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The role of Signal Transducer and Activator of Transcription 5 (Stat5) and the Glucocorticoid Receptor (GR) in hepatocyte function

Conducted at the Ludwig-Boltzmann-Institute for Cancer Research (LBI-CR) under supervision of Dr. Richard Moriggl

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1. Summary

Signal transducer and activator of transcription 5 (Stat5) represents a cytokine- and growth factor-dependent transcription factor regulating important biological processes such as postnatal body growth, erythropoiesis and the expression of milk protein genes. The glucocorticoid receptor (GR) binds Stat5 in a cofactor fashion and was reported to modulate Stat5 target gene specificity. In this study we aimed to elucidate the role of Stat5 in pivotal hepatocyte processes such as the regulation of postnatal body growth, fatty acid metabolism and liver fibrogenesis, taking into account its propensity to interact with the GR. The major findings of this study are summarized in the following:

(i) Hepatic loss of Stat5, the GR or both resulted in comparable defects in postnatal body growth. This was accompanied by quantitatively similar changes in transcription profiles, affecting predominantly growth-related Stat5 target genes (e.g. IGF-1 and Socs2). These observation suggested the existence of a linear Stat5-GR signal transduction pathway essential for regulating postnatal body growth. Using protein interaction studies the Stat5 aminoterminus was identified as docking platform for the GR. Luciferase reporter assays confirmed the Stat5-GR-dependent synergism in vitro exemplarily for transactivation of the Socs2 promoter.

(ii) Hepatic loss of Stat5 in mice leads to the pathological accumulation of lipids in hepatocytes (steatosis). This coincides with perturbations of hepatic lipid metabolism such as hepatic insulin resistance and induction of de novo lipogenesis mediated by lipogenic master regulators PPARgamma and SREBP-1c. Compound loss of Stat5 and GR in hepatocytes severely aggravates this phenotype, leading to late-stage hepatopathologies such as severe steatosis and liver fibrosis already in young animals. At older ages, these mutants additionally exhibit depletions of peripheral fat stores (lipodystrophy) leading to an increased lipid flux into hepatic tissues and thus exacerbating disease. The two-step pathogenesis observed in double knockout animals closely mimics the onset and progression of human non-alcoholic fatty liver disease (NAFLD).
(iii) Hepatic loss of Stat5 in a murine model for proinflammatory liver carcinogenesis (MDR2⁻ model) leads to extrahepatic cholestasis (bile duct obstruction) causing secondary liver fibrosis at young ages. The pathogenesis in these animals comprises major hallmarks observed in fibrotic states such as the activation of hepatic stellate cells and remodelling of the extracellular matrix. Ablation of Stat5 in hepatocytes leads to a direct loss of Stat5-dependent basolateral bile acid transporter transcription in hepatocytes and thus coincides with perturbations in hepatic bile acid circulation and recycling. This may eventually cause hepatocyte damage and, finally, liver fibrosis and cirrhosis.

Taken together, two published first-author manuscripts and one first-author manuscript in preparation resulted from this (see section 8).
Zusammenfassung


2. Introduction

2.1. Metabolic homeostasis and the liver

Obesity and its underlying causes are major concerns in the industrialized world affecting both adults and children \(^1\). Little physical activity and calory excess are associated with the onset of obesity and constitute the second-most preventable source of death in the US \(^2\). Nutrition in the industrialized world is based predominantly on saturated fatty acids which leads to increasing body fat content, especially in concert with carbohydrate enriched, high-calory diets \(^3\). Obesity is highly correlated to the Metabolic Syndrome, a cluster of nutrition-derived pathologies such as coronary hypertension, insulin resistance and Type 2 Diabetes (T2D) \(^4\). Another epidemiologic feature associated with obesity and T2D is a condition called non-alcoholic fatty liver disease (NAFLD), usually coined to circumscribe a pathology reminiscent of liver damage caused by alcohol abuse. The liver as central metabolic organ possesses essential functions in synthesis and catabolism of nutrients. Postprandially, carbohydrates, lipids, vitamins and mineral salts are resorbed by the intestinal tract, funneled into the liver and metabolized therein. During fasting periods, the liver releases stored nutrients in order to ensure proper functioning of metabolite-sensitive extrahepatic tissues like the brain. The liver also controls the body’s protein, glucose and lipid metabolism \(^5\) and synthesises essential lipid carrier proteins such as lipoproteins and albumins which are abundantly secreted into the circulation \(^6\). Another hepatic function is the synthesis and secretion of bile, which aids in emulsifying and thus resorbing dietary lipids. In addition, the liver possesses important detoxification properties as it clears xenobiotics and metabolic waste such as bilirubin, an abundantly heme degradation product. Finally, it removes peptide hormones from the circulation and exhibits immunomodulatory as well as vitamin storage functions \(^7\).

2.1.1. Uptake and systemic distribution of lipids

The liver is crucial for metabolizing dietary lipids. These lipids are composed primarily of free fatty acids (FFA) and triglycerides (TG), a tricarboxylic acid esterified with
saturated and unsaturated fatty acids (FA). A three step process is necessary for proper absorption of FFA and TG in the intestinal lumen and subsequent transport into the liver:

(i) Nutritional triglycerides are emulsified by endogenous detergents such as bile acids (BA) and enzymatically cleaved by the pancreatic lipase (PL) in the intestinal tract yielding free fatty acids and monocarboxylic acid esters. Other dietary sources of fatty acids are hydrolysed phospholipids and cholesterol esters.

(ii) Triglyceride cleavage products are subsequently absorbed by the intestinal epithelium in the distal jejunum and ileum via facilitated transport across the epithelial cell layer.

(iii) Finally, triglycerides are resynthesised and loaded onto lipoprotein particles (chylomicrons) which enter the bloodstream and efficiently deliver dietary triglycerides into hepatic, adipose and other target tissues. In the liver, hepatic lipase (HL) cleaves triglycerides off the lipoprotein particle surface and translocates fatty acids into the cytoplasm. Subsequently, fatty acids are catabolically degraded by the mitochondrial and peroxisomal beta-oxidation machinery leading eventually to synthesis of ATP energy equivalents. Alternatively, lipids are incorporated into newly synthesised triglycerides for long-term storage. Of note, the hepatic fat-storage capabilities are limited; the most abundant lipid store is represented by the peripheral white adipose tissue (WAT). Moreover, fatty acids are utilized for synthesis of hepatic inflammation mediators such as prostaglandins and leukotrienes. Finally, via enzymatic remodelling of the fatty acid carbon backbone, fatty acids cleavage products indirectly serve as substrates for synthesis of glucose (gluconeogenesis).

The liver possesses potent lipogenic properties: Triglycerides are synthesized in hepatocytes by esterification of glycerol and fatty acids hailing from two sources: Non-esterified fatty acids (NEFA) derived from lipolysis of peripheral fat stores (adipose tissues) and dietary fatty acids.

Newly synthesised triglycerides and excess dietary TG are packaged onto very-low density lipoparticles (VLDL) and shuttled into the periphery. Subsequently, the lipoprotein lipase (LPL), which is abundantly expressed on adipocytes in the periphery efficiently cleaves triglycerides from VLDL particles and releases glycerol and fatty acids into the adipocyte cytoplasm. Thus, in the periphery, consecutive triglyceride hydrolysis of lipoproteinparticles leads to an increase in their protein/lipid ratio. So called ‘remnants’ of hydrolysed lipoproteinparticles are recycled in the liver.
via clathrin-mediated endocytosis. Alternatively, excess lipoparticles are conveyed to the hepatic proteasome machinery for protein degradation.

2.1.2. Glycolysis and hepatic beta-oxidation

The liver constitutes the key regulator of energy homeostasis in the body. Two major substrates can be metabolised by hepatic tissue to yield energy equivalents such as ATP and pyruvat eventually utilised to catalyze energy-consuming processes in the body:

(i) Glucose, (a six-carbon (6-C) carbohydrate) is enzymatically processed in an oxygen-independent process called glycolysis yielding two pyruvat molecules (3-C). Pyruvat is decarboxylated into a two-carbon residue (2-C, Acetyl residue) esterified to Coenzyme A (Acetyl-CoA). The Acetyl-CoA pool is constantly replenished by oxidative catabolism of proteins, lipids and carbohydrates. Subsequently, Acetyl-CoA is funnelled into the cytoplasmic citrate cycle, where two consecutive decarboxylations of the acetyl residue yield eight reduced reduction equivalents (NADH/H and FADH$_2$). These reduction equivalents are used to replenish cellular ATP stores via oxidative phosphorylation of ADP, a reaction catalyzed by respiratory complexes localized in the outer mitochondrial membranes.

(ii) Fatty acids, long-chain carbohydrates hailing either from hydrolysed triglycerides or hepatic de novo lipogenesis are catabolised by beta-oxidation in mitochondria and peroxisomes. Mitochondria are specialised in beta-oxidation of saturated short, medium and long-chain fatty acids. In contrast, peroxisomes accommodate enzymes specialised in metabolising more exotic and branched fatty acids. Prior to beta-oxidation, fatty acids are activated by esterification to Coenzyme A yielding high energy Acyl-(n)-CoA thioesters. Subsequently, repetitive cleavage of two-carbon residues from the Acyl carbon backbone leads to Acetyl-CoA and truncated Acyl-(n$^2$)-CoA molecules. Acetyl-CoA is processed via translocation across the mitochondrial membranes and funnelled into the citrate cycle (see above). Alternatively, Acetyl-CoA is utilised for biosynthesis of cholesterol. Under conditions of energy deprivation, Acetyl-CoA may serve as substrate for the biosynthesis of ketone bodies. These compounds serve as energy carriers nourishing glucose-dependent tissues (e.g. the brain) under conditions of glucose deprivation.
2.1.3. Hepatic de novo lipogenesis

The biosynthesis of fatty acids takes place in the cytoplasm of most somatic cells. Enzymatic carboxylation of Acetyl-CoA residues yields activated three-carbon-CoA compounds (Malonyl-CoA); a reaction catalyzed by the Acetyl-CoA Carboxylase (ACC), the rate limiting enzyme of hepatic de novo lipogenesis. The biosynthesis of fatty acids is orchestrated by a multi-enzyme complex called fatty acid synthase (FAS). FAS catalyses the condensation of long-chain, saturated fatty acids under consumption of Malonyl-CoA substrates. During fatty acids synthesis, precursor thioesters (Acyl-CoA) get elongated by two carbon residues (2-C) in each step, leading to saturated 16-C (palmityl-CoA) or 18-C (stearyl-CoA) fatty acids. Branched and unsaturated fatty acids are essential for high membrane fluidity and are generated by enzymatic oxidation of saturated fatty acids. So-called ‘essential fatty acids’ have to be provided by the nutrition as they cannot be synthesised in the body. Hepatic de novo lipogenesis of fatty acids is tightly controlled by allosteric inhibition of rate limiting enzymes (e.g. ACC). Independently, metabolic master regulators like insulin lead to increased transcription of lipogenic enzymes (see below); whereas others repress lipogenesis indirectly by inhibiting FAS through elevated intracellular cAMP concentrations (e.g. elicited by glucagon or catecholamines). Long-chain, polyunsaturated fatty acids also inhibit FAS activity, constituting an efficient negative feedback loop essential for times of abundant fatty acid levels 12.

2.2. Transcription factors and hepatic lipid metabolism

Eukaryotic chromatin consists of long stretches of DNA intertwined to spatially compact structures called nucleosomes. Each nucleosome consists of octamer nucleoprotein (histones) complexes bound to chromosomal DNA 13. Efficient transcription of exonic DNA sequences into complementary messenger RNA (mRNA) strands thus depends on conformational rearrangement of nucleosomes facilitating binding to basal transcription machinery components such as RNA polymerase II.

Transcription factors are DNA-binding proteins characterized by strong binding affinities towards defined DNA sequences (responsive elements; RE). Transcription factors are essential for the recruitment of additional transcription cofactors like p300/CBP which possess potent histone acetylases (HAT) activity crucial for
removing acetyl residues from nucleosome lysine moieties. These modifications lead to electrostatic repulsion of nucleoproteins resulting in accessible, ‘open chromatin’ structures \(^{14, 15}\). Histone Deacetylases (HDAT) reverse this process leading to ‘closed chromatin’ and abrogated gene expression \(^{16}\). Classical transcription factors are activated by ‘upstream kinases’, which itself are triggered by extracellular stimuli such as cytokines or growth factors binding to cognate receptors. Alternately, nuclear hormone transcription factors are activated directly via binding of membrane-permeable ligands. Generally, activated transcription factors integrate extracellular signals, leading to specific gene expression changes. This flexibility enables the cell to react to everchanging extracellular nutrient supplies. Aberrant transcription factor activity and concomitant dysregulation of target gene expression was demonstrated to account for a rising number of metabolic disorders \(^{17}\).

### 2.2.1. Transcriptional regulation of hepatic beta-oxidation

Beta-oxidation of fatty acids takes place in subcellular organelles such as mitochondria and peroxisomes. In addition, the endoplasmatic reticulum harbours high levels of cytochrome p450 family members (e.g. CYP4a) which catalyze the so-called omega-oxidation, the hydroxylation and subsequent catabolic breakdown of saturated and unsaturated fatty acids yielding Acetyl-CoA and dicarboxylic acids in times of nutrient excess. Dicarboxylic acids in turn constitute potent ligands for PPARalpha, a member of the PPAR class of transcription factors. This class of nuclear hormone receptors was initially discovered for binding compounds known to induce proliferation of peroxisomes \(^{18}\). The PPAR class of transcription factors consists of four isoforms, which exhibited distinct functions in hepatic lipid metabolism \(^{19, 20}\). Whereas PPARgamma constitutes the key regulator for lipid biosynthesis in liver and adipose tissue (section 2.2.2 and \(^{21}\)), PPARalpha regulates the beta-oxidation of lipids in hepatocytes in concert with PPARbeta and PPARdelta \(^{22}\). PPARalpha was identified as key transcriptional regulator for beta-oxidation of fatty acids in hepatocytes \(^{23}\). PPARalpha senses extracellular serum levels of dietary lipids and, under satiety conditions, stimulates transcription of lipid-degrading enzymes \(^{24}\). Upon prolonged fasting, fatty acids get shuttled from peripheral fat depots into hepatocytes, leading to activation of PPARalpha and induction of beta-oxidation in order to fulfil the body’s energy demands \(^{25}\). PPAR isoforms require
heterodimerization with the retinoic acid receptor (RXR) in order to initiate transcription of PPAR target genes. PPAR-RXR complexes specifically recognize and bind DNA elements containing AGGT_CAnAGGTCA sequences termed PPAR responsive elements (PPRE) in the 5'-promoter region of target genes. Most rodent and human genes, which possess crucial functions in transport or catabolism of fatty acids contain at least one PPRE in their promoter sequence (reviewed in ). Experimentally validated PPAR target genes encode apolipoproteins, fatty acid transflocases, lipases, cytochrome p450 family members and Acyl-CoA oxidases, yet many putative PPAR target genes still lack experimental proof. Very-long chain fatty acids represent the most established in vivo ligand of PPARalpha. The importance of PPARalpha in regulating fatty acid catabolism is underpinned by the phenotypes observed in PPARalpha knockout mice. These animals are unable to translocate fatty acids into the hepatocyte cytoplasm and exhibit defects in clearance of fatty acids accumulated in hepatocytes upon prolonged fasting. The same phenotype is observed upon feeding of special diets known to channel the flux of lipids into the liver. The same hold true for mice harbouring a genetic abrogation of PPARalpha downstream targets (e.g. ACOX1).

2.2.2. Transcriptional regulation of hepatic de novo lipogenesis

Three nuclear hormone transcription factors, which are independently regulated by glucose and insulin orchestrate the hepatic biosynthesis of fatty acids (de novo lipogenesis):

(i) Insulin induces the de novo lipogenesis of fatty acids by activating the sterol regulatory element binding protein 1-c (SREBP-1c), a membrane-tethered member of the basic helix-loop-helix leucine zipper family of transcription factors (bHLH-Leu-Zip). Upon triggering by insulin, SREBP-1c translocates into the nucleus and initiates the transcription of lipogenic target genes. Hepatic insulin resistance, the insensitivity of the liver to respond to insulin action leads to unbalanced increases in serum insulin levels (hyperinsulinaemia) and concomitant rises in lipogenic SREBP-1c activity. Thus, insulin resistance is characterized by a high biosynthesis of fatty acids contributing to the development of steatosis. The significance of SREBP-1c-dependent lipogenesis has been demonstrated in vivo by generating SREBP-1c transgenic mice. These mutants exhibit a severe accumulation of
intrahepatic fat \(^{38}\). On the contrary, loss of SREBP-1c in hepatocytes leads to significant reductions of the hepatic lipid load \(^{39}\).

(ii) The carbohydrate response element binding factor/protein (ChREBP), another bHLH-Leu-Zip transcription factor mediates hepatic responses to varying plasma glucose levels \(^{40}\). Briefly, glucose induces binding of ChREBP to the promoter region of rate-limiting glycolytic enzymes such as pyruvat kinase (PK), in turn leading to an increased catabolism of glucose, the cleavage products of which are metabolized in the tricarbon cycle. Studies utilizing ChREBP knockout mice pinpointed towards additional lipogenic properties of ChREBP under hyperglycaemic conditions \(^{41}\). High serum glucose levels (hyperglycaemia, closely associated with insulin resistant states) thus lead to ChREBP-1c-mediated induction of hepatic lipid biosynthesis and a concomitant accumulation of intrahepatic lipids.

(iii) PPARgamma, a member of the PPAR nuclear hormone receptor class of transcription factors exhibits key functions in the biosynthesis of fatty acids in liver. Abundantly expressed in adipocytes, PPARgamma was initially thought to be solely implicated in the differentiation of peripheral adipose tissues \(^{42, 43}\). Later, it was recognized that PPARgamma is expressed at low levels in hepatocytes. Interestingly, loss of hepatic PPARgamma expression interferes with the development of steatosis in respective mouse models \(^{44}\), whereas PPARgamma overexpression leads to development of fatty liver disease \(^{45, 46}\). Strikingly, a rising number of steatotic mouse models display increased hepatic PPARgamma transcript and protein levels \(^{47}\). Finally, an indirect crossactivation of PPARgamma-dependent transcription by SREBP-1c-mediated biosynthesis of PPARgamma ligands was demonstrated, thereby exacerbating fatty liver disease \(^{48}\).

### 2.3. Insulin signaling and insulin resistance

Binding of insulin to the heterotypic insulin receptor (IR) triggers insulin-dependent signal transduction. Two intracellular beta-chains and two extracellular alpha-chains \((\text{alpha}_2\text{beta}_2)\) form the functional IR. Upon ligand binding, insulin induces conformational changes in the IR leading to autophosphorylation of specific tyrosine (Tyr\(^ {960}\)) residues in the IR beta-subunit (IRb) and subsequent exposure of docking sites for the Insulin Receptor Substrate 1 (IRS-1) adaptor protein \(^ {49}\). IRS proteins (in humans comprising the IRS-1, IRS-2, and IRS4 isoform) are activated by IR-
mediated tyrosine phosphorylation leading to simultaneous recruitment of specific effector proteins such as Grb2 and Shc, which are responsible for triggering non-canonical IR-dependent signal transduction $^{50, 51}$, e.g. the Mitogen Activated Protein Kinase (MAPK) and Stat5 pathways upon insulin challenge $^{52-54}$. MAPK eventually phosphorylates IRS-1 at specific serine residues leading to abrogation of IR-dependent signal transduction $^{55}$. In contrast, the canonical insulin-dependent signaling are conveyed via activation of phosphatidylinositol-3-kinase (PI3K) effector proteins, which in turn synthesise phosphatidylinositol (3,4,5) trisphosphate (PIP$_3$), a potent intracellular second messenger $^{56}$. PIP$_3$ triggers activation of the AKT family of serine and threonine kinases leading to the translocation glucose transporters to the plasma membrane (e.g. GLUT4).

Studies using IR knockout animals shed further light onto the role of individual IR signal transduction components: Hepatocyte-specific loss of IR proteins led to severely insulin resistant states and concomitant hyperinsulinaemia, decreased insulin clearance and adenoma formation $^{57}$. Deletion of either IRS-1 or IRS-2 in the liver surprisingly had little effects $^{58, 59}$, yet combined loss of IRS-1 and IRS-2 was embryonically lethal $^{60}$. Thus, redundant roles of IRS-1 and IRS-2 in hepatic metabolism were assumed $^{61}$ although this concept recently came under scrutiny as refined genetic analyses rather claimed distinct functions for IRS-1 in the regulation of hepatic glucose metabolism and for IRS-2 in regulation of hepatic lipid metabolism $^{51}$.

The term insulin resistance was coined to circumscribe a condition frequently observed in metabolic disorders such as obesity and NAFLD $^{62}$. Insulin resistant states are generally characterized by blunted responses towards insulin action and harbour molecular impairments in IR-dependent signal transduction. Clinically relevant, insulin resistance often precedes severe metabolic conditions like T2D and NAFLD for decades and is associated with high risks for contracting cardiovascular diseases $^{63}$. Interestingly, an inverse correlation exists between hepatic insulin sensitivity and the accumulation of intrahepatic lipids (steatosis) $^{64}$, yet the causal relationship between steatosis and insulin resistance remains enigmatic $^{65, 66}$. It is undisputed that central and visceral obesity constitute a risk factor for developing insulin resistance. Surprisingly, serious insulin resistance is also observed among patients suffering from dystrophies of peripheral adipose tissues (lipodystrophy) $^{67}$.
2.4. Insulin resistance and liver steatosis

The development of fatty liver disease is closely associated with chronic alcohol abuse (alcoholic fatty liver disease; AFLD)\(^{68}\) as well as calorie-excess; both leading to obesity and insulin resistance and NAFLD\(^ {22, 69}\). NAFLD comprises a wide spectrum of diseases, ranging from the simple accumulation of intrahepatic lipids (steatosis) to inflammatory processes such as non-alcoholic steatohepatitis (NASH), fibrosis and degenerative processes such as liver cirrhosis and hepatocellular carcinoma (HCC)\(^ {70}\). Steatosis as component of NAFLD was initially regarded as a benign condition, but was recently recognized to lead to more severe liver pathologies such as steatohepatitis and hepatic fibrosis. The incidence of NAFLD is considerable in the industrialized world, affecting up to 25% of the population\(^ {71}\), more than 50% of chronically obese and up to 90% of morbidly obese patients\(^ {72, 73}\). The significance of NAFLD is reflected in the observation that steatosis constitutes the leading cause of pathological liver tests in the US\(^ {70}\). In addition, liver biopsies of one third of adult US citizens exhibit steatosis\(^ {74}\). Microscopically, steatosis is diagnosed by the appearance of abundant cytoplasmic lipid droplets\(^ {75}\). Macroscopically, steatosis is diagnosed whenever the amount of intrahepatic fat exceeds >5% of total liver weight\(^ {76}\).

The causal relationship between steatosis and insulin resistance remains uncertain (see section 2.2 and 2.3), although a significant correlation between insulin resistance and fatty liver disease exists\(^ {77, 78}\). A similar correlation is observed in several mouse models for steatosis (section 2.4.1). Mechanistically, hepatic insulin resistance-derived hyperinsulinaemia was proposed to trigger SREBP-1c-mediated lipogenesis which subsequently induces steatosis\(^ {79, 80}\) (see section 2.2.2 and Figure 1). An alternative notion, the clinical manifestation of insulin resistance secondary to liver steatosis remains provocative, although recently researchers reported that transgenic overexpression of DGAT1, the rate-limiting enzymes of hepatic lipogenesis induces steatosis without accompanying insulin resistance\(^ {65}\).
Introduction

Figure 1. Interplay between insulin resistance and liver steatosis
Metabolic perturbations leading to the hepatic accumulation of triglycerides (steatosis) in insulin resistant states: One aspect of hepatic insulin resistance is the concomitant hyperinsulinaemia leading to increases in gluconeogenesis and induction of ChREBP-mediated lipogenesis. Insulin resistance in the adipose tissue triggers the activity of hormone-sensitive lipase (HSL), leading to hydrolysis of triglycerides in adipocytes and increased serum levels of free fatty acids which are eventually stored in the liver. High serum levels of insulin trigger SREBP-1c activation and results in increased hepatic lipogenesis. In concert with ChREBP, SREBP-1c activates enzymes essential for hepatic lipogenesis (ACL, ACC, FAS and SCD). Another aspect of insulin resistance is the impaired capability of hepatocytes to remove excess FFA by beta-oxidation.

2.4.1. Mouse models of NAFLD

NAFLD affects approximately one quarter of the healthy population in the western world\textsuperscript{81}, constituting a significant risk factor for contracting more severe liver diseases. Therefore it represents a logic disease stage applicable for therapeutic intervention. Apart from obvious modification of lifestyle habits this could also involve pharmacological manipulation. The need for animal models mimicking the complex onset and progression of NAFLD through genetic and environmental factor is apparent. The laboratory mouse, \textit{Mus Musculus} shares many physiological, anatomical and metabolic features with humans and has been accepted as the species of choice in metabolic research. Transgenic mice harbouring specific genetic manipulations leading to defined metabolic phenotypes are well-suited for therapeutic interventions in defined, homogenous populations. Generally, murine models for NAFLD are subdivided into two classes:
(i) Mouse models harbouring specific genetic modifications leading to deletion or overexpression of lipid metabolism-related genes. This strategy is exemplified in the ablation of anorexic genes (e.g. leptin) leading to hyperphagia and weight gain \((ob/ob\) mouse \(^{82}\); \(db/db\) mouse \(^{83}\)). An alternative approach is based on the systemic relocation of fatty acids from peripheral adipose tissues to the liver (CD36\(^{-/-}\) mouse \(^{84}\)) or on increased of hepatic \textit{de novo} lipogenesis by transgenic overexpression of lipogenic transcription factors (PPARgamma\(^{tg}\) \(^{45, 46}\), SREBP-1\(^{ctg}\) \(^{85, 86}\) and ChREBP\(^{tg}\) \(^{41}\)). Finally, the hepatic combustion of fatty acids can be disrupted, leading to the defective clearance of lipids by beta-oxidation and the development of steatosis (ACOX\(^{-/-}\) mouse; \(^{27}\)).

(ii) Mice develop NAFLD-like symptoms upon feeding high-calory, carbohydrate-rich diets \(^{87}\). Moreover, specific diets, e.g. deficient for methionine and choline (MCD diet) interfere with the hepatic beta-oxidation machinery \(^{88}\) leading to steatosis, late-stage steatohepatitis and hepatic fibrosis. The exact mechanism underlying pathogenesis remains elusive, yet an increase in reactive oxygen species (ROS) was noted \(^{89}\).

Taken together, the rising morbidity of diseases like insulin resistance, obesity and NAFLD make animal models closely mimicking human disease imperative. Genetic and nutritional mouse models for NAFLD are well-suited for dissecting the complex metabolic processes underlying NAFLD pathogenesis.

### 2.5. Fibrosis of the liver

Liver fibrosis constitutes an abundantly diagnosed intermediate step in the progression of chronic liver diseases. It is widely believed to be secondary to multiple viral, bacterial and metabolic liver insults \(^{90}\). One hallmark of liver fibrosis is the remodelling of extracellular matrix (ECM) constituents leading to abundant collagen deposition around hepatic lobes. High amounts of collagen eventually impede vital hepatic processes and lead to degenerative processes like fibrosis, cirrhosis and HCC \(^{91}\). These processes are accompanied by activation and transdifferentiation of non-epithelial, mesenchymal cells termed hepatic stellate cells (HSC) \(^{92}\). A multitude of stimuli such as high ROS level, immunological activation by parasites \((Schistosoma mansony)\) or intrahepatic accumulation of lipids was demonstrated to activate HSCs which in turn transdifferentiate into pro-fibrogenic cells called
myofibroblasts (MFB). This is accompanied by the appearance of mesenchymal differentiation markers such as alpha-smooth-muscle actin (SMA) and the subsequent deposition of collagens and proteoglycans in the space of Disté secondary to perturbations in ECM turnover such as an altered matrix metalloproteinase (MMPs) and tissue inhibitor of MMPs (TIMPs) secretion. Following transdifferentiation, MFB are rendered responsive to pro-fibrogenic signals such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGFβ). This in turn leads to activation of MAPK signaling, further proliferation of MFB and aggravation of disease. If left untreated, liver fibrosis cumulates in cirrhotic organ failure, liver carcinoma and death (Figure 2).

![Diagram](image)

**Figure 2. Canonical pathogenesis of liver fibrosis (fibrogenesis)**

According to the "canonical principle of fibrogenesis" primary hepatocyte damage leads to cell death and activation of HSC. The latter undergo epithelial-mesenchymal transition (EMT) into MFB. This process is accompanied by synthesis and secretion of ECM components such as collagen. Repetitive damage of fibrotic tissues leads to necrotic processes such as liver cirrhosis if left untreated. The MFB pool is replenished by bone marrow-derived fibrocytes, circulating monocytes or transdifferentiation hepatocytes and bile duct cells. Constantly refueled, the MFB pool actively synthesizes ECM components, a process mediated by canonical fibrosis mediators such as platelet derived growth factor (PDGF), IGF-1, endothelin 1 (ET-1) or ROS. Abbreviations: ASH – alcoholic steatohepatitis; HBV/HCV, hepatitis B/C virus.

### 2.5.1. Molecular mechanisms underlying liver fibrogenesis
Specific cellular events accompany hepatic fibrogenesis: Firstly, HSC undergo transdifferentiation into MFB, upon which they actively secrete collagen into the intercellular space of Dissé. Secondly, activated MFB possess high proliferation capacities leading to elevated numbers of fibrogenic cells. These processes are mirrored in activation of two distinct PDGF-dependent mitogenic signaling pathways.

(i) PDGF receptor (PDGFR) activation triggers activation of Raf, Mitogen Induced Extracellular Kinase (MEK) and Extracellular Signal Regulated Kinase (ERK) signaling. Simultaneous activation of multiple MAPK pathways leads to accelerated MFB cell cycle progression and cellular proliferation. The significance of PDGFR-driven cell proliferation is reflected in the observation that pharmacological targeting of MAPK family members delays MFB proliferation and decelerates progression of liver fibrosis.

(ii) In addition, PDGFR phosphorylates Focal Adhesion Kinase (FAK) leading to active PI3K signaling. FAK and PI3K then synergistically drive MFB proliferation. Furthermore, phosphorylated PI3K activates AKT, an essential survival regulator stimulating cellular proliferation leading to phosphorylation of p70 kinase. Taken together, these cellular events, result in sustained protein synthesis and the concomitant growth of fibrogenic cells. The paramount significance of PDGF-FAK-PI3K-AKT pathway activation for fibrogenesis was confirmed by studies demonstrating that pharmacological inhibition of pathway constituents severely impairs MFB proliferation and ameliorates liver disease.

Transforming growth factor beta (TGFβ) constitutes another crucial profibrogenic cytokine. Upon ligand binding to the TGFβ receptor (TGFβR), TGFβ-derived signals are relayed to the nucleus by activation of cytoplasmic SMAD2 and SMAD3 transcription factors. SMAD2 and SMAD3 are closely associated with the TGFβR, constituting activated core complexes. Secondary association with SMAD4 yields trimeric transcription-competent complexes initiating RNA transcription of profibrogenic genes like type I and III collagens (COL1 and COL3).

Taken together it is widely recognized that activation of PDGF signaling leads to potent HSC proliferation which triggers multiple cellular signaling cascades simultaneously (e.g. the MAPK and FAK-PI3K-AKT-p70 axes). MFB which have undergone the process of transdifferentiation from quiescent HSC require autocrine TGFβ stimuli to sustain their fibrogenic properties.
2.6. Signal transducer and activator of transcription 5 (Stat5)

Signal transducer and activator of transcription 5 (Stat5) constitutes a critical transcription factor involved in controlling developmental and differentiation processes throughout the body. A component of linear Jak (Janus Kinase)/Stat signal transduction pathway, Stat5 is essential for the signal transduction of external stimuli triggered by upstream cytokine and growth factor receptor binding (Figure 3). Mechanistically, ligand binding induces conformation changes in receptors which in turn activate Jak kinases (Jak2 in the case of Stat5). This leads to phosphorylation and nuclear translocation of latent cytosolic Stat transcription factors. Activated Stat5 proteins recognize and bind specific DNA sequence motifs (so-called Stat5 responsive elements or Stat5 RE) located in the 5’ promoter region of Stat5 target genes. Additional cofactors essential for transcriptional initiation are recruited, leading to the formation of transcription-competent protein complexes and transcription of genes implicated in cell proliferation, development, differentiation and survival (reviewed in 109). A schematic illustration of growth factors and cytokines known to activate Stat5 is given in (Figure 3). Latent Stat5 monomers form homodimers upon phosphorylation, yet heterodimeric Stat3/Stat5 complexes have been reported, putatively in order to modulate Stat5 target gene specificity 110. Stat5 comprises two distinct isoforms, Stat5a and Stat5b, encoded by two different genes, precursors of which are already found in Drosophila 111. The Stat5a/b isoforms readily form heterodimers and thus complicate the understanding of Stat5 biology. Stat5a is predominantly expressed in the hematopoietic system and mammary epithelia, whereas Stat5b constitutes the most frequent isoform expressed in hepatocytes. Because of this tissue-specific pattern, we focussed predominantly on the role of Stat5b in the current work.
Introduction

Figure 3. Mechanisms of Stat5 activation by growth factors and cytokines
Stat5 is activated by ligands binding to cytokine and growth factor receptors. In this representation, cytokines and growth factor were clustered according to structural and functional homologies. After ligand binding, receptors undergo conformational changes leading to recruitment of Jak2 molecules in turn phosphorylating monomeric Stat5 molecules in the cytosol. Following nuclear translocation phosphorylated Stat5 dimers bind to Stat5 RE found in Stat5 target gene promoters. Among others, Stat5 target genes regulate postnatal body growth, cytochrome p450-dependent detoxification and hepatic lipid metabolism. Stat5 hyperactivation, e.g. by transformed tyrosine kinases such as Bcr-Abl, can induce leukaemic transformation. Hypoactived Stat5 results in defective erythropoiesis and anaemia. Finally, Stat5 molecules possess a propensity to form oligomeric structures inducing conformational chromatin changes (DNA loop formation).

Like most Stat molecules, Stat5 consists of a flexible aminoterminus domain (N-terminus) followed by a alpha-helical coiled-coil (CCD) and a src homology 2 (SH2) domain; the latter is located in vicinity to the tyrosine residue critical for activation by Jak kinases such as Jak1-3. The carboxyterminal transactivation domain (TAD) constitutes the most divergent part of Stat5. A schematic illustration of the protein structure of Stat5a is given in Figure 4.

Figure 4. Protein structure of Stat5a
Stat5a proteins are encoded by seventeen exons leading to translation of five functionally discriminable domains: The N-terminal domain (NTD), responsible for cofactor interaction, (e.g. with the glucocorticoid receptor (GR, see below)), the coiled-coil domain (CCD), essential for lateral diffusion of Stat5 molecules on the DNA. Efficient DNA binding is also warranted by the DBD. The SH2 domain is indispensable for Stat5 protein interaction with its upstream kinase Jak2. The C-terminal transactivation domain (TAD) harbours critical tyrosine and serine residues, which are phosphorylated by Jak2 and lead to efficient dimerization and nuclear translocation of Stat5 molecules. Transcriptional coactivators like CBP/p300 also interact with this domain.

Unphosphorylated Stat5 forms rigid, antiparallel dimers with the N-terminal domain retaining its flexibility leading to protrusion from the dimeric Stat5 core complex. Formation of higher order complexes of Stat5 (particular Stat5 tetratomers) depends on the Stat5 N-terminus. In addition, the Stat5 N-terminus constitutes the docking platform for essential transcription cofactors like TUDOR domain coactivators \(^{112}\), CBP/p300 \(^{113}\) and (as revealed in this work), the glucocorticoid receptor (GR) \(^{114}\). A schematic representation of established Stat5 protein interactors is given in Figure 5.

![Figure 5. Stat5 oligomerization and cofactor interaction](image)

**Figure 5. Stat5 oligomerization and cofactor interaction**

Oligomeric Stat5 molecules (Stat5 tetratomers) potently rearrange the chromatic architecture and induce DNA loop formation. In addition, Stat5 tetratomers recruit transcriptional cofactors/corepressors which in turn modulate Stat5 target gene specificity. Several components of the basal transcription machinery are reported to interact with Stat5 and are illustrated to the right.

### 2.6.1. Stat5 in health and disease

Among others, Stat5 possesses critical functions in regulating hematopoiesis and hepatic lipid metabolism. Hyperactivation of Stat5 is associated with severe conditions such as myeloproliferative diseases \(^{115,116}\), inflammation \(^{117}\), autoimmunity \(^{118}\) and cancer \(^{119}\). However, Stat5 hypoactivation can cause diseases such as myeloid hyperplasia and anaemia \(^{120,121}\), dwarfism \(^{122,123}\), immunodeficiency and metabolic problems \(^{124}\). One important aspect of this work was unravelling the significance of hepatic Stat5 signaling in regulating the postnatal body growth and
hepatic lipid metabolism. Hitherto known functions of Stat5 in controlling body growth and lipid metabolism are summarized in the following (section 2.6.1.1 and 2.6.1.2).

2.6.1.1. Stat5 and postnatal body growth

Serum Growth hormone (GH) potently activates hepatic Stat5 which in turn mediates the effects of GH on longitudinal body growth by transcription of bioactive Insulin-Like growth factor (IGF-1) \(^{125}\). Using transgenic mouse models, which express dysfunctional GH receptors (GHR) \(^{126}\) or harbours deletions of hepatic Stat5 \(^{123,127}\), it was confirmed that GH signal transduction is largely mediated via Stat5 activation \(^{122,124}\). Impaired secretion of GH or mutations of the GHR leads to dwarfism secondary to reduced levels of IGF-1, a condition termed Laron’s syndrome \(^{128}\). In line with this, human patients harbouring Stat5 missense mutations are severely growth retarded \(^{129}\). Interestingly, a role for the glucocorticoid receptor (GR) in synergistic transcription of Stat5 target genes \(^{114,122}\) was demonstrated.

2.6.1.2. Stat5 and hepatic lipid metabolism

Stat5 signal transduction in the liver differs from other tissues in the regard that hepatocytes express predominantly the Stat5b isoform transcribing Stat5 target genes. An essential role of hepatic Stat5b, among others, is transcription of growth-promoting genes (e.g. IGF-1 and ALS) upon rhythmic GH pulses \(^{129,130}\). Strikingly, hepatic loss of Stat5b leads to impairments of postnatal body growth and elevated levels of serum GH \(^{123}\). Studies involving GHR knockout mice elegantly demonstrated that elevated levels of lipolytic GH lead to substantial reductions of peripheral adipose tissues (lipodystrophy), an effect mediated by Stat5 in adipocytes \(^{131}\). Further proof for the role of hepatic Stat5 in indirectly influencing adipose tissues came from mice expressing aminoterminally truncated Stat5 proteins (termed Stat5\(^{\Delta N}\) mice) \(^{127}\). These animals harboured reduced lipid reservoirs (lipodystrophy) \(^{122,127,132}\) and in this regard resembled GHR knockout mice., The lipodystrophy was attributed to impaired negative feedback signaling via IGF-1 In both strains (GHR\(^{-/-}\) and Stat5\(^{\Delta N}\)) \(^{126,133}\), the main feedback inhibitor of GH secretion in the anterior pituitary gland.
Recently, the ablation of hepatic Stat5 was reported utilizing Albumin-Cre-mediated recombination of floxed Stat5 alleles (Stat5<sub>AlbCre</sub>)<sup>124</sup>, a targeting approach leading to an approximate 80% reduction in protein levels. Missing defects in postnatal body growth were attributed to incomplete losses of Stat5 in hepatocytes. Interestingly, mild perturbations of hepatic fat metabolism leading to mild liver steatosis were reported. Compensatory activation of Stat1 and Stat3 signaling was another consequence of incomplete hepatic Stat5 loss.

### 2.7. The glucocorticoid receptor (GR)

The GR transcription factor is expressed in most somatic cell types and is activated by compounds called glucocorticoids (GC), which are secreted under stress conditions by the adrenal gland. The GR constitutes an important executor of the neuroendocrine hypothalamus-pituitary-adrenal (HPA) axis. After GC binding, cytoplasmic GR dissociates from bound heat shock proteins (e.g. Hsp90), translocates into the nucleus and induces the transcription of GR-target genes<sup>134, 135</sup>. Loss of hepatic GR signaling leads to well-studied defects in glucose metabolism such as impaired gluconeogenesis leading to hypoglycaemia under fasting conditions<sup>136</sup>. This was attributed to blunted transcription of key gluconeogenic enzymes like phosphoenol pyruvat carboxykinase (PEPCK)<sup>137</sup> and glucose-6-phosphatase (G6P)<sup>138</sup>. Adrenal release of GC is negatively regulated by serum GC itself. Impaired negative feedback leads to hypercortisolic conditions which are characterized by pathological lipid accumulation in peripheral adipose tissue or dermis<sup>139</sup>. Structurally, murine GR is composed of several unique domains forming structural units: An aminoterminal TAD domain (termed AF-1) was shown to be responsible for cooperation with Stat5<sup>140</sup>. The AF-1 function is followed by a DBD, which comprises two zinc finger domains, warranting specific GR binding to hormone response elements. The carboxyterminal ligand binding domain (LBD) guarantees ligand specificity and contains amino acid sequences important to homogenous dimerization, nuclear localisation signals and possesses another TAD termed AF-2 (Figure 6).
2.8. Linear Stat5-GR signal transduction: Two transcription factor pathways converge

Activated GR not only potently binds onto GRE sequences leading to activation or repression of GR target genes. Strikingly, the GR was demonstrated to bind as cofactor to other transcription factors, thus modulating their transcriptome. This process does not require DNA binding of the GR 141. The existence of a linear Stat5-GR signal transduction pathway was initially found in mammary epithelia, in which efficient Stat5-GR protein interaction was demonstrated to be a prerequisite for transcription of milk proteins during lactation (e.g. beta-Casein) 142. Interestingly, loss of hepatic GR leads to stunted postnatal body growth reminiscent of mice deficient for hepatic Stat5 114. Thus, it has been postulated that Stat5-GR protein interaction is mandatory for transcription of hepatic target genes essential for postnatal growth.

2.8.1. Growth hormone, glucocorticoids and body composition

Growth hormone and GCs, two major effectors of the neuroendocrine HPA axis potently activate Stat5 and GR in various organs. Both compounds have profound effects on body composition 143, 144. In adipose tissues, GCs possess anti-lipolytic properties and stimulate fat accumulation via LPL-mediated triglyceride hydrolysation 145, whereas GH exerts lipolytic effects by accelerating basal lipolysis in adipocytes 143. In addition, GH blunts fatty acid synthesis in adipose tissues 146 and impairs sensitivity towards insulin action, thereby leading to a reduction of transcript and protein levels of crucial lipogenic genes 147.

Thus, single administration of GH and GC leads to opposite effects on peripheral lipolysis 148. Intriguingly, combined stimulation with GH and GC in vivo leads to the dramatic mobilisation of peripheral adipose tissues exceeding GH-mediated lipolysis.
alone \textsuperscript{149}. This concept was confirmed by \textit{in vitro} reports demonstrating dramatic lipolytic drive in rat adipocytes elicited by co-administration of GH and Dexamethasone (DEX, a synthetic GR agonist extensively used in clinics) \textsuperscript{150}.

2.9. Conditional gene deletion using the Cre/LoxP system

The Cre/LoxP recombination system allows tissue-specific gene deletion in defined species such as the laboratory mouse \textsuperscript{151}. First described in bacteriophages, the Cre/LoxP system consists of two components \textsuperscript{152}:

(i) DNA sequences essential for the function/structure of targeted proteins are flanked by palindromic loxP sequences (yielding a ‘floxed allele’).

(ii) The tissue-specific expression of the Cre proteins induces DNA recombination of floxed alleles resulting in the excision or inversion of respective DNA sequences, depending on either parallel or anti-parallel orientation of the loxP sites \textsuperscript{153}. The tissue-specificity of Cre expression is warranted by respective tissue-specific situated upstream of the Cre expression cassette. This restricts Cre activity solely to promoter-responsive tissues. Usually, gene-sequences are floxed which do not interfere with overall expression of the protein; rather small domains essential for protein function are flanked by loxP sites. For example, for conditional inactivation of the GR, only the first zinc finger in the DNA binding domain is targeted, leading to transactivation-defective GR molecules. Another strategy is flanking whole genes loci with loxP sites. This is exemplified by conditional deletion of Stat5 by flanking the whole chromosomal locus of 110 kb containing Stat5a and Stat5b. Upon Cre activity, both Stat5 isoforms are excised. This representative example is illustrated in Figure 7.
Figure 7. Tissue-specific loss of Stat5 using Cre/LoxP technology
The complete murine Stat5a/b locus, spanning 110kb of genomic DNA on chromosome 17, is flanked by loxP sites ('floxed' locus, Stat5\(^{fl/fl}\)). The crossing of Stat5\(^{fl/fl}\) mice with animals expressing Cre recombinase in a tissue-specific manner results in the excision of floxed DNA sequences.
2.10. Aim of the thesis

Stat5 possesses well-established functions critical for mediating cytokine and growth factors responses in the hematopoietic system. In contrast, the role of Stat5 in solid organs such as the liver remains poorly understood. It is widely recognized, that Stat5 in hepatocytes regulates postnatal body growth by transactivation of growth-mediating target genes such as IGF-1. Apart from classical functions as cytokine and growth factor responsive transcription factor, Stat5 interacts with the glucocorticoid receptor (GR), together constituting a linear Stat5-GR signal transduction axis leading to modulation of the Stat5 transcriptome.

Deletion of hepatic Stat5 alone leads to

(i) severe perturbations of postnatal body growth
(ii) an increased biosynthesis of lipids leading to fat accumulation in hepatocytes and
(iii) defects in bile uptake leading to cholestasis, jaundice and secondary liver fibrosis.

In this study we aimed to investigate the underlying molecular processes leading to aforementioned phenotypes observed upon hepatic ablation of Stat5. Using knockout mice in concert with molecular biological techniques such as expression profiling, quantitative mRNA analysis, protein biochemistry and immunohistochemistry we aimed to shed further light on the role of hepatic Stat5 in health and disease. In addition, we put special emphasis on addressing the question whether above phenotypes were solely the effect of loss of hepatic Stat5 signaling or necessitated additional ablation of the GR.
3. Results

3.1. A linear Stat5-GR axis controls postnatal body growth

Hepatic Stat5 influences postnatal body growth via transcription of growth-related genes like IGF-1 and ALS upon GH action\(^\text{154}\). Bioactive IGF-1 consists of IGF-1•ALS complexes, necessary for longitudinal growth of extremities such as bones and internal organs\(^\text{155}\). Interestingly, hepatic deletion of the GR (GR\(^\text{AlfpCre}\)) leads to similar growth retardations as observed for Stat5 knockout mice (Stat5\(^\text{AlfpCre}\))\(^\text{156}\). In addition, classical GH-triggered Stat5 target genes like IGF-1 and ALS were significantly reduced in GR\(^\text{AlfpCre}\) animals. Furthermore, Stat5 and the GR were reported to physically interact in the liver and the GR binds to Stat5 REs located in the promoter sequences of IGF-1 and ALS\(^\text{114}\). Collectively, this indicates that Stat5 and the GR are constituents of a linear signal transduction pathway in hepatocytes necessary for full transcription of growth hormone regulated Stat5 target genes like IGF-1 and ALS. This however stands in stark contrast to the hypothesis that postnatal growth is mediated by local production of IGF-1 in bone, cartilage and muscles upon GH action and that GH action in liver is dispensable\(^\text{157}\). However, the possibility remains that the dwarfism observed in GR\(^\text{AlfpCre}\) mice is due to indirect consequences independent of Stat5, e.g. metabolic disturbances. To address the question whether (i) GH action in the liver is necessary for postnatal growth and (ii) whether and how this phenomenon involves physical Stat5-GR interaction, we generated mice harbouring deletions of Stat5 (Stat5\(^\text{AlfpCre}\)), the GR (GR\(^\text{AlfpCre}\)) or both (Stat5/GR\(^\text{AlfpCre}\)) in hepatocytes.

3.1.1. Loss of hepatic Stat5 and/or the GR signaling leads postnatal growth deficiency
Results

3.1.2. Loss of hepatic Stat5 and/or the GR signaling leads to similar changes in gene expression profiles

We aimed to further underpin our hypothesis of a linear signal transduction axis comprising Stat5 and GR controlling growth-related genes upon GH action. In
addition, we questioned the fraction of the transcriptome co-dependently regulated by Stat5 and GR. We analysed livers from control, Stat5AlfpCre and GRAlfpCre animals and undertook Affymetrix whole genome expression analysis. Interestingly, the gene signatures significantly downregulated by ablation of either Stat5 or the GR overlap to a high extend (Figure 9A, left). The significance criterion was defined as either (i) \( p<0.001 \) or (ii) \( p<0.05 \) and fold change \( >2^{1} \). According to these criteria, 42 % of genes downregulated in GRAlfpCre mice were concomitantly downregulated in Stat5AlfpCre animals. Correspondingly, 26 % of genes downregulated in Stat5AlfpCre mice were also downregulated in GRAlfpCre mutants. The genes significantly upregulated in both mutants were far less abundant (Figure 9A, right). The high extend of overlapping genes was in the same range if less stringent criteria were applied (e.g. \( p<0.005 \) or \( p<0.01 \), not shown). To further corroborate the coordinate change of Stat5-GR co-dependent genes, we determined their expression changes in Stat5/GRAlfpCre double knockout animals. As expected, we observed that genes significantly changed in a given direction in the single mutants were changed in the same direction with the same magnitude in Stat5/GRAlfpCre mutants (Table 1A).

Figure 9. Substantial similarities in expression profiles from Stat5AlfpCre, GRAlfpCre and Stat5/GRAlfpCre mice
(A) Venn diagrams showing the overlap of genes significantly changed in livers from mice with hepatocyte-specific deletion of Stat5 or GR. As criterion for calling a gene significantly changed we
Results

used p < 0.001, or the combined criterion of p < 0.0025 and a log ratio >1 or less than −1 (fold change >2 or <0.5). Note the substantial overlap of genes downregulated by the two mutations. (B) Comparison between Stat5^AlfpCre and GR^AlfpCre mutants of the expression changes for genes significantly changed in both mutants. All of these genes are changed in the same direction in the two mutants and the magnitude of change correlates tightly. (C) Comparison of expression changes in Stat5^AlfpCre and Stat5/GR^AlfpCre livers for the same genes as in B. There is no additional effect of deleting GR in the absence of Stat5 for the expression levels of these genes.

The genes found to be significantly dependent on both, GR and Stat5, are listed in (Table 1), the complete list of genes significantly changed in a single knockout is accessible online at: [http://www.genesdev.org/cgi/content/full/21/10/1157/DC1](http://www.genesdev.org/cgi/content/full/21/10/1157/DC1) (Supplementary Table 1).

| Table 1. Genes and functional groups of genes that are regulated by Stat5-GR interaction |
|-----------------------------------|-------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| (A) Individual genes | Down in GR^AlfpCre and Stat5^AlfpCre | Stat5^AlfpCre | GR^AlfpCre | Stat5/GR^AlfpCre | Stat5^AlfpCre | Functional set |
| Gene name | Changes (log2 ratios) | | | | | |
| IGF-binding protein, acid labile subunit | −0.7 | −2.6 | −7.1 | −1.9 | s, g |
| Insulin-like growth factor 1 | −6.3 | −2.2 | −5.7 | −1.1 | s, g |
| Epidermal growth factor receptor | −5.3 | −5.1 | −5.1 | −5.4 | g, m |
| Cysteine sulfenic acid decarboxylase | −5.0 | −2.6 | −4.8 | −3.3 | g |
| Hydroxysteroid dehydrogenase-5 | −4.8 | −5.5 | −6.5 | −4.0 | g, m, d |
| Suppressor of cytokine signaling 2 | −4.6 | −5.1 | −5.1 | −2.8 | g |
| Peroxisome 4 | −4.5 | −4.6 | −4.6 | −3.6 | g |
| DNA segment, human D4S114 | −4.2 | −2.1 | −3.2 | −2.5 | g |
| Serine proteinase inhibitor A3K (SP12) | −3.5 | −2.7 | −3.0 | −2.5 | g |
| Flavin-containing monoxygenase 3 | −3.0 | −2.5 | −3.8 | −3.6 | g |
| Hydroxysteroid 17B dehydrogenase 2 | −2.4 | −1.4 | −2.3 | −0.9 | d |
| Hydroxysteroid dehydrogenase-2 | −1.8 | −1.6 | −2.1 | −0.9 | d |
| β-chain-associated protein laeas | −1.7 | −1.8 | −1.8 | −0.6 | d |
| Acidic ribosomal phosphoprotein P0 | −0.9 | −0.6 | −0.9 | −0.4 | d |
| Leukemia inhibitory factor receptor | −0.8 | −0.8 | −1.0 | −1.0 | d |
| Cytochrome P450, 4H3 | −0.7 | −0.5 | −0.7 | −0.1 | d |
| Ribosomal protein L17a | −0.6 | −0.4 | −0.5 | −0.3 | d |

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<th>(B) Groups of genes</th>
<th>p-value</th>
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Table 1. Genes and functional groups of genes that are regulated by Stat5-GR interaction

(A) Genes significantly downregulated in Stat5^AlfpCre, GR^AlfpCre, Stat5/GR^AlfpCre and Stat5^AlfpCre mice. Expression changes are given as log2 ratios of fold changes. If they belong to any of the significantly changes gene sets (see below, B) it is indicated in the far right bar. Abbreviations indicate (s) Somatomedin mediators; (g) GH responsive genes; (m) male predominant genes; (d) steroid dehydrogenase; (r) genes encoding ribosomal proteins. (B) Functional gene sets in which changed genes are enriched, determined using MAPPfinder software (see Methods).

The extensive overlap between expression changes observed in either single-deletion knockout mouse could be explained by (i) a direct functional interaction of the two transcription factors acting in a linear signal transduction fashion or (ii) may
be due to independent regulation of the same set of genes. To distinguish between those two putative mechanisms, we argued the following way: If there is a direct physical interaction between Stat5 and the GR, the magnitude of change of a given gene should correlate in Stat5$^{AlfpCre}$ and GR$^{AlfpCre}$ mice. If, in contrast, this is not the case, the actions are probably independent. To address this issue, we plotted the fold change as log2 ratio of significantly changed genes in Stat5$^{AlfpCre}$ and GR$^{AlfpCre}$ against each other (Figure 9B). The positive correlation of fold changes between the two single mutants indicated the presence of a ‘positive’ interaction. In contrast to the general tendency, a small subset of genes was less affected in the GR$^{AlfpCre}$ mutants, indicating that Stat5 action on these Stat5-dependent promoters is not totally dependent on the GR. To further expand the interpretation of our data, we similarly plotted the expression changes of coregulated genes in Stat5/GR$^{AlfpCre}$ versus Stat5$^{AlfpCre}$ (Figure 9C). The similar positive correlation indicated that the GR is not able to significantly alter the expression changes under conditions in which Stat5 is lacking. We concluded that the activating effect of the GR is completely dependent on protein-protein interaction with Stat5. Collectively, these findings strongly support a model of Stat5 and GR regulated expression of specific gene subsets via direct interaction with each other.

Next, we aimed to elucidate which functional groups of genes are predominantly affected by Stat5-GR interaction in the liver. We utilized the MAPPfinder software to find gene groups with coordinate changes$^{159}$. This approach can detect low-amplitude changes common to many genes attributed to a functional group. This is superior to detecting high-amplitude changes given for a single gene as compensatory mechanisms exerted by functionally related genes are not taken into account. We found that Stat5-GR interaction is important for many gene sets (Table 1B). Noteworthy was the strong dependency of the gene sets designated to “fatty acid metabolism” and “genes repressed by GH”. The coordinate expression changes elicited by Stat5 and the GR correlate closely with those found for various truncations of the GH receptor (GHR)$^{160}$. The observed changes for male-predominant genes were in line with earlier reports from the Waxman group$^{161}$ which reported sex-specific gene expression to be dependent on GH action and hepatic Stat5.

3.1.3. The N-terminus of Stat5 constitutes the docking platform for the GR
To shed further light on the nature of Stat5-GR protein interaction in hepatocytes, we utilized livers from mice harbouring N-terminally truncated versions of Stat5a and Stat5b proteins (Stat5$^{\Delta N}$ mice). The N-terminus of Stat5 was reported to be of importance for oligomerization of Stat5 proteins $^{162}$. Initially designed as complete Stat5 knockout mouse, Stat5$^{\Delta N}$ mice still express considerable amounts of N-terminally truncated Stat5 proteins, approximately 30-40% of control protein levels $^{115}$. We further utilized Stat5$^{\Delta N}$ mice because they displayed growth retarding comparable to Stat5$^{AlfpCre}$, GR$^{AlfpCre}$ and Stat5/GR$^{AlfpCre}$ mutants $^{114}$, putatively due to similar molecular perturbations in the liver. Collectively, these mice are regarded as an in vivo model for studying the physiological consequences which are secondary to the loss of the Stat5b N-terminus. As Stat5b is the predominant Stat5 isoform expressed in the liver, no considerable amounts of Stat5a were detected (not shown). We thus detected only the low-molecular weight Stat5b$^{\Delta N}$ isoform in livers of Stat5$^{\Delta N}$ mutants, which were fully tyrosine phosphorylated upon GH stimulation (Figure 11A). Also, GR$^{AlfpCre}$ mice exhibited normal tyrosine phosphorylation properties upon GH challenge. To further investigate whether GH-induced tyrosine phosphorylation led to normal DNA binding of Stat5b$^{\Delta N}$ proteins we performed electrophoretic mobility shift assays (EMSA) on classical Stat5 responsive promoter elements (beta-Casein elements). Stat5b$^{\Delta N}$ proteins fully bound beta-Casein elements upon GH activation compared with full-length Stat5b (Figure 10).

![Figure 10](image)

**Figure 10. Stat5$^{\Delta N}$ proteins fully bind to Stat5 responsive elements upon GH challenge in vivo**

Liver extracts from control and Stat5$^{\Delta N}$ mice were subjected to EMSA analysis in order to assess the DNA-binding properties of Stat5 isoforms in vivo upon GH stimulation. Tyrosine phosphorylation of Stat5b$^{\Delta N}$ proteins resulted in a comparable DNA binding activity on classical Stat5 RE from the beta-Casein promoter compared with full-length Stat5b.
Next, we performed chromatin-immunoprecipitation (ChIP) assays to investigate the chromatin-binding properties of Stat5b\textsuperscript{AN} proteins on classical GH-triggered Stat5 targets. GH stimulation led to significant binding of Stat5b as well as Stat5b\textsuperscript{AN} proteins to specific Stat5 responsive elements in the promoter regions of IGF, ALS and Socs2. N-terminally truncated Stat5b\textsuperscript{AN} proteins retained an approximately 70-80% binding capacity to these chromatin regions compared with full-length Stat5b proteins (Figure 10B). To further investigate whether the N-terminus of Stat5b is essential for protein interaction with GR, we performed immunoprecipitation (IP) assays (Figure 10C). Reciprocal co-immunoprecipitation with anti-GR or anti-Stat5 specific antibodies revealed Stat5-GR protein interaction in both directions. However, the specific Stat5-GR interplay was lost in Stat5b\textsuperscript{AN} mice.

Thus, we concluded that the N-terminus of Stat5b constitutes the docking platform for the GR in hepatocytes.

Figure 11. Stat5-GR interaction is dependent on the Stat5b N-terminus
(A, top) Liver extracts were prepared from untreated or GH injected mice, followed by quantitative anti-Stat5b immunoprecipitation (IP) and subsequent immunoblotting with an anti-pY-Stat5 antiserum. (A, bottom) Blots were stripped and reprobed with an anti-Stat5b-specific antiserum. Stat5b and Stat5b\textsuperscript{AN} proteins are fully tyrosine-phosphorylated upon GH stimulation. The amounts of total Stat5b were reduced to ~40% in Stat5b\textsuperscript{AN} mice. (B) In vivo binding of Stat5 to proximal enhancers of ALS, Socs2 and IGF-1. The fold enrichment for each DNA fragment upon immunoprecipitation by anti-Stat5 followed by quantitative PCR is illustrated as bar graphs (mean ± SEM). Upon GH stimulation, wild-type Stat5b is highly bound to chromatin. Stat5b\textsuperscript{AN} displays ~50–70% of the
DNA binding capacity of full-length Stat5b. (C) The N-terminus of Stat5b is essential for Stat5b-GR interplay. Reciprocal immunoprecipitation of liver extracts using anti-Stat5b (left) or anti-GR (right) antisera. (Left) Stat5b\(^{\Delta N}\) as well as full-length Stat5b proteins are efficiently immunoprecipitated with anti-Stat5b antisera (bottom), but only wild-type Stat5b interacts with the GR (top). (Right) GR proteins are comparably pulled down (bottom), but only full-length Stat5b is co-immunoprecipitated (top). Four similar experiments were performed and one representative analysis is shown.

Expression profiling of Stat\(^{\Delta N}\) livers revealed that some of those genes previously identified as Stat5 responsive genes were expressed at normal levels while some others were downregulated. Notably, most genes that were exclusively dependent on Stat5 but not on interaction between Stat5 and the GR were unchanged in Stat\(^{\Delta N}\) mice. In contrast, most genes co-dependent on Stat5-GR interaction displayed compromised expression in Stat\(^{\Delta N}\) animals (Figure 12A, B). In addition, Stat5 responsive genes that were only partially dependent on the interaction with GR were concomitantly only partially dependent on the N-terminus of Stat5b (Figure 12C). These results supported our hypothesis that the N-terminus of Stat5 is important for protein interaction between Stat5 and the GR. Moreover, the overlapping expression changes observed in Stat5\(^{AlfpCre}\), GR\(^{AlfpCre}\), Stat5/GR\(^{AlfpCre}\) and Stat5\(^{\Delta N}\) mutants are likely due to the disruption of Stat5-GR protein complexes.
Collectively, we could show that physical protein interaction between Stat5 and the GR in hepatocytes is necessary for ensuring proper postnatal growth. Using conditional mouse models, whole genome expression profiling (Affymetrix analysis) and biochemical methods we could corroborate that physical Stat5-GR interaction is necessary for full transcription of specific gene subsets. Functional gene groups depending on Stat5-GR interplay include genes responsible for fatty acid metabolism (not shown herein) and postnatal body growth. The latter includes genes like IGF-1, ALS and Socs2, important genes implicated in regulation of longitudinal body growth. In addition, we could identify the N-terminus of Stat5b as docking platform for the GR.
in hepatocytes. Growth defects observed in mice harbouring N-terminally truncated Stat5 proteins (Stat5\textsuperscript{ΔN} mice) are likely due to an impaired Stat5-GR protein interaction. GH-induced tyrosine phosphorylation and DNA binding of Stat5\textsuperscript{ΔN} proteins were unaltered and thus could not account for the dwarfism seen in Stat5\textsuperscript{ΔN} mice.

### 3.1.4. Soc2 as example for Stat5-GR co-dependent gene transcription

Suppressor of cytokine signaling 2 (Socs2) belongs to the Socs family of proteins which are transcribed upon cytokine action and serve as potent negative feedback regulators, e.g. for signals leading to activation of the Jak/Stat pathway \textsuperscript{163}. Although Socs proteins are rather promiscuous in their upstream specificity, the transcription of the Socs2 isoform is widely recognized to be driven by GH-induced Stat5 activation alone \textsuperscript{164, 165}. A growing body of evidence supports the notion of Socs2 serving as major negative regulator of the GH-Stat5 axis \textsuperscript{166}. Genetic studies underpinned the pivotal role of Socs2 in growth and development: Socs2 knockout mice exhibit gigantism, a severe increase in longitudinal growth due to sustained Stat5 activation and elevated IGF-1 levels. Recent reports revealed roles for activated glucocorticoid receptor (GR) in supporting Stat5-mediated transcription of Socs2 mRNA \textsuperscript{114}. In addition, a substantial decrease of Socs2 mRNA transcription in knockout mice lacking either Stat5 (Stat5\textsuperscript{AlfpCre}; log2ratio (L2R) -4.6 vs. controls), the GR (GR\textsuperscript{AlfpCre}; L2R -5.1 vs. controls) or both (Stat5/GR\textsuperscript{AlfpCre}; L2R -5.1 vs. controls) in hepatocytes was observed using our whole genome expression analysis (Table 1). Moreover, Socs2 mRNA levels were severely decreased in mice which lack the aminoterminal Stat5 domain, which is critical for interaction with the GR (Stat5\textsuperscript{ΔN}; L2R -2.8 vs. controls). Thus, we presumed that the decrease in Socs2 transcription was due to disrupted Stat5-GR protein interaction serving as example for genes regulated by linear Stat5-GR signal transduction. Interfering with either Stat5 or GR signaling thus results in decreased levels of Socs2 mRNA.

#### 3.1.4.1. The Soc2 promoter harbours putative Stat5-GR responsive elements
It is widely recognized that ample transcription of Stat5 target genes requires oligomerization/tetramerization of Stat family members, exemplified for Stat5a/b proteins. Interestingly, those genes demonstrated to depend on functional Stat5-GR protein interaction often harbour tandem Stat5 responsive elements (Stat5 tetramer sites, Stat5 2xRE) in their 5’ promoter regions. Promoters with several Stat52xRE were especially found in growth-related genes such as IGF-1, ALS and Socs2. To investigate this phenomenon in more detail we utilized bioinformatics screening of the Socs2 promoter region and identified three Stat5 2xRE therein (Accession No. NT_039500, Figure 13). All tandem sites were spaced in close vicinity (<30 bp), which could allow for DNA loop formation as a prerequisite for Stat5 tetramerization.

Figure 13. The Socs2 promoter (-3443 to +1 bp) harbours three tandem Stat5 RE (Stat5 2xRE) Tandem Stat5 REs (Stat5 2xRE) were identified in the 5’ promoter region of Socs2 by bioinformatics screening for two tandem linked Stat5 RE (TTC(T/C)N(G/A)GAA) in close vicinity (n<30 bp; gray). Underlined sequences were used for EMSA analysis (see Figure 15). Black arrows indicate Stat5 2xRE#1 (-3351/-3329 bp upstream of ATG; top), Stat5 2xRE #2 (-2976/-2931 bp; middle) and Stat5 2xRE #3 (-1964/-1944 bp; bottom). Additional GR responsive elements found in the Socs2 promoter are capitalized.

* = 3443bp 5’ of translation start [ATG]

** = 1832bp 5’ of translation start [ATG]
To test whether transactivation of the Socs2 promoter requires synergistic Stat5 and GR signaling, we designed three Luciferase (LUC) reporter constructs harbouring truncated versions of the proximal Socs2 promoter. Each of these constructs (termed I-III) harboured:

(i) A core region of the Socs2 promoter containing the three Stat5 2xRE (-3443 bp/-1832 bp)

(ii) Additional 5’- and 3’ regions flanking the three Stat5 2xRE-containing core region to assess the requirement for putative enhancer sequences.

Promoter constructs I-III were amplified by polymerase chain reaction (PCR) as described in Methods (section 5.2.2.1) using 5’ overhang primers containing restriction sites for enzymes MluI, BgIII and HindIII. Subsequently, PCR products were cloned by appropriate restriction enzyme digestion and T4 ligation into the multiple cloning site (MCS) of a LUC reporter vector under the control of a 5’-minimal TA promoter (Figure 14 and Methods).

![Figure 14. Socs2 promoter regions cloned for LUC reporter assays](image)

Socs2 reporter constructs No. I-III containing the three Stat5 2xREs (Stat5 2xRE#1-3; left) were cloned into the MCS of a LUC reporter vector under the control of a minimal TA promoter (right). Additional 5’- and 3’- regions of the Socs2 promoter were included in Construct I+III to assess for putative enhancer elements. Correct assembly of vector constructs was confirmed by restriction analysis and DNA sequencing.

3.1.4.2. Putative Stat5-GR DNA binding sites in the proximal Socs2 promoter are Stat5 responsive
First, we investigated whether the three Stat5 2xREs located in murine Socs2 promoters are recognized activated Stat5 molecules. Figure 15 shows an EMSA analysis illustrating the Stat5 binding properties in vitro and in vivo on all three Stat5 2xRE located in the Socs2 promoter. First, human HEK 293 cells were transfected with Stat5b and the erythropoietin (Epo) receptor (EpoR), a cytokine receptor structurally related to the GHR triggering potent activation of Stat5 upon Epo ligand binding. Subsequent stimulation with 25 U/ml Epo for 30 min led to significant binding of Stat5 protein onto all three Stat5 2xRE#1-3 in vitro, albeit to a less extend onto Stat5 2xRE #2. No DNA binding of Stat5 molecules was observed under basal, non-stimulated conditions. Stat5 specificity was confirmed via supershift analysis using high affinity Stat5b-specific antisera. This led to the specific retention of $^{32}$P-labeled oligomer-Stat5-antibody complexes (Figure 15; upper panel). Similar as observed upon Epo stimulation of HEK293 cells in vitro, GH potently activated hepatic Stat5 in vivo leading to high affinity binding of Stat5 onto Stat5 2xRE#1-3 elements in the murine Socs2 promoter. Interestingly, Stat5 2xRE #2 recruited significantly less phosphorylated Stat5 molecules, corroborating observations for in vitro stimulated Stat5. All complexes bound to $^{32}$P-labeled oligomers contained Stat5 molecules as confirmed by supershift analysis (Figure 15; lower panel). Evidence for Stat5b tetramerisation on the Socs2 binding sites was not predominant; rather Stat5b dimers were detected.
Figure 15. Activated Stat5 binds to Stat5 2xRE in the Socs2 promoter in vitro and in vivo
Electromobility shift assay (EMSA) analysis of Stat5 2xRE#1-3 (as introduced in Figure 13). (Top) HEK 293 cell extracts were transfected with the EpoR and Stat5 and Epo-stimulated for 30 min. Extracts were analysed for binding of activated Stat5 onto Stat5 2xRE#1-3 located in the murine Socs2 promoter. (Bottom) Mice were stimulated with GH for 30 min and liver homogenates analysed for DNA binding properties of activated Stat5 on Stat5 2xRE#1-3. Fast-migrating complexes represent Stat5 dimers bound to 32P-labeled oligomers. Stat5-specific binding was confirmed via Stat5 antibodies yielding high molecular weight complexes migrating slower on respective oligomers (ss, supershift). Beta-Casein was used as positive control for a Stat5 responsive promoter sequence.

Thus, Epo treatment stimulated high affinity binding of Stat5 onto all three Stat5 2xRE located in the murine Socs2 promoter in vitro. In vivo analysis of GH activated Stat5 confirmed these results.

3.1.4.3. The proximal Socs2 promoter is synergistically transactivated by Stat5-GR interplay

We demonstrated that the Stat5 2xRE#1-3 located in the proximal Socs2 promoter are recognized and bound by phosphorylated Stat5 dimers. Subsequently, we studied the transactivation properties of promoter fragments containing all three Stat5 2xRE when placed in front of a LUC reporter construct (Reporter constructs I-III, see Figure 14). For this, pMSCV plasmids expressing murine Stat5, GR and EpoR were transiently transfected into COS7 cells and after 48 h stimulated for 30 min with 25
U/ml Epo and/or the glucocorticoid agonist Dexamethasone (Dex) at a final concentration of 10 nM. As shown in Figure 16 (bars 1-8), no LUC activity was observed upon Stat5-GR transactivation of the two distal Socs2 promoter fragments No. I+II. Contrarily, the 3′-most proximal Socs2 promoter fragment No. III exhibited LUC activity already under non-stimulated conditions (bar 9). Stimulation with Epo led to ~2fold increases in LUC activity (bar 10). No effect of Dex treatment alone was observed (bar 12). Strikingly, co-activation of Stat5 and GR signaling by simultaneous Epo and Dex treatment led to significant increases in LUC activity (~5fold, bar 11).

Thus, Stat5 and GR signaling synergistically transactivated the Socs2 promoter. This effect was demonstrated for the proximal Socs2 promoter, whereas distal promoter fragments did not show these synergies. This pinpointed towards additional 3′- enhancer elements, either located in the proximal Socs2 promoter itself or in Socs2 intron sequences.

Figure 16. The proximal Socs2 promoter is synergistically activated by Stat5-GR signaling

Synergistic Stat5-GR transactivation properties of three Socs2 LUC constructs (No. I-III, as schematically shown in Figure 14) were determined. Briefly, COS7 cells were transfected with pMSCV plasmids expressing murine Stat5b, GR and EpoR. After 48 h, cells were stimulated with 25 U/ml Epo and/or 10 nM Dex. Cellular extracts were prepared and the LUC activity measured as described in Methods. Equal transfection efficiency was determined by co-transfection of a beta-Galactosidase (beta-Gal) expression plasmid and normalization of LUC activity to beta-Gal activity. Assays were performed in triplicate.

3.1.4.4. Full transactivation of the Socs2 promoter depends on Stat5 and GR

We identified the proximal Socs2 promoter to be strongly responsive to synergistic Stat5-GR transactivation. Next, we addressed the question whether this effect
required Stat5 and GR proteins. Thus, we compared conditions of Stat5 and GR overexpression and subsequent cytokine stimulation by Epo and/or Dex with mock-transfected COS7 cells. COS7 cells were chosen since they lack endogenous Stat5 expression. Untransfected COS7 cells exhibited a mere ~1.5fold induction in LUC activity upon Epo/Dex co-stimulation (Figure 17, bar 3). Epo treatment alone had no effect (Figure 17, bars 1-2). Dex treatment led to ~2fold increases in LUC activity compared with untreated cells. In strong contrast, overexpression of Stat5 and GR proteins led to potent increases in LUC activity (Figure 17, bars 5-8). A modest ~2fold increase of LUC activity was observed upon Epo stimulation alone. Combinatorial stimulation of Stat5 and GR proteins led to approximately 5fold increases in LUC activity, thereby significantly bypassing the effects of Epo treatment alone.

Thus, we confirmed that the observed synergism of Stat5 and GR signaling in transactivating the proximal Socs2 promoter depends on Stat5-GR interaction.

![Graph](image)

**Figure 17.** Synergistic Epo/Dex-dependent transactivation of the Socs2 promoter depends on Stat5 and GR proteins

The Stat5-GR co-dependent transactivation of Socs2 LUC construct No. III (as schematically shown in Figure 14) was determined. Briefly, COS7 cells were transfected with pMSCV plasmids expressing murine Stat5b, GR and EpoR. After 48 h, cells were stimulated with 25 U/ml Epo and/or 10 nM Dex. Cellular extracts were prepared and the LUC activity measured as described in Methods. Equal transfection efficiency was determined by co-transfection of a beta-Galactosidase (beta-Gal) expression plasmid and normalization of LUC activity to beta-Gal activity. Assays were performed in triplicate.

**3.1.4.5.** Synergistic Stat5 and GR signaling is required for full Socs2 promoter transactivation.
Synergistic activation of Stat5 and GR signaling led to a pronounced transactivation of the proximal Socs2 promoter. This effect was dependent on Stat5 and GR proteins. We next followed the question whether two distinct Stat5 and GR or rather one linear Stat5-GR signal transduction pathway was responsible for the observed synergism. We previously identified the Stat5b N-terminus as the GR-docking domain disrupting the Stat5-GR protein interaction upon N-terminal truncation of Stat5b \(^{122}\). We took advantage of these findings and transiently transfected cells with pMSCV plasmids encoding murine GR, EpoR and full-length Stat5b as well as aminoterminally (Stat5b\(_{\Delta N}\)) or carboxyterminally (Stat5b\(_{\Delta 754}\)) truncated Stat5b isoforms. The latter isoform lacks the carboxyterminal transactivation domain essential for cofactor recruitment of the basal transcription machinery. Transfection of full length Stat5b together with GR led to potent, synergistic Socs2 transactivation following Epo/Dex treatment (Figure 18, bar 3). Interestingly, this effect was severely impaired upon transfection with plasmid encoding Stat5b\(_{\Delta N}\) instead of full-length Stat5b (Figure 18, bar 7). Transfection of the Stat5b\(_{\Delta 754}\) isoform almost completely abrogated Socs2 transcription upon Epo or Epo/Dex treatment (Figure 18, bar 11).

Thus, synergistic Stat5-GR co-dependent transactivation of the proximal Socs2 promoter required Stat5-GR protein interaction, the disruption of which led to severe decreases in Socs2 transcription. Abrogation of Stat5 transactivation properties resulted in the complete shutdown of Socs2 transcription.

Figure 18. Socs2 transactivation depends on physical Stat5-GR protein interaction
The Stat5-GR co-dependent transactivation of Socs2 LUC construct No. III (as schematically shown in Figure 14) was determined. Briefly, COS7 cells were transfected with pMSCV plasmids expressing murine Stat5, Stat5<sup>ΔN</sup>, Stat5<sup>Δ754</sup>, GR and EpoR. After 48 h, cells were stimulated with 25 U/ml Epo and/or 10 nM Dex. Cellular extracts were prepared and the LUC activity measured as described in Methods. Equal transfection efficiency was determined by co-transfection of a beta-Galactosidase (beta-Gal) expression plasmid and normalization of LUC activity to beta-Gal activity. Assays were performed in triplicate.

3.1.4.6. A linear Stat5-GR pathway controls hepatic Socs2 transcription

We demonstrated the existence of a linear Stat5-GR signaling pathway essential for full transactivation of murine Socs2 in a standard epithelial cell line (COS7 cells). As Stat5 functions depend largely on the cellular context we wanted to further investigate the extent of Stat5-GR synergism in transactivating the Socs2 promoter in other, more physiologically relevant cells such as hepatocytes. For this, we utilized primary hepatocytes isolated from p16/19<sup>ARF</sup> deficient mice (MIMp16/19ARF-/-). Growth factor supplementation with a defined panel of growth factors (IGF-II, TGFβ and insulin) rendered these cells immortal in vitro, yet an epithelial polarity and expression of hepatocyte markers was kept<sup>169</sup>. A variant of this cell line was generated via genomic integration of the Ras proto-oncogene (MIMp16/19ARF-/-Ras), rendering these cells independent of growth factors stimuli and facilitating handling of cells<sup>170</sup>. Transfection of pMSCV plasmids encoding murine Stat5b, the GR, the EpoR and subsequent cytokine stimulation with 25 U/ml Epo and 10 nM Dex for 30 min induced significant increases in LUC activity in both cell lines: The increase in LUC activity was more pronounced MIMp16/19ARF-/-Ras cells (~6.5fold; Figure 19 bar 7) compared with MIMp16/19ARF-/- cells (~4.5fold; Figure 19, bar 3). Stimulation with 25 U/ml Epo alone led to limited inductions of LUC activity in both cell lines (~2.5fold; Figure 19, bar 2 and 6). Treatment with 10 nM Dexamethasone led to increases in LUC activity of borderline significance.

Thus, we demonstrated, that full Socs2 transactivation is regulated by linear Stat5-GR signaling also in hepatocytes. As the liver represents the primary site of action for GH-mediated Stat5 activation, potent GR signaling as observed under stress conditions might modulate the magnitude of Stat5-derived Socs2 transcription in vivo and thus affecting liver function, metabolism and body growth.
Results

Figure 19. Full Socs2 promoter transactivation in primary hepatocytes is dependent on the Stat5-GR interaction

The Stat5-GR co-dependent transactivation of Socs2 LUC construct No. III (as schematically shown in Figure 14) was determined. Briefly, MIMp16/19ARF/-/ and MIMp16/19ARF/-/Ras cells were transfected with pMSCV plasmids encoding for murine Stat5b, GR and EpoR. After 48 h, cells were stimulated with 25 U/ml Epo and/or 10 nM Dex. Cellular extracts were prepared and LUC activity measured as described in Methods. Equal transfection efficiency was determined by co-transfection of a beta-Gal expression plasmid and normalization of LUC activity to beta-Gal activity. Assays were performed in triplicate.

3.2. Compound loss of hepatic Stat5 and GR signaling leads to steatosis

Fat accumulation in the liver (steatosis) is either the result of excessive fatty acid biosynthesis relative to oxidative clearance or alternatively impaired hepatic release of lipids and thus net deposition of fat in hepatocytes. Fatty acid synthesis, clearance and release are regulated by multiple endocrine factors including hypothalamic and adrenal effectors like GH and glucocorticoids. Stat5 mediates the body’s response to numerous cytokines and growth factors including GH. Both, gain- and loss-of-function studies have shown a critical role of hepatic Stat5 in regulation of postnatal body growth, sexual dimorphism and detoxification control. These GH-derived effects are largely attributed to activated Stat5 efficiently transcribing IGF-1. We could demonstrated in vivo that the GR binds as essential cofactor to the aminoterminus of Stat5b in order to fully drive IGF-1 synthesis upon GH action. IGF-1 in turn inhibits GH secretion in the anterior pituitary, constituting an auto-inhibitory feedback loop preserving appropriate pathway activation. In addition to the existence of a Stat5b knockout line, mice harbouring N-terminal truncations in both Stat5 isoforms were reported (Stat5$^{\Delta N}$ mice). Both Stat5 knockout strains exhibit reduced peripheral lipid reservoirs resembling in
many ways the lipodystrophic phenotype of growth hormone receptor deficient (GHR\(^{-}\)
\(^{-}\)) mice. This and once again supports the notion of GH-Stat5 negative feedback
inhibition\(^{133}\).

In contrast to GH and Stat5, adrenally derived glucocorticoids such as corticosterone
signal through the glucocorticoid receptor (GR) and trigger multiple downstream
effects including inhibition of peripheral lipolysis. Glucocorticoids constitute a critical
arm of the HPA axis, a neuroendocrine system which integrates stress signals to
modulate multiple processes including digestion, immune activation, and energy
supply. Like GH, glucocorticoids tightly regulate their own secretion through an
autocrine negative feedback loop, the impairment of which leads to one of several
hypercortisolic conditions characterized in part by pathological accumulation of lipids
in peripheral adipose tissues\(^{139}\). Deletion of the GR in hepatocytes leads to well-
studied defects in hepatic glucose metabolism such as impaired gluconeogenesis
leading to fasting-induced hypoglycaemia\(^{136}\). In this study, we addressed the
question whether Stat5 and GR proteins act separately in regulating hepatic lipid
homeostasis or rather require a functional Stat5-GR signaling axis. In vitro studies
claimed an inhibitory role of Stat5 during adipogenesis\(^{172}\). Thus, we generated mice
lacking Stat5 and/or the GR in hepatocytes.

By macroscopic examination we discovered a remarkable dependence on Stat5 and
GR proteins in regulating hepatic lipid homeostasis: Preliminary macroscopic
analyses at sacrifice revealed graded hepatic pathologies between genotypes. Whereas both control and GR\(^{AlfpCre}\) mice displayed normal liver morphology, hepatic
steatosis and hepatomegaly were observed in both Stat5\(^{AlfpCre}\) and Stat5/GR\(^{AlfpCre}\)
mutants as early as 4 weeks postnatally. Strikingly, while the extend of steatosis in
Stat5\(^{AlfpCre}\) single mutants remained mild and stable at all time points analysed,
Stat5/GR\(^{AlfpCre}\) mutant mice displayed a marked and progressive pathology exhibiting
3-4 fold increases in liver mass by 12 months of age (Figure 20A, B) and ~8 fold
increases in the triglyceride content as early as 2 months of age (Figure 20C).
Further, whereas control and GR\(^{Alfpcre}\) animals displayed only mild pathologies with
time, detailed histopathological analysis revealed marked age-dependent micro- and
macrovesicular steatosis in livers from both Stat5\(^{AlfpCre}\) (moderate) and Stat5/GR\(^{AlfpCre}\)
(extreme) mice (Figure 21B) suggesting a critical role for Stat5 in the regulation of
hepatic lipid homeostasis. As an additional testament to the severity of disease in
Stat5/GR^{AlfpCre} double mutants, maximal fatty degeneration of hepatocytes was detected as early as 8 weeks of age (Figure 21B) in these mutants.
Results

(A) Macroscopic appearance of steatosis in 12-months-old mutant mice. (B) Livers from 8-weeks-old and 12-months old male mice of the indicated genotypes (top). Liver/Body Weight (LW/BW) ratio in male mice at indicated time points (bottom). Data represent mean ± SEM (n=8/genotype/timepoint). (C) Hepatic triglyceride content as measured by colorimetric assays in 8-weeks-old male mice. Data represent mean ± SEM (n=5/genotype).

Also observed with a consistent graded severity between genotypes were elevated serum ALT levels (Table 3) and extensive lobular and portal fibrosis histopathologies (Figure 21A). Thus, hepatic Stat5 deficiency caused mild, stable steatosis that, when coupled to a GR loss, initiated overt progressive hepatosteatosis reminiscent of human NAFLD.

Figure 21. Histological steatosis in Stat5AlfpCre and Stat5/GRAlfpCre mice
(A) Histological analyses of liver sections from 8-weeks and 12-months-old mutant mice. Tissue sections were fixed and stained with HE (row 1 and 2). Lipid accumulation was visualized by Sudan III on cryosections (row 3). Electron microscopy analysis of subcellular fat distribution in liver samples (row 4). (B) Histological degree of steatosis in HE-stained livers was scored semi-quantitatively by two certified pathologists [in collaboration].

3.2.1. Loss of hepatic Stat5 signaling leads to increased lipogenesis

Since hepatic steatosis can either result from an imbalance in fatty acid synthesis and oxidation or from an imbalance in build-up versus shuttling of lipids out of hepatocytes we examined these processes in more detail. As no apparent changes in lipoprotein particle-mediated shuttling of TG were observed (Figure 27, see below), we postulated that Stat5\textsuperscript{AlfpCre} and Stat5/GR\textsuperscript{AlfpCre} mutant phenotypes were caused either by an induction of hepatic \textit{de novo} lipid synthesis or by inhibition of hepatic fatty acid catabolism. Affymetrix whole-genome expression and subsequent molecular signature analysis identified a marked induction of genes controlled by the lipogenic master regulators SREBP-1c and PPARgamma in Stat5\textsuperscript{AlfpCre} and Stat5/GR\textsuperscript{AlfpCre} mice (Table 2).
### Results

**Table 2. Analysis of transcription factor signatures upregulated in Affymetrix profiling**

Lipid metabolism-related genes significantly deregulated under either knockout condition (Stat5\textsuperscript{AlfpCre} or Stat5\textsuperscript{AlfpCre} or Stat5/GR\textsuperscript{AlfpCre}). Genes are clustered according to upstream transcription factor activation (PPARalpha/gamma) or gene function (lipogenic, catabolic or others). Other major liporegulatory master genes are included for interpretation of the metabolic phenotype.

<table>
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<th>Symbol</th>
<th>Name</th>
<th>TF pathway</th>
<th>PPARalpha</th>
<th>PPARgamma</th>
<th>Stat5\textsuperscript{AlfpCre}</th>
<th>Stat5\textsuperscript{AlfpCre}</th>
<th>GR\textsuperscript{AlfpCre}</th>
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<td>0.1964</td>
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Table 2. Analysis of transcription factor signatures upregulated in Affymetrix profiling

Lipid metabolism-related genes significantly deregulated under either knockout condition (Stat5\textsuperscript{AlfpCre} or Stat5\textsuperscript{AlfpCre} or Stat5/GR\textsuperscript{AlfpCre}). Genes are clustered according to upstream transcription factor activation (PPARalpha/gamma) or gene function (lipogenic, catabolic or others). Other major liporegulatory master genes are included for interpretation of the metabolic phenotype.
Indeed, quantitative RT-PCR (qPCR), Western blot, and immunohistochemical analyses confirmed induction of both critical liporegulatory transcription factors at both the transcript and protein levels (Figure 22A, B and Figure 23A, B). Importantly, upregulation of gene expression of lipogenic SREBP-1c (FASN, SCD1 and SCD2) and PPARgamma targets (DGAT1, DGAT2, CD36, FABP4 and MOD1) were shown to be increased over 2-fold in both Stat5-deficient lines indicating a strong underlying role for SREBP-1c/PPARgamma-regulated de novo lipogenesis in the observed phenotype. Though somewhat counterintuitive in the context of steatosis, transcription of catabolic genes regulated by PPARalpha also appeared upregulated in Stat5\textsuperscript{AlfpCre} and Stat5/GR\textsuperscript{AlfpCre} animals. Expression profiling (Table 2) and qPCR (Figure 22C) analyses revealed significant increases in PPARalpha expression as well as the downstream targets ACOX1, EHHADH, ACADM, ACADL, ACADVL and CPT1, important mediators of peroxisomal and mitochondrial beta-oxidation. Also noteworthy, the typically adipose tissue specific transcription factors C/EBPalpha and C/EBPbeta which control adipogenesis and lipogenesis in concert with PPARgamma showed similar elevations in both Stat5\textsuperscript{AlfpCre} and Stat/GR\textsuperscript{AlfpCre} further supporting the idea of an induced lipogenic/adipogenic program (Figure 22D). Interestingly, neither the PPARgamma coactivators PGC1alpha and RXRalpha, nor the hepatocyte transcriptional marker HNF1alpha showed evidence of altered expression (Figure 22D and Figure 23A).

Thus deletion of Stat5 in the liver causes a marked induction of lipogenic and adipogenic programs including de novo lipogenesis.
Results

Figure 22. Induction of de novo lipogenesis in Stat5^AlfpCre and Stat5/GR^AlfpCre mice
Relative hepatic mRNA levels were quantified by qPCR in 8-weeks-old mice of indicated genotypes. Ct values were normalized to endogenous GAPDH levels (ΔCt method). n=6/genotype; *, p<0.05; **, p<0.01, ***, p<0.001. Error bars represent mean ± SEM. Genes with known functions in hepatic lipid metabolism were subdivided into the following four groups: (A) Lipogenic genes transcriptionally regulated by SREBP-1c. (B) Lipogenic (left) or other (right) genes transcriptionally regulated by PPARgamma. (C) Lipolytic genes transcriptionally regulated by PPARalpha. (D) Other lipid metabolism-related genes.
Results

Figure 23. Increased SREBP-1c/PPARgamma protein expression upon Stat5 deletion in liver
(A) Representative immunoblots showing expression of nuclear SREBP-1 and PPARgamma protein in liver. HNF1alpha served as hepatocyte-specific marker. HSC70 was used as loading control. (B) Immunohistochemical staining for PPARgamma protein expression in liver.

3.2.2. Loss of hepatic Stat5 signaling induces hepatic insulin resistance

One hallmark of most steatotic disorders is an impaired insulin responsiveness of the liver (hepatic insulin resistance). Preliminary measures of fasting and fed glucose in concert with significant hyperinsulinaemia and elevated levels of serum resistin pinpointed towards insulin resistance in Stat5AlfpCre and Stat5/GRAlfpCre mice (Table 3). Administration of 0.75 U/kg body weight insulin intraperitoneally revealed blunted hypoglycaemic responses in both Stat5AlfpCre and Stat5/GRAlfpCre mutants (Figure 24A), corroborating the notion of insulin resistance in Stat5-deficient animals. Next, glucose tolerance tests were performed to assess overall glucose homeostasis. They yielded similar findings: GRAlfpCre and Stat5AlfpCre mice displayed mild glucose intolerance in response to 1 g/kg body weight oral glucose, while double mutant mice exhibited overt diabetes. Of note, Stat5AlfpCre and Stat5/GRAlfpCre mice displayed marked fasting and glucose-challenged hyperinsulinaemia further suggestive of significant insulin resistance (Figure 24A). The significantly mitigated insulin sensitivity observed in Stat5/GRAlfpCre double knockouts in comparison to Stat5AlfpCre mutants may reflect the additive and independent effects of pronounced insulin resistance (due to deletion of Stat5) and established glucose intolerance (due to
deletion of GR). Finally, Western blot analysis of Stat5\textsuperscript{AlfpCre} and Stat5/GR\textsuperscript{AlfpCre} liver revealed severely impaired insulin receptor (IR) downstream signaling 15 min after intraperitoneal insulin administration. Tyrosine phosphorylation of the insulin receptor beta-subunit (IRbeta), insulin-response substrates -1 and -2 (IRS-1 and IRS-2) and the downstream effector kinase AKT were reduced in Stat5/GR\textsuperscript{AlfpCre} livers (Figure 24B). Once again, changes in the Stat5\textsuperscript{AlfpCre} mice were more modest with reductions in phosphorylation only observed in IRS-1 and Akt.

Thus, hepatocyte-specific deletion of Stat5 alone or in combination with GR leads to marked hepatic insulin resistance.

Table 3: Biochemical and metabolic serum parameters in male mutant mice

<table>
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<tr>
<th>Parameter</th>
<th>Control</th>
<th>GR\textsuperscript{AlfpCre}</th>
<th>Stat5\textsuperscript{AlfpCre}</th>
<th>Stat5/GR\textsuperscript{AlfpCre}</th>
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<td></td>
<td>2 months</td>
<td>12 months</td>
<td>2 months</td>
<td>12 months</td>
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<tr>
<td>ALT (U/l)</td>
<td>7</td>
<td>57 ± 5</td>
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<td>ND</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>7</td>
<td>45 ± 11</td>
<td>59 ± 3</td>
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<td>TG (mg/dl)</td>
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<td>174 ± 34</td>
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<td>Cholesterol</td>
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<td>158 ± 23</td>
<td>118 ± 10</td>
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<tr>
<td>(mg/dl)</td>
<td>FFA (mmol/l)</td>
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<td>130 ± 21</td>
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<td>after 4h fast</td>
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<td>182 ± 9</td>
<td>ND</td>
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<td>after 16h fast</td>
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<td>Insulin (pg/ml)</td>
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Eight weeks old male mice were fed with given chow and sacrificed by carbon dioxide asphyxiation. Results are expressed as mean ± SEM for indicated number of mice from each genotype. ALT, alanine transaminase; ALP, alkaline phosphatase; FFA, free fatty acids; TG, triglycerides; TNFa, tumour necrosis factor alpha; ND = not determined. All values represent mean ± SEM. \(^{a} = p\text{-Value <0.05 vs. Control, }^{b} = p\text{-Value <0.01 vs. Control, }^{c} = p\text{-Value <0.001 vs. Control}
Results

Male mice were analysed at 8-weeks-of age for Insulin Tolerance Tests (ITT) and Oral Glucose Tolerance Tests (OGTT). (A) ITT: Insulin was injected intraperitoneally following a 4h fast. Blood glucose levels were determined at given time points (row 1). OGTT: Mice were fasted overnight and glucose orally administered. Blood glucose levels were determined at given time points (row 2). Plasma insulin levels following OGTT challenge. The insulin levels were determined by ELISA (row 3). For ITT/OGTT analyses: n=6-8/genotype; *, *p<0.05; **, *p<0.01, ***, *p<0.001. Error bars in ITT/OGTT assays represent mean ± SEM. (B) Phosphorylation of proteins downstream of the insulin receptor upon insulin challenge in 8-weeks-old mice. Levels of phosphorylated and total protein were determined 15 min after intraperitoneal insulin injection by immunoprecipitation and phospho-tyrosine-specific immunoblot analysis.

Figure 24. Impaired Glucose and Insulin Metabolism in Control, GR<sup>AlfpCre</sup>, Stat5<sup>AlfpCre</sup> and Stat5/GR<sup>AlfpCre</sup> mice
3.2.3. Compound loss of hepatic Stat5 and GR signaling leads to peripheral lipodystrophy.

Interestingly, despite the divergent steatotic phenotypes observed between Stat5Alfpcre single and Stat5/GRAlfpcre double knock-outs, SREBP-1c/PPARgamma-dependent de novo lipogenesis appeared equally induced in both sets of animals. These findings suggested an additional independent stimulus for lipid deposition in Stat5/GRAlfpcre livers and prompted us to examine peripheral fat stores as a potential source of additional lipid fuels. Interestingly, while the independent single mutants exhibited either modest increases (+29 %; GRAlfpCre) or decreases (-28 %; Stat5AlfpCre) in white fat pad mass, double mutant animals displayed a clear depletion of both white and brown adipose tissue stores (-58 %, Stat5/GRAlfpCre; Figure 25A, B). Histological analysis of Stat5/GRAlfpCre white (WAT) and brown adipose tissues (BAT) confirmed peripheral lipodystrophy characterized by grossly reduced lipid droplet size (Figure 25C). Interestingly, measurement of multiple serum lipid regulators revealed elevated levels of GH and decreased levels of IGF-1 in Stat5AlfpCre and Stat5/GRAlfpCre mice (Figure 26A, B). Consistent with systemically elevated serum GH levels, Stat5 phosphorylation in WAT was significantly increased (Figure 26C). In addition, in GRAlfpCre and Stat5/GRAlfpCre mutants, serum corticosterone and ACTH were elevated (Figure 26D, E) consistent with increased nuclear translocation of the GR in WAT (Figure 26F) and the lipodystrophic phenotype in the double mutants. Thus, Stat5/GRAlfpCre mice displayed peripheral lipodystrophy driven by the compound effects of hepatocyte non-responsiveness towards GH action. This eventually led to elevated lipolytic drive through additive effects of elevated GH and corticosterone levels mobilising lipids in the adipose tissue.
Figure 25. Atrophy of peripheral adipose tissues (lipodystrophy) in Stat5/GR\textsuperscript{AlfpCre} mice
For all subsequent panels, male mice at 12 months of age were analyzed. (A) Macroscopic appearance of intrascapular BAT and epidydimal WAT. (B) WAT/Body Weight (WAT/BW) and BAT/Body Weight (BAT/BW) ratios. n=8/genotype; *, p<0.05; **, p<0.01, ***, p<0.001. Error bars represent mean ± SEM. (C) Histology of BAT and WAT. Tissues were fixed and stained with HE. Inlets show higher magnification (top row) or quantification of cell density in adipose tissue. Adipocyte circumference versus area (Cell density) in WAT as determined by HistoQuest software (bottom) was plotted on y-Axis. X-Axis data represent haematoxylin staining intensity and mean X values were arbitrarily set =1.
Figure 26. GH resistance and impaired GC negative feedback regulation in Stat5/GRAlfpCre mice
For all subsequent panels, male mice at 12 months of age were taken. (A, B) Levels of GH (A) and IGF-1 (B) were determined by ELISA. For all subsequent serum analyses: n=8/genotype; *, p<0.05; **, p<0.01, ***, p<0.001. Error bars represent mean ± SEM. (C) GH-mediated activation of Stat5 in epidydimal WAT. Levels of phosphorylated Stat5 (pY-Stat5), total-Stat5 and total-GR were analysed using immunoblot analysis. (D, E) Levels of corticosterone (D) and ACTH (E) were determined by RIA. (F) Nuclear translocation of the GR in white adipose tissue (WAT) was analysed by immunohistochemistry.
3.2.4. Compound loss of hepatic Stat5 and Glucocorticoid Receptor signaling does not affect lipoprotein particle-mediated lipid distribution

Apart from imbalances in fatty acids synthesis and breakdown, liver steatosis can result from altered shuttling of lipids to and from the liver. Thus, we examined these processes in more detail. We isolated the hepatic organic phase, prepared density fractions using FPLC and determined the qualitative lipoprotein composition (see Methods). No apparent differences in lipoprotein-particle-mediated shuttling of triglycerides were observed, although the triglyceride and cholesterol content in serum of GR$^{\text{AlfpCre}}$ mice was generally lower (Figure 27). Thus, we concluded that the phenotypes observed in Stat5$^{\text{AlfpCre}}$ and Stat5/GR$^{\text{AlfpCre}}$ mutants were rather caused by induction of hepatic de novo lipid synthesis as shown above (Figure 22 and Figure 23) then by dysfunctional lipoprotein-mediated shuttling of lipids.

![Figure 27. Unaltered lipoprotein particle profile in 8-weeks old male mice](image)

Figure 27. Unaltered lipoprotein particle profile in 8-weeks old male mice
Lipoprotein distribution was analysed by FLCP fractionation and subsequent TG and total cholesterol quantification in each fraction (n=5/genotype).

3.2.5. Loss of Stat5 is not compensatory counteracted by Stat1 and Stat3 activation

A recent publication reported the limited onset of liver steatosis in mice conditionally deleted for hepatic Stat5 signaling using Albumin Cre recombinase mediated excision of Stat5 alleles (Stat5$^{\text{AlbCre}}$) $^{124}$. Compensatory activation of Stat1 and Stat3 transcription factors accompanied the fatty accumulation. Researchers proposed mechanistically that Stat1 and Stat3 proteins were compensatory recruited to the GHR without mechanistic proof. Moreover, aberrantly activated interferon signaling pathways normally associated with antiviral, immunomodulatory and antitumor activities were present in Stat5$^{\text{AlbCre}}$ mutants. In contrast to the strategy followed by
Cui et al 2007, we warranted excision of floxed Stat5 alleles using AlfpCre recombinase which recombines more efficiently in liver hepatocytes and biliary cells. Supportive of the findings observed by Cui et al., loss of hepatic Stat5 proteins mediated by AlfpCre recombinase led to significant increases in total Stat1 and a slight increase in total Stat3 levels (Figure 28; row 1, 3 and 5). In stark contrast to Cui et al., we observed no increased phosphorylation of Stat1 or Stat3 proteins in Stat5\textsuperscript{AlfpCre} and Stat5/GR\textsuperscript{AlfpCre} mice (Figure 28, row 2 and 4). Thus, we explained the accumulation of lipids upon hepatic loss of Stat5 using AlfpCre as being a direct consequence of Stat5 deletion in the liver. The metabolic syndrome-like phenotype observed in Stat5\textsuperscript{AlfpCre} and Stat5/GR\textsuperscript{AlfpCre} was thus irrespective of compensatory Stat1 and Stat3 signaling.

![Figure 28. Loss of hepatic Stat5 is not compensatory counteracted by Stat1 and Stat3 activation](image)

Figure 28. Loss of hepatic Stat5 is not compensatory counteracted by Stat1 and Stat3 activation
Representative immunoblots of liver homogenates from 8-weeks-old male mice. Amount of phosphorylation and total levels of Stat family members were determined using specific antibodies. HSC70 served as loading control.

3.2.6. Development of steatosis is independent of Jak2 upstream kinase

Upstream stimuli for Stat5 activation are conveyed by Janus kinases upon cytokine and growth factor stimulation; specifically Jak2 was shown to activate Stat5 upon GH action. As shown before, ablation of hepatic Stat5 signaling leads to inductions of hepatic \textit{de novo} lipogenesis and concomitant onset of insulin resistance under steady-state conditions. We aimed to shed further light on the role of Jak2 in steatogenesis. To achieve hepatic losses of Jak2 proteins, we crossed mice harbouring floxed Jak2 alleles with mice expressing the AlfpCre recombinase
(Jak2AlfpCre). Interestingly, Jak2AlfpCre mutants had macroscopically normal livers (Figure 29A), exhibited neither increases in the LW/BW ratio (~5 % LW/BW) nor other obvious alterations upon macroscopic examination of livers. Histopathological analysis confirmed these observations: In contrast to Stat5AlfpCre and Stat5/GRAlfpCre animals, Jak2AlfpCre mutants exhibited no lipid accumulation in hepatic tissues. In addition, no fibrotic or necrotic processes could be observed (Figure 29B). Thus, we concluded that Jak2-dependent signal transduction is dispensable for the Stat5-mediated steatogenesis. Rather, this pinpointed towards direct repressive functions of Stat5 proteins on hepatic lipid biosynthesis. Interestingly, Jak2AlfpCre animals displayed perturbations in normal postnatal growth (not shown herein), further underpinning the importance of the GH-Jak2-Stat5-GR axis in regulation body growth.

![Figure 29. No liver steatosis upon loss of hepatic Jak2 signaling in Jak2Alfpcre mice](image)

(A) Liver/Body Weight (LW/BW) ratio in 6-months-old male mice. (B) Histological analyses of liver sections from 6-months old male mice. Tissue sections were fixed and stained with HE.

### 3.3. Loss of hepatic Stat5 leads to severe liver fibrosis in MDR2⁻ mice

The transgenic MDR2⁻ mouse line strain harbours genetics deletions of the multi-drug resistance protein 2 (MDR2), a liver-specific P-glycoprotein responsible for bile acid transport across the apical hepatocyte membrane. The MDR2⁻ strain is widely used for studying the onset and progression of inflammatory liver cancer. To
investigate possible roles of hepatic Stat5 during liver carcinogenesis, we crossed MDR2\(^-\) mutants with mice specifically deleted for Stat5 in hepatocytes (Stat5\(^{AlfpCre}\)). By respective intercrossing we obtained four genotypes (Controls, MDR2\(^-\), Stat5\(^{AlfpCre}\) and MDR2\(^-\)/Stat5\(^{AlfpCre}\)) which were born at Mendelian ratios. Postnatally, both Stat5-deficient lines, Stat5\(^{AlfpCre}\) and MDR2\(^-\)/Stat5\(^{AlfpCre}\) exhibited profound defects in postnatal growth (Figure 30A, D), an effect caused by impairments in Stat5-mediated transcription of growth promoting genes such as IGF-1 (see section 3.1). In addition, livers of Stat5\(^{AlfpCre}\) and MDR2\(^-\)/Stat5\(^{AlfpCre}\) mutants were yellowish and enlarged starting with four weeks of age (Figure 30B). Again this reflects the crucial role of Stat5 in hepatic lipid metabolism demonstrated earlier (see section 3.2). Strikingly, livers from MDR2\(^-\)/Stat5\(^{AlfpCre}\) compound knockouts were texturized and sclerotic, pinpointing towards increased wound-healing and scar-formation processes leading to increased collagen deposition, a process widely observed during liver fibrogenesis (Figure 30B). Severe jaundice (Figure 30C) was observed in MDR2\(^-\)/Stat5\(^{AlfpCre}\) double mutants and hinted towards perturbed bile flux across hepatocyte membranes or towards defective bile secretion into the intestine due to obliterated bile ducts (cholestasis). Serum levels of bile acids and bilirubin were increased dramatically (~19fold and ~23fold vs. controls). These are two parameters frequently elevated in cholestatic disorders such as PBC (primary biliary cirrhosis) and PSC (primary sclerosing cholangitis). Additional serum parameters indicative of extrahepatic cholestasis like high ALP and ALT (increased ~4.2fold and ~7fold respectively) were observed (Table 4). To investigate these processes in more detail, we undertook histopathological analyses of tissue specimens obtained from all four genotypes: HE staining of eight weeks old mice revealed no histopathologies in control animals and, interestingly, MDR2\(^-\) mutants. Stat5\(^{AlfpCre}\) knockout animals displayed significant steatosis as described earlier (see section 3.2). Interestingly, MDR2\(^-\)/Stat5\(^{AlfpCre}\) mutant livers displayed marked perilobular fibrosis and a profound disruption of normal liver architecture (Figure 30E, top panel). Chromotrop Anilinblue (CAB) staining as collagen-specific marker revealed massive perivenular and perilobular collagen accumulation, indicative of severe liver fibrosis. Closer examination revealed marked pericellular collagen deposition (chicken wire fibrosis), an indicator for late-stage liver fibrosis (Figure 30E, bottom panel). Periodic acid-Schiff (PAS) staining as hepatic glycogen marked revealed defects in hepatocyte functions leading to defective synthesis and storage of glycogen in livers from MDR2\(^-\)/Stat5\(^{AlfpCre}\) mutants.
Significant PAS negative areas were indicative of degenerative processes such as necrosis (Figure 30E, bottom panel). Thus, MDR2/Stat5AlfpCre knockouts exhibited severe liver fibrosis observed on the macroscopic and microscopic level. Fibrosis was accompanied by extensive extrahepatic cholestasis, jaundice and hepatomegaly. The respective single mutants exhibited mild histopathologies, in part reported before (see section 3.2).
Results

Figure 30. Loss of hepatic Stat5 in MDR2⁻ mice leads to cholestasis, jaundice and liver fibrosis
(A) Macroscopic appearance and (B) Livers and (C) serum from 8-weeks-old male mice of the indicated genotypes. (D) Body Weight (left) and Liver/Body Weight (LW/BW) ratio (right) in 8-weeks-old male mice. Data represent mean ± SEM (n=8/ genotype/timepoint). (E) Histological analyses of liver sections from 8-weeks old mutant mice. Tissue sections were fixed and stained with HE (row 1). Collagen deposition was visualized by CAB staining. Inlets show higher magnification of vascular structures (row 2). Hepatic glycogen was visualized by PAS staining (row 3).

Table 4: Biochemical and metabolic serum parameters in male mutant mice

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Eight weeks old male mice were fed with chow and sacrificed by carbon dioxide asphyxiation. Concentrations of given serum parameters are expressed as mean ± SEM for indicated number of mice from each genotype. ALT, alanine transaminase; ALP, alkaline phosphatase; TG, triglycerides. a = p-Value <0.05 vs. Control, b = p-Value <0.01 vs. Control, c = p-Value <0.001 vs. Controls.

### 3.3.1. Deletion of MDR2 proteins causes hepatic Stat5b activation

Tetracarbonchloride (CCl₄) treatment leads to artificially induced liver fibrosis in mice. Recently, a report demonstrated important roles for hepatic Stat5 in CCl₄-induced fibrogenesis. Insensitivity to GH action (GH resistance) and blunted Stat5-mediated signal transduction accompanied the disease. Thus, Stat5 was claimed to constitute an importer repressor of liver fibrosis in mice.

To address the question whether Stat5 similarly counteracts MDR2-driven liver fibrogenesis, we isolated livers of all genotypes, prepared liver homogenates and determined the activation status of Stat5 and other important Stat family members using immunoblot analysis. Strikingly and in line with previous observations (section 3.2.5), loss of hepatic Stat5 leads to significant increases in basal Stat1 (strong) and Stat3 (mild) protein levels (Figure 31). Notably, no compensatory Stat1 or Stat3 hyperphosphorylation was observed. Interestingly, MDR2⁻animals expressed significantly elevated levels of phosphorylated Stat5 (pY-Stat5). Thus, we reasoned that Stat5 might exhibit fibrogenesis-repressing functions during development or progression of liver fibroses.
3.3.2. MDR2⁻/Stat5AlfpCre mice display autocrine growth factor loops

Hepatic stellate cells (HSC) under normal conditions perform important vitamin A storage functions, but upon a variety of liver insults transdifferentiate into profibrogenic and pro-inflammatory myofibroblasts (MFB) (176). MFB upon liver damage proliferate and accumulate at the site of tissue repair and synthesize ECM components like collagen. Kupffer cells, macrophage-like liver cells of mesenchymal origin secrete mitogenic PDGF and TGFβ under fibrotic conditions, further favouring HSC transdifferentiation and ECM synthesis. To evaluate the impact of TGFβ and/or PDGFβ growth factor circuits in MDR2⁻/Stat5AlfpCre-associated fibrosis, we isolated hepatic RNA and quantified TGFβ and PDGFβ mRNA levels by qPCR analysis. Interestingly, we observed only modest increases (~1.5fold vs. controls) of TGFβ mRNA levels in MDR2⁻/Stat5AlfpCre mutants. In contrast, TGFβR mRNA levels were drastically increased (~3.5fold vs. controls) in MDR2⁻/Stat5AlfpCre mutants, thereby triggering potent TGFβR-dependent signal transduction even under conditions of moderately elevated TGFβ (Figure 32, left). Unambiguously, PDGFβ (~150fold vs. controls) and PGFβR mRNA (~40fold vs. controls) were elevated in MDR2⁻/Stat5AlfpCre animals (Figure 32, right). Thus, autocrine PDGFβ and, less pronounced
TGFβ signaling is activated in fibrotic MDR2\(^{-}/\)Stat5\(^{AlfpCre}\) double knockout animals. These observations reflect the current notion of an essential role of PDGFβ and TGFβ during hepatic fibrogenesis.

![Figure 32. MDR2\(^{-}/\)Stat5\(^{AlfpCre}\) mutants harbour hyperactive TGFβ and PDGFβ growth factor circuits](image)

Quantitative PCR (qPCR) analysis of hepatic mRNA expression in male mice of indicated genotypes. For qPCR analysis, mice were sacrificed at eight weeks of age. Hepatic mRNA was isolated using Trizol, transcribed into cDNA and analysed by qPCR using intron-spanning, gene specific primers. Ct values were normalized to GAPDH mRNA levels using the ΔCt method. Data represent mean ± SEM. N=6/genotype were used.

### 3.3.3. Fibrogenesis in MDR2\(^{-}/\)Stat5\(^{AlfpCre}\) mice is accompanied by ECM remodelling

Fibrosis is believed to secondarily occur due to repetitive liver insults and concomitant wound-healing processes. Following acute injury, the liver parenchyma undergoes intensive cellular proliferation in order to replace degenerative hepatocytes, accompanied by inflammatory processes and moderate deposition of collagen. Finally, the sum of liver insults leads to overt fibrosis, which can progress to late-stage liver pathologies such as cirrhosis, defined by the circumlobular deposition of collagen (Figure 30E). Molecularly, the massive build-up of ECM hails from imbalances between synthesis (predominantly collagen 1 and 3 (COL1 and COL3)) and proteolysis (predominantly Matrixmetalloproteases (MMP) members, especially MMP2, MMP3 and MMP13) of ECM components. Most MMPs are synthesized as latent pro-enzymes and require proteolytic activation by MMP14 and MMP15 to liberate their proteolytic properties. Tissue inhibitors of MMP1 and 2 (TIMP1 and TIMP2) effectively quench MMP proteolysis. Transcriptional activation of TIMPs and decrease of MMP mRNA synthesis is frequently observed during fibrotic processes. To assess the extend of ECM remodelling, we isolated hepatic mRNA of all four...
Results

Fibrogenesis in MDR2−/Stat5AlfpCre mice is accompanied by potent ECM remodelling.

Quantitative PCR (qPCR) analysis of hepatic mRNA expression in male mice of indicated genotypes. For qPCR analysis, mice were sacrificed at eight weeks of age. Hepatic mRNA was isolated using Trizol, transcribed into cDNA and analysed by qPCR using intron–spanning, gene specific primers. Ct values were normalized to GAPDH mRNA levels using the ΔCt method. Data represent mean ± SEM. N=6/genotype were used.

Thus, fibrogenesis in MDR2−/Stat5AlfpCre animals is accompanied by imbalances in ECM turnover. Potent synthesis of COL1 and COL3 in concert with strong inhibition of ECM proteolysis exerted by TIMP1 and TIMP2 was observed, leading to a massive build-up of collagen deposition. Interestingly, MDR2−/Stat5AlfpCre mutant mice were also characterized by significant increases in MMP-dependent proteolysis. Notably, although MDR2− animals exhibit modest liver fibrosis at eight
weeks of age, no significant remodelling of the ECM was observed on the transcript level.

3.3.4. Loss of hepatic Stat5 in MDR2\(^{-}\) mice leads to impaired bile acid circulation

An essential function for hepatocytes is the synthesis and recycling of bile. The underlying hepatobiliary transport processes are crucial for proper secretion and flux of bile\(^{177, 178}\). These processes are regulated by an array of bile transporters located either on the basolateral (facing the hepatic sinusoids) or apical side (protruding into the bile canaliculi) of hepatocytes (reviewed in\(^{179}\)). Dysfunctional bile transporters expression is frequently observed in cholestatic diseases artificially induced by bile-duct ligation (BDL)\(^{180}\). Common to animal models characterized by cholestatic-induced fibroses is the reduction of two major basolateral bile pumps; sodium independent organic anionic transporter (OATP1) and sodium dependent bile acid transporter (NTCP1). Dysregulation basolateral bile pump expression was also observed upon artificial induction of liver fibrosis\(^{181}\). Interestingly, NTCP1\(^{182}\) and OATP1\(^{183}\) are experimentally confirmed Stat5 target genes; a finding pinpointing towards essential roles of Stat5 in bile circulation homeostasis. We thus addressed the question whether loss of hepatic Stat5 in MDR2\(^{-}\) mutants leads to dysfunctional bile acid flux due to aberrant NTCP1/OATP1 transporter expression. As shown in Figure 34A, NTCP1 and OATP1 transcript levels were severely downregulated in both Stat5-deficient mouse strains. In contrast, apical bile transporters (i.e. MRP2, MRP3, MDR1b and BESP) did not differ significantly in their transcript levels, although modest reductions in OATP2 and BSEP mRNA could be observed (Figure 34B). To confirm our findings, we prepared liver homogenates and probed with antibodies specific for afore mentioned bile transporters (Figure 34C). In line with reduced mRNA levels, OATP1 protein levels were reduced to below the detection limit in mice devoid of Stat5 (Figure 34C, row 3). NTCP1 was residually expressed in Stat5\(^{AlfpCre}\) single mutants, yet significantly lower compared to control animals and undetectable in MDR2\(^{-}/\)Stat5\(^{AlfpCre}\) animals (Figure 34C, row 4). Protein levels of MRP2 and MRP3 were unaltered (Figure 34C, row 1, 2). Thus, we could show that hepatic Stat5 loss in the MDR2\(^{-}\) background led to the downregulation of two major basolateral bile transporters, notably NTCP1 and OATP1 at the transcript and protein
Results

This presumably results in impaired bile flux across the hepatocyte and retention of bile in the serum (Table 4). Residual NTCP1 expression in Stat5AlfpCre animals might suffice to ensure physiological bile flux, reflected in the observation that serum bile acids are only moderately elevated (Table 4).

Figure 34. Loss of hepatic Stat5 defective NTCP1/OATP1-mediated bile flux

(A, B) Quantitative PCR (qPCR) analysis of hepatic mRNA expression in male mice of indicated genotypes. For qPCR analysis, mice were sacrificed at eight weeks of age. Hepatic mRNA was isolated using Trizol, transcribed into cDNA and analysed by qPCR using intron–spanning, gene specific primers. Ct values were normalized to GAPDH mRNA levels using the ΔCt method. Data represent mean ± SEM. N=6/genotype were used. (C) Immunoblot analysis of liver homogenates from

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male mutant mice using specific antibodies. Endogenous beta Actin (bACTIN) served as loading control. Three livers of each genotype were analysed and representative images shown.
4. Discussion

4.1. A linear Stat5-GR axis controls postnatal body growth

We could demonstrate the critical role of GR–Stat5 protein interactions in regulating postnatal body growth and expression of maturation-related genes. Our hypothesis that this functional interaction is a direct consequence of physical Stat5-GR interaction is supported by the following results:

(i) Affymetrix expression profiling revealed an extensive overlap of gene expression changes induced by the loss of either hepatic Stat5 or GR (Figure 9A).

(ii) A tight correlation of the magnitude of expression changes for Stat5-GR coregulated genes was demonstrated for the single mutants (Stat5^AlfpCre and GR^AlfpCre, Figure 9B).

(iii) Combined ablation of GR and Stat5 transcription factors did not affect postnatal body growth or the expression of the Stat5-GR coregulated genes more than loss of Stat5 only (Figure 8).

(iv) Aminoterminal truncation of Stat5 disrupted the protein–protein interaction of Stat5 with GR (Figure 11C,D) but led to similar growth retardations. Moreover, it resulted in abrogated transcription preferentially of those Stat5-responsive target genes that are also dependent on GR cofactor recruitment (Figure 12).

This is further supported by previous data, showing that GR is present on Stat5-dependent regulatory regions of growth hormone induced genes and that mice with a mutation in the dimerization domain of the GR (GR^dim mice) leading to defective DNA binding of GR, display normal growth and intact transcription of GH-responsive genes^{114}. Collectively, these data support a model in which GH-activated Stat5 binds to Stat5 responsive DNA elements and uses the GR as a cofactor to promote body growth by inducing appropriate gene transcription. Our results have a clear implication for the long-standing discussion on the role of the liver in postnatal growth. According to the original “somatomedin hypothesis” from 1956, GH triggers the hepatocyte to secrete a secondary mediator (later suggested to be IGF-1) promoting postnatal growth. This hypothesis was later contradicted since it was shown that peripheral administration of GH promotes growth^{154} and that deletion of IGF-1 in hepatocytes does not cause any major growth perturbations^{157}. It was also
shown recently that mice harbouring a muscle-specific loss of Stat5 show an approximate 20% reduction in growth \(^{184}\). However, researchers \(^{155}\) showed that mice lacking ALS in all cells and IGF-1 exclusively in hepatocytes display abrogated growth behaviour. This suggested a role of the hepatocyte in somatic growth, although the conclusions of the study are somewhat limited since ALS was also deleted in non-hepatic tissues, such as in the developing cartilage. In addition, the confounding effect of IGF-1 deletion on general body growth was small and transient. We provide unequivocal evidence for the importance of Stat5 and GR in hepatocytes for postnatal growth. Thus, GH triggers growth both through endocrine factors secreted by the liver and through paracrine mediators produced in the target organs. In the study by Yakar et al., the blunted growth of the ALS/IGF-1 double-mutant mice was suggested to result from the fact that they had even lower levels of circulating IGF-1 than the liver-specific IGF-1 mutants. However, the growth disturbance of the mice in our study might depend on additional GH-target genes, further with actions different from simply regulating levels of circulating IGF-1. Thus, IGF-1 serum levels are only moderately reduced in the growth-retarded GR mutants (28% reduction) \(^{114}\), while it is much lower in liver-specific IGF-1 mutants (75% reduction), which show no overt growth defect. This implies that other genes found to be dependent on GR-Stat5 interactions in our study are actively involved in growth promotion. Identification of those additional hepatic GH-induced mediators of growth remains to be done and will probably be challenging since simultaneous and hepatocyte-specific deletions of several genes might be necessary. The second major finding was the unexpectedly high fraction of transcriptional activation mediated by GR and Stat5 dependent on a physical Stat5-GR interaction (42% and 26%, respectively). We could also demonstrate that the GR is not a mere general positive modulator of Stat5 action since the whole Stat5 transcriptome was divided in relatively clear sets of GR-dependent and GR independent genes. Interestingly, these sets fall into different functional groups. Thus, the genes dependent on Stat5–GR protein interactions were preferentially enriched in functional groups related to growth and maturation. However, a small group of Stat5-responsive genes are not totally, but still partially, dependent on GR. We show that the N-terminal domain of Stat5b constitutes the docking domain for the GR, while it is also involved in chromatin binding. Accordingly, it is required for the expression of genes dependent on physical Stat5-GR interaction in vivo and for maintaining physiological postnatal growth. We have recently
demonstrated that this domain is also important for the oligomerisation of Stat5. The AF-1 domain of GR may constitute its cognate binding partner, since it has been shown to be necessary for Stat5-GR interaction. In conclusion, we show an unexpectedly prominent role for the GR in acting as coactivator for Stat5 in hepatocytes. We provide in vivo evidence that this Stat5-GR interaction is not an unselective requirement for all Stat5 signaling processes but rather preferentially affects gene sets involved in growth and maturation. In line with this, we show that Stat5-GR protein interactions in hepatocytes are instrumental for maintaining physiological postnatal growth and that the coactivating action of GR involves the amino terminus of Stat5. This provided insight into the mechanism of growth control and we solved and supported the challenged somatomedin hypothesis at the transcriptional level, which postulated a critical role for hepatic GH signaling in postnatal growth.

As an example for Stat5-GR synergism in gene transactivation we chose Suppressor of Cytokine Signaling 2 (Socs2). Using Luciferase (LUC) reporter assays we could demonstrate that Stat5-mediated transactivation of the Socs2 promoter also necessitates GR signaling. We confirmed the dependence of synergistic Stat5- and GR signaling in a standard cell line (COS7) as well as in immortalized primary hepatocytes.

Generally, Stat5 signaling plays a key role in mediating cytokine and growth factor action. Constitutive signaling leads to malignant transformation and thus has to be tightly controlled. Stat5 feedback inhibition is in part mediated by transcription of Socs2 proteins leading to Jak2 or GHR degradation by ubiquitin-mediated proteolysis. No reports have so far demonstrated a role for GR signaling in modulating Stat5-mediated transactivation of Socs2. Interestingly, the estrogen receptor (ER), a closely related nuclear hormone receptor was shown to negatively regulate GH signaling by inducing Socs2 transcription. Although the findings presented in this study are reminiscent to results presented in the former report, no protein interaction between the ER and Stat5 were observed, putatively due to differences in the AF-1 domain, which is poorly conserved among nuclear hormone receptors, but it is crucial for Stat5-GR interaction. We thus conclude that Stat5-GR-dependent transactivation of the Socs2 promoter represents an unrelated finding. The significance of synergistic Stat5-GR co-dependent Socs2 transcription might open new avenues for keeping unbalanced Stat5 activity at bay. This is physiologically relevant, as hyperactivation
of Stat5 might lead to malignant transformation under certain conditions. Pharmacological activation of GR signaling in diseases, e.g. by established GR agonists (e.g. Prednisolone or Dex) would result in potent transactivation of the Socs2 promoter, leading to indirect suppression of Jak2-Stat5 signaling, presumably ameliorating disease.

4.2. Compound loss of hepatic Stat5 and GR signaling causes steatosis

Deletion of Stat5 in hepatocytes causes modest, non-progressive steatosis. Surprisingly, combined ablation of Stat5 and the GR induced severe, progressive steatosis, and steatohepatitis. Thus, we propose a two-step model of steatogenesis in Stat5^AlfpCre and Stat5/GR^AlfpCre mice.

(i) Hepatocyte-deletion of Stat5 leads to insulin resistance and steatosis due to induction of hepatic de novo lipogenesis. This is reflected by the phenotype observed in young Stat5^AlfpCre and Stat5/GR^AlfpCre mutants ('1st step'). Peripheral adipose tissues are unaltered at this time point.

(ii) Additional deletion of the GR mobilizes lipids from peripheral fat stores which eventually accumulate in the liver of older animals. This is reflected by the dramatic phenotype seen in older Stat5/GR^AlfpCre mice ('2nd step').

Triglyceride synthesis in the liver is mainly controlled by two transcription factors: Firstly, SREBP-1c controls cholesterol and fatty acid synthesis \(^{188,189}\). Secondly, the nuclear hormone receptor PPARgamma promotes lipogenesis in concert with C/EBPalpha \(^{43,190}\). In addition, SREBP-1c potentiates activation of the adipogenic PPARgamma/C/EBPalpha axis \(^{48}\). Overexpression of SREBP-1c or PPARgamma factor induced steatosis in transgenic mice \(^{45,46,191}\). Molecular signature analysis revealed a potent upregulation of SREBP-1c/PPARgamma-dependent de novo lipogenesis in Stat5^AlfpCre and Stat5/GR^AlfpCre animals. This was in line with a rising number of mouse models for liver steatosis which are characterized by SREBP-1c \(^{47,192}\) and/or PPARgamma upregulation \(^{47,193,194}\). The importance of PPARgamma and SREBP-1c has been underscored by their deletion in steatotic ob/ob mice. In both cases a reversion of hepatic fat accumulation was observed \(^{39,44}\). PPARalpha constitutes another important PPAR family member regulating beta-oxidation of fatty acids. High PPARgamma levels can lead to aberrant transactivation of the PPARalpha promoter \(^{195}\). The induction of PPARalpha-dependent peroxisomal and
mitochondrial catabolism could be caused by this cross reactivity. Another, more speculative explanation for combined induction of PPARgamma and PPARalpha signaling pathways could be putative repressive functions of Stat5 in PPARalpha/gamma transcription. Indeed, Stat5 responsive elements were found in the human PPARgamma promoter \(^{196}\) and an inhibitory function of Stat5 on PPARalpha/gamma-dependent transcription was demonstrated \textit{in vitro} \(^{197}\). Recently, a report underlined the significance of Stat5/PPARgamma interplay in hematopoietic disorders \(^{198}\). Yet, the molecular nature of Stat5/PPAR interplay in epithelia remains enigmatic without further investigation.

In most cases, a tight correlation exists between steatosis and hepatic insulin resistance \(^{64, 199}\). The hyperinsulinaemic conditions subsequently lead to increased \textit{de novo} lipogenesis via SREBP-1c \(^{200}\) and a blunted suppression of FFA release from adipose tissues via HSL. High circulating levels of FFA can induce steatosis by aberrant deposition of lipids in hepatocytes. Increased plasma levels of FFA, resistin and insulin in Stat5\(^{AlfpCre}\) and Stat5/GR\(^{AlfpCre}\) mice suggested insulin resistance. This was confirmed by functional glucose and insulin tolerance tests. A molecular defect in phosphorylating the IR-adaptor proteins IRS-1 and IRS-2 upon insulin challenge was concomitantly observed. Two possible mechanisms might explain the insulin resistance observed in Stat5\(^{AlfpCre}\) and Stat5/GR\(^{AlfpCre}\) animals:

(i) The insulin resistance is secondary to the lipid accumulation caused by increased SREBP-1c/PPARgamma-dependent \textit{de novo} lipogenesis. A similar effect is seen upon short term high-fat diet feeding of mice. These animals develop primary steatosis without concomitant insulin resistance \(^{201}\). Hepatic insulin resistance develops later due to impaired IRS-1 and IRS-2 phosphorylation, as high levels of SREBP-1c are correlated to impaired transcription of IRS-2. Indeed, reduced levels of IRS-2 protein in concert with high SREBP-1c levels are observed in Stat5\(^{AlfpCre}\) and Stat5/GR\(^{AlfpCre}\) mice (Figure 24B and Figure 23A).

(ii) Hepatic insulin resistance in Stat5\(^{AlfpCre}\) and Stat5/GR\(^{AlfpCre}\) animals could be due to a putative IR-docking function of Stat5b. This was reported by multiple studies relying e.g. on Yeast 2-Hybrid screens \(^{52, 54}\). Insulin-dependent activation of Stat5 was demonstrated to be independent of Jak2 upstream kinases \(^{202}\). Our data support the latter notion, especially as mice harbouring conditional deletions of Jak2 in hepatocytes (Jakt\(^{AlfpCre}\)) did not develop steatosis (Figure 29A, B). Thus, conditional deletion of Stat5 in hepatocytes might lead to primary defects in IR-dependent signal
transduction. The aggravated insulin resistance phenotype of Stat/GR\textsuperscript{AlfpCre} mice compared with Stat5\textsuperscript{AlfpCre} animals was a reflection of the combination of both, pronounced insulin resistance (due to deletion of Stat5) and glucose intolerance (due to deletion of GR) (Figure 24A). This condition might be due to either increased internalisation or prolonged cytoplasmic retention of the IR, a compensatory mechanism described \textit{in vitro}\textsuperscript{203}. This was reflected in the reduced phosphorylation of the IR itself upon insulin action in Stat5/GR\textsuperscript{AlfpCre} animals. Surprisingly, we could not detect any significant \textit{in vivo} tyrosine phosphorylation of Stat5b upon insulin injection over different time points and doses. Our data currently suggest a cytoplasmic role for Stat5b proteins in insulin signaling, but further proof would be required to clarify the role of Stat5b proteins in the IR signal transduction cascade.

Steatosis is not only found in obese but also in lean patients suffering under depletion of peripheral adipose tissues (lipodystrophy). The extend of hepatic fat accumulation correlates well with degeneration of peripheral fat depots\textsuperscript{204}. The homeostasis of adipose tissues is usually tightly controlled. Among others, GH possesses potent lipolytic properties. High levels of GH reduce adipose tissue mass via increased basal lipolysis\textsuperscript{205} and leads to increased levels of FFA\textsuperscript{148}. This effect is dependent of Stat5 activation in adipose tissues\textsuperscript{206}.

Glucocorticoids in contrast have anti-lipolytic properties and favour fat accumulation in adipose tissue via stimulation of LPL\textsuperscript{145}. Interestingly, a combination of both (GH+ glucocorticoids) leads to dramatic lipolysis exceeding the lipolysis induced by GH alone\textsuperscript{148, 149}. The secretion of GH is controlled via negative inhibition by IGF-1. Hepatic deletion of Stat5 reduced the synthesis of IGF-1 which was followed by high systemic levels of GH (GH resistance)\textsuperscript{126}. This liver-neuroendocrine feedback mechanism was reflected in the modest depletion of fat storages in Stat5\textsuperscript{AlfpCre} knock-outs\textsuperscript{123, 127} and was consistent with previous reports showing high levels of circulating GH levels upon hepatic Stat5 deletion\textsuperscript{207}. The GH-mediated lipolysis was reflected in high phosphorylation of Stat5 in WAT of Stat5\textsuperscript{AlfpCre} and Stat5/GR\textsuperscript{AlfpCre} animals.

In contrast, GR\textsuperscript{AlfpCre} mice harboured enlarged fat pads and showed signs of hypercorticolism (preliminary data, not shown). We could show that high levels of corticosterone in GR\textsuperscript{AlfpCre} and Stat5/GR\textsuperscript{AlfpCre} animals were caused by elevated concentrations of ACTH. High levels of corticosterone subsequently led to increased nuclear translocation of the GR in WAT. A liver-mediated inhibitor of glucocorticoid
secretion (as seen for IGF-1 regulating GH secretion) was so far not described. Yet, mouse models with defects in hepatic gluconeogenesis (as seen in GR\textsuperscript{AlfpCre} mice\textsuperscript{136}) showed elevated levels of ACTH and corticosterone\textsuperscript{208}. We excluded ectopic expression of the AlfpCre recombinase in the pituitary gland due to the reported obesity phenotype observed upon Stat5 deletion using Nestin-Cre., which deletes also in the pituitary\textsuperscript{209}. We can currently not exclude that mechanisms upstream of the pituitary might contribute to ACTH release upon hepatic GR or Stat5/GR deletion. Taken together, Stat5/GR\textsuperscript{AlfpCre} animals harboured a hyperactive HPA axis leading to elevated levels of GH and glucocorticoids. This led to potent lipolysis of adipose tissues resulting in peripheral lipodystrophy. Increased mobilisation of triglycerides by combined GH and glucocorticoid action subsequently resulted in the massive flux of FFA into the liver of Stat5/GR\textsuperscript{AlfpCre} mice (‘2\textsuperscript{nd} hit’). This aggravated the mild steatosis caused by increased \textit{de novo} lipogenesis upon Stat5 deletion in hepatocytes (‘1\textsuperscript{st} hit’).

4.3. Loss of hepatic Stat5 signaling leads to severe liver fibrosis in the MDR2\textsuperscript{+} mouse model of inflammatory liver cancer

Liver fibrosis constitutes a severe liver pathology induced by constant liver damage and concomitant wound-healing processes. Repetitive liver insults such as chronic alcohol abuse or viral infections result in abundant collagen deposition around hepatic lobes, a process in which dynamics and magnitude of fibrotic damages are orchestrated by the nature of the damaging agent. It is widely established that bile flux perturbations and impairments in hepatic bile uptake result in cholestasis, in turn constituting a major risk factor for developing liver fibrosis. In various mouse models for cholestasis, the expression of NTCP1 and OATP1, two major basolateral bile pumps are severely compromised\textsuperscript{179, 180}. Whether these observations were causative for the development of cholestasis or mere compensatory consequences secondary to elevated serum bile levels could not be fully answered. NTCP1 and OATP1\textsuperscript{183} are established Stat5 target genes, transcribed upon prolactin action. It was thus conceivable that loss of hepatic Stat5 would result in profound defects of bile flux across the hepatocyte. Interestingly, this did not induce cholestasis in Stat5\textsuperscript{AlfpCre} mutants alone. We hypothesized that additional insults due to loss of the apical MDR2 bile transporter are necessary to induce overt cholestasis as observed
in MDR2/Stat5<sup>AlfpCre</sup> double mutants. Generally, cholestasis and the concomitant retention of toxic bile in hepatocytes leads to constant hepatocyte damage, transdifferentiation of HSC, ECM remodelling and degeneration of hepatocytes. Thus, MDR2/Stat5<sup>AlfpCre</sup> mutants constitute a valuable murine model for liver fibrosis and could assist in further investigating molecular mechanisms underlying hepatic fibrogenesis. One especially intriguing aspect could be the pharmacologically induction of Stat5-dependent bile transporters putatively leading to alleviation of the disease.

In summary, my work on the Stat5GR transcription factor gene regulatory network contributes to our molecular understanding of postnatal body growth, the regulation of hepatic lipid metabolism and fibrogenesis in the liver. Hopefully, these results will lead to therapeutic inventions leading to amelioration of disease.

5. Materials and Methods

5.1. Materials

5.1.1. Chemicals and enzymes:

In general, chemicals, materials and enzymes were commercially obtained from the following companies:

Chemicals/Materials:
VWR international, Darmstadt, Germany
Sigma-Aldrich, Vienna, Austria
Lactan, Graz, Austria
Eppendorf, Vienna, Austria
Amersham, Vienna, Austria
Fisher Scientific, Vienna, Austria
Sarstedt, Wr. Neudorf, Austria
Qiagen, Vienna, Austria
Biorad, Vienna, Austria
PAA, Pasching, Austria
Applied Biosystems, Foster City, USA
Neolab, Heidelberg, Germany
BD Biosciences, Franklin Lakes, USA
Novo Nordisk Pharma, Vienna, Austria
MP Biomedicals, Illkirch, France
IBL Hamburg, Hamburg, Germany
Enzymes:
Invitrogen, Karlsruhe, Germany
Roche, Mannheim, Germany
New England Biolabs, Schwalbach, Germany
Fermentas, St. Leon-Rot, Germany
DAKO, Glostrup, Denmark

Radionucleotides:
Amersham, Vienna, Austria

5.1.2. Standard solutions

10 x Phosphate buffered saline (PBS):
80 g NaCl
2 g KCl
14.4 g Na₂HPO₄
2.4 g KH₂PO₄
→ adjust pH to 7.4, fill up with H₂O to 1 L and autoclave

10 x TBE (Tris borate EDTA) buffer:
108 g Tris
55 g boric acid
40 ml 0.5 M EDTA
→ fill up with H₂O to 1 L and autoclave

50 x TAE (Tris acetate EDTA) buffer:
242 g Tris
57.1 ml Acetic acid
100 ml 0.5 M EDTA (pH 8.0)
→ fill up with H₂O to 1 L and autoclave

TE buffer:
10 mM Tris/HCl pH 8.0
1 mM EDTA pH 8.0

5.1.3. Media for bacterial cultivation

LB medium:
10 g Bacto-Trypto1
5 g Yeast Extract
5 g NaCl
--> fill up with H₂O to 1 L, set pH =7.2 using NaOH and autoclave

LB agar:
1 x LB medium
1.5 % (w/v) bacto agar
5.1.4. Plasmid constructs

**pMSCV:**
The pMSCV (Murine Stem Cell Virus) plasmids, originally designed as expressed vectors optimised for efficient protein expression in stem cell lines, are widely used in other mammalian cell lines. pMSCV plasmids harbour mutated MSCV-derived long terminal repeats (LTR) ensuring high transcriptional activity, driving abundant protein expression of cloned target genes. In addition, pMSCV plasmids harbour ampicillin resistance cassettes for selection of transformed bacteria. Hygromycin (hygro) resistance cassettes allow stable transfection of mammalian cell lines. A green fluorescent protein (GFP) is situated at the 3' end of the cloned target gene under the control of an internal ribosomal entry site (IRES) allowing for quantification of transfection efficiency using FACS (fluorescence activated cell sorting) analysis. The multiple cloning site (MCS) harbours restriction sites for standard restriction endonucleases used in molecular biology.

**pE2F-Luc reporter plasmids:**
pE2F-Luc was originally designed for monitoring E2F-promoter-dependent (a major target gene of the retinoblastoma (Rb) tumour suppressor, a critical cell-cycle regulator) transcriptional activation. To adapt the pE2F-Luc vector for quantifying Stat5-dependent transactivation of the Socs2 promoter, respective 5' promoter regions were cloned 3' of the TA minimal promoter (Figure 14). Thus, Luciferase transcription is driven by (i) the minimal TA promoter in concert with E2F enhancer elements and (ii) a variable Socs2 promoter region.

5.2. Molecular biology

5.2.1. Standard techniques

5.2.1.1. DNA electrophoresis

Routinely, DNA fragments were analysed by separation according to their molecular weight using agarose gel electrophoresis. Briefly, 1-2 % (w/v) low-melting agarose (Roche Cat. No. 1388991) was dissolved in 1 x TAE buffer, boiled in a microwave oven and allowed to cool down to approximately 40-50 °C. Five μl of ethidium bromide (10 mg/ml) was added per 100 ml agarose solution, the gel cast in plastic trays and allowed to solidify at RT. Depending on the size and agarose concentration of the gel a voltage between 80-130 V was applied. The migratory behaviour of the DNA fragments was visualised using a short-wave UV lamp.

5.2.1.2. Extraction of DNA fragments from agarose gels

Following size-separation by agarose gel electrophoresis, gel slices containing desired DNA fragments were excised under a UV lamp using a sterile blade. The DNA was separated from the agarose and purified using the QIAquick Gel Extraction Kit (Qiagen Cat. No. 28704) according to vendor’s instructions. After column-based purification, the DNA was eluted with H₂O(dd) and stored at -20 °C until needed.
5.2.1.3. Transformation of bacteria

Chemically competent E.coli DH5alpha Library efficiency (LE, Invitrogen Cat. No. 18263012) were used for standard plasmid amplification and transformation. For transformation, 200 μl of chemically competent LE were thawed on ice and 100 μl/reaction transferred to a prechilled 15 ml Falcon tube. Depending on the application either (i) 100 ng of plasmid DNA or (ii) 2 μl of ligation reaction mixture were added on ice and mixed gently. The reaction was incubated on ice for 20-30 min followed by a heat shock at 42 °C for exactly 90 s. Subsequently, the tube was allowed to chill on ice for 2 min and 900 μl of prewarmed LB medium added. The reaction was incubated at 37 °C for 60 min under shaking at 225 rpm and various dilutions of the reaction mixture plated on appropriated agar selection plates overnight. Positive single cell clones were picked using a sterile tip and transferred to a 15 ml Falcon tube containing 4 ml LB medium plus antibiotics.

5.2.2. Vector cloning

Cloning of truncated Socs2 promoter fragments (see Figure 14) into the pE2F-Luc Luciferase vector was performed using established protocols from Sambrook et al. (1989). Briefly, respective genomic sequences were amplified using primers containing appropriate restriction endonucleases sites. The PCR fragments were then cut by restriction enzymes, dephosphorylated using calf intestine phosphatase and ligated using T4 ligase into appropriately cut vectors. Purification of DNA fragments was carried out using the QIAquick Gel-Extraction Kit (Qiagen) according to supplier’s protocol. Chemically competent DH5alpha E.coli was heatshock transformed and plated on agar selection plates. Positive clones were confirmed by restriction analysis and DNA sequencing.

5.2.2.1. Cloning strategy for Socs2 reporter constructs

In the following, DNA sequences of primers are given which were used for PCR amplification of Socs2 promoter regions for subsequent cloning into the pE2F-Luc Luciferase reporter vectors:

<table>
<thead>
<tr>
<th>PrimerID</th>
<th>Sequence (Localisation 5’ of Socs2 mRNA transcription start)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2Clon1</td>
<td>GCG CGG CCA CGC GTT CTG TGC AAA GGC CCA GGA GGG (bp -4696 to -4675 5’ of Socs2 mRNA ATG)</td>
</tr>
<tr>
<td>S2Clon2</td>
<td>GCG CGG CCA CGC GTG CAT CCT GCT AAG TTA CTC CC (bp -3402 to -3364 5’ of Socs2 mRNA ATG)</td>
</tr>
<tr>
<td>S2Clon3b</td>
<td>GCG CGG CCA GAT CTA GTG TGC GCG CGA CTT CCC AG (bp -1845 to -1865 5’ of Socs2 mRNA ATG)</td>
</tr>
<tr>
<td>S2Clon4b</td>
<td>GCG CGG CCA GAT CTC GGA GAT GAG TCA ACA CGT CC (bp 1 to -20 5’ of Socs2 mRNA ATG)</td>
</tr>
</tbody>
</table>

Mlu I site
5.2.3. DNA isolation and analysis

5.2.3.1. Isolation of plasmid DNA from bacteria

Plasmid DNA was isolated using commercially available plasmid isolation kits (Qiagen Cat. No. 27106) according to vendor's manual. Briefly, bacteria were lysed, cellular debris removed and the supernatant given onto a DNA-binding matrix column. The column was rinsed extensively with ethanol and the DNA eluted using nuclease-free H$_2$O$_{dd}$. The DNA concentration was determined using UV/VIS spectrophotometry: The OD at 260 nm was used for calculation of DNA concentration and the OD ration of 260 nm/280 nm served as indicator of DNA purity. Generally, DNA exhibiting a 260 nm/280 ratio = 1.8-2.0 were used for subsequent experiments. The DNA was used instantly for cloning purposes or stored at -20 °C for long-term storage.

5.2.3.2. Isolation of tail DNA for genotyping

Pubs were weaned between day 17-21 post natum and identified using ear tags. In addition, a 1-2 mm tail piece was clipped using sterile scissors, transferred into a 1.5 ml reaction tube and digested in 200 μl of NID buffer containing 2 μl of Proteinase K (stock solution 10 mg/ml) overnight at 56 °C. The next morning, the proteolytic activity was inactivated by heating to 95 °C for 10 min. Two μl of supernatant used as template for genotyping the mice using gene-specific genotyping PCR.

NID buffer:

- 50 mm KCl
- 10 mM Tris-HCl (pH 8.3)
- 2 mM MgCl$_2$
- 0.1 mg/ml Gelatine
- 0.45 % NP-40
- 0.45 % Tween-20

5.2.3.3. Genotyping PCR

To determine the allele status of specific genes, 2 μl of tail DNA were analysed by gene-specific PCR using Taq-polymerase (Roche) following manufacturer’s instructions. The cycling conditions used for all genotyping PCR were as follows:

- 95 °C 5 min Initial denaturation (1 cycle)
- 95 °C 20 s Denaturation
- 55 °C 20 s Annealing
- 72 °C 90 s Elongation
Materials and Methods

(40 cycles)

72°C 10 min Final elongation
(1 cycle)

Following PCR amplification, the reaction mixture was diluted with DNA loading buffer and analysed using 2 % agarose gel electrophoresis containing 5 μl ethidium bromide (10 mg/ml) per 100 ml gel.

In the following, DNA sequences of primers used for gene-specific genotyping PCR are given. All oligonucleotides were obtained commercially (MWG Biotech, Munich).

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR flox1</td>
<td>5’ – GGC ATG CAC ATT ACG GCC TTC T – 3’</td>
</tr>
<tr>
<td>GR flox4</td>
<td>5’ – GTG TAG CAG CCA GCT TAC AGG A – 3’</td>
</tr>
<tr>
<td>GR flox8</td>
<td>5’ – CCT TCT CAT TCC ATG TCA GCA TGT – 3’</td>
</tr>
</tbody>
</table>

Amplicons:

- GR<sup>wt</sup> 225 bp
- GR<sup>flox</sup> 275 bp
- GR<sup>null</sup> 390 bp

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>S5del1685</td>
<td>5’ – GAA AGC ATG AAA GGG TTG GAG – 3’</td>
</tr>
<tr>
<td>S5fl1686</td>
<td>5’ – AGC AGC AAC CAG AGG ACT AC – 3’</td>
</tr>
<tr>
<td>S5fl1842</td>
<td>5’ – AAG TTA TCT CGA GTT AGT CAG G – 3’</td>
</tr>
</tbody>
</table>

Amplicons:

- Stat5<sup>wt</sup> 450 bp
- Stat5<sup>flox</sup> 200 bp

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Cre1</td>
<td>5’ – CGG TCG ATG CAA CGA GTG ATG AGG – 3’</td>
</tr>
<tr>
<td>Cre2</td>
<td>5’ – CCA GAG ACG GAA ATC CAT CGC TCG – 3’</td>
</tr>
</tbody>
</table>

Amplicons:

- AlfpCre<sup>+</sup> 700 bp

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR2for</td>
<td>5’ – GCT GAG ATG GAT CTT GAG – 3’</td>
</tr>
<tr>
<td>MDR2rev</td>
<td>5’ – GTC GAG TAG CCA GAT GAT GG – 3’</td>
</tr>
</tbody>
</table>

Amplicons:

- MDR2<sup>wt</sup> 362 bp

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEOgeno1</td>
<td>5’ - ATC CAA AGC CAG ACC GG – 3’</td>
</tr>
<tr>
<td>NEOgeno4</td>
<td>5’ – TTT GTC ACG TCC TGC ACG ACG C – 3’</td>
</tr>
</tbody>
</table>

Amplicons:

- MDR2<sup>+</sup> 700 bp
5.2.4. RNA isolation and analysis

5.2.4.1. Isolation of messenger RNA from murine livers

High quality mRNA was isolated from frozen organs (e.g. liver, adipose tissues). Approximately 50 mg of frozen tissue were transferred into a prechilled 14 ml round bottom Falcon tube containing 5 ml of Trizol reagent (Fisher Scientific Cat. No. VX15596018). Tissue specimens were homogenized directly in Trizol using a rotating blade homogenator. The resulting homogenate was incubated for 2-3 min at RT to sequester RNA from bound nucleoprotein complexes and transferred into a new nuclease-free reaction tube. Chloroform was added (200 μl per initial 1 ml of Trizol reagent), vigorously shaken and centrifuged at 12,000 rpm for 15 min at 4 °C. The upper aqueous phase, containing hydrophilic RNA was transferred into a new tube, carefully ensuring no cross-contamination with the lower organic phase. For precipitation, 0.7 x (v/v) 2-propanol was added and incubated for 10 min at RT. Two μl of glycogen were added to facilitate pellet handling at later stages. Centrifugation at 12,000 for 10 min led to pelleting of precipitated RNA, which was washed two times with 75 % DEPC-Ethanol. Finally, the solvent was removed, remaining ethanol droplets carefully removed using a pipette and 100 μl of DEPC-H2O added for solubilization of the RNA. The solubilization was facilitated by incubating the tube at 37 °C for 10 min. Isolated RNA was used for Affymetrix expression profiling or reversely transcribed into cDNA for quantitative RT-PCR (qPCR) analysis. Samples were stored at -80 °C and repeated freeze-thawing avoided by appropriate aliquoting. The RNA quality and quantity was determined using agarose 1 % RNAse-free 1x MOPS gel electrophoresis: For this, 500 ng of isolated RNA were diluted in 10 μl RNA denaturation buffer and incubated at 65 °C for 10 min. Following denaturation, samples were loaded onto the gel and separated at low voltage (< 80 V). High RNA quality could be ascertained by three clearly discriminable bands for 28 S, 18 S and 5.8 S of ribosomal RNA (rRNA). The ratio between 28 S to 18 S RNA bands should be approximately 2:1.

**H2O/DEPC**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 l</td>
<td></td>
</tr>
<tr>
<td>1 ml</td>
<td>Diethylpyrocarbonate (DEPC)</td>
</tr>
</tbody>
</table>

The mixture was stirred, incubated overnight at RT and autoclaved

**RNA denaturation buffer:**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μl</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>0.5 μl</td>
<td>10x MOPS buffer</td>
</tr>
<tr>
<td>5 μl</td>
<td>Formamide</td>
</tr>
<tr>
<td>1.75 μl</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>0.55 μl</td>
<td>Nuclease-Free H2O</td>
</tr>
</tbody>
</table>

**MOPS buffer:**

<table>
<thead>
<tr>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>41.8 g</td>
<td>[N-Morpholino] propanesulfonic acid (MOPS)</td>
</tr>
<tr>
<td>6.8 g</td>
<td>Sodium Acetate</td>
</tr>
<tr>
<td>20 ml</td>
<td>0.5 M EDTA</td>
</tr>
</tbody>
</table>

→ fill up with DEPC-H2O to 1 L, autoclave and store at 4 °C in the dark.
In addition, the concentration of isolated RNA was quantified using UV/VIS spectrophotometry at 260 nm. The 260 nm/280 nm ratio was an indicator of RNA purity and should be >1.8.

5.2.4.2. Reverse transcription

For synthesis of complementary DNA (cDNA) from respective mRNA samples, 1 μg of total RNA were used. The cDNA synthesis was carried out using commercially available kits (Applied Biosystems). Total RNA was diluted to 2 μl and incubated with MuLV reverse transcriptase (Applied Biosystems Cat. No. N8080018) buffer, oligo-dT primers (Applied Biosystems Cat. No. N8080128), RNase inhibitor (Applied Biosystems Cat. No. N8080119) and DEPC-H₂O for 5 min at 70 °C for annealing of oligo-dT primers onto poly-A sequences of the mRNA. Afterwards, the samples were incubated on ice for >2 min and 1 μl MuLV reverse transcriptase (Applied Biosystems Cat. No. N8080018) were added, followed by an incubation for 60 min at 42 °C. A final heat inactivation step at 70 °C for 10 min removed residual reverse transcriptase activity. The cDNA was diluted 1:5 and was used instantly or stored at -20 °C.

5.2.4.3. Quantitative RT-PCR (qPCR)

Five μl of diluted cDNA samples (section 5.2.4.2) were used for quantification of gene-specific transcript levels via quantitative RT-PCR (qPCR). For this, the diluted cDNA was amplified using commercially available Taq polymerase kits (Eppendorf Cat. No. 0032007724) according to manufacturer’s instructions. Intron-spanning primers ensured amplification of specific mRNA transcripts. Cross-contamination of genomic DNA (gDNA) was excluded by regular testing of mRNA samples not reversely transcribed. The SYBR green method was applied for determining concentrations of double stranded PCR products obtained after PCR using intron-spanning primers. The PCR cycle in which a given mRNA specific fluorescent signal crossed an arbitrary threshold was defined as C_T.cycle. The difference of a gene-specific C_T value to that of an endogenous housekeeping gene (GAPDH) was called ΔC_T value. Individual ΔC_T for all genotypes were calculated and compared relatively to controls. The primer sequences were designed manually and DNA oligonucleotides obtained from a commercial supplier (MWG Biotech, München). The DNA sequences of primers used for qPCR analysis are given in (Table 5).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Accession No.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Srebp-1</td>
<td>NM_011480</td>
<td>for: GCG TGG TTT CCA ACA TGA TGT GCA TCA CAG CTC AGC TGT AGT GC</td>
</tr>
<tr>
<td>Fasn</td>
<td>NM_007988</td>
<td>for: TGT CTG ACA CTG GCA ATC TGA TGG GTA TCA TGG TAG GGA CGC TGT TTT TAT GTA AGC</td>
</tr>
<tr>
<td>Scd1</td>
<td>NM_009127.3</td>
<td>for: CGC TCT TTA CCC TTT GCT GAA CAC GAC TAC TAC TAC TAC</td>
</tr>
<tr>
<td>Scd2</td>
<td>NM_009128.1</td>
<td>for: ATC TGC AGG ATT GCC TCT GGG TCT CAT GCT CAC TAC CCG</td>
</tr>
<tr>
<td>Acaca</td>
<td>NM_133360.2</td>
<td>for: GGA CTT CAT GAA TTT GCT GAT TCT CAG TT ACC TAC TCA TAT GAT TAC TAC</td>
</tr>
</tbody>
</table>

93
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Primer 1</th>
<th>Primer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acacb</td>
<td>NM_133904.1</td>
<td>for: AGT GTG TAA GAA GCT CGT GGG</td>
<td>rev: CCT CCA GTA GAA GAA GGT GCG</td>
</tr>
<tr>
<td>Pparg</td>
<td>NM_011146.2</td>
<td>for: ACC CAA TGG TTG CTG ATT AC</td>
<td>rev: CGG GAA GGA CTT TAT GTA TGA G</td>
</tr>
<tr>
<td>Dgat1</td>
<td>NM_010046.2</td>
<td>for: TGT GGT GAT GCT GAT CCT GAG</td>
<td>rev: GGA TAG GAT CCA CCA GGA TGC</td>
</tr>
<tr>
<td>Dgat2</td>
<td>NM_026384.3</td>
<td>for: TGG CAT AAG GCC CTA TTT GG</td>
<td>rev: ATG GTG TCT CGG TTG ACA GG</td>
</tr>
<tr>
<td>Cd36</td>
<td>NM_007643.3</td>
<td>for: CCT TAC TTG GGA TGG GAG TGG</td>
<td>rev: CGG TTT TAC CAA AGA TGT AGC C</td>
</tr>
<tr>
<td>Fabp4</td>
<td>NM_024406.1</td>
<td>for: TTC GAT GAA ATC ACC GCA GA</td>
<td>rev: AGG GCC CGG CCA TCT</td>
</tr>
<tr>
<td>Mod1</td>
<td>NM_008615.1</td>
<td>for: AGT TGG TCT TGG GGT GGT GGC</td>
<td>rev: TAG TGC TGT ACA TCT GGG AGG</td>
</tr>
<tr>
<td>Ppara</td>
<td>NM_011144.3</td>
<td>for: ATC GGG TAC GGC AAT GGC TTT A</td>
<td>rev: GCC TCG GAG GTC CCT GAA CAG</td>
</tr>
<tr>
<td>Acox1</td>
<td>NM_015729.2</td>
<td>for: CGG CCT ATG CCT TCC ACT TTC TC</td>
<td>rev: CGC CAC TTC CTT GCT CTC CTT GT</td>
</tr>
<tr>
<td>Ehhadh</td>
<td>NM_023737.2</td>
<td>for: CGG GTC AAT GCC ATC AGT CC</td>
<td>rev: GGT AGA AGC TGC GTT CCT CTT GC</td>
</tr>
<tr>
<td>Acadm</td>
<td>NM_007382.2</td>
<td>for: ATG ACG GAG GCA CTA AGT A</td>
<td>rev: ATG GCC GCC ACA TCA GA</td>
</tr>
<tr>
<td>Acadl</td>
<td>NM_007381.3</td>
<td>for: AGC TGA TGG CAA GAC AGA TCG</td>
<td>rev: AGA ATC CGC ATG AGC TCG ATG</td>
</tr>
<tr>
<td>Acadl</td>
<td>NM_007366.2</td>
<td>for: CCC GGT TCT TTG AGG AAG TGA A</td>
<td>rev: ATG GTC GTC CTC CAC CTT CTG</td>
</tr>
<tr>
<td>Cpt1</td>
<td>NM_009948.1</td>
<td>for: CGA GGA TTC TCT GGA ACT GC</td>
<td>rev: GTG CGC TTC TTC AAG GTC TG</td>
</tr>
<tr>
<td>Pgc1a</td>
<td>NM_008904.1</td>
<td>for: CCG ATC ACC AT CTT CAG GT</td>
<td>rev: GTG TGC GGT GTC TGT AGT GG</td>
</tr>
<tr>
<td>Cebpa</td>
<td>NM_007678</td>
<td>for: ATG GAG TAC AGC GGT GAG TAT TC</td>
<td>rev: TGC GCC ACT GCA CAT CTC TTC</td>
</tr>
<tr>
<td>Cebpb</td>
<td>NM_009883.2</td>
<td>for: GGT GGA CAA GCT GAG CGA GTA</td>
<td>rev: AAC AAG TCG CCA AGG GTG CTG AG</td>
</tr>
<tr>
<td>Hnf1a</td>
<td>NM_009327.2</td>
<td>for: CCA CTT TCA CGA ACA CGG GCC GC</td>
<td>rev: TGG AGG CCT CTG TGT CTG AGG TG</td>
</tr>
<tr>
<td>Rxra</td>
<td>NM_011305.3</td>
<td>for: CGA GCC ATC GTC CTC TTT CCC CCA GC</td>
<td>rev: TGG TTT GAT GTG GTG CCT CTC CCA GC</td>
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<tr>
<td>Tgfb1</td>
<td>NM_011577.1</td>
<td>for: GGC ACC GGA GAG CCC TGG ATG TA</td>
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</tr>
<tr>
<td>Tgfbr</td>
<td>NM_009370.2</td>
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</tr>
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<td>Pdgf1</td>
<td>NM_011057.3</td>
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</tr>
<tr>
<td>Pdgfr</td>
<td>NM_008809.1</td>
<td>for: TCA AGC ACT CAC CAG TGC TC</td>
<td>rev: TTC AGA GGC AGG TAG GTG CT</td>
</tr>
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<td>Col1</td>
<td>NM_007742.3</td>
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<td>rev: TGG CGG GCT GAT GAA CTA GT</td>
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<td>Col3</td>
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<td>rev: CAG GTG AAC CCG GCA AGA ACG</td>
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<tr>
<td>Timp1</td>
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<td>for: TCC TCT TGT TGC TAT CAC TTA TAG TCT</td>
<td>rev: CGG TGG TAT AAG GTG GTG TGG TGG</td>
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<tr>
<td>Timp2</td>
<td>NM_011594.3</td>
<td>for: GGG ACA CGT TTA GCA TCA CCC</td>
<td>rev: GAA GAA CTT GGC GTG TGG CCC</td>
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<td>Mmp2</td>
<td>NM_008610.2</td>
<td>for: TCC CTG ATG ACC TGG ATG CCG</td>
<td>rev: GCA GGC ATG AAG ATG ATG GGG C</td>
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<td>rev: GCT TCA AAG ACA GCA TCC ACC C</td>
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<td>Mmp13</td>
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</table>
Materials and Methods

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
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<td>GGG TGT TTG ACG AAG CCT CCC</td>
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<tr>
<td>Mmp15</td>
<td>NM_008609.3</td>
<td>ATC CCC ACC TCT CCC AAA GGG</td>
<td>TCC CGC AGG ATG GAT TTG GGG</td>
</tr>
<tr>
<td>Ntcp1</td>
<td>NM_011387.1</td>
<td>ACC TTC CCC CCT GAA GTC ATT GG</td>
<td>GGA TTA TTC CCG TTG TGG GTA CC</td>
</tr>
<tr>
<td>Oatp1</td>
<td>NM_013797.2</td>
<td>GTT TCA TCT TCT CAC TCG CC</td>
<td>TTG ATC CTC TCA CTG CTG CAG GC</td>
</tr>
<tr>
<td>Bsep</td>
<td>NM_021022.3</td>
<td>CAA CAC CAA AGA GAT CTC CGT GG</td>
<td>CAA TGA CAA TAC AGG TCC GAC CC</td>
</tr>
<tr>
<td>Mdr1b</td>
<td>NM_011075.1</td>
<td>TGT GGA GGG CAG CCA AGG AGG CC</td>
<td>CAC AAT GCA GGT GCC GCC TAC CC</td>
</tr>
<tr>
<td>Mrp2</td>
<td>NM_013806.2</td>
<td>TTG TGG CTG GCC TGC AAC TTG GG</td>
<td>CAA TCT TGC CGC TGT CTA GGA CC</td>
</tr>
<tr>
<td>Oatp2</td>
<td>NM_178235.2</td>
<td>GAA TTG AAA GTA CTT GGA ATG CC</td>
<td>TTA AAG CTA TAC TCA ATG AAC CCA CC</td>
</tr>
<tr>
<td>Mrp3</td>
<td>NM_029600.3</td>
<td>GCA GGC CTG GAT TTG CAG TGC GC</td>
<td>ATT CAG CTA CTA CTC CTT TGT CC</td>
</tr>
</tbody>
</table>

Table 5. Primer sequences used for qPCR experiments

5.2.4.4. Whole genome expression profiling (Affymetrix) and bioinformatics

For each microarray, three livers were pooled. Total RNA was isolated using RNeasy kits with DNaseI digest on column (Qiagen). RNA quality was assessed using the Bioanalyzer 2100 Lab-on-chip system (Agilent Technologies). Ten μg of isolated RNA were labelled and hybridised onto mouse U74Av2 arrays (Affymetrix), containing 12,500 sequences. We used three arrays for each mutant group (Stat5AlfpCre, GRAlfpCre, Stat5/GRAlfpCre and Stat5ΔN) and nine arrays for the control group (littermates to the mutant mice). Arrays were normalized using GCRMA. Genes with a mean expression of <25 in all of the groups were excluded. Significance levels were calculated in affylmGUI. As criterion for calling a gene significantly changed we used p < 0.001, or the combined criterion of p < 0.0025 and a log ratio >1 or less than -1 (fold change >2 or <0.5). Investigation of coordinated changes in functional gene-sets was done using MAPPfinder. We limited the analysis to approximately 100 predefined groups, of which some were custom made. The two criteria for inclusion of genes were p < 0.01 and p < 0.05. Gene sets with p-value <0.05 after correction for multiple comparisons were considered significant.

5.2.4.5. Molecular signature analysis of Affymetrix expression data

We performed a specific molecular signature analysis for genes involved in lipid metabolism on raw data from microarray analysis previously described (section 5.2.4.4). In order to find molecular signatures in the data we looked for coordinated changes in functional gene-sets using MAPPFinder. We limited the analysis to around one hundred hand-picked, pre-defined groups, of which some were custom-made from papers describing profiles from the livers of mice lacking different transcription factors or administered with specific agonists or antagonists. Many lists were also taken from the GSEA software and consist of genes with certain motifs in their promoter regions as identified in a systematic study of regulatory motifs in mammals. The two criteria for inclusion of genes in the MAPPFinder-analysis were p<0.001 or p<0.05. This was done to pick up high magnitude changes of small
groups of genes and low-amplitude changes in large groups, respectively. Gene-sets with p-value <0.05 after correction for multiple comparisons were considered as significant.

5.3. Protein chemistry

5.3.1. Protein isolation

5.3.1.1. Preparation of liver homogenates

For preparation of murine liver homogenates, approximately 100 mg of tissue were transferred to a 15 ml round-bottom tube loaded with 1 ml of IP buffer containing Proteinase/phosphatase inhibitors (IP+ buffer). Using a tissue homogenator with rotating blades, liver specimens were solubilised directly in IP+ buffer until no tissues clumps were visible. Foam generation should be avoided to minimize protein denaturation. The homogenate was transferred to a fresh 1.5 ml reaction tube and cellular debris removed by centrifugation at maximum speed for 15 min at 4 °C in a table micro centrifuge. The supernatant was again transferred to a new tube and centrifugation repeated until the solutions turned translucent. The supernatant was either mixed instantly with 4xSB (reducing sample buffer) for SDS-PAGE, incubated with antibodies for immunoprecipitation purification or shock frozen in liquid N₂ and stored at -80 °C.

**IP buffer:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES pH 7.5</td>
<td>25 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
</tr>
<tr>
<td>Tween-20</td>
<td>0.1 %</td>
</tr>
<tr>
<td>NP-40</td>
<td>0.5 %</td>
</tr>
<tr>
<td>beta-Glycerophosphate</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

**IP+ buffer = IP buffer containing:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₃VO₄</td>
<td>1 mM</td>
</tr>
<tr>
<td>NaF</td>
<td>1 mM</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>10 μg/ml</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>10 μg/ml</td>
</tr>
<tr>
<td>PMSF</td>
<td>1 mM</td>
</tr>
<tr>
<td>Complete protease inhibitor cocktail (Boehringer Cat. No. 1697498)</td>
<td>1 tablet</td>
</tr>
</tbody>
</table>

**4 x SDS sample buffer (reducing):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris/HCl pH 6.8</td>
<td>240 mM</td>
</tr>
<tr>
<td>DTT</td>
<td>40 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>8 %</td>
</tr>
<tr>
<td>Bromphenol blue</td>
<td>0.01 %</td>
</tr>
<tr>
<td>Glycerol</td>
<td>40 %</td>
</tr>
</tbody>
</table>

→ aliquoted and stored at -20 °C
5.3.1.2. Preparation of cell line extracts

Cellular extracts from HEK293 cells used for EMSA analysis were prepared using whole cell extract (WCE) buffer. If not indicated otherwise, cells were harvested from Petri dishes using a sterile blade. Cells were washed two times with ice-cold PBS, followed by centrifugation at 12,000 rpm for 15 min at 4 °C and solubilisation in twice the cell pellet volume in WCE buffer. Disruption of cells was facilitated by incubation for 1 h at 4 °C under vigorous shaking using a horizontal shaker. Subsequently, cellular debris was removed by centrifugation at maximum speed for 15 min at 4 °C using a tabletop micro centrifuge. The supernatant was either directly used in EMSA analysis or shock frozen in liquid N2 and stored at -80 °C.

<table>
<thead>
<tr>
<th>WCE buffer:</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM Hepes pH 7.9</td>
</tr>
<tr>
<td>20 % Glycerol</td>
</tr>
<tr>
<td>50 mM KCl</td>
</tr>
<tr>
<td>1 mM EDTA</td>
</tr>
<tr>
<td>1 mM DTT</td>
</tr>
<tr>
<td>400 mM NaCl</td>
</tr>
<tr>
<td>5 μg/ml Leupeptin</td>
</tr>
<tr>
<td>5 mM Beta-Glycerophosphate</td>
</tr>
<tr>
<td>1 mM PMSF</td>
</tr>
<tr>
<td>10 mM NaF</td>
</tr>
<tr>
<td>5 mM Na3VO4</td>
</tr>
</tbody>
</table>

→ aliquot and store at -20°C

5.3.1.3. Protein quantification with the Bradford method

Protein concentration in liver homogenates were quantified using Bradford reagent (Bio-Rad Cat. No. 500-0006). Briefly, a standard curve including protein concentrations ranging from 1 to 20 μg/μl was prepared in microcuvettes according to manufacturer's instructions. The unknown protein sample was pipetted into a separate cuvette. When necessary, 1/10 dilutions of the unknown samples were prepared in the respective homogenisation buffer. Finally, the standards and samples were diluted in 1 ml of a 1:5 mixture of water and Bradford reagent, incubated for 5 min and the absorption of measured at 550 nm. The protein concentration of unknown samples was calculated using the OD values of the standard curve.

5.3.2. Immunoprecipitation

For certain applications, enrichment of proteins was necessary prior to immunoblot analysis due to low expression of the respective protein. Immunoprecipitation utilised high-affinity binding of Sepharose bead-bound antibodies to the respective protein epitope. Briefly, 1000-2000 μg of tissue homogenate in IP+ buffer were diluted with IP+ buffer to a final volume of 600 μl. One μg of antibody/antiserum was added and antibody-antigen complexes allowed to form overnight under constant agitation using a wheel shaker. Subsequently, 40 μl of pre-swollen protein A Sepharose beads (GE Healthcare Cat. No. 17-0780-01) were added and incubated for 1 h at 4 °C on the
wheel shaker. The Fc region of the utilised antibody was bound with high affinity by the Protein A and stable Bead-Antibody-Epitope complexes were formed. After incubation, the Sepharose beads were rinsed three times with 700 μl of IP+ buffer and spun down in a micro centrifuge at 2,000 rpm for 1 min at 4 °C. After a final spin at 12,000 rpm for 5 min at 4 °C, the supernatant was discarded and pelleted Sepharose beads dissolved in 40μl of reducing SDS sample buffer. Finally, the mixture was heated to 95 °C for 5 min and the supernatant transferred into a new 1.5 reaction tube. It was subsequently analysed instantly using Western Blot analysis or stored at -20 °C. Antibodies were used for immunoprecipitation as indicated in

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS-1</td>
<td>Up (#06248)</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>IRS-2</td>
<td>Up (#06506)</td>
<td>Rabbit polyclonal</td>
</tr>
<tr>
<td>IR beta chain (IRb)</td>
<td>Sc (sc-711)</td>
<td>Rabbit polyclonal</td>
</tr>
</tbody>
</table>

Table 6. Antibodies used for immunoprecipitation of respective proteins

5.3.3. Western Blot

5.3.3.1. Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

Cellular extracts or tissue homogenates diluted in 4x SDS sample buffer were boiled for 5 min prior to SDS-PAGE analysis including 5 % (v/v) of fresh beta-Mercaptoethanol. According to original protocols introduced by Laemmli, proteins were separated in a discontinuous gel consisting of
(i) A stacking gel with a low acrylamide percentage for focusing of migratory bands.

<table>
<thead>
<tr>
<th>Stacking gel (4 % acrylamide, 4 ml for two gels):</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 ml</td>
<td>1.0 M Tris (pH 6.8)</td>
</tr>
<tr>
<td>0.67 ml</td>
<td>30 % Acrylamide Mix</td>
</tr>
<tr>
<td>20 μl</td>
<td>20 % SDS</td>
</tr>
<tr>
<td>40 μl</td>
<td>10 % APS</td>
</tr>
<tr>
<td>4 μl</td>
<td>TEMED</td>
</tr>
<tr>
<td>2.7 ml</td>
<td>H₂O dd</td>
</tr>
</tbody>
</table>

(ii) A separation gel with variable acrylamide percentages for separation of proteins according to molecular weight. To facilitate polymerisation, the gel is overlaid with 2-Propanol. Routinely, a 6 cm Minigel system was used (Bio-Rad Cat. No. 170-3940). The following separation gels were routinely casted:

<table>
<thead>
<tr>
<th>8 % gel (20 ml for 2 x 1.5 mm gels):</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ml</td>
<td>1.5 M Tris (pH 8.8)</td>
</tr>
<tr>
<td>5.3 ml</td>
<td>30 % Acrylamide</td>
</tr>
<tr>
<td>100 μl</td>
<td>20 % SDS</td>
</tr>
<tr>
<td>200 μl</td>
<td>10 % APS</td>
</tr>
<tr>
<td>12 μl</td>
<td>TEMED</td>
</tr>
<tr>
<td>9.3 ml</td>
<td>H₂O dd</td>
</tr>
</tbody>
</table>
Materials and Methods

10 % gel (20 ml for 2 x 1.5 mm gels):
6.7 ml 1.5 M Tris (pH 8.8)
5 ml 30 % Acrylamide
100 μl 20 % SDS
200 μl 10 % APS
8 μl TEMED
7.9 ml H₂O(dd)

10 x Laemmli buffer:
25 mM Tris-HCl (pH 8.3)
192 mM Glycine
1 % (w/v) SDS
Æ fill up with H₂O(dd) to 1 L and store at RT

SDS-PAGE was carried out in 1 x Laemmli buffer running at 20-30 mA/gel until the dye in the loading buffer was running out of the gel. After electrophoretic separation gels were subjected to immunoblotting (section 5.3.3.2).

5.3.3.2. Protein transfer onto Nylon membranes

Protein transfer was carried out using Bio-Rad semi-dry blotting or wet blotting devices and proteins blotted onto 0.45 μm nitrocellulose membranes (GE Healthcare Cat. No. RPN303E) pre-equilibrated in transfer buffer. A current of 0.8 mA/cm² was applied and proteins were allowed to transfer for 90 min (semi-dry transfer, RT) or 3 h (wet transfer, 4 °C). Following protein transfer, the membranes were rinsed with H₂O(dd) and transfer efficiency controlled via staining with Ponceau S solution for 5 min. Excess Ponceau staining was removed with multiple washes of H₂O(dd).

Ponceau S solution:
0,1 % Ponceau S dye
5 % Acetic Acid

Transfer buffer:
48 mM Tris
39 mM Glycine
0.037 % (v/v) SDS
20 % Methanol

5.3.3.3. Immunoblot analysis (antibody staining)

Following Ponceau S staining, nitrocellulose membranes were washed twice with H₂O(dd) and blocked for 1 h in blocking solution (5 % BSA in 1x TBST). The membrane was sealed in plastic foil and incubated overnight with respective primary antibodies diluted in TBST + 1 % BSA. Subsequently, the membrane was rinsed three times for 10 min in 1x TBST and incubated for 30-45 min with an appropriate species-specific antibody conjugated to horseradish-peroxidase (HRP) in a dilution of 1:2000 to 1:5000. Afterwards, membranes were washed three times with 1x TBST. For specific
detection of bands corresponding to the utilized antibody, an enhanced chemiluminescence (ECL) system (Amersham Biosciences Cat. No. RPN 2132) was used to expose ECL films (THP Pierce Cat. No. PI-34091). The exposition times varied between the antibodies used. Primary antibodies were utilised as indicated in (Table 7). Membranes could be stripped of the primary antibody by immersion in stripping buffer for 30 min at 55 °C under constant agitation. Afterwards, the membrane could be blocked and re-incubated using different antibodies.

Stripping buffer:
6.25 ml  1 M Tris-HCl pH 6.8
0.675 ml  beta-Mercaptoethanol
10 ml    20 % SDS
⇒ fill up with H₂O to 100 ml

10 x TBST:
500 mM Tris-HCl
1.5 M NaCl
0.5 % Tween-20
⇒ adjust to pH 8 and fill up with H₂O to 2 L

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Species</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti HSC-70</td>
<td>SC (sc-7298)</td>
<td>Mouse monoclonal</td>
<td>1:20,000</td>
</tr>
<tr>
<td>Anti AKT</td>
<td>SC (sc-8312)</td>
<td>Rabbit polyclonal</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Anti pS-AKT</td>
<td>CS (#9271S)</td>
<td>Rabbit polyclonal</td>
<td>1:250</td>
</tr>
<tr>
<td>Anti phosphothreonine</td>
<td>Up (12-302)</td>
<td>Mouse monoclonal</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Anti PPARGamma</td>
<td>SC (sc-7273)</td>
<td>Mouse monoclonal</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Anti SREBP-1c</td>
<td>SC (sc-13551)</td>
<td>Mouse monoclonal</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Anti Stat1</td>
<td>BD (#610115)</td>
<td>Mouse monoclonal</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Anti pY-Stat1</td>
<td>CS (#9167)</td>
<td>Rabbit monoclonal</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Anti Stat3</td>
<td>BD (#610189)</td>
<td>Mouse monoclonal</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Anti pY-Stat3</td>
<td>CS (9131)</td>
<td>Rabbit polyclonal</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Anti Stat5</td>
<td>Anti Stat5 aa775-788</td>
<td>Rabbit polyclonal</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Anti GR</td>
<td>SC (sc-1004)</td>
<td>Rabbit monoclonal</td>
<td></td>
</tr>
<tr>
<td>Anti MRP2</td>
<td>Kindly provided by Dr. James Boyer, Yale</td>
<td>1:2,000</td>
<td></td>
</tr>
<tr>
<td>Anti MRP3</td>
<td>University, New Haven, USA</td>
<td>1:2,000</td>
<td></td>
</tr>
<tr>
<td>Anti NTCP1</td>
<td>Kindly provided by Dr. Bruno Stieger, Zurich, Switzerland</td>
<td>1:2,000</td>
<td></td>
</tr>
<tr>
<td>Anti OATP1</td>
<td>Switzerland</td>
<td>1:2,000</td>
<td></td>
</tr>
<tr>
<td>Anti beta Actin</td>
<td>Sigma AC-15</td>
<td>1:2,000</td>
<td></td>
</tr>
<tr>
<td>Anti mouse-HRP</td>
<td>Dako (P0447)</td>
<td>1:5,000</td>
<td></td>
</tr>
<tr>
<td>Anti rabbit-HRP</td>
<td>Dako (P0217)</td>
<td>1:5,000</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: BD, Becton Dickinson; CS, Cell Signaling; Dako, DakoCytofomation; SC, Santa Cruz Biotechnologies; Up, Upstate Biotech.

Table 7. Primary and secondary antibodies used in immunoblot analysis

5.3.4. EMSA

Electromobility shift assays (EMSA) constitute a widely utilised technique for studying in vitro the interaction between DNA binding proteins (e.g. transcription factors) and respective DNA elements. Briefly, radiolabeled DNA oligomers migrate along an electric current. The migration behaviour is influenced by interaction of DNA-binding protein interacting with respective DNA sequences and decelerating their migratory velocity (‘shift’). Additional binding of specific antibodies leads to further deceleration of the DNA-binding protein velocity (‘supershift’). For analysis of the DNA binding properties of full-length and N-terminally truncated Stat5 isoforms in vivo, we
prepared liver homogenates from PBS and GH-treated control as well as Stat5ΔN mice directly in IP+ buffer. The binding properties of GH-activated Stat5 were tested on classical Stat5 responsive DNA elements from the bovine beta Casein promoter. Similarly, the putative binding properties of GH-activated Stat5 on three putative Stat5 responsive elements found in the Socs2 promoter (Socs2 Stat5 RE #1-3) were assessed. To verify our findings in cell culture, we transfected HEK293 cells with pMSCV expression plasmids encoding for Stat5 and the erythropoietin receptor (EpoR). Subsequent Epo stimulation for 30 min led to potent activation and phosphorylation of cytoplasmic Stat5 molecules. The binding properties of Epo-activated Stat5 on putative Stat5 responsive elements in the Socs2 were assessed using EMSA analysis. Single stranded DNA oligonucleotides applied for EMSA analysis (Table 8) were annealed at equimolar concentrations (~100 μM/oligomer) in 200 μl annealing buffer (10 x = 0.625 x PCR buffer II (Roche); 9.4 mM MgCl₂) by heating to 95 °C for 10 min, followed by slow cooling to RT and subsequent incubation on ice for > 15 min. Annealed double stranded DNA oligos were diluted to 2.5 μM for radiolabeling. The labelling reaction (5 pmol of annealed dsDNA incubated with 10 U of polynucleotide kinase (PNK) and 5 μl of ATP ³²P (Amersham Biosciences; 8,000 cpm/μl) were incubated at 37 °C for 1 h. Radiolabeled oligonucleotides were purified on size exclusion columns (Bio-Rad Cat. No. 7326221) and stored at -20 °C until needed. For analysis of Stat5-DNA binding interaction using EMSA, 20 μl reaction mixtures were prepared for each sample, containing 2 μl BSA solution, 2 μl poly-dI-dC (Roche Cat. No. 108812), 4 μl 5x binding buffer and 1 μl of radiolabeled oligonucleotides. Usually 20-30 μg of liver homogenate or cellular extract were used per reaction. Protein extracts were incubated for > 5 min at RT before loading of the gel. For supershift analysis, the reaction mixture was incubated for >10 min with 1 μl of a rabbit polyclonal anti Stat5 specific antiserum. Complexes were separated on non-denaturing 4 % acrylamide gels containing 0.25 x TBE and run in 0.25 x TBE running buffer at 200 V for 2-3 h. The reaction was stopped after the Orange G dye, which migrates with a comparable velocity as unbound oligos, was running out of the gel. The gel was dried on a vacuum drier at 80 °C for 2 h and subjected to autoradiography. Coated Kodak MR films (Sigma Cat. No. Z350370) were exposed > 1 d at -80°C.

**BSA solution:**
- 10 mg/ml
- 50 mM
- 0.1 mM
- 5 %

**5 x Binding buffer:**
- 50 mM
- 5 mM
- 1 mM
- 0.5 mM
- 25 %
- 250 mM
- 0.5 %

- BSA in 20 mM KPO₄
- NaCl
- EDTA
- Glycerol
- Tris
- DTT
- PMSF
- Glycerol
- NaCl
- KPO₄
Table 8. DNA sequence of Stat5 responsive oligonucleotides used in EMSA analysis

<table>
<thead>
<tr>
<th>DNA oligomer</th>
<th>Promoter</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta Casein Stat5 RE</td>
<td>Bovine beta Casein</td>
<td>AGATTCTAGGAATTCAATC (sense) GATTGTGCTCTAGAATCT (antisense)</td>
</tr>
<tr>
<td>Socs2 Stat5 2xRE #1</td>
<td>Murine Socs2</td>
<td>AAGATTTCCAGGAAAACA (sense) TGTTTTCTCGGATTCTT (antisense)</td>
</tr>
<tr>
<td>Socs2 Stat5 2xRE #2</td>
<td>Murine Socs2</td>
<td>GTATTCCAAGAAAAAT (sense) ATTTTTCTTTGAAAAAT (antisense)</td>
</tr>
<tr>
<td>Socs2 Stat5 2xRE #3</td>
<td>Murine Socs2</td>
<td>CGGATTCTGGAAAAAGTT (sense) AACTTTCAGGAAATCCG (antisense)</td>
</tr>
</tbody>
</table>

Abbreviations: RE, responsive element

5.3.5. Tissue analysis and paraffin embedding

Experimental mice were killed by asphyxiation or cervical dislocation and isolated organs cut in 2-3 mm thick slides, fixed for 48 h in phosphate-buffer 10% formaldehyde solution and dehydrated using graded ethanol. The dehydrated organs were washed three times with xylene, replaced with liquid paraffin and finally cast into metal moulds and let solidify. Solid paraffin blocks were stored at RT until needed for histological or immunohistochemical analyses. Two μm thick sections of paraffin blocks were generated using a microtome (Leica) and subsequent smoothening in a 50 °C water bath. Finally, the tissue sections were transferred onto poly-Lysine coated Superfrost glass slides (Lacton Cat. No. 193515000) and let dry at RT overnight. Tissue sections can be stored at RT for several weeks until needed for histological or immunohistochemical analyses.

5.3.5.1. Histopathology

For standard histology using haematoxylin/eosin (HE) staining, paraffin sections of 2 μm thickness were prepared, deparaffinised by incubation for 2x5 min in xylene and dehydrated using a decreasing ethanol (EtOH) gradient (2x2 min 100 % EtOH; 20 dips in each 95 % EtOH, 95 % EtOH, 70 % EtOH, 50 % EtOH, H2Odd, H2Odd). Subsequently, sections were incubated in Mayer’s haematoxylin solution (VWR Cat. No. 1092490500) and rinsing with tap for 5-10 min to differentiate the staining intensity. Slides were counterstained with eosin solution (Roth Cat. No. 7089.2), dipped in H2Odd rehydrated using the aforementioned graded ethanol in reverse order. After a final incubation in xylene, tissue sections were mounted on Superfrost glass slides using Eukitt mounting medium (Lactan Cat. No. 361894G).

Eosin solution:
30 ml 1 % Eosin
270 ml 70 % Ethanol
3 ml Glacial acetic acid

5.3.5.2. Immunohistochemistry

For immunohistochemical analysis of PPARgamma and GR protein expression, overnight-dried paraffin sections were heated for 2 h at 60 °C or 30 min at 70 °C to
remove excess paraffin. Finally paraffin slides were rehydrated using graded ethanol (2x2 min 100 % EtOH; 20 dips in each 95 % EtOH, 95 % EtOH, 70 % EtOH, 50 % EtOH, H$_2$Odd, H$_2$Odd). Subsequently, the sections treated with 3 % Tris-buffered hydrogen peroxide for 15 min to block endogenous peroxide activity. Endogenous avidin- and biotin-activity was masked using a commercially available blocking kit (Eubio Cat. No. SP-2001). Slides were blocked with 5 % Tris-buffered BSA and incubated overnight with an antibody recognising mouse/human PPARgamma (sc-7273, dilution 1:100, Santa Cruz Biotech.) or murine GR (sc-1004; dilution 1:1000, Santa Cruz Biotech.). The slides were rinsed three times using 1x TBST and incubated with a polyvalent biotinylated antibody and streptavidin-HRP each for 10 min (both ID labs Cat. No. IDST1007). Specific signals were detected using a AEC-substrate detection kit (IDLabs Cat. No. IDST1007). After appropriate staining intensity had developed the reaction was stopped by immersion in H$_2$Odd and mounted on Superfrost glass slides using aqueous Aquatex mounting medium (Lactan Cat. No. 1085620050).

5.3.6. Electron microscopy

For electron microscopy, mouse livers were cut into 1-2 mm thin pieces and fixed overnight in 1.6 % glutaraldehyde. Images were recorded using various magnifications (1,500 x – 4,000 x) using a standard transmission electron microscope (TEM).

5.4. Cell biology

5.4.1. Cell lines

Cultivation of cell lines was carried out in incubators maintaining a temperature of 37 °C, humidity of 95 % and 5 % CO$_2$. Cells were passaged and handled under sterile conditions.

5.4.2. Handling of cell lines and transient transfection

The following cell lines were routinely used for in vitro transfection experiments:

**HEK 293 cells**

Human embryonic kidney cells (HEK 293 cells) were cultivated and propagated in Dulbecco’s Modified Eagle Medium with high glucose (DMEM; Invitrogen Cat. No. 41965-062); 10 % fetal calf serum (FCS; PAA Cat. No. 41965-062), 1 mM L-Glutamine and 1 % penicillin / streptomycin. Transient transfection were carried using a modified version of the Sambrook Ca$_3$(PO$_4$)$_2$ method: Briefly, HEK 293 cells were seeded out at a final concentration of 1x10$^5$ cells/ml medium in 100 mm Petri dishes and were allowed to adhere to the dish for 2-6 h. 10 µg of total plasmid DNA were diluted in 450 µl sterile water. Subsequently, 50 µl of 2.5M CaCl$_2$ solution were added, followed by drop wise addition of 500 µl 2x HBS buffer under constant vortexing. The solutions were incubated for 1 min until precipitation of calcium phosphate crystals was observed by a foggy appearance of the reaction mixture,
which was given onto the adherent cells in a swirling manner. Proper precipitation of crystals was confirmed by microscopy. After 18 h, the medium was replaced and transfected cells from one 100 mm Petri dish were distributed onto 2x6well dishes to allow for multiple cytokine stimulation conditions. Cells were allowed to adhere overnight and were stimulated with various cytokines. Subsequently, culture dishes were rinsed two times with ice-cold PBS, scraped off using a sterile blade and pelleted by centrifugation for 1 min at 6,000 rpm. Finally, cells were taken up in WCE buffer for EMSA analysis.

2 x HBS buffer:
50 mM Hepes pH 7.05
10 mM KCl
280 mM NaCl
1.5 mM Na$_2$PO$_4$

$\Rightarrow$ dissolve in 950 ml H$_2$O$_{dd}$ and adjust pH to 7.1 with 1 N HCl. Fill up with H$_2$O to 1 L and sterilize using 0.22 $\mu$m filters. Aliquot in 50 ml reaction tubes and store at -20 $^\circ$C.

MIMp16/19ARF-/-Ras and MIMp16/19ARF-/- cells

MIMp16/19ARF-/- and MIMp16/19ARF-/-Ras cells are primary hepatocytes isolated from mice deleted for p16/19ARF proteins and exhibited sustained proliferative capabilities in cell culture. Both cell lines were maintained and extended in RPMI medium (Sigma Cat. No. R0883-6x500ml), supplemented with 10 % FCS, 1 mM L-Glutamine and 1 % penicillin / streptomycin in Petri dishes coated with rat collagen (BD Cat. No. 354236). Moreover, MIMp16/19ARF-/- were dependent on growth factor stimulation with 80 ng/ml TGFalpha (Sigma Cat. No. # T-7924), 60 ng/ml IGF-II (Sigma Cat. No # I-2526) and 2.8 $\mu$M insulin (Novo Nordisk). MIMp16/19ARF-/-Ras were stably transfected with the Ras proto-oncogene to allow for factor-independent growth. Both MIMp16/19/- cell lines were transiently transfected with the Lipofectamine 2000® reagent (Invitrogen Cat. No. 11668-027) using a modified version of the vendor’s instructions: Briefly, cells were seeded out a final concentration of 1x10$^5$ cells/ml medium one day prior to transfection. Before transfection the medium was removed and replaced with RPMI containing only 1 mM L-Glutamine (RPMI+). The next morning, 8 $\mu$g of total plasmid DNA were mixed with 750 $\mu$l of RPMI+ and 40 $\mu$l Plus® reagent (Mix I). Simultaneously, 60 $\mu$l of Lipofectamine 2000® reagent was diluted in 800 $\mu$l RPMI+ and incubated for 15 min (Mix II). Subsequently, Mix I and Mix II were pooled, mixed gently and incubated at room temperature (RT) for 15 min. The pooled reaction mixture was added to the cells under constant agitation. After 3 h, RPMI+ was removed and replaced with RPMI containing FCS, L-Glutamine and penicillin / streptomycin. After 18 h, the medium was replaced and transfected cells from one 100 mm Petri dish were distributed onto 2x6well dishes to allow for multiple cytokine stimulation conditions. Cells were allowed to adhere overnight and were stimulated with various cytokines after 48 h. Subsequently, culture dishes were rinsed two times with ice-cold PBS, scraped off using a sterile blade and pelleted by centrifugation for 1 min at 6,000 rpm. Finally, cells were taken up in potassium phosphate buffer for reporter assay analysis.

COS7 cells
COS7 are epithelial African green monkey kidney cells immortalized using an origin-defective mutant of SV40. COS7 are widely used in reporter assay analyses due to their rather basal transcription machinery. COS7 cells were maintained and propagated in DMEM (high glucose) supplemented with FCS, L-Glutamine and penicillin/streptomycin. For transient transfection experiments, one fully growth T75 cell culture flask was trypsinised and detached cells allocated onto five 100 mm Petri dishes one day prior to transfection. At the day of transfection, 544 µl of DMEM was pipetted into a 1.5 ml reaction tube and mixed with 48 µl of Fugene reagent (Roche Cat. No. 11814443001), flicked once and allowed to incubate for 5 min. Subsequently, 8 µg of total plasmid DNA was added to the reaction mixture, flicked and incubated for 5 min. The transfected reagent:DNA complex was given onto the cells in a swirling and drop wise manner. After 18 h, cells from one transfected 100 mm Petri dish were distributed onto one 24 well dish to allow for multiple cytokine stimulations. COS7 cells were allowed to adhere overnight and were stimulated with various cytokines after 48 h. Subsequently, culture dishes were rinsed two times with ice-cold PBS, scraped off using a sterile blade and pelleted by centrifugation for 1 min at 6,000 rpm. Finally, cells were taken up in KH₂PO₄ lysis buffer for reporter assay analysis.

5.4.3. Luciferase reporter assay

Luciferase reporter assays are used to quantify the ability of a given promoter sequence to drive the transcription of the firefly Luciferase gene. Luciferase itself is an enzyme catalysing the reaction a reaction in which Luciferin is transformed into its decarboxylated isoform leading to emission of photons. As under standard conditions the amount of luciferin is supplied in excess, the amount of emitted light is proportional to the amount of Luciferase and constitutes an indirect measurement for the transactivation capability of a cloned promoter sequence. To determine the Luciferase activity in transfected cell lines, the pelleted cells were taken up in 50-100 µl of potassium chloride lysis buffer. The pellets were pipetted up and down repeatedly and put on a horizontal shaker for 30 min to facilitate lysis of cells. Insoluble debris was removed by centrifugation for 15 min at 12,000 rpm in a cooled micro centrifuge. The supernatant was shock-frozen and stored at -80°C or used instantly. For measuring Luciferase activity, 20 µl of supernatant was transferred to a opaque 96 well microtiter plate and diluted with 50 µl of assay buffer (4 mM ATP, 200 mM MgSO₄ in Gly-Gly buffer). The plate was transferred into a luminometer equipped with a dispenser, automatically adding 50 µl of injection buffer (0.25 mM luciferin in Gly-Gly buffer) into each well. The subsequent light emission was detected at 560 nm and counted for 2 s.

The efficiency of each transfection was quantified via co-transfection of 0.5 µg plasmid vector DNA constitutively expressing beta Galactosidase. To assess the enzymatic Galactosidase activity as an indicator for efficiency of DNA transfection into cells, the complete Luciferase reaction mixture (120 µl) was transferred onto a fresh, translucent microtiter plate and diluted with 50 µl of assay buffer (4 mM ATP, 200 mM MgSO₄ in Gly-Gly buffer). The plate was transferred into a luminometer equipped with a dispenser, automatically adding 50 µl of injection buffer (0.25 mM luciferin in Gly-Gly buffer) into each well. The subsequent light emission was detected at 405 nm and counted for 2 s.

For relative quantification of the transactivitation activity of a given promoter elements...
each sample the Luciferase values were measured and normalised against the beta Galactosidase values as an indicator of transfection efficiency.

\[
\text{KH}_2\text{PO}_4 \text{ lysis buffer:}
\]
\[
13.6 \text{ g} \quad \text{KH}_2\text{PO}_4
0.1 \% \quad \text{Triton X-100}
\rightarrow \text{fill up with } \text{H}_2\text{O}_\text{dd} \text{ to } 1 \text{ L, adjust pH to 7.8 using NaOH}
\]

\[
\text{CPRG solution:}
\]
\[
27.1 \text{ mg} \quad \text{CPRG}
0.5 \% \quad \text{BSA}
\rightarrow \text{fill up with } 20 \text{ ml PBS, aliquot and store at -20 °C}
\]

5.5. Colony breeding and management

Mice were kept at the Decentralized Biomedical Facilities, Medical University of Vienna (MUW) under standardized conditions. All animal experiments were carried out according to an ethical animal license protocol and contract approved by the MUW and the Austrian Ministry BMBWK authorities. Mice are maintained on a 12 h light-dark cycle.

5.5.1. Transgenic mouse lines

(i) Mice harbouring a hepatocyte-specific deletion of the glucocorticoid receptor (GR\text{\textsuperscript{AlfpCre}}) were generated by intercrossing with animals with a floxed third exon of the GR\textsuperscript{211} with mutants carrying the Cre recombinase under the control of the albumin promoter and alpha-fetoprotein enhancer sequences\textsuperscript{158}. Animals deleted for hepatic Stat5 were generated by crossing mice harbouring the AlfpCre recombinase with mutants carrying a floxed Stat5a/b allele (Stat5\textsuperscript{AlfpCre})\textsuperscript{119}. Stat5/GR\textsuperscript{AlfpCre} compound knockout animals were generated by respective intercrossing. Stat5\textsuperscript{ΔN} mice were generated as described\textsuperscript{127}. All animals were maintained on a mixed C57/BL6 and 129SvEv background and genotyped as indicated.

(ii) Mice deleted for MDR2 (MDR2\textsuperscript{-}) were purchased from Jackson Laboratories. MDR2\textsuperscript{-} mice were initially maintained on a FVB/N background. Due to appropriate intercrossing MDR2\textsuperscript{+/-}, MDR2\textsuperscript{-}, Stat5\textsuperscript{AlfpCre} and MDR2/Stat5\textsuperscript{AlfpCre} animals were generated and experimental mice were regarded to be on a mixed background.

5.5.2. Metabolic function tests

5.5.2.1. Glucose tolerance test

To assay the capability of transgenic mice to metabolize orally administered glucose, we performed oral glucose tolerance tests (OGTT). For this, mice were fasted overnight in a fresh cage prior to glucose gavage to obtain comparable basal glucose levels. The next morning, the body weight was determined and basal glucose levels analysed. For this, the tail vein was nicked and one droplet venous blood measured...
using a glucometer (OneTouch Ultra, LifeScan). Glucose was orally administered at a concentration of 10 µl/g body weight using a gavage syringe. The measurement of glucose levels was done by sampling venous tail blood at indicated time points. Immediately after the experiment, mice were transferred into a fresh cage and provided with food ad libitum.

5.5.2.2. Insulin tolerance test

Insulin tolerance tests were performed with animals initially fasted for 4 h in a fresh cage. Subsequently, the body weight was determined and insulin (Novo Nordisk) injected peritoneally at a final concentration of 0.75 U/kg body weight. Blood samples were taken from the tail vein at indicated time points and glucose levels determined using a glucometer (OneTouch Ultra, LifeScan). Plasma insulin concentrations were analysed using a rat insulin ELISA (Crystal Chem Cat. No. INSKR020) following vendor's instruction manual.

5.5.3. Blood sampling and serum parameter quantification

To determine serum concentration of cytokines and growth factors or biochemical parameters, blood was harvested from mice using heart puncture and collected in tubes precoated with EDTA. Blood samples were subsequently centrifuged at 5,000 rpm for 5 in at RT to remove insoluble, coagulated blood components. The plasma (supernatant) was transferred into a new tube and analysed instantly using various techniques or shock frozen in liquid N₂ and stored at -80 °C.

Concentrations of important biochemical and metabolic serum parameters were determined using a Reflotron Plus analyszer (Roche). Briefly, 30 µl of serum or plasma were pipetted onto an analyte-specific strip and inserted into the machine. Final concentration of were calculated taken possible pre-dilutions into account.

5.5.3.1. Lipoprotein profile

Plasma samples of five fed mice of each genotype were pooled and compared with five control littermates. Lipoproteins were isolated by FPLC. 200 µl pooled plasma samples were subjected to FPLC analysis and lipoproteins were eluted with 10 mM Tris-HCl, 1 mM EDTA, 0.9 % NaCl, and 0.02 % NaN₃ (pH 7.4). Fractions of 0.5 ml each were collected and lipids (triglycerides and total cholesterol) assayed enzymatically using commercially available kits. To enhance sensitivity, reaction buffers were supplemented by the addition of sodium 3, 5-dichloro-2-hydroxy-benzenesulfonate.

5.5.4. Immuno assays

Immuno assays (particularly enzyme linked immuno sorbent assays (ELISA) and radio immunoassay (RIA)) detect the concentration of an antigen in serum samples with high reproducibility and accuracy. Briefly, an antigen-specific antibody was immobilised and scavenges the desired antigen. Finally, the amount of trapped
antigen was converted to a signal using radiolabel led secondary antibodies (RIA) or a chromogenic enzyme activity coupled to the secondary antibody (ELISA).

5.5.4.1. Radio Immunoassay (RIA)

Serum corticosterone (MP Biochemicals Cat. No. 07-120102) and ACTH (MP Biochemicals Cat. No. 07-106102) levels were determined using commercially available RIA following instructions from the vendor. Briefly, a standard curve was prepared and subsequently the displacement of Corticosterone/ACTH-specific antibodies bound to radiolabel led Corticosterone/ACTH (Cort+ /ACTH+) by serum Corticosterone/ACTH quantified using a gamma counter (Beckmann). Thus, extensive displacement of Corticosterone/ACTH antibodies from Cort+ /ACTH+ due to high levels of serum Corticosterone/ACTH led to a low radioactivity. The exact concentrations of serum Corticosterone/ACTH was deduced using the previously prepared standard curve.

5.5.4.2. Enzyme Linked Immuno Sorbent Assay (ELISA)

Plasma GH (Millipore Cat. No. EZRMGH-45K), IGF-1 (IBL Hamburg Cat. No. UK45021), Leptin, TNFalpha and Resistin concentrations (all Millipore Cat. No. MADPK-71K-04) were determined using ELISA kits. Briefly, plastic dishes precoated with the respective growth factor/cytokine specific antibody were incubated with serum samples according to manufacturer’s instructions. A secondary antibody coupled to an enzymatic function was added. Finally, the development of the chromogenic reaction was stopped and the concentration of the respective analyte in the serum determined measuring the OD at 450 nm using a spectrophotometer. The exact concentrations were calculated using an analyte standard curve.

5.5.5. Colorimetric assays

5.5.5.1. Quantification of free fatty acids (FFA)

The concentration of serum FFA was determined using a colorimetric assay system (Wako Cat. No. 999-65406). Briefly, the assay relied on the catalytic oxidation of free carbonic acids such as FFA (R-COOH) by (i) Acyl-CoA synthase, (ii) Acyl-CoA oxidase and (iii) peroxidase:

(i) \[ \text{R-COOH + ATP + CoA-SH} \rightarrow \text{Acyl-CoA + AMP + PP_i} \]
(ii) \[ \text{Acyl-CoA + O_2} \rightarrow \text{2,3-Enoyl-CoA H_2O}_2 \]
(iii) \[ \text{H_2O}_2 + 4 \text{H}_2\text{N-phenanzon + 3-CH}_3\text{-N-Ethyl-N (beta-hydroxyethyl)-aniline} \rightarrow \text{quinoneimine (color) + 4 H}_2\text{O} \]

A standard curve of oleic acid was prepared and 4 µl serum sample used for quantification. The experiment was carried out as given in the vendor’s instructions. The OD was measured at 550 nm and the final concentration of FFA calculated taken possible pre-dilutions into account.
5.5.5.2. Isolation and quantification of hepatic triglycerides

Hepatic lipids were isolated from frozen liver tissues using the original method of Stanley and Folch. For this, approximately 100 mg were weighted and transferred into a 15 ml reaction tube pre-equipped with 2 ml of a chloroform/methanol (2:1 v/v) mixture. The tissue was homogenized until no tissues clumps were visible, followed by incubation for 20-25 min on a rotating wheel at RT. Cell debris and insoluble fragments were removed by centrifugation at 4,000 rpm for 10 min. The resulting supernatant was transferred into a fresh reaction tube and the organic phase extracted two times 0.9 % NaCl solution, followed by careful aspiration of the aqueous phase. Then, 50 µl of the lipophillic phase were incubated with 10 µl of a TX-100/chloroform (1:1 v/v) mixture in a fresh reaction tube. Vigorous shaking was followed by evaporation of the solvent in a Speed vac. The remaining pellet, containing the organic phase of the liver was taken up in 50 µl of H₂Oₐ₉ and stored at -20 °C.

To determine the concentration of triglycerides in hepatic tissues, this colorimetric assay (Sigma Cat. No. TR0100) relied on the enzymatic quantification of glycerol liberated from cleaved triglycerides (tricarboxylic acid esters of glycerol) since the stochiometric ratio between triglycerides and glycerol equals one. Four µl of isolated triglycerides from liver tissues were pipetted onto a 96 well microtiter plate. Glycerol at a concentration of 2.5 mg/ml (Sigma Cat. No. G7793) was included as positive control whereas blank values include water. 100 µl Free Glycerol Reagent is pipetted into each well and incubated for 5 min at 37 °C. The amount of glycerol not incorporated into triglycerides was determined by spectrophotometry measured at 540 nm. A second 96 well plate containing the same samples was pipetted and 100 µl of Triglyceride Reagent added. The plate was incubated for 5 min at 37 °C and the OD at 540 nm measured again. This time, the OD 540 nm was proportional to the total amount of glycerol (free glycerol and glycerol incorporated in triglycerides) in the respective sample. Subtraction of the two values and normalization blank values yielded the concentration of triglycerides in the respective sample.
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7. **Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>µ</td>
<td>micro ((10^{-6}))</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AF</td>
<td>activating function</td>
</tr>
<tr>
<td>AF-1</td>
<td>Activation function 1</td>
</tr>
<tr>
<td>AflpCre</td>
<td>Albumin alpha fetoprotein Cre</td>
</tr>
<tr>
<td>ALS</td>
<td>acid-labile subunit</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BDL</td>
<td>bile-duct ligation</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSEP</td>
<td>bile salt export pump</td>
</tr>
<tr>
<td>CAB</td>
<td>chromotrope anilinblue</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCD</td>
<td>coiled-coil domain</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>COL</td>
<td>collagen</td>
</tr>
<tr>
<td>CPRG</td>
<td>chlorophenolred-beta-D-galactopyranoside</td>
</tr>
<tr>
<td>Cre</td>
<td>cyclization recombination (Cre) recombinase</td>
</tr>
<tr>
<td>CbP</td>
<td>CREB binding protein</td>
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<tr>
<td>CYP</td>
<td>cytochrome p450</td>
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<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DTT</td>
<td>dithioretil</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immuno sorbent assay</td>
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<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
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<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin 1</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acids</td>
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<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
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<td>FAK</td>
<td>focal adhesion kinase</td>
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<td>fatty acid synthases</td>
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<td>free fatty acid</td>
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<td>G6P</td>
<td>glucose-6-phosphatase</td>
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<td>glyceraldehyde-3-phosphate-dehydrogenase</td>
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<td>GC</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>growth hormone</td>
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<td>GRE</td>
<td>GR responsive element</td>
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<td>HAT</td>
<td>histone acetylase</td>
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PDGFb: platelet derived growth factor beta
PDGFbR: platelet derived growth factor beta receptor
PEPCK: phosphoenol pyruvat carboxykinase
pH: pH value
PI3K: phosphatidylinositol-3-kinase
PIP$_3$: phosphatidyl-inositol 3, 4, 5 trisphosphate
PK: pyruvate kinase
PL: pancreatic lipase
PPAR: peroxisome proliferator-activated receptor
PSC: primary sclerosing cholangitis
RE: responsive element
RIA: radioimmunoassay
RNA: ribonucleic acid
ROS: reactive oxygen species
rpm: rotations per minute
RT: room temperature
RXR: retinoic acid receptor
SDS: sodiumdodecylsulfate
SH2: src homology domain
SMA: smooth muscle actin
SOCS: Suppressor of cytokine signaling
SREBP: sterol regulatory element binding protein
Stat: signal transducer and activator of transcription
ss: supershift
T2D: type II diabetes
TAD: transactivation domain
TAE: tris acetate EDTA buffer
TBE: tris borate EDTA buffer
TE: tris ETDA
TG: triglycerides
TGFb: tumor growth factor beta
TGFbR: tumor growth factor beta receptor
TIMP: tissue inhibitor of matrix metalloprotease
U: enzymatic unit
UV: ultraviolet
V: volt
VLDL: very-low density lipoprotein
8. Bibliography

Overexpression of TACE and TIMP3 in squamous cell carcinomas of the head and neck: Association with tumor development and progression (in preparation)

Expression of activated STAT5 in neoplastic mast cells in systemic mastocytosis: subcellular distribution and role of the transforming oncoprotein KIT D816V (in preparation)

Loss of transcription factors Stat5 and GR in hepatocytes leads to severe steatosis and peripheral lipodystrophy Cell Metab (submitted)

Engblom D*, Kornfeld JW*, Schwake L, Tronche F, Reimann A, Beug H Hennighausen L, Moriggl R# and Schütz G#


Riethdorf S, Reimers N, Assmann V, Kornfeld JW, Terracciano L, Sauter G and Pantel K
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### Academic degrees
Curriculum Vitae

July 2003

Biochemistry (Diploma)
University Hannover, Germany

Research institutions

2006-
Graduate Studies
Ludwig Boltzmann Institute for Cancer Research (LBI-CR)
Supervisor: Prof. Richard Moriggl, PhD
Project: The Role of Signal Transducer and Activator of transcription (Stat5) and the glucocorticoid receptor (GR) in hepatocyte function

2003-
Fulltime Research Associate
Department of Tumor Biology, Hamburg, Germany
Supervisor: Sabine Riethdorf, PhD
Project: The Role of Tumor Necrosis Factor alpha Cleaving Enzyme (TACE) in squamous cell carcinoma of the Head and Neck

2003-
Diploma studies (masters equivalent)
Hannover Medical School (MHH)
Supervisor: Prof. Reinhard Schwinzer, PhD
Project: Glycosylation and compartmentalisation of variantly expressed isoforms of the protein tyrosine phosphatase CD45RA

Further research experience

2003-
Graduate Study Program in Molecular Biology
Center for molecular neurobiology (ZMNH), Hamburg
Supervisor: Prof. Melitta Schachner, PhD

October 2000-
Scientific Internship at the Siberian Institute of Plant Physiology and Biochemistry (SIFIBR), Irkutsk, Russia
Supervisor: Prof. Natalya Igorevna, PhD

June 2001-
Scientific Internship at the University of Montreal

October 2001
School of Medicine (CHUM), Montréal, Canada
Supervisor: Prof. Yves Raymond, PhD

Invited talks

Symposium of the Medical University of Vienna (MUW), Vienna, Austria, 2007
Curriculum Vitae

Laboratoire d’Immunologie, CHU d’Amiens, Amiens, France, 2007

Ludwig Boltzmann Institute for traumatology, Vienna, Austria, 2007

Institute for zoology and developmental biology, Kiel, Germany, 2007

Internal Seminars of the special research council (SFB), Vienna, Austria 2006-2007

Internal Seminars of the department of tumor biology (SFB), 2003-2006

Poster presentations

Hepatocyte-Specific deletion of Stat5 and GR leads to severe fatty liver disease and liver cancer
- International conference: “Invasion and Metastasis”, Berlin, Germany, 2008

Hepatocyte-Specific Deletion of Stat5 and GR leads to severe fatty liver disease and liver cancer
- Recess of the Medical university of Vienna (MUW), Vienna, Austria, 2007

Scientific publications

STAT3 PROTECTS FROM BILE ACID-INDUCED LIVER INJURY AND CIRRHOSIS Nature Med (submitted)

Overexpression of TACE and TIMP3 in squamous cell carcinomas of the headand neck: Association with tumor development and progression (in preparation)

Expression of activated STAT5 in neoplastic mast cells in systemic mastocytosis: subcellular distribution and role of the transforming oncoprotein KIT D816V (in preparation)


131
Loss of transcription factors Stat5 and GR in hepatocytes leads to severe steatosis and peripheral lipodystrophy (in preparation)

The different functions of Stat5 and chromatin alteration through Stat5 proteins *Front Biosci* 1;13:6237-54 (2008)


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## References

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