UNIVERSITAT DE BARCELONA

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Additionally, four extra scientific papers have been published during the development of the doctoral thesis and are attached in the annex section. These were obtained as a result of participating in other projects that do not correspond to the scope of the results presented in this thesis:

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Abbreviations

ACC	Acetyl-CoA carboxylase
ACOX	Acyl-coenzyme A oxidase
ADP	Adenosine diphosphate
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
Akt2	Serine/threonine protein kinase 2
ALK	Activin receptor-like kinases
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AMPKK	AMP-activated protein kinase kinase
AP	Area postrema
AP1	Activator protein 1
APRT	Adenine phosphoribosyltransferase
ARTN	Artemin
AS160	Akt substrate of 160 kDa
ATGL	Adipose triglyceride lipase
ATF3	Activating transcription factor 3
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
Bip/GRP78	Binding immunoglobulin protein/78 kDa glucose-regulated protein
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CAMKK	Calcium/calmodulin-dependent protein kinase kinase
cAMP	Cyclic-AMP
CBP	Cyclic AMP response element binding protein
CBS	Cystathionine beta-synthase
CD36	Cluster of differentiation 36
CHOP	C/EBP homologous protein
COX	Cyclooxygenase

CPT1	Carnitine palmitoyl transferase 1
CRE	cAMP response element
CREB	cAMP response element binding protein
CREBH	cAMP-response element-binding protein H
CRIF1	CD6-interacting factor-1
CRM1	Chromosome region maintenance 1
CRP	C-reactive protein
CRTC2	Regulated transcription coactivator 2
DAG	Diacylglycerol
DBD	DNA binding domain
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DNL	*De novo* lipogenesis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGR1	Early growth response 1
eIF2α	Eukaryotic initiation factor 2α
ELISA	Enzyme-Linked Immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
ERK1/2	Extracellular signal–regulated kinases 1/2
ERSE	Endoplasmic reticulum stress elements
FATP	Fatty acid transport proteins
FAS	Fatty acid synthase
FGF21	Fibroblast growth factor 1
FBS	Fetal bovine serum
Fbxo40	Muscle-specific ubiquitin
FOXO1	Forkhead box protein O1

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN2	General control non-derepressible kinase 2
GDFs	Growth and differentiation factors
GDF15	Growth differentiation factor 15
GDNF	Glial cell line-derived neurotrophic factor
GFRAL	GDNF family receptor alpha-like
GLP	Glucagon-like peptide
GLUT4	Glucose transporter 4
G6Pase	Gucose-6-phosphatase
GPD	Glycerophosphate dehydrogenase
GS	Glycogen synthase
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
GTT	Glucose tolerance test
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HFD	High fat diet
HNF4	Hepatocyte nuclear factor 4
HRI	eIF2α kinase heme-regulated inhibitor
HSL	Hormone-sensitive lipase
HSP	Heat-shock protein
HUVEC	Human umbilical vein endothelial cells
IDF	International Diabetes Federation
IFN	Interferon
IKKβ	IκB kinase β
IκBα	Inhibitor of κB α
IL	Interleukin
Insig1	Insulin induced gene 1
IRβ	β subunit of insulin receptor
IRE1	Inositol-requiring kinase 1
IRS	Insulin receptor substrates

JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
LBD	Ligand binding domain
LDL	Low-density lipoproteins
LKB1	Liver kinase B1
LPS	Lipopolysaccharide
MAD	Mothers against decapentaplegic
MAPK	Mitogen-activated protein kinase
MATE1	Multidrug and toxin extrusion 1
MCAD	Medium-chain acyl-coenzyme A dehydrogenase
MCP-1	Monocyte Chemoattractant Protein 1
MDMX	Murine double minute X
MEK	MAPK-ERK kinase
MIC-1	Macrophage inhibitory cytokine 1
MKO	Skeletal muscle-specific *Crif1*-knockout model
MMLV	Moloney murine leukemia virus
mRNA	Messenger ribonucleic acid
mTORC1	Mammalian target of rapamycin complex 1
NAG-1	NSAID-activated gene 1
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NPC	Nuclear pore complex
NRTN	Neurturin
NSAIDs	Non-steroidal anti-inflammatory drugs
NTS	Nucleus *tractus solitarius*
OCT (*1*)	Optimal cutting temperature
OCT (*2*)	Organic cation transporter
OxPhos	Oxidative phosphorylation

OvNa	Sodium orthovanadate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer solution
PCSK	Proprotein convertase subtilisin/kexin
PDK4	Pyruvate dehydrogenase kinase 4
PEPCK	Phosphoenolpyruvate carboxykinase
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1α
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKB/Akt	Protein kinase B/Akt
PKC	Protein kinase C
PKR	Protein kinase R
PLAB	Placental bone morphogenetic protein
PLC	Phospholipase C
PMAT	plasma monoamine transporter
PMSF	Phenylmethylsulphonyl fluoride
PSPN	Persephin
PTGFB	Placental transforming growth factor beta
PTP1B	Protein tyrosine phosphatase 1B
PPARs	Peroxisome proliferator-activated receptors
PP2A	Phosphatase protein 2C
PPRE	PPAR response element
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
RET	Rearranged during transfection
rGDF15	Recombinant GDF15
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RXR	Retinoid X receptor
SCAP	Cleavage-activating protein
SCD1	Stearoyl-CoA desaturase 1
SDS	Sodium dodecyl sulphate
SGLT2	Sodium-glucose co-transporter 2
SH2	Src-homology-2
siRNA	Small interference RNA
SIRT1	Sirtuin 1
Smad	Referred to the homologies of *Caenorhabditis elegans* SMA ("small" worm phenotype)
SNP	Single nucleotide polymorphism
SOCS	Suppressors of cytokine signalling
Sp1	Specificity protein 1
SREBP1	Sterol regulatory element-binding protein
STAT	Signal transducer and activator of transcription
sXBP1	Spliced x-box binding protein 1
T2D	Type 2 diabetes
TBC1D1	2/Bub2/Cdc16 (TBC1) Tre-Domain Family Member 1
TBP	TATA-binding protein
TBR	Transforming growth factor β receptor
TBS	Tris-buffered saline solution
TGF-β	Transforming growth factor β
Th2	Type 2 T helper cell
TLR	Toll-like receptors
TNFα	Tumor necrosis factor α
TRAF2	Tumour necrosis factor receptor-associated factor 2
TRB3	Tribbles 3
TRIP-Br2	Transcriptional regulator interacting with the PHD-bromodomain
TZD	Thiazolidinediones

UPR	Unfolding protein response
UTR	Untranslated region
VLDL	Very low-density lipoproteins
VLDLr	VLDL receptor
WB	Western blot
WT	Wild type

SUMMARY

Type 2 diabetes (T2D) is a multifactorial disease that comprises metabolic defects in multiple organs. Several studies have shown that the presence of a chronic low-state inflammatory process induces the development of insulin resistance, a pathology in which the organism fails to respond to the hormone insulin. This condition precedes and predicts the development of T2D. Likewise, the presence of endoplasmic reticulum (ER) stress induced by a lipid overload in obesity states contributes to the development of insulin resistance through multiple processes, including the activation of inflammatory pathways.

At present, T2D has become a chronic disease that affects more than 400 million people worldwide, reaching epidemiological rates. In addition to the metabolic defects, T2D progression can lead to further complications, including blindness, cardiovascular disease, kidney failure, or limb amputation. Despite its growing incidence, the current available drugs for the treatment of T2D show a limited efficacy and significant side effects that are not fully controlled. For that reason, there is an urgent need to discover new therapeutic targets that can control the complexity of the disease. In this regard, PPARβ/δ agonists have been proven effective as a therapy against insulin resistance and T2D, by improving lipid-induced ER stress and inflammation, as well as glucose and lipid homeostasis. However, PPARβ/δ agonists are not currently available for clinical therapy. On the other hand, metformin has been the most prescribed oral drug for T2D in the past years. Nevertheless, the molecular mechanisms by which this drug exerts its antidiabetic actions are not fully understood. Interestingly, many of the antidiabetic effects of PPARβ/δ activation and metformin on lipid metabolism, inflammation or insulin signalling rely on AMPK activation, and present similarities with the actions of growth differentiation factor 15 (GDF15), a stress response cytokine that regulates energy metabolism through different mechanisms.

In the present thesis, we show that pharmacological PPARβ/δ activation increases GDF15 levels, which contribute to the antidiabetic effects of PPARβ/δ on glucose intolerance, fatty acid oxidation, ER stress, inflammation and insulin signalling. Additionally, we report that the AMPK-p53 pathway is involved in the PPARβ/δ-mediated increase in GDF15, which in turn is necessary to maintain the

activation of AMPK. Similarly, metformin treatment increases GDF15 levels through an AMPK-ATF3 pathway. Interestingly, we report that GDF15 mediates the AMPK-mediated antidiabetic effects of metformin on fatty acid oxidation, inflammation and insulin signalling independently of changes in food intake or body weight. Importantly, one of the most remarkable findings is that GDF15 activates AMPK in skeletal muscle independently of central nervous system or the glial cell line-derived neurotrophic factor (GDNF) family receptor α-like (GFRAL), the neuronal receptor for GDF15.

Overall, the results of the present doctoral thesis shed light on the mechanism of action of two well-known antidiabetic agents and indicate that GDF15 may become a new therapeutic target for the potential treatment of metabolic disorders.

RESUMEN

La diabetes tipo 2 es una enfermedad multifactorial que engloba defectos metabólicos en múltiples órganos. Diversos estudios ponen de manifiesto que la presencia de un proceso inflamatorio crónico de baja intensidad induce el desarrollo de la resistencia a insulina, una patología metabólica en la cual el organismo no responde correctamente a la hormona insulina. Esta condición precede y predice el desarrollo de la diabetes tipo 2. Asimismo, la presencia de estrés en el retículo endoplásmico (RE) inducida por una sobrecarga lipídica en estados de obesidad contribuye al desarrollo de la resistencia a insulina a través de múltiples procesos, incluyendo la activación de vías inflamatorias.

En la actualidad, la diabetes tipo 2 se ha convertido en una enfermedad crónica que afecta a más de 400 millones de personas en todo el mundo, alcanzando tasas epidemiológicas. Además de los defectos metabólicos inherentes a la enfermedad, la progresión de ésta puede desembocar en futuras complicaciones, incluyendo ceguera, enfermedades cardiovasculares, fallo renal, o amputación de extremidades. A pesar del crecimiento de su incidencia, los fármacos actualmente disponibles para el tratamiento de la diabetes tipo 2 muestran una eficacia limitada y efectos secundarios significativos que no están completamente controlados. Por esta razón, existe una urgente necesidad de definir nuevas dianas terapéuticas que permitan controlar la complejidad de esta enfermedad. En este sentido, los agonistas de PPARβ/δ han demostrado ser una terapia eficaz contra la resistencia a insulina y la diabetes tipo 2, mejorando la inflamación y el estrés del RE inducido por lípidos, así como la homeostasis glucídica y lipídica. Sin embargo, los agonistas de PPARβ/δ no están actualmente disponibles para la terapia clínica. Por otro lado, la metformina ha sido el fármaco oral más prescrito contra la diabetes tipo 2 en los últimos años. A pesar de ello, los mecanismos moleculares por los que este fármaco ejerce sus funciones antidiabéticas no están completamente descritas. Resulta interesante que varios de los efectos antidiabéticos de la activación de PPARβ/δ y de la metformina sobre el metabolismo lipídico, la inflamación, o la señalización de la insulina dependen de la activación de AMPK, y presentan similitudes con las acciones de GDF15 (*growth differentiation factor* 15), una citoquina de respuesta a estrés que regula el metabolismo energético a través de diferentes mecanismos.

En la presente tesis, demostramos que la activación farmacológica de PPARβ/δ aumenta los niveles de GDF15, y que este aumento contribuye a los efectos antidiabéticos de PPARβ/δ sobre la intolerancia a la glucosa, la oxidación de ácidos grasos, el estrés del RE, la inflamación y la señalización de la insulina. Además, reportamos que la vía AMPK-p53 está involucrada en el aumento de GDF15 mediado por PPARβ/δ, y que este aumento de GDF15 es necesario para mantener la activación de AMPK. De modo similar, el tratamiento con metformina aumenta los niveles de GDF15 a través de la vía AMPK-ATF3. Asimismo, este aumento de GDF15 media los efectos antidiabéticos de la metformina regulados por AMPK sobre la oxidación de ácidos grasos, la inflamación y la señalización de la insulina en ausencia de cambios en la ingesta de comida o en el peso corporal. Finalmente, uno de los descubrimientos más relevantes es que GDF15 activa AMPK en el músculo esquelético independientemente del receptor neuronal de GDF15 GFRAL (GDNF *family receptor α-like*) y sin mediación del sistema nervioso central.

En resumen, los resultados obtenidos en la presente tesis doctoral permiten definir en mayor profundidad el mecanismo de acción de dos agentes antidiabéticos ampliamente conocidos e indican que GDF15 podría constituir una nueva diana terapéutica para el potencial tratamiento de distintas enfermedades metabólicas.

I. INTRODUCTION

I.1 Type 2 Diabetes and Insulin Resistance

Type 2 diabetes (T2D) is a chronic disease that has reached epidemiological rates and, at present, affects more than 400 million people all over the world. Besides, according to the International Diabetes Federation (IDF), 1 in 2 adults with diabetes are undiagnosed and its treatment cost represents 10% of global health expenditure ($760 billion). T2D represents around the 90-95% of the total diabetes cases and the number of patients is expected to reach over 600 million by 2040. This pathology can result in different complications, including blindness, cardiovascular disease, kidney failure, or limb amputation. In addition, these long-term complications substantially impact quality of life of patients and could represent a worse outcome for young people, which are experimenting an increasing incidence of this disease (The, 2018).

The major risk factor for the development of T2D, independently of age, is the presence of obesity. A wide range of factors have been implicated in the development of T2D, including unhealthy nutrition, sedentary lifestyle, genetics, or hormonal imbalance. Indeed, these factors contribute to a marked increase in obesity rates.

The molecular mechanisms underlying the onset of diabetes have not been completely delineated. However, a broad amount of studies have proven that the presence of a chronic low-state inflammatory process induces the development of insulin resistance, which precedes and predicts the development of T2D (Grundy, 2008). Likewise, the presence of endoplasmic reticulum (ER) stress induced by a lipid overload contributes to the development of insulin resistance through multiple processes, including the activation of inflammatory pathways.

In an insulin-resistant state, the body fails to respond to insulin, leading to an attenuated insulin signalling and the increase of blood glucose levels. Pancreatic islets usually respond by increasing insulin secretion to maintain normoglycemia, a process known as β cell compensation. However, if this situation persists, it can cause the exhaustion of the pancreas and the inability to cope with the insulin production required to achieve a physiological response, which can cause higher blood glucose levels or hyperglycaemia.

According to the IDF, the most commonly prescribed oral medication for the treatment of T2D is metformin, which increases insulin sensitivity through several mechanisms, some of them not yet well-known. However, despite the growing incidence of the disease, the current available drugs for the treatment of T2D show a limited efficacy and significant side effects that are not fully controlled (Ghasemi and Norouzirad, 2019). Because of this, there is an urgent need for developing new drugs to prevent and treat T2D.

I.1.1 Insulin signalling pathway

Insulin is an anabolic hormone produced and secreted by the pancreas β-cells. It acts through the insulin receptor located in the membrane of target cells and regulates glucose homeostasis and lipid metabolism, among other metabolic processes. Insulin binding to its receptor elicits a wide range of signalling cascades (**Figure 1**), through insulin receptor substrates (IRS) and the canonical phosphoinositide 3-kinase (PI3K) and extracellular signal–regulated kinases 1/2 (ERK) cascades. This intracellular signalling generates pleiotropic actions of insulin and promotes glucose uptake, glycogen synthesis, cell proliferation and growth, lipogenesis, or protein synthesis, whereas inhibits gluconeogenesis, lipolysis or apoptosis (De Meyts, 2000).

The insulin receptor is a transmembrane protein formed by α and β subunits. This receptor belongs to the subfamily of receptor tyrosine kinases and forms a homodimer (Taniguchi et al., 2006). Insulin binds to the α subunit of the homodimer in the extracellular part of the receptor. In response, the β subunits are autophosphorylated and induce a conformational change in the receptor, which allows the phosphorylation of target proteins in order to promote the intracellular signalling of insulin (Kahn and White, 1988). Next, IRS are phosphorylated on up to 20 potential tyrosine-phosphorylation residues and bind to intracellular molecules that contain Src-homology-2 (SH2) domains (Shaw, 2011). This allows further cascade signalling. Importantly, phosphorylation of IRS on serine residues inhibits the downstream signalling of insulin and is a key regulatory mechanism that is involved in the development of insulin resistance. For example, IRS activates a

kinase cascade that results in the activation by phosphorylation of the mitogen-activated protein kinases (MAPK)-ERK kinase (MEK1/2) - ERK1/2 pathway, which promotes cell differentiation and growth. However, ERK1/2 is also involved in a negative feedback loop with insulin action by phosphorylating IRS on serine residues and causing its inhibition (Taniguchi et al., 2006). These mechanisms will be further explored in the next sections.

PI3K is an enzyme that plays a pivotal role in insulin signalling. In fact, inhibition of this enzyme blocks some of the most important actions of insulin (Kurtz and Ray-Coquard, 2012). The SH2 domains of the regulatory subunits of PI3K bind to the IRS and subsequently PI3K moves closer to the cell membrane, where it can catalyse the conversion of phosphatidylinositol 2 phosphate (PIP2) to PIP3 (Taniguchi et al., 2006). Next, PIP3 binds to the 3-phosphoinositide-dependent protein kinase 1 (PDK1) and promotes the phosphorylation of its substrates, such as the protein kinase B (PKB), more frequently referred to as Akt, or protein kinase C (PKC) isoforms (Mora et al., 2004).

Akt is involved in several biological processes, including glucose homeostasis, apoptosis, cell proliferation or cell migration (Taniguchi et al., 2006). Akt can be activated by several mechanisms (Gao et al., 2014). Once activated, Akt mediates most of the metabolic actions of insulin by phosphorylating several substrates (**Figure 1**). Glycogen synthase kinase 3 (GSK3) phosphorylation by Akt decreases its inhibitory activity towards the enzyme glycogen synthase (GS) (Ali et al., 2001). Therefore, Akt activity promotes glycogen synthesis in different peripheral tissues. Importantly, Akt also phosphorylates the Akt substrate of 160 kDa protein (AS160). This impairs the inhibitory activity of this protein on the Rab-GTPase-activating protein, which is involved in the vesicular formation and translocation of the glucose transporter type 4 (GLUT4) to the plasma membrane (Kane et al., 2002). Hence, the inhibition of AS160 by Akt promotes the GLUT4-mediated glucose uptake in skeletal muscle and adipose tissue (Mîinea et al., 2005).

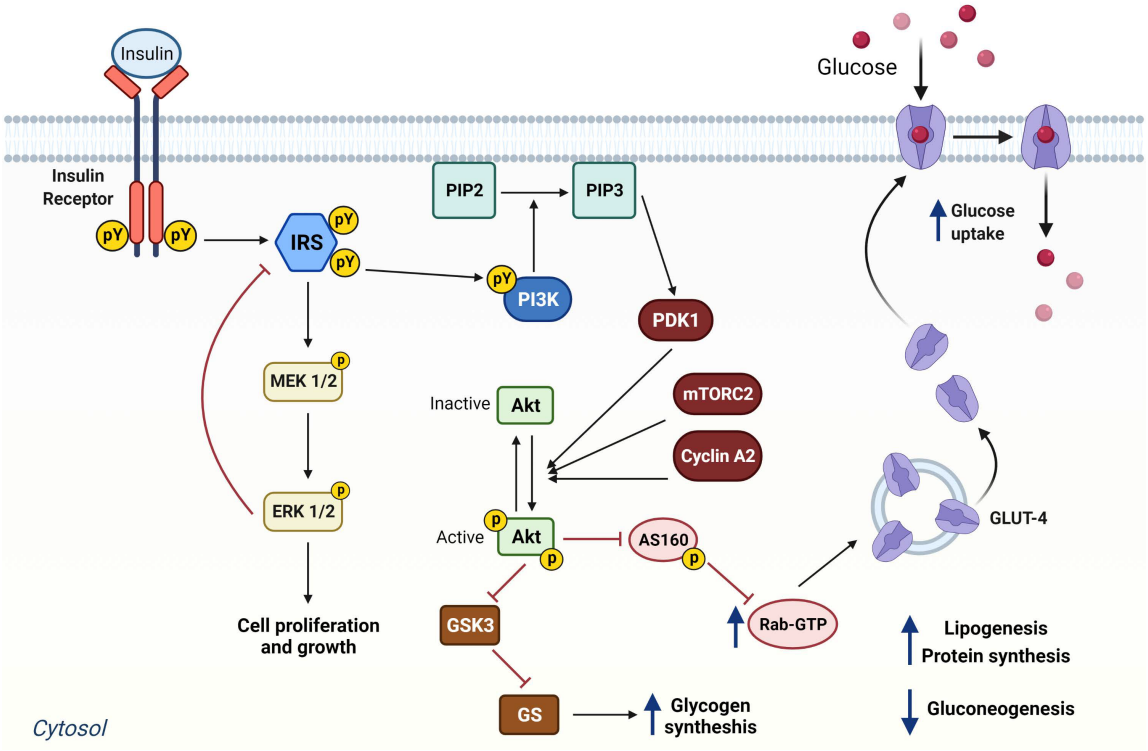


Figure 1. Insulin signalling pathway. Insulin binding to the transmembrane insulin receptor elicits the phosphorylation of the β subunits of the receptor and its substrates on tyrosine (pY) residues. Downstream signalling regulates the activation of the MEK/ERK pathway that promotes cell proliferation and growth, and the activation of Akt, which promotes glycogen synthesis and glucose uptake. In addition, insulin signalling promotes lipid and protein biosynthesis and inhibits gluconeogenesis. Adapted from (Jung and Choi, 2014).

I.1.2 Insulin Resistance

In normal homeostatic conditions, insulin released from pancreas in response to an increase in blood glucose levels promotes glucose uptake in cells to cope with the metabolic requirements of each tissue or organ. However, as a result of different cellular stresses, the organism develops a pathological condition known as insulin resistance, in which normal plasma insulin levels cannot induce a glucose-lowering response in target tissues. This attenuates the effects of insulin leading to a decrease in glycogen synthesis; gluconeogenesis is not inhibited and lipogenesis is increased (Kahn et al., 2006; Reaven, 1997), causing an energetic imbalance that force a higher production of insulin. As a result, β-cells in the pancreas secrete

higher amounts of insulin to compensate, causing hyperinsulinemia (Reaven, 2012) and an increase in fasting insulin levels (Czech, 2017).

Many authors point out that insulin resistance is secondary to obesity, as a response to the increase of triglycerides and lipid intermediates and its infiltration in non-adipose tissues (Coughlan et al., 2013; de Ferranti and Mozaffarian, 2008). However, insulin resistance may also appear in non-obese individuals (DeFronzo and Tripathy, 2009), which indicates that the development of this alteration can be multifactorial and its development may not be attributable to single events.

The mechanisms involved in the development of insulin resistance are not fully understood. However, it is clear that the main peripheric tissues and organs differentially contribute to the development of insulin resistance in unfavourable situations, and that molecular events involving inflammation, lipid accumulation and metabolism or ER stress in the different organs and tissues play a major role in the inhibition of insulin signalling and the development of insulin resistance.

I.1.2.1 Insulin resistance in adipose tissue

Obesity is highly related to insulin resistance. In fact, nearly half of obese patients show a deficiency in insulin signalling (Chen et al., 2013), primarily due to abnormally high levels of triglycerides and free fatty acids and its accumulation in liver and skeletal muscle, among other peripheral tissues (Boden and Shulman, 2002). This accumulation is a consequence of a dysfunctional adipose tissue that is unable to cope with the metabolic requirements to process all these lipids, causing an increase in the release of pro-inflammatory adipokines and free fatty acids to the bloodstream, which eventually infiltrate and accumulate into peripheral tissues.

In adulthood, the number of total adipocytes remains without significant variation, resulting in an hypertrophy of adipocytes as adipose tissue expands due to the accumulation of triglycerides (Gustafson et al., 2015a). These adipocytes show a proinflammatory state and a decreased insulin sensitivity, as immune cells contribute to local inflammation and the polarization of the M2 anti-inflammatory macrophages onto M1-like proinflammatory macrophages occurs (Castoldi et al., 2015). The production of anti-inflammatory cytokines by M2 macrophages such as

interleukin (IL)-10, IL-4 and IL-13 is significantly reduced, and this also contributes to an insulin-resistant state (Hong et al., 2009).

Normally, insulin action decreases the activity of hormone-sensitive lipase (HSL), an enzyme that promotes the release of stored fatty acids (Jaworski et al., 2007). However, in an insulin-resistant condition, visceral adipose tissue is unable to cope with the storage requirements of the lipids excess, resulting in the release of this overload and the ectopic accumulation of triglycerides and free fatty acids in the liver, skeletal muscle or pancreas (Spalding et al., 2017; Virtue and Vidal-Puig, 2010). This process is called lipotoxicity and results in a low-grade inflammatory state in peripheral tissues, decreasing insulin sensitivity (Biden et al., 2014).

Insulin sensitivity in peripheral tissues is regulated by circulating factors, including plasma lipids, hormones or cytokines. Adipose tissue is responsible for the regulation of the levels of circulating free fatty acids and glycerol or by storing them in form of triglycerides. Adipocytes are also responsible for the production of different hormones called adipokines, such as leptin and adiponectin, that can positively regulate insulin sensitivity in peripheral tissues (Yadav et al., 2013). Other adipokines, such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) or monocyte chemoattractant protein 1 (MCP-1) can contribute to insulin resistance.

The implication of adipose tissue in the development of insulin resistance makes obese patients more prone to lose insulin sensitivity (Arner, 2002; Coughlan et al., 2013; Eckardt et al., 2011). However, as previously mentioned, this pathology can also appear in non-obese individuals (DeFronzo and Tripathy, 2009).

M1 macrophage polarization, hypoxia due to impaired vascularization of adipocytes, and mitochondrial dysfunction cause adipose tissue dysfunction that leads to an increase of the inflammatory process and ER stress that attenuate insulin signalling. Consequently, glucose uptake and glycolysis are reduced, and fatty acid release is increased, as an effect of the reduction of triglyceride synthesis and the increase of the lipolysis rate, causing lipotoxicity and glucotoxicity in peripheral tissues (**Figure 2**).

I.1.2.2Insulin resistance in skeletal muscle

Skeletal muscle plays a primary role in whole-organism glucose homeostasis as it is responsible for around 80% glucose clearing from blood in response to insulin stimulation (Abdul-Ghani and DeFronzo, 2010). As a result, it has been observed that loss of insulin sensitivity in skeletal muscle is the major defect in the pathogenesis of T2D (Wu and Ballantyne, 2017a).

Under insulin resistance conditions, skeletal muscle is unable to uptake circulating glucose for its utilisation in glycolysis or its conversion into glycogen, causing an increase in blood glucose concentrations that surpass the physiological range. One of the main effectors involved in this metabolic process is GLUT4. GLUT4 is the transporter responsible for the internalization of glucose in different cell types, but more importantly in skeletal muscle cells in response to insulin. Due to the importance of this transporter in glucose metabolism, many authors have suggested that the main cause of insulin resistance in skeletal muscle is a loss in the functionality of GLUT4. Accordingly, the levels of this transporter negatively correlate with an insulin-resistant state accompanied by high levels of inflammatory markers (Leguisamo et al., 2012) and its disruption or impairment of its translocation generates insulin resistance in skeletal muscle (Mueckler, 2001; Xu et al., 2015).

Data have shown that accumulation of triglycerides in skeletal muscle cells correlate negatively with insulin sensitivity (Pan et al., 1997). In fact, there is growing evidence that accumulation of lipid intermediates, including ceramides, long-chain fatty acyl-CoAs, diacylglycerols (DAG) or saturated fatty acid products negatively impact on insulin sensitivity (Chavez and Summers, 2003; Straczkowski et al., 2007).

A strong correlation between chronic inflammation and insulin resistance in skeletal muscle has been also established. Since adipose tissue in insulin-resistant states releases a higher amount of lipids and proinflammatory cytokines, this is in part responsible for a loss of insulin sensitivity in skeletal muscle. Thus, T2D patients show infiltration of inflammatory cells into skeletal muscle, such as M1

macrophages (Wellen and Hotamisligil, 2005), as well as increased levels of TNF-α or IL-6, among other cytokines.

IL-6 has been reported to inhibit the transcriptional activity of IRS-1, GLUT4 and peroxisome proliferator-activated receptor γ (PPARγ) (Wei et al., 2008). In addition, IL-6 also promotes IRS-1 serine phosphorylation, resulting in an increase in its ubiquitination and its subsequent reduction. The increase in the production of TNF-α also induces apoptotic and inflammatory responses. This cytokine is increased in skeletal muscle in patients with insulin resistance or diabetes (Lappas et al., 2005) and plays a direct role in the reduction of insulin sensitivity.

The increase in glucose levels and the compromised ability of skeletal muscle to obtain energy from glucose in insulin resistance results in a switch in the metabolism towards fatty acid oxidation in this organ. Nevertheless, in insulin-resistant muscle cells, the rate of fatty acid oxidation is markedly lower than the rate of fatty acid uptake, causing an accumulation of lipid intermediates. Consequently, mitochondrial function and its oxidative capacity is compromised (Schrauwen-Hinderling et al., 2007). The main effects of these events are the reduction of glucose uptake, glycolysis and glycogen synthesis and the increase in lipid accumulation (**Figure 2**). Notably, the accumulation of lipid intermediates from circulating fatty acids in skeletal muscle can only come from other tissues, since skeletal muscle cells lack the molecular machinery needed to synthesize fatty acids from carbon precursors (Tumova et al., 2016).

I.1.2.3 Insulin resistance in the liver

The liver is one of the most crucial organs in the regulation of whole-body energy homeostasis. This organ coordinates the production and disposal of glucose by regulating the molecular pathways of gluconeogenesis and glycogenolysis in fasting conditions for other tissues, including skeletal muscle (Czech, 2017). In physiological conditions, insulin promotes the suppression of hepatic gluconeogenesis while stimulating the synthesis of cholesterol and triglycerides (Ferris and Kahn, 2016).

Insulin resistance impairs the hepatic switch from glucose production to glucose storing in form of glycogen, which causes an uncontrolled hepatic glucose production and a marked increase in hyperglycemia. As well, glucagon secretion from the pancreas is not correctly regulated and this event also contributes to higher glucagon levels in blood, which ultimately activates hepatic gluconeogenesis (Moore et al., 2003)

Insulin-resistant mice created by liver-specific knockout of the insulin receptor (LIRKO mice) show increased gluconeogenesis and impaired hepatic lipogenesis, resulting in severe glucose intolerance, hyperinsulinemia and increased lipid accumulation (Michael et al., 2000). However, in T2D, while hepatic gluconeogenesis is elevated, consistent with insulin resistance, the liver also exhibits a high rate of cholesterol and triglyceride synthesis, suggesting that these pathways are not affected by insulin desensitization (Brown and Goldstein, 2008; Miao et al., 2014). This apparently contradictory mechanism has been termed as the paradox of selective insulin resistance in the liver (Ferris and Kahn, 2016). This may be explained by a selective mechanism of insulin resistance that fails to suppress hepatic glucose production in exchange of sustaining hepatic lipogenesis and triglyceride accumulation, contributing to hepatic steatosis and hypertriglyceridemia (Otero et al., 2014).

Substrate availability plays a key role in this increase of triglyceride synthesis. In diabetes, increased lipolysis from insulin-resistant adipose tissue generates sufficient fatty acids to form additional triglycerides, while mice that are liver-specific resistant to insulin show adipose tissue that respond normally to insulin, suppressing lipolysis (Vatner et al., 2015)

As previously mentioned, due to an increased release of free fatty acid into the bloodstream by adipose tissue and a higher production of proinflammatory cytokines, extrahepatic factors also play an important role in the development of insulin resistance in hepatocytes.

Long-chain fatty acids are transported inside the hepatocytes through cluster of differentiation 36 (CD36) and it has been reported that elevated levels of the soluble form of this membrane protein are detected in diabetic patients (Yokoi and

Yanagita, 2016). Additionally, fatty acids derived from lipolysis are internalized in the liver through fatty acid transport proteins (FATPs), mainly the 2/5 isoforms (Falcon et al., 2010). Increased internalization of free fatty acids produces an increase in the levels of lipid intermediates, including DAG and ceramides. Consequently, hepatic DAG increases the translocation of PKC in the liver (Samuel et al., 2007), which in turn results in the inhibition of the activity of the kinase domain of the hepatic insulin receptor. This leads to a reduction in the insulin-stimulated phosphorylation of IRS-2, which is associated with a reduction with PI3K activity and phosphorylation of serine/threonine protein kinase 2 (Akt2) (Perry et al., 2014), resulting in increased gluconeogenesis and reduced glycogen synthesis. Hepatic insulin resistance leads to an increase in gluconeogenesis, an inhibition of glycogen synthesis and glycolysis and a general increase of glucose levels (**Figure 2**).

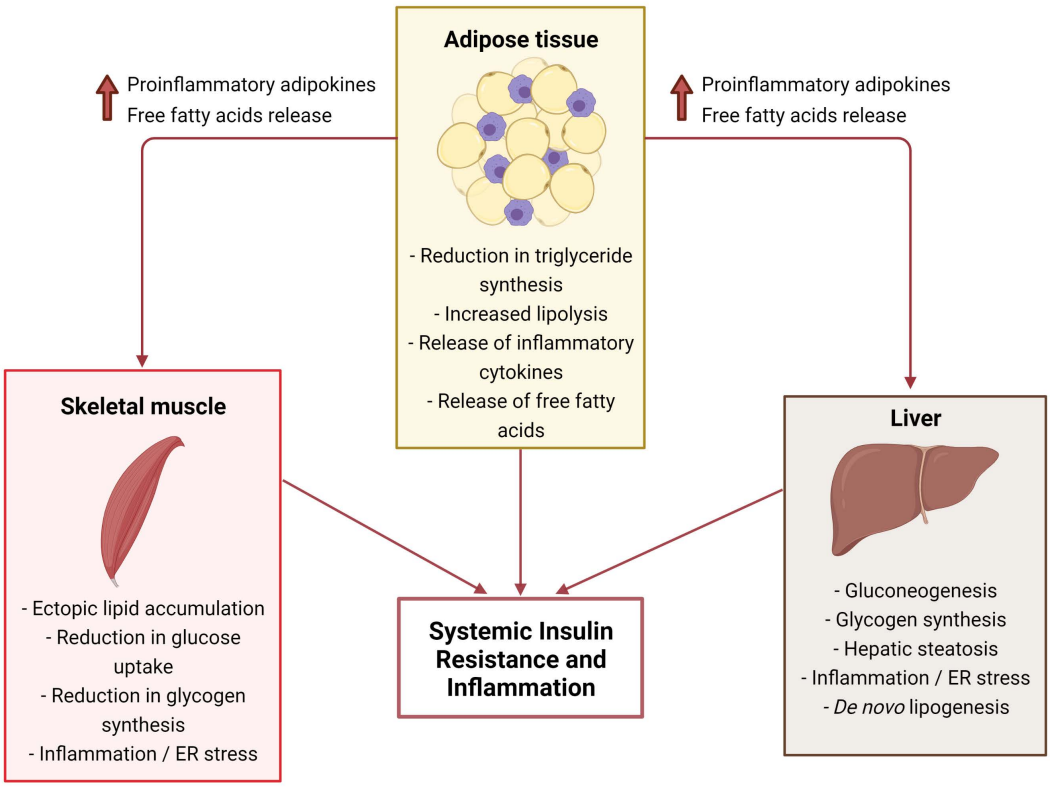


Figure 2. Insulin resistance in peripheral tissues. In an obesity and insulin resistance, adipose tissue is hypertrophic and present macrophage infiltration. The release of proinflammatory cytokines and free fatty acids from this adipose tissue cause ectopic lipid accumulation in skeletal muscle and liver. Skeletal muscle presents

a reduction in glucose uptake and glycogen synthesis as well as inflammation caused by cytokines and ER stress. Hepatic gluconeogenesis is increased, as well as hepatic steatosis and *de novo* lipogenesis. All these events contribute to the progression of insulin resistance. Adapted from (Clària et al., 2011).

I.1.3 Pathophysiology of insulin resistance

Probably, the development of insulin resistance in different tissues does not respond to a unique event or is induced by solely one effector. During the past years, it has been demonstrated that it is a multifactorial pathology involving a defective functioning of insulin and the molecular pathways initiated by its union to the insulin receptor. The molecular causes develop simultaneously in time, and it has been well documented that an inflammatory environment, the oxidative stress, ER stress and a dysfunctional processing of fatty acids induce a harmful feedback that contributes to the increase of negative factors that attenuates the insulin signalling, promoting insulin resistance.

I.1.3.1 The role of ER stress in insulin resistance

ER is an important cell organelle that regulates a variety of intracellular processes, being particularly important the synthesis, folding, posttranslational modifications and export of proteins. Attending to its physical and functional differences, it can be divided into rough ER and smooth ER. Smooth ER controls the synthesis of lipids and steroidal precursors from cholesterol, as well as calcium homeostasis and detoxification.

The rough ER contains the ribosomes, which are primarily responsible for the synthesis of proteins, and controls protein folding and maturation. It is located next to the nucleus, which allows a rapid signalling between both organelles. The ER lumen provides a specialized environment for protein modifications and maturation, however, different stress factors that alter this environment can contribute to the accumulation of misfolded or unfolded proteins caused by a dysfunctional ER, which causes the activation of a signalling cascade termed unfolding protein response (UPR). This is an adaptative response elicited to restore

ER function, by reducing protein synthesis and enhance the folding capacity of the ER through chaperones. If this adaptative response fails to restore the balance upon a long-term stress and misfolded proteins accumulate, the mechanism undergoes an apoptotic pathway to trigger cell death and eliminate dysfunctional cells (Ren et al., 2021; Xu et al., 2012).

I.1.3.1.1 Unfolded protein response (UPR)

The canonical UPR comprises three pathways that are mediated by three transmembrane proteins embedded in the ER membrane: the protein kinase R-like ER kinase (PERK), the inositol-requiring kinase 1 (IRE1) and the activating transcription factor 6 (ATF6) (**Figure 3**) (Ozcan et al., 2006). In homeostatic conditions, these three proteins remain in an inactive state though the binding of 78-kDa glucose-regulated protein/immunoglobulin heavy chain-binding protein (GRP78/BiP)(Back and Kaufman, 2012), an ER chaperone that is considered as a master regulator of ER stress because of its ability to control the activation of UPR and its antiapoptotic properties (Wang et al., 2009). Accumulation of misfolded proteins leads to the recruitment of BiP, releasing the previously mentioned proteins and leading to the activation of UPR (Hotamisligil, 2010). BiP is a well characterized marker of metabolic diseases (Girona et al., 2019) and its involvement in the generation of ER stress previous to insulin resistance has been further demonstrated. Heterozygous mice for this gene exhibit an adaptative response upon high-fat diet (HFD) that is protective against insulin resistance (Ye et al., 2010). Furthermore, exercise in obese subjects attenuates the ER stress response by reducing the expression and release of this chaperone (Khadir et al., 2016).

- ATF6

Upon activation, ATF6 is cleaved by regulated intramembrane proteolysis and translocated into the nucleus, promoting the transcription of genes regulated by ER stress elements (ERSE) and cAMP response elements (CRE) (Scheuner et al., 2005) to increase ER folding capacity. Additionally, ATF6 regulates the expression of the chaperone C/EBP homologous protein (CHOP), an apoptotic protein that can

regulate the expression of different proapoptotic genes, and transactivates genes encoding ER chaperones, ER-associated degradation (ERAD) components, and protein foldases (Flamment et al., 2012).

- PERK

Upon activation, PERK phosphorylates the Ser^{51} residue of the α subunit of eukaryotic translation initiation factor 2 (eIF2α), resulting in its activation. This leads to a decrease in the mRNA translation rate, therefore reducing the amount of newly synthesized proteins. p-eIF2α also interacts with the activating transcription factor 4 (ATF4), which promotes the transcription of genes involved in protein synthesis or apoptosis, including tribbles homolog 3 (TRB3), CHOP or BiP (Flamment et al., 2012). Moreover, phosphorylated eIF2α can result in the activation of the pro-inflammatory pathway of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Salvado et al., 2015). This branch of the UPR can also be regulated by different factors; eIF2α serine residue can be phosphorylated by three other kinases: protein kinase R (PKR), general control non-derepressible kinase 2 (GCN2), and heme-regulated inhibitor kinase (HRI) (Ron and Walter, 2007; Schröder and Kaufman, 2005). Since the eIF2α/ATF4 branch can be activated by alternative mechanisms independent of the UPR, this pathway has been termed additionally as the integrated stress response (IRS).

In hyperinsulinemia conditions, the activation of the phosphorylation of eIF2α and the increase of CHOP expression leads to the overproduction of hepatic glucose and the upregulation of the gluconeogenic genes Phosphoenolpyruvate carboxykinase (PEPCK) and Glucose 6-phosphatase (G6Pase) (Choudhury et al., 2011). Additionally, mice that had the *Chop* gene ablated and were induced to an obesity state exhibited a correct response to glucose and normal insulin sensitivity, suggesting that the increase in pro-inflammatory cytokines caused by the interconnexion between ER stress and inflammation is dependent on CHOP and mediate insulin resistance (Maris et al., 2012).

IRE1

ER stress leads to the autophosphorylation of IRE1, a kinase that can act as an endoribonuclease to carry out the alternative splicing of the X box-binding protein 1 (XBP1) mRNA. This leads to an unconventional form of XBP1 termed as spliced XBP1 (sXBP1), which can act as a transcription factor after translation (Flamment et al., 2012). sXBP1 can regulate in association with ATF6 the production of chaperones and proteins involved in ER biogenesis or the degradation and exportation of proteins, resulting in the activation of the most important UPR pathways aimed to restore the ER folding capacity upon stress (Hotamisligil, 2010)

IRE1 also activates c-jun N-terminal kinase (JNK) by associating with the tumour necrosis factor receptor-associated factor 2 (TRAF2) (Schröder and Kaufman, 2005) and activates pro-inflammatory and pro-apoptotic pathways. The activation of the JNK pathway is directly associated with the development of insulin resistance, since the IRE1α-dependent activation of JNK cause the subsequent phosphorylation of IRS-1 on serine residues (Kim et al., 2015a).

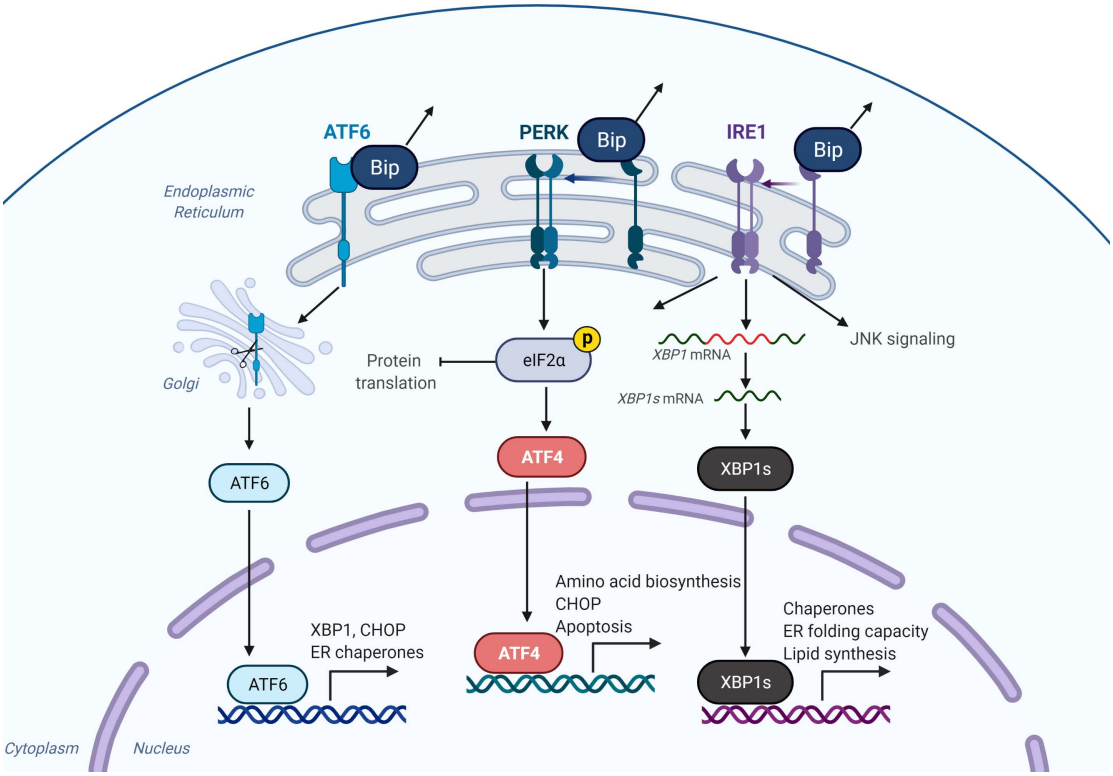


Figure 3. Canonical UPR pathways. The dissociation of the chaperone Bip from ATF6, PERK and IRE1 promotes the activation of the unfolded protein response. ATF6 is processed in the Golgi apparatus to promote the transcription of XBP1, CHOP and

ER chaperones. PERK induces the phosphorylation of eIF2α, blocking protein translation and increasing ATF4, which increases CHOP levels and apoptosis. The spliced isoform of XBP1 induced by IRE1 regulate the production of chaperones and the ER folding capacity. IRE1 additionally activates JNK signalling. Adapted from BioRender.com

When the UPR is sustained chronically, it can lead to the development of different diseases, including obesity, T2D, non-alcoholic fatty liver disease (NAFLD) or atherosclerosis, among others (Lee and Ozcan, 2014). ER stress was first described in mice in 2006, and it was reported as a risk factor for insulin resistance (Ozcan et al., 2006). The implication of ER stress in hepatocytes and myocytes will be the main points addressed, since the liver and skeletal muscle are the organs more extensively analysed in the present thesis.

I.1.3.1.2 ER Stress in skeletal muscle

Activation of ER stress response by the ER stressors tunicamycin or thapsigargin results in the phosphorylation of IRS proteins on serine residues via the activation of the IRE-1/JNK pathway, which causes insulin resistance in cultured myotubes (Hwang et al., 2012; Salvadó et al., 2013). In fact, ER stress is tightly involved with the mechanisms relating saturated fatty acid-induced inflammation in insulin resistance. For example, the phosphorylation of eIF2α inhibits protein translation and causes a decrease in the inhibitor of κB α (IκBα)/nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) ratio, leading to the upregulation of proinflammatory genes (Palomer et al., 2016). Genetic models of diabetes or feeding a HFD to rodents also elicit the ER stress response in skeletal muscle (Peng et al., 2011). Importantly, while the saturated fatty acid palmitate induces ER stress in myotubes, co-incubation with the unsaturated fatty acid oleate prevented this effect, improving insulin resistance (Salvadó et al., 2013). The AMP-activated protein kinase (AMPK) mediates this effect (Salvadó et al., 2013) and shows multiple prevention effects on inflammation and insulin resistance, as well as in attenuating

ER stress (Dong et al., 2010). The role of AMPK in the development and prevention of insulin resistance will be addressed in the next sections.

Further mechanisms also involve the activation of TRB3 or protein tyrosine phosphatase 1B (PTP1B) after ER stress induction. While ER stressors enhance the expression of TRB3 and cause insulin signalling attenuation and impaired glucose uptake in skeletal muscle, the genetic deficiency of this protein in mice reverts this detrimental effect (Koh et al., 2013). As well, PTP1B hydrolyses the insulin receptor β subunit and IRS-1 (Panzhinskiy et al., 2013). Moreover, the tunicamycin-induced phosphorylation of eIF2α and JNK was attenuated in a genetic model of mice that lacked the presence of PTP1B.

In general, ER stress is promoted in skeletal muscle as an adaptative response to particular stresses, including exercise or diet alteration. However, an uncontrolled and pathological ER stress that becomes chronic against long-term alterations may result in the formation of autophagosomes and the activation of pro-inflammatory pathways, which causes insulin resistance and even more destructive outcomes, such as apoptosis and necrosis, if maintained during a long period of time (Rayavarapu et al., 2012).

Additionally, the elevated levels of very-low density lipoproteins (VLDL) observed in diabetic states can contribute to the development of ER stress in skeletal muscle cells. More specifically, apolipoprotein CIII is the fraction responsible for the activation of the ER stress response through Toll-like receptor (TLR)2 and the inhibition of extracellular signal-regulated kinase (ERK)1/2 (Botteri et al., 2017).

I.1.3.1.3 ER Stress in the liver

The liver regulates the production of glucose in homeostatic conditions. However, ER stress induces hepatic insulin resistance and causes differential regulation of the transcription of gluconeogenic genes. The cAMP-response element-binding protein H (CREBH), which is the liver-specific homolog of ATF6, increases the expression of PEPCK and G6Pase, two key elements in gluconeogenesis, along with the proinflammatory marker C-reactive protein (CRP) (Lee et al., 2010a).

The intracellular accumulation of lipid intermediates because of increased lipogenesis may also contribute to ER stress-induced hepatic insulin resistance. For instance, DAG accumulation plays a pivotal role for ER stress-mediated insulin resistance and hepatic steatosis, which can be partially reversed by PPARα activation that leads to an increased fatty acid oxidation and a reduction in lipid accumulation (Chan et al., 2013). The PERK-eIF2α pathway is involved in this type of response, since hyperglycemia or exposure to palmitic acid promotes the activation of the sterol regulatory element binding protein 1 (SREBP-1), a lipogenic transcription factor regulated by eIF2α and sXBP1 (Li et al., 2014b)

SREBP-1c is induced in the liver of obese mice and activates the transcription of different lipogenic genes, including fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) (Kammoun et al., 2009). This transcription factor is also regulated by the mammalian target of rapamycin complex 1 (mTORC1), which promotes its expression, inducing hepatic insulin resistance and lipid accumulation (Li et al., 2014b).

The UPR can also reduce insulin sensitivity directly. Activation of IRS proteins can be blocked through the phosphorylation of serine residues, which can be mediated by the kinases JNK and inhibitor of κ kinase (IKK), recruited after IRE1 activation in UPR (Turban and Hajduch, 2011). PKR is also capable of phosphorylating IRS1 on serine residues, directly or indirectly through JNK (Flamment et al., 2012).

Additionally, fibroblast growth factor 21 (FGF21) is a metabolic hormone that regulates a wide variety of effects. It is activated via the IRE1-XBP1 and PERK pathways upon ER stress (Jiang et al., 2014). Interestingly, the administration of this hormone leads to a reduction in tunicamycin-induced ER stress and steatosis, and enhances glucose tolerance (Jiang et al., 2014). In fact, the administration of eIF2α kinase heme-regulated inhibitor (HRI) activators have been proposed as a promising strategy for the treatment of T2D and NAFLD, by promoting endogenous production of FGF21 and increasing its hepatic and circulating levels (Zarei et al., 2019b). Next, **Figure 4** shows the molecular interconnections between ER stress and the development of insulin resistance:

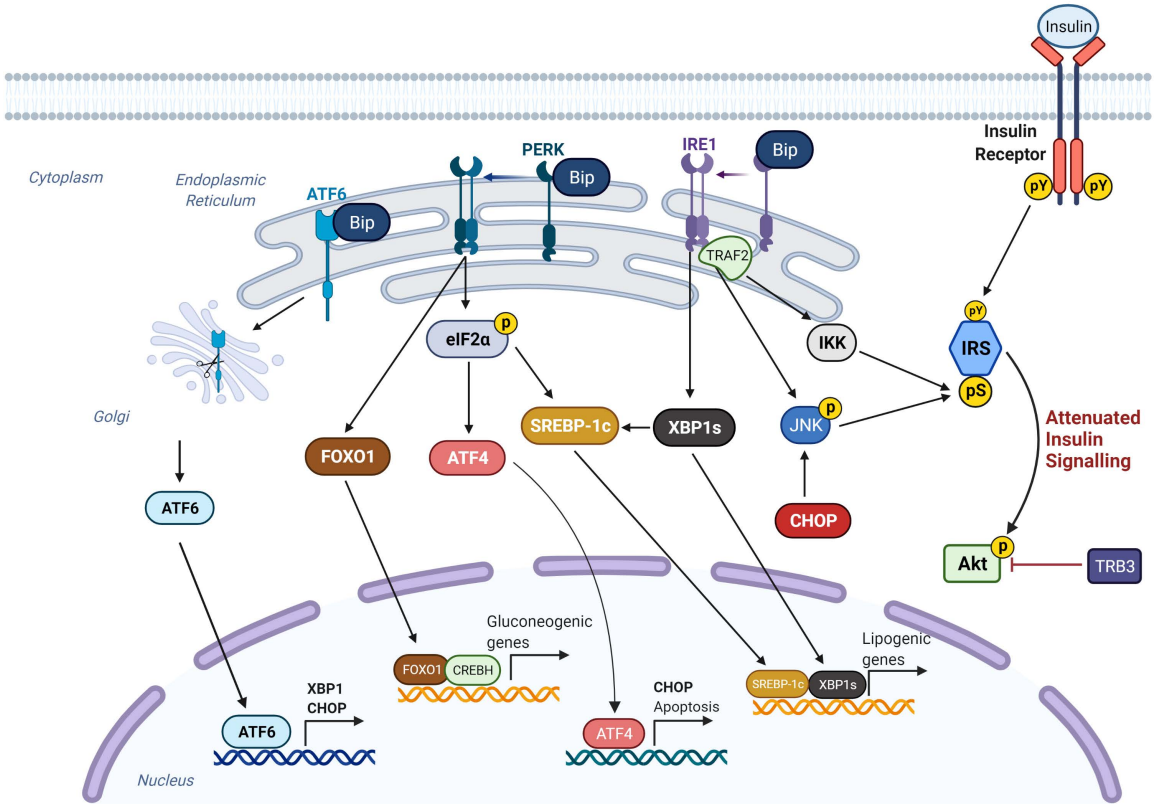


Figure 4. ER stress molecular pathways involved in insulin resistance. PERK activation leads to the increase of hepatic FOXO1 and the formation of FOXO1-CREBH complex, which upregulates gluconeogenic genes. At the same time, eIF2α is activated by PERK and increases SREBP-1c, which promotes the upregulation of lipogenic genes upon the interaction with XBP1s. IRE1 activates IKK and phospho-JNK, increasing the phosphorylation of IRS serine residues and attenuating the insulin signalling pathways. The increase in CHOP caused by different UPR pathways exacerbate these effects and altogether contribute to the development of insulin resistance in different tissues. Adapted from (Salvado et al., 2015).

I.1.3.2 The role of inflammation in insulin resistance

Although it has been recently described that insulin resistance may contribute to an inflammatory process in adipose tissue (Shimobayashi et al., 2018), a number of studies show the contribution of a chronical low-level inflammatory state to the development of insulin resistance. Additionally, there is a tight regulation involving inflammation and ER stress, and many of the pathways involved in these processes are interconnected. The development of these negative

processes is usually related with overnutrition and the consequent chronic accumulation of toxic intermediaries. For example, hyperglycemia can trigger the JNK pathway and high levels of glucose derived from muscle-specific inactivation of GLUT4 may induce oxidative stress and inflammation (Evans et al., 2005; Kim et al., 2001).

Different tissues can contribute to the development of chronic inflammation. For example, hepatic inflammatory pathways can be triggered in obesity as a result of steatosis (de Luca and Olefsky, 2008). A macrophage-like type of liver-resident cell called Kupffer cells can be activated in response to steatosis, which exacerbate inflammation by releasing cytokines. This induces insulin resistance in the liver, which causes a malfunction in hepatic inhibition of gluconeogenesis. If the steatosis and inflammation become chronic, it causes liver fibrosis and the progression to more severe pathologies, including NAFLD, non-alcoholic steatohepatitis (NASH) or even cirrhosis.

An increased accumulation of free fatty acids also takes place in skeletal muscle in overnutrition. Long-chain saturated fatty acids robustly induce inflammation in this organ, which subsequently activate NF-κB and JNK pathways (Wu and Ballantyne, 2017b). This leads to an overproduction of inflammatory myokines and makes skeletal muscle a secretory organ of inflammatory molecules, such as IL-6, IL-8, TNF-α or FGF21. However, although a chronic release of these cytokines may contribute to a systemic inflammatory state, some can positively regulate insulin sensitivity, as many of them are upregulated upon exercise (Pedersen and Febbraio, 2012).

On the other hand, adipose tissue represents a major secretory organ of inflammatory cytokines and chemokines. Although in homeostatic conditions this tissue constitutes a fat storage, elevated levels of fatty acids and triglyceride accumulation caused by obesity provokes a high release of lipids to the circulation (Lumeng and Saltiel, 2011), which causes ectopic fat deposition and insulin resistance in target tissues, like skeletal muscle or the liver. Adipose tissue is composed mainly by adipocytes, more specifically about 90%. However, it also contains millions of cells different from adipocytes that regulate the function of this tissue, including immune cells, endothelial cells or pre-adipocytes. Immune cells are

subjected to change from an anti-inflammatory state to a pro-inflammatory state, a process that is driven by different factors like obesity. Within the lymphocyte group of immune cells, macrophages are in charge of the phagocytosis of infected cells, attracted to the infection or injury site by an acute inflammation process. However, if this process becomes chronic, like in obesity, the switch from M2 anti-inflammatory macrophages to the M1 pro-inflammatory type and the secretion of adipokines like MCP-1 produces the infiltration of more monocytes into the adipose tissue and its differentiation into macrophages (Kosteli et al., 2010). Furthermore, these M1 type macrophages produce inflammatory mediators such as IL-6, IL-1β, TNF-α or resistin (Lumeng et al., 2007), which promote apoptosis and the inhibition of insulin signalling pathway and glucose uptake (Lu et al., 2020).

Next, many of the pathways reported to be involved in the development of insulin resistance caused by inflammation are described.

I.1.3.2.1 IKK/NF-κB pathway

NF-κB is a transcription factor formed by up to five subunits: p65/relA, relB, c-Rel, p50 and p52. The NF-κB complex is usually formed by homodimers or heterodimers, being the most common the heterodimer p50/p65. This factor is involved in the regulation of the inflammatory and the immune response after injury and in pathological situations (Rahman and McFadden, 2011; Sakurai et al., 2003). In a physiological state, NF-κB is normally bound to IκB proteins that prevent the nuclear translocation of NF-κB by masking the nuclear localization signal of this complex and maintaining it in an inactive state. After pathogenic stimuli, like obesity, the IKK complex becomes activated and induces the phosphorylation of IκB-α (Chen et al., 2015a), resulting in its ubiquitination and the liberation of NF-κB and its translocation into the nucleus, where it becomes transcriptionally active.

Once in the nucleus, NF-κB acts as a transcription factor enhancing the expression of pro-inflammatory cytokines, including TNF-α, IL-6, MCP-1 and IL-1β (Lee and Lee, 2014). In fact, TNF-α exerts a positive feedback regulatory mechanism towards NF-κB activation (Hayden and Ghosh, 2014). Although TNF-α can stimulate the activation of AMP-activated protein kinase (AMPK) pathways, it triggers insulin

resistance by the activation of JNK in visceral adipose tissue (Fernández-Veledo et al., 2009). This cytokine negatively regulates the functions of IRS-1, since it promotes its phosphorylation in serine residues and decreases tyrosine phosphorylation (Bouzakri and Zierath, 2007). In fact, mice genetically lacking TNF-α function are less prone to develop obesity-induced insulin resistance (Steinberg et al., 2006) and mice receiving inhibitors directed to this cytokine showed improved insulin sensitivity (Hotamisligil et al., 1993). NF-κB is a recurrent target for treating insulin resistance, since its inhibition or deletion is widely reported to improve insulin sensitivity (Yekollu et al., 2011). In adipocytes, IKKβ deficiency results in a reduction in the expression of TNF-α and IL-6 caused by free fatty acids (Jiao et al., 2011).

Conversely, the activation of IKK induces de phosphorylation of IRS-1 on serine residues, leading to a decrease in insulin sensitivity (Gao et al., 2002). MCP-1 overexpression by NF-κB activation in obesity is responsible for the recruitment and infiltration of macrophages in different tissues, which promotes insulin resistance (Kouyama et al., 2008), (Nguyen et al., 2007). Moreover, IL-6 activates the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling pathway, increasing the expression of suppressor of cytokine signalling 3 (SOCS3), which causes a reduction in the expression of GLUT4 and IRS-1 (Serrano-Marco et al., 2012a). The effects of NF-κB are not restricted to adipose tissue, since overexpression of NF-κB in the liver of healthy mice provoked liver and muscle insulin resistance (Cai et al., 2005). These studies confirm the major role of NF-κB in the development of insulin resistance.

I.1.3.2.2 MAPK/ERK pathway

MAP kinases, originally known as ERK constitute a family of serine-threonine kinases involved in cell cycle progression and proliferation. JNK and other MAP kinases are activated by TNF-α, IL-1β or by ER stress and saturated fatty acids (Wu and Ballantyne, 2020). Activation of JNK by proinflammatory cytokines drives insulin resistance by mediating the direct phosphorylation of IRS1 on serine 307, attenuating the insulin signalling (Lee et al., 2003). Conversely, the insulin-

stimulated phosphorylation of serine 307 was prominently inhibited in cells lacking JNK1 and JNK2, which promoted Akt phosphorylation and glucose uptake.

Additionally, the MAP kinase ERK1/2 plays an important role in the regulation of inflammation and insulin resistance. Inhibition of this protein suppresses the NF-κB activity in adipose tissue, leading to a reduction in proinflammatory cytokines (Rodríguez-Calvo et al., 2008). This inhibition of NF-κB is mediated by the phosphorylation and activation of IKK (Zhang et al., 2014a). Moreover, ERK1/2 is directly activated after insulin signalling stimulation, which leads to the phosphorylation of IRS-1 on serine residues, as a control mechanism (Taniguchi et al., 2006). Interestingly, ERK1/2 can also inhibit the phosphorylation of AMPK, and this inhibition is reciprocal, which constitutes a negative crosstalk regulation between these proteins (Du et al., 2008). In fact, the reduction of ER stress caused by PPARβ/δ activation requires AMPK activation and ERK1/2 inhibition in skeletal muscle to prevent insulin resistance (Salvadó et al., 2014), bringing out the importance of ERK1/2 in the regulation of AMPK and insulin resistance progression.

I.1.3.2.3 JAK/STAT pathway

The JAK/ STAT pathway is initiated by the JAK receptor, which is activated by different molecules, including interferon (IFN)γ or cytokines. The signalling pathway is elicited by binding of ligands to the JAK receptors in the membrane, which leads to the dimerization of this receptor and the activation of downstream kinases (Croker et al., 2008). JAK activation induces de phosphorylation of the tyrosine residues of the receptors and the phosphorylation of STAT proteins, which dimerize and translocate to the nucleus where they act as transcription factors (Chen et al., 2015a). Phosphorylation of STAT proteins is related with insulin resistance progression; in skeletal muscle, constitutive STAT3 phosphorylation on tyrosine 705 induces a decrease in insulin sensitivity (Mashili et al., 2013). Conversely, palmitate-induced insulin resistance was prevented by downregulation of STAT3. In fact, STAT3 activation by a HFD stimulates the expression of muscle-specific ubiquitin (Fbxo40), which drives the degradation of IRS-1 (Zhang et al.,

2020a). Additionally, muscle-specific ablation of STAT3 also resulted in an improvement of glucose tolerance, highlighting the importance of skeletal muscle in systemic glucose homeostasis.

One of the key target genes of this dimer are SOCS1 and SOCS3, whose transcription is robustly enhanced by STAT proteins (Lebrun and Van Obberghen, 2008). In T2D obese patients, the expression of these cytokines is increased (Rieusset et al., 2004) and can cause insulin resistance through direct inhibition of tyrosine phosphorylation of IRS proteins (Ueki et al., 2004). SOCS1 and SOCS3 can also block insulin signalling by the ubiquitin-mediated degradation of IRS-1 and IRS-2 (Rui et al., 2002) or even interrupt the interaction of the insulin receptors with IRS proteins by competing with its binding site, inhibiting its downstream signalling (Tanti et al., 2012). SOCS3 protein levels are increased in obesity and metabolic syndrome (Boucher et al., 2014), whereas muscle-specific deletion of SOCS3 attenuates insulin resistance in mice models of induced obesity (Jorgensen et al., 2013). On the contrary, SOCS3 overexpression promotes insulin resistance and overweight in mice (Lebrun et al., 2009).

I.1.3.2.4 PKCs

In overnutrition or obesity, an excess of free fatty acids accumulates and infiltrate into peripheral tissues, entering the cells by diffusion or through transporter proteins. When the oxidative capability of the cells is surpassed, fatty acids undergo different metabolic outcomes, such as the production of DAG and ceramide (Petersen and Shulman, 2017). When DAG levels increase in response to an overload of lipids, these lipid intermediates activate different isoforms of PKC. Rats fed a HFD show increased levels of PKC isoforms and a decrease in glucose uptake (Schmitz-Peiffer et al., 1997), and palmitate treatment causes insulin resistance by a mechanism involving PKC and NF-κB activation (Jové et al., 2006). PKC can impair insulin signalling by inducing the phosphorylation of the insulin receptor and IRS proteins on serine residues, which causes the reduction of GLUT4 translocation to the membrane(Li et al., 2004; Schmitz-Peiffer and Biden, 2008). In fact, specific ablation of a single PKC isoform can protect mice from fat-induced

insulin resistance (Kim et al., 2004). Moreover, it has been reported a negative regulation of AMPK through PKC activation, resulting in the impairment of AMPK-mediated inhibition of NF-κB and ERK1/2 and the enhancement of inflammatory pathways (Salminen et al., 2011).

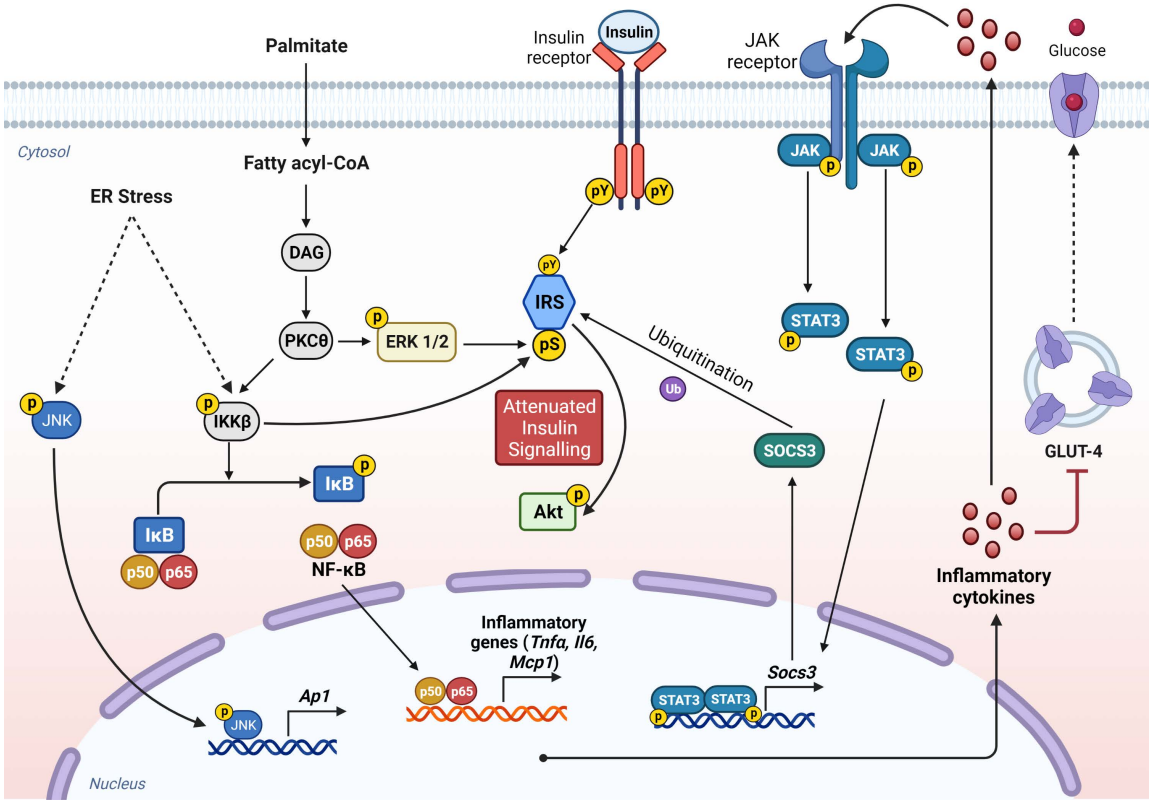


Figure 5. Inflammation pathways involved in the development of insulin resistance. Different processes, including ER stress or exposure to saturated fatty acids activate several kinases, including IKKβ, JNK, ERK1/2 that are responsible for the phosphorylation of IRS on serine residues. Moreover, IKKβ promotes the activation of NF-κB by releasing it from the inhibitor IκB via phosphorylation. Production of proinflammatory cytokines inhibit GLUT4, reducing the internalization of glucose, and activate cytokine receptors, promoting inflammatory pathways like JAK/STAT, which increases SOCS3 levels and induce serine phosphorylation and ubiquitination of IRS. These processes contribute to the development of insulin resistance. AP1: Activator protein 1.

I.2 AMPK

Lipid metabolism is essential to maintain a long-term energy disposal in the cell to cope with the requirements of the different biological processes that take place in the organism. Adenosine triphosphate (ATP) obtained from glucose represents a fast source of energy that can be used for immediate needs. However, lipid metabolism provides approximately 30-35% of total calories in a healthy diet and generates more energy than carbohydrates in terms of calories, although the energy obtention process from lipids is slower. Specifically, a complete β-oxidation process of a fatty acid generates a net value of 129 ATP, approximately 6-fold higher than the energy obtained from an equivalent amount of glycogen (Bebernitz and Schuster, 2002).

Maintaining a balanced metabolism requires the existence of an equilibrium between the energy provided by dietary intake and the energy expenditure of the organism. This balance is regulated primarily by an enzyme known as adenosine monophosphate (AMP)-activated protein kinase (AMPK).

AMPK is a cell energy sensor that responds to the AMP:ATP ratio inside cells and regulates the molecular mechanisms involved in the production and utilization of ATP, primarily by promoting mitochondrial fatty acid β-oxidation. This enzyme has a kinase activity that is activated in response to a decrease of the AMP:ATP ratio, which induces a metabolic switch towards catabolic pathways that generates ATP instead of anabolic pathways that consume ATP (Hardie et al., 2003).

I.2.1 AMPK structure and regulation

AMPK is a heterotrimeric enzyme formed by three subunits: the catalytic α1/α2 subunit and the regulatory β1/β2 and γ1/γ2/γ3 subunits. Each subunit shows multiple isoforms encoded by different genes in mammals (Hardie, 2007). The α subunits contain serine/threonine kinase domains at the N-terminus, which are functionally active only when upstream kinases phosphorylate the threonine 172 residue in the catalytic domain (Gowans and Hardie, 2014).

AMPK is allosterically active when AMP binds to its γ regulatory subunit. This binding induces a conformational change in the complex that blocks the access of threonine 172 residues to phosphatase protein 2A (PP2A), consequently inhibiting the dephosphorylation and inactivation of the enzyme. This conformational change additionally allows upstream kinases with AMPK kinase (AMPKK) activity to phosphorylate α subunit, therefore increasing AMPK net activation (**Figure 6**). This phosphorylation is mainly driven by a complex containing the tumour suppressor liver kinase B1 (LKB1) (Woods et al., 2003). An increase in the intracellular content of Ca^{2+} also provides a nucleotide-independent regulation of AMPK, by which calmodulin-activated protein kinase kinases (CaMKKs), especially CaMKKβ may also phosphorylate AMPK on threonine residues (Hawley et al., 2005). The activation of AMPK results in an increase in the activity of catabolic pathways and an inhibition of anabolic pathways (**Figure 6**).

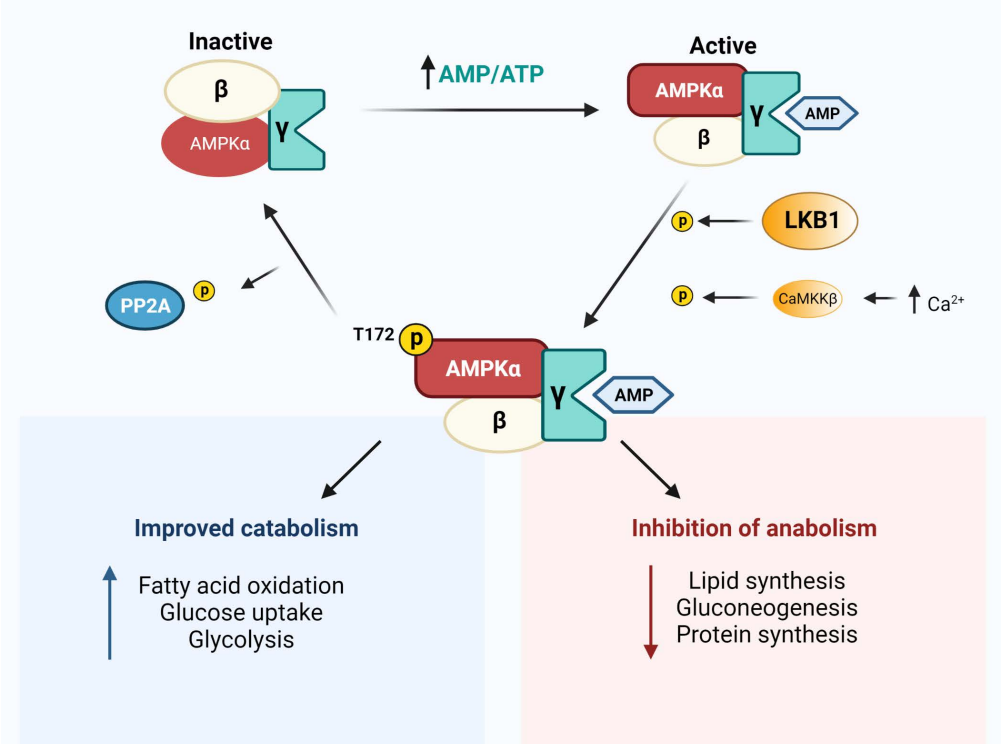


Figure 6. Allosteric and functional activation of AMPK. AMPK remains in an inactive state when AMP/ATP ratio is low. AMP binding to the γ subunit allosterically activates AMPK by inducing a conformational change in the enzyme, allowing LKB1 to phosphorylate AMPK and becoming functionally active. An increase in intracellular Ca^{2+} may also induce AMPK phosphorylation by CaMKKβ. Active AMPK positively regulates catabolic pathways that produce ATP and inhibit anabolic pathways that consume ATP.

In a normal energy state, ATP is competitively bound to the γ subunit, which allows the access of phosphatases to T172. The γ subunits contain a sequence motif known as cystathionine beta-synthase (CBS) repeat that constitutes the binding sites for the nucleotides AMP, adenosine diphosphate (ADP) and ATP, which bind competitively to these tandem repeats (Gowans and Hardie, 2014). Therefore, AMPK becomes active after any stimuli or cellular stress that causes and increase in the AMP/ATP ratio, including physical exercise, long fasting periods, pharmacological treatments, or hypoxia (Salminen and Kaarniranta, 2012).

AMPK activation may also be regulated by other factors. For instance, ERK1/2 is involved in a negative crosstalk with this kinase. In cardiac fibroblasts, serum growth factors can inhibit AMPK phosphorylation by an ERK-dependent mechanism and, conversely, AMPK activation by 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) also induces a significant inhibition of serum-induced ERK phosphorylation (Du et al., 2008). Moreover, ER stress inducers tunicamycin or thapsigargin increase ERK phosphorylation and drastically reduces endogenous AMPK activity in skeletal muscle (Hwang et al., 2013). This ER stress-mediated reduction in AMPK is recovered when ERK function is blocked by its selective inhibitor U0126. The negative regulatory mechanism between AMPK and ERK represents a key therapeutic target in ER stress-induced insulin resistance.

Other mechanisms have been established on the regulation of AMPK activity. Insulin may inhibit AMPK by directly phosphorylating its serine 48 residues in the α subunit, preventing its activation by upstream kinases (He et al., 2017). Insulin inhibits AMPK also by inducing its indirect phosphorylation on serine 485 by Akt and blocking the access of threonine 172 to upstream kinases (Hawley et al., 2014). Furthermore, the increase in the lipid intermediate DAG in hyperglycemic and hyperlipidemic states inhibits AMPK by directly phosphorylating its serine 491 of the α2 subunit by a PKC isoform (Coughlan et al., 2016), blocking the action of upstream kinases.

I.2.2 AMPK in lipid metabolism

The main downstream target of AMPK is the enzyme acetyl-CoA carboxylase (ACC), which catalyses the rate-limiting step in fatty acid synthesis by converting acetyl-CoA into malonyl-CoA, a molecule that inhibits the mitochondrial enzyme carnitine palmitoyl transferase 1 (CPT1). CPT1 is essential in the fatty acid oxidation process since it allows the transport of long-chain fatty acids into the mitochondrial space by catalysing the transfer of acyl-CoA to L-carnitine. When AMPK is activated, it phosphorylates and inhibits ACC, thereby preventing the production of malonyl-CoA, and therefore inhibiting endogenous fatty acid synthesis and promoting fatty acid oxidation (Jeon, 2016b). Moreover, AMPK induces the inhibitory phosphorylation of SREBP1c, a transcription factor that promotes the expression of lipogenic enzymes like ACC1 and fatty acid synthase (FAS) (Hardie and Pan, 2002; Li et al., 2011).

As well, AMPK controls the translocation of the fatty acid transporter CD36 to the plasma membrane through a non-defined mechanism, increasing fatty acid uptake in cardiomyocytes (Habets et al., 2009). AMPK is also able to affect CPT1 activity by inducing its transcription, apart from the indirect mechanism involving ACC inhibition. Administration of AICAR, a drug that produces an analogue of AMP (ZMP) and activates AMPK, increases the expression of PPARγ co-activator (PGC)-1α and simultaneously coactivates PPARα (Lee et al., 2006e). As a result, this complex acts as a transcription factor that increases the expression of genes implicated in fatty acid oxidation in the mitochondria, mainly CPT1 and medium-chain acyl-coenzyme A dehydrogenase (MCAD) (Vega et al., 2000).

Kim et al. showed that mice with adipose-tissue-specific knockout of both the α1 and α2 catalytic subunits of AMPK did not change adipose triglyceride lipase (ATGL) total levels but reduced its phosphorylation at S406, resulting in a decrease in its triglyceride hydrolase activity. However, in the same study these mice also showed a defective phosphorylation of hormone sensitive lipase (HSL) at S565, resulting in an increase of its hydrolase activity and lipolysis (Kim et al., 2016). The net activity of fatty acid oxidation was increased because of an enhanced lipolysis that causes the activation of PPARβ/δ. The role of AMPK in lipolysis remains controversial. While some authors state that AMPK is able to activate HSL, other

studies show that phosphorylation of HSL by AMPK impairs its further activation by PKA (Daval et al., 2005; Garton and Yeaman, 1990).

Overall, these studies indicate that AMPK exerts its role in lipid metabolism controlling the amount of circulating free fatty acids through its consumption by mitochondrial fatty acid β-oxidation and by inhibiting lipolysis and lipogenesis simultaneously (Jeon, 2016a).

I.2.3 AMPK in glucose metabolism

Apart from the stimulation of lipid catabolic pathways, AMPK also regulates glucose transport and metabolism, primarily on skeletal muscle. AMPK increases the activity of Rab family G proteins by inhibiting the AS160 paralog 2/Bub2/Cdc16 (TBC1) Tre-Domain Family Member 1 (TBC1D1), which induces the fusion of GLUT4 vesicles with the plasma membrane (Taylor et al., 2008) and increases glucose uptake into the cell. Furthermore, pharmacological AMPK activators like AICAR increase glucose uptake and GLUT4 translocation to the plasma membrane (Merrill et al., 1997). The increase in glucose transport reported after exercise in skeletal muscle or after contraction *in vitro* and the concomitant activation of AMPK by AMP elevation suggests an implication of this kinase in the contraction-induced glucose uptake (Hutber et al., 1997). Further studies using specific AMPK subunit isoforms-knockout models show that α2 subunit ablation partially impairs contraction-stimulated glucose uptake (Mu et al., 2001) while other subunits deletion did not affect this process (Long and Zierath, 2006). However, LKB1-knockout models present an impaired contraction-stimulated increase of glucose transport (Sakamoto et al., 2005).

Glycolysis and glycogen synthesis are regulated by AMPK in different cell types. Phosphorylation and activation by AMPK of the enzyme that produces fructose-2,6-bisphosphate, allosterically actives the glycolytic enzyme phosphofructokinase-1 (Marsin et al., 2002). Furthermore, AMPK activation increases the levels of hexokinase, which catalyses the first step in the glycolytic pathway (Winder and Thomson, 2007)

AMPK also inhibits glycogen synthesis through phosphorylation and inactivation of GS (Hunter et al., 2011). However, this process can be reversed by a chronic activation of AMPK.

AMPK is also activated in a low-glucose state independently of the AMP/ATP or ADP/ATP ratios (Zhang et al., 2017). Glucose deprivation has been reported to elicit the formation of a multimeric complex involving different proteins in the lysosomal surface, promoting the activation of AMPK by LKB1 (Zhang et al., 2013).

Gluconeogenesis in the liver is a critical process regulating glucose levels in plasma. AMPK inhibits hepatocyte nuclear factor 4 (HNF4) and cAMP response element-binding (CREB) regulated transcription coactivator 2 (CRTC2), two transcription factors that control the expression of the gluconeogenic enzymes PEPCK and G6P, therefore inhibiting gluconeogenesis in the liver (Koo et al., 2005), (Leclerc et al., 2001). Additionally, AMPK can induce the export from the nucleus of class IIa histone deacetylases, which cause the activation of the nuclear factor forkhead box protein O (FOXO) and promotes the expression of gluconeogenic enzymes in fasting conditions (Mihaylova et al., 2011).

Overall, AMPK plays a critical role in the increase of ATP production by enhancing the rapid disposal of glucose and fatty acids as fuel for mitochondrial oxidation in skeletal muscle and other tissues and decreasing the glucose production in liver. Thus, AMPK becomes an important therapeutic target for the treatment of T2D and insulin resistance.

I.2.4 AMPK and insulin resistance

Chronic exposure to an excess of nutrients leads to the development of insulin resistance in the most relevant tissues that regulate metabolism. Although AMPK is widely expressed in the organism, heart and skeletal muscle account for the major expression and protein levels of this kinase.

In macrophages and adipose tissue, the lipid overload and accumulation caused by overnutrition leads to the triggering of proinflammatory responses caused by the activation of TLR and its downstream targets, causing insulin

resistance (Shi et al., 2006). AMPK activity is reduced in obese individuals that display a chronic inflammatory state (Gauthier et al., 2011). In fact, TNF-α overexpression and lipid accumulation-stimulated upregulation of IL-6 and SOCS3 decrease AMPK phosphorylation and protein levels (Ko et al., 2009; Steinberg et al., 2006).

Fatty acid accumulation and internalization in peripheral tissues increase the levels of lipid intermediates, such as DAG and ceramide. DAG is a potent activator of different PKC isoforms, which can ultimately cause the inhibition of the hepatic insulin receptor (Samuel et al., 2007). Ceramide accumulation is mainly associated with an increase in the content of palmitic acid. Although the role of ceramide in the induction of insulin resistance is controversial, it negatively affects insulin signalling by a direct inhibition of insulin-induced Akt2 activation (Stratford et al., 2004). The complex formed in the liver by the packaging of ceramides and low-density lipoproteins (LDL) are also capable of eliciting inflammatory responses in skeletal muscle by activating NF-κB (Kirwan, 2013). As previously discussed, AMPK increases fatty acid oxidation through direct inhibition of ACC, causing an increase in CPT1 activity. Sustained activation of AMPK in hepatocytes derived from transgenic mice overexpressing AMPK robustly promotes fatty acid oxidation and leads to higher rates of oxidation using palmitate as fuel (Garcia et al., 2019). This leads to a decrease in hepatic steatosis, a downregulation of inflammation-related genes and an improved glucose tolerance and insulin sensitivity. The cellular location of lipin 1 determines whether fatty acids undergo mitochondrial oxidation or are incorporated in the synthesis of triglycerides and phospholipids. In the cytoplasm, lipin 1 catalyses the conversion of phosphatidic acid to DAG (Reue, 2009). In the nucleus, lipin 1 transcriptionally coactivates the expression of genes involved in mitochondrial fatty acid oxidation by forming a complex with PPARα and PGC-1α (Lee et al., 2006d). This complex can be promoted by the activation of hepatic AMPK after the pharmacological induction of PPARβ/δ, which mediates the prevention of HFD-induced hypertriglyceridemia and inflammation, improving insulin sensitivity (Barroso et al., 2011a).

I.2.4.1 AMPK in ER stress and inflammation

Most of the biological processes regarding lipid metabolism occur in the ER, where many of the enzymes controlling these processes are located. An excessive exposure to saturated fatty acids and lipid intermediates results in the inability of the ER to maintain homeostasis, resulting in ER stress and lipotoxicity (Fu et al., 2012). Chronic AMPK activation by AICAR administration results in a decrease in p-PERK and p-eIF2α levels in the liver of mice exposed to a HFD (Li et al., 2014a) mediated by mTORC1 inhibition, an improved response to insulin and a reduction in hepatic lipid accumulation.

Furthermore, AMPK activation in diet-induced models of insulin resistance improves insulin sensitivity by restoring tyrosine phosphorylation of the insulin receptor β subunit (IRβ) and IRS-1, along with an increased phosphorylation of the p85 subunit of PI3K and serine phosphorylation of Akt, resulting in an increase of GLUT4 translocation (Radika and Anuradha, 2015).

The downregulation of the NF-κB system by AMPK activation has been extensively reported (Bai et al., 2010; Choi et al., 2016; Huang et al., 2015; Sag et al., 2008; Xiang et al., 2019; Yang et al., 2010b). AMPK indirectly suppresses NF-κB by acting through downstream mediators such as SIRT1, FOXO family or PGC-1α, which repress the inflammatory response (Salminen et al., 2011). As well, AMPK activation inhibits NF-κB transactivation induced by palmitate and the increase of TNF-α (Cacicedo et al., 2004). Additionally, the lack of specific AMPK components compromises the anti-inflammatory effects of this kinase. For instance, knockdown of AMPK α1 subunit abolishes the inhibition of TNF-α-induced IκB kinase phosphorylation caused by metformin treatment in endothelial cells (Huang et al., 2009). Constitutive activation of AMPK α1 subunit abolishes fatty acid-induced inflammation and NF-κB function in macrophages, which was reversed by AMPK α1 subunit knockout (Yang et al., 2010b).

SIRT1-AMPK regulation mechanism represents a positive feedback loop aimed to enhance cell survival in energy-deficient states (Salminen et al., 2011). AMPK can increase cellular NAD^+ levels and therefore activate SIRT1 deacetylase (Cantó et al., 2009), which controls the acetylation of the RelA/p65 subunit of NF-

κB (Yeung et al., 2004). Therefore, SIRT1-induced deacetylation of the NF-κB complex inhibits its activity and the activation of proinflammatory pathways.

p53 is a transcription factor involved in apoptosis, metabolism and inflammation (Komarova et al., 2005; Vousden and Ryan, 2009). In fact, lipin 1 gene transcription is upregulated by p53 upon nutritional stresses such as glucose deprivation in cultured myotubes, increasing fatty acid oxidation (Assaily et al., 2011). AMPK stabilizes and activates p53 (Chen et al., 2015b; He et al., 2014), through the inhibitory phosphorylation of murine double minute X (MDMX), a transcriptional inhibitor of p53 (Yu et al., 2020). AMPK can also target p53 phosphorylation sites on serine 15 and 20, which are linked to the inflammatory process (Goodman et al., 2004).

Furthermore, AMPK-stimulated reduction of lipid intermediates including DAG and ceramide directly affects NF-κB regulation., since DAG activates PKC isoforms in skeletal muscle that are responsible for the activation of IKKβ (Nath and Isakov, 2014) and the posterior activation of NF-κB signalling pathway. Both kinases phosphorylate IRS proteins on serine residues, attenuating the response to insulin in peripheral tissues (Vázquez-Carrera, 2016).

As mentioned above, an inhibitory crosstalk regulates AMPK and ERK1/2 phosphorylation (Hwang et al., 2013). In this study, pharmacological inhibition of ERK1/2 prevented the development of ER stress and insulin resistance in *db/db* mice by increasing the activation of AMPK and rescuing the phosphorylation of Akt. Furthermore, it has been reported that the insulin-induced serine phosphorylation of the IRS may respond to a mechanism involving ERK1/2 (Fritsche et al., 2011), and that the sustained activation of the ERK pathway is involved in the pathogenesis of T2D, therefore becoming a potential target for the treatment of insulin resistance and related metabolic disorders (Ozaki et al., 2016)

I.3 PPARβ/δ and metformin in insulin resistance and T2D

T2D is considered an epidemic disease that, at present, affects more than 400 million people all over the world. Currently, it is necessary a combination of changes in lifestyle and pharmacological treatment to achieve a good metabolic control for diabetes (Marín-Peñalver et al., 2016) and prevent the onset of other related pathologies like cardiovascular disease, retinopathy or neuropathy. Since most T2D patients show a certain degree of overweight and obesity that favour the appearance of factors that induce insulin resistance and the consequent development of T2D, dietary intake control and promotion of physical exercise are key to initiate a better glycemic control. Nevertheless, drug therapy becomes necessary for many patients with long-term complications and metabolic singularities. Although insulin resistance and diabetes therapies have been widely investigated in the past years, the current treatments are unable to prevent all the complications derived from this pathology, being necessary in most cases the combination of different drugs or approaches. Some of the current strategies available for the treatment of insulin resistance and diabetes are shown in **Table 1**:

Treatment	Mechanism of action	Main effects	Side effects
Metformin	Inhibition of gluconeogenesis by reduction of G6Pase and PEPCK. Activation of AMPK that promotes fatty acid oxidation and the reduction of factors that induce insulin resistance	Fasting blood glucose lowering Body weight reduction Reduction of inflammation and lipid-induced ER stress	Gastrointestinal: anorexia, nausea, diarrhea. Possible lactic acidosis
Sulfonylureas and meglitinides	Target pancreatic beta-cell potassium channels to induce its closure and the accumulation of calcium, which causes a higher insulin secretion.	Most cost-effective glucose-lowering agents. High glucose-lowering effectiveness for sulfonylureas and lower for meglitinides	Loss of efficacy, hypoglycemia, weight gain
Glucagon-like peptide (GLP) 1 receptor agonists	Stimulation of GLP-1 receptors in the pancreas, inducing insulin release and inhibiting glucagon secretion in hyperglycemia states.	Combination with insulin significantly improves glycemic control and reduction in body weight.	Nausea, vomiting
Thiazolidinediones (TZD)	TZDs bind to PPARγ and enhances its action as a transcription factor of genes implicated in regulation of glucose metabolism, fatty acid storage, and adipocyte differentiation	Increase of adiponectin Decrease of hepatic gluconeogenesis Increase of GLUT4 expression	Weight gain, heart failure, hepatotoxicity, macular edema, bladder tumours. No longer authorized in Europe.
Sodium-glucose co-transporter 2 (SGLT2) inhibitors	iSGLT2 inhibit the reabsorption of glucose in kidney, therefore increasing its excretion and reducing the levels of glucose in diabetic patients.	Body weight reduction Plasma glucose levels reduction Modest improvement of glycemic control	Increased urinary tract infections, hypotension. Not effective for severe insulin resistance
Alpha-glucosidase inhibitors	Reversible inhibition of the hydrolase enzymatic activity of membrane-bound intestinal alpha-glucosidases, causing delayed carbohydrate absorption and reducing hyperglycemia.	Reduction of postprandial triglycerides. Low blood glucose reduction	Low efficacy compared to other options. Gastrointestinal problems. Contraindicated in patients with intestinal disorders or high creatinine

Concentrated insulin products	Help to improve the delivery of insulin when large doses are needed in the situation of severe insulin resistance. Stimulation of glucose uptake in insulin-resistant tissues	Significant improvement of glycemic control compared to regular insulin.	Possibility of dosing errors Necessity of other complementary treatments

Table 1. Summary of the pharmacological strategies currently used for the treatment of type 2 diabetes (Marín-Peñalver et al., 2016), (Church and Haines, 2016).

As depicted in **Table 1**, the currently available therapies for T2D and insulin resistance present a low efficacy and limited tolerability, as well as significant side effects that could worsen other pathologies. For instance, most T2D patients require an intensification of the treatment for the control of hyperglycemia and, in clinical practice, only 30% of patients treated with insulin achieve a minimal control of A1c levels (Nichols et al., 2012).

In the past years, new therapeutic targets have been described for the treatment of diabetes, insulin resistance and other metabolic pathologies. For example, the inhibition of ACC, an enzyme that controls the balance between fatty acid synthesis and oxidation has been shown to reduce the synthesis of fatty acids and switch its fate onto oxidation (Harriman et al., 2016). The compound is known as ND-630 and reduces hepatic steatosis and improves insulin sensitivity and dyslipidemia in rats. Resveratrol also induces a robust increase of SIRT1, which leads to an amelioration of inflammation and oxidative stress, and the activation of AMPK (Timmers et al., 2011). FGF21 has been well established as a therapeutic target for the treatment of metabolic diseases by extensive research (Gimeno and Moller, 2014; Kharitonenkov and DiMarchi, 2015). This has led to the development of FGF21 analogues for the treatment of disorders such as obesity or T2D (Kim et al., 2017). To overcome the problems of these analogues, new strategies regarding administration issues have been addressed, and the development of oral activators of HRI, which increases hepatic and circulating FGF21 levels allowed a new strategy to improve HFD-induced glucose tolerance and hepatic steatosis (Zarei et al., 2019a).

As previously described, AMPK is another important therapeutic target for insulin resistance, due to its pleiotropic actions in multiple metabolic target tissues and the beneficial effects of this kinase regarding the enhancement of fatty acid oxidation or the reduction of inflammation and lipid-induced ER-stress. In fact, in the past few years new-generation AMPK activators have been shown to supress hepatic steatosis (Boudaba et al., 2018), or promote glucose disposal and glucose homeostasis (Cokorinos et al., 2017b; Steneberg et al., 2018).

Interestingly, most of the effects caused by PPARβ/δ, a transcription factor whose activation prevents ER stress-associated inflammation and insulin resistance in different tissues (Vázquez-Carrera, 2016) rely on the activation of AMPK (Salvadó et al., 2014). The pharmacological activation of this nuclear receptor has been shown to produce a strong antidiabetic effect in different models of fatty acid or HFD-induced insulin resistance (Barroso et al., 2011b; Doktorova et al., 2017; Salvadó et al., 2014; Salvado et al., 2015; Serrano-Marco et al., 2012a).

I.3.1 PPARβ/δ

PPARs belong to the nuclear hormone receptors superfamily and act as transcription factors in response to the binding of a ligand. PPARs regulate the expression of genes involved in the regulation of lipid and glucose metabolism, inflammatory response, or control of cell growth and differentiation. There are three different identified isoforms that show diverse functions: PPARα was the first discovered in rodent hepatocytes (Issemann and Green, 1990), while PPARβ/δ and PPARγ were subsequently identified and characterized (Dreyer et al., 1992), (Kliewer et al., 1994).

PPARβ/δ presents a virtually ubiquitous expression. However, organs/tissues that are implicated in fatty acid catabolism show higher levels of this transcription factor, including the liver (Sanderson et al., 2010), brown and white adipose tissue (Wang et al., 2003) and skeletal muscle cells (Giordano et al., 2009). Moreover, PPARβ/δ is also significantly present in keratinocytes, enterocytes, brain, macrophages or cardiomyocytes (Giordano Attianese and Desvergne, 2015b).

I.3.1.1 Structure and mechanism of action

PPARs present the same modular structure as the rest of the nuclear hormone receptors. This transcription factor is composed by an A/B domain in the N-terminus part of the peptide; a C domain with DNA binding function or DNA binding domain (DBD), a D domain involved with the interaction with cofactors for the regulation of transcription, and the E domain that forms the ligand binding domain (LBD) for the union with endogenous or exogenous ligands. The DBD and the LBD are the most conserved regions.

In the case of PPARβ/δ, the LBD allows the binding of a broad spectrum of ligands, including fatty acids, eicosanoids or synthetically produced agonists that allow the activation of this transcription factor and the recruitment of the retinoid X receptor (RXR) to form a heterodimer. The two-zinc finger-like structure of the DBD allows the binding of this heterodimer to peroxisome proliferator response elements (PPREs) located in the promoter region of PPARβ/δ target genes, regulating their transcription. PPARβ/δ follows a tight and complex regulatory mechanism. While its ligand binding pocket is considered as relatively large and allows the interaction with different ligands, a helical structure at the C-terminal of the LBD presents its open conformation when no ligand is available and allows the repression of PPARβ/δ by corepressor molecules. By contrast, ligand binding induces a conformational change that results in the dissociation of corepressor factors and allows the binding of coactivator molecules and the regulation of the transcription of target genes (Tan et al., 2016a). This mechanism is known as transactivation.

However, PPARβ/δ also regulates gene expression through the interaction with other transcription factors through a mechanism known as transrepression. Transrepression is responsible for most of the anti-inflammatory effects of PPARβ/δ (Vázquez-Carrera, 2016) and is driven by three different mechanisms. In a situation of limited levels of coactivators, PPARβ/δ may prevent the binding of these coactivators to other transcription factors by competition, inhibiting its activity. The activated heterodimer formed by PPARβ/δ and RXR can also interrupt the binding of other transcription factors to their target genes regulatory elements by physical interaction and sequestration. Finally, PPARβ/δ may prevent the phosphorylation

of target proteins by masking it phosphorylation sites, thereby inhibiting its downstream signalling.

As previously mentioned, crystallization of the PPAR proteins show a large ligand-binding pocket in the three isoforms (Giordano Attianese and Desvergne, 2015a), which may explain the wide diversity of natural and synthetic ligands for these receptors, including saturated or unsaturated fatty acids, eicosanoids, vitamin B3 or synthetic ligands like TZDs or fibrates. In the case of PPARβ/δ, extensive research has been developed by using high-affinity and highly specific synthetic ligands of this receptor, like GW501516 or GW0742 in order to elucidate specific actions of PPARβ/δ in different tissues by activating only this receptor and no other isoforms (Barroso et al., 2011a; Salvadó et al., 2014). Interestingly, three selective PPARβ/δ agonists have reached clinical studies: MBX-8025, KD-3010 and CER-002 (Vázquez-Carrera, 2016), however, none of them is currently available for therapy. GW501516 has been extensively used in investigation for elucidating the roles of PPARβ/δ in metabolism and other processes., however, it never reached clinical trials since it promoted intestinal polyps growth in mice predisposed to intestinal polyposis (Gupta et al., 2004).

I.3.1.2 PPARβ/δ activation in adipose tissue

One of the main mechanisms leading to inflammation and the development of insulin resistance in adipose tissue is the infiltration of macrophages and its polarization towards the proinflammatory type macrophage or M1. This occurs as a consequence of expansion of abdominal fat and obesity (Glass and Olefsky, 2012). For instance, macrophages found in the adipose tissue of non-obese individuals show increased levels of type 2 T helper cell (Th_2) cytokines, which promote the switch of macrophages towards the anti-inflammatory or M2 state. PPARβ/δ constitutes an important determinant in the polarization of M1 macrophages into the M2 type, which ameliorates insulin sensitivity (Kang et al., 2008) (**Figure 7**). In fact, PPARβ/δ mediates the IL-13 and IL-4-induced polarization of macrophages to the anti-inflammatory phenotype. Moreover, fructose-induced infiltration of

macrophages, inflammation and glucose intolerance is exacerbated in the adipose tissue of PPARβ/δ-null mice (Barroso et al., 2015).

Triglyceride accumulation and the inflammatory pathways elicited by lipid intermediates like DAG induce the direct and indirect phosphorylation of IRS on serine residues, leading to the attenuation of insulin signalling. PPARβ/δ activation increases the oxidation rates of fatty acids in adipose tissue (Roberts et al., 2011), a tissue that usually promotes fat storage pathways, while transgenic mice that overexpress PPARβ/δ are more resistant to HFD-induced obesity by activating fat metabolism and reducing the levels of triglycerides and circulating free fatty acids (Wang et al., 2003). TNF-α, which can attenuate the PPARβ/δ transcriptional activity, may be responsible, at least in part, for the reduced levels of PPARβ/δ in obese individuals (Serrano-Marco et al., 2012b).

The growth of visceral adipose tissue generates an increase in the rate of lipolysis, thus increasing saturated fatty acid levels and activating inflammatory mediators like IKKβ or JNK/AP1 pathways (Glass and Olefsky, 2012). Toll-like receptor 4 (TLR4) is a main mediator of the proinflammatory effects of saturated fatty acids or lipopolysaccharide (LPS), produced by the gastrointestinal tract upon metabolic stress. Removal of either TLR4 or its endogenous ligand fetuin A prevents lipid-induced insulin resistance. The PPARβ/δ ligand GW501516 prevents the LPS and TLR4-induced increased activity of NF-κB by neutralizing the activity of ERK1/2 (Rodríguez-Calvo et al., 2008) (**Figure 7**). Studies with GW501516 also show inhibition of the IL-6-induced activation of the JAK-STAT pathway, by preventing the union of activated STAT proteins to heat-shock protein 90 (HSP90), which is necessary for its full activation in order to translocate into the nucleus and promote the transcription of SOCS3 (Serrano-Marco et al., 2011). Conversely, the association of STAT3 with HSP90 is enhanced in PPARβ/δ knockout mice.

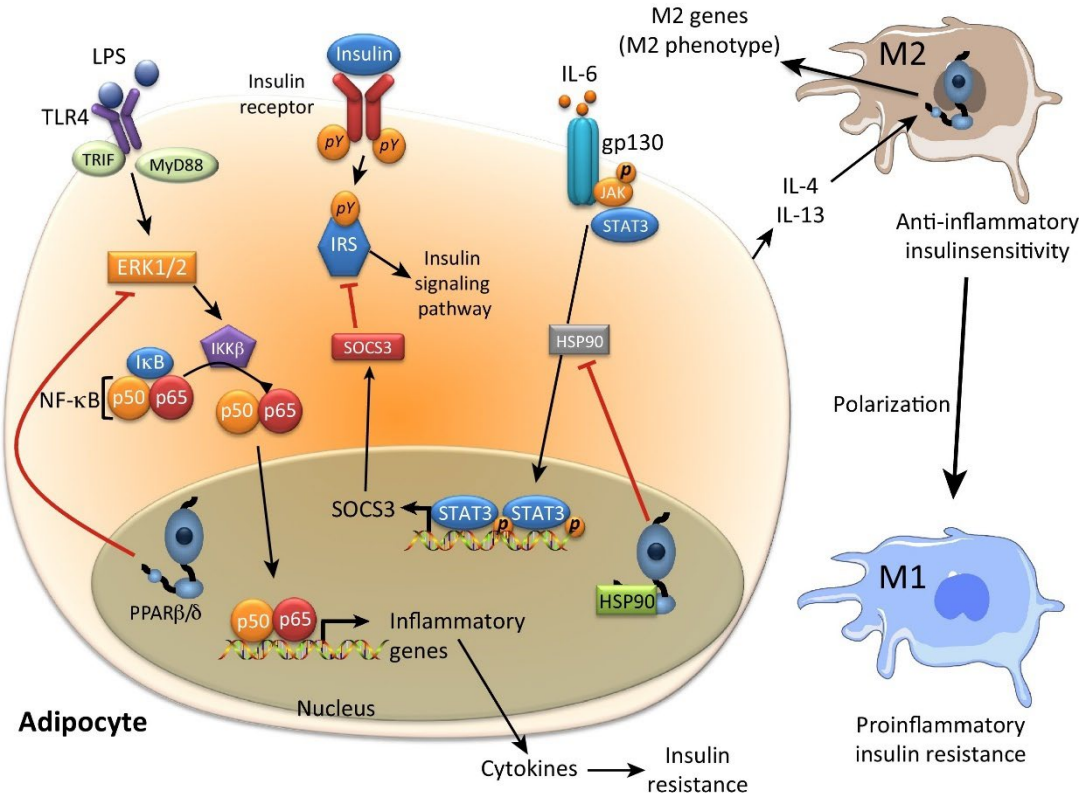


Figure 7. Effects of PPARβ/δ activation on adipocytes and macrophages. PPARβ/δ polarizes macrophages toward the anti-inflammatory M2 state. In adipocytes, PPARβ/δ prevents LPS-induced activation of NF-κB by inhibiting ERK1/2. Moreover, PPARβ/δ attenuates IL-6-induced insulin resistance and the induction of SOCS3, improving insulin sensitivity. (Vázquez-Carrera, 2016)

I.3.1.3 PPARβ/δ activation in the liver

The liver is an essential organ for glycemic control in the organism. Hepatic steatosis is the consequence for lipid accumulation in this organ and is present in more than 40% of T2D patients (Haas et al., 2016). When insulin resistance is present, hepatic production of glucose and lipid synthesis is altered, leading to hyperglycemia and hypertriglyceridemia in a paradoxical manner that disrupts energy balance in the organism (Santoleri and Titchenell, 2019). This lipid accumulation is a consequence of an increased activity of SREBP-1, and the downregulation of AMPK, which promotes fatty acid oxidation (Vázquez-Carrera, 2016). PPARβ/δ promotes the expression of the insulin-induced gene 1 (*Insig1)*, whose protein product prevents the SREBP activity by keeping this protein

complexed with the cleavage-activating protein (SCAP) in the ER (Qin et al., 2008) (**Figure 8**). Additionally, administration of the PPARβ/δ agonist GW501516 promotes via the activation of AMPK the nuclear localization of Lipin 1 and the increase of the levels of the PPARα endogenous ligand 16:0/18:1-phosphatidylcholine. These effects amplify the Lipin1-PGC-1α-PPARα axis, leading to the increase of fatty acid oxidation in the liver of mice fed a HFD (Barroso et al., 2011a).

The PPARβ/δ-mediated polarization to the M2 type of hepatic resident macrophages known as Kupffer cells improves the insulin sensitivity in HFD-induced insulin resistant mice, whereas its deficiency provokes hepatic dysfunction and insulin resistance in obese mice (Odegaard et al., 2008). Additionally, in a human liver cell line, PPARβ/δ activation can prevent IL-6-induced insulin resistance by inhibiting STAT3 (Serrano-Marco et al., 2012a).

In humans, PPARβ/δ agonists improve plasma markers of the liver function and reduce steatosis (Vázquez-Carrera, 2016). Although clinical evidence of these agonists is still scarce, PPARβ/δ agonists could be a treatment for chronic liver diseases in humans. In fact, they have been proposed as a treatment for NAFLD and NASH, and in in the past few years some of these agonists were under clinical development for the treatment of these hepatic pathologies (Zarei et al., 2021).

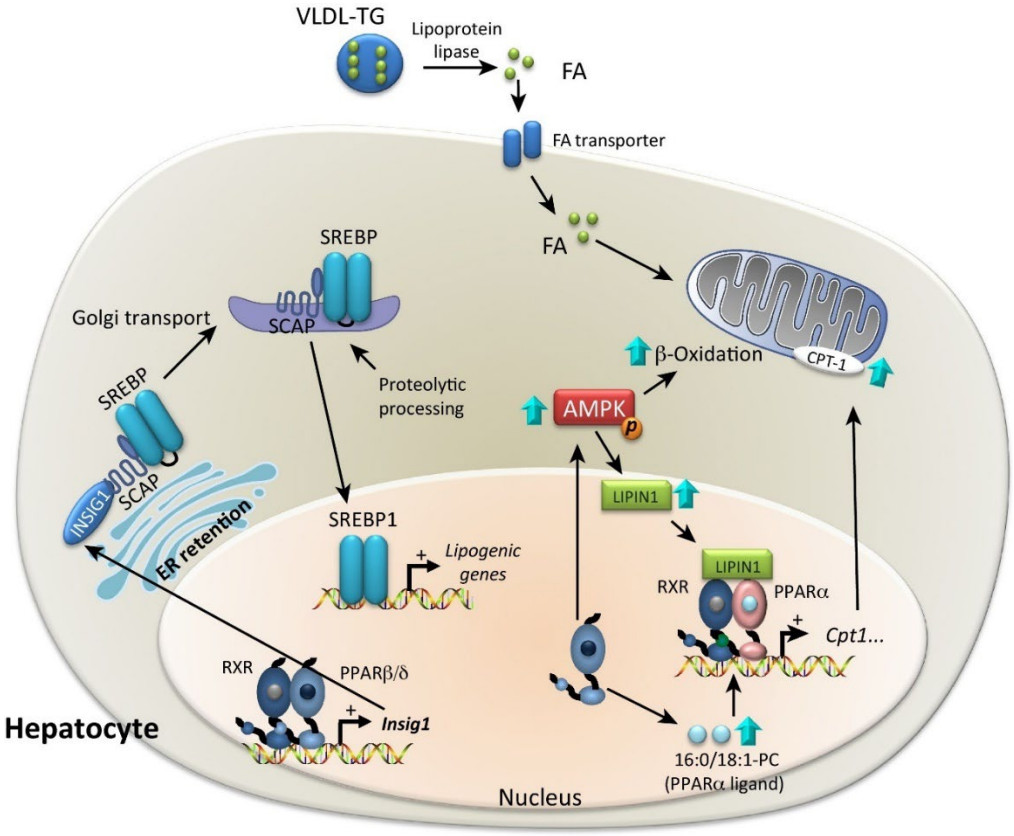


Figure 8 - Effects of PPARβ/δ activation on hepatocytes. PPARβ/δ activation induces the expression of *Insig1*, which reduces the processing of SREBP1 and the upregulation of lipogenic genes, ameliorating hepatic steatosis in obesity and diabetes. Additionally, PPARβ/δ activates AMPK and promotes the lipin1-PPARα-PGC-1α, which exacerbates mitochondrial fatty acid oxidation by upregulating genes such as *Cpt1.* (Vázquez-Carrera, 2016)

I.3.1.4 PPARβ/δ activation in skeletal muscle

Skeletal muscle is a markedly relevant organ in the regulation of glucose homeostasis, since it accounts for most insulin-stimulated glucose uptake and is the primary site for the development of systemic insulin resistance (Gustafson et al., 2015b). The accumulation and infiltration of fatty acids originated by the excess of lipids and adipokines released from adipose tissue mediates insulin resistance development mechanisms in skeletal muscle. Apart from the TLR-mediated lipotoxicity (Glass and Olefsky, 2012), the overload of fatty acids surpasses the β-oxidation capability of myocytes, accumulating fatty acid derivatives like DAG and

ceramide that activate PKC, ultimately leading to the phosphorylation of IRS proteins on serine residues (Nath and Isakov, 2014).

PPARβ/δ has been shown to upregulate the expression of fatty acid oxidation genes in skeletal muscle (Giordano Attianese and Desvergne, 2015a), which may reduce lipid overload and prevent insulin resistance. Pyruvate dehydrogenase kinase 4 (PDK4) is a direct target upregulated by PPARβ/δ activation and is implicated in fatty acid metabolism. In fact, PDK4 deficiency leads to the inhibition of fatty acid oxidation in exchange for a higher glucose utilization (Zhang et al., 2014b). The CPT1 isoform gene in skeletal muscle, CPT1b, is transcriptionally upregulated by the binding of PPARβ/δ to its PPRE in the promoter region (Toral et al., 2015). The transport of long-chain fatty acids into mitochondria by CPT1 represents the rate-limiting step in β-oxidation. Additionally, pharmacological activation of PPARβ/δ enhances the activation of AMPK, thereby inhibiting ACC, attenuating the inhibition of CPT1 (Galic et al., 2018). Consequently, PPARβ/δ activation has been shown to prevent the palmitate-induced increase of DAG and the PKC-mediated activation of NF-κB, ameliorating insulin sensitivity (Coll et al., 2010a). Since etomoxir, a CPT1 selective inhibitor, prevents this effect of PPARβ/δ, fatty acid oxidation has been considered a main driver of these beneficial effects.

PPARβ/δ activation also prevents lipid-induced ER stress in myocytes exposed to palmitate, effects that rely on AMPK activation (Salvadó et al., 2014). The negative crosstalk regulating AMPK and ERK1/2 activity may also be implicated in the PPARβ/δ agonists effects on ER stress, since ERK1/2 also elicits ER stress pathways (Hwang et al., 2013). Additionally, transgenic mice overexpressing PPARβ/δ show better exercise performance as a result of a interaction between PPARβ/δ and the exercise-inducible kinase AMPK, which promotes a general improvement of the metabolic profile and an increase in insulin sensitivity (Gan et al., 2011b). It is worth noting that AMPK accounts for many of the exercise-derived effects on glucose uptake and lipid metabolism in skeletal muscle (Friedrichsen et al., 2013), and this kinase mediates many of the antidiabetic effects shown when PPARβ/δ is activated.

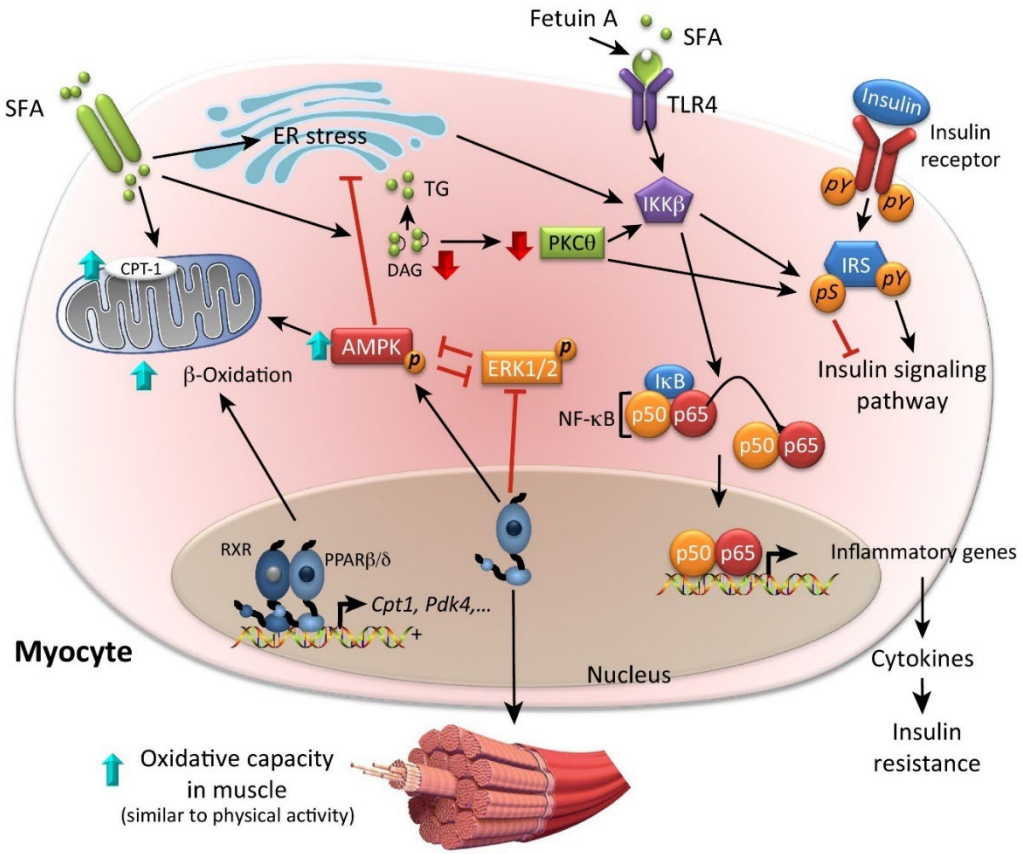


Figure 9 - Effects of PPARβ/δ activation in skeletal muscle. PPARβ/δ activation increases the expression of genes involved in fatty acid oxidation, reducing the availability of fatty acids and preventing the activation of the DAG-PKCθ-NF-κB pathway that attenuate the insulin signalling pathway by several mechanisms. PPARβ/δ also activates AMPK, which reduces phospho-ERK1/2 levels and prevents ER stress by increasing mitochondrial fatty acid oxidation, among other mechanisms. Finally, PPARβ/δ provokes an increase in the oxidative capacity of the skeletal muscle. (Vázquez-Carrera, 2016)

Extensive studies using genetical or pharmacological approaches in order to evaluate PPARβ/δ implication in insulin resistance and other metabolic disorders show that PPARβ/δ activation represents a potential strategy to address these metabolic disorders. However, although PPARβ/δ ligands have reached clinical trials, none of these compounds are available for therapeutic use and several issues remain to be clarified in terms of safety and therapeutic efficacy.

For instance, the role of PPARβ/δ in carcinogenesis remains controversial, since conflicting studies show that PPARβ/δ activation can lead both to inhibition and promotion of tumorigenesis in animal models (Peters et al., 2015). In fact, PPARγ and PPARα ligands that are available for clinical use have been reported to increase the incidence of different types of cancer in animal models (Vázquez-Carrera, 2016). Given that PPAR expression is lower in humans, the use of these clinical agonists has not been associated with an increased incidence of tumorigenesis in most of the studies performed (Levin et al., 2015; Youssef and Badr, 2011). However, safety and efficacy remain to be a pending subject for PPARβ/δ, since the mechanism underlying its antidiabetic actions is not fully understood and additional studies must be performed in order to shed light on its mechanism of action and assess the implication of AMPK and consequent reduction of ERK1/2 activity in this function. In addition, the discovery of new determinants implicated in PPARβ/δ actions could constitute an alternative approach to find new therapeutic targets for the treatment of insulin resistance and derived metabolic disorders.

I.3.2 Metformin

Metformin has become the most prescribed oral antidiabetic agent worldwide, according to the European Association for the Study of Diabetes (Inzucchi et al., 2015). The herbaceous plant *Galega officinalis* was used from the 18th century as a traditional medicine in Europe to treat symptoms of diabetes (Bailey, 2017). In the beginning of the 20th century, the phytochemical product guanidine that was found in the extract of *G. officinalis* was reported to lower blood glucose in animals, and years later the first biguanides were synthesized, which set

the fundaments for the synthesis of metformin (dimethylbiguanide hydrochloride) in 1922. However, it was not until years later when other groups reported the glucose-lowering activities of metformin and other guanidine derivatives, like phenformin and buformin. Nevertheless, while some of these derivatives were withdrawn in most countries because of the production of lactic acidosis, metformin has been proved as an effective glucose-lowering agent that shows little contraindications, no expensive production and high benefit/risk ratio, which has turned this drug into the first-line approach for the pharmacological treatment of T2D.

Additionally, metformin improves glycaemic control in type 1 diabetes patients, reducing the insulin doses necessary for its treatment, as well as it prevents the progression from glucose intolerance states to the development of insulin resistance and T2D (Hostalek et al., 2015).

I.3.2.1 Pharmacokinetics

In animal models or humans, metformin is not metabolized and is excreted without modifications through kidneys. Metformin is orally administered and delivered to the intestine, where it is absorbed by enterocytes through the plasma monoamine transporter (PMAT) and organic cation transporter 3 (OCT3) (He and Wondisford, 2015). The transport outside the enterocytes is mediated by OCT1 in the basolateral membrane and then it is delivered through the portal vein to the liver, where it is incorporated through OCT1-OCT2. While concentrations in the portal vein reach up to 40-70 μM of metformin when patients receive a maximum approved daily dose of 35 mg/kg body weight, metformin accumulates in the liver and reach up to 1-1.5 mM concentrations (Chandel et al., 2016; Gormsen et al., 2016), which is consistent with the high expression of organic cation transporters in this tissue. In fact, ablation of OCT1 and OCT2 impairs the hepatic distribution of metformin (Jensen et al., 2016). However, metformin is rapidly secreted through the multidrug and toxin extrusion 1 (MATE1) transporter and its time of accumulation is relatively low. After hepatic uptake, the remaining concentration of metformin in

the plasma reaches 10-40 μM. Then, metformin is absorbed into renal epithelial cells by OCT2, from where it will be ultimately secreted within urine flux.

Interestingly, an *in vivo* imaging study of metformin labelled with ^{11}C showed the biodistribution of metformin in peripheral organs. The results revealed that an acute administration of metformin was primarily taken up by the liver, the kidneys and the urinary bladder, and to a lesser extent by salivary glands, skeletal muscle and intestines (Gormsen et al., 2016). Uptake rates in the skeletal muscle were low and the concentration of metformin in skeletal muscle was slightly higher than in plasma, however, an increasing activity over time was observed in the skeletal muscle of individuals that took oral metformin. This reveals that metformin is slowly absorbed by this tissue, but a prolonged accumulation of metformin would be facilitated by the transport mediated by low-affinity OCTs (Gormsen et al., 2016). This is consistent with the reported activation of AMPK caused by metformin in the skeletal muscle of patients with T2D (Musi et al., 2002). The intestines, more specifically the jejunum shows a great accumulation of metformin as well, with values ranging 30-300-fold compared to metformin plasma levels (Bailey et al., 2008).

One of the main mechanisms of metformin glucose-lowering actions is through the inhibition of hepatic gluconeogenesis. However, the pathways underlying this action are not clear and additional metabolic actions have been attributed to metformin.

I.3.2.2 Metformin and the control of mitochondrial respiration

The energy-demanding process of hepatic gluconeogenesis requires the production of ATP to compensate with this expenditure, and this is primarily provided by the mitochondria (Rena et al., 2017). Metformin carries a strong positive charge, which favours its internalization into the cytoplasm and subsequent internalization into the mitochondria with negative charges inside, facilitating its accumulation in this organelle and reaching concentrations up to 1000-fold higher than in the extracellular medium (Bridges et al., 2014). There exists a persistent

controversy regarding the mitochondrial action of metformin of inhibiting the complex I of the respiratory chain.

Blocking complex I activity leads to an increase in the AMP/ATP or ADP/ATP ratios, which causes the indirect activation of AMPK and some authors attribute the antidiabetic effects of metformin to the inhibition of this complex (Bridges et al., 2014; Owen et al., 2000). Nevertheless, some authors report that a significant inhibition of complex I of the mitochondrial respiratory chain only takes place when concentrations greater than 5 mM are used in culture (El-Mir et al., 2000; Madiraju et al., 2014), while ≈2 mM concentrations of metformin is required to maximally elevate the AMP/ATP ratio and more potently activate AMPK (Foretz et al., 2010b). In addition, low concentrations of metformin (≤50 μM) are also capable of activating AMPK without affecting mitochondrial respiration or changing AMP/ATP levels (Cao et al., 2014; He and Wondisford, 2015), which are more consistent with the concentrations of metformin found in the portal vein. In fact, at this concentration, ATP levels appear to slightly increase probably induced by the direct activation of AMPK, whose activation mechanism remains currently unclear. These lower concentrations of metformin can also drive the AMPK-mediated phosphorylation of CREB-binding protein (CBP) at Ser436, which leads to the disassembly of the CREB co-activator complex and the inhibition of gluconeogenic genes expression (He and Wondisford, 2015).

Collectively, these studies indicate that metformin may act through different mechanisms that are not fully understood to activate AMPK depending on the concentrations used and having different contributions to the inhibition of gluconeogenesis (**Figure 10**).

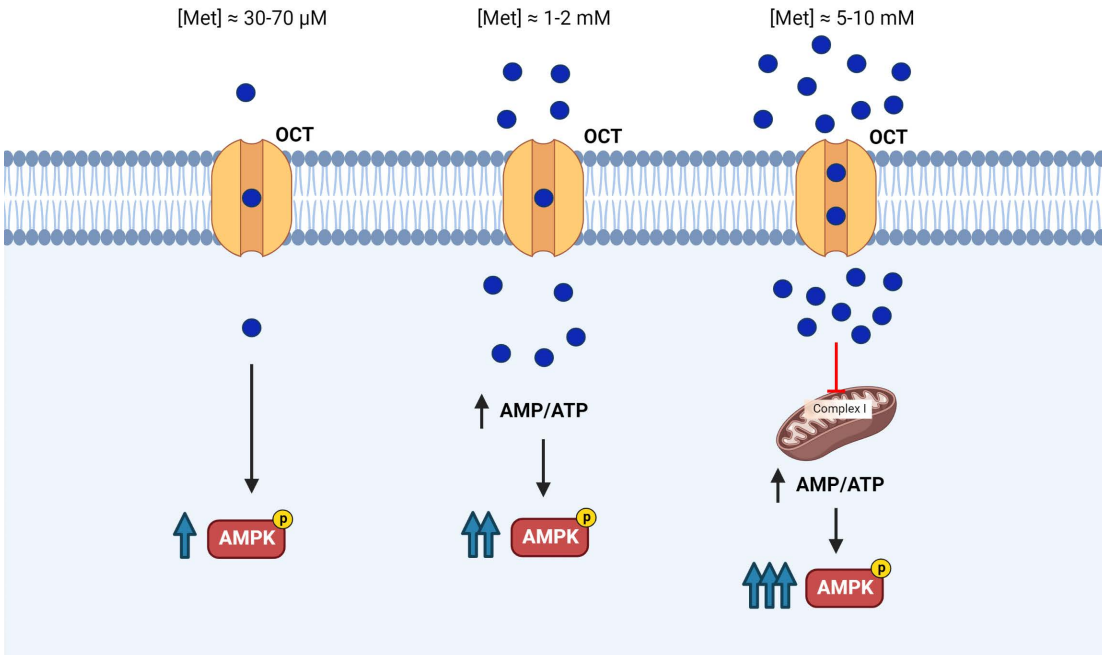


Figure 10 - Dose-dependent mechanism of activation of AMPK caused by metformin treatment. At low concentrations (≈50 µM), metformin can activate AMPK promoting its phosphorylation independently of changes in the AMP/ATP ratio. From 250 µM, metformin increases the AMP/ATP ratio and reaches its maximal effect at 2 mM, inducing AMPK activation by this energy unbalance. Concentrations higher than 5 mM are reported to significantly inhibit complex I of the mitochondrial respiratory chain, increasing the AMP/ATP ratio and potently inducing AMPK activation.

Furthermore, an alternative mitochondrial target for metformin action has been proposed. In cell-free assays, metformin could inhibit mitochondrial glycerophosphate dehydrogenase (GPD) (Madiraju et al., 2014), which impaired the re-oxidation of cytoplasmic NADH and reduced the endogenous glucose production in the liver. Consistent with this, the knockout model of mice lacking GPD were not able to respond to metformin-induced lowering of plasma glucose and endogenous production of glucose (Rena et al., 2017). However, the real influence of the inhibition of complex I and mitochondrial GPD on metformin glucose-lowering effects remain to be further characterized.

I.3.2.3 AMPK in the antidiabetic actions of metformin

The activation of AMPK by metformin is one of the most studied mechanisms of this antidiabetic drug. However, the significance of AMPK activity in the actions of metformin is also a cause of controversy. When mitochondrial function is impaired by metformin, the increase in the AMP/ATP and the ADP/ATP ratios leads to the activation of AMPK in order to switch on catabolic pathways and generate energy to restore the balance (Ross et al., 2016). In 2001, activation of AMPK by metformin treatment was reported in cultured hepatocytes and rat liver (Zhou et al., 2001). Here, activation of AMPK by metformin resulted in the reduction of the activity of ACC, leading to fatty acid oxidation induction and the suppression of lipogenesis. AMPK inhibition blunted the inhibitory effect of metformin on hepatic glucose production. In skeletal muscle, metformin stimulated the AMPK-mediated increase of glucose uptake, explaining the pleiotropic effects of this agent in the treatment of metabolic disorders.

The hypothesis that metformin and other biguanides activate AMPK through variating the intracellular AMP/ATP or ADP/ATP ratios is supported by the observation that the introduction of a genetic modification to generate an AMP-insensitive AMPK complex impairs the AMPK activation caused by metformin (Hawley et al., 2010). However, the idea that AMPK activation is responsible for the anti-gluconeogenic effect of metformin is put in doubt, since metformin (≥250 μM) could directly suppress gluconeogenesis in a mice model lacking the AMPK catalytic subunits (Foretz et al., 2010a). Additionally, the AMP increase might have an independent effect of AMPK, by which it reduces cAMP levels, thus leading to the reduction of the phosphorylation of CREB protein, and preventing the recruitment of the CBP and p300 to subsequently inhibit the expression of gluconeogenic genes (Miller et al., 2013). Activation of AMPK by AMP analogues or by A769662 does not mimic the inhibitory effect of metformin on gluconeogenesis (Agius et al., 2020). However, AMP may allosterically inhibit the gluconeogenic enzyme fructose-1,6-bisphosphatase in acute treatments without the implication of AMPK (Foretz et al., 2010a). Moreover, a short-length clinical trial revealed that in human adipose tissue and skeletal muscle, AMPK activation is not elicited after acute metformin treatment for 1 nor 3 days (Kristensen et al., 2019).

While there is controversy regarding the implication of AMPK in metformin actions, it appears that a division in acute versus long treatments of metformin show differential involvement of AMPK in the effects of this drug. The acute effects of metformin on hepatic glucose production appear to be AMPK-independent in the mentioned studies (Rena et al., 2017).

However, the clinically relevant effect of metformin relates more to long-term studies in which hepatic and systemic insulin sensitivity is enhanced and AMPK plays a major role. Mice generated with a knock-in modification in the ACC isoforms 1 and 2 that presented mutated AMPK-targeted phosphorylation sites and prevented the inactivation of ACC by this kinase showed lower fatty acid oxidation and increased lipogenesis, which contributed to the development of insulin resistance, glucose intolerance and NAFLD (Fullerton et al., 2013). This establishes that the reduction of lipid levels and the insulin-sensitizing effect of metformin after 6 weeks of treatment on mice fed a HFD is mainly driven by the AMPK-mediated phosphorylation of ACC and that this kinase is essential to improve insulin signalling pathways. Moreover, ACC phosphorylation has been reported to require the LKB1-AMPK actions, since knockout of these kinases completely abolishes ACC phosphorylation. Additionally, studies in humans carried out by the Diabetes Prevention Program manifest that at the genetic level, single nucleotide polymorphisms (SNPs) affecting AMPK subunits and LKB are associated with the clinical response to metformin (Jablonski et al., 2010).

Moreover, a 4-week treatment of orally administered metformin to *ob*/*ob* mice retarded body weight gain, improved insulin sensitivity and reduced the intramyocellular lipid accumulation (Wang et al., 2014). The lipid accumulation was also prevented in C2C12 cells by metformin after triglyceride synthesis induction caused by oleic acid treatment. These effects on fatty acid oxidation genes upregulation and the reduction of lipid droplets in skeletal muscle was attributed to the activation of AMPK and the improvement of catabolic pathways by this kinase involving lipids. Interestingly, a recent publication revealed that the accumulation of lipid droplets and impaired autophagic flux caused by saturated fatty acids in endothelial cells was prevented by the treatment with metformin (Kim et al., 2020). AMPK was shown to drive this effect, since the knockdown of this kinase expression

by siRNA transfection blunted this action. AMPK also mediated the metformin-induced reduction of pro-inflammatory responses caused by the ectopic lipid accumulation in vascular endothelium.

In general, the reduction in the accumulation of lipids and their intermediates leads to an attenuation of the inflammatory response and its implication on insulin sensitivity. However, metformin can also suppress the expression of NF-κB or MCP-1 after negative stimuli (Gu et al., 2014). In fact, a short treatment of metformin for 3 hours caused the inhibition of the TNF-α-induced degradation of IκB in mouse primary hepatocytes, mimicking the effects of a pharmacological inhibitor of IKKβ (Cameron et al., 2016). Additionally, metformin supressed the LPS-induced TNF-α and IL-6 expression upregulation (Kim et al., 2014a), which is mediated by the action of AMPK and activating transcription factor 3 (ATF3).

Collectively, these studies manifest the involvement of AMPK activation in the long-term effects of metformin, which mediate the activation of fatty acid oxidation and the reduction of inflammation, leading to an insulin-sensitizing effect.

I.4 Growth differentiation factor 15 (GDF15)

Growth differentiation factor 15 (GDF15), also known as macrophage inhibitory cytokine 1 (MIC-1), placental bone morphogenetic protein (PLAB), placental transforming growth factor beta (PTGFB), or nonsteroidal anti-inflammatory drug (NSAID)-activated gene 1 (NAG-1), is a stress response cytokine that regulates a wide variety of biological processes. In the year 1997, three independent labs discovered this protein simultaneously and named it depending on the context of each research. Subtractive cloning technique allowed Bootcov *et al.* to identify a novel gene transcript expressed in activated monocytoid human cells, one of them being MIC-1 (Bootcov et al., 1997). Its expression is induced by macrophage activation and acts as an autocrine regulator by inhibiting proinflammatory cytokines involved in this process, such as TNF-α. Two separate studies performed to discover novel members of the transforming growth factor β (TGF-β) superfamily in placenta identified and cloned the same gene, termed as

PLAB (Hromas et al., 1997) and PTGFB (Lawton et al., 1997). Later, a different study conducted to elucidate the molecular mechanisms underlying cyclooxygenase (COX) inhibitors identified NAG-1 (Baek et al., 2001b).

All these first studies provided an initial insight of the variety of functions that GDF15 may be involved in. In the metabolic context, this anti-inflammatory cytokine promotes fatty acid oxidation in the liver, improves glucose tolerance and insulin sensitivity and can also regulate food intake and body weight by binding to its neuronal receptor glial cell line-derived neurotrophic factor (GDNF) family receptor α-like (GFRAL), all these actions contributing to the maintaining of metabolic homeostasis in the organism.

GDF15 is secreted into the plasma as a 25 kDa homodimer and its serum levels are increased in response to different stresses including metabolic disorders, different types of cancer, cardiovascular diseases, inflammation, or mitochondrial dysfunction, constituting and important marker for different pathologies.

I.4.1 TGF-β superfamily

The TGF-β superfamily is constituted by a group of proteins that share structural homology and are involved in the regulation of diverse cellular processes, including cell growth, proliferation, adhesion, or apoptosis. TGF-β superfamily proteins are synthesized as precursor proteins located in the extracellular matrix (ECM). After environmental stimuli, the pre-protein undergoes dimerization and is subjected to cleavage by a furin-like protease at a specific site, becoming functionally active.

There are different subfamilies attending to its protein structure or its function (Mueller and Nickel, 2012): the activin/inhibin subfamily, the subfamily of TGF-β, the bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs) subfamily, and the distant members of the TGF-β superfamily **(Table 2)**.

Subfamily	Members of the family	Biological function	Receptors
Activin/Inhibin	Activin A/B Inhibin C/E/A Nodal, Lefty1, Lefty 2	Involved in mesoderm induction, inflammation or immunity, required for body pattern determination and cell stemness	Alk4, Alk7, Cripto ActR-II/IIB
TGF-β	TGF-β1, TGF-β2 and TGF-β3	The smallest family, proliferation and differentiation regulators in multiple cell types. Dual role in its function in cancer progression.	Alk1/Alk5 TβR-II
BMPs and GDFs	BMP-2/4, BMP-5/6/7/8, BMP-9/10 GDF-5/6/7, GDF-8/11, GDF-1/3 GDF-10/BMP-3	BMPs can induce bone growth with different potency depending on cell type. Involved in heart development, skeletogenesis, neurogenesis, or muscle growth control	Alk1, Alk2, Alk3, Alk4, Alk5, Alk6, Cripto BMPR-II, ActR-II, ActR-IIB
Distant members	Anti-Muellerian Hormone (AMH) GDNFs (GDNF, Artemin, Persephin, Neurturin) GDF15	GDNFs act as neurotrophic factors that promote neuron survival and control dopamine uptake. GDF15 is an anti-inflammatory cytokine with no affinity to other family receptors, becoming the most distant member.	Alk3/6, GFRa GFRAL AMHR-II RET

Table 2: TGF-β subfamily composition and members.

TGF-β superfamily ligands bind to two different types of transmembrane receptors for signal transduction through serine/threonine kinase, termed as activin receptor-like kinase (Alk) receptors (type I) and type II receptors. Binding of the TGF-ligands as dimers to these receptors recruits the other type and assembles an heterotetrameric receptor complex formed by two type I receptors and two type II receptors that elicit downstream signalling. Phosphorylation of the type I receptors by the type II complex allows the phosphorylation of the Smad (abbreviation that refers to the homologies to the *Caenorhabditis elegans* SMA

("small" worm phenotype) and MAD family ("Mothers Against Decapentaplegic")) proteins which, once activated, form a complex that is translocated into the nucleus and can act as transcription factors (Morikawa et al., 2016).

GDF15 shares structural homology with the rest of members of the TGF-β superfamily cytokines, and it shares a highly conserved distribution of cysteine residues in the mature domain which is present in other TGF-proteins. However, this cytokine present significant differences that will be further addressed in the next section. For instance, sequence similarity is low with the TGF-β superfamily and the closest relationship is with the GDNF family, the most distant subfamily (**Figure 11**). Moreover, the disulphide bonds that form an intrachain present a different arrangement compared to other 9-cysteine members of the family. Finally, GDF15 ligand only has been reported to bind to a specific receptor, GFRAL, which was considered an orphan receptor until 2017 and has no reported affinity for other TGF-β members. These differences have contributed to the consideration of GDF15 as a divergent member of the TGF-β superfamily (Assadi et al., 2020). More specifically, based on the crystal structures showing the binding of mature GDF15 to GFRAL, it was reclassified as a member of the GDNFs (Hsu et al., 2017b).

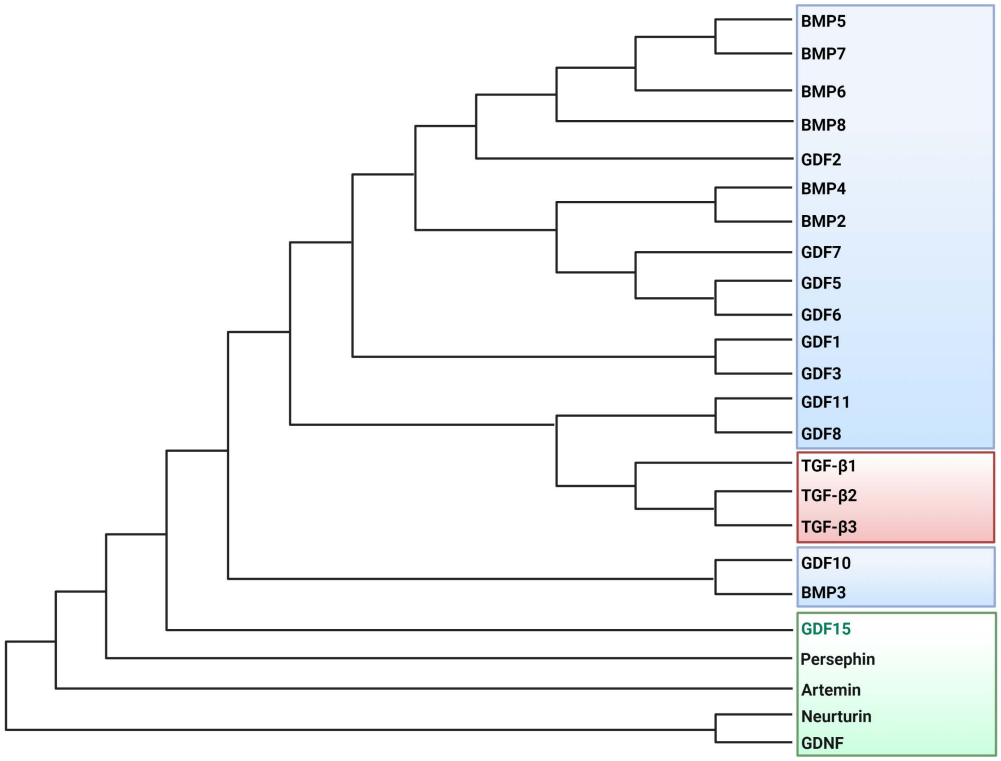


Figure 11. Phylogenetic tree of TGF-β superfamily. 24 members of the TGF-β superfamily arranged according to their sequences homology, including GDF15 as a

distant member, showing the closest relationship to the GDNF family. Blue boxes include BMP/GDF family, red box includes TGF-β family and green box includes GDNF family. Adapted from Assadi et al., 2020.

I.4.2 Molecular biology of GDF15

The GDF15 gene is located in the chromosome 19, in the region p13.11 in humans, while in mice it is located in the chromosome 8, sharing only a 70% of sequence homology (Böttner et al., 1999). GDF15 gene is formed by two exons and one intron of 3,020 bp length, which codes for a transcription product that presents a conserved structure with a sequence of approximately 1200 bp (**Figure 12a**). Of note, the sequence contains 4 instability motifs "ATTTA" in the 3'-UTR region, which are more characteristic of cytokines structure as described by Bootcov et al., who named the protein macrophage inhibitory cytokine 1 (MIC-1). The GDF15 promoter contains several transcription factor binding sites and its expression is regulated by different proteins, including specificity protein 1 (Sp1), p53, CHOP, XBP1, early growth response-1 (EGR-1), ATF3, or PPARγ. The regulation of GDF15 expression by these transcription factors will be further discussed.

The splicing product is translated into a monomer that consists of 308 amino acids in humans and 303 amino acids in mice (Bootcov et al., 1997). This GDF15 pro-protein includes a signal peptide, a pro-peptide at the N-terminus and a mature region at C-terminus (Fairlie et al., 2001), which corresponds to the distinctive structure of the members of TGF-β superfamily. The inclusion of GDF15 as a TGF-β superfamily member relies on the sequence homology and a conserved region of 9 cysteines (Hsu et al., 2017b). The cysteine knot forms four intrachain disulphide bonds and the remaining cysteine residue forms a disulphide bond with the cysteine residue of another GDF15 monomer, undergoing dimerization in the ER. However, this homodimer differs in the disulphide bond organisation compared to the rest of the TGF-β members. Whereas GDF15 forms disulphide bonds from cysteines 1 and 2 and cysteines 3 and 7, the other 9 cysteine TGF-β members form these two bonds

between cysteine 1 and 3 and cysteine 2 and 7, respectively. This confers a unique orientation of the crystal structure of GDF15 (Lockhart et al., 2020).

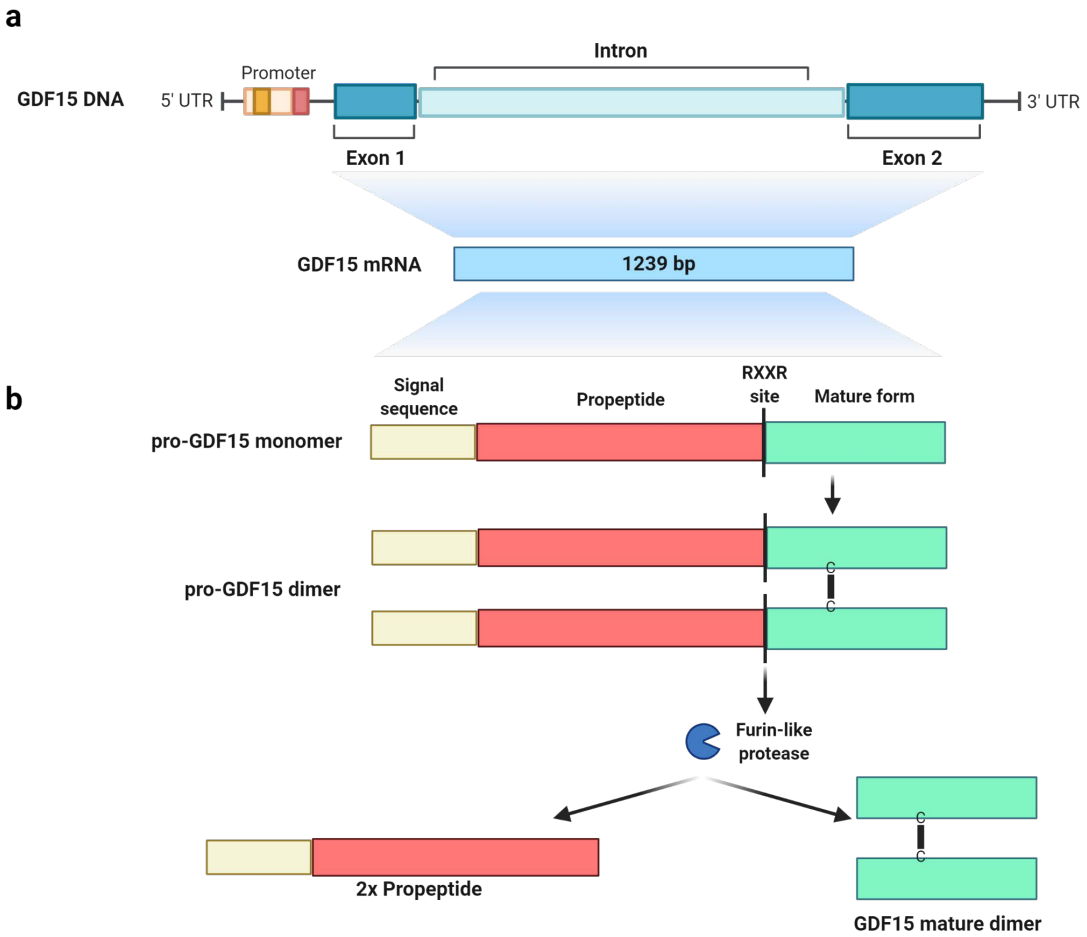


Figure 12. From DNA to mature dimer. Representation of the GDF15 gene, formed by 2 exons and 1 intron, spliced into a 1239 mRNA product and translated into a pro-GDF15 monomer. This pro-GDF15 undergoes dimerization via disulphide bonds and is cleaved by a furin-like protease by its RXXR site, being separated into the propeptide and the GDF15 mature dimer that can be secreted. Adapted from Assadi et al., 2020

The dimerized GDF15 precursor is translocated to the Golgi apparatus from the ER for its posterior processing by a furin-like protease that cleaves the RXXR site of GDF15 (**Figure 12b**), therefore giving rise to multiple forms of GDF15: the pro-GDF15 monomer, the pro-GDF15 dimer, the mature dimer and the N-terminal pro-peptide cleaved from the mature form. Other proteases, including proprotein convertase subtilisin/kexin (PCSK)3, 5 and 6, have been reported to drive the maturation of GDF15 through its cleavage (Li et al., 2018). At this point, mature

GDF15 constitutes a 25 kDa homodimer that is secreted into circulation and is assumed to be the active form of GDF15. Its secondary structure is formed by 6 β-sheets and 1 α-helix per monomer, which are bound by a disulphide link.

The remaining pro-GDF15 monomer has a high affinity for the extracellular matrix (Bauskin et al., 2005) , where it is assumed to rapidly accumulate as a reservoir that can be released upon environmental stimuli, such as inflammation. This characteristic is not present in the mature form (Bauskin et al., 2010). However, pro-GDF15 contains a signal peptide in the N-terminus that determines its nuclear localization. In fact, it was recently reported that pro-GDF15 is able to translocate into the nucleus and control transcriptional regulation (Baek and Eling, 2019). This machinery includes a nuclear pore complex (NPC) by which pro-GDF15 is internalized in an energy-dependent manner (**Figure 13**). Once inside, pro-GDF15 is able to block the binding of the SMAD complex (Smad 2/3 and Smad4 associated with cofactors) to DNA and interrupt the signalling pathway that is promoted by TGF-β1, a pro-tumorigenic factor. Indeed, the accumulation of full-length GDF15 reported by different studies and the resulting inhibition of the SMAD complex transcriptional activity may explain the reduction in tumours observed in mice expressing GDF15 (Min et al., 2016). Nuclear export of pro-GDF15 is carried out by chromosome region maintenance 1 (CRM1), which is an essential step in pro-GDF15 processing, since CRM1 inhibition results in an impairment in the secretion of mature GDF15 (Min et al., 2016).

These recent discoveries suggest a complex regulation of GDF15 from its proteolytic processing until its secretion. Mature GDF15 binding to GFRAL and downstream effects are currently being extensively investigated and will be further discussed in the next sections. However, Min et al. has reported a transcriptional regulation activity of pro-GDF15, and a critical inflexion point by which nuclear internalization and further export is necessary for the secretion of GDF15. Moreover, the possible biological activity of the different unprocessed GDF15 isoforms has not been further explored and may have specific functions depending on diverse factors, including cellular type or receptors involved.

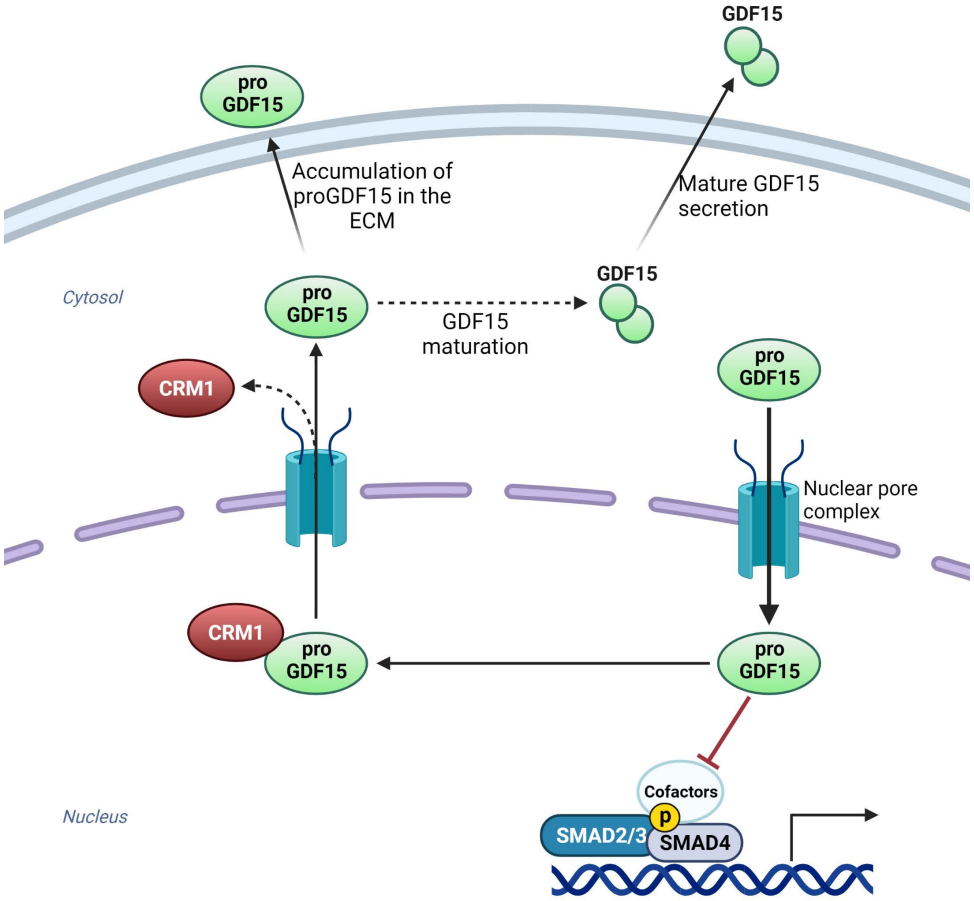


Figure 13. GDF15 translocation process. Dimeric pro-GDF15 is translocated into the nucleus via the nuclear pore complex in an energy-dependent manner. In the nucleus, pro-GDF15 can inhibit the transcriptional activity of the SMAD2/3/4 complex, and binds to CRM1 for translocation again to the cytosol. In the cytosol, pro-GDF15 can be secreted and accumulated in the extracellular matrix (ECM) or be processed to the dimeric mature GDF15 form, which can be secreted into the plasma. Adapted from Baek et al., 2019

I.4.3 GDF15 expression and transcriptional regulation

GDF15 is a widely expressed cytokine in the organism. However, under physiological conditions, the amount of mRNA expressed in the majority is low. Placenta accounts for the highest expression site of *GDF15* (Moore et al., 2000), not without reason was named PLAB by Hromas et al. and PTGFB by Lawton et al. in 1997. It is expressed in the trophoblast cells of the placenta and it increases significantly in the first semester of gestation. The function of GDF15 in this process is believed to inhibit the production of proinflammatory cytokines in order to

promote placentation and maintain pregnancy (Marjono et al., 2003). The liver, lung, kidney, and gastrointestinal tract also express high levels of *GDF15*, as well as prostate in males. Other tissues, like skeletal muscle or adipose tissue show relatively low levels of *GDF15,* which can be robustly promoted after stimuli.

In basal conditions its circulation levels reach 30-100 pg/mL in mice, while humans show unusually high levels for a cytokine, with a range of 200-1000 pg/mL approximately (Tsai et al., 2018). When major pathological conditions are present, like cancer, GDF15 serum concentrations rise up to 10,000-100,000 pg/mL in humans and up to 5000 pg/mL in mice (Assadi et al., 2020). Several studies have proven that GDF15 is a general cell stress-response cytokine, and its tissue expression and serum levels are robustly increased after injury, inflammation, mitochondrial dysfunction, tumour growth and cancer or in metabolic disorders, including obesity, insulin resistance and T2D.

In the liver, GDF15 is upregulated after pathological conditions like NASH (Kim et al., 2018) or liver cirrhosis (Liu et al., 2015) and constitutes a valuable tool for diagnosis and prediction of the severity of chronic liver diseases (Lee et al., 2017). NASH patients generally suffer from obesity, insulin resistance or diabetes. Kim et al. reported that GDF15 is upregulated in mice and human subjects with NASH and involves ER stress induction.

In the gastrointestinal tract, GDF15 has been shown to be implicated in the pathogenesis of colorectal cancer formation (Zimmers et al., 2010) and in the mediation of anti-tumor effects of NSAIDs. Studies with transgenic mice overexpressing GDF15 also allowed to assess the implication of this cytokine against HFD-induced obesity and inflammation by regulating gut microbiota, function of gut barrier and bile acid metabolism (Sang et al., 2019). Moreover, Coll et al. demonstrated that GDF15 mediates the effects of metformin related to energy intake and body weight at least in part by its interaction with GFRAL. The source of GDF15 production was mainly attributed to small intestine, colon and kidney, showing a strong induction of the cytokine in crypt enterocytes (Coll et al., 2020b). However, this study may present some controversy, since the dose of oral metformin used was 600 mg/kg, very far from therapeutic doses, and the *in-situ*

hybridization experiments used to analyse the distribution of GDF15 were carried out after one single administration.

The source production of GDF15 in metabolic disorders and exogenous administration of recombinant GDF15 effects are commonly attributed to the liver, kidney or intestine. However, an emerging evidence is growing about the implication of skeletal muscle in the production, release, and secretion of GDF15. The impairment of the oxidative phosphorylation (OxPhos) process in skeletal muscle mitochondria results in a GDF15-induced improvement of insulin sensitivity and HFD-induced obesity (Chung et al., 2017b). Moreover, recombinant GDF15 (rGDF15) increased lipolysis in cultured adipocytes and elevated insulin sensitivity, mitochondrial fatty acid oxidation and lipolysis in the liver, skeletal muscle and adipose tissue of *ob/ob* mice.

GDF15 has also been designated as an endocrine myokine (Ito et al., 2018), since taurine transporter knockout mice show a marked increase of *Gdf15* expression in skeletal muscle of old mice, contrary to other TGF-β family proteins. Aging-related factors also elicit stress-related induction of GDF15 secretion.

Opposed results have been reported regarding the involvement of exercise in GDF15 production and secretion and the implication of skeletal muscle in this process. Different models of exercise simulation by electrical pulse stimulation and contraction of human cultured myotubes promote the increase of GDF15 protein levels and secretion, in a time-dependent manner (Laurens et al., 2020a). Moreover, both exogenous rGDF15 and endogenously increased GDF15 in conditioned media by electrical stimulation of myotubes caused an increase in the lipolysis rate in cultured human adipocytes. Additionally, maintained exercise increases circulating GDF15 in human studies (Klein et al., 2021a), which is reproducible in mice and show increased *Gdf15* expression in the liver and skeletal muscle also in a time-dependent manner. In this case, the physiological increase of GDF15 is not sufficient to mimic the pharmacological effects of rGDF15 administration on appetite.

Although a significant increase in skeletal muscle production of GDF15 has been reported (Laurens et al., 2020b), the liver levels of GDF15 in a basal state remained 20-fold higher compared to skeletal muscle, casting doubts about the

physiological relevance of skeletal muscle-derived GDF15 in these effects (Gil et al., 2019; Kleinert et al., 2018). Multiple transcription sites and regulation mechanisms have been described for GDF15. We will focus on the main ones:

Effector	Type of regulation	Description	Reference
Sp1/Sp3	Transcription factor	Regulation of the basal transcription of *GDF15*. Binding studies show in HCT-116 the union of Sp1-BC element at the sequence between -73 and -44 in the *GDF15* promoter	(Baek et al., 2001a)
COUP-TF1	Transcription factor	Regulation of the basal transcription of *GDF15*. Binds to GC box located within -133 bp of the *GDF15* promoter.	(Baek et al., 2001a)
p53	Transcription factor	Main regulator of *GDF15* expression upon cancer development, DNA damage and apoptosis, also known as "guardian of the genome". 2 p53 binding sites localized in the *GDF15* promoter.	(Osada et al., 2007)
EGR-1	Transcription factor	EGR-1 binding sites located within the region -73 and -51 in the *GDF15* promoter. Mediation of the response to PPARγ activators and COX inhibitors	(Baek et al., 2005; Baek et al., 2004b)
ATF3	Transcription regulator	Overexpression of *ATF3* increases *GDF15* promoter activity. Also acts as a cofactor involving GSK3β and C/EBPβ activity	(Lee et al., 2010b; Lee et al., 2006c)
ER stress / ISR	Transcription regulator	The PERK branch of UPR activated upon ER stress or ISR promotes ATF4 and CHOP production. The latter promotes the upregulation of *GDF15* by binding to its promoter	(Coll et al., 2020a; Chung et al., 2017a)
XBP1s	Transcription factor	IRE1α-XBP1s branch promotes *GDF15* expression upon metabolic stress to increase hepatic β-oxidation	(Zhang et al., 2018)
PGC-1α - KLF5	Coactivator complex	PGC-1α complex formation with KLF5, which is acetylated and enhances *GDF15* promoter activity	(Dominy et al., 2010)
Demethylation	Epigenetic regulation	Demethylation with the inhibitor of methylation 5-aza-2'-deoxycytidine	(Tsui et al., 2015)

		increase GDF15 mRNA and protein levels in a dose-dependent manner	
Metformin	Pharmacological activation	GDF15 was designated as a novel biomarker for metformin use in a clinical study in 2017. Moreover, GDF15 has been reported to mediate the body weight lowering effects of metformin	(Coll et al., 2020a; Gerstein et al., 2017)
NSAIDs	Pharmacological activation	Antitumorigenic activities of NSAIDs are mediated by GDF15 increase caused by COX-2 inhibition	(Wang et al., 2011a)
Thiazolidinediones	Pharmacological activation	PPARγ-dependent and independent activation of *GDF15* expression in the use of TZDs in cancer treatment	(Baek et al., 2003; Yamaguchi et al., 2006)
Resveratrol	Dietary induction	p53-mediated induction of GDF15 independent of its anti-oxidant activity	(Baek et al., 2002)
Metabolic pathologies	Biomarker	GDF15 has been reported to increase its levels upon mitochondrial dysfunction, NASH, obesity, chronic inflammation, or insulin resistance	(Tsai et al., 2018)

Table 3: Regulation mechanisms of GDF15.

I.4.4 GDF15 in metabolic regulation

GDF15 is involved in many pathologies and its expression is enhanced upon diverse stimuli and controlled by different transcription factors **(Table 3)**. Before the discovery of the brain receptor GFRAL, several studies brought out the implication of GDF15 in metabolic pathways.

GDF15 released from different tissues can act as a metabolic regulator. In fact, it has been described as an adipokine, since Ding et al. identified GDF15 as a secretory product of adipocytes by different stimuli (Ding et al., 2009) and its expression was enhanced in adipose tissue of diabetic patients. The induction of GDF15 has been associated with different metabolic pathologies, including obesity, insulin resistance and diabetes (Adela and Banerjee, 2015). Transgenic overexpression of MIC-1/GDF15 in mice produced a lean phenotype that was associated with a decrease in food intake (Macia et al., 2012). The overexpression of

this cytokine also improved glucose tolerance both in normal and HFD-fed mice. Consequently, GDF15 knockout mice showed an increase in body mass, adiposity, and appetite, with decreased energy expenditure (Tsai et al., 2013). Exogenous administration of rGDF15 from human origin was sufficient to reverse these effects. Other models of HFD-induced obesity that lack GDF15 showed as well increased fat accumulation, higher glucose and insulin levels and worsened glucose tolerance than wild-type littermates (Tran et al., 2018). In this case, male knockout mice exhibited lower locomotor activity, whereas this reduction did not reach statistical significance in females.

Mice constitutively overexpressing GDF15 are leaner, show less white adipose tissue accumulation and are more resistant to diet-induced obesity (Chrysovergis et al., 2014). Interestingly, these mice also display higher glucose tolerance, as well as reduction in the HFD-induced increase of the levels of leptin and insulin. In white adipose tissue, ATGL and HSL are increased, as well as thermogenesis, which mediate the reversion of obesity in mice exposed to a high fat diet.

High concentrations of glucose also elicit a response from GDF15. Human umbilical vein endothelial cells (HUVEC) and hepatocyte cell lines challenged with a high glucose level (Li et al., 2013) or with insulin stimulation (Schernthaner-Reiter et al., 2016) increase GDF15 levels.

In fact, GDF15 ablation in diet-induced NASH in mice models worsens triglyceride accumulation, fibrosis, results in a lower response to insulin and higher transaminase levels compared to WT mice (Lee et al., 2017),. The increase in GDF15 after challenging mice with a NASH-inducing diet seems to be an adaptative response to the enhanced ER stress (Kim et al., 2018). Overexpression of *Gdf15* negatively regulates the expression of fibrosis-related genes and alleviates metabolic deterioration.

In fasting conditions or upon ketogenic feeding, GDF15 mediates the fatty acid β-oxidation process in the liver by an ER stress-induced mechanism (Zhang et al., 2018). The GDF15 promoter contains an ER stress-response element that constitutes an sXBP-1 binding site, which mediates the upregulation of GDF15 upon

nutritional stresses. Liver-specific silencing of GDF15 reduced GDF15 serum levels and the consequent reduction of fatty acid oxidation. Adenovirus-mediated induction of GDF15 promotes insulin sensitivity in *db/db* mice, showing increased lipolysis and fatty acid oxidation, as well as a reduction in hepatic triglyceride levels (Zhang et al., 2018).

Since many of the metabolic characterization studies of GDF15 previously mentioned were reported before characterizing GFRAL as a specific receptor for GDF15, the mechanisms involved in these actions are not fully described. Since the discovery of GFRAL as a specific receptor for GDF15 by Mullican, Emmerson, Hsu and Yang groups, all efforts have been put into elucidating the interactions between this neuronal receptor and GDF15 to shed light on the intrinsic mechanisms by which GDF15 exerts its metabolic actions. The next sections will be focused in exploring the implication of GFRAL in the metabolic effects of GDF15.However, some of the metabolic regulations reported of GDF15 appear to be cell-specific, and autocrine and paracrine effects of this cytokine have been reported in the absence of central nervous system (CNS) mediation.

I.4.4.1 GFRAL: a specific receptor for GDF15

Since the discovery of GDF15 more than 20 years ago, no specific receptor was assessed for this cytokine until recent dates. Different studies reported the activation of TGF-β-related receptors by exogenous GDF15 administration. However, the presence of very small amounts of contaminating TGF-β in the preparations of recombinant GDF15 derived from human expression systems resulted in findings that should be revised (Olsen et al., 2017). This will be addressed in the discussion section.

In 2017, four independent groups from pharmaceutical companies identified GFRAL as the specific receptor for GDF15 (Emmerson et al., 2017; Hsu et al., 2017a; Mullican et al., 2017b; Yang et al., 2017). Hsu et al. used a screening approach with a library of 4,000 cDNAs encoding cell-surface proteins. Only GFRAL generated a robust fluorescent signal by binding to FC-labelled GDF15 and a FC-binding fragment containing a fluorescent tag. However, 10 constructs also demonstrated a

2-fold increase compared to control construct, although this increase was 1 order of magnitude lower than GFRAL.

GFRAL was previously classified as a distant homolog of the GDNF receptors family, but it was considered an orphan receptor since its identification and characterization (Li et al., 2005). This receptor is composed by 393 amino acid with a molecular weight of 44 kDa and presents a 70% of homology structure between human and mouse. The cytosolic domain represents only 23 amino acids and contains no signalling motifs and therefore no kinase activity. This suggests that this motif only confers anchoring function, and that recruitment of another receptor is needed for its downstream effects. On its discovering as a GDF15 receptor in 2017, it was defined as a brainstem-restricted receptor (Hsu et al., 2017b), since GFRAL mRNA and protein location is limited to a specific subset of neurons in the area postrema (AP) in all the analysed species (Mullican and Rangwala, 2018). Previously, Tsai et al. already reported that AP and the nucleus *tractus solitarius* (NTS) in the brainstem was required for GDF15 anorectic effect (Tsai et al., 2014).

The GDNF family consists of four proteins that do not bind and elicit signalling pathways through type I or II TGF-β receptors and is formed by GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN). These ligands bind with high affinity to the GDNF family of receptors: GFRA1, GFRA2, GFRA3 and GFRA4, respectively, but do not bind GFRAL. Similarly, GDF15 binds with high affinity to GFRAL but it is not capable of binding other GDNF family of receptors, making this union strongly specific (Mullican and Rangwala, 2018).

The canonical pathway elicited by the union of GDNF family ligands to GFRA1-4 and GFRAL receptors requires the participation of the tyrosine kinase co-receptor RET (rearranged during transfection). Binding of GDF15 to GFRAL provokes the recruitment and binding of the homodimer, which is actively arranged for the co-activation of RET. GFRAL-GDF15 complex binds to the tyrosine kinase co-receptor RET, which is subsequently phosphorylated and leads to the downstream activation of Akt, ERK1/2 and phospholipase C (PLC) (**Figure 14**), but not the SMAD pathway regulated by TGF-β (Assadi et al., 2020). This pathway has been described for GFRAL-Ret co-transfected cells; in the mouse hindbrain, GDF15 can induce Fos immunoreactivity and SP6 activation (Tsai et al., 2014). This is in contrast to other

non-distant members of the TGF-β superfamily that bind either TGF-β receptor I (TBRI) or TBRII (also including the denomination activin receptor-like kinases (ALKs) 1-7), which induce trans-phosphorylation of the receptor and activate the canonical Smad signalling pathway (Zhang, 2017).

Taken together, these studies report the anti-obesity and metabolic effects of GDF15 on weight regulation and food intake in mice, rats, and nonhuman primates. However, the full implication of the brainstem-restricted GFRAL receptor in the metabolic effects of GDF15 is not clearly defined, as this cytokine may act through other mechanisms that are not fully understood.

I.4.4.1.1 GFRAL-dependent actions of GDF15:

In 2013, Tsai et al. explored the actions of GDF15 in the context of appetite regulation, since GDF15 is believed to drive anorexia and cachexia by its overproduction in cancer. As previously mentioned, *Gdf15*-knockout mice manifest increased body weight and adiposity and augmented food intake (Tsai et al., 2013). Conversely, infusion of human rGDF15 reversed these effects. Since the GDF15-driven appetite reduction is thought to depend on hypothalamus and brainstem regulation (major centres of appetite regulation), one year later Tsai et al. analysed the neuronal activation location after demonstrating that rGDF15 intracerebroventricular injection also caused a reduction in appetite and body weight. Systemic administration of rGDF15 induced Fos in the AP and the NTS of the brainstem (Tsai et al., 2014). Interestingly, ablation of the AP and the NTS of the brainstem completely abolished the anorectic effect of GDF15. However, ablation of the AP solely resulted in a slight inhibition. These studies provided a first insight into the regulation of GDF15-induced reduction in appetite and consequent body weight decrease, but the identification of a receptor remained elusive.

This and previous studies allowed the identification and characterization of GFRAL as a brainstem-restricted receptor for GDF15. The binding between GDF15 and the neuronal receptor GFRAL triggers an intracellular signalling in the brainstem neurons that induce anti-obesity effects in the organism (**Figure 14**). This

effect is limited to central mechanisms, since GFRAL is only expressed in hindbrain neurons and not in peripheral tissues (Yang et al., 2017).

Since its discovery as a direct receptor for GDF15, GFRAL has been strictly restricted to the AP and the NTS of the brainstem. Nevertheless, recently it has been reported that abdominal adipose tissue in humans show substantial expression of GFRAL accompanied by the presence of its coreceptor RET (Laurens et al., 2020a). Interestingly, this location appears to be species-specific, since adult female mice lack expression of GFRAL in white adipose tissue. Moreover, neither human nor mice show detectable levels of GFRAL in whole muscle tissue, contrary to RET, which is widely expressed but is also involved in the regulation of other pathways. Laurens et al. brought out that cultured myotubes increase the production and secretion of GDF15 upon electrical stimulation, thereby resembling exercise. Conditioned media from these myocytes elicited a lipolytic response in human cultured adipocytes, which was mediated by GFRAL-RET activation, as stated in this study. However, the intrinsic mechanism was not described and the mediation of GFRAL in GDF15-induced lipolysis in adipocytes remains hypothetical, implying that other receptors may be involved in this action.

Mitochondrial dysfunction is known to promote *Gdf15* upregulation. A transgenic mouse model that presents muscle-specific overexpression of *Ucp1* showed compromised oxidative capacity in the mitochondria (Ost et al., 2020). This elicited the upregulation of *Gdf15* and brought out its major role as a myokine, since the increase of this cytokine showed a daytime-restricted induction of anorexia, which promoted enhanced systemic metabolism, insulin sensitivity and white adipose tissue browning.

Additionally, the administration of an antagonist of GFRAL prevented the GDF15-mediated dimerization with RET in tumor-bearing mice that caused excessive lipid consumption and protected them against anorexia and cachexia (Suriben et al., 2020). It was reported that the development of cachexia in these mice was driven by an overexpression of ATGL and HSL by the signal transduction elicited by GFRAL. Ablation of the *Atgl* gene impaired the loss of adipose tissue caused by the treatment of rGDF15.

Importantly, different studies revealed that a decrease in body weight and fat mass may not be exclusively dependent on a reduction of food intake (Baek and Eling, 2019). The transcriptional regulator interacting with the PHD-bromodomain (TRIP-Br2) is involved in the regulation of lipid metabolism and energy expenditure and its ablation causes resistance to obesity and prevention of insulin resistance, mainly due to an enhanced expression of lipolytic genes and an increase of adipocyte thermogenesis and oxidative metabolism (Liew et al., 2013). Interestingly, some of these effects mimic the metabolic actions of GDF15 on lipid processing and oxidation. However, these events were produced in the absence of appetite suppression. In the case of GDF15, GFRAL may regulate the lipolytic pathways implicated in the mobilization of fatty acids and the appetite suppression. Nevertheless, the metabolic actions of GDF15 on fatty acid oxidation and the prevention of insulin resistance may involve other mechanisms.

I.4.4.1.2 GFRAL-independent actions of GDF15:

GDF15 was first characterized as MIC-1 by Bootcov et al. in 1997 and was classified as a distant member of the TGF-β superfamily and closer in sequence similarity to the BMP subfamily. In human monocytoid cell lines, GDF15 was not expressed, but macrophage chemical activation induced a great expression increase. TNFα or IL-1β induce the expression of GDF15 (Bootcov et al., 1997). These cytokines are secreted by activated macrophages and, conversely, LPS-stimulated increase of TNFα in macrophages is reduced in a dose-dependent manner by recombinant GDF15, suggesting an autocrine/paracrine regulation of this cytokine in the peripheral system (**Figure 14**). Until date, no GFRAL expression has been documented in activated macrophages.

In line with this, GDF15-knockout mice studies showed that lack of GDF15 increased inflammatory response and worsened renal and cardiac injury induced by LPS (Abulizi et al., 2017). In parallel, GDF15 overexpression had a protective effect on these organs upon LPS-induced dysfunction. Although the intrinsic mechanism for this function was not provided, LPS-induced apoptosis was diminished by recombinant GDF15 in primary kidney tubular cells, where GFRAL expression was

likely null. Moreover, in a physiological condition, GDF15 is reported to act as an immunosuppressor in placenta to reduce fetal exposure to endogenous cytokines (Corre et al., 2013).

Additionally, other mechanisms involving GDF15 action have been described. GDF15 is able to inhibit the norepinephrine-induced increase of epidermal growth factor receptor (EGFR) by a mechanism involving β-arrestin1. This decreases Akt and ERK1/2 phosphorylation as downstream targets for EGFR in cultured cardiomyocytes (Xu et al., 2014a). This is interesting as it is opposed to the canonical downstream effects described after GDF15-GFRAL binding, since this union elicits an increase response of the Akt and ERK1/2 pathways regulated by RET.

In HUVECs, a high glucose stimulus induces the expression and the protein levels increase of GDF15 (Li et al., 2013). This adaptative response of GDF15 is reactive oxygen species (ROS) and p53-dependent and is essential as an anti-apoptotic mechanism in this type of cells. This is mediated by the GDF15 regulation of the PI3K/Akt/endothelial nitric oxide synthase (eNOS) signalling pathway responsible for the protection against ROS-induced cell injury. Furthermore, GDF15 expression was shown to attenuate the NF-κB and JNK activation upon ROS activation, demonstrating the implication of GDF15 in the reduction of inflammation in a cell line that has not been reported to express GFRAL.

Additionally, brown adipose tissue has been reported to secrete GDF15 after thermogenic stimulus (Campderrós et al., 2019), in parallel to the increase in UCP1 mRNA levels. This increase appears to be mediated by an autocrine regulation involving FGF21. In turn, GDF15 released by brown adipocytes showed an anti-inflammatory response in targeted macrophages previously induced to a proinflammatory M1 state after LPS treatment.

The source production of GDF15 in metabolic disorders and exogenous administration of rGDF15 effects are commonly attributed to the liver, kidney or intestine. However, an emerging evidence is growing about the implication of skeletal muscle in the production, release, and secretion of GDF15. In 2015, Kim et al. designed a mouse model that lacked CD6-interacting factor-1 (CRIF1) in islet beta cells (Kim et al., 2015b), that results in OxPhos deficiency *in vivo*. Later, a skeletal

muscle-specific *Crif1*-knockout model (MKO) was implemented in order to impair OxPhos in this tissue, since CRIF1 is a mitoribosome that is responsible for the production of the OxPhos components in mitochondria, and resulted in the improvement of insulin sensitivity and a protection from HFD-induced obesity (Chung et al., 2017a). This was accompanied by a robust increase of *Gdf15* expression, secretion, and plasma levels, therefore being denominated as myomitokine. This increase was associated with the increase of CHOP and its binding to two putative responsive elements in the human *GDF15* promoter. Double *Gdf15*$^{-/-}$/MKO mice showed a worsened metabolic phenotype compared to MKO mice regarding body weight, blood glucose and hepatic steatosis. Moreover, pharmacological effects of GDF15 caused by administration of rGDF15 were assessed in order to elucidate its effects on obesity and insulin sensitivity. Administration of rGDF15 at a dose of 0.5 mg/kg for 3 weeks caused body weight reduction, independently of changes in food intake (Chung et al., 2017a).

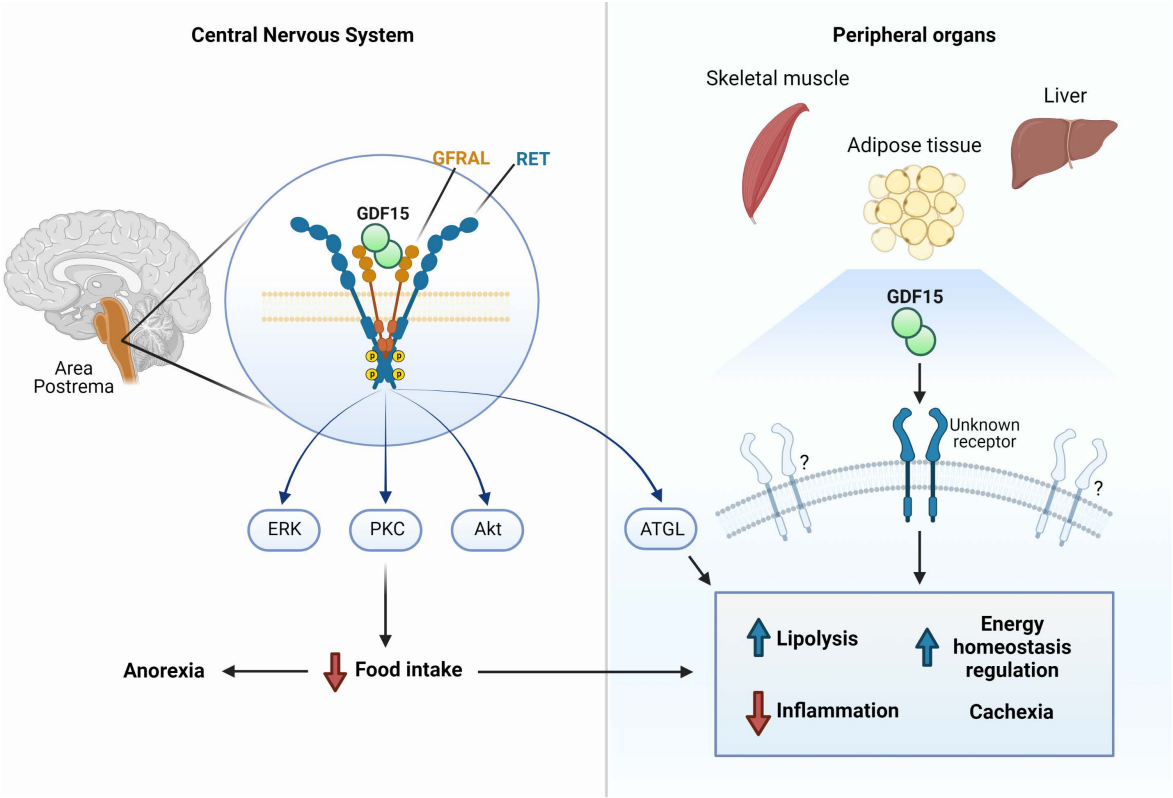


Figure 14. GDF15 effects in central and peripheral systems. GDF15 binds to the brain receptor GFRAL located mainly in the area postrema of the brainstem, which then forms a homodimer. GFRAL homodimer recruits RET and induces its dimerization, subsequently activating the downstream targets ERK1/2, PKC/PLC and Akt, and therefore reducing food consumption. In addition, GFRAL/RET activate ATGL in peripheral tissues. Additionally, several effects of GDF15 in peripheral tissues have been reported without the mediation of GFRAL. GDF15 may act through an unknown receptor or different regulatory mechanisms, and combination of these effects and those from GFRAL regulate lipolysis, energy homeostasis, inflammation and cachexia in target tissues.

Further metabolic studies regarding metabolic pathways of GDF15 need to be conducted in order to determine whether these effects on lipid metabolism observed in different studies are mediated by GFRAL or whether other mechanisms or receptors are involved in the metabolic actions of GDF15.

II. OBJECTIVES

T2D is a multifactorial disease that comprises metabolic defects in multiple organs (DeFronzo, 2009). Despite its growing incidence, the current available drugs for the treatment of T2D show a limited efficacy and significant side effects that are not fully controlled (Ghasemi and Norouzirad, 2019). For that reason, there is an urgent need to discover new therapeutic targets that can control the complexity of the disease.

In this regard, PPARβ/δ is a transcription factor that regulates glucose and lipid metabolism, as well as inflammation and insulin signalling (Da'adoosh et al., 2019). Treatment of insulin resistance and T2D with PPARβ/δ agonists has been proven effective (Vázquez-Carrera, 2016), however, clinical trials using PPARβ/δ agonists are scarce and its relationship with cancer development is controversial (Müller, 2017). On the other hand, metformin is the first-line oral drug treatment for T2D (Inzucchi et al., 2015), however, the molecular mechanisms by which this drug exerts its antidiabetic actions are not fully understood (Rena et al., 2017). Interestingly, many of the antidiabetic effects of PPARβ/δ activation and metformin rely on AMPK activation and present similarities with the actions of GDF15 on lipid metabolism, inflammation and insulin signalling (Baek and Eling, 2019).

Considering this, the general aim of the present doctoral thesis has been to shed light on the mechanism of action of two well-known antidiabetic agents in order to characterize a new therapeutic target for the potential treatment of insulin resistance and T2D. To accomplish this aim, the following objectives were set up and divided into two differentiated parts:

Part I - Implication of GDF15 in the antidiabetic actions of PPARβ/δ

- To evaluate whether the antidiabetic effects of PPARβ/δ agonists on lipid metabolism, ER stress, inflammation and insulin resistance are mediated by GDF15.
- To define the potential molecular mechanism by which PPARβ/δ increases GDF15 levels.
- To examine whether the potential involvement of GDF15 in PPARβ/δ actions requires central mechanisms.

- To explore whether AMPK activation by PPARβ/δ requires GDF15.

Part II - Implication of GDF15 in the AMPK-mediated metabolic actions of metformin

- To define the molecular mechanism by which metformin increases GDF15 levels.
- To evaluate whether the metabolic effects of metformin treatment on lipid metabolism, ER stress, inflammation and insulin resistance are mediated by GDF15.
- To evaluate the role of GDF15 in the activation of AMPK by metformin.
- To determine the involvement of central mechanisms in the GDF15-mediated effects of metformin.

III. MATERIALS AND METHODS

III.1 Reagents

The chemicals, compounds, reagents, commercial detection kits and recombinant proteins used in the present thesis are summarized in the next table:

Product	Manufacturer	Reference
A769662	Tocris Bioscience	3336
β-Hydroxybutyrate detection kit	Sigma-Aldrich	MAK041
Compound C	Santa Cruz	sc-200689
Eosin Y	Merck	230351
GDF15 ELISA detection kit	Biorbyt	Orb391081
GDF15 (human, recombinant)	PeproTech	120-28
GDF15 (murine, recombinant)	R&D Systems	8944-GD
GDF15 neutralizing antibody	R&D Systems	AF957
GW501516	Sigma-Aldrich	SML1491
Hematoxylin Solution	Merck	MHS32
Lipofectamine 2000	Thermo-Fisher	11668027
Dimethylbiguanide hydrochloride (Metformin)	Sigma-Aldrich	D150959
Pifithrin alpha p-nitro, cyclic (for cell culture)	Millipore	506154
Pifithrin alpha p-nitro (for *in vivo* studies)	Santa Cruz	sc-222176
SB431542	PeproTech	3014193
siRNA Control	Santa Cruz	sc-37007

siRNA ATF3	Santa Cruz	sc-29758
siRNA PPARβ/δ	Santa Cruz	sc-36306
siRNA AMPK1/2	Santa Cruz	sc-45313
siRNA p53	Santa Cruz	sc-29436
siRNA GDF15	Santa Cruz	sc-39799
Triglyceride determination kit	Spinreact	41031

Table 4. List of reagents used for cell and *in vivo* experiments

III.2 Animal experiments

Male WT C57BL/6J mice (Envigo, Barcelona, Spain) and *Gdf15* null (*Gdf15*$^{-/-}$) mice with their WT littermates (C57BL/6/129/SvJ background) were housed and maintained under constant conditions of lighting (12 hours light-dark cycles), temperature (22 ± 2°C) and humidity (55%). The mice were constantly fed a standard diet and supplied with freshwater *ad libitum* and were randomly distributed into different groups in cages after 1 week of acclimatization.

This research complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th edition: National Academies Press; 2011). All procedures were approved by the Bioethics Committee of the University of Barcelona, as stated in Law 5/21 July 1995 passed by the Generalitat de Catalunya.

All experimental procedures carried out in animals were performed in accordance with the ARRIVE Guidelines (McGrath and Lilley, 2015), maximizing the Replacement, Refinement and Reduction in these studies when possible. All animals received a humane treatment, always trying to minimize the suffering in the experimental procedures.

III.2.1 PPARβ/δ agonist (GW501516) administration

12-week-old male C57BL/6J mice were randomly distributed into six different experimental groups (n=5). The control groups received a daily administration of carboxymethylcellulose (CMC) 0,5% w/v (vehicle) by oral gavage and the treated groups received a daily dose of 3 mg/kg/day of the PPARβ/δ agonist GW501516 dissolved in CMC for 6 days, at a final volume of administration of 10 ml/kg. This dose of the agonist has been previously reported to activate specifically PPARβ/δ over other PPAR isoforms (Lee et al., 2006b). Treatments were carried out for 2, 4 or 6 days and after the end of the experiments mice were sacrificed by cervical dislocation under isoflurane (IsoFlo, Esteve) anaesthesia and blood, liver and skeletal muscle (gastrocnemius) samples were collected. Blood samples were stored in blood collection tubes to later separate and obtain the serum, whereas tissue samples were snap-frozen in liquid nitrogen and then stored at -80°C.

III.2.2 PPARβ/δ agonist (GW0742) administration

12-week-old male C57BL/6J mice were randomly distributed into two different experimental groups (n=5). The control group received a daily administration of carboxymethylcellulose (CMC) 0,5% w/v (vehicle) by oral gavage and the treated groups received a daily dose of 3 mg/kg/day of the PPARβ/δ agonist GW0742 dissolved in CMC for 6 days, at a final volume of administration of 10 ml/kg. This experiment was carried out in order to discern the specificity of GDF15 increase with different PPARβ/δ agonists . After 6 days of treatment, mice were sacrificed by cervical dislocation under isoflurane (IsoFlo, Esteve) anaesthesia and skeletal muscle (gastrocnemius) samples were collected. Tissue samples were snap-frozen in liquid nitrogen and then stored at -80°C.

III.2.3 GDF15 neutralizing antibody on GW501516-treated mice

12-week-old male C57BL/6J mice were randomly distributed into four different groups (n=5). The control group was fed a standard diet (Teklad 2018, Envigo) and received a daily oral administration of CMC 0,5% w/v (vehicle). A

second group was fed a Western-type high fat diet (HFD) (45% of Kcal from fat, product D12451 from Research Diets Inc., New Brunswick, NJ) and received a daily oral administration of vehicle. The third and fourth group were fed a HFD and were treated with 3 mg/kg/day of the PPARβ/δ agonist GW501516 dissolved in the vehicle, administered by oral gavage. These conditions were maintained for 21 days. Three days before the end of the treatment, mice in the first three groups received an intraperitoneal injection of murine IgG (40µg/mouse) as control and the last group received an intraperitoneal injection of a neutralizing antibody (40µg/mouse) against GDF15, as reported elsewhere (Sasahara et al., 2017) to block GDF15 functions. Two days after the antibody or IgG administration, a glucose tolerance test (GTT, later described) was performed. After the 21-days treatment, mice were sacrificed by cervical dislocation under isoflurane (IsoFlo, Esteve) anaesthesia and blood, liver and skeletal muscle (gastrocnemius) samples were collected. Food consumption and body weight were regularly measured throughout the experiment.

III.2.4 GDF15 neutralizing antibody efficacy

12-week-old male C57BL/6J mice were randomly distributed into three different groups (n=5) and each group distributed into three different cages, all receiving a standard diet. Mice were intraperitoneally injected with the GDF15 neutralizing antibody or IgG 48h before receiving 0.1 mg/kg of recombinant GDF15 or saline solution administered via subcutaneous injection, in order to assess the efficacy of the GDF15 neutralizing antibody. The administration of recombinant GDF15 was performed at 18:00 h and measurement of food consumption was performed the next day at 8:00 h. This was performed considering that serum GDF15 reaches maximal levels at ≈4h after injection of the recombinant GDF15 and differences in food intake are significant between 8 and 48 hours after the administration (Borner et al., 2020).

III.2.5 Pifithrin-α p-nitro

12-week-old male C57BL/6J mice were randomly distributed into three different groups (n=5), all receiving a standard diet. Control group received a daily oral dose of CMC 0,5% w/v (vehicle) plus an intraperitoneal administration of 1:10 solution of dimethyl sulfoxide (DMSO) in phosphate buffered saline (PBS) every two days. A second group received a daily oral dose of 3 mg/kg/day GW501516 plus the DMSO:PBS solution ant the third group received the oral dose of 3 mg/kg/day GW501516 plus an intraperitoneal administration of the p53 selective inhibitor pifithrin-α, p-nitro (2.2 mg/kg) dissolved in the DMSO:PBS solution every two days. After the 6 days of treatment, mice were sacrificed by cervical dislocation under isoflurane and liver and skeletal muscle (gastrocnemius) samples were collected. Tissue samples were snap-frozen in liquid nitrogen and then stored at -80°C.

III.2.6 Recombinant GDF15

12-week-old male C57BL/6J mice were randomly distributed into two different groups (n=6), both fed a standard diet. The control group received a subcutaneous injection of sterile physiological saline solution (vehicle) and the treated group received a subcutaneous injection of 0.05 mg/kg of recombinant GDF15 protein (cell culture derived, from human origin) in sterile physiological saline solution, twice a day for two days. Previously, lyophilized GDF15 was reconstituted in sterile 4 mM HCl. The animals were sacrificed two hours after the last injection by cervical dislocation under isoflurane and skeletal muscle samples were collected. Tissue samples were snap-frozen in liquid nitrogen and then stored at -80°C.

III.2.7 *Gdf15*$^{-/-}$ mice treated with a GW501516

Gdf15 null mice and their wild-type littermates were kindly donated by Dr. Se-Jin Lee, from the Johns Hopkins University School of Medicine. The generation of the knockout mice for the *Gdf15* gene was described by Se-Jin Lee and colleagues (Hsiao et al., 2000). These mice present a truncated variation of the *Gdf15* gene that

ablates its function. The *Gdf15* null mice and their wild-type littermates were maintained on a hybrid C57BL/6/129/SvJ background.

Gdf15 null mice and their WT littermates were randomly distributed respectively into different groups (n=5) and fed either a standard or a high fat diet. From the WT mice, one control group received a daily oral dose of CMC 0,5% w/v (vehicle). A second group was fed a HFD and received a daily oral dose of vehicle. The third group was fed a HFD and was treated daily with 3 mg/kg of GW501516 administered by oral gavage. The *Gdf15* null mice were also distributed into three different groups and the treatment received was identical. Three days before the end of the treatment, a GTT was performed. At the day 21, mice were sacrificed, and blood, skeletal muscle (gastrocnemius) and liver samples were collected. For histological staining studies, liver sections were collected in paraformaldehyde (PFA) 4% in PBS or embedded in a cryomold containing optimal temperature cutting (OCT) compound, for cryopreservation and later cryosectioning. Food consumption and body weight were regularly measured throughout the experiment.

III.2.8 *Gdf15*$^{-/-}$ mice treated with metformin

Gdf15 null mice and their WT littermates were randomly distributed respectively into different groups (n=5) and fed either a standard or a high fat diet. From the WT mice, one control group received a daily oral dose of CMC 0,5% w/v (vehicle). A second group was fed a HFD and received a daily oral dose of vehicle. The third group was fed a HFD and was treated daily with 100 mg/kg of metformin administered by oral gavage. The *Gdf15* null mice were also distributed into three different groups and the treatment received was identical. Three days before the end of the treatment, a GTT was performed. At the day 21, mice were sacrificed, and blood, skeletal muscle (gastrocnemius) and liver samples were collected. For histological staining studies, liver sections were collected in paraformaldehyde (PFA) 4% in PBS or embedded in a cryomold containing optimal temperature cutting (OCT) compound, for cryopreservation and later cryosectioning. Food consumption and body weight were regularly measured throughout the experiment.

III.2.9 *Gfral* detection in brain and peripheral tissues

12-week-old male C57BL/6J mice were fed a standard diet (n=5). Brainstem dissection was performed as previously described (Whitebirch, 2020) and skeletal muscle (gastrocnemius) and liver were obtained and snap-frozen in liquid nitrogen for further characterization of *Gfral* expression.

III.3 Glucose tolerance test (GTT)

Three days before the sacrifice, a glucose tolerance test was conducted on mice that were fasted for 6 hours. Mice body weight was measured before the start of the test, in order to administer 2 g/kg of glucose to each animal by intraperitoneal injection. Blood was collected from the tail vein after 0, 15, 30, 60 and 120 minutes from the glucose injection and measured at the same time by a blood glucose meter (Accu-Chek) in mg/dl. Area under the curve (AUC) was calculated from the values obtained in the measurements and were used for the statistical analysis of the different groups.

III.4 β-hydroxibutyrate, GDF15 and triglyceride determination

Serum obtained from blood collection in the different studies was used to determine the levels of β-hydroxybutyrate, GDF15 and triglycerides present in the different samples, according to manufacturer instructions.

III.5 Liver histology

For histological staining studies, liver sections were collected in 4% paraformaldehyde in PBS (for hematoxylin and eosin staining) or embedded in a cryomold containing optimal temperature cutting (OCT, Tissue-Tek) compound for cryopreservation and later cryosectioning to perform Oil Red O (ORO) staining. Liver sections were deparaffinized and rehydrated by consecutive washes with xylene and ethanol at different concentrations and hematoxylin and eosin staining was performed as described elsewhere (Feldman and Wolfe, 2014). Microscopy

images of ORO-stained histological sections were quantified using the IHC profiler plugin of the Image J software.

III.6 *Ex vivo* experiment

Soleus muscles were dissected from anesthetized mice, using isoflurane, and incubated *in vitro* as previously described (Gumà et al., 1988) with some modifications as following indicated. The soleus muscles of each mice were incubated in a single vial containing 4 mL of the incubation medium for a recovery period of 10 minutes at 34 ºC. Incubation medium consists of Krebs-Hepes buffer supplemented with 0.2% dialysed bovine serum albumin (BSA) and glucose 5 mM, in order to sustain a physiological range of energy levels. After that, muscles were placed in new vials containing 3 mL of the incubation medium in the presence or absence of the respective treatments, GW501516 or rGDF15, for 90 minutes. Afterwards, two consecutive replacements of the muscles were done in vials containing fresh medium and maintaining the respective treatments for a period of 90 and 60 minutes respectively, in order to ensure the viability of the soleus muscles. At the end of the treatment, soleus muscles were washed with fresh saline buffer and snap-frozen in liquid nitrogen for posterior processing.

III.7 Cell Culture

III.7.1 C2C12

The mouse myoblast cell line C2C12 (ATCC) was maintained in Dulbecco's modified Eagle's Medium (DMEM, Thermo-Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Sigma), 50 units/ml penicillin and 50 μg/ml streptomycin (Thermo-Fisher Scientific). Cell passages were performed every 2 days using trypsin/EDTA solution before reaching more than 60% confluence to detach cells from the surface and were either reseeded at approximately 1:20 dilution or stored at -80° in a solution containing 1:10 DMSO in culture medium. For the different treatments performed, when cells reached 90-100% confluence, medium was changed to differentiation medium containing DMEM and 5% horse

serum (Thermo-Fisher Scientific) plus penicillin/streptomycin; medium was changed every 2 days. After 4 days from the medium switch, C2C12 cells were differentiated and had fused into myotubes.

III.7.2 HUH7

The human hepatoma cell line HUH7 (kindly donated by Dr. Mayka Sanchez from the Josep Carreras Leukemia Research Institute) was maintained in DMEM supplemented with 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin. Cell passages were performed every 3-4 days, always before cells reached confluence. Different treatments were performed when cells reached 80-90% confluency.

III.7.3 Primary hepatocytes

Primary mouse hepatocytes were isolated from non-fasting male C57BL/6 mice (10–12 weeks old) by perfusion with collagenase as described elsewhere (Benveniste et al., 1988).

After the corresponding treatment, cells were collected from plate wells in PBS 1X (Sigma) by a cell scratcher and centrifuged at 10.000 g for 2 minutes and at 4°C. Supernatant was discarded and pellet was kept for protein extraction. For RNA extraction, cells were collected by direct homogenization with Trisure (Bioline) reagent to isolate RNA.

III.7.4 Cell treatments

<u>GW501516 and A769662</u>

For all the cell treatments, 1 hour before treating the cells medium was changed to DMEM containing 50 units/ml penicillin and 50 µg/ml streptomycin, in the absence of horse or fetal bovine serum. Myotubes were then incubated in serum-free DMEM in the presence of the PPARβ/δ agonist GW501516 or the AMPK

activator A769662 at a final concentration of 10 μM or 60 μM respectively or in the presence of DMSO at the same concentrations for control groups, during 24h before collecting the cells for RNA or protein extraction. A GW501516 additional treatment was carried out in C2C12 myotubes for 1, 3, 6, 12 and 18 hours.

GSK3787

The PPARβ/δ antagonist GSK3787 was added into the culture medium 1 hour before the corresponding treatment.

Compound C

The AMPK inhibitor Compound C (Santa Cruz) was used at a final concentration of 30 μM and incubated alone or co-incubated with GW501516 or A769662, for 24h.

siRNA transfection

For RNA silencing experiments, the medium of differentiated myotubes was changed one day before transfection to DMEM without serum or antibiotics, to avoid interferences with the siRNAs. Lipofectamine 2000 (7 μl/1.5 ml well, Thermo-Fisher) was used as the transfection agent to conjugate the Control siRNA (Santa Cruz) or the siRNA oligomers against PPARβ/δ, AMPK1/2, ATF3, GDF15 or p53 (70 μM, Santa Cruz) in Opti-MEM medium (Sigma) at a final volume of 500 μl for each well, according to the manufacturer's instruction. These complexes were incubated for 30 minutes at room temperature and then added to the wells containing 1 ml of incubation medium. After 8 hours from the initial step of the transfection, medium was changed to DMEM containing antibiotics and treatments with the different compounds assayed were performed after 24 h of transfection up to a total time of 48 hours from the first step. This process guaranteed 60-70% of gene expression knockdown.

Pifithrin-α

The selective inhibitor of p53, pifithrin-α p-nitro cyclic (Merck) was used to inhibit the expression of this transcription factor in differentiated myotubes. A 6-hour pre-treatment was performed by adding 5 μM of pifithrin-α to the culture medium without serum, and then myotubes were exposed to DMSO (control cells), 10 μM GW501516 or the combination of 10 μM GW501516 + 8 μM pifithrin-α. Cells were collected after 24 hours.

Recombinant GDF15 and SB431542

Differentiated myotubes were exposed to 100 ng/ml of cell culture-derived recombinant human GDF15 or *E. coli*-derived murine recombinant GDF15 diluted in sterile PBS. Previously, lyophilized GDF15 was reconstituted in sterile 4 mM HCl. Cells were collected after 24h of exposure. The ALK4/5/7 inhibitor SB-431542 was co-incubated with GW501516 or recombinant GDF15 when indicated at 5 μM.

Metformin

Metformin (powder) was resuspended in sterile distilled water and added to C2C12 myotubes, HUH7 cells or primary hepatocytes at concentrations ranging 0.5-10 mM and from 24 to 72h.

Palmitate incubation

Lipid-containing medium was prepared by conjugation of non-esterified fatty acids with fatty acid-free BSA. Palmitic acid was dissolved in 96% ethanol and diluted 1:100 in DMEM containing 2% (w/v) fatty acid-free BSA. Differentiated myotubes were incubated in serum-free DMEM containing 2% BSA in either the presence or the absence of palmitate for 16 hours. This was performed alone or with the combination of a pre-treatment with metformin or GW501516 for 24h and a total time of 40h.

III.8 Tissue Processing

Skeletal muscle

Gastrocnemius muscle was dissected from mice after sacrifice in the different experimental procedures and snap-frozen in liquid nitrogen for later storing at -80°C. For further processing, skeletal muscle was subjected to a micronization process using a mortar and liquid nitrogen. Micronization ensures optimal yield for protein and RNA extraction and allows processing of all parts of the tissue.

Liver

Whole liver was dissected from mice after sacrifice in the different experimental procedures and snap-frozen in liquid nitrogen for later storing at -80°C. For further processing, 30 mg of a liver section was weighed and submerged into cold PBS 1X (Sigma) in glass test tubes. The tissue was then homogenized by a Polytron homogenizer (Fisher Scientific) and transferred to a 1.5 ml Eppendorf tube. All homogenized samples were then centrifuged at 10.000g for 2 minutes at 4°C and the supernatant was removed. Processed tissue was ready for protein or RNA extraction.

III.9 Immunoblotting

Total protein extraction

Cell or tissue pellets were resuspended in a solution containing RIPA buffer (Sigma), adding a cocktail of protease and phosphatase inhibitors containing 1 mM sodium orthovanadate (OvNa), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM sodium fluoride (NaF), 2.78 ml/ml aprotinin and 20 mM leupeptin. This was incubated for 1 hour (cell samples) or 2 hours (tissue samples) on a microtube rotating system at 30 rpm and 4°C. Next, samples were centrifuged for 20 minutes at 10,000 g and 4°C. Supernatant fraction containing the total protein extract was collected and stored.

Nuclear and cytosolic protein extraction

For nuclear and cytosolic protein isolation, the extraction consisted in two steps. First, the pellet was resuspended in a solution containing the components described above plus 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT. After 45 minutes of incubation on a microtube rotating system at 30 rpm and 4°C, samples were centrifugated at 10,000 *g* for 10 min at 4°C. The supernatant that contained the cytosolic fraction was collected and stored at -80°C. The remaining pellet was resuspended in a second buffer containing 25% glycerol, 420 mM NaCl and 0.2 mM EDTA and incubated for 30 additional minutes on a microtube rotating system at 30 rpm and 4°C. Afterwards, samples were centrifugated at 10,000 *g* for 10 min at 4°C. At the end, supernatant contained the nuclear fraction and was collected and stored at -80°C.

Protein quantification

Protein quantification was performed using a Bradford-based colorimetric method with a protein assay kit (Bio-Rad). Diluted samples and bovine serum albumin (BSA) standards were mixed in a 1:20 dilution with the dye reagent and incubated for 5 minutes. Absorbance was read at 595 nM and the protein concentration of each sample was calculated by extrapolation of the BSA standard curve ranging 0.1-0.6 mg/ml.

SDS-PAGE

Nuclear, cytosolic or total protein extracts were separated by molecular weight in a sodium-dodecyl-sulphate (SDS, Sigma) polyacrylamide gel electrophoresis (PAGE). The gel contained 8-12% of polyacrylamide, SDS and Tris-HCl solution. 20-40 µg of the protein extract (depending on the type and source of the protein) were run into the gel on a electrophoresis at 120 Volts for 70-90 minutes, depending on the size of the protein and the percentage of polyacrylamide used.

Once migrated, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) which was previously activated by rinsing in methanol

for 1 minute and washing in distilled water for 1 additional minute. After transference, proteins in the membrane were incubated for 1 hour in the commercial solution West Vision (Palex) to block non-specific binding of the proteins. The membrane was incubated with the primary antibody in West Vision solution (see Table 5 for dilutions) over night at 4°C, then washed 5 times for 5 minutes with Tris-buffered saline (TBS) solution containing 0.1% Tween® 20 detergent (Sigma) and later incubated with secondary antibody solution (1:5000 to 1:10000 dilution) that was species-specific to bind the primary antibody used, in TBS solution containing 5% of BSA and 0.1% Tween® 20. The membranes were washed three times for 5 minutes to remove excessive amount of antibody and ECL kit (Millipore) was used to detect the protein by chemiluminescence. Precision Plus Protein Dual Color Standard (Bio-Rad) was used as protein marker to assess the molecular weight to the bands detected and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, α-tubulin (total and cytosolic protein), lamin B, TATA-binding protein (TBP) or Histone H3 (nuclear protein) were used as loading control to normalize the levels of the proteins detected. Band detection and imaging were obtained with the ChemiDoc XRS+ system (Bio-Rad) after membrane expositions ranging 5 sec to 2 min.

Antibody	Manufacturer	Reference	WB Dilution
β-Actin	Sigma	A5441	1:2000
Akt	Cell Signaling	#9272	1:1000
Phospho-Akt Ser^{473}	Cell Signaling	#9271	1:1000
AMPK	Cell Signaling	#2532	1:1000
Phospho-AMPK Thr^{172}	Cell Signaling	#2531	1:500
Secondary Anti-goat	Santa Cruz	sc-2020	1:5000
Secondary Anti-mouse	Thermo Fisher	A-11001	1:10000
Secondary Anti-rabbit	Thermo Fisher	A-11034	1:5000
ATF4 (CREB-2)	Santa Cruz	sc-390063	1:500
CHOP	Cell Signaling	#2895	1:1000
eIF2a	Cell Signaling	#9722	1:1000

Phospho-eIF2a Ser^{51}	Cell Signaling	#9721	1:500
ERK1/2 (p44/42 MAPK)	Cell Signaling	#9102	1:1000
Phospho-ERK1/2 (p42/44 MAPK) Thr^{202}/Tyr^{204}	Cell Signaling	#9101	1:1000
GAPDH	Millipore	MAB374	1:2000
GDF15	Santa Cruz	sc-515675	1:1000
Histone H3	Santa Cruz	sc-10809	1:1000
IκBα	Santa Cruz	sc-371	1:1000
Insulin receptor β	Cell Signaling	#3025	1:1000
IRS-1	Cell Signaling	#2382	1:1000
Phospho-IRS-1Ser^{307}	Cell Signaling	#2381S	1:1000
IRS-2	Cell Signaling	#4502	1:1000
Lamin B	Santa Cruz	sc-6216	1:1000
Lipin 1	Santa Cruz	sc-98450	1:1000
NF-κB p65	Santa Cruz	sc-109	1:1000
p53	Cell Signaling	#2524T	1:500
Phospho-p53 Ser^{15}	Cell Signaling	#9284T	1:500
PGC-1α	Abcam	ab54481	1:1000
PPARα	Santa Cruz	sc-1985	1:1000
SOCS3	Santa Cruz	sc-9023	1:1000
STAT3	Santa Cruz	sc-482 X	1:1000
Phospho-Stat3 Tyr^{705}	Cell Signaling	#9131	1:1000
Phospho-Stat3 Ser^{727}	Cell Signaling	#9134	1:1000
TBP	Santa Cruz	Sc-74596	1:1000
α-Tubulin	Sigma	T6074	1:1000

Table 5. List of antibodies used for immunoblotting

III.10 Quantitative PCR (qPCR)

RNA extraction

Total RNA extraction for tissue samples (skeletal muscle, liver) and cell culture samples (C2C12, HUH7 and HepG2) was performed using TRIsure™ reagent (Bioline) and based on the method described by Chomczynski et al. (Chomczynski and Sacchi, 1987). Chloroform was used to isolate RNA from DNA and proteins and isopropanol was added to precipitate RNA. The obtained pellet was washed with 75% ethanol to remove salts. Quantification of RNA was done with NanoDrop 1000 (Thermo Scientific) and purity was assessed by the ratio of the absorbance at 260 and 280 nm (A260/280) and the A260/230 ratio. Ratios over 1.8-1.9 were considered as acceptable.

Reverse transcription

Isolated RNA was reverse transcribed to obtain 1 µg of complementary DNA (cDNA) using Random Hexamers (Thermo Scientific), 10 mM deoxynucleotide (dNTP) mix and the reverse transcriptase enzyme derived from the Moloney murine leukemia virus (MMLV, Thermo Fisher). The protocol was run in a thermocycler (BioRad) and consisted in a program with different steps and temperatures: 65°C for 5 min., 4°C for 5 min., 37°C for 2 min., 25°C for 10 min., 37°C for 50 min. and 70°C for 15 min.

Real time quantitative polymerase chain reaction (qPCR)

Quantitative gene expression of mice tissues and cell culture samples was evaluated by real time PCR technique in a Mini-48 well T100™ thermal cycler (Bio-Rad) using SYBR Green Master Mix (Applied Biosystems). The samples contained a final volume of 20 µl, with 25 ng of total cDNA, 0.9 µM of primer mix and 10 µl of 2x SYBR Green master mix. The thermal cycler protocol for real time PCR included a first step of denaturation at 95°C for 10 min and 40 repetitive cycles with three steps for denaturation, primer annealing and amplification: 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Primers sequences were designed using the Primer-BLAST tool (NCBI), based on the full mRNA sequences to find optimal primers for amplification and evaluated with the Oligo-Analyzer tool (Integrated DNA Technologies) to ensure optimal melting temperature (Tm) and avoid the formation of homo/hetero-dimers or nonspecific structures that can interfere with the interpretation of the

results. The primer sequences were designed specifically spanning the junction between exons (**Table 6**).

Gene	**Primer Sequence**	
mAcadm	for	5'-TGACGGAGCAGCCAATGA-3'
	rev	5'-ATGGCCGCCACATCAGA-3'
mAcox	for	5'-TCTGGAGATCACGGGCACTT-3'
	rev	5'-TTTCCAAGCCTCGAAGATGAG-3'
mAmpk 1	for	5'-CCGCACCAGAAGTCATTTCA-3'
	rev	5'-CCCGCTGCTCCAGATGTC-3'
mAmpk 2	for	5'-TGGAATATGTGTCTGGAGGTG-3'
	rev	5'-GCGCGCTTCCACCTCTT-3'
mAprt	for	5'-CAGCGGCAAGATCGACTACA-3'
	rev	5'-AGCTAGGGAAGGGCCAAACA-3'
mAtf3	for	5'-CTGGAGATGTCAGTCACCAAGTCT-3'
	rev	5'-TTTCTCGCCGCCTCCTTT-3'
hATF3	for	5'-AAGAACGAGAAGCAGCATTTGAT-3'
	rev	5'-TTCTGAGCCCGGACAATACAC-3'
mBip	for	5'-CAGATCTTCTCCACGGCTTC-3'
	rev	5'-GCAGGAGGAATTCCAGTCAG-3'
mChop	for	5'-CGAAGAGGAAGAATCAAAAACCTT-3'
	rev	5'-GCCCTGGCTCCTCTGTCA-3'
mCpt1a	for	5'-GCAGAGCACGGCAAAATGA-3'
	rev	5'-GGCTTTCGACCCGAGAAGAC-3'
mCpt1b	for	5'-GGCTGCCGTGGGACATT-3'
	rev	5'-TGCCTTGGCTACTTGGTACGA-3'

mFgf21	for	5’-CAGGGAGGATGGAACAGTGGTA-3’
	rev	5’-TGACACCCAGGATTTGAATGAC-3’
mGapdh	for	5’-TGTGTCCGTCGTGGATCTGA-3’
	rev	5’-CCTGCTTCACCACCTTCTTGA-3’
hGAPDH	for	5’-GGCCTCCAAGGAGTAAGACC-3’
	rev	5’-AGGGGTCTACATGGCAACTG-3’
hGDF15	for	5’-TCAGATGCTCCTGGTGTTGC-3’
	rev	5’-GATCCCAGCCGCACTTCTG-3’
mGdf15	for	5’-GAGCTACGGGGTCGCTTC-3’
	rev	5’-GGGACCCCAATCTCACCT-3’
mGfral	for	5’-GCTGTGAGCAGTCATGGAGA-3’
	rev	5’-TTCCACCAAAGCCTGGATAC-3’
mIl6	for	5’- ACACATGTTCTCTGGGAAATCGT-3’
	rev	5’- AAGTGCATCATCGTTGTTCATACA-3’
mp53	for	5’-ACTCAGACTGACTGCCTCTG-3’
	rev	5’-TCTCAGCCCTGAAGTCATAA-3’
mPdk4	for	5’-CACCACATGCTCTTCGAACTCT-3’
	rev	5’-AAGGAAGGACGGTTTTCTTGATG-3’
mPgc1a	for	5’-AACCACACCCACAGGATCAGA-3’
	rev	5’- TCTTCGCTTTATTGCTCCATGA-3’
mPpara	for	5’-CAAGGCCTCAGGGTACCACTAC-3’
	rev	5’-GCCGAATAGTTCGCCGAAA-3’
mPparβ	for	5’-GCCACAACGCACCCTTTG-3’
	rev	5’-CCACACCAGGCCCTTCTCT-3’
mSocs3	for	5’-ATGGTCACCCACAGCAAGTTT-3’

	rev	5'-TCCAGTAGAATCCGCTCTCCT-3'
mTnfa	for	5'-ATGGCCCAGACCCTCACA-3'
	rev	5'-TTGCTACGACTGGGCTACA-3'
mTrb3	for	5'-CGTGGCACACTGCCACAA-3'
	rev	5'-CCTCTCACAGTTGCTGAAGACAA-3'
mVldlr	for	5'-TCCAATGGCCTAATGGAATTACA-3'
	rev	5'-AGCATGTGCAACTTGGAATCC-3'

Table 6. Primer sequences designed for qPCR. h: human genes, m: mouse genes.

III.11 Statistical Analysis

Results are expressed as means ± s.e.m. of at least four independent experiments or at least four different animals per group. Results were analysed by Student's t-test or one-way analysis of variance (ANOVA), according to the number of groups compared, using the GraphPad Prism program (V6.01) (GraphPad Software Inc., San Diego, CA). When significant variations were found by ANOVA, the Tukey-Kramer multiple comparison post-hoc test was performed only if F achieved a P value <0.05 and there was no significant variance inhomogeneity. Differences were considered significant at $P<0.05$. Specific statistical tests done for experiments are listed under the corresponding figure.

III.12 Illustrations

The images used to illustrate the introduction and discussion parts were performed using the online design tool BioRender (BioRender.com) and are published under license. For use permission, contact the author.

IV. RESULTS

IV.1 PART I - GDF15 mediates the metabolic effects of PPARβ/δ by activating AMPK

IV.1.1 PPARβ/δ activation by GW501516 increases GDF15 levels

In order to determine whether PPARβ/δ activation increases GDF15 levels, we treated C2C12 myoblasts differentiated into myotubes with the PPARβ/δ agonist GW501516. Treatment with this agonist for 24h induced a significant increase in *Gdf15* mRNA levels (**Fig. 15a**), as well as its protein levels (**Fig. 15b**) and its secretion into the culture media (**Fig. 15c**). To demonstrate that this effect was specifically dependent on PPARβ/δ, myotubes were co-incubated with GW501516 and the PPARβ/δ antagonist GSK3787. GW501516 increased *Gdf15* mRNA levels and co-incubation with GSK3787 attenuated this increase (**Fig. 15d**). Additionally, knockdown of PPARβ/δ by siRNA transfection mitigated the increase in *Gdf15* expression (**Fig. 15e**). Mice treated for 6 days with 3 mg/kg/day of GW501516 also showed an increase in *Gdf15* expression (**Fig. 15f**), GDF15 protein levels (**Fig. 15g**) and serum GDF15 levels (**Fig. 15h**). Additionally, **Supplementary Fig. 1** shows that the PPARβ/δ agonist GW0742 also increases GDF15 protein levels.

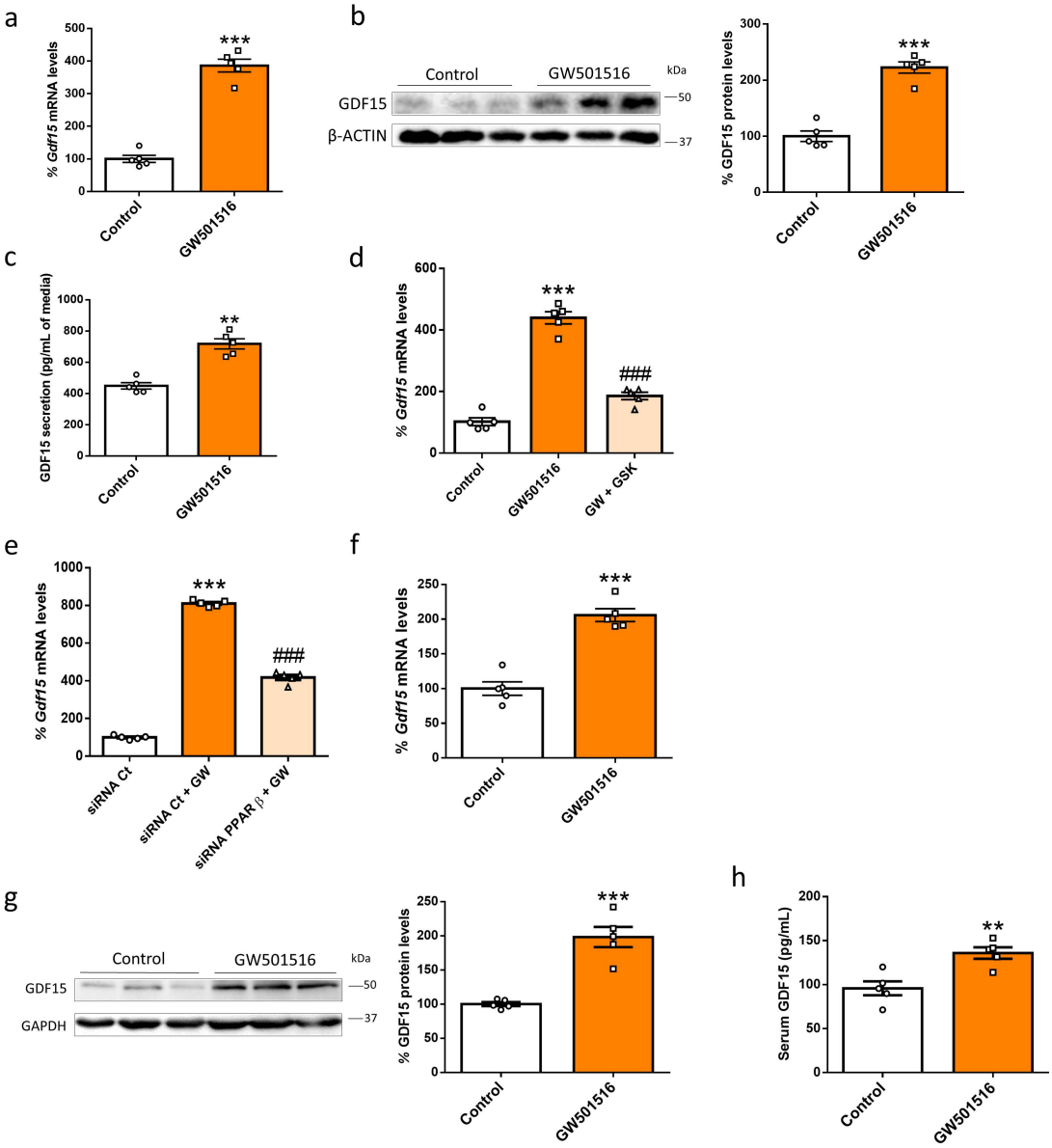


Figure 15 - PPARβ/δ activation increases GDF15 levels. a) *Gdf15* mRNA levels, **(b)** GDF15 protein levels and **(c)** GDF15 secreted into the culture medium of C2C12 myotubes exposed to 10 µM of the PPARβ/δ agonist GW501516 for 24 h (n = 5 per group). **d)** *Gdf15* mRNA levels in C2C12 myotubes exposed to 10 µM of GW501516 for 24 h in the presence or absence of 10 µM of the PPARβ/δ antagonist GSK3787 or **(e)** transfected with control siRNA or PPARβ/δ siRNA for 48 h (n = 5 independent cell culture experiments). **f)** *Gdf15* mRNA levels and **(g)** GDF15 protein levels in the skeletal muscle of mice (n = 5 animals) treated with vehicle or 3 mg/kg/day of GW501516 for 6 days. **h)** GDF15 serum levels in mice (n = 5 per group) treated with GW501516 for 6 days. Data are presented as the mean ± s.e.m. **p < 0.01, and ***p < 0.001 *versus* control. ### p < 0.001 *versus* GW501516-treated cells. p-values determined by one-way ANOVA with Tukey's post hoc test (d and e) and two-tailed unpaired Student's t-test (a, b, c, f, g, h).

IV.1.2 In skeletal muscle, GDF15 neutralization reverts the metabolic effects of PPARβ/δ activation

Since we observed an increase in GDF15 levels caused by GW501516 treatment and given the implication of GDF15 in lipid metabolism, inflammation and insulin resistance, we evaluated whether the antidiabetic effects of PPARβ/δ activation on HFD-fed mice depended on GDF15 by using a neutralizing antibody against this cytokine to block its actions.

Mice receiving a HFD for 3 weeks showed reduced glucose tolerance than mice fed a standard diet (**Figs. 16a-b**). This was improved by GW501516 treatment, but this beneficial effect was inhibited by the blockade of GDF15 actions caused by the administration of the neutralizing antibody. GFRAL was recently described as the neuronal receptor for GDF15 and the mediator of its weight loss function (Hsu et al., 2017b), (Mullican et al., 2017a). However, in our study, neither GW501516 nor antibody treatment affected food intake or body weight (**Figs. 16c-d**). The molecular complex formed by PGC-1α, Lipin 1 and PPARα regulates the expression of genes involved in fatty acid oxidation (Barroso et al., 2011a). In our study, the expression of these target genes involved in fatty acid oxidation, as well as very-low density lipoprotein (VLDL) uptake were examined in skeletal muscle. Consistent with the previously reported regulation of these genes by PPARβ/δ (Vázquez-Carrera, 2016), (Tan et al., 2016b), GW501516 increased the expression of *Pdk4, Cpt1b, Acox, Acadm* and *Vldlr* (**Fig. 16e**). However, GDF15 neutralization abolished the increase in *Acox, Acadm* and *Vldlr* expression, implying that this PPARβ/δ-dependent increase was mediated by enhanced GDF15 levels. The levels of *Cpt1b* were partially, but not totally, reverted by the antibody, suggesting that other complementary mechanisms are involved in this increase. Lastly, the increase in *Pdk4* expression caused by GW501516 was not modified by the GDF15 neutralizing antibody, suggesting that the regulation of this gene by PPARβ/δ is independent of GDF15.

The cellular location of lipin 1, which regulates metabolic homeostasis, determines whether fatty acids are incorporated into triglycerides or undergo mitochondrial β-oxidation. In the cytoplasm, lipin 1 is a phosphatase enzyme that promotes triglyceride accumulation and phospholipid synthesis, whereas in the nucleus lipin 1 acts as a transcriptional coactivator regulating the induction of PPAR-γ coactivator 1 (PGC)-1α-PPARα-target genes implicated in fatty acid oxidation (Finck et al., 2006). As previously reported, PPARβ/δ activation promotes the PGC-1α-Lipin 1-PPARα axis in liver through AMPK activation (Barroso et al., 2011a). The levels of PPARα and PGC-1α in skeletal muscle of HFD-fed mice were decreased compared to standard diet-fed control mice, but this reduction was blocked by GW501516 (**Fig. 16f**). The antibody also reduced GW501516-induced nuclear translocation of lipin 1 and increased its cytosolic levels, suggesting that GDF15 promotes lipin 1 nuclear internalization.

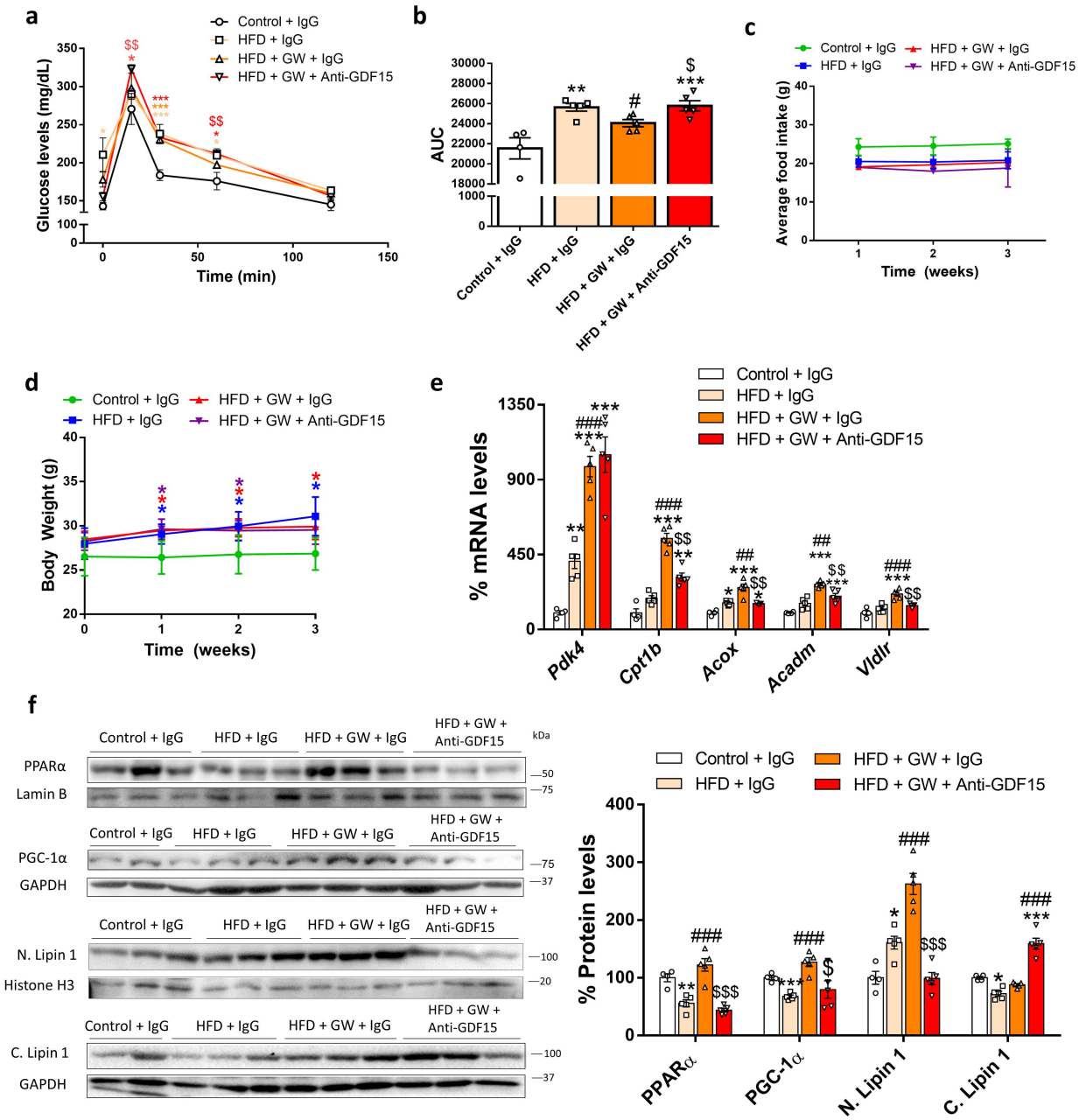


Figure 16 - GDF15 neutralization reverts the metabolic effects of PPARβ/δ activation a) Glucose tolerance test and **(b)** area under the curve (AUC) of mice (n = 5 animals; except for control mice where n = 4) fed standard chow (control), a HFD or a HFD and treated with GW501516 (GW) for 3 weeks. Three days before the end of the 3-week treatment, mice were injected once intraperitoneally with either IgG or a neutralizing antibody against GDF15. **c)** Food intake. **d)** Body weight. **e)** mRNA levels of *Pdk4, Cpt1b, Acox, Acadm* and *Vldlr* in skeletal muscle. **f)** Skeletal muscle cell lysate extracts were assayed via Western blot analysis with antibodies against PPARα, PGC-1α, and nuclear (N) and cytosolic (C) extracts with antibody against lipin-1. Data are presented as the mean ± s.e.m. *p < 0.05, **p < 0.01 and ***p < 0.001 *versus* control + IgG. #p < 0.05, ##p < 0.01, ###p < 0.001 vs. HFD + IgG group. $p < 0.05, $$p < 0.01, and

$^{\$\$\$}p < 0.001$ *versus* GW501516-treated mice injected with IgG. p-values determined by one-way ANOVA with Tukey's post hoc test.

IV.1.3 In skeletal muscle, GDF15 neutralization reverts the beneficial effects of PPARβ/δ activation on, ER stress, inflammation and insulin signalling

As previously mentioned, ER stress is induced by HFD and its development is correlated with the induction of proinflammatory pathways and the initiation of insulin resistance (Salvado et al., 2015). Additionally, in peripheral organs, proinflammatory cytokines mediate the increase of SOCS3 via the induction of the JAK-STAT signalling pathway (Wunderlich et al., 2013), which inhibits insulin signalling through several mechanisms (Ueki et al., 2004).

PPARβ/δ activation prevents lipid-induced ER stress, inflammation and insulin resistance in skeletal muscle (Salvadó et al., 2014). In the current study, mice fed a HFD showed increased expression of the ER stress markers *Bip* and *Chop*, as well as an increase in the mRNA levels of the proinflammatory cytokine *Tnfa,* and *Socs3* (**Fig. 17a**). As expected, the activation of PPARβ/δ by GW501516 attenuated these changes. However, treatment with the GDF15 neutralizing antibody reverted these effects. In agreement with the increase in ER stress caused by the HFD, activating transcription factor 4 (ATF4) protein levels and the phosphorylated levels of eukaryotic translation initiation factor 2α (eIF2α) were increased, which was blocked by GW501516 (**Fig. 17b**). However, this protective effect of GW501516 was abrogated when the HFD-fed mice were co-treated with the GDF15 neutralizing antibody. HFD-fed mice treated with GW501516 also exhibited an increase in the protein levels of IκBα, which keeps the pro-inflammatory transcription factor NF-κB in the cytosol, preventing its translocation into the nucleus to act as a transcription factor. Indeed, GW501516 treatment of HFD-fed mice inhibited the internalization of the p65 subunit of NF-κB into the nucleus, and co-treatment with the GDF15 neutralizing antibody attenuated this effect (**Fig. 17b**).

Regarding the insulin signalling pathway, GW501516 treatment increased the protein levels of the β subunit of the insulin receptor (IRβ). This increase was completely suppressed by the GDF15 neutralizing antibody (**Fig. 17c**). TNFα has been shown to activate c-Jun N-terminal kinase causing serine phosphorylation of IRS1 (Hirosumi et al., 2002). In agreement with the activation of the NF-κB pathway by the HFD, IRS-1 phosphorylation at Ser307 was increased. GW501516 blocked this increase, but this protective effect was abolished by the GDF15 neutralizing antibody (**Fig. 17c**).

AMPK activation has been extensively reported to improve insulin sensitivity in diet-induced insulin-resistant individuals (Cokorinos et al., 2017b), whereas ERK1/2 negatively regulates the insulin signalling pathway as a response to insulin binding to its receptor. More specifically, this kinase is activated via its phosphorylation by insulin receptor cascade, which in turn phosphorylates IRS1 serine residues (Taniguchi et al., 2006). Consistent with a previous report (Salvadó et al., 2014), GW501516 induced the activation of AMPK via its phosphorylation, and reduced the HFD-mediated increase in the levels of phospho-ERK1/2, which is involved in a negative crosstalk with AMPK (Hwang et al., 2013) (**Fig. 17d**). However, these changes were not observed in mice treated with the GDF15 neutralizing antibody (**Fig. 17d**). Collectively, the data presented here indicate that part of the beneficial effects of pharmacological PPARβ/δ activation in skeletal muscle, including lipid metabolism, ER stress, inflammation and insulin signalling are attenuated or abolished when GDF15 actions are blocked by a neutralizing antibody.

Importantly, the activity of the neutralizing antibody was tested by analysing food intake in mice that were treated with recombinant GDF15. Treatment with this cytokine has been previously reported to reduce food intake (Borner et al., 2020). Here, recombinant GDF15 caused a decrease in food intake that was partially reverted when mice also received the neutralizing antibody against GDF15 (**Supplementary Fig. 2**), indicating that the antibody function was efficient.

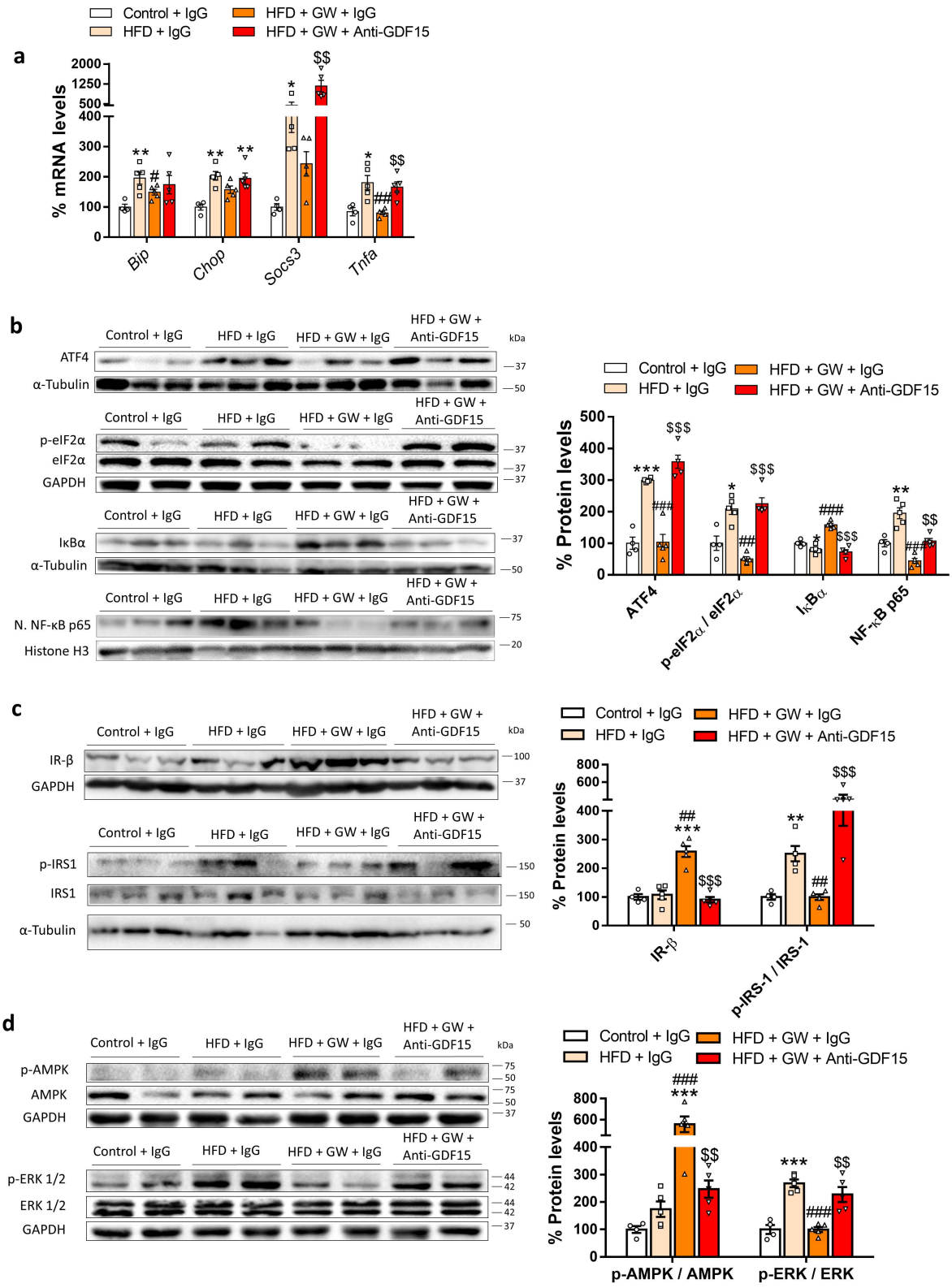


Figure 17. Neutralization of GDF15 attenuates the beneficial effects of PPARβ/δ activation on ER stress and inflammation in skeletal muscle. a) mRNA levels of *BiP/Grp78*, *Chop, Socs3* and *Tnfa* in skeletal muscle of mice (n = 5 animals; except for control mice where n = 4) fed standard chow, a HFD or a HFD and treated with GW501516 for 3 weeks. Three days before the end of the 3-week treatment, mice were injected once intraperitoneally with either IgG or a neutralizing antibody against GDF15. **b)** Skeletal muscle cell lysate extracts were assayed via Western blot analysis

with antibodies against ATF4, total and phospho-eIF2α, IκBα and the p65 subunit of NF-κB. **c)** Total and phospho-IRS1 (Ser307), and IRβ levels. **d)** Total and phospho-AMPK and total and phospho-ERK1/2 levels. Data are presented as the mean ± s.e.m. *p < 0.05, **p < 0.01, and ***p < 0.001 *versus* Control + IgG. #p < 0.05, ##p < 0.01, and ###p < 0.001 *versus* HFD + IgG group. $p < 0.05, $$p < 0.01 and $$$p < 0.001 *versus* GW501516-treated mice injected with IgG. p-values determined by one-way ANOVA with Tukey's post hoc test.

IV.1.4 In the liver, GDF15 neutralization attenuates the beneficial effects of PPARβ/δ activation on lipid metabolism, ER stress and the insulin signalling pathway

Mice treated with GW501516 for 6 days also showed increased *Gdf15* expression and protein levels in the liver (**Figs. 18a-b**). Furthermore, the expression of genes involved in fatty acid oxidation (*Pdk4, Cpt1a, Acadm* and *Acox*) and of the hormone *Fgf21* was slightly increased by the HFD, with GW501516 treatment exacerbating this increase (**Fig. 18c**). GDF15 neutralization did not reduce *Pdk4* as in skeletal muscle, confirming that this gene is not regulated by GDF15. Similarly, although *Cpt1a* expression showed a reduction trend when GDF15 was neutralized, it did not reach statistical significance, suggesting that, in contrast to its effect in skeletal muscle, the increase in *Cpt1a* expression caused by GW501516 is not affected by GDF15 in these conditions in the liver. By contrast, the increased expression of *Acadm* and *Acox* caused by GW501516 was reduced by GDF15 neutralization. As previously mentioned, FGF21 is a stress-response hormone that has been defined as a biomarker for insulin resistance and metabolic disturbances (Tanajak et al., 2018). Interestingly, the induction of the expression of *Fgf21* caused by GW501516 was strongly increased by GDF15 neutralization (**Fig. 18c**), indicating an increased HFD-induced metabolic stress in the absence of GDF15 activity.

Consistent with the GW501516-mediated increase in the expression of genes involved in fatty acid oxidation, the levels of serum β-hydroxybutyrate, a product of ketogenesis used as a marker of hepatic fatty acid oxidation, were significantly elevated in HFD-fed mice receiving GW501516 (**Fig. 18d**). This was abolished by the neutralizing antibody against GDF15. The HFD increased the hepatic expression of the ER stress markers *Bip* and *Chop* (**Fig. 18e**). GW501516 partly protected against this increase, but this effect was lost in mice that received the GDF15 neutralizing antibody injection. Hepatic *Socs3* mRNA levels showed a similar trend and were highly increased when GDF15 was neutralized.

In agreement with a previous study showing AMPK activation in the liver by PPARβ/δ activation (Barroso et al., 2011a), GW501516 increased phospho-AMPK levels and decreased phospho-ERK1/2 levels (**Fig. 18f**), which is consistent with the negative crosstalk between these kinases (Hwang et al., 2013). Blocking GDF15 with the neutralizing antibody abrogated this effect.

The increase of SOCS3 via the induction of the JAK-STAT signalling pathway (Wunderlich et al., 2013), promotes the ubiquitination and degradation of IRS-1 and IRS-2 (Rui et al., 2002). When we explored the phosphorylation of STAT3, the transcription factor that regulates *Socs3* expression, we observed that the HFD increased the phosphorylated levels of STAT3 at both Tyr705 and Ser727 residues (**Fig. 18g**), which allows its translocation into the nucleus and further activity as a transcription factor. This was accompanied by increased SOCS3 protein levels. Interestingly, GW501516 prevented these increases, whereas in HFD-fed mice treated with GW501516 and the GDF15 neutralizing antibody, the protective effect of the PPARβ/δ agonist was attenuated. In line with the increased SOCS3 levels in the liver of HFD-fed mice, there was a slight decrease in IRS-1 and a marked reduction in IRS-2 protein levels (**Fig. 18g**). These changes were blocked by GW501516, but this inhibition was lifted by the GDF15 neutralizing antibody (**Fig. 18g**). Collectively, these data indicate that many of the beneficial effects that promote the recovery of the insulin signalling by PPARβ/δ activation in the liver depend on GDF15.

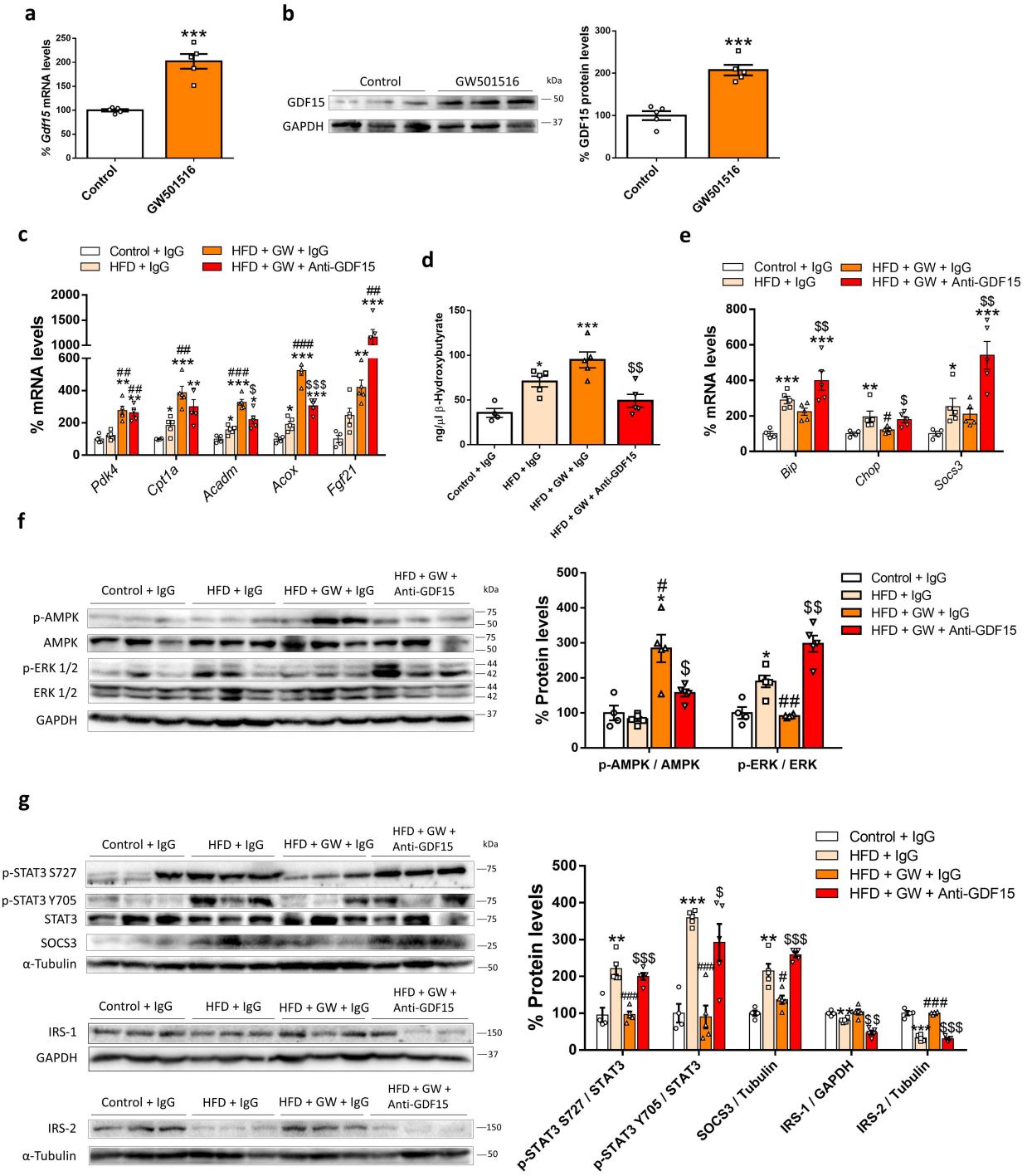


Figure 18. Neutralization of GDF15 reverts the beneficial effects of PPARβ/δ activation on ER stress, inflammation and insulin signalling in the liver. **a)** *Gdf15* mRNA levels and **(b)** GDF15 protein levels in the liver of mice (n = 5 animals) treated with vehicle or 3 mg/kg/day of GW501516 for 6 days. **c)** mRNA levels of *Pdk4, Cpt1a, Acadm, Acox* and *Fgf21* in the liver of mice (n = 5 animals; except for control mice where n = 4) fed standard chow, a HFD or a HFD plus GW501516 for 3 weeks. Three days before the end of the 3-week treatment, mice were injected once intraperitoneally with either IgG or a neutralizing antibody against GDF15. **d)** Plasma

levels of β-hydroxybutyrate. **e)** mRNA levels of *Bip, Chop and Socs3* in the liver. **f)** Liver cell lysate extracts (n = 5 animals; except for control mice where n = 4) were assayed via Western blot analysis with antibodies against total and phospho-AMPK and total and phospho-ERK1/2. **g)** Total and phospho-STAT3 (Ser727 and Tyr705), SOCS3, total IRS1 and IRS2 levels. Data are presented as the mean ± s.e.m. *p < 0.05, **p < 0.01, and ***p < 0.001 *versus* control + IgG. $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, and $^{\#\#\#}$p < 0.001 *versus* HFD + IgG group. $^{\$}$p < 0.05, $^{\$\$}$p < 0.01, and $^{\$\$\$}$p < 0.001 *versus* GW501516-treated mice injected with IgG. p-values determined by one-way ANOVA with Tukey's post hoc test (c, d, e, f, g) and two-tailed unpaired Student's t-test (a, b).

IV.1.5 *Gdf15* null mice are less responsive to the metabolic effects of PPARβ/δ activation on HFD

Our previous results showed the implication of GDF15 in the metabolic effects of pharmacological PPARβ/δ activation by using a neutralizing antibody to block GDF15 actions. Nevertheless, to clearly demonstrate the involvement of GDF15 in PPARβ/δ actions, we reproduced the same experimental conditions on mice lacking the functional *Gdf15* gene compared to wild-type (WT) mice.

WT and *Gdf15*$^{-/-}$ animals were fed either a standard or a HFD (45% Kcal from fat) and received a daily dose of vehicle (CMC) or 3mg/kg of GW501516. Feeding WT mice with the HFD caused glucose intolerance that was significantly ameliorated with the PPARβ/δ agonist treatment (**Fig. 19a**). On the contrary, this beneficial effect of GW501516 was not observed in *Gdf15*$^{-/-}$ mice, which, in fact, showed a significant increase in glucose intolerance compared to WT mice treated with GW501516 (**Fig. 19a**). Contrary to other studies reporting a reduction in food intake and body weight by the GDF15-GFRAL interaction (Hsu et al., 2017a), (Mullican et al., 2017a), our treatment with GW501516 did not significantly affect the increase in body weight caused by the HFD in WT and *Gdf15*$^{-/-}$ mice **(Supplementary Fig. 3),** as observed in the previous study. As expected, GW501516 reduced serum triglyceride levels (-20%) in WT mice, but this effect was significantly attenuated (-12%) in *Gdf15*$^{-/-}$ mice (**Fig. 19b**).

In skeletal muscle, administration of the PPARβ/δ agonist significantly increased *Pdk4* mRNA levels in both WT and *Gdf15*$^{-/-}$ mice with no differences between these groups (**Fig. 19c**). *Cpt1b* and *Acox* expression was also upregulated by GW501516 in WT mice, but this effect was not observed in *Gdf15*$^{-/-}$ mice (**Fig. 19c**). Similarly, PPARβ/δ activation reduced the increase in the expression of *Bip*, *Socs3* and *Mcp1* caused by HFD in WT mice, although without reaching statistical significance in the case of *Bip*. In *Gdf15*$^{-/-}$ mice the reduction in the expression of *Bip, Socs3* and *Mcp1* caused by pharmacological treatment was attenuated or abolished (**Fig. 19c**). Two proteins strongly induced by PPARβ/δ activation in skeletal muscle of WT mice, IκBα and IRβ, involved in the inhibition of NF-κB proinflammatory pathways and maintaining insulin signalling, respectively, remained unchanged in *Gdf15*$^{-/-}$ mice (**Fig. 19d**). Remarkably, phospho-AMPK levels were lower in skeletal muscle of *Gdf15*$^{-/-}$ mice than in WT, suggesting that GDF15 is involved in the regulation of phospho-AMPK levels. GW501516 restored the phosphorylated levels of this kinase in WT mice fed the HFD, but it failed to achieve it in *Gdf15*$^{-/-}$ mice (**Fig. 19d**). The involvement of GDF15 in AMPK activation will be more extensively analysed in the next sections. Consistent with the negative crosstalk between AMPK and ERK1/2, phosphorylated levels of the latter were increased in skeletal muscle of *Gdf15*$^{-/-}$ mice compared with WT mice. Likewise, HFD feeding increased the levels of phospho-ERK1/2 and this effect was prevented by drug treatment in WT mice, but not in *Gdf15*$^{-/-}$ mice.

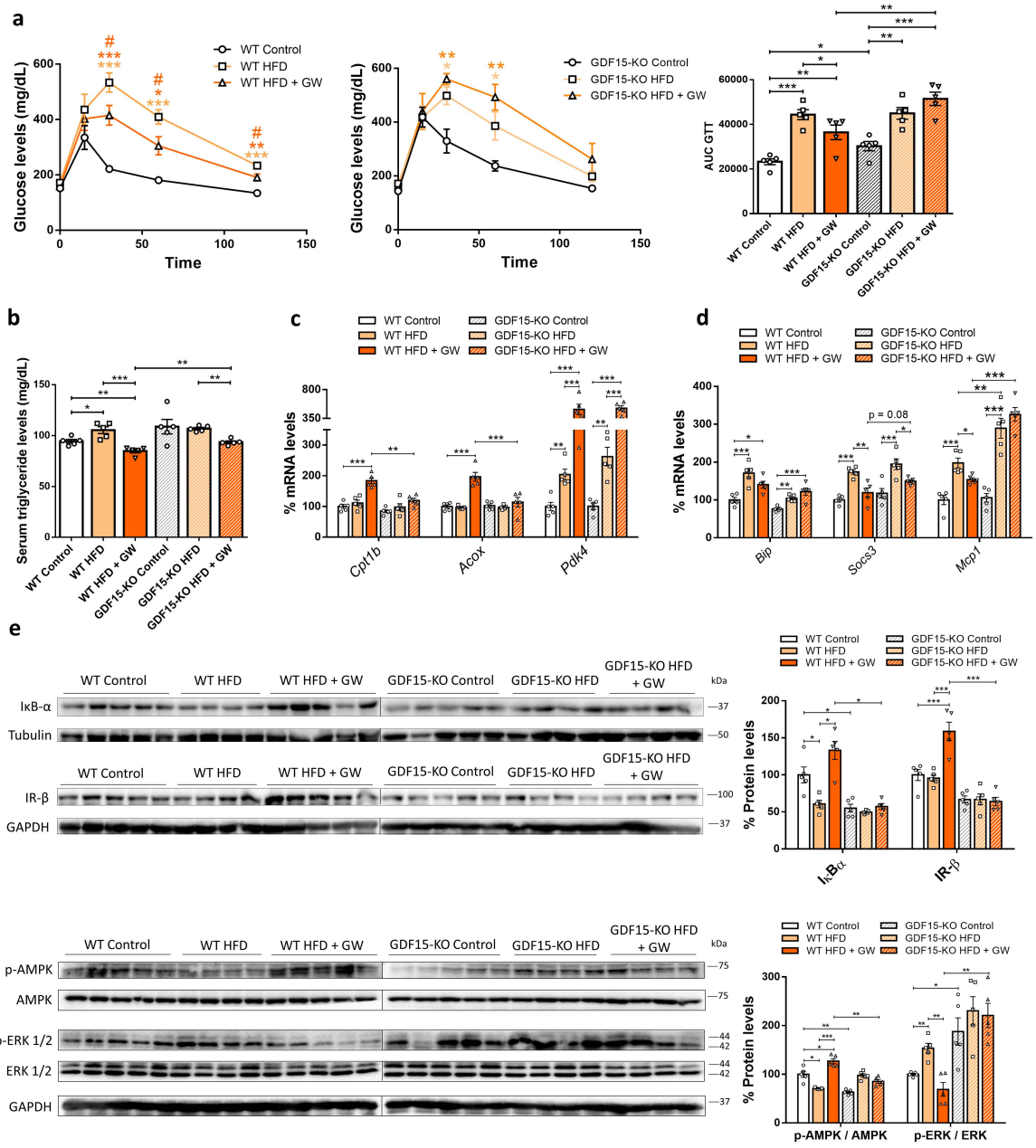


Figure 19. The beneficial effects of PPARβ/δ activation are attenuated in *Gdf15*$^{-/-}$ mice. a) Glucose tolerance test and area under the curve (AUC) of wild-type (WT) and *Gdf15*$^{-/-}$ mice (n = 5 animals) fed standard chow, a HFD or a HFD plus GW501516 for 3 weeks. **b)** Serum triglyceride levels (n = 5 animals). **c)** mRNA levels of *Pdk4*, *Cpt1b*, *Acox*, *Bip/Grp78*, *Socs3* and *Mcp1* in skeletal muscle (n = 5 animals). **d)** Skeletal muscle cell lysate extracts were assayed via Western blot analysis (n = 5 animals) with antibodies against IκBα, IRβ, total and phospho-AMPK, and total and phospho-ERK1/2 levels. Data are presented as the mean ± s.e.m. *p < 0.05, **p < 0.01, and ***p < 0.001 *versus* WT or GDF15-KO control mice. #p < 0.05 *versus* WT or GDF15-

KO mice fed a HFD. p-values determined by one-way ANOVA with Tukey's post hoc test.

In the liver, hematoxylin-eosin and oil red O (ORO) staining showed that the HFD caused a significant hepatic lipid accumulation that was reduced (p=0.06) by the PPARβ/δ agonist (**Figs. 20a-b**). This modest reduction is consistent with the effect of short treatments with PPARβ/δ agonists that increase the expression of genes involved in fatty acid oxidation, but also of genes implicated in hepatic lipid deposition (Vázquez-Carrera, 2016), (Tan et al., 2016b). Hepatic steatosis was remarkably higher in HFD-fed *Gdf15*$^{-/-}$ mice and it was exacerbated by GW501516. The lipid content increase showed in the last group suggest that GW501516 actions involved in hepatic lipid deposition remain functional, but the lack of GDF15 impairs its utilisation in catabolic processes.

Pharmacological activation of PPARβ/δ increased the expression of *Gdf15* in the liver of WT mice (**Fig. 20c**). Moreover, consistent with the results using the neutralizing antibody against GDF15, GW501516 increased the hepatic expression of *Pdk4* in both WT and *Gdf15*$^{-/-}$ mice (**Fig. 20c**), confirming that the regulation of this gene by PPARβ/δ is independent of GDF15. By contrast, the hepatic expression of *Cpt1a* and *Acox* was upregulated by GW501516 in WT mice, but this increase was significantly attenuated in HFD-fed *Gdf15*$^{-/-}$ mice treated with the PPARβ/δ agonist (**Fig. 20c**). Interestingly, we observed that the mRNA levels of hepatic *Cpt1a* were significantly downregulated in all the *Gdf15*$^{-/-}$ mice groups (**Fig. 20c**). The increase in the expression of both *Bip* and *Socs3* caused by the HFD in WT mice was significantly decreased by GW501516 (**Fig. 20d**), and the reduction of *Socs3* was attenuated in *Gdf15*$^{-/-}$ mice while *Bip* mRNA levels showed a dramatic decrease in every groups of mice lacking *Gdf15*. The same trend was observed in hepatic SOCS3 protein levels (**Fig. 20e**). In agreement with the neutralizing antibody study, the HFD reduced the protein levels of IRS2 in WT mice, an effect that was prevented by the PPARβ/δ agonist (**Fig. 20e**), whereas in *Gdf15*$^{-/-}$ mice the levels of IRS2 were significantly lower than in WT mice and GW501516 did not affect its levels (**Fig.**

20e). As in skeletal muscle, GW501516 increased the hepatic levels of phospho-AMPK and reduced those of phosho-ERK1/2 in WT mice, whereas no effect was observed in *Gdf15*$^{-/-}$ mice (**Fig. 20e**). These data confirm that many of the beneficial effects caused by pharmacological activation of PPARβ/δ in the liver, including the reduction in hepatic steatosis, depend on GDF15.

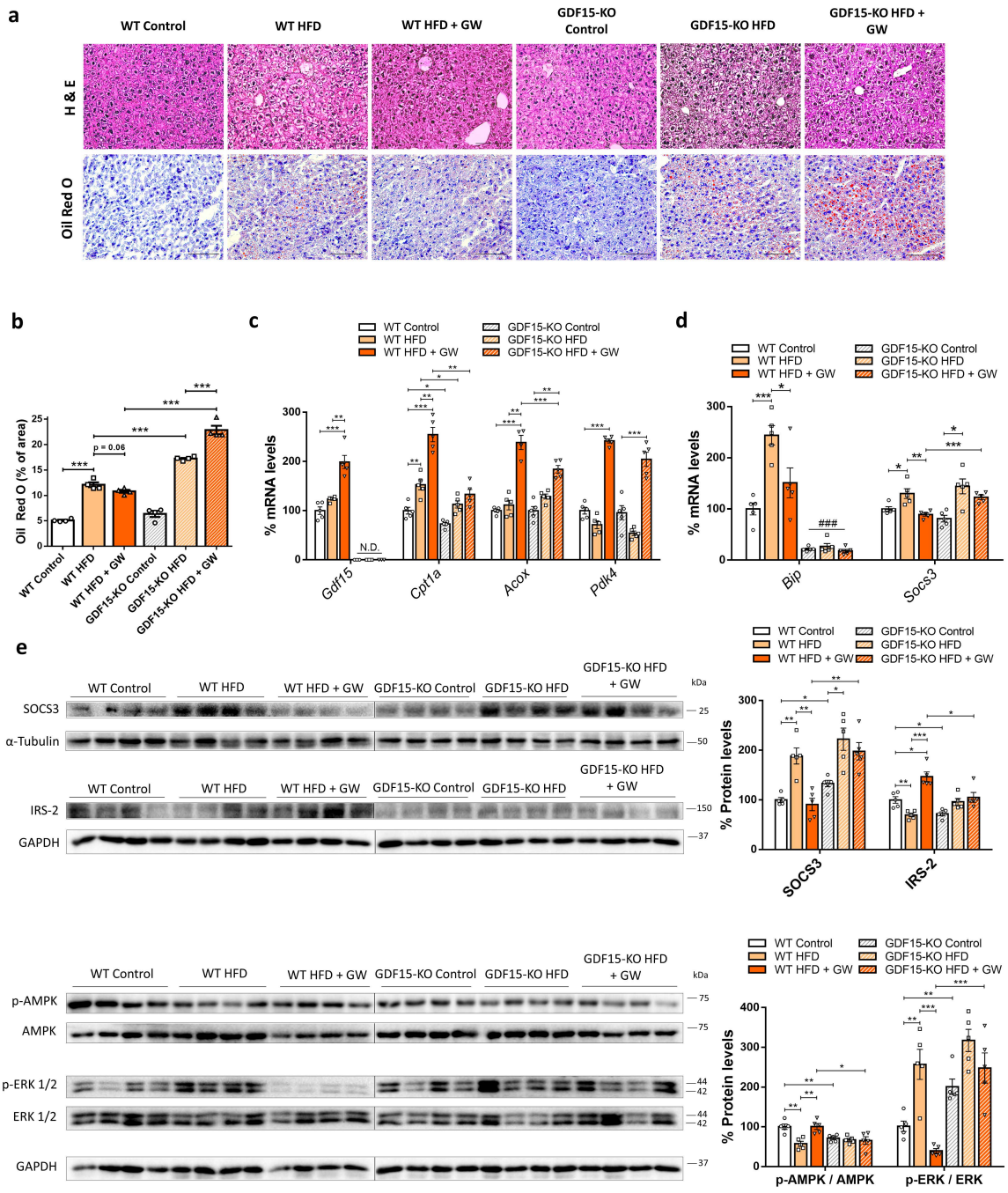


Figure 20. The beneficial effects of PPARβ/δ activation are reversed in the liver of *Gdf15*$^{-/-}$ mice. a) Hematoxylin–eosin (H&E), Oil Red O (ORO) staining of livers. Scale bar: 100µm. **b)** Quantification of ORO staining (n = 4 animals). **c)** mRNA levels of *Gdf15, Pdk4*, *Cpt1b* and *Acox* in liver of wild-type (WT) and *Gdf15*$^{-/-}$ mice fed standard chow, a HFD or a HFD plus GW501516 for 3 weeks (n = 5 animals). **d)** mRNA levels of *Bip* and *Socs3* in liver (n = 5 animals). **e)** Liver cell lysate extracts were assayed via Western blot analysis with antibodies against IRS-2, total and phospho-AMPK and total and phospho-ERK1/2 (n = 5 animals). Data are presented as the mean

± s.e.m. ND = Not Detected. *p < 0.05, **p < 0.01, and ***p < 0.001 *versus* indicated group. p-values determined by one-way ANOVA with Tukey's post hoc test.

IV.1.6 PPARβ/δ activation increases GDF15 levels through an AMPK-dependent mechanism

Next, we examined the potential mechanism by which PPARβ/δ activation increases GDF15 levels. Many of the anti-inflammatory and antidiabetic effects of PPARβ/δ activation in skeletal muscle are dependent on AMPK activation (Salvadó et al., 2014) a key metabolic sensor in energy homeostasis. Likewise, the administration of PPARβ/δ ligands to mice fed a HFD increases fatty acid oxidation through AMPK activation, resulting in glucose-lowering activity (Lee et al., 2006a). Given the similarities observed between the functions of GDF15 and AMPK regarding the induction of lipid catabolic pathways and insulin sensitivity, we first explored whether the activation of this kinase was involved in the increase of GDF15 levels.

Treatment of C2C12 myotubes with the AMPK allosteric activator A769662 strongly increased the mRNA and protein levels of GDF15, which was blocked by co-incubating the cells with the AMPK inhibitor compound C (**Figs. 21a-b**). Likewise, compound C blocked the increase in GDF15 mRNA and protein levels, as well as the phosphorylation of AMPK caused by the activation of PPARβ/δ by GW501516 (**Figs. 21c-d**), suggesting that the PPARβ/δ-mediated increase in GDF15 involves AMPK activation. To confirm this, C2C12 myotubes were transfected with a specific siRNA against both *Ampk1/2* to knockdown both genes (**Supplementary Fig. 3a-b**), which significantly attenuated the increase in *Gdf15* mRNA levels caused by GW501516 (**Fig. 21e**). All together, these results confirm the implication of AMPK in the increase of GDF15 caused by GW501516.

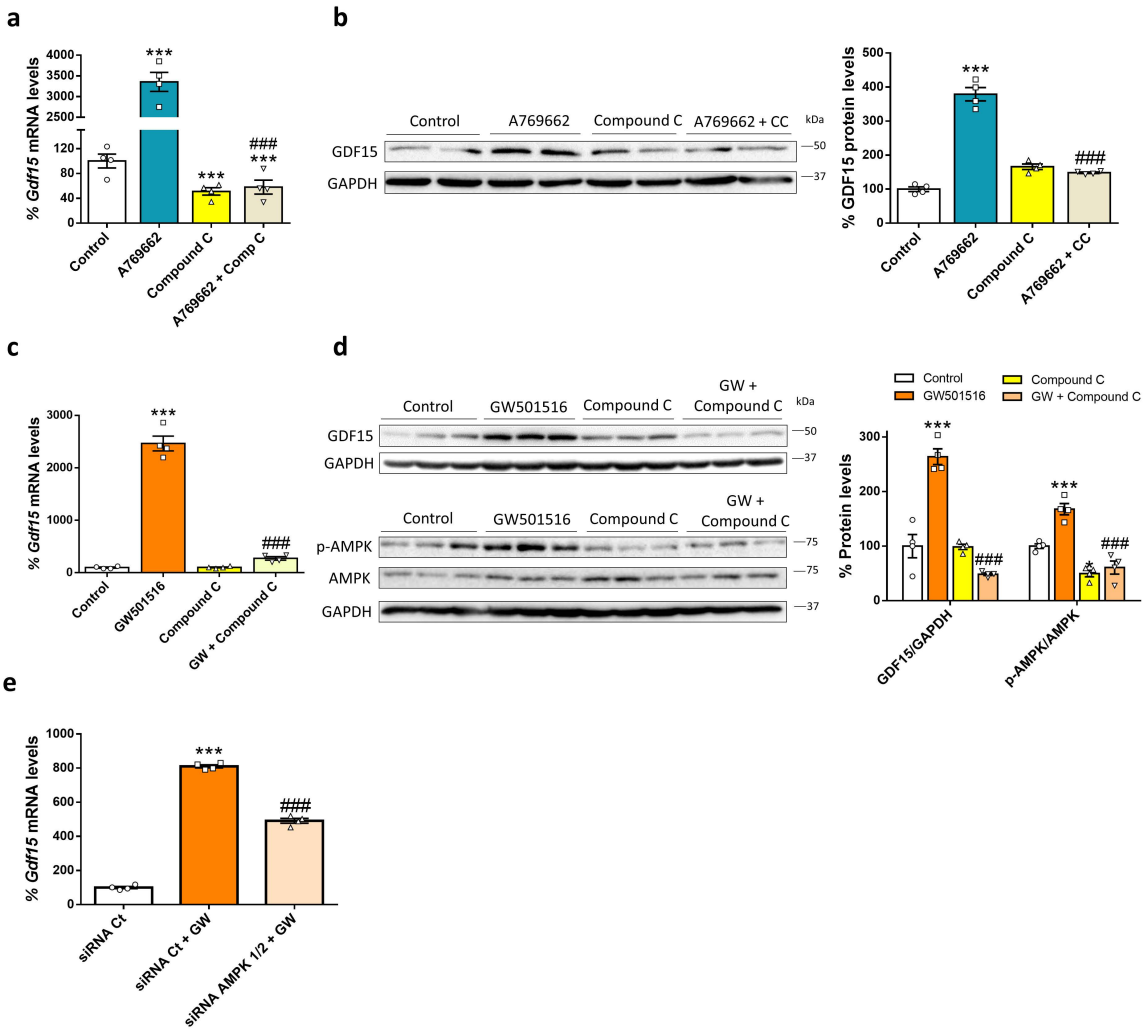


Figure 21. AMPK activation is involved in the increased GDF15 expression caused by PPARβ/δ activation. **a)** *Gdf15* mRNA levels and **(b)** GDF15 protein levels in C2C12 myotubes exposed to 60 µM of the AMPK activator A769662 in the presence or absence of 30 µM of the AMPK inhibitor compound C (CC) for 24 h (n = 4). **c)** *Gdf15* mRNA levels and **(d)** GDF15 and total and phospho-AMPK protein levels in C2C12 myotubes exposed to 10 µM of the PPARβ/δ agonist GW501516 for 24 h in the presence or absence of 30 µM of compound C (CC) (n = 4 independent cell culture experiments). **e)** *Gdf15* mRNA levels in C2C12 myotubes transfected with control siRNA or *Ampk1/2* siRNA for 48h (n = 4 independent cell culture experiments). Data are presented as the mean ± s.e.m. **p < 0.01 and ***p < 0.001 *versus* control. ###p < 0.001 *versus* A769662- or GW501516-treated cells. p-values determined by one-way ANOVA with Tukey's post hoc test.

IV.1.7 p53 mediates the increase of GDF15 caused by PPARβ/δ and AMPK activation

GDF15 expression is regulated by different transcriptions factors, including p53 (Kannan et al., 2000), (Tan et al., 2000), (Osada et al., 2007), which is negatively regulated by murine double minute X (MDMX), an MDM2 homolog that binds to p53 and inhibits its transcriptional activity (Yu et al., 2020). Given that AMPK inhibits MDMX through its phosphorylation, resulting in the stabilization and activation of p53 (He et al., 2014), (Chen et al., 2015b), we evaluated the involvement of p53 in the increased levels of GDF15 following PPARβ/δ activation.

First, we examined whether under our conditions, AMPK also increased p53 levels. AMPK activation by A769662 in myotubes increased *p53* expression, which was prevented by the AMPK inhibitor compound C (**Fig. 22a**). Interestingly, GW501516 treatment increased p53 protein levels in myotubes (**Fig. 22b**) and this increase was blocked by the PPARβ/δ antagonist GSK3787 (**Fig. 22c**) and by the knockdown of *Ampk1/2* (**Fig. 22d**). Similar to these myotubes studies, mice treated with GW501516 showed increased p53 protein levels in both the skeletal muscle (**Fig. 22e)** and liver (**Fig. 22f**). Treatment of myotubes with GW501516 caused an increase in the protein levels of both p53 and GDF15 that was completely blocked by the selective p53 inhibitor pifithrin-α (**Fig. 22g**). Similarly, treatment of mice with GW501516 increased both the mRNA and protein levels of p53 and GDF15 in skeletal muscle and that was blocked by pifithrin-α (**Figs. 22h-i**). Moreover, a genetic approach using a specific siRNA to knockdown the expression of *p53* in myotubes completely inhibited the increase in GDF15 caused by GW501516 (**Fig. 22j**). Collectively, these findings indicate that PPARβ/δ activation increases GDF15 levels through activation of the AMPK-p53 pathway.

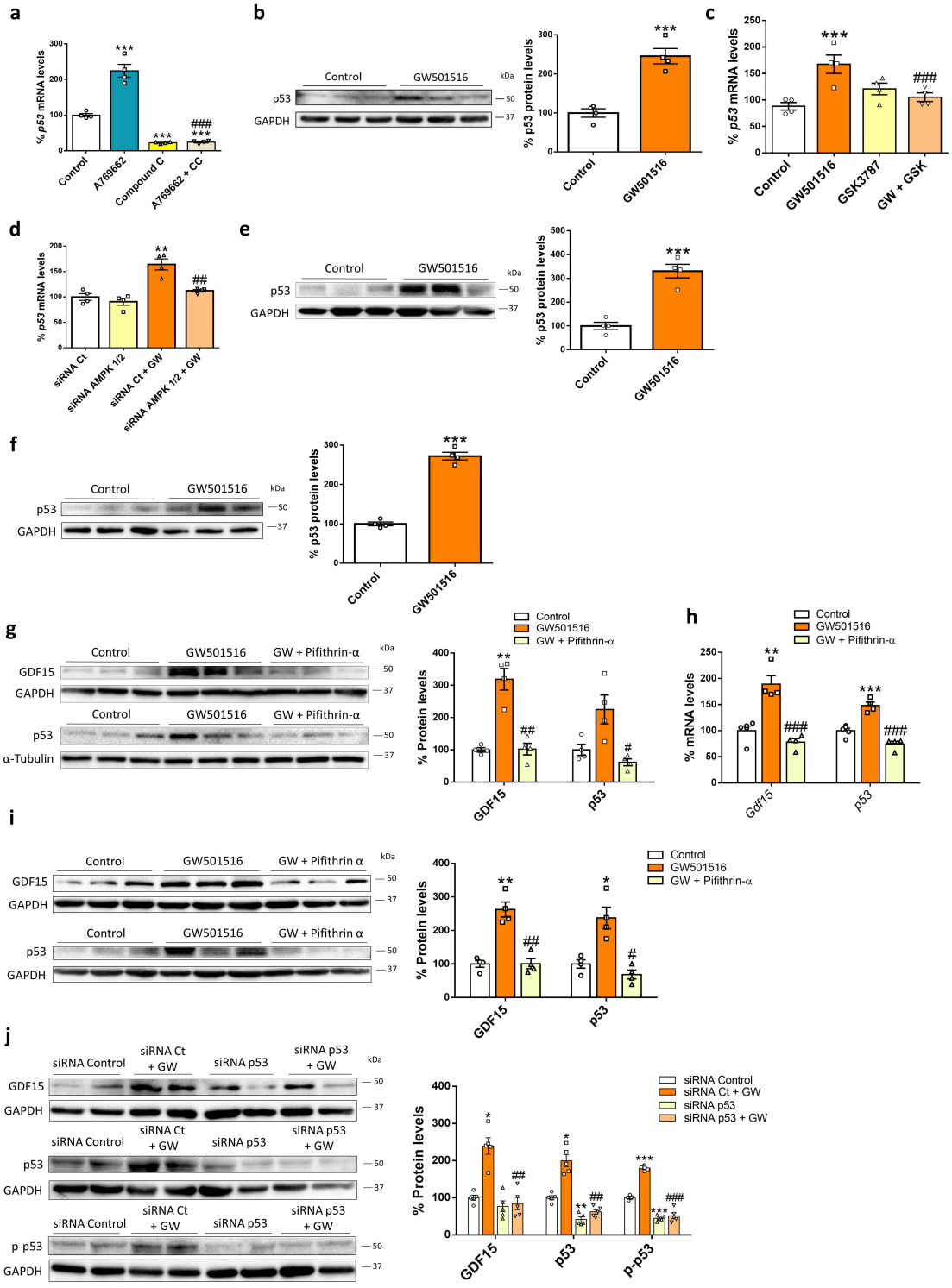


Figure 22. PPARβ/δ activation upregulates GDF15 levels through p53. a) *p53* mRNA levels in C2C12 myotubes exposed to 60 μM of the AMPK activator A769662 for 24 h in the presence or absence of 30 μM of the AMPK inhibitor compound C (CC) (n = 4 independent cell culture experiments). **b)** p53 protein levels in C2C12 myotubes exposed to 10 μM of the PPARβ/δ agonist GW501516 for 24 h (n = 4 independent cell culture experiments). **c)** *p53* mRNA levels in C2C12 myotubes exposed to 10 μM of GW501516 for 24 h in the presence or absence of 10 μM of the PPARβ/δ antagonist GSK3787 or **(d)** transfected with control siRNA or AMPK1/2

siRNA for 48h (n = 4 independent cell culture experiments). **e)** p53 protein levels in the skeletal muscle and **(f)** the liver of mice (n = 4 animals) treated with vehicle or 3 mg/kg/day of GW501516 for 6 days. **g)** GDF15 and p53 protein levels in C2C12 myotubes exposed to 10 μM of GW501516 for 24 h in the presence or absence of 10 μM of the p53 inhibitor pifithrin-α (n = 4 independent cell culture experiments). **h)** *Gdf15* and *p53* mRNA levels and (**i**) GDF15 and p53 protein levels in the skeletal muscle of mice (n = 4 animals) treated with either vehicle plus GW501516 for 6 days or GW501516 plus pifithrin-α. **j** GDF15, phospho-p53 and p53 levels in C2C12 myotubes exposed to 10 μM of GW501516 for 24 h and transfected with control siRNA or p53 siRNA for 48h (n = 4 independent cell culture experiments). Data are presented as the mean ± s.e.m. *p < 0.05, **p < 0.01, and ***p < 0.001 *versus* control. $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 *versus* A769662- or GW501516-treated cells or animals. P-values determined by one-way ANOVA with Tukey's post hoc test (a, b, c, d, g, h, i, j) and two-tailed unpaired Student's t-test (e, f).

IV.1.8 The increase in GDF15 caused by PPARβ/δ activation results in AMPK activation and does not require central effects

Since the administration of the PPARβ/δ agonist GW501516 for 21 days did not affect food intake or body weight on mice fed a HFD, consistent with previous studies carried out with this ligand (Barroso et al., 2011b), we postulated that the effects caused by the PPARβ/δ-induced increase in GDF15 levels might be peripheral. In order to test a possible GDF15 effect independent of central system, we exposed cultured myotubes to GW501516. We observed an increased expression of *Gdf15* and, more importantly, of three genes involved in fatty acid oxidation, *Cpt1b, Acadm* and *Acox* (**Fig. 23a**). The stimulation of these genes caused by GW501516 was abolished when *Gdf15* was knocked down (**Fig. 23a**). These results suggest that the increased expression of these genes that control lipid metabolism is mediated by GDF15, without implicating the central receptor GFRAL, as previously reported (Chung et al., 2017b). Co-incubation with the saturated fatty acid palmitate also increased the expression of these genes caused by the treatment with the PPARβ/δ activator (**Supplementary Figs. 5b-d**).

Since activation of AMPK plays a pivotal role in the effects of PPARβ/δ agonists and our findings show that the GDF15 neutralization and *Gdf15* deficiency prevents the increase in phospho-AMPK levels caused by PPARβ/δ activation (**Figs. 17d, 18f, 19d and 20c**), together with the reduction in phospho-AMPK in skeletal muscle of *Gdf15*$^{-/-}$ mice compared with WT mice (**Fig. 19d**), we examined whether the increased GDF15 levels caused by PPARβ/δ activation affected AMPK. Interestingly, the GW501516-mediated increase in phospho-AMPK levels in the myotubes was inhibited by the knockdown of *Gdf15*, with an opposite trend observed for ERK1/2 phosphorylation (**Fig. 23b and Supplementary Fig. 5a**), which agrees with the negative crosstalk between AMPK and ERK1/2. These results indicate that the activation of AMPK by PPARβ/δ ligands requires increased GDF15 levels to maintain its activation.

Next, to evaluate whether the direct action of GDF15 could induce the activation of AMPK, we treated myotubes with recombinant GDF15 derived from human cells for 24h and observed that this cytokine significantly increased phospho-AMPK levels, and reduced phospho-ERK1/2 levels (**Fig. 23c**). Due to recent studies reporting contamination of TGF-β in recombinant GDF15 purified from human cells expression systems (Olsen et al., 2017), (Tsai et al., 2018) and used in GDF15 function studies (Chung et al., 2017b), we additionally exposed myotubes to murine recombinant GDF15 obtained from *E. coli*, a bacterial species that lack constitutive expression of TGF-β. Treatment of myotubes with this cytokine increased phospho-AMPK levels and reduced p-ERK levels, corroborating a direct action of GDF15. Since some of the effects of recombinant GDF15 in cultured cells seem to involve activin receptor-like kinase (ALK) receptors 4/5/7 (ALK4/5/7) (Chung et al., 2017b), we exposed cells to the ALK4/5/7 inhibitor SB431542 to examine whether these ALK isoforms were involved in the effects of GDF15 on AMPK and ERK1/2. SB431542 did not prevent the effects of either recombinant GDF15 or GW501516 on the phosphorylation status of AMPK and ERK, making unlikely the involvement of ALK4/5/7 isoforms in the observed changes (**Supplementary Figs. 6a-b**).

Consistent with the effect observed in cultured myotubes, mice receiving a subcutaneous administration of recombinant GDF15 showed an increase in

phospho-AMPK in skeletal muscle, whereas phospho-ERK1/2 was reduced (**Fig. 23d**), confirming that GDF15 activates AMPK in skeletal muscle, possibly without the intervention of the central receptor GFRAL, as shown above. Nevertheless, to clearly confirm that the effect of GDF15 was independent of central effects, soleus muscle isolated from WT littermates and *Gdf15*$^{-/-}$ mice were incubated with GW501516 or recombinant GDF15 (**Fig. 23e**). Both treatments increased phospho-AMPK and reduced phospho-ERK1/2 levels in WT, but not in *Gdf15* null mice. Based on the PPARβ/δ-induced secretion of GDF15 by myotubes and increased GDF15 serum levels in mice, we speculate that GDF15 acts via autocrine or paracrine signalling. These results are consistent with the recently reported absence of GFRAL in whole muscle tissue in mice (Laurens et al., 2020b).

Although the presence of the neuronal receptor GFRAL in the C2C12 cell line has been previously excluded by other authors (Yang et al., 2017), we evaluated whether this receptor could be expressed in our cell line in order to corroborate our findings. We observed that *Gfral* expression was virtually absent in C2C12 cells, as well as in skeletal muscle and the liver obtained from WT mice (**Supplementary Fig. 7**), implying that the GDF15-mediated activation of AMPK in isolated skeletal muscle and cultured myotubes seems not to involve this receptor. Since the area postrema and the nucleus of the solitary tract located in the brainstem are the only areas that have been extensively described as of the GFRAL receptor (Yang et al., 2017), (Hsu et al., 2017b), we used brainstem as a positive control for this experiment.

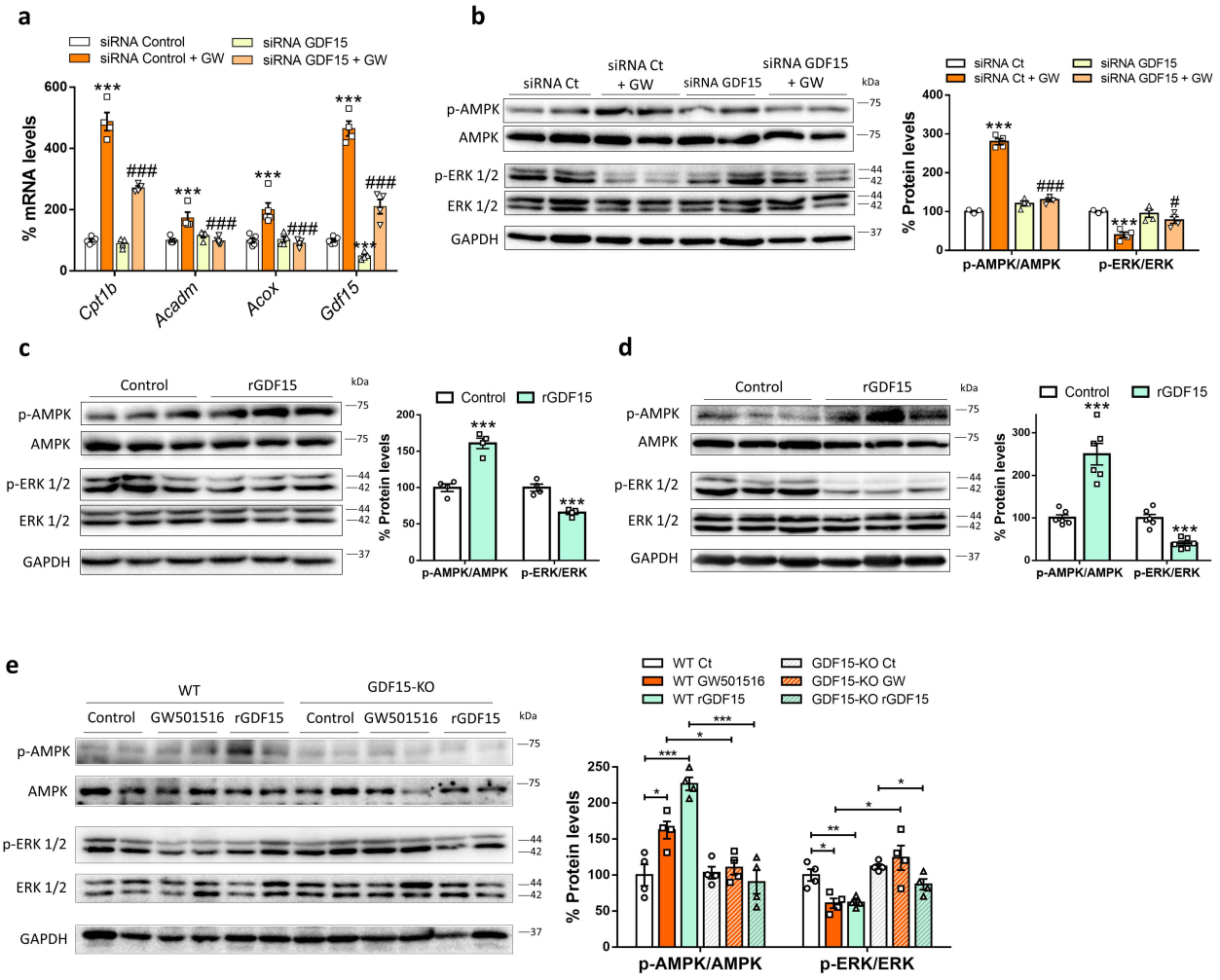
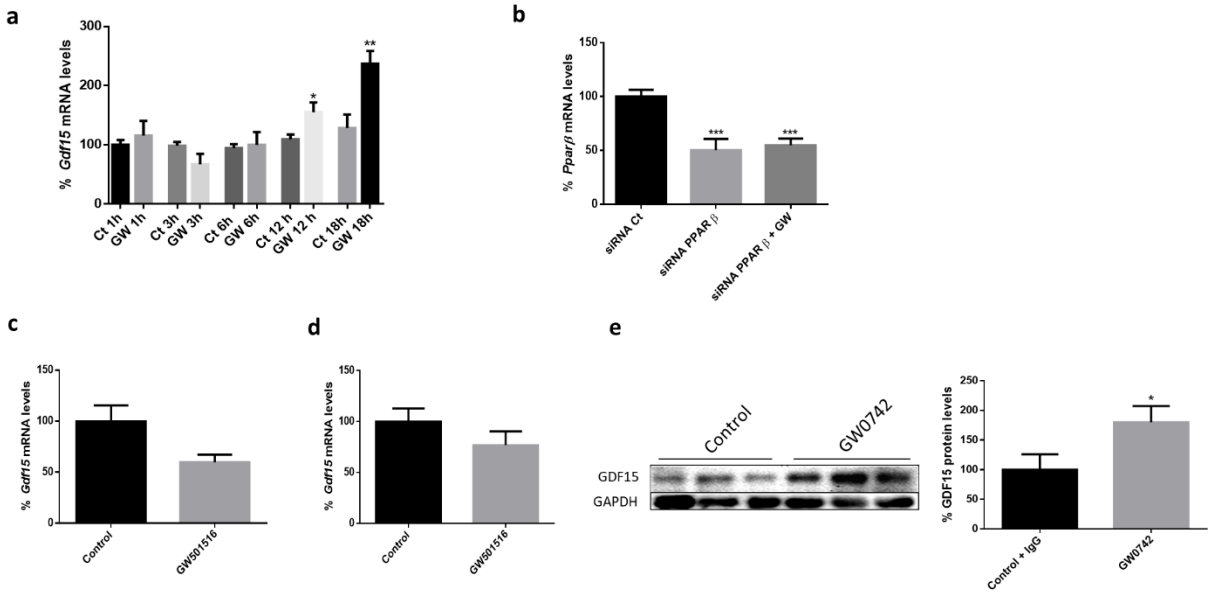


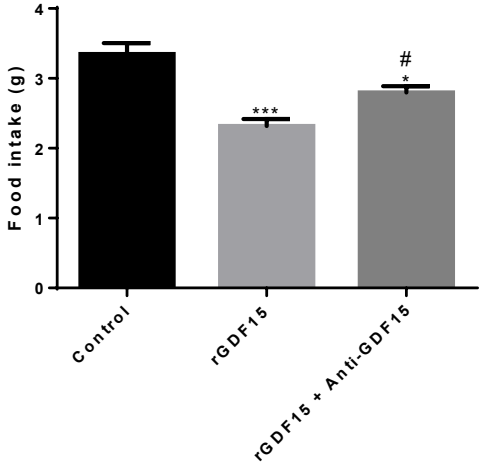
Figure 23. PPARβ/δ activation requires increased GDF15 expression to maintain the increase in phospho-AMPK levels. **a)** *Cpt1b, Acadm, Acox* and *Gdf15* mRNA levels in C2C12 myotubes in the presence or absence of 10 μM of the PPARβ/δ agonist GW501516 for 24 h and transfected with control siRNA or GDF15 siRNA for 48 h (n = 4). **b)** Total and phospho-AMPK and total and phospho-ERK1/2 protein levels in C2C12 myotubes in the presence or absence of 10 μM of GW501516 for 24 h and transfected with control siRNA or GDF15 siRNA (n = 4). **c)** Total and phospho-AMPK and total and phospho-ERK1/2 protein levels in C2C12 myotubes exposed to human GDF15 (100 ng/L) for 24 h (n = 4). **d)** Immunoblot analysis of total and phospho-AMPK and total and phospho-ERK1/2 protein in in skeletal muscle of mice (n = 6 animals) treated with GDF15 (two subcutaneous injections of 0.05 mg/kg GDF15 for 2 days). **e)** Total and phospho-AMPK and total and phospho-ERK1/2 protein levels in isolated soleus muscles from WT and *Gdf15* null mice incubated with 10 μM GW501516 or 500 ng/ml of recombinant mouse GDF15 for 4 h (n = 4 muscles). Data are presented as the mean ± s.e.m. ***p < 0.001 *versus* control. ###p < 0.001 and #p < 0.05 *versus* siRNA control cells treated with GW501516. p-values determined by

one-way ANOVA with Tukey's post hoc test (a, b) and two-tailed unpaired Student's t-test (c, d).

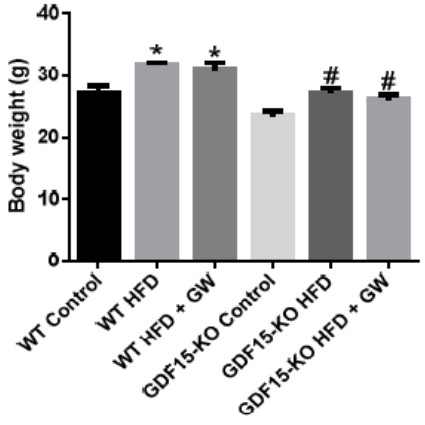
IV.1.9 Supplementary information related to Part I:



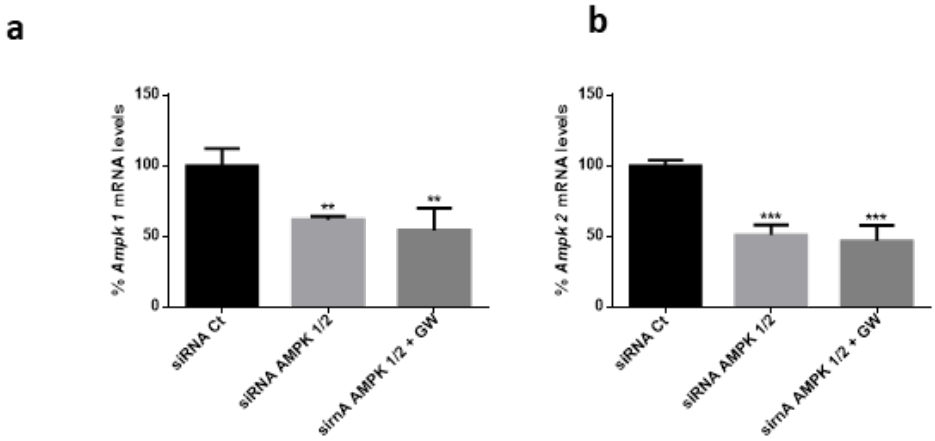
Supplementary Fig. 1. The increase in GDF15 caused by PPARβ/δ is a family effect and is time dependent (**related to main Figure 1**). **a)** *Gdf15* mRNA levels in C2C12 myotubes treated with 10 µM GW501516 at different time points. **b)** *PPARβ/δ* mRNA levels in C2C12 myotubes transfected with control siRNA or PPARβ/δ siRNA. Data are presented as the mean ± s.e.m. (n=4 per group). *Gdf15* mRNA levels in skeletal muscle of mice treated with vehicle or 3 mg/kg/day/ of GW501516 for 2 **(c)** or 4 days **(d). e)** Immunoblot analysis of GDF15 in skeletal muscle of mice treated with vehicle or 3 mg/kg/day of GW0742 for 6 days. Data are presented as the mean ± s.e.m. (n=4 per group). *p<0.05, **p<0.01 and ***p<0.001 *versus* control.



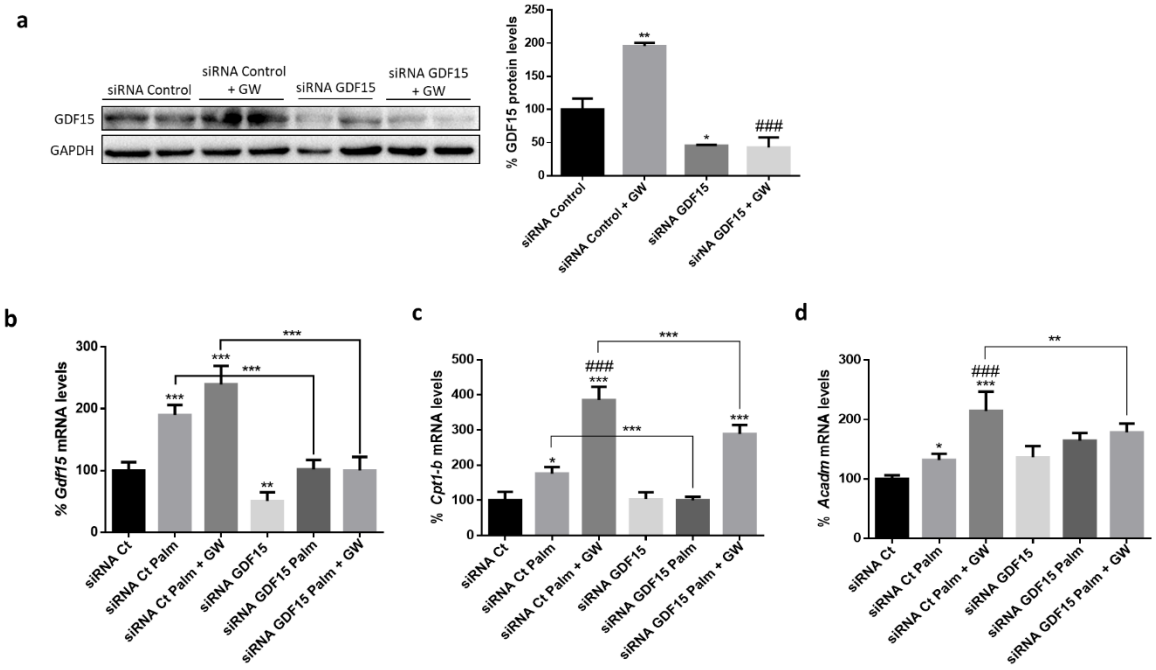
Supplementary Figure 2. The GDF15 neutralizing antibody attenuates the reduction in food intake caused by recombinant mouse GDF15 (rGDF15) (**related to main Figure 2**). Mice were treated with GDF15 neutralizing antibody or IgG 48 h before receiving 0.1 mg/kg rGDF15 or vehicle (at 18.00 h.) and food intake was determined 16 h later. Data are presented as the mean ± s.e.m. (n = 5 per group). *p<0.05 and ***p<0.001 *versus* control. #p<0.05 *versus* mice treated with rGDF15.



Supplementary Figure 3. GW501516 treatment does not affect body weight (**related to main Figure 5**). GW501516 treatment does not significantly affect body weight either in wild-type or in *Gdf15*$^{-/-}$ mice. Data are presented as the mean ± s.e.m. (n = 5 per group). *p<0.05 *versus* WT control. #p<0.05 *vs.* GDF15 KO control.



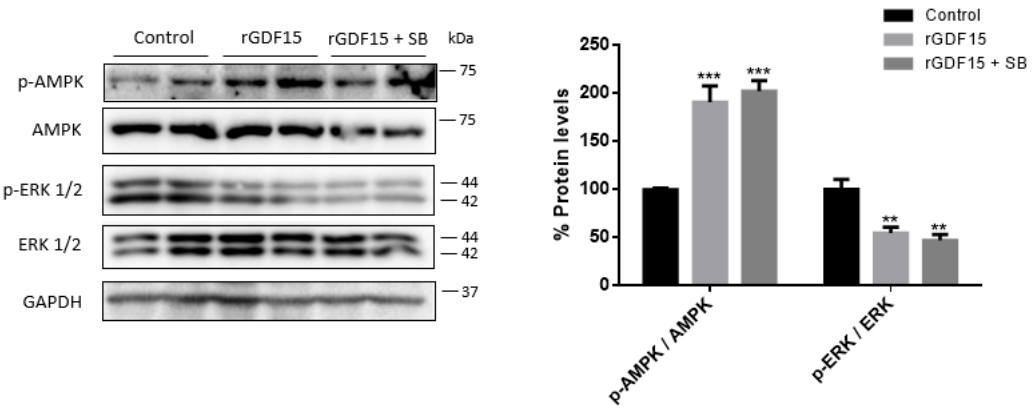
Supplementary Figure 4. *Ampk1* and *Ampk2* mRNA levels (**related to main Figure 7**). *Ampk1* (**a**) and *Ampk2* (**b**) mRNA levels in C2C12 myotubes transfected with control siRNA or Ampk1/2 siRNA in the presence or absence of 10 µM of GW501516. Data are presented as the mean ± s.e.m. (n=4 per group). **p<0.01 and ***p<0.001 *versus* control.



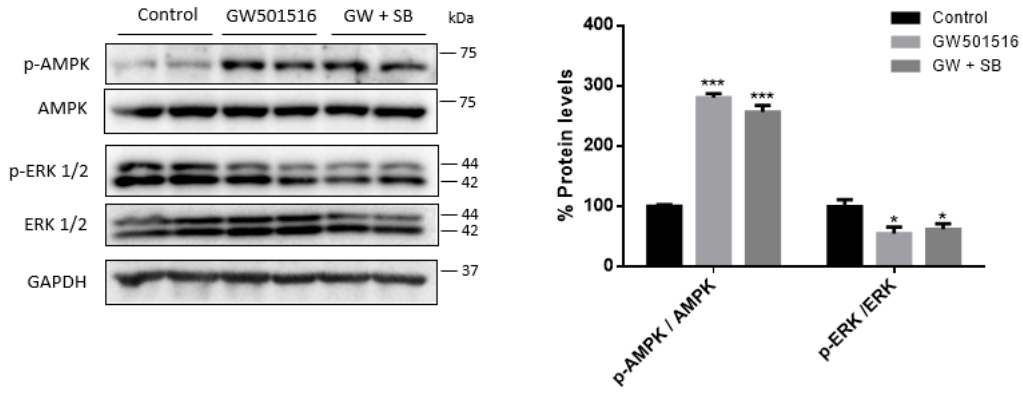
Supplementary Figure 5. GDF15 regulates the expression of genes implicated in fatty acid oxidation (**related to main Figure 9**). **a)** Immunoblot analysis of GDF15 in C2C12 myotubes transfected with control siRNA or *Gdf15* siRNA. **b-d** *Gdf15* **(b)**, *Cpt-1b* (**c**) and *Acadm* **(d)** mRNA levels in C2C12 myotubes exposed to palmitate (0.5 mM), in the presence or absence of 10 µM of GW501516 for 24 h and transfected with

control siRNA or GDF15 siRNA. Data are presented as the mean ± s.e.m. (n= 4 per group). *p<0.05, **p<0.01 and ***p<0.001 *versus* control or between the indicated groups. #p<0.05, ##p<0.01, and ###p<0.001 *versus* siRNA control cells exposed to either palmitate or GW501516.

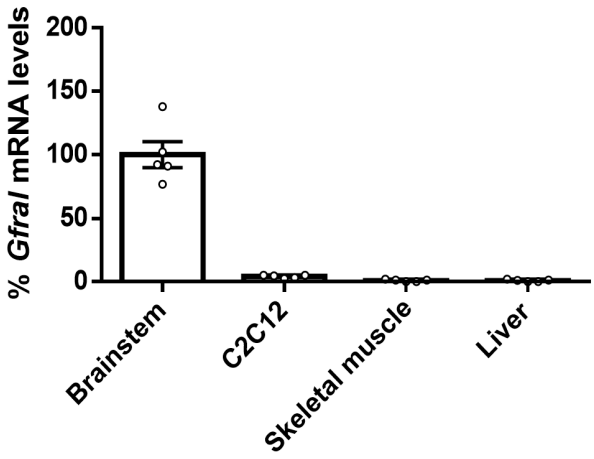
a



b



Supplementary Figure 6. Recombinant mouse GDF15 and GW501516 activate AMPK in C2C12 myotubes (**related to main Figure 9**). Total and phospho-AMPK and total and phospho-ERK1/2 protein levels in C2C12 myotubes exposed to either recombinant mouse GDF15 (rGDF15) (100 ng/L) **(a)** or 10 µM GW501516 **(b)** for 24 h in the presence or in the absence of 5 µM SB431542 (ALK4/5/7 inhibitor). Data are presented as the mean ± s.e.m. (n= 4 per group). *p<0.05, **p<0.01 and ***p<0.001 *versus* control.



Supplementary Figure 7. ***Gfral*** **expression is absent in C2C12 myotubes, skeletal muscle and the liver**. *Gfral* mRNA levels in brainstem, skeletal muscle, and the liver of mice and in C2C12 myotubes. Data are presented as the mean ± s.e.m. (n= 5 per group).

IV.1.2 PART II - GDF15 is involved in the AMPK-mediated effects of metformin

IV.2.1 Metformin treatment increases GDF15 levels in different cell lines

Our previous study showed that PPARβ/δ increases GDF15 levels through a molecular pathway involving the activation of AMPK. Since metformin promotes the activation of AMPK (Hawley et al., 2002), and a clinical study carried out in 2017 revealed that metformin increased the serum levels of GDF15 in a dose-dependent manner (Gerstein et al., 2017), we evaluated if treatment with this drug could increase GDF15 levels in different cell lines. For that purpose, C2C12 myotubes were treated with different concentrations of metformin. *Gdf15* mRNA levels were significantly increased after the treatment of 1 mM and 2 mM metformin for 24h (**Fig. 24a**). In addition, GDF15 secretion into the culture media was increased by the treatment of 2 mM metformin for 24h (**Fig. 24b**). This is consistent with the fact that approximately 2 mM metformin is required to maximally elevate the AMP/ATP ratio for AMPK activation (Foretz et al., 2010b). However, this concentration is lower than the 5 mM required to inhibit complex 1 *in vitro* (He and Wondisford, 2015). In agreement with the uptake and accumulation of metformin in skeletal muscle (Gormsen et al., 2016), myotubes exposed to 0.5 mM metformin for 3 days showed increased *Gdf15* expression (**Fig. 24c**). A concentration-response study in the Huh-7 human hepatoma cell line showed that metformin also increased *GDF15* expression in the cells with a liver origin. (**Fig. 24d**). Furthermore, in a mouse primary culture of hepatocytes metformin also increased *Gdf15* expression significantly at 1 mM and 2 mM (**Fig. 24e**).

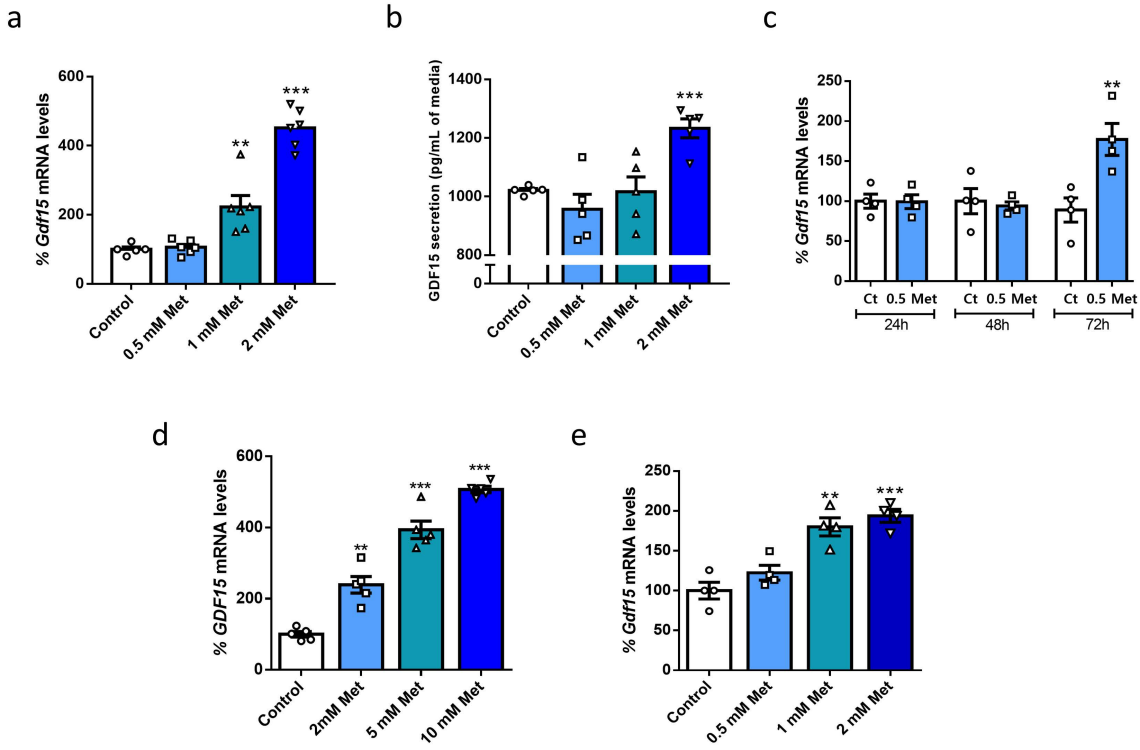


Figure 24. Metformin increases GDF15 levels. a) *Gdf15* mRNA levels, and **b)** GDF15 secreted into the culture medium in C2C12 myotubes exposed to different concentrations of metformin (Met) for 24 h. **c)** *Gdf15* mRNA levels in C2C12 myotubes exposed to 0.5 mM metformin (Met) for 24, 48 or 72 h. **d)** *GDF15* mRNA levels in Huh-7 human hepatoma cells exposed to different concentrations of metformin (Met) for 24 h. **e)** *Gdf15* mRNA levels in mouse primary hepatocytes exposed to different concentrations of metformin (Met) for 24 h. n = 4-5 per group. Data are presented as the mean ± SEM. P values: one-way ANOVA and Student's t test. **p < 0.01 and ***p < 0.001 *versus* control.

IV.2.2 Metformin increases GDF15 levels through an AMPK-dependent mechanism

Consequent with our previous findings, a recent study also showed that AMPK activation increases GDF15 levels (Townsend et al., 2021). Considering that many of the antidiabetic actions of metformin at the molecular level involve AMPK activation (Santomauro Júnior et al., 2008) (Hawley et al., 2002) (Rena et al., 2017), we hypothesized that AMPK could also be involved in the increase of GDF15 levels caused by metformin. To test this, we transfected cultured myotubes with a specific

siRNA against AMPK (**Supplementary Fig. 8a**), which resulted in a complete prevention of the increase in *Gdf15* mRNA levels caused by metformin (**Fig. 25a**), indicating that this effect was mediated by AMPK. In mouse primary culture of hepatocytes, *Ampk* knockdown (**Supplementary Fig. 8d**) significantly reduced *Gdf15* basal expression and prevented *Gdf15* upregulation in cells exposed to metformin (**Fig. 25b**). Likewise, the AMPK inhibitor compound C prevented the increase in *Gdf15* mRNA levels caused by metformin in primary hepatocytes (**Fig. 25c**). In fact, compound C blocked both the phosphorylation of AMPK and the increase in GDF15 protein levels caused by metformin in primary hepatocytes (**Fig. 25d**). To further confirm that this increase of GDF15 levels is directly caused by AMPK, we exposed primary hepatocytes to the AMPK activator A769662. Treatment with this compound increased the mRNA levels of *Gdf15*, as previously reported (Townsend et al., 2021), but this effect was blocked by co-incubating the cells with compound C (**Supplementary Fig. 8e**). These results confirm that metformin increases GDF15 levels through an AMPK dependent mechanism.

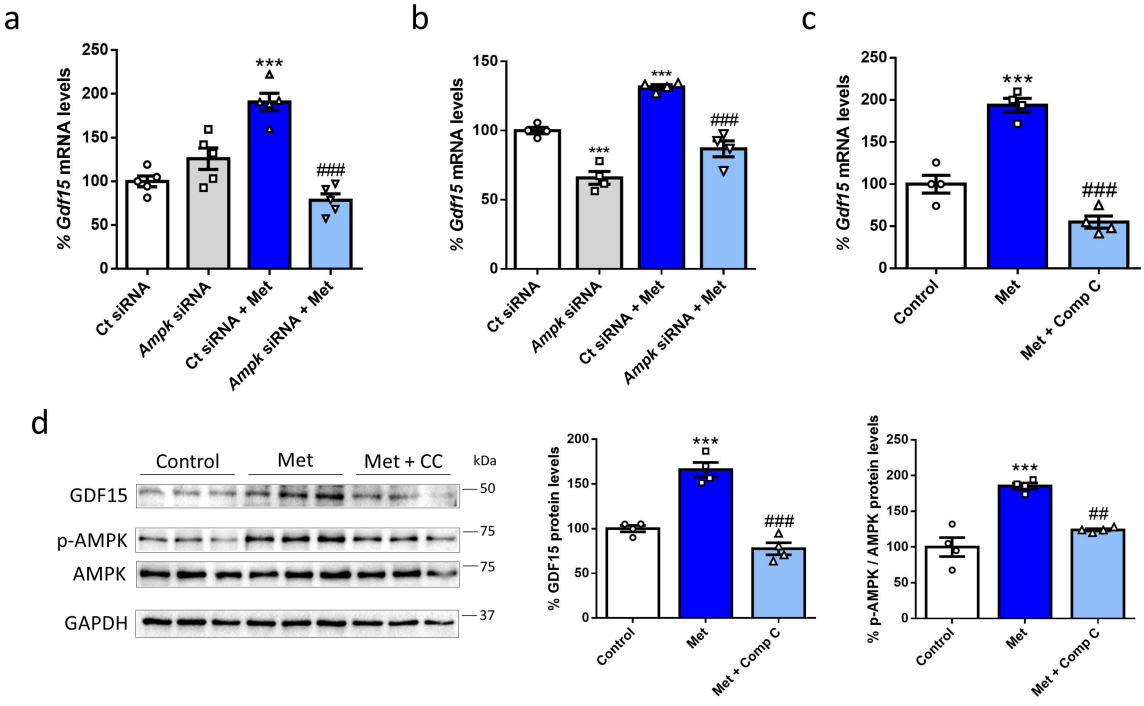


Figure 25. Metformin increases GDF15 levels via AMPK activation. **a)** *Gdf15* mRNA levels in C2C12 myotubes transfected with control (Ct) (scramble) siRNA or AMPK siRNA and treated with 2 mM metformin fo 24h. **b)** *GDF15* mRNA levels in

mouse primary hepatocytes transfected with control (Ct) (scramble) siRNA or AMPK siRNA and treated with 2 mM metformin fo 24h. **c)** *Gdf15* mRNA levels in primary hepatocytes exposed to 2 mM metformin (Met) for 24 h in the presence or absence of 30 μM compound C (Comp C). **d)** Immunoblot analysis of GDF15, and total and phospho-AMPK in primary hepatocytes exposed to 2 mM metformin (Met) for 24 h in the presence or absence of 30 μM compound C (Comp C). n = 4-5 per group. Data are presented as the mean ± SEM. P values: one-way ANOVA and Student's t test. ***p < 0.001 *versus* control. ##p < 0.01 *versus* Met ###p < 0.001 *versus* Ct siRNA + Met or Met.

IV.2.3 GDF15 increase by metformin involves ATF3

GDF15 expression is positively regulated by different transcription factors, including Sp1, EGR1 CHOP, p53 or ATF3. According to our previous study, AMPK activation increases GDF15 levels through p53, therefore, we evaluated whether this transcription factor was involved in the increase of GDF15 caused by metformin. However, it is unlikely that p53 was involved in the observed increase in *Gdf15* expression, since knockdown of *p53* did not affect the metformin-induced stimulation of *Gdf15* (**Supplementary Fig. 8b**).

Since activation transcription factor 3 (ATF3) has been reported to mediate the metformin-induced suppression of the inflammatory response against lipopolysaccharide (LPS) treatment (Kim et al., 2014b), a process dependent on AMPK, and this transcription factor has the ability to bind to the promoter of GDF15 and increase its expression, we next explored its involvement in metformin-mediated *Gdf15* upregulation. First, metformin increased *Atf3* mRNA at 2 mM concentration in cultured myotubes (**Fig. 26a**). Consistent with the increase of *GDF15* expression in Huh-7 cells caused by metformin at 2 mM concentration, this drug also increased the expression of *ATF3* at this concentration (**Fig. 26b**). The increase of *Atf3* expression levels caused by metformin was attenuated by *Ampk* knockdown in cultured myotubes (**Fig. 26c**), demonstrating the involvement of this kinase. Finally, *Atf3* knockdown in myotubes (**Supplementary Fig. 8c**) significantly

attenuated the increase caused by metformin in *Gdf15* (**Fig. 26d**), confirming its participation. Overall, these findings confirm that metformin increases GDF15 levels through AMPK phosphorylation by a mechanism involving ATF3 upregulation

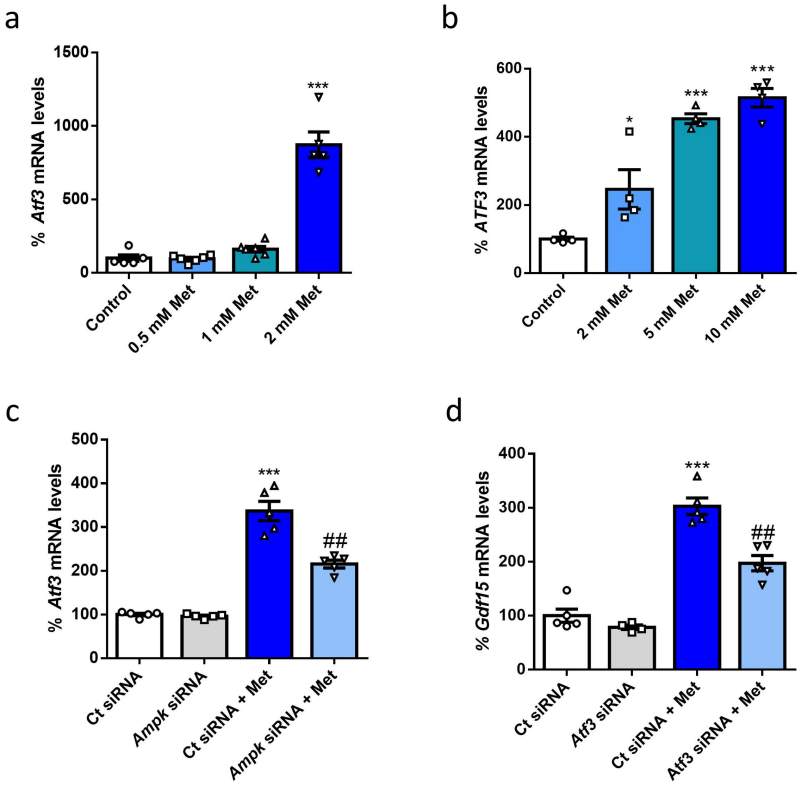


Figure 26. ATF3 is upregulated by metformin and is involved in the increase of GDF15 levels. *Atf3* mRNA levels in C2C12 myotubes **(a)** or Huh-7 cells **(b)** exposed to different concentrations of metformin (Met) for 24 h. **c)** *Atf3* mRNA levels in C2C12 myotubes transfected with control (Ct) (scramble) siRNA or AMPK siRNA and treated with 2 mM metformin for 24 h. **d)** *Gdf15* mRNA levels in C2C12 myotubes transfected with control (Ct) (scramble) siRNA or Atf3 siRNA and treated with 2 mM metformin for 24 h. n = 4-5 per group. Data are presented as the mean ± SEM. P values: one-way ANOVA and Student's t test. *p < 0.05 and ***p < 0.001 *versus* control. ##p < 0.01 *versus* Ct siRNA + Met.

IV.2.4 Recombinant GDF15 induces metabolic changes independently of GFRAL

The administration of recombinant GDF15 to *ob/ob* mice was shown to reduce body weight independently of food intake; these effects were attributed to increased oxidative metabolism and lipid mobilization in the liver, muscle, and

adipose tissue (Chung et al., 2017b). To demonstrate that GDF15 regulates the expression of these genes in the absence of the central receptor GFRAL, we treated C2C12 myotubes with recombinant GDF15, as we have shown that this cell line lacks the central receptor GFRAL (**Supplementary Fig. 7**), which is consistent with previous studies (Yang et al., 2017). This cytokine increased the expression of *Cpt1b, Acadm* and *Acox*, whereas *Pgc1a* mRNA levels were not altered (**Fig. 27a**). In addition, recombinant GDF15 reduced the mRNA levels of the pro-inflammatory cytokines *Tnfa* and *Il6*, whereas no changes were observed in the ER stress marker *Trb3* (Fig. 4a). Recombinant GDF15 also increased the protein levels of PGC-1α, PPARα, and the β unit of the insulin receptor (IRβ), thereby suggesting that it can influence insulin signalling in addition to fatty acid oxidation (**Fig. 27b**). In fact, recombinant GDF15 treatment significantly increased insulin-stimulated Akt phosphorylation in myotubes compared to cells that were not exposed to insulin (**Fig. 27c**). Collectively, these data indicate that GDF15 has peripheral effects on the levels of proteins involved in fatty acid β-oxidation and in insulin signalling in cultured myotubes and we suggest that these effects are not dependent on GFRAL signalling.

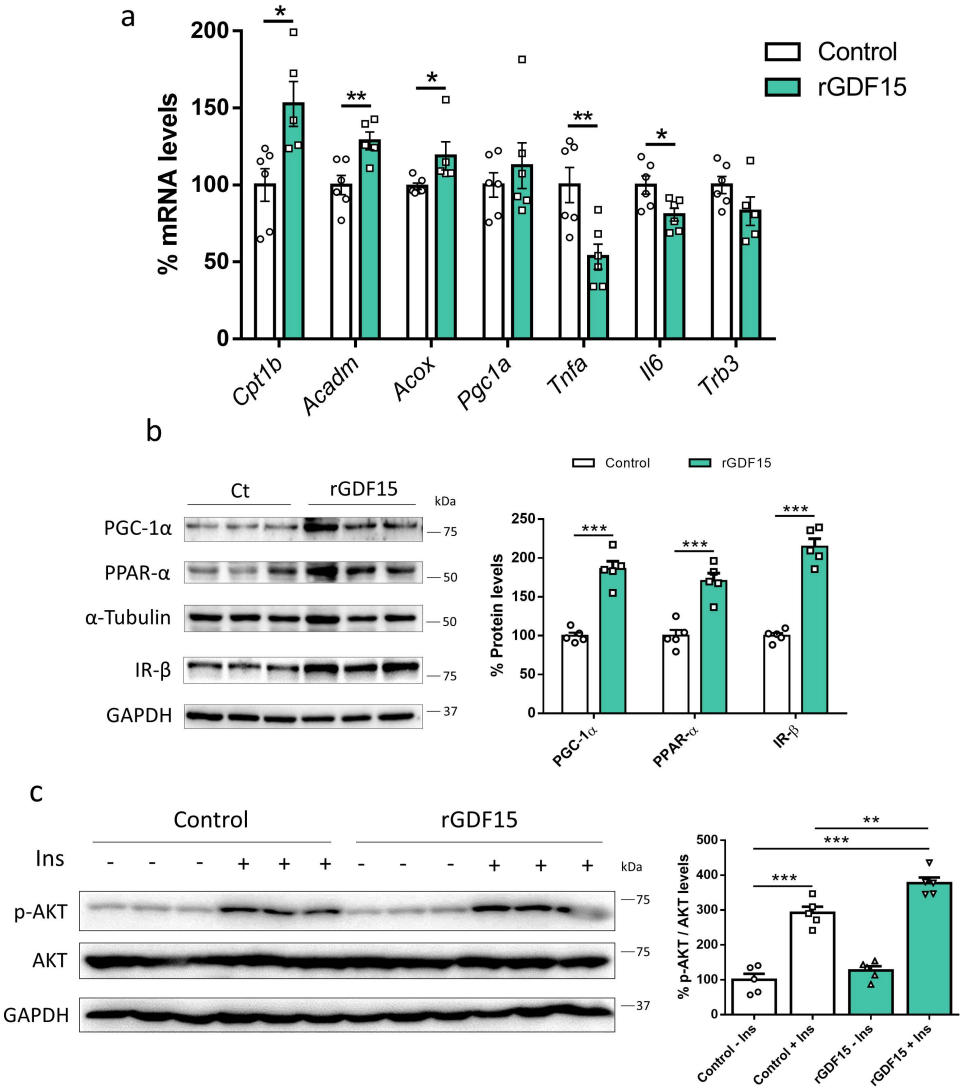


Figure 27. Metabolic effects of recombinant GDF15 administration in cultured myotubes. a) mRNA levels of *Cpt1b, Acadm, Acox, Pgc1a, Tnfa, Il6* and *Trb3* in C2C12 myotubes exposed to 100 ng/ml of recombinant GDF15 (rGDF15) for 24 h. **b)** Immunoblot analysis of PGC-1α, PPARα, and IRβ in C2C12 myotubes exposed to 100 ng/ml of recombinant GDF15 (rGDF15) for 24 h. **c)** Immunoblot analysis of total and phospho-Akt (Ser473) in C2C12 myotubes exposed to 100 ng/ml of rGDF15 for 24 h. Where indicated, cells were incubated with 100 nmol/l of insulin (Ins) 10 minutes before collection. n = 5 per group. Data are presented as the mean ± SEM. P values: (a-b) Student's t test and (c-g) one-way ANOVA. *p < 0.05, **p < 0.01 and ***p < 0.001 *versus* indicated groups.

IV.2.5 *Gdf15* knockdown attenuates metformin-mediated AMPK activation in the absence of GFRAL

Next, we evaluated whether metformin contributed to AMPK activation and the regulation of the proteins involved in lipid metabolism and insulin signalling in cultured myotubes in the absence of GFRAL. First, *Gdf15* was knocked down by siRNA transfection, which blunted the phosphorylation of AMPK by metformin, indicating that GDF15 is required for metformin-mediated AMPK activation (**Fig. 28a**). Similarly, *Gdf15* knockdown partially prevented the increase in *Pgc1a* expression and abrogated the increase in *Acox* expression and the decrease in *Mcp1* mRNA levels caused by metformin (**Fig. 28b, Supplementary Fig. 9b**). Second, it is well known that the saturated fatty acid palmitate lowers PGC-1α and phospho-AMPK levels and increases inflammation in myotubes and skeletal muscle (Palomer et al., 2018). In line with this, incubation of myotubes with palmitate decreased *Pgc1a* mRNA levels, but co-incubation with metformin reverted this situation and caused a notable increase in the expression of this gene, which was attenuated by *Gdf15* knockdown (**Fig. 28c**, **Supplementary Fig. 9b**). The strong induction of *Il6* expression caused by palmitate was mitigated by co-incubation with metformin, whereas *Gdf15* knockdown significantly increased the expression of this cytokine in cells exposed to palmitate and in those co-incubated with metformin (**Fig. 28c**). Palmitate treatment also reduced phospho-AMPK levels, whereas treatment with metformin caused a large increase in the phosphorylated levels of this kinase that was attenuated by *Gdf15* knockdown (**Fig. 28d**, **Supplementary Fig. 9c**). The increase in the protein levels of PPARα, IRβ and the nuclear factor (NF)-κB inhibitor IκBα caused by metformin was also prevented by *Gdf15* knockdown (Fig. 5e). Finally, the reduction in PGC-1α protein levels caused by palmitate was prevented by metformin treatment, but this effect of metformin was abolished by *Gdf15* knockdown (**Fig. 28e**). These findings indicate that GDF15 upregulation by metformin contributes to AMPK activation and the effects on the levels of proteins involved in fatty acid oxidation, inflammation, and insulin signalling, independent of central mechanisms or the intervention of GFRAL.

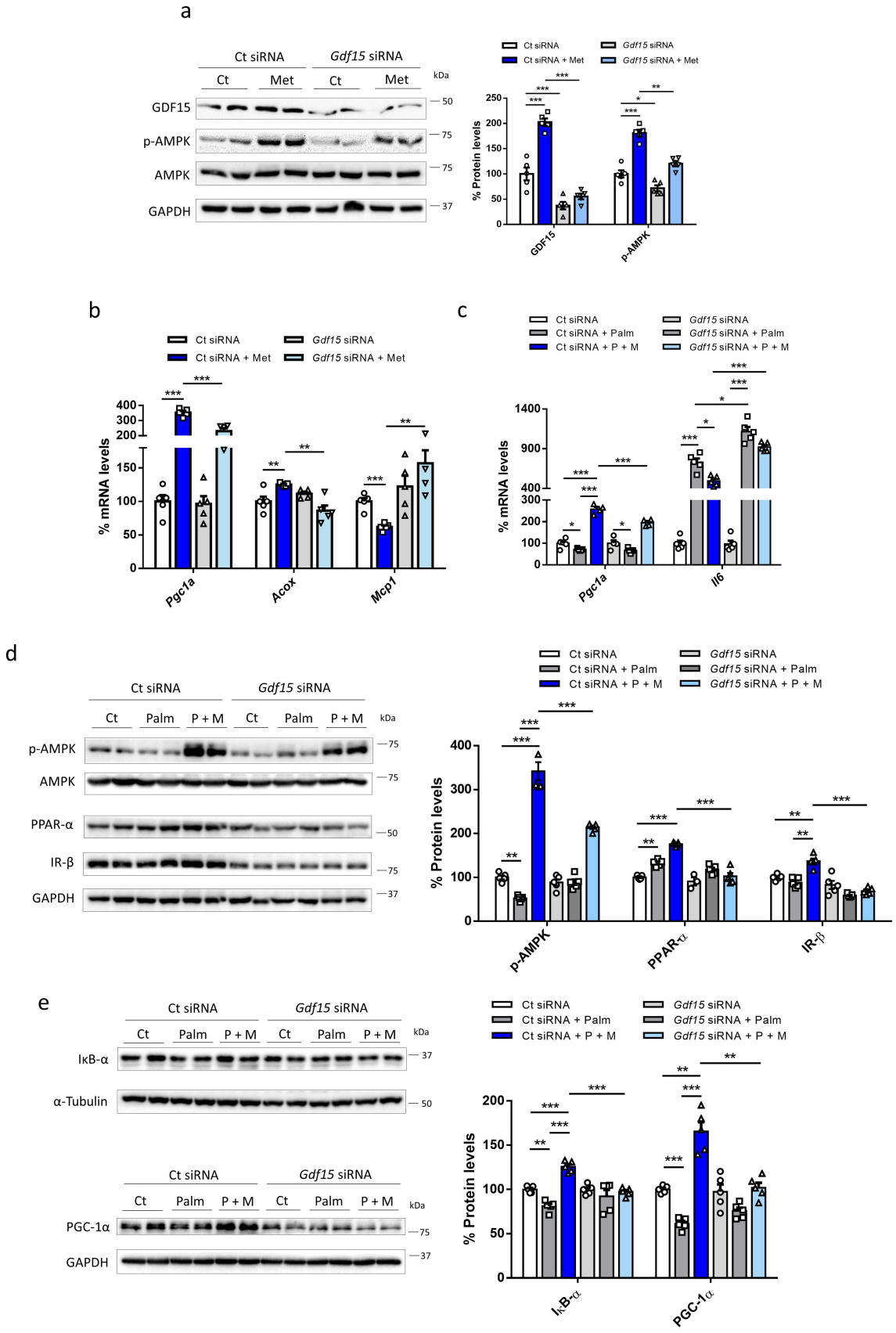


Figure 28. ***Gdf15*** **knockdown prevents metformin-mediated AMPK activation. a)** Immunoblot analysis of GDF15, and total and phospho-AMPK in C2C12 myotubes transfected with control (Ct) (scramble) siRNA or GDF15 siRNA and treated with 2

mM metformin for 24 h. **b)** mRNA levels of *Pgc1a*, *Acox* and *Mcp1* in C2C12 myotubes transfected with control (Ct) (scramble) siRNA or GDF15 siRNA and treated with 2 mM metformin for 24 h. **c)** mRNA levels of *Pgc1a* and *Il6* in C2C12 myotubes transfected with control (Ct) (scramble) siRNA or GDF15 siRNA and exposed to 0.5 mM palmitate or palmitate plus 2 mM metformin for 24 h. Immunoblot analysis of total and phospho-AMPK, PPARα and IRβ **(d)** or IκBα and PGC-1α **(e)** in C2C12 myotubes transfected with control (Ct) (scramble) siRNA or GDF15 siRNA and exposed to 0.5 mM palmitate or palmitate plus 2 mM metformin for 24 h. (a-g) n = 5 per group. Data are presented as the mean ± SEM. P values: (a-b) Student's t test and (c-g) one-way ANOVA. *p < 0.05, **p < 0.01 and ***p < 0.001 *versus* the indicated group.

IV.2.6 *Gdf15* null mice present an attenuated response to metformin in the liver

To clearly demonstrate that the metabolic effects of metformin were dependent on GDF15, we used a *Gdf15*-knockout (*Gdf15*$^{-/-}$) murine model and their wild-type (WT) littermates. Mice were fed either a standard diet or a HFD and treated with vehicle or metformin at a low dose (100 mg/kg/day) or vehicle as control for 21 days. On purpose, the metformin dose was lower than that used in previous studies demonstrating weight loss through GFRAL signalling (Coll et al., 2020b), (Day et al., 2019). WT mice on the HFD showed a significantly increased body weight, but the administered dose of metformin did not cause a significant reduction of weight gain (**Fig. 29a**). In *Gdf15*$^{-/-}$ mice, no significant differences in body weight were observed compared to WT mice (**Fig. 29a**). WT mice fed the HFD displayed increased epididymal white adipose tissue (eWAT) referenced to each animal total body weight, and this was attenuated by metformin treatment, whereas no changes in eWAT were observed in *Gdf15*$^{-/-}$ mice (**Fig. 29b**). Contrary to other studies reporting a decrease in food intake caused by metformin treatment at higher doses (Coll et al., 2020b), (Day et al., 2019), metformin treatment did not affect cumulative food intake in both the WT and *Gdf15*$^{-/-}$ mice, with an increase only observed in the *Gdf15*$^{-/-}$ mice fed the standard diet (**Fig. 29c**). As expected, metformin ameliorated glucose intolerance in WT mice, but this effect was

completely abolished in *Gdf15*$^{-/-}$ mice (**Fig. 29d**). Remarkably, the AUC from *Gdf15*$^{-/-}$ mice fed the standard diet was significantly higher than that from the WT mice. HFD feeding caused a slight increase in serum triglyceride levels that was strongly reverted by metformin in WT mice (**Fig. 29e**). However, this reduction was significantly lower in *Gdf15*$^{-/-}$ mice exposed to the same treatment (-30%). Interestingly, WT mice fed the HFD and treated with metformin had significantly higher levels of serum GDF15 than those fed the HFD and treated with vehicle (**Fig. 29f**), whereas serum from *Gdf15*$^{-/-}$ animals did not reach the lower detection limit of the assay (5.6 pg / mL) (data not shown).

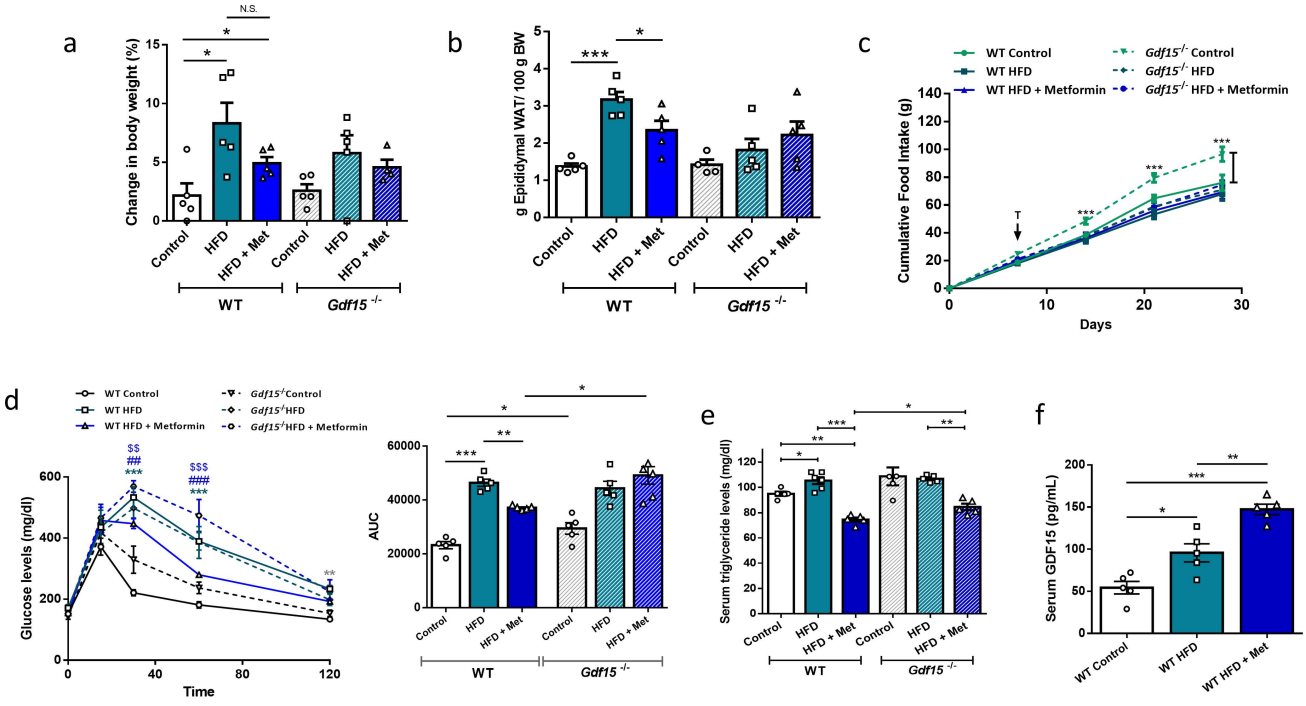


Figure 29. **Metformin improves the metabolic phenotype of HFD-fed mice, but not in the absence of GDF15. a)** Body weight gain in wild-type (WT) and *Gdf15*$^{-/-}$ mice fed a standard diet (control) or a high-fat diet (HFD) and treated with vehicle or 100 mg/kg/day of metformin for 3 weeks. **b)** Epididymal adipose tissue expressed per 100 g of total body weight. **c)** Cumulative food intake (g). **d)** Glucose tolerance test and area under the curve (AUC). **e)** Serum triglyceride levels (mg/dl). **f)** Serum GDF15 levels expressed as pg/mL. n = 5 per group. Data are presented as the mean ± SEM. P values: one-way ANOVA and Tukey post-test. *p < 0.05, **p < 0.01 and ***p < 0.001 *versus* the indicated group.

In the liver, hematoxylin-eosin staining, and Oil Red O (ORO) staining and quantification showed that the HFD caused significant hepatic lipid accumulation that was reduced by metformin treatment (**Fig. 30a**). Hepatic steatosis was remarkably higher in the HFD-fed *Gdf15*$^{-/-}$ mice compared to WT mice and, in the case of WT and *Gdf15*$^{-/-}$ mice that received a metformin treatment, the knockout animals did not revert the accumulation of lipids caused by the HFD. A reduction in lipid droplets was observed compared to *Gdf15*$^{-/-}$ mice fed a HFD, but quantification showed similar lipid levels.

In WT mice, metformin treatment increased the hepatic expression and protein levels of GDF15 (**Figs. 30b-c**). Metformin has been reported to activate AMPK by different mechanisms that have not been fully described (Hawley et al., 2002), (Foretz et al., 2010b), (He and Wondisford, 2015), but it is well established that this kinase promotes fatty acid oxidation in different cell types (Yoon et al., 2006), (Boudaba et al., 2018). Consistent with this, metformin administration increased the hepatic expression of genes involved in fatty acid β-oxidation (*Cpt1a*, *Acadm* and *Acox*), and reduced the expression of the pro-inflammatory cytokine gene *Il6* in WT mice (**Fig. 30c**). By contrast, these effects of metformin were attenuated or abolished in *Gdf15*$^{-/-}$ mice. The HFD decreased hepatic phospho-AMPK levels in WT mice, but this reduction was prevented by metformin treatment (**Fig. 30d**), as expected. Remarkably, phospho-AMPK levels in the liver of *Gdf15*$^{-/-}$ mice were lower than in the livers of their WT littermates, which is in line with the regulation of this kinase by GDF15. Metformin did not increase the phosphorylated levels of AMPK in the liver of *Gdf15*$^{-/-}$ mice.

Next, we examined the effects of the HFD and metformin on SOCS3, which is under the control of STAT3, and inhibits insulin signalling (Howard and Flier, 2006). WT mice fed the HFD showed increased hepatic levels of phosphorylated STAT3 at Ser^{727}, indicating increased activity of this transcription factor, as confirmed by the higher protein levels of its target gene SOCS3 (**Fig. 30d**). These changes were prevented by metformin treatment in WT mice, but *Gdf15*$^{-/-}$ mice showed significantly increased levels of Ser^{727} p-STAT3 in all groups that lacked

Gdf15 compared to WT groups. Additionally, metformin did not revert the increase in the phosphorylation of STAT3 caused by HFD in *Gdf15*$^{-/-}$ mice. SOCS3 protein levels showed a similar trend than the observed in the phosphorylation levels of STAT3 at Ser^{727} (**Fig. 30d**). Consistent with the increase in the levels of SOCS3, which inhibits insulin signalling through several mechanisms, WT mice fed the HFD exhibited a reduction in hepatic IRS2 protein levels (**Fig. 30d**). Conversely, HFD-fed mice treated with metformin showed a marked increase in IRS2 protein levels, presenting higher values than control mice. Again, this beneficial effect of metformin was absent in *Gdf15*$^{-/-}$ mice.

Since CHOP is a key transcription factor upregulating GDF15 expression (Patel et al., 2019) and it has been reported that metformin increases GDF15 levels through a CHOP-dependent mechanism to exert its actions through GFRAL (Coll et al., 2020b); (Day et al., 2019), we examined the protein levels of this factor. WT mice fed the HFD showed increased hepatic CHOP protein levels, which is consistent with the presence of lipid-induced ER stress (**Fig. 30d**). However, metformin treatment reduced hepatic CHOP protein levels in WT mice fed the HFD, thereby suggesting that the increase in CHOP was not responsible for the upregulation of GDF15. This is consistent with the induction of AMPK activation caused by metformin, which reduces ER stress pathways (Dong et al., 2010) (Wang et al., 2011b). Additionally, this agrees with the findings of previous reports showing that metformin decreases ER stress (Duan et al., 2017) (Cheang et al., 2014). A different trend was observed in *Gdf15*$^{-/-}$ mice, in which metformin did not reduce the HFD-induced increase in CHOP protein levels. Overall, these findings indicate that GDF15 function is necessary for the metformin-mediated reduction of hepatic steatosis, increase of fatty acid oxidation and general amelioration of insulin signalling, a process that is independent of CHOP upregulation.

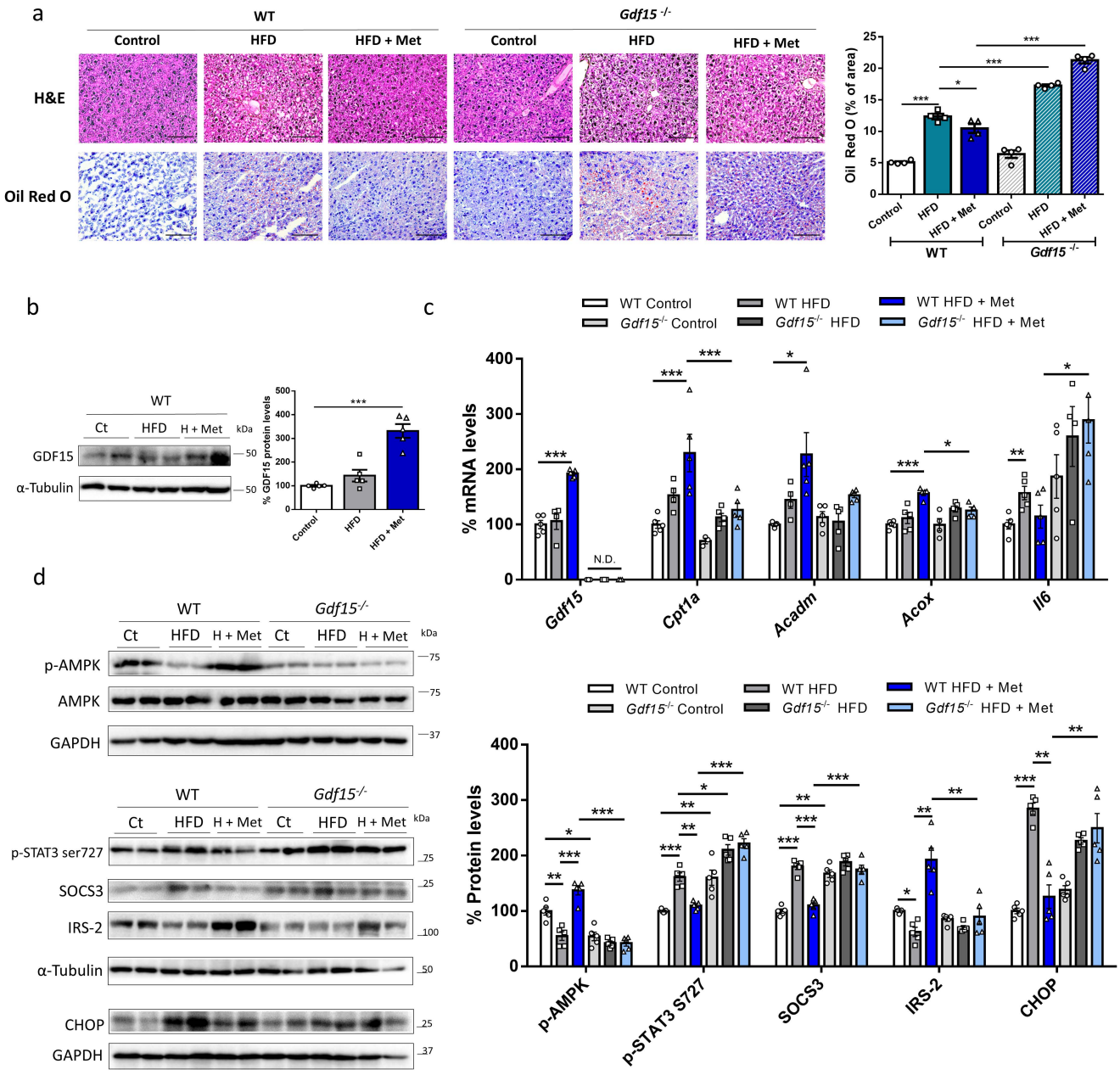


Figure 30. Metformin requires GDF15 to activate AMPK and improve the metabolic profile in the liver of mice. a) Hematoxylin–eosin (H&E) and Oil Red O (ORO) staining of the livers and the quantification of ORO staining. Scale bar: 100µm. **b)** Immunoblot analysis of GDF15 in the liver of WT and *Gdf15*$^{-/-}$ mice fed a standard diet (Ct) or a high-fat diet (HFD) and treated with vehicle or 100 mg/kg/day of metformin for 3 weeks. **c)** mRNA levels of *Gdf15*, *Cpt1a*, *Acadm*, *Acox* and *Il6* in the liver. **d)** Immunoblot analysis of total and phospho-AMPK, phospho-STAT3 (Ser^{727}), SOCS3, IRS2, and CHOP in the liver. n = 5 per group. Data are presented as the mean ± SEM. P values: (a-j) one-way ANOVA. *p < 0.05, **p < 0.01 and ***p < 0.001.

IV.2.7 *Gdf15* null mice present an attenuated response to metformin in skeletal muscle

In the skeletal muscle of WT mice, metformin treatment increased *Gdf15* expression (**Fig. 31c**) and protein levels (**Fig. 31a**). Given that AMPK is a major energy regulator in skeletal muscle (Kjøbsted et al., 2018), an organ submitted to higly variable energy turnover, and since AMPK is activated after metformin administration in skeletal muscle (Kristensen et al., 2014), we evaluated the levels of p-AMPK in skeletal muscle of this mice. Metformin treatment significantly increased the phosphorylation of AMPK, whereas this effect was absent in *Gdf15*$^{-/-}$ mice (**Fig. 31b**). Interestingly, *Gdf15*$^{-/-}$ mice fed a standard (control) diet showed lower p-AMPK levels compared to WT mice, as reported in the previous study. This confirms that GDF15 is necessary to maintain the activation of AMPK in skeletal muscle, consistent with our previous findings demonstrating the direct action of GDF15 in the activation of AMPK in cultured myotubes and skeletal muscle of mice.

The increase p-AMPK in WT mice was accompanied by enhanced mRNA levels of genes involved in fatty acid β-oxidation, including *Cpt1b, Ppara* and *Acox*, whereas this effect of metformin was significantly reduced in *Gdf15*$^{-/-}$ mice (**Fig. 31c**). As expected, the HFD increased the skeletal muscle expression of the markers of ER stress *Bip* and *Chop*, and inflammation (*Mcp1*), with metformin abrogating these increases in WT mice (**Fig. 31d**). However, this reduction was not achieved in *Gdf15*$^{-/-}$ mice. Remarkably, the HFD caused a larger increase in *Mcp1* mRNA levels in *Gdf15* null mice than in WT mice (**Fig. 31d**).

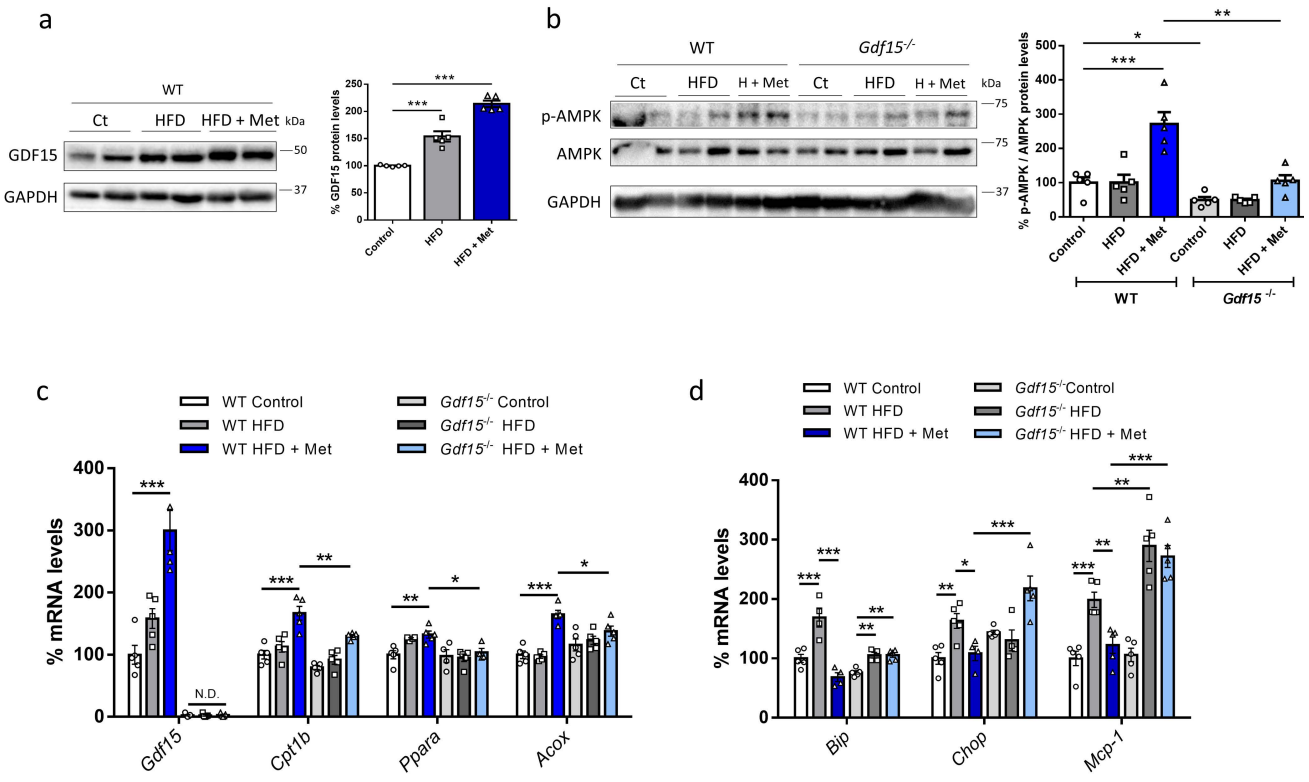


Figure 31. **Metformin activates AMPK and regulates fatty acid oxidation, ER stress and inflammation markers in the skeletal muscle of WT mice, but not in *Gdf15*$^{-/-}$ mice. a**) Immunoblot analysis of GDF15 in skeletal muscle of wild-type (WT) mice fed a standard diet (Control) or a high-fat diet (HFD) and treated with vehicle or 100 mg/kg/day of metformin for 3 weeks. **b**) Immunoblot analysis of total and phospho-AMPK in skeletal muscle of wild-type (WT) and *Gdf15*$^{-/-}$ mice fed a standard diet (Control) or a HFD and treated with vehicle or 100 mg/kg/day of metformin for 3 weeks. **c**) mRNA levels of *Gdf15, Cpt1b*, *Ppara and Acox* in the skeletal muscle. **d**) mRNA levels of *Bip, Chop* and *Mcp1* in skeletal muscle. n = 5 per group. Data are presented as the mean ± SEM. P values: one-way ANOVA. *p < 0.05, **p < 0.01 and ***p < 0.001 *versus* the indicated group.

Similarly, PGC-1α protein levels were increased by metformin only in the skeletal muscle of WT mice (**Fig. 32a**). In line with the changes in the expression of the NF-κB target gene *Mcp1*, WT mice fed the HFD showed reduced IκBα protein levels (**Fig. 32a**), which were accompanied by an increase in the levels of the p65 subunit of NF-κB (**Fig. 32b**). These changes were prevented by metformin in WT mice, but not in *Gdf15*$^{-/-}$ mice. PPARα protein levels showed a significant increase in mice that received metformin treatment; however, this effect was not shown in mice lacking *Gdf15*.

Metformin also increased the protein levels of IRβ in WT mice, whereas no such increase was observed in *Gdf15*$^{-/-}$ mice (**Fig. 32c**). Finally, CHOP protein levels were increased by the HFD in WT mice, but metformin restored basal levels, indicating that the increased levels of this transcription factor were not responsible for the increase in GDF15 levels caused by metformin in skeletal muscle (**Fig. 32c**). This effect of metformin on CHOP was absent in mice lacking *Gdf15* (**Fig. 32c**). Altogether, these findings suggest that the increase in GDF15 levels caused by metformin in the liver and skeletal muscle does not depend on CHOP upregulation. Furthermore, it has been demonstrated that metformin requires GDF15 actions to exert its metabolic effects on AMPK activation, fatty acid oxidation, ER stress, inflammation and insulin signalling.

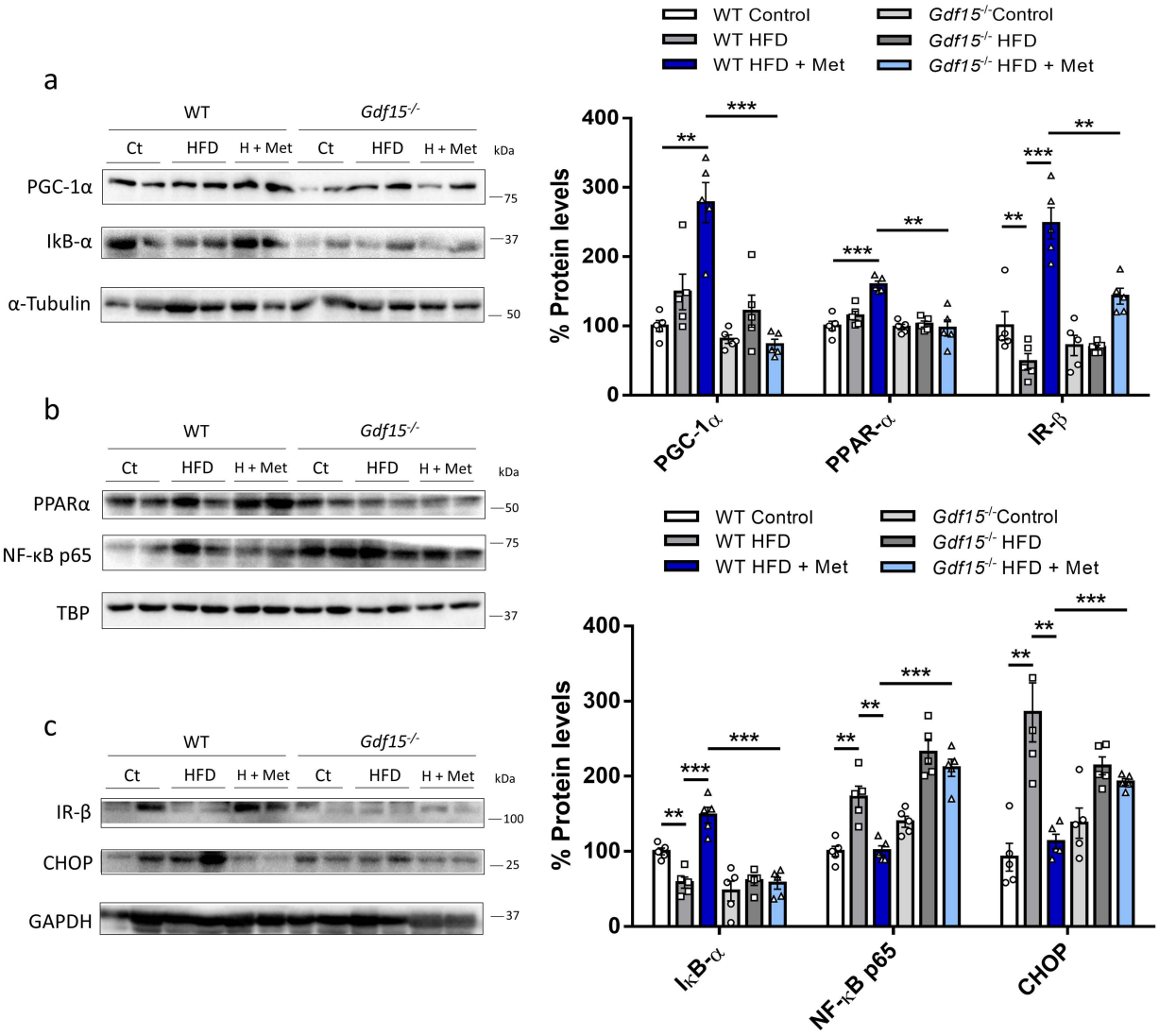
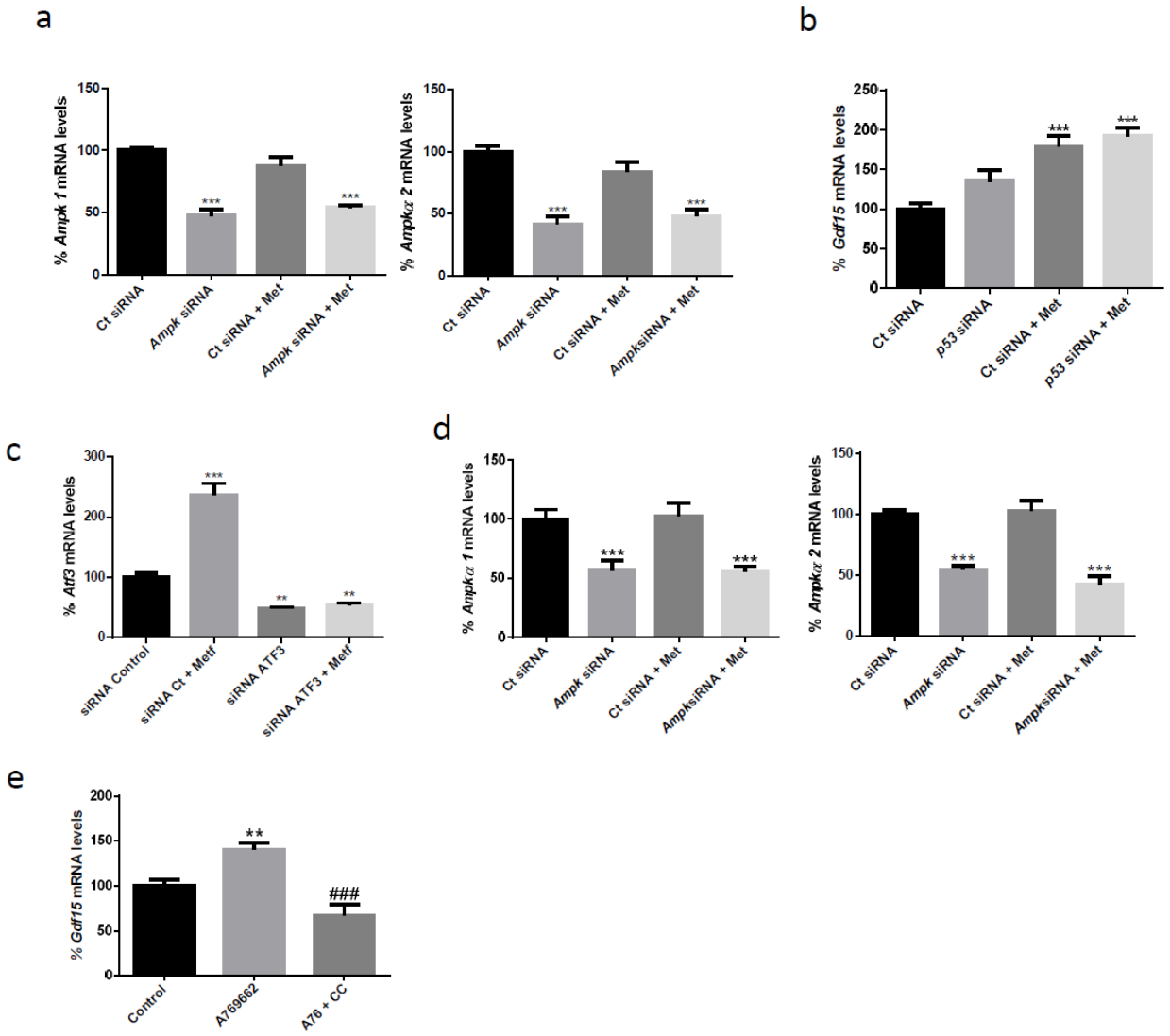
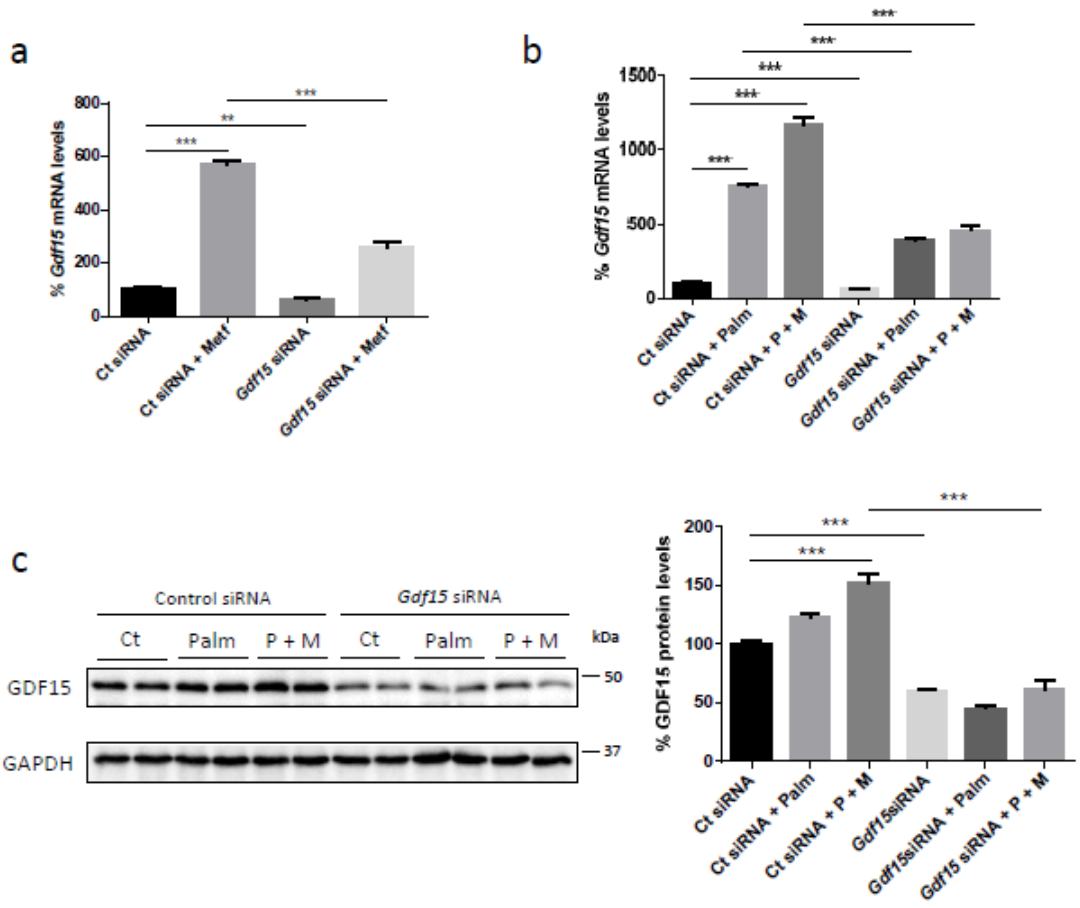


Figure 32. Metformin increases GDF15 levels independently of CHOP and regulates fatty acid metabolism and inflammation in the skeletal muscle of WT mice, but not *Gdf15*$^{-/-}$ mice. Immunoblot analysis of PGC-1α, IκBα **(a)** PPARα, the p65 subunit of NF-κB **(b)**, IRβ and CHOP **(c)** in skeletal muscle of wild-type (WT) mice fed a standard diet (Control) or a high-fat diet (HFD) and treated with vehicle or 100 mg/kg/day of metformin for 3 weeks. Quantification is grouped according to function similarity. n = 5 per group. Data are presented as the mean ± SEM. P values: one-way ANOVA. **p < 0.01 and ***p < 0.001.

IV.2.8 Supplementary information related to part II



Supplementary Figure 8. **a)** *Ampk1* and *Ampk2* mRNA levels in C2C12 myotubes transfected with scrambled (Control, Ct) siRNA or Ampk1/Ampk2 siRNA and treated with vehicle or 2 mM metformin (Met) for 24 h. **b)** *Gdf15* mRNA levels in C2C12 myotubes transfected with scrambled siRNA or p53 siRNA and treated with vehicle or 2 mM metformin for 24 h. **c)** *Atf3* mRNA levels in C2C12 myotubes transfected with scrambled siRNA or ATF3 siRNA and treated with vehicle or 2 mM metformin for 24 h. **d)** *Ampk1* and *Ampk2* mRNA levels in mouse primary hepatocytes transfected with scrambled siRNA or Ampk1/Ampk2 siRNA and treated with vehicle or 2 mM metformin for 24 h. **e)** *Gdf15* mRNA levels in mouse primary hepatocytes exposed to 60 µM A769662 for 24 h in the presence or absence of 30 µM compound C (CC). Data are presented as the mean ± s.e.m. (n=4 per group). **p<0.01 and ***p<0.001 *versus* control siRNA. ###p<0.001 *versus* A769662-treated cells.



Supplementary Figure 9. **a)** *Gdf15* mRNA levels in C2C12 myotubes transfected with scrambled siRNA (Control, Ct) or GDF15 siRNA and treated with vehicle or 2 mM metformin (Met) for 24 h. *Gdf15* mRNA **(b)** and GDF15 protein levels **(c)** in C2C12 myotubes exposed to palmitate (Palm or P) (0.5 mM), in the presence or absence of 2 mM of metformin (M) for 24 h and transfected with scrambled siRNA (Control, Ct) or

GDF15 siRNA. Data are presented as the mean ± SEM (n= 4 per group). **p<0.01 and ***p<0.001 *versus* control or between the indicated groups.

V. DISCUSSION

T2D has become a major concern in the past years, given its accelerated spread worldwide and the lack of an effective therapy to treat all the complications derived from this pathology (Ghasemi and Norouzirad, 2019). The major changes experimented in lifestyle factors mainly in developed countries have turned this pathology into a global epidemic in the 21st century. Moreover, a chronic low-state inflammatory process derived from lipid accumulation causes insulin resistance, which is known to precede and predict the development of T2D. Considering that the current pharmacological therapies available for these pathologies show limited efficacy and significant side effects, it becomes essential to uncover new therapeutic targets for the treatment of insulin resistance and T2D. In the present study, we evaluated the involvement of GDF15 in the antidiabetic actions of two well-known compounds: PPARβ/δ agonists and metformin.

GDF15 is a secreted cytokine released from different tissues upon stimuli, including mitochondrial dysfunction, obesity, or cancer. Importantly, GDF15 can act as a metabolic regulator that governs energy homeostasis, promoting lipid catabolism, increasing thermogenesis and improving glucose tolerance along with insulin sensitivity (Chrysovergis et al., 2014) , (Chung et al., 2017b). Consequently, ablation of the *Gdf15* gene causes an increase in body mass, fat accumulation and higher glucose and insulin levels in different murine diet-induced models of obesity and diabetes (Tsai et al., 2013), (Tran et al., 2018). Interestingly, many of the metabolic effects of GDF15 are similar to those elicited by the treatment with PPARβ/δ agonists (Vázquez-Carrera, 2016), (Tan et al., 2016b); or by the treatment with metformin (Gu et al., 2014), (Wang et al., 2014) on animal and human studies. For this reason, we considered that GDF15 could be implicated in the effects caused by these two antidiabetic agents.

V.1 PART I - GDF15 mediates the metabolic effects of PPARβ/δ by activating AMPK

PPARβ/δ plays a key role in the regulation of glucose and lipid metabolism as well as in energy homeostasis. Mechanistically, PPARβ/δ acts as a ligand-inducible transcription factor and regulates the expression of different genes implicated in metabolism (Vázquez-Carrera, 2016). Originally, it was explored as a mediator of exercise due to its involvement in mitochondrial metabolism in skeletal muscle (Barish et al., 2006). In fact, the PPARβ/δ ligand GW501516 has been extensively studied as an exercise mimetic as it promotes endurance during exercise (Dressel et al., 2003). Given its implication in exercise and the reported transcriptional upregulation of a relevant set of genes that promote oxidative metabolism (Giordano Attianese and Desvergne, 2015b), (Neels and Grimaldi, 2014) the transcriptional activity of this receptor was considered for long as the main mechanism of action of PPARβ/δ to regulate metabolic homeostasis (Palomer et al., 2018). Nevertheless, it is worth mentioning the implication of AMPK activation in PPARβ/δ metabolic actions. For instance, AMPK activators exhibit an exercise mimetics activity as well, which is consequent with its high expression levels in skeletal muscle, like PPARβ/δ. Importantly, PPARβ/δ and AMPK synergistically upregulate target genes linked to oxidative metabolism (Narkar et al., 2008) and are involved in a positive feedback loop that contributes to the glucose uptake process in skeletal muscle (Koh et al., 2019). Moreover, many of the antidiabetic effects of PPARβ/δ in the liver, such as the amplification of the lipin1 - PGC-1α - PPARα axis that leads to increased fatty acid oxidation (Barroso et al., 2011a), or the prevention of lipid-induced ER stress in skeletal muscle, rely on the activation of AMPK (Salvadó et al., 2014).

As previously mentioned, GDF15 is implicated in the regulation of several metabolic and inflammatory processes. In fact, overexpression or exogenous administration of this cytokine results in an improved metabolic state and prevention of insulin resistance in different models of obesity or insulin resistance (Macia et al., 2012), (Tsai et al., 2013), (Chrysovergis et al., 2014), (Chung et al., 2017b), (Tran et al., 2018). Remarkably, these effects of GDF15 are similar to those elicited by PPARβ/δ activation.

In the present study we demonstrate that treatment with the PPARβ/δ agonist GW501516 increases GDF15 levels through an AMPK-dependent mechanism. It is well known that PPARβ/δ agonists induce the activation of AMPK (Coll et al., 2010b), and different mechanisms have been suggested to describe how PPARβ/δ induction results in this activation. For example, PPARβ/δ promotes the Sp1-mediated enhancement of SIRT1 promoter activity (Okazaki et al., 2010), which may contribute to AMPK activation due to the regulation of this kinase by SIRT1 (Lee and Kim, 2010). In skeletal muscle, PPARβ/δ overexpression leads to an increase of the levels of CaMKKα (Gan et al., 2011a), a kinase that can activate AMPK by a different mechanism independent of LKB1 (Hawley et al., 2005). Likely, the combination of several mechanisms is responsible for the activation of AMPK caused by PPARβ/δ. Furthermore, AMPK phosphorylates and inhibits the activity of MDMX, which in turn induces p53 (He et al., 2014), a transcription factor that upregulates GDF15 expression (Osada et al., 2007). In our study, inhibition of AMPK or p53 blocked the increase of GDF15 caused by PPARβ/δ. Interestingly, lipin1 was previously described as a p53-responsive gene that is induced upon glucose deprivation or DNA damage (Assaily et al., 2011), and was found responsible for the regulation of fatty acid oxidation in skeletal muscle cells. This latter p53-mediated effect of lipin 1 may explain part of the reported metabolic actions of PPARβ/δ in fatty acid oxidation. In fact, p53 functional mutations are found in approximately 50% of cancers (Freed-Pastor and Prives, 2012), where lipid metabolism is impaired, provoking the accumulation of these lipids and leading to cancer progression (Parrales and Iwakuma, 2016).

Interestingly, we revealed that the beneficial effects of pharmacological PPARβ/δ activation on glucose intolerance, fatty acid oxidation, ER stress, inflammation and activation of AMPK were abrogated by a neutralizing antibody against GDF15 and in *Gdf15*$^{-/-}$ mice, indicating that these PPARβ/δ effects were mainly mediated by this cytokine. The GDF15 neutralizing antibody counteracted the PPARβ/δ-dependent increased levels of the transcription factor PPARα and the transcriptional co-activators PGC-1α and lipin 1, which form a protein complex that translocates into the nucleus to promote the transcription of genes involved in fatty acid oxidation (Vázquez-Carrera, 2016). This agrees with the observed reduction in

serum β-hydroxybutyrate levels, an indirect marker of β-oxidation. Consistent with the effects shown on proteins that regulate lipid metabolism at genetic level, the increased expression of genes involved in fatty acid oxidation (*Cpt1a/b, Acox* and *Acadm*) was blocked by the GDF15 neutralizing antibody and in *Gdf15*$^{-/-}$ mice, in skeletal muscle and the liver. This provides evidence for GDF15 strongly contributing to the increased fatty acid metabolism caused by PPARβ/δ activation and underscores the critical role of this cytokine in the process. This is in agreement with previous studies showing an upregulation of fatty acid oxidation genes in response to exogenous administration of recombinant GDF15 (Chung et al., 2017b). Interestingly, some of the analysed genes implicated in fatty acid oxidation present in their promoters regulatory regions that are responsive to PPARs, known as PPRE (Song et al., 2010). Further analysing the expression trend, we observed that GDF15 neutralization did not completely revert the upregulation of *Cpt1* or *Acox* caused by the treatment with GW501516. Conversely, the mRNA levels of fatty acid oxidation genes exhibited a reduction in the *Gdf15*$^{-/-}$ group; however, GW501516 provoked a partial increase on these levels. This indicates that, complementary to the pivotal role of GDF15 observed on PPARβ/δ metabolic actions, other regulatory mechanisms may control the mRNA and protein levels of different factors derived from the treatment with PPARβ/δ agonists.

Although the improvements in glucose intolerance, obesity and body weight caused by GDF15 have been linked to a reduction in food intake caused by the binding of GDF15 to its neuronal receptor GFRAL (Emmerson et al., 2017; Hsu et al., 2017b; Mullican et al., 2017a; Yang et al., 2017), we did not observe changes in either food intake or body weight after the treatment with GW501516 for 21 days, as reported in previous studies using this pharmacological ligand (Barroso et al., 2011a). This is consistent with a previous study revealing that exogenous administration of recombinant GDF15 at an elevated dose did not cause changes in food intake (Chung et al., 2017), thereby making this improvement unlikely to be the result of central effects. However, further studies with genetic models of mice or neutralization experiments against GFRAL should be conducted to utterly confirm this hypothesis. It becomes relevant to point out that experiments carried out with recombinant GDF15 usually represent an extreme elevation of the serum levels of

this cytokine, ranging 20-200-fold the physiological concentration of GDF15 in plasma.

In the past few years, most of the so far reported metabolic effects of GDF15 have been attributed to its binding to GFRAL, which appears to be a brainstem-restricted receptor, and the consequent triggering of central pathways that lead to the phosphorylation of ERK1/2, Akt and PLC-γ1, and a decrease in food consumption (Mullican *et al.*, 2017), (Mullican and Rangwala, 2018). In the current study, *Gdf15* knockdown in C2C12 myotubes in culture demonstrated that the increased expression of the genes involved in fatty acid oxidation *Cpt1b, Acox* and *Acadm* caused by PPARβ/δ activation is dependent on the effects of the GDF15 increase that takes place in these cells. Such peripheral effects have previously been attributed to the activation of a range of TGFβ receptors also known as the ALK receptors (ALK1-7) (Ago and Sadoshima, 2006), (Johnen et al., 2007), (Artz et al., 2016), which support a possible GDF15 autocrine signalling mechanism in the cultured myotubes. However, due to recent studies reporting contamination of TGF-β in recombinant GDF15 purified from human cells expression systems (Olsen et al., 2017), the interaction of GDF15 with these receptors should be confirmed in the previously mentioned studies. In fact, our findings exclude the involvement of ALK4/5/7 in the regulation of AMPK by GDF15 or PPARβ/δ agonists, since blockade of these receptors did not revert the effects of either recombinant GDF15 or PPARβ/δ agonists. Further studies should be conducted in order to elucidate the potential mechanisms involved. For instance, the transcriptional inhibition of the nuclear complex Smad2/3/4 caused by the non-mature GDF15 (or pro-GDF15) also remains as a possibility to explain its GFRAL-independent functions (Min et al., 2016).

Hypertriglyceridemia is correlated with the development of insulin resistance and β cell function (Ma et al., 2020). In our study, PPARβ/δ activation caused a hypotriglyceridemic effect; however, the reduction in triglyceride levels in serum caused by GW501516 was attenuated in *Gdf15*$^{-/-}$ mice. In agreement with this, hepatic steatosis was markedly increased in *Gdf15*$^{-/-}$ mice compared to WT mice. In fact, treatment with GW501516 exacerbated the lipid accumulation in the liver of

Gdf15$^{-/-}$ mice fed a HFD. This event could be explained by the lipid-internalization activity of GW501516 (Barroso et al., 2011a), which, in the absence of GDF15, fails in the utilisation of lipids as fuel, provoking its accumulation. Furthermore, it was recently reported a hepatic triglyceride sympathetic outflow function carried out by GDF15 by controlling adrenergic signalling in this organ (Luan et al., 2019). In our *Gdf15* null mice, this function may be compromised and this event could lead to a lipid overload and increased insulin resistance in the liver of *Gdf15*$^{-/-}$ mice, accompanied with higher rates of gluconeogenesis, which may explain the similar trend observed in the glucose tolerance test.

Importantly, we found that the GDF15-mediated PPARβ/δ-dependent regulation of the genes involved in fatty acid oxidation is modulated by the activity of AMPK, a crucial metabolic sensor in energy homeostasis that switches between anabolic towards catabolic pathways in response to an energy demand, promoting the generation of ATP. Indeed, activation of this kinase plays a pivotal role in the effects of PPARβ/δ agonists. For instance, the activation of the lipin 1 - PGC-1α - PPARα is mediated by AMPK activation, which is necessary for the antidiabetic effects of PPARβ/δ on ER stress and inflammation, thereby leading to an increase in insulin sensitivity (Barroso et al., 2011a; Salvadó et al., 2014). Consistent with this, we show that pharmacological PPARβ/δ activation increased the phosphorylated levels and thereby the activity of AMPK in skeletal muscle and the liver, which was abolished by the neutralizing antibody against GDF15 and in *Gdf15*$^{-/-}$ mice. Moreover, and in line with the reported inhibitory cross talk between AMPK and ERK1/2 (Du et al., 2008; Hwang et al., 2013), the increased phospho-AMPK levels caused by PPARβ/δ activation was associated to reduced phospho-ERK1/2 levels, an effect abrogated by the GDF15 neutralizing antibody and in *Gdf15*$^{-/-}$ mice. This suggests that the increase in phospho-AMPK levels and the reduction in phosho-ERK1/2 levels caused by PPARβ/δ activation are dependent on the action of GDF15.

The development of ER stress caused by lipid accumulation and the consequent induction of inflammation are key factors for the development of insulin resistance and T2D (Kim et al., 2015a), (Flamment et al., 2012), (Salvado et al.,

2015). Our findings show that feeding mice a HFD increases the expression of the markers of ER stress and inflammation, with PPARβ/δ activation attenuating these increases. However, the GDF15 neutralizing antibody or the deficiency of this hormone suppresses the PPARβ/δ protective effects. The beneficial effect of PPARβ/δ activation on ER stress and inflammation has been attributed to the activation of AMPK and the reduction of phospho-ERK1/2 levels (Coll et al., 2010a; Salvadó et al., 2014). In fact, ER stress increases ERK1/2 phosphorylation in myotubes (Hwang et al., 2013; Salvadó et al., 2014), whereas ERK1/2 inhibition restores AMPK activity and insulin signalling. Now, our findings suggest that these beneficial effects of PPARβ/δ activation on ER stress, inflammation and insulin resistance are dependent on GDF15.

Several cytokines and hormones associated with insulin resistance induce the production of SOCS proteins, which inhibit insulin signalling through different mechanisms (Rui et al., 2002), (Ueki et al., 2004), (Serrano-Marco et al., 2012a). SOCS3 expression is under the transcriptional control of STAT3, which is activated by phosphorylation. Phosphorylation of STAT3 at Tyr^{705} is dependent on JNKs that are activated by cytokines. In addition to Tyr^{705} phosphorylation, STAT3 also requires phosphorylation at Ser^{727} to achieve maximal transcriptional activity. Several kinases can phosphorylate STAT3 at Ser^{727}, including ERK1/2 (Derek and Kovarik, 2000). It has been previously reported that pharmacological PPARβ/δ activation suppresses IL-6-induced STAT3 activation by inhibiting ERK1/2 phosphorylation and preventing the reduction in phospho-AMPK levels (Serrano-Marco et al., 2012). In our treatment, the induction of this pathway by a HFD was reverted by GW501516 administration. Importantly, this beneficial effect was absent in mice treated with the GDF15 neutralizing antibody and in *Gdf15*$^{-/-}$ mice. This has a direct impact on the phosphorylation and total levels of IRS-1 and IRS-2, which were decreased when GDF15 was blocked or absent. Defective IRS-1 and IRS-2 has been proven crucial for the pathogenesis of insulin resistance (Shehata, 2009). In addition, IRS-2 downregulation appears to contribute to the onset of selective hepatic insulin resistance in other metabolic disorders like NAFLD (Honma et al., 2018). The findings of the current study demonstrate that the effect of PPARβ/δ on STAT3 phosphorylation, and SOCS3 and IRS protein levels depend on the increase

of GDF15 levels, highlighting the crucial role of GDF15 in the prevention of insulin resistance.

Intriguingly, despite having determined that PPARβ/δ increases GDF15 levels by previously activating AMPK, we observed that the increase in phospho-AMPK levels caused by pharmacological activation of PPARβ/δ is suppressed in skeletal muscle and the liver of mice treated with a neutralizing antibody against GDF15 and in *Gdf15*$^{-/-}$ mice, accompanied by the consequent increase of phospho-ERK1/2 levels. Moreover, *Gdf15*$^{-/-}$ mice fed a standard diet also presented lower levels of phospho-AMPK compared to WT mice fed a standard diet. In support of a potential regulatory mechanism by which GDF15 activates AMPK, treatment of C2C12 myotubes and WT mice directly with recombinant GDF15 revealed that this cytokine activated AMPK in both C2C12 and in skeletal muscle of these mice, accompanied by a reduction of phospho-ERK1/2 levels, which is supported by previous studies showing a reduction of the ERK1/2 pathway by GDF15 treatment in cardiac muscle tissue (Xu et al., 2014b). This finding is of crucial relevance, since C2C12 was reported to lack *Gfral* expression, among other 30 cell lines analysed (Yang et al., 2017). Nevertheless, a qPCR was performed to assess the levels of expression of *Gfral* in our C2C12 cell line. Consistent with Yang et al., brainstem exhibited a normal basal expression of *Gfral* in WT mice, and we observed that skeletal muscle and the liver from these mice, along with C2C12 myotubes showed virtually null expression of *Gfral* (Supplementary Figure 7). This is also consistent with other studies reporting null expression of this central receptor in mice whole muscle tissue (Laurens et al., 2020b) or undetectable mRNA of *Gfral* in peripheral tissues (Hsu et al., 2017b).

However, in the *in vivo study,* although we observed null expression of *Gfral* in skeletal muscle, we could not exclude the involvement of central effectors in the actions of recombinant GDF15 on AMPK activation. Incubation of isolated skeletal muscle obtained from WT and *Gdf15* null mice showed that the PPARβ/δ agonist or recombinant GDF15 treatment led to an increase in phospho-AMPK levels and a decrease in phospho-ERK1/2 levels. Altogether, these results indicate that GDF15 activates AMPK independently of central mechanisms involving the action of GFRAL.

However, the mechanism by which GDF15 increases phospho-AMPK levels remains to be elucidated.

Overall, the findings of this study highlight a novel regulatory mechanism by which the activation of the AMPK-p53 pathway by PPARβ/δ ligands results in increased levels of GDF15, which in turn mediates the metabolic effects of these ligands by prolonging AMPK activation through a non-defined mechanism, preventing inflammation, ER stress and insulin resistance (**Figure 33**). Since activation of PPARβ/δ-AMPK enhances physical performance (Koh et al., 2019; Narkar et al., 2008) and ameliorates inflammation, ER stress and insulin resistance (Vázquez-Carrera 2016; Tan et al., 2016), the findings of this study identify a new player in these pathways: GDF15. Additionally, AMPK has been reported to induce PPARβ/δ (Koh et al., 2019), and are involved in a positive feedback loop; GDF15 could contribute to a general amplification of this molecular pathway. Furthermore, the novel identification of a GDF15-mediated activation of AMPK sheds light on the possible mechanisms that could turn GDF15 into a promising therapeutic target for the treatment of metabolic disorders.

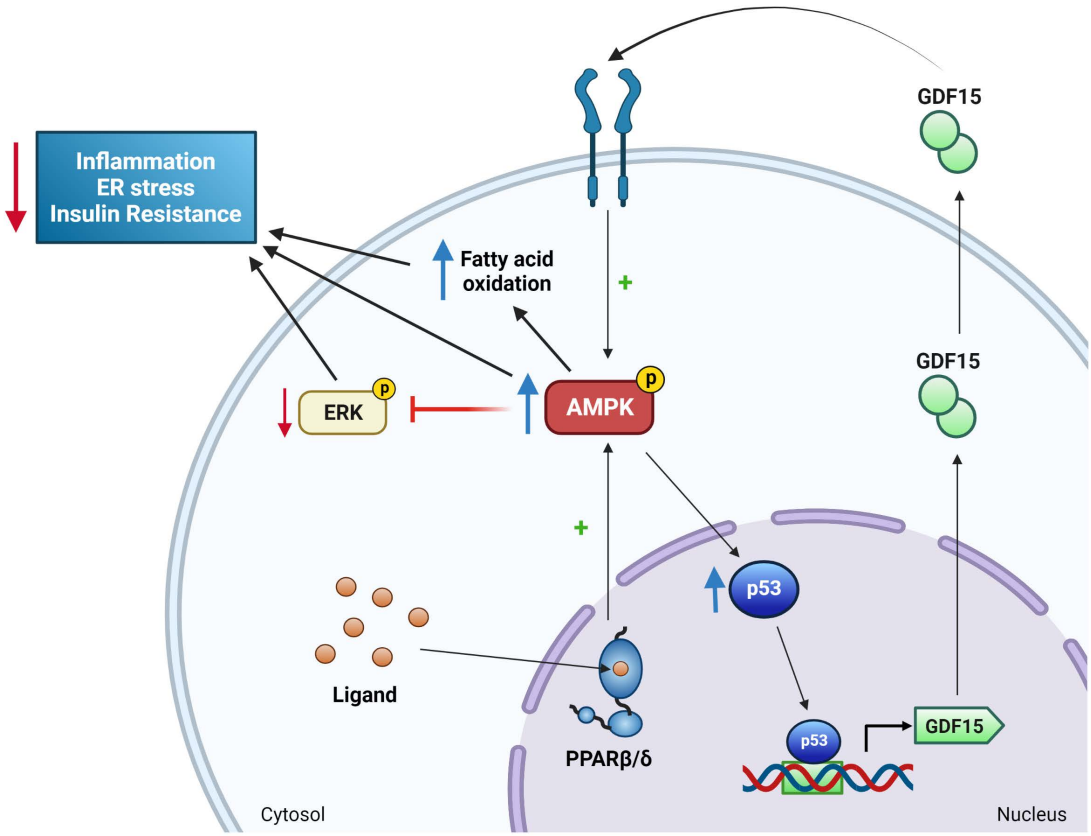


Figure 33 - Proposed mechanism for the GDF15-mediated antidiabetic actions of PPARβ/δ. Ligands (GW501516) activate PPARβ/δ in the nucleus, which in turn activates AMPK by promoting its phosphorylation. GDF15 transcription is enhanced by the AMPK-mediated increase of p53 in the nucleus. The increase in GDF15 levels sustains AMPK activation by an unknown mechanism and cause the subsequent reduction in phospho-ERK levels, increasing fatty acid oxidation and reducing inflammation, ER stress and insulin resistance.

V.2 PART II - GDF15 is involved in the AMPK-mediated effects of metformin

In the previous study, we described a novel regulatory effect of GDF15, which activates AMPK by a non-defined mechanism. AMPK is a central regulator of multiple metabolic pathways and is of therapeutic interest for the treatment of insulin resistance, T2D, NAFLD, and cardiovascular disease (Day et al., 2017). Indeed, AMPK has long held promise as a therapeutic target for these pathologies due to its pleiotropic actions in multiple tissues and, in the past few years, many authors have designed and synthesized new-generation AMPK activators in order to prevent different metabolic disorders, including hepatic steatosis or glucose homeostasis deficiencies (Boudaba et al., 2018), (Cokorinos et al., 2017a), (Steneberg et al., 2018), (Esquejo et al., 2018).

AMPK is a target for metformin, the first-line drug for the treatment of T2D. How metformin activates this kinase remains only partially understood, yet the activation of AMPK is one of the most studied mechanisms of action of this drug and its implication is a cause of controversy (Rena et al., 2017). However, it turns out difficult to elucidate the canonical mechanism of action of metformin, since the concentrations used in cell and animal studies are widely variable (He and Wondisford, 2015). Low concentrations of metformin (<80 μM) are reported to significantly activate AMPK independently of changes in the AMP/ATP ratio (Cao et al., 2014), which are consistent with the concentrations of metformin present in the portal vein in patients receiving the maximal therapeutic dose for this drug (He and Wondisford, 2015). The AMP/ATP ratio is maximally elevated at concentrations ranging 1-2 mM (Foretz et al., 2010a), which relate to the hepatic concentrations reached in humans treated with metformin due to the accumulation of this drug (Gormsen et al., 2016) (Chandel et al., 2016). Nevertheless, this is lower than the 5 mM concentrations needed to significantly inhibit the complex I of the mitochondrial respiratory chain (Madiraju et al., 2014), which also results in the increase of the AMP/ATP ratio and the subsequent activation of AMPK.

In the present study, we show that metformin increases GDF15 levels in different cell lines at concentrations ranging 1-2 mM. In this context, the AMP/ATP

ratio is reported to be maximal (Foretz et al., 2010a), whereas 5-10 mM metformin is needed to inhibit complex I of the respiratory chain (Madiraju et al., 2014). The increase in GDF15 levels caused by metformin did not reach statistical significance at lower concentrations; however, the 3-day treatment of C2C12 myotubes with 0.5 mM metformin showed an increase on the third day, in agreement with previous studies that report metformin accumulation in skeletal muscle (Gormsen et al., 2016). It is important to take into consideration that an increase in the AMP/ATP ratio is not an exclusive consequence of the inhibition of complex I. Apart from different natural processes, mitochondrial uncoupling may also cause a reduction in the respiratory rate of this organelle, leading to the reduction of ATP. Uncoupling proteins (UCPs) are transmembrane proteins located in the inner membrane of the mitochondria that control the ion transport across the mitochondrial membrane (Jacobsson et al., 1985). It functions "uncoupling" the proton transport in order to induce thermogenesis instead of generating ATP, which causes a reduction in the ATP/AMP ratio. Interestingly, transgenic mice overexpressing UCP1 in skeletal muscle show activation of AMPK, and double transgenic showing this modification and the ablation of *Gdf15* revert the activation of AMPK (Ost et al., 2020), which is consistent with the regulatory effect of GDF15 on AMPK that we have reported. Moreover, treatment of mice or rats with metformin has been reported to increase the levels of UCP1 (Breining et al., 2018), (Tokubuchi et al., 2017). UCP1 is mainly expressed in brown adipose tissue, however, Tokubuchi et al. reported the upregulation of UCP3 in the skeletal muscle. Other studies also report upregulation of UCP3 in skeletal muscle by metformin (Jäger et al., 2007), and of the ubiquitously expressed UCP2 in adipocytes (Anedda et al., 2008). The upregulation of uncoupling proteins could constitute a feasible explanation for the increase of the AMP/ATP ratio exhibited after metformin treatments with concentrations lower than 5 mM when the mitochondrial complex I is not significantly inhibited. Nevertheless, this potential regulation needs to be further explored.

Moreover, different forms of autophagy activation have been reported as a consequence of mitochondrial uncoupling (Demine et al., 2019), which can reduce the triglyceride content in cultured adipocytes independent of the action of ATGL and HSL (Demine et al., 2018). Exploring the role of metformin in the autophagy-

mediated induction of lipolysis could represent an interesting issue, since treatment with this drug can induce autophagy in different cell types (Gao et al., 2020), (Bharath et al., 2020).

In the present study, we show that AMPK activation by metformin is responsible for the increase of GDF15, which in turn is required to promote the activation of AMPK by this drug. Our previous study showed that GDF15 administration alone is able to activate AMPK in cultured myotubes and in skeletal muscle of mice. Here, we observed that *Gdf15*$^{-/-}$ mice exhibited reduced phospho-AMPK levels in skeletal muscle and liver, and that the increase in the phosphorylation levels of this kinase caused by metformin treatment in WT mice was not present in *Gdf15*$^{-/-}$ mice. Moreover, many of the reported AMPK-mediated effects of metformin on the levels of proteins involved in fatty acid oxidation, inflammation, ER stress and insulin signalling were attenuated or abolished by *Gdf15* knockdown. For instance, in the liver, the metformin-mediated upregulation of *Cpt1a, Acadm* or *Acox* was prevented in *Gdf15* null mice. The *Il6* levels reduction (although not significant) caused by metformin was inhibited in mice that lacked *Gdf15*, and all the *Gdf15*$^{-/-}$ groups exhibited an increased trend of this cytokine expression. IL-6 is known to elicit the JAK/STAT pathway (Garbers et al., 2012), which is consistent with our results, showing increased phosphorylation levels of STAT and SOCS3 in the *Gdf15*$^{-/-}$ mice. Consequently, the HFD-mediated reduction of IRS-2 total levels in WT mice was prevented with the treatment of metformin. However, this effect was absent in *Gdf15*$^{-/-}$ mice, which agrees with the worsened response to glucose in this group, where metformin could not prevent the glucose intolerance development caused by HFD. Hepatic steatosis reduction by metformin in HFD-mice was additionally impaired in mice that lacked *Gdf15*, contributing to the worsened metabolic state of these mice. A similar trend was observed in skeletal muscle of these mice, where the nuclear levels of the p65 subunit of NF-κB were not reversed by metformin when *Gdf15* expression was ablated. Consistent with this, a worsened inflammatory state accompanied the presence of ER stress and the reduction of fatty acid metabolism and insulin signalling in skeletal muscle, whereas metformin effect was absent in mice lacking *Gdf15.*

Importantly, we reported that treatment of C2C12 myotubes with recombinant GDF15 increased the expression of genes implicated in fatty acid oxidation, while reducing the levels of proinflammatory cytokines and improving insulin signalling, thus mimicking some of the beneficial effects caused by either PPARβ/δ activation in the previous study or metformin treatment here. Given the similarity of the results obtained by either metformin or recombinant GDF15 treatment in C2C12 myotubes where *Gfral* expression is virtually null (Supplementary Figure 7); (Yang et al., 2017), compared to the effects of metformin treatment on HFD-fed mice and the lack of responsiveness to this drug in *Gdf15* null mice, we speculated that the beneficial effects of metformin observed in skeletal muscle and liver of HFD-fed mice were dependent on the activation of AMPK promoted by the increase of GDF15 levels. In fact, we have observed in cultured myotubes and in the analysed mouse tissues that knockdown or ablation of *Gdf15* respectively lowers the activation of AMPK caused by metformin, although further loss-of-function studies would be necessary to fully confirm this hypothesis. Nevertheless, this would be consistent with the observation that some authors (Rena et al., 2017) point: the fact that the clinically relevant effects of metformin might take place in long-term treatments, where the activation of AMPK becomes essential for the lipid-lowering, anti-inflammatory and insulin-sensitizing effects of this drug.

The involvement of AMPK in the metformin-mediated increase in GDF15 levels provides a new mechanism in addition to those recently reported in studies showing that metformin upregulates GDF15 by increasing CHOP levels (Coll et al., 2020a), (Day et al., 2019). These studies report that the reduction in body weight observed after administration of metformin is caused by the increase in GDF15 levels and the subsequent activation of GFRAL. Consequently, GDF15 interaction with GFRAL would mediate the reported reduction of food intake. It is important to remark that the doses of metformin administered to carry out the mentioned experiments range from 250 to 600 mg/kg, overreaching therapeutic doses. The experiments carried out by Coll et al. to elucidate the mechanism of action of metformin were performed after single injections of 600 mg/kg of this drug. Moreover, phenformin was the drug used to assess the role of the integrated stress

response (ISR) in the biguanide-induced *Gdf15* upregulation. Phenformin differs structurally from metformin by changing a dimethyl group to a phenyl group. While the glucose-lowering effects are similar between these two drugs, the clinical use of phenformin was withdrawn from many countries due to the propension of the patients to develop lactic acidosis (Yendapally et al., 2020), which could indicate that the functions of mechanism of action of phenformin may not be comparable to the functions of metformin. In our study, we administered a dose of 100 mg/kg/day of metformin to HFD-fed mice for 21 days. We did not observe a significant reduction neither in body weight nor in cumulative food intake, suggesting that GFRAL may not be involved in the metabolic effects observed. Additionally, other studies have reported that the effects of GDF15 on metabolism are independent of changes in food intake, thereby implying the presence of peripheral effects of this cytokine (Chung et al., 2017b). Of note, a supplementary figure from Coll et al. study showed that one treatment of 11 days with metformin administered at 300 mg/kg/day did not reduced food intake, which is consistent with our results.

Although cumulative food intake was not reduced by metformin treatment, this drug did cause a significant reduction in the amount of epididymal fat compared to mice fed a HFD and treated with vehicle; this could be an explanation to the trend observed of body weight reduction caused by metformin, despite it did not reach statistical significance. Interestingly, this reduction effect on fat mass by metformin has been also reported (Coll et al., 2020b), and has been attributed to GFRAL, since neutralization of this receptor with a neutralizing antibody partially reverted this effect. A recent study revealed that ATGL and, to a lower extent, HSL expression in white adipose tissue is controlled by the GFRAL-GDF15 sympathetic signalling (Suriben et al., 2020). Interestingly, recombinant GDF15 administration to *Atgl*$^{-/-}$ mice did not cause a reduction in fat mass or body weight, despite the anorectic action of GDF15 through GFRAL was intact. This reveals that the loss of epididymal fat mass observed in our study has been previously reported to be independent of food consumption, as we reported. The reduction in fat mass caused by metformin on HFD-fed mice in our study is consistent with the increase in the expression of the genes involved in fatty acid oxidation in the liver and skeletal muscle, which reduces the amounts of fatty acids for storage in the adipose tissue, as demonstrated by the

reduction in serum and hepatic triglyceride levels. In fact, our experiments in isolated cells, without the influence of central GFRAL, indicate that both metformin and GDF15 increase the expression of these genes through peripheral receptors whose nature remains to be determined.

Day et al. (2019) showed that metformin increased GDF15 secretion in primary hepatocytes from AMPKβ1$^{-/-}$ mice, suggesting that AMPK activation by metformin was not responsible for the increase in GDF15 levels. Consulting the data from the article where the generation of these mice was described (Dzamko et al., 2010), AMPKβ1$^{-/-}$ mice showed no reduction of the activity of AMPK in skeletal muscle or heart. In the liver, these mice exhibit a reduction of the protein levels of 65% and 45% for the α1 and the α2 subunits, respectively. However, γ1 subunit, which is responsible for the allosteric regulation of the enzyme and responds to variations of the levels of AMP, ADP or ATP (Yan et al., 2018), appears unchanged in all the tissues. Furthermore, although the reduction of the activity of AMPK α1 and α2 subunits reached 75-90%, the serine 79 phosphorylation of the AMPK target protein ACC is only reverted up to 60% compared to WT mice. Importantly, these comparative studies were done without any external pharmacological treatment. This indicates that AMPK functions could not be totally compromised when challenged with external agents, like metformin treatment. In addition, Day et al. do not show the phosphorylation levels of any target protein of AMPK. In our study, AMPKα1/α2 knockdown in primary hepatocytes and C2C12 myotubes prevented the increase in GDF15 levels caused by metformin, indicating that this drug increases GDF15 levels through AMPK. Supporting this proposition, a recent study showed that activation of AMPK by A769662 increased hepatic GDF15 levels independently of CHOP (Townsend et al., 2021). Furthermore, we observed that phospho-AMPK levels were reduced in the liver and skeletal muscle of mice lacking *Gdf15*, confirming the activation of AMPK by GDF15.

Although the increase in *Gdf15* expression caused by metformin has been reported to be dependent on CHOP (Coll et al., 2020b; Day et al., 2019), in our study, administration of a lower dose of metformin to WT mice did not increase CHOP protein levels as caused by the HFD in the liver and skeletal muscle. In fact,

metformin significantly decreased CHOP protein levels in HFD-fed mice. These observations make unlikely that, in our conditions, CHOP might be involved in the increase in GDF15, in line with the above-mentioned study (Townsend et al., 2021). Although it has been reported that AMPK activation upregulates p53 (He et al., 2014), a transcription factor reported to regulate *Gdf15* expression (Osada et al., 2007), the present findings discard the involvement of p53 in metformin-mediated upregulation of GDF15. By contrast, our findings suggest that AMPK activation increases the expression levels of *Atf3*, which in turn upregulates the expression of *Gdf15*. In fact, ATF3 has been involved in the upregulation of *Gdf15* by non-steroidal anti-inflammatory drugs and compounds, such as resveratrol, green tea catechins and capsaicin (Baek et al., 2004a), (Lee et al., 2010c), (Yang et al., 2010a). Moreover, ATF3 has been previously reported crucial for the anti-inflammatory effects of metformin, and its upregulation is dependent on AMPK activation (Kim et al., 2014a), also mediating the prevention of the LPS-induced MAPK phosphorylation.

Our findings in C2C12 myotubes indicated that metformin promoted GDF15 peripheral effects independent of GFRAL. The involvement of ALK receptors in the peripheral effects of GDF15 have not yet been discarded (Chung et al., 2017a), (Kim and Lee, 2021) and some authors state that GDF15 may bind to other receptors besides GFRAL, implying that GFRAL-independent GDF15 effects may be involved in the improvement of metabolic disorders like NAFLD/NASH or lung/kidney fibrosis (Kim and Lee, 2021). Some of the peripheral metabolic effects of metformin require the presence of GDF15; however, in our previous study, pharmacological blockade of ALK receptors did not revert the activation of AMPK caused by recombinant GDF15 treatment in myotubes, making unlikely the involvement of these receptors in the GDF15-mediated activation of AMPK. Furthermore, *Gdf15* knockdown in myotubes attenuated the effects of metformin on AMPK activation, as well as its effects on the proteins involved in fatty acid oxidation and inflammation. This is consistent with our results showing that recombinant GDF15 treatment alone is able to regulate some of these effectors related to fatty acid oxidation, inflammation and insulin signalling.

Our current study also uncovers an important role of skeletal muscle in the action of metformin. Although the liver is central for the effect of metformin and this is the organ with the highest expression of *Gdf15* (Assadi et al., 2020), (Luan et al., 2019), other tissues may also contribute to the effects of metformin with increased *Gdf15* expression. In fact, the lower small intestine and colon have been reported to be a major site of metformin-induced *Gdf15* expression (Coll et al., 2020b). Treatment with metformin has been also previously reported to increase the insulin-stimulated glucose uptake in skeletal muscle in patients with T2D (Widén et al., 1992), (Galuska et al., 1994). We show here that a low dose of metformin increased *Gdf15* expression in skeletal muscle, which was accompanied by an increased expression of the genes involved in fatty acid oxidation, and a decreased expression of ER stress and inflammatory markers. Moreover, the experiments carried out in C2C12 myotubes suggest that these are autocrine and paracrine effects of GDF15 in skeletal muscle, and may contribute to the improvement of glucose intolerance and other metabolic disorders. This is further supported by the findings obtained in myotubes exposed to palmitate, where the beneficial effects of metformin were attenuated by *Gdf15* knockdown. In addition, a potential systemic effect of muscle-derived GDF15, as previously reported (Chung et al., 2017a), (Laurens et al., 2020a), (Ost et al., 2020) remains possible.

Overall, the most remarkable finding of the present study is that metformin requires GDF15 to achieve full AMPK activation. Two mechanisms have been reported to date to explain the activation of AMPK by metformin: pharmacological concentrations of metformin directly activate AMPK, whereas supra-pharmacological concentrations (approximately ≥ 5 mM in *in vitro* studies) or high doses *in vivo* act indirectly by inhibiting the mitochondrial respiratory chain complex I, causing an increase in the AMP/ATP ratio, which in turn activates AMPK (He and Wondisford, 2015). However, this inhibition of mitochondrial respiration by supra-pharmacological doses of metformin may result in oxidative and ER stress and CHOP induction, leading to increased *Gdf15* expression. Our findings now suggest that AMPK activation by metformin increases the levels of GDF15, which in turn is involved in achieving and sustaining full AMPK activation (**Figure 34**).

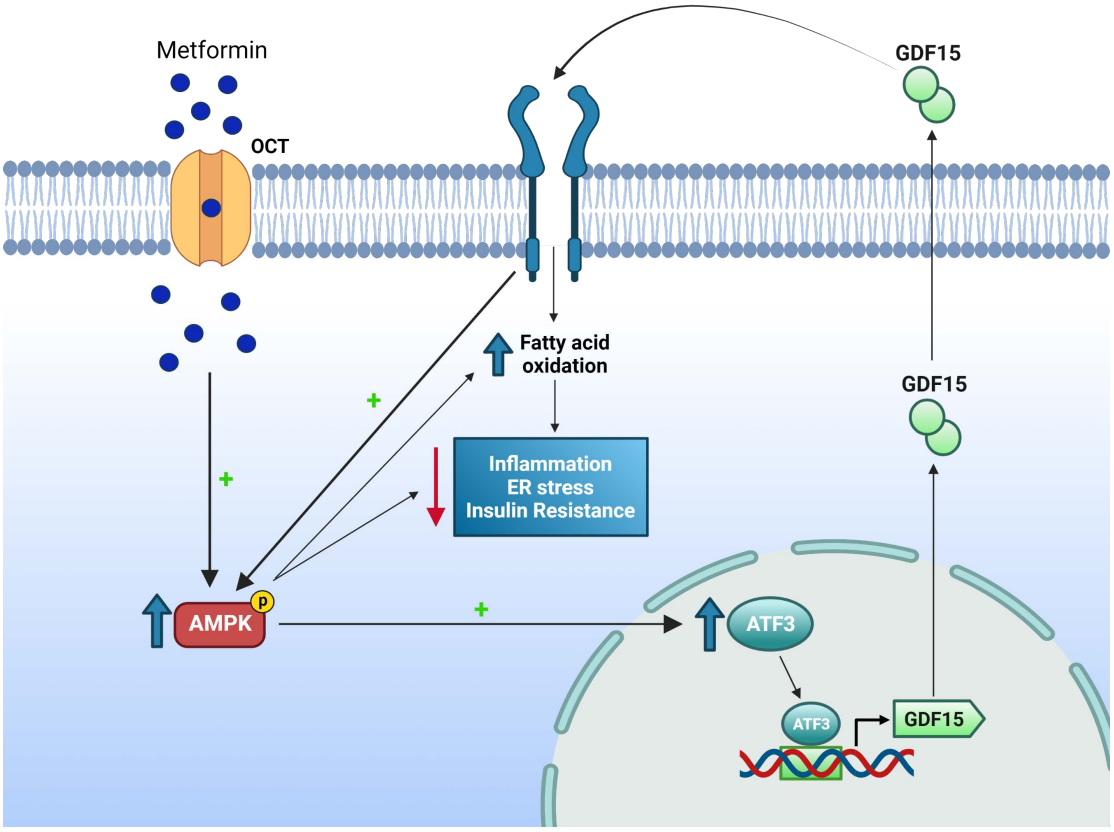


Figure 34. Proposal of the mechanism of action of the GDF15-mediated antidiabetic effects of metformin. Metformin treatment induces the activation of AMPK and the increase of ATF3 nuclear levels. The ATF3-mediated increase of GDF15 is responsible for the prolongation of the activation of AMPK, which induces fatty acid oxidation and contributes to the reduction of inflammation, ER stress and insulin resistance. OCT: organic cation transporter.

V.3 Integrated discussion

Since the identification of GFRAL as the specific receptor for GDF15 in 2017 (Emmerson et al., 2017) (Hsu et al., 2017b) (Yang et al., 2017) (Mullican et al., 2017b), many of the metabolic effects of the overexpression or administration of this cytokine have been attributed to the reduction of food consumption caused by the signals elicited by GFRAL-RET. Initially, this receptor was described as brainstem-restricted and many authors pointed out that it was only expressed in this part of the brain. However, some authors do report expression and activity of GFRAL in specific peripheral tissues. For example, Laurens et al. reported *Gfral* expression in human abdominal subcutaneous adipose tissue explants, whereas in adult female mice adipose tissue it was absent (Laurens et al., 2020a). Interestingly, the lipolytic effect of GDF15 assessed in this study was attributed to its binding to GFRAL in adipose tissue and the result of a paracrine effect of GDF15 release from skeletal muscle cells; however, the involvement of GFRAL was not fully explored. The presence of GFRAL has been additionally determined in human and mouse liver and, to a lesser extent, in cultured hepatocytes or hepatic cell lines (Zhang et al., 2020b). In this case, GDF15 treatment resulted in the induction of GFRAL/RET downstream signalling. Nevertheless, studies neutralizing or ablating the presence of GFRAL receptor were not conducted.

In both of our studies we have reported metabolic effects of GDF15 in the absence of reduction in food intake or body weight. Recently, it was pointed out that pharmacological GDF15 reduces food consumption and motivated exercise (Klein et al., 2021b). Interestingly, pharmacological administration of this cytokine had no adverse effect on exercise performance; however, voluntary exercise resulted compromised. In addition, even with the elevated doses administered to mice, a reduction in food consumption was only significant in the first day, whereas following days this factor was unaffected. Physiological induction of GDF15 after exercise did not cause any effect on food intake or voluntary running. Noteworthy, in cancer states, GDF15 levels in serum are abnormally elevated, ranging from 5 to 50-fold higher than control individuals, depending on the type of cancer and stage of the pathology, (Vocka et al., 2018), (Assadi et al., 2020), and this event has been

proposed as an explanation for the anorexia and cachexia development in this pathology (Suriben et al., 2020). Here, in an induced model of cancer, neutralization of GFRAL by administering a neutralizing antibody reversed the GDF15-mediated cachexia in mice. In fact, inhibition of GFRAL is currently being investigated in phase I trials for the treatment of cancer cachexia. Furthermore, this study reveals that ATGL and, to a lower extent, HSL expression in white adipose tissue is controlled by the GFRAL-GDF15 sympathetic signalling. A combination of these previously reported central effects and the peripheral effects of GDF15 that we report could be responsible for the systemic actions of GDF15 on metabolism.

Considering that abnormally elevated levels of GDF15 (~2000 pg/mL) in serum are responsible for the reduction of food consumption and that this event is mainly driven by nausea and emesis (Borner et al., 2020), we hypothesize that the administration of GDF15 carried out in most studies reporting the food intake-lowering activity of this cytokine could result toxic, whereas the GDF15 induction achieved in our treatments would be within an tolerable range, obtaining values between 150-200 pg/ml of GDF15, approximately 3 or 4-fold higher than control animals. In fact, Tsai et al. reported in 2014 that the 16 ng/mL of GDF15 in serum obtained after exogenous administration resembled the levels reported of serum GDF15 when anorexia and cachexia were present in cancer states in mice (Johnen et al., 2007) or in human (Tsai et al., 2012). Coll et al. administered 600 mg/kg of metformin to mice, resulting in 1,000-1,500 pg/mL of GDF15 serum levels, which, in fact, are values also reached in some cancers (Assadi et al., 2020).

Overall, one of the most remarkable findings of the present thesis is that GDF15 activates AMPK in the absence of GFRAL. Previous to the identification of GFRAL, several studies have described GFRAL-independent actions of GDF15 (Bootcov et al., 1997), (Corre et al., 2013), (Li et al., 2013), (Abulizi et al., 2017) (Chung et al., 2017a), (Campderrós et al., 2019), suggesting that other receptors or mechanisms may be involved in GDF15 actions. As previously mentioned, *Gfral* expression is virtually null in most of the cell lines analysed, and peripheral tissues show null or very low expression of this receptor (Yang et al., 2017). In addition, since GFRAL-RET axis induce the phosphorylation of ERK1/2, which is involved in a

negative crosstalk with AMPK (Hwang et al., 2013), and we report an activation of AMPK upon the treatment with recombinant GDF15, the involvement of GFRAL on this action would result misguided. Notably, reviewing the source data of one of the original articles identifying GFRAL, 50 constructs analysed reported higher signals than the control construct. Among them, some showed 5 to 8-fold higher signal than the control, although the GFRAL-transfected cells exhibited 1 order of magnitude greater than the signal of the receptors mentioned before (Hsu et al., 2017b). Nevertheless, some of these receptors may be implicated in the action of GDF15 activating AMPK reported in the present work. For instance, the highest signal for GDF15 binding in this study comes from the catalytic alpha-subunit of the ATPase enzyme, which is responsible for the hydrolysis of ATP. This enzyme is highly active in skeletal muscle (Pirkmajer and Chibalin, 2016) and its activation would theoretically reduce the AMP/ATP ratio, which could induce AMPK activation. Supposing that GDF15 is able to activate this ATPase in skeletal muscle, this could provide an explanatory mechanism for the activation of AMPK by GDF15. Nevertheless, further experiments are required to test this hypothesis.

AMPK has been long held a promise for the treatment of insulin resistance, due to its actions on fatty acid oxidation, inflammation and the insulin-sensitizing effects this kinase exerts (Vega et al., 2000), (Hwang et al., 2013), (Huang et al., 2015), (Garcia et al., 2019). The unveiling of a novel regulatory action of GDF15 activating AMPK constitutes an important advance in the comprehension of the metabolic actions of GDF15 and how this cytokine may improve the metabolic profile of obese and insulin resistant individuals. For that reason, we are currently developing GDF15 pharmacological inductors that endogenously activate this cytokine and cause an increase of serum levels that does not result toxic, ideally activating AMPK and not relying on the anorexic actions of GDF15. Future *in vivo* studies will be conducted in order to test this potential GDF15 inductors as treatments for metabolic diseases.

VI. CONCLUSIONS

The results obtained in the present doctoral thesis led to the following conclusions:

I.1 Pharmacological PPARβ/δ activation increases GDF15 levels.

I.2 The increase in GDF15 caused by PPARβ/δ is mediated by a molecular mechanism involving the AMPK-p53 pathway.

I.3 GDF15 mediates the antidiabetic effects of PPARβ/δ agonists on lipid metabolism, hepatic steatosis, ER stress, inflammation pathways and insulin signalling on mice exposed to a high fat diet.

I.4 The AMPK activation caused by PPARβ/δ requires increased GDF15 levels to maintain its activation.

I.5 GDF15 induces the activation of AMPK in the absence of GFRAL and independently of central nervous system.

II.1 Metformin treatment increases GDF15 levels via the AMPK-ATF3 pathway.

II.2 The metabolic effects of metformin on C2C12 myotubes exposed to the saturated fatty acid palmitate on AMPK activation, fatty acid oxidation, ER stress, inflammation and insulin signalling are mediated by GDF15 in the absence of GFRAL.

II.3 The metabolic effects of metformin on mice exposed to a high fat diet on AMPK activation, lipid metabolism, ER stress, inflammation and insulin signalling require GDF15 and are independent on changes in food consumption and body weight.

II.4 GDF15 contributes to the activation of AMPK by metformin through a mechanism independent of the central nervous system.

VII. BIBLIOGRAPHY

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Review

PPARβ/δ: A Key Therapeutic Target in Metabolic Disorders

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Abstract: Research in recent years on peroxisome proliferator-activated receptor (PPAR)β/δ indicates that it plays a key role in the maintenance of energy homeostasis, both at the cellular level and within the organism as a whole. PPARβ/δ activation might help prevent the development of metabolic disorders, including obesity, dyslipidaemia, type 2 diabetes mellitus and non-alcoholic fatty liver disease. This review highlights research findings on the PPARβ/δ regulation of energy metabolism and the development of diseases related to altered cellular and body metabolism. It also describes the potential of the pharmacological activation of PPARβ/δ as a treatment for human metabolic disorders.

Keywords: PPARβ/δ; obesity; dyslipidaemia; type 2 diabetes mellitus; non-alcoholic fatty liver disease

1. Introduction

Acquired metabolic disorders, particularly obesity and its associated co-morbidities currently pose a risk to human health on a global scale. These metabolic disorders are closely related to adipose tissue dysfunction, one of the primary defects observed in obesity that may link this condition to its co-morbidities such as non-alcoholic fatty liver disease (NAFLD), atherogenic dyslipidaemia, type 2 diabetes mellitus and cardiovascular disease [1–3]. In fact, up to a third of obese subjects are metabolically healthy and they do not develop obesity-related metabolic or cardiovascular disorders [4], probably because of the preservation of normal adipose tissue architecture and function. Adipose tissue dysfunction in obese patients results from the interactions of genetic and environmental factors that lead to the presence of hypertrophic adipocytes, which have a pro-inflammatory, insulin-resistant phenotype compared to small adipocytes [5]. In addition, a critical factor contributing to the difference between metabolically healthy and unhealthy obese individuals is the anatomical distribution of the adipose tissues. Expansion of the visceral adipose tissue, which is considered a dysfunctional adipose tissue unable to store excessive levels of lipids, to a greater extent than that of subcutaneous adipose tissue, is associated with metabolic alterations [6]. Failure to store surplus lipids into visceral adipose tissue causes a chronic elevation of circulating fatty acids (FA), which can reach toxic levels in non-adipose tissues, such as skeletal muscle, the liver and the pancreas [7]. The deleterious effect of lipid accumulation in non-adipose tissues is known as lipotoxicity. This surplus of fatty acids (FAs),

especially saturated FA and their derived metabolites, such as diacylglycerol and ceramides, induces chronic low-grade inflammation and has harmful effects on multiple organs and systems.

Given the enormous stress on global health services caused by the increasing incidence of obesity and its co-morbidities on global health services, there is a need to better understand the mechanisms behind the relationship between obesity and the development of metabolic disorders to prevent and to improve the outcomes of these diseases. Peroxisome proliferator-activated receptor (PPAR)β/δ is a nuclear receptor that exerts many metabolic effects. Its activation may prevent and improve the outcome of obesity-related metabolic disorders. In this review, we will summarize the molecular features of PPARβ/δ and the benefits of using its agonists to treat obesity and its related co-morbidities.

2. Basic Overview of the Molecular Features of PPARβ/δ

PPARβ/δ is a member of the nuclear receptor (NR) superfamily of ligand-inducible transcription factors and belongs to the PPAR family, which comprises three isotypes: PPARα (NR1C1, according to the unified nomenclature system for the NR superfamily); PPARβ/δ (NR1C2); and PPARγ (NR1C3) [8,9]. PPARβ/δ was initially called PPARβ when it was first cloned in *Xenopus laevis*. However, when cloned in other species it was not clearly identified as being homologous to the Xenopus PPARβ and it was alternatively called NUC-1 in humans and PPARδ in mice. Currently, it is accepted that Xenopus PPARβ is homologous to murine PPARδ, giving rise to the terminology PPARβ/δ [8]. PPARβ/δ consists of four major functional domains: The N-terminal ligand-independent transactivation domain (A/B domain), often known as activation function 1 (AF-1); the DNA binding domain (DBD or C domain); the hinge region (D domain); and the carboxy-terminal E domain or AF-2, which includes the ligand-binding domain and the ligand-dependent transactivation domain [8,9]. The major physiological functions of PPARβ/δ result from its activity as a transcription factor, modulating the expression of specific target genes. Through this mechanism, PPARβ/δ regulates lipid metabolism and glucose homeostasis [8–11]. In addition, PPARβ/δ can regulate inflammation [12]. The involvement of PPARβ/δ in all these functions depends on its tissue distribution, ligand binding and the recruitment of co-activators or co-repressors.

PPARβ/δ is ubiquitously expressed, although it is most abundant in metabolically active tissues, especially in those organs/cells associated with FA metabolism, such as skeletal and cardiac muscle, hepatocytes and adipocytes. It has also been particularly characterized in macrophages. Compared to other NRs, PPARs present a large ligand-binding pocket (≈1300 $Å^3$) [8], which directly contributes to the ability of PPARs to bind a great variety of endogenous and synthetic ligands. FAs are considered endogenous PPAR ligands but they show little selectivity for the different PPAR isoforms. Although all-trans retinoic acid has been reported to be a PPARβ/δ agonist [13], this has not been confirmed by other groups [14,15] and therefore remains controversial. To elucidate PPARβ/δ functions, synthetic ligands with high affinity and specificity (GW501516, GW0742 and L-165041) that only activate PPARβ/δ at very low concentrations both in vivo and in vitro have been developed [8]. At present, there are no clinically available drugs targeting PPARβ/δ but three PPARβ/δ agonists have reached clinical trials: Seladelpar (MBX-8025) (CymaBay Therapeutics) [16]; KD-3010 (Kalypsys) [17]; and CER-002 (Cerenis).

To activate transcription, PPARβ/δ forms an obligate heterodimer with retinoid X receptor (RXR or NR2B) and binds to peroxisome proliferator response elements (PPREs) located at the promoter regions of target genes, thereby increasing gene transcription in a ligand-dependent manner (transactivation) [8]. In the absence of a ligand, the PPARβ/δ-RXR heterodimer is bound by nuclear co-repressor proteins, which block transcriptional activation by preventing the binding of the heterodimer to the promoter. Ligand binding induces a conformational change within PPARβ/δ, resulting in the dissociation of the co-repressors and the recruitment of co-activators, which subsequently lead to PPARβ/δ-RXR binding to PPREs to initiate transcription [8]. PPARβ/δ also regulates gene expression independently of DNA binding, via cross-talk with other types of transcription factors, thus influencing their function through

a mechanism termed receptor-dependent transrepression [8]. Most of the anti-inflammatory effects of PPARs probably occur through this mechanism [12].

3. PPARβ/δ as a Major Regulator of Metabolic Disorders

3.1. Obesity

PPARβ/δ-deficient mice exhibited a marked reduction in adiposity compared to wild-type mice levels [18]. This effect, however, cannot be reproduced in mice harbouring an adipose tissue-specific deletion of PPARβ/δ, indicating that PPARβ/δ elicits peripheral functions in systemic lipid metabolism. In fact, PPARβ/δ activation prevents weight gain in diet- or genetically-induced animal models of obesity by increasing fat burning in different tissues [19,20] or switching muscle fibre type, which, in turn, increases the muscle oxidative capacity [21] (Figure 1). A recent study also suggests that intestinal PPARβ/δ protects against diet-induced obesity, since intestinal epithelial cell-specific deletion of PPARβ/δ in mice results in increased amounts of omental white adipose tissue [22].

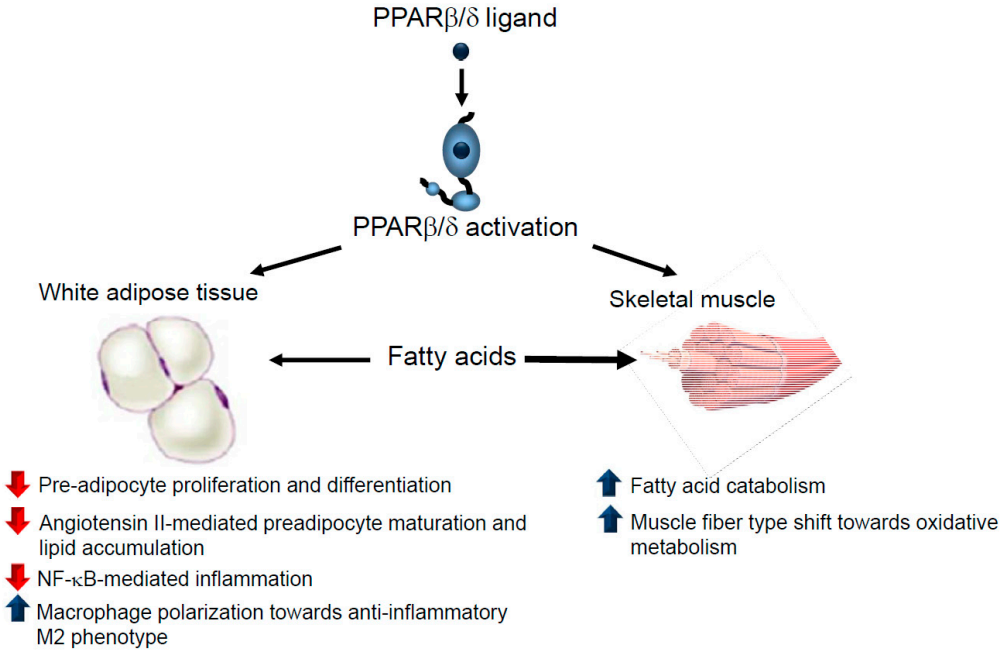

Figure 1. PPARβ/δ activation prevents obesity through several mechanisms. PPARβ/δ activation reduces pre-adipocyte proliferation and differentiation, attenuates angiotensin II-mediated dysfunctional hypertrophic adipogenesis and inhibits inflammation in adipose tissue. PPARβ/δ ligands reduce the availability of fatty acids to be stored in adipose tissue since these drugs induce fat burn in skeletal muscle by either increasing fatty acid oxidation or switching muscle fibre type towards oxidative metabolism. Blue arrow: increases. Red arrow: decreases.

Additional PPARβ/δ-mediated mechanisms can also contribute to the reduction in adiposity, since PPARβ/δ also regulates preadipocyte proliferation and differentiation through different mechanisms [23–25], such as by regulating of the expression of PPARγ, a key regulator of terminal adipocyte differentiation. Moreover, PPARβ/δ ligands prevent angiotensin II-induced adipocyte growth and lipid accumulation [26]. Angiotensin II increases levels of reactive oxygen species (ROS), which attenuate the canonical Wnt signalling pathway, leading to dysfunctional hypertrophic adipogenesis. PPARβ/δ agonists prevent oxidative stress and the reduction in Wnt signalling pathway induced by angiotensin II by increasing the expression of heme oxygenase 1 in adipose tissue. Consequently, PPARβ/δ activation delays preadipocyte maturation and lipid accumulation, leading to increased numbers of smaller adipocytes with an improved adipocytokine profile. Thus, overall,

PPARβ/δ activation prevents oxidative stress and dysfunctional adipogenesis under conditions of overactive renin-angiotensin system [26].

In humans, PPARβ/δ expression is reduced in both the subcutaneous and in visceral adipose tissues of morbidly obese patients compared to non-obese subjects [27]. This might result in adipose tissue dysregulation since PPARβ/δ has anti-inflammatory effects in white adipose tissue. PPARβ/δ activation inhibits lipopolysaccharide (LPS)-induced cytokine expression and secretion by preventing nuclear factor (NF)-κB activation in adipocytes via the activation of mitogen-activated protein kinase (MAPK)–extracellular signal-regulated kinase (ERK)1/2 (MEK1/2) activation [28]. Furthermore, adipose tissue inflammation is characterized by increased infiltration and an altered polarization of the macrophages from the anti-inflammatory M2 phenotype towards the pro-inflammatory M1 phenotype, with PPARβ/δ a crucial signalling molecule that activates polarization towards the anti-inflammatory M2 phenotype [29]. Consistent with the effects of PPARβ/δ in adipose tissue, it has been reported that overweight patients with mixed dyslipidaemia who were administered the PPARβ/δ agonist MBX-8025 for 8 weeks presented favourable trends in their body fat percentage, lean body mass and waist circumference, although the differences did not reach statistical significance [30].

3.2. Dyslipidaemia

Atherogenic dyslipidaemia, often observed in patients with obesity, insulin resistance, metabolic syndrome and type 2 diabetes mellitus, is a significant risk factor for cardiovascular disease. This dyslipidaemia is characterized by the presence of low high-density lipoprotein (HDL) cholesterol levels, elevated triglyceride (TG)-rich very low-density lipoprotein (VLDL) amounts and an increased proportion of small and dense low-density lipoprotein (LDL) particles. It is presently accepted that atherogenic dyslipidaemia is initiated by insulin resistance through the overproduction of TG-rich VLDL [31]. Under conditions of insulin resistance, adipose tissue lipolysis is enhanced, leading to an increase in plasma non-esterified FA (NEFA). The subsequent increase in the flux of NEFA into the liver overcomes the oxidative capacity of hepatocytes and NEFA are then esterified for TG production, causing hepatic steatosis and VLDL over secretion in the plasma [31]. Finally, in the presence of hypertriglyceridemia, the cholesterol-ester content of LDL and HDL decreases, whereas these lipoproteins are enriched in their TG content through the activity of cholesteryl ester transfer protein. These TG-enriched particles are then hydrolysed by hepatic lipase, leading to the formation of small, dense LDL and to the decrease in HDL-cholesterol levels [31].

PPARβ/δ agonists show a strong TG-lowering action in vivo. Given that the main factor affecting hepatic TG secretion is FA availability, the hypotriglyceridaemic effect of PPARβ/δ activators has been attributed, at least in part, to their ability to induce FA β-oxidation in the liver [32] and other tissues [21,33] (Figure 2). In the liver, this role of PPARβ/δ involves the increased expression of the genes involved in FA oxidation via amplification of the lipin 1/PPARγ-coactivator 1α (PGC-1α)/PPARα signalling system and increased levels of the hepatic endogenous ligand for PPARα, 16:0/18:1-phosphatidylcholine [32]. Moreover, the increased FA β-oxidation caused by PPARβ/δ activators might be due to the activation of AMP kinase (AMPK), probably through an increase in the AMP:ATP ratio in hepatocytes [32]. Likewise, the effects of PPARβ/δ on the expression of several genes (*VldlR*, *ApoA5*, *ApoA4* and *ApoC1*) involved in lipoprotein metabolism can contribute to its hypotriglyceridaemic effect [32,34]. In accordance with these effects of PPARβ/δ, mice deficient in this receptor fed a high-fat diet (HFD) show increased plasma TG levels due to hepatic VLDL overproduction. Moreover, these mice also exhibited reduced activity of the enzyme lipoprotein lipase, which catalyses the hydrolysis of the TG component of circulating chylomicrons and VLDL.

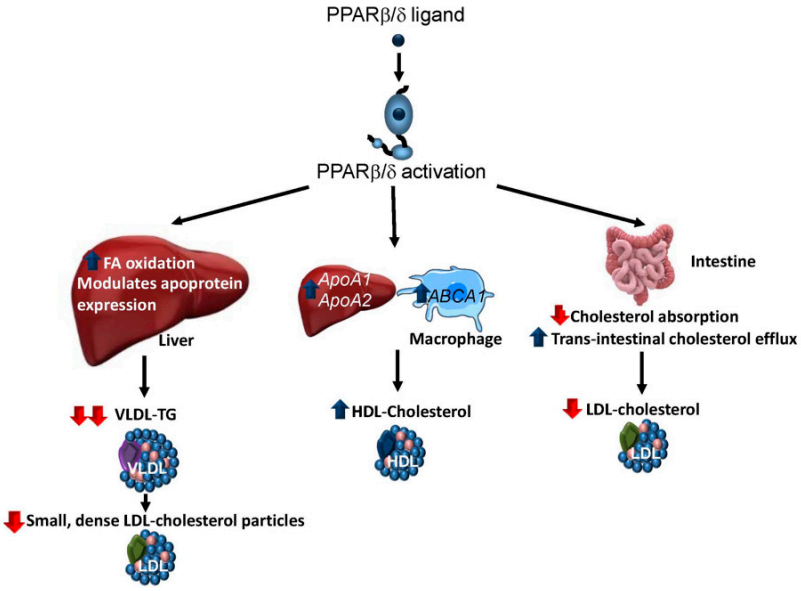

Figure 2. Effects of PPARβ/δ activation in dyslipidaemia. PPARβ/δ activation ameliorates atherogenic dyslipidaemia by reducing the amounts of very low-density lipoprotein (VLDL)-triglyceride (TG) and small dense low-density lipoprotein (LDL) particles and increasing the levels of high-density lipoprotein (HDL)-cholesterol. PPARβ/δ ligands reduce VLDL-TG by increasing hepatic fatty acid (FA) oxidation, which decreases the availability of this lipid for TG synthesis and changing the expression of several apoproteins. PPARβ/δ ligands increase HDL-cholesterol levels by elevating the amounts of the main apopoproteins of these lipoproteins (ApoA1 and ApoA2) in the liver and raising the levels of ATP-binding cassette A1 (ABCA1) in macrophages. Reduced LDL-cholesterol levels results from a decrease in cholesterol absorption and an increase in faecal excretion that are mediated by PPARβ/δ activation. Blue arrow: increases. Red arrow: decreases.

PPARβ/δ agonists also increase plasma HDL-cholesterol levels and reduce LDL-cholesterol and NEFA levels in both animal models and humans [30,33–36]. The increase in plasma HDL-cholesterol levels following PPARβ/δ activation has been linked to an increased expression of the two major apolipoproteins of HDL, *ApoA1* and *ApoA2* [36], in the liver. In addition, PPARβ/δ activation increases the expression in macrophages of the reverse cholesterol transporter, ATP-binding cassette A1 (*ABCA1*) [36,37], which is crucial for the formation of HDL particles through its transport of cholesterol and phospholipid to apolipoprotein acceptors in the bloodstream. Furthermore, PPARβ/δ agonists also regulate the expression of hepatic phospholipid transfer protein (*Pltp*), which regulates the size and the composition of HDL and plays an important role in controlling plasma HDL levels [38]. More recently, it has been reported that the absence of intestinal PPARβ/δ abolishes the ability of its agonists to increase HDL-cholesterol plasma levels [22].

Regarding the reduction in LDL-cholesterol levels, PPARβ/δ agonists have been shown to decrease the efficiency of intestinal cholesterol absorption possibly by reducing the intestinal abundance of the cholesterol absorption protein, Niemann-Pick C1-like 1 (NPC1L1) [39]. Furthermore, PPARβ/δ activation also stimulates faecal excretion of cholesterol in mice, primarily by the two-fold increase in trans-intestinal cholesterol efflux [40], a non-hepatobiliary-related route that transports cholesterol from the blood to the intestinal lumen directly via enterocytes.

The assessment of PPARβ/δ agonists in several small-scale clinical trials mainly for the treatment of atherogenic dyslipidaemia has confirmed that these drugs reduce plasma TG levels, increase the amounts of HDL-cholesterol and decrease the levels of small dense LDL particles in humans, indicating that treatment with these drugs initiates a transition towards a less atherogenic lipoprotein profile [30,37,41–43].

3.3. Type 2 Diabetes Mellitus

More than 90% of patients with type 2 diabetes mellitus are overweight or obese, since obesity is associated with insulin resistance. Insulin resistance, which is defined as a defect in the ability of insulin to drive glucose into its target tissues, predicts and precedes the development of type 2 diabetes mellitus [44]. However, patients with insulin resistance do not develop hyperglycaemia and type 2 diabetes mellitus until the pancreatic β cells fail to secrete sufficient amounts of insulin to meet the increased metabolic demand for this hormone. Adipose tissue expansion in obese individuals releases increased amounts of NEFAs, hormones, pro-inflammatory cytokines and other factors that contribute to the development of insulin resistance. Most of these molecules cause a chronic low-level inflammation, which contributes to insulin resistance and type 2 diabetes mellitus [45].

PPARβ/δ agonists improve glucose tolerance and insulin sensitivity in animal models [20,46]. The antidiabetic effects of these drugs are exerted in different tissues. For instance, macrophage infiltration into adipose tissue and polarization towards the pro-inflammatory M1 phenotype promotes inflammation and correlates with the degree of insulin resistance [47]. As mentioned above, PPARβ/δ activates polarization towards the anti-inflammatory M2 phenotype in macrophages [29] (Figure 3). In accordance with this, myeloid-specific PPARβ/$\delta^{-/-}$ mice show adipocyte dysfunction and insulin resistance [29]. Interestingly, a link exists between metabolism and function in macrophages. Thus, M2 macrophages require oxidative metabolism for their responses, whereas M1 macrophages depend on aerobic glycolysis [48,49]. In fact, blocking oxidative metabolism leads to the polarization of macrophages from the M2 to the M1 phenotype. Similarly, forcing oxidative metabolism in an M1 macrophage potentiates the M2 phenotype [50,51]. Given that PPARβ/δ activation increases β-oxidation in macrophages [52], this effect might also contribute to the polarization to the M2 phenotype caused by the agonists of this receptor. Macrophages also play a key function in a specialized phagocytic process called efferocytosys [53] that contributes to promoting the resolution of inflammation and PPARβ/δ activation enhances this process [53,54]. Since a defective efferocytosys has emerged as a causal factor in the etiopathogenesis of atherosclerosis [55], the increase in this process caused by PPARβ/δ activation might contribute to its beneficial effects in atherosclerosis.

Interleukin (IL)-6 is one of the inflammatory mediators released by adipose tissue that correlates most strongly with obesity and insulin resistance, predicting the development of type 2 diabetes mellitus [56]. PPARβ/δ activation prevents IL-6-induced insulin resistance by inhibiting the signal transducer and activator of transcription 3 (STAT3) pathway in adipocytes, whereas this pathway is over activated in PPARβ/δ-null mice compared to wild-type animals [57].

Skeletal muscle is the primary site of insulin resistance in obesity and type 2 diabetes mellitus since it displays the highest level of insulin-stimulated glucose utilization [5]. Increased plasma levels of saturated NEFAs, caused by the expansion of adipose tissue, promote inflammation and insulin resistance through several mechanisms: the synthesis of FA-derived complex lipids such as diacylglycerol and ceramides; the impairment of the function of cellular organelles (endoplasmic reticulum [ER] stress and mitochondrial dysfunction); and the activation of pro-inflammatory pathways through membrane receptors, such as toll-like receptor 4 (TLR4). PPARβ/δ activation the decrease in insulin sensitivity by suppressing the FA-induced increase in diacylglycerol levels and the subsequent activation of protein kinase C (PKC)θ and NF-κB by enhancing the expression of the genes involved in FA oxidation via PGC-1α and by increasing AMPK phosphorylation [58,59]. Furthermore, PPARβ/δ overexpression in the skeletal muscle of mice has been reported to promote the interaction between PPARβ/δ and AMPK, which enhances glucose uptake, FA oxidation and insulin sensitivity [60]. PPARβ/δ agonists also prevent palmitate-induced ER stress in myotubes through a mechanism involving AMPK activation [61]. Overall, it is believed that PPARβ/δ activation in skeletal muscle produces changes that resemble the effects of exercise training [61], making it a potential candidate for mimicking the effects of exercise to treat metabolic diseases [62].

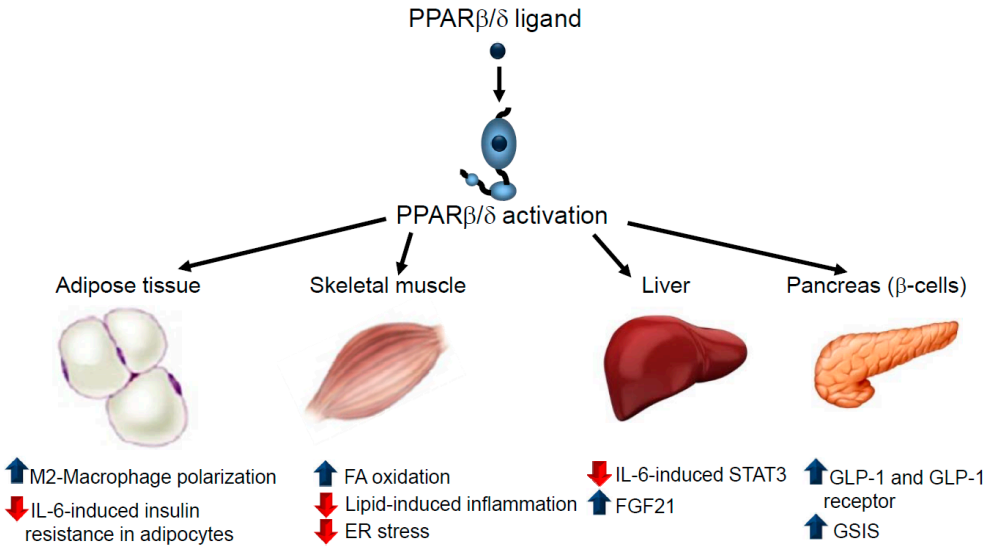

Figure 3. Effects of PPARβ/δ in type 2 diabetes mellitus. This figure depicts the effects of PPARβ/δ ligands in adipose tissue, skeletal muscle, the liver and pancreatic β cells that contribute to the attenuation of type 2 diabetes mellitus. In adipose tissue, PPARβ/δ activation switches macrophage polarization towards the anti-inflammatory M2 phenotype and prevents IL-6-induced insulin resistance by inhibiting STAT3. In skeletal muscle, PPARβ/δ ligands induce FA oxidation, reducing their availability for the synthesis of deleterious complex lipids involved in inflammation and prevent endoplasmic reticulum (ER) stress by activating AMPK. PPARβ/δ activation in hepatocytes blocks the effects of IL-6 by inhibiting the STAT3 pathway through several mechanisms and increasing FGF21 levels. PPARβ/δ activators promote the beneficial effects of GLP-1 in the pancreas and enhance GSIS.ER, endoplasmic reticulum; FA, fatty acid; GLP-1, glucagon-like peptide 1; GSIS, glucose-stimulated insulin secretion; IL-6, interleukin 6; STAT3, signal transducer and activator of transcription 3. Blue arrow: increases. Red arrow: decreases.

In the liver, IL-6 induces insulin resistance by activating STAT3-suppressor of cytokine signalling 3 (SOCS3) pathway [63]. We have previously reported that PPARβ/δ activation prevents the attenuation of the insulin signalling pathway in human liver cells by preventing IL-6-induced STAT3 activation through a mechanism that inhibits ERK1/2 phosphorylation and suppresses the reduction in phospho-AMPK levels [64]. More recently, the inhibitory effect of PPARβ/δ on STAT3 was confirmed, with a new mechanism described involving a T cell protein tyrosine phosphatase 45 (TCPTP45) isoform [65]. According to this recent study, short-term PPARβ/δ activation prevents IL-6-induced insulin resistance as a result of PPARβ/δ forming a complex with nuclear TCPTP45 and retaining it in the nucleus, thereby deactivating the STAT3-SOCS3 signalling [65]. Fibroblast growth factor 21 (FGF21) is a liver-derived circulating hormone that has emerged as an important regulator of glucose and lipid metabolism, making it a promising agent for the treatment of insulin resistance and type 2 diabetes mellitus [66]. Since PPARβ/δ activators increase the plasma levels of FGF21 in humans [67], some of the antidiabetic effects of these drugs might be mediated by the increased levels of this protein.

β-cell failure, a result of the progressive decline in pancreatic β cell function and mass, impairs insulin secretion and contributes to the development of type 2 diabetes mellitus [68]. PPARβ/δ activation in the small intestine potentiates the production of glucagon-like peptide (GLP)-1, which preserves β cell morphology and function, thereby increasing systemic insulin sensitivity [69]. This is consistent with a recent study reporting that intestinal PPARβ/δ protects against diet-induced obesity and insulin resistance [26]. Moreover, PPARβ/δ activation protects pancreatic β cells from palmitate-induced apoptosis by upregulating the expression of the receptor for GLP-1 [70]. PPARβ/δ agonists also increase mitochondrial oxidation in β cells, enhance glucose-stimulated insulin secretion (GSIS) from pancreatic islets and protect GSIS from the adverse effects of prolonged FA exposure [71].

In fact, PPARβ/δ is critical for the expression of the genes involved in mitochondrial function and consequently ATP production, in β cells, which is required for GSIS [72].

3.4. Non-Alcoholic Fatty Liver Disease (NAFLD)

NAFLD encompasses a spectrum of liver disorders ranging from simple steatosis (non-alcoholic fatty liver, NAFL) to non-alcoholic steatohepatitis (NASH) and liver fibrosis. It is closely linked to obesity and metabolic syndrome, predisposing susceptible individuals to cirrhosis, hepatocellular carcinoma and cardiovascular disease [73]. At present, there are no approved pharmacological therapies for NAFLD. In animal models, long-term treatment with PPARβ/δ agonists attenuates hepatic steatosis by enhancing FA oxidation, reducing lipogenesis and improving insulin sensitivity [74–77]. PPARβ/δ activation and overexpression inhibit lipogenesis in hepatocytes by inducing the expression of insulin-induced gene-1 (*INSIG-1*), an ER protein that blocks the activation of sterol regulatory element-binding protein-1 (SREBP-1), a pivotal transcription factor controlling lipogenesis in hepatocytes [77]. However, short treatments with PPARβ/δ agonists might result in a transient increase in hepatic TG levels [78] but without hepatotoxicity, since PPARβ/δ increases the number of monounsaturated FAs but reduces the levels of saturated FAs [79]. In addition, PPARβ/δ might affect hepatic TG levels by regulating the abundance of the VLDL receptor [80]. In humans, PPARβ/δ agonists reduce hepatic fat content and elicit improvements in the plasma markers of liver function [30,33]. These and additional findings point to PPARβ/δ, similarly to PPARα, as a master regulator of hepatic intermediary metabolism. During fasting conditions, hepatic metabolism is programmed to oxidize FA and both, PPARα and PPARβ/δ are thought to promote ketogenesis by inducing FGF21 [67] and the expression of genes involved in fatty acid oxidation [81] in rodents. Interestingly, as mentioned before, PPARβ/δ activation in mice increases the hepatic levels of the hepatic endogenous ligand for PPARα, 16:0/18:1-phosphatidylcholine, leading to amplification of the PGC-1α-PPARα pathway [32], suggesting the presence of a cooperation between both nuclear receptors in the regulation of hepatic metabolism.

The progression from NAFL to NASH involves the development of inflammation and signs of hepatocellular damage [65]. Both the hepatic expression of inflammatory genes [74,76,82] and hepatic ER stress [76], which contribute to the activation of inflammatory pathways, are reduced by PPARβ/δ ligands. This hepatoprotective effect of PPARβ/δ ligands might also involve Kupffer cells, resident liver macrophages that play a critical role in maintaining liver functions. Hematopoietic deficiency of PPARβ/δ selectively impairs the alternative activation of Kupffer cells in obese mice, leading to reduced oxidative metabolism and hepatic dysfunction [83]. Additional studies are required to conclusively determine the effects of PPARβ/δ agonists on liver fibrosis due to the inconsistent results currently reported in the literature [17,84].

4. Safety of PPARβ/δ Agonists

Despite the promising data of PPARβ/δ agonists in metabolic disorders in preclinical studies, the discovery that mice treated with GW501516 developed adenocarcinoma [85] halted further development of this drug and undermined the potential use of these drugs in human therapeutics. However, subsequent attempts to assess the role of PPARβ/δ in cancer have demonstrated that this receptor both inhibits and promotes tumorigenesis, as it has been extensively reviewed previously [10,86,87], becoming one of the most controversial effects of PPARβ/δ. The conflicting results about PPARβ/δ in cancer might indicate that the activity of this receptor in cancer development is influenced by the mutational status of the tumour cell and the tumour environment [10]. Moreover, it has been proposed that the high-level expression of PPARβ/δ in normal cells suggest an antitumour effect for this receptor but the reduction in its expression or the presence of endogenous antagonists or inverse agonists might lead to a protumorigenic role for PPARβ/δ [86].

Although there are only a few clinical trials assessing the safety of PPARβ/δ agonists, the administration of these drugs to humans seems to be safe and generally well-tolerated, at least for the short periods evaluated [30]. Thus, no study subjects were withdrawn because of adverse effects from

a study evaluating the administration of seladelpar for 8 weeks to dyslipidaemic overweight patients at doses showing beneficial metabolic effects [30]. Treatment with this PPARβ/δ agonist slightly but significantly decreased red blood cell count, haemoglobin and haematocrit. In a more recent study, the effect of seladelpar was assessed in patients with primary biliary cholangitis [88]. Drug treatment for 12 wk. elicited anti-cholestatic effect but three patients showed rapidly reversible alanine aminotransferase elevations and the study was interrupted before completion. The authors of the study suggest that this effect might be specific for these patients with primary biliary cholangitis due to the biliary excretion of seladelpar ant its metabolites, leading to increased hepatic drug concentrations.

5. Conclusions

Over the past 20 years, a substantial body of preclinical evidence has demonstrated that PPARβ/δ activation is a promising therapeutic strategy for treating obesity-associated co-morbidities. This has led to the assessment of PPARβ/δ agonists in clinical trials, although these studies on the efficacy and safety of PPARβ/δ agonists in humans are scarce. Safety issues have been raised regarding the role of PPARβ/δ ligands in carcinogenesis [87]. However, there are conflicting findings on the role of PPARβ/δ as a tumour suppressor or tumour promoter [87], the latter being mostly observed in animal models. Further studies are required to obtain conclusive data on the role of PPARβ/δ in human cancer given that although mouse models are invaluable tools for investigating basic tumour biology, they show significant limitations when compared to human beings. For instance, PPARs are expressed at lower levels in human than in rodent cells and gene expression is also regulated differently by PPARs in human and rodent cells [9]. These differences might explain why long-term treatment with PPARα ligands have been shown to induce carcinogenesis in rodents but carcinogenesis has not been observed in humans treated with the PPARα agonists fibrates for dyslipidaemia over the decades [89]. However, this needs to be studied for PPARβ/δ too.

In summary, although further studies are required to confirm the safety of PPARβ/δ ligands, these drugs have demonstrated that modulation of PPARβ/δ activity shows efficacy in preclinical studies and in a few clinical trials in the treatment of dyslipidaemia, type 2 diabetes mellitus and NAFLD.

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Reviews

Revealing the role of peroxisome proliferator-activated receptor β/δ in nonalcoholic fatty liver disease

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD), a form of chronic liver disease that occurs in individuals with no significant alcohol abuse, has become an increasing concern for global health. NAFLD is defined as the presence of lipid deposits in hepatocytes and it ranges from hepatic steatosis (fatty liver) to steatohepatitis. Emerging data from both preclinical studies and clinical trials suggest that the peroxisome proliferator-activated receptor (PPAR)β/δ plays an important role in the control of carbohydrate and lipid metabolism in liver, and its activation might hinder the progression of NAFLD. Here, we review the latest information on the effects of PPARβ/δ on NAFLD, including its capacity to reduce lipogenesis, to alleviate inflammation and endoplasmic reticulum stress, to ameliorate insulin resistance, and to attenuate liver injury. Because of these effects, activation of hepatic PPARβ/δ through synthetic or natural ligands provides a promising therapeutic option for the management of NAFLD.

Contents

Abbreviations: AMPK, AMP-activated protein kinase; CCl_4, carbon tetrachloride; DBD, DNA-binding domain; DNL, *de novo* lipogenesis; ER, endoplasmic reticulum; ERK1/2, extracellular-related kinase 1/2; FGF, fibroblast growth factor; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; HFD, high-fat diet; HRI, heme-regulated eukaryotic translation initiation factor 2α (eIF2α) kinase; HSC, hepatic stellate cells; LBD, ligand-binding domain; LDL, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD Activity Score; NASH, nonalcoholic steatohepatitis; NEFA, non-esterified fatty acids; PPAR, peroxisome proliferator-activated receptor; RXR, 9-cis retinoic acid receptors; TCPTP45, T-cell protein tyrosine phosphatase 45; TG, triglyceride; PPRE, peroxisome proliferator response element; SOCS3, suppressor of cytokine signaling 3; SREBP-1, sterol regulatory element-binding protein-1; STAT3, signal transducer and activator of transcription 3; VLDL, very-low-density lipoprotein.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver damage worldwide [1]. This disorder encompasses a group of conditions in which the main characteristic is the presence of triglyceride (TG) accumulation or steatosis in >5% of hepatocytes in the livers of people who drink little or no alcohol. NAFLD is categorized into two pathologically distinct conditions depending on their prognoses: nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). NAFL refers to hepatic steatosis with no hepatocyte injury in the form of hepatocyte ballooning. In NASH, the hepatic steatosis is accompanied by inflammation and hepatocyte injury (ballooning) with or without fibrosis. This latter condition may result in the development of severe diseases, including fibrosis, cirrhosis and hepatocellular carcinoma (HCC).

NAFLD development and progression is strongly associated with obesity, type 2 diabetes mellitus and dyslipidemia [1], and its global prevalence is currently estimated to be 24% [2], which rises up to 90% in subjects with morbid obesity [3]. It has been estimated that NASH affects 2%–3% of the general population [4], but this incidence raises up to 37% in subjects with morbid obesity [3].

The molecular mechanisms involved in TG accumulation and subsequent hepatocellular damage in NAFLD are multiple and not completely understood. Initially, the two-hit hypothesis suggested that hepatic TG accumulation sensitizes the liver to a second insult, such as oxidative stress, resulting in NASH. A more accurate explanation of NAFLD pathogenesis is provided by the current "multiple-hit" hypothesis, which considers that following TG accumulation, multiple insults act together on genetically predisposed subjects to induce NAFLD. Those hits include hepatic alterations (mitochondrial dysfunction, endoplasmic reticulum stress, oxidative stress), but they also involve other organs such adipose tissue (adipokine production imbalance), altered regulation of immunity or compositional changes of the microbiota. Whilst NAFL in the absence of significant fibrosis is considered a relatively harmless and reversible condition [5], the development of fibrosis predicts both the progression of the disease and the presence of liver-related complications [6]. Current strategies for treating NAFLD aim to ameliorate obesity and insulin resistance, the main drivers of NAFLD in humans, through dietary intervention and increased physical activity. These interventions are not always sufficient and sustained results are difficult to achieve. Therefore, there is a need for pharmacological treatment. However, although a great number of therapeutic targets have been reported, there are currently no approved specific pharmacological drugs for treatment of NASH.

In recent years, considerable advances have been made clarifying the mechanisms of NAFLD progression, from liver steatosis to more advanced liver inflammation and fibrosis. The peroxisome proliferator-activated receptor (PPAR)β/δ has emerged as a therapeutic target to alleviate NAFLD. PPARβ/δ is expressed in major liver cell types (hepatocytes, Kupffer cells, cholangiocytes and hepatic stellate cells) [7–9] and it is critically involved in the organization of tissue development and repair, regeneration, insulin sensitivity, inflammation, mitochondrial function, energy expenditure, and both lipid and carbohydrate metabolism. In this review, we adopt a multifaceted approach to the role of PPARβ/δ in NAFLD, including its therapeutic potential in the treatment of this condition and how impairment of PPARβ/δ expression or activity may contribute to the progression of this disease.

2. Molecular features of PPARβ/δ

PPARβ/δ (also referred to as hNUC1 and FAAR and known as NR1C2: nuclear receptor subfamily 1, group C, member 2) is one of the three PPARs in the nuclear hormone receptor superfamily. It acts as a ligand-inducible transcription factor. It was first cloned in *Xenopus laevis* under the name of PPARβ. Later on, it was cloned in humans and mice, and named hNUC-1 and PPARδ, respectively [10–12]. However, it has been discovered that there are no differences between these three and it is now most commonly called PPARβ/δ.

Similarly to other PPAR isotypes and other nuclear hormone receptors, PPARβ/δ protein shows a common structure formed by four domains: A/B, C, D, and E/F [13]. The amino-terminal region (domain A/B) contains a ligand-independent activation function 1 (AF-1). The DNA-binding domain (DBD) (C) is structurally conserved over the nuclear receptor superfamily and is folded into two zinc fingers conferring DNA binding specificity. The DBD binds to DNA sequences located in the promoter region of target genes known as peroxisome proliferator response elements (PPREs). A short hinge region (D) connects the DBD to the C-terminal trans-activating domain (E/F), also known as the ligand-binding domain (LBD). Apart from binding to ligands, this domain is also involved in heterodimerization with 9-cis retinoic acid receptors (RXRs) and interactions with transcriptional cofactors [14].

Following ligand binding, PPARβ/δ heterodimerizes with RXR and the resultant heterodimer modulates gene transcription by binding to PPREs. Despite this, PPARβ/δ modulates biological functions independently of binding to PPREs; in fact, most anti-inflammatory effects of PPARβ/δ are mediated by this PPRE-independent action [15].

Although PPARs are constitutively and predominantly present in the nucleus, their biological functions are regulated by a dynamic shuttle between the nucleus and the cytoplasm in response to external signals [16]. Nuclear transport of PPAR involves at least two nuclear localization signals in the DBD-hinge and AF1 regions, and the receptors mediating this transport include importin α/β, importin 7, and an unidentified receptor. PPARs also contain two nuclear export signals in the DBD and LBD regions that are recognized by distinct export receptors: calreticulin and chromosomal maintenance 1 [16].

Moreover, the shuttle between the nucleus and the cytoplasm of PPARs is also regulated by respective PPAR ligands and Ca^{2+} concentration. In addition, several fatty acids and other ligands promote nuclear translocation of fatty acid-binding protein 5 (FABP5), where it is thought to engage with PPARβ/δ, allowing for the channeling of the ligand into the binding pocket of the nuclear receptor [17].

PPARβ/δ is expressed at high levels in liver [18]. However, the expression and the activity of this transcription factor has been reported to be reduced in the liver of patients suffering NAFLD, compared to healthy subjects [19]. In mice fed a high-fat diet (HFD), the hepatic protein levels of PPARβ/δ were reduced [20,21], while incubation of human hepatocytes with the saturated fatty acid palmitate resulted in a decrease of the levels of this nuclear receptor. This suggests that lipids are responsible for this downregulation of PPARβ/δ [20]. Moreover, a reduction of PPARβ/δ protein levels has been reported in HCC and murine liver regeneration, compared to adjacent non-tumor liver tissue [22].

The use of synthetic PPARβ/δ ligands (L-165041, GW501516, and GW0742) that selectively activate with high affinity and specificity this receptor at very low concentrations both *in vivo* and *in vitro* has allowed the elucidation of PPARβ/δ functions [23]. At present, drugs targeting PPARβ/δ are not clinically available, although three PPARβ/δ agonists have been assayed in clinical trials: seladelpar (MBX-8025) [24], KD-3010 [25], and CER-002. In addition, the dual PPARα and β/δ agonist elafibranor (GFT505) has reached phase 3 clinical trial, which is expected to be completed in December 2021 [26].

3. Effects of PPARβ/δ in cellular and animal models of NAFLD

3.1. The role of PPARβ/δ in hepatic steatosis

The hallmark of NAFLD is the accumulation of liver TGs, which are synthesized from fatty acids. Hepatic lipid content is regulated by the complex interplay between the delivery of fatty acids to the liver and the processes of synthesis, hepatic lipid uptake, oxidation and secretion. Hepatic fatty acids come from several different sources: 59% are derived from serum non-esterified fatty acids (NEFAs), 26% from the *de novo* lipogenesis (DNL) pathway, and 15% from diet [27].

In insulin-resistant states, adipose tissue does not respond to the antilipolytic effect of insulin and the release of NEFAs is increased [28], favoring hepatic TG accumulation. PPARβ/δ activation attenuates insulin resistance and reduces serum levels of NEFAs in animal models of obesity and type 2 diabetes mellitus [29,30], suggesting that this action can contribute to amelioration of fatty liver. The reduction in serum NEFAs caused by PPARβ/δ activation is also the result of an increase in fatty acid oxidation in liver and other tissues such as skeletal muscle [30–32]. Muscle fatty acid uptake in skeletal muscle seems to depend on hepatic PPARβ/δ activation [33]. Interestingly, PPARβ/δ activation by GW501516 increases the hepatic levels of 16:0/18:1-phosphatidilcholine, an endogenous ligand for PPARα [31] (Fig. 1). In turn, this 16:0/18:1-phosphatidilcholine is regulated by diurnal hepatic PPARβ/δ activity, and it reduces postprandial lipid levels while increasing fatty acid use through muscle *via* activation of PPARα [33]. PPARβ/δ also regulates hepatic levels of PPAR-γ coactivator (PGC)-1α, a master regulator of mitochondrial biogenesis [31], that regulates fatty acid oxidation.

The PPARβ/δ ligand GW501516 also prevents the reduction caused by a HFD in hepatic AMP-activated protein kinase (AMPK) phosphorylation and activity [31], a kinase that stimulates fatty acid oxidation, caused by the exposure to a HFD. More recently it has been reported that PPARβ/δ attenuates hepatic steatosis through autophagy-mediated fatty acid oxidation [21]. Autophagy is a degradative pathway that promotes cell survival by supplying energy in times of stress or by removing cellular debris after injury. Accumulating evidence suggests that hepatic autophagy is decreased during the progression of NAFLD [34]. Interestingly, PPARβ/δ ligands induce autophagy in primary hepatocytes and the autophagy-lysosomal pathway is required for the stimulation of fatty acid oxidation by conditional hepatic PPARβ/δ overexpression [21]. Consistent with this, inhibition of autophagy attenuated the beneficial effects of PPARβ/δ ligands on fatty acid oxidation and hepatocyte TG accumulation. The increase in autophagy attained by PPARβ/δ ligands seems to involve the activation of AMPK and the reduction of the mammalian target of rapamycin (mTOR) [21], two kinases involved in the regulation of autophagy.

The liver uses excess glucose for glycogen synthesis during feeding. Hepatic PPARβ/δ activation also contributes to improving hyperglycemia by increasing hepatic glycogen and glucose utilization [35]. In fact, the glucose-lowering effect of hepatic PPARβ/δ is partially driven through upregulation of hepatic glucokinase, an enzyme that increases glucose flux into glycogen synthesis.

Hepatic DNL, the synthesis of new fatty acids from non-lipid sources, plays a key role in the development of NAFLD, and fatty acid sensors, such as PPARs, regulate this process. Sterol regulatory element-binding protein-1c (SREBP-1c) is a key transcription factor that regulates genes in the glycolytic and DNL pathways. It has been clearly established that SREBP-1 is significantly increased in the liver of animal models of obesity and type 2 diabetes, as well as in obese patients, where it contributes to hepatic TG accumulation and insulin resistance [36,37]. SREBP-1c is synthesized as an endoplasmic reticulum (ER)-anchored precursor form and is associated with the SREBP-1c cleavage activating protein (SCAP) and the ER retention protein called insulin-induced gene (Insig) [38,39]. Insulin activates SREBP-1c by stimulating the dissociation of the SREBP-SCAP complex from Insig and by promoting its migration to the Golgi apparatus. In this organelle, SREBP-1c is sequentially cleaved by site 1 (S1P) and site 2 (S2P) proteases, releasing the N-terminal cytosolic portion of the protein, which enters the nucleus where it acts as an active transcription factor [40]. PPARβ/δ activation

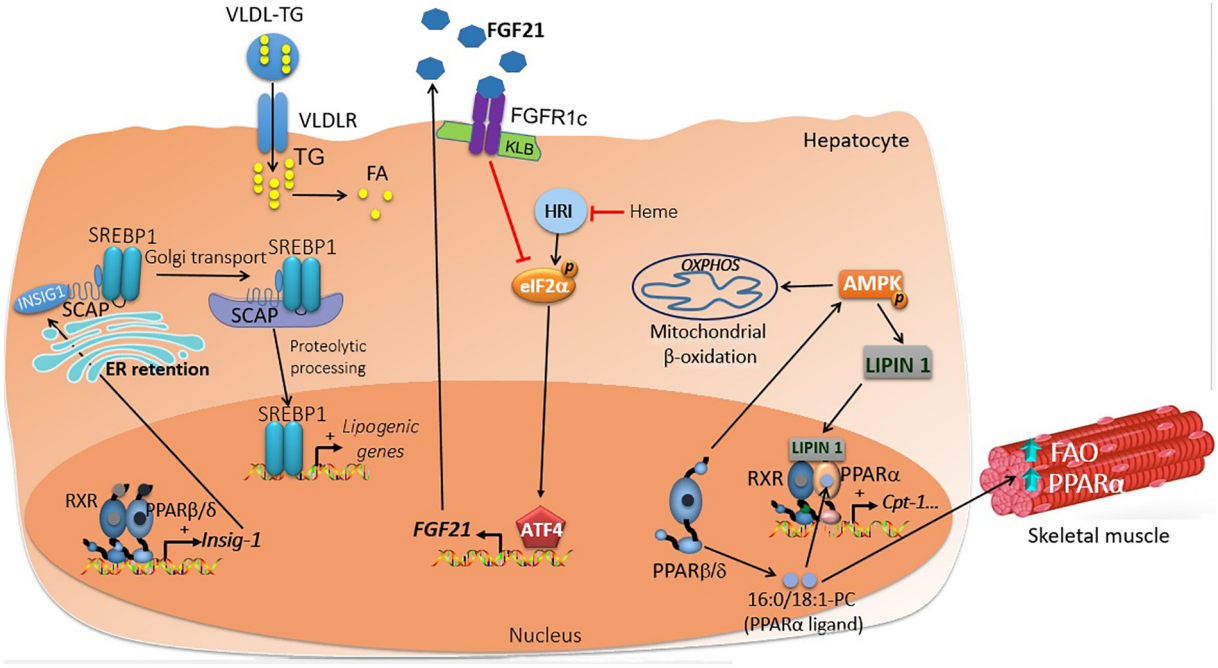

Fig. 1. Potential mechanisms of PPARβ/δ involvement in hepatic TG accumulation. Blue arrows. indicate upregulation following PPARβ/δ activation. PPARβ/δ activation decreases non-esterified fatty acid levels by upregulating the expression of genes involved in fatty acid oxidation (FAO), such as *Cpt-1*, in liver and skeletal muscle. SREBP-1c, a transcription factor involved in lipogenesis, is activated by the dissociation of the SREBP-SCAP complex from Insig and by promoting its migration to the Golgi apparatus. In this organelle, SREBP-1c is cleaved and enters the nucleus to upregulate the expression of genes involved in lipogenesis. Hepatic PPARβ/δ activation reduces SREBP-1c proteolytic cleavage by upregulating Insig-1. PPARβ/δ deficiency causes a reduction in hemin levels that activates HRI, leading to the stimulation of the eIF2α-ATF4 pathway, which increases the expression of FGF21 and VLDLR. In addition, through a negative feedback loop, FGF21 inhibits eIF2α phosphorylation. PPARβ/δ agonists prevent the downregulation of AMPK in liver caused by a HFD and amplify the PGC-1α-Lipin 1-PPARα pathway while also increasing the levels of the PPARα endogenous ligand 16:0/18:1-phosphatidylcholine. All these effects increase FAO. Abbreviations: AMPK, AMP-activated protein kinase; ATF 4, activating transcription factor 4; ER, endoplasmic reticulum; FA, fatty acid; FAO, fatty acid oxidation; FGF21, fibroblast growth factor 21; HRI, heme-regulated eukaryotic translation initiation factor 2α (eIF2α); Insig-1, insulin-induced gene; KLB, β-Klotho; OXPHOS, oxidative phosphorylation; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; SCAP, SREBP cleavage-activating protein; SREBP, sterol regulatory element-binding protein; TG, triglyceride; VLDLR, very-low-density lipoprotein receptor; 16:0/18:1-PC; 16:0/18:1-phosphatidylcholine;

suppresses proteolytic cleavage of SREBP1-c through induction of Insig-1 (Fig. 1), eventually improving hepatic steatosis in a genetic mouse model of obesity, insulin resistance, and NAFLD (db/db mice) [41]. Moreover, PPARβ/δ$^{-/-}$ mice display SREBP-1c upregulation compared to wild-type animals [42], supporting the notion that overexpression or activation of PPARβ/δ ameliorates hepatic steatosis by suppressing the action of SREBP-1c in DNL. In addition to its role in fatty acid oxidation, AMPK is known to regulate DNL [43], and it has been reported that PPARβ/δ activation by GW501516 may attenuate fatty acid synthesis through activation of this kinase [44].

Surprisingly, it has been reported that a short treatment with a PPARβ/δ agonist causes a transient increase in liver TG levels [45], but without reaching hepatotoxicity, since PPARβ/δ increases the amount of monounsaturated fatty acids, but it reduces the levels of saturated fatty acids, thus protecting the liver against lipotoxicity [35]. This initial increase in hepatic TG levels caused by the administration of PPARβ/δ ligands might be the result of an increase in the glucose flux through the pentose phosphate pathway, ultimately causing enhanced fatty acid synthesis [30]; or of the increase in hepatic lipid deposition due to the modulation of the abundance of the very-low density lipoprotein (VLDL) receptor [19]. An increase in the levels of VLDL receptor following treatment with PPARβ/δ agonists may contribute to the hypotriglyceridemic effect of these drugs, as previously reported for the PPARα ligand fenofibrate [46]. In fact, the promoter of the VLDL receptor gene contains a PPRE that may respond to both PPARα and PPARβ/δ ligands. In contrast, longer treatments with PPARβ/δ agonists cause a reduction in the accumulation of hepatic TGs [45], suggesting that the raise in fatty acid oxidation and the reduction in DNL exceeds the mechanisms that initially potentiate non-toxic hepatic lipid deposition.

PPARβ/δ activation can also affect the progression of NAFLD by regulating the levels of hepatokines. These are proteins secreted by hepatocytes that can influence metabolic processes through autocrine, paracrine and endocrine actions [47]. One hepatokine with an important role in NAFLD is fibroblast growth factor 21 (FGF21), a member of the FGF family, which acts as a hormone with important actions on carbohydrate and lipid metabolism [48]. Currently, targeting FGF21 has emerged as a novel therapeutic target for the treatment of NASH [49].

Serum FGF21 levels are liver-derived [50] and they correlate with hepatic expression [51]. Different studies in genetic and diet-induced models of obesity have demonstrated that pharmacological FGF21 administration reverses a large number of metabolic alterations [49,52–55], including hyperglycemia and hypertriglyceridemia, liver steatosis and NASH. Despite these pharmacological effects, serum FGF21 levels are paradoxically increased in obesity, both in rodents [56–59] and humans [60–62]. The increase in circulating FGF21 levels in obesity and other metabolic alterations probably reflects the deposition of fat in liver [63] and, consistent with this, serum FGF21 levels correlate with NAFLD in humans [64]. Hepatic FGF21 expression is upregulated by PPARα following long exposure to fasting [65,66]. Interestingly, PPARβ/δ agonists increase circulating FGF21 in humans [67], suggesting that some of the effects of these drugs might be mediated by this hepatokine. Surprisingly, serum levels and hepatic expression of FGF21 are also enhanced in PPARβ/δ-null mice [20]. Increased levels of FGF21 in the liver of PPARβ/δ-deficient mice appears to depend on a reduction in the content of PGC-1α and the subsequent reduction in heme content, which finally activates the heme-regulated eukaryotic translation initiation factor 2α (eIF2α) kinase (HRI). This kinase phosphorylates eIF2α, which in turn increases activating transcription factor (ATF) 4 levels, a transcription factor which is essential for FGF21-induced expression [68], resulting in enhanced expression of this hepatokine.

ER stress plays a crucial role in both the development of liver steatosis and progression to NASH. ER stress occurs to restore homeostasis when the cell is stressed due to the accumulation of unfolded or misfolded proteins. However, if this adaptive response fails to restore homeostasis, sustained ER stress may have pathological consequences, including lipid accumulation, inflammation, insulin resistance, and apoptosis; all of which are involved in the pathogenesis of NAFLD [69]. It has been reported that GW501516 corrects hepatic steatosis induced by a high-fat cholesterol-containing diet, and this was accompanied by a reduction in the expression of several ER stress markers [44]. More recently, it has been demonstrated that treatment of mice fed a HFD with the PPARβ/δ agonist GW0742 prevented excessive weight gain and glucose intolerance, reduced hepatic inflammation and favored hepatic β-oxidation over lipogenesis [70]. Of note, these changes were associated with an improvement in ER ultrastructure and reduced ER stress and apoptosis in liver of mice treated with the PPARβ/δ agonist. According to the authors, PPARβ/δ activation attenuated ER stress by improving insulin sensitivity and by favoring hepatic β-oxidation over lipogenesis [70].

3.2. PPARβ/δ, lipoprotein metabolism and NAFLD

Lipoprotein metabolism is an integral part of lipid homeostasis and is involved in the pathogenesis and treatment of NAFLD. In fact, dyslipidemia, a constellation of abnormalities in plasma lipoproteins, is linked to NAFLD. In addition, alterations in the assembly and secretion of lipoproteins in liver may contribute to NAFLD [71]. Very often, NAFLD patients show atherogenic dyslipidemia, characterized by the presence of high levels of TG, small-dense low-density lipoprotein (LDL), and low levels of high-density lipoprotein (HDL) cholesterol. This atherogenic dyslipidemia is a major risk factor for cardiovascular disease, the leading cause of morbidity and mortality in NASH patients.

PPARβ/δ agonists cause a significant reduction in plasma TG levels. Part of the hypotriglyceridemic effect of PPARβ/δ agonists might be the result of their effects on the expression levels of several genes involved in lipoprotein metabolism (*VldlR*, *ApoAV*, *ApoAIV*, and *ApoCI*) [31,72]. This is consistent with the increase in serum TG levels observed in PPARβ/δ-null mice fed a HFD due to hepatic VLDL overproduction.

Alterations in hepatic cholesterol homeostasis and liver free cholesterol accumulation may also contribute to the development of NAFLD. Hepatic free cholesterol accumulation in NAFLD results from disturbances of intracellular cholesterol transport, activation of cholesterol biosynthetic pathways, increased cholesterol de-esterification, and attenuation of cholesterol export [73]. PPARβ/δ agonists raise plasma HDL-cholesterol levels and decrease LDL-cholesterol [74]. The increase in plasma HDL-cholesterol has been linked to the upregulation in the hepatic expression of the two major HDL apolipoproteins: ApoAI and ApoAII [74]. The reduction in plasma LDL-cholesterol levels by PPARβ/δ agonists has been attributed to the reduction in the intestinal abundance of Niemann-Pick C1-like 1 (NPC1L1), a protein implicated in cholesterol absorption [75]. In addition, activation of PPARβ/δ stimulates fecal cholesterol excretion in mice, through an increase in transintestinal cholesterol efflux [76].

3.3. PPARβ/δ in the progression from NAFL to NASH

The progression from NAFL to NASH involves the development of inflammation and signs of hepatocellular damage [77]. PPARβ/δ ligands reduce the hepatic expression of inflammatory genes [44,78,79] and attenuate hepatic ER stress [44]; a process that contributes to the activation of inflammatory pathways. Interestingly, PPARβ/δ promotes alternative activation of Kupffer cells, macrophages resident in the liver, towards the anti-inflammatory M2 phenotype and preserves hepatic function in the presence of IL-4 stimulation (Fig. 2). In contrast, Kupffer cells deficient in PPARβ/δ show a marked impairment of alternative activation, resulting in hepatic dysfunction and insulin resistance [80]. A recent study confirmed that PPARβ/δ activation in different animal models of NASH attenuates hepatic inflammation and macrophage activation [81]. In addition, the PPARβ/δ activator GW501516 prevents IL-6-induced activation of signal transducer and activator of transcription 3 (STAT3), by inhibiting extracellular-related kinase 1/2 (ERK1/2)

phosphorylation and restoring the reduction in phospho-AMPK levels [82]. More recently, the inhibitory effect of PPARβ/δ on STAT3 was confirmed, with the report of a new mechanism involving T-cell protein tyrosine phosphatase 45 (TCPTP45) isoform [83]. According to this study, short-term PPARβ/δ activation in hepatic cells prevents IL-6-induced insulin resistance due to a nuclear protein-protein interaction between PPARβ/δ and TCPTP45 that retains the latter in the nucleus, thereby deactivating the STAT3-(suppressor of cytokine signaling 3) SOCS3 signaling pathway [83].

The dual PPARα and β/δ agonist elafibranor, initially indicated for dyslipidemia and type 2 diabetes mellitus, also shows protective effects on steatosis, inflammation and fibrosis in different animal models of NAFLD/NASH and liver fibrosis [84]. In PPARα knockout mice, elafibranor also prevented Western diet-induced liver steatosis and inflammation, suggesting that these actions might be attributed to PPARβ/δ activation. More recently, it has been reported that seladelpar (MBX-8025), a potent selective PPARβ/δ agonist (EC_{50} = 2 nM), improves insulin sensitivity and reverses dyslipidemia and hepatic storage of lipotoxic lipids, thereby improving NASH pathology in atherogenic diet-fed obese diabetic mice [85].

Chronic liver inflammation leads to fibrosis, which is accompanied by persistent alterations in the mechanical environment as a consequence of pathological matrix deposition and stiffening. The effects of PPARβ/δ agonists on liver fibrosis are inconsistent. Thus, it has been reported that GW501516 stimulates hepatic stellate cells (HSC) proliferation which causes enhanced fibrotic and inflammatory responses. The mechanism responsible for this effect is the increase in the phosphorylation of p38 and c-Jun N-terminal kinases through the phosphoinositide-3 kinase/protein kinase-C alpha/beta mixed-lineage kinase-3 pathway [86]. By contrast, the PPARβ/δ agonist KD-3010, but not GW501516, mitigates liver injury induced by carbon tetrachloride (CCl_4) injections [25]. The hepatoprotective and antifibrotic effect of KD-3010 was also observed in a model of cholestasis-induced liver injury and fibrosis using bile duct ligation. KD-3010, but not GW501516, preserved primary hepatocytes from CCl_4-induced cell death, in part by reducing reactive oxygen species production [25].

Hepatic NOD-like receptor protein 3 (NLRP3) inflammasome activation, which promotes caspase-1-dependent interleukin (IL)-1β production, is present in patients with NASH, and evidence from genetic mouse models indicates that activation of the inflammasome plays a key role in NAFLD progression [87]. Notably, PPARβ/δ agonists reduce fatty acid-induced inflammation and liver steatosis by attenuating inflammasome activation [88].

4. PPARβ/δ as a therapeutic target for treating NAFLD in humans

Despite the significant continuously increasing prevalence of NAFLD and of its aggressive form NASH, there are no approved drugs specifically tailored to treat these conditions. Therefore, there is a need for novel therapeutic approaches based on the mechanisms involved in the development of NAFLD, to halt disease progression to advanced fibrosis or cirrhosis and cancer.

Administration of PPARβ/δ agonists to patients suffering NAFLD has demonstrated that these compounds alleviate fatty liver and multiple abnormalities associated with the metabolic syndrome [89–91]. They show multiple functions in the liver and other organs that contribute to ameliorate NAFLD, including increased fatty acid oxidation, improved insulin sensitivity, reduced glucose production, decreased macrophage activation and inflammation and antifibrotic effects.

Despite no PPARβ/δ activator having been approved for human use, several of these compounds that target NAFLD, NASH, and associated diseases are under clinical development, at different stages.

Thus, KD-3010 (Kalypsys) is being considered as a therapeutic agent for patients with chronic liver diseases, and it is presently being

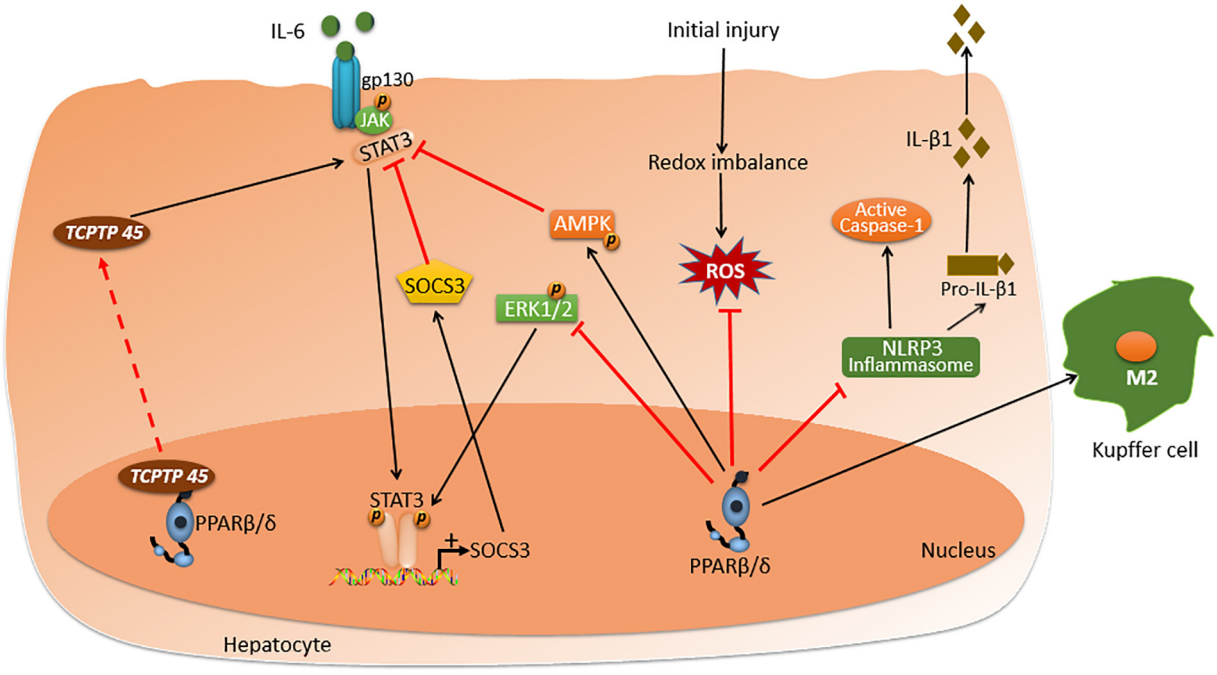

Fig. 2. Potential effects of PPARβ/δ on NASH. PPARβ/δ activation alleviates chronic liver inflammation through its involvement in several mechanisms: 1. Decreasing proinflammatory cell recruitment by attenuating NLRP3. 2. Reducing ROS generation, which decreases liver injury. 3. Preventing IL-6-induced insulin resistance by increasing phospho-AMPK and reducing phospho-ERK1/2 levels. IL-6 signals through glycoprotein gp130, which activates JAKs, with subsequent phosphorylation and activation of STAT3. Once in the nucleus, STAT3 is also phosphorylated by ERK1/2. STAT3 promotes insulin resistance in liver by increasing the expression of SOCS3, a negative regulator of cytokine signaling. 4. PPARβ/δ forms a complex with nuclear TCPTP45 and retains the latter in the nucleus, thereby deactivating the STAT3-SOCS3 signaling pathway. 5. Switching Kupffer cells towards an anti-inflammatory M2 phenotype, thereby preserving hepatic function and insulin sensitivity. Abbreviations: AMPK, AMP-activated protein kinase; ERK1/2, extracellular-related kinase 1/2; IL, interleukin; JAK, Janus Tyrosine Kinases; NLRP3, NOD-like receptor protein 3; PPARβ/δ, peroxisome proliferator-activated receptor β/δ; ROS, reactive oxygen species; RXR, retinoid X receptor; SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; TCPTP45, T-cell protein tyrosine phosphatase 45.

evaluated for liver fibrosis in clinical trials [25]. Likewise, administration of seladelpar to overweight men and women with mixed dyslipidemia reduced liver enzyme levels, inflammation marker levels, insulin resistance, and atherogenic dyslipidemia. It also decreased levels of LDL-cholesterol, non-HDL-cholesterol, Apo-B100, high-sensitivity C-reactive protein (hs-CRP), and hepatic TGs [92]. However, although seladelpar improved NASH in animal models (85), it has been recently reported that seladelpar failed to reduce liver fat as quantified by magnetic resonance imaging in a phase 2 trial (NCT03551522).

The beneficial effects of elafibranor on the levels of liver enzymes in patients with combined dyslipidemia and/or type 2 diabetes mellitus [93,94] prompted to study its efficacy in patients with NASH. In a phase 2 clinical trial (GOLDEN-505), elafibranor administration (120 mg/day) for one year reached the primary outcome, reversal of NASH without worsening of fibrosis for patients with NAFLD Activity Score (NAS) ≥ 4 [95]. Compared to placebo, elafibranor administration (120 mg/day) significantly ameliorated liver enzymes, lipids and glucose, hepatocyte ballooning and lobular inflammation, with a trend towards improvement in steatosis, but failed to demonstrate any significant effect on fibrosis [95]. Subgroup analysis of GOLDEN-505 demonstrated that elafibranor significantly improved glycated hemoglobin (Hb1Ac) and HOMA-IR (Homeostatic Model Assessment for Insulin Resistance) in 40% of NASH patients with type 2 diabetes mellitus [95]. Currently, elafibranor is being assessed in a phase 3 clinical trial (RESOLVE-IT) (NCT02704403), with a goal to enroll 2000 patients with NAS ≥4 and stage 1–3. The estimated primary completion date is in December 2021.

To date no single compound has been able to control all the aspects of NAFLD (steatohepatitis and fibrosis), suggesting that the best treatment for a complex disease like this may be the use of compounds activating several targets or the combination of drugs targeting different mechanistic pathways [96]. Recently, it has been reported that the use of a pan PPAR agonist, activating PPARα, β/δ and γ, counteracts inflammation and NASH progression more potently than selective PPAR agonists for PPARα, β/δ or γ [81]. In this study, PPARβ/δ activation mainly reduced liver macrophage infiltration. However, the main challenge of this pharmacological strategy in the treatment of NAFLD is the increased risk of side effects.

Overall, these findings indicate that activating PPARβ/δ represents a good strategy through which to prevent fatty liver and the development of NASH. However, drugs activating only PPARβ/δ are unlikely to resolve NASH, since no single agent is likely to control all aspects of this complex liver disease.

5. The long and winding road in the development of drugs against NASH

There are many pharmacotherapies emerging for the treatment of NASH that predominantly target metabolic pathways (*e.g.* PPAR and farnesoid X receptor agonists), immune-related mechanisms (*e.g.* inhibition of C—C chemokine receptors type 2/5, CCR2/5), apoptosis (*e.g.* pan-caspase inhibitors) and fibrosis (*e.g.* galectin-3 antagonist). However, demonstrating the efficacy of new drugs for NASH in clinical studies has been challenging, and findings from animal models could not fully be reproduced in clinical trials. This has also been the case for PPARβ/δ agonists. Several factors can contribute to explain why promising preclinical drugs have not performed as expected once tested in humans. Some of these factors are common for all drugs in development [97], whereas others are specific for PPARβ/δ agonists. Among the former, differences in disease initiating mechanisms between mice and humans and a high heterogenicity in NASH patients has been reported [98]. In addition, many pathways contribute to NASH and, as mentioned above, targeting only one of these pathways might result in a modest effect or it might be attenuated by the activation of compensatory mechanisms. For instance, cenicriviroc, an inhibitor of CCR2/5 receptors, failed to improve necroinflammation in human trials [99], likely as the result of redundancy of the pathways activated by these receptors and the presence of alternate ligands [100]. This supports the use of drug combinations for the treatment of NASH to attain a robust effect and to avoid the risk of activation of compensatory mechanisms.

In the case of PPARβ/δ agonists additional factors might also contribute. Gene expression is differentially regulated by PPARs in human *versus* rodent cells [101]. In addition, PPARs are known to be expressed at lower levels in human than in rodent cells [102], and the presence of marked steatosis can reduce the expression and activity of PPARβ/δ [103]. Likewise, the reduction in the expression of human PPARβ/δ in liver can upregulate the expression of the VLDL receptor [103], which is involved in hepatic steatosis development and might attenuate the effects of PPARβ/δ agonists.

Although animal models are crucial to understand disease mechanisms and for offering a systemic perspective on organ crosstalk, their limitations have led to a growing interest in human *in vitro* and *ex vivo* liver models. For instance, *in vitro* methodologies have successfully reproduced molecular and cellular features of human NASH and it has been demonstrated to be a sensitive testing tool for the investigation of novel drugs for its treatment [104]. Recently, Boeckmans et al. [105] have reproduced NASH features *in vitro* by exposing primary human hepatocytes and several human cell lines to multiple stimuli that play a role in the onset of NASH. This human *in vitro* approach was used to evaluate the effects of several PPAR agonists. The findings obtained were used to generate a scoring system to grade the potency against NASH, showing that elafibranor had the strongest effect among all the PPAR agonists tested against NASH. Remarkably, the findings obtained were in line with the clinical data, indicating the importance of this approach in the development of drugs for the treatment of NASH. The combination of *in vivo* and human *in vitro* models might increase the chances of clinical efficacy for new drugs, including PPARβ/δ agonists, in the race for developing drugs for the treatment of NASH.

In addition to efficacy, the other key aspect in the development of any drug is safety. Despite the promising effects of PPARβ/δ agonists in the treatment of NAFLD/NASH, the discovery that GW501516 administration to mice resulted in the development of adenocarcinoma wrecked the development of this compound [106]. However, since then, subsequent studies have reported conflicting data showing that this receptor both inhibits and promotes tumorigenesis [107], the latter being mostly observed in *in vivo* studies. The potential effects of PPARβ/δ agonists on carcinogenesis in humans might be prevented by the lower expression of PPARs and its different regulation in human compared to rodent cells [102,103], as it has been observed for PPARα agonists. Thus, synthetic PPARα agonists induce carcinogenesis in rodents, whereas no cancer incidence has been reported in humans treated with fibrates, PPARα agonists used for treating hypertriglyceridemia [108].

Although the safety of PPARβ/δ agonists was initially evaluated in a scarce number of clinical trials for very short periods of time, elafibranor development has allowed to evaluate the effect of activating PPARβ/δ in a higher number of patients for longer periods. The GOLDEN-505 study evaluated the safety of elafibranor for 52 weeks in 274 patients with NASH [95]. Elafibranor was well tolerated and only a recurrent elevation in serum creatinine levels was observed, that was associated to the activation of PPARα, since it has been previously reported for fibrates [109]. Completion of RESOLVE-IT should confirm the safety of elafibranor in a higher number of patients.

6. Conclusions

Findings involving PPARβ/δ ligands in preclinical animal models and in humans show promise for the use of these compounds in the prevention and treatment of NAFLD. In general, PPARβ/δ activation mitigates hepatic TG accumulation and protects the liver against aberrations caused by lipotoxicity in NAFLD and other related metabolic disorders. However, more studies are necessary to completely characterize the effects of these drugs, paying special attention to their side effects caused

by chronic administration. In addition, due to the complexity of the pathophysiology of NASH, drugs activating only PPARβ/δ or another single target, have not achieved sufficient efficacy [110]. Treatment of this disease will require the engagement of several targets to improve the efficacy attained so far with monotherapy. This combination therapy will have to face new challenges, including the risk of increased side effects.

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CRediT authorship contribution statement

MZ & DAR: Investigation, Writing - original draft. XP: Writing - review & editing. MVC: Conceptualization, Investigation, Writing – original draft, Review & editing, Coordination.

Declaration of competing interest

The authors have no conflict of interest to declare.

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REVIEW ARTICLE

Uncovering the role of apolipoprotein C-III in insulin resistance

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KEYWORDS
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Abstract Apolipoprotein C-III (apoC-III) is a small protein that is predominantly synthesized in the liver and mainly resides at the surface of triglyceride-rich lipoproteins. Its expression is upregulated by glucose and reduced by insulin, with enhanced apoC-III promoting hypertriglyceridemia and inflammation in vascular cells. The protein is also elevated in patients with diabetes, suggesting that enhanced apoC-III levels might contribute to the development of type 2 diabetes mellitus. The present review focuses on the key mechanisms by which apoC-III could promote type 2 diabetes mellitus, including exacerbation of insulin resistance in skeletal muscle, activation of β-cell apoptosis, promotion of weight gain through its effects on white adipose tissue and hypothalamus, and attenuation of the beneficial effects of high-density lipoproteins on glucose metabolism. Therapeutic strategies aimed at reducing apoC-III levels may not only reduce hypertriglyceridemia but also might improve insulin resistance, thus delaying the development of type 2 diabetes mellitus.

Abbreviations: ApoC-III, apolipoprotein C-III; DM, diabetes mellitus; FOXO1, forkhead box protein O1; HDL, high-density lipoproteins; LDL, low-density lipoproteins; LPL, lipoprotein lipase; NAFLD, non-alcoholic fatty liver disease; NF-κB, nuclear factor κB; PGC-1β, PPARγ coactivator-1β; PPAR, peroxisome proliferator-activated receptor; TLR, toll-like receptor; TRL, triglyceride-rich lipoproteins; VLDL, very low-density lipoproteins.

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PALABRAS CLAVE
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Descubriendo el papel de la apolipoproteína C-III en la resistencia a la insulina

Resumen La apolipoproteína C-III (apoC-III) es una pequeña proteína predominantemente sintetizada en el hígado y que se encuentra principalmente en la superficie de las lipoproteínas ricas en triglicéridos. Su expresión es aumentada por la glucosa y reducida por la insulina, y sus niveles elevados promueven la hipertrigliceridemia, así como la inflamación en células vasculares. Esta proteína también se encuentra elevada en los pacientes diabéticos, lo que sugiere que el aumento de esta apoproteína podría contribuir al desarrollo de la diabetes mellitus de tipo 2. Esta revisión aborda los mecanismos clave por los que la apoC-III podría promover la diabetes mellitus tipo 2, entre los que se encuentran la exacerbación de la resistencia a la insulina en el músculo esquelético, la activación de la apoptosis en la célula β, la promoción del aumento de peso por sus efectos sobre el tejido adiposo blanco y el hipotálamo, y la atenuación de los efectos beneficiosos de las lipoproteínas de alta densidad sobre el metabolismo de la glucosa. Las estrategias terapéuticas dirigidas a disminuir los niveles de apoC-III no sólo podrían reducir la hipertrigliceridemia, sino también mejorar la resistencia a la insulina y retrasar el desarrollo de la diabetes mellitus de tipo 2.

Introduction

Common to insulin resistant states in patients with obesity and type 2 diabetes mellitus (DM) is the presence of atherogenic dyslipidemia, which is known to amplify cardiovascular risk.[1,2] Atherogenic dyslipidemia is characterized by elevated triglyceride levels, reduced high-density lipoprotein (HDL) cholesterol levels, and the appearance of small dense low-density lipoprotein (LDL).[1,2] Interestingly, atherogenic dyslipidemia usually precedes a diagnosis of type 2 DM by several years, suggesting that lipoprotein abnormalities are an early event in the disease's development.[3] In insulin resistant states, atherogenic dyslipidemia is initiated by an overproduction of triglyceride-enriched very low-density lipoproteins (VLDLs) that triggers the observed sequence of lipoprotein changes.[1,2]

In addition to triglycerides, VLDLs also contain apolipoproteins, of which apolipoprotein C-III (apoC-III) is one of the most abundant.[4] It is well-known that the increase in apoC-III contributes to elevated triglyceride levels[5,6] and cardiovascular risk.[7,8] In fact, individuals with loss-of-function mutations in the *APOC3* gene present lower triglyceride levels and a reduced risk of cardiovascular disease.[9,10] ApoC-III promotes atherosclerotic plaque formation not only through its indirect effects on triglyceride levels but also through its direct pro-inflammatory effects on vascular cells.[11] However, the effects of apoC-III on insulin resistance are less well-known.

Plasma apoC-III levels are elevated in patients with DM,[4] which in turn, are associated with insulin resistance.[12] This suggests that the increase in apoC-III during the development of atherogenic dyslipidemia might exacerbate insulin resistance, thereby accelerating the development of type 2 DM. Consistent with this, mice overexpressing apoC-III have been shown to be more prone to insulin resistance and DM induced by high-fat diet (HFD).[13,14]

In this manuscript, we aim to review the possible mechanisms that lead to apoC-III-induced insulin resistance.

ApoC-III: features and regulation

ApoC-III, which is encoded by the gene *APOC3*, is a major component of triglyceride-rich lipoproteins (TRL) (chylomicrons and VLDL) that is also present in LDL and HDL. Predominantly synthesized in the liver, and to a lesser extent in the intestine,[15] its hepatic expression is negatively regulated by insulin through forkhead box protein O1 (FOXO1).[16] This transcription factor binds to a consensus site in the *APOC3* promoter and upregulates its expression. Insulin then phosphorylates and prevents FOXO1 translocation to the nucleus, thereby downregulating apoC-III expression. In animal models of insulin deficiency or resistance, a reduction in the effects of this hormone leads to unrestrained apoC-III production that provides potential mechanism for increased apoC-III levels, hypertriglyceridemia, and atherogenic dyslipidemia in diabetic patients. By contrast, glucose upregulates apoC-III expression in primary rat hepatocytes and in immortalized human hepatocytes *via* the transcription factors carbohydrate response element-binding protein (ChREBP) and hepatocyte nuclear factor-4α.[17] This regulation of apoC-III by glucose may help to explain the link between hyperglycemia, hypertriglyceridemia, and cardiovascular disease in patients with type 2 DM. In addition, because peroxisome proliferator-activated receptor (PPAR)α activation by fibrates reduces the expression of *APOC3* by decreasing hepatocyte nuclear factor-4α levels and promoting the displacement of this transcription factor from the *APOC3* promoter,[18] this mechanism provides an explanation for the reduction in plasma apoC-III levels with fibrate treatment. Farnesoid X receptor agonists also reduce hepatic *APOC3* expression, an effect that may contribute to the hypotriglyceridemic effect of these compounds.[19]

Finally, the increase in plasma saturated fatty acids levels might result in hypertriglyceridemia by upregulating apoC-III expression *via* the PPARγ coactivator-1β (PGC-1β).[20]

ApoC-III exists as three isoforms that differ in terms of glycosylation: apoC-III_0 (no sialic acid bound to the protein), apoC-III_1 (one sialic acid residue), and apoC-III_2 (two sialic acid residues). Each isoform contributes approximately 10, 55 and 35%, respectively, of the total circulating apoC-III levels.[21] ApoC-III glycosylation seems to be under metabolic control since a reduction in the apoC-III_1 to apoC-III_2 ratio has been reported after weight loss by caloric restriction,[22] whereas an increase in apoC-III_0 has been observed in combined hyperlipidemia.[23]

ApoC-III in cardiovascular disease

ApoC-III is considered an independent risk factor for cardiovascular disease because of its close association with hypertriglyceridemia and inflammation in vascular cells.[6] The effects of apoC-III on triglyceride levels can be explained by several mechanisms. First, it is a potent inhibitor of lipoprotein lipase (LPL), a key enzyme for the lipolysis of triglyceride from VLDL and chylomicrons.[24] Second, it favors hypertriglyceridemia by attenuating the apoE-mediated hepatic uptake of TRL remnants by LDL receptors and LDL receptor-related protein 1.[25–27] This indicates that apoC-III modulates triglyceride levels through LPL-dependent and LPL-independent mechanisms. More recent studies have shown that lowering apoC-III levels in the absence of apoE did not improve TRL clearance, but that it significantly decreased serum triglyceride levels.[28] The hypotriglyceridemic effect in the absence of apoE resulted from improved LPL activity in white adipose tissue, rather than in all LPL-target tissues. Moreover, apoC-III stimulates the assembly and secretion of VLDL.[29,30] Finally, *in vitro* studies using apoC-III complexes with higher apoC-III_2/apoC-III_1 ratios show attenuated inhibition of VLDL uptake by hepatic cells and LPL-mediated lipolysis, providing possible functional explanations for the inverse association between a higher apoC-III_2/apoC-III_1 ratio and hypertriglyceridemia and cardiovascular risk.[31]

The above mechanisms mean that elevated levels of apoC-III result in hypertriglyceridemia. Consistent with this, volanesorsen, a second-generation antisense oligonucleotide that inhibits apoC-III synthesis by coupling to the *APOC3* messenger RNA, reduces serum triglycerides levels by 68% and increases HDL cholesterol levels by 40% in patients with DM, familial chylomicronemia syndrome, or with moderately high triglyceride levels.[32] Genomic studies have also demonstrated the influence of apoC-III on cardiovascular risk. The SstI polymorphism, that results in increased apoC-III levels, carries a higher risk of hypertriglyceridemia and cardiovascular disease.[33] By contrast, loss-of-function mutations in the *APOC3* gene have been shown to reduce non-fasting triglyceride levels by 44% and the incidence of ischemic heart disease by 36%.[9]

Regarding inflammation in vascular cells, apoC-III-rich lipoproteins or apoC-III itself can activate monocytes and stimulate their adhesion to vascular endothelial cells, thereby promoting atherosclerosis.[7,8] Moreover, apoC-III upregulates the expression of vascular cell adhesion molecule-1 in endothelial cells to increase monocyte adhesion.[7] These cells later differentiate into tissue macrophages, leading to inflammation, transformation in foam cells, and atherogenesis. Notably, the stimulation of inflammatory pathways can exacerbate the increase in apoC-III levels because activation of the pro-inflammatory transcription factor nuclear factor κB (NF-κB) upregulates the expression of apoC-III.[34]

ApoC-III can also affect a critical step in the pathogenesis of atherosclerosis, the deposition and retention of lipoproteins by vascular extracellular matrix molecules, particularly proteoglycans.[35] Although, apoC-III does not bind directly to proteoglycans, it has been reported that increases in apoC-III content alters the lipid composition in LDL from diabetic patients with a high endogenous apoC-III/apoB molar ratio, which allows apoB to acquire a conformation that is more favorable for proteoglycan binding.[11]

The marked heterogenicity in apolipoprotein content in HDLs affects their function. ApoC-III is present in 4% of HDL in subjects of normal weight, but it increases to 10% in subjects who are obese.[36] This may be responsible for attenuating some of the beneficial effects of HDL, because it is known that there is a higher risk of cardiovascular disease than when compared to HDL that lacks apoC-III.[37] Similarly, HDLs from patients with coronary artery disease have been shown to stimulate endothelial proapoptotic pathways because of the higher content of apoC-III; by contrast, HDL with less apoC-III has been shown to inhibit apoptosis.[38] These findings indicate that HDL containing apoC-III may be dysfunctional.

ApoC-III and insulin resistance

Insulin resistance and β-cell failure are the two major pathophysiologic abnormalities that contribute to the development of type 2 DM. Insulin resistance precedes and predicts the development of type 2 DM and is initiated when overnutrition results in elevated levels of glucose and/or lipids in the serum, inducing low-grade inflammation and activating various transcriptional and metabolic pathways. This results in the induction of various pro-inflammatory mediators that provoke the pathogenesis of tissue-specific insulin resistance by attenuating insulin signaling pathways.[39] Once insulin resistance develops, it promotes β-cell failure, and impaired insulin secretion, ultimately resulting in overt hyperglycemia.

Skeletal muscle insulin resistance is the primary defect that precedes β-cell failure and overt hyperglycemia by decades.[40] In fact, the primary site of insulin-stimulated glucose disposal is skeletal muscle, accounting for up to 90% of glucose clearance.[41] Consequently, loss of skeletal muscle insulin sensitivity is critical to the pathogenesis of type 2 DM.[42] Although the mechanisms involved in the development of insulin resistance are currently unclear, accumulating evidence points to the presence of a chronic low-level inflammatory process.[39] Endoplasmic reticulum (ER) stress[43] and toll-like receptors (TLRs)[44] have been implicated in the activation of pro-inflammatory kinases, such as the inhibitor of κB (IκB) kinase β (IKK-β) in the NF-κB pathway. Together with other kinases, this phosphorylates the insulin receptor substrate 1 (IRS-1) in serine residues and attenuate

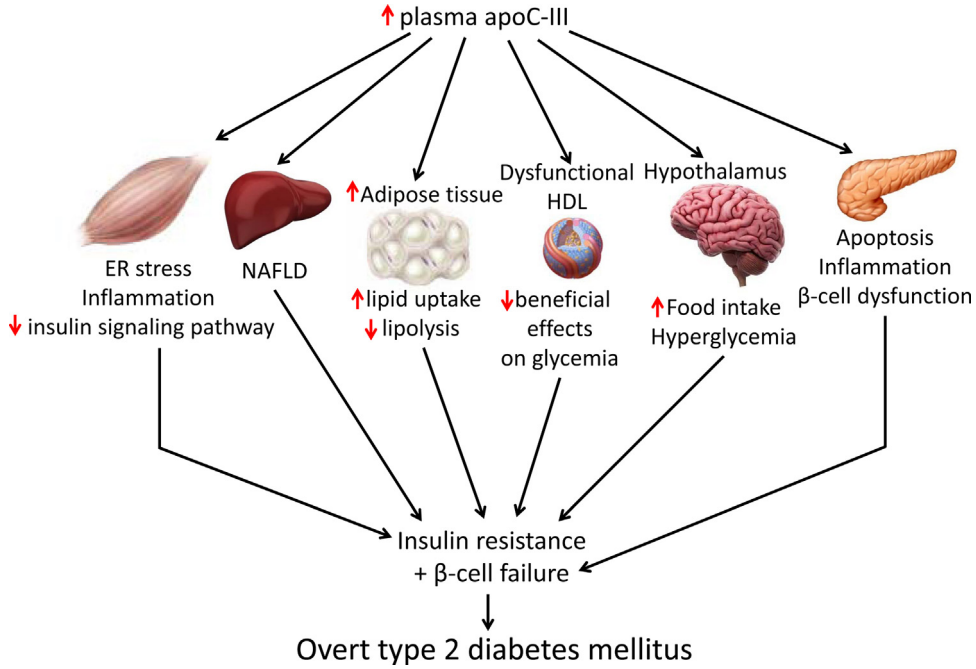

Figure 1 Insulin resistance mechanisms induced by apoC-III. ApoC-III, apolipoprotein C-III; ER, endoplasmic reticulum; HDL, high-density lipoprotein; NAFLD, non-alcoholic fatty liver disease.

the insulin signaling pathway. Likewise, pro-inflammatory pathways upregulate the expression of multiple inflammatory mediators that also facilitate insulin resistance.[39] We have previously reported that skeletal muscle from transgenic mice overexpressing apoC-III show increased levels of ER stress and inflammatory markers (Fig. 1).[45] Activation of ER stress by apoC-III in myotubes stimulated the NF-κB pathway, attenuated insulin signaling by increasing the phosphorylation of IRS-1 in serine residues, and upregulated the expression levels of inflammatory mediators, including interleukin 6, tumor necrosis factor α, and monocyte chemoattractant protein-1. Remarkably, these effects were prevented by incubation with a neutralizing antibody against TLR-2, indicating that some of the effects of apoC-III might be mediated by this receptor. Given that apoC-III is the most abundant apolipoprotein in the VLDL of people with DM,[4] these results suggest that their elevated VLDL may affect skeletal muscle and thereby exacerbate insulin resistance.

Non-alcoholic fatty liver disease (NAFLD) is the principal manifestation of liver disease in insulin resistant states such as obesity and metabolic syndrome. There are conflicting results for the effects of apoC-III in NAFLD. Transgenic mice overexpressing apoC-III fed a standard diet showed NAFLD-like features compared with non-transgenic control littermates, with these changes being exacerbated by feeding a HFD.[46] Treatment with the PPARα activator fenofibrate reversed several of the effects caused by elevated apoC-III levels but failed to normalize inflammatory markers even when the increase in liver lipid accumulation was completely abolished.[44] However, in a study where transgenic mice overexpressing apoC-III were fed a HFD for up to 10 months, the authors concluded that apoC-III was not a predisposing factor for linking overnutrition to NAFLD in obesity.[47] In humans, carriers of *APOC3* variant alleles develop increased plasma levels of apoC-III that is associated with NAFLD and insulin resistance.[12,48] By contrast, several studies show no causal relationship between *APOC3* variants linked to hypertriglyceridemia and the hepatic triglyceride content[49] or linking *APOC3* promoter region polymorphisms to liver damage (severity of steatosis, non-alcoholic steatohepatitis, and moderate/severe fibrosis).[50]

Transgenic mice overexpressing apoC-III have also been used to confirm the role of this apolipoprotein in obesity. Diet-induced obesity was exacerbated in these mice due to the increased availability of free fatty acids from postprandial TRLs, the greater adipose capacity for lipid uptake, and the reduced lipolysis in adipose tissue.[51]

ApoC-III also links insulin resistance and β-cell failure in type 2 DM. Local insulin resistance in β-cells reduces FOXO1 nuclear exclusion and increases apoC-III levels. The increase in islet apoC-III then causes apoptosis by increasing cytoplasmic free Ca^{2+} concentrations and promoting a local inflammatory milieu.[52] Supporting this, antisense oligonucleotide treatment decreased *in vivo* apoC-III and improved glucose tolerance. ApoC-III knockout islets transplanted into diabetic mice with high systemic levels of apoC-III have also been shown to induce normal cytoplasmic free Ca^{2+} concentrations in the absence of inflammation. In the setting of islet insulin resistance, these findings indicate that locally produced apoC-III impairs β-cell function and contributes to the development of type 2 DM. In type 1 diabetic patients, increased activity of voltage-gated L-type Ca^{2+}-channels in β-cells results in increased cytoplasmic free Ca^{2+}, leading to β-cell apoptosis. Remarkably, it has been reported that in these patients apoC-III stimulates the activity of L-type voltage-gated Ca^{2+}-channels in insulin-producing cells, ultimately leading to an increase in cytoplasmic free Ca^{2+} and β-cell apoptosis.[53]

Research indicates that HDL ameliorates glucose metabolism by modulating insulin secretion by pancreatic β-cells,[54–56] and insulin-independent glucose uptake by skeletal muscle,[56,57] as well as by inhibiting inflammation.[58] Although results conflict, the presence of apoC-III in HDL may attenuate the beneficial effects of these lipoproteins on glucose metabolism. Thus, dysfunctional HDL containing apoC-III was reported to be a major independent predictor of new-onset type 2 DM.[59] A more recent study confirmed that the presence of apoC-III on HDL also diminished the protective association of HDL with incident DM.[60] In another study, however, HDL without apoC-III was inversely associated with risk of DM, but no such association was shown for HDL with apoC-III.[61]

ApoC-III might also affect food intake by inhibiting LPL in the hypothalamus. This lipase promotes fatty acid uptake in this brain tissue, ultimately downregulating orexigenic neuropeptide expression, which in turn, reduces food intake and inhibits glucose production.[62,63] ApoC-III is expressed in the hypothalamus, and its intracerebroventricular injection can suppress hypothalamic LPL activity and stimulate night-time food intake.[64] These findings suggest that increased apoC-III levels in the hypothalamus can promote food intake, leading to obesity and altered glucose metabolism.

Finally, genetic and pharmacological approaches have provided robust evidence of the involvement of apoC-III in the development of insulin resistance. Data show that subjects with a genetic mutation in the *APOC3* gene, who have life-long reduced apoC-III levels, are healthier overall and have increased insulin sensitivity.[65] Moreover, a small study showed that volanesorsen improved dyslipidemia and insulin sensitivity when used to treat patients with type 2 DM.[66] Although the cohort was small, a strong relationship was found between enhanced insulin sensitivity and both plasma apoC-III and triglyceride suppression.

Targeting apoC-III

ApoC-III has a key role in lipid metabolism and inflammation, but it can also contribute to the development of type 2 DM by promoting insulin resistance in skeletal muscle and triggering β-cell apoptosis. It also contributes to obesity through effects on white adipose tissue and hypothalamus and attenuates the beneficial effects of HDL on glucose metabolism. Therefore, lowering apoC-III reduces not only cardiovascular risk but also insulin resistance, which in turn is involved in the development of atherogenic dyslipidemia and serves as a risk factor for type 2 DM and cardiovascular disease. Several therapeutic tools are available for reducing apoC-III, including the PPARα activators fibrates, which reduce hepatic apoC-III expression,[19] or the insulin sensitizer pioglitazone, a PPARγ activator used in the treatment of type 2 DM.[67] A meta-analysis of randomized clinical trials also reported that statins can decrease apoC-III levels, helping to explain their hypotriglyceridemic action.[68] However, directly targeting *APOC3* mRNA with volanesorsen is the only treatment that induces a robust reduction in apoC-III levels.

The clinical efficacy and safety of volanesorsen as a lipid-lowering drug has recently been reported in phase 2 and phase 3 clinical studies.[32] It induced a strong reduction in serum triglyceride levels and a significant increase in HDL cholesterol. However, larger clinical trials are needed to validate the use of volanesorsen as a lipid-lowering drug for eliminating residual cardiovascular risk. Concerning safety issues, the APPROACH trial in patients with familial chylomicronemia syndrome revealed that volanesorsen caused thrombocytopenia in 76% of treated patients.[69] However, no significant differences have been reported between carriers and non-carriers of an inactivating mutation in *APOC3* in platelet count or in the prevalence of thrombocytopenia.[70] This suggests that this side effect is drug- or class-specific rather than a consequence of apoC-III inhibition.

A monoclonal antibody targeting lipoprotein-bound human apoC-III has also been developed.[71] In mice expressing human APOC3, its administration promoted circulating apoC-III clearance and enhanced TRL catabolism. To date, its effects on insulin sensitivity has not been evaluated.

Conclusions

The available evidence suggests that apoC-III is not only relevant to lipoprotein metabolism and inflammation but also to insulin resistance, with evidence that pharmacological targeting its reduction might improve this latter condition. Drugs reducing apoC-III levels or activity might be key in type 2 diabetic patients, since cardiovascular disease remains the principal cause of death among these patients. Additional research is needed to confirm the efficacy of volanesorsen in patients with type 2 DM because only a single study with a small number of patients has examined this potential new indication. Moreover, safety concerns remain given the effects of volanesorsen on the platelet count and thrombocytopenia.

Authors' contributions

All authors contributing to the writing, editing, and approval of the manuscript.

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Conflict of interest

The authors declare no competing interests.

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Review

Sirtuins: To Be or Not To Be in Diabetic Cardiomyopathy

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Diabetic cardiomyopathy is the leading cause of death among people with diabetes. Despite its severity and poor prognosis, there are currently no approved specific drugs to prevent or even treat diabetic cardiomyopathy. There is a need to understand the pathogenic mechanisms underlying the development of diabetic cardiomyopathy to design new therapeutic strategies. These mechanisms are complex and intricate and include metabolic dysregulation, inflammation, oxidative stress, fibrosis, and apoptosis. Sirtuins, a group of deacetylase enzymes, play an important role in all these processes and are, therefore, potential molecular targets for treating this disease. In this review, we discuss the role of sirtuins in the heart, focusing on their contribution to the pathogenesis of diabetic cardiomyopathy and how their modulation could be therapeutically useful.

Highlights

Diabetic cardiomyopathy (DCM) is a chronic disease characterized by metabolic dysregulation that is often accompanied by local inflammation, oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, cardiomyocyte apoptosis, and fibrosis.

No specific drugs have yet been approved for treating DCM, the leading cause of death among people with diabetes.

The pathogenetic mechanisms of DCM are characterized not only by changes in protein levels, but also by their post-translational modifications, which are of utmost importance. One such modification is lysine residue acetylation, which has a crucial role in metabolic homeostasis through the regulation of multiple cellular processes. The deacetylase group of enzymes named sirtuins are key players in controlling reversible acetylation of proteins.

Accumulating evidences suggest that sirtuins play an important role in several of the mechanisms involved in DCM development and progression. Several studies have demonstrated their efficacy in both animal models and humans with DCM and, thus, strategies aimed at modulating sirtuin activity have become of clinical interest.

Diabetic Cardiomyopathy (DCM): The Not-So-Silent Disease

DCM is defined as the occurrence of myocardial dysfunction in people with diabetes that is not directly attributable to coronary artery disease, hypertension, or valve disease. It is a chronic disease characterized by metabolic dysregulation, in which hyperglycemia and hyperinsulinemia play an indispensable pathogenic role, and that is often accompanied by local inflammation, oxidative stress, mitochondrial dysfunction, **endoplasmic reticulum (ER) stress** (see Glossary), cardiomyocyte apoptosis, and **fibrosis** [1]. A detailed description of the mechanisms underlying DCM is presented in Box 1. As a result of DCM, the heart develops left ventricular hypertrophy, contractile dysfunction, and dilated cardiomyopathy, which impair cardiac output and eventually lead to heart failure. DCM typically manifests initially with **diastolic dysfunction**, although later it may also evolve with systolic dysfunction [1]. DCM is the leading cause of death among diabetic people, although its prevalence differs among studies, ranging from 20% to 60%, regardless of whether they suffer from type 1 diabetes (T1D) or type 2 diabetes (T2D) [2]. Thus, DCM is a harmful and common condition in diabetic patients, but it displays a silent development, particularly in its earlier stages. As a result, and due to the few diagnostic tools available, DCM is frequently underdiagnosed.

Despite the severity and poor prognosis of DCM, there are currently no formal guidelines regarding its management or approved specific pharmacological drugs to treat it. Chronic diseases like diabetes are characterized not only by changes in protein levels, but also by post-translational modifications, which are of the utmost importance [3]. One such modification is lysine acetylation, which regulates a myriad of cell processes [3]. Sirtuins (SIRT) are a group of enzymes that catalyze the reversible deacetylation of proteins. Accumulating evidence suggests that they play an important role in several of the mechanisms involved in DCM. In recent years, some reviews addressing the role of sirtuins on overall cardiovascular diseases [including **cardiac hypertrophy**, ischemia/reperfusion (I/R) injury, heart failure, and atherosclerosis], metabolic syndrome, and other diabetes-related diseases have been published. However, despite DCM sharing some pathophysiological mechanisms with other

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Box 1. Mechanisms Underlying Diabetic Cardiomyopathy

Metabolic Dysregulation

Despite free FAs being the preferred energy substrate in the adult heart, cardiac cells may use alternative fuel sources, including glucose, lactate, or ketone bodies. In diabetes, due to the prevailing hyperglycemia and/or insulin resistance, there is a shift towards increased mitochondrial FA β-oxidation, at the expense of glucose, as the sole fuel source, which limits ATP production. Dysregulation of the transcriptional machinery controlled by the PPAR family of nuclear receptors is fundamental in this process. Thus, the activity of one of its target genes, the insulin-induced GLUT4 that controls the uptake of glucose in the heart, is downregulated, thereby contributing to the abovementioned substrate shift [1]. PGC-1α, a coactivator of PPARs and other transcription factor receptors (ERRα, NFE2L2, or NRF1 and NRF2), is a master regulator in controlling fuel utilization in the heart, since it regulates mitochondrial biogenesis, promotes FA oxidation, and shuts down glucose oxidation [12,16]. Regardless of the increased FA oxidation rate, intracellular lipid accretion and cardiac steatosis are hallmarks of the diabetic heart, resulting in lipotoxicity, since the accumulation of toxic lipid intermediates activates the proinflammatory transcription factor NF-κB and induces ER stress and mitochondrial dysfunction, which are all linked to cardiomyocyte apoptosis, myocardial fibrosis, and contractile dysfunction [1].

Inflammation and Fibrosis

Both elevated plasma levels of free FAs and hyperglycemia may trigger the cardiac transcriptional activity of NF-κB, thus increasing the secretion of cytokines and chemokines, which carry out numerous autocrine activities via the downstream activation of AP-1, NFAT, and NF-κB itself [30]. All of them are involved in reducing cardiac contractility and the subsequent progression to heart failure in DCM, as well as in cardiac hypertrophy [1]. Inflammation harms myocardial tissues and causes **cardiac remodeling**, which is characterized by interstitial fibrosis, in a process regulated by AP-1 and NF-κB, among others. Hyperglycemia-induced formation of AGEs in cardiomyocytes also independently contributes to NF-κB activation [109], increasing interstitial fibrosis, myocardial stiffness, impaired cardiac relaxation, and diastolic dysfunction [2].

Oxidative Stress and Apoptosis

The imbalance between glucose and FA oxidation in the heart causes the mitochondria to produce ROS, which accumulate in cardiomyocytes. The resulting oxidative stress stimulates proinflammatory transcription factors, promotes cell death, and elicits ER stress, which contribute to all stages of DCM. AGEs significantly aggravate intracellular oxidative stress. Apoptosis is hastened by hyperglycemia and ROS accumulation in the heart through the activation of MAPK, involving the proapoptotic JNK and p38 and the antiapoptotic ERK1/2.

Other Pathophysiological Mechanisms

ER stress plays an important role in determining the fate of cardiomyocytes in DCM. If it persists, the activation of the NF-κB, p38 MAPK, and JNK pathways will bring on ER stress-mediated cardiomyocyte apoptosis [10,110]. AGEs inhibit cardiac SERCA2a expression and promote ER stress in cardiomyocytes [111]. When ER stress arises, cardiomyocyte calcium handling is also altered, thus aggravating diastolic dysfunction [2]. The ensuing acute rise in the intracellular calcium concentration results in mitochondrial calcium accumulation, which leads to ROS formation and apoptosis [112].

cardiac diseases, it is regarded as a distinct disease entity. For these reasons, in this review we thoroughly discuss the role of sirtuins in the pathogenesis of DCM to better clarify how their modulation could be therapeutically useful (see Clinician's Corner).

Sirtuins: A Tale with Seven Intricate Main Characters

The sirtuin family encompasses a group of evolutionarily conserved enzymes that couple the deacetylation of both histone and non-histone lysine residues to nicotinamide adenine dinucleotide $(NAD)^+$ hydrolysis. The resulting dependence on the NAD^+/NADH ratio explains why their activity is closely associated with the energy and redox status of the cell [4]. Seven mammalian sirtuin orthologs have been described (SIRT1–7), with characteristic tissue and subcellular distributions. SIRT1, SIRT6, and SIRT7 are located in the nucleus, SIRT2 is primarily found in the cytoplasm, while SIRT3, SIRT4, and SIRT5 reside mainly in the mitochondrial matrix. However, some sirtuins may shuttle between different subcellular compartments and even display different locations depending on the cell type [5–8].

All sirtuins possess conserved NAD^+-binding and catalytic domains, but their different flanking N and C terminal regions contribute to specific differences in subcellular localization, enzymatic activity, and

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substrate specificity. SIRT1, SIRT2, and SIRT3 display deacetylase and long-chain deacylase activities, while SIRT4 exhibits ADP-ribosyltransferase, deacylase, substrate-specific deacetylase, and lipoamidase activities. SIRT5 shows strong deacylase activity, while SIRT6 presents deacetylase, ADP-ribosyltransferase, and long-chain deacylase activities. SIRT7 primarily mediates deacetylation, histone desuccinylation, and long-chain deacylation responses. The deacylation catalyzed by sirtuins includes, besides deacetylase activity, desuccinylase, demalonylase, deglutarylase, demyristoylase, and depalmitoylase activities [4,9]. Sirtuins regulate important cell functions associated with physiological as well as pathological conditions. Mitochondrial sirtuins share some functional redundancy and, together, coordinate numerous aspects of mitochondrial function, including the redox balance, metabolism homeostasis, and dynamics.

The Plot: Sirtuins Are Multitalented Proteins

SIRT1: Cardioprotection beyond Metabolism Regulation

SIRT1 is highly expressed in the heart. Protein substrates of SIRT1 include histones, transcription factors, DNA repair proteins, and factors associated with **autophagy** [10], through which it modulates cardiac metabolism, stress responses, apoptosis, DNA repair, inflammation, and mitochondrial function [11]. Several types of pathophysiological stress modulate SIRT1 expression and activity in the heart, either through the regulation of the transcription factors controlling its mRNA expression [E2F transcription factor 1 (E2F1), Forkhead box class O (FOXO)1 and FOXO3] [12] or by the direct control of its enzymatic activity via post-translational modifications (methylation, nitrosylation, phosphorylation, and sumoylation) [13].

By regulating the activity of many cytoplasmic proteins [phosphoinositide 3-kinase (PI3K)/AKT, AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor (PPAR)α, PPARγ coactivator-1α (PGC-1α), and protein tyrosine phosphatase (PTP)1B], SIRT1 ameliorates metabolism in diabetes, thereby contributing to improve DCM [1,13–15]. SIRT1 exerts a strong protective effect against oxidative stress by attenuating the production of reactive oxygen species (ROS) and, consequently, reducing cardiomyocyte apoptosis. Mitochondrial manganese-dependent superoxide dismutase (SOD2), the major ROS detoxifying enzyme, is transcriptionally upregulated by SIRT1 through deacetylation and the subsequent nuclear translocation of FOXO1, as well as through the activities of hypoxia-inducible factor (HIF)-2α and FOXO4 [16]. SIRT1 also attenuates oxidative stress by upregulating thioredoxin 1 and catalase [12]. SIRT1 prevents cardiomyocyte apoptosis by reducing caspase-3 activity and the expression of the proapoptotic protein BCL2-associated X protein (BAX) through FOXO activation [17] and through the deacetylation and inhibition of **poly (ADP-ribose) polymerase (PARP)** activity, which preserves NAD^+ levels [18]. Furthermore, SIRT1 protects cardiomyocytes via the expression of autophagy-related genes in a process that depends on the activation of FOXO1 and FOXO3 [12].

SIRT1 displays anti-inflammatory activity by promoting the binding of PPARα to the proinflammatory transcription factor nuclear factor-κB (NF-κB) [19], as well as through the deacetylation of the K310 residue of the NF-κB p65 subunit, which results in the inhibition of its transcriptional activity [20].

SIRT2: Friend or Foe in Cardiac Pathophysiology?

SIRT2 is abundantly expressed in metabolically active tissues [21], but its role in the heart is yet to be elucidated. SIRT2 attenuates apoptosis, oxidative stress damage, and inflammation, the latter probably due to K310 deacetylation and the subsequent deactivation of p65/NF-κB [22–24]. This sirtuin also protects mice from angiotensin II-induced cardiac hypertrophy and fibrosis through the deacetylation of liver kinase B1 (LKB1), which promotes AMPK activity [25], and nuclear factor in activated T cells (NFAT) [26]. In contrast, SIRT2 overexpression renders cardiomyocytes more susceptible to cell death during I/R injury [27], and genetic or pharmacological inhibition of SIRT2

Glossary

Advanced glycation end products (AGEs): a diverse group of proteins and lipids that become glycated and oxidized as a consequence of persistent exposure to hyperglycemia and are causatively associated with the complications of diabetes because they are highly oxidative.
Autophagy: a homeostatic process that involves the degradation of unnecessary or dysfunctional cytoplasmic components ranging from protein aggregates to whole organelles through the action of lysosomes. Its main objective is to recycle cell components, removing damaged mitochondria and other organelles to protect the tissue.
Cardiac hypertrophy: abnormal thickening of the heart muscle that results from the enlargement of cardiomyocytes and changes in the extracellular matrix, increasing ventricular dimensions and myocardial dysfunction. Initially, it is an adaptive response to compensate for hemodynamic stress by enhancing cardiac performance (physiological hypertrophy), but can evolve into pathological hypertrophy in conditions of chronic stress.
Cardiac remodeling: a set of molecular, cellular, and interstitial changes resulting from an imbalance between pro- and antifibrotic factors that promotes the deposition of extracellular matrix proteins. The resulting cardiac fibrosis impairs cardiomyocyte contractility and, ultimately, leads to cardiac stiffness and heart failure.
Diastolic dysfunction: this occurs when ventricles do not properly relax and their filling is impaired, thus resulting in a higher end-diastolic pressure for a given end-diastolic volume and causing blood accumulation in other parts of the body.
Endoplasmic reticulum (ER) stress: the ER organelle is responsible for protein folding and maturation. The ER found in myocytes, the so-called sarcoplasmic reticulum, stores calcium ions that are crucial regulators of the excitation–contraction coupling process. Any disturbance that alters ER function may result in the accumulation of unfolded or misfolded proteins, thus activating the unfolded protein response (UPR). The UPR aims to promote cell survival by alleviating the adverse effects of ER stress, which is attained by

in insulin-resistant myotubes has been shown to activate AKT and increase insulin-stimulated glucose uptake [21,28], suggesting that its downregulation might improve insulin sensitivity.

SIRT3: A Polyvalent Enzyme at the Crossroads of Mitochondrial Function and Cardiac Metabolism

SIRT3 exhibits robust deacetylase activity on proteins associated with the oxidative balance, fatty acid (FA) oxidation, glycolysis, amino acid metabolism, the tricarboxylic acid (TCA) cycle, the electron transport chain (ETC), and mitochondrial turnover and biogenesis [13,29]. Recent studies indicate that, at least in the heart, SIRT3 localizes in the mitochondria, the cytoplasm, and the nucleus, where it also displays enzymatic activity [30].

Homozygous SIRT3-knockout mice have been reported to exhibit a marked hyperacetylation of mitochondrial proteins, which is not observed in SIRT4- or SIRT5-knockout mice [31]. These mice display reduced rates of FA oxidation, glucose oxidation, oxygen consumption, the mitochondrial respiration rate, ATP synthesis, and the activity of oxidative phosphorylation complexes in the heart [32,33]. Unexpectedly, SIRT3-knockout mice show normal cardiac function, although this worsens after the induction of cardiac hypertrophy. The hearts of these mice display increased glycolysis, abnormal lipid accumulation, energy depletion, impaired contractile function, and fibrosis upon stress [32,34]. SIRT3 promotes the TCA cycle and the generation of ATP [35]. SIRT3 activates the first enzyme of the pyruvate dehydrogenase complex (PDC), pyruvate dehydrogenase E1α (PDHA1) [29], which catalyzes the oxidation of pyruvate into acetyl-CoA that subsequently enters the TCA cycle and regulates anaerobic glycolysis by deacetylating and activating lactate dehydrogenase A (LDHA), a key enzyme in determining the metabolic fate of pyruvate, the end-product of glycolysis [36]. In cardiomyocytes, SIRT3 promotes AMPK activity by LKB1 deacetylation, thus inhibiting the phosphorylation of glycogen synthase kinase (GSK)3β and upregulating glucose transporter 4 (GLUT4) [37].

SIRT3 deacetylates FOXO3, subsequently increasing the expression of the antioxidant enzymes SOD2 and catalase in the heart [33]. Indeed, it is capable of directly deacetylating several lysine residues of SOD2, thereby increasing its activity [38]. SIRT3 indirectly reduces ROS production by increasing the efficiency of the ETC and promoting the TCA cycle, which increases NADPH production in the mitochondria [29]. NADPH is necessary to form reduced glutathione, an essential cofactor for mitochondrial glutathione peroxidase in the scavenging of ROS [16]. In the heart, SIRT3 deacetylates cyclophilin D [12], which inhibits the opening of the mitochondrial permeability transition pore (mPTP), thereby reducing ATP depletion and the release of proapoptotic factors from the mitochondria, subsequently preventing cardiomyocyte cell death [13,16].

Overall, the data suggest that SIRT3 acts as a redox-sensitive rheostat that is required for preserving oxidative metabolism and increasing energy production (ATP synthesis) in the mitochondria for the maintenance of proper function in the heart. SIRT3 can also have important cardioprotective effects through mitochondrial ROS detoxification and carrying out anti-inflammatory, antifibrotic, and antiapoptotic actions [39–41]. These actions may result in the inhibition of prohypertrophic transcription factors, GATA-binding protein 4 (GATA4) and NFAT, and translation factors [29], which might explain why SIRT3 overexpression prevents cardiac hypertrophy whilst SIRT3 knockout causes interstitial fibrosis and cardiac hypertrophy in mice [33].

SIRT4: The Great Unknown in the Heart

SIRT4 is known to interact with fewer proteins than the other sirtuins, even though it has been associated with several pathways controlling oxidative balance, FA metabolism, glycolysis, and amino acid catabolism. It shows very low NAD^+-dependent deacetylase activity, instead displaying

reducing general mRNA translation, increasing protein degradation and inducing the synthesis of chaperones that are involved in protein folding. When ER homeostasis is not reestablished by the UPR activation, inflammation and apoptosis are induced.

Fibrosis: pathological wound healing resulting from undue accumulation of extracellular matrix proteins, which arises in response to chronic tissue injury and inflammation. It may lead to tissue remodeling and the formation of scar tissue that disrupts the organ architecture and normal function.

Poly (ADP-ribose) polymerase (PARP): an NAD^+-dependent polymerase regulating DNA double-strand break repair, chromatin remodeling, gene transcription, and energy metabolism that is often activated under conditions of oxidative stress and in diabetic cardiomyopathy. It also activates NF-κB and diverts glucose metabolism from its usual glycolytic pathway.

lipoamidase activity and strong ADP-ribosyltransferase activity [29,42]. The role of SIRT4 in the heart has been poorly investigated, but studies performed with knockout mice have demonstrated that it is tightly associated with energy metabolism, inflammation, oxidative stress, steatosis, and fibrosis in other tissues [43–46].

SIRT5: The Missing Link in Metabolic Dysregulation in DCM?

SIRT5 is ubiquitously expressed, although its expression in the heart is comparatively high relative to other tissues [47]. It is regarded as a mitochondrial matrix protein, but it may also localize to the cytosol and nucleus [47–49]. This sirtuin shows weak deacetylase activity and is primarily known for carrying out NAD^+-dependent deglutarylation, demalonylation, and desuccinylation [29].

SIRT5 represses the activity of PDC directly by desuccinylating several of its subunits [48], as well as indirectly by deacetylating signal transducer and activator of transcription 3 (STAT3) [50]. In the liver, SIRT5 regulates the activities of diverse enzymes to increase ketone body synthesis [51]. This is important, since ketone bodies are an important source of energy for the heart under fasting conditions. SIRT5-mediated desuccinylation also inhibits the activity of cardiac succinate dehydrogenase (SDH) within the TCA cycle, which contributes to the protection of the heart from I/R injury due to reduced superoxide production [52]. It is noteworthy that SIRT3 and SIRT5 cooperate in deacylating very long-chain acyl-CoA dehydrogenase (ACADVL) to promote FA oxidation [53]. SIRT5 demalonylates glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and other glycolytic enzymes to promote the glycolytic flux [48]. SIRT5 deficiency has also been linked with a decrease in ATP production and subsequent AMPK activation, a fact which contributes to cardiac protection in left ventricular hypertrophy models [54].

Given that succinyl-CoA is the most abundant acyl-CoA molecule in the heart [49], it is not surprising that SIRT5 plays an important role in cardiac function. Studies on SIRT5-knockout mice have shown significant protein hyperacylation and hypersuccinylation in the heart, although only mild cardiac dysfunction is observed in the absence of any stress [49,52]. However, these mice display severe cardiac hypertrophy with aging or in response to chronic pressure overload [47,49]. This is probably due to a reduction in both FA and glucose oxidation and a decrease in the mitochondrial NAD^+/NADH ratio and ATP production, which result in a greater impairment of systolic function and favor the development of pathological cardiac hypertrophy. In cardiomyocytes, SIRT5 boosts the cell antioxidant capacity and prevents apoptosis by desuccinylating and activating copper- and zinc-dependent superoxide dismutase (SOD1), increasing NADPH generation through the activation of isocitrate dehydrogenase 2 (IDH2) and decreasing the activity of SDH by desuccinylation, and increasing glucose-6-phosphate dehydrogenase (G6PD) activity via deglutarylation [55,56]. Another study suggested that SIRT5 could deacetylate FOXO3 in lung epithelial cells and, thus, promote the expression of additional antioxidant genes [57]. The deacetylation of cytochrome c and peroxiredoxin by SIRT5 further reinforces its antiapoptotic role in the heart [12].

SIRT6: A Negative Endogenous Regulator of Cardiac Hypertrophy and Heart Failure

SIRT6 regulates chromatin remodeling, genome stability, and gene transcription through its mono-ADP-ribosyltransferase and histone deacetylase activities [13]. It is highly expressed in the myocardial tissue, where it regulates glucose and lipid homeostasis and plays a protective role [4]. It acts as a negative endogenous regulator of cardiac hypertrophy and heart failure by suppressing JUN transcriptional activity, which dampens the prohypertrophic insulin-like growth factor (IGF)-AKT signaling pathway [58], and by suppressing the expression and activity of NFATc4 [59]. SIRT6 improves cardiomyocyte stress resistance through several mechanisms: AMPKα activation, B cell lymphoma 2 (BCL2) upregulation, as well as reductions in AKT activity,

cell oxidative stress, and inflammation [60]. This anti-inflammatory effect depends on the histone H3 deacetylation at the gene promoter of the NF-κB p65 subunit [61]. SIRT6 prevents cardiomyocyte apoptosis by activating GATA4 transcription factor in a deacetylase-independent manner [62] and through the ADP-ribosylation of PARP [63], which increases its activity and, consequently, stimulates DNA double-strand break repair under oxidative stress. An antifibrotic role for SIRT6 has also been evidenced, since its deficiency resulted in the hyperactivation of transforming growth factor (TGF)β and subsequent deposition of collagen and other extracellular matrix proteins in the heart [64].

SIRT7: The Last Sirtuin Discovered

SIRT7 is widely expressed throughout the body, but is significantly expressed in the heart and liver. It is the only sirtuin that predominantly localizes in the nucleoli, where it binds to RNA polymerase I and activates ribosomal rRNA-encoding DNA (rDNA) transcription [65]. Deletion of SIRT7 in mice has been shown to reduce the mean lifespan and yielded a multiorgan phenotype [66], but it has been reported to make mice resistant to high-fat diet (HFD)-induced fatty liver, obesity, and glucose intolerance [67]. Interestingly, SIRT7-knockout mice have been observed to display AKT hyperphosphorylation and increased activity [68].

Sirtuins in DCM

The expression and activity of sirtuins in the heart are significantly modified in animal models of diabetes, although the extent and direction of these changes depends on the species, gender, age, tissue, and the model of diabetes analyzed, among other factors [3,69–71]. As an example, the mRNA and protein levels of all sirtuins was reduced in the heart of the rat model of streptozotocin (STZ)-induced T1D, except for SIRT2, expression of which was increased [3]. By contrast, in the high-fructose diet-induced T2D model, SIRT1 and SIRT2 were reported to be reduced and SIRT3 increased [3]. Strikingly, immunoblot analysis revealed increased acetylation of both cytoplasmic and nuclear proteins in the heart of the T1D model, but increased acetylation of only the nuclear proteins in the T2D model, suggesting the existence of a complex sirtuin signaling network. Sirtuins may modulate DCM by acting on oxidative stress, calcium homeostasis, metabolism, inflammation, fibrosis, and apoptosis. In the following section, we will outline an overall framework of the intricate role of sirtuins in DCM.

Metabolism Dysregulation and Mitochondrial Function

Cardiomyocytes have abundant mitochondria and mitochondrial function notably impacts on heart physiology by regulating cell energy metabolism, redox signaling, apoptosis, calcium handling, and cardiac contraction. Therefore, their deterioration or malfunction alters energy production, favoring oxidative stress and increasing cardiomyocyte apoptosis, thereby contributing to the pathogenesis of many cardiovascular diseases [11,42]. Since sirtuins regulate mitochondrial function, their activity is unequivocally associated with the onset and progression of DCM (Figure 1). Mitochondrial sirtuins are responsible for most of the changes in lysine acetylation that are observed in diabetes, but the other sirtuins can also intervene.

Reduced SIRT3 activity has been linked to the development of diabetes in rodent models of T2D (Table 1) [72] and humans [31]. In agreement with this, a genetic polymorphism in the human SIRT3 gene that reduces its activity was found to be associated with the metabolic syndrome [73]. Decreased SIRT3 expression in T1D cardiomyocytes impairs mitochondrial energetics and reduces ATP production [74]. Conflicting information is found regarding the acetylation status of the enzymes involved in FA oxidation, which might depend on the tissue, the specific enzyme, and the different lysine residues that may be acetylated. However, at least in skeletal muscle and in the heart, hyperacetylation of mitochondrial proteins in SIRT3 knockout mice is

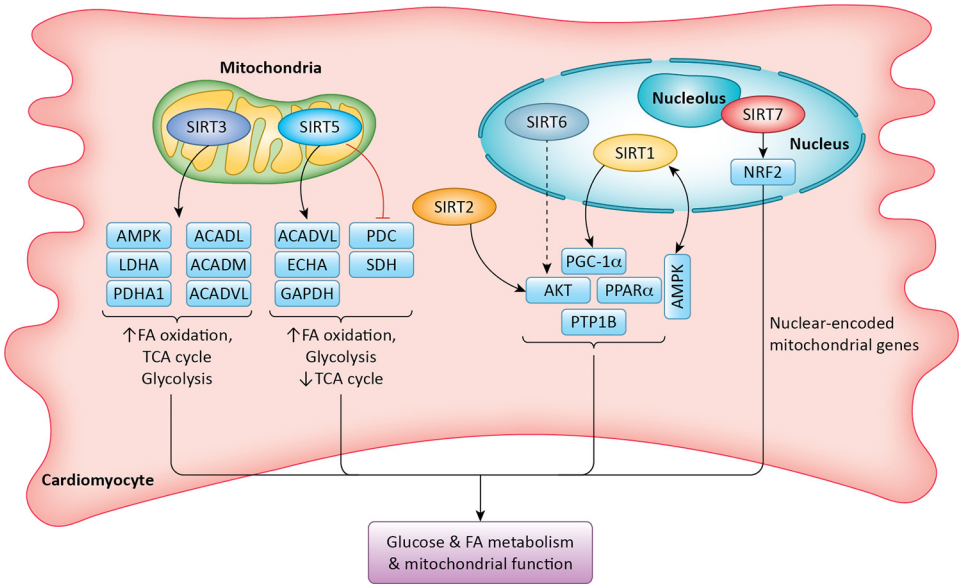

Figure 1. Metabolic Effects of Sirtuins in the Diabetic Heart. SIRT1 improves metabolism by activating AKT, peroxisome proliferator-activated receptor (PPAR)α, and protein tyrosine phosphatase (PTP)1B and favors mitochondrial dynamics by deacetylating PPARγ coactivator-1α (PGC-1α). AMP-activated protein kinase (AMPK) and SIRT1 regulate each other to maintain metabolic homeostasis. SIRT2 is required for optimal AKT activation. SIRT3 promotes fatty acid (FA) oxidation by deacetylating mitochondrial long-chain (ACADL), medium-chain (ACADM), and very long-chain (ACADVL) acyl-CoA dehydrogenases. It promotes the tricarboxylic acid (TCA) cycle and activates the first enzyme of the pyruvate dehydrogenase complex, pyruvate dehydrogenase E1α (PDHA1), which catalyzes the formation of acetyl-CoA that subsequently enters the TCA cycle. SIRT3 regulates glycolysis by activating lactate dehydrogenase A (LDHA) and promotes LKB1-mediated AMPK activation, thus upregulating glucose uptake. SIRT5 promotes FA oxidation by deacetylating ACADVL acyl-CoA dehydrogenase and by positively regulating enoyl-CoA hydratase α-subunit (ECHA) and demalonylates glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to promote the glycolytic flux. SIRT5 represses the activity of pyruvate dehydrogenase complex (PDC) and inhibits the activity of cardiac succinate dehydrogenase (SDH) within the TCA cycle. SIRT6 activates the AKT signaling pathway to prevent mitochondrial degeneration and lipid accumulation in the heart, while SIRT7 improves mitochondrial function by inducing the nuclear respiratory factor (NRF)2. Overall, these effects result in an increase in FA and glucose oxidation, thus attenuating lipid accumulation in the heart.

associated with increased FA oxidation rates [75,76]. Likewise, HFDs reduce SIRT3 expression in the heart and this is accompanied by reduced glucose utilization, enhanced FA oxidation, increased ROS formation, and impaired HIF-1α signaling, leading to impaired cardiac function [75,77]. By contrast, SIRT3 activation represses HFD-induced obesity [29] and attenuates lipid accumulation in cardiomyocytes [34]. Overall, the data suggest that the increased FA oxidation in the heart in response to HFDs depends, at least in part, on the downregulation of SIRT3 activity and the resulting increased acetylation of mitochondrial β-oxidation enzymes [75]. Treatment of cardiomyocytes with palmitate or feeding mice a diet rich in fat and sucrose downregulated the expression of SIRT3 and SIRT6, which contributed to obesity and the development of diabetes [70]. Systemic activation of SIRT6 in transgenic mice fed the same diet inhibited insulin resistance, reduced lipid accumulation, and sustained cardiac mitochondrial function [70]. In accordance with this, SIRT6 knockdown in mice hampers the insulin-sensitizing action of the antidiabetic rosiglitazone [78], with its cardiac-specific suppression resulting in mitochondrial degeneration and lipid accumulation in the heart [58]. The latter phenotype could arise from the blockade of IGF-AKT signaling. Kanwal *et al*. [70] elegantly demonstrated that SIRT3 and SIRT6 regulate each other to prevent the development of cardiomyopathy under diabetic conditions, with SIRT3 preventing a decline in SIRT6 expression by reducing oxidative stress and SIRT6

Table 1. Preclinical Studies Addressing the Role of Sirtuins in Murine Diabetic Cardiomyopathy Models[a]

Sirtuin	DCM model	Key findings	Refs
SIRT1	Rat model of STZ-induced T1D	The decrease in the AMPK/SIRT1 pathway activity in the heart during diabetes contributes to DCM progression.	[80]
		SIRT1 prevents proinflammatory and profibrotic phenotypic alterations in heart tissue by inhibiting NF-κB and the transcriptional activation of MMP9.	[85]
		SIRT1 deacetylates and inhibits p300, thus reducing TGFβ and preventing fibrosis and heart failure.	[86]
		SIRT1 attenuates ER stress-induced cardiomyocyte apoptosis via PERK/eIF2α, ATF6/CHOP, and IRE1α/JNK-mediated pathways.	[10]
	Rat model of HFD + STZ-induced T2D	SIRT1 reduces oxidative stress via activating eNOS in the diabetic heart.	[93]
	Fructose-fed T2D rat model	Activation of SIRT1 leads to deacetylation of both NF-κB-p65 and H3, thereby attenuating cardiac oxidative stress and inflammation.	[92]
	Mouse model of STZ-induced T1D	SIRT1 favors mitochondrial dynamics, boosts ATP generation, improves glucose metabolism, and reduces insulin resistance, thus improving cardiac function and reducing cardiac hypertrophy.	[11]
		SIRT1 activation increases autophagy in the myocardium by inducing FOXO1-dependent Rab7 gene transcription.	[102]
		Activation of SIRT1 upregulates sarcoplasmic calcium ATPase and improves contractile function.	[103]
	T2D db/db mouse model (+ angiotensin II)	Activation of SIRT1 improves mitochondrial function, alleviates oxidative stress and fibrosis, and blunts proinflammatory pathways.	[14]
		PARP-1 inhibition protects the diabetic heart through activation of SIRT1-PGC-1α axis.	[18]
SIRT2	Rat model of STZ-induced T1D	SIRT2, through the deacetylation of α-tubulin in microtubules, prevents cardiomyocyte hypertrophy and contractile impairment.	[104]
SIRT3	Rat model of STZ-induced T1D	SIRT3 preserves mitochondrial function and ATP production in the heart, resulting in reduced cardiomyocyte apoptosis, cardiac atrophy, and fibrosis.	[74]
	Mouse model of HFD-induced T2D	SIRT3 inhibition impairs cardiac function and favors cardiac hypertrophy due to enhanced fatty acid oxidation, lipid accumulation, and increased ROS formation.	[75,77]
		SIRT3 attenuates oxidative stress by regulating the acetylation status of mitochondrial FA β-oxidation enzymes.	
		SIRT3 deficiency, through NF-κB activation, stimulates inflammation and fibrosis, thus disrupting contractile function and impairing both systolic and diastolic functions.	[39]
	Mouse model of STZ-induced T1D	Suppressed SIRT3-FOXO3A-Parkin signaling-mediated downregulation of mitophagy plays a vital role in the development of DCM. SIRT3 deficiency prompts interstitial fibrosis, cardiomyocyte apoptosis, and mitochondrial injury. SIRT3 prevents the suppression of the autophagy.	[81]
		SIRT3 deficiency exacerbates DCM via necroptosis enhancement and NLRP3 activation.	[100]
	T2D db/db mouse model	SIRT3 improves mitochondrial function, decreases cardiac fibrosis, and inhibits apoptosis.	[71]
SIRT6	Rat model of HFD + STZ-induced T2D	SIRT6 restores normal mitochondrial function and biogenesis during DCM through the activation of the AMPK-PGC-1α-AKT signaling pathway.	[82]
	Mouse model of high-fat and high-sucrose diet-induced T2D	SIRT6 inhibits insulin resistance, reduces lipid accumulation, and sustains cardiac mitochondrial function.	[70]
		SIRT3 and SIRT6 regulate each other's activity.	
	Mouse model of HFD + STZ-induced T2D	SIRT6 prevents cardiac fibrosis through the inhibition of the endothelial-to-mesenchymal transition.	[89]

[a]Abbreviations: AMPK, AMP-activated protein kinase; ATF6, activating transcription factor; CHOP, CCAAT/enhancer-binding protein homologous protein; DCM, diabetic cardiomyopathy; eIF2α, eukaryotic translation initiation factor α; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; FA, fatty acid; FOXO3A, Forkhead box class O; HFD, high-fat diet; IRE1α, inositol-requiring enzyme 1α; JNK, JUN N terminal kinase; MMP9, matrix metalloproteinase 9; NF-κB, nuclear factor-κB; NLRP3, NOD-, LRR-, and pyrin domain-containing protein 3; PARP, poly (ADP-ribose) polymerase; PERK, protein kinase R-like endoplasmic reticulum kinase; PGC-1α, peroxisome proliferator-activated receptor (PPAR)γ coactivator-1α; ROS, reactive oxygen species; T1D, type 1 diabetes; T2D, type 2 diabetes; TGFβ, transforming growth factor β.

maintaining SIRT3 expression levels by inducing its nuclear factor erythroid-derived 2-like 2 (NFE2L2)-dependent transcription, a key player in the antioxidant defense.

Mice with SIRT6 haploinsufficiency also demonstrate an important role for this sirtuin in the regulation in cardiomyocytes of glucose channeling into the TCA cycle [79]. According to these authors, SIRT6 transcriptionally represses pyruvate dehydrogenase kinase 4 (PDK4) in a FOXO1-dependent manner [79]. PDK4 is an essential enzyme for glucose oxidation and, therefore, SIRT6 deficiency results in cardiac lactate accumulation, compromised mitochondrial glucose oxidation, and lesser ATP production [79].

SIRT5 might contribute to DCM and the progression of cardiac lipotoxicity through desuccinylation and the subsequent inhibition of SDH or by the activation of the hydroxyacyl-CoA dehydrogenase α subunit [47]. A lack of SIRT5 has been reported to impair FA metabolism in the hearts of mice during energy-demanding conditions due to the reduced activity of the enoyl-CoA hydratase α-subunit (ECHA) [49]. This leads to an accretion of long-chain acyl-CoAs and a decline in cardiomyocyte ATP levels [29], since ECHA desuccinylation by SIRT5 increases its activity and promotes the oxidation of long-chain acyl-CoAs.

Energy store depletion in diabetes increases intracellular NAD^+ levels, consequently activating AMPK. AMPK, in turn, activates SIRT1, which increases the AMP/ATP ratio and allows AMP to bind to the regulatory γ subunit of AMPK [80]. SIRT1 deacetylates and activates LKB1, an upstream positive regulator of AMPK [15]. Thus, AMPK and SIRT1 regulate each other and share molecular targets that contribute to the maintenance of metabolic homeostasis in DCM. In fact, SIRT1 activation improves cardiac function in DCM by reducing insulin resistance, while its suppression in mice induces cardiac hypertrophy and dysfunction, insulin resistance, and anomalous glucose metabolism [11]. AMPK-mediated phosphorylation of SIRT2 also plays a role in insulin signaling and the development of insulin resistance, since the activity of this sirtuin is required for optimal AKT activation [28].

SIRT1 favors mitochondrial dynamics and boosts ATP generation by deacetylating PGC-1α, which coactivates the mitochondrial regulatory transcription factors estrogen-related receptor (ERR)α, nuclear respiratory factor (NRF)1, NRF2, and mitochondrial transcription factor (TFAM) [11]. Treatment with resveratrol has been shown to increase the activity of TFAM, which is a downstream target of both SIRT1 and SIRT3 [11,74], resulting in normalized mitochondrial function as well as reducing cardiomyocyte apoptosis, cardiac atrophy, and fibrosis in a T1D rat model [74]. SIRT3 contributes to the preservation of mitochondrial function in diabetes by removing damaged mitochondria in cardiomyocytes, probably through the stimulation of FOXO3-Parkin-mediated mitophagy [81]. In a similar way to SIRT1, SIRT6 is capable of restoring normal mitochondrial function and biogenesis during DCM through the activation of the AMPK-PGC-1α-AKT signaling pathway [82]. Finally, low SIRT7 activity has been linked to mitochondrial dysfunction and cardiomyopathy, which probably arises from the deacetylation of distinct lysine residues in NRF2 [4,83].

Inflammation and Fibrosis

SIRT1 inhibits the activity of two important mediators of inflammation, p38 mitogen-activated protein kinase (MAPK) and NF-κB (Figure 2) [84]. As a result, there is a reduction in the expression of proinflammatory cytokines, which attenuates cardiac inflammation and apoptosis.

The transcription factor activator protein-1 (AP-1), which is a heterodimer composed of proteins from the FOS, JUN, and activating transcription factor (ATF) families, induces fibrosis of the interstitial substance and cardiomyocyte hypertrophy by increasing the deposition of collagen and the

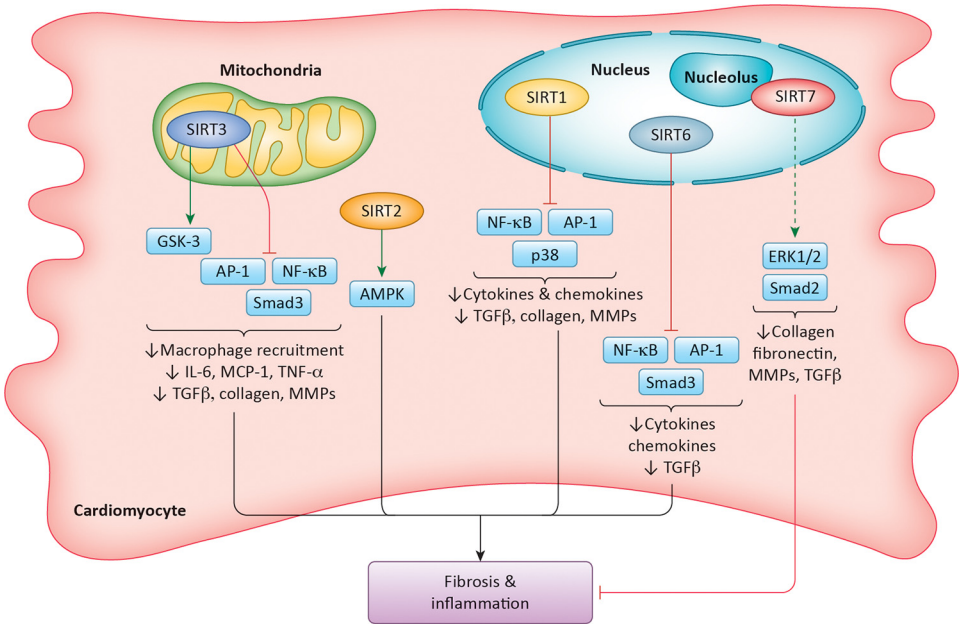

Figure 2. Sirtuin Effects on Inflammation and Fibrosis in the Diabetic Heart. SIRT1 inhibits inflammation by reducing nuclear factor-κB (NF-κB) activity and downregulating p38 mitogen-activated protein kinase (MAPK) activity. As a result, there is a reduction in the expression of proinflammatory cytokines and chemokines. SIRT1 blunts fibrosis through the deacetylation-dependent inhibition of activator protein-1 (AP-1), which reduces the transcription of matrix metalloproteinase (MMP)9 and transforming growth factor (TGF)β. SIRT3 reduces both inflammation and fibrosis through the inhibition of NF-κB activity and subsequent monocyte chemoattractant protein 1 (MCP-1) expression; the blockade of the TGFβ/Smad3 pathway; the activation of glycogen synthase kinase (GSK)3β, which blocks TGFβ signaling; and the inhibition of AP-1 transcriptional activity. Overall effects result in a reduction in macrophage recruitment and a diminution in the secretion of cytokines and chemokines [interleukin (IL)-6, tumor necrosis factor (TNF)-α, MCP-1], collagen, MMPs, and TGFβ. SIRT2 reduces myocardial fibrosis in a process that involves AMP-activated protein kinase (AMPK) activation. SIRT6 represses the activities of AP-1 and NF-κB, thus reducing the expression of cytokines and chemokines, and deacetylates Smad3 and histone H3 at the promoter of the TGFβ gene to repress its transcription. In contrast, SIRT7 induces the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and Smad2, which promote the differentiation of cardiac fibroblasts into myofibroblasts. Myofibroblasts increase the deposition of extracellular matrix components (collagen, fibronectin, MMPs, and TGFβ) to promote fibrosis.

synthesis of endothelin-1, fibronectin, and TGFβ. JUN deacetylation by SIRT1 reduces fibrosis by inhibiting the transcriptional activation of matrix metalloproteinase (MMP)9 [85]. Furthermore, in diabetes, hyperglycemia induces the transcriptional coactivator p300, which increases TGFβ levels. SIRT1 deacetylates and inhibits p300 and, thus, prevents fibrosis and heart failure in DCM [86]. Likewise, SIRT3 activation prevents collagen deposition and improves cardiac function in response to hypertrophic stimuli, in a process mediated by the inhibition of the TGFβ/Smad3 pathway [87]. Deacetylation by SIRT3 activates GSK3β, which blocks TGFβ signaling [88]. In a mouse model of T1D, SIRT3 suppression was shown to enlarge the area of myocardial interstitial fibrosis and aggravate cardiac dysfunction [81]. These deleterious effects of SIRT3 deficiency were mediated, at least in part, by the activation of FOS transcription through specific histone H3 lysine acetylation at its promoter [30]. Similarly, SIRT3 deficiency, through NF-κB activation, stimulated the expression of monocyte chemoattractant protein 1 (MCP-1), a chemotactic factor that promoted the recruitment of macrophages into the myocardium [39]. These macrophages secreted proinflammatory cytokines [interleukin (IL)-6, tumor necrosis factor (TNF)-α, and TGF] and augmented collagen deposition, with the resulting fibrosis disrupting contractile function and impairing both systolic and diastolic functions, thus hastening the progression of heart failure [39].

Although less known, there is also a potential role for other sirtuins in cardiac fibrosis. Suppression of SIRT5 [47,49], SIRT6 [58], and SIRT7 [66,83] in knockout mice has been reported to induce cardiac hypertrophy, inflammation, fibrosis, apoptosis, and downregulated cardiac performance compared with their wild type littermates. Transgenic mice overexpressing SIRT6 display the exact opposite [58]. The repression of HIF-1α, AP-1, and NF-κB activities by SIRT6 might account for its favorable effects [58,61]. SIRT6 deficiency resulted in the hyperactivation of TGFβ and the subsequent deposition of collagen and other extracellular matrix proteins in the heart [64]. Mechanistically, this was explained by SIRT6 binding to (and deacetylating) Smad3 and histone H3 at the promoter of the TGFβ gene, which repressed its transcription. SIRT6 prevents fibrosis in the heart through the inhibition of the endothelial-to-mesenchymal transition, a key process in the conversion of cardiac microvascular endothelial cells to myofibroblasts, which are responsible for most of the extracellular matrix deposition and perivascular fibrosis in DCM [89].

The hyperacetylation and subsequent activation of the prohypertrophic AKT, GATA4, and p53 signaling pathways in the absence of SIRT7 activity might explain its effects in the heart [83]. SIRT7 might also regulate cardiac fibrosis by promoting the differentiation of fibroblasts into myofibroblasts, a highly active cell type that increases the deposition of extracellular matrix components [90].

Oxidative Stress and Apoptosis

Oxidative stress is a fundamental mechanism underlying DCM, since it induces cardiomyocyte hypertrophy, apoptosis, and interstitial fibrosis. Activation of SIRT1 by caloric restriction in a mouse model of DCM was shown to improve mitochondrial function, alleviate oxidative stress and fibrosis, and blunt proinflammatory pathways, all of which contributed to the improvement of cardiomyopathy in these mice (Figure 3) [14]. These effects were mediated by the activation of PGC-1α and the subsequent increase in SOD2 protein levels. Similar results have been reported after activation of SIRT1 by resveratrol. Thus, activation of SIRT1 by resveratrol treatment attenuated cardiac injury in rats with STZ-induced diabetes through the improvement of mitochondrial function and the reduction of oxidative stress, in a process that was partly mediated through the deacetylation of PGC-1α [91]. Likewise, SIRT1 activation by resveratrol ameliorated cardiac hypertrophy, electrocardiographic abnormalities, and oxidative stress in the fructose-fed diabetic rat heart [92], although the latter study pointed to an additional mechanism entailing the deacetylation of NF-κB and histone H3 proteins, which led to the upregulation of SOD2. Another consequence of NF-κB inhibition was a reduction in the transcription of the NADPH oxidative subunits NOX1 and NOX2 [92]. SIRT1 activation also preserves endothelial nitric oxide synthase (eNOS or NOS3) activity by reducing its acetylation state, which contributes to its antioxidant effects, since increased NO production inhibits NADPH oxidase-dependent superoxide formation [93]. Fibroblast growth factor (FGF)21-induced expression of uncoupling proteins (UCP2 and UCP3) and SOD2 might account for some of the antioxidant activity of SIRT1, as FGF21 expression itself is, in turn, under the control of the SIRT1-PPARα pathway [94].

Activation of SIRT3, in a mouse model of angiotensin II-induced cardiac hypertrophy, was observed to restore mitochondrial function and reduce intracellular ROS levels through the upregulation of the SOD2 and catalase genes in a FOXO3-dependent manner [33], protecting cells from diabetes-induced oxidative stress. In a similar way, SIRT1 promotes the expression of the FOXO target genes involved in oxidative stress resistance and decreases the transcription of genes involved in apoptosis [95]. By contrast, SIRT4 overexpression promotes mitochondrial ROS generation, increases fibrosis, aggravates hypertrophy, and worsens cardiac function in a mouse model of angiotensin II-induced cardiac hypertrophy [96]. Surprisingly, these harmful effects of SIRT4 involve the inhibition of SIRT3-mediated deacetylation of SOD2 [96]. Nevertheless, the antioxidant effects of sirtuins extend beyond SOD2 and catalase activation. For instance, studies

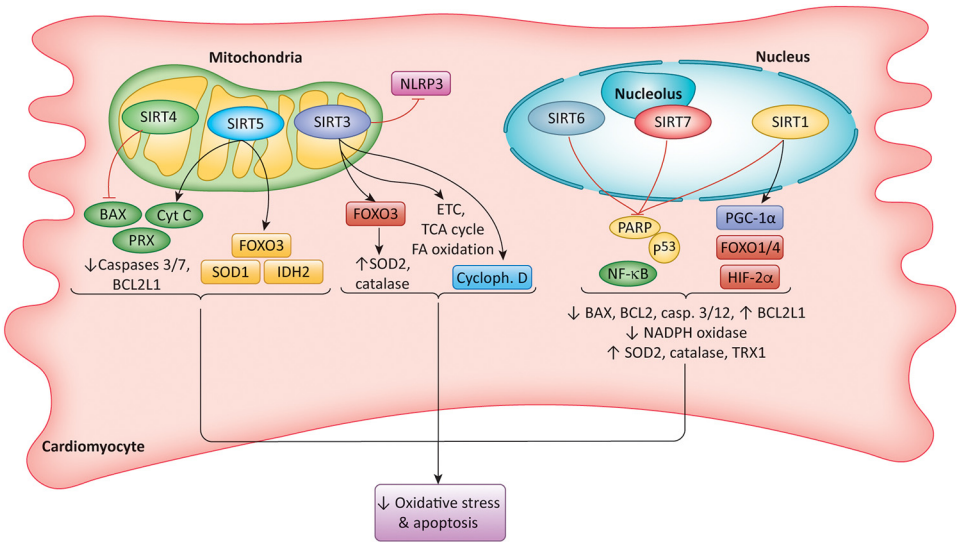

Figure 3. Sirtuins Protect against Oxidative Stress and Apoptosis in the Diabetic Heart. SIRT1 upregulates antioxidant thioredoxin 1 (TRX1), catalase, and manganese-dependent superoxide dismutase (SOD2), the latter through the activation of Forkhead box class O (FOXO)1, FOXO4, hypoxia-inducible factor (HIF)-2α, and peroxisome proliferator-activated receptor (PPAR)γ coactivator-1α (PGC-1α), and reduces the activity of NADPH oxidase by inhibiting the nuclear factor-κB (NF-κB). SIRT1 inhibits p53, caspases 3/12, and poly (ADP-ribose) polymerase (PARP) activities, reduces the protein levels of the proapoptotic BCL2-associated X (BAX) and B cell lymphoma 2 (BCL2), and increases the expression of the antiapoptotic BCL2 like 1 protein (BCL2L1). SIRT3 increases the activity of SOD2 by directly deacetylating several lysine residues and by increasing its expression through FOXO3 deacetylation. SIRT3 indirectly reduces ROS production by increasing the efficiency of the electron transport chain (ETC), promoting the tricarboxylic acid (TCA) cycle and regulating fatty acid (FA) oxidation, and prevents cardiomyocyte apoptosis by deacetylating cyclophilin D. SIRT3 also prevents necroptosis in a NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3)-dependent manner. SIRT5 activates copper- and zinc-dependent superoxide dismutase (SOD1) as well as isocitrate dehydrogenase 2 (IDH2) and stimulates the expression of FOXO3-dependent antioxidant genes. SIRT4 prevents cardiomyocyte apoptosis by suppressing BAX translocation, whereas SIRT5 acts through BCL2L1, cytochrome c (CytC), peroxiredoxin (PRX), and caspases 3/7. SIRT6 inhibits PARP and BCL2 and SIRT7 regulates p53 and PARP to prevent apoptosis.

in knockout mice suggest that SIRT3 may also attenuate oxidative stress by regulating the acetylation status of mitochondrial FA β-oxidation enzymes [β-hydroxyacyl-CoA dehydrogenase (HAD) and ACADL] [29,32,77]. Also, SIRT3 expression negatively correlates with ROS production in human AC16 cardiomyocytes under hyperglycemia conditions and this is due to the down-regulation of JUN N terminal kinase (JNK) phosphorylation [97]. The same study demonstrated that SIRT3 is a downstream target of PPARα, a fact which might account for the ability of the latter in maintaining antioxidant defense and oxidant equilibrium in cardiomyocytes [97].

ROS overproduction by mitochondria favors the release of cytochrome c and other proapoptotic proteins, which trigger caspase activation and apoptosis. Excess free FAs also trigger apoptosis in cardiac cells [98]. Thus, metabolism and oxidative stress are narrowly inter-related to apoptosis and, as a consequence, sirtuins are also involved in regulating cell death. For instance, an inverse relationship has been reported for SIRT1 and PARP activity in the hearts of T2D mice, which exhibit increased fibrosis, inflammation, and oxidative stress [18]. Similarly, the saturated FA palmitate increases oxidative stress and induces apoptosis in cultured neonatal mouse cardiomyocytes, which depends on SIRT1 inhibition [98]. The antiapoptotic properties of sirtuins may rely on several mechanisms. SIRT1 activation decreases cardiomyocyte apoptosis by preventing BCL2 down-regulation and ROS production [98], positively regulating the transcription of the antiapoptotic protein BCL2 like 1 protein (BCL2L1) [99], suppressing ER stress (see the following section) [10],

Clinician's Corner

Diabetic cardiomyopathy (DCM) is a chronic, prevalent, and complex disease characterized by metabolic dysregulation, which is often accompanied by local inflammation, oxidative stress, mitochondrial dysfunction, cardiomyocyte apoptosis, extracellular matrix remodeling, and fibrosis.

DCM is the leading cause of death among diabetic people and affects both patients suffering from type 1 or type 2 diabetes, although with different pathogenesis and time course. Women with diabetes are at higher risk of developing DCM than their male counterparts.

The quality of glycemic control is relevant for the development of DCM and heart failure. Each one precentral point increment in glycated hemoglobin (HbA1c) promotes parallel increases of 30%, for T1D patients, and 8%, for T2D patients, in the risk of developing heart failure.

The clinical picture of DCM is different in T1D and T2D. Patients with T1D develop myocardial remodeling with myocyte loss, interstitial fibrosis, left ventricular chamber dilation, and reduced systolic function, featuring arrhythmias and anterograde heart failure symptoms such as fatigue. Symptoms in T2D patients appear rather insidiously, as pulmonary and systemic congestion, owing to diffuse myocardial fibrosis, left ventricular concentric hypertrophy, and diastolic dysfunction with, at least initially, preserved ejection fraction (HFpEF).

The common final pathway for DCM, both in patients with T1D and T2D, is dilated cardiomyopathy with impaired systolic function (HFrEF) and chamber dilation, although this scenario appears later in the course of the disease in T2D patients.

New pharmacological approaches such as SGLT2 inhibition or treatment with GLP1 receptor agonists appear to tackle diabetes-induced metabolism disturbances and have been already shown to reduce cardiovascular mortality and incident heart failure hospitalizations in diabetic patients.

and deacetylating and inactivating p53, thus preventing the recruitment of transcription cofactors to the promoter region of the PUMA and BAX proapoptotic genes [16,99]. SIRT7 has an antiapoptotic role in the heart, with some studies suggesting that this sirtuin might act synergistically with SIRT1 to prevent oxidative stress and apoptosis by regulating p53 [83].

Less is known about the role of SIRT3 on cardiac cell death, although it may regulate necroptosis, a programmed cell death pathway different from necrosis and apoptosis that is associated with the high inflammation state occurring in DCM [100]. The absence of SIRT3 in knockout mice is associated with an increase in the expression of the inflammasome-related protein NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3), which promotes proinflammatory cell recruitment, caspase 1 activation, and proinflammatory cytokine secretion, ultimately exacerbating DCM in these mice [100]. Concerning the role of other mitochondrial sirtuins in apoptosis, SIRT4 exerts a cytoprotective effect against hypoxia-induced apoptosis of H9c2 cardiomyoblasts, mostly by suppressing mitochondrial BAX translocation [42]. In a similar way, SIRT5 is inhibited in cardiomyocytes upon oxidative stress. Studies in knockout mice have demonstrated that SIRT5 can reduce oxidative stress-induced apoptosis in cardiomyocytes through its interaction with BCL2L1, which dampens the uncoupling of the mitochondrial respiratory chain, thereby decreasing superoxide levels in the mitochondria [55]. Moreover, SIRT5 suppression decreases the viability of H9c2 cardiomyoblasts by promoting caspase 3/7 activity and apoptosis [13].

Sirtuins are a group of deacetylase enzymes that, according to preclinical studies, play an important role in regulating oxidative stress, calcium homeostasis, metabolism, inflammation, fibrosis, and apoptosis, all of which are mechanisms involved in the pathogenesis of DCM. Therefore, they are promising molecular targets for the development of specific therapeutics for this life-limiting complication of diabetes mellitus.

Other Pathophysiological Mechanisms

The link between SIRT1 and ER stress in DCM is relevant. SIRT1 activation protects cardiac cells from the apoptosis induced with ethanol or under hyperglycemic conditions by preventing caspase 12 activation and ER stress [10]. The genetic suppression of SIRT1 significantly increases the expression of ER stress markers and inhibits its antiapoptotic effect [10]. In fact, SIRT1 attenuates the ER stress pathways mediated by protein kinase R-like endoplasmic reticulum kinase (PERK)/eukaryotic translation initiation factor (eIF)2α, ATF6/CCAAT/enhancer-binding protein homologous protein (CHOP), and inositol-requiring enzyme 1α (IRE1α)/ JNK [10]. Another ER stress-related protein, the transcription factor X-box binding protein-1 (XBP1), which is activated by IRE1α, may also be inactivated directly by SIRT1 deacetylation [101]. The initiation of the unfolded protein response during ER stress, which involves the ATF6, IRE1α, and PERK pathways, aims to protect cells by halting mRNA translation, enabling protein degradation, improving protein folding, and potentiating autophagy. However, if ER stress is not mitigated, apoptosis occurs instead of autophagy in order to dispose of the damaged cells. This explains why reduced autophagy is often associated with DCM [81]. In mice with STZ-induced diabetes, SIRT1 activation increases autophagy in the myocardium by inducing FOXO1-dependent Rab7 gene transcription, which contributes to the maturation of autophagosomes and their fusion with lysosomes [102]. Similar to SIRT1, SIRT3 overexpression in cultured cardiomyocytes prevents the suppression of the autophagy and mitophagy observed in mice with STZ-induced diabetes [81].

Sirtuins may also improve calcium homeostasis in cardiomyocytes. SIRT1 activation restores sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) protein levels in the heart of a mouse model of T1D, probably by activating specificity protein 1 (Sp1), which normalizes cardiac cell contraction and ventricular dysfunction [103]. Activation of SIRT1 in DCM also contributes to the improvement in Ca^{2+} homeostasis by increasing the expression of HOMER1a in a process that requires the phosphorylation and subsequent activation of extracellular signal-regulated kinase (ERK)1/2 [103]. HOMER1a is a scaffold protein that modulates the release of Ca^{2+} from the ER in cardiac myocytes and acts as a calcium-dependent endogenous ROS scavenger, thus presenting antioxidative properties. In DCM, there is an intracellular overload of Ca^{2+} and Na^{+} due to the overall increase in the levels of **advanced glycation end products (AGEs)**,

which impairs the activity of the Na^+/K^+-ATPase in the sarcolemma [80]. This worsens the energy transduction from the intracellular membrane [80] and can also lead to ROS generation and oxidative stress, which both correlate with the contractile dysfunction of the diabetic heart [80]. Interestingly, AMPK, by activating SIRT1, can restore Na^+/K^+-ATPase activity [80].

Finally, SIRT2 has a unique protective role in DCM in a model of STZ-induced T1D that involves the deacetylation of α-tubulin in microtubules [104]. Microtubules are cytoskeletal heterodimers containing α- and β-tubulin proteins that, in the myocardium, are involved in intracellular mRNA and protein transport and subcellular organization. Tubulin acetylation stabilizes microtubules and promotes cardiomyocyte hypertrophy and contractile impairment, thus contributing to the progression of DCM.

Concluding Remarks

DCM is associated with high morbidity and mortality rates [105], making it very important to discover new targets for the development of more efficient drugs. Some of the main problems linked to DCM are: (i) its asymptomatic character, particularly at the early stages of the disease, (ii) its atypical and diverse signs and symptoms that hamper its evaluation in clinical practice, and (iii) its complex and devious etiopathogenic mechanisms [2,105]. Sirtuins mediate all these cell processes, thus making them potential targets for treating this disease (Box 2).

Box 2. Pharmacological Modulation of Sirtuin Activity

More than 3500 SIRT1-activating compounds have been identified to date [12]. The most investigated is resveratrol (3,4′,5-trihydroxystilbene), a phenolic compound naturally found in the skin of grapes and berries. In a T1D model, resveratrol normalizes the protein levels and activities of SIRT1, SIRT2, SIRT3, and SIRT5, but not of SIRT4, SIRT6, and SIRT7 [3]. By contrast, the administration of resveratrol to a T2D rat model only normalizes SIRT1 levels and stimulates SIRT5 protein expression [3]. To further complicate the story, resveratrol promotes the deacetylase activity of SIRT5, but, at the same time, it inhibits its desuccinylase activity in a substrate-specific way [113]. Resveratrol ameliorates cardiac dysfunction in DCM through its antioxidant properties, as well as by alleviating metabolic dysregulation and the inflammatory response, improving calcium homeostasis, attenuating ER stress and the impaired autophagy, and reducing apoptosis [3,10,91,92,114–116]. The activation of SIRT3 by resveratrol could also contribute to the improvements in these processes [87,117]. However, the beneficial effects of resveratrol and many other SIRT1-activating molecules (SRT1720, SRT2183, and SRT1460) on the heart also depend on their effects on other sirtuins and on sirtuin-independent activities that improve oxidative stress and inflammation [12,118].

At the molecular level, resveratrol mostly acts through AMPK activation and by modulating NFE2L2 and the receptor for AGEs (RAGE) in T1D. In T2D, resveratrol mostly has anti-inflammatory effects [119]. Resveratrol restores mitochondrial function, increases glucose uptake, and inhibits NF-κB activity [20,91,92,120–122]. As a consequence, there are decreases in the expression of NADPH oxidase, the generation of superoxide, and the activity of inducible NOS (iNOS), thus reducing oxidative and nitrative stress [116]. Resveratrol also reduces inflammation through the regulation of the MAPK-dependent pathways (ERK1/2, p38) and high mobility group box 1 (HMGB1), a proinflammatory molecule released by immune cells in hyperglycemia [123,124]. Its antioxidant activity is dependent on NFE2L2, although reductions in FA oxidation, together with improved pyruvate dehydrogenase activity and decreased glucose oxidation, could also be involved [115,121,125]. Moreover, its antifibrotic and antiapoptotic effects can occur through the suppression of the ERK1/2 [125], TGFβ/Smad3 [126], and FGF2 [127] signaling pathways, as well as via the activation of UCP2 [128].

Honokiol, a natural lignan isolated from the bark and leaves of *Magnolia* trees, acts as a selective SIRT3 activator [88]. It displays prophylactic and therapeutic activities against cardiac hypertrophy and fibrosis in animal models [88]. Drugs that selectively inhibit some specific sirtuin activities could also be of interest. Selective SIRT4 inhibitors, such as ZINC12421989, have been proposed to be suitable candidates for the treatment of cardiac hypertrophy and heart failure, or T2D [129]. Although several SIRT5 modulators have been described, they exhibit poor potency and/or low selectivity, which hinders their application [130].

Finally, some drugs belonging to currently approved clinical therapeutic groups may exert some of their beneficial effects by modulating sirtuin activity. For instance, the antihypertensive losartan exerts anti-ischemic effects, at least in part, by normalizing SIRT3 activity in the heart [131], while the phosphodiesterase-5 inhibitor sildenafil and the strong natural antioxidant curcumin mediate antioxidant cardioprotective activities by activating SIRT1 [13].

Outstanding Questions

Will it be possible in the near future to prevent diabetic cardiomyopathy (DCM) or even to treat it once established by targeting sirtuin activity? Should we selectively target sirtuin activity in the heart or impact also the whole body?

Will it be feasible to modulate the activity of each sirtuin separately? Since most sirtuins are cardioprotective, will only their pharmacological activators be useful for clinical purposes or also the selective inhibitors for a specific sirtuin?

It is peremptory to develop novel cellular and animal models where we can modulate the acetylation of specific residues and in a context-specific manner to unequivocally elucidate the functional consequences of each post-translational modification (acetylation, succinylation, malonylation) on a single target protein, carried out by each sirtuin, and to comprehend how sirtuins interplay between them and regulate each other's action.

Mitochondrial sirtuins (SIRT3, SIRT4, and SIRT5) would deserve special attention, as this organelle is essential for cardiac function and its impairment strongly contributes to DCM and heart failure. Since mitochondrial sirtuins share some functional redundancy and, together, coordinate numerous aspects of mitochondria, would the targeting of mitochondrial sirtuins be the best approach to treat DCM? Should we selectively target each mitochondrial sirtuin separately or consider them as a whole?

How would potential side effects influence the development of sirtuin modulators for the treatment of DCM? How will we be able to selectively avoid these adverse effects without affecting their cardioprotective properties? Will these sirtuin modulators be safe in chronic treatments for DCM?

Most data currently available are based on preclinical studies. Thus, further studies and clinical trials are still required to unequivocally elucidate the role of sirtuins and to develop and validate novel compounds that provide a fine-tuned, tissue-specific modulation of the activity of these enzymes.

Data found in the literature indicate that all the sirtuins regulate one another in a complex network, coordinating cardiac physiology and preserving their proper function. However, it has not been completely elucidated yet how this interplay operates in DCM. SIRT1 activation seems to be a promising tool in the protection of the diabetic heart. However, despite its recognized cardioprotective effects, some studies indicate that SIRT1 could also behave as a prohypertrophic molecule [106–108]. For this reason, the selective activation of SIRT1 in the heart to treat DCM would be inadvisable. In fact, it is probable that its protective effects actually rely on the coactivation of other sirtuins. Unfortunately, much less is known about the other sirtuins in DCM. Mitochondrial sirtuins, which are regarded as the watchmen of mitochondrial function, deserve a special mention. Available studies on SIRT3 suggest that its activation might be useful in treating metabolic diseases that exert deleterious effects on the heart, as is the case with DCM [30]. However, more data are needed to unequivocally demonstrate that these effects arise from the selective modulation of SIRT3. Modulation of SIRT4 and SIRT5 has also emerged as an interesting strategy. Moreover, improving mitochondrial function would positively affect not only cardiac function, but also whole-body metabolic homeostasis in metabolic diseases.

Despite all this knowledge, the relative contribution of each sirtuin is yet to be completely elucidated. Many issues still remain far from resolved (see Outstanding Questions). It is important to know their specific interactome to deepen our knowledge on how their physical associations with other proteins regulate cardiac physiology and to comprehend how sirtuins regulate one another, since they share many overlapping functions. Many potential targets have been identified and, thus, it would be desirable to unequivocally elucidate the functional consequences of each post-translational modification (e.g., acetylation, succinylation, and malonylation) on a single target protein and in a context-specific manner to shed light on the functional significance of each sirtuin. Moreover, the functional consequences of post-translational modifications on the same target protein may give rise to opposite effects, depending on the overall acylation pattern, the tissue or the cell environment [31,75]. Of course, the development and validation of novel compounds that fine-tune and provide tissue-specific modulation of any sirtuin analog will also be very helpful. Even so, most of the results presented herein are based on preclinical data. Therefore, further preclinical studies and clinical trials are required before these therapeutic approaches reach clinical practice.

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Declaration of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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