokine TNF- $\alpha$ . Circulating concentration of TAG and FFA are significantly decreased by adiponectin treatment (Xu et al., 2003). In other tissues such as skeletal muscle,

adiponectin binding to the adiponectin receptor improves insulin sensitivity and promotes glucose uptake utilization (Patel et al., 2012). Taken together adiponectin has anti-inflammatory properties and can improve the detrimental phenotype obesity and T2D.

Interestingly, some of the most robust effects of FGF21 administration involves FGF21 action on adipose tissue. Interestingly, adiponectin is essential to the insulin sensitizing effects of FGF21 in liver, as adiponectin knockout mice are refractory to the beneficial effects of FGF21 administration on hepatic insulin sensitivity. However, weight loss is not affected in these mice suggesting that the FGF21-adiponectin axis primarily targets the liver. Adipocytes in FGF21 global knockout mice are larger than in wild type mice and exhibit decreased expression of the target genes of the transcription factor PPAR $\gamma$  (*Hsl* and *Atgl*) as well as reduced secretion of adiponectin (Adams et al., 2013a; Badman et al., 2009; Dutchak et al., 2012). PPAR $\gamma$  regulates the FGF21-white adipose tissue axis in a feed forward fashion by enhancing expression of the FGF21 co-receptor  $\beta$ -Klotho. FGF21 signaling is then able to prevent PPAR $\gamma$  sumoylation restoring its effect of adipocyte differentiation and expression of its target genes (Dutchak et al., 2012). Recently it was published that the previously reported beneficial effect of FGF21 treatment, such as increased glucose uptake and adiponectin secretion were propagated by FGF21-ERK dependent activation of mTORC1 adipocyte. Use of specific inhibitors of either Akt or MEK (the kinase immediately upstream of ERK) demonstrated that only MEK inhibition prevented expression of downstream FGF21 targets (Minard et al., 2016)

Figure 24:

The Adiponectin-FGF21 axis

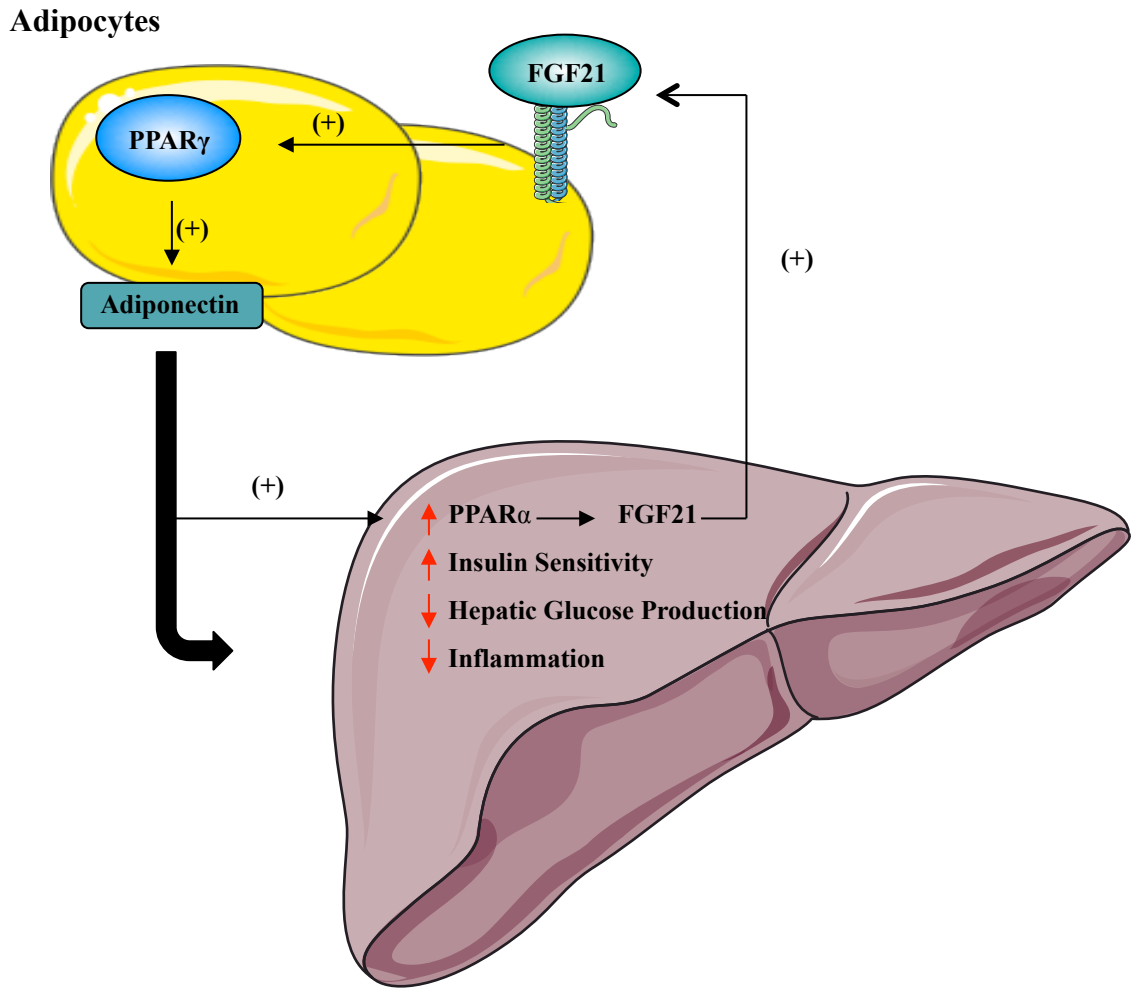


Figure 24: Adiponectin is a key regulator of whole body energy balance with an important anti-inflammatory role. In white adipose tissue, PPAR $\gamma$  up regulates adiponectin in response to TZD and FGF21 signaling. Adiponectin is able to travel to the liver and induce FGF21 in a PPAR $\alpha$ -dependent manner, in turn improving insulin sensitivity, reducing hepatic glucose production and inflammation. FGF21 released from the liver positively regulates adiponectin creating a positive feed back loop between the white adipose tissue and the liver.

## ***C/Role of FGF21 in brown adipose tissue***

Brown adipose tissue is another key site of FGF21 metabolic effects. Cold exposure increases circulating FGF21 and brown adipose tissue *FGF21* mRNA expression in humans and mice. FGF21 is able to induce the brown adipose tissue thermogenic program and induce brown adipose tissue like gene expression profiles in white adipose tissue including induction of uncoupling protein 1 (*Ucp1*) essential for “browning” of white adipose tissue (Fisher et al., 2012; Lee et al., 2013). FGF21 is indeed able to increase glucose uptake in brown adipose tissue and browning of visceral white adipose tissue in diet induced obese mice (Emanuelli et al., 2014) The pharmacological relevance of FGF21 on weight loss and glucose lowering seems to be partially due to the increased rate of energy expenditure and improved storage of fatty acids by white adipose tissue and brown adipose tissue (Fisher and Maratos-Flier, 2016).

Transgenic FGF21 over expressing mice increases brown adipose tissue content in the thymus and improves T-cell populations leading to a 30 to 40% longer life span (Youm et al., 2016). The thymus is essential to the rejuvenation of immunity T-cells and it was hypothesized that its ability to sustain a healthy T-cell population is related to an improved and prolonged lifespan (Dorshkind et al., 2009). Interestingly, transgenic activation of FGF21 delays aging and mortality while protecting from diet induced obesity and improves insulin sensitivity (Youm et al., 2016; Zhang et al., 2012b).

## ***D/FGF21 in the control of growth and fertility***

The beneficial effects of transgenic FGF21 activation are also associated with growth restriction and female infertility. FGF21 blunts the growth hormone (GH) and insulin-like growth factor (IGF-1) signaling pathway and consequent activation of STAT5 and its transcripts (Inagaki et al., 2008). While down regulation of this pathway is a mechanism in which FGF21 has also been suggested to increase life span, the GH/IGF-1 pathway is important for growth and FGF21 mice are significantly smaller than wild type litter mates (Zhang et al., 2012b). Additionally, female FGF21 over expressing transgenic mice were reported to be infertile (Inagaki et al., 2007). This was first thought to be a direct effect of FGF21 action on the hypothalamic–pituitary–adrenal axis. Indeed, 48-hours of fasting or exogenous FGF21 administration suspends estrus in mice by suppression of the vasopressin-kisspeptin signaling cascade in the hypothalamus, responsible for the release of pituitary hormones essential to estrus including luteinizing hormone and follicle-stimulating hormone (Owen et al., 2013). However, it was later determined that this is an indirect effect, as fertility of FGF21 over expressing transgenic mice is rescued by a HFD (Singhal et al., 2016). These results suggest that FGF21 does indeed act on the vasopressin-kisspeptin cascade, however, caloric intake, body composition and other factors including light cycle and estrogen are able to blunt the suppressive effects of FGF21.

## ***IV- FGF21 as a therapeutic target***

Over the past years, FGF21 has appeared as a potential interesting target for metabolic therapies. FGF21 administration to diabetic rhesus monkeys causes a decline in fasting plasma

glucose in a dose dependent manner 3 weeks after start of daily treatment (Kharitononkov et al., 2007). This may suggest that FGF21 reduces plasma glucose in non-human primates indirectly rather than by a direct increase in glucose uptake as was suggested by the early 3T3-L1 studies. However, of particular note from the study was an important decrease in plasma TAG (70%) in the highest doses and improved markers of cardiovascular health (Kharitononkov et al., 2007). Improved lipid profiles may have been the cause for the delayed blood glucose improvement as high plasma FFA are a direct cause of insulin resistance (Randle et al., 1963). In addition, mouse studies provided support that FGF21 administration could correct diet-induced obesity (Berglund et al., 2009; Coskun et al., 2008) Following the promising studies a more biopharmaceutical optimal FGF21 variant was developed (Kharitononkov et al., 2013). The FGF21 variant LY2405319 lowered blood glucose, body weight, insulin, cholesterol and plasma triglyceride in both non-human primates and humans (Adams et al., 2013b; Gaich et al., 2013). To confirm that the variant worked in a similar fashion as the endogenous FGF21 protein, *ob/ob* mice on an MCD were treated with LY2405319. Three weeks of LY2405319 treatment reduced inflammation by blunting activation of the JNK 1/2 pathway, and reversed fibrosis. Using primary hepatocytes the authors suggest that LY2405319 also restored mitochondrial function by up regulation of genes important to  $\beta$ -oxidation (Lee et al., 2016) (Figure 25).

### ***V-Transcriptional regulation of FGF21 in liver***

### ***A/PPAR $\alpha$ is a key regulator of FGF21 during fasting and ketogenic diet feeding***

The first descriptions of FGF21 transcriptional regulation were identified using microarray analysis of mice on ketogenic diets (Badman et al., 2007). Because of the beneficial



effects of ketogenic diets, including weight loss and reduced cardiovascular risk factors, the diet has been commonly prescribed by physicians despite poor understanding of the physiological mechanism (Paoli, 2014). A microarray analysis of wild type mice identified sets of genes highly enriched by a ketogenic diet. Of these genes, most significantly up regulated, was FGF21 followed by transcripts known to be direct PPAR $\alpha$  target genes (Badman et al., 2007). Further analysis identified two PPRE sequences located between -1093 bp and -1057 bp and between -98 bp and -53 bp on the human *FGF21* promoter in which PPAR $\alpha$  and its partner RXR $\alpha$  were able to bind (Inagaki et al., 2007; Lundåsen et al., 2007). Hepatic FGF21 knockdown prevents up regulation of down stream PPAR $\alpha$  target genes central to ketogenesis and gluconeogenesis in response to prolonged fasting. Unable to clear FFA due to a disruption in  $\beta$ -oxidation and suppressed lipid transport, livers of FGF21 knockdown mice become engorged with TAG resulting in inflammation (Badman et al., 2009) (Figure 26).

### ***B/A PPAR $\alpha$ network control of FGF21 during fasting, stress and circadian rhythms***

#### **B-1/ Cooperative response during fasting**

Several nuclear receptors work in synergy with PPAR $\alpha$  to induce FGF21 action for inhibition of gluconeogenesis and to halt the production of lipids. Both the cAMP-responsive element binding protein (CREBH) and FXR are able to bind to the *Fgf21* promoter synergistically stimulating *Fgf21* expression in a PPAR $\alpha$  dependent manner (Cyphert et al., 2012; Girer et al., 2016; Kim et al., 2014; Zhang et al., 2012a). In addition to lipid regulation, CREBH has been shown to promote gluconeogenesis during fasting by regulating key glycogenic genes including *Pepck* and *G6pase*. During fasting, build-up of toxic bile acids in the liver activate FXR. Metabolic action

**Figure 25:**

**FGF21's analog LY2405319 effect across species**

**(A) Mouse studies of diet induced and genetic obesity**



- ↓ Plasma Glucose
- ↓ Body weight
- ↓ Non-alcoholic steatohepatitis
- ↑ Life span
- ↑ Glucose tolerance

**(B) Non-human diabetic primate studies**



- ↓ Plasma glucose
- ↓ Body weight
- ↓ Triglycerides
- ↑ HDL
- ↑ Glucose tolerance

**(C) Clinical trials for obesity and T2D**

- ▬ Plasma glucose
- ↓ Body weight
- ↓ Triglycerides
- ↑ HDL
- ↑ Glucose tolerance

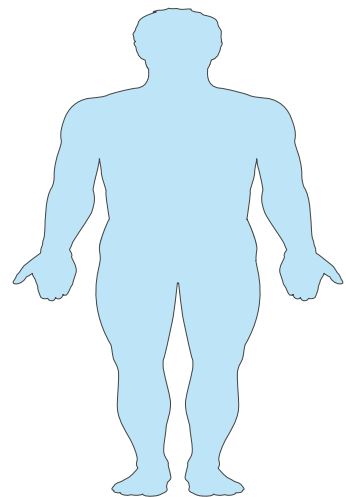


Figure 25: (A) Results from i) diet-induced obese (DIO) male C57/BL6 mice, ii) male *ob/ob* mice given subcutaneous injections for two weeks of LY2405319 (Kharitononkov et al., 2013), iii) Methionine and choline deficient mice fed LY240531 for 4 weeks (Lee et al. 2016). (B) Results from daily subcutaneous injections for a total of 7 weeks to hyperinsulinemic and obese Rhesus monkeys (Adams et a., 2013). (C) Results from daily subcutaneous injections for 28 days to obese and type 2 diabetes patients (Gaich et al. 2013).

of FXR is primarily exerted via FGF19 which activates phosphorylation of the MAP kinase signaling cascades down regulating *Cyp7a1*, the rate limiting enzyme for bile acid production (Kliwer and Mangelsdorf, 2015). However, experiment completed in mice fed a high-fat low-carbohydrate diet reported that FXR was able to bind to the *Fgf21* promoter which includes a FXR binding domain, FXR response element (-1231 bp to -1196 bp). It is suggested that the beneficial effects observed by treatment of mice with a FXR agonist, such as improved insulin sensitivity and reduced hypertriglyceridemia (Thomas et al., 2008), are in part due to FGF21 (Cyphert et al., 2012). Future experiments using FXR agonist in hepatocyte-specific FGF21 knockout mice would help differentiate the independent roles of FGF19 and FGF21.

Glucocorticoids work in synergy with PPAR $\alpha$  in order to sustain gluconeogenesis during stress (Liang et al., 2014; Marino et al., 2016). Glucocorticoids activate FGF21 via the glucocorticoid receptor that is able to bind directly to the DR3 region (+4388 bp and +4374 bp) of the *Fgf21* promoter (Patel et al., 2014). This creates a feed-forward loop in which FGF21 endocrine action in the brain further promotes production of glucocorticoids. Binding of FGF21 to its co receptors FGFR1-c and  $\beta$ -klotho on the hypothalamus activates the ERK1/2 signaling pathway propagating release of corticotropin-releasing hormone (CRH) into the hypophyseal portal system connected to the anterior pituitary (Liang et al., 2014). Once in the anterior pituitary CRH stimulates release of adrenocorticotrophic hormone (ACTH) (Lehnert et al., 1998). The neurohormone ACTH activates production and secretion of the glucocorticoid cortisol from the adrenal glands that can then target metabolically active tissues promoting the breakdown of energy stores and also circulating to the liver to activate glucocorticoid receptor, positively driving FGF21 production (Swaab et al., 2005).

Figure 26:

## Structure of the FGF21 mouse promoter

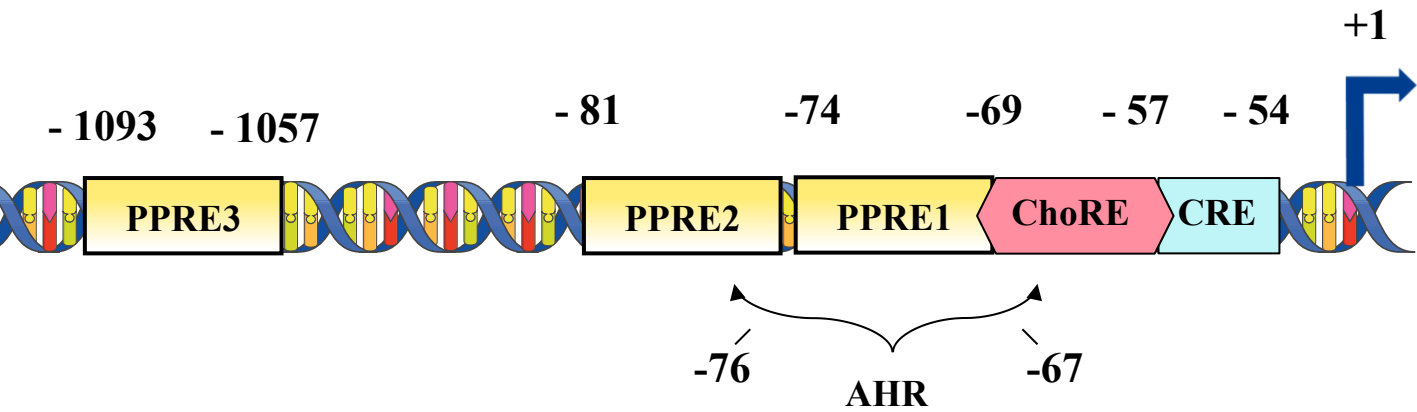


Figure 26: The FGF21 mouse promoter has three PPRE. One distal, PPRE3, (-1093 to -1057) and two proximal PPRE 2 (-81 to -76) and PPRE 1 (-74 to -69). Overlapping the PPRE1 is a ChoRE region from (-72 to -52). The CREB binding site (CRE) overlaps the ChoRE region at (-58 to -54). This region is highly conserved across species. An AHR region important for FGF21 inhibition can also be found over lapping the PPRE1 PPRE2 and ChoRE region from (-76 to -67).

In addition to nutritional signals and stress the CNS is able to regulate FGF21 expression in a circadian manner. FGF21 plasma concentrations diurnally peak during early morning hours and again in the afternoon in healthy human males (Yu et al., 2011). The rhythmic expression of *FGF21* is regulated through two key transcription factors of the central circadian clock, retinoic acid receptor-related orphan receptors (ROR $\alpha$ ) and reverse erythroblastosis virus  $\alpha$  (Rev-ERB $\alpha$ ) (Estall et al., 2009; Wang et al., 2010). Located in the SCN, the central circadian clock prepares the body to respond to perceived environmental factors such as sunlight, temperature and food availability (Delezie et al., 2016; Solt et al., 2011). Several circadian clock targets have been linked to coordinating energy metabolism including leptin, cortisol and FGF21 (Andersen et al., 2011; Kalra et al., 2003; Weitzman et al., 1971). ROR $\alpha$  and Rev-ERB $\alpha$  both bind the RORS binding domain to regulate gene transcription by alternating promoter occupancy of their target genes (Froy, 2009). The nocturnal rise of sterols, the endogenous ROR $\alpha$  ligand, activates FGF21 expression and secretion by direct ROR $\alpha$  binding to the ROR binding domain (-1223 bp to -1213 bp) in a PPAR $\alpha$  dependent manner (Oishi et al., 2008; Wang et al., 2010). Alternately, *Fgf21* is suppressed by Rev-ERB $\alpha$  binding to the same R1 binding domain as ROR $\alpha$ . Strongly regulated by clock genes Rev-ERB $\alpha$  is able to suppress activation of FGF21 by binding RORS and recruiting HDAC3 and co repressors hepatic nuclear factor 6 (HNF6) and NCoR1 (Chavan et al., 2016; Estall et al., 2009). This oscillation in both ROR $\alpha$  and Rev-ERB $\alpha$  activity on the *Fgf21* promoter is suggested to allow for the preparation of lipid oxidation and transport important during anticipated fasting and feasting periods.

## B-2/ PPAR $\alpha$ independent regulation of FGF21

Independently of PPAR $\alpha$ , reports have documented that FGF21 is up regulated by activation of the glucagon receptor (Arafat et al., 2013). These findings supported earlier observation of increased FGF21 protein levels in response to glucagon in humans (Arafat et al.,

2013; Habegger et al., 2013). Whole body deletion of the glucagon receptor suppresses the ability of glucagon to increase hepatic *Fgf21* mRNA expression through the AMPK pathway during fasting (Cyphert et al., 2014). However, the fact that FGF21 is absent in the plasma of fasted hepatic PPAR $\alpha$  knockout mice (Montagner et al., 2016) suggests that glucagon may work by a posttranslational mechanism and that FGF21 is an important mediator of the biological activity of glycogen (Cyphert et al., 2014).

### B-3/ Repression of the *fgf21* promoter

Direct suppression of the *Fgf21* promoter is mediated by the recruitment of the HDAC3 and by co repressors LXR $\beta$  and which binds directly to the *Fgf21* promoter upon cholesterol feeding or treatment with an LXR agonist, respectively (Archer et al., 2012; Uebanso et al., 2012). LXR $\beta$ , acting as a cholesterol sensor, heterodimerizes RXR $\alpha$  and binds within the LXR response element located on the human *FGF21* promoter at (-388 bp to -372 bp) (Uebanso et al., 2012). In addition LXR $\beta$  agonist GW3965, enhanced recruitment of the co repressors complex HDAC3, Rev-erb $\alpha$  and ROR $\alpha$ . Rev-erb $\alpha$  is tightly regulated by circadian rhythms and cited for the circadian suppression of FGF21 (Estall et al., 2009). However, deletion of LXR $\beta$  in mice blunted binding of the HDAC3, Rev-erb $\alpha$ /ROR $\alpha$  complex to the ROR response element region of the *Fgf21* promoter (-93 bp to -63 bp mouse), suggesting that LXR $\beta$  is required for HDAC3 mediated suppression of FGF21 in response to LXR $\beta$  agonist (Archer et al., 2012). The role of LXR $\beta$  during circadian suppression of *Fgf21* was not detailed. A recent report has established the importance of PPAR $\alpha$  presence on the *Fgf21* promoter for the suppression of HDAC3 as PPAR $\alpha$  knockout mice display a 10-fold increase in HDAC3 binding (Rando et al., 2016).

## ***C/Up-regulation of FGF21 by glucose and fructose via ChREBP***

Interestingly, novel work has reported that FGF21 is also activated in response to glucose and fructose in rodents and in humans (Herman et al., 2012; Iizuka et al., 2009; Sánchez et al., 2009; Uebanso et al., 2011). In the liver, the transcriptional response to dietary carbohydrates is mediated through ChREBP. The identification of the ChREBP binding region, ChoRE, on the FGF21 promoter in both mice (-74 to -52 bp) and humans (-380 to -366 bp) (Iizuka et al., 2009; Uebanso et al., 2011) identified FGF21 as a direct ChREBP target gene. The physiological significance of ChREBP-induced FGF21 was brought to light by a series of recent publications which reported that ChREBP-induced FGF21 improves whole body glucose homeostasis by targeting the paraventricular nucleus of the hypothalamus blocking sugar-seeking behavior and intake of mice (von Holstein-Rathlou et al., 2016; Talukdar et al., 2016) (Figure 22).

## **Conclusion of Part 5**

FGF21 is a hepatokine that acts directly on the white adipose tissue, brown adipose tissue, CNS and liver to improve hepatic insulin sensitivity, reduce glucose output and correct dyslipidemia. Up regulated by PPAR $\alpha$  during fasting, FGF21 is a stress hormone, and is regulated by several other stress factors in a PPAR $\alpha$  dependent manner. Recently it has been shown that in addition to fasting and stress, carbohydrate overload induces FGF21 via direct ChREBP binding.





***RESULTS***

***RESEARCH SUBJECT 1***



## ***Research Objectives:***

There is a current need for effective Type 2 diabetes and NAFLD/NASH therapies. Understanding the elaborate inter-organ network and the regulation of proteins secreted by the liver in response to injury could identify key hormones (hepatokines) that protect against the life threatening consequences of these metabolic disorders. The current report includes two distinct research subjects. Research subject 1 addresses the reciprocal role of two seemingly opposing transcription factors, ChREBP and PPAR $\alpha$ , in the regulation of the hepatokine FGF21. Research subject 2 reflects on previous publications from the laboratory, which has shown that hepatic ChREBP over expression has beneficial effects on glucose metabolism and insulin resistance (Benhamed et al., 2012). The research subject 2 was to identify the role of hepatic ChREBP-mediated FGF21 expression in  $\beta$ -cell signaling and glucose tolerance.

The objectives of the current work are as followed:

- 1.) Determine if ChREBP directly regulates FGF21 in response to glucose in vivo and in primary hepatocytes.
- 2.) Determine whether a cross-talk between ChREBP and PPAR $\alpha$  exists for the regulation of FGF21 in response to high glucose.
- 3.) Identify the physiological significance of ChREBP-mediated FGF21 secretion on FGF21 signaling in pancreatic INS1 cells and glucose homeostasis.

# ***Research Subject 1: A specific ChREBP and PPAR $\alpha$ cross-talk is required for the glucose-mediated FGF21 response***

## ***I-Project background***

Discovery of the hepatokine FGF21 and its effect on glucose homeostasis has highlighted the importance of the liver in development of novel diabetic therapies (Kharitononkov and Adams, 2014). While the physiological benefits of FGF21 have been documented, little information is available on the regulation of FGF21 in the context of glucose overload. New developments have reported the importance of the glucose sensitive transcription factor ChREBP for the regulation of FGF21 in response to glucose (Uebanso et al., 2011). Interestingly, a physiological role for the ChREBP-FGF21 axis was recently revealed in experiments showing that, in response to sugar consumption, ChREBP-enhanced FGF21 secretion from the liver blocked sugar-seeking behavior in mice and primates by targeting the paraventricular nucleus of the hypothalamus (von Holstein-Rathlou et al., 2016; Talukdar et al., 2016a).

FGF21 is mostly known to be a direct target of the nuclear transcription factor PPAR $\alpha$  in response to fasting (Badman et al., 2007b; Lundåsen et al., 2007). Understanding the mechanism by which FGF21 is transcriptionally regulated in liver in response to the nutritional status is currently an important research point. One of the challenges of understanding FGF21 regulation is differentiating the role of two assumingly contradictory transcription factors PPAR $\alpha$  and ChREBP, which act during fasting and carbohydrate feeding, respectively. Few publications have proposed a ChREBP-PPAR $\alpha$  dialogue for the coordination of energy metabolism, but some

examples of cross-talk and regulatory mechanisms have been described for lipid metabolism in brown adipose (Iizuka et al., 2013) and for function of pancreatic  $\beta$  cells (Boergesen et al., 2011).

**Hypothesis:**

- Is FGF21 is strictly dependent on ChREBP control in response to a glucose stimulus?
- Could ChREBP and PPAR $\alpha$  be mutually required for the glucose-mediated FGF21 response?

## **Major results**

We have identified a major transcriptional node in the control of hepatic FGF21 in response to glucose. Microarray analysis in liver reveals that only few genes respond in the same unique fashion as FGF21 to fasting and carbohydrate feeding. Our study shows that PPAR $\alpha$  is required together with ChREBP, to balance the glucose-mediated FGF21 response. *In vitro* and *in vivo* studies reported lack of FGF21 in ChREBP<sup>-/-</sup> mice. Rescue of ChREBP activity in liver of ChREBP<sup>-/-</sup> mice through adenoviral gene transfer fully restored FGF21 expression and secretion in these mice. Unexpectedly, carbohydrate challenge of liver specific PPAR $\alpha$  knockout mice, demonstrates a PPAR $\alpha$ -dependent glucose-mediated FGF21 response. *In vivo* CHIP experiments reveal that in the absence of PPAR $\alpha$ , ChREBP binding to the *Fgf21* ChoRE binding site is decreased demonstrating, for the first time, a PPAR $\alpha$ -ChREBP crosstalk in response to glucose. Stimulation of mouse and human primary hepatocytes with a combination of glucose and Wy-14643, a PPAR $\alpha$  agonist, resulted in a synergetic effect on *Fgf21* mRNA. Physiologically, decreased circulating FGF21 levels in liver specific PPAR $\alpha$  knockout mice led to increased sucrose preference.

## **Conclusion**

Altogether, our study reveals that the glucose sensor ChREBP requires PPAR $\alpha$  for the induction of FGF21 in response to dietary sugar. This is a unique collaboration between transcription factors that are activated in response to distinct nutritional conditions, high glucose for ChREBP versus fasting for PPAR $\alpha$  (Figure 27). This article was submitted to Cell Reports on December 20<sup>th</sup> 2016 and is currently under revision.

Figure 27:

## A ChREBP and PPAR $\alpha$ cross-talk is required for the glucose-mediated FGF21 response

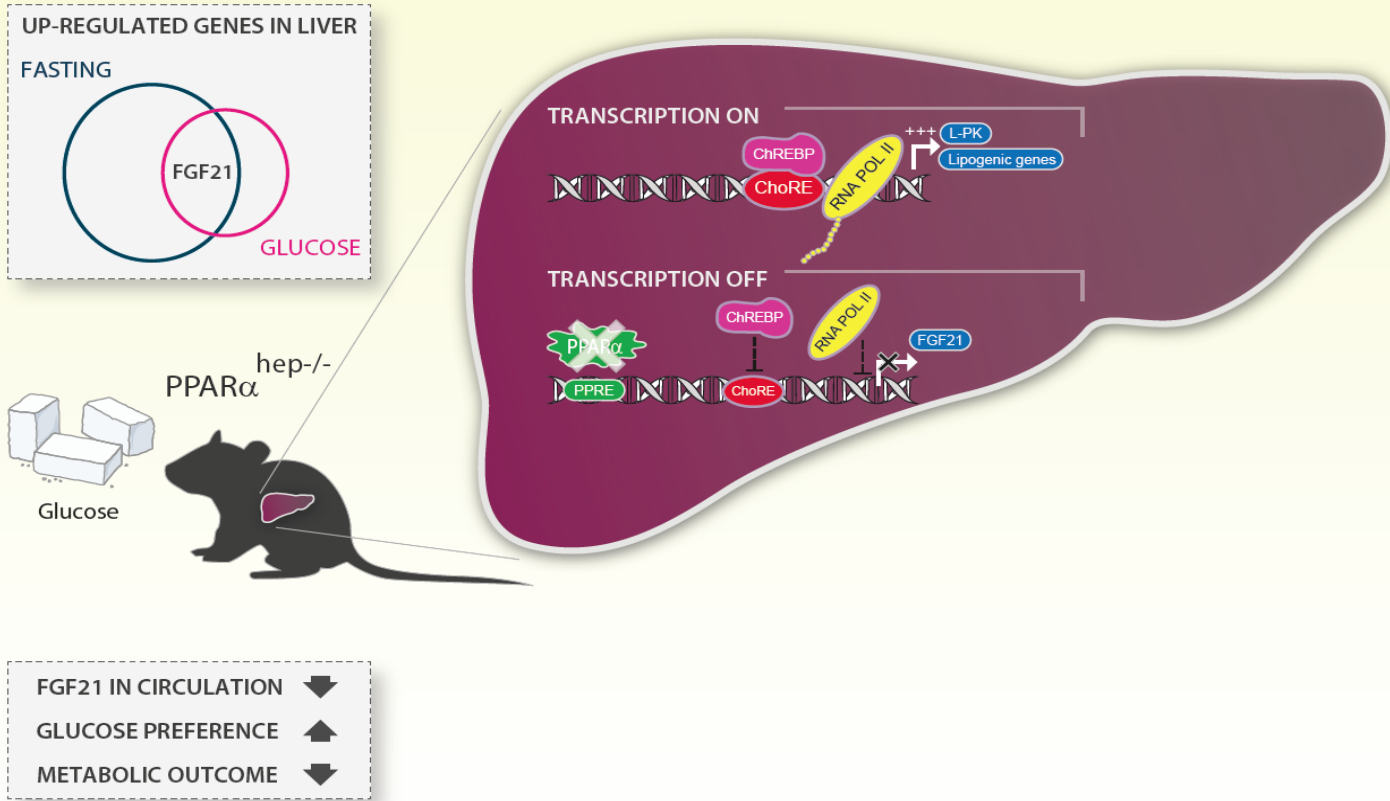


Figure 27: *Fgf21* is up regulated in response to both fasting and feeding signals. In response to carbohydrate intake ChREBP is responsible for the direct up regulation of FGF21 by binding to its promoter. However, ChREBP-mediated regulation of FGF21 is PPAR $\alpha$  dependent as the the FGF21 response to carbohydrate intake is lost in liver specific PPAR $\alpha$  knockout mice.





***RESEARCH ARTICLE***



**A specific ChREBP and PPAR $\alpha$  cross-talk is  
required for the glucose-mediated FGF21 response**

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**Abbreviations used are:** ACC, Acetyl CoA carboxylase, Acox1, Acyl-CoA oxidase 1; ChIP, chromatin immunoprecipitation; ChoRE, carbohydrate responsive element; ChREBP, carbohydrate responsive element binding protein; FAS, fatty acid synthase; FGF21, Fibroblast Growth Factor 21; G6Pase, glucose-6-phosphatase; GFP, green fluorescent protein; GRACE, glucose responsive activation conserved element; HDAC3, histone deacetylase; LID, low glucose inhibitory domain; L-PK, L-pyruvate kinase; NAFLD, non-alcoholic fatty liver disease; PGC1 $\beta$ , peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\beta$ ; Pol II, Polymerase II; PPAR $\alpha$ , peroxisome proliferator-activated receptor alpha; PPAR $\gamma$ , peroxisome proliferator-activated receptor gamma; PPRE, peroxisome proliferator response element; Scd1, Stearoyl CoA desaturase.

## Summary

Discovery of the hepatokine FGF21 and its effect on glucose homeostasis has highlighted the importance of the liver in development of novel anti-diabetic therapies. While the physiological benefits of FGF21 in response to fasting have been documented, little information is available on the regulation of FGF21 in the context of glucose overload. New developments have reported the importance of the glucose sensitive transcription factor ChREBP for the regulation of FGF21 in response to glucose and fructose. Microarray analysis in liver confirms that few genes respond in the same unique fashion as FGF21 to fasting and glucose feeding. Here we report that PPAR $\alpha$ , a key nuclear receptor for the adaptive fasting response, is required together with ChREBP, to balance the glucose-mediated FGF21 response. *In vitro* and *in vivo* analyses reported lack of FGF21 in ChREBP<sup>-/-</sup> mice. Rescue of ChREBP activity in liver of ChREBP<sup>-/-</sup> mice through adenoviral gene transfer fully restored FGF21 expression and secretion in these mice. Unexpectedly, carbohydrate challenge of liver specific PPAR $\alpha$  knockout mice, demonstrates a PPAR $\alpha$ -dependent glucose-mediated FGF21 response associated with increased sucrose preference. *In vivo* CHIP experiments reveal that in the absence of PPAR $\alpha$ , ChREBP binding to the *Fgf21* ChoRE binding site is decreased demonstrating, for the first time, a PPAR $\alpha$ -ChREBP crosstalk in response to glucose. Stimulation of mouse and human primary hepatocytes with a combination of glucose and Wy-14643, a PPAR $\alpha$  agonist, resulted in a positive synergetic increase of *Fgf21* mRNA. In conclusion, our study reports that ChREBP and PPAR $\alpha$  co-dependently regulate the glucose-mediated induction of FGF21.

## Introduction

The liver has been exclusively considered an organ involved in the uptake and release of glucose. Recently a new function of the liver has emerged, the production of hepatokines, particularly FGF21, coordinating energy homeostasis. Encompassing numerous metabolic effects, FGF21 is currently a promising target for the development of metabolic therapies (Kharitononkov and Adams, 2014). Originally targeted for its glucose lowering properties in both rodents and primates (Berglund et al., 2009; Fisher et al., 2011; Kharitononkov et al., 2007), FGF21 secreted by the liver is able to improve insulin sensitivity, lipid homeostasis, reduce arterial blood pressure and induce weight loss (Coskun et al., 2008; Markan et al., 2014).

FGF21 is known to be a direct target of the nuclear transcription factor Peroxisome Proliferator-Activated Receptor  $\alpha$  (PPAR $\alpha$ ) in response to fasting (Badman et al., 2007; Lundåsen et al., 2007). Activated by free fatty acids derived from lipolysis (Jaeger et al., 2015; Montagner et al., 2016), PPAR $\alpha$  is essential to liver health as its deletion promotes the development of non-alcoholic fatty liver disease (NAFLD) and hypercholesterolemia during ageing (Montagner et al., 2016). The observed beneficial role of PPAR $\alpha$  in response to dyslipidemia is thought to be mediated, at least in part, through FGF21 (Ong et al., 2012). Indeed, anti-diabetic therapies and PPAR $\alpha$  agonists such as Fenofibrate or Wy-14653 significantly induce liver derived circulatory FGF21 in both mouse and human plasma (Christodoulides et al., 2009; Gälman et al., 2008; Lundåsen et al., 2007).

Hepatocyte PPAR $\alpha$  plays an essential role during fasting, which triggers strong transcriptional regulation for the maintenance of glycemia and the production of ketone

bodies through fatty acid catabolism for use as an alternative energy source. In this process, several transcription factors sense the hormonal and metabolic challenges that occur in food-deprived animals (Goldstein and Hager, 2015). Hepatocyte PPAR $\alpha$  has been shown to play a major role in this adaptation, as PPAR $\alpha$ -deficient mice exhibit impaired fatty acid oxidation and ketogenesis that promotes hepatic steatosis during fasting (Kersten et al., 1999; Kroetz et al., 1998; Leone et al., 1999; Montagner et al., 2016).

Interestingly, novel work has reported that, in addition to PPAR $\alpha$  and its pharmacological agonists, FGF21 is also activated in response to glucose and fructose in rodents and in humans (Herman et al., 2012; Iizuka et al., 2009; Sánchez et al., 2009; Uebanso et al., 2011). Highly enriched in the liver, the transcription factor Carbohydrate Responsive Element Binding Protein (ChREBP), mediates the hepatic response to dietary carbohydrates. The ChREBP protein contains a low glucose inhibitory domain (LID) and a glucose responsive activation conserved element (GRACE) located in its N-terminus (Li et al., 2006). Activation of the GRACE domain by glucose promotes ChREBP transcriptional activity and binding to the ChoRE element of its target genes including L-pyruvate kinase (*L-pk*), a rate-limiting enzyme in glycolysis, Fatty acid synthase (*Fas*), Acetyl CoA carboxylase (*Acc*) or Steroyl CoA desaturase (*Scd1*), key enzymes of *de novo* lipogenesis (Kawaguchi et al., 2001). Recently, another isoform of ChREBP, ChREBP $\beta$ , originating from an alternative first exon promoter, was identified in adipose tissue and in liver (Herman et al., 2012). This alternative splicing results in a constitutively active and potent ChREBP isoform lacking the LID domain that is associated with inhibition of ChREBP activity (Herman et al., 2012). A physiological role for the ChREBP-FGF21 axis was recently revealed in experiments showing that, in response to sugar consumption,

ChREBP-enhanced FGF21 secretion from the liver blocked sugar-seeking behavior in mice and primates by targeting the paraventricular nucleus of the hypothalamus (Talukdar et al., 2016; von Holstein-Rathlou et al., 2016).

Understanding the mechanism by which FGF21 is regulated is currently a research focal point. One of the challenges of understanding FGF21 regulation is differentiating the role of two assumingly contradictory transcription factors PPAR $\alpha$  and ChREBP, which act during fasting and carbohydrate feeding, respectively. Few publications have proposed a ChREBP-PPAR $\alpha$  dialogue for the coordination of energy metabolism, but some examples of cross-talk and regulatory mechanisms have been described for lipid metabolism in brown adipose (Iizuka et al., 2013) and for function of pancreatic  $\beta$  cells (Boergesen et al., 2011). Through the use of ChREBP knockout mice, we report here that ChREBP is required for the expression and secretion of hepatic FGF21 in response to carbohydrate intake. Inversely, studies in hepatocyte-specific PPAR $\alpha$  knockout mice demonstrate a novel physiological role for PPAR $\alpha$  in the context of glucose challenge, as ChREBP is unable to induce FGF21 in the absence of hepatic PPAR $\alpha$ . Altogether, our results suggest that FGF21's glucose-mediated response is dependent on both ChREBP and PPAR $\alpha$ .



## Results

**FGF21 is strongly induced by both fasting and after a glucose challenge.** To characterize gene expression during fasting and glucose challenge, a microarray analysis was conducted using liver samples from wild-type mice (Fig.1). The expression of genes sensitive to Fasting or Glucose and markedly different from the Fed group were incorporated into a heat map (Fig. 1a). This illustrates that fasting and glucose induce different transcriptional changes. Sixty-seven genes were significantly induced by Glucose in comparison to Fed (Log FC>1) and 675 genes were significantly different between Fed and the Fasted groups (Log FC>1). Gene ontology analysis revealed that pathways identified as specifically impacted by Glucose and not by Fasting are involved in pyruvate and insulin-sensitive metabolism (Fig. 1b). Gene ontology revealed that pathways specifically sensitive to fasting and not to glucose, are involved in PPAR signaling (Fig. 1c). Therefore, Glucose and Fasting induce different transcriptional responses that control distinct metabolic pathways. However, among the genes significantly induced (Log FC>1;  $p \leq 0.01$ ) by either Glucose or Fasting 3 genes (*Fgf21*, *Fut1*, *Atf5*) were significantly up-regulated in both the Fasted and Glucose groups as compared to Fed mice (Fig. 1d). Moreover, when the stringency of the selection was increased to a Log FC>2 (Fig. 1e), *Fgf21* was left to be the sole gene up-regulated by both fasting and Glucose Challenge, (Log FC>3,9 and Log FC>3,7 respectively ( $p \leq 0.01$ )). In support of our results the *Fgf21* promoter contains overlapping PPRE and ChoRE binding units (-88 to -54bp) suggesting a potential cross-talk between the two paradoxical transcription factors (Girer et al., 2016; Uebanso et al., 2011). In order to define the mechanism by which FGF21 is regulated by carbohydrate signaling experiments were designed to explore the role of ChREBP and PPAR $\alpha$  in the glucose-mediated FGF21 response.

**ChREBP is necessary for glucose mediated expression and secretion of hepatic FGF21.** Experiments in mouse primary hepatocytes first confirmed that glucose positively induced ChREBP recruitment onto the *Fgf21* promoter leading to its increased expression and secretion (Fig. S1a-h). To determine if ChREBP recruitment was mandatory for up-regulation of FGF21 in response to glucose, similar experiments were completed in hepatocytes lacking ChREBP (ChREBP<sup>-/-</sup>) (Fig.2). Mice lacking exon 9 through 15 of the *ChREBP* gene were generated through homologous recombination as described in the *Experimental procedures* section (Fig. S2). The primers used to measure *ChREBP* expression were designed to detect the two ChREBP isoforms,  $\alpha$  and  $\beta$ . A specific primer to detect only the  $\beta$  isoform was also used (Supplemental Table 1). Absence of the ChREBP protein ( $\alpha$  isoform, 94 kDa) in ChREBP<sup>-/-</sup> hepatocytes was validated by Western Blot (Fig.2a). Under these conditions, the ChREBP  $\beta$  protein (72 kDa) could not be detected (*not shown*). Similarly to *ChREBP*, *ChREBP*  $\beta$  and target genes *L-pk* and *Scd1*, *Fgf21* robustly responded to 25 mM glucose stimulation in wild-type hepatocytes, however, this response was lost in *ChREBP*<sup>-/-</sup> hepatocytes (Fig. 2b-f). Furthermore, while FGF21 protein in culture medium collected from *ChREBP*<sup>+/+</sup> hepatocytes exposed to 25 mM glucose reached concentrations of 600 pg/ml, FGF21 concentration in medium from ChREBP<sup>-/-</sup> hepatocytes remained low averaging 40 pg/ml (Fig. 2g). These findings report that ChREBP is mandatory for the glucose-mediated expression and secretion of FGF21 by hepatocytes.

**FGF21 dose not respond to glucose challenge *in vivo* in the absence of ChREBP.** To complement our *in vitro* experiment, ChREBP<sup>+/+</sup> and ChREBP<sup>-/-</sup> mice were given 24-hour access to a bottle of glucose-free water (Fed) or a bottle containing a 20% Glucose (Glucose Challenge) (Fig. 3). Blood glucose was similar between Fed ChREBP<sup>+/+</sup> and Fed

ChREBP<sup>-/-</sup> mice. While blood glucose tended to increase in glucose-challenged ChREBP<sup>+/+</sup> mice a significant elevation in blood glucose was observed in glucose-challenged ChREBP<sup>-/-</sup> mice (Fig. 3a). ChREBP protein was not detectable in protein extracted from liver tissue in ChREBP<sup>-/-</sup> mice (Fig. 3b). In response to glucose, a significant increase in *ChREBP*, *ChREBP β*, *L-pk* and *Scd1* mRNA expression was observed in liver of ChREBP<sup>+/+</sup> mice compared to fed ChREBP<sup>+/+</sup> mice. In the absence of ChREBP, *L-pk* and *Scd1* mRNA levels were significantly diminished in liver of glucose-challenged ChREBP<sup>-/-</sup> mice as compared to ChREBP<sup>+/+</sup> (Fig. 3 c-f). Consistent with results reported *in vitro*, *Fgf21* mRNA increased in glucose-challenged ChREBP<sup>+/+</sup> mice, however, this response was lost in ChREBP<sup>-/-</sup> mice despite elevated blood glucose (Fig. 3g). Similarly, plasmatic FGF21 protein concentrations were increased in glucose-challenged ChREBP<sup>+/+</sup> mice while low levels were detected in ChREBP<sup>-/-</sup> mice under similar conditions (Fig. 3h). Altogether, our results reveal that in a context of glucose overload ChREBP activity is necessary for FGF21 expression and production.

**Liver specific active ChREBP expression rescues FGF21 plasma concentrations in ChREBP knockout mice.** We next determined whether liver-specific re-expression of ChREBP activity in a context of global ChREBP deficiency could rescue FGF21 expression and production (Fig. 4). ChREBP<sup>+/+</sup> and ChREBP<sup>-/-</sup> mice were injected with an adenovirus vehicle containing the GFP protein or a truncated isoform of ChREBP lacking the LID domain (Li et al., 2006) which corresponds to a constitutively active version of ChREBP (ChREBP<sup>CA</sup>). Mice were exposed to a 20% glucose solution for a 24-hour period before sacrifice. Blood glucose from GFP treated ChREBP<sup>-/-</sup> mice was higher than GFP ChREBP<sup>+/+</sup> mice (Fig. 4a). Injection of ChREBP<sup>CA</sup> to ChREBP<sup>-/-</sup> lowered blood glucose to concentrations comparable to that of controls. Western Blot analysis confirmed the

absence of native ChREBP protein while detecting ChREBP<sup>CA</sup> (72 kDa) in ChREBP<sup>-/-</sup> ChREBP<sup>CA</sup> treated mice (Fig. 4b). Analysis of liver tissue revealed that ChREBP<sup>CA</sup> rescued the expression of *ChREBP/ChREBP β* mRNA, exceeding expression levels observed in GFP treated ChREBP<sup>+/+</sup> (Fig. 4c-d). Transcriptional activity of ChREBP<sup>CA</sup> was confirmed by up-regulation of both *L-pk* and *Scd1* mRNA expression (Fig. 4e-f). Similarly to *L-pk* and *Scd1*, *Fgf21* expression was low in liver of GFP treated ChREBP<sup>-/-</sup> but was significantly restored by ChREBP<sup>CA</sup> injection in ChREBP<sup>-/-</sup> mice (Fig. 4g). Circulating concentrations of FGF21 were equally increased (6-fold) in ChREBP<sup>+/+</sup> and after ChREBP rescue (Fig. 4h). Our findings report that ChREBP<sup>CA</sup> administration rescued ChREBP activity in liver of ChREBP<sup>-/-</sup> mice and was sufficient in rescuing FGF21 gene expression and protein secretion.

**Glucose stimulation or ChREBP overexpression is unable to rescue FGF21 expression or secretion in the absence of PPAR $\alpha$ .** To investigate the impact of PPAR $\alpha$  silencing on glucose-mediated FGF21 induction, primary hepatocytes from liver-specific PPAR $\alpha$  knockout mice (PPAR $\alpha$ <sup>hep<sup>-/-</sup></sup>) and their littermates, PPAR $\alpha$ <sup>hep<sup>+/+</sup></sup>, were stimulated by glucose in a dose dependent manner (Fig. 5). A similar induction in *ChREBP*, *ChREBP β*, *L-pk* and *Scd1* mRNA was observed between PPAR $\alpha$ <sup>hep<sup>-/-</sup></sup> and PPAR $\alpha$ <sup>hep<sup>+/+</sup></sup> hepatocytes in response to increasing glucose concentrations (Fig. 5a-d). In contrast, expression and production of FGF21 in culture medium was significantly reduced in PPAR $\alpha$ <sup>hep<sup>-/-</sup></sup> hepatocytes (Fig. 5e-f). We next addressed whether ChREBP overexpression could rescue FGF21 in the absence of PPAR $\alpha$ . Hepatocytes from PPAR $\alpha$ <sup>hep<sup>-/-</sup></sup> mice and their controls, PPAR $\alpha$ <sup>hep<sup>+/+</sup></sup>, were infected with the ChREBP<sup>CA</sup> adenovirus for 24-hours. The expression of *ChREBP* and *ChREBP β* was significantly

increased in response to ChREBP<sup>CA</sup> in both PPAR $\alpha$ <sup>hep+/+</sup> and PPAR $\alpha$ <sup>hep-/-</sup> hepatocytes (Fig. 6a,b). As a result, *L-pk* and *Scd1* mRNA expression was significantly increased (Fig. 6c,d). However, while ChREBP overexpression increased *Fgf21* expression in PPAR $\alpha$ <sup>hep+/+</sup> hepatocytes, it failed to significantly induce *Fgf21* expression in PPAR $\alpha$ <sup>hep-/-</sup> hepatocytes (Fig. 6e). FGF21 measured in the medium of cell culture confirmed *Fgf21* mRNA expression profiles (Fig. 6f). These results show that ChREBP is not efficient in rescuing *Fgf21* gene expression or protein production in the absence of PPAR $\alpha$ .

### **FGF21 synergistically responds to glucose and a pharmacological PPAR $\alpha$ activator**

**in both mouse and human hepatocytes.** To determine whether ChREBP and PPAR $\alpha$  could act in synergy to regulate *Fgf21* gene expression, mouse and human hepatocytes were stimulated during 24 hours with low (5 mM) or high glucose concentrations (25 mM), in the presence of the PPAR $\alpha$  agonist Wy-14643 (10  $\mu$ M) (Fig. S3). Expression of *ChREBP* and *ChREBP*  $\beta$  mRNA confirmed a positive glucose response in both mouse and human hepatocytes (Fig. S3a,b,e,f). Activation of PPAR $\alpha$  by Wy-14643 was validated by a significant up-regulation in the expression of the PPAR $\alpha$  target gene *Acox1* (Fig. S3c, g). *Fgf21* mRNA levels were drastically increased when hepatocytes (mouse and human) were incubated in the combined presence of high glucose (25 mM) and Wy-14643 (Fig. S3d,h) Altogether, these results confirm that ChREBP and PPAR $\alpha$  can act synergistically to induce Fgf21 in mouse and in human liver cells.

### **Hepatocyte PPAR $\alpha$ is required for the FGF21 secretion in response to glucose *in***

***vivo* and for control of sweet preference.** To investigate whether PPAR $\alpha$  is involved in the glucose-mediated induction of *Fgf21*, *in vivo* a glucose challenge was performed

using PPAR $\alpha$ <sup>hep+/+</sup> and PPAR $\alpha$ <sup>hep-/-</sup> mice. Two nutritional conditions were assigned to both genotypes: i) mice fasted 24 hours with free access to water (Fasted group); ii) mice fed ad libitum with access to standard chow diet and a 20% glucose solution in water (Glucose challenge group) (Fig. 7). Before harvest, blood glucose concentrations were significantly elevated in response to glucose challenge in comparison to Fasted mice regardless of the genotype (Fig. 7a). Western Blot analysis confirmed the significant induction of ChREBP and L-PK protein levels in response to glucose in liver of both PPAR $\alpha$ <sup>hep+/+</sup> and PPAR $\alpha$ <sup>hep-/-</sup> mice (Fig. 7b). No significant differences were observed for the expression of *ChREBP*, *ChREBP*  $\beta$  and their target genes (*L-pk* and *Scd1*) in liver of PPAR $\alpha$ <sup>hep-/-</sup> versus PPAR $\alpha$ <sup>hep+/+</sup> mice (Fig. 7c-f). The up-regulation of hepatic *Fgf21* during fasting was lost in PPAR $\alpha$ <sup>hep-/-</sup> mice. In addition, the glucose-mediated induction of *Fgf21* was also significantly reduced in the absence of PPAR $\alpha$  (Fig. 7g). FGF21 in circulation matched mRNA levels in the liver revealing a significant up-regulation of FGF21 in plasma of PPAR $\alpha$ <sup>hep+/+</sup> that was significantly reduced in both Fasted and Glucose Challenged PPAR $\alpha$ <sup>hep-/-</sup> mice (Fig. 7h). Circulating FGF21 in the plasma is able to cross the blood brain barrier. It was recently demonstrated that its action on the paraventricular nucleus of the hypothalamus blocked sugar-seeking behavior and intake of mice (Talukdar et al., 2016; von Holstein-Rathlou et al., 2016). Using a similar protocol, we investigated sucrose preference in liver specific PPAR $\alpha$  knockout mice. PPAR $\alpha$ <sup>hep+/+</sup> and PPAR $\alpha$ <sup>hep-/-</sup> mice were given free choice between a bottle containing a 10 % sucrose solution or water. Consumption was measured daily during three days which demonstrated that PPAR $\alpha$ <sup>hep-/-</sup> mice consumed 30% more sucrose solution than PPAR $\alpha$ <sup>hep+/+</sup> mice while water intake remained similar between genotypes (Fig. 7i).

**ChREBP and PPAR $\alpha$  work in concert to regulate transcriptional of *Fgf21* in response to elevated blood glucose.** Since *Fgf21* is an unusual gene regulated by fasting and high glucose, co-dependently by PPAR $\alpha$  and ChREBP respectively, we questioned whether a specific cross-talk between the two transcription factors may control *Fgf21* expression. CHIP experiments were completed on Fasted and Glucose Challenged PPAR $\alpha^{\text{hep}+/+}$  and PPAR $\alpha^{\text{hep}^-/-}$  mice. ChREBP binding onto the *L-pk* and *Fgf21* mouse promoter ChoRE was analyzed (Fig.7 j,k). Presence of Polymerase II (Pol II) on the *L-pk* promoter agreed with mRNA expression of *L-pk*, as it was significantly elevated in response to glucose and comparable in both Glucose groups (Fig. 7j). Interestingly, recruitment of ChREBP onto the ChoRE of the *Fgf21* promoter was significantly reduced in liver of PPAR $\alpha^{\text{hep}^-/-}$  Glucose Challenged mice. While Pol II recruitment was most elevated in Fasted PPAR $\alpha^{\text{hep}+/+}$  mice it was lower in Glucose PPAR $\alpha^{\text{hep}+/+}$  mice and almost undetectable in Glucose PPAR $\alpha^{\text{hep}^-/-}$  mice (Fig. 7k).

## Discussion

Characterizing the regulation of FGF21 in liver has been complex due to the paradoxical regulation of this key hepatokine by fasting and glucose signals. In the current study we uncover a novel cross-talk between ChREBP and PPAR $\alpha$  for the induction of hepatic FGF21 in response to a glucose challenge. The main finding of our study is that hepatic PPAR $\alpha$  is necessary for the glucose-mediated induction of *Fgf21* by the transcription factor ChREBP.

Key to  $\beta$ -oxidation and ketogenesis, FGF21 mobilizes energy in the liver for peripheral use protecting against dyslipidemia and hepatosteatosis (Inagaki et al., 2007; Potthoff et al., 2009). First described as a fasting hormone regulated by PPAR $\alpha$ , transcriptional regulation of FGF21 in the context of excessive blood glucose has only recently been explored despite early identification of the glucose sensing region, ChoRE, on the *Fgf21* promoter in both mouse (-74 to 52 bp) and human (-380 to -366 bp) (Iizuka et al., 2009). Microarray analysis highlights the gene uniqueness of *Fgf21*'s flexibility to respond to distinct nutritional challenges including fasting and carbohydrate signaling. Our experiments confirm that FGF21 is significantly expressed and released by cultured mouse and human hepatocytes in response to glucose upon ChREBP binding to the ChoRE (Iizuka et al., 2009; Uebanso et al., 2011). Following clues from the microarray, we hypothesized the existence of a cross-talk between the two transcription factors for the regulation of *Fgf21* in response to glucose.

Our study, through several approaches, underlines the importance of ChREBP for the glucose-induced regulation of FGF21. CHIP experiments show that ChREBP directly



binds to the ChoRE region of the *Fgf21* promoter. We also demonstrate that deletion of ChREBP completely abolished both transcription and secretion of hepatic FGF21 in response to glucose *in vitro* and *in vivo*. Importantly, hepatic rescue of ChREBP in global ChREBP knockout mice was sufficient to restore *Fgf21* mRNA in the liver and protein in circulation demonstrating the specificity of hepatic ChREBP activity on the induction of FGF21 in response to glucose challenge. Interestingly, ChREBP rescue in liver of ChREBP<sup>-/-</sup> mice significantly normalized blood glucose concentrations to control levels. This suggests that hepatic ChREBP activity and not peripheral ChREBP is essential for glucose homeostasis maintenance. This result is consistent with a previous publication in which hepatic ChREBP over-expression improved impaired glucose tolerance of high fat diet challenged mice (Benhamed et al., 2012). However, the role of ChREBP in normalizing blood glucose seems paradoxical since it was recently shown that ChREBP activation by fructose regulates glucose-6-phosphatase (G6Pase) expression and in turn increases hepatic glucose production, aggravating hyperglycemia (Kim et al., 2016). The contribution of ChREBP to whole body energy balance may lie in its regulation of lipid species and/or hepatokine production that could contribute to inter organ coordination of lipid storage.

Activated in response to free fatty acids liberated from adipocytes during fasting, a role for PPAR $\alpha$  in response to glucose has not yet been identified. Unexpectedly, despite normal hepatic ChREBP activity, we report that liver-specific PPAR $\alpha$  is necessary for ChREBP induction of FGF21 in response to glucose. In the absence of PPAR $\alpha$ , ChREBP binding to the *Fgf21* ChoRE is significantly reduced, in turn blunting the FGF21 inhibitory response on sucrose intake. Further studies of chronic glucose challenge in the context of PPAR $\alpha$  deficiency could reveal the importance of the glucose-PPAR $\alpha$ -

FGF21 axis in energy metabolism. One possible lead to explain decreased binding of ChREBP in liver of glucose-challenged PPAR $\alpha$  knockout mice could be linked to decreased *Fgf21* promoter accessibility and/or reduced histone acetylation. The fact that Pol II recruitment at the *Fgf21* promoter locus was also decreased under glucose conditions and PPAR $\alpha$  deficiency may be in favor of this hypothesis. Interestingly, it was recently reported that, in the absence of PPAR $\alpha$ , the histone deacetylase HDAC3 prevents transcription at the *Fgf21* locus in liver (Rando et al., 2016). The inhibition of HDAC3 in a context of PPAR $\alpha$  deficiency could help clarify the potential role of this histone deacetylase in the glucose-mediated induction of *Fgf21*.

Importantly, our study reveals that the synergic induction of *Fgf21* by glucose and the PPAR $\alpha$  agonist Wy-14643 also occurs in human hepatocytes. We hypothesize that this cross-talk is specific to FGF21 as we found no effect on other ChREBP target genes including *L-pk* or *Scd1*. The specificity of their interaction may be due to the proximity of their promoter regions, as both a peroxisome proliferator response element (PPRE) and a ChoRE region coexist in the proximal region of the *Fgf21* promoter (-88 to -54 bp) (Girer et al., 2016). Microarray analysis comparing the regulation of genes during fasting and feeding highlight a small subset of genes that are up-regulated both in fasting and glucose overload. Of those genes *Fgf21* is the most significantly induced. It is not the first example of a link between the hepatic lipid regulators ChREBP and PPAR $\alpha$ . In the liver, it was previously described that the PPAR $\alpha$  co-factor, peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\beta$  (PGC-1 $\beta$ ), also acts as a co-activator of ChREBP in response to glucose. PGC-1 $\beta$  is known to activate genes responsible for fatty acid oxidation and hepatic gluconeogenesis during fasting (Vega et al., 2000; Yoon et al., 2001), and it was also reported to up-regulate *de novo* lipogenic genes during glucose

stimulation in a ChREBP dependent manner (Chambers et al., 2013). We cannot exclude that the proposed cross-talk between ChREBP and PPAR $\alpha$  may be due to both direct and indirect mechanisms. Indeed, during periods of over-nutrition, lipids synthesized by lipogenesis through a ChREBP dependent manner in liver, could be later liberated during fasting and impact PPAR $\alpha$  activity despite little ChREBP activity. Interestingly, ChREBP was shown to promote adipocyte differentiation, in part, through its control of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), a major actor of adipogenesis (Witte et al., 2015). The ChREBP mediated-induction of PPAR $\gamma$  activity was reported to rely (i) on fatty acid synthase (FAS) activity, which provides ligands of lipid origin for PPAR $\gamma$  and (ii) on direct trans-activation of the PPAR $\gamma$  ligand-binding domain. Collectively, these studies suggest that ChREBP may be a direct and/or indirect regulator of PPAR nuclear factors.

In conclusion, we identify a novel transcriptional node in the control of hepatic FGF21 in response to glucose. The glucose sensor ChREBP requires PPAR $\alpha$  for induction of FGF21 in response to dietary sugar. This is a unique collaboration between transcription factors that are activated in response to distinct nutritional conditions, high glucose for ChREBP versus fasting for PPAR $\alpha$ . These data implies that drug targeting of PPAR $\alpha$  may exert in part beneficial effects on metabolic homeostasis by supporting the ChREBP-induced loop controlling sweet preference via FGF21.

## Experimental procedures

### Generation of ChREBP knockout mice

A vector containing the ChREBP sequence with the addition of two Lox-P sites flanking the coding exons 9-15 and a FRT-flanked hygromycin-resistant cassette was constructed (Fig. S2). Clones were then selected after validation by 3 PCR sequences targeting i) 5' region homologue ii) 3' region homologue iii) floxed fragment. Homologous recombination of the target construct at the appropriate site was confirmed by Southern blot analysis followed by sequencing. Validated ES cells containing the target construct were injected in C57BL/6N derived blastocysts. Male agouti progeny were then bred with female C57BL6/J "flippase" mice to remove the hygromycin-resistant cassette. F1 progeny were crossed with CRE recombinase mice to remove the Lox-P floxed exons 9 to 15. Mice were genotyped using specific primers in Supplemental Table. 1. Loss of ChREBP expression was confirmed by RT-PCR and by western blot. Correspondence regarding ChREBP<sup>-/-</sup> mice should be addressed to renaud.dentin@inserm.fr and catherine.postic@inserm.fr.

### Animals

Adult C57BL6/J, ChREBP<sup>+/+</sup>, ChREBP<sup>-/-</sup> (8-10 weeks old) female mice were used for primary cultures of hepatocytes. C57BL/6J, ChREBP<sup>+/+</sup>, ChREBP<sup>-/-</sup>, PPAR $\alpha$ <sup>hep+/+</sup> and PPAR $\alpha$ <sup>hep-/-</sup> male adult mice (10-12 weeks old) were used for *in vivo* experiments. PPAR $\alpha$ <sup>hep+/+</sup> and PPAR $\alpha$ <sup>hep-/-</sup> are described in (Montagner et al. 2016). Procedures were carried out according to the French guidelines for the care and use of experimental animals. Mice were maintained in a 12 hours light/dark cycle with water and standard diet (65% carbohydrate, 11% fat, and 24% protein) unless specified. Fasted mice were fasted starting at 11pm and sacrificed 24 hours later. All fasted animals had free access

to drinking water. Glucose treated mice were allowed ad libitum access to standard diet in addition to a 20% glucose solution (Sigma, G7021).

*Fasting vs glucose experiment:* 10 weeks-old male mice from wild-type (WT) genotypes were fed ad libitum or fasted from ZT14 for 24 hours or fed a standard diet with a 20% glucose solution. All mice were sacrificed at ZT14. n=6 mice/genotype/experimental condition. To address the physiological impact of PPAR $\alpha$  ablation in response to sucrose intake, PPAR $\alpha^{\text{hep}+/+}$  and PPAR $\alpha^{\text{hep}-/-}$  mice were given free choice between a bottle containing a 10 % sucrose solution or water. Consumption was measured daily during three days.

### **Injection of adenovirus *in vivo***

GFP and ChREBP<sup>CA</sup> adenovirus (*Laboratoire de thérapie génique, Nantes, France*; requests to R. Dentin (renaud.dentin@inserm.fr)) were delivered through penis vein injection ( $3.10^9$ [pfu]/mouse). Four days later, the nutritional protocol was performed. Livers were flash-frozen and stored at  $-80^{\circ}\text{C}$ .

### **Primary cultures of human and mouse hepatocytes**

Human hepatocytes were prepared from lobectomy segments, resected from adult patients for medical reasons unrelated to our research program, under approval of the National Ethics Committee. The use of human specimens was approved by the French National Ethics Committee. Written informed consent was obtained from each patient prior to surgery. The tissue encompassing the tumor was dissected by the surgeon and sent for anatomopathological studies, while the remaining tissue was used for hepatocyte preparation and primary culture according to the described procedure (Pichard et al., 2006). Human hepatocytes were incubated with insulin (100  $\mu\text{M}$ ) in

presence of low glucose concentrations (5 mM) and high glucose concentrations (25 mM). Mouse hepatocytes were isolated and cultured as described (Dentin et al., 2004). Mouse hepatocytes were incubated under low glucose concentration (5 mM) and infected with specific adenovirus (3 pfu/cell) for 24 h. Glucose treated cells were incubated with insulin (10 uM) in the presence of either low (5 mM) or high (25 mM) glucose concentrations for 24 hours. Stimulation of primary human or mouse hepatocytes with DMSO used as a control or Wy-14643, 100 uM for human and 10 uM for mice, was done in combination with glucose stimulation 48 hours after seeding.

### **ChIP analysis**

Liver proteins were cross-linked to DNA by addition of 1% formaldehyde for 10 minutes at room temperature. Crosslinking was stopped by a 5 minutes incubation in 0.125 M glycine. Hepatocytes were scrapped and liver lysates were homogenized in low salt PIPES buffer and the nuclei fraction was enriched by a 10 minutes centrifugation. Sonication was performed in 10 seconds pulses, 15 times. Immunoprecipitation was performed with a ChREBP antibody (NB400–135; Novus Biologicals) and an IgG control (Cell Signaling). Immune complexes were captured with magnetic beads (Protein A-ChIP-Ademtech) and a saturated buffer. The eluted fraction was re-immunoprecipitated using anti-lysine antibody (Cell signaling). DNA fragments, shown on agarose gels, were quantified by qPCR, using primers described in Supplemental Information Table 1. ChIP experiments presented in Fig.7 were performed by Active Motif (Carlsbad, CA).

### **Gene expression analysis**

Total cellular RNA was extracted using the SV total RNA isolation system (Promega).

For real-time quantitative polymerase chain reaction (qPCR), total RNA samples (2  $\mu$ g) were reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Courtaboeuf, France). Primers for SYBR Green assays are presented in Supplemental Information Tables 2,3. Amplifications were performed on an ABI Prism 7300 Real Time PCR System (Applied Biosystems). qPCR data were normalized by TATA-box binding protein (TBP) messenger RNA (mRNA) levels or 18S ribosomal (18S) for human samples and analyzed with LinRegPCR.22. Transcriptomic profiles were obtained using Agilent SurePrint G3 Mouse GE 8x60K 5Design 074809 following the manufacturer's instructions. Microarray data and all experimental details are available in the Gene Expression Omnibus (GEO) database (accession GSE92502).

### **Transcriptomic data analysis.**

All data were analyzed using R ([www.r-project.org](http://www.r-project.org)). Microarray data were processed using Bioconductor packages ([www.bioconductor.org](http://www.bioconductor.org), v 2.12, (Gentleman, Carey et al. 2004)) as described in GEO entry GSE26728. A model was fitted using the `limma lmFit` function (Smyth, 2004). A correction for multiple testing was then applied using False Discovery Rate (Benjamini et al. 1995). Probes with adjusted p-value  $\leq 0.05$  were considered differentially expressed between conditions (n=6). Hierarchical clustering was applied to the samples and the differentially expressed probes using 1-Pearson correlation coefficient as distance and Ward's criterion for agglomeration. The resulting dendrogram were illustrated as a heatmap. The enrichment of Gene Ontology (GO) Biological Processes was evaluated using a conditional hypergeometric test (GOstats package, Falcon S and Gentleman R (2007). "Using GOstats to test gene lists for GO term association." *Bioinformatics*, 23(2), pp. 257-8.).

### **Western blotting analysis**

Proteins from hepatocytes and liver tissue were extracted from whole cell lysates. Proteins were subjected to 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Rabbit polyclonal ChREBP (1:1000) and L-PK (1:1500) antibodies (Novus Biologicals and a gift from Dr. Axel Kahn respectively) were used. Protein  $\beta$ -actin (1:5000) (Cell Signaling) was used to normalize data.

### **FGF21 measurement in plasma and conditioned medium**

Plasma concentrations of FGF21 were determined using a commercially available ELISA (Millipore) for either human or mouse samples. The mouse ELISA (Millipore) kit was used to measure FGF21 in cell culture medium. For mouse culture medium samples 30  $\mu$ l of sample was used for FGF21 detection.

### **Statistical Analysis**

Data represent at least three independent experiments, are reported as means  $\pm$  S.E.M, and were analyzed with analysis of variance using GraphPad Prism 5 (Prism 5.0, GraphPad) software. The Mann and Whitney test was used as a *post hoc* where indicated in figure legend. Significance for all other figures is based on 2-way ANOVA followed by Bonferroni used *post hoc*,  $p \leq 0.05$  (\*),  $p \leq 0.01$  (\*\*),  $p \leq 0.001$  (\*\*\*)).

### **Author Contributions**

AI designed experiments, performed experiments, analyzed the data and wrote the manuscript.

AM, FB, CP and HG designed experiments, performed experiments, analyzed the data,



and contributed to the critical review of the manuscript.

EA, AP, EF, MR, CL, VF, SM performed experiments and analyzed the data

ET, MDC, FL, RD generated and characterized the ChREBP knockout mice

RD constructed the ChREBP<sup>CA</sup> adenovirus

MDC and SGC provided human hepatocytes

YL performed microarray analysis and biostatics analysis

RD, SG, AFB, JG, WW provided critical comments.

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## Figure legends

**Figure 1. FGF21 is highly induced by both fasting and glucose challenge.** Wild-type (WT) male 10-week-old mice were fed ad libitum, fasted for 24 hours, or fed for 24 hours a standard diet with addition of 200g/L sucrose in drinking water. All mice were killed at ZT14 (14 after start of light period in animal housing unit), and serum and livers were collected. Transcriptomic analysis was performed on livers (n=6 per condition) using gene expression microarray. (a) Heat map was performed based on differentially expressed probes (FDR>5%) (b) KEGG categories corresponding to functions impacted by glucose and (c) fasting respectively. (d) Venn diagram presenting overlap between glucose and fasting induced gene expression (log FC>1; adjusted p<0.01). (e) Venn diagram presenting overlap between glucose and fasting induced gene expression (log FC>2; adjusted p<0.01).

**Figure 2. ChREBP is necessary for glucose mediated expression and secretion of FGF21 *in vitro*.** Primary hepatocyte cultures derived from female adult global ChREBP<sup>-/-</sup> mice, littermate ChREBP<sup>+/+</sup> mice were used as controls. Hepatocytes were stimulated one day after plating for 24 hours with cell culture medium containing 5 or 25 mM glucose. (a) Western Blot analysis of protein extracted from whole cell lysate of hepatocytes. Two representative samples are presented (n=3).  $\beta$  actin was used as loading control. (b-f) gene expression determined by RT-qPCR as described in the *Methods* section. (g) ELISA quantification of FGF21 protein in medium collected at end of glucose stimulation reported in pg/ml. Figures are presented as means  $\pm$  SEM from 4 independent cultures. Each culture was completed in triplicate. Significance is based on 2-way ANOVA followed by Bonferroni post test, p $\leq$ 0.01 (\*\*), p $\leq$  0.001 (\*\*\*).

**Figure 3. FGF21 is unable to respond to a glucose challenge without ChREBP.** Adult male ChREBP<sup>-/-</sup> mice and their ChREBP<sup>+/+</sup> littermates were allowed access to a 20% glucose drinking water solution as well as standard chow diet ad lib for 18 hours overnight. Fed mice received drinking water from the same water source used to make the glucose solution. (a) Blood glucose in mg/dl recovered at time of harvest from tail snip. (b) Western Blot analysis of protein extracted from whole liver lysate.  $\beta$  actin was used as loading control. Three representative samples are presented. (c-g) Relative

expression of hepatic genes determined by RT-qPCR. (h) ELISA quantification of FGF21 protein in plasma reported in pg/ml. Figures are presented as means  $\pm$  SEM from 4 to 6 individual mice. Significance is based on 2-way ANOVA followed by Bonferroni post test,  $p \leq 0.05$  (\*),  $p \leq 0.01$  (\*\*),  $p \leq 0.001$  (\*\*\*) or not detectable (nd).

**Figure 4. Liver specific ChREBP expression rescues FGF21 plasma concentrations in ChREBP knockout mice.** Adult male ChREBP<sup>+/+</sup> and ChREBP<sup>-/-</sup> mice were injected intravenously with a single dose of  $3 \times 10^9$  pfu GFP or ChREBP<sup>CA</sup> adenovirus on Day 1. Four days later, liver and plasma samples were harvested for analyses. (a) Blood glucose in mg/dl recovered at time of harvest from tail snip. (b) Western Blot analysis of protein extracted from whole liver lysate.  $\beta$  actin was used as loading control. Three representative samples are presented. (c-g) Relative expression hepatic genes determined by RT-qPCR. (h) ELISA quantification of FGF21 protein in plasma reported in pg/ml. Figures are presented as means  $\pm$  SEM from 8 to 12 individual mice. Significance is based on 2-way ANOVA followed by Bonferroni post test,  $p \leq 0.05$  (\*),  $p \leq 0.01$  (\*\*),  $p \leq 0.001$  (\*\*\*) or not detectable (nd).

**Figure 5. Liver PPAR $\alpha$  is required for the FGF21 glucose response in primary hepatocytes.** Primary hepatocyte cultures, derived from adult male PPAR $\alpha$ <sup>hep+/+</sup> or PPAR $\alpha$ <sup>hep-/-</sup> mice. Hepatocytes were stimulated one day after plating for 24 hours with medium containing 5, 10, 15, 20, 25, or 30 mM glucose. (a-e) Relative gene expression was determined by RT-qPCR. (f) ELISA quantification of FGF21 protein in medium collected at end of glucose stimulation reported in pg/ml. Figures are presented as means  $\pm$  SEM from 4 independent cultures. Each culture was completed in duplicate. Significance is based on 2-way ANOVA followed by Bonferroni post test to address differences between PPAR $\alpha$ <sup>hep+/+</sup> (control) and PPAR $\alpha$ <sup>hep-/-</sup> hepatocytes  $p \leq 0.05$  (\*),  $p \leq 0.01$  (\*\*),  $p \leq 0.001$  (\*\*\*) or not detectable (nd).

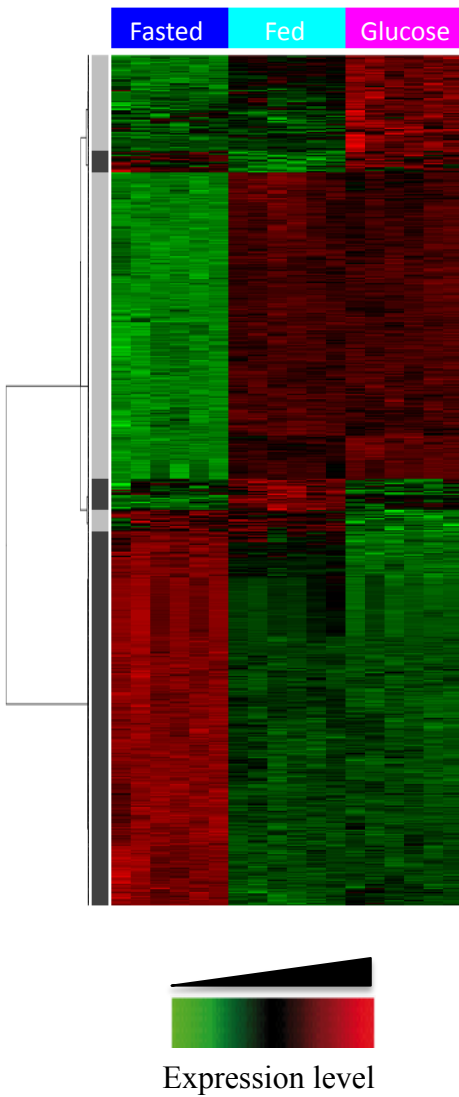
**Figure 6. ChREBP overexpression is unable to rescue FGF21 expression or secretion in the absence of PPAR $\alpha$ .** Primary hepatocytes derived from adult male mice PPAR $\alpha$ <sup>hep+/+</sup> (control) or PPAR $\alpha$ <sup>hep-/-</sup> were stimulated 6h after plating for 24 hours with  $3 \cdot 10^9$  pfu GFP or ChREBP<sup>CA</sup> adenovirus at a glucose concentration of 5 mM. (a-e) Relative gene expression was determined by RT-qPCR. (f) ELISA quantification of FGF21



protein in medium collected at end of adenoviral stimulation reported in pg/ml. Figures are presented as means  $\pm$  SEM from 4 independent cultures. Each culture was completed in duplicate. Significance is based on 2-way ANOVA followed by Bonferroni post test  $p \leq 0.05$  (\*),  $p \leq 0.01$  (\*\*),  $p \leq 0.001$  (\*\*\*) or not detectable (nd).

**Figure 7. PPAR $\alpha$  is required for the glucose dependent expression and secretion of FGF21 by the liver.** Adult male PPAR $\alpha^{\text{hep-/-}}$  and littermates PPAR $\alpha^{\text{hep+/+}}$  (controls) were assigned one of two treatment groups: i) mice fasted 24 hours with free access to drinking water (Fasted); ii) mice fed ad libitum with access to a 20% glucose drinking water solution for 24 hours (Glucose Challenge). (a) Blood glucose in mg/dl recovered at time of harvest from tail snip. (b) Western Blot analysis of protein extracted from whole liver lysate.  $\beta$  actin was used as loading control. Two representative samples are presented. (c-g) Relative hepatic gene expression was determined by RT-qPCR. (h) ELISA quantification of FGF21 protein in plasma reported in pg/ml. (i) A separate cohort of mice was used for sucrose intake. Mice were given a four-day adaptation period with two water bottles. A 10% sucrose solution was added to one of the water bottles for 3 days following adaptation and intake of sucrose solution and water was recorded daily for 3 days. Figures are presented as means  $\pm$  SEM from 5 to 6 individual mice. Significance is based on 2-way ANOVA followed by Bonferroni post test,  $p \leq 0.05$  (\*),  $p \leq 0.001$  (\*\*\*). (j-k) ChIP analysis followed by RT-qPCR of whole mouse liver tissue. IP conducted with ChREBP antibody for the binding region (ChoRE) or polymerase II. IgG was used as a binding control. ChIP analysis was conducted by Active Motif (Carlsbad, CA).

a.



b. Pathways induced by glucose (67 genes  $\log FC > 1$ ;  $p < 0.01$ )

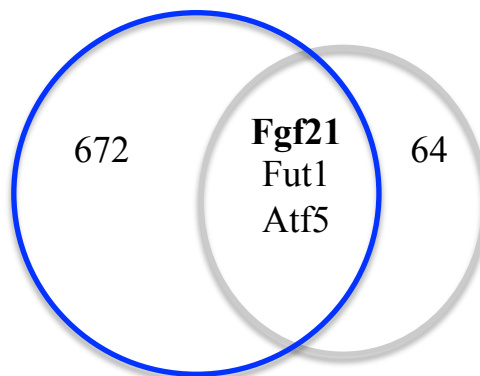
KEGG Pathways			
Pathway ID	Pathway description	Count (in gene set)	False discovery rate
00030	Pentose phosphate pathway	4	3,67 <sup>e-05</sup>
00620	Pyruvate metabolism	4	0,000122
04910	Insulin signaling pathway	5	0,000607
00970	Aminoacyl-tRNA biosynthesis	3	0,00468
00010	Glycolysis/Gluconeogenesis	3	0,0111
00670	One carbon pool by folate	2	0,0222

c. Pathways induced by fasting (675 genes  $\log FC > 1$ ;  $p < 0.01$ )

KEGG Pathways			
Pathway ID	Pathway description	Count (in gene set)	False discovery rate
03320	PPAR signaling pathways	15	2,71 <sup>e-08</sup>
00071	Fatty acid degradation	9	5,71 <sup>e-05</sup>
00270	Cysteine and methionine metabolism	8	7,78 <sup>e-05</sup>
00062	Fatty acid elongation	6	0,00069
04920	Adipocytokine signaling pathway	9	0,0111
01212	Fatty acid metabolism	7	0,00348
00250	Alanine, aspartate and glutamate metabolism	6	0,00361
00830	Retinol metabolism	8	0,0124
04068	FoxO signaling pathway	10	0,0193
01040	Biosynthesis of unsaturated fatty acids	4	0,0426

d.

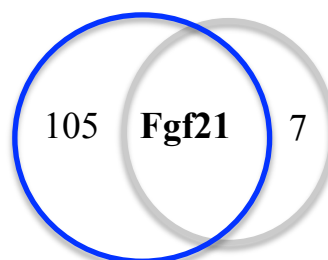
Genes induced by fasting  
( $\log FC > 1$ ;  $p < 0.01$ )



Genes induced by glucose  
( $\log FC > 1$ ;  $p < 0.01$ )

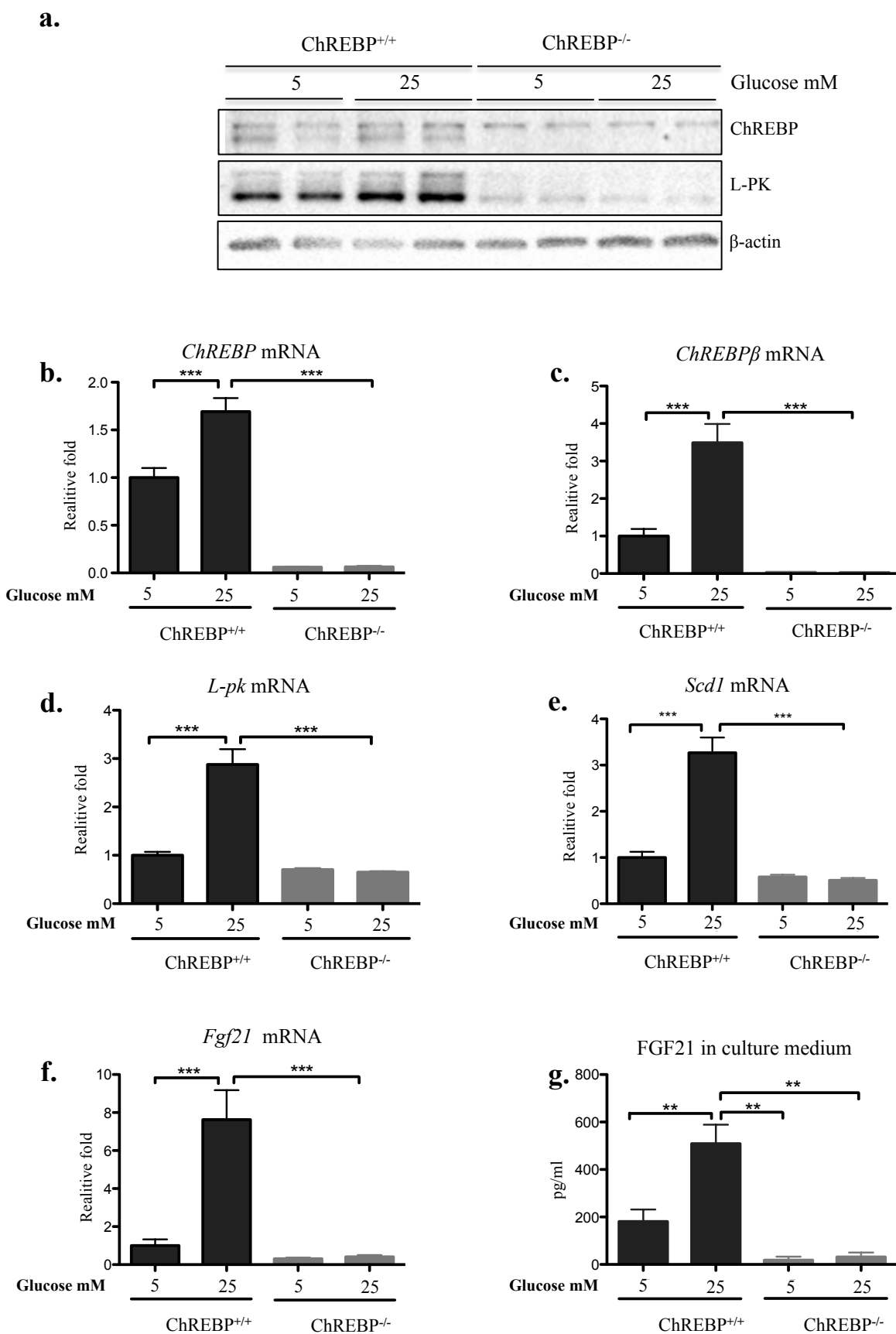
e.

Genes highly induced by fasting  
( $\log FC > 2$ ;  $p < 0.01$ )

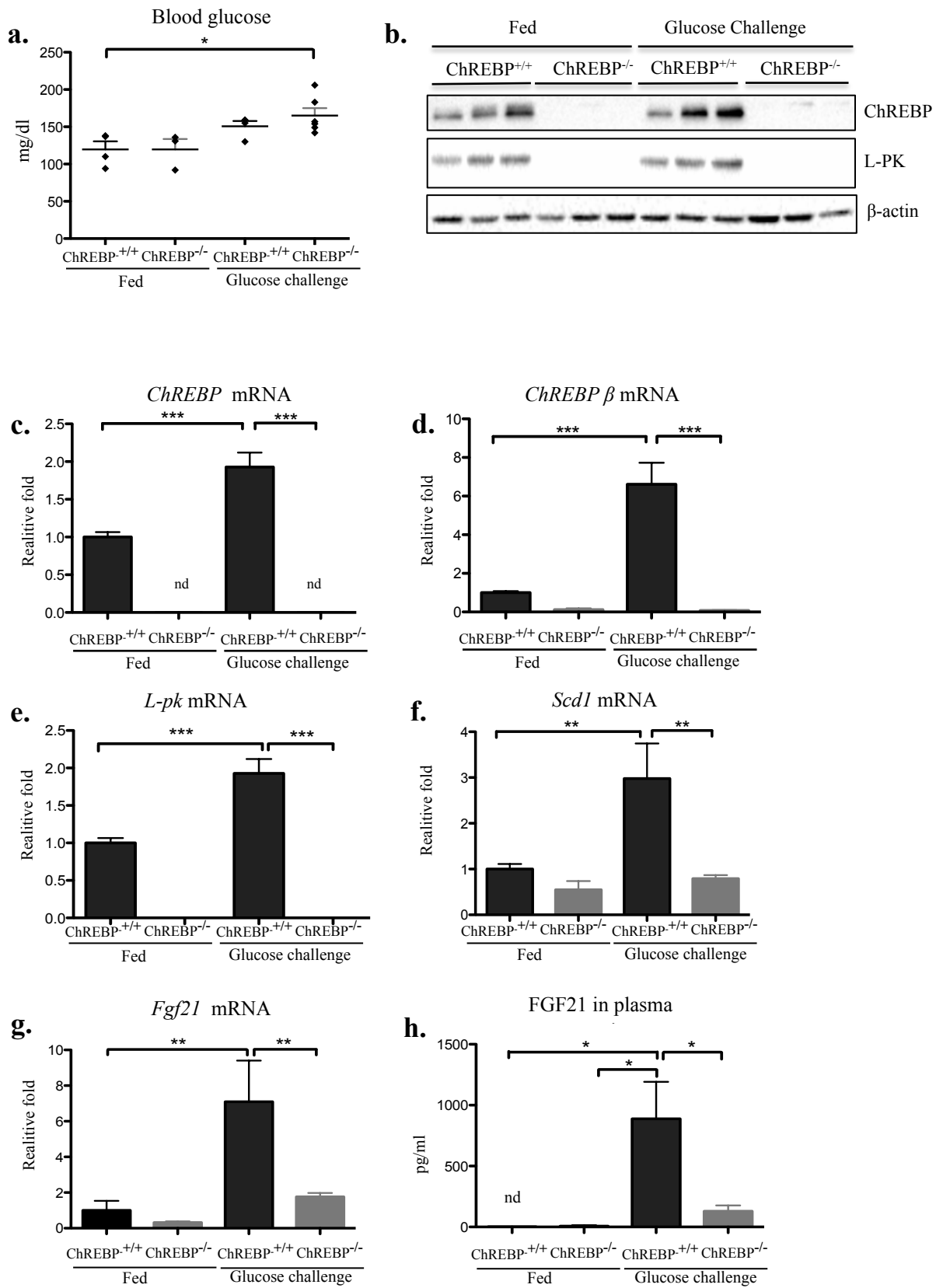


Genes highly induced by glucose  
( $\log FC > 2$ ;  $p < 0.01$ )

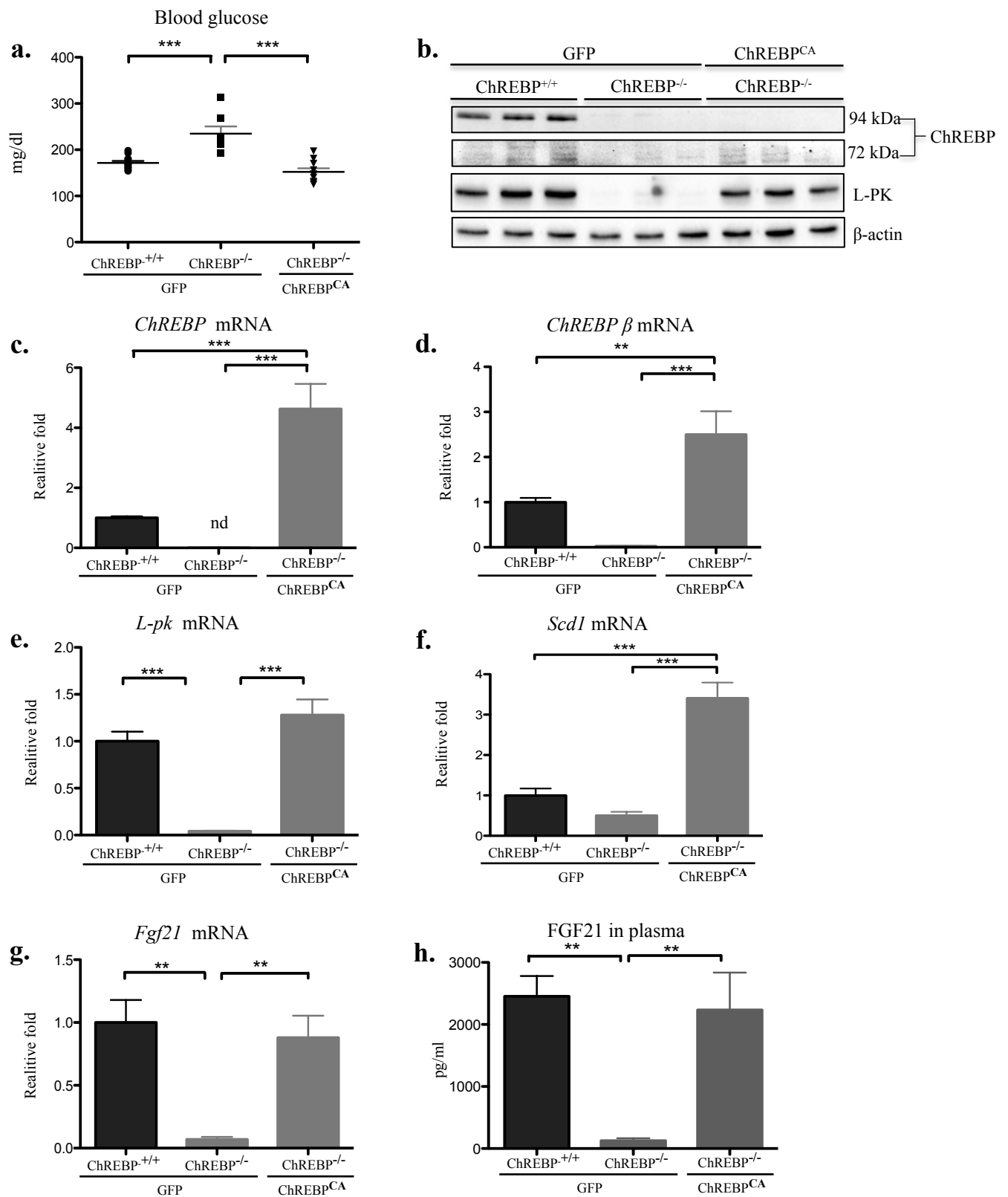
Figure 1.



**Figure 2.**

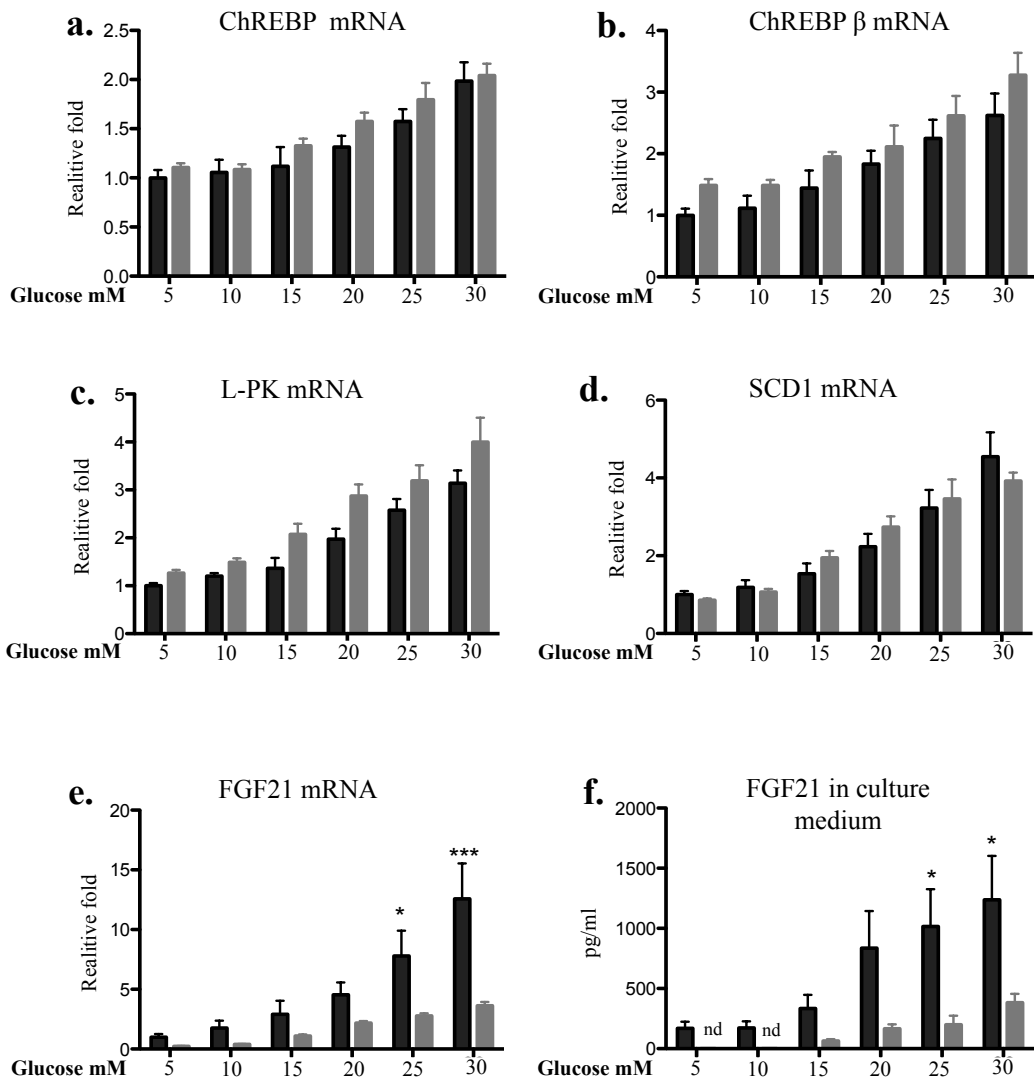


**Figure 3.**

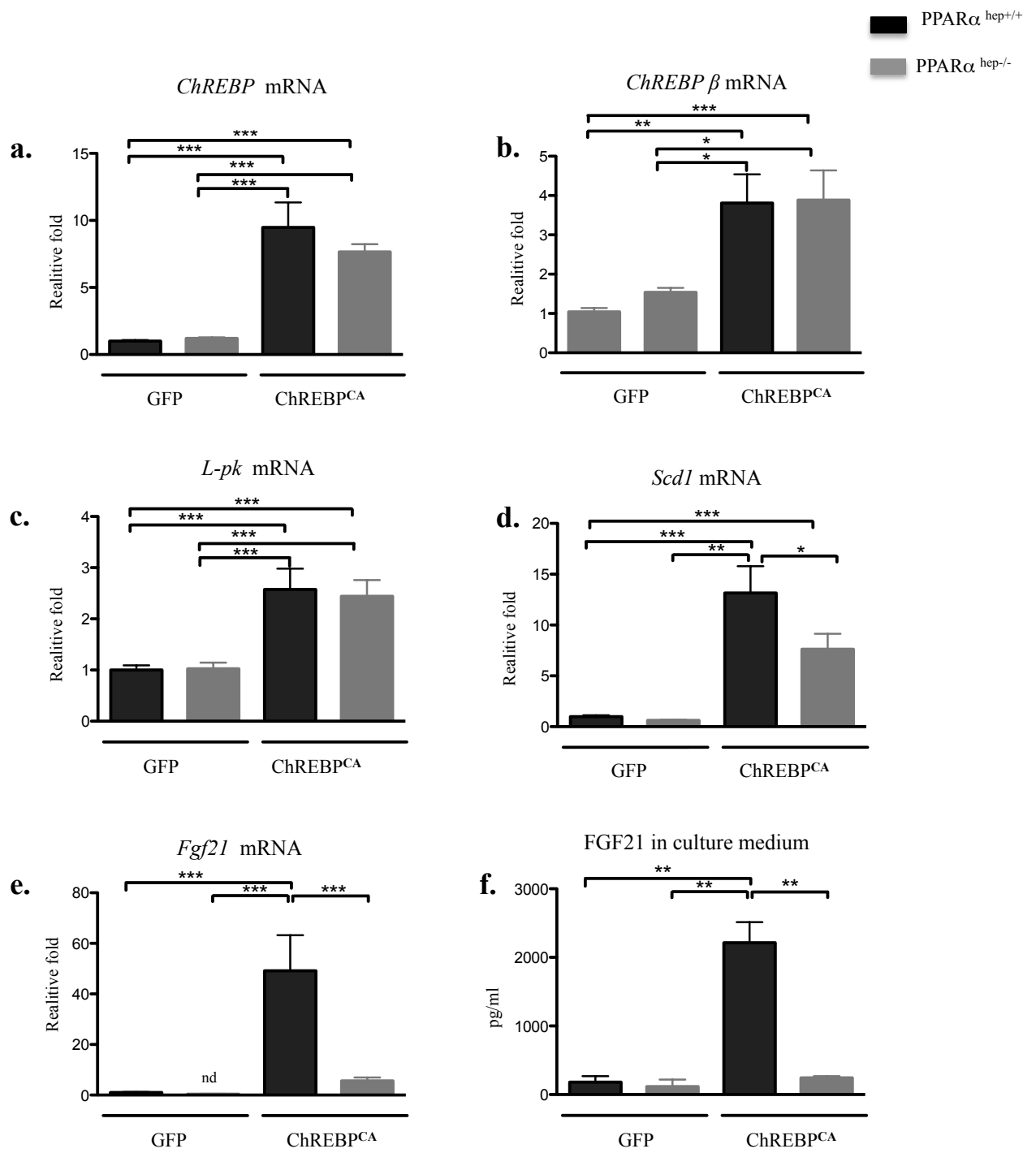


**Figure 4.**

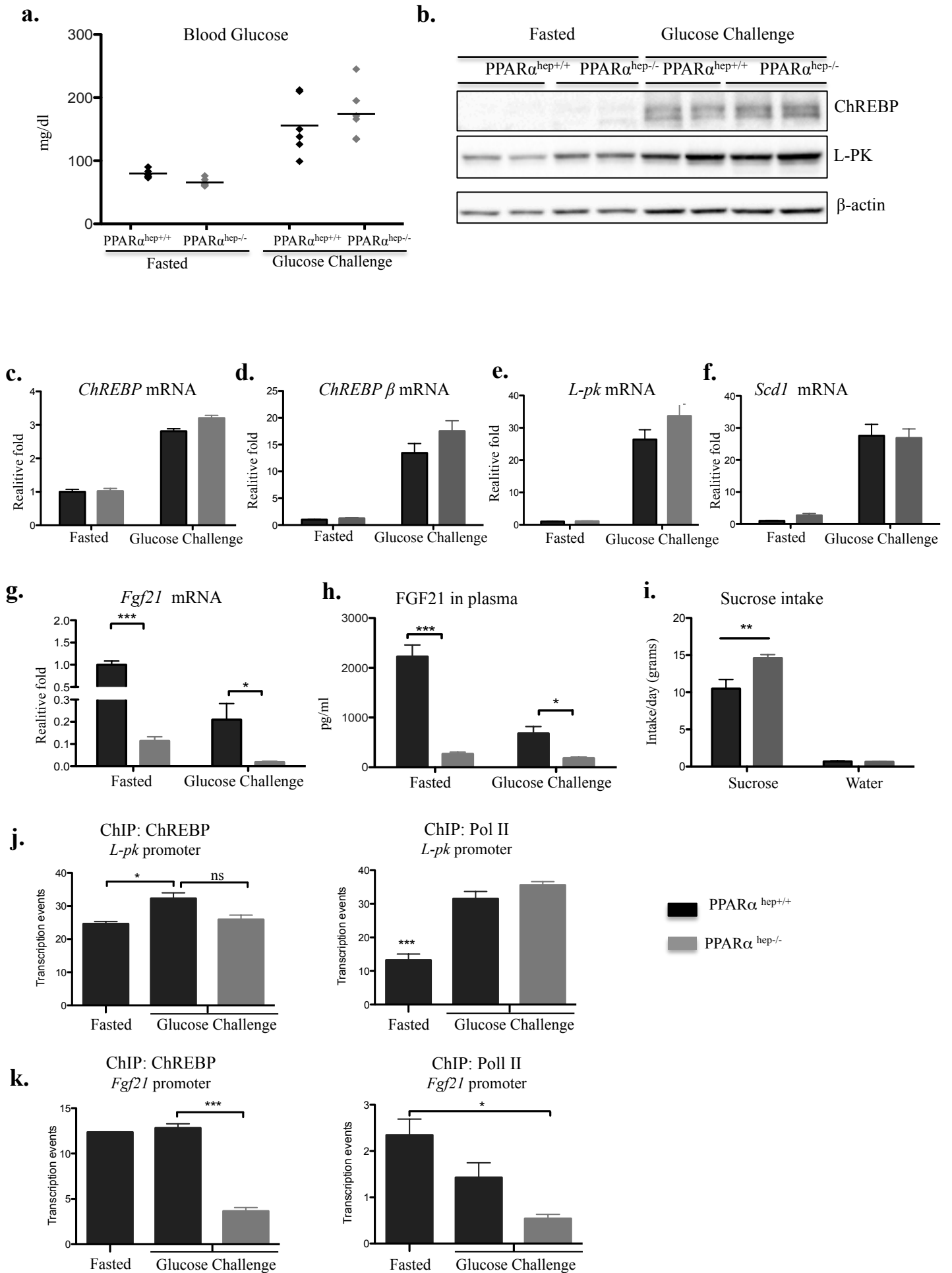
PPAR $\alpha$ <sup>hep+/+</sup>  
 PPAR $\alpha$ <sup>hep-/-</sup>



**Figure 5.**



**Figure 6.**



**Figure 7.**



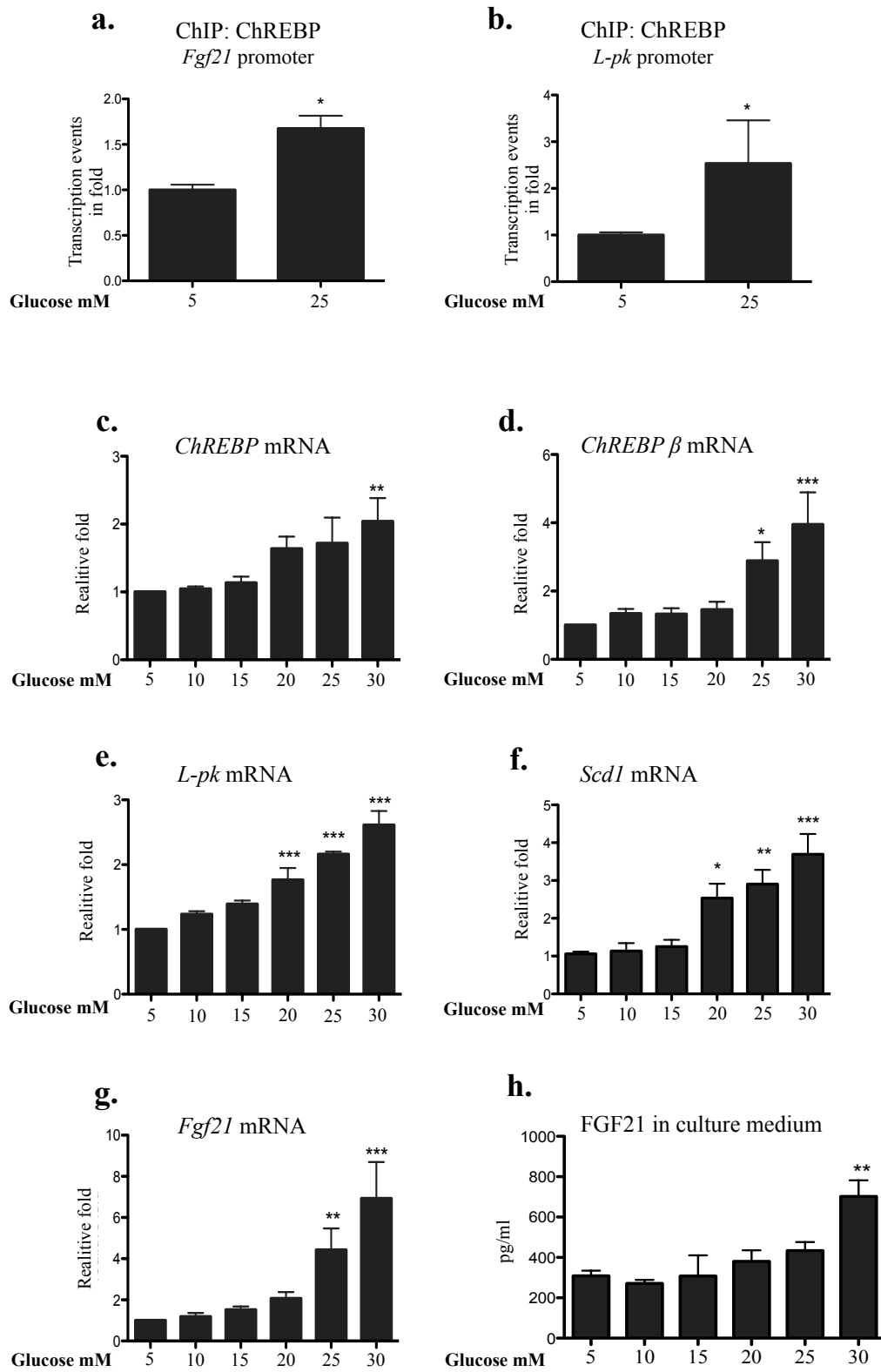
## Supplemental Information

**Supplemental Figure S1. FGF21 is expressed and secreted in response to glucose in a dose dependent manner in mice and in response to glucose in human primary hepatocytes. Related to Figure 2.** Primary hepatocytes derived from adult male C57BL/6J mice. Hepatocytes were stimulated one day after plating for 24 hours with medium containing 5, 10, 15, 20, 25, or 30 mM glucose. (a-b) CHIP analysis followed by RT-qPCR of primary mouse hepatocytes challenged by 5 or 25 mM glucose for 24 hours. Mann-Whitney test  $p \leq 0.05$  (\*) (n=3). (c-g) Relative gene expression was determined by RT-qPCR. (h) ELISA quantification of FGF21 protein in medium collected at end of glucose stimulation reported in pg/ml. Figures are presented as means  $\pm$  SEM from 4 independent cultures. Each culture was completed in triplicate. Significance is based on 2-way ANOVA followed by Dunnett's multiple comparisons (5 mM glucose as control),  $p \leq 0.05$  (\*),  $p \leq 0.01$  (\*\*),  $p \leq 0.001$  (\*\*\*)

**Supplemental Figure S2. Generation of global ChREBP knockout mice. Related to Experimental procedures generation of knockout mice.** (a) Wild type and recombined alleles are indicated. Exons 9-15 were floxed to generate *ChREBP* conditional mutant allele. (b) PCR analysis was used to identify offspring that carry Cre-mediated recombination between loxP1 and loxP2 using primers Fwd1 and Rev1 for wild type allele: 319 bp and for the allele including LoXP site: 375 pb. Primer Fwd1 and Rev2: 477 bp were used for mutant allele). (c) The first lanes is a DNA sample from a wild type mice (+/+) detecting the 319 bp band, while the second lane is a DNA sample from a heterozygous mouse (+/-) detected the 375 bp band and the third lane is a ChREBP knockout mouse (-/-) detected at 477 bp band.

**Supplemental Figure S3. FGF21 synergistically responds to glucose and a pharmacological PPAR $\alpha$  activator in both mouse and human hepatocytes. Related to Figure 7.** Primary mouse or human hepatocytes stimulated 24 hours with medium containing 5 or 25 mM glucose and of 100uM Wy-14643, 10ul of DMSO was used as a control. (a-h) Relative gene expression was determined by RT-qPCR. Mouse data are presented as means  $\pm$  SEM from 4 independent cultures. Each culture was completed in triplicate. Significance is based on 2-way ANOVA followed by Bonferroni post test

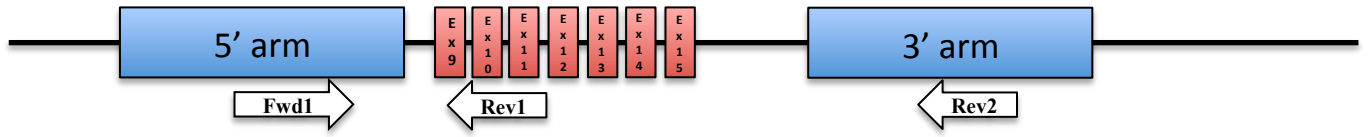
$p \leq 0.01$  (\*\*),  $p \leq 0.001$  (\*\*\*). Human hepatocyte data are presented as means  $\pm$  SEM from 1 culture in triplicate assays.



**Supplemental Figure S1.**

**a.**

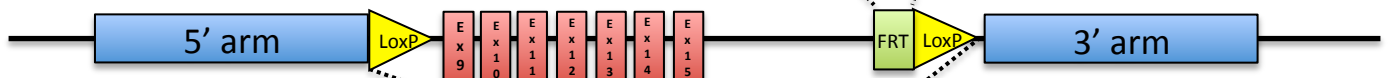
Wild type allele



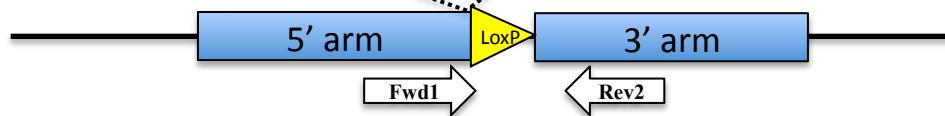
Recombined allele



Recombined allele after FLP recombinase



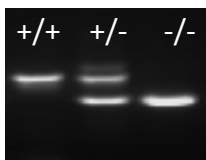
Null allele after Cre recombinase



**b.**

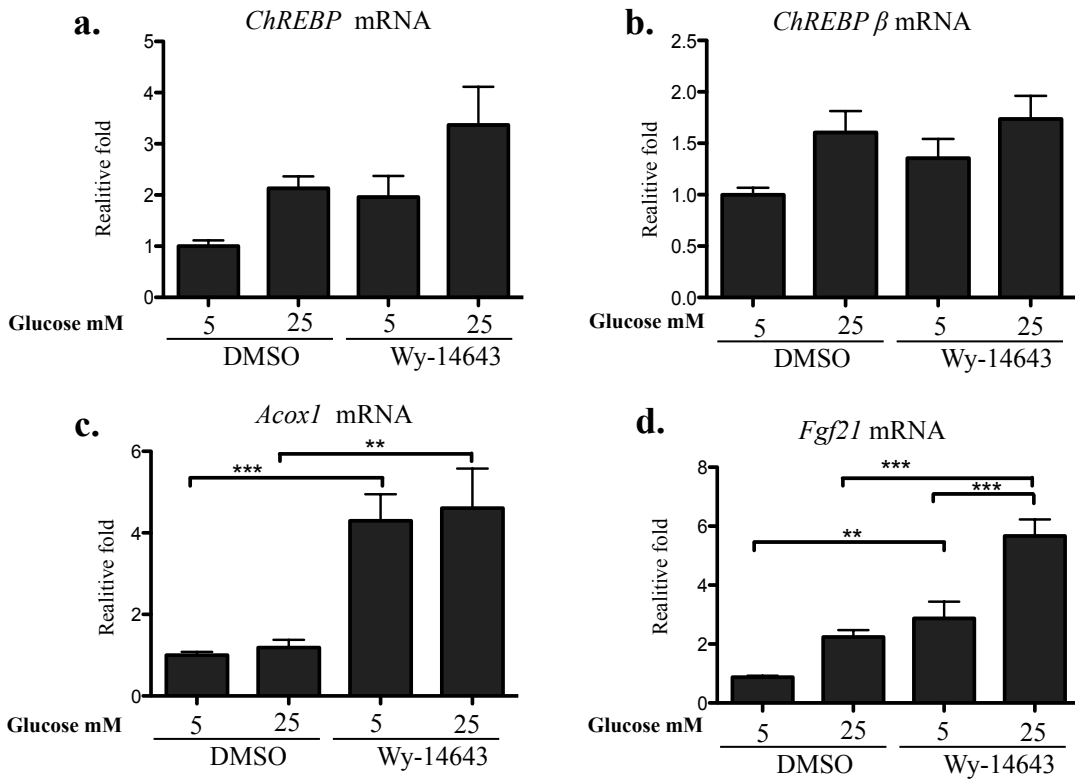
Primer	Sequence	Position
Fwd1	CCACCCCTATGGAATGGTTC	Arm 5'
Rev1	GCACCCATTACCAACTTAGTC	floxable zone
Rev2	TCCACATCTCTAGGCTCAG	Arm 3'

**c.**

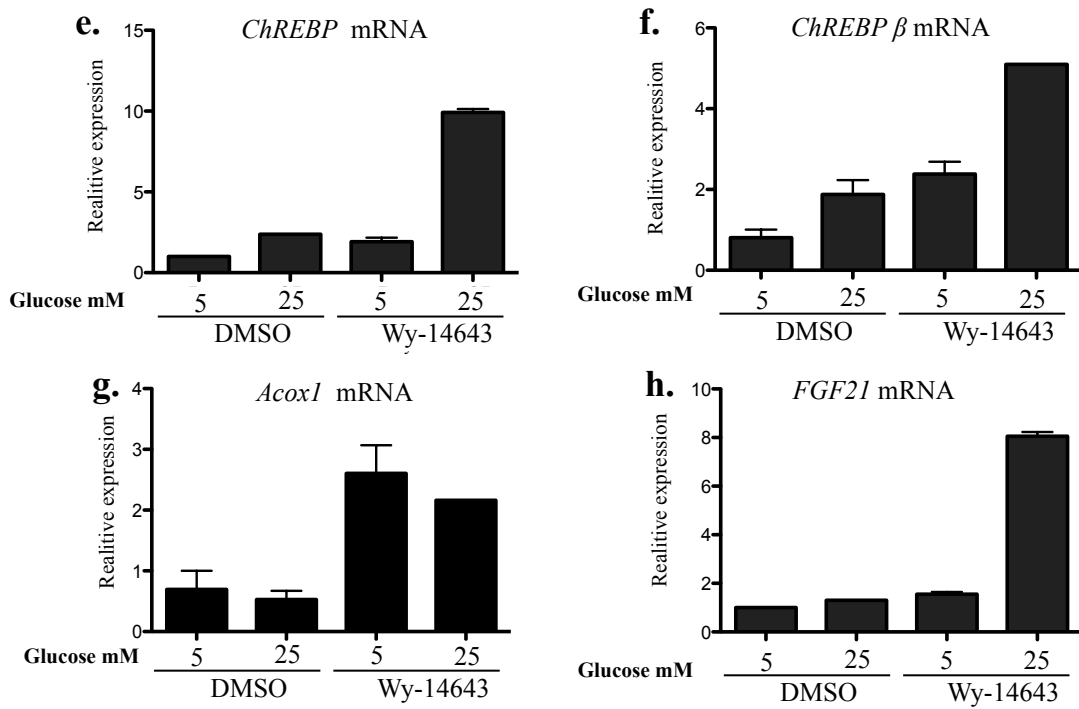
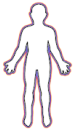


**Supplemental Figure S2.**

Mouse



Human



Supplemental Figure S3.

Mouse Genes		Sequences for ChIP	T fusion °C
ChoRE <i>Fgf21</i>	forward	TAGCCCTTTTCATTCAGCCCCT	60
	reverse	CTCTGTGTTGAACTCCCAGCTGA	
ChoRE <i>L-pk</i>	forward	GTCCCACACTTTGGAAGCAT	60
	reverse	CCCAACACTGATTCTACCC	

**Supplementary Table 1. Primers for ChIP analysis by qRT-PCR.  
Related to Figure 7 and Supplemental figure 1.**

Mouse Genes		Sequences for qPCR	T fusion °C
<i>ChREBP</i>	forward	AATGGGATGGTGTCTACCGC	58
	reverse	GGCGAAGGGAATTCAGGACA	
<i>ChREBP β</i>	forward	TCTGCAGATCGCGTGGAG	58
	reverse	CTGTCCCGGCATAGCAAC	
<i>L-pk</i>	forward	CTTGCTCTACCGTGAGCCTC	58
	reverse	ACCACAATCACCAGATCACC	
<i>Scd1</i>	forward	CCGGAGACCCCTTAGATCGA	58
	reverse	TAGCCTGTAAAAGATTTCTGCAAA	
<i>Fgf21</i>	forward	TACAATGTGTACCAGTCTGAAG	58
	reverse	ACAGCCCTAGATTCAGGA	
<i>Acox1</i>	forward	CCGCCTATGCCTTCCAATTTC	60
	reverse	CAAGCCATCCGACATTCTTCG	
<i>TBP</i>	forward	CCCACAACCTCTTCCATTCT	58
	reverse	GCAGGAGTGATAGGGGTCAT	

**Supplementary Table 2 . Primers for mouse mRNA analysis by qPCR. Related to Figure 2-7 and Supplemental Figure 1 and 3.**

Human Genes		Sequences for qPCR	T fusion °C
<i>ChREBP</i>	forward	CCA GCC TCA AGG TGA GCA AA	58
	reverse	CAC GCT CCT GCT GTA GCA	
<i>ChREBP β</i>	forward	AGCGGATTCCAGGTGAGG	58
	reverse	TTG TTCAGGCGGATCTTGTC	
<i>L-pk</i>	forward	TGGGCCTCATGCCTCTGACA	58
	reverse	TCCTGGGTCAGTTGGGCCAC	
<i>Fgf21</i>	forward	GGATTCGGACTGGTAAACAT	58
	reverse	GGGAGTCAAGACATCCAGGT	
<i>Acox1</i>	forward	GTGGGCTTGGAAGACTTCA	58
	reverse	CCGATGTCACCAACGGTAAT	
<i>18S</i>	forward	CCATCCAATCGGTAGTAGCG	58
	reverse	GTAACCCGTTGAACCCCAT	

**Supplementary Table 3 . Primers for human mRNA analysis by qRT-PCR. Related to Supplemental Figure 3.**





***RESEARCH SUBJECT 2***



## ***Research Subject 2: ChREBP improves glucose tolerance via FGF21***

### ***I-Project background***

The liver has become a target for novel therapeutic strategies through the identification of liver derived circulating proteins, hepatokines. Over expression of ChREBP in the liver of diet induced obesity (DIO) mice dissociates hepatic steatosis from insulin resistance and is associated with a reduction of blood glucose and parallels with increased concentrations of circulating FGF21 (Benhamed et al., 2012a). Stimulated by metabolites of glucose, the nuclear transcription factor ChREBP translocates to the nucleus and binds to the ChoRE region of target genes (Dentin et al., 2005; Kawaguchi et al., 2001a). Interestingly, FGF21 (Fibroblast Growth Factor 1), was reported to have a ChoRE region in which ChREBP could functionally bind (Iizuka et al., 2009). A leading target for diabetic therapies, FGF21, exerts beneficial metabolic actions on peripheral tissues including glucose-lowering, weight loss and  $\beta$ -cell protection in rodents (Wente et al., 2006). Several FGF21 benefits have translated to non-human primates and obese T2D patients (Kharitonov et al., 2007; Talukdar et al., 2016b).

#### **Hypothesis:**

- Can ChREBP-induced FGF21 secretion from hepatocytes promotes FGF21 like-signaling in the pancreatic  $\beta$ -cells?
- Is FGF21 in part responsible for the beneficial blood glucose lowering effect observed following hepatic ChREBP over expression?

### ***II-Materials and Methods***

## ***A/Animals***

All animal procedures were approved by the French guidelines for the care and use of experimental animals. Adult male C57BL/6J were used for all following experiments. Mice were housed in colony housing, exposed to a 12h light/dark cycle, with free access to water and a control chow diet (65% carbohydrate, 11% fat, 24% protein). Fasted mice were allowed free access to water without chow for 24 hr prior to sacrifice. Animals designated as refed were fasted with free access to water 42 hr during a 24 hr period followed by an 18 hr refeeding period before sacrifice were mice had abundant access to chow and 20% glucose water. FGF21 global knockout mice (FGF21<sup>-/-</sup>) mice (Potthoff et al., 2009) were obtained through a collaboration with D Srs Fabienne Rajas and Gilles Mithieux (Inserm U855, Université Claude Bernard, Lyon).

## ***B/Adenoviruses***

ChREBP overexpression was performed by an adenovirus expressing a truncated form of ChREBP lacking the first 196 amino acids, ChREBP<sup>CA</sup>, under the control of the cytomegalovirus promoter and co-expressing GFP. An adenovirus co expressing GFP only was used for a control (laboratoire de thérapie génique, Nantes, France). ChREBP expression was inhibited by using an adenovirus expressing a short hairpin against ChREBP, shChREBP. An shControl adenovirus was used as a control (genecust).

### ***C/Primary culture of hepatocytes and treatment with recombinant adenovirus***

Fed mice at 7 to 8 weeks of age consuming a control diet were used for hepatocyte culture. Mice were anesthetized with a 10:1 ketamine-xylazine mixture by intra-peritoneal injection, following anesthetized mice were transferred for cell culture preparation and liver was exposed by ventral incision. Livers were perfused by the portal vein with Hank's balanced salt solution followed directly by perfusion with collagenase. Viability of isolated hepatocytes was calculated using the trypan blue exclusion test and then plated on 60mm diameter plates at a concentration of 2 millions cells per plate. Cells were plated in Medium M199 5mM glucose with earl salts (Invitrogen) supplemented with 100µg/ml streptomycin, 100 units/ml of 2.4 mM penicillin, L-glutamine (2,4mM) 0,1% Bovine serum albumin and 2,5 % neu-serum (BD Bioscience). Following a 24 hr incubation at 37°C 5% CO<sub>2</sub>, M199 medium was replaced and cells were treated with recombinant adenovirus GFP or ChREBP<sup>CA</sup> at 2 pfu/cell or small interfering RNA 2 pfu/cell. Following a 24 hr incubation with the adenovirus, medium was changed for either a low glucose (G5mM) or high glucose (G25mM) medium. Insulin and dexamethasone (Actrapid, Novonordisk at 100mM respectively) were added in addition in addition to the glucose. Twenty-four hours after low or high glucose stimulation, medium was recuperated for conditioned medium and analyzed by ELISA for FGF21 protein. Cell were rinsed with PBS (Invitrogen) and recuperated.

### ***D/Pancreatic INS1 cell line culture and conditioned medium***

INS1 cells were cultured in RMPI 1640 (Life technology) supplemented with 10% fetal calf serum (Life Technology), 100U/ml penicillin, 100mg/ml streptomycin, 1mM sodium

pyruvate, 10mM Hepes and  $\beta$ -mercaptoethanol. Medium collected from conditioned hepatocytes was frozen and stored at -20°C until use. The conditioned medium warmed in a water bath before use and 1 ml of conditioned medium was placed into cell wells for 10 minutes. Medium was then removed and cells were washed with cold PBS to stop reaction and directly frozen until analysis.

### ***E/Injection of adenovirus in vivo***

Four days before sacrifice, fed mice were anesthetized using isoflurane (Belamont, Paris, France) and injected with  $3 \times 10^9$  pfu of either GFP or ChREBP<sup>CA</sup> adenovirus for overexpression trials and shChREBP or shcontrol for knockdown trials. Injection was delivered by penis vein injection diluted in sterile NaCl 9% (Invitrogen).

### ***F/RNA extraction of primary hepatocytes and liver tissue***

PBS of washed cells was removed and 175 $\mu$ l of RNA lysis buffer (Promega) was added at time of cell recovery. Plated cells were then removed by scraping into a tube. Liver tissue, snap frozen in liquid nitrogen at time of necropsy, was crushed into a homogenized powder. Approximately 10mg of liver powder was then distributed to tubes containing 175 $\mu$ l of the previously mentioned RNA lysis buffer. The RNA for hepatocytes or tissue was isolated using the SV Total RNA Isolation System (Promega). Generation of cDNA was done by reverse transcriptase. Samples of cDNA were diluted by 1/30<sup>e</sup> and analyzed using the Lightcycler Fast Start DNA master SYBR Green I (Roche). Expression quantified was corrected by expression of cyclophilin as a control. All primers are described below (Article Supplementary Table 2).

## ***G/Western Blot analysis of protein expression***

Proteins of cell lysates for both hepatocytes and homogenized liver powder and pancreas were prepared in buffer (20mM Tris HCL,pH 7.5; 150mM NaCl; 5mM EDTA; 50mM NaF; 30mM NaP; 1% triton X-100; pepstatine, 1ug/ml; leupeptine, 2ug/ml; PMSF, 1mM; aprotinine, 2.5ug/ml and orthovanadate, 1mM). Proteins in cleared lysate of samples were separated by molecular weight using an acrylamide gel at 10 or 12% followed by a liquid transfer to a nitrocellulose membrane (GE Healthcare). Membranes were blocked 1 hr in 5% milk and then incubated over night in their respective primary antibodies. After incubation membranes underwent a wash series in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20). Membranes were then incubated in a secondary antibody containing a peroxidase-conjugated anti-rabbit antibody (1:10000) recognized by the primary antibody for 1 hr followed again by a final TBST wash series. Proteins were revealed using ECL (Pierce) according to the manufacturer's protocol. Protein  $\beta$ -actin (1:5000) was used to normalize data.

## ***H/Enzyme Linked Immunosorbent Assay (ELISA)***

A sandwich ELISA (Millipore) was used to quantify concentration of FGF21 in both serum and in conditioned medium. Serum concentrations of FGF21 were measured according to the manufacturer's protocol. Conditioned medium was measured by adding 50ml conditioned medium as total sample volume and excluding the kits assay buffer normally added in addition to each sample. Quantification of FGF21 was preformed in Prism GraphPad Software, Inc. Interpolation of results were analyzed using a nonlinear regression third order polynomic.



## ***I/Chromatin Immunoprecipitation***

Frozen liver samples were suspended in 2ml of PBS and histones were cross linked to DNA by addition of 15ml of 36% formaldehyde for a final concentration of 1%. Glycine was used at a final concentration of 0.125 mM to block additional cross linking. Extraction of cytosolic and nuclear fraction was performed. DNA was then sheared into lengths approximately 500 basepairs by sonification 15 times for 10 second with a 1 min cooling time in ice between each sonification. DNA sonification efficiency was visualized by migration of sonification product in a 1% agarose gel. Product of sonification was immunoprecipitated with antibodies with a control, IgG, and ChREBP antibody over night at 4°C using the magnetic beads Protein A for ChIP (Ademtech) and a standard buffer which was added to collect the histone antibody complex. Beads for in vivo samples were washed using 3 buffers each for 10 min as follows; low salt buffer high salt buffer, and TE buffer. Hepatocyte samples were washed using the Thermo-Fisher in collaboration with Ademtech beads (Protein A/G). Elute buffer of 0.1 M sodium bicarbonate using 1% SDS prepared at time of use. Cross linking was then reversed over night at 65°C. Cross linking reversal was continued using a high salt buffer with a high concentration of SDS and proteinase K. DNA was recovered by phenol/chloroform extraction. Quantification of bound DNA was performed by PCR using the IgG as a control and normalized using the input. Primers used for ChIP experiments are listed in Article Supplementary Table 1.

## ***J/Statistical Analysis***

All statistics were performed in Prism GraphPad Software, Inc using one-way ANOVA followed by a Bonferroni post test in order to compare all columns. When applicable for groups of only two data sets an unpaired two-tailed t-test was conducted. A 95% confidence interval was used for all statistical measurements.

### ***III-Results***

#### ***A/FGF21 is elevated in mouse plasma following an oral glucose bolus***

To investigate the rate of FGF21 response following an oral glucose bolus male C57BL/6J mice were orally administered by gavage a 20% glucose solution in 15- minute intervals. Blood glucose was measured before glucose gavage and then at 15, 30, 40, 60, 90 and 120 minutes after the second glucose bolus. Four mice were sacrificed at each time point 0, 15, 45, 60 and 120 minutes. Plasma and liver was harvested for analysis. Following glucose bolus blood glucose of C57BL/6J mice was significantly higher at 15 minutes and 30 minutes after bolus and gradually started to return to pre-gavage concentrations at 45 minutes until reaching pre-gavage concentrations at 90 and 120 minutes post bolus (Figure 28a). As expected *ChREBP* and *ChREBPβ* mRNA expression as well as its target gene *L-pk* was significantly increased at 120 minutes after glucose bolus (Figure 28b-d). The ChREBP target gene *Fgf21* was elevated before glucose, possibly due to stress of animal handling and dropped at 15 minutes following glucose bolus and then, like *ChREBP*, *ChREBPβ* and *L-pk*, tended to increase over time (Figure 28e). However FGF21 protein concentrations measured in plasma were significantly increased at 120 minutes (Figure 28f). Western blot analysis of whole liver lysates confirmed a normal hepatic glucose response, as phosphorylation of Akt was increased 60 to 120 minutes following bolus (Figure 28g). This test suggested that FGF21 in circulation is increased in response to glucose in vivo however future experiments implemented different glucose challenge strategies in order to prevent animal stress.

## ***B/The refeeding-mediated FGF21 response is ChREBP dependent***

To determine if the FGF21 glucose response *in vivo* was ChREBP dependent a shChREBP was delivered to the liver of C57BL/6J mice in order to knockdown ChREBP expression and activity. As reported in previous publications ChREBP knockdown reduced refeed blood glucose (Figure 29a). The shChREBP adenovirus effectively reduced *ChREBP* and *ChREBPβ* mRNA in liver tissue of shChREBP treated mice as compared to shControl mice resulting in similar expression levels as shControl fasted mice (Figure 29b-c). *L-pk* mRNA expression was significantly increased by refeeding in shControl mice but not in shChREBP (Figure 29d). Like *L-pk*, *Fgf21* mRNA expression and FGF21 in plasma was significantly up regulated by refeeding as compared to fed shControl mice. ChREBP knock down significantly blunted the *Fgf21* refeeding response to levels similar as fed mice (Figure 29e). However, in plasma, ChREBP knockdown only tended to decrease FGF21 in refeed mice transfected with shChREBP (Figure 29f). To confirm that both transcription and protein activity of ChREBP had been affected, western blot analysis was completed for ChREBP and two of its target genes L-PK, used as a control, and fatty acid synthesis (FAS) a key enzyme of lipid synthesis. The reduced intensity of L-PK and FAS protein in shChREBP treated mice confirmed that ChREBP protein activity was effectively reduced (Figure 29g).

## ***C/Over expression of hepatic ChREBP induces FGF21 secretion and FGF21-like signaling in the pancreas***

A previous publication from our lab reported that over expression of ChREBP dissociated hepatic steatosis from insulin resistance (Benhamed et al., 2012a). To understand how hepatic ChREBP could improve glucose metabolism male C57BL/6J mice were injected with an

adenovirus vehicle containing the GFP protein or a truncated isoform of ChREBP lacking the LID domain (Li et al., 2006) which corresponds to a constitutively active version of ChREBP (ChREBP<sup>CA</sup>). Four days following injection mice were harvested and blood, liver and pancreas was collected for sample analysis. Analysis of liver tissue confirmed that ChREBP<sup>CA</sup> expression significantly induced *ChREBP* mRNA, exceeding expression levels observed in GFP treated mice (Figure 30a). Transcriptional activity of ChREBP<sup>CA</sup> was confirmed by up regulation of *L-pk* mRNA expression (Figure 30b). *Fgf21* mRNA and circulating FGF21 concentrations were equally increased (6-fold) (Figure 30c-d). Our findings report that ChREBP<sup>CA</sup> restored ChREBP activity in liver induces FGF21 gene expression and protein secretion. Whole pancreas lysates were analyzed by western blot to monitor FGF21-like signaling. In whole pancreas tissue, including both the endocrine and exocrine pancreas phosphorylation of FRS2 was more important in ChREBP<sup>CA</sup> treated mice. Following downstream of the FGF21 pathway pERK, important for insulin secretion was also visually increased as compared to GFP-treated mice (Figure 30e). Because the whole pancreas was used and FGF21 is known to stimulate ERK signaling in exocrine acinar cell (Coate et al., 2017) we moved into a cell line model to isolate FGF21's role on  $\beta$ -cell signaling.

### ***D/Conditioned medium of ChREBP<sup>CA</sup> expressing hepatocytes enhances the phosphorylation of ERK and Akt in INS1 cells in a FGF21 dependent manner***

Following *in vivo* experiments, *in vitro* experiments using primary hepatocytes and the  $\beta$ -cell line INS1 were used to investigate the role of ChREBP induced hepatic secretory factors on  $\beta$ -cell signaling. Primary mouse hepatocytes harvested from male C57BL/6J mice or FGF21 global knockout (FGF21<sup>-/-</sup>) mice from a similar genetic background were transfected with the ChREBP<sup>CA</sup> adenovirus or a GFP control vector. We confirmed that transfection was effective as

expression of *ChREBP* mRNA and its target gene *L-pk* was significantly increased following ChREBP<sup>CA</sup> transfection as compared to GFP treated hepatocytes. *ChREBP* and *L-pk* mRNA expression was similar between genotypes (Figure 31a-c). In similarity to *in vivo* experiments *Fgf21* mRNA paralleled *L-pk* expression in C57BL/6J mice and was increased in response to ChREBP<sup>CA</sup> (Figure 31d). FGF21 protein was a direct reflection of *Fgf21* mRNA profiles, as it was significantly more concentrated in medium of ChREBP<sup>CA</sup> treated hepatocytes than GFP treated hepatocytes harvested from C57BL/6J mice (Figure 31e). As expected, *Fgf21* mRNA and FGF21 protein in secretion was undetectable in hepatocytes harvested from FGF21<sup>-/-</sup> mice (Figure 31e). Medium collected of treated hepatocytes was collected and then used to stimulate INS1 cells in order to determine if a secretory factor from ChREBP<sup>CA</sup> transfection hepatocytes could induce differences in signaling in important  $\beta$ -cell insulin secretion (ERK) and survival pathways (Akt). Indeed, a 10-minute incubation with medium from ChREBP<sup>CA</sup> transfected hepatocytes harvested from C57BL/6J mice noticeably induced ERK and Akt phosphorylation as compared to INS1 incubated with GFP treated C57BL/6J hepatocytes. INS1 cells were also incubated with medium from ChREBP<sup>CA</sup> or GFP treated hepatocytes harvested from FGF21<sup>-/-</sup> mice. Phosphorylation of ERK was enhanced in INS1 cells treated with GFP transfected FGF21<sup>-/-</sup> hepatocytes as compared to GFP transfected C57BL/6J hepatocytes but was not as intense as observed in INS1 stimulated with ChREBP<sup>CA</sup> transfected C57BL/6J hepatocyte medium. This suggests that in the absence of the stress induced hormone FGF21, hepatocytes may have different secretory profiles in response to an injury such as adenovirus transfection. This effect was not observed however for Akt phosphorylation. Regardless, a difference between the intensity of pERK and pAkt between INS1 stimulated with medium from GFP or ChREBP<sup>CA</sup> transfected FGF21<sup>-/-</sup> hepatocytes was not detectable (Figure 31f). Quantification analysis of both phosphorylation of ERK and Akt was significantly increased in INS1 cells stimulated with medium from ChREBP<sup>CA</sup> transfected C57BL/6J hepatocytes as compared to INS1 cells stimulated with the other three culture mediums (Figure 31g).

## ***E/FGF21 is necessary for the improved glucose tolerance observed in mice over expressing ChREBP in the liver***

Previous experiments of hepatic ChREBP over expression in C57BL/6J showed improved glucose tolerance as ChREBP<sup>CA</sup> expression reduced blood glucose following an oral glucose challenge (data not shown). To address the role of FGF21 in the observed glucose lowering effect, ChREBP<sup>CA</sup> or GFP was transfected into male C57BL/6J mice or male FGF21<sup>-/-</sup> mice. Fed blood glucose was significantly lower at time of sacrifice in mice treated with the ChREBP<sup>CA</sup> over expressing adenovirus and was similar between genotypes (Figure 32a). ChREBP<sup>CA</sup> transfection was successful and similar between genotypes with a significant up regulation of both hepatic *ChREBP*, *ChREBPβ* and *L-pk* mRNA (Figure 32b-d). Hepatic expression and plasma concentration of FGF21 was confirmed to be significantly increased in C57BL/6J mice treated with ChREBP<sup>CA</sup> as compared to GFP treated C57BL/6J mice. FGF21 was not detectable in either ChREBP<sup>CA</sup> or GFP transfected FGF21<sup>-/-</sup> mice (Figure 32e-f). Adiponectin is known to be responsible for some of the beneficial effect of FGF21 on insulin sensitivity and glucose utilization (Holland et al., 2013). In this study, adiponectin concentration in plasma was not different between groups (Figure 32g). When an oral glucose tolerance test (OGTT) was conducted only C57BL/6J ChREBP<sup>CA</sup> expressing mice and not FGF21<sup>-/-</sup> ChREBP<sup>CA</sup> expressing mice had reduced blood glucose 15 minutes and 30 minutes after oral glucose gavage. This suggests that FGF21 may be necessary for an insulin sensitizing effect of ChREBP (Figure 32h). Blood insulin concentration varied highly within groups and was not interpretable (data not shown).

## **Conclusion**

In conclusion, *fgf21* expression is increased in the liver following oral glucose intake, and, hepatic knockdown of ChREBP by shRNA reduced hepatic *fgf21* expression in response to

carbohydrate intake. We demonstrated that over expression of ChREBP in hepatocytes induces FGF21 secretion into the culture medium. This medium induces FGF21-like signaling including; phosphorylation of ERK and Akt, in the  $\beta$ -cell line INS1. Conditioned medium experiments strengthen previous reports that the liver is able to communicate with the pancreas in order to regulate energy metabolism. Furthermore, hepatic ChREBP over expression in C57BL/6J mice reduced blood glucose following a glucose bolus in an FGF21 dependent manner. These data suggests that hepatic ChREBP is responsible for the secretion of FGF21 and potential other hepatokines that improve glucose tolerance (Figure 33).

**Figure 28:** FGF21 is elevated in mouse plasma following an oral glucose bolus

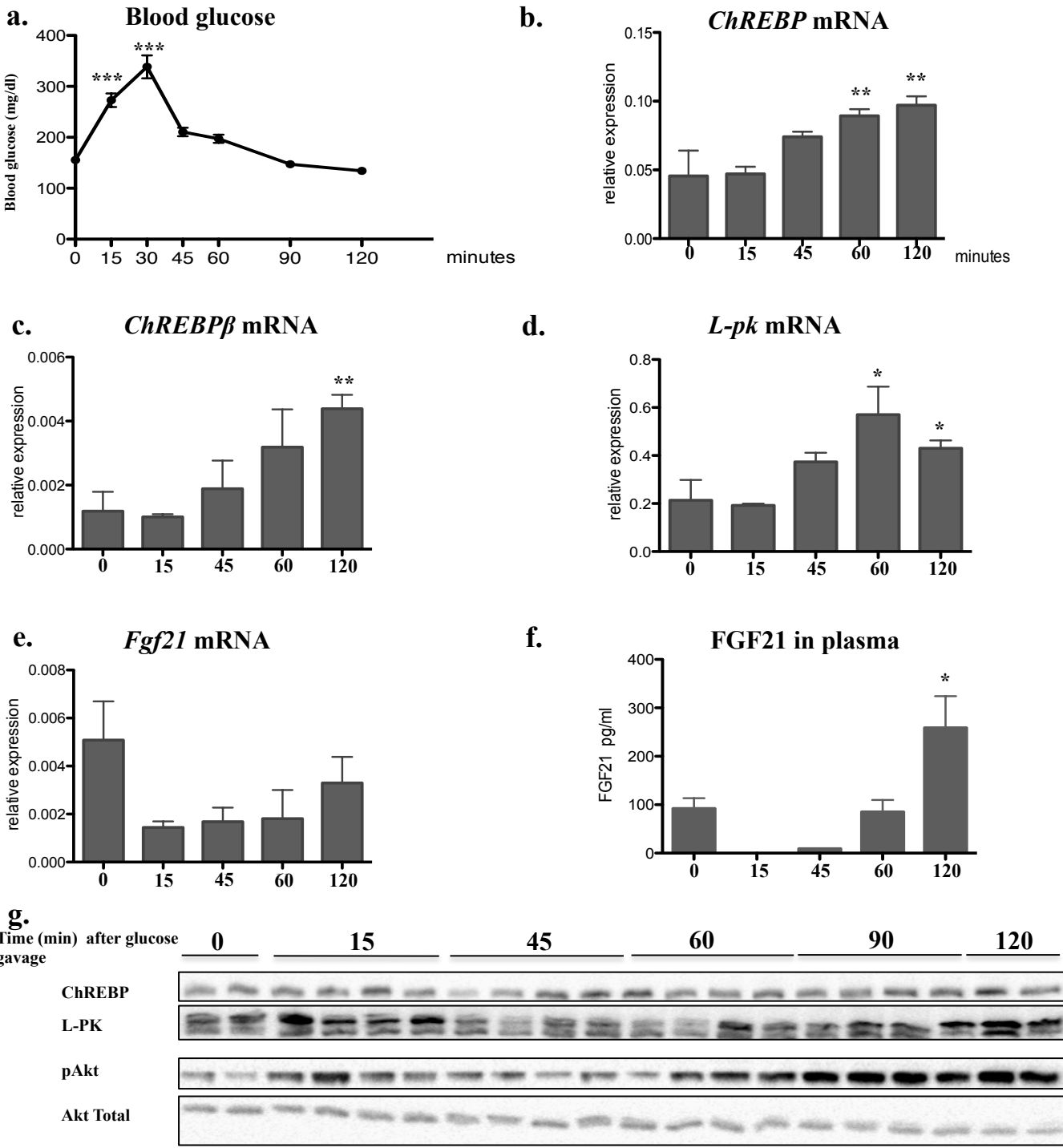


Figure 28: (a) Blood glucose of wild type mice administered a 20% glucose solution orally 3 times in fifteen minute intervals. (b-e) Liver gene expression determined by RT-qPCR 15, 45, 60 or 120 minutes after second glucose gavage. Time zero mice were not submitted to oral gavage. (f) FGF21 in plasma quantified by ELISA after the second glucose gavage. (g) Western blot of whole liver homogenates (40µg/ul) n=4. 2-way ANOVA followed by Dunnett's multiple comparisons Means + s.e.m plotted, p<0.05 (\*), p<0.01 (\*\*). (n=4)



**Figure 29: The refeeding-mediated FGF21 response is ChREBP dependent**

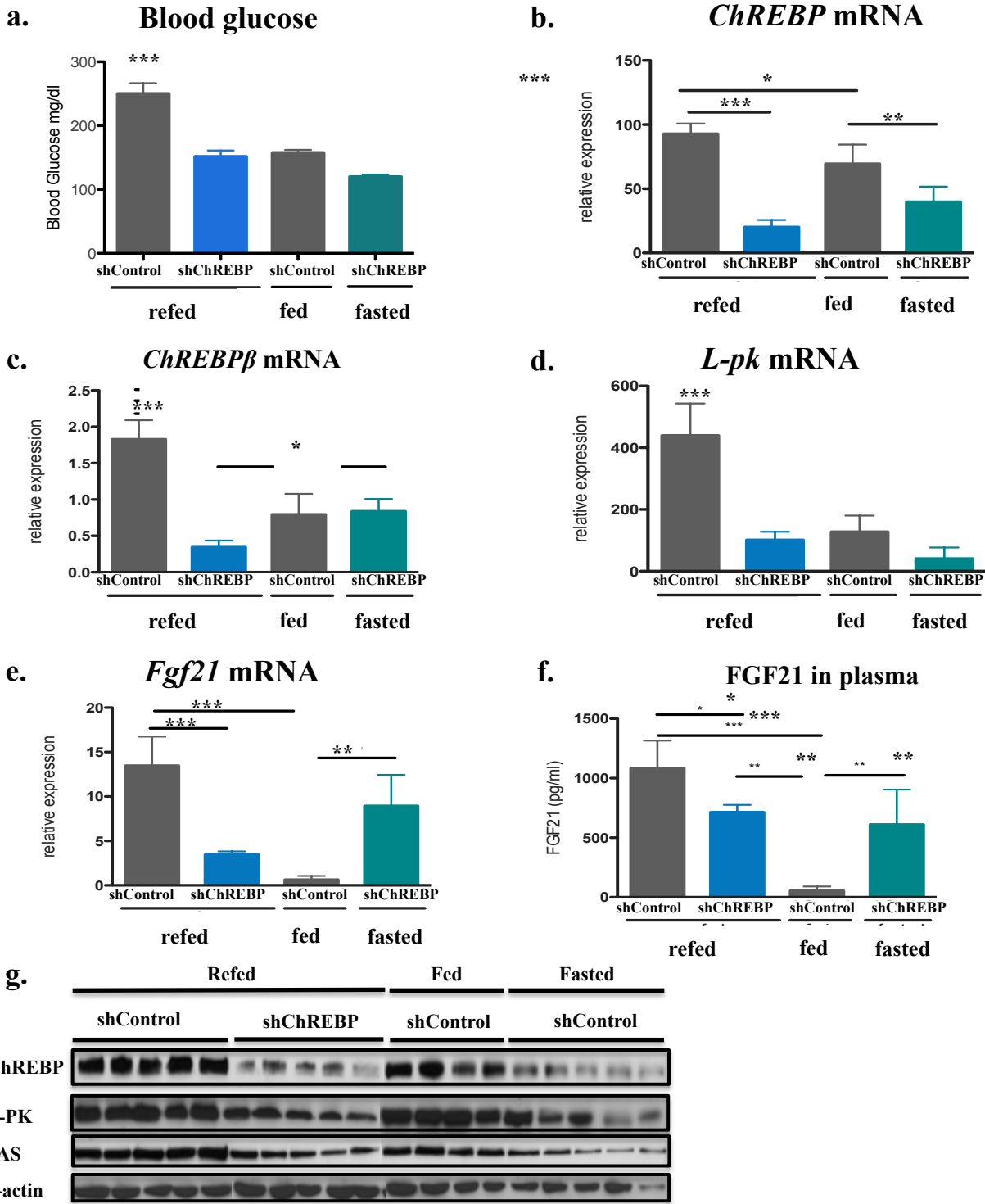


Figure 29: (a) Blood glucose at time of sacrifice. (b-e) Liver gene expression determined by RT-qPCR normalized using a housekeeping gene (cyclophilin). (f) Plasma collected at tissue harvest and quantified for FGF21 by ELISA. (g) Western blot of whole liver lysates collected at time of tissue harvest controlled by β-actin. Means + s.e.m plotted p≤0.05 (\*) p≤0.01 (\*\*) p≤ 0.001 (\*\*\*) (n=5) Adenoviral injection 3x10<sup>9</sup> pfu/mouse

**Figure 30: Over expression of ChREBP in liver induces FGF21 secretion and FGF21-like signaling in the pancreas**

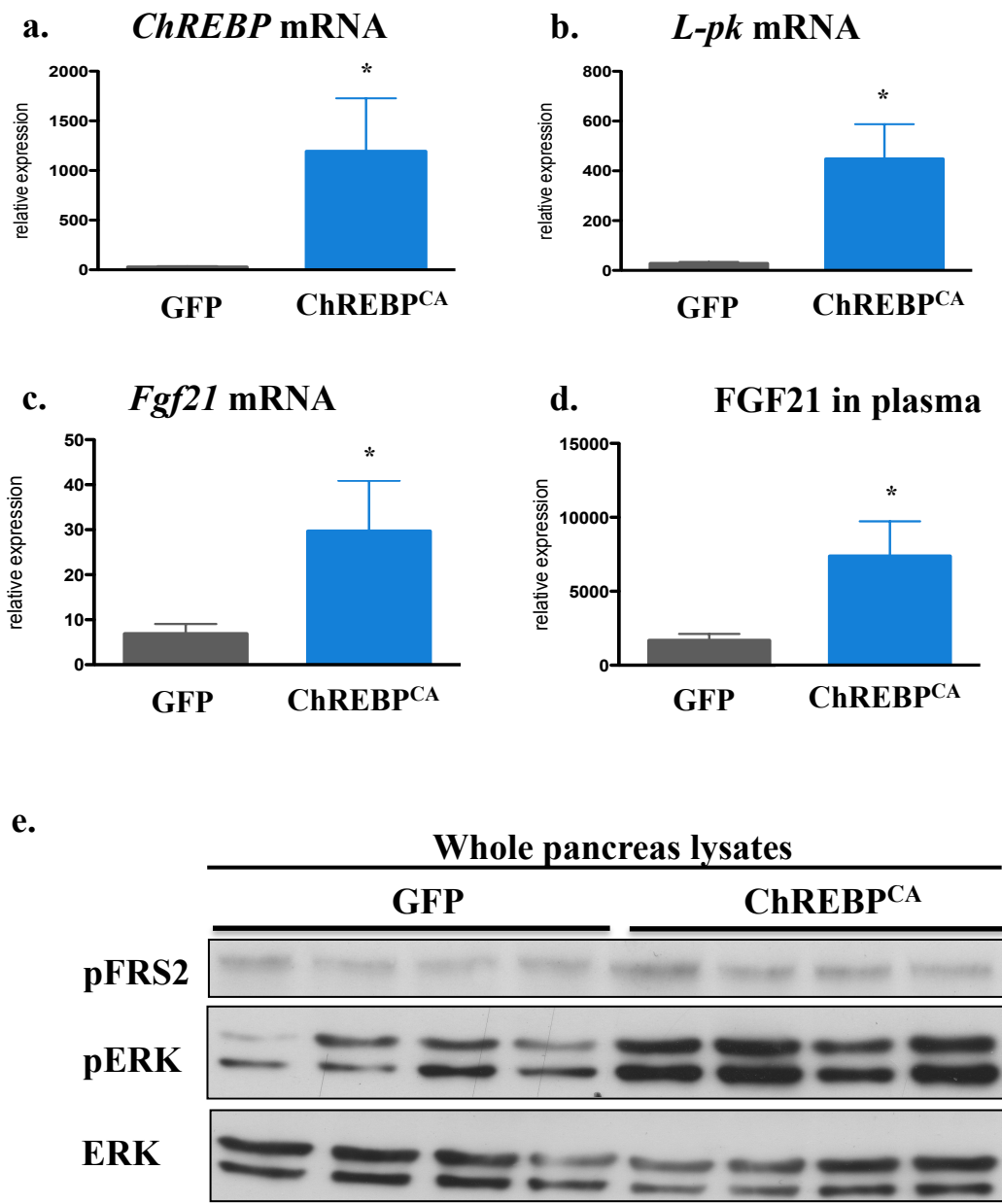


Figure 30: (a-c) Liver gene expression determined by RT-qPCR normalized using a housekeeping gene (cyclophilin). (d) Plasma collected at tissue harvest and quantified for FGF21 by ELISA. (e) Western blot of whole pancreas lysates collected at time of tissue harvest (fed) and analyzed for FGF21 like signaling. Mann-Whitney test, means + s.e.m plotted  $p \leq 0.01$  (\*\*),  $p \leq 0.001$  (\*\*\*) (n=4) Adenoviral injection  $3 \times 10^9$  pfu/mouse.

**Figure 31: Effect of conditioned medium of ChREBP<sup>CA</sup> expressing WT and FGF21 KO hepatocytes on INS1 cells**

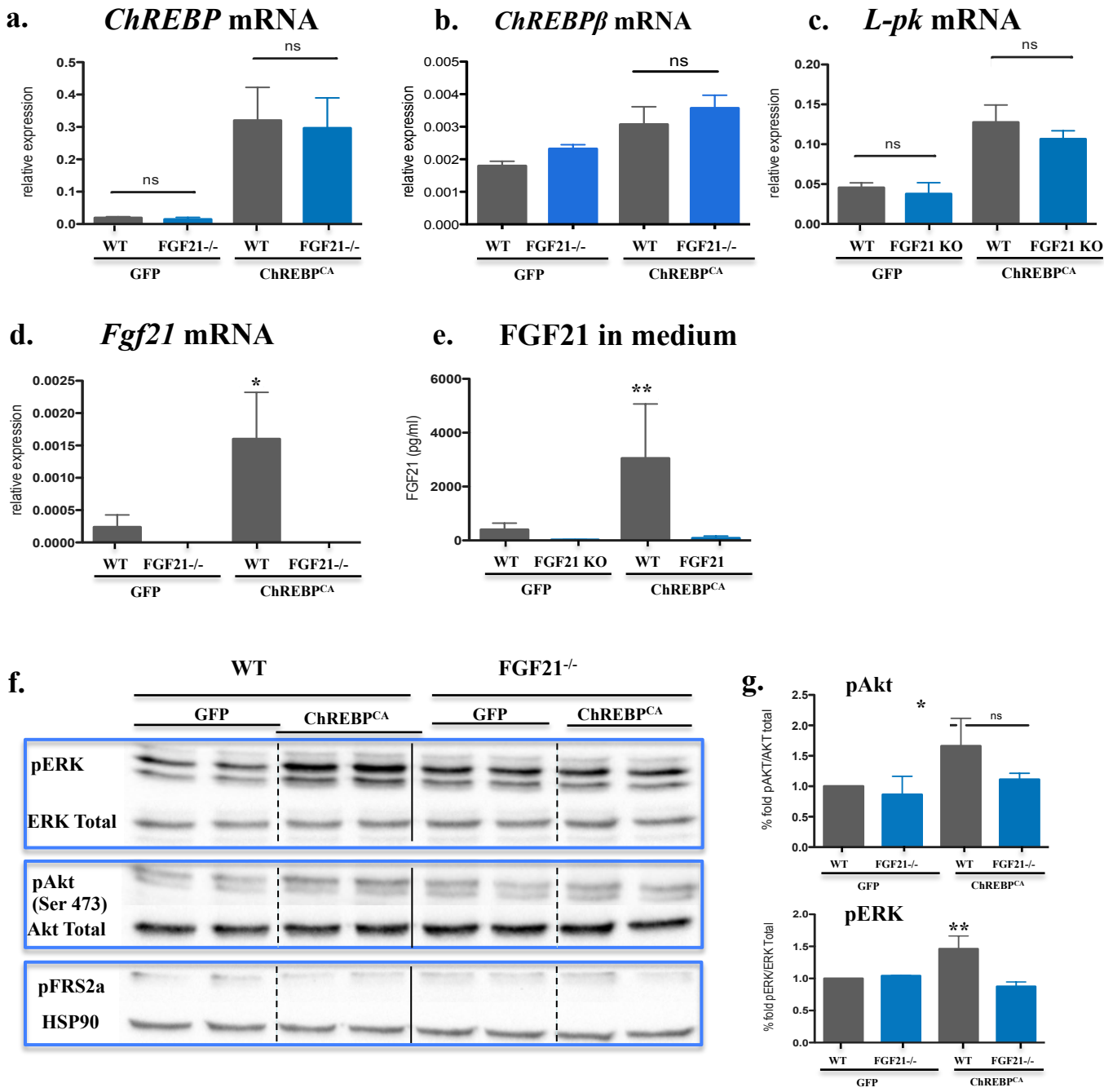


Figure 31: (a-d) Gene expression determined by RT-qPCR of hepatocytes from WT or FGF21<sup>-/-</sup> mice infected 6h after seeding by control (GFP) or ChREBP<sup>CA</sup> containing adenovirus. Medium was changed 24 h after transfection and cells collected following a 24 h resting period with 5mM glucose M199 medium. (e) Medium collected following 24 h glucose challenge of transfected cells and quantified by ELISA. (f) Western blot of INS1 cells incubated for 10 minutes with medium of hepatocytes stimulates by GFP or ChREBP<sup>CA</sup> (3x10<sup>9</sup> pfu) for FGF21 like-signaling. (g) Quantification of Akt and ERK phosphorylation over total Akt and total ERK protein content. Analysis conducted using image J software. ANOVA followed by Bonferroni post test Means + s.e.m plotted, p≤0.05 (\*), p≤0.01 (\*\*)(n=4)

**Figure 32: Glucose tolerance in WT and FGF21<sup>-/-</sup> mice expressing ChREBP<sup>CA</sup>**

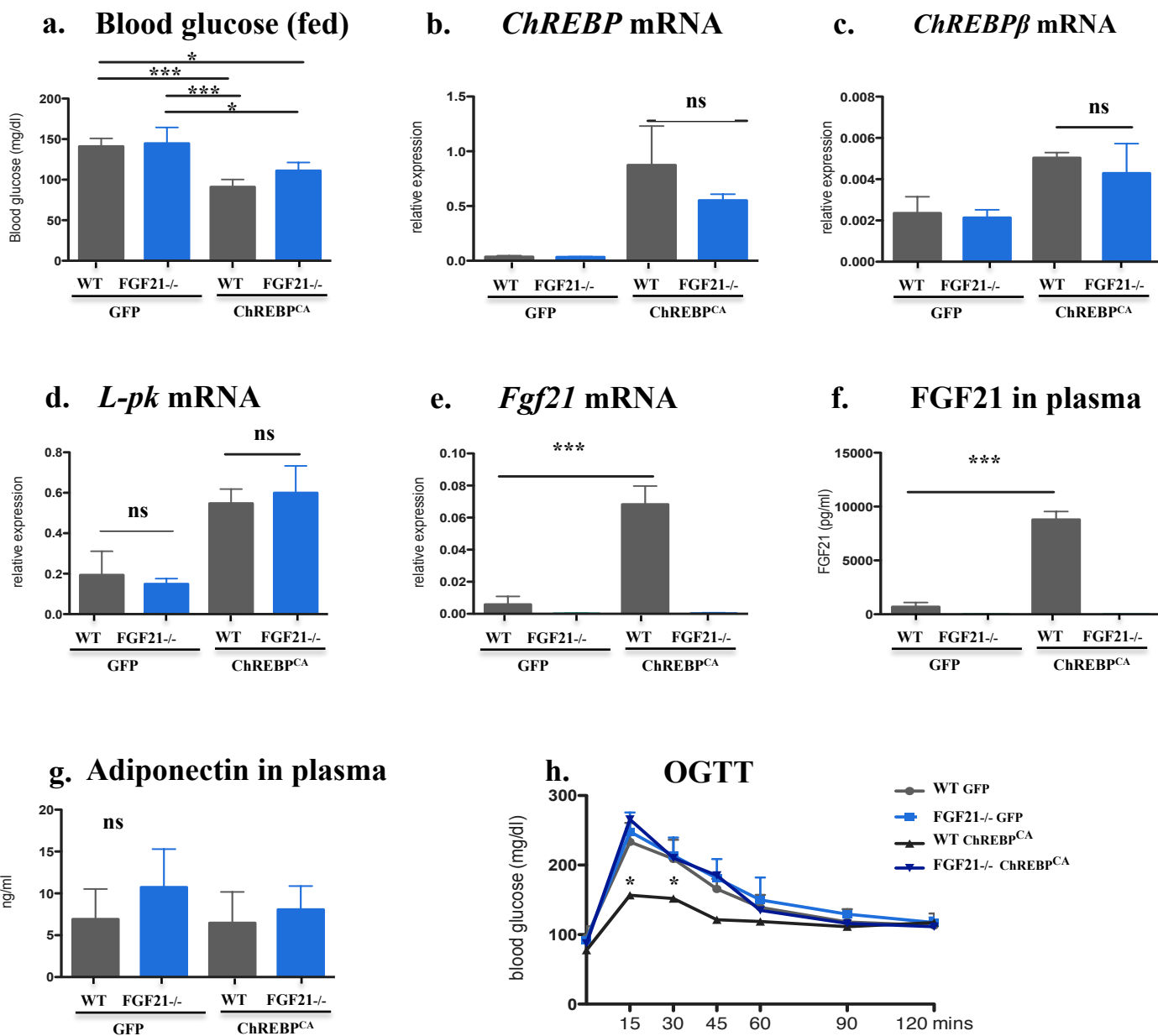


Figure 32: (a-e) Liver gene expression determined by RT-qPCR normalized using a housekeeping gene. (f) Plasma collected at tissue harvest and quantified for FGF21 by ELISA. (g) Plasma collected at tissue harvest and quantified for adiponectin by ELISA. (h) Oral Glucose Tolerance Test (OGTT, 2g/kg glucose) of male mice performed in GFP or ChREBP (3x10<sup>9</sup> pfu/mouse) expressing mice 4 days after adenoviral gene transfer. Blood glucose levels were measured by tail tip bleed at 15, 30, 60, 90 and 120 minutes post glucose gavage. Means + s.e.m plotted p≤0.05 (\*) p≤0.01 (\*\*) p≤ 0.001 (\*\*\*) (n=5)

**Figure 33: ChREBP improves glucose tolerance in a FGF21 dependent manner**

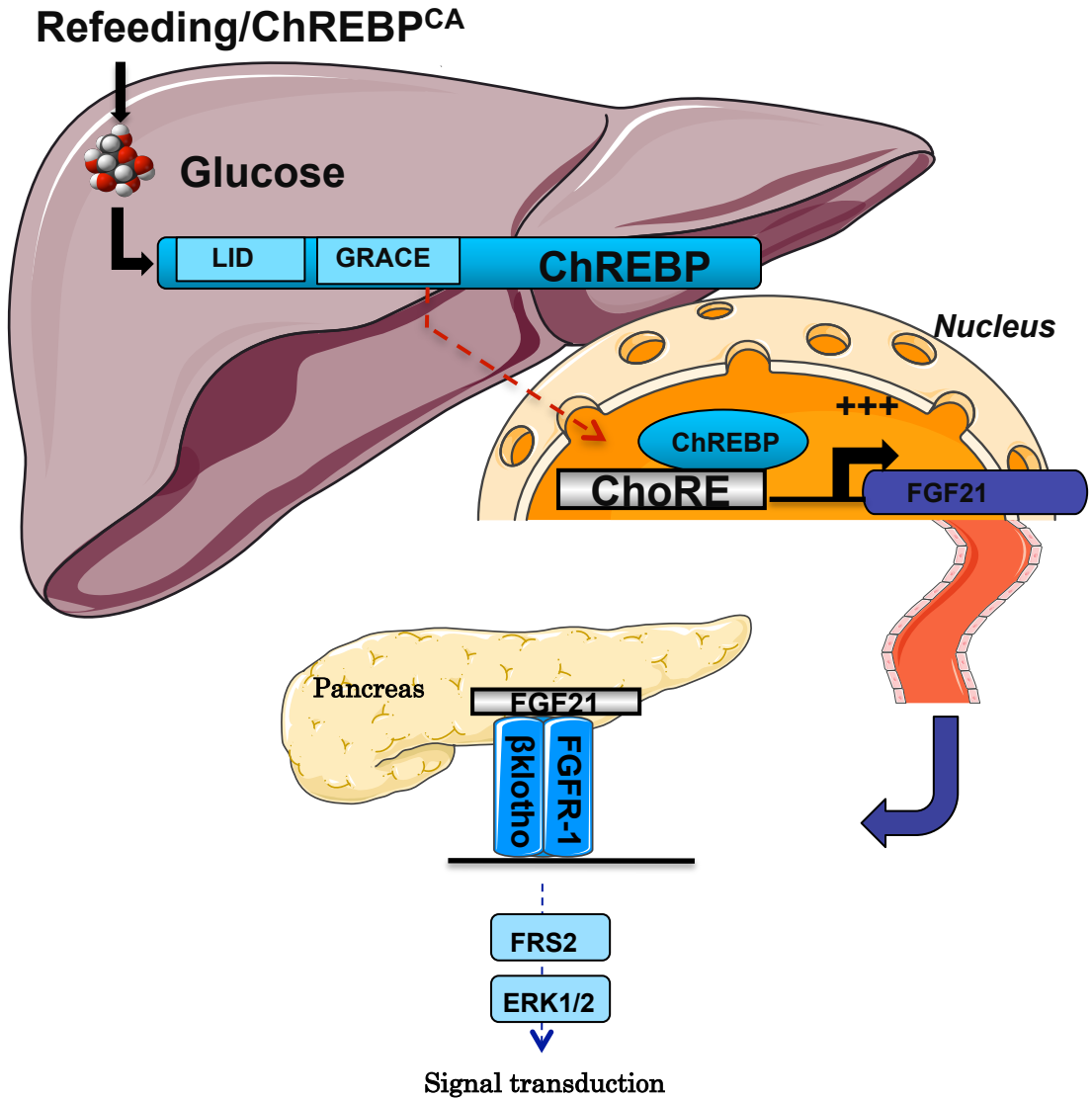


Figure 33: In response to glucose or hepatic ChREBP over expression FGF21 is secreted from the liver after direct binding of ChREBP to the *fgf21* promoter of. Proteins secreted from ChREBP-expressing hepatocytes are able to induce signaling of key  $\beta$ -cell survival pathways. This signaling effect seems, in part, dependent on FGF21.

## ***GENERAL DISCUSSION AND PERSPECTIVES***



## ***General discussion and perspectives***

### ***I-Hepatic ChREBP is necessary for the glucose-mediated induction of FGF21***

A hallmark of NAFLD is the accumulation of TAG in the liver with 26.1% derived from *de novo* lipogenesis (Donnelly et al., 2005). ChREBP and *de novo* lipogenesis are indeed increased in livers of obese and NAFLD patients (Eissing et al., 2013; Hurtado del Pozo et al., 2011; Lambert et al., 2014). However, recent research has revealed a more complex role for *de novo* lipogenesis. Mice fed a HFD lacking the lipogenic enzyme ACC in the liver have decreased *de novo* lipogenesis but display increased hepatic TAG concentrations due to decreased fatty acid oxidation (Chow et al., 2014). Similarly, hepatic FAS<sup>-/-</sup> mice fed a fat-free diet also show decreased *de novo* lipogenesis rates but suffer from hypoglycemia and increased NASH (Chakravarthy et al., 2005). On the opposite end, increasing *de novo* lipogenesis by ChREBP over expression in the liver of HFD diet mice improves glucose tolerance despite increasing fatty acid accumulation in the liver. The protective effect against insulin resistance and liver toxicity was attributed, in this study, to a change in hepatic lipid composition (a shift in the ratio from SFA to MUFA). Collectively, these studies suggest that *de novo* lipogenesis plays an important and complex role in supporting pathways of metabolic homeostasis (Solinas et al., 2015). Interestingly, the study of Benhamed et al. 2012 also reports that in response to ChREBP overexpression, even in a context of HFD, circulating levels of FGF21 were significantly increased. In this study, the direct contribution of FGF21 to the improved phenotype was not addressed (Figure 34). Guided by these observations, our laboratory also conducted experiments to investigate the role of ChREBP in glucose tolerance in a T1D diabetic model. Male C57Bl6/J mice, treated with STZ to destroy  $\beta$ -cells, were injected with GFP or ChREBP<sup>CA</sup> adenovirus. Analysis of these mice two weeks following adenoviral injection revealed that hepatic ChREBP<sup>CA</sup> expression reduced hyperglycemia and



**Figure 34: Hepatic ChREBP overexpression dissociates steatosis from insulin resistance**

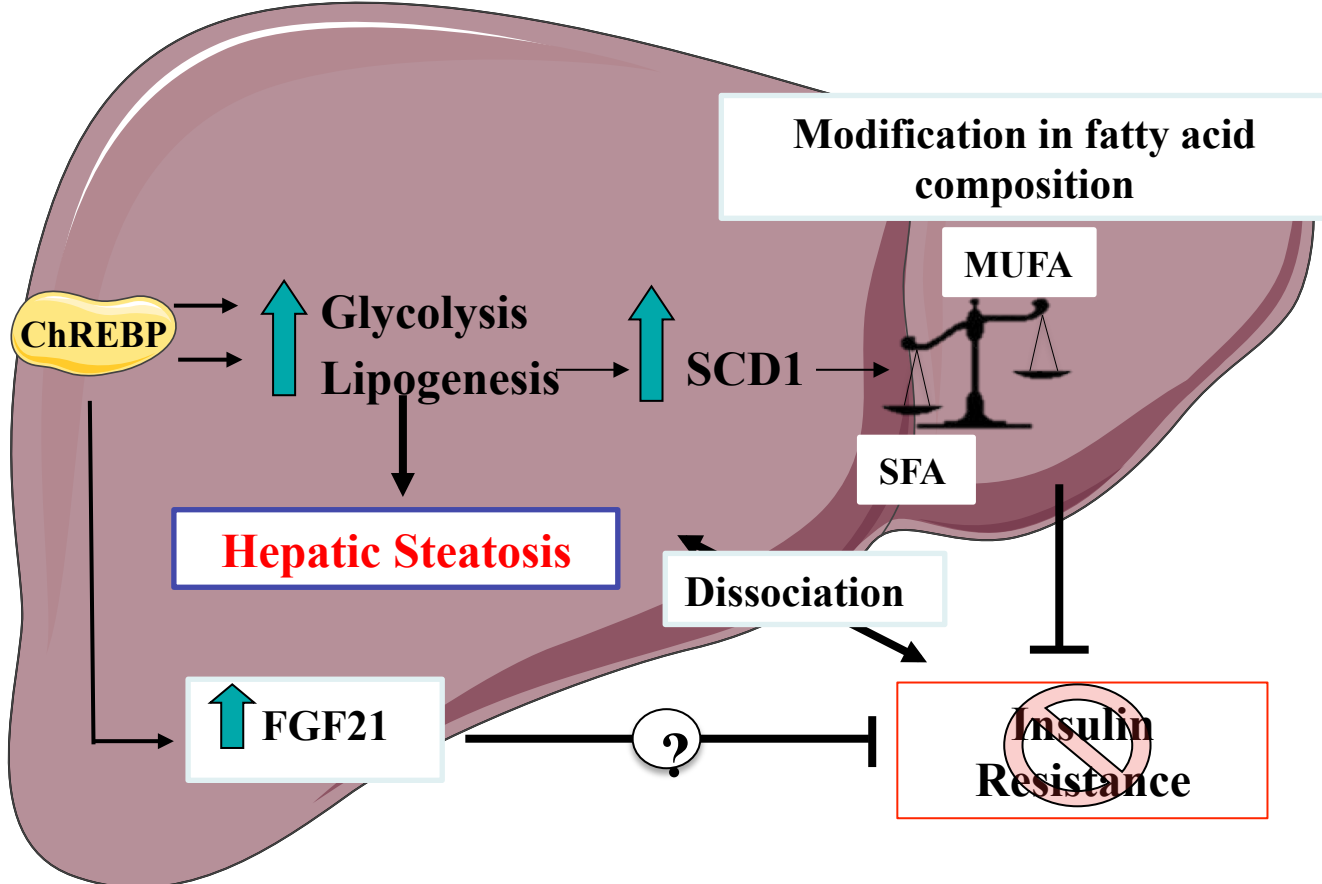


Figure 34: ChREBP overexpression in liver stimulates lipogenic genes, including SCD1, and leads to hepatic steatosis onset. ChREBP-mediated steatosis is however dissociated from insulin resistance due, in part, to a modification in fatty acid composition (a shift to the SFA to MUFA balance). Interestingly, a parallel increase in FGF21 expression and concentrations are also observed in response to ChREBP overexpression. The contribution of FGF21 to the protection of insulin resistance was not addressed in the study by Behamed et al. JCI 2012.

tended to increase blood insulin concentrations. Interestingly, a parallel increase in FGF21 concentrations was also observed. In addition, visual difference in insulin staining of islet sections was observed (A. Poupeau, thesis 2013). At the time of the study, the potential direct regulation of FGF21 by ChREBP was only emerging with the identification of the ChoRE on the *Fgf21* promoter (Iizuka et al. 2009). This paper, in combination with preliminary work in the laboratory, led to the original objectives of the current thesis, which was to determine if FGF21 responded to glucose in a ChREBP dependent manner and to investigate the impact of hepatic ChREBP activity on whole body glucose metabolism.

Our first studies reported that FGF21 was up regulated in plasma following a 24-hour fast 18-hour refeeding protocol. However, our early fasting/refeeding protocol left suspicion into whether the observed FGF21 measured 18 hours after refeeding was, in fact, residual FGF21 induced during the 24-hour fast. For this reason, we adapted an *in vivo* glucose bolus protocol. Oral glucose gavage (50mg/kg) significantly increased FGF21 in plasma measured 120 minutes following the first glucose bolus (Research subject 2 Figure 28). However, in two separate experiments, we noticed that *Fgf21* mRNA and protein in plasma tended to be elevated at time 0 of the experiment (Research subject 2 Figure 28f). We speculated that this early FGF21 induction was due to handling and experimental stress since FGF21 is highly sensitive to acute stress. Within the first hour of a physical stress, the glucocorticoid corticosterone is significantly increased in the plasma of C57BL/6 mice (Ahn et al., 2016). Circulating corticosterone is able to activate the glucocorticoid receptor in the liver, a potent inducer of *Fgf21*, by binding directly to the distal end of the *Fgf21* promoter (Patel et al., 2014)(Alexandrová and Farkas, 1992). To ensure that the glucose-mediated FGF21 response was not stress induced we adopted a new model of glucose challenge: a 20% glucose solution provided by water bottle overnight. Using this glucose challenge model, we demonstrated that hepatic FGF21 responds to glucose in C57Bl6/J mice. The use of global ChREBP<sup>-/-</sup> mice confirmed the necessity of ChREBP for the

induction of FGF21 in response to glucose, as plasma FGF21 protein concentrations were increased in glucose-challenged ChREBP<sup>+/+</sup> mice while low levels were detected in ChREBP<sup>-/-</sup> mice under similar conditions (Article Figure 3j;h). These findings are also supported in other publications (von Holstein-Rathlou et al., 2016; Iizuka et al., 2009).

Importantly, hepatic rescue of ChREBP in global ChREBP<sup>-/-</sup> mice was sufficient to restore *Fgf21* mRNA in the liver and protein in circulation demonstrating the specificity of hepatic ChREBP activity on circulating FGF21 in response to glucose challenge (Article Figure 4g;h). Figure 38). Interestingly, ChREBP re-induction in the liver of ChREBP<sup>-/-</sup> mice significantly rescued blood glucose concentrations to the control levels (Article Figure 4a). Inversely, hepatic knockdown of ChREBP using shRNA significantly reduced *Fgf21* expression and protein in the plasma (Research Subject 1 Figure 29i;f). These results suggest that hepatic ChREBP activity and not peripheral ChREBP is essential for FGF21 production and glucose homeostasis. This result is consistent with a previous publication in which hepatic ChREBP over expression improved impaired glucose tolerance of HFD challenged mice (Benhamed et al., 2012). However, the role of ChREBP in normalizing blood glucose seems paradoxical since it was recently shown that ChREBP activation by fructose regulates G6Pase expression and in turn increases hepatic glucose production, aggravating hyperglycemia (Kim et al., 2016). FGF21 may be the link by which ChREBP is able to regulate metabolic pathways when challenged with nutrient overload.

## ***II-Hepatic ChREBP over expression improves glucose tolerance in mice and FGF21-like signaling in pancreatic INS1 cells.***

To investigate the physiological impact of the ChREBP-FGF21 axis on whole body glucose metabolism we measured FGF21-like signaling in several metabolic organs, including skeletal

muscles and in the pancreas of hepatic ChREBP over expressing mice. Western blot analysis of whole pancreas lysate revealed an increase in phosphorylation of FRS2 and ERK (Research subject 2 Figure 30e). While this initial study was promising that hepatic ChREBP activity could have an effect on endocrine pancreatic function it is important to note that FGF21 signaling is almost 20 times more important in acinar cells of the exocrine pancreas than in  $\beta$ -cells of the endocrine pancreas (Singhal et al., 2016; Wente et al., 2006). In this context, to test the effects of the ChREBP-FGF21 pathway on  $\beta$ -cell function, conditioned medium experiments were performed using naïve rat islets or pancreatic INS1 cells. For this purpose, primary hepatocytes harvested from FGF21<sup>+/+</sup> or FGF21<sup>-/-</sup> mice were transfected with GFP or ChREBP<sup>CA</sup>. The medium of these cells was collected and used to stimulate naïve INS1 cells. Following a 10-minute incubation, we noted an increase in phosphorylation of ERK and Akt in INS1 incubated with culture medium from ChREBP<sup>CA</sup> expressing FGF21<sup>+/+</sup> that was absent in INS1 cells treated with the culture medium from ChREBP<sup>CA</sup> expressing FGF21<sup>-/-</sup> hepatocytes (Research subject 2 Figure 31f ;g). Akt is especially important for  $\beta$ -cell survival and growth. Studies using transgenic mice expressing with a constitutively active Akt linked to the *insulin* gene promoter in  $\beta$ -cell, reported an increase in  $\beta$ -cell mass, which rescued the diabetic phenotype induced by low dosed STZ administration (Bernal-Mizrachi et al. 2001). In addition, it is well established that ERK phosphorylation stimulates insulin gene transcription in  $\beta$ -cells (Benes et al., 1998). Previous publications have reported that FGF21 induces both ERK and Akt signaling in INS1 cells. The positive FGF21-like signal in INS1 cells we observed in the current study is in agreement with other published studies in which direct FGF21 stimulation on rat pancreatic islets or INS1 cells increased ERK and Akt phosphorylation (Adams et al., 2012; Wente et al., 2006). It has also been suggested that FGF21 may stimulate  $\beta$ -cell proliferation through ERK signaling, however, this remains controversial and it is more likely that FGF21 provides a protective effect by reducing  $\beta$ -cell glucolipotoxicity thus preserving  $\beta$ -cell function (Singhal et al., 2016; Wente et al., 2006) (Figure 35).

In parallel of these *in vitro* experiments, we addressed if ChREBP-mediated FGF21 induction could induce a glucose lowering effect *in vivo*. FGF21<sup>+/+</sup> and FGF21<sup>-/-</sup> mice were injected with GFP or ChREBP<sup>CA</sup> adenoviruses. ChREBP<sup>CA</sup> activity significantly lowered blood glucose levels in both FGF21<sup>+/+</sup> and FGF21<sup>-/-</sup> mice, suggesting that FGF21 is not necessary for the glucose lowering effect of ChREBP. However, an OGTT demonstrated that FGF21<sup>+/+</sup> mice expressing ChREBP<sup>CA</sup> had improved glucose tolerance as compared to FGF21<sup>-/-</sup> mice expressing ChREBP<sup>CA</sup> (Research Subject 2 Figure 32a ;h). The pancreas of mice was then assessed for histological changes. Despite promising results from INS1 experiments conducted *in vitro* of increased ERK and Akt phosphorylation, FGF21<sup>+/+</sup> mice expressing ChREBP<sup>CA</sup> did not reveal any marked differences in  $\beta$ -cell insulin content, as measured by immunostaining, or a change in  $\beta$ -cell mass (data not shown). Further studies using liver specific FGF21<sup>-/-</sup> mice would help determining the mechanism in which the ChREBP-FGF21 axis improves glucose tolerance.

It should be also discussed that the importance of ChREBP-FGF21 axis may lie in the liver and not at the level of the pancreas. Indeed, following 8 weeks of high-fructose diet, FGF21 in circulation is increased in a ChREBP dependent fashion in C57BL/6 mice. Interestingly, fructose-fed FGF21<sup>-/-</sup> mice demonstrate liver atrophy and fibrosis accompanied with an increase in markers of inflammation, which was not observed in fructose fed control mice (C57BL/6) (Fisher et al., 2016) (Figure 35). We also noted a significant increase in hepatic TNF- $\alpha$  mRNA concentrations in livers of FGF21<sup>-/-</sup> mice expressing ChREBP<sup>CA</sup>, however, plasma concentrations of transaminases (ALT and AST), markers of liver injury, remained similar in FGF21<sup>+/+</sup> or FGF21<sup>-/-</sup> mice expressing ChREBP<sup>CA</sup> (data not shown). Finally, we cannot exclude that in response to glucose or fructose ChREBP may induce other hepatokines linked to glucose tolerance. During the course of the current thesis project, proteomic analysis of culture medium collected from ChREBP<sup>CA</sup> expressing C57Bl6/J hepatocytes was conducted. Preliminary analysis confirms that FGF21 is highly enriched in culture medium from ChREBP<sup>CA</sup> expressing hepatocytes. Detailed bioinformatics analysis could reveal novel ChREBP-mediated hepatokines that could possibly affect  $\beta$ -cell function and whole body glucose homeostasis.

Figure 35:

# Physiological relevance of ChREBP-mediated FGF21

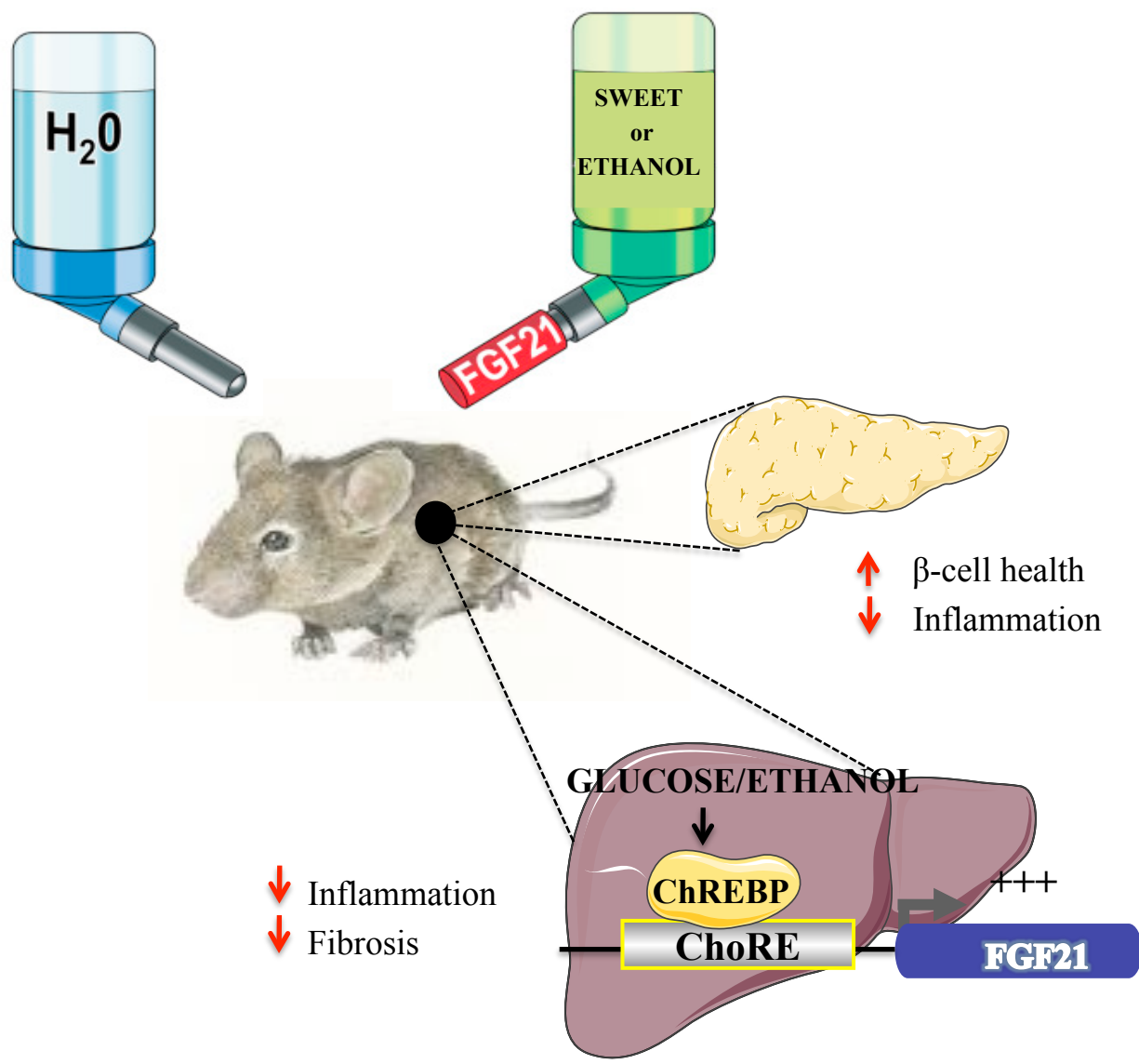


Figure 35: FGF21 is induced in response to sweet intake and ethanol intake in a ChREBP dependent manner by binding to the ChoRE of the FGF21 promoter. Hepatic FGF21 in circulation has been shown to improve β-cell health and reduce inflammation. The absence of FGF21 in liver aggravates inflammation and fibrosis.

The important role of FGF21 in protecting the liver and pancreas from glucose toxicity FGF21 has been shown to work in a negative feed back loop to regulate carbohydrate intake. The first clues to a role for FGF21 in carbohydrate intake came from genetic screenings that identified SNPs in and around the *Fgf21* gene, which caused increased carbohydrate consumption and decreased fat and protein intake (Chu et al., 2013; Tanaka et al., 2013). FGF21 has been identified to acts centrally on the paraventricular and the suprachiasmatic nucleus of the hypothalamus, these regions includes  $\beta$ -klotho and FGFR1-c receptors in which FGF21 is able to bind (Bookout et al., 2013). Studies in mice and humans have identified FGF21 as a regulator of sweet intake and gave physiological relevance to the ChREBP-mediated FGF21 regulation. These publications reported that in response to sucrose intake FGF21 was ChREBP dependently induced in the liver, creating a negative feedback loop to the paraventricular nucleus of the hypothalamus blocking sugar-seeking behavior and improving whole-body glucose homeostasis of mice (von Holstein-Rathlou et al., 2016; Talukdar et al., 2016). Ethanol intake is signaled to the brain in a similar fashion as sweet intake. Ethanol consumption drives *de novo* lipogenesis and causes inflammation, and insulin resistance (Lustig, 2013). In response to ethanol intake in male C57BL/6 mice hepatic ChREBP acetylation and its recruitment onto target gene promoters are increased (Marmier et al., 2015). Activation of FGF21 in the liver of ChREBP also induced a negative feedback loop via dopamine signaling in the hypothalamus reducing ethanol intake (Talukdar et al., 2016). In this context, it would be interesting whether liver specific ChREBP<sup>hep-/-</sup> show modification of sweet and/or ethanol preference.

### ***III-Dynamic regulation of FGF21 by two opposing transcription factors ChREBP and PPAR $\alpha$***

Despite advances in understanding the physiological role of FGF21 in response to carbohydrates, few publications have addressed the transcriptional regulation of FGF21 and the potential importance of a cross regulation by the transcription factor ChREBP and PPAR $\alpha$ . By pooling together publications from 2009 identifying a ChoRE on the FGF21 promoter at -72 to -54 bp (Iizuka et al., 2009) and a 2014 publication placing a PPRE at -74 to -69 bp from the transcription start site (TSS) (Kim et al., 2014), we noticed that these two sites were overlapping. This observation was later confirmed (Girer et al., 2016). The overlapping binding region led us to question the role of PPAR $\alpha$  in the ChREBP-dependent FGF21 glucose response. With the use of PPAR $\alpha^{\text{hep-/-}}$  mice, we unexpectedly observed *in vitro* and *in vivo* that in the absence of PPAR $\alpha$  the glucose-mediated FGF21 response was lost (Article Figures 5 and 7). ChIP experiments confirmed that the loss of *Fgf21* induction in response to glucose was due to a decrease in ChREBP binding to the *Fgf21* ChoRE. We initially hypothesized that the specificity of their interaction may be due to the proximity of their promoter regions. While immunoprecipitation experiments in HEK cells confirmed a direct interaction of ChREBP and PPAR $\alpha$  (data not shown) we are currently investigating the role of the histone deacetylase HDAC3, an important regulator of FGF21 promoter activity, to explain the lack of induction of FGF21 in response of glucose in the context of PPAR $\alpha$  deficiency (Rando et al. 2016).

HDAC3 has been identified as a key regulator of lipid synthesis and hepatic glucose production (Sun et al., 2012). Like studies of hepatic ChREBP over expression, HDAC3 inhibition dissociates fatty liver from insulin resistance. Liver specific deletion of HDAC3 shunts energy away from hepatic glucose production by up regulating genes important for lipogenesis (*Scd1*, *Fas*) and NADH production (citrate synthase). Up regulation of Perilipin 2, a protein that coats



**Figure 36: Regulation of the *Fgf21* promoter by ChREBP and PPAR $\alpha$**

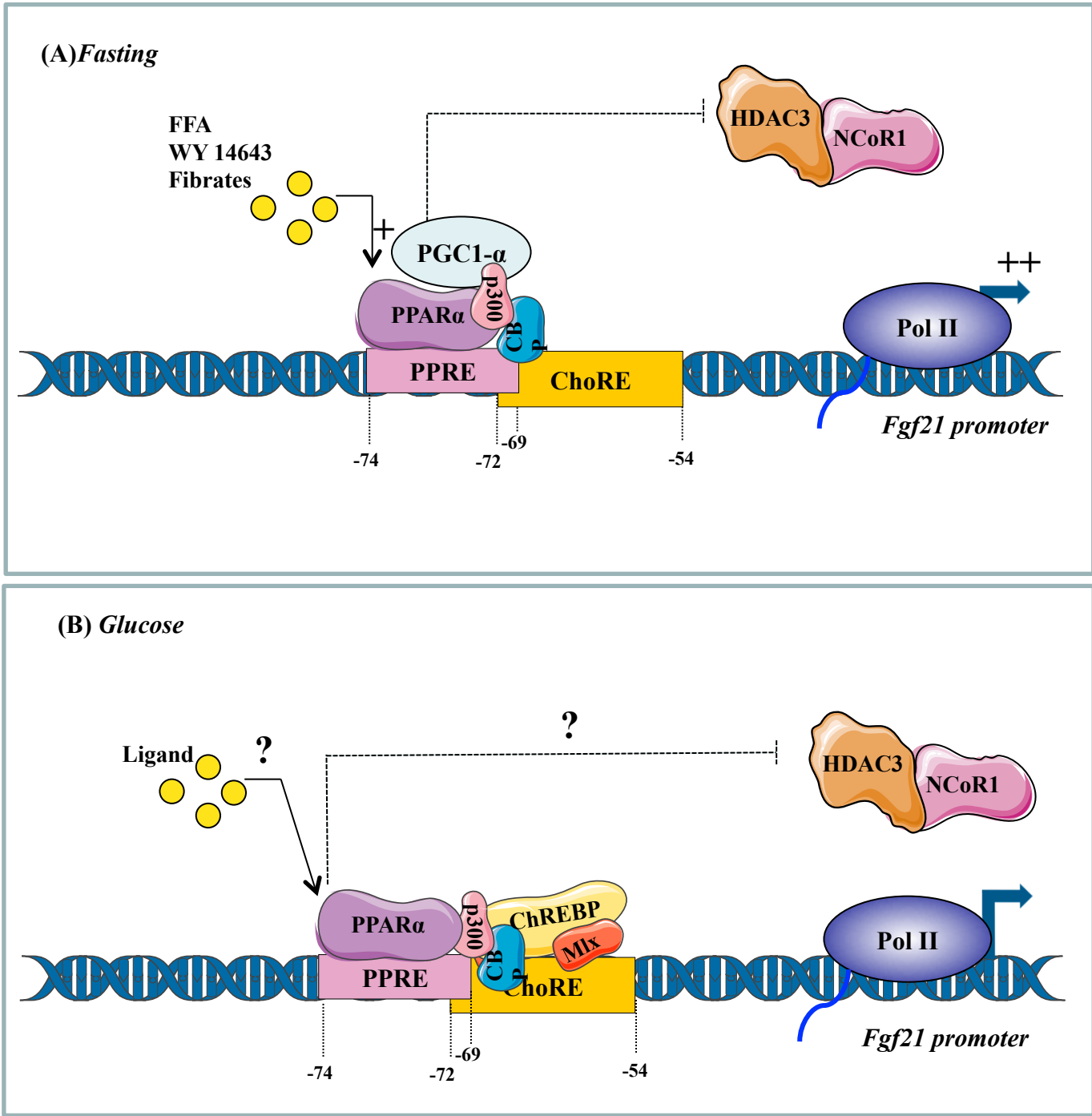


Figure 36 : Binding of PPAR $\alpha$  ligands allows for recruitment of coactivator PGC1- $\alpha$  and HAT proteins p300/CBP during fasting and blocks corepressor NCoR1 and recruitment of HDAC3. Glucose-mediated *Fgf21* expression requires both ChREBP and PPAR $\alpha$ . During glucose challenge PPAR $\alpha$  may be necessary to block HDAC3 recruitment to the *Fgf21* promoter, however, the signal and mechanism in which PPAR $\alpha$  would block the recruitment of corepressors has to date not been explored.

lipid droplets, was also reported in liver-specific HDAC3<sup>-/-</sup> mice which displayed improved hepatic lipid storage. While hepatic HDAC3 knockout did lead to severe hepatosteatosis mice showed improved insulin sensitivity and glucose tolerance (Sun et al., 2012). It was first suggested that HDAC3 might be a regulator of FGF21 when a HDAC3 blocking agent, sodium butyrate, increased FGF21 in the plasma of C57BL/6J fed a HFD (Li et al., 2012). HDAC3 deacetylase activity requires interaction with several nuclear receptors, including PPAR $\alpha$  and co-repressor NCoR1 in the fed state (Feng et al., 2011; Guenther et al., 2001). NCoR1 works on a phosphorylation-dependent switch to suppress lipogenesis during fasting and fatty acid oxidation during refeeding. Insulin activated Akt phosphorylates NCoR1 on ser1460 which favors NCoR1 interaction with PPAR $\alpha$  and suppresses induction of its target gene (*Acox1*) (Mottis et al., 2013). In the fasted state, NCoR1 represses lipogenesis by a mechanism implying the HDAC3-mediated suppression of SREBP-1c (Jo et al., 2015; Papazyan et al., 2016). During fasting, binding of PPAR $\alpha$  ligands allows for recruitment of co-activator PGC1- $\alpha$  and HAT proteins p300/CBP. ChIP assay revealed that HDAC3 interacts with PPAR $\alpha$  at the distal PPRE of the *Fgf21* promoter (Figure 36a). HDAC3 inhibition increased recruitment of both PPAR $\alpha$  and polymerase II onto the *Fgf21* promoter in C57BL/6J mice fed a HFD (Li et al., 2012). In our study we showed that recruitment of ChREBP onto the ChoRE of the *Fgf21* promoter was significantly reduced in the liver of PPAR $\alpha^{\text{hep-/-}}$  glucose challenged mice. In line with the current body of evidence suggesting that HDAC3 inhibition improves *Fgf21* expression in the literature, we are currently investigating the role of HDAC3 in the ChREBP-mediated induction of *Fgf21* expression. However, this does not answer the question as to why the presence of PPAR $\alpha$  is necessary for ChREBP binding to the *Fgf21* promoter. To address this issue we are currently conducting ChIP analysis of the *Fgf21* promoter region that includes the overlapping ChoRE and PPRE regions. Assuming that PPAR $\alpha$  is always bound to the *Fgf21* PPRE as would suggest its regulation by NCoR1 and HDAC3 it is possible that PPAR $\alpha$  is necessary to hold open the DNA for ChREBP binding (Figure 36b). The proximity of their binding regions on the *Fgf21* promoter

**Figure 37: The ChREBP and PPAR $\alpha$  cross talk: direct and indirect mechanisms**

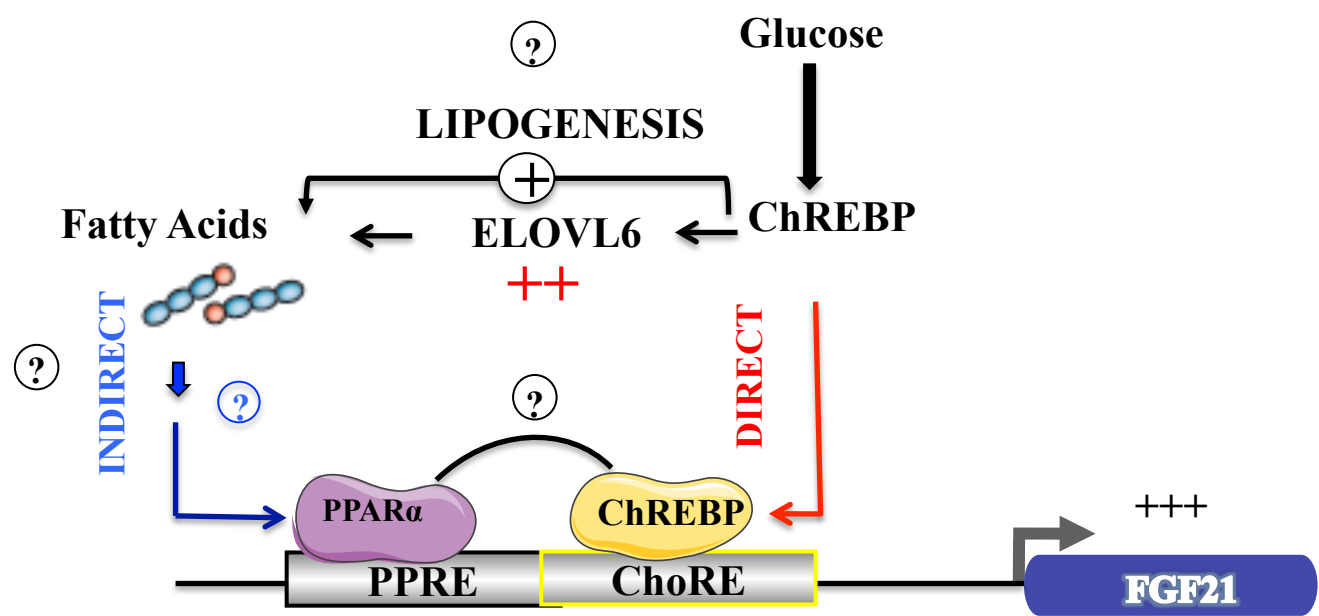


Figure 37: The proposed cross-talk between ChREBP and PPAR $\alpha$  may be due to both direct and indirect mechanisms. During periods of over-nutrition, ChREBP dependent lipogenesis in the liver may produce specific lipid species that are sent to the white adipose tissue for storage. During fasting these lipid species are liberated and impact PPAR $\alpha$  activity in the liver despite little ChREBP activity. Directly, on the *Fgf21* promoter, ChREBP and PPAR $\alpha$  have an overlapping binding region and both transcription factors may interact for induction of FGF21.

would also explain their physical interaction observed by immunoprecipitation identifying a direct relationship between ChREBP and PPAR $\alpha$  for the dynamic regulation of FGF21.

In addition, we cannot exclude that the proposed cross-talk between ChREBP and PPAR $\alpha$  may be due to both direct and indirect mechanisms. Indeed, during periods of over-nutrition, lipids synthesized by lipogenesis through a ChREBP-dependent manner in the liver, could be later liberated during fasting and impact PPAR $\alpha$  activity despite little ChREBP activity (Figure 37). Interestingly, ChREBP was shown to promote adipocyte differentiation, in part, through its control of PPAR $\gamma$ , a major actor of adipogenesis (Witte et al., 2015). The indirect ChREBP-mediated induction of PPAR $\gamma$  activity was reported to rely on FAS activity, which provides ligands of lipid origin for PPAR $\gamma$  and by direct trans-activation of the PPAR $\gamma$  ligand-binding domain. Studies by Semenkovich and co-workers suggested a FAS-dependent activating PPAR $\alpha$  pathway (Chakravarthy et al., 2009). Collectively, these studies suggest that ChREBP may be a direct and/or indirect regulator of PPAR nuclear factors. Future studies will be conducted in ChREBP liver-specific knockout mice, currently under development in our lab to determine how hepatic ChREBP-driven lipogenesis can modulate FFA released from white adipose tissue during fasting and their effects on hepatic PPAR $\alpha$  activity.

## **General conclusion**

The current thesis dissertation contributes to the growing body of evidence that places ChREBP as a key regulator of energy balance in response to over nutrition and highlights the necessity of ChREBP for the expression of the anti-diabetic hepatokine FGF21. Interestingly the work completed during this thesis revealed an unexpected cooperation between the two transcription factors ChREBP and PPAR $\alpha$ , independently regulated by opposing nutritional states, in preserving whole body energy balance.



## ***ANNEX***



# Hepatokines: unlocking the multi-organ network in metabolic diseases

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**Abstract** In the face of urbanisation, surplus energy intake, sedentary habits and obesity, type 2 diabetes has developed into a major health concern worldwide. Commonly overlooked in contemporary obesity research, the liver is emerging as a central regulator of whole body energy homeostasis. Liver-derived proteins known as hepatokines are now considered attractive targets for the development of novel type 2 diabetes treatments. This commentary presents examples of three leading hepatokines: fetuin-A, the first to be described and correlated with increased inflammation and insulin resistance; angiopoietin-like protein (ANGPTL)8/betatrophin, initially proposed for its action on beta cell proliferation, although this effect has recently been brought into question; and fibroblast growth factor 21 (FGF21), an insulin-sensitising hormone that is an appealing drug target because of its beneficial metabolic actions. Novel discoveries in hepatokine research may lead to promising biomarkers and treatments for metabolic disorders and type 2 diabetes. This is one of a series of commentaries under the banner ‘50 years forward’, giving personal opinions on future perspectives in diabetes, to celebrate the 50th anniversary of *Diabetologia* (1965–2015).

**Keywords** Beta cell · Glucose homeostasis · Hepatokines · Liver · Metabolic diseases · NAFLD · Type 2 diabetes

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## Abbreviations

ANGPTL	Angiopoietin-like protein
FGF21	Fibroblast growth factor 21
LECT2	Leucocyte cell-derived chemotaxin 2
LIRKO	Liver-specific insulin receptor knockout
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis

## Introduction

The metabolic syndrome is a leading health concern in the wake of urbanisation, surplus energy intake, obesity and sedentary habits. It is defined by a constellation of interconnected physiological and biochemical factors that increase the risk of cardiovascular disease and type 2 diabetes, namely, insulin resistance, visceral adiposity, dyslipidaemia, fatty liver, endothelial dysfunction and elevated blood pressure. Hyperglycaemia in type 2 diabetes is the result of impaired insulin secretion and increased insulin resistance in target tissues (liver, muscle, adipose tissue). Interestingly, several proteins that are exclusively or predominantly secreted by the liver are now known to directly affect energy metabolism. Analogous to the adipokines (from adipose tissue) and myokines (from skeletal muscle), these liver-derived proteins, named hepatokines, have ambivalent roles, either promoting insulin resistance or improving metabolic variables of type 2 diabetes [1]. For example, fetuin-A, plays a major pathogenic role in metabolic diseases, while fibroblast growth factor 21 (FGF21) has recently emerged as a novel hormone, leading to beneficial effects on glucose metabolism and lipid homeostasis, in addition to promoting rapid body weight loss in rodents [2]. Several FGF21 benefits have translated to non-human primates and obese type 2 diabetic patients [3].



From an historical point of view, and in order to illustrate the roles of hepatokines in whole system biology, we will briefly review the current knowledge on three major hepatokines: (1) fetuin-A; (2) angiopoietin-like protein (ANGPTL)8, also known as betatrophin; and (3) FGF21. Keeping in mind the spirit of the task at hand, we will also speculate on how hepatokines may in the future represent novel biomarkers and therapeutic approaches for metabolic disorders and type 2 diabetes.

While our environment in Western societies has played a major role in the exponential growth of metabolic-related diseases, the notion that the sole cause of obesity is behaviour was first challenged in 1969 by the results of the parabiosis studies conducted by Douglas Coleman [4]. The experiments involved mice with two distinct diabetic mutations, *ob/ob* mice, which display moderate glucose intolerance and islet hypertrophy, and *db/db* mice, characterised by a shortened life expectancy and islet apoptosis. When paired to a wild-type mouse, a significant food intake-limiting effect was reported on the *ob/ob* partner; conversely, the *db/db*–wild-type pairing induced severe feeding apathy in the wild-type, leading to its starvation and death [4]. Coleman concluded that the observations were the result of a circulatory satiety factor, with the *ob/ob* mouse producing insufficient factor to regulate its food intake and the *db/db* mouse producing factor that it was unresponsive to. These findings not only provided the first clues to a genetic component of obesity but also—of particular relevance to this commentary—opened a new role for organ crosstalk in the control of energy homeostasis. While Coleman's work revitalised the importance of circulatory factors in the field of diabetes, the theory of diabetes as a consequence of multi-organ dysfunction was first established during the mid-19th century. Claude Bernard, professor and physiologist at the College of France in Paris (1855–1878) founded the theory of internal secretion and first illustrated diabetes mellitus as a multi-organ disorder. His discovery of endogenous glucose production by the liver highlighted the critical role of this organ in glucose homeostasis and diabetes. Development of the liver-specific insulin receptor knockout mouse (LIRKO) by Ronald Kahn's laboratory in 2000 provided the first opportunity to explore the consequences of selective hepatic insulin resistance on systematic glucose tolerance [5]. From the results of classic physiological experiments such as parabiosis and newly developed genetic mouse models, the role of hepatokines can be unlocked.

### Fetuin-A

The first liver-derived factor suggested to regulate metabolic balance through integrated organ crosstalk was fetuin-A, a 64 kDa glycoprotein encoded by the *Ahsg* gene. Circulating levels of fetuin-A are increased in obesity and related

disorders such as the metabolic syndrome and type 2 diabetes. Fetuin-A concentrations are tightly correlated with impaired insulin sensitivity and glucose intolerance as it directly inhibits the downstream phosphorylation events of the insulin signalling cascade and the translocation of the glucose transporter GLUT4 in insulin target tissues. Interestingly, this liver-derived protein is also associated with fatty liver and correlates with non-alcoholic fatty liver disease (NAFLD) in humans [1]. Identified as an adaptor protein for saturated fatty acid-mediated activation of Toll-like receptor 4, this fetuin-A–fatty acid synergy may induce inflammatory signalling and insulin resistance—two important driving forces behind type 2 diabetes [6]. Pioglitazone, one of the thiazolidinedione class of type 2 diabetes agents, significantly reduces serum fetuin-A levels in patients with type 2 diabetes. However, other insulin-sensitising therapies, such as metformin and aerobic exercise, do not seem to affect fetuin-A levels [7]. Although its regulation is still largely unknown, fetuin-A still represents an attractive target for the development of type 2 diabetes treatments.

### ANGPTL8/betatrophin

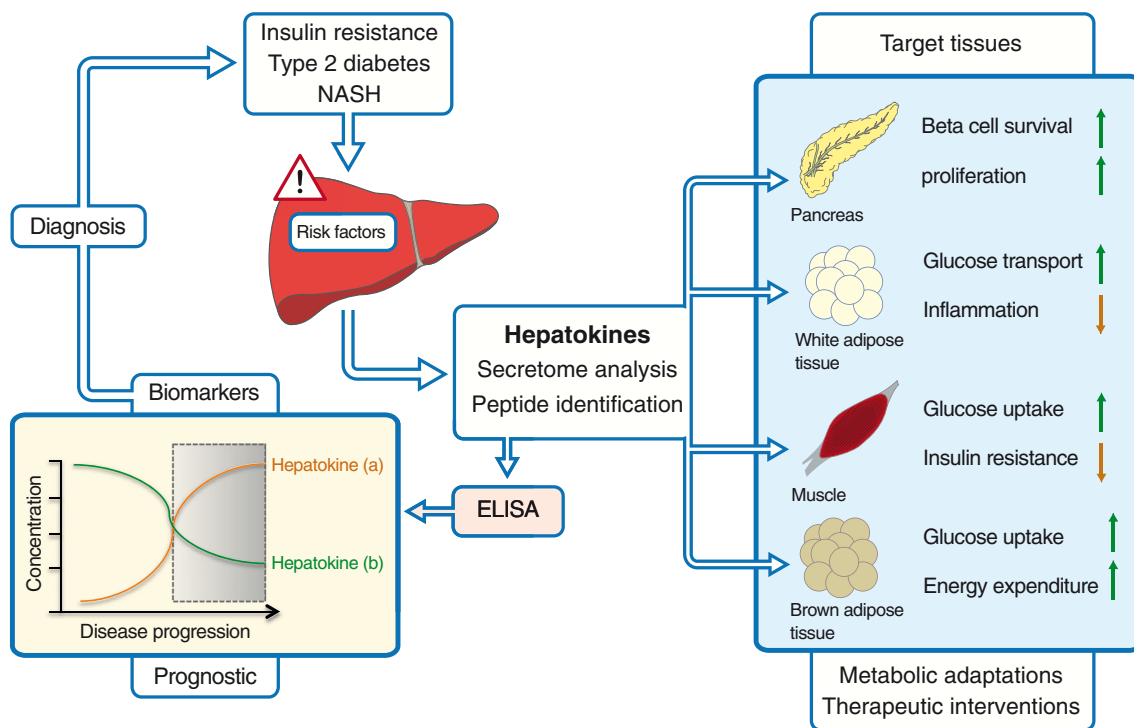
Since the discovery of a glucose-lowering agent in pancreatic extracts by Banting and Best in 1922 [8], improving pancreatic function, particularly endogenous insulin production by beta cells, has been the focus of pharmaceutical research. The notion of a hepatokine that could improve beta cell proliferation was based on the findings of a parabiosis study in which LIRKO mice were paired with lean controls [9]. Control partners of LIRKO mice showed increased plasma insulin concentrations associated with beta cell proliferation. Importantly, serum from LIRKO mice increased beta cell proliferation in cultured human islets, indicating that the secretome of an insulin-resistant liver is able to induce physiological changes in beta cell function/mass [9]. However, despite extensive analysis of known circulating factors, a potential candidate was not identified. Shortly following the description of liver-derived secretory proteins secretion in LIRKO mice, a separate team proposed a potential candidate, ANGPTL8, renaming it betatrophin [10]. Through the use of the insulin receptor antagonist S961, the authors credited this hepatokine for the increased beta cell proliferation and mass observed under conditions of whole body insulin resistance. While direct stimulation of mouse beta cells with S961 *in vitro* had no effect on morphology, systemic treatment strikingly increased beta cell proliferation and mass. Initially promising, the role of ANGPTL8 as a hepatokine controlling beta cell growth has been challenged. *Angptl8* knockout mice, while exhibiting profoundly reduced triacylglycerol levels, show no abnormalities in terms of glucose homeostasis and/or in beta cell expansion in response to insulin resistance resulting from either a high-fat diet or from the administration of S961 [11].

Overexpression of ANGPTL8 in the liver of mice did not affect beta cell expansion [12]. Importantly, ANGPTL8 was reported to be increased in serum of type 1 diabetic patients, suggesting that hepatic insulin resistance is not necessary for ANGPTL8 release. In addition, despite increased serum ANGPTL8 concentrations, ANGPTL8 was unable to rescue the massive beta cell apoptosis characteristic of type 1 diabetic patients [13]. Regardless of current criticism, the fact that plasma triacylglycerol levels seem to be correlated with ANGPTL8 expression in mice supports the possibility that inhibiting ANGPTL8 may represent an efficient therapeutic strategy for the prevention of hypertriacylglycerolaemia.

### FGF21

FGF21 has been noted as a promising pharmacological target because of its ability to reduce blood glucose independently of insulin by increasing the expression of glucose transporter GLUT1 in human primary adipocytes [2]. In addition, FGF21 decreases circulating triacylglycerol levels by reducing lipolysis and increasing  $\beta$ -oxidation in the liver and adipose tissue [3]. Classically observed to be upregulated by the

nuclear receptor peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) during fasting [14], recent studies have demonstrated that FGF21 is also upregulated in hepatocytes stimulated by high glucose and that its increased expression is directly induced by the glucose-sensitive transcription factor known as carbohydrate responsive element-binding protein (ChREBP) [15]. This suggests an unexpected role for FGF21 in the response to glucose overload. A cohort of both lean adults and adults with the metabolic syndrome experienced a marked increase in plasma levels of FGF21 60 min after a fructose and glucose bolus [16], making FGF21 the first hepatokine to be identified as being regulated by nutritional status. In addition, hepatic expression and circulating concentrations of FGF21 are increased in patients with NAFLD [17]. These observations point to a central role of FGF21 in metabolic regulation and suggest that carbohydrate-induced FGF21 secretion may be instrumental in preventing nutritional-induced lipotoxicity by partitioning excess nutrients. In line with this, mice deficient in hepatic glucose 6-phosphatase (a key enzyme in glucose production) fed a high-fat/high-sucrose diet, are resistant to obesity and type 2 diabetes, despite the onset of hepatic steatosis, owing to the secretion of liver-derived proteins such as



**Fig. 1** Hepatokines as biomarkers and therapeutic approaches for metabolic diseases. The liver plays a central role in orchestrating whole body energy metabolism through the secretion of hepatokine(s) in response to stress signals (insulin resistance, type 2 diabetes, NASH). Combining proteomic techniques and bioinformatics software platforms may identify variations in pathogenic or beneficial hepatokine(s) (lines a and b) as potential biomarkers for the progression of diseases such as type 2

diabetes and/or NASH. Ideally, hepatokine discovery would also lead to the development of therapeutics to control energy homeostasis, insulin sensitivity and glucose uptake in peripheral tissues, while reducing resistance and inflammation in target tissues. Improvement of specific tissue function could then reduce the pancreatic workload and normalise hepatic glucose production

FGF21 and ANGPTL6 and the subsequent activation of peripheral glucose metabolism and thermogenesis [18].

## Hepatokine potential

Evaluation of insulin resistance (or sensitivity) and beta cell function is key to understanding diabetes progression and for decision-making regarding the choice of pharmacological treatment(s). The gold standard method for assessment of insulin sensitivity is the hyperinsulinaemic–euglycaemic clamp, but this approach remains costly and time consuming and is therefore mostly limited to clinical research. HOMA-IR, calculated from fasting plasma glucose level and immunoreactive insulin, is, in contrast to the glucose clamp, a simple method for assessing insulin sensitivity. However, HOMA-IR is not always reliable and is difficult to apply to patients with poor glycaemic control, severe beta cell dysfunction and/or receiving insulin therapy. In this context, hepatokines may have the potential to provide simple and effective strategies for disease detection/progression and patient care, and may serve as therapeutic targets for various metabolic diseases, including type 2 diabetes (Fig. 1).

Another field of interest is NAFLD, the hepatic component of the metabolic syndrome. NAFLD ranges from simple benign steatosis to non-alcoholic steatohepatitis (NASH). Distinguishing simple steatosis from NASH is crucial, since the latter can progress to end-stage liver disease, like fibrosis, cirrhosis and hepatocellular carcinoma. Liver biopsy, considered the gold standard for NASH diagnosis, is invasive and costly. Imaging studies (ultrasonography or magnetic resonance imaging) have limited sensitivity for steatosis detection and are currently not able to distinguish steatosis from NASH. Therefore, reliable non-invasive biomarkers are needed for NASH patients, to establish diagnosis, follow disease progression and implement efficient therapeutic strategies. Proteomic techniques combined with mass spectrometry and bioinformatics software should help to identify and quantify ‘networking’ proteins in the secretome of patients at risk for type 2 diabetes or NASH (Fig. 1). In this context, one potential interesting lead is leucocyte cell-derived chemotaxin 2 (LECT2), a secreted molecule mainly produced by hepatocytes that exerts an anti-inflammatory effect in  $\beta$ -catenin-induced hepatic carcinoma [19]. According to recent observations in a cohort of type 2 diabetic patients, circulating levels of LECT2 positively correlate with BMI and hepatic steatosis [20]. These data suggest that LECT2 could be a new player in the mechanisms of insulin resistance and may constitute a biomarker for patient follow-up, notably in those at risk for developing type 2 diabetes. However, circadian rhythms need to be taken into account before considering a liver-secreted protein as a potential biomarker. In particular, the fact that

baseline concentrations of circulating FGF21 are driven by circadian control and that the early morning FGF21 peak observed in lean individuals is completely lost in obese patients [21], highlights the importance of fully characterising the circadian relationship of future hepatokines in order to determine their suitability as potential biomarkers.

From a therapeutic point of view, LY2405319, an FGF21 analogue that is already in clinical trial, has shown a significant and positive impact on reducing body weight and fasting insulin in obese and diabetic patients after only 28 days of daily subcutaneous injections [22]. Over the study period, although glycaemia only showed a trend towards a decrease, body weight was significantly reduced. These results could be promising since weight reduction has substantial beneficial effects on the metabolic syndrome. A recent publication using insulin-deficient diabetic mice (resulting from streptozotocin treatment) reported the importance of multiple tissue networking in regulating blood glucose [23]. Improvement of hyperglycaemia following LY2405319 treatment was due to the utilisation of glucose by brown adipose tissue through upregulation of the glucose transporters GLUT1 and GLUT4. Removal of brown adipose tissue obliterated the glucose-lowering effect of LY2405319 [23]. Personalised therapies that could specifically target glucose utilisation in peripheral tissues (adipose tissue, muscle) could greatly improve the quality of life of patients by reducing the need for insulin injections. A better understanding of the molecular basis of FGF21 expression in the liver represents a necessary step for efficient therapeutic approaches.

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**Contribution statement** All authors were responsible for drafting the article and revising it critically for important intellectual content. All authors approved this version to be published.

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