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Licenciatura em Bioquímica

Fsp27/CIDEC is a CREB target gene induced during fasting and regulated by fatty acid oxidation rate

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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"*Por que foi que cegámos, Não sei, talvez um dia se chegue a conhecer a razão, Queres que te diga o que penso, Diz, Penso que não cegámos, penso que estamos cegos, Cegos que vêem, Cegos que, vendo, não vêem.*"

José Saramago "*Ensaio sobre a Cegueira*"

“Why did we go blind, I don’t know, maybe one day we’ll find out, Do you want me to tell you what I think, Yes, I don't think we did go blind, I think we are blind, Blind but seeing, Blind people who can see, but do not see.”

José Saramago, “*Blindness*”

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Abstract

Fsp27 (*CIDEC* the human homologue) is a lipid droplet protein that when overexpressed down regulates fatty acid oxidation (FAO). Previous results of this group showed *that Fsp27/CIDEC* expression is regulated by fasting in liver in a time-dependent manner. The present study aimed to elucidate the mechanism by which *Fsp27/CIDEC* is mediating fasting adaptation and regulated by FAO rate in liver. We showed that induction of *Fsp27/CIDEC* expression during fasting is not regulated by PPARα. Pharmacological inhibition of FAO by etomoxir induces *Fsp27/CIDEC* in fasting conditions and this regulation is not mediated by PPARγ – a master regulator in triglyceride accumulation. The early response to fasting can be explained by a canonical PKA-CREB-CRTC2 signaling pathway, since *CIDEC* expression was increased by forskolin which effect was lost when a vector containing a dominant negative of CREB construct (KCREB) was co-transfected in HepG2 cells, and, consistently, Fsp27 promoter activity was increased by CREB. Also, CIDEC expression was up-regulated by specific *Sirt1* depletion by siRNA in HepG2 cells. Our data demonstrate that *Fsp27/CIDEC* is a CREB target gene that could be up-regulated when FAO is reduced and that fluctuations in SIRT1 activity, in response to nutrient availability, mediate this mechanism.

The peroxissome proliferator-activated receptor gamma coactivator-1α (PGC-1α) induces and coordinates gene expression that stimulates metabolic pathways linked to the fasted response in liver including gluconeogenesis. We observed that *Pgc-1α* expression was increased in late fasting in the liver of mice previously subjected to a leucin deprived diet. These conditions also enhanced transcription from *Foxa2* gene. This study showed that the mechanism regulating the induction of *Pgc-1α* expression under these conditions is not mediated by the recruitment of CREB by *Foxa2* to the *Pgc-1α* or the *Pepck* promoters, since both promoters activities were not enhanced by the co-transfection of FOXA2 with CREB in HepG2 cells.

Keywords: CIDEC/Fsp27; Fasting; FAO; CREB; SIRT1; Amino-acid deprivation; PGC-1α; FOXA2.

Resumo

Fsp27 (CIDEC, o homologo humano) é uma proteina associada às gotas lipídicas que quando sobre-expressada inibe a oxidação dos ácidos gordos (FAO). Recentemente, resultados deste grupo demonstraram que a sua expressão é regulada durante o estado de jejum no fígado no decurso do tempo. Neste estudo pretende-se elucidar o mecanismo pelo qual *Fsp27/CIDEC* medeia a adaptação ao jejum e a sua regulação pela taxa de FAO no fígado. Demonstrou-se que a sua indução durante o jejum não é regulada por PPARα e a indução registada pela inibição farmacológica da FAO por etomoxir não é mediada por PPARγ – regulador crucial na acumulação de triglicéridos. A resposta à fase inicial do jejum explica-se pela via de sinalização PKA-CREB-CRTC2 uma vez que a expressão de *CIDEC* aumentou com forskolina e este efeito foi atenuado com a transfecção de um dominante negativo de CREB em células HepG2. Consistentemente, a actividade do promotor *Fsp27* aumentou com CREB. A indução da expressão de CIDEC observou-se com a supressão específica de *Sirt1* por siRNA em células HepG2. Estes resultados demonstram que *Fsp27/CIDEC* é um gene alvo de CREB cuja expressão aumenta com redução da FAO e este processo é mediado pela actividade de SIRT1.

PGC-1α induz e coordena a expressão genética que estimula processos metabólicos relacionados com a resposta ao jejum no fígado incluindo a gluconeogenese. Observou-se que a sua expressão aumenta na fase tardia do jejum no fígado de ratos previamente sujeitos a uma dieta sem leucina, condições que induziram a transcrição do gene *Foxa2*. Este estudo demonstrou que o mecanismo que regula a indução da expressão de *Pgc-1α* nestas condições não é mediada pelo recrutamento de CREB por *Foxa2* para os promotores *Pgc-1α* e *Pepck*, uma vez que ambas as actividades promotoras não foram induzidas pela transfecção simultânea de FOXA2 com CREB em células HepG2.

Palavras Chave: CIDEC/Fsp27; Jejum; FAO; CREB; SIRT1; Deprivação de aminoácidos; PGC-1α; FOXA2.

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List of Abbreviations

AAR	Amino acid response
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATF4	Activating transcription factor 4
BAT	Brown adipose tissue
CACT	Carnitine Acylcarnitine Translocase
cAMP	Cyclic Adenosine monophophate
CARE	CCAAT-enhancer binding protein-activating transcription factor C/EBP-ATF response elements
CBP	CREB binding protein
C/EBP	CCAAT/enhancer binding protein
ChIP	Chromatin Immunoprecipitation
CIDEC	Cell death inducing DFF45-like effector C
CPT-I	Carnitine palmitoytranferase I
CPT-II	Carnitine palmitoyltransferase II
CRTC2	CREB-regulated transcriptional co-activator 2
CREB	cAMP responsive element binding protein
CMV	Cytomegalovirus
CoA	Coenzyme A
DMSO	Dimetylsulfoxid
DNA	Deoxyribonucleic Acid
E.Coli	Escherichia Coli
eIF2	Eukaryotic Initiation factor 2
ERR	Estrogen related receptor
EDTA	Ethylenediamine tetraacetic acid
FFA	Free fatty acids
FADH2	Flavine adenine dinucleotide
FOXA	Forkhead box protein A
FOXO1	Forkhead box protein O1

FSP27	Fat specific protein 27
GCN2	General Control Nonderepressible protein 2 kinase
G6P	Glucose-6-phosphate
GFP	Green fluorescent protein
GR	Glucocorticoid receptors
HAT	Acetyl transferases
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
HMGCS2	3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase 2
HNF3	Hepatic nuclear factor 3
HNF4α	Hepatic nuclear factor-4α
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KCREB	Expression vector (pcDNA3-KCREB) containing a dominant negative CREB construct
LB	*Lysogeny Broth* (growth media for bacteria)
LCAS	Long-chain acyl-CoA synthetase
LD	Lipid Droplet
(-) leu	Leucin deficient
Luc	Luciferase gene, from *Photinus pyralys*
MEM	Minimum Essential Medium
mut	Mutated
µ	Micro (10^{-6})
NAD	Nicotinamide adenine dinucleotide, oxidizing agent
NADH	Nicotinamide adenine dinucleotide, reducing agent
ng	Nanogram(s)
PBS	Phosphate buffered-saline
PCR	Polymerase Chain Reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1α	Peroxissome proliferator-activated receptor gamma coactivator- 1α
PKA	Protein kinase A
PPARs	Peroxissome proliferator-activated receptors
PPRE	PPAR response element

PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
RNA	Ribonucleic Acid
SIRT1	Sirtuin 1
SRC-1	CBP/p300 and steroid receptor coactivator
Tat	Tyrosine aminotransferase
TG	Triglycerides
TORC2	Transducer of regulated CREB protein 2
TZD	Thiazolidinedione
WAT	White adipose tissue
WT	Wild type
VLDL	Very low density lipoprotein

1. INTRODUCTION

1.1. Fasting

Mammals have evolved a metabolic response system that allows them to survive during long periods of energy deprivation. The overall metabolic response to fasting operates at numerous levels. One prominent feature is the gradual shift in whole-body fuel utilization from carbohydrates and fat in the fed state to almost exclusively fat after a day of fasting [1]. This adaptation is particularly striking in the brain, an obligate glucose utilizer in the fed state, which is able to acquire energy predominantly from ketone bodies after prolonged fasting [2,3]. The main metabolic fuels of the human body are glucose, fatty acids and ketone bodies. Its principal energy stores are liver glycogen, adipose tissue triglyceride and muscle protein. Most of the actual inter-conversions in energy substrates occur in liver, which plays a central role in the adaptive response to fasting [4].

Prolonged fasting is characterized by low insulin concentrations, high glucagon, glucocorticoids and epinephrine concentrations in plasma (Figure 1.1). In feeding/fasting conditions, these counter regulatory hormones will maintain blood glucose levels [5].

In this state liver degrades glycogen into glucose. In addition, it receives glucogenic amino acids such as alanine and lactate (from muscle) and glycerol (from adipose tissue) which are precursors for gluconeogenesis or *de novo* synthesis of glucose [4]. Gluconeogenesis it is a highly polarized metabolic pathway involving biochemical reactions in different compartments such as the cytoplasm, mitochondria, endoplasmic reticulum and plasma membrane. The rate of gluconeogenesis is controlled by three key enzymes: phosphoenol-pyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase and glucose-6-phosphatase (G6P). Hormonal control of these enzymes is achieved at the transcriptional level. The main positive signals to turn on gluconeogenesis are activated during fasting, stress and diabetes. As already referred, among these signals is glucagon that produces an increase in intracellular cAMP and glucocorticoids, endogenous ligands for the glucocorticoid receptor [5].

The fasting liver also receives non-esterified fatty acids (mainly long chain fatty acids), released from adipose tissue triglycerides [5]. The hormonal profile of the fasted state promotes the hydrolysis of triacylglycerol in adipose tissue, thereby increasing the concentration of free fatty acids (FFA) in plasma. The fatty acids are taken up by the liver, where they are either re-esterified to triacylglycerol and secreted as very-light-density lipoproteins (VLDL) or oxidized in the mitochondria via β-oxidation. The majority of fatty acids are only partially oxidized to acetyl-coenzyme A (acetyl-CoA), which then condenses with itself to form ketone bodies, an important fuel to the brain [6].

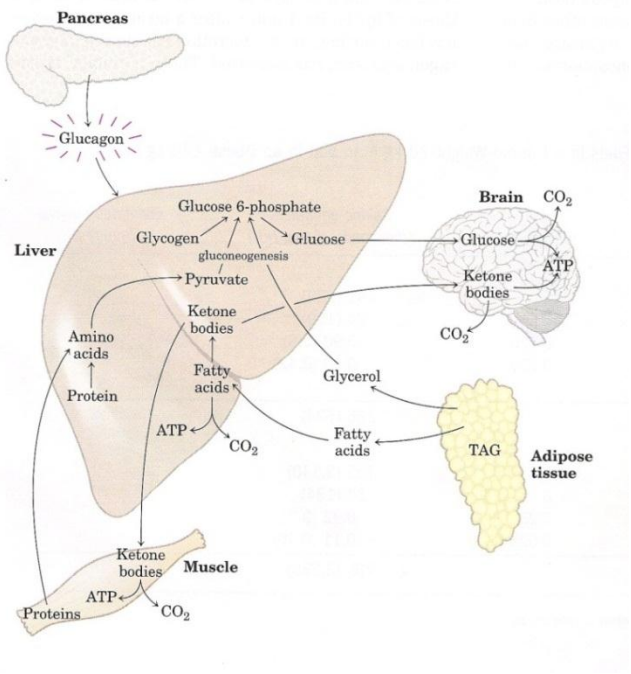


Figure 1.1. The fasting state: The gluconeogenic liver.

In fasting conditions, the liver becomes the principle source of glucose for the brain. Liver glycogen is brake down, and the glucose 1-phosphate produced is converted to glucose 6-phophate, then to free glucose, which is released into the blood stream. Amino acids from the degradation of proteins and glycerol from the breakdown of triglycerides in adipose tissue are used for gluconeogenesis. The liver uses fatty acids as its principal fuel, and excess acetyl-CoA is converted to ketone bodies for export to other tissues for fuel; the brain is especially dependent on this fuel when glucose is in short supply. Adapted from *Lehninger, Principles of Biochemistry 2005* [5].

1.2. Molecular mechanisms regulating adaptation to fasting

1.2.1. CREB/CRTC2 Signaling Pathway

Circulating glucose levels reflect a balance between glucose production by the liver and glucose utilization by skeletal muscle. The liver, a key target of both insulin and glucagon signaling, contributes to the control of glucose metabolism by facilitating glucose uptake and output depending on the physiological condition [7]. Under fasting conditions, mammals initiate a whole body metabolic response that limits glucose and favors fatty acid oxidation. In these conditions the liver is central in the metabolic response to glucagon, epinephrine and glucocorticoids, which act to stimulate hepatic glucose via glycolysis and gluconeogenesis in order to prevent extend periods of hypoglycemia [8].

The gluconeogenic program is largely regulated at the transcriptional level, by the cyclic AMP (cAMP) responsive element binding protein (CREB) and its ability to activate PGC-1α (PPAR gamma coactivator- 1α) [9]. This response is dependent on the phosphorylation of CREB at Ser133 mediated by protein kinase A (PKA) and the subsequent recruitment of the coactivators CREB binding protein (CBP)/p300 to promoters of gluconeogenic genes (such as PEPCK and G6P) [9]. The simplicity of this model, has been challenged with the discovery of the CREB-regulated transcription coactivator 2 (CRTC2) [10] which has been implicated as the foremost mediator driving CREB target expression [11].

In fact, CRTC2 was described as a critical component of the early transcriptional fasting response in the liver by acting up-stream of PGC-1α in the gluconeogenic pathway [12, 13].

In the early state of fasting, circulating glucose and insulin levels are low, glucagon and/or epinephrine signaling results in the dephosphorylation of CRTC2 and its translocation to the nucleus where it drives the transcription of several genes implicated in gluconeogenesis by specifically interacting with CREB and facilitating recruitment of the coactivators CBP and p300 to CREB target sequences. Following long-term stimulation, it has been proposed that CRTC2 is deacetylated by SIRT1 and targeted for degradation in proteasome (Figure 1.2) [13]. Sirtuin 1 (SIRT1) is a type III NAD^+ dependent deacetylase (its role will be further explored in the next section).

CRTC2 activity is also controlled by insulin signaling which results in CRTC2 phosphorylation and exclusion from the nucleus where it can no longer impact gene expression [14]. Interestingly, whereas the increase in SIRT1 activity that accompanies prolonged fasting attenuates CRTC2 signal, it actually improves PGC-1α's ability to increase hepatic gluconeogenesis [15]. In fact, maintenance of the gluconeogenic response through prolonged fasting will be mediated by PGC-1α and Forkhead box O1 (FOXO1) (Figure 1.2) [13].

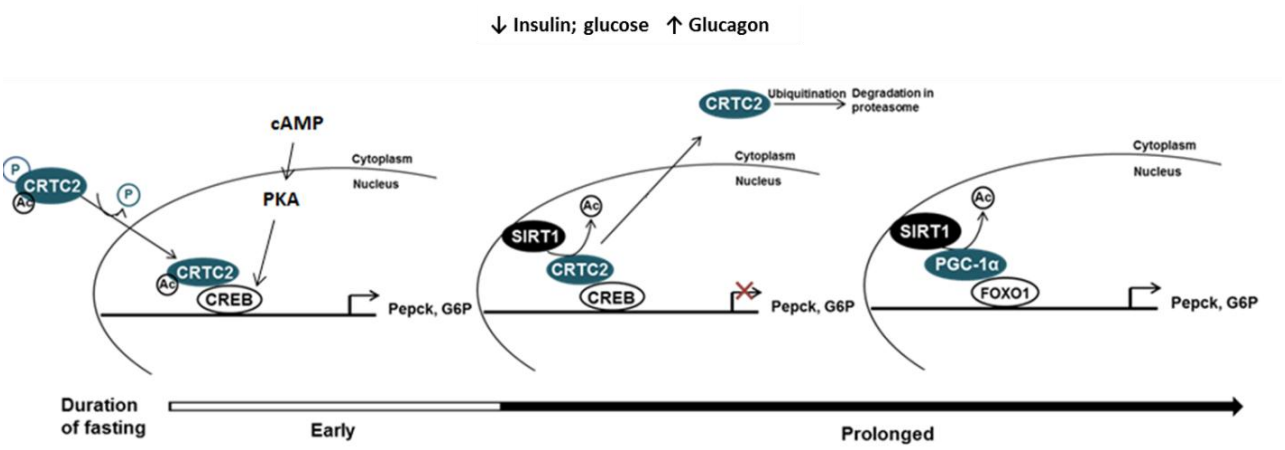


Figure 1.2. Schematic diagram of the inducible switch that modulates gluconeogenesis through the CREB/CRTC2 signaling pathway.

Fasting corresponds to a metabolic state where insulin levels are low and glucagon levels are high and it can be dissected in two distinct phases, the early phase (<12-18h) and late phase (>12-18h). CRTC2 – CREB regulated transcription coactivator 2; CREB - cAMP responsive element binding protein, FOXO1 - Forkhead box protein O1 , PGC-1α - Peroxissome proliferator-activated receptor gamma coactivator- 1α, SIRT1 – Sirtuin 1.

1.2.2. SIRT1

Caloric restriction causes many changes in glucose metabolism [16]. Recently, it was suggested a model in which protein acetylation regulates metabolic strategy and also helps store energy in cells [17]. In the cell, acetylation is carried out by histones acetyl transferases (HATs) and deacetylation by class I, II and III deacetylases. The type III, NAD^+ dependent deacetylase is termed sirtuins [17]. Among sirtuins, SIRT1 – which modulates ageing in several species [18, 19] – appears to be involved in a great number of physiological pathways, including gluconeogenic/glycolytic pathways in liver in response to fasting signals through the transcriptional coactivator PGC-1α [20]. Also, SIRT1 is known to target MyoD, p53 and forkhead transcription factors for deacetylation [21, 22].

SIRT1 seems to be involved in pathways affected directly by caloric restriction in mammals [23]. Several studies showed that SIRT1 activity is low in growth conditions of glucose excess and high during energy limitation [15, 16]. This behavior is due to SIRT1 being a NAD-dependent deacetylase. In energy abundance conditions, there is ATP generation trough glycolysis and conversion of NAD to NADH in mitochondria and therefore SIRT1 activity is expected to decrease. This behavior suggests a feed forward mechanism, in which diet drives glycolysis, convert NAD to NADH, inactivate sirtuins, and increase acetylation and activity of glycolytic enzymes [17]. Conversely, under fasting or low nutrient conditions, limit glycolysis, activate SIRT1 triggering protein deacetylation in mitochondria. These conditions, fit with one of the main outputs of SIRT1 activity, the deacetylation of PGC-1α to promote mitochondrial biogenesis and oxidative metabolism [15] (Figure 1.2).

This evidence, suggest protein acetylation as a regulatory mechanism, as well as energy-storage mechanism when energy is in excess. The regulatory aspects include histone acetylation to regulate gene transcription, acetylation of metabolic enzymes to favor glycolysis for ATP production, and acetylation of several transcription factors such as p53, FOXO1, PGC-1α, nuclear receptors to adjust expression levels of pathways for oxidative versus glycolytic metabolism [24]. Regarding the transcription factors, SIRT1 has mainly been linked to negative regulation of gene expression through protein deacetylation (e.g. p53) [25, 26]; however it can act both positively and negatively to control gene expression as PGC-1α [15] in response to NAD^+ fluctuations in accordance to nutrient availability [13, 15, 16, 23].

The storage aspect of acetylation may be important in transitioning from energy access to energy limitation. Under these conditions, the acetate generated by SIRT1 deacetylation of many proteins would be a substract for acetyl-CoA synthethase. Along with the oxidation of fatty acids, would drive the citric acid cycle and oxidative phosphorylation to yield ATP and CO_2 [17].

1.2.3. PGC-1α

One of the components of the signal transduction pathway involved in metabolic reprogramming is the peroxissome proliferator-activated receptor gamma coactivator-1α – PGC-1α. This transcriptional co-activator is able to coordinate the expression of a wide array of genes involved in glucose and fatty acid metabolism [3].

Molecular mechanisms of PGC-1α function

PGC-1α was first identified as a binding partner and co-activator of the transcriptional activity of PPARγ [27]. It is known to bind and consequently modulate the activity of a wide range of transcription factors such as PPARα, glucocorticoid receptor, hepatic nuclear factor-4α (HNF4α), members of the estrogen related receptor (ERR) family, Foxo1, among others [28, 29].

Once it is activated, PGC-1α is recruited to the chromatin through interaction with transcription factors [30]. After docking to a transcription factor, PGC-1α undergoes a conformational change that allows interaction with histone acetyl transferases (HAT) such as CBP/p300 and steroid receptor coactivator (SRC-1). This set of proteins interacts at the PGC-1α N-terminal region that contains a potent transcriptional activation domain. Although they enhance the activity of PGC-1α, SRC-1 and CBP/300 do not acetylate PGC-1α. In fact, acetylation of PGC-1α, is correlated to a decrease in its activity [15].

This data reveal that the regulation of gene expression by PGC-1α is achieved by a sequential and dynamic recruitment of different set of proteins making a functional multiprotein complex that transcribes specific genes.

From a physiological standpoint, the co-activation of these transcription factors by PGC-1α has important metabolic repercussions. Specifically, this set of transcription factors to which PGC-1α binds, controls the expression of genes involved in adaptive thermogenesis, gluconeogenesis, glycolysis, lipogenesis, mitochondrial fatty acid oxidation, and mitochondrial respiration efficiency [3]. Thus, PGC-1α can single coordinate gene expression of multiple energy pathways. This point is based in studies where PGC-1α knockout mouse, shows a reduced respiratory capacity, reduces rates of hepatic gluconeogenesis and β-oxidation, hepatic steatosis under fasting conditions and hypoglycemia [31, 32].

PGC-1α in the adaptation to food deprivation

In terms of its contribution to the diet-dependent maintenance of energy homeostasis in mammals, there is a body of evidence to suggest that the co-transcriptional activity of PGC-1α is important to the compensatory metabolic responses that occur during food deprivation [3, 33].

During fasting, gluconeogenesis is profoundly up regulated primarily at the level of transcription. As already described (in CREB/CRTC2 signaling pathway section) a compelling argument can be made for dissecting the up regulation of gluconeogenesis into two temporally distinct phases [13]. Regarding the adaptation to the maintenance of the gluconeogenic response through prolonged fasting is thought to be mediated by PGC-1α and its transcription factors [3]. Indeed, in the late state of fasting, the elevated levels of PGC-1α facilitate increased hepatic glucose output by promoting the expression of gluconeogenic genes [29]. This regulation is mediated by the hormones that signal fasting such as glucagon and insulin [34] but also by SIRT1 mediated deacetylation [15].

As discussed above, during fasting glucagon activate via cAMP, the CREB transcription factor, an important regulator of PGC-1α gene expression [9]. In addition, glucocorticoids strongly synergize with cAMP to induce PGC-1α levels. Under this state, of lower nutrient availability, intracellular NAD^+ levels increase which leads to the deacetylation of PGC-1α by SIRT1 [35]. The change in PGC-1α acetylation coincides with an increased activation of PGC-1α which binds and co-activates different transcription factors such as HNF4α and FOXO1 and glucocorticoid receptors (GR) to coordinate expression of gluconeogenic genes [33, 36]. Insulin, a dominant-negative signal suppresses PGC-1α expression in liver (Figure 1.3) [34].

FOXO1 acts as a cellular sensor of insulin signaling via Akt. Akt phosphorylates FOXO1 and induces cytoplasmic localization and degradation. PGC-1α requires FOXO1 to bind and localize to the promoter chromatin region of gluconeogenic genes [33]. This mechanism, corresponds with the re-feeding state were PGC-1α activity decreases.

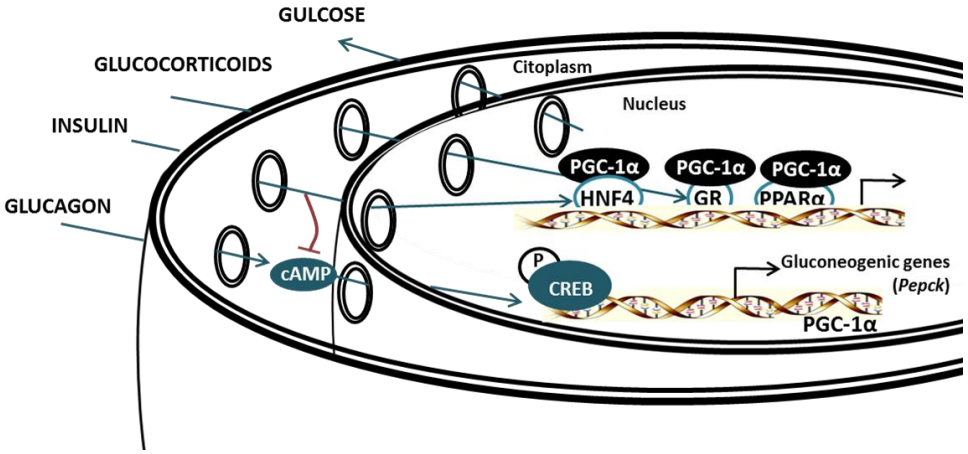


Figure 1.3. Transcriptional regulation of hepatic gluconeogenesis by PGC-1 alpha.

Hepatic glucose production in the liver is tightly controlled by hormones. Glucagon and catecholamines stimulate the cAMP pathway and CREB, which activates PGC-1α gene expression. PGC-1α is then recruited to different transcription factors that bind to the promoter of gluconeogenic genes such as PEPCK. Glucocorticoids induce a specific interaction between PGC-1α and GR-activating PEPCK. Insulin represses cAMP activation on gluconeogenic genes, but how it blocks PGC-1α gene expression is unknown. Adapted from Puigserver et al., 2003 [34].

1.2.4. FOXA2

A complex network functions to maintain whole body homeostasis of metabolites such as glucose and amino acids. One group of such genes is FOXA (forkhead box A) family, also known as hepatic nuclear factor 3 (HNF3) [32]. The mammalian FOXA family consists of three members, FOXA 1, FOXA2 and FOXA3, which contribute to embryonic development [37] and to hormonal control of gene expression [38]. FOXA proteins bind to similar recognition sequences and the proteins are proposed to have redundant function in regulating some of their target genes [39]. The *Foxa* genes appear to be involved in the protection of the organism from hypoglycemia [40, 41]. However, each FOXA protein has distinct functions.

Foxa2 is involved in the mechanisms regulating fasting and amino acid deprivation.

In the liver, some Foxa target genes identified are involved in glucose homeostasis, particularly in the response to fasting [42]. Examples include the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (Pepck), glucose-6-phosphatase (G6p) and tyrosine aminotransferase (Tat) [39].

The role of Foxa2 in hepatic glucose homeostasis was evaluated by Zhang et al [42], by genetic approach using a mouse model in which Foxa2 was deleted specifically from hepatocytes. Although these mice were normoglycemic, they fail to fully activate Pepck and Tat in response to fasting. Furthermore, this group suggested that Foxa2 mediates activation of guconeogenic genes by enabling recruitment of CREB and glucocorticoid receptors to their target sites in chromatin [42]. The results of this study suggest that Foxa2 is required for execution of hepatic gluconeogenic program, by integrating the transcriptional response of the hepatocyte to hormonal regulation during fasting.

Recent work from Wolfrum and colleagues [43, 44] has introduced a new concept regarding *Foxa2* in nutrient metabolism. They have shown that in cultured cells the transcriptional activity of Foxa2 is blocked by treatment with insulin, and that this regulation requires an intact phosphorylation site for the insulin-activated kinase of mouse Foxa2. This phosphorylation is reported to result in nuclear exclusion of Foxa2, thereby providing an appealing molecular model in which Foxa2's gluconeogenic activity is blocked via feeding-induced insulin secretion [43, 44].
The authors proposed a model in which Foxa2 is nuclear only in the starved state, during which it activates multiple genes driving increased hepatic glucose utilization, fatty acid oxidation, and ketogenesis.

Further, it was observed that under amino acid deprivation of HepG2 hepatoma cells transcription of the Foxa2 gene was enhanced [45]. However, this activation did not required activation of the transcription factor 4 (ATF4), a critical component of the conventional amino acid response (AAR) pathway (this pathway will be discussed in ‘’Amino Acid deprivation” section), but Foxa2 induction was partially dependent on CCAAT/enhancer-binding protein β (which is induced upon amino acid deprivation) [45]. The results presented in this study suggested that Foxa family of genes is differentially regulated by amino-acid availability.

1.3. Fatty Acid Signal Transduction Pathway

1.3.1. Fatty Acid Oxidation

In the transition from the fed to the fasted state the liver switches from an organ of carbohydrate utilization and fatty acid synthesis to one of fatty acid oxidation and ketone body production. The fasted state is associated with mobilization of stored lipid in white adipose tissue and increase in fatty acid flux through the mitochondrial β-oxidation [46].

β-oxidation is the major process by which fatty acids are oxidized, thus providing a major source of energy for the heart and skeletal muscle [47, 48]. β-oxidation is stimulated during starvation or endurance exercise [6] .

Fatty Acids are Activated and Transported into Mitochondria

The β-oxidation of activated fatty acids occurs within the mitochondrial matrix and is catalyzed by the sequential action of four enzyme families (acyl CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl- CoA thiolase) each with different substrate specificity for short-, medium, and long-chain acyl-CoAs. Long-chain fatty acids are activated on the mitochondrial outer membrane by the long-chain acyl-CoA synthetase (LCAS) but the mitochondrial inner membrane is not permeable to these acyl-CoAs. The carnitine dependent transport of these activated fatty acids precedes their β-oxidative chain shortening [49].

This transport system consists of three proteins: carnitine palmitoytranferase I (CPT-I), carnitine acylcarnitine translocase (CACT) and carnitine palmitoyltransferase II (CPT-II), each with a different submitochondrial localization. As a first step, acyl CoAs formed by the catalytic action of LCAS in the mitochondrial outer membrane are converted to acylcarnitines. This transterification is catalyzed by CPT-I also localized in the mitochondrial outer membrane. The reaction products, long-chain acylcarnitines, are then translocated into the mitochondrial matrix in an exchange reaction catalyzed by CACT, an integral inner membrane protein. Within the matrix the acylcarnitines are then reconverted to the respective acyl-CoAs by CPT-II, an enzyme associated with the mitochondrial inner membrane (Figure 1.4) [50].
The carnitine-mediated entry process is the rate limiting step for oxidation of fatty acids in mitochondria and it is an important point of regulation [5].

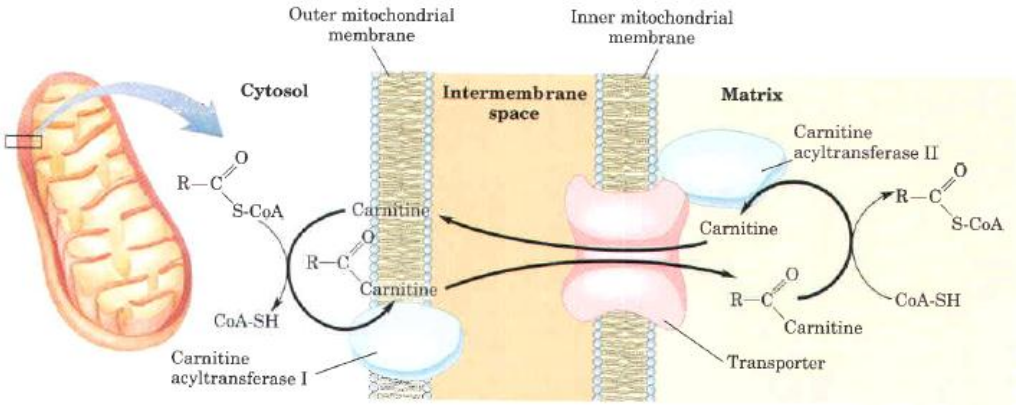


Figure 1.4. Fatty acid entry into mitochondria via acyl carnitine/carnitine transporter.

After fatty acyl-carnitine is formed at the outer membrane in the intermembrane space, it moves into the matrix by facilitated diffusion through the transporter in the inner membrane. In the matrix, the acyl group is transferred to mitochondrial coenzyme A, freeing carnitine to return to the inter-membrane space through the same transporter. Acyl transferase I is inhibited by malonyl CoA. This inhibition prevents the simultaneous synthesis and degradation of fatty acids. Adapted from *Lehninger, Principles of Biochemistry 2005* [5].

Oxidation of Fatty Acids

Mitochondial β-oxidation of fatty acids takes place in three stages. In the first stage (β-oxidation) the fatty acids undergo oxidative removal of successive two carbon units in the form of acetyl-CoA [50].

In the second stage of fatty acid oxidation, the acetyl groups of acetyl CoA are oxidized to CO_2 in the citric cycle, which take place in the mitochondrial matrix. The first two stages of fatty acid oxidation produce the reduced electron carriers NADH and FADH2, which in the third stage donate electrons to the mitochondrial respiratory chain, through which the electrons pass to oxygen with the concomitant phosphorylation of ADP to ATP. The energy released by fatty acid oxidation is conserved as ATP [6].

Hepatic regulation of mitochondrial β-oxidation

Much of the control and regulation of the rate of hepatic mitochondrial β-oxidation appears to reside at the level of the entry of acyl groups into mitochondria.
Malonyl-CoA is the first intermediate in the cytosolic biosynthesis of long-chain fatty acids from acetyl-CoA, increases in concentration whenever the body is well supplied with carbohydrate; excess glucose that cannot be oxidized or stored as glycogen is converted in the cytosol into fatty acids for storage as triacylglycerol. The inhibition of CPT-I by malonyl CoA ensures that the oxidation of fatty acids is inhibited in the fed state [6, 50].

1.3.2. Ketogenesis

Ketogenesis becomes significant under conditions of food (carbohydrate) deprivation. Provision of peripheral tissues, such as skeletal muscle and heart, with ketone bodies as an alternative fuel for energy production, results in glucose sparing for organs depending on glucose as an energy source [51, 52]. The ketone bodies play an important role in this adaptation: starvation induces an increase in plasma ketone bodies concentration and turnover serving as an important fuel for the central nervous system [53].

In fasting conditions the liver needs to maintain the Krebs cycle for ATP production and gluconeogenesis for supplying glucose for extrahepatic tissues. Since these two processes require the same intermediates, having oxaloacetate as rate-limiting enzyme, both begin to slow down. As a result acetyl CoA accumulates in the mitochondria [54]. Through a serie of condensation reactions (Figure 1.5), ketone bodies (acetoacetate, β-hydroxybutyrate and acetone) are produced [53].

The sum of these reactions is β-hydroxybutyrate, formed by the reduction of acetoacetate in the mitochondrial matrix with the generation of NAD^+ (Figure 1.5) [55].

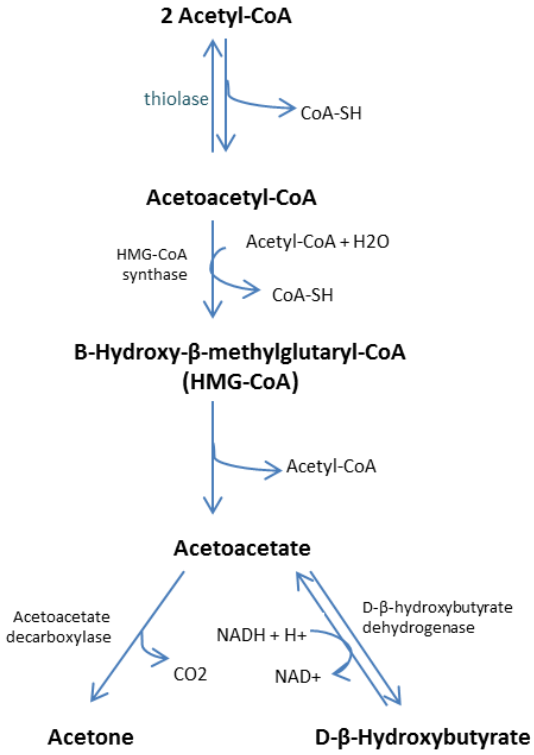


Figure 1.5. Formation of ketone bodies from acetyl-CoA. Adapted from Lehninger, Principles of Biochemistry 2005 [6].

Although fatty acids are by far the major carbon donors, for ketone body synthesis, the catabolism of some (ketogenic) amino acids also provides carbon atoms for ketogenesis, either via acetyl-CoA or directly by yielding acetoacetate [52].

Control of ketogenesis is exerted by transcriptional regulation of mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase 2 (HMGCS2) [51, 52, 56]. The transcriptional rate of this rate-limiting enzyme increases in response to cAMP, starvation, fat feeding and diabetes while insulin and refeeding repress it [57, 58]. Regarding to its transcriptional control, results of this group suggested that HMGCS2 expression is affected by the PPARα-mediated response and it is also involved in the induction of β-oxidation [59].

1.3.3. PPARs

Nutrient availability impact gene expression mainly by activating or suppressing specific transcription factors [60]. The most important group of transcription factors involved in mediating the effect of nutrients and their metabolites one gene transcription is the superfamily of nuclear receptors [61]. Several studies have shown that a group of nuclear hormone receptors, the peroxisome proliferator-activated receptors (PPARs), have an important role in fatty acid metabolism [62]**.**

Nuclear receptors govern gene expression via several distinct mechanisms that involve both activation and repression of DNA transcription. PPARs are members of the nuclear hormone superfamily. [63]. Transcriptional regulation by PPARs requires heterodimerization with the retinoid X receptor (RXR) which is also part of the nuclear receptor superfamily. When activated by a ligand, the PPAR/ RXR heterodimer stimulates transcription via binding to DNA response elements – PPAR response elements (PPRE) – present in the promoter of target genes [64].

The PPAR family consists in three isotypes: α, δ and γ that can be distinguished and characterized by different biological roles (Table 1.1) [65].

Table 1.1. Simplified overview of current understanding of the metabolic roles of the 3 PPAR isoforms. Adapted from Semple et al. 2006 [66].

	PPARα	PPARγ	PPARδ
Sites of highest expression	Liver, kidney, heart	Adipose tissue, macrophages	Adipose tissue, skin, brain, but widespread
Cellular processes activated	Fatty acid β-oxidation, lipoprotein synthesis, amino acid catabolism	Adipocyte differentiation, triglyceride synthesis	Fatty acid β-oxidation
Physiological function	Coordination of metabolic response to fasting	Differentiation of adipocytes, FA trapping	Muscle fiber type determination?
Examples of target genes	*Carnitine palmitoyl transferase I, HMG CoA synthase 2, apoA-I*	*Fatty acid–binding protein 4, lipoprotein lipase, adiponectin*	*Acyl-CoA oxidase, carnitine palmitoyl transferase I*
Metabolic phenotype of knockout mice	Fasting hypoglycemia, hypothermia, hypoketonemia, and hepatic steatosis	–/– Lethal, –/+ more insulin sensitive at baseline	Reduced base-line adiposity; increased obesity on high-fat feeding

1.3.3.1. *PPARα*

PPARα plays a crucial role in the control of lipid metabolism in response to nutritional, hormonal and environmental stimuli. It was described to be highly expressed in organs that carry out significant catabolism of fatty acids such as the liver, brown adipose tissue, heart, intestine and kidney [67, 68]. Therefore, identification of PPARα target genes has concentrated mainly on cellular lipid metabolism in the context of the hepatocyte. Indeed, the first PPARα target gene identified was acyl-coenzyme A oxidase [69] involved in fatty acid β-oxidation. Numerous genes involved in hepatic fatty acid oxidation were shown to be induced by PPAR α. Results from this group have been relevant for the identification and characterization of functional PPREs in key regulatory genes such as HMGCS2 (rate-limiting enzyme of ketogenesis); CPT1 and CPT2 (involved in mitochondrial β-oxidation).

PPARα can be activated by at least 2 different mechanisms. First, during fasting, the expression of PPARα in liver increases, an effect mediated by glucocorticoids that results in an increased amount of PPARα protein. Second the fatty acids that are liberated from the adipose tissue and travel to the liver may serve as ligands for PPARα. By means of such mechanism, the organism assures that the enzymatic system of the liver responds appropriately to the increased demands for fatty acid oxidation.

1.3.3.2. PPARγ

PPARγ is a master regulator for adipocyte differentiation [64, 70-72]. PPARγ also regulates lipid metabolism, insulin sensitivity, cardiovascular disease, inflammation, organ development and tumor formation [73, 74]. PPARγ has two isoforms, PPARγ1 and PPARγ2, which are created by alternative splicing [75]. PPARγ1 is expressed in several tissues including liver, whereas PPARγ2 is exclusively expressed in adipocytes, where it is key orchestrator of the cascade underlying adipocyte differentiation along with the CCAAT enhancer binding protein (C/EBP) [74]. The currently favored hypothesis is that C/EBP induces expression of PPARγ, which provides the initial trigger for the adipogenic program [74].

PPARγ also plays a key role in the switch of adipose tissue lipid metabolism to nutritional state. Its expression is highest postprandially [76] and its activation leads to the up-regulation of genes that mediate fatty acid up-take [77, 78]. PPARγ may also promote a futile cycling in adypocytes between triglyceride esterification and de-esterification [79].

Given the central role of PPARγ in the promotion of adipogenesis and the link between adiposity and insulin resistance, the discovery that PPARγ is the target for the thiazolidinedione (TZD) antidiabetic drugs was unexpected [66]. In fact, several lines of evidence support the conclusion that TZDs exert their biological effects on insulin sensitivity through binding to PPARγ (Figure 1.6).

TZDs appear to coordinately activate gene expression leading to an increase in net lipid partitioning into adipocytes. Target genes directly regulated by PPARγ that are involved in this pathway include lipoprotein lipase [77] and fatty-acid transport protein [80] which all favor adipocyte uptake of circulating fatty acids; phosphoenolpyruvate carboxykinase [81], glycerol kinase [79], which promote recycling rather than export of intracellular fatty acids.
Together, these pathways lead to the net flux of fatty acids from the circulation and other tissues into adipocytes.

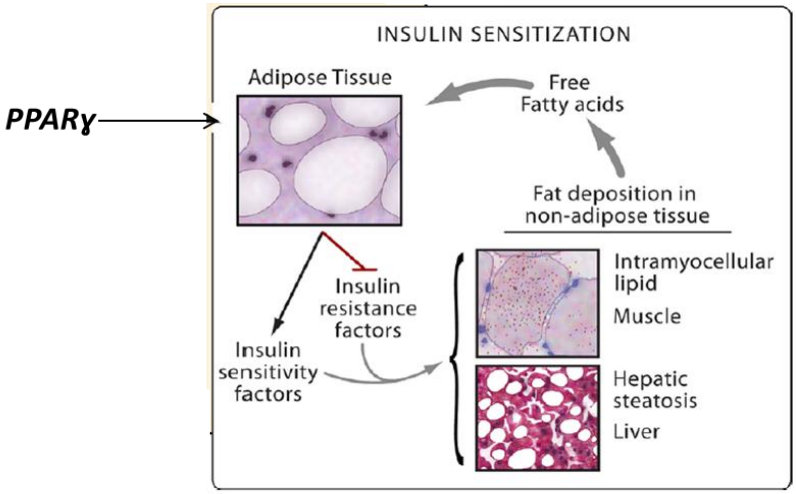


Figure 1.6. The insulin sensitization action of PPAR gamma. Adapted from Lehrke et al., 2005 [73].

1.3.4. Fsp27/CIDEC

Fat specific protein 27 (FSP27) or cell death inducing DFF45-like effector C (CIDEC), the human homologue of FSP27 belong to the CIDE family [82, 83]. *FSP27/CIDEC* was described as an important regulator of energy homeostasis and its functions are closely linked to the development of metabolic disorders including obesity, diabetes and liver steatosis [72, 84].

The CIDE family consists into three isoforms: CIDEA, CIDEB and FSP27/CIDEC. CIDEA is highly expressed in brown adipose tissue and CIDEB in liver [82]. Regarding to the tissue distribution of *FSP27/CIDEC* it was described that it was highly and specifically expressed in brown and white adipose tissue [85]. Interestingly, *FSP27* is also highly expressed in the fatty liver of typical diabetes or obese model *ob/ob* mouse and in the high fat-inducible fatty liver.

In eukaryotic cells the excess of lipids are store in the white adipose tissue in lipid droplets. *FSP27/CIDEC* was identified as a new lipid droplet associated protein that was shown to be involved in the formation and modulation of the size of unicular lipid droplets through their protection from lipolysis and it is required for the efficient accumulation of triacylglycerols in mature adipocytes [85, 86].

The expression of many adipocyte specific genes is affected by diettary manipulation. It was demonstrated that long-term intermittent fasting, significantly up-regulated *FSP27* gene expression in WAT [86] and that PPARγ2, C/EBPα, and insulin could be involved in its nutritional regulation.

The role of *FSP27* in promoting lipid droplet formation is not restricted to adipocytes. In fact, although *FSP27/CIDEC* is not expressed in normal liver, it was described to be highly expressed during hepatic steatosis since the excess of lipids stimulate the expression of lipid droplet associated proteins [72].
In hepatic steatosis, the expression of *Fsp27/CIDEC* is induced by proliferator-activated receptor γ (PPARγ). Serially deleted *FSP27* luciferase reporter plasmids and chromatin immunoprecipitatation (ChIP) assays revealed the presence of a functional PPAR response element and direct binding of PPARγ to this element. Corroborating these findings, the expression of the mouse promoter *FSP27* was markedly increase by the treatment with thiazolidinedione (TDZ), rosiglitazone – a specific ligand for PPARγ. However, *FSP27* was not activated by specific ligands of PPARα or PPARδ [72].

Moreover, Matsusue and colleagues [72] have also demonstrated that repression of mitochondrial β-oxidation activity was associated with an increase in *FSP27* expression. These data suggest that the accumulation of triglycerides and the increase of lipid droplets by *FSP27*

may be due to impaired β-oxidation activity and reduced triglyceride turnover. Whether how FSP27 suppresses β-oxidation activity remains unclear, however it was proposed that since FSP27 suppresses lipolysis there will be a lack of fatty acyl-CoA released from TG and this event will in turn impair mitochondrial β-oxidation.

Recent data of this laboratory demonstrated that *Hmgcs2,* which regulates ketone body production, also regulates fatty acid oxidation [59]. Also, specific depletion of this enzyme correlates with 25% decrease in serum ketone bodies in fasted animals, and altered gene expression. By, microarray analysis (Table 1.2) it was showed that the gene most-up regulated in fasted animals when ketogenesis and FAO were blocked was *FSP27/CIDEC.*

Table 1.2. Genes that are up regulated by HMGCS2 knock down. List of differentially expressed genes (up regulated >1.6 fold) in a microarray Affymetrix® (GeneChip Mouse Gene 1.0ST) performed with RNA of liver of shRNA control or shRNA Hmgcs2 treated mice (n=3). (Unpublished data from Haro D. and Marrero P., School of Pharmacy, University of Barcelona)

Ontology (up regulated)	Gene	FI	p-Value
Sulfotransferase family 1E, estrogen-preferring, member 1	Sult1e1	**2.106**	0.017
ND	Gm129	**2.037**	0.069
Lipocalin 13	Lcn13	**1.810**	0.030
Calcium ion binding	Cib3	**1.753**	0.013
Ion channel activity	Accn5	**1.748**	0.036
Induction of Apoptosis	CIDEC/Fsp27	**1.738**	0.036
G-protein coupled receptor	Sucnr1	**1.692**	0.026
Intermediate filament	Krt23	**1.660**	0.213
DNA binding (transcription regulator)	Bhlhe41	**1.613**	0.147
Basic helix-loop-helix domain containing, class B, 9	Bhlhb9	**1.613**	0.010
ND	Gm15441	**1.610**	0.025
G-protein coupled receptor protein signaling	Defb1	**1.604**	0.021

This finding prompted this group to further elucidate the role of *FSP27/CIDEC* during adaptation to fasting. It was demonstrated that during the early stages of fasting (<12-18h) *FSP27* expression was significantly increased (Figure 1.7A) and this expression decreased during the later phase of fasting (>12-18h) while *Hmgcs2* and other FAO genes (*Cpt1a, Cact, Cpt2*), showed maximal expression (Figure 1.7B). At this point, the results suggested a kinetic mechanism of auto-regulation between short- and long-term fasting by which free fatty acids that arrive to the liver during early fasting are fated to accumulation/exportation by *FSP27/CIDEC*, while over longer periods of fasting the fatty acids are oxidized through the CPT system in mitochondria.

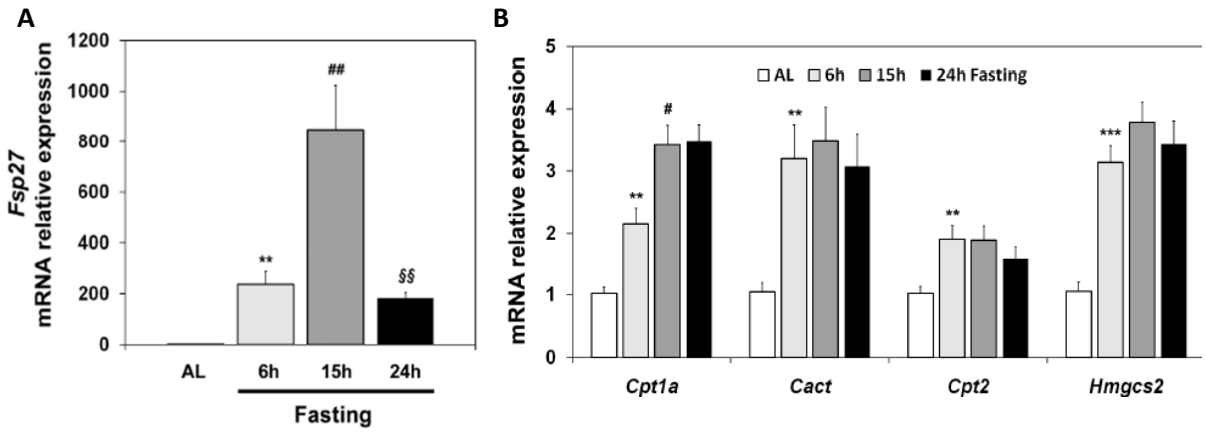


Figure 1.7. Hepatic *Fsp27* expression is induced by early fasting.

Mice fed Ad Libitum (AL) or subjected to 6h, 15h, or 24h fasting. (A) Fsp27, (B) Hmgcs2 and other fatty acid oxidation genes (Cpt1a, Cact and Cpt2) mRNA levels in liver. Results are means ± SEM for each group (n=5) ** P <0.01, *** P<0.001 relative to AL; # P<0.05, ## P <0.01 relative to 6h fast; §§ P<0.01 relative to 15h fasting.

1.4. Amino acid deprivation

Mammals have evolved a wide range of adaptive mechanisms to detect and respond to fluctuations in dietary protein nutrients. At the level of individual cells, dietary protein limitation is manifested as amino acid deprivation, which activates an amino acid response (AAR) signal transduction pathway. Amino acid limitation regulates numerous steps in gene expression including chromatin structure, transcription start site, transcription rates, mRNA splicing and RNA export, turnover and translation [87].

Amino acid response pathway

Limiting the extracellular supply of an essential amino acid or blocking the synthesis of an otherwise nonessential one results in an increase in uncharged tRNA that binds to and activates the general control nonderepressible protein 2 (GCN2) kinase. In this manner, the GCN2 protein serves as a sensor of amino acid insufficiency. Thus depletion at the cellular level of any individual amino acid can trigger AAR. Once activated, GCN2 kinase phosphorylates the translation factor eIF-2α that suppresses general protein synthesis but promotes a paradoxical increase in translation of mRNA species. Among these are several activating transcription factors such as ATF4. ATF4 triggers increased transcription by binding to (CCACT-enhancer binding protein-activating transcription facto C/EBP-ATF response elements (CARE)), so named because they are composed of a half-site for the C/EBP family and a half-site for the ATF family transcription factors. The products of these CARE -containing genes modulate a wide spectrum of cellular events designed to adapt to dietary stress [88].

2. THESIS GOAL

Currently, whereas the role of *Fsp27/CIDEC* begins to be better understood in adipose tissue, little is known about its physiology in liver. As discussed above, previous results of this group showed that the *Fsp27/CIDEC* gene expression is regulated in a time-dependent manner during fasting in liver, being induced in the early steps of fasting and decreased after a longer period.

These previous results and the importance of *Fsp27/CIDEC* in regulating energy homeostasis, including its modulation by FAO prompted the present study where we aimed to further explore the physiologic role and to provide mechanistic insight of *Fsp27/CIDEC* regulation in adaptation to fasting response in HepG2 cells. We aimed to specifically answer two questions: 1) Why *CIDEC* expression decreases during prolonged fasting and 2) How FAO affects *CIDEC* expression.

In the second part of the present study we aimed to elucidate if the mechanism by which *Pgc-1α* becomes activated in response to prolonged fasting and amino acid deprivation is somehow mediated by *Foxa2*. This notion is based in the suggestion that *Foxa2* could be involved in the regulation of metabolic adaptation to low nutrient conditions such as fasting and amino acid deprivation in hepatocytes. This hypothesis, is further based in recent results of this group demonstrated that in these particular nutrient restriction conditions, both *Foxa2* and *Pgc-1α* expression are increased in mice liver.

3. MATHERIALS AND METHODS

3.1. Plasmid DNA preparation

To obtain the plasmid DNA used in this study, the interest mouse promoters (Pgc-1α and Fsp27) were amplified by PCR, with Taq polymerase (Biotools), dNTPS, specific primers (see plasmid construct below) and mouse genomic DNA. The amplified product was run in an agarose gel to verify the product size. The band of interest was cut from the gel and purified with the *Wizard SV Gel and PCR Clean System kit* (Promega), according to the manufacturer's instructions.

After quantification of the purified product on *NanoDrop-1000* (NanoDrop Technologies, Inc. Thermo Scientific) the product was inserted in a pGEM-T vector, according to the protocol *pGEM-T and pGEM-T Easy Vector* (Promega).

The plasmid DNA was transformed into competent cells *E.coli* DH5α, previously prepared by salt washes ($CaCl_2$) to permeabilize them, and frozen at -80^0C, and then plated on indicator agar plates (AIX plates: 100 mg/L ampicillin, 8 mg/L IPTG, and 40 g/L XGal) for selection.

The plates were incubated overnight at 37ºC; recombinants were selected by blue/white secreening on indicator plates. Positive white colonies are incapable to hydrolyze the substracte X-Gal because the insert causes the loss of the β-galactosidase activity, while colonies that have incorporated the empty vector (negative colonies) are blue due to the presence of the X-Gal hydrolysis products. The white positive colonies were left to grow overnight in 3 mL LB inoculums, with selection antibiotic (ampicillin).

The plasmid DNA from inoculums was obtained with a commercial kit *PureYield Plasmid Miniprep System* (Promega). The plasmid DNA was sequenced (Macrogen sequencing service).

After sequencing, the insert was released from pGEM-T, with restriction enzymes specific for the sequence amplified (see plasmid construct below), and ran in an agarose gel. The bands were purified (with the *Wizard SV Gel and PCR Clean System kit* (Promega)) and the insert was subcloned into the pGL3b vector.

The plasmid DNA was transformed in competent cells *E.coli* DH5α and plated in agar plates with the selection antibiotic for pGL3b (ampicillin). Selection of positive colonies was performed through PCR screening. The DNA of the positive colonies selected was obtained with a commercial kit *PureLink HiPure Plasmid Filter Maxiprep Kit* (Invitrogen).

3.2. Plasmid construct and site directed mutagenesis

3.2.1. Mouse PGC1-α promoter

The *PGC-1α* promoter (nt -2010/-51 to the relative transcription start site) was amplified by PCR from mouse genomic DNA using Taq polymerase (Biotools) with the oligonucleotides forward (**DH1384**) and reverse (**DH1385**) containing the sequence of the restriction sites *MluI* and *XhoI* respectively (italicized and bolded in the primer sequence, see Primer Design, “Cloning” section in Annex). The PCR products were cloned in the vector pGEM-T and sequenced (Macrogen sequencing service). This plasmid was digested with *MluI* and *XhoI* and subcloned into the promoter firefly luciferase reporter gene vector pGL3-Basic (Promega).

3.2.2. Mouse Fsp27 promoter

The *Fsp27 promoter* (nt -2054/+18 to the relative transcription start site) was amplified by PCR from mouse genomic DNA using Taq polymerase (Biotools) with the oligonucleotides forward (**DH1303**) and reverse (**DH1304**) containing the sequence of the restriction sites *MluI* and *XhoI* respectively (italicized and bolded in the primer sequence see Primer Design, “Cloning” section in Annex). The PCR products were cloned in the vector pGEM-T and sequenced (Macrogen sequencing service). This plasmid was digested with *MluI* and *XhoI* and subcloned into the promoter firefly luciferase reporter gene vector pGL3-Basic (Promega).

1.2.2.3. QuikChange™ Site-Directed Mutagenesis Kit

The mutations in the CREB identified sequences in the mouse *Fsp27* promoter were generated by site directed mutagenesis, carried out by a commercial kit - *QuickChange™ Site-Directed Mutagenesis* (Stratagene) following the manufacturer’s instructions. The mutants were generated by point mutations replacing the original sequences **TGACTTCA** (CRE1 site, -375/-366) and **CGTCA** (CRE2 half site, -1792/-1787) by **TGAGTATC** and **ATCGC** respectively in both sense and antisense orientations, following the manufacturer’s instructions (see primer design, “Site direct mutagenesis” section in Annex).

The QuikChange site-directed mutagenesis kit is used to make point mutations, switch amino acids, and delete or insert single or multiple amino acids. The QuikChange site-directed mutagenesis method is performed using PfuTurbo™ DNA polymerase II and a thermal temperature cycler PfuTurbo DNA polymerase replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each

complementary to opposite strands of the vector, are extended during temperature cycling by using PfuTurbo DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with Dpn I. The Dpn I endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA.

3.2.3. Automatic sequenciation of DNA

The sequences and orientations of the constructions were verified by sequencing – Macrogen Inc. (Macrogen sequencing service).

3.2.4. DNA oligonucleotide (primers)

All DNA oligos were synthesized by Sigma-Aldrich with technology ultra-high base coupling efficiency, combined with optimized cartridge purification and 100% quality control by mass spectrometry (Designed primer sequences are listed in the Annex).

3.3. Cell culture

Cell Line	Description	Reference
HepG2	Human liver carcinoma (*Hommo sapiens*)	ATCC No. HB-8065

HepG2 are adherent, epithelial-like cells growing as monolayers and in small aggregates. HepG2 cell line was derived from the liver tissue of fifteen year old male with differentiated hepatocelullar carcinoma.

The human hepatocellular carcinoma cells HepG2 were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 4 mM glutamine, 100 µg/ml streptomycin sulfate, 100 units/ml penicillin G, and 10% (v/v) fetal bovine serum (FBS). Cells were incubated at 37°C in humidified atmosphere containing 5% CO_2. Culture medium was discarded and changed every 2-3 days. To passage, cells were briefly washed twice with 1xPBS and 0.05% Trypsin-EDTA solution was added. The cell layer dispersed at 37°C for 5 min, trypsin was deactivated by adding complete growth medium. Cells were split 1:4 dilution every 5 days or counted (Trypan Blue stain method) and plated according the final experiment. Cells were replenished with fresh medium before all experiments. To induce the fasting response, cells were incubated for 16 hours in a serum-free medium – Opti-MEM (Invitrogen) and supplemented with or without 10 µM Etomoxir (6 hours); 10 µM Etomoxir plus 10 µM of GW9662 for 6 hours; 10 µM of Forskolin

(6 hours); 10 µM of Forskolin (6 hours) and pretreatment with 50 µM of H89 (1 hour); 10 µM of Wy14643 (24 hours). In the controls of these experiments, cells were also incubated for 16h in Opti-MEM (Invitrogen) and the vehicles (water or DMSO) of the specific reagents used in each experiment were added – see "Reagents used in cell culture specific treatments" below.

3.4. Reagents

3.4.1. Reagents used in cell culture maintenance

Regent	Reference
Minimum Essential Media (MEM)	GIBCO, 61100-087
Fetal Bovine Serum (FBS)	GIBCO, 10270-106
Pen Strep (Penicillin-Streptomycin)	GIBCO, 15140-122
L-Glutamine 200mM	GIBCO, 25030-024
Sodium bicarbonate solution 7.5%	GIBCO, 15140-122
OPTI-MEM® I	GIBCO, 31985-047
Tripsin-EDTA 10X	GIBCO, 15400-054

3.4.2. Reagents used in cell culture specific treatments

Reagent	Reference	Description	Vehicle
GW9662	Sigma M6191	PPARγ antagonist	Dissolved in DMSO to 10 mM.
ETOMOXIR	Sigma E1905	CPT1 inhibitor	Dissolved in water to 10 mM.
FORSKOLIN	Sigma F6886	cAMP agonist	Dissolved in DMSO to 10 mM.
H89	Sigma B1427	PKA inhibitor	Dissolved in water to 10 mM.
Wy14643	Sigma C7081	PPARα agonist	Dissolved in DMSO to 10 mM.

Following reconstitution stock solutions aliquots were stored at -20°C.

3.4.3. Reagents used in cell culture transfection

Reagent	Reference
Lipofectamine LTX Reagent	Invitrogen, 15338-100
DharmaFECT®4 Transfection Reagent	Thermo Scientific, T-2004-01

3.5. Transient transfection and gene reporter assays

HepG2 cells were plated at a density of 1.5 x 10^5 cells/well on 24-well plates and cultured for 24h in culture medium (MEM). Cells were transiently transfected using Lipofectamine LTX (Invitrogen) at a ratio of 2.5 µl to 1 µg of DNA, following the manufacturer's instructions. For each transfection 0.4 µg of reporter gene construct (pGL3b-Fsp27 or pGL3b-Pgc-1α) and different amounts of the transcription factors expression vectors (pcDNA3, CREB or FOXA2) were co-transfected (indicated in the respective graphs). The plasmid pRL-CMV (10ng/well) was included as an internal transfection control. The total amount of transfected DNA was kept constant among experimental groups by the addition of pcDNA3 empty plasmid.

At approximately 48 h following transfection, cellular extracts were prepared for analysis of luciferase activity, by washing the cells with phosphate-buffered saline (PBS 1x), and harvested in 100 µl of 1x Passive lysis buffer (E194A, Promega). The lysates were collected and a 10 µl aliquot was used for Firefly luciferase assays using the Dual-Luciferase Reporter Assay System (E1960, Promega). *Firefly* and Renilla luciferase activities were dertermined in a Berthold Sirius Luminometer. Relative luciferase activity was given as the ratio of relative luciferase unit/relative Renilla unit.

3.6. siRNA Transfection

Specific human SIRT1 (T2004-01) and siControl nontargeting (D-001210-01) siRNAs were purchased from Dharmacon (Thermo Fisher). HepG2 cells were seeded in 6 well plates at density 4.5 x 10^5 cell/well in MEM and grown for 24h. Transfection was performed according to the instructions of Dharmacon using 5 µl of DharmaFECT®4 and 25 nM per well final siRNA concentration. At 72h following transfection, cells were rinsed with PBS, and total RNA was isolated and analyzed by real time PCR.

3.7. KCREB Transfection – Fluorescence assay

HepG2 cells were seeded in 6 well plates at density 4.5 x 10^5 cell/well in MEM and grown for 24h. Cells were transiently transfected using Lipofectamine LTX (Invitrogen) at a ratio of 2.5 µl to 1 µg of DNA, following the manufacturer's instructions. The constructs GFP (500 ng) and pcDNA3-KCREB (500 ng) were co-transfected. The experiment controls only contained the GFP construct transfected. At 24h following transfection, cells were visualized and photographed with a confocal laser microscope *LEICA DMI 4000 B* in order to access transfection efficiency. Cells were then incubated for 16 hours (overnight) in a serum-free medium – Opti-MEM (Invitrogen) and then treated for 6h with 10 µM of forskolin and controls were supplemented with vehicle (DMSO). Following the 6h forskolin treatment, cells were rinsed with PBS, and total RNA was isolated and analyzed by real time PCR.

3.8. Isolation and analysis of RNA

3.8.1. Isolation of total RNA

Total RNA was extracted from HepG2 cells using TRI Reagent Solution (Ambion) according to manufacturer's instructions. Cultured cells were washed twice with cold PBS 1x and isolated by scrapping. TRI Reagent maintains the integrity of the RNA due to highly effective inhibition of RNAse activity while disrupting cells and dissolving cell components during sample

homogenization. Furthermore, this reagent is free of protein and DNA contamination. However, RNA was pretreated with *DNaseI* (Ambion) to eliminate genomic DNA contamination.
RNA was dissolved in DEPC-treated water (Sigma-Aldrich) and the concentration and purity of each sample was obtained from $A_{260/A280}$ and $A_{260/230}$ measurements in a micro-volume spectrophotometer *NanoDrop-1000* (NanoDrop Technologies, Inc. Thermo Scientific).

3.8.2. RT-PCR

Reverse transcription polymerase chain reaction is used to synthetized cDNA from one microgram of total RNA. Total RNA was reversed transcribed using random hexamers (Roche Diagnostics), dNTPs (Attend Bio) and M-MLV reverse transcriptase (Invitrogen) following manufacturer's instructions.

3.8.3. Real time PCR

cDNA was subjected to real time PCR analysis using TaqMan universal PCR master mix (Invitrogen) and the specific gene expression Taqman probes from Applied Biosystems and normalized against those of 18S ribosomal RNA determined by Eukaryotic 18S rRNA endogenous control (Applied Biosystems). The following human gene probes were used: CIDEC Hs00535723_m1, PEPCK Hs00159918_m1, PGC-1α Mm00447181_m1, FOXA2. Reactions were measured in duplicate. The reactions were performed in *ABI PRISM® 7700 Sequence detection Systems* (Applied Biosystems) in 96-well plates. The thermal cycling conditions were, an initial activation step for 2 min at 50 °C and 10 min at 95 °C and 40 cycles of melting ate 95 °C for 15 sec and annealing/extend for 1 min ate 60 °C [89].
Results were obtained by the comparative Ct method and expressed as fold of the experimental control. The comparative CT (ΔΔCt) method [90] was used to determine the relative target quantity in the samples.

3.9. Statistical analysis

Results are expressed as means ± standard error mean (SEM) from n=2 to n=6. The statistical significance of differences found between groups was assessed by one-way analysis of variance. An unpaired Student's t test was use to compare the groups. $P<0.05$ was considered as statistically significant difference.

3.10. Information technologic tools

Entrez-Pubmed, National Center for Biotechnology Information (NCBI, USA):
http://www.ncbi.nlm.nih.gov/pubmed/

TFSEARCH: Searching Transcription Factor Binding Sites:
http://www.cbrc.jp/research/db/TFSEARCH.html

3.11. Additional Information

Any missing information regarding the experimental procedures, which also cannot be found in Annex, means that the procedure was performed according to the detailed information given by “Molecular Cloning, a Laboratory Manual” (Sambrook, Fritsch and Maniatis), or following the protocols provided in the instructions of the commercial kits used.

4. RESULTS

4.1. Down regulation of FAO increases CIDEC expression.

To elucidate whether the FAO rate affected *CIDEC* expression, we have treated HepG2 cells with 10 µM of etomoxir (amount previously optimized), a CPT1 inhibitor that represses FAO [91]. Figure 4.1A shows that this treatment significantly increased, approximately six-fold, *CIDEC* (left panel) and *PEPCK* (right panel) mRNA levels in HepG2 cells when compared to the control.

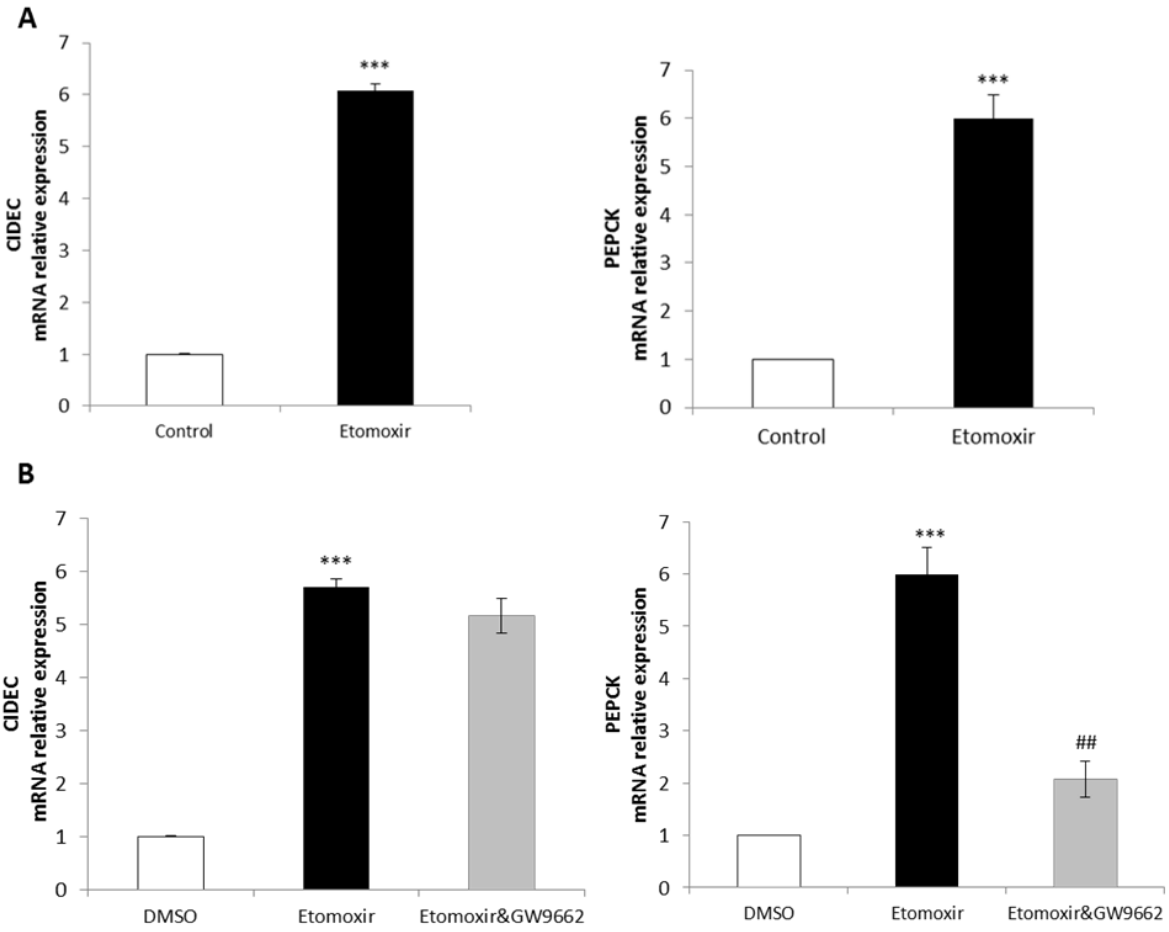


Figure 4.1. CIDEC is induced by etomoxir.

(A) *CIDEC* (left panel) and *PEPCK* (right panel) mRNA levels in HepG2 cells, serum-starved for 16 h and treated with 10 µM of etomoxir for an additional 6 h. **(B)** *Effect of synthetic PPARγ antagonist (GW9662) in CIDEC expression*. *CIDEC* (left panel) and *PEPCK* (right panel) mRNA levels in HepG2 cells, serum-starved for 16 h and treated with 10 µM of etomoxir and with or without 10 µM of GW9662 (6 hours). Results are means ± SEM of three independent measurements. ***, P<0.001, relative to control (HepG2 cells treated with vehicle - DMSO); ##, P<0.01 relative to the etomoxir treatment.

Previously it was suggested that impairment of FAO leads to triacylglyceride accumulation that stimulates lipid droplet associated proteins like *Fsp27/CIDEC* and that this mechanism was specifically mediated by PPARγ [64, 72].

Having this into account and to further study whether PPARγ was involved in *Fsp27/CIDEC* expression changes affected by FAO rate, we used a specific PPARγ antagonist (GW9662). As shown in Figure 4.1B, treatment of HepG2 cells with 10 μM of GW9662 had no effect on *CIDEC* expression (left panel) while the same treatment resulted in a significant reduction of *PEPCK* expression (right panel). *PEPCK* was used as a positive control for these experiments since it has been described as a direct target of PPARγ [92].

These data suggest that *CIDEC* expression is regulated by mitochondrial FAO rate, but the mechanism by which *CIDEC* is upregulated when FAO is impaired is not mediated by PPARγ.

4.2. The induction of CIDEC expression is not mediated by PPARα during fasting.

Under physiological conditions, the function of PPARα is mainly evoked during fasting, which is associated with increased hepatic PPARα mRNA expression and increased FFA levels that are natural ligands of PPARα [93]. Based on this data, we investigated whether it could be one of the signals triggering the induction of *CIDEC* in fasting conditions. Figure 4.2 shows that *CIDEC* expression (left panel) is unchanged upon treatment with 10 μM of specific PPARα ligand Wy14643 (Sigma) while, under the same conditions, *HMGCS2* mRNA levels (right panel) were significantly increased in HepG2 cells, as expected, since *HMGCS2* is a target gene of PPARα [59].

These results suggest that the fasting expression pattern of *CIDEC* is not modulated by the activity of PPARα in HepG2 cells.

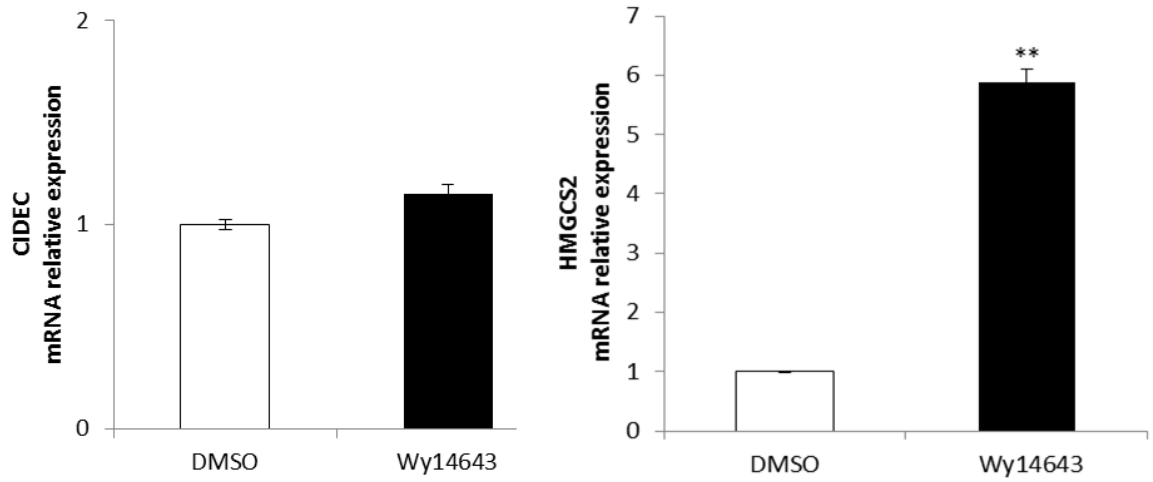


Figure 4.2. Effect of specific PPARα agonist (Wy14643) in CIDEC expression.

CIDEC (left panel) and *HMGCS2* (right panel) mRNA levels in HepG2 cells treated with 10 µM of Wy14643 (Sigma) for 24 hours. Results are means of two independent experiments. **, P<0.01 relative to control (HepG2 cells treated with vehicle - DMSO).

4.3. Fsp27 is activated by CREB and its induction by fasting is repressed by SIRT1.

The liver contributes to glucose homeostasis by promoting either storage or production of glucose depending on the physiological state. During early fasting, the increase in circulating pancreatic glucagon stimulates the gluconeogenic program through the activation of the cAMP pathway leading to the upregulation of gluconeogenic genes [9, 13, 34].

To further investigate the expression pattern of *Fsp27/CIDEC* during adaptation to fasting response we treated HepG2 cells with 10 µM of forskolin (amount previously optimized), an adenilate cyclase activator which leads to an increase of intracellular levels of cAMP. Figure 4.3A shows that *CIDEC* (left panel) is markedly induced by forskolin treatment as well as *PEPCK* (right panel) that shows the expected effect of this cAMP stimulator.

Cyclic AMP (cAMP) regulates the expression of numerous gluconeogenic genes through phosphorylation of CREB, mediated by protein kinase A (PKA) [9]. To further establish that the induction of *CIDEC* expression is regulated by the cAMP-mediated activation of CREB activity, corroborating the specificity of the forskolin treatment, we repeated the forskolin experiment with the addition of a protein kinase A (PKA) inhibitor, H89 (Figure 4.3B). In the presence of the PKA inhibitor H89*, CIDEC* expression was decreased approximately two-fold when compared to forskolin induction (Figure 4.3B, left panel). As expected, *PEPCK* (Figure 4.3B, right panel) expression was markedly diminished, approximately eight-fold, by the addition of H89.

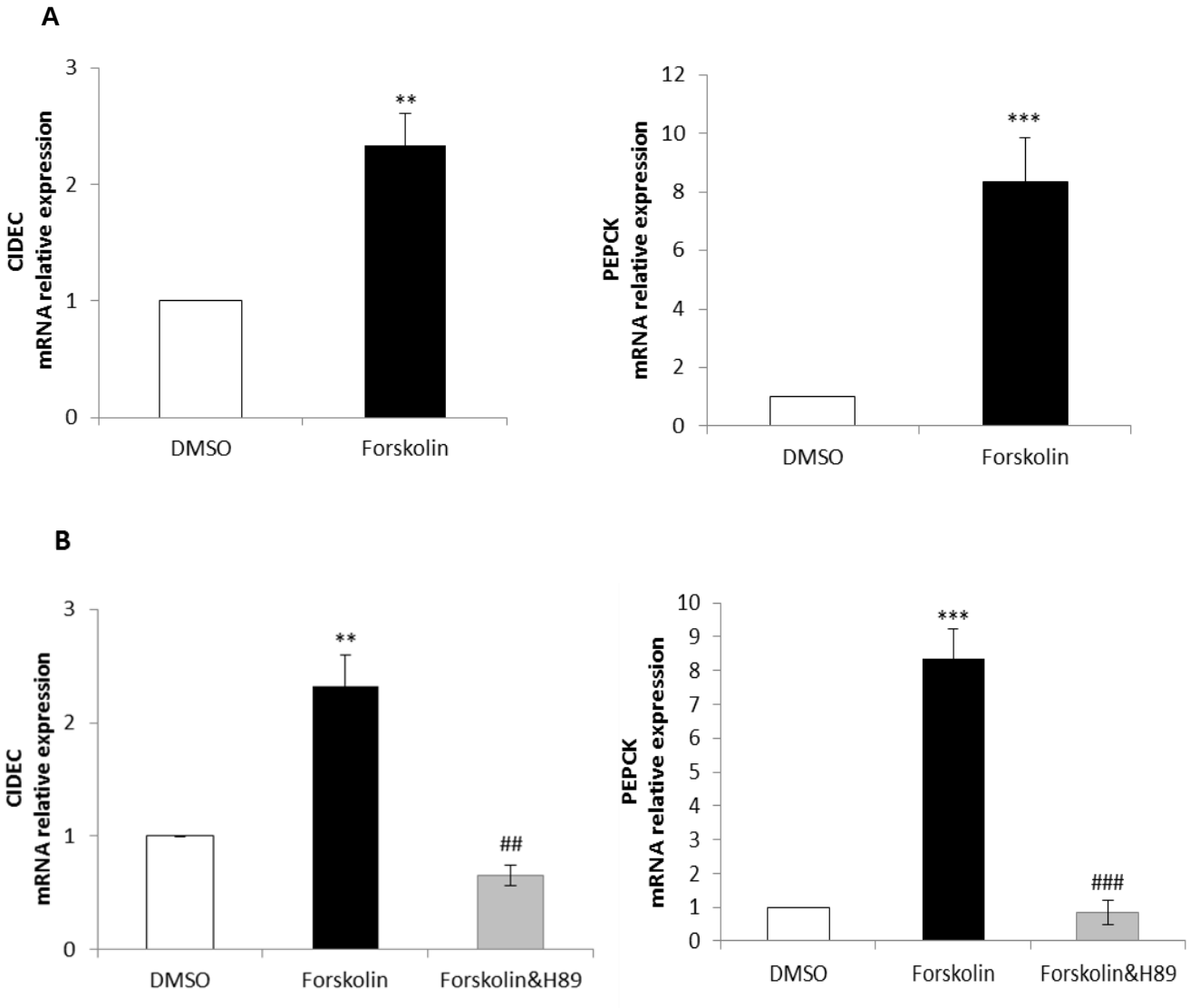
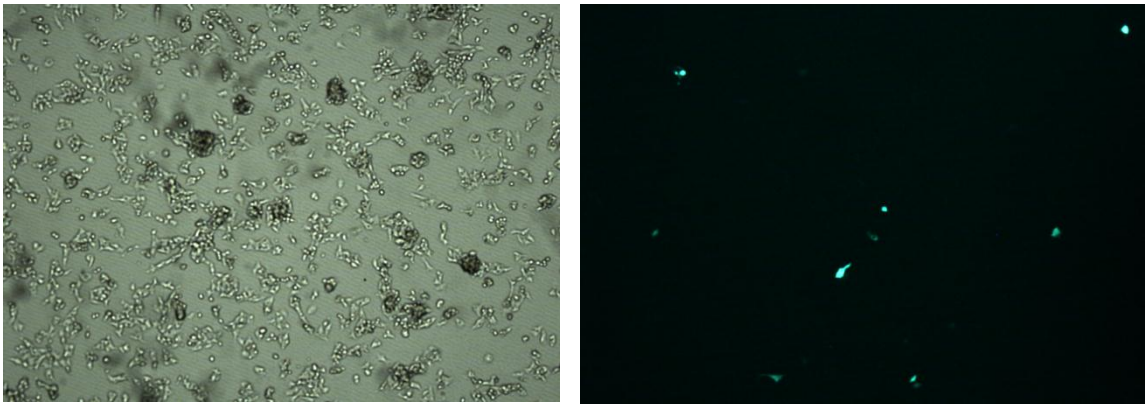


Figure 4.3. CIDEC is induced by Forskolin and repressed by PKA inhibitor H89.

(A) *CIDEC* (left panel) and *PEPCK* (right panel) mRNA levels in HepG2 cells treated with 10 µM of forskolin in OPTI-MEM (Invitrogen) for 6 h (mean of three independent experiments). **(B)** The effect of treatment with forskolin 10 µM for 6h and/or pretreatment with H89 (50 µM) for 1 hour in *CIDEC* (left panel) and *PEPCK* (right panel) mRNA levels in HepG2 cells. Cells were in OPTI-MEM (Invitrogen), or vehicle (DMSO). Data represent the means ± SEM of three independent experiments. **, P<0.01; ***, P<0.001, relative to the control; ##, P<0.01, ###, P<0.001 relative to forskolin activation of *CIDEC* and *PEPCK* mRNA levels.

To further support these data and to provide additional information for the mechanism regulating *CIDEC* expression in the adaptation to fasting, a vector containing a dominant repressor of CREB activity – KCREB expression vector – was transfected in HepG2 cells and treated with 10 µM of forskolin, the transfection was followed by visible fluorescence from a co-expressed green fluorescent protein (GFP). Although not very efficient, about 20% (Figure 4.4A), the transfection of KCREB expression vector significantly decreased the induction of *CIDEC* mRNA levels by cAMP (Figure 4.4B, left panel). *PEPCK* expression, as expected, was also decreased in the presence of dominant negative of CREB expression vector relatively to the forskolin induction (Figure 4.4B, right panel).

A



B

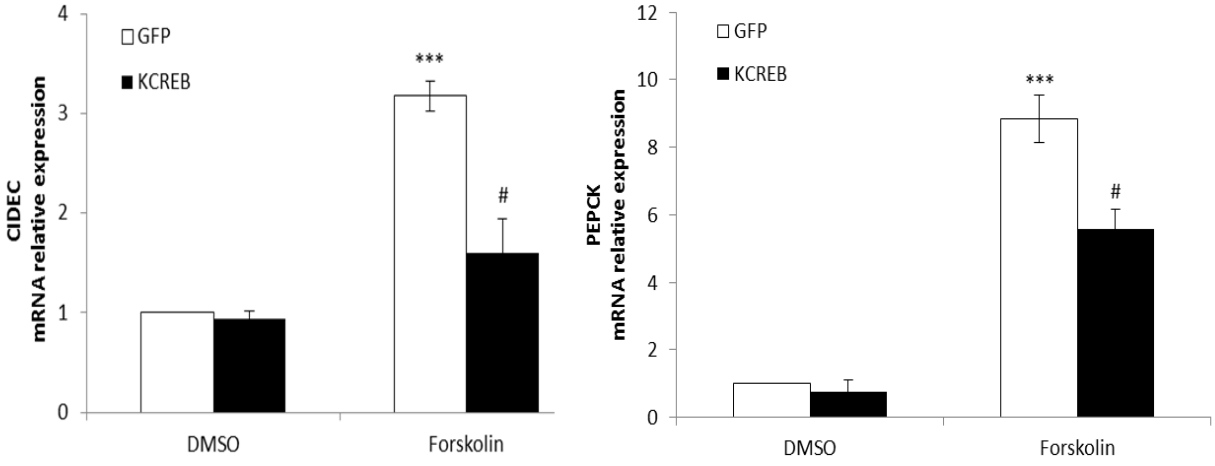


Figure 4.4. CIDEC is induced by Forskolin and repressed by a negative dominant of CREB – KCREB.

(A) HepG2 appearance (left panel) and transfection assessment in HepG2 cells by green fluorescence protein (GFP) (right panel). **(B)** *CIDEC* (left panel) and *PEPCK* (right panel) mRNA levels in HepG2 cells transfected with GFP (green fluorescent protein), or GFP plus KCREB (500 ng) expression vector and treated with 10 µM of Forskolin (in OPTI-MEM - Invitrogen) for 6h. Data represent the means ± SEM of three independent experiments. ***, P<0.001, relative to the control; #, P<0.05, relative to forskolin activation of *CIDEC* and *PEPCK* mRNA levels.

To elucidate the molecular mechanism underlying *Fsp27/CIDEC* fasting mediated expression in HepG2 cells and in liver, the transcriptional regulation of *Fsp27* through CREB was investigated.

By searching the TFSEARCH database (http://www.cbrc.jp), two putative CREB response elements (CRE) in the 5'-flanking region of the mouse *Fsp27* gene (nt -2054/+18 relative to the transcriptional start site) were revealed at positions -1787/-1792 (CRE2) and -366/-375 (CRE1) from the transcription start site (Figure 4.5A).

The upstream site (CRE2) is a conserved half-site motif (CGTCA) while the downstream site (CRE1) is an eight-base-pair element (TGAC**T**TCA) partially conserved from the canonical palindrome (TGACGTCA).

To elucidate whether the Fsp27 gene was induced by CREB, reporter gene transfection studies were carried out. Several constructs were produced with the luciferase gene as a reporter and transfected in HepG2 cells (Figure 4.5B). The same cells were co-transfected with an expression vector for CREB. Figure 4.5C, shows that CREB expression significantly induced (thirty-fold) the wild type reporter and this induction was diminished when single CREs were mutated (mut 1 or in a higher extend mut 2) or completely abolished, when both elements were simultaneously mutated (mut x2). These results identify the sequences CRE1 and CRE2 as CREB responsive elements in Fsp27 mouse gene.

Taken together, these data support that *CIDEC* is a direct target of CREB activity in hepatocytes and this process is mediated by the cAMP axis activation during the early fasting state.

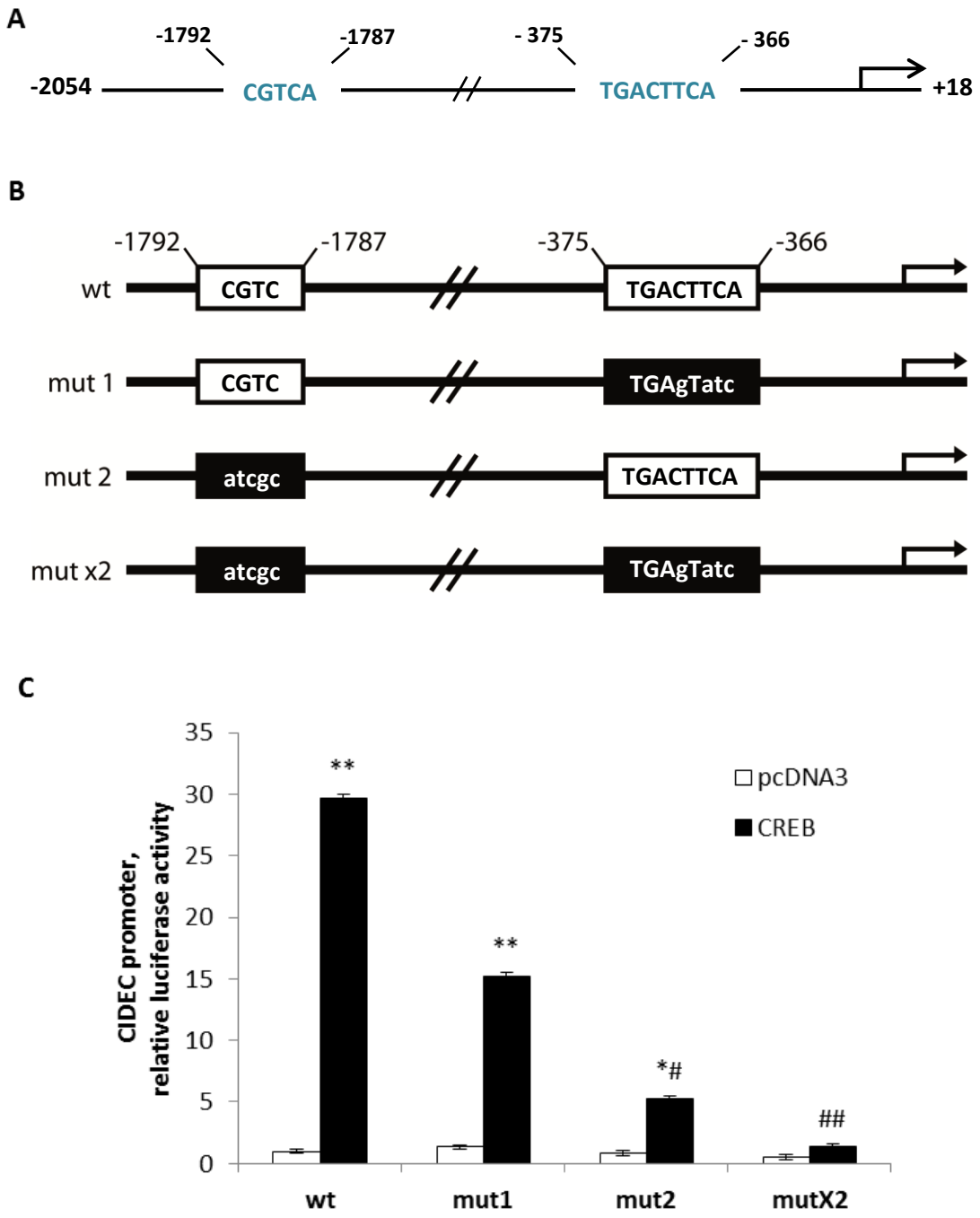


Figure 4.5. *Fsp27* mouse promoter activity is induced by CREB transcription factor.

(A) 5'-flanking region of the mouse *Fsp27* gene with the nucleotide sequences of the putative CREB sites - CRE1 and CRE2. **(B)** The subsequent constructs have the correspondent mutated elements inside the boxes bellow the *wild type* construct. The original sequence is shown as a line. **(C)** HepG2 cells transfected for 48 h with *Fsp27* promoter constructs cloned in pGL3basic and co-transfected with either pcDNA3 or pcDNA3-CREB (150 ng) expression vectors. Results are represented by fold activation to the WT promoter construct. pGL3basic activation was subtracted from each condition. Data represent the means ± SEM of four independent experiments performed in duplicate. *, P<0.05; **, P<0.01; relative to wt construct; #, P<0.05, ##; P<0.01 relative to CREB activation of WT promoter.

Once gluconeogenic gene expression is activated, the nutrient-sensing deacetylase SIRT1 reaches a maximal activity promoting a fasting-inducible switch between the early and late signaling programs [13]. Having this into account, and to elucidate the role of increased levels of SIRT1 in the regulation of *CIDEC* during fasting adaptation, *SIRT1* activity was disrupted by siRNA in HepG2 cells. Figure 4.6 shows that *CIDEC* mRNA expression was induced by siRNA mediated down-regulation of *SIRT1*, which suggests that *SIRT1* activity could repress the expression of *CIDEC* during the fasting-inducible switch. Furthermore, arguing in this favor, previous findings of this group, showed that *CIDEC* expression is decreased during the late phase of fasting, coincident with the increased levels of *SIRT1.*

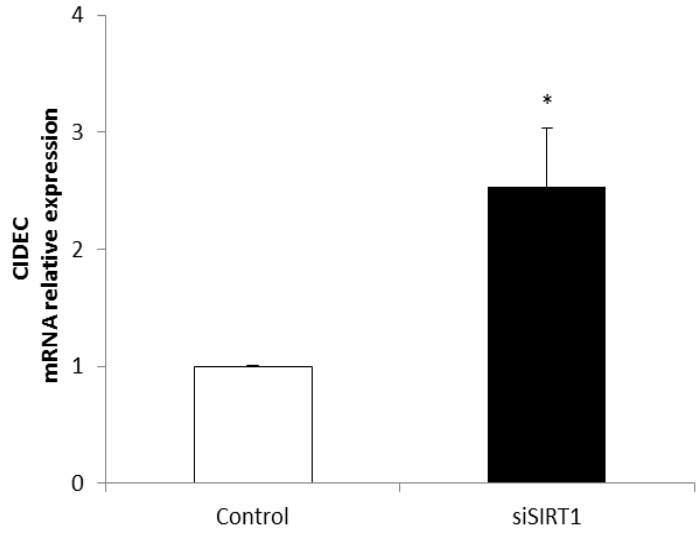


Figure 4.6. *CIDEC* is induced by SIRT1 depletion.

CIDEC mRNA levels in HepG2 cells treated with siRNA control or siRNA of *SIRT1*. Data represent the means ± SEM of three independent experiments. *, P<0.05 relative to the siRNA control.

4.4. Effect of fasting and amino acid deprivation in PGC-1α expression.

During late fasting, glucagon effects are attenuated, CRTC2 is down-regulated due to SIRT1-mediated deacetylation and its contribution to the fasting co-transcriptional response is significantly diminished [15]. The maintenance of gluconeogenic response through prolonged fasting is mediated by *FOXO1* and *PGC-1α* [13, 34]. In fact, *PGC-1α* co-transcriptional activity is important for the compensatory metabolic responses that occur during food deprivation [15]. Previous results of this group showed an interesting expression pattern of *Pgc-1α* in response to dietary manipulations in mice such as fasting and amino acid deprivation (leucin deprived diet). Figure 4.7A shows that hepatic levels of *Pgc-1α* of mice subjected to a leucin deprived diet (*Ad Libitum* (-) leu) were unchanged, whereas under fasting conditions hepatic levels of *Pgc-1α* were significantly increased, as expected. Interestingly, in livers of fasted mice previously treated with a leucin deprived-diet *Pgc-1α* is induced more than 15-fold.

In these conditions of limit nutrient availability, a complex gene network functions to maintain whole body homeostasis of metabolites such glucose and amino acids. One of these genes is *FOXA* (forkhead box A) family. Recently, was described that this gene mediates the induction of *Pepck* in fasting conditions [42] and also that *Foxa2* expression is induced upon amino acid deprivation [45]. So, to explore the mechanism mediating the significant increase of hepatic *Pgc-1α* levels in response to fasting and to a leucine deprivation we studied the gene expression of *Foxa2* in the same animals. Figure 4.7B shows that liver of fasted animals previous treated with a leucin deprived diet significantly enhanced *Foxa2* expression.

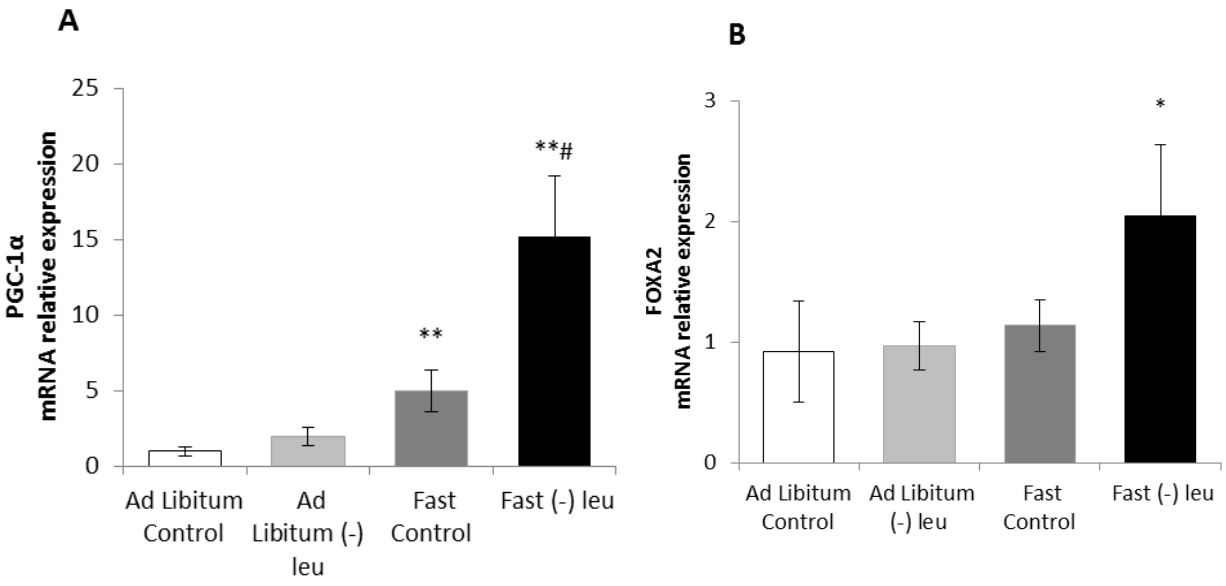


Figure 4.7. *Pgc-1α* and *Foxa2* are induced upon leucin deprived diet in fasting conditions.

(A) *Pgc-1α* mRNA levels and **(B)** *Foxa2* mRNA levels in liver of mice fed *Ad Libitum* (*Ad Libitum* Control), fed with a leucin deprived diet (*Ad Libitum* (-) leu, fasted (Fast control) or fasted and previously subjected to a leucin deprived diet (Fast (-) leu). Results represent the means ± SEM of six independent experiments. *, P<0.05; **, P<0.01; relative to the Ad Libitum control group; # P<0.05 relative to the fast control group.

These data, and previous findings suggesting that the genes that regulate hepatic response are blunted in the absence of *Foxa2* [42], prompted us to elucidate if *Foxa2* was mediating the *Pgc-1α* induction observed during fasting and amino acid deprivation (Figure 4.7A). Towards this end, Pgc-1α mouse promoter was cloned (nt -2010/-51 to the relative transcription start site) in a plasmid which drives the expression of a luciferase reporter gene (pGL3b) and, reporter gene transfection studies were carried out. Since previous studies suggested that Foxa2 allows chromatin access for hormone dependent transcription factors that are activated during food deprivation [42], we transfected HepG2 cells with either Pepck or Pgc-1α promoters and different concentrations of expression vectors for CREB and FOXA2 (indicated in the graph). Figure 4.8 shows that CREB expression significantly induced both Pepck (left panel) and Pgc-1α (right panel) promoters, while single addition of FOXA2 had no effect in the activity of the promoters. Then, when co-tranfected with CREB and FOXA2 both Pepck and Pgc-1α promoter activities were induced however in a lesser extend then the activation observed for CREB itself.

These results suggest that indeed Foxa2 plays a role in the hepatic response in the adaptation to amino acid deprivation and fasting although the mechanism by which Foxa2 is mediating this response is not by allowing CREB binding to both Pepck and Pgc-1α promoters in HepG2 cells.

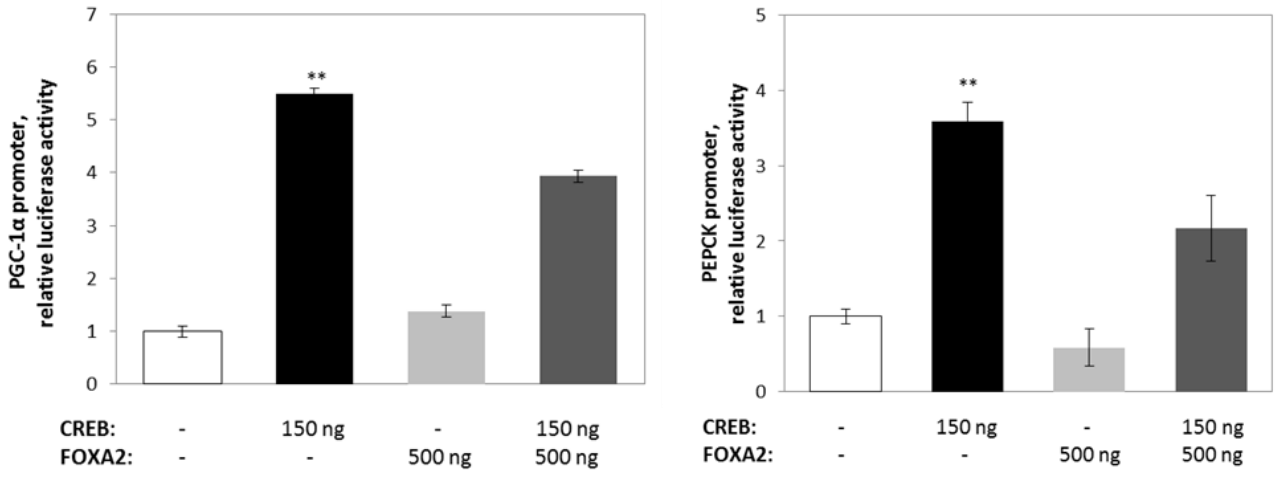


Figure 4.8. Pgc-1α and Pepck promoter activities are induced by CREB but not by FOXA2 transcription factors.

Transient assay of HepG2 cells transfected for 48 h with pGL3b (control) or the *Pgc-1α* and *Pepck* promoters and co-transfected with either pcDNA3 and/or pcDNA3-CREB, pcDNA3 and/or pcDNA3-FOXA2 or pcDNA3-CREB plus pcDNA3-FOXA2 expression vectors, represented by fold activation to pGL3b. pGL3b activation was subtracted from each condition. Data represent the means ± SEM of three independent experiments performed in duplicate. **, P<0.01 relative to pcDNA3.

5. DISCUTION

Fat specific protein of 27 kDa (FSP27) or the cell death-inducing DNA fragmentation factor 45-like effector C (CIDEC, the human homologue of FSP27) belong to the CIDE family [83, 84]. The FSP27 was previously reported to be a lipid droplet-associated protein [84-86], which is expressed in white adipose tissue (WAT) [85, 86] or in steatotic liver [72]. Moreover, as previous described, results of this group showed that *Fsp27/CIDEC* expression was induced in fasting liver.

The previous findings prompted us to further investigate the mechanism by which *Fsp27/CIDEC* is mediating fasting adaptation and regulated by FAO rate in liver. Towards this end, we use an *in vitro* model – the assays were carried out in a human liver carcinoma cell line, HepG2 – where the fasting condition was mimed by serum-starvation of cultured cells.

It was described that *Fsp27* expression in hepatocytes significantly decreases the activity of mitochondrial β-oxidation and reduces triglyceride turnover [72, 84]. The present study showed that pharmacological inhibition of FAO (etomoxir, Figure 4.1A) in HepG2 cells induces *CIDEC* expression. This data suggests a direct link between impaired FAO and *CIDEC* induced expression. However, the mechanism mediating this link is not so clear.

Previously it was suggested that an impairment in FAO leads to triglyceride accumulation which stimulates lipid droplet-associated proteins such as FSP27/CIDEC and also that this TG accumulation mediated by *Fsp27* gene is specifically regulated by PPARγ and its specific ligands [72, 75]. This data prompted us to speculate that the missing link between induction of *CIDEC* and FAO inhibition could be mediated by PPARγ. However, analysis of *CIDEC* mRNA levels in HepG2 cells treated simultaneously with etomoxir and GW9662, an antagonist of PPARγ (Figure 4.1B) didn't show any effect in CIDEC expression. This data suggests that inhibition of FAO could alter gene expression through a different mechanism from that of increased levels of free fatty acids, which are natural ligands of the PPARs.

Regarding, to the induction of *Fsp27* during fasting, the data present in this study suggests that PPARα, a master regulator of fasting liver [65], is not one of the signals triggering this induction (Figure 4.2). In accordance with these findings, a previous report showed that Fsp27 promoter is not activated by ligands of PPARα nor PPAR δ [72]. Also, these results are corroborated by a preceding suggesting that induction of *CIDEC* was not mediated by PPARα [94].

Interestingly, *CIDEC* gene expression was up regulated by forskolin a cAMP agonist in HepG2 cells (Figure 4.3A). Consistently, mouse *Fsp27* promoter was induced approximately 30-fold by CREB. Indeed, the present study demonstrates two function CRE binding sites that directly interact with CREB *in vitro*. These results suggest that *Fsp27/CIDEC* could be direct target of hepatic CREB. To demonstrate additional evidence for this mechanism and to prove the

specificity of forskolin treatment we showed that impairment of PKA (by its inhibitor H89) resulted in the decrease of *CIDEC* mRNA levels, suggesting that indeed forskolin is inducing CREB activity and in turn CREB is activating *CIDEC.* Furthermore, the effect of KCREB expression vector (Figure 4.4B) confirms that *Fsp27/CIDEC* is a direct target of CREB in HepG2 cells.

CIDEC mRNA levels were increased in HepG2 cells when *SIRT1* activity was impaired by siRNA. Recently, it has been suggested a switch from early gene activation via CRTC2 to late activation of FOXO1 being critical for transcriptional regulation in fasting [13]. CRTC2 is a coactivator responsible for CREB-mediated transcriptional activation of the hepatic gluconeogenic program [11, 12]. However, it has also been proposed that the transcriptional response to fasting is only transiently dependent on CRTC2 with the demonstration that it is deacetylated by the NAD^+ dependent enzyme *SIRT1* which allows its ubiquitination and degradation in proteasome after long term fasting, suppressing CREB-CRTC2 signaling [95]. These data, together with the previous results demonstrating that *Fsp27* is induced in early fasting, fit with the model suggested by Liu et al. [13] in which CREB mediated expression is attenuated in long fasting periods.

Also, *SIRT1* regulation seems to be the missing link that could explain the induced expression of *CIDEC* when FAO is impaired (Figure 4.1). Recently *SIRT1* has been suggested has a link between protein acetylation and metabolism [17]. Vilà-Brau et al., [59] suggested a feed-forward mechanism in which the oxidative metabolism (FAO and ketogenesis), through NADH and ATP supply, would be expected to pull forward an anabolic process like gluconeogenesis, that would in turn generate NAD^+ and stimulate *SIRT1* (a NAD^+ consuming enzyme) activity. Arguing in favor of this hypothesis, and validating our model, it was described that inhibition of mitochondrial fatty acid oxidation with etomoxir, the CPT1 inhibitor used in the experiments present in this study, decreases the NAD^+/NADH ratio [96]. Therefore, once *Fsp27/CIDEC* is a gene that is repressed by *SIRT1* (Figure 4.6) will be up regulated by an impaired FAO.

Our results argue for a model of temporal regulation of *Fsp27/CIDEC* expression in response to fasting adaptation and FAO rate (Figure 5.1).

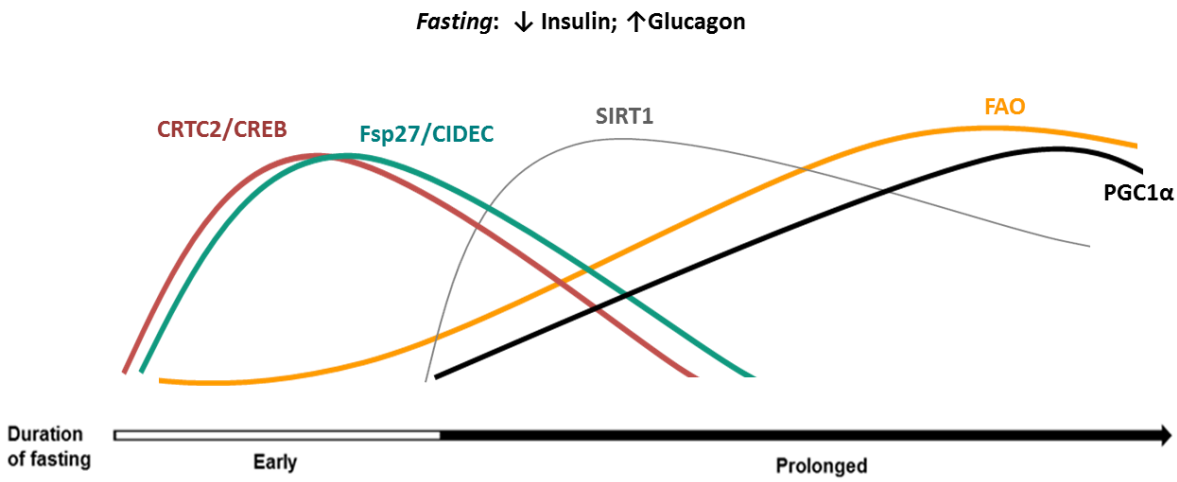


Figure 5.1. Temporal regulation of *Fsp27/CIDEC* expression in response to fasting adaptation and FAO rate. At the onset of fasting glucagon circulating levels are increased and insulin levels decreased. This hormonal change produces an increase in both the levels and activity of the transcriptional coactivator CRTC2, which by activating CREB increases the expression of hepatic *Fsp27/CIDEC*. With sustained fasting (>12-18h) SIRT1 becomes activated and deacetylates CRTC2. This event allows the ubiquitination of the protein targeting it for degradation. Furthermore, these conditions promote the switching to the utilization of free fatty acids and thereby FAO rate increases. This increment results in the increase of NAD^+/NADH ratio which will further activate SIRT1 (a NAD^+ consuming enzyme activity). The activation of SIRT1 results in an impairment of *CIDEC* expression. SIRT1 activation also outcomes the deacetylation of FOXO1 and its coactivator PGC-1α which is associated with an increase in their activities, providing a further mechanism for gluconeogenesis maintenance in response to prolonged fasting.

Effect of fasting and amino acid deprivation in PGC-1α expression.

Survival during prolonged food deprivation depends on the activation of hepatic gluconeogenesis which is mediated by the cAMP response element binding protein (CREB). As discussed above, in the late fasting state, the maintenance of gluconeogenic response through prolonged food deprivation conditions is mediated by *FOXO1* and *PGC-1α* [13]. Accordingly, Figure 4.7A shows that *PGC-1α* expression is increased in livers of mice subjected to prolonged fasting conditions. Interestingly this increase is even higher (15-fold) when the same animals are previously treated with a leucin deprived diet.

Also, these conditions induced *Foxa2* mRNA levels (Figure 4.7B). It was previously suggested that *Foxa2* is required for activation of hepatic gluconeogenic program during fasting [42] by cAMP and glucocorticoids once it allows chromatin access for hormone-dependent transcription factors that are activated during the fasting response [42, 97, 98]. This notion is based on the aforementioned proximity of *Foxa2* binding sites to CREs and GREs in cis-regulatory elements of several gluconeogenic genes [99, 100]. Also, it has been reported that *Foxa2* increases the efficacy of CREB binding to Pepck promoter [42].

Having this into account, and a previous report showing that *Foxa2* expression was induced upon amino acid restriction [45] we speculated that the induction of *Pgc-1α* expression observed in both fasting and amino acid deprived conditions would be mediated by *Foxa2* activity, since under these two dietary manipulations there were be more *Foxa2* available which would increase and facilitate the binding of CREB to the promoter.

The transient assays performed in HepG2 cells confirmed that both *Pepck* and *Pgc-1α* promoters are indeed direct targets of CREB [9] but, when co-transfected along with FOXA2 the induction was not significant (Figure 4.8) suggesting that *Foxa2* is not facilitating the binding of CREB to the promoters. These data did not confirm the previous findings described by Zhang et al., [42] or other reports that identified a Foxa binding site in Pepck promoter [99, 101]. Contrarily, corroborating our results, Wolfrum et al., [43] reported that Foxa2 did not bind to the conserve Hnf3/Foxa site in the Pepck promoter, suggesting that Pepck promoter is not a target of Foxa2 in fasting conditions.

Our results of induction of *Pgc-1α* and *Foxa2* gene expression in fasting and amino-acid deprived conditions are supported by other findings implicating *Pgc-1α* and *Foxa2* in the adaptation of gluconeogenic genes during food deprivation conditions [15, 42, 45]. Still, the transient assays in HepG2 cells suggested that *Foxa2* is not mediating the CREB targeting to the Pgc-1α neither the Pepck promoters. These results suggest that this is not the mechanism by which Foxa2 is regulating the activation of the hepatic transcriptional program of gluconeogenesis during adaptation to limit food supply.

6. CONCLUSIONS

To conclude, in the first part of this study we showed that a lipid droplet-associated protein, *Fsp27/CIDEC*, was expressed in HepG2 cells in a physiological situation like fasting and that this induction was not mediated by PPARα.

This study provides significant mechanistic advance for the regulation of *Fsp27/CIDEC* in adaptation to fasting response in HepG2 cells, since we demonstrated that *Fsp27/CIDEC* is a CREB target gene (positively regulated by the PKA-CREB-CRTC2 pathway) that could be up-regulated when FAO is impaired and that the fluctuations in *SIRT1* activity, in response to nutrient availability, mediate this mechanism.

In the second part of this study, we showed that *Pgc-1α* and *Foxa2* expression are increased in livers of mice subjected to prolonged fasting and previously treated with a leucin deprived diet. Further we show that under our experimental conditions the activation of the hepatic transcriptional program of gluconeogenesis during adaptation to limit food supply (fasting and amino acid deprivation) appears not to be regulated by the recruitment of CREB by Foxa2 for the *Pgc-1*α or the *Pepck* promoters.

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8. ANNEX

8.1. Vectors

8.1.2. Cloning vectors

pGEM®-T Vector

The pGEM®-T Vector Systems are convenient systems for the cloning of PCR products.
T-overhangs for Easy PCR Cloning: This vector is a linearized vector with a single 3´-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases.

Blue/White Selection of Recombinantes: T7 and SP6 RNA polymerase promoters flank a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates (AIX plates: 100mg/L ampicilin, 8mg/L IPTG, and 40mg/L XGal).

Choice of Restriction Sites for Release of Insert: The pGEM®-T vector contains numerous restriction sites within the multiple cloning region.

8.1.3. Promoter vectors

The Reporter Vectors provide a basis for the quantitative analysis of factors that potentially regulate mammalian gene expression. The backbone of the Reporter Vectors is designed for increased expression, and contains a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells.

pGL3-Basic Vector

The **pGL3-Basic Vector** (Promega, Ref. E1751) lacks eukaryotic promoter and enhancer sequences, allowing maximum flexibility in cloning putative regulatory sequences. Expression of luciferase activity in cells transfected with this plasmid depends on insertion and proper orientation of a functional promoter upstream from *luc*+. Potential enhancer elements can also be inserted upstream of the promoter or in the BamHI or SalI sites downstream of the *luc*+ gene.

pRL-CMV Vector

The pRL Reporter Vectors contain a cDNA (*Rluc*) encoding *Renilla* luciferase, which was originally cloned from the marine organism *Renilla reniformis*. These vectors intended for use as an internal control reporter vector and may be used in combination with any experimental reporter vector to co-transfect mammalian cells. The **pRL-CMV Vector** (Promega, Ref.E2261) was used in the transfection assays of this study. This vector contains the CMV enhancer and immediate/early promoter elements to provide high-level expression of *Renilla* luciferase in co-transfected mammalian cells.

8.1.4. Expression vectors

pcDNA3 – Eukaryotic expression empty vector.

pcDNA3-CREB - The pcDNA3-CREB expression plasmid was subcloned from pSVCREB in pcDNA3 (Invitrogen) with *HidIII*/ *XbaI* restriction sites.

pcDNA3-KCREB – The pcDNA3-KCREB expression plasmid was subcloned from pSVKCREB in pcDNA3 with *HidIII*/ *XbaI* restriction sites.

pcDNA3-FOXA2 – The pcDNA3-FOXA2 expression plasmid was subcloned from pSV-FOXA2 in pcDNA3 with *HidIII*/ *BamHI* restriction sites.

8.2. Primer Design (5’ - 3’)

8.2.1. Cloning

Mouse PGC1- α promoter constructs

Wild type (-2010;-51)

Forward (DH1384)

5’ - ***ACGCGT***TGATCCCAGGGTTGTCTCTC - 3’ **(-2010;-1990)**

Reverse (DH1385)

5’ - ***CTCGAG***CACACAGCACACACTCATGC - 3’ **(-71;-51)**

Mouse Fsp27 promoter constructs

Wild type (-2054;+1)

Primer Forward (DH1303)

5’ – TTA***ACGCGT***CTGCAACTCATTCTGTAGCCC - 3’ **(-2054;-2075)**

Primer Reverse (DH1304)

5’ – TTA***CTCGAG***GGCAATACCGCGTGGCCAG - 3’ **(-27;+1)**

8.2.2. Site Directed Mutagenesis

Mutagenic primers introduce specific experimental mutations. The mutagenic oligonucleotide primers must be designed individually according to the desired mutation. The following considerations should be made for designing mutagenic site and selection primers:

1. Both the mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.

2. Primers should be between 25 and 45 bases in length, and the melting temperature (*T*m) of the primers should be greater than or equal to 78°C. The following formula is commonly used for estimating the *T*m of primers:

$$T_m = 81.5 + 0.41(\%GC) - 675 / N - \%\ \text{mismatch}$$

where *N* is the primer length in base pairs.

3. The desired mutation (deletion or insertion) should be in the middle of the primer with ~10–15 bases of correct sequence on both sides.

4. The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

5. Primers need not be 5´ phosphorylated but must be purified either by fast polynucleotide liquid chromatography (FPLC) or by polyacrylamide gel electrophoresis (PAGE). Failure to purify the primers results in a significant decrease in mutation efficiency.

6. It is important to keep primer concentration in excess.

CIDEC CREB site (mut1)

Wild type

5'- GCTCTGAGCCACCA TACC**TGACTTCA**A CATGAAC GTTACTCCTGAC - 3'

Primer Forward (DH1392)

5' - GCTCTGAGCCACCA TACC**TGAgTatc**A CATGAAC GTTACTCCTGAC - 3' **(-384;-430)**

Primer Reverse (DH1393)

5' - CATTAGTAAC GTTCATG T**gatAcTCA**GGTA TGGTGGCTCAGAGC - 3' **(-384;-430)**

CIDEC CREB half site (mut2)

Wild type

5' – GGTGATGTTTGTG**CGTCA**GCCTCTGGAGGCTCCGGGTGAGAGAGAGGGG - 3'

Primer Forward (DH1394) (-1799;-1848)

5' – GGTGATGTTTGTG**atcgc**GCCTCTGGAGGCTCCGGGTGAGAGAGAGGGG - 3'

Primer Reverse (DH1395) (-1799;-1848)

5' - CCCCTCTCTCTCACCCGGAGCCTCCAGAGGC**gcgat**CACAAACATCACC - 3'