From THE DEPARTMENT OF MOLECULAR MEDICINE AND SURGERY
Karolinska Institutet, Stockholm, Sweden

STUDIES ON THE MECHANISMS OF ACTION AND PHYSIOLOGICAL RELEVANCE OF SOCS PROTEINS

Fahad AL-Zadjali

Karolinska Institutet

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“LOVE FOR OTHER PEOPLE WHAT YOU LOVE FOR YOURSELF"
Imam Ali will to his son

To my family

ABSTRACT

Understanding systemic biological pathways and the key cellular mechanisms that dictate disease states, drug response, and altered cellular function in metabolic disorders is a significant challenge. Research in the last 20 years have made it clear that tissue communication, through the actions of endocrine, paracrine or autocrine factors play a significant role in pathogenesis of complex multiorgan diseases such as the metabolic syndrome. The actions of these factors are governed both at the site of production and through mechanisms that regulate the sensitivity of target tissues.

The Suppressors of Cytokine Signaling (SOCS) proteins act as negative regulators of the main cytokine and growth factor signaling pathways in multiple tissues and as such have important physiological functions. The molecular basis for SOCS actions as well as their role in the pathogenesis of metabolic diseases is just starting to be understood. All SOCS proteins (SOCS1 to 7 and CIS) are characterized by the presence of structural motifs called SOCS box and a SH2 domain. SOCS are thought to act as substrate recognition subunits of multimeric Cullin/RING E3 ubiquitin ligase complexes. It has been proposed that the SH2 domain mediates the recognition of tyrosine phosphorylated signaling proteins to be targeted for ubiquitination and subsequently, proteasomal degradation, thereby inhibiting cytokine signaling.

In this thesis we investigated the mechanisms of action of SOCS2 and SOCS6 in the inhibition of cellular signaling and the physiological consequence of their actions. We demonstrated that both SOCS2 and SOCS6 assemble a canonical ECS (Elongin/Cullin/SOCS) complex through the interaction of SOCS box with Elongin B and C, cullin 5 and Rbx2. We also demonstrated that SOCS2 and SOCS6 exert E3-ligase activity towards the growth hormone (GH) receptor and cKIT proteins, respectively. Our structural and binding studies confirmed the existence of substrate binding motifs mainly in the SH2 domains and the N-terminal domain of both SOCSs. The C-terminus harbours the cullin 5 recognition domain that controls both E3 ligase activity of the complex as well as the SOCS stability. We proposed that extended target recognition domain in the SOCS proteins may serve to broaden their specificity toward various targets and hence their ability to regulate various signaling pathways. On the other hand, the existence of a degradation signal within the Elongin C interacting domain of SOCS proteins may serve to secure their timely actions avoiding competition from SOCS that are not engaged in active E3 ubiquitin ligase complexes.

The patho-physiological role of SOCS2 was studied in SOCS2 knockout ($SOCS2^{-/-}$) mice. In line with the in vitro studies (paper I), we observed an increased GH sensitivity in $SOCS2^{-/-}$ mice, demonstrated by low plasma GH/IGF1 ratio. In the liver, this enhanced sensitivity was manifested through increased VLDL secretion and reduced hepatic triglycerides levels. $SOCS2^{-/-}$ showed reduced hepatic steatosis upon high fat feeding as compared to wild type littermates but also exhibited increased adiposity and fat deposition in the skeletal muscles accompanied by profound systemic insulin resistance. We also demonstrated the involvement of SOCS2 in the regulation of inflammatory pathways. $SOCS2^{-/-}$ mice showed an exacerbated response to a high fat diet, with increased expression of inflammatory cytokines such as IL-6, RANTES,

IL1β both in the liver and adipose tissue. We also identified possible mechanisms to explain these phenomena by demonstrating that macrophages isolated from SOCS2$^{-/-}$ mice showed higher phagocytic activity and higher LPS-induced NF-κB activity; indicative of SOCS2 negative regulation of TLR4 signals.

Given the short half-life of SOCS proteins in the cells, the regulation of SOCS gene transcription is an important mechanism to control their function. The nuclear receptor LXR has regulatory functions on hepatic lipid metabolism that overlap with those controlled by the GH. Therefore, in order to understand the molecular basis for possible crosstalks between these two pathways, we studies how LXR ligands regulate the GH receptor signaling in liver. We showed that LXR agonist downregulates STAT5b protein levels and suppresses GH receptor activity in hepatocytes through a mechanism involving SREBP1. The regulation by SREBP1 occurs through the modulation of STAT5b protein stability and results in reduced expression of GH target genes such as SOCS2. These results provide a plausible explanation for the hepatosteatosis observed upon LXR agonist treatment in vivo.

In conclusion, through structural, *in vivo* and *in vitro* studies, we provide mechanistic and functional data on SOCS2 and SOCS6; information that may lead to a better understanding of the distinct physiological functions of these proteins. Given our demonstration on the key role of ubiquitination on SOCS functions, future mechanistic studies of SOCS2 and SOCS6 function should focus on the identification of ubiquitination targets of these proteins. Are the physiological functions ascribed to these proteins a result of the degradation of a few target proteins or do they have many targets? If the later is the case, how is SOCS target-specificity structurally determined and how is their activity regulated in time and cellular location? The demonstration that SOCS2 regulate both GH receptor and TLR4 signaling offers system where these questions can be addressed. At physiological levels, the SOCS2$^{-/-}$ mice constitute a novel model system for the study of the metabolic syndrome with unique features that are relevant to the human disease. In the SOCS2$^{-/-}$ mice, insulin resistance and production of inflammatory cytokine are exacerbated by high fat feeding and associated with obesity and deposition of triacylglycerides in the muscle. In these conditions reduced accumulation of TG in liver is observed. This model presents opportunities for future investigations aiming to distinguish between steatotic versus inflammatory causes for insulin resistance.

LIST OF PUBLICATIONS

I. Vesterlund M, **Zadjali F**, Persson T, Nielsen M.L, Kessler B.M, Norstedt G and Flores-Morales A*. **The SOCS2 Ubiquitin Ligase Complex Regulates Growth Hormone Receptor Levels.**
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II. **Zadjali F**, Santana-Farre R, Vesterlund M, Carow B, Mirecki-Garrido M, Hernandez-Hernandez I, Flodström-Tullberg M, Parini P, Rottenberg M, Norstedt G, Fernandez-Perez L and Flores-Morales A*. **SOCS2 inactivation protects against hepatic steatosis but worsens insulin resistance in high fat diet fed mice.**
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III. **Zadjali F***, Santana-Farre R, Mirecki Garrido M, Ellis E, Norstedt G, Fernandez-Perez L and Flores-Morales A. **Liver X Receptor agonist Downregulates Hepatic Growth Hormone Signaling**
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IV. **Zadjali F**, Pike AC, Vesterlund M, Sun J, Wu C, Li SS, Rönnstrand L, Knapp S, Bullock AN, Flores-Morales A*. **Structural basis for c-KIT inhibition by the suppressor of cytokine signaling 6 (SOCS6) ubiquitin ligase.**
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* Corresponding author

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LIST OF ABBREVIATIONS

ABCD	Avidin, biotin, complex, DNA
ACC	Acetyl-CoA carboxylase
AOX	Acyl-CoA oxidase
ATGL	Adipocyte triacylglyceride lipase
BRL	Bufflo Rat Liver
BTB	Bric-a-Brac, Tramtrack and Broad complex
cAMP	Cyclic adenosine monophosphate
CAND	Cullin-Associated and Neddylation- Dissociated
ChREBP	Carbohydrate response element-binding protein
CIS	Cytkine induced SH2 protein
CoA	Co-enzyme A
COX	Cyclooxygenase
CRL	Cullin ring ligase
CUL	Cullin
CYP	Cytochrome P450
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DHA	Docosahexaenoic acid
DNL	*De novo* lipogenesis
E1	Ubiquitin activating enzyme
E2, UBC	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
ECS	Elongin/Cullin/SOCS
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FABP	Fatty acid binding protein
FAS	Fatty acid synthase
FFA	Free fatty acid
GH	Growth hormone
GHD	Growth hormone Defeciency
GHR	Growth hormone receptor
GSH	Glutathione
HA	Hemagglutinin
HDL	High-density lipoprotein
HETES	Hydroxyeicosatetraenoic acid
HFD	High fat diet
HGF	Hepatic growth factor
HOMA	Homeostasis model of assessment
HSL	Hormone sensitive lipase
HSP	Heat shock proteins
ICAM	Inter-Cellular Adhesion Molecule
IGF/IGFBP	Insulin-Like growth factor/binding proteins
IHC	Immunohistochemistry
IL	Interleukin

INF	Interferon
ipGTT	Intraperitoneal glucose tolerance test
ipITT	Intraperitoneal insulin tolerance test
IRS	Insulin receptor substrate
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
KC	Kupffer cells
KIR	Kinase inhibitory region
LDL	Low density lipoprotein
LO	Lipoxygenase
LPL	Lipoprotein Lipase
LPS	Lipopolysaccaride
LRR	Leucine Rich Repeats
LXA	Lipoxins
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
MCD	Methionine-Choline defecient
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein-
mTORC	Mammalian target of rapamycin complex
MTTP	Microsomal triacylglyceride transfer protein
NAFLD	Non-Alcoholic FattyLliver Disease
NASH	Non-Alcoholic Steatohepatitis
NEDD	Neural precursor cell Expressed Developmentally Down-regulated protein
NF- kB	Nuclear factor-kB
NK	Natural killer cells
NO, NOS	Nitric oxide, nitric oxide synthase
NOX	NADPH oxidase
NSAID	Non-Steroidal Anti-Inflammatory drugs
PC, LPC	Phosphatidyl choline, Lysophosphatidyl choline
PCR	Polymerase chain reaction
PDK	Protein kinase D
PERK	Protein kinase R-like endoplasmic reticulum kinase
PGE	Prostaglanding E
PI_3, PI3K	Phosphoinositol triphosphate, PI_3 Kinase
PKB	Protein kinase B, Akt
PKC	Protein kinase C
PNPLA	Patatin-like phospholipase domain containing protein
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acids
RANTES	Regulated on activation, normal T cell expressed and secreted chemokine
RING	Really Interesting New Gene
ROS	Reactive oxygen species
RXR	Retenoid acid receptor
SCD	Stearoyl-CoA desaturase
SCF	Skp1-Cullin-F-box

SH2	Src-homology domain 2
SNP	Single nucleotide polymorphism
$SOCS2^{-/-}$	SOCS2 knockout
SREBP	Sterol regulatory element-binding protein
STAT	Signal Transducer and Activator of Transcription
TAG	Triacylglycerides
TGF	Tumour growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPN	Total parenteral nutrition
UCP	Uncoupling protein
VEGF	Vascular endothelial growth factor
VLDL	Very-low density lipoprotein
βTrCP	β-transducin repeat-containing protein

1 INTRODUCTION

Fatty liver disease also known as hepatic steatosis is a pathological condition characterized by the retention of lipids in the hepatocytes exceeding 5% of the liver weight[1] that may result from impairment in the synthesis or mobilization of triacylglycerides (TAG)[2]. Fatty liver disease is classically divided into alcoholic fatty liver disease in individuals with ethanol intake >20g/day and non-alcoholic fatty liver disease (NAFLD) [3].

Patients with NAFLD are usually asymptomatic although fatigue and upper abdominal discomforts are commonly reported[4]. The prevalence of NAFLD in the general population is estimated to be 20–30% in the Western countries [2] and it is more frequent among individuals with type 2 diabetes (50%) and obesity (76%) [5, 6]. The histological spectrum of NAFLD spans from simple steatosis to hepatocellular inflammation with cellular damage (nonalcoholic steatohepatitis, or NASH) that may be further complicated to fibrosis and can ultimately progress to cirrhosis. The extent and pattern of fat deposition in the hepatocytes further subdivides hepatic steatosis into microvesicular and macrovesicular steatosis [7]. In the latter case, the fat vacuoles are large enough to displace the cytoplasm and push the nucleus to the periphery. This histological classification provides an indication about the duration of lipid accumulation (chronic or acute) and also about the etiopathogenesis. The most common form of NAFLD is primarily caused by mediators of metabolic syndrome, such as insulin resistance and obesity. Secondary causes of NAFLD are listed in Table 1 and reviewed elsewhere [2, 8].

NAFLD is clinically important since it occurs frequently in the general population and it may potentially progress to become an advanced hepatic and extrahepatic disorder. Identification and understanding of the predisposing factors leading to NAFLD gives better prognostic information in an individual patient [9]. Due to the high risk for development of NAFLD-related complications, the mortality rate for patients with NAFLD is higher than for age and sex-matched controls [10, 11]. Retrospective studies indicate that 38% of patients with NAFLD with histological necroinflammation progress to liver fibrosis [12] and 30-70% of patients with cryptogenic cirrhosis had end-stage NASH [9]. Furthermore, 13% of hepatocellular carcinoma cases were attributed to NASH-related cirrhosis [13]. NAFLD is also known to occur after normal liver transplant surgery to patients with NASH [14]. Taken together, these statistics show the importance to further study the molecular basis of NAFLD.

1.1 PATHOGENESIS OF NAFLD AND NASH:

Mechanisms that govern the progression of simple hepatic steatosis to NASH are multifactorial. The two-hit hypothesis has been suggested by Day and James[15] to explain how this process occurs. Accumulation of hepatic lipids, caused by changes in uptake, synthesis, and secretion of lipids, constitutes for the first insult. A variety of factors have been implicated as the second “hit” driving the progression to NASH. These include the actions of inflammatory cytokines, oxidative stress, mitochondrial dysfunction and gut-derived bacterial endotoxins [15, 16]. The two-hit hypothesis suggests that TAG accumulation acts as an inert bystander. Hence, an alternative hypothesis was presented first by Heribert Thaler that considers the central role of lipotoxicity from

TAG and fatty acid metabolites in the hepatocellular injury observed in NAFLD [17, 18]. Figure 1.1 summarizes the contributing factors involved in the development of NALFD and NASH and these will be discussed further in this chapter.

Table 1: Secondary factors contributing to the development of non-alcoholic fatty liver disease

Genetic/metabolic Wilson's disease Lipodystrophy Cholesterol ester storage disease Hemochromatosis Reye's Syndrome Hypobetalipoproteinemia Wolman's disease	**Drug induced** Glucocorticoids Estrogen NSAID'S Methotrexate Hypervitaminosis A Tamoxifen Calcium antagonists Anti-retroviral drugs Tetracycline
Nutritional Total parental nutrition Intestinal bypass Rapid weight loss Prolonged starvation High carbohydrate diet Protein malnutrition	**Toxins** Toxic mushrooms Petrochemicals Phosphorus
Extrahepatic causes Cardiac failure Irritable bowl syndrome Infected diverticulosis Hypothyroidism GH deficiency Pregnancy Polycystic ovarian syndrome	**Infection** Hepatitis C Hepatitis B Intestinal bacterial growth

1.1.1 Factors enhancing lipid delivery to the liver

Non-alcoholic fatty liver disease is a multifactorial disorder linked to hypertriglyceridemia, insulin resistance and obesity. The primary cause of hepatic steatosis is an elevated supply of fatty acids to the liver, either from nutritional sources, excessive adipose tissue lipolysis, defective lipid mobilization or increased hepatic *de novo* lipogenesis (DNL). The relative importance of these factors depends on their primary cause. For example, rapid weight loss as well as obesity is related to the development of fatty liver, in the first cases it is due to increased lipolysis in adipose tissue while in obesity, the dietary intake has a prominent role.

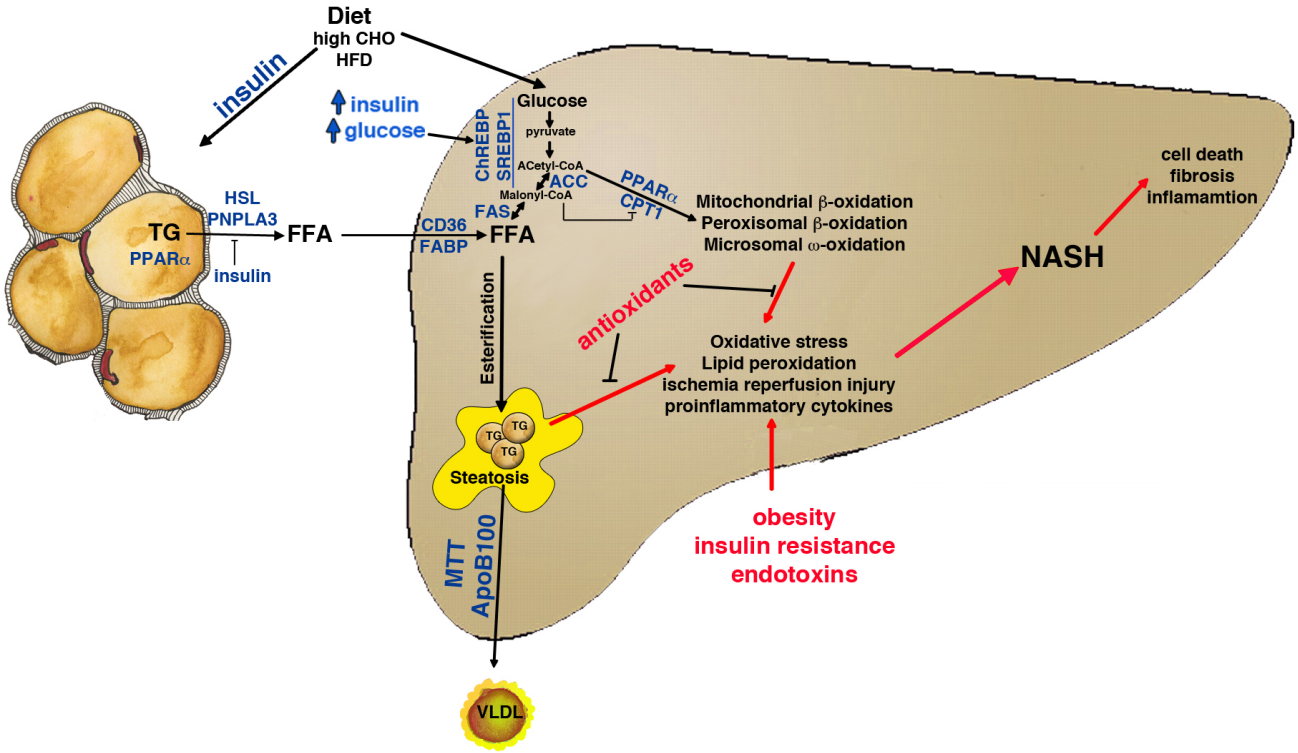

Figure 1.1: The two hit theory of the development of non-alcoholic steatohepatitis (NASH). Factors involved in the development of the first hit (blue) include enhanced fatty acid delivery to the liver, enhanced *de novo* lipogenesis and impaired disposal of triacylglycerides (TAG). Factors involved in the development of the second hit are in red. Further details are in the text.

1.1.1.1 Nutritional factors

Diet plays a critical role in the development of NAFLD in humans. Fatty liver is the most common histological finding in patients receiving total parenteral nutrition (TPN) [19]. TPN increases the load of glucose, lipid or total calories and leads to fat accumulation in the liver[20]. Other nutritional factors include an imbalanced diet of high carbohydrate consumption, which in excess get converted into fat and predispose to obesity and insulin resistance [21]. Collectively, an increased dietary supply of calories enhances the delivery of fat or the synthesis in the liver. NAFLD is also observed in extreme malnutrition. Kwashiorkor, a form of severe protein deficiency, is associated with fatty liver most likely due to the accompanied insulin resistance[22]. Deficiencies in both carnitine, involved in fat oxidation, and choline, involved in VLDL assembly, as a result of malnutrition or post-TPN have been associated with NAFLD [23,24]. Gastrointestinal bariatric bypass surgical procedures causes profound fasting, protein calorie malnutrition and rapid weight loss which increases the flux of free fatty acids into the liver, which even in short term may cause deterioration of liver functions [25]. However, these effects are transient in which numerous observational studies have demonstrated that bariatric surgery may cause regression of NAFLD in line with improved whole body insulin sensitivity and enhanced mitochondrial function [25, 26].

In rodents, methionine-choline deficient (MCD) diet impairs VLDL secretion due to inhibition of phosphatidyl choline synthesis and can induce fatty liver within 2-4 weeks of feeding [27]. However, rodents fed with this diet do not develop obesity and insulin resistance in contrast to what is observed in humans with NASH and other diet-induced NAFLD[28, 29]. On the other hand, the choline-deficient diet induces dyslipidemia, insulin resistance and results in a less severe fat accumulation, inflammation and necrosis in the liver when compared to methionine-choline deficient diet [30, 31]. High fat diet (HFD) is also well known to induce obesity and insulin resistance in rodents. It increases

hepatic lipid content quite rapidly, before a significant increase in fat accumulation in the peripheral tissues is observed. However, HFD in rats induces a milder steatosis when compared to the methionine-choline deficient diet and the rats show fewer signs of inflammation and fibrosis [32, 33].

1.1.1.2 Increased peripheral lipolysis

The sources of hepatic free fatty acids are primarily adipocyte triacylglyceride lipolysis and hepatocyte *de novo* lipogenesis (DNL). In normal individuals, fasting DNL contributes to about 5% of the total free fatty acids (FFA) but it increases up to 26% in patients with NAFLD [34, 35]. Other minor contributors include the uptake of short chain fatty acids delivered through portal circulation and lysosomal breakdown of lipoprotein remnants and autophagosomes, containing limited TAG[34]. The liver lipoprotein lipase (LPL) is normally not expressed in adults but its expression increases as a response to infection, cancer cachexia and cytokine stimulation [36]. Liver specific expression of LPL increases hepatic uptake and accumulation of TAG [37]. Circulating triacylglycerides are not a direct source of fat for the liver and therefore pharmacological reduction of serum TAG levels might not have a significant direct beneficial effects on the liver [34].

Peripheral lipolysis is regulated by hormonal, neural and pharmacological stimuli that releases FFA into the circulation and later deposits them in the liver and skeletal muscles. Table 2 summarizes the factors that regulate adipocyte lipolysis (reviewed in ref. [38]). Genetic mouse models of high plasma FFA levels such as CD36 knockout mice and muscle specific deletion of LPL develops hepatosteatosis due to increased FFA flux to the liver [39, 40]. It is worth to mention that the impact of increased peripheral lipolysis associated with prolonged starvation or insulin resistance on the liver is modulated by the oxidative capacity of the muscle. Muscle specific lipoprotein lipase (LPL) knockout mouse models are spared from the deleterious effect of high plasma FFA on the muscle but show severe hepatic steatosis[39]. Similarly, the SJL/J mouse strain tolerates fasting induced hepatic steatosis because of the enhanced compensatory muscle fat oxidation[41]. This collectively emphasizes the role of enhancing peripheral fatty acid oxidation through exercise in the therapeutic management of NAFLD.

1.1.2 Increased hepatic *de novo* lipogenesis

Hepatic *de novo* lipogenesis refers to the process where excess glucose is converted to fatty acids. Enhanced DNL, has been demonstrated in both humans and murine models of steatosis [42]. Sterol regulatory element-binding protein-1c (SREBP-1c) is a key transcription factor that mediates insulin stimulation of hepatic lipid synthesis [43]. Similarly, elevated glucose levels also activates DNL via the carbohydrate response element-binding protein (ChREBP) [44]. ChREBP, triggered by high plasma insulin or glucose, enhances the expression of liver pyruvate kinase, a key regulatory enzyme in glycolysis. A third transcription factor that participates in the development of hepatic steatosis in rodents is PPAR-γ. In human, hepatic PPARγ expression is very low compared to rodents [45, 46]. In animal models, hepatic expression of PPARγ is low but significantly increases in conditions of insulin resistance and fatty liver [47]. Hepatic knockout of PPAR-γ in livers of obese mice markedly attenuates the development of hepatic steatosis, independent of the presence of hyperinsulinemia or hyperglycemia [48]; while viral mediated hepatic overexpression of PPARγ in mice leads to hepatic steatosis with upregulation of a wide range of PPARγ-responsive genes. PPARγ exerts other functions, especially those relevant to the development of NASH. PPARγ shows antifibrogenic actions in which it reverses the activation of hepatic stellate cells toward a resting state [49, 50].

Table 2: Factors that regulates adipose tissue lipolysis

Effect on lipolysis	Mechanism
Decrease lipolysis	
Insulin	Activates Akt , lowers cAMP and inhibits HSL
PPARγ	Promotes lipid droplet formation and stabilization
Adipocyte PLA2	Releases arachidonic acid for PGE2 synthesis
Increase lipolysis	
Catecholamines	Enhances camp through adrenergic receptors
Glucagon	Enhances cAMP
Growth Hormone	Enhances HSL activity
ACTH	Enhances cAMP
Methylxanthines	Enhances cAMP and releases catecholamines
Adipocyte triacylglyceride lipase (ATGL or PNPLA2)	Hydrolysis of TAG
Adiponutrin (PNPLA3)	Hydrolysis of TAG
HSL	Hydrolysis of TAG
Perilipins	Activates ATGL
TNFα	Activates JNK and induces insulin resistance
JNK	Serine phosphorylation of insulin receptor substrate-1

ACTH, adrenocorticotropic hormone; cAMP, cyclic adenosine monophosphate; HSL, hormone sensitive lipase; PPAR, peroxisomal proliferator activated receptor; JNK, c-Jun N-terminal kinase

1.1.3 Impaired disposal of hepatic triacylglycerides

1.1.3.1 Decreased oxidative capacity

Fatty acids are oxidized to carbon dioxide and water through β-oxidation (mitochondrial and peroxisomal) and ω-oxidation (endoplasmic reticulum). All these oxidative pathways are strongly regulated by the PPARα nuclear receptor [51]. PPARα serves as a sensor for a broad array of lipophilic species and it heterodimerize with the retinoid X receptor to induce genes that regulate fatty acid oxidation [51]. On the other hand, the nuclear receptor PPARγ, which also senses lipophilic species, promotes the formation and export or storage of fatty acids as triacylglycerides.

The shuttling of FFA for either oxidation or formation of TAG is tightly controlled. In the liver, β-oxidation is inhibited in the fed state as a result of insulin actions. Cellular levels of malonyl-CoA and ATP are potent inhibitors of β-oxidation. Deletion of the ACC2 gene or Adenoviral-mediated expression of malonyl-CoA decarboxylase in mice lowers the cellular levels of malonyl-CoA which in turn enhances β-oxidation and therefore these mouse models are protected from diet-induced NAFLD [52, 53]. Animal models of reduced oxidative capacity includes the carnitine deficient mouse, i.e. juvenile visceral steatosis (JVS), that develop severe hepatic steatosis within days after birth [54].

Mitochondrial dysfunction, genetic or acquired, is a well-known observation in patients with NASH [55] Peroxisomal β-oxidation can take over when the mitochondrial oxidation is dysfunctional. Acyl-CoA oxidase (AOX) catalyzes the first step of peroxisomal β-oxidation. Substrates of AOX include very long chain fatty acids, arachidonic acid metabolites, leukotriene B4, and 8(S)-hydroxyeicosatetraenoic acid. Inhibition of peroxisomal β-oxidation leads to NASH and stimulation of peroxisomes by PPARα agonists protects againsts NASH [56-58]. The AOX deficiency disease (pseudoneonatal adrenoleukodystrophy) manifests in the neonatal period with hypotonia and children die between ages 4-5 years [59]. AOX knockout mice show defects in peroxisomal β-oxidation with severe age-progressive hepatic steatosis [29].

Microsomal ω-oxidation via the smooth endoplasmic reticulum cytochrome P450 (CYP) oxidizes a variety of lipid compounds including the FFA. Upregulation of various CYP isoforms, e.g. CYP4A11, have been reported in patients with NASH [60]. CYP enzymes catalyze the first step of ω-hydroxylation forming dicarboxylic acids which then are conjugated to CoA by the action of the microsomal acyl-CoA synthetase. Dicarboxylic acids can impair mitochondrial function by uncoupling oxidative-phosphorylation [61, 62]. Yet, the importance of microsomal oxidation in the progression to NASH has not been confirmed.

Triacylglyceride in tissue are hydrolyzed under the action of lipases. In adipose tissues, TAG is hydrolyzed by adipose triacylglyceride lipase (ATGL or desnutrin) and DAG is hydrolyzed by HSL [63]. In the liver, the expression levels of ATGL are much lower compared to adipose tissue [63]. Mice with ATGL deletion display higher adipose tissue mass and knockdown of liver-ATGL causes steatosis [64]. PNPLA3 or adiponutrin is another TAG lipase predominantly expressed in the adipose tissue and liver. It has very high homology with ATGL and it is involved the re-esterification of TAG in the hepatocytes [64, 65]. The hepatic expression of PNPLA3 in mice is upregulated by high fat feeding and fasting downregulates its expression [66]. This suggests that PNPLA3 has important function in the hepatic lipid mobilization. Indeed, point mutations in PNPLA3 genes are strongly associated with NAFLD in children and adults [67, 68].

1.1.3.2 Decreased TAG assembly and secretion

Hepatic export of TAG, as very low-density lipoprotein (VLDL), is accomplished by a regulated protein machinery. Studies on livers from patients with NASH showed disturbed VLDL metabolism and secretion[69]. The rate-limiting step of VLDL assembly is controlled by the microsomal TAG transfer protein (MTTP), located in the endoplasmic reticulum. Mutations in this protein in human are responsible for the autosomal recessive condition abetalipoproteinemia, which is characterized by severe hepatic steatosis and cirrhosis[70, 71]. Several commonly used drugs (e.g. doxycycline and tetracycline) are known to affect β-oxidation and inhibit MTTP activity in animal models of drug-induced steatosis [72].

1.1.4 Progression of hepatic steatosis to NASH. Introducing the 2nd hit

Extensive studies have been carried out to understand the mechanisms of how intrahepatic lipid accumulation progresses to the development of inflammation and fibrosis. One third of patients with hepatic steatosis are estimated to progress to steatohepatitis [73]. NASH is characterized by cumulative defects in cellular organelles (e.g. mitochondria), elevated systemic and local levels of cytokines and recruitment of

inflammatory cells. Fatty acid accumulation results in increased generation of reactive oxygen species (ROS), induction of cellular stress responses [e.g., activation of protein kinase C (PKC), mitogen-activated protein kinase (MAPK), Jun N-terminal kinase (JNK), nuclear factor-κB (NF- κB)], and subsequent expression of pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) [74]. These cytokines are involved in the activation of Kupffer cells and also the transformation of stellate cells to a fibromyoblastic cell type, contributing to the progression from steatosis to steatohepatitis [75]. In this section we will further discuss the molecular basis of lipid toxicity and the development of inflammation.

1.1.4.1 Lipotoxicity

Lipotoxicity describes cellular injury and death caused by free fatty acids, their metabolites, and even by lipid droplets and triacylglycerides, although the later two are found to be generally inert.

Triacylglycerides: TAG accumulation is associated with tissue injury and insulin resistance in the muscle and liver [76]. However, current data suggest that this association lacks causality. Diacylglycerol acyltransferase 2 (DGAT2) catalyzes the last step of TAG synthesis. In mice, overexpression of DGAT2 leads to hepatic TAG accumulation and endoplasmic reticulum (ER) stress, shown by the enhanced phosphorylation of PERK (protein kinase R-like endoplasmic reticulum kinase) [77]. However, the accumulated TAG did no increase the activity of proinflammatory pathways such as JNK and NF-κB [77]. In contrast, blocking of DGAT2 causes lower hepatic TAG accumulation but increased FFA levels resulting in lipotoxic cell injury [78]. Furthermore, cellular injuries or inflammatory responses were not observed after TAG accumulation caused by blocking the MTTP-mediated TAG incorporation to VLDL [79]. In β–cells, overexpression of perilipin increases TAG accumulation and protects against palmitate-induced suppression of glucose mediated insulin secretion [80]. Overall, these studies do not support a direct role of TAG in lipid mediated liver injury but do not exclude that the stored TAG could serve as a source of other lipotoxic intermediates.

Other studies have suggested that lipid droplets by themselves have metabolic actions in regulating cellular signaling; in particular those that regulate lipid storage and disposal. Lipid droplets construct a continuous intracellular membrane system (membrane flow hypothesis) that enables exchanges between lipid droplet and lipid mobilizing organelles such as peroxisomes and mitochondria [81]. Studies have confirmed the role of lipid droplets in the regulation of lipid oxidation [81, 82]. Reduction of adipophilin, a component of lipid droplets, leads to enhanced expression of lipogenic genes in mouse models of NAFLD [83]. The close proximity between lipid droplet and these organelles regulates the substrate availability for oxidation. In fact, studies on yeast shown that some lipid droplets contain extension of peroxisome and hence named as pexopodia [81].

Free fatty acids: FFA has deleterious effects on biological systems and hence is found bound to proteins such as albumin in circulation and fatty acid binding proteins (FABP) inside the cells. Studies on mice show that knockout of liver-FABP can block TAG accumulation after treatment with a MTTP inhibitor [84, 85]. The net flux of FFA into the liver is relatively more important than the actual concentration for the development of NASH [34]. Cellular concentrations of FFA in the liver remain fairly constant despite the

increase in circulating levels in patients with NASH [86]. In the cells, FFA can induce cellular damage by activating inflammatory and apoptotic pathways, some of which can be initiated by vinding of saturated fatty acids to Toll-like receptor 4 (TLR4) [87]. Furthermore, FFAs induce the translocation of the proapoptotic Bax protein to the lysosome and subsequently enhances lysosome permeability and the release of cathepsin B which initiates the cellular death program, (Figure 1.2) [88]. Beside mitochondrial dysfunction, cathepsin B induces NF-kB activity and subsequent TNFα expression[88]. FFAs also serve as ligands for PPARγ and PPARα to regulate storage or disposal of lipids[89]. Additional cytotoxic effects of FFA occur via the formation of acyl-CoA[90]. Inhibition of FFA esterification with CoA by triacsin C significantly blockes palmitate induced pancreatic β-cell deaths [90]. In hepatocytes, triacsin C treatment also blockes the palmitate-induced inhibition of insulin signaling. All together, these data suggest that palmitoyl CoA ester formation is critical in mediating the cytotoxic effects of FFA[91].

Ceramides: Ceramides are mainly found in the plasma membrane and are composed of sphingosine and fatty acids. They serve as cellular signals for proliferation, differentiation and apoptosis, most importantly activation of protein kinase Cθ leading to an inhibitory serine/threonine phosphorylation of IRS1 and 2 [92, 93]. Cellular levels of ceramide are increased in livers of patients with NASH [94]. However, blocking the ceramide synthesis does not abolish the palmitate-induced steatohepatitis [92, 93]. Further studies are required to determine the importance of ceramides in the development of NASH.

Diacylglycerol (DAG): DAG acts as an important second messenger that modulates the function of several signaling proteins. The plasma membrane levels of DAG increase upon stimulation of a multitude of cellular signaling pathways. DAG is primarily synthesized by the action of PI_2-specific phospholipase C isoenzymes, which are activated by G-coupled protein receptors, Ca^{2+}, protein receptor tyrosine kinases and Ras. Phospholipase C produces DAG and PI_3, which in turn lead to Ca^{2+} mobilization and further activation of the DAG synthesis and downstream signaling.. DAG, in synergy with Ca^{2+}, binds to and regulates the function of various downstream signaling molecules in particular protein kinase C (PKC), a serine/threonine kinase. PKC activates JNK, ERK and NF-κB which all activate the transcription of inflammatory cytokines [95]. DAG has been proposed to contribute in the progression of NAFLD. Serine/threonine activity of PKC induces insulin resistance and indeed the content of DAG and its activation of PKCε are found to be the best predictor of hepatic insulin resistance in human liver samples [96]. Although the hepatic cellular levels of DAG are found to be high in biopsy samples obtained from patients with NASH [86, 97], no differences are observed when comparing to simple steatosis [86].

Lysophosphatidyl choline: phosphatidyl choline or lecithin is synthesized from DAG and it is a major component of plasma membrane, lipid droplet, and VLDL particles. Lysophosphatidyl choline (LPC) is formed from lecithin under the action of phospholipase A2. Hepatocytes may undergo steatosis if the DAG→TAG pathway prevails or may enter apoptosis if the DAG→PC→LPC pathway predominates [93]. The balance of these two pathways might depend on a "second hit," such as inflammatory cytokines [93]. LPC is known to cause direct cellular injury and stimulation of immune cells, thus mediates the progression to NASH [98]. Similar to DAG, LPC also activates PKC in cells and induces apoptosis [93, 99]. Unlike DAG's inhibition, the inhibition of LPC synthesis blockes the palmitate-induced apoptosis in cultured and primary human

hepatocytes [93]. Studies have shown that LPC impairs mitochondrial permeability and causes the release of cytochrome c, which ultimately activates caspase reaction and initiates apoptosis [93], Figure 1.2. LPC is also known to activate JNK and subsequently enhance proinflammatory pathways [93, 100].

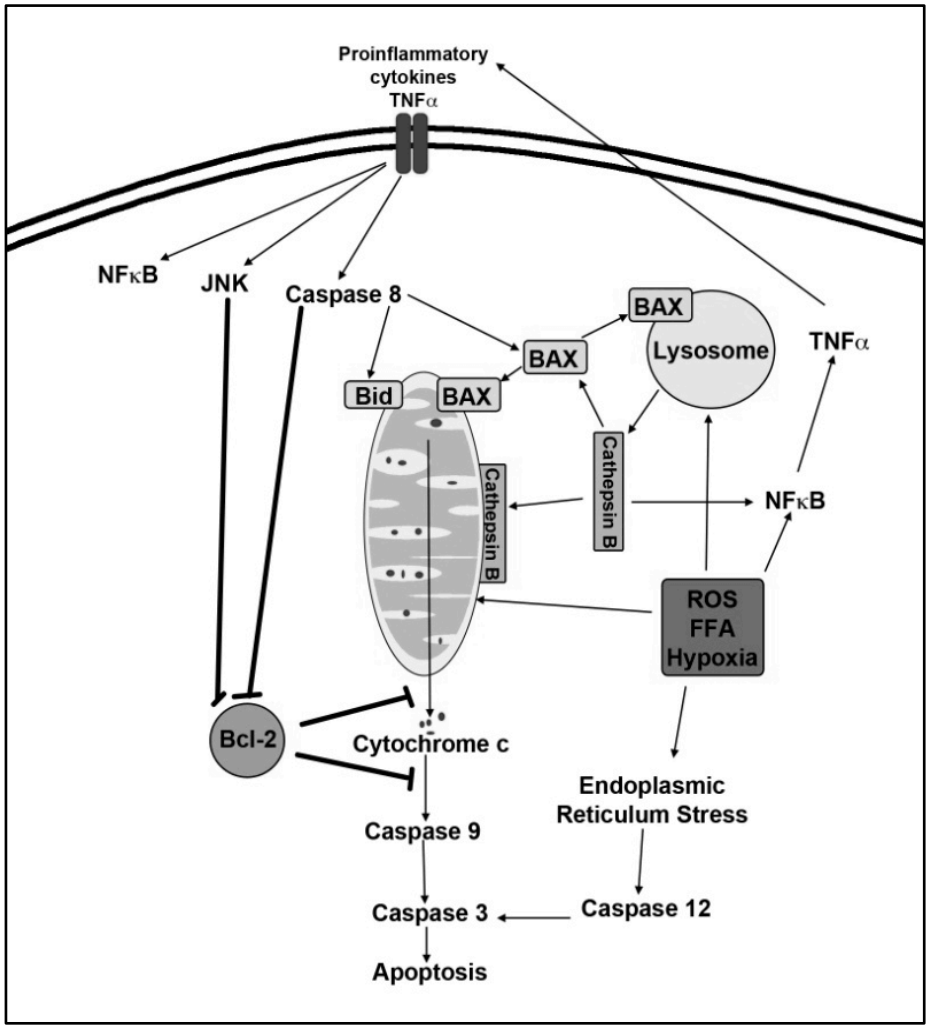

Figure 1.2: Cellular apoptotic pathway. Mitochondrial damage and increased membrane permeability causes release of cytochrome c that activates the caspase pathway which ultimately activates apoptotic cell damage pathways. Mitochondrial damage is achieved by binding to Bid, BAX and cathepsin B molecules. BAX is activated by proinflammatory signals from circulating cytokines or by the lysosomes. Free fatty acids (FFA), reactive oxygen species (ROS) and hypoxia induced by hepatic steatosis introduces damage to lysosomes and mitochondria and activate endoplasmic reticulum stress.

1.1.4.2 Oxidative stress and mitochondrial dysfunction

Oxidative stress has been implicated as an etiological factor in many progressive liver diseases including alcoholic steatohepatitis, Wilson's disease, hepatitis C and toxin induced liver failure. Reactive oxygen species (ROS), including superoxide ($O2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), and reactive nitrogen species, including nitric oxide (NO) and peroxynitrite ($ONOO^-$) are physiologically generated in normal hepatocytes and at low concentrations are critical for normal physiological processes, including oxidative respiration, apoptosis, and microsomal defense pathways [101]. In macrophages or Kupffer cells, ROS are also generated by the action of NADPH oxidase and myeloperoxidase[101]. ROS cellular toxicity encompasses their interaction with cellular macromolecules, denaturing or inactivating enzymes and causing DNA damage.

Lipid peroxidation can lead to the destruction of biological membranes and to production of reactive aldehydic products such as malondialdehyde or 4-hydroxynonenal. These lipid hydroperoxides have longer half lives than ROS and diffuse more to introduce damage to distant intracellular organelles and extracellularly [102]. Mechanisms by which these hydroperoxides potentiate development of NASH include ER stress, mitochondrial dysfunction, glutathione depletion, and the attenuation of VLDL secretion [103]. These products can also directly activate hepatic stellate cells and are chemotactic for neutrophils inducing further hepatocyte necrosis, inflammation, and liver fibrosis

ROS are direct cause of mitochondrial dysfunction due to their interference with oxidative phosphorylation and damage of the mitochondrial DNA [104]. Mitochondrial dysfunction blocks the normal electron flow for the reduction of oxygen to water. This will results in the transfer of free electrons to oxygen or lipids enhancing the generation of ROS.

Mitochondrial dysfunction in NASH pathogenesis may also result from the action of inflammatory cytokines. TNFα, produced by Kupffer cells, hepatocytes or adipocytes, activates Bax translocation to the mitochondria, which enhances the permeability of the outer membrane and blocks the electron flow[105], Figure 1.2. In addition, TNFα activates caspase 8 which enhances the release of cathepsin B from the lysosomes. Cathepsin B acts on the mitochondria and increases their membrane permeability and blocks the electron flow by releasing cytochrome c [106], Figure 1.2. The enhanced ROS production further leads to damage of mitochondrial DNA, membranes and proteins [101]. Collectively, enhanced mitochondrial permeability and cytochrome c release leads to the release of caspase 3 which later initiates cellular processes of apoptosis[106]. Apoptosis then further stimulates Kupffer cells activity and thus generates a vicious cycle. Oxidative stress is also known to promote the proliferation of stellate cells, and expression of adhesion molecules, which promote infiltration of immune cells [107, 108]. It is noteworthy that serum levels of ICAM-1, an adhesion molecule, are significantly elevated in patients with NASH [109].

Excessive ROS production is normally counteracted by enzymatic and non-enzymatic antioxidants. These enzymes include: superoxide dismutase, catalase, and glutathione (GSH) peroxidase. Another adaptive mechanism is the increased expression of uncoupling protein-2 (UCP-2) which reduces the cellular levels of ROS[104, 110]. Uncoupling of oxidative phosphorylation by UCP-2 allows the backflow of H^+ to the mitochondrial matrix and thereby oxygen tension is reduced. Upregulation of hepatic UCP-2 has been confirmed in liver biopsies from NASH patients[110]. However, this adaptive response may increase the hepatic susceptibility to other stressors such as hypoxia, TNFα- and drug-induced toxicity [104, 110, 111]. Hypoxia may result from impaired microcirculation observed in severe steatosis and this may further increase liver injury [112].

Non-enzymatic antioxidants include GSH, Vitamin E and C, and ubiquinone[101]. GSH, an endogenous antioxidant, is present at high concentrations in hepatocytes and mitochondria which helps to eliminates reactive molecules generated during oxidative stress[113]. Thus, the balance between oxidative stress and GSH levels may influence the development of NASH. Yet, the depletion of hepatic glutathione has not been reliably shown to occur in NASH [113]. However, treatment with antioxidants, e.g., vitamin E, β-carotene, and n-3 polyunsaturated fatty acids (PUFAs), alleviates hepatic steatosis and the extent of liver injury in humans and animal models [114, 115].

1.1.4.3 Proinflammatory cytokines

Together with the hepatocyte damage and fibrosis, inflammation is a marker of progression from simple steatosis to steatohepatitis and a great potential target for therapeutics. Inflammation in the liver results from the cross talk between parenchymal and nonparenchymal cells through circulating and paracrine cytokines. These cytokines are involved in the activation of hepatocytes, Kupffer cells and the transformation of stellate cells to the fibromyoblastic cell types[75]. Furthermore, pro-inflammatory cytokines and adipocytokines have been shown to play a role in the pathogenesis of NAFLD by modulating insulin resistance and lipid oxidation [101, 116]. Plasma adiponectin and resistin levels are negatively correlated with hepatic steatosis and inflammation in NASH [117]. TNFα is positively correlated with ongoing liver damage and inflammation [118]. Treatment with TNFα leads to functional and morphologic alterations in mitochondria while treatment with anti-TNFα antibodies improved mitochondrial function, inflammation, and alleviated hepatic steatosis in mouse models of NASH [119, 120]. Proinflammatory cytokines also induce gene expression and secretion of chemokines, which attract and activate other immune cells. These cells include T-cells and natural killer cells that upon activations cause cytotoxic damage. In the non-inflamed human liver, several chemokines, including the regulated on activation, normal T cell expressed and secreted chemokine (RANTES), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1) and IL-8, can be detected in vascular endothelium of portal tracts and their expressions increase markedly with inflammation [121, 122].

TNFα and IL-6 activate specific intracellular pathways that can enhance peripheral lipolysis and mitochondrial dysfunction. NF-κB homodimers and heterodimers are generated by association of five members of the Rel family that become activated upon assembly of a kinase complex formed by two catalytic subunits, IKK1 (IKKα) and IKK2 (IKKβ), and a regulatory subunit, IKKγ (NEMO) [123]. The IKK kinase complex phosphorylates a group of inhibitory proteins, named IκB, leading to their degradation through the ubiquitin–proteasome pathway[124]. The NF-kB dimers migrate to the nucleus and regulate gene transcription. NF-κB pathway is activated in the livers of rodent models and in patients with NASH[123]. Experimental activation of NF-κB is sufficient to induce low-grade inflammation and insulin resistance [125]. On the other hand, inhibition of IKKβ enhances insulin sensitivity [126]. The other inflammatory signal mediator is JNK which belongs to a family of intracellular mitogen activated protein (MAP) kinases. JNK mediates phosphorylation and inactivation of Bcl-2 and promotes apoptosis. In fat-induced cell toxicity, JNK also leads to Bax activation and initiation of apoptosis [127], Figure 1.2. TNFα can activate JNK directly, early activation, or indirectly and persistently by TNFα-induced ROS in the late persistent phase[128]. The persistant activation of JNK by TNFα may contribute to the liver damage[128]. Further evidence for the role of JNK in NASH development comes from studies on JNK1 null mice. Methionine-choline defiecient (MCD) diet-fed JNK1 null mice have significantly lower hepatic TAG accumulation, inflammation, CYP2E1 expression, lipid peroxidation, liver injury, and apoptosis compared to the wild-type littermates [129].

Toll-like receptors (TLR) are pattern recognition receptors that recognize microbial pathogens leading to the activation of innate immune system. Liver tissue from TLR4 knockout mice showed absence of inflammatory gene expression after high fat feeding [130]. This supports the role of TLR4 in the pathogenesis of diet induced NASH. In Kupffer cells, medium chain fatty acids activate TLR4 signaling, whereas the

polyunsaturated fatty acids and docosahexaenoic acid inhibit activation of various TLRs [131]. Palmitic acid activation of TLR4 triggers the activation of NF-κB and upregulation of its target genes TNFα, type I interferon, NO, IL-1 and IL-6 in leukocytes and Kupffer cells, all together contributing to the development of NASH [132].

IL1β is another cytokine implicated in the development of NASH. IL1β is primarily produced by the Kupffer cells and its expression increases after TLR4 or TLR9 activations [133]. Deletions in mice of either TLR9 or its signaling mediator Myd88 suppress the release of IL1β and produces phenotypes which resembles the one observed in mice lacking the IL1β receptor [133]. All these mice models are protected from diet induced NASH development [133]. Furthermore, treatment of hepatocytes with IL1β causes accumulation of TAG and induces cellular injury [133]. In addition, IL1β also stimulates hepatic stellate cells and induces fibrogenesis.

IFNγ in the liver is mainly produced by T-cells and exerts anti-proliferative and growth inhibitory actions on hepatocytes. IFNγ receptors are also present in Kupffer cells and stellate cells [134, 135]. IFNγ has been shown in numerous reports to be anti-fibrogenic, both inhibiting the activation of hepatic stellate cells in culture and attenuating drug-induced liver fibrosis[136]. IFNγ is currently being used in trials as an anti-fibrogenic therapeutic agent in patients with chronic liver disease [137, 138]. However, studies on mice fed with a choline-deficient diet demonstrated that liver fibrosis is enhanced when mice were treated with IFNγ and reduced in IFNγ-deficient mice [139, 140]. This finding is crucially important when applying IFNγ as a therapeutic target.

IL6 is generally elevated in conditions of NASH, insulin resistance and obesity. IL-6 in circulation is produced by adipose tissue, skeletal muscle and liver parenchymal and Kupffer cells [141]. Presently, the role of IL-6 in insulin resistance is unclear and still the subject of debate [141]. Long-term administration of IL-6 to lean rodents causes hepatic insulin resistance in mice but increases whole body insulin sensitivity and glucose tolerance in rats[142, 143]. Overexpression of IL-6 in mice causes a pronounced proinflammatory state but does not cause hepatic steatosis [144]. In mice, IL-6 deficiency causes insulin resistance and obesity. However, when fed with high fat diet these mice showed no differences in insulin sensitivity and adiposity when compared to the wild type littermates but developed exacerbated HFD-induced hepatic insulin resistance and inflammation [145,146, 147]. Similarly, deletion of the liver IL-6/gp130-STAT3 axis leads to hepatic steatosis and inflammation [148].

1.1.5 Role of non-parenchymal cells in development of NASH

Hepatocytes constitute the largest proportion of liver mass. Sinusoidal structures are lined with endothelial cells and the hepatic resident macrophages, know as Kupffer cells (KC). KC constitute 5% of liver cells and 31% of the non-parenchymal subpopulation of cells[149]. The biliary canaliculi are formed by cholangiocytes and stellate cells of mesenchymal origin, and it lies scattered in the space of Disse, Figure 1.3. Cross talk and exchange of proinflammatory cytokines between these cells is thought to contribute to the pathogenesis of NASH. Other circulating lymphocytes also play a role in the inflammatory-mediated progression of NASH and in particular polymorphonuclear leukocytes, T-cells and natural killer (NK) cells, Figure 1.2.

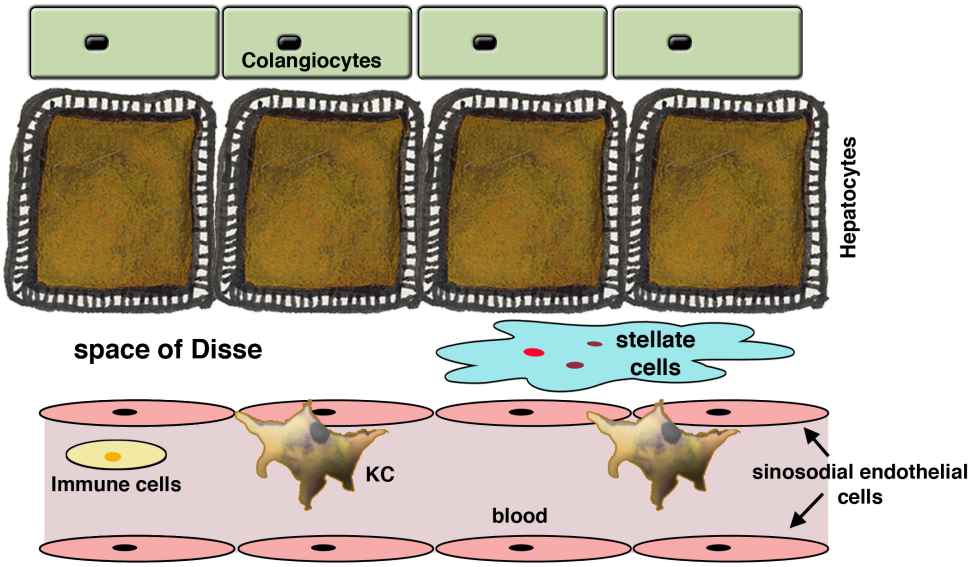

Figure 1.3: Subpopulation of cells in the liver. Liver is composed of parenchymal cells (hepatocytes) and nonparenchymal cells including endothelial cells, stellate cells, cholangiocytes and Kupffer cells (KC). Other immune cells (T-cells, NK cells, or leukocytes) can infiltrate the liver in disease conditions such as NASH.

Sinusoidal endothelial cells are generally activated in NAFLD due to increased circulating levels of proinflammatory cytokines, endotoxins, ROS and VEGF [150]. Activation of endothelial cells leads to the production of cytokines, which further can activate other immune cell [151]. Furthermore, local release of chemotactic molecules and cytokines from damaged hepatocytes activate the expression of adhesion molecules (ICAM, CD31, and E-selectin) in endothelial cells leading to infiltration of leukocytes [152].

Kupffer cells activation is positively correlated with NASH [153, 154]. Activation of KC in hepatic steatosis is achieved by multiple pathways. Circulating endotoxin and alarmins released from necrotic hepatocytes such as DNA fragments, heat shock proteins (HSPs) and nuclear high mobility box protein 1(HMBP1) activate toll-like receptors on KC [155], Figure 1.4. Other activating factors also include ROS, FFA, lipid hydroperoxides, hypoxia, MCP-1 and complement factors C3 and C5a [155]. Most recently, prostaglandin E2 produced by KC has been shown to stimulate the secretion of oncostatin M from KC which induces SOCS3 expression in the hepatocyte and causes hepatic insulin

resistance and steatosis[156]. Upon activation, KC express adhesion molecules (CD11b/CD18), which attract and recruit leukocytes to the liver [75]. Further, KC releases cytokines such as TNFα, IL-1β, nitric oxide and IL-6, which activate other immune cells and amplify the immune response [75]. Direct activation of NADPH oxidase (NOX-2) in KC and leukocytes leads to the production of ROS which causes further damage and stimulation of proinflammatory signaling in the liver [75]. It is noteworthy to mention that KC also a play critical role in the resolution of inflammation by clearing out the infiltrated leukocytes [157].

Additional cross talks originate from the stellate cells. Release of TNFα and TGFβ1 triggers transformation of stellate cells from the vitamin A-storing phenotype to the myofibroblastic phenotype, resulting in production of collagen types I and III and development of fibrosis [105]. Stellate cells can also be activated by long-chain polyunsaturated fatty acids and ROS, which may explain the observed low-grade hepatic fibrosis associated with obesity, insulin resistance and after high fat feeding [105].

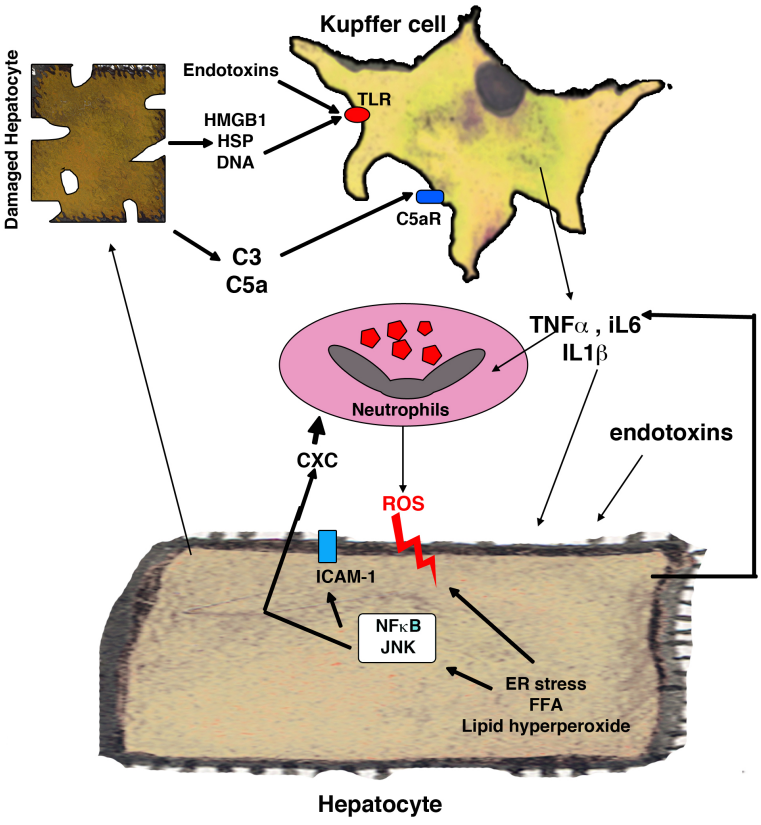

Figure 1.4: Cross talk between Kupffer cell and hepatocyte in the pathogenesis of NASH. Proinflammatory cytokines and molecules generated from stressed and damaged hepatocytes stimulate Kupffer cells to release further cytokines. Furthermore, these cytokines attract other immune cells to the liver and amplify the damage further. HSP: heat shock proteins. CXC: chemotactic substances

1.1.6 Role of anti-inflammatory pathways in development of NASH

Eicosanoids (prostaglandins, prostacyclins, thromboxanes and leukotrienes) are signaling molecules that are synthesized from arachidonic acid, docosahexaenoic acid (DHA) or eicosapentaenoic acid by the action of two families of enzymes cyclooxygenase (COX) and lipooxygenase [158]. They display anti-inflammatory actions and play a major role in the interaction between hepatocytes and endothelial cells with KC and other immune cells.

The enzymatic action of COX 1-2 (targets of NSAIDs drugs) leads to the formation of prostaglandin H_2 which is later converted into different types of prostaglandins and thromboxanes [158, 159]. The COX-derived PGE_2 from Kupffer cells was demonstrated to inhibit basal and TGFβ1-mediated collagen synthesis by the stellate cells [160]. More positive effects of PGE_2 are evident in the improvement of liver microcirculation and suppression of LPS-induced TNFα, NO, and ROS release through the stimulation of PGE_2 receptor EP4 [161-163]. However, contradicting findings were obtained from a study in which *in vivo* treatment with a COX-2 inhibitor attenuated the progression to NASH in a steatotic mouse model [164]. In this model, COX2 inhibitor treatment caused decreased hepatic TAG accumulation via the upregulation of PPARα [165, 166], hence eliminating the first hit. However more studies should be conducted before considering the therapeutic role of PGE_2 in the development of NASH. Studies have shown some unwanted effects of PGE_2 on hepatocytes. PGE_2 attenuated insulin signaling in rat hepatocytes by an EP3-R mediated activation of ERK1/2 that causes serine phosphorylation of IRS [167]. PGE_2 also stimulates the secretion of oncostatin M from Kupffer cells which acts on hepatocytes and induces insulin resistance and steatosis through the activation of lipogenesis and inhibition of VLDL secretion [156].

Lipoxins are generated from arachidonic acid via the lipoxygenase-mediated transcellular biosynthesis. The latter term describes the process of synthesis of a metabolic intermediate by a donor cell and the formation of the active metabolite by another cell [168, 169]. In the liver, this process has been shown in the interactions between KCs, endothelial cells and hepatocytes. Hepatocytes express the 15-lipoxygenase enzyme that generates 15-hydroxyeicosatetraenoic acid (15-HETE) from arachidonic acid. 15-HETES is then catalyzed to lipoxin LXA4 by the Kupffer cell enzyme 5-lipooxygenase (5-LO) [170]. In addition, in the KCs 15-HETE is also generated by the action of aspirin-acetylated COX-2 enzyme which later gets transformed by the KC or neutrophil 5-LO into the aspirin triggered 15-epi-LXA4 [170]. 15-epi-LXA4 and LXA4 have been found to modulate the actions of transcription factors (e.g., PPARα) and regulate cytokine production and chemotactic properties of hepatic cells [171-174]. Furthermore, lipoxins and aspirin-triggered leukotrienes inhibit proinflammatory actions and the recruitments of neutrophils to the liver [171]. Recently, it has been shown that lipoxins stimulate lipid disposal by enhancing the expression of PPARα in hepatocytes suggesting additional NAFLD protective actions[173].

1.1.7 Insulin resistance in NASH progression

The action of insulin is initiated through its binding to the extracellular α-subunit of the insulin receptor which activates the intracellular tyrosine kinase domain of the β-subunit [175]. Receptor autophosphorylation results in insulin receptor substrate (IRS-1 and 2) phosphorylation and IRS-associated phosphatidylinositol 3 phosphate kinase (PI3K) activation. PI3K consists of a regulatory subunit, p85, and a catalytic subunit p110. Phosphatidylinositol (3,4,5)-trisphosphate, generated by PI3K, recruits the

protein kinase B (PKB or Akt) to the plasma membrane, which then gets activated through the phosphorylation of serine 473 by mTORC2 and threonine 308 by PDK1 [175]. Akt activation mediates the downstream effects of insulin such as inhibition of gluconeogenesis and glycogenolysis. Insulin resistance results from defects in the insulin signaling cascade and is commonly observed in patients with NAFLD and NASH. In inflammatory conditions, serine phsophorylation of the insulin receptor or IRS inhibits signal transduction and activation of downstream Akt[176]. Figure 1.5 summarizes some of the factors which inhibit the insulin signaling cascade including ceramides, DAG activated PKC isoforms and ROS [177-180].

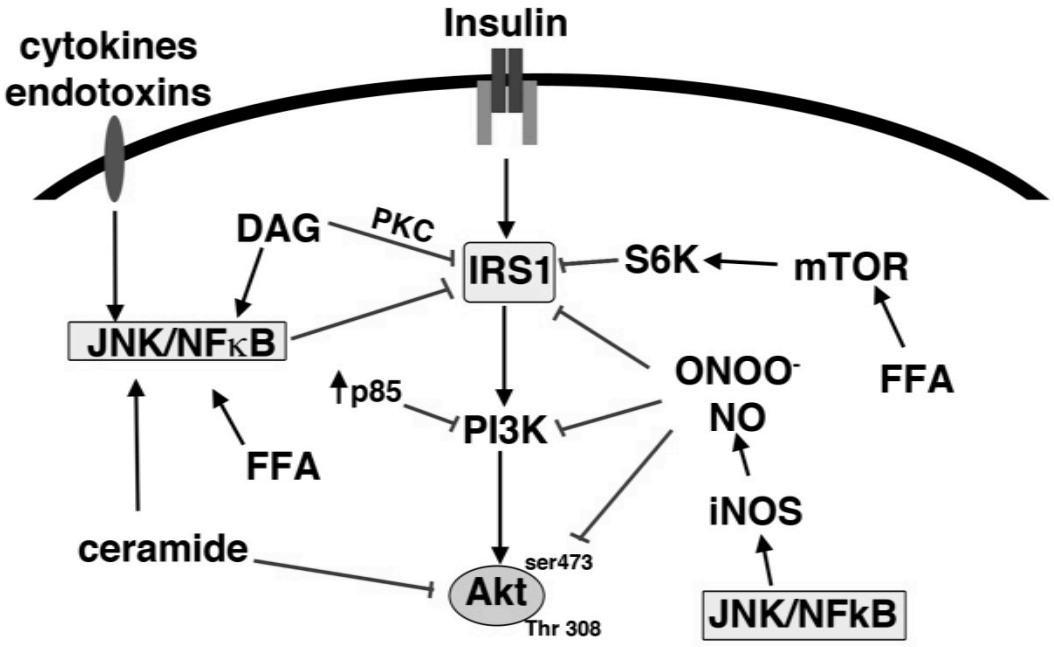

Figure 1.5: Mechanism of cellular insulin resistance. Excess lipid intermediates in the TAG biosynthetic pathway, such as diacylglycerol (DAG) and free fatty acid (FFA) cause insulin resistance by activating several serine/threonine kinases, including protein kinase C (PKC), mammalian target of rapamycin/p70 S6 kinase (mTOR/S6K), and c-Jun N-terminal kinase (JNK), which inhibits insulin signaling either directly through serine phosphorylation of insulin receptor substrates (IRS)-1 and IRS-2 or indirectly through a series of transcriptional events mediated by nuclear factor NFkB. This, in turn, results in reduced insulin activation of phosphatidylinositol (PI) 3-kinase and Akt. Ceramide can also impair insulin action through interactions with Akt. Increased levels of monomeric p85 competitively inhibit the binding of the p85/p110 heterodimer (PI3K) to the phosphorylated IRS-1.

Peripheral insulin resistance results in enhanced lipolysis and impaired lipid storage as a result of reduced inhibition of hormone-sensitive lipase (HSL) and reduced activity of transcription factors involved in lipid droplet formation, such as PPARγ [181]. High plasma insulin, FFA and glucose all together enhance DNL in the liver and cause hepatic steatosis [107]. Insulin resistance in obesity is also accompanied by elevated levels of plasma proinflammatory cytokines TNFα, IL-6 or IL-1β, which promote liver toxicity as described earlier [182]. This data indicates that insulin resistance could be considered a causative factor for NAFLD. However, several mouse models have shown that NAFLD or NASH can occur without major deterioration in whole body insulin sensitivity [183]. Such models include those with defective uptake of lipid in peripheral tissues like CD36 and skeletal muscle specific-LPL deletion. In addition, mouse models of reduced hepatic β-oxidation or VLDL secretion are non-obese but present with hepatic steatosis; these include the MCD-fed mice and mice with deletion of PPARα, MTTP, or ACOX [39, 183]. In humans, SNPs identified in the DGAT2 and PNPLA3

genes, involved in TAG packaging, has been shown to be associated with fatty liver but not with insulin resistance [184, 185]. This suggests that the pattern of lipid mobilization and partitioning plays a major role in the dissociation/association between insulin resistance and hepatic steatosis.

1.2 ROLE OF GROWTH HORMONE IN REGULATION OF HEPATIC LIPID CONTENT

Growth Hormone (GH) is the key regulator of body size in mammals and an important regulator of lipid and glucose metabolism. GH is secreted by the pituitary gland and binds the GH receptor in target cells where it activates various signaling pathways including JAK2, STAT5b, PI3K, MAP kinase, PKC, and phospholipase A2 [186]. In the liver, the GH signal cascade induces the transcription of specific genes, such as IGF-1, IGF-binding proteins (IGFBPs), the acid-labile subunit (ALS), and SOCS proteins [186]. GH metabolic actions can be exerted directly or indirectly, by IGF-1 or adipocytokines, or can be part of GH antagonism of insulin signaling [186]. The main actions of GH are anabolic in muscle resulting in increased lean body mass from the promotion of protein synthesis combined with a reduction of nitrogen excretion. By promoting adipose tissue lipolysis and hepatic lipid mobilization GH increases circulating fatty acids and promotes their utilization in muscle at the expense of glucose. The end result is that GH has hyperglycemic (diabetogenic) actions. The lipolytic actions of GH in adipose tissue results from enhancing hormone-sensitive lipase activity and stimulation of the β-adrenergic receptors [187]. GH does not increase the lipoprotein lipase (LPL) activity in the adipose tissue which suggests no direct effects of GH on triacylglyceride uptake in adipose tissue [188]. Finally, GH also reduces circulating leptin and increases resistin levels, while the effect on adiponectin remains controversial [188]. In liver and unlike its action on adipose tissue, GH induces triacylglyceride uptake by increasing LPL, hepatic lipase and LDL receptor expression [188]. Furthermore, GH induces hepatic DNL through the induction of fatty acid synthase (FAS) and Stearoyl-CoA desaturase (SCD1) expression. These activities are counteracted by enhanced hepatic VLDL secretion [189, 190] in connection to GH induction of apoB48 and MTTP proteins levels[189, 190]. The end result of GH actions in liver is reduction of TAG accumulation. Patients with NAFLD have lower plasma GH levels than controls [191]. Adult GH deficiency (GHD) is associated with NAFLD [192, 193] and patients characteristically develop abdominal obesity with increased total plasma cholesterol and triacylglycerides and decreased high-density lipoprotein (HDL)-cholesterol levels, resembling the metabolic syndrome [194]. In addition, 29% of children with GH deficiency developed NAFLD after cessation of GH therapy [194]. Reduced steatosis is observed in GH transgenic mice and after GH replacement therapy in adults with GH deficiency.

The protective action of GH on liver steatosis has also been highlighted in genetic studies showing hepatic inactivation of the GH receptor (GHR) [195, 196], its associated kinase, JAK2 [197] or its downstream signaling intermediary STAT5b [198] leads to hepatic steatosis. Due to the disruption of hepatic IGF-1 production, the negative feedback regulation on GH secretion is disrupted and these mice exhibit elevated circulating levels of GH. A very recent study showed that abrogation of GH rescued the development of fatty liver in the JAK2 knockout mice [199]. In this context, increased steatosis was attributed to the GH-augmented adipose tissue lipolysis and subsequent increase in circulating FFA levels[197]. The net effects of GH on hepatic lipid metabolism might be influenced by GH antagonism of insulin signaling [200]. Compared

to treatment with GH alone, in vivo co-stimulation with insulin results in lower hepatic TAG content despite lower VLDL secretion [201]. These findings suggest that the effect of GH on hepatic triacylglyceride secretion is dependent on the GH antagonism of insulin actions in the liver while hepatic TAG content may also be influenced by the GH antagonism of insulin anti-lipolytic actions in adipose tissue.

1.3 METABOLIC ACTIONS OF SOCS PROTEINS

The suppressor of cytokine signaling (SOCS) protein family comprises eight different intracellular proteins (SOCS 1-7 and CIS) whose functions are to regulate cellular sensitivity to cytokine and growth factor signals. SOCS regulate JAK kinase associated cytokine receptors (e.g. those for leptin, GH, interferons and erythropoetin) but also tyrosine kinase receptors such as those responsive to insulin, IGF-1, SCF and EGF [186, 202]. Studies using genetic inactivation of different SOCS proteins in mice have demonstrated that individual SOCS protein have specific physiological functions.

All SOCS proteins are characterized by a centrally located SH2 domain, an amino-terminal domain of variable length and sequence, and a carboxyl-terminal 40-amino-acid module known as the SOCS box. The function of the SOCS box is the recruitment of an ubiquitin-transferase system. The SOCS box interacts with Elongins B and C, Cullin5, Rbx 2 and E2 ubiquitin conjugating enzymes to form an E3 ubiquitin ligase complex. Thus, CIS/SOCS family proteins, as well as other SOCS-box-containing molecules, probably function as E3 ubiquitin ligase to mediate the ubiquitination and possibly the proteasomal degradation of targeted proteins [203]. The recognition of targeted proteins by SOCS is thought to be mediated by the SH2 domain binding to tyrosine phosphorylated targets. Additional mechanisms may also be involved SOCS regulation of cellular signaling. Both SOCS1 and SOCS3 exhibit homology within a 12-residue sequence known as the kinase inhibitory region (KIR) [204]. The KIR binds to the activated JAK and can act as a JAK pseudosubstrate preventing the access of other legitimate substrates to the JAK catalytic pocket, thus inhibiting its activity. .

1.3.1 SOCS-1 and SOCS-3:

In multiple cell lines, SOCS1 and SOCS3 have been shown to bind IRS-1 and IRS-2 and promote their ubiquitination and proteosomal degradation [205]. Mutations in the SOCS box domain of SOCS1 inhibit the ubiquitination and degradation of IRS-1 and IRS-2 [205]. Hepatic expression of SOCS1 and SOCS3 has been reported to be elevated in rodent treated with GH and in models of obesity and insulin resistance [206]. Furthermore, *db/db* mice treated with SOCS1 and SOCS3 antisense oligonucleotides show improvement in insulin signaling and hepatic lipid accumulation [206]. In addition, hepatic short-term overexpression of SOCS1 or SOCS3 attenuated STAT3 activity which causes induction of lipogenesis via SREBP1c activation[206]. *In vivo* over-expression of SOCS3 in adipocytes leads to a reduction in IRS-1 protein which causes diminished glucose uptake and reduced lipogenesis [207, 208]. In addition, these SOCS3 transgenic-mice were resistant to the development of diet-induced obesity and its associated insulin resistance. On the other hand, TNF-α-induced IRS-1 and IRS-2 degradation was suppressed in adipocytes of SOCS3 deficient mice[209]

The previous results suggest that SOCS3 negatively regulates insulin signaling contributing to hepatic steatosis. However, opposite findings were observed when this

concept was tested in SOCS3 liver specific knockout mice. These mice developed hepatic steatosis after high fat diet feeding [210]. The author hypothesized that the differences observed are due to unknown hypothalamic effects of liver specific SOCS3 deletion that result in increased food intake and decreased energy expenditure [210], which were absent in other short-term *in vivo* or cell culture based studies [205 206].

SOCS1 exerts negative inhibition of the INFγ signaling and SOCS1$^{-/-}$ mice die before weaning from multiorgan inflammatory disease that is manifested in liver by severe steatohepatitis. This phenotype could be rescued by concurrent deletion of the INFγ gene [211]. In the liver, SOCS1 also downregulates hepatic growth factor (HGF) signaling and displays anti-oncogenic properties in the liver [212]. Inhibition of hepatic SOCS1 expression in *db/db* mice enhances hepatic insulin sensitivity and reduces of hepatic steatosis [206] suggesting a role for SOCS1 in the pathogenesis of NASH.

1.3.2 SOCS 6 and 7:

Recent studies have shown the involvement of SOCS 6 and 7 in insulin signaling [213, 214]. SOCS6 has been shown to bind to the monomeric p85 regulatory subunit of PI3 kinase enhancing the activity of the catalytic subunit and increasing insulin sensitivity [213]. Accordingly, over-expression of SOCS6 in mice results in improved glucose tolerance and insulin sensitivity [213]. On the other hand SOCS6$^{-/-}$ mice do not display any abnormalities in glucose homeostasis [215] suggesting that SOCS6 activity on insulin signaling may be redundant. SOCS7 is a close homolog of SOCS6, sharing greater than 50% identity in the SH2 domain, and it has also been reported to interact with PI-3 kinase. SOCS7$^{-/-}$ mice display improved glucose tolerance and enhanced insulin sensitivity compared to the wild-type mice [214]. These mice display increased islet size due to enhanced insulin sensitivity at the β-cells level.

1.3.3 SOCS2:

GH receptor activity is counteracted through a negative feedback loop exerted by SOCS2, which interacts and inhibits GH receptor signaling [216, 217]. The gigantism observed in SOCS2$^{-/-}$ mice is not observed in other mouse models of SOCS inactivation and identifIes this proteins as the key negative regulator of GH receptor signaling *in vivo* [186]. In agreement, overgrowth in SOCS2$^{-/-}$ mice was attenuated in the double SOCS2/STAT5b knockout mice or in SOCS2$^{-/-}$ mice made GH deficient [218]. GWAS studies have identified SNPs in the vicinity of SOCS2 associated to increased postnatal growth [219]. A single patient has been identified with a mutation within the SH2 domain of SOCS2, he presented with acromegaly with normal plasma GH levels and plasma IGF1 in the upper limit of normal [220]. In a Japanese cohort, several SNPs in the SOCS2 gene promoter region were found to be associated with increased risk of type 2 diabetes [221]. Further studies are needed to elucidate the exact mechanism whereby SOCS2 acts on insulin signaling.

Despite the increased GH activity in SOCS2-deficient mice (SOCS2$^{-/-}$), these mice show normal glucose tolerance and insulin sensitivity, which may be attributed to the increased lean body mass and reduced levels of GH observed in these mice [222]. Under normal dietary conditions, SOCS2$^{-/-}$ mice exhibit reduced TAG accumulation in the liver. SOCS2 has been shown to have other functions related to neuronal development, bone growth, estrogen and glucocorticoid signaling and inflammation [223]. The later SOCS2 function is relevant in our discussion of the role of inflammation in the progression of NAFLD. SOCS2 has shown anti-inflammatory properties and its mRNA

and protein levels were induced by anti-inflammatory mediators such as lipoxin (LXA4) [223]. In addition, aspirin's anti-inflammatory actions and inhibition of NF-κB signaling is found to be SOCS2 dependent in mice [223]. Furthermore, SOCS2$^{-/-}$ mice have abnormal leukocyte infiltration, an uncontrolled production of proinflammatory cytokines leading to higher mortality observed after infection [223]. Recently, SOCS2 was implicated in the acceleration of dendritic cell (DC) maturation, an antigen-presenting cell of the innate immune system [224]. Furthermore, SOCS2 deletion in DC enhanced TLR signaling and increased the STAT3 mediated expression and secretion of IL-1β and IL-10 [225] [224]. Overall, the anti-inflammatory role of SOCS2 might suggest a protective role in the development of NASH.

1.3.4 Cullin-RING-Ligase system

SOCS are thought to target cellular signaling intermediates for degradation via the ubiquitin-proteasome system. Ubiquitin, a small conserved protein of 76 amino acids, is first conjugated to the E1-ubiquitin activating enzyme. This is an ATP-requiring thioesterification reaction between the C-terminal glycine residue of ubiquitin and the sulfahydryl group of cysteine residue in the E1. Ubiquitin is then transferred from the E1 to a cysteine residue of the E2 (ubiquitin conjugating enzyme, UBC) enzyme. Almost 30-40 UBCs exist in the human genome. Finally, through the action of a ubiquitin ligase (E3), ubiquitin is transferred to a lysine residue in the target protein, Figure 1.6. The Lys48-linked polyubiquitination chain on target proteins makes them a substrate for the 26S proteasome [226].

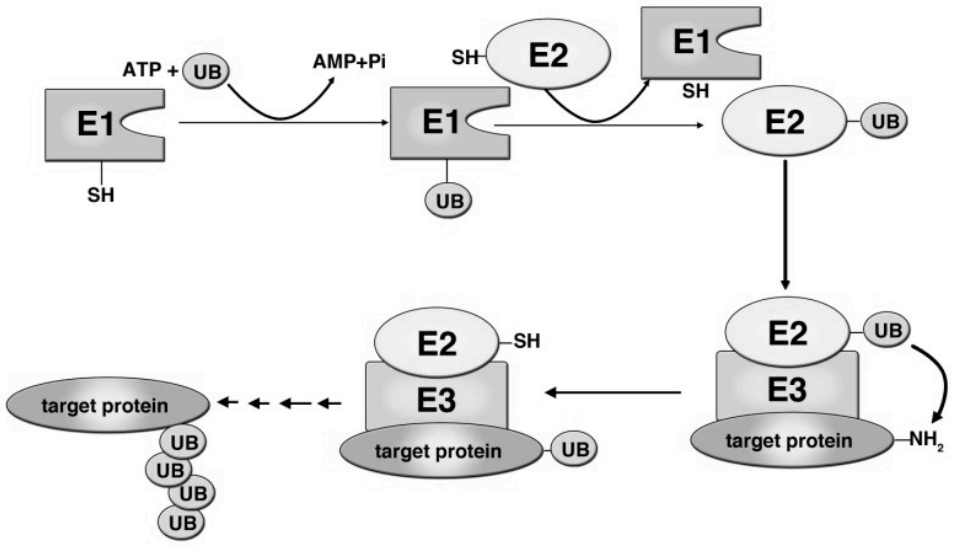

Figure 1.6: Protein ubiquitination system. Ubiquitin (UB) molecule is activated by the ubiquitin activating enzyme (E1) and then conjugated to the ubiquitin conjugating enzyme (E2). The ubiquitin ligase (E3) recognizes the target substrate and catalyzes the transfer of UB molecule.

Two main classes of E3 are evident at the primary sequence level: those bearing a HECT (Homologous to E6-AP C-Terminus)-type catalytic domain and a prominent class containing a RING (Really Interesting New Gene) domain. In contrast to HECT-type E3 enzymes, which contain an active cysteine residue and form an obligate thioester bond with ubiquitin prior to transfer to the substrate. RING-type E3s serve as a docking site for the ubiquitin-activated E2, which provides the catalytic activity [226].

The largest E3 superfamily consists of the multisubunit Cullin-RING ligases (CRLs).

CRLs consist of an extended cullin scaffold interacting with a catalytic RING-containing protein, either Rbx1 or Rbx2 [226]. The human genome encodes at least seven cullins including CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5 and CUL7. They form around 300 distinct CRL complexes in different subfamilies (CRL1 containing CUL1, CRL2 containing CUL2, etc.). SOCS2-7 and CIS interact with the Cullin5/Rbx2 complex while SOCS1 interacts with Cullin2/Rbx1.

1.3.4.1 Substrate recognition by CRL

Even though CRL subclasses contain the same catalytic core, they use different substrate-recognition modules. In most cases these modules use a BTB (Bric-a-Brac, Tramtrack and Broad complex)-fold adaptor to interact with the amino-terminal domains of cullins, and thus provides specificity for the interaction with the various substrate-recognition modules [227]. In the classical SCF (Skp1-Cullin-F-box), the BTB-fold adaptor SKP1 connects the CUL1 to F-box proteins, which recognize their substrates through various substrate interaction motifs, such as WD40 motifs or Leucine Rich Repeats (LRR). In CRL2 and CRL5 complexes, the BTB-fold adaptor Elongin C connects CUL2 and CUL5 to BC-box proteins (e.g. SOCS box), which in turn binds their substrates primarily through LRR, ankyrin repeats or SH2 domains[228]. Despite the fact that both CUL2 and CUL5 use the same adaptor, they engage different classes of substrate-recognition subunits, termed VHL-box and SOCS box proteins respectively [228]. The ubiquitin-like protein Elongin B is also part of CRL2 and CRL5. In CRL3 complexes, a single polypeptide fulfils the function of SKP1/ElonginC and F-box/BC-box dimers and bridges CUL3 to substrates [227]. Illustrations of these examples are displayed in Figure 1.7.

1.3.4.2 Catalytic domain of CRL

The C terminal of CRL consists of the same catalytic core composed of a cullin-RBX1 heterodimer bound to the E2-ubiquitin, except for CUL5, which also binds RBX2. In the CRL complex, the ubiquitin molecule on the E2 is spatially distant from the substrate and hence to activate the ubiquitin transfer the cullin protein gets covalently neddylated at a conserved lysine residue present in all cullin family members, Figure 1.8. Neddylation requires the ubiquitin-like protein NEDD8 (Neural precursor cell Expressed Developmentally Down-regulated protein 8) [229]. NEDD8 attachment stimulates multiple CRL ubiquitin E3 activities by promoting the binding to E2-ubiquitin, enhancing ubiquitin transfer from the E2 active site, and by positioning the E2 active site adjacent to the substrate. The NEDD8 conjugation is catalyzed by two NEDD8-specific E2s, Ubc12 and UBE2F, that interact with the two different RBX proteins to modify specific cullins [230]. For example, UBE2F and Rbx2 neddylate CUL5. Cullins get deneddylated by a multisubunit enzyme named the COP9 Signalosome (CSN) [229]. The catalytic component, CSN5, is a member of the zinc metalloproteinase family. Another mechanism of CRL activation might include dimerization through the substrate recognition motif or the NEDD8-cullin; although CUL2 and CUL5 CRL complex has only been observed as monomers [231, 232]. It is worth mentioning that CRL can be inhibited via CAND1 (Cullin-Associated and Neddylation- Dissociated 1). CAND1 binds specifically to free cullin-Rbx complexes lacking NEDD8 and inhibits CRL assembly and NEDD8 activation [233].

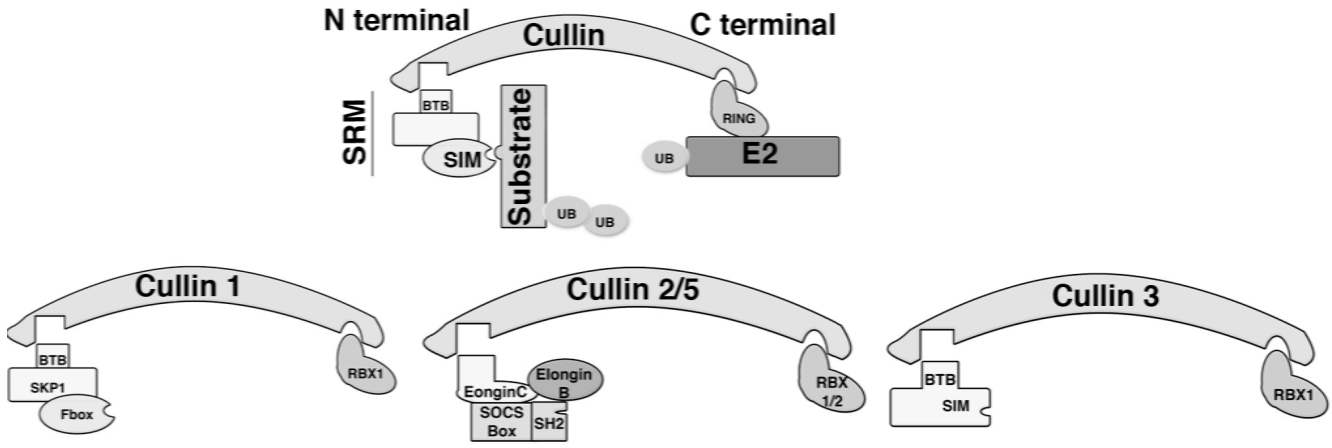

Figure 1.7: Composition of cullin-RING E3-ligases (CRLs). CRLs are formed around a cullin scaffold protein, which recruits the substrate-recognition module (SRM) through its N-termini and the catalytic site through its C-terminal part. The basic composition of a CRL with CUL1, CUL2, CUL3 and CUL5 are illustrated and described in the text. The catalytic module contains the RING domain (Rbx1 or Rbx2) and hosts the E2 enzyme which catalyzes the transfer of ubiquitin to the substrate. The N-terminal of cullin contains a docking site for a BTB domain present in the adaptor molecules. In CRL2 and 5, the substrate interaction motif (SIM) represents the SH2 domain in SOCS protein which specifically bind to phosphorylated substrates

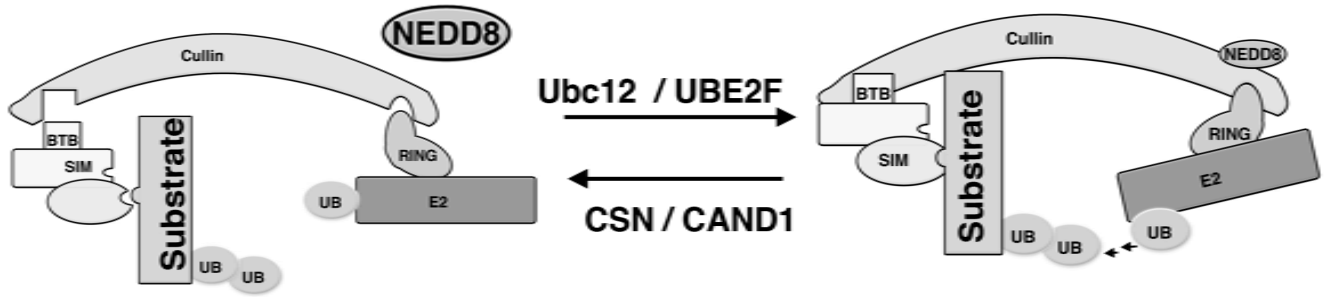

Figure 1.8: Regulation of the CRL system. Neddylation of CRL is catalyzed by conjugation of NEDD8 via E2 enzymes Ubc12 and UBE2F. CSN and CAND1 are involved in the deactivation of the CRL complex.

2 AIM OF THE STUDY

The main goal of this thesis is to evaluate the mechanism of actions and physiological significance of SOCS2 and SOCS6 proteins. We hypothesised that SOCS2 and SOCS6 play important roles in the regulation of hepatic insulin and GH signalling and also hepatic lipid content. We therefore aimed to:

- To explore the E3-ligase activity of SOCS proteins toward cytokine and growth factor receptors. Paper 1 and 4.
- To investigate the physiological of role of SOCS2 in the control of hepatic metabolism in conditions of dietary stress. Paper 2
- To investigate the role of growth hormone-SOCS2 axis in understanding the pharmacological actions of the LXR ligands on hepatic lipid metabolism. Paper 3
- To characterize the structural binding of SOCS6 to target proteins. Paper 4

3 MATERIALS AND METHODS

3.1 ANIMAL AND CELL MODELS

In the studies presented in this thesis we have used the SOCS2$^{-/-}$ mice (C57BL/6J) previously described [234]. Animal experiments were held in the animal facilities at the university Las Palmas of Gran Canaria, Canary Islands, Spain and Karolinska Institutet, Sweden. To induce diet-induced insulin resistance and obesity, mice fed a regular chow diet (CD) (SAFE-diet A04, Panlab SLU, Barcelona, Spain) or high fat diet (HFD, OpenSource DIETS, D12492) *ad libitum* for 2 and 4 months. The high-fat diet contained 34.9% fat, 26.3 % carbohydrate, and 26.2 % protein per unit weight. Bone marrow derived macrophages were isolated from SOCS2$^{-/-}$ mice, cultured and used to study TLR4 receptor signalling. Primary human hepatocytes were isolated from healthy human liver tissue from donors in hepatic transplants. All human and animal experimental protocols were according to the Karolinska Institutet and University of Las Palmas Gran Canaria ethical guidelines (ethical permits No. 2010/678-31/3, N415/08, N46/11 and N363/08). Isolated hepatocytes were obtained from the liver cell laboratory, KI-CLINTEC, Huddinge University Hospital. Cells were maintained in Williams E medium as described previously [235]. GH signaling was studied in BRL4 cells, which are stably transfected with the rat GHR gene and express endogenous JAK2 and STAT5b [236] (paper III). To study the ubiquitin ligase activity of SOCS2 and SOCS6, transient transfection of GHR and c-KIT were performed in HEK293T cells (paper I and IV).

Table 3: Calorie contents of diets used in the study (% of total calories)

	Control diet (CD)	High fat diet (HFD)
Fat	3 %	60%
Carbohydrates	60%	20 %
Proteins	16%	20%

3.2 MEASUREMENT OF WHOLE BODY INSULIN SENSITIVITY

Insulin sensitivity was assessed *in vivo* by measurement of fasting blood glucose, fasting plasma insulin, intraperitoneal glucose tolerance test (ipGTT), intraperitoneal insulin tolerance test (ipITT), and homeostatic model assessment of insulin resistance (HOMA-IR). Plasma insulin levels were measured using commercial ELISA kits. For ipGTT, mice were fasted 16 h overnight followed by an i.p. injection of D (+)-glucose (20% in 0.9% NaCl) at a dose of 2g/kg body weight. Blood glucose levels were measured using a glucometer (Roche Diagnostics, Basel, Switzerland). For ipITT, human insulin was intraperitoneally injected into 4-h fasted mice at a dose of 0.75 U/kg body weight and glucose levels were determined as explained above. HOMA-IR was calculated as follows: fasting insulin (ng/mL) × fasting glucose (mM).

3.3 ASSESSMENT OF PANCREATIC FUNCTION

We characterized the SOCS2$^{-/-}$ pancreatic phenotype by measuring plasma insulin at fasting and 10 minutes after ipGTT (index of insulin secretion). We also analyzed pancreatic insulin content and insulin/glucagon ratio by immunohistochemistry (IHC).

Insulin extractions were carried out overnight on pre-weighed homogenized pancreas at 4°C under constant shaking followed by centrifugation. Supernatants were diluted 1000 fold and insulin concentrations were determined using an ELISA kit (Ultra. Sensitive Rat Insulin ELISA; Crystal Chem, Downers. Grove, IL, USA). Paraffin embedded tissue was sectioned for insulin and glucagon immunostaining. Primary antibodies were detected with an appropriate biotinylated secondary antibody in conjunction with Vectastatin ABC peroxidase kit (Vector Laboratories, Carpenteria, CA) and chromogen diaminobenzidine (Sigma, St. Louis, MO). Slides were counterstained in hematoxylin and eosin. We further calculated HOMA-β cell function as follows: 20 × fasting insulin (μU/mL)/fasting glucose (mmol/L) − 3.5.

3.4 *IN VIVO* HEPATIC TRIACYLGLYCERIDE SECRETION ASSAY

Triacylglyceride secretion rate *in vivo* was measured by intravenous administration of Triton WR-1339 (Sigma, St. Louis, MO, USA) which blocks the peripheral hydrolysis of triacylglycerides. The animals were fasted for 6 h to avoid the influence of chylomicrons from the intestine. Thereafter, they were injected intravenously with Triton WR-1339 diluted in saline (200 mg/ml) via the tail vein (500 mg/kg body weight). Blood samples were taken before the injection (baseline fasting triacylglyceride concentration) and 30, 60 and 90 min after Triton WR-1339 administration. The triacylglyceride accumulation was linear during this time period. Total plasma content of triacylglycerides was calculated using the published plasma volume in male mice (0.071 ml/g). Hepatic triacylglyceride secretion rate, expressed as total μmol TAG/min was calculated from the slope of the curve.

3.5 MULTIPLEX IMMUNOASSAY

Multiplex technology allows the simultaneous qualitative or quantitative analysis of analytes using color-coded microspheres in a single tube or well. We utilized flow cytometric multiplex arrays, also known as bead-based multiplex assays. Different bead sets employ properties (e.g. size or fluorescence), which are distinguishable under flow cytometry. Each bead set is coated with a specific capture antibody, and fluorescence or streptavidin-labelled detection antibodies bind to the specific cytokine-capture antibody complex on the bead set. This allows the identification and quantification of various analytes using one sample volume.

In our study we employed multiplex technology to quantify cytokine levels IL1β, IL-6, interferon (IFN) γ, TNFα, and RANTES. Cytokine levels were measured both in plasma and media collected from cell cultures. The multiplex assay was also used to quantify AKT, JNK , and IRS1 phosphorylation in tissue homogenates. Traditional single analyte ELISA kit were used to measure plasma GH, IGF1, leptin and insulin levels.

3.6 PLASMID CONSTRUCTION AND IN VITRO MUTAGENESIS

In paper IV, we constructed the pFlag-SOCS6 expression plasmid using PCR-based technology and ligation, Figure 3.1. The human SOCS6 open reading frame was obtained from a commercial source (Invitrogen, Carlsbad, CA). A DNA fragment containing the full length human SOCS6 was amplified by PCR using a high fidelity *Pfu* DNA polymerase and cloned into the pFlag-CMV2 vector (Sigma-Aldrich) to express a N-terminally Flag-tagged version of hSOCS6 in mammalian cells. To insert a

mutation in the cloned gene, we applied site-directed mutagenesis. Two complementary primers are designed to carry the mutation of interest. Thereafter, these primers anneal with the wild type methylated plasmid and create a mismatch at the mutation site. Then PCR reaction is carried out using a high fidelity DNA polymerase to synthesize a new non-methylated plasmid from the two primers carrying the mutation. Before cellular transformation, the wild type plasmid is removed by the action of the *Dpn1* enzyme, which digests the methylated DNA only, Figure 3.1. Insert and mutant sequences were then confirmed by DNA sequencing.

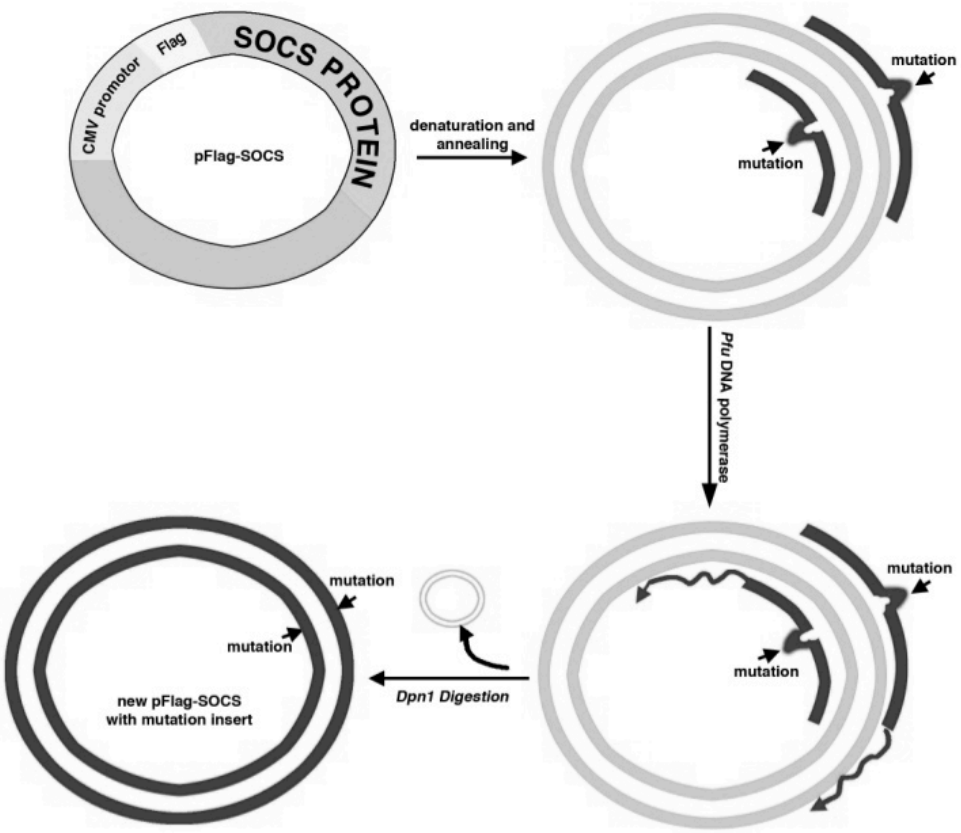

Figure 3.1: *In vitro* site directed mutagenesis. The wild type plasmid contains the SOCS gene of interest tagged with a Flag peptide sequence at the N terminal. The expression of the Flag-SOCS gene is driven by a strong cytomegalovirus promoter (CMV). Mutant plasmid can be generated by PCR reaction using set of complementary primers (red). Newly *in vitro* synthesized plasmid (red) will be non-methylated while the methylated-wild type plasmid (blue) will be digested by the enzyme *Dpn1*.

3.7 *IN VITRO* E3-LIGASE ASSAY

To determine the E3 ligase activity of SOCS2 and SOCS6, cells were transiently transfected with Flag-tagged SOCS2 or SOCS6. Elongin B and C were co-transfected to increase the stability of the SOCS proteins. Complexes of SOCS proteins and their associated proteins were immunoprecipitated by anti-Flag-agarose beads (Sigma-Aldrich). The E3 ligase assay was carried out on the precipitated SOCS protein complexes by adding the other components of ubiquitination reaction in an appropriate buffer: E1 enzyme (human recombinant, Enzo Life Sciences, Inc.), E2 (UbcH5b), ubiquitin, HA-tagged ubiquitin, ATP, $MgCl_2$, creatine phosphate, rabbit skeletal muscle creatine kinase (Calbiochem, La Jolla, CA). Reactions were carried at 37 ^{o}C for 30 min

and stopped by the addition of protein electrophoresis sample buffer, followed by SDS-PAGE. An anti-HA antibody was used to detect incorporation of ubiquitin into high molecular weight protein conjugates. The intensity of this signal was used to evaluate ubiquitin ligase activity. To detect polyubiquitination of specific target proteins, a parallel set of experiments was carried and reactions were stopped by addition of EDTA (to chelate Mg^{+2} ions), followed by elution with Flag peptide (Sigma-Aldrich). This is to recover the SOCS6-containing complexes from the anti-flag agarose beads. Then target proteins were immunoprecipitated from the elution samples using specific antibodies followed by immunoblotting with anti-HA to detect ubiquitin or a specific antibody to detect the shift in molecular weight of the target protein. Figure 3.2 illustrates the steps of the E3 ligase assay.

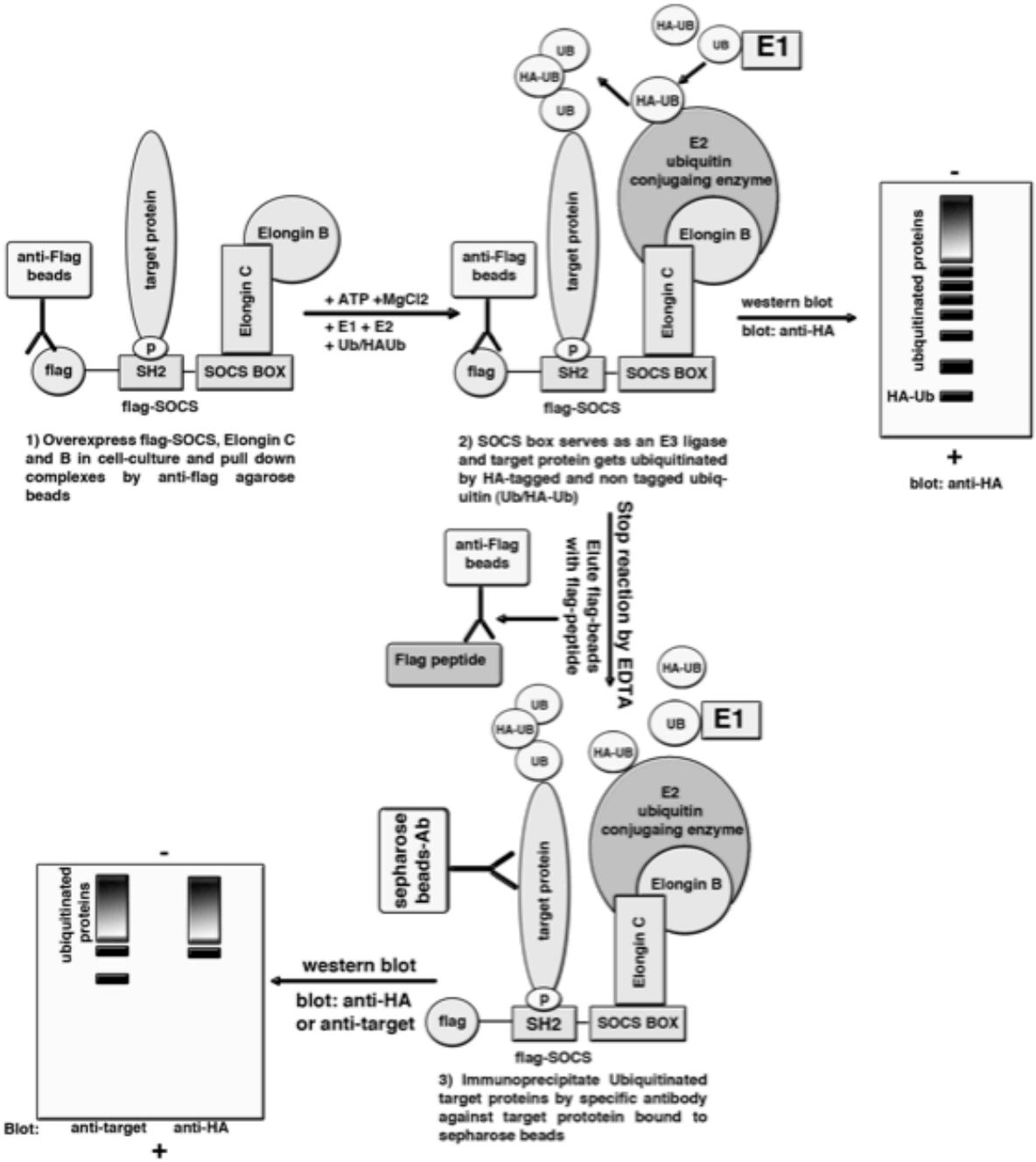

Figure 3.2: In vitro E3-Ligase activity. Western blot against anti-HA is used to detect HA-tagged ubiquitin (HA-Ub) in the ubiquitinated target proteins. E3 ligase activity can be stopped by EDTA and flag-beads are eluted by flag peptide. Ubiquitination of a specific target protein can be detected after immunoprecipitation and immunoblotting against the target protein or against HA-ubiquitin

3.8 LUCIFERASE ACTIVITY ASSAY

The luciferase reporter assay has been widely used to study gene promoter activity. The pGL2 luciferase reporter vectors are designed for quantitative analysis of factors that potentially regulate mammalian gene expression. These vectors carry the coding region of wild-type firefly (*Photinus pyralis*) luciferase. In our study, the luciferase activity of pGL2 vectors was driven by the GH response element of the human SOCS2 gene promoter region, containing a SREBP1 binding site (E-box) and two STAT5b binding sites in close proximity [237]. After the vector was transfected into cultured cells, the luciferase activity was measured using a luminometer. The light intensity reflects the amount of luciferase, which reflects the transcriptional activity of the inserted SOCS2 promoter region in relation to specific experimental condition. The luciferase activity was normalized to the protein content or to the β-galactosidase from a cotransfected vector expressing the β-galactosidase gene.

3.9 CYCLOHEXIMIDE CHASE EXPERIMENTS

Cycloheximide chase is an experimental tool used to determine the half-life of a specific protein. In cells, proteins are constantly being synthesized and degraded. Cycloheximide inhibits protein synthesis by interfering with the translation-elongation step. Cells are homogenized after treatment with cycloheximide at indicative time points. Protein cellular levels drops after cycloheximide treatment and are detected by western blot using specific antibodies. In this thesis cycloheximide chase experiments were used to examine the regulation of SOCS protein stability by Elongin C and B. In addition, stability of SOCS target proteins, GH receptor and c-KIT was studied after overexpression of SOCS2 or SOCS6 respectively.

3.10 TRANSCRIPTION FACTOR BINDING ABCD ASSAY

The ABCD assay (avidin, biotin, complex, DNA) is an experimental tool to study the binding of proteins such as transcriptional factors to a DNA molecule. It is an alternative to chromatin immunoprecipitation, in which protein-DNA complexes are immunoprecipitated using a specific antibody and then followed by the detection of target DNA elements by polymerase chain reaction. In the ABCD assay, protein-DNA complexes are immobilized by biotinylated oligonucleotides to a streptavidin bead matrix followed by detection of the bound transcription factors using a specific antibody by western blotting. Biotinylated oligonucleotide containing the binding site for a transcription factor is mixed with lysates from cells treated under different conditions. The mixtures were incubated overnight to allow the binding of transcription factors to the DNA element. This was then followed by the addition of streptavidin agarose beads to pull down the biotinylated oligonucleotide-protein complexes followed by detection of bound proteins by western blotting. In this thesis, we used the ABCD assay to study the binding and competition of SREBP1 and STAT5b to their response elements in the SOCS2 promoter.

4 RESULTS AND DISCUSSION

4.1 PAPER I

The SOCS2 Ubiquitin Ligase Complex Regulates Growth Hormone Receptor Levels

Some of SOCS2 physiological functions are exerted through feedback inhibition of GHR signaling. GH activated STAT5b binds the promoter of SOCS2 to induce its expression, in turn SOCS2 binds at least two phosphorylated tyrosines on the GHR to negatively regulate JAK2 and STAT5b activation [218, 237]. The molecular basis for these actions of SOCS2 is not entirely clear. In this study we tested whether SOCS2 can assemble an E3 ubiquitin ligase complex that targets the GHR and can modulate its turnover.

We have demonstrated that SOCS2 interacts with elongin B and C, cullin5 and Rbx2 to form a canonical ECS (elongin/Cullin/SOCS) complex. (Figure 4.1A). *In vitro* assays demonstrate that this complex has intrinsic ubiquitin ligase activity and can ubiquitinate the GHR. This strongly suggests that this is the mechanism whereby SOCS2 controls GH sensitivity (Figure 4.1B). In support of this conclusion, we show that GHR levels decreased in cells overexpressing SOCS2 as a result of accelerated GHR degradation. Accordingly, elevated GHR levels were observed following SOCS2 reduction in cells or in livers from SOCS2$^{-/-}$ mice. Our data provide a mechanistic explanation for the negative actions of SOCS2 on the GH activated signaling pathways.

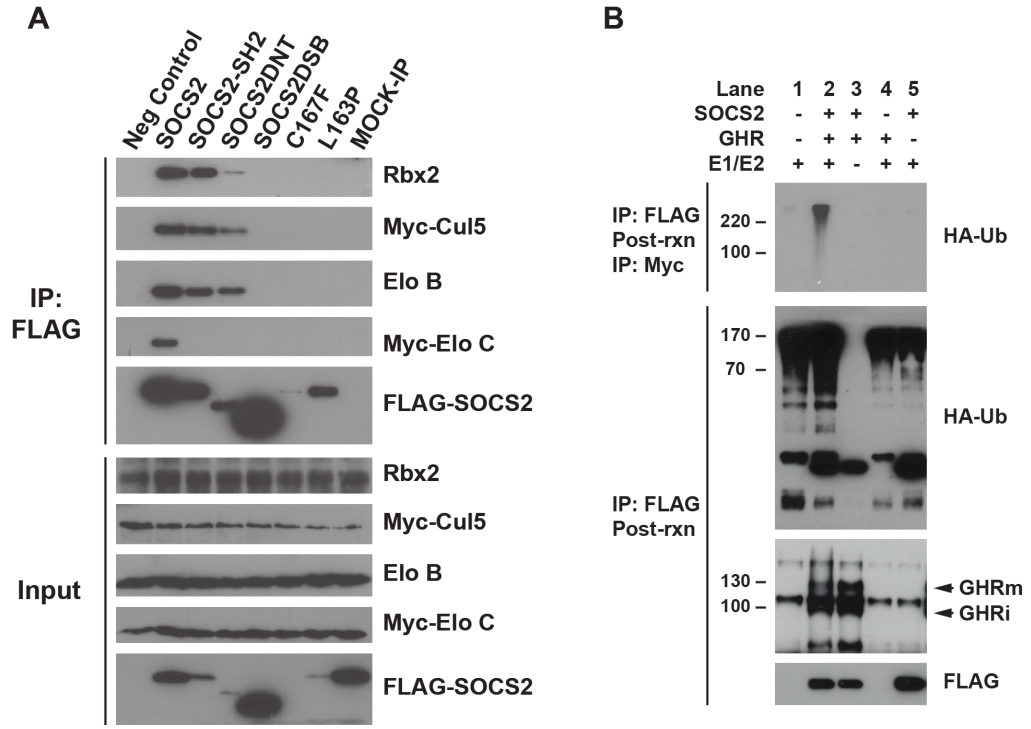

Figure 4.1: SOCS2 forms a stable complex with CRL5 and mediate ubiquitination of GHR. A, HEK293T cells were transfected with Elongin B, Elongin C and *Myc*-Cullin5 and either an empty vector (lane 1), wild type (lane 2 and 8) or a mutant form of FLAG-SOCS2 (lanes 3-7) as denoted in the picture. B, *In vitro* E3 ligase assays shows enhanced protein ubiquitination of target proteins in samples overexpressing SOCS2. Upper panel demonstrate the ubiquitination of GHR after E3 ligase reaction

Unliganded GHRs undergo constant endocytosis via clathrin-coated vesicles and are subsequently transported to lysosomes for degradation[238]. Endocytosis of the GHR is dependent on ubiquitination. A motif has been identified in the cytosolic portion of the receptor (UbE motif) that controls the GHR ubiquitination level. Mutations in this region hinder GHR endocytosis and degradation causing enhanced cellular sensitivity to GH [239]. Interestingly basal GHR endocytosis can proceed even when all Lysine residues in the intracellular domain are mutated to Arginine [240], indicating that ubiquitination of associated proteins but not the GHR itself may drive this process. The F-box protein β-transducin repeat-containing protein (βTrCP) serves as the substrate recognition subunit in the SCF (βTrCP) ligase (Skp1, Cul1 and Rbx1) and has been shown to mediate GHR recognition through the UbE motif and promote its ubiquitination [241]. βTrCP seems to be involved in the continuous and constitutive ubiquitin-dependent endocytosis of GHR [242] . In fact, the close proximity of the JAK2 (box-1) and βTrCP (UbE) binding sites on GHR seems to preclude the regulation of activated GHr by this ligase. Indeed, upon GH stimulation, the phosphorylation and binding of JAK2 inhibits the βTrCP mediated-GHR ubiquitination and endocytosis [242]. Mice where βTrCP gene has been inactivated do not show obvious disruption in growth rate implicating that either basal turnover of GHR contribute little to GH sensitivity or that additional E3 ligases can target the GH receptor complex in the absence of βTrCP.

Endocytosis of the GHR is accelerated by ligand binding indicating the existence of a second E3 ligase preferentially targeting active GH receptors. Our finding that SOCS2 mediated GHR degradation depends on the phosphorylation of Tyr487 suggest its involvement in the GHR downregulation after GH stimulation. This is in agreement with a study showing that phosphorylation of cytosolic tyrosine residues is important for the GH-induced GHR endocytosis [243]. The importance of SOCS2 in the control of GH sensitivity is validated by the gigantism observed in SOCS2$^{-/-}$ mice. The ability of SOCS2 to regulate the levels of the active mature GHR suggests that it has a pivotal role in conditions of altered GH sensitivity such as chronic wasting, infection and hyperinsulinemia [244].

4.2 PAPER II

SOCS2 inactivation protects against hepatic steatosis but worsens insulin resistance in high fat diet fed mice

Non-alcoholic fatty liver disease (NAFLD) is associated with a wide spectrum of clinical and pathological manifestations. Accumulation of fat in the liver is strongly related to insulin resistance and associated metabolic alterations e.g. abnormal glucose tolerance and alterations of serum lipids. The underlying mechanisms for the association between insulin resistance, obesity and hepatic steatosis are not fully understood but recent studies show that a reduction of hepatic steatosis in obese mice is sufficient to restore insulin sensitivity [53].

Growth hormone (GH) is a key metabolic regulator of liver triacylglyceride (TAG) content. Hepatic steatosis is a prominent feature in GH-deficient patients and can be corrected by GH replacement therapy [192]. These effects are due to direct effects of GH on the liver as recently demonstrated by liver specific inactivation of the GH receptor and its downstream signaling intermediaries, JAK2 and STAT5, which in all cases lead to severe steatosis [245]. The Suppressor of Cytokine Signalling 2 (SOCS2) is a subunit of an E3 ubiquitin ligase responsible for the downregulation of GH receptor signalling *in*

vivo [218]. Deficiency of SOCS2 leads to an increased GH sensitivity and therefore SOCS2-deficient ($SOCS2^{-/-}$) mice grow larger without elevation of GH circulating levels. In contrast to GH transgenic mice or GH-treated patients, $SOCS2^{-/-}$ animals do not exhibit deterioration of insulin sensitivity. Sensitization to GH through SOCS2 inhibition may provide a novel approach to regulate hepatic lipid load avoiding some of the limitations associated with direct GH treatment. Here we tested this concept by analyzing the metabolic response of $SOCS2^{-/-}$ mice to high-fat feeding.

We found that $SOCS2^{-/-}$ mice are protected from high-fat diet (HFD)-induced hepatic steatosis through mechanisms that involve enhanced expression of genes involved in TAG synthesis coupled to increased hepatic TAG secretion. Paradoxically, we found that the effect of HFD on reducing systemic and hepatic insulin sensitivity was substantially more notable in $SOCS2^{-/-}$ mice. When investigating possible reasons why $SOCS2^{-/-}$ animals have an increased sensitivity to dietary stress, we observed enhanced expression of genes encoding inflammatory cytokines (IL-6, INFγ, and RANTES) in the liver and adipose tissue and elevated levels of these cytokines where also detected in blood from HFD fed $SOCS2^{-/-}$ mice. Measurements of macrophage cell markers and histological examination of $SOCS2^{-/-}$ mice livers did not detect any overt inflammatory cell infiltration, suggesting that enhanced cytokine production is due to over activity of the liver resident macrophages. This notion is further supported by findings that NFκB activity is enhanced in liver tissue of $SOCS2^{-/-}$ fed a HFD. By using bone marrow derived macrophages we could show that SOCS2 inactivation enhanced phagocytic activity, augmented NF-κB activation and increased production of inflammatory cytokines upon lipopolysaccaride (LPS) stimulation. Therefore, our findings indicate that SOCS2 has an important role in the regulation of hepatic homeostasis under conditions of dietary stress through actions on hepatic TAG secretion and in the control of toll-like receptor 4 signalling. These findings may be of relevance for the pathogenesis of NAFLD given that SOCS2 gene expression is reduced in liver from obese individuals [246]. The reduced levels of liver SOCS2 mRNA, may be a consequence of reduced GH secretion that is commonly associated with obesity. Likewise, stimulation of SOCS2 expression by GH may be a contributing factor to the reversal of hepatic inflammation during replacement treatment in GH deficiency [192].

The recognition that SOCS2 acts on macrophages to limit cytokine production in response to LPS suggest that SOCS2 could be a link for cross talk between GH receptors and Toll-like 4 receptors and this provides a novel mechanism for GH to modulate the inflammatory activity of macrophages [218, 222]. Possibly, it may also help to explain why GH treatment exacerbates inflammation in LPS-treated rodents and can be life threatening when given to critically ill patients [247].

In conclusion, we identified SOCS2 as a novel regulator of hepatic homeostasis in conditions of dietary stress. The HFD-fed $SOCS2^{-/-}$ mice provide a novel model to understand the complex relationship between inflammation and steatosis in the control of hepatic glucose homeostasis. Future use of this model may help to outline the contribution of different mechanisms related to lipid overload or inflammatory stress in the development of fat-induced hepatic insulin resistance.

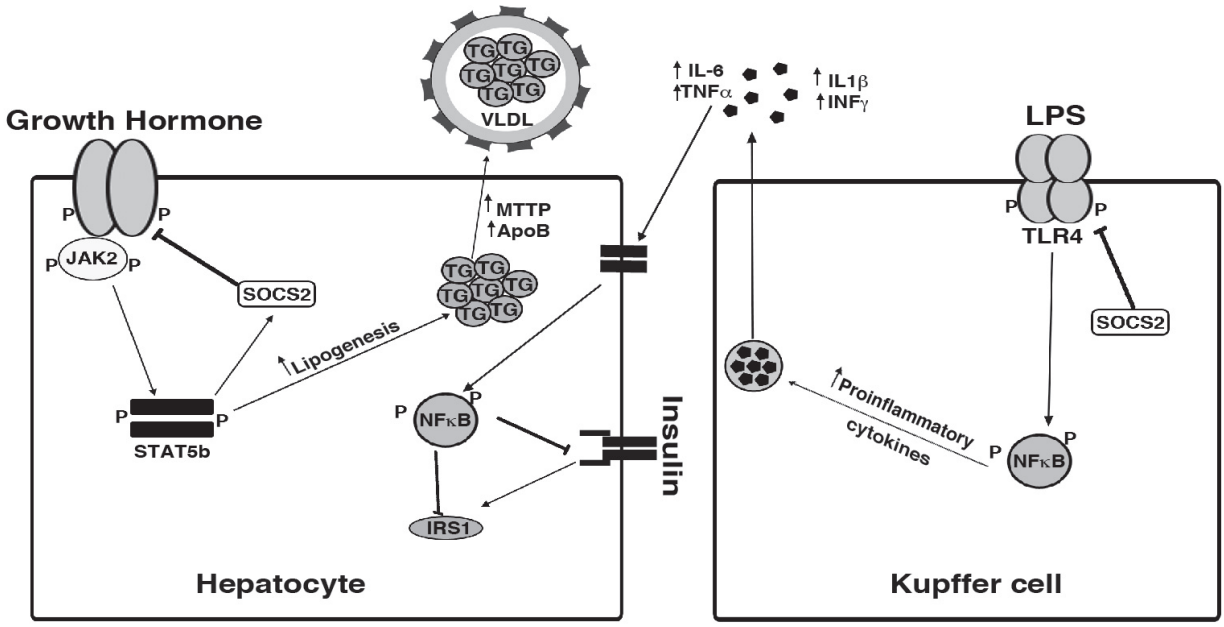

Figure 4.2: Effect of SOCS2 deletion on hepatic lipid content and development of insulin resistance. Enhanced growth hormone sensitivity results in enhanced hepatic *de novo* lipogenesis coupled with enhanced VLDL secretion resulting in less hepatic lipid accumulation. Enhanced TLR-4 signaling in Kupffer cells or hepatocyte enhances the expression of inflammatory cytokines predisposing to insulin resistance.

4.3 PAPER III

A Liver X Receptor agonist Downregulates Hepatic Growth Hormone Signaling

The nuclear receptor Liver X receptor-α (LXRα) controls a complex transcriptional network involved in the control of hepatic intracellular non-esterified cholesterol levels. Known LXR target genes include membrane transporters promoting cholesterol efflux, enzymes involved in cholesterol catabolism and genes involved in *de novo* lipogenesis[248]. Treatment of animals with the LXR agonist T0901317 reduces circulating cholesterol levels, improves insulin sensitivity but also causes severe hepatic steatosis and hypertriglyceridemia [249]. On the other hand LXRα/β knockout mice ($LXR^{-/-}$) are resistant to hepatic steatosis induced by high fat diet feeding [250, 251]. In contrast GH actions promote lipid mobilization from the liver, fatty acid oxidation in muscle and can lead to insulin resistance. Because of the apparent antagonistic actions of LXR agonist and GH, in this study we analyzed how hepatic GH signaling is affected by LXR activation.

We showed that LXR activation impairs GH signaling in hepatocytes. LXR agonist treatment attenuated GH transcriptional activity in BRL-4 cells while inducing the expression of SREBP1c, SREBP1a and SREBP2. The inhibitory effect of the LXR agonist can be recapitulated by overexpression of the LXR downstream-regulated factors SREBP1a and SREBP2 in hepatic cells. Likewise, the activity of a reporter vector, driven by the STAT5b response element of the SOCS2 gene, was inhibited by a simultaneous treatment with LXR agonist or by overexpression of SREBP1a or SREBP2. Additional experiments show that SREBPs induce the degradation of STAT5b. Furthermore, LXR activation of primary hepatocytes results in reduced levels of GH receptor parallel to the down regulation of STAT5b. Collectively, these findings

suggest that the mechanisms of LXR mediated inhibition of GH signaling involve the downregulation of the transcriptional factor STAT5b and the GH receptor.

The empirical findings of LXR interference with hepatic GH signaling assist in our understanding of the mechanisms of GH insensitivity observed after feeding or during conditions such as in hyperinsulinemia. Previous studies have shown that insulin inhibits growth hormone activation of JAK2 and STAT5b in hepatoma cell lines [252, 253]. Insulin is a known inducer of LXR-dependent SREBP1 expression and this may contribute to antagonize the diabetogenic actions GH in fed-states [252, 254]. Overall, these findings have important implications for designing a new LXR agonist treatment protocols for the management of hypercholesterolemia and insulin resistance since GH antagonism may have wide physiological implications in multiple tissues. More broadly, additional research is needed to determine the *in vivo* pharmacological effect of LXR activation on hepatic growth hormone signaling.

4.4 PAPER IV

Structural Basis for c-KIT Inhibition by the Suppressor of Cytokine Signaling 6 (SOCS6) Ubiquitin Ligase

SOCS6 was first described to associate to the IRS-4/PI3 kinase complex [213]. Later studies have shown that SOCS6 positively regulates insulin signaling *in vivo* and *in vitro*. In addition, SOCS6 [255], as well as SOCS1 [256, 257], SOCS2, SOCS3[258] can inhibit SCF induced cKIT-mediated cell proliferation. Furthermore, SOCS6 expression is downregulated in various human cancers [259] and tumour cell lines, suggesting that SOCS6 may also act as a possible tumour suppressor.

Tyrosine kinase signaling from the SCF receptor, c-KIT is essential for the proliferation of mast cells, but must be tightly regulated to avert the onset of multiple human tumours [260, 261]. We have demonstrated that SOCS6, via its SH2 domain, interacts with c-KIT to promote its ubiquitination and regulates c-KIT receptor turnover. The interaction site in the juxtamembranous region, c-KIT pY568, binds to the SOCS6 SH2 domain with high affinity and conforms to the preferred recognition sequence determined for SOCS6 (pYVYI) [215]. The SH2 loop arrangement is similar to SHP2, which also binds c-KIT pY568, but displays striking differences to other SOCS family members, including SOCS3, which target other cytokine receptors.

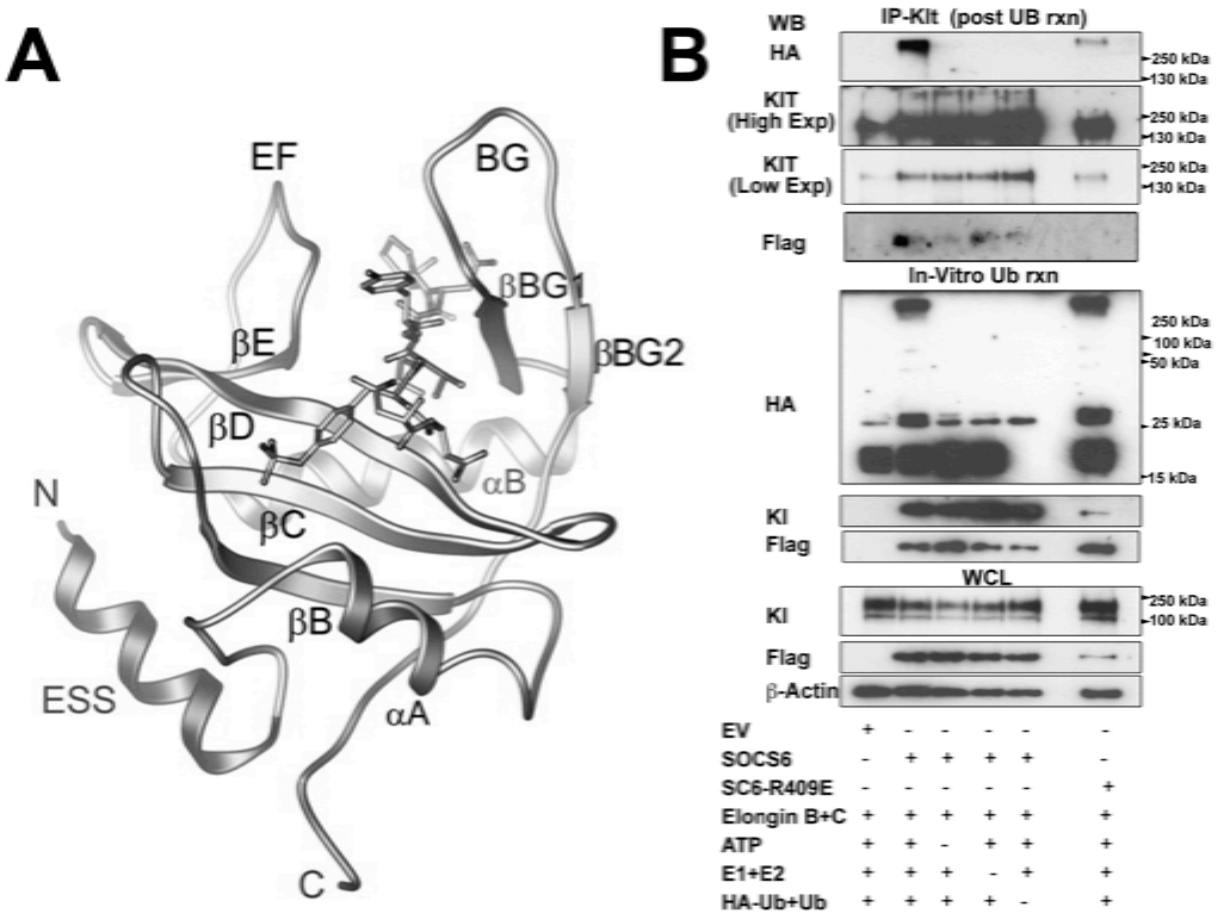

Figure 4.3 Binding of cKIT to SOCS6 and its ubiquitination. A, Ribbon structural demonstration of cKIT peptide binding to the SH2 domain pocket in SOCS6. cKIT pY568 residue is projected into a positive pocket between the βD and βC loops. B, *In vitro* E3 ligase activity of SOCS6 and subsequent ubiquitination of cKIT after the ubiquitination reaction.

All SOCS family members are thought to be part of Cullin/Ring ubiquitin ligase complexes, were they act as the substrate recognition subunits (see Fig 1.7). Within the SOCS proteins, the SH2 domain is thought to recognize tyrosine phosphorylated substrates allowing the preferential targeting of signaling active proteins. The distinction of active and inactive targets (tyrosine phosphorylated or not) by SOCS is essential if they are to act as negative feedback regulators as is widely believed. E3 ligase recognition of posttranslationally-modified substrates provides temporal and spatial specificity to their actions that may have important implications for their biological function. This is well exemplified by the differences between βTRCP and SOCS2 in the control of GH sensitivity but less understood for other SOCS family members [262].

A key issue to understand the SOCS system is how substrates are recognized. In this study, we show that the SH2 domain of SOCS6 binds the cKIT pY568 peptide with a strong affinity (Kd = 0.3 μM) comparable to the binding of SOCS4 to the pY1092 in the EGFR (Kd = 0.5 μM) [263] or of SOCS3 to the pY595 of the gp130 cytokine receptor subunit (Kd=0.152 μM) [264]. Accordingly, SOCS6 ubiquitinates c-KIT in an SH2 dependent manner. On the other hand, several other substrates have been proposed for SOCS6 actions, including the phosphatidylinositol 3-kinase regulatory subunit (PIK3R)

1 and 2, p56LCK and STAT3 [265, 266]; all proteins with regulatory role in metabolism and inflammation. A close inspection of the known tyrosine phosphorylated residues in the PIK3R1 and PIK3R2 fails to recognize sites that conform to the preferred recognition sequence for the SOCS6-SH2 domain (pYVYI). This suggests that either SOCS6 can recognize substrates through lower affinity interactions, as it is the case for SOCS2 (Kd=1.6 μM for GHR Tyr 595) [262] or additional domains may contribute to substrate recognition. Indeed, the N-terminal domain of SOCS6 has been implicated to bind to p56LCK and STAT3[265, 266] while similar domain in SOCS4 participate in the binding to the EGFR [263]. The N-terminal domain of SOCS2 is also necessary for full activity towards the GH receptor [267]. Additional studies are needed in order to define the structure of full-length SOCS proteins bound to substrates are needed, as this information will lead to a better understanding of the distinct physiological functions of these proteins.

5 CONCLUSIONS AND GENERAL DISCUSSION

1. SOCS proteins exert an E3-ligase activity toward cytokine and growth factor receptors.

In this thesis, we characterized the ubiquitin ligase activity of SOCS2 and SOCS6. Both SOCS2 and SOCS6 were demonstrated to interact with Elongin B and C, cullin5 and Rbx2 assembling a canonical ECS (Elongin/Cullin/SOCS) complex and they exert E3-ligase activity towards GHR and cKIT proteins, respectively. The E3-ligase activity results in degradation of these receptor and reduction in the cellular sensitivity toward GH and SCF. We have also displayed the importance of SH2 domains of both SOCSs in the binding to their target substrate. However, single point mutation in the SH2 domain did not completely abolish the binding of SOCS6 to cKIT or SOCS2 to GHR suggesting that additional binding sites exist outside the SH2 domain that contribute to substrate recognition. This may provide SOCS proteins with a broader flexibility for targets recognition and hence widens their ability to regulate various signalling pathways. The physiological implication of this hypothesis is illustrated by the ability of SOCS2 of modulate GHR and TLR4 signalling, modulating the physiological response to high fat feeding.

2. SOCS2 is in control of hepatic metabolism in conditions of dietary stress.

We observed that SOCS2$^{-/-}$ mice on a high fat diet exhibit higher fat deposition in adipose tissue and skeletal muscle. This indicates a shift in fat mobilization to peripheral tissues in these mice. This effect could be a consequence of higher GH sensitivity, which results in a low plasma GH/IGF1 ratio. In this context, reduced circulating GH levels would cause a reduction in adipose tissue lipolysis and muscle lipid oxidation that cannot be compensated by the increase in GHR activity derived from SOCS2 deletion. In contrast, the increased hepatic GH sensitivity in SOCS2$^{-/-}$ mice results in enhanced VLDL secretion. The overall effect is a reduction of TAG accumulation in the liver; protecting mice from HFD induced steatosis. This model of control of lipid metabolisms would implicate that certain tissue such as muscle and fat may be less sensitive to SOCS2 inactivation. In other words, it seems that in muscle and adipose tissue, GH actions are primarily determined by its serum levels while GH liver actions can be modulated to a larger extent by SOCS2 regulation of GHR responsiveness. In support of this hypothesis is data showing comparatively higher expression of SOC2 mRNA in liver than in adipose tissue and muscle [268].

Interestingly, the low hepatic steatosis was associated with significantly higher systemic insulin resistance indicated by both glucose and insulin tolerance tests. This was also associated with local insulin resistance observed in adipose (not published), skeletal muscle and the liver. It is possible therefore, that the observed insulin resistance in SOCS2$^{-/-}$mice may result from the diabetogenic effect of enhanced GH actions in liver, alone or in combination with the proinflammatory pathways that are enhanced after SOCS2 deletion. We detected higher phagocytic activity and higher LPS-induced NF-kB activity in macrophages isolated from SOCS2, implying a role for SOCS2 in the regulation TLR4 signaling. No exogenous GH was added in this *in vitro* assays, suggesting that other SOCS2 targets than the GHR exist in the TLR4 signaling pathway. On the other hand, GH is a component of the fetal bovine sera used to culture the cells and we cannot exclude paracrine production of GH by bone marrow derived macrophages. It is still possible that increased GH receptor content or

activity in macrophages of SOCS2$^{-/-}$ mice may crosstalk with TLR4 activated signaling pathways to enhance cytokine production. This is certainly a possibility *in vivo* and further experimentd in SOCS2$^{-/-}$ mice are warranted to ascertain the possible role of GH in the immunoregulatory actions of SOCS2[224].

3. The growth hormone-SOCS2 axis is perturbed by LXR ligands.

SOCS are cytokine inducible proteins with a short half-life and their activity depends on their transcriptional activation. Therefore, SOCS actions can be altered by signaling factors regulating gene transcription. In the case of SOCS2, factors that modulate the GH activation of STAT5b would modulate SOCS2 function. We have demonstrated that LXR and SREBP1 significantly downregulates growth hormone signaling by targeting the STAT5b activity in hepatocytes resulting in reduced SOCS2 expression. A similar effect has been observed in macrophages where LXR activation interfere with LPS induction of SOCS3 and SOCS1 expression [269]. However, It is unclear how SOCS2 is regulated in macrophages by LXR activation and if STAT5 downregulation may contribute to the known anti-inflammatory actions of LXR agonist in this tissue. It is worth noting that GM-CSF utilizes the JAK2/STAT5 signal transduction pathway and it is therefore possible that LXR may modulate macrophage differentiation triggered by this factor [270].

An altered sensitivity towards hormonal/cytokine signals is a common disease causing mechanism, best exemplified by the insulin resistance associated to type 2 diabetes. It will be interesting to learn how the SOCS system relates to insensitivity syndromes towards GH or pro-inflammatory cytokines in patients. In the case of GH, an inactivating mutation in the SOCS2 gene has been recently found in a human patient [220]. In line with our findings in the SOCS2$^{-/-}$ mice, this patient exhibits an increased GH sensitivity defined by low GH and high IGF-1. A similar pattern of GH and IGF-1 is observed in obese patients [271] suggesting that SOCS2 downregulation may contribute to obesity. Genetic association studies have identified polymorphisms in SOCS2 that influence postnatal growth rate [219]. It will be interesting to observe whether these individuals exhibit increased sensitivity to develop metabolic disorders with increased food intake and if those with metabolic syndrome will exhibit less NAFLD. It is yet an open question if there are pathological situations where the SOCS2 system is activated to reduce GH sensitivity. GH insensitivity is found in patients with GHR mutations but also after treatment with glucocorticoids, estrogen or androgens which induce SOCS2 expression [223, 272]. GH resistance is also associated with kidney disorders [273] and certain metabolic conditions [274] where the role of SOCS2 deserves further studies. It will be interesting to identify subpopulation of patients with unique disease features that can be attributed to SOCS protein actions.

6 FUTURE PERSPECTIVES

The findings in this thesis increase our knowledge about the SOCS2 and SOCS6 mechanism of action and of the physiological role of these proteins. This in turn has generated novel questions for further studies to gain a better understanding of how SOCS proteins control metabolism and their role in the pathogenesis of other human diseases.

- Our study suggests an anti-inflammatory role of SOCS2 through its regulation of TLR4 signaling. Further investigations are needed to define the signaling molecules within the TLR4 pathway that are targeted by SOCS2 and the possible role of GH signaling in this process.

- Further studies are needed to understand the contribution of GH signaling to the SOCS2$^{-/-}$ reactivity to high fat diet. One possible approach is to cross breed the SOCS2$^{-/-}$ mice with GH deficient mice in order to understand GH-independent functions of SOCS2. An alternative approach is to use systemic treatment with GH or GH receptor antagonist in order to reverse or enhance the effects observed in SOCS2$^{-/-}$ mice.

- High fat diet induces hepatic inflammation and insulin resistance in SOCS2 knockout mice. Therefore, we need to further investigate whether there is a causal relationship between inflammation and the development of insulin resistance. Experiments that involve Kupffer cell ablation in SOCS2$^{-/-}$ mice and its substitution by SOCS2 WT macrophages will create chimeric mice where the role of hepatic inflammation can be studied. Other approaches may include systemic treatment of SOCS2$^{-/-}$ mice with NFκB inhibitors.

- Our structural models of SOCS6-KIT binding have revealed some of the structural elements involved in substrate recognition. It will be very interesting to obtain similar data for the interaction between SOCS6 and the p85 regulatory subunit of the PI3 kinase complex. This would allow an increased understanding of how a single SOCS protein interacts with different substrates. Likewise, a complete understanding of SOCS2 actions on the GH receptor would require structural information regarding their interaction.

- Recently a missense mutation in the SOCS2 gene has been identified in human. Other genome wide associated studies have identified polymorphisms in the vicinity of SOCS2 that are related to increased longitudinal growth. The knowledge accumulated in these studies using mice models may help to interpret the phenotype of these subjects and contribute to their clinical management.

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