The Role of STAT5 and GR Signaling in Non-alcoholic Fatty Liver Disease and Hepatocellular Carcinoma

Doctoral thesis at the Medical University of Vienna for obtaining the academic degree

Doctor of Philosophy

Submitted by

Dipl. Ing. (FH) Kristina M. Mueller

Supervisor:

Prof. Dr. DI Richard Moriggl

Ludwig Boltzmann Institute for Cancer Research

Waehringerstraße 13a
1090 Vienna

Vienna, 05/2014
Declaration

The majority of experiments presented in this thesis were performed at the

Ludwig Boltzmann Institute for Cancer Research (LBI-CR)
Waehringerstraße 13a, 1090 Vienna, Austria

I hereby declare that the publication “Impairment of Hepatic Growth Hormone and Glucocorticoid Receptor Signaling Causes Steatosis and Hepatocellular Carcinoma in Mice” (Mueller et al., Hepatology 2011) which is the experimental basis of this thesis, is my original work but includes as well data that originates from collaborative research.

JW Kornfeld (Max Planck Institute for Neurological Research, University of Cologne, Cologne, Germany) and I designed and performed the majority of experiments/data analyses presented in Figures 1-3 and Supporting Figures 1-2.
I designed and carried out the majority of experiments/data analyses presented in Figures 4-5 and Supporting Figures 3-6.
I performed data interpretation and wrote the manuscript under the supervision of JW Kornfeld and R Moriggl.

Provision of essential technical support, data analyses and interpretation:
G Haemmerle (Institute of Molecular Biosciences, University of Graz, Graz, Austria) and D Kratky (Institute of Molecular Biology and Biochemistry, Center of Molecular Medicine, Medical University of Graz, Graz, Austria) performed the determination of hepatic triglyceride content.
S Haindl and A Kozlov (Ludwig-Boltzmann-Institute for Experimental and Clinical Traumatology, Vienna, Austria) carried out the isolation of liver mitochondria and subsequent measurement of ROS.
G Egger (Clinical Institute of Pathology, Medical University of Vienna, Vienna, Austria) helped me to design ChIP strategies and performed subsequent ChIP analyses.
H Esterbauer (Department of Laboratory Medicine, Medical University Vienna, Vienna, Austria) and A Pospisilik (Max-Planck-Institute of Immunobiology, Freiburg, Germany) performed insulin and glucose tolerance tests and provided help for GSEA and MAPPFinder analyses.
L Kenner (Ludwig-Boltzmann-Institute for Cancer Research, Vienna, Austria) performed pathologic analysis/tumor grading of liver tumors.
MH Heim (Department of Biomedicine, Division of Gastroenterology and Hepatology, University Hospital Basel, Basel, Switzerland) and L Terracciano (Institute of Pathology, University Hospital Basel, Basel, Switzerland) conducted pathologic analyses of non-tumor bearing livers.

The review article Mueller et al., Molecular and Cellular Endocrinology, 2012 which is included in the Introduction (section 1.4) was mainly prepared and written by me under the supervision of R Moriggl. The manuscript was thereafter critically revised by the co-authors.

In addition, I declare the following contributions to the second publication Friedbichler et al., Hepatology, 2012 (1) and unpublished projects (2):
(1) I was involved in designing and performing experiments (Figure 5B, C, D and E, Supporting Figures 1, 2A, B, E and 5D) as well as in the analyses and interpretation of data. Further, I supervised M Themanns during the revision of this manuscript and critically revised the manuscript prior to submission and resubmission.
(2) I designed and performed the experiments, data analyses and interpretation presented for adipose tissue-specific Gr knockout mice. The insulin and glucose tolerance tests were performed at the LBI-CR under the guidance of H Esterbauer and S Amann (Department of Laboratory Medicine, Medical University Vienna, Vienna, Austria). I performed the insulin and glucose tolerance tests with assistance of D Kaltenecker, designed the experiments and provided assistance for data analyses as well as interpretation presented for adipose tissue-specific Stat5 knockout mice during my supervision of the master thesis “STAT5 signalling in adipose tissue function” of D Kaltenecker.

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All projects were supported by the research grant SFB-F28 from the Austrian Science Fund (FWF).
“The truth is more magical - in the best and most exciting sense of the word - than any myth or made-up mystery or miracle. Science has its own magic: the magic of reality.”

- Richard Dawkins
Table of Contents

Declaration........................................................................................................................................ii
Table of Contents .............................................................................................................................v
List of Figures ...................................................................................................................................viii
List of Tables ......................................................................................................................................x
Abstract...........................................................................................................................................xi
Zusammenfassung...............................................................................................................................xii
Publications arising from and during this thesis ...........................................................................xiv
Abbreviations ....................................................................................................................................xvi
Acknowledgments ............................................................................................................................xix

CHAPTER ONE: INTRODUCTION ...............................................................................................1
  1.1 Liver Metabolism: Physiology and Pathology ..........................................................................1
    1.1.1 Hepatic Lipid Metabolism .................................................................................................2
    1.1.2 Hepatic Steatosis and Insulin Resistance ..........................................................................6
    1.1.3 Non-Alcoholic Fatty Liver Disease ....................................................................................10
    1.1.4 Hepatocellular Carcinoma ...............................................................................................10
  1.2 The JAK-STAT Pathway .........................................................................................................13
    1.2.1 Growth Hormone and STAT5 ..........................................................................................15
  1.3 Glucocorticoids and the Glucocorticoid Receptor ..................................................................17
  1.4 Review: Hepatic Growth Hormone and Glucocorticoid Receptor Signaling in Body Growth, Steatosis and Metabolic Liver Cancer Development. ...........................................19
  1.5 Aims of this Thesis ...............................................................................................................30

CHAPTER TWO: RESULTS ............................................................................................................31
  2.1 Prologue ..................................................................................................................................31
    2.1.1 Manuscript: Impairment of Hepatic Growth Hormone and Glucocorticoid Receptor Signaling Causes Steatosis and Hepatocellular Carcinoma in Mice ..............................32
    2.1.2 Supporting Information ..................................................................................................45
  2.2 Interlude ...................................................................................................................................55
    2.2.1 Manuscript: Growth-Hormone-induced Signal Transducer and Activator of Transcription 5 Signaling Causes Gigantism, Inflammation, and Premature Death but Protects Mice from Aggressive Liver Cancer .................................................................56
    2.3.2 Supporting Information ..................................................................................................69
  2.3 Interlude ...................................................................................................................................78
    2.3.1 Adipose Tissue-Specific Deficiency of GR or STAT5 Signaling Causes Diminished Lipid Mobilization and Improves Insulin Sensitivity in Mice ..............................................79

CHAPTER THREE: DISCUSSION .....................................................................................................86
3.1 Disruption of Hepatic GH-STAT5 Signaling Causes a Lipogenic Gene Expression Profile and Steatosis Independently of Functional GR Signal Transduction ........................................86
3.2 Effects of Hepatic STAT5 and GR Deficiency on Adipose Tissue: Combined hepatic GH Resistance and Hypercortisolism Stimulates Adipose Tissue Lipid Mobilisation and Hepatic Fatty Acid Influx........................................................................................................90
3.3 Effects of Hepatic STAT5 and GR Deficiency on Glucose Homeostasis ............93
3.4 Disruption of Hepatic GH-STAT5 Signaling Sensitizes the Liver to Metabolic Toxicity and Facilitates the Development and Progression of HCC .........................................................95
3.5 Conclusion....................................................................................................................99

4. CHAPTER FOUR: MATERIALS & METHODS ...............................................................100

4.1 Materials .................................................................................................................. 100
4.2 Transgenic Mouse Lines ...........................................................................................104
   4.2.1 Mouse Breeding and Genotyping ........................................................................105
   4.2.2 Oral Glucose Tolerance Test ...............................................................................105
   4.2.3 Intraperitoneal Insulin Tolerance Test ................................................................106
   4.2.4 Mouse Tissue and Blood Collection ....................................................................106
   4.2.5 Growth Hormone, Interleukin-6 and Interferon-γ Administration .......................106
   4.2.6 Dexamethasone and RU486 Treatment ..............................................................107
4.3 Plasma Biochemistry..................................................................................................107
   4.3.1 Liver Damage Parameter and Plasma Metabolites .............................................107
   4.3.2 Radio Immunoassay and Enzyme-Linked Immunosorbent Assay ......................107
   4.3.3 Quantification of Non-Esterified Fatty Acids .......................................................108
4.4 Isolation and Quantification of Hepatic Triglycerides..................................................108
4.5 Histology and Immunohistochemistry.........................................................................108
   4.5.1 Haematoxylin and Eosin Staining........................................................................108
   4.5.2 Oil Red O Staining ............................................................................................109
   4.5.3 Periodic Acid Schiff Staining ............................................................................109
   4.5.4 Trichrome Chromotrope Anilin Blue Staining .....................................................109
   4.5.5 Immunohistochemistry.......................................................................................109
   4.5.6 Quantification of Cell Size and IHC Positive Cells.............................................110
4.6 Electron Microscopy ...................................................................................................110
4.7 RNA Extraction and Quantitative mRNA Expression Analysis ..................................................110
4.8 Protein Biochemistry..................................................................................................111
   4.8.1 Protein Extraction ............................................................................................111
   4.8.2 SDS-PAGE and Immunoblotting .......................................................................111
   4.8.3 Immunoprecipitation .......................................................................................112
   4.8.4 Quantification of Western Blot Signal Intensity ..................................................112
List of Figures

CHAPTER ONE: INTRODUCTION

1.1 Liver Metabolism: Physiology and Pathology
Figure 1. Fatty acid shuttling and hepatic lipid metabolism......................................................3
Figure 2. Fatty acid metabolism, insulin resistance and hepatic steatosis.................................8
Figure 3. Development of HCC in NAFLD..............................................................................12

1.4 Review: Hepatic Growth Hormone and Glucocorticoid Receptor Signaling in Body
Growth, Steatosis and Metabolic Liver Cancer Development
Figure 1. Signal transduction pathways induced by GH and GCs. ........................................21
Figure 2. STAT5 and GR protein-protein interaction in postnatal body growth......................22
Figure 3. Schematic illustrations demonstrating the development of liver phenotypes follow-
ing combined hepatic STAT5/GR deletion and STAT5 deletion in GH transgenic mice. ....25

CHAPTER TWO: Results

2.1.1 Manuscript: Impairment of Hepatic Growth Hormone and Glucocorticoid Receptor
Signaling Causes Steatosis and Hepatocellular Carcinoma in Mice.
Figure 1. DKO mice develop severe steatosis, hepatomegaly and lipodystrophy. ...............36
Figure 2. The combination of hepatic GH resistance and hypercortisolism causes peripheral
lipodystrophy in DKO animals. ...............................................................................................37
Figure 3. Impact of GR agonist or antagonist treatment on WAT lipolysis. .........................38
Figure 4. Spontaneous development of liver tumors in DKO mice........................................38
Figure 5. Oxidative stress dependent hepatocyte damage and tumor-promoting signaling in
DKO livers. .............................................................................................................................41
Supporting Figure 1. Deletion of STAT5 and GR in hepatocytes and histological scoring of
steatosis. ................................................................................................................................45
Supporting Figure 2. Hepatic deletion of STAT5 using Alfp-Cre results in insulin resistance
and impaired hepatic insulin receptor signaling. ....................................................................46
Supporting Figure 3. Induction of PPARγ/SREBP-1 mediated lipogenesis upon hepatic
STAT5 deficiency. ..................................................................................................................47
Supporting Figure 4. Tumorigenesis in DKO mice. .................................................................48
Supporting Figure 5. Relative mRNA expression levels of Serpina6 and 11βHsd1 in livers
from 2-month-old mice. .........................................................................................................49
Supporting Figure 6. Compensatory STAT1 and STAT3 activation upon hepatic deletion of
STAT5 using Alfp-Cre. ..........................................................................................................50
2.2.1 Manuscript: Growth-Hormone-induced Signal Transducer and Activator of Transcription 5 Signaling Causes Gigantism, Inflammation, and Premature Death but Protects Mice from Aggressive Liver Cancer.

Figure 1. STAT5 is essential for postnatal body growth..........................................................60
Figure 2. High serum levels of GH reduce life expectancy......................................................61
Figure 3. Changes in body composition and metabolic parameters secondary to altered GH-STAT5 signaling..................................................................................................................62
Figure 4. Loss of hepatic STAT5 reverses GH-mediated changes in hepatocytes, but leads to increased fat deposition. ........................................................................................................63
Figure 5. Loss of hepatic STAT5 promotes HCC progression in GHtg mice........................65
Figure 6. Aberrant signaling accelerates HCC development in GHtgSTAT5Δhep mice. .........66
Supporting Figure 1. Long bone growth in mice depends on GH-JAK2-STAT5 signaling. ....69
Supporting Figure 2. Hepatic alterations resulting from GH overexpression. ......................70
Supporting Figure 3. Pathologic changes in GHtg hepatocytes are reversed by STAT5 deletion.......................................................................................................................72
Supporting Figure 4. Histologic analysis of livers.................................................................73
Supporting Figure 5. Loss of hepatic STAT5 in GHtg mice is associated with increased lipid synthesis and decreased expression of hepatoprotective factors........................................74
Supporting Figure 6. Schematic diagram illustrating the development of phenotypes following hepatic STAT5 deletion in GHtg mice. ....................................................................................75

2.3.1 Adipose Tissue-Specific Deficiency of GR or STAT5 Signaling Causes Diminished Lipid Mobilization and Improves Insulin Sensitivity in Mice

Figure 1. Adipocyte-specific GR- or STAT5-deficient mice display reduced fasting-induced lipid mobilization of white adipose tissues..........................................................80
Figure 2. Impairment of adipose tissue lipid mobilization upon adipocyte-specific GR or STAT5 deficiency is associated with different alterations in the lipolytic pathway. .............82
Figure 3. Increased insulin sensitivity upon adipocyte-specific GR or STAT5 deficiency. ......84
CHAPTER TWO: Results

2.1 Manuscript: Impairment of hepatic growth hormone and glucocorticoid receptor signaling causes steatosis and hepatocellular carcinoma in mice.

Supporting Table 1. Detailed histology analysis of mice at indicated time-points. ..................51
Supporting Table 2. Transcription factor signature analysis of genes statistically upregulated in Affymetrix expression profiling.................................................................54

2.2.1 Manuscript: Growth-Hormone-induced Signal Transducer and Activator of Transcription 5 Signaling Causes Gigantism, Inflammation, and Premature Death but Protects Mice from Aggressive Liver Cancer.

Supporting Table 1. Overview of phenotypic characteristics..............................77

4. CHAPTER FOUR: MATERIALS & METHODS

4.1 Materials

Table 1. Standard buffer.................................................................................................100
Table 2. Primer sequences ..........................................................................................101
Table 3. Antibodies.......................................................................................................102
Abstract

Lipid and carbohydrate metabolism are, amongst others, under the control of the neuroendocrine factors growth hormone (GH) and glucocorticoids (GC). A deregulation of either pathway has been associated with metabolic disorders including non-alcoholic fatty liver disease (NAFLD) in mouse models and humans. GH-activated signal transducer and activator of transcription (STAT) 5 and the glucocorticoid receptor (GR) were further shown to synergistically mediate the transcription of distinct GH target genes involved in hepatic sexual dimorphism. Here, we aimed to characterize STAT5’s functions in liver metabolism, NAFLD and liver cancer development using two different approaches: 1) Targeted deletion of Stat5, Gr or both transcription factors in murine liver to investigate STAT5’s function, particularly in context of an interaction with the GR. 2) Targeted deletion of hepatic Stat5 in a GH transgenic mouse model of inflammation-related hepatocellular carcinoma (HCC).

Liver-specific GR deficiency was not associated with apparent alterations in hepatic lipid metabolism despite pronounced systemic hypercortisolism. Hepatic STAT5 deficiency, however, resulted in an early onset but stable steatotic phenotype associated with hepatic GH resistance and decreased insulin sensitivity. Genes involved in de novo lipogenesis and fatty acid uptake were induced, likely due to aberrant expression of their upstream activators proliferator activated receptor gamma (PPARγ) and sterol regulatory element binding protein 1c (SREBP-1c). GH was further shown to down-regulate Srebf mRNA expression and to mediate STAT5 binding to responsive elements within Srebf promoter regions in vivo. The stable steatotic phenotype was severely aggravated upon co-deficiency with hepatic GR or upon GH overexpression. More than 50% of 12-month-old STAT5 and GR co-deficient mice presented spontaneous HCC, while STAT5 deficiency strongly accelerated HCC development and progression in the GH transgenic background (100% penetrance, 40 versus 52-60 weeks of age). The aggrevated phenotype of both mouse models could be linked to enhanced adipose tissue lipid mobilization in response to high circulating GC and/or GH level and a drastic increase in ectopic lipids in liver. The hereby induced metabolic toxicity led to hepatocellular and DNA damage associated with oncogenic c-Jun N-terminal kinase 1 (JNK1) and STAT3 activation, whereas tumor-suppressive p53 activity was diminished. In two follow-up studies, we could further demonstrate that genetic inactivation of GC-GR or GH-STAT5 signaling in mouse adipose tissue diminished the lipolytic response of white adipocytes, ameliorated fasting-induced hepatic lipid deposition and increased insulin sensitivity.

Thus, our data suggest a protective role of hepatic STAT5 signaling in NAFLD development and progression independently of GR function. Impairment of either transcription factor in white adipose tissue, vice versa, protects from ectopic hepatic lipid deposition.
Zusammenfassung


Eine Ko-Defizienz mit dem GR beziehungsweise die GH-Überexpression bewirkte eine drastische Verschärfung des steatotischen Phänotyps. Über 50% der 12 Monate alten STAT5 und GR Ko-defizienten Mäuse entwickelten spontan HCC, während die STAT5 Defizienz die Entstehung und Progression der GH-transgenen HCCs beschleunigte (100% Penetranz, 40 gegenüber 52-60 Wochen). Der Phänotyp beider Mausmodelle konnte auf eine erhöhte, durch GC- und/oder GH-induzierte Mobilisierung von Fettgewebslipiden und eine drastische Anreicherung von ektopen Lipiden in der Leber zurückgeführt werden. Die hierdurch induzierte metabolische Toxizität führte letztendlich zur Leber- und DNA-Schädigung sowie zu einer erhöhten Aktivierung der onkogenen c-Jun N-terminal Kinase 1 (JNK1) und STAT3 Signaltransduktion, während die Tumor-supprimierende Aktivität von p53
vermindert war. In zwei Folgestudien konnte weiterhin gezeigt werden, dass die fettgewebsspezifische Inaktivierung der GC-GR oder der GH-STAT5 Signaltransduktion, die durch Fasten-induzierte Lipolyse von weißen Fettzellen als auch eine ektope hepatische Lipidablagerung stark vermindert sowie zu einer erhöhten Insulinsensitivität führt.
Publications arising from and during this thesis


Submitted manuscripts and manuscripts in preparation:


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>β-HSD1</td>
<td>11β-Hydroxysteroid dehydrogenase type 1</td>
</tr>
<tr>
<td>ACAD</td>
<td>Acyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACOX</td>
<td>Fatty acyl-CoA oxidase</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AF</td>
<td>Activating function (N-terminal transactivation domain)</td>
</tr>
<tr>
<td>AKT</td>
<td>v-Akt murine thymoma viral oncogene homolog</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Aminotransferase</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>Angiopoietin-like 4</td>
</tr>
<tr>
<td>ATGL</td>
<td>Adipose triglyceride lipase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosin triphosphate</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine 3',5'-monophosphate</td>
</tr>
<tr>
<td>CBG/ Serpina 6</td>
<td>Corticosteroid-binding globulin</td>
</tr>
<tr>
<td>CCD</td>
<td>Coiled-coil domain</td>
</tr>
<tr>
<td>CCl₄</td>
<td>Carbon tetra chloride</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>Cdkn</td>
<td>Cyclin dependent kinase inhibitor</td>
</tr>
<tr>
<td>ChREBP</td>
<td>Carbohydrate responsive element binding protein</td>
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<tr>
<td>CM</td>
<td>Chylomicrons</td>
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<tr>
<td>CoA</td>
<td>Coenzyme A</td>
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<tr>
<td>CPT</td>
<td>Carnitine palmitoyltransferase</td>
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<tr>
<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>DEN</td>
<td>Diethyl-nitrosamine</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
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<tr>
<td>DG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DGAT</td>
<td>Diacylglycerol acyltransferase</td>
</tr>
<tr>
<td>DNL</td>
<td>De novo lipogenesis</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELOVL</td>
<td>ELOVL fatty acid elongase</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FAS/Fasn</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FATP</td>
<td>Fatty acid transporter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid(s)</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GHRH</td>
<td>GH releasing hormone</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GNG</td>
<td>Gluconeogenesis</td>
</tr>
<tr>
<td>GPAT</td>
<td>Glycerol-3-phosphate acyltransferase</td>
</tr>
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<td>Gapdh</td>
<td>Glycerol-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRB2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GRE</td>
<td>GC responsive element</td>
</tr>
<tr>
<td>GS</td>
<td>Glycogen synthase</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HES-1</td>
<td>Hairy and enhancer of split-1</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>Hmgs2</td>
<td>3-Hydroxy-3-methylglutaryl-CoA synthase 2</td>
</tr>
<tr>
<td>Hnf6</td>
<td>Hepatocyte Nuclear Factor 6</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
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<td>HSL</td>
<td>Hormone-sensitive lipase</td>
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<td>HSP</td>
<td>Heat-shock protein</td>
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<td>IGF</td>
<td>Insulin-like growth factor</td>
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<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
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<td>Lifr</td>
<td>Leukemia inhibitory factor receptor</td>
</tr>
<tr>
<td>Lpin1</td>
<td>Lipin 1</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MetS</td>
<td>Metabolic syndrome</td>
</tr>
<tr>
<td>MG</td>
<td>Monoacylglycerol</td>
</tr>
<tr>
<td>MGL</td>
<td>Monoacylglycerol lipase</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
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<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NASH</td>
<td>non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acid</td>
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<td>NH2</td>
<td>N-terminal domain</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
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<tr>
<td>PDE3B</td>
<td>Phosphodiesterase 3B</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>PEPCK</td>
<td>Pyruvate carboxykinase</td>
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<td>PI3K</td>
<td>Phosphoinositol 3-kinase</td>
</tr>
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<td>PIAS</td>
<td>Protein inhibitors of STATs</td>
</tr>
<tr>
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<td>Pyruvate kinase</td>
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<td>Plin</td>
<td>Perilipin 1</td>
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<td>Prolactin receptor</td>
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<tr>
<td>SHC</td>
<td>Src homology 2 domain-containing-transforming protein</td>
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<td>SOCS</td>
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<td>SRC</td>
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<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<td>Type 2 diabetes mellitus</td>
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<td>TAD</td>
<td>Transactivation domain</td>
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<td>Citric acid cycle</td>
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<td>Triglyceride</td>
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<td>Tumor growth factor</td>
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<tr>
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<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TYK</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
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<tr>
<td>WAT</td>
<td>White adipose tissue</td>
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</tbody>
</table>
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CHAPTER ONE: INTRODUCTION

1.1 Liver Metabolism: Physiology and Pathology

Metabolic homeostasis is vital for organisms to satisfy energy demands and to maintain overall physiologic stability. The liver is one of the body’s key detoxifying and metabolic organ, which has the ability to control protein, lipid and carbohydrate metabolism via the absorption, conversion, storage and release of various substrates and metabolites (Bechmann et al, 2011; Canbay et al, 2007; Rui, 2014). In response to nutritional and endocrine stimuli, such as insulin, glucagon and catecholamines, it performs critical opposite metabolic processes to meet the body’s energy demand. In the postprandial phase (increased insulin), the liver removes excess glucose from blood either converts it into glycogen or feeds it into oxidative pathways to generate energy and lipogenic substrates. Conversely, in the postabsorptive phase (increased glucagon and catecholamines), when blood glucose level decrease, glycogen stores are mobilized for glucose production and release to maintain blood glucose levels. This is particularly important to provide energy for the brain and other glucose-requiring tissues. When glycogen stores are exhausted, hepatic gluconeogenesis from glucogenic precursors ensures continuous glucose supply. Hepatic lipid metabolism is the body’s central source for synthesis and distribution of fatty acids (FA), triglycerides (TG) and cholesterol. TG and cholesterols are assembled together with apolipoproteins into very low density lipoprotein (VLDL) particles and redistributed to peripheral tissues for energy generation and storage. In addition, the liver synthesizes bile acids from cholesterol that are essential for efficient intestinal absorption, subsequent metabolism and redistribution of dietary lipid species in form of a second class of lipoproteins, the so-called chylomicrons (CM). The clearance and recycling of lipoproteins remnants as well as bile acids are also achieved by the liver. In the postabsorptive phase and fasting periods, when glucose must be conserved for glucose-requiring tissues, increased levels of non-esterified fatty acids (NEFA) derived from adipose tissue lipolysis are transported to the liver. Here, NEFA are oxidized to acetyl-CoA which undergoes further oxidation for energy production or is used to generate ketone bodies that serve as energy source for extra-hepatic tissues.

Persistent dysfunctions of these metabolic pathways and processes, which might result from dietary insults, obesity and/or genetic defects, are major contributors to the development of liver diseases that are characterized by a progressive fatty degeneration of hepatocytes (Angulo, 2002; Bechmann et al, 2011). This pathologic TG accumulation in hepatocytes, termed hepatic steatosis, is a common characteristic of liver pathologies, which are summarized under the term non-alcoholic fatty liver disease (NAFLD). The pathologic
accumulation of TG in the cytoplasm of hepatocytes may occur due to disproportional TG synthesis from de novo lipogenesis and/or NEFA uptake as well as TG clearance caused by impaired VLDL export and/or oxidative metabolism of TG.

In the following paragraphs I will focus on reviewing processes and pathways which are involved in hepatic lipid metabolism and their potential contribution to excess TG accumulation, the development of NAFLD and its progression to liver cancer.

1.1.1 Hepatic Lipid Metabolism

Under physiological conditions the liver does not function as a lipid storage depot. The TG content in hepatocytes is relatively low, which is achieved by a delicate equilibrium of FA and TG synthesis/breakdown as well as shuttling between the liver and the periphery in accordance to the body's energy demand (Fig. 1). The shuttling of TG and FA between different organs for energy production or storage is achieved via the lymphatic and vascular system. In principal, there are two mechanisms by which FA are transported: in form of TG, esterified to glycerol, as the main lipid species of circulating lipoproteins such as CM and VLDL or as albumin-bound NEFA.

In the postprandial phase, ingested lipids are emulsified by bile acids before they are hydrolyzed by pancreatic lipase to NEFA, monoacylglycerols (MG) and cholesterol within the intestinal lumen. Thereafter, lipid hydrolyzation products translocate into enterocytes where they are re-esterified to form lipids, which are packed together with apolipoproteins into CM (Iqbal & Hussain, 2009). To initially bypass the liver, CM are released via the lymphatic into the vascular system. White adipose tissue (WAT) is the body’s most abundant lipid storage depot. In capillary beds of WAT, lipoprotein lipase binds to CM and facilitates the uptake of NEFA into adipocytes by hydrolyzation of TG (Merkel et al, 2002). NEFA are re-esterified in white adipocytes to form TG for energy storage, while the amounts that are not taken up bind to albumin and contribute to the circulating NEFA pool (Faraj et al, 2004). The partially TG-depleted, cholesterol-rich CM remnants are transported to the liver, where they are endocytosed, further processed by lysosomal hydrolysis to NEFA, glycerol and cholesterol before recycled in form of newly synthesized VLDL particles (Canbay et al, 2007; Sundaram & Yao, 2010). Subsequently, VLDL particles are secreted to deliver lipids for storage in adipose tissue.

The circulating NEFA pool is predominantly composed of WAT-derived FA. In WAT, TG synthesis for energy storage and TG mobilization via lipolysis are regulated in accordance to the actual energy demand (Bhathena, 2006; Lass et al, 2011; Yu & Ginsberg, 2005). The major hormones in the control of these processes are catecholamines (mobilization under fasting conditions) and insulin (storage during external energy supply).
Figure 1. Fatty acid shuttling and hepatic lipid metabolism. Fatty acid (FA) and lipid uptake: Dietary lipid species are metabolized within the intestinal tract to form chylomicron (CM) lipoprotein particles which are released into the circulation to deliver FA to extrahepatic tissues such as adipose tissue. CM remnants (CR) are endocytosed by the liver, where remaining lipids are recycled in form of newly synthesized very low density lipoprotein particles (VLDL; VL). VLDL are released into the circulation to deliver FA to extrahepatic tissues, and its remnants (VR) are subsequently recycled within the liver. Hepatic uptake of circulating non-esterified fatty acids (NEFA) derived from adipose tissue lipolysis is mediated by fatty acid transporters such as FATP and CD36. In the cytosol, taken up FA are activated to form fatty acyl-CoA. Fatty acid oxidation: Acyl-CoA is transported into mitochondria in a CPT-dependent manner; oxidized to form acetyl-CoA which may be used for ketone body synthesis or enters the citric acid cycle (energy and lipogenic precursor production). De novo lipogenesis: Palmitic acid is newly synthesized from glucose-derived acetyl-CoA by a series of enzymatic reactions. Within the cytosol, ACC and FAS catalyze the rate-limiting and final steps, respectively. The subsequent elongation (ELOVL6) and desaturation (SCD1) involves the activation of palmitic acid (palmitoyl-CoA). Triglyceride (TG) synthesis: The newly formed fatty acyl-CoA derivates are esterified to glycerol-3-phosphate to form TG by depicted sequential enzymatic reaction catalyzed by the enzymes GPAT, Lipin and DGAT. TG are either stored in lipid vacuoles within hepatocytes or packed into VLDL particles within the endoplasmic reticulum (ER).
Catecholamine-induced β-adrenergic stimulation of lipolysis leads to the recruitment of the lipolytic machinery and the stepwise hydrolysis of TG to NEFA and glycerol, which is chiefly conducted by three lipases: adipose triglyceride lipase (ATGL) catalyzes the conversion of TG to diacylglycerol (DG), hormone-sensitive lipase (HSL) cleaves DG to MG which then is hydrolyzed by monoacylglycerol lipase (MGL) (Lass et al, 2011; Zechner et al, 2012). Stimulation of β-adrenergic receptors activates adenylyl cyclase, which increases intracellular cyclic adenosine 3’,5’-monophosphate (cAMP) levels and activates cAMP-dependent protein kinase A (PKA). PKA phosphorylates and activates HSL in addition to the lipid droplet protein perilipin 1 (Miyoshi et al, 2007; Wang et al, 2009). Perilipin 1 associates with the cytoplasmic side of the lipid vacuoles (Brasaemle, 2007). Under basal conditions, perilipin 1 is thought to suppress lipolysis by blocking lipase access and/or by sequestering the ATGL co-activator comparative gene identification 58 (CGI-58) (Brasaemle et al, 2000; Granneman et al, 2009; Lass et al, 2006). Upon a lipolytic stimulus, PKA phosphorylates perilipin 1, which results in the releases of CGI-58, the subsequent association with and activation of ATGL (Granneman et al, 2007). Conversely, insulin acts as a potent inhibitor of lipolysis to circumvent TG mobilization in the presence of external energy supply. This is accomplished by the downregulation of catecholamine-induced β-adrenergic signaling cascade and a consequent decreased activity of the lipolytic machinery (Zechner et al, 2009). In times of TG mobilization from adipose tissue, the released NEFA are transported via their circulating pool to the liver. Here, the uptake involves the dissociation of NEFA from albumin (Doege et al, 2006), which are then transported across the plasma membrane into hepatocytes. The uptake occurs mainly in a facilitated manner (Berk, 2008), mediated by plasma membrane transporters such as fatty acid transporters (FATPs) and CD36 (Doege et al, 2006; Falcon et al, 2010; Su & Abumrad, 2009). In hepatocytes, NEFA are directly converted into their fatty acyl-CoA derivatives by esterification to Coenzyme A (CoA). Depending on the energy status, acyl-CoA is either used for FA and TG synthesis or shuttled into mitochondrial β-oxidation for subsequent ATP and ketone body production (Sidossis et al, 1996). During fasting periods, FA as well as TG synthesis is suppressed and β-oxidation is activated, in which fatty acyl-CoA derivatives are consecutively catabolized to acetyl-CoAs by repetitive enzymatic reactions (Eaton et al, 1996). Thereby produced Acetyl-CoA feeds into the TCA cycle to produce energy equivalents for ATP synthesis by the electron transport chain. Alternatively, when excess amounts of acetyl-CoA are produced, these are used for ketone body synthesis (McGarry & Foster, 1980). A major transcriptional regulator of these processes is the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR) α (Issemann & Green, 1990). PPARα is a key integrator in the feeding to fasting transition (Kersten et al, 1999) and participates in additional lipid metabolic processes, such as FA uptake, peroxisomal β-oxidation and TG storage (Rakhshandehroo et al, 2009;
Rakhshandehroo et al, 2010). PPARα is activated, amongst others, by FA themselves as well as FA derivatives and its transcriptional activity requires the formation of heterodimers with the nuclear receptor RXR (Keller et al, 1993; Kliwer et al, 1997; Wan et al, 2000). PPARα signaling is indispensable for the activation of genes that control FA import to the mitochondria (e.g. Cpt1 and 2) as well as mitochondrial fatty acyl-CoA carboxylase (Acad) and peroxisomal fatty acyl-CoA oxidase (Acox1), both coding for enzymes that catalyze the initial step of the respective β-oxidation pathway (Aoyama et al, 1998; Dreyer et al, 1992; Kersten et al, 1999; Rakhshandehroo et al, 2009). Further, PPARα transcriptionally regulates 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (Hmgcs2) (Le May et al, 2000; Rakhshandehroo et al, 2009), a mitochondrial enzyme that catalyzes the rate-limiting step of ketogenesis as well as fibroblast growth factor 21 (Fgf21) that promotes FA oxidation and ketone body synthesis, at least in mice, during periods of fasting (Fisher et al, 2011; Inagaki et al, 2007).

FA that are used for hepatic TG synthesis can originate, as described above, from NEFA uptake in addition to lysosomal hydrolysis of CM remnants but may also be endogenously produced via de novo lipogenesis. This process is mainly controlled on the transcriptional level in response to the postprandial rise of glucose and insulin in the circulation. Vice versa both pathways are negatively controlled by the fasting-induced rise in glucagon (Ferre & Foufelle, 2010; Nguyen et al, 2008; Timlin & Parks, 2005). Hence, the fed state induces a switch in lipid metabolism from TG oxidation to synthesis, thereby allowing its storage and distribution. FA are newly synthesized from glucose which requires its conversion to acetyl-CoA by glycolysis and subsequent oxidation of the hereby produced pyruvate. Lipogenesis starts with acetyl-CoA carboxylase (ACC) facilitated formation of the 3-carbon compound malonyl-CoA (Abu-Elheiga et al, 2005; Munday, 2002). This is then converted to palmitic acid; a 16-carbon saturated fatty acid, by the multifunctional enzyme fatty acid synthase (FAS) through the sequential addition of two carbons which are donated by malonyl-CoA (Smith et al, 2003). Before esterification to glycerol-3-phosphate and formation of TG, palmitic acid is further processed by elongation and unsaturation. These microsomal-associated reactions are mediated, amongst others, by long chain fatty acid elongase (ELOVL) 6 (Jakobsson et al, 2006) and stearoyl-CoA desaturase (SCD) 1 (Miyazaki et al, 2001). Subsequent formation of TG by sequential esterification of three FA to the glycerol backbone is catalyzed by the enzymes glycerol-3-phosphate acyltransferase (GPAT), Lipin-1 and diacylglycerol acyltransferase (DGAT) (Coleman & Lee, 2004). Master transcriptional regulators of these processes in response to external energy supply are the glucose-responsive carbohydrate response element binding protein (ChREBP) and the insulin-activated sterol regulatory element binding protein 1c (SREBP-1c) by controlling glycolytic and lipogenic gene expression (Ferre & Foufelle, 2010). Activation of SREBP-1c by insulin
involves its transcriptional up-regulation in a phosphoinositide 3-kinase (PI3K)-dependent manner as well as the cleavage of the ER membrane-bound precursor SREBP-1c to release the mature and transcriptionally active protein (Ferre & Foufelle, 2010; Fleischmann & Lynedjian, 2000; Hegarty et al, 2005; Shimomura et al, 1999). Lipogenic SREBP-1c target genes include \textit{Acc}, \textit{Fasn}, \textit{Scd1} and \textit{Lpin1} (Horton et al, 2003; Ishimoto et al, 2009; Magana et al, 1997). Glucose activates ChREBP by inducing its expression and by regulating its relocation from the cytosol into the nucleus (Dentin et al, 2005; Ishii et al, 2004; Kawaguchi et al, 2001). Activation of ChREBP is required for full induction of lipogenesis through the control of \textit{Acc} and \textit{Fasn} expression (Dentin et al, 2005; Ishii et al, 2004). In addition, ChREBP activation stimulates the transcriptional up-regulation of rate-limiting glycolytic enzymes such as pyruvate kinase (PK) (Kawaguchi et al, 2001; Uyeda & Repa, 2006), thereby enhancing glucose catabolism to ensure substrate supply for FA and TG synthesis.

1.1.2 Hepatic Steatosis and Insulin Resistance
Hepatic steatosis describes the pathologic accumulation of TG in the cytoplasm of hepatocytes and consequent formation of lipid vacuoles that may lead to an enlargement of the liver (hepatomegaly). Steatosis, which is commonly defined as TG content in liver exceeding 5%, is a hallmark morphological feature and the initial stage of NAFLD (Angulo, 2002; Feldstein, 2010). A standardized approach for its diagnosis is the Kleiner score (Kleiner et al, 2005). This scoring system defines three stages of steatosis with TG content between 5-33% as stage one, 34-66% as stage two and above 66% as stage three. Two different types of TG accumulation exist which are classified as macro- and microvesicular steatosis by the size of existing lipid vacuoles (Aly & Kleiner, 2011). Macrovesicular steatosis classically displays single large lipid droplets that often lead to the displacement of the nucleus to the cell boundary. Microvesicular steatosis is characterized by enlargement of hepatocytes, centrally located nuclei and several small lipid vacuoles. While macrovesicular lipid accumulation is a result of imbalances between FA availability and disposal, microvesicular lipid vacuoles are associated with mitochondrial injury and dysfunction (Begriche et al, 2011). Mixed forms of macro- and microvesicular steatosis exist as well in NAFLD (Aly & Kleiner, 2011; Tandra et al, 2011).
Hepatic steatosis is frequently associated with resistance to insulin and it is thought to be a central indicator for systemic insulin resistance independently of overall body fat content and visceral fat mass (Bugianesi et al, 2005a; Korenblat et al, 2008; Seppala-Lindroos et al, 2002). Under physiologic conditions, postprandial secreted insulin is indispensable for proper substrate utilization by regulating carbohydrate and lipid metabolism (Saltiel, 2001). In WAT, it enables glucose uptake by inducing the translocation of glucose transporter 4 (GLUT4) to the plasma membrane (Ng et al, 2008; Stenbit et al, 1997), it enhances NEFA uptake and
TG synthesis, while it suppresses TG mobilization from lipolysis (Arner et al, 2011). In liver, insulin controls intrahepatic in addition to blood glucose level through inhibition of gluconeogenesis and stimulation of glycogen synthesis. This is accomplished through suppression of key gluconeogenic enzymes such as phosphoenol pyruvate carboxykinase (Pepck) and glucose-6-phosphatase (G6p) and the induction of glycogen synthase (GS) activity (Frame & Cohen, 2001; Puigserver et al, 2003; Saltiel & Kahn, 2001; Vidal-Puig & O’Rahilly, 2001). As discussed in the previous section, insulin induces hepatic de novo lipogenesis and TG synthesis by activation of lipogenic signaling pathways. At the same time, malonyl-CoA produced in the lipogenic process inhibits mitochondrial FA oxidation and combustion (Mao et al, 2006; Savage et al, 2006). Insulin's functions are mediated by the activation of insulin receptor signaling and subsequent induction of two major pathways: the canonical PI3K pathway and, non-canonically, the mitogen-activated protein kinase (MAPK) signaling cascade (Saltiel & Kahn, 2001; Taniguchi et al, 2006). Briefly, insulin binding to the insulin receptor (IR) initiates phosphorylation of insulin receptor substrates (IRS) that activate the p85 regulatory subunit of PI3K. This event leads to activation of AKT-dependent downstream signaling cascades via formation of phosphatidylinositol 3,4,5-trisphosphate. In contrast, the non-canonical IR signaling through MAPK pathway is induced by activation of its effector proteins such as GRB2 and SHC by IRS. Insulin resistance, the inability of tissues to properly respond to insulin stimulation, is characterized by impairments in IR-dependent signal transduction (Saltiel, 2001; Saltiel & Kahn, 2001). Loss of sensitivity to insulin is linked to hyperinsulinemia in addition to hyperglycemia and, as mentioned before, intrahepatic TG accumulation (Saltiel, 2001; Samuel & Shulman, 2012). Intrahepatic TG accumulation might be a result of deregulated lipid as well as carbohydrate metabolic pathways all of which, at least in part, overlap. Further, it is suggested that the development of steatosis greatly relays on an excess influx of NEFA into the liver. In this regard, it was demonstrated that in human steatotic patients approximately 60% of hepatic TG arise from circulating NEFA influx, while 15 and 25% are derived from diet and de novo lipogenesis, respectively (Donnelly et al, 2005). A reduced capability of FA storage in adipocytes (Medina-Gomez et al, 2007; Roden, 2006; Samocha-Bonet et al, 2012) as well as unrestrained basal lipolysis in insulin resistant WAT might be causative of an increased NEFA availability. Besides the increased availability, the rate of hepatic NEFA uptake is additionally enhanced by alterations in the expression of FA transporters (Berk, 2008; Bradbury & Berk, 2004; Chabowski et al, 2007). Elevated expression of CD36 and the correlation with augmented steatosis and insulin resistance was demonstrated in human patients (Greco et al, 2008; Miquilena-Colina et al, 2011). These findings are supported by murine models that display increased liver TG content upon overexpression of hepatic CD36 (Koonen et al, 2007) and, conversely, a reduction of liver TG upon adenoviral knockdown of
FATPs in high fat diet mouse models (Doege et al, 2008; Falcon et al, 2010). In addition to the circulating NEFA pool, unrestrained gluconeogenesis and elevated rates of de novo lipogenesis contribute to FA and TG accumulation in insulin resistant livers (Donnelly et al, 2005). Pathological elevation of de novo lipogenesis is frequently mediated by increased expression and activity of ChREBP and SREBP-1c along with the induction of downstream lipogenic signaling pathways in human subjects and in several steatotic animal models (Pettinelli & Videla, 2011; Postic & Girard, 2008; Smith & Adams, 2011).

Figure 2. Fatty acid metabolism, insulin resistance and hepatic steatosis. In insulin resistant states, adipose tissue glucose uptake is diminished, while lipolysis is elevated leading to an increase in circulating non-esterified fatty acids (NEFA) and subsequent hepatic NEFA uptake. The increased hepatic NEFA uptake is further supported by elevated expression of fatty acid (FA) transporters (e.g. CD36) which are, in part, regulated by FA and FA derivates activated PPARs. At the same time, unrestrained hepatic gluconeogenesis (GNG), hyperglycemia and hyperinsulinemia contribute to greater rates of hepatic de novo lipogenesis (DNL) by stimulating key lipogenic transcription factors ChREBP and SREBP-1c, respectively. Together with aberrantly expressed lipogenic PPARγ, these processes lead to an increase in hepatic FA content and triglyceride (TG) synthesis which ultimately results in fatty degeneration of hepatocytes. In addition, the insulin resistant state may impair the livers competence to metabolize excess FA by oxidation within mitochondria. Peroxisomal and microsomal FA oxidation may increase which is associated with the formation of toxic metabolites.

Further, hepatic overexpression of either transcription factor is sufficient to induce the entire lipogenic program and steatosis in transgenic animals (Benhamed et al, 2012; Shimano et al,
1997), while the deletion of either factor in the obese ob/ob mouse model improves hepatic steatosis and related metabolic alterations (Dentin et al, 2006; Iizuka et al, 2006; Yahagi et al, 2002).

Interestingly, increased activation of SREBP-1c signaling frequently occurs despite insulin resistance. This inability of insulin to inhibit gluconeogenesis while continuously promoting lipogenesis has been described as “selective insulin resistance” (Brown & Goldstein, 2008) and detailed molecular mechanisms behind are still under investigation. Activation of the insulin/Akt substrate and nutrient-sensing mammalian target of rapamycin complex 1 (mTorC1) pathway (Laplante & Sabatini, 2009a; Laplante & Sabatini, 2009b) was recently shown to stimulate hepatic SREBP-1c and de novo lipogenesis (Li et al, 2010b; Yecies et al, 2011). Insulin-mediated changes of Pepck and Srebf mRNA expression were blocked by inhibitors of PI3K and AKT (Li et al, 2010b). Yet, the mTORC1 inhibitor rapamycin suppressed insulin-mediated induction of Srebf, but not insulin-mediated suppression of Pepck (Li et al, 2010b; Yecies et al, 2011). The existence of these parallel pathways was suggested to uncouple lipogenesis from gluconeogenesis (Laplante & Sabatini, 2010). These findings were recently substantiated by the discovery that inhibition of the Notch receptor (Fortini, 2009) uncouples AKT activation from hepatic lipid accumulation by decreasing mTorC1 stability (Pajvani et al, 2013). Hepatic Notch signaling was shown to stabilize and activate mTorC1 and to be permissive for diet-induced de novo lipogenesis and steatosis in insulin resistant livers. Vice versa, Notch-mediated hepatic steatosis was shown to be rapamycin-sensitive (Pajvani et al, 2013). A second proposed possibility is that ER stress, which is frequently present in insulin-resistant livers, leads to increased cleavage and activation of immature SREBP-1c. Thereby, ER stress may activate SREBP-1c signaling in an insulin-independent manner (Ferre & Foufelle, 2010; Kammoun et al, 2009).

An additional, lipogenic factor that is implicated in intrahepatic TG accumulation is PPARγ. This transcription factor constitutes a second member of the PPAR class of nuclear hormone receptors and can be activated by a wide range of ligands, including endogenous fatty acids, in addition to synthetic ligands such as thiazolidinediones (Rosen & Spiegelman, 2001). Under physiologic conditions, PPARγ is abundantly expressed in adipose tissue where it exerts crucial functions in adipose tissue metabolism and it is required for proper adipogenesis (Rosen & Spiegelman, 2014; Rosen et al, 2000). However, in contrast to PPARα, its expression levels are low in healthy livers (Vidal-Puig et al, 1997). In livers of steatotic patients and animal models PPARγ expression and activity is highly increased (GavriloVA et al, 2003; Matsusue et al, 2003; Matsusue et al, 2008; Memon et al, 2000; Pettinelli & Videla, 2011; Rahimian et al, 2001; Westerbacka et al, 2007) and is associated with the presence of a lipogenic transcription program. This comprises, for instance, the up-regulation of proteins involved in FA uptake, such as CD36 (Memon et al, 2000), but also
coincides with SREBP-1c activation and the corresponding induction of lipogenic genes *Fasn*, *Acc* and *Scd1* (Matsusue et al, 2003; Moran-Salvador et al, 2011; Pettinelli & Videla, 2011). The lipogenic effects of hepatic PPARγ and its involvement in the maintenance of steatotic phenotypes is further emphasized by its liver-specific knockout in genetically or diet-induced murine obesity models as well as lipoatrophic mice that result in strongly attenuated steatosis (Gavrilova et al, 2003; Matsusue et al, 2003; Moran-Salvador et al, 2011).

**1.1.3 Non-Alcoholic Fatty Liver Disease**

NAFLD is the most common chronic liver disease in adults and children worldwide. NAFLD develops in the absence of excessive alcohol abuse but is frequently associated with overnutrition, diabetes and central obesity (Angulo, 2002; Cohen et al, 2011; Feldstein et al, 2009). Further, it represents a major health issue due to its worldwide increasing incidence, affecting up to 30% of the general population, more than half of chronically obese and almost 90% of morbidly obese patients (Day, 2010; Lazo & Clark, 2008). It is suggested that NAFLD is the hepatic manifestation of the metabolic syndrome (MetS) as one third of NAFLD patients present every MetS criteria and almost all of them display at least one feature (Marchesini et al, 2001; Marchesini et al, 2003). Accordingly, NAFLD is not only associated with insulin resistance and type 2 diabetes mellitus (T2D), but also dyslipidemia and hypertension and, at the same time, confers a risk factor to development these conditions. Noteworthy, similar to obese and diabetic subjects, lipodystrophic patients, who are characterized by partial or complete absence of adipose tissue, display NAFLD in addition to MetS features such as insulin resistance (Gorden et al, 2010). The term NAFLD describes a spectrum of liver pathologies which range from isolated steatosis to its progressive form non-alcoholic steatohepatitis (NASH) (Cohen et al, 2011). NASH is classified as steatosis in conjunction with hepatocellular ballooning (swollen hepatocytes, aberrations in cytoskeleton and nuclei), inflammation (parenchymal and portal) and generalized liver cell injury, which can be accompanied by variable degrees of fibrotic degeneration (Kleiner et al, 2005). While isolated steatosis correlates mostly with a relatively favorable clinical outcome, its progression to NASH (approximately 20% of NAFLD cases) severely increases the risk of advanced fibrosis, cirrhosis and eventually liver cancer (Baffy et al, 2011; Cohen et al, 2011).

**1.1.4 Hepatocellular Carcinoma**

Liver cancer is the fifth most common cancer with increasing incidence and the third leading cause of cancer death worldwide (Jemal et al, 2011). Hepatocellular carcinoma (HCC) is the main subtype of primary liver cancer (El-Serag, 2011) and, in most cases, develops in cirrhotic livers resulting from chronic viral hepatitis as well as from alcohol-induced liver injury
(Baffy et al, 2011; El-Serag & Rudolph, 2007). HCC occurrence has been additionally linked to obesity and related metabolic disorders such as T2D as well as NAFLD. Data from epidemiological studies and meta-analysis of cohort studies showed a 4.5 times higher risk of HCC in obese men and an increase in the relative risk of HCC from 117% for overweight to 189% for obese patients, respectively (Calle et al, 2003; Larsson & Wolk, 2007). The presence of T2D was linked to a three-fold risk of HCC in a population-based study, while large cohort-based studies provided evidence for HCC occurrence in the background of T2D regardless of the presence of other risk factors (Davila et al, 2005; El-Serag et al, 2004). In NAFLD, the progression from isolated steatosis to NASH with the potential to progress to cirrhosis substantially increases the risk of HCC although not to the degree that is reported for chronic viral hepatitis (Baffy et al, 2011; Torres & Harrison, 2012). In spite of the etiology of the underlying chronic liver disease, HCC development is typically a stepwise process from cirrhosis to dysplasia to carcinoma which might takes decades to evolve (Farazi & DePinho, 2006; Thorgeirsson & Grisham, 2002). During this time chronic liver injury followed by compensatory proliferation in response to metabolic stress, oxidative toxicity and inflammation create an environment that is susceptible to genetic alterations and malignant transformation. A critical event in this transformation process is the clonal selection of premalignant hepatocytes (Paradis et al, 1998). The final transition form premalignant lesions to HCC comprises multiple epigenetic and genetic alterations including point mutations as well as gain of function and loss of function mutations ultimately resulting in complex and heterogeneous tumors (Minguez et al, 2009; Villanueva et al, 2007). The genomic alterations in HCC frequently comprises activation and increased expression of various growth factors (e.g. VEGF, PDGF and FGF), developmental pathways (e.g. WNT/β-Catenin and c-Met/HGF), several proliferative and survival signaling cascades (e.g. Ras/MAPK and PI3K/AKT) as well as suppression of cell cycle regulators and tumor suppressors that confers insensitivity to anti-growth signals and checkpoint disruption (e.g., p53, RB and cyclin D1) (Breuhahn et al, 2006; Llovet & Bruix, 2008; Minguez et al, 2009; Villanueva et al, 2007). Even though HCC develops in most cases in the background of cirrhotic livers, in recent years, HCC has been additionally reported to occur in the presence of NASH without underlying cirrhosis (Baffy et al, 2011; Torres & Harrison, 2012). In rare cases, HCC development further was reported to occur in subjects who display NAFLD and features of MetS but no apparent steatohepatitis and fibrosis (Guzman et al, 2008; Paradis et al, 2009; Sanyal et al, 2010). The mechanisms causing HCC in the background of NAFLD and MetS, particularly, in non-cirrhotic and non-fibrotic livers, are still poorly understood. However, data from human studies and animal models already have led to the proposal of some risk factors and key events (Baffy et al, 2011; Michelotti et al, 2013; Torres & Harrison, 2012). These include older age and features of NAFLD/NASH itself, such as insulin resistance, increased
levels of inflammatory cytokines, lipotoxicity, and oxidative stress. Lipotoxicity is characterized by cellular damage and dysfunction resulting from ectopic FA deposition in non-adipose tissues such as the liver (Neuschwander-Tetri, 2010; Unger et al, 2010). This condition may arise when the amount of FA taken up and produced by the liver exceeds the clearance and/or storage capacity which might leads to the formation of toxic metabolites (Lelliott & Vidal-Puig, 2004; Neuschwander-Tetri, 2010). In this regard, the synthesis of TG seems to be a detoxification mechanism (Medina-Gomez et al, 2007). In support of this notion, blocking DGAT2 expression and, thereby, TG synthesis ameliorated hepatic steatosis in genetically obese mice when fed a NASH-inducing diet. However, the block in TG synthesis was also associated with enhanced liver injury and fibrosis which was attributed to increased hepatic FA accumulation, microsomal FA oxidation, oxidative stress and lipid peroxidation (Yamaguchi et al, 2007).

**Figure 3. Development of HCC in NAFLD.** NAFLD is closely associated with obesity and co-morbid conditions such as the metabolic syndrome and type 2 diabetes. Key processes which are thought to be involved in NAFLD progression are indicated (lower box, left). NAFLD progression usually follows a sequential process through its different stages: from primary steatosis to NASH (fibrotic; non-fibrotic) to cirrhosis which may progress to HCC (dark blue arrows). HCC may also occur at any stage of non-cirrhotic or non-fibrotic NAFLD with above indicted key process as suggested risk factors (light blue arrows). Background: grayscale hematoxylin and eosin staining of a steatotic mouse liver.

Accelerated rates of FA oxidation (peroxisomal, microsomal and mitochondrial) cause the formation of free radicals and lipid peroxides that, in turn, may cause mitochondrial dysfunction, oxidative injury of DNA and proteins, ER stress and cell death (Alkhouri et al, 2009; Browning & Horton, 2004; Li et al, 2008). These processes in conjunction with inflammatory responses in affected livers may create an environment that promotes hepatocarcinogenesis. This includes chronic liver damage-induced compensatory
proliferation of otherwise quiescent hepatocytes which is thought to be a major contributing mechanism to HCC development (Fausto, 1999; Thorgeirsson & Grisham, 2002). Increased proliferation was also reported for NASH-associated HCC in a mouse model of diet-induced obesity as well as in steatotic livers of genetically obese mice. This suggests that, at least in this model system, hyperproliferative response can occur already at early stages of NAFLD (Hill-Baskin et al, 2009; Yang et al, 2001). Interestingly, mRNA profiles of diet-induced HCCs clustered separately from regenerating and developing liver tissue further indicating that NASH-associated HCC represent a distinct class of hyperproliferation (Hill-Baskin et al, 2009).

Insulin resistance and obesity not only favor hepatocyte damage by FA overflow, but are also frequently associated with an overall low-grade chronic inflammation. This inflammatory state is mediated by proinflammatory cytokines such as TNF-α and IL-6, which are increasingly released from dysfunctional adipose tissue (Marra & Bertolani, 2009; Rosen & Spiegelman, 2014). TNF-α and IL-6 are well-known activators of pathways contributing to tumorigenesis (Grivennikov & Karin, 2011), further IL-6 potently induces STAT3 activity and subsequent proliferative and anti-apoptotic signals (Subramaniam et al, 2013). Hence, it is suggested that TNFα and IL-6 contribute to the pathogenesis of NASH and its progression to HCC. Accordingly, in murine models of genetic and dietary obesity, both cytokines are elevated in the circulation and are increasingly expressed in liver (Arkan et al, 2005; Vansaun et al, 2013). Thereby, they support HCC formation induced by the carcinogen diethyl-nitrosamine (DEN) (Park et al, 2010). This was attributed to increased liver inflammation, liver injury, and increased proliferation but reduced apoptosis in the presence of highly activated STAT3 and MAPK family members extracellular-signal-regulated kinases (ERK) and c-Jun N-terminal kinase (JNK). Conversely, impairment of IL-6 or TNF-α signaling pathways as well as the impairment of both attenuated the steatotic, inflammatory phenotype and suppressed obesity-enhanced HCC development in carcinogen-treated mice (Park et al, 2010). Furthermore, hyperinsulinemia in insulin resistant states might directly contribute to activation of proliferative cell signaling that remains responsive to insulin action (Siddique & Kowdley, 2011). In this regard, hyperinsulinemia is associated with increased insulin-like growth factor (IGF-1) expression and IRS-1 activity, thereby stimulating cellular proliferation and survival through activation of respective downstream pathways such as MAPK signaling cascades (Gallagher & LeRoith, 2011).

1.2 The JAK-STAT Pathway

The evolutionarily highly conserved Janus kinase (JAK)/STAT signaling pathway mediates the rapid transduction of numerous extracellular signals, such as cytokines, hormones and growth factors, into molecular and cellular responses (Kiu & Nicholson, 2012; Stark &
Darnell, 2012). These responses and the tight control of their duration/magnitude are crucial for a variety of developmental and homeostatic processes. Conversely, unbalanced JAK/STAT signal transduction is associated with development of various pathologies ranging from inflammatory diseases to cancer (Decker et al, 2012; Ferbeyre & Moriggl, 2011; Li et al, 2011; Santos & Costa-Pereira, 2011). In mammals, four JAK tyrosine kinases (JAK1-3 and TYK2) and seven STAT transcription factors (STAT1-4, 5A, 5B and 6) are involved in the transduction of signals from transmembrane receptors, which lack intrinsic tyrosine kinase activity (Kiu & Nicholson, 2012; Schindler et al, 2007). STATs can also be directly tyrosine phosphorylated either by receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR) and by non-receptor tyrosine kinases such as SRC. STAT transcription factors share a similar structure which consist of several modular domains (section 1.4., Fig. 2): an N-terminal domain (NH2), followed by a coiled-coil domain (CCD) and a central DNA binding domain (DBD), a linker region, the highly conserved src homology 2 (SH2) domain which is close to the single tyrosine residue required for STAT phosphorylation and activation, and a C-terminal transactivation domain (TAD), which shows the highest divergence and thereby contributes to STAT specificity (Schindler et al, 2007). The NH2 domain is involved in protein–protein interactions between chromatin-bound STAT dimers, thereby aids STAT tetramer formation (Kornfeld et al, 2008). It is additionally involved in dimer formation of unphosphorylated STATs (Mertens et al, 2006). Further, cofactor interactions which positively or negatively modulate STAT transcriptional activity also involve the NH2 domain, but additionally occur through the CCD and TAD (Kornfeld et al, 2008; Schindler et al, 2007). The SH2 domain is indispensable for efficient recruitment of STATs to specific phosphotyrosine motifs in the cytoplasmic domains of their receptors and the subsequent association with activating JAKs, as well as the dimerization of phosphorylated STATs (Kiu & Nicholson, 2012).

Activation of the canonical pathway is initiated by binding of an extracellular ligand to its cognate receptor, leading to trans-phosphorylation of receptor-associated JAKs which then phosphorylate tyrosine residues in the cytoplasmic receptor domain. These tyrosine residues serve as docking sites for unphosphorylated, anti-parallel STAT dimers (Mertens et al, 2006). STAT association with the receptor via the SH2 and NH2 domain, results in their phosphorylation by JAKs on the conserved C-terminal tyrosine residue and the formation of active, parallel STAT dimers. Phosphorylated STATs translocate more efficiently into the nucleus and bind as dimers or as tetramers to specific consensus responsive elements in the promoter regions of target genes to regulate gene transcription (Kiu & Nicholson, 2012; Kornfeld et al, 2008) (section 1.4., Fig. 1). The “fine-tuning” of the JAK/STAT-mediated signaling duration and magnitude is essential to ensure physiologic homeostasis. This is achieved by three major negative regulatory mechanisms (Wormald & Hilton, 2004): de-
phosphorylation of receptor tails and STATs by phosphotyrosine phosphatases; direct inhibition of STAT function via protein inhibitors of STATs (PIAS) (Shuai, 2006) and inhibition by suppressor of cytokine signalling (SOCS) proteins via interference with JAK-mediated phosphorylation in addition to SOCS-induced receptor degradation (Krebs & Hilton, 2001).

1.2.1 Growth Hormone and STAT5

Growth hormone (GH) is a single chain peptide synthesized and released into the circulation by the anterior pituitary somatotrophs. GH might also be expressed in other tissues, possibly acting in an auto or paracrine manner on the local environment (Harvey & Hull, 1997). At the pituitary level, GH is secreted in a pulsatile manner mainly in response to hypothalamic growth hormone releasing hormone (GHRH) and stomach-derived ghrelin (Brazeau et al, 1973; Lengyel, 2006), while it is negatively controlled by hypothalamic somatostatin (Thorner et al, 1990). Further, GH regulates its own secretion via a negative feedback loop involving its major downstream effector IGF-1 (Wallenius et al, 2001) that stimulates hypothalamic somatostatin secretion and by acting directly on pituitary somatotrophs (Berelowitz et al, 1981). Additional factors reported to counteract GH release comprise obesity and hyperglycemia, whereas dietary restriction/fasting, hypoglycaemia, acute stress and exercise are associated with an increased GH secretion (Flores-Morales et al, 2006; Veldhuis et al, 2001). First and foremost, GH is essential for postnatal body growth (Brooks & Waters, 2010; Laron et al, 1966; Rosenfeld et al, 2007) but it continuously plays a crucial role in several vital processes such as regeneration, cellular reproduction, substrate utilization and energy metabolism throughout life time. GH’s metabolic functions include optimization of body composition and adaptation to energy shortage; enhancing adipose tissue lipolysis and overall fatty acid oxidation, while promoting protein synthesis or decreasing protein break down depending on the actual energy status (Moller & Jorgensen, 2009a; Vijayakumar et al, 2010). Further, GH influences systemic glucose metabolism both directly and by antagonizing insulin function, leading to the inhibition of glucose oxidation, and, simultaneously, to the induction of hepatic gluconeogenesis (Moller & Jorgensen, 2009b; Vijayakumar et al, 2010). On the cellular level, several signaling pathways have been shown to mediate GH’s diverse homeostatic functions. GH signaling is initiated by its binding to the broadly expressed GH receptor (GHR) and subsequent activation of the receptor-associated kinase JAK2 (section 1.4. Fig.1). This event triggers the activation of downstream signaling molecules including the MAPK family member ERK and JNK, the PI3K cascade as well as STATs (Rowland et al, 2005; Waters et al, 2006). Even though STAT1 and STAT3 have been shown to be activated by GHR, STAT5 is believed to be the major downstream target of GH (Teglund et al, 1998; Waters et al, 2006).
Signal transduction through STAT5 is implicated in the control of numerous homeostatic processes throughout the body involving most notably the immune system, mammary and liver epithelial cells (Hennighausen & Robinson, 2008). As already mentioned STAT5 consists of two isoforms, STAT5A and STAT5B, which are encoded by two closely located genes and harbour 96% similarity at the protein level with the highest divergence in their TAD domain. Both proteins are activated not only by GH, but also in response to a variety of extracellular signaling molecules including, prolactin, erythropoietin, and several interleukins (Hennighausen & Robinson, 2008; Kornfeld et al, 2008). STAT5A and STAT5B are both broadly expressed and share not only high sequence similarity but also similar responsive elements in the promoter regions of target genes which suggest overlapping and redundant roles. However, data from human studies and from mouse models have revealed different expression pattern in some tissues as well as non-redundant roles in their functions (Hwa et al, 2011; Teglund et al, 1998). In this regard, the prototypical example lies in mammary and liver epithelial cells. STAT5A is the predominant isoform in mammary tissue and preferentially conveys prolactin-mediated development of secretory mammary alveolar epithelia during pregnancy and lactation (Liu et al, 1998; Liu et al, 1997; Teglund et al, 1998). However, in the absence of functional STAT5A, elevated STAT5B expression and partial rescue of mammary alveolar development and function was only observed after several rounds of pregnancies (Liu et al, 1998). Conversely, STAT5B is more abundant in liver epithelial cells where it was shown to confer many of GH’s functions and is indispensable for the expression of several GH-controlled sexual dimorphic genes (Holloway et al, 2007; Rowland et al, 2005; Udy et al, 1997). GH signaling mediates a plethora of vital processes in liver. As discovered by the use of mice either harboring different Ghr mutations or an liver-specific Stat5 knockout, several hundred transcripts comprising genes involved in body growth, proliferation, and differentiation but also cellular metabolism, protein turnover and electron transport are under the control of hepatic GHR signaling (Barclay et al, 2011; Cui et al, 2007; Engblom et al, 2007; Rowland et al, 2005). Amongst these, GH-activated STAT5 is pivotal for the transcription of genes such as components of the cytochrome p450 detoxifying system, the negative-feedback regulator Socs2 as well as Igf1 and acid labile subunit (Als) (Greenhalgh et al, 2005; Ooi et al, 1998; Rotwein, 2012; Woelfle et al, 2003). The latter two proteins complexed with IGF-1 binding protein 3 form bioactive IGF-1 (Dai & Baxter, 1994; Jones & Clemmons, 1995), which highly impacts numerous events that contribute to cellular growth. In addition, through the expression of IGF-1 and SOCS2, hepatic STAT5 indirectly controls systemic GH availability and the liver’s sensitivity to GH, respectively (Flores-Morales et al, 2006; Vesterlund et al, 2011). Recent studies using liver-specific STAT5 knockout mice have further provided substantial evidence that GH-activated STAT5 has important functions in the control of hepatic lipid metabolism and hepatocyte regeneration.
Thereby, it potentially contributes to liver disease development (reviewed in more detail under 1.4).

1.3 Glucocorticoids and the Glucocorticoid Receptor

Glucocorticoids (GC) are steroid hormones which are produced and secreted by the adrenal cortex as important integrators in the body’s adaptation to stress and the maintenance of metabolic homeostasis (Oakley & Cidlowski, 2013; Rose & Herzig, 2013). Under basal and under stress conditions, GC release is controlled by the hypothalamic-pituitary-adrenal (HPA) axis, an adaptive neuroendocrine system (Smith & Vale, 2006). Vice versa, GC constitute key negative feedback regulators of the HPA axis at the hypothalamic and pituitary level (Cole et al, 1995). Further, glucocorticoid action is additionally modulated at the pre-receptor level within target tissues by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (Walker & Andrew, 2006). 11β-HSD1 converts inactive 11-dehydrocorticosterone (human: cortisone) to active corticosterone (human: cortisol) and thereby amplifies local GC action (Seckl & Walker, 2001). On the cellular and molecular level, GC actions are largely conducted by activation of the glucocorticoid receptor (GR), a transcription factor belonging to the nuclear hormone receptor superfamily (Aranda & Pascual, 2001). The murine GR is encoded by the Nr3c1 gene, which gives rise to two protein products, termed GRα and GRβ, by alternate exon usage (Kassel & Herrlich, 2007; Yudt & Cidlowski, 2001). GRα (hereafter referred to as GR) is almost ubiquitously expressed in tissues and organs and exerts most of GC actions, while GRβ is thought to act as dominant negative isoform and thereby function as an inhibitor of GC signaling (Oakley et al, 1999; Yudt et al, 2003).

The GR consists of several modular domains (section 1.4., Fig. 2): an N-terminal transactivation domain (AF-1), a DNA-binding domain followed by a hinge region which contains a nuclear localization signal (NLS), and a C-terminal ligand-binding domain that is additionally involved in GR dimerization and includes a second transactivation domain (AF-2). Glucocorticoids diffuse freely from the circulation across plasma membranes into the cytoplasm, where the GR is retained in a ligand-receptive conformation complexed with several chaperone proteins such as heat shock proteins (HSP) 70 and 90 (Pratt et al, 2006). Upon ligand binding, the activated GR dissociates from the chaperones, translocates into the nucleus where it induces or represses target gene transcription either via direct DNA binding to glucocorticoid responsive elements (GRE) or through protein-protein interaction with other transcription factors and co-regulators (Kassel & Herrlich, 2007; Oakley & Cidlowski, 2013). GC effects on metabolism are of anabolic as well as catabolic nature and involve changes in the expression of various genes involved in TG and glucose metabolism in metabolically active tissues such as liver and WAT (Rose & Herzig, 2013; Vegiopoulos & Herzig, 2007). One important function is the induction of hepatic gluconeogenesis, in part, through the
transcriptional induction of key gluconeogenic enzymes by the GR, such as *Pepck* and *G6p* (Opherk et al, 2004; Rose & Herzig, 2013). At the same time, GC signaling is associated with the inhibition of insulin-stimulated glucose uptake and utilization in extra-hepatic, non-glucose requiring organs (Rose & Herzig, 2013; Vegiopoulos & Herzig, 2007). In this regard, GC treatment was shown to interfere with insulin-induced glucose uptake in adipose tissue via inhibiting the translocation of GLUT4 to the plasma membrane (Sakoda et al, 2000). Further, GC potently stimulate adipose tissue TG mobilization by up-regulating the expression of all three lipases (Campbell et al, 2011; Yu et al, 2010) and enhancing catecholamine-induced lipolysis. For the latter two independent mechanisms were reported: (1) GC-GR signaling mediated transcriptional upregulation of angiopoietin-like 4 (*Angptl4*) (Gray et al, 2012; Koliwad et al, 2009), which encodes for a secreted protein that increases fasting- and catecholamine-induced lipolysis by increasing intracellular cAMP level and subsequent activation of the lipolytic cascade; (2) GC stimulated transcriptional downregulation of phosphodiesterase 3B (*Pde3b*) (Gray et al, 2012; Xu et al, 2009a), which encodes for an insulin-activated enzyme that rapidly degrades cAMP and thereby decreases the activation of the lipolytic cascade. These functions, particularly in fasting periods, are a mechanism for mobilization and supply of non-glucose energy substrates (NEFA, ketone bodies) to spare glucose for central nervous system utilization. While physiologic GC signaling enables the body to adapt appropriately to changes in energy status, excess GC exposure disrupts metabolic homeostasis which is associated with liver disease development (reviewed in more detail under 1.4).
1.4 Review: Hepatic Growth Hormone and Glucocorticoid Receptor Signaling in Body Growth, Steatosis and Metabolic Liver Cancer Development.

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Review

Hepatic growth hormone and glucocorticoid receptor signaling in body growth, steatosis and metabolic liver cancer development

Kristina M. Mueller a, Madeleine Themanns a, Katrin Friedbichler a, Jan-Wilhelm Kornfeld b, Harald Esterbauer c, Jan P. Tuckermann d,e, Richard Moriggl a,∗

a Ludwig Boltzmann Institute for Cancer Research, Vienna, Austria
b Institute for Genetics, Department of Mouse Genetics and Metabolism, University of Cologne, Cologne, Germany
c Department of Laboratory Medicine, Medical University Vienna, Vienna, Austria
d Institute for General Zoology and Endocrinology, University of Ulm, Ulm, Germany

Abstract

Growth hormone (GH) and glucocorticoids (GCs) are involved in the control of processes that are essential for the maintenance of vital body functions including energy supply and growth control. GH and GCs have been well characterized to regulate systemic energy homeostasis, particular during certain conditions of physical stress. However, dysfunctional signaling in both pathways is linked to various metabolic disorders associated with aberrant carbohydrate and lipid metabolism. In liver, GH-dependent activation of the transcription factor signal transducer and activator of transcription (STAT) 5 controls a variety of physiologic functions within hepatocytes. Similarly, GCs, through activation of the glucocorticoid receptor (GR), influence many important liver functions such as gluconeogenesis. Studies in hepatic Stat5 or Gr knockout mice have revealed that they similarly control liver function on their target gene level and indeed, the GR functions as a cofactor of STAT5 for GH-induced genes. Gene sets, which require physical STAT5–GR interaction, include those controlling body growth and maturation. More recently, it has become evident that impairment of GH-STAT5 signaling in different experimental models correlates with metabolic liver disease, ranging from hepatic steatosis to hepatocellular carcinoma (HCC). While GH-activated STAT5 has a protective role in chronic liver disease, experimental disruption of GC-GR signaling rather seems to ameliorate metabolic disorders under metabolic challenge. In this review, we focus on the current knowledge about hepatic GH-STAT5 and GC-GR signaling in body growth, metabolism, and protection from fatty liver disease and HCC development.

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Contents

1. Introduction ............................................................... 2
2. Mechanism of GH-STAT5 and GC-GR action ................... 2
2.1. GH signaling and STAT5 ........................................... 2
2.2. GC signaling and GR ............................................... 2
2.3. STAT5 and GR synergy in hepatocytes ......................... 3
3. STAT5 and GR function in body growth ......................... 4
4. GH-STAT5 and GR function in metabolism and hepatic steatosis ............................................. 5
4.1. Glucose metabolism ................................................. 5
4.2. Lipid metabolism and hepatic steatosis ....................... 6
5. GH-STAT5 signaling and HCC ....................................... 8
6. Concluding remarks .................................................. 9
Acknowledgments ....................................................... 9
References ............................................................... 9

∗ Corresponding author. Address: Ludwig Boltzmann Institute for Cancer Research, Waeldlergasse 13a, 1080 Vienna, Austria. Tel.: +43 14277 64111; fax: +43 14277 6441.
E-mail address: richard.moriggl@bkr.bmg.ac.at (R. Moriggl).

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19
1. Introduction

The liver plays an important role in whole-body metabolism and energy homeostasis. Depending on the body’s needs, hepatocytes coordinate these processes by regulating gene expression programs in response to various humoral signals. Imbalances in this control system are associated with a variety of liver pathologies ranging from hepatic steatosis to end-stage liver disease including hepatocellular carcinoma (HCC) (Feldstein, 2010; Bechmann et al., 2011).

Growth hormone (GH) and glucocorticoids (GCs) are necessary for normal growth and development, as well as immune functions (Sapolsky et al., 2002; Lea et al., 2002). Further, both factors are important regulators of whole-body energy homeostasis (Veg中原opoulos and Herzog, 2007; Moller and Jorgensen, 2008; Vijayakumar et al., 2010). At the cellular level, GH action is mediated amongst others by the signal transducer and activator of transcription (STAT) 5 pathway. Through STAT5, GH controls many features of liver physiology, including regulation of genes associated with somatic growth and maturation (Hennighausen and Robinson, 2006). GCs and other stress hormones modulate the expression of cytokines, GH receptor (GHR), and the glucocorticoid receptor (GR). The GR acts as a transcriptional regulator of distinct target genes via direct DNA binding or through protein–protein interactions with other transcription factors (Kassel and Herrlich, 2007).

Whole-body STAT5- or GR-knockout mice are not viable, demonstrating the importance of both transcription factors for development and survival (Cote et al., 1995; Cui et al., 2004). Therefore, conditional knockouts were key to explore the functions of both transcription factors in liver physiology. Hepatic deficiency of STAT5 or the GR results in a comparable retardation of postnatal body growth (Tronche et al., 2004; Engblom et al., 2007). From these studies, it became evident that transcription of distinct STAT5-target-gene subsets requires binding of the GR onto the STAT5-binding motif, the major STAT5 isoform expressed in the liver. This interaction preferentially affects gene sets involved in somatic growth and maturation (Engblom et al., 2007). Yet, although both transcription factors are important for the maintenance of energy homeostasis, the phenotypes obtained upon conditional deletion of either gene in mice with regard to lipid and glucose metabolism are rather distinct. Interference with hepatic GC–GR signaling is associated with defects in gluconeogenesis leading to fasting hypoglycemia, with no effects on hepatic lipid homeostasis under basal conditions (Tronche et al., 2004; Mueller et al., 2011; Friedrich et al., 2012).

In the following, we will summarize the consequences of genetic GR or Stat5 deletion for body growth, metabolic homeostasis, hepatic steatosis and HCC development.

2. Mechanism of GH-STAT5 and GC-GR action

2.1. GH signaling and STAT5

GH is an important regulator of postnatal body growth. In contrast to a nuclear hormone receptor steroid ligand it is a peptide and belongs to the superfamily of cytokines. GH controls gene regulation and cellular reproduction. In addition, GH exerts important functions in energy metabolism (Moller and Jorgensen, 2009; Vijayakumar et al., 2010) and influences the immune system (Lea et al., 2002). GH is part of the somatotropic axis and it is synthesized and secreted by the anterior pituitary gland. Its secretion is under strict hormonal control. Hypothalamic growth hormone–releasing hormone (GHRH) is the central stimulator of GH synthesis, while hypothalamic somatostatin exerts strong inhibitory effects (Schneider et al., 2003). Other factors stimulating GH release are acute stress and energy deprivation (Moller and Jorgensen, 2009), whereas overnutrition and obesity inhibit its secretion (Scacchi et al., 1998; Flores-Morales et al., 2006).

At the cellular level, GH action is mediated via the GH receptor (GHR), which is widely expressed in many tissues such as liver, muscle and adipose tissue. GH signaling is very similar both from receptor binding and signal transduction to proline, erythropoietin and thrombopoietin signaling. These four cytokine signaling receptors all make homodimers upon cytokine binding, and their main tyrosine kinase responsible for signal transduction is the cytoplasmic Janus kinase 2 (JAK2). GHR binding leads to the activation of multiple signaling pathways, including the RAS/RAF/MEK, the PI3K and the JAK/STAT pathways (Lanning and Carter-Staunton, 2006; Waters et al., 2006). Although STAT1 and STAT3 can be activated through GHR signaling, STAT5 activation is the major target (Zhu et al., 2001) (Fig. 1). Yet, in the absence of STAT5 expression, increased GR, and the nuclear hormone receptor family (Kassel and Herrlich, 2007; Mueller et al., 2011). STAT5 consists of two different but highly homologous isoforms STAT5A and STAT5B (referred to as STAT5), which are encoded by two juxtaposed genes on mouse chromosome 11 and human chromosome 17. Both STAT5 isoforms differ in their tissue distribution (Hennighausen and Robinson, 2006). Activated GHR brings two JAK2 molecules into proximity, which causes autoactivation, and subsequent tyrosine phosphorylation of preactivated STAT5 molecules. Subsequently, activated STAT5 translocates to the nucleus, where it binds specific DNA binding response elements (REs), usually an inverted repeat of TICN4GAA, to modulate target gene transcription (Zhu et al., 2001; Kornfeld et al., 2008). STAT5 was also shown to be an efficient chromatin regulator and it can induce loop formation through oligomerization via the N-terminus (Nortieg et al., in press; Kornfeld et al., 2008). Whether hepatic STAT5B participates in oligomerization is controversial, yet, multiple STAT5 REs in target genes as in Socs2 and Igf1-s suggests that possibility (Laz et al., 2006). Amongst other functions, the classical STAT5 target genes Igf1 and Socs2 serve to down-regulate GH signaling, thereby establishing a negative feedback loop (Flores-Morales et al., 2006; Cui et al., 2007).

2.2. GC signaling and GR

Main biological functions of the GC-GHR pathway include the suppression of inflammation (Webster et al., 2002) and the control of energy metabolism in metabolically active organs (Veg中原opoulos and Herzog, 2007). Secretion of GCs by the adrenal cortex is under control of the hypothalamic–pituitary–adrenal (HPA) axis, a neuroendocrine feedback system. Activation of the HPA axis is influenced by various stresses, such as energy deprivation, by the circadian clock and inflammation (Tsigos and Chrousos, 2002; Buckley and Schatzberg, 2005; Lamia et al., 2011). The subsequent release of hypothalamic corticotropin releasing hormone (CRH) stimulates synthesis and secretion of pituitary adrenocorticotropic hormone (ACTH) and the following ACTH-induced stimulation of adrenal GC synthesis. GCs, in turn, control the regulation of basal HPA axis activity, thereby establishing a regulatory feedback loop.

Cellular action of GCs is attributed to their binding to intracellular GR, a member of the superfamily of cytokines. GCs controls gene regulation and cellular reproduction. In addition, GH exerts important functions in energy metabolism (Moller and Jorgensen, 2009; Vijayakumar et al., 2010) and influences the immune system (Lea et al., 2002). GH is part of the somatotropic axis and it is synthesized and secreted by the anterior pituitary gland. Its secretion is under strict hormonal control. Hypothalamic growth hormone–releasing hormone (GHRH) is the central stimulator of GH synthesis, while hypothalamic somatostatin exerts strong inhibitory effects (Schneider et al., 2003). Other factors stimulating GH release are acute stress and energy deprivation (Moller and Jorgensen, 2009), whereas overnutrition and obesity inhibit its secretion (Scacchi et al., 1998; Flores-Morales et al., 2006).

At the cellular level, GH action is mediated via the GH receptor (GHR), which is widely expressed in many tissues such as liver, muscle and adipose tissue. GH signaling is very similar both from receptor binding and signal transduction to proline, erythropoietin and thrombopoietin signaling. These four cytokine signaling receptors all make homodimers upon cytokine binding, and their main tyrosine kinase responsible for signal transduction is the cytoplasmic Janus kinase 2 (JAK2). GHR binding leads to the activation of multiple signaling pathways, including the RAS/RAF/ERK, the PI3K and the JAK/STAT pathways (Lanning and Carter-Staunton, 2006; Waters et al., 2006). Although STAT1 and STAT3 can be activated through GHR signaling, STAT5 activation is the major target (Zhu et al., 2001) (Fig. 1). Yet, in the absence of STAT5 expression, increased GR, and the nuclear hormone receptor family (Kassel and Herrlich, 2007; Mueller et al., 2011). STAT5 consists of two different but highly homologous isoforms STAT5A and STAT5B (referred to as STAT5), which are encoded by two juxtaposed genes on mouse chromosome 11 and human chromosome 17. Both STAT5 isoforms differ in their tissue distribution (Hennighausen and Robinson, 2006). Activated GHR brings two JAK2 molecules into proximity, which causes autoactivation, and subsequent tyrosine phosphorylation of preactivated STAT5 molecules. Subsequently, activated STAT5 translocates to the nucleus, where it binds specific DNA binding response elements (REs), usually an inverted repeat of TICN4GAA, to modulate target gene transcription (Zhu et al., 2001; Kornfeld et al., 2008). STAT5 was also shown to be an efficient chromatin regulator and it can induce loop formation through oligomerization via the N-terminus (Nortieg et al., in press; Kornfeld et al., 2008). Whether hepatic STAT5B participates in oligomerization is controversial, yet, multiple STAT5 REs in target genes as in Socs2 and Igf1-s suggests that possibility (Laz et al., 2006). Amongst other functions, the classical STAT5 target genes Igf1 and Socs2 serve to down-regulate GH signaling, thereby establishing a negative feedback loop (Flores-Morales et al., 2006; Cui et al., 2007).
Fig. 1. Signal transduction pathways induced by GH and GCs. GH binding to a GH receptor induces a conformational change that activates two JAK2 molecules which results in phosphorylation of multiple cytoplasmic residues in the cytoplasmic domain of the JAK. The activated JAK2 tyrosine kinase phosphorylates STAT3 proteins by tyrosine phosphorylation. Activation of STAT3 allows GH to elicit diverse biologic and physiological effects. STAT3 targets include anti-apoptotic genes and genes that promote cell cycle progression as well as inhibition. STAT5 promotes also regulate expression of genes involved in growth, differentiation, RNA biogenesis and metabolism. In addition, GH-activated JAK2 activates multiple signaling proteins and pathways including STAT1, MAPK and PI3K signaling. The binding of GH to GHR may also activate IC, protein kinase, initiating other signaling pathways. The IC is retained in the cytoplasm as part of a chaperone-containing multiprotein complex. GCs can diffuse freely across the plasma membrane. Upon ligand binding, GC dissociates from its chaperone complex and translocates into the nucleus, where it exerts transcriptional effects via direct DNA binding at GREs or through a GRE-independendent distinct protein–protein interaction mechanism. GC target genes include amongst others some limiting enzymes of glucocorticoids. Additionally, the GC regulates GH–STAT3–dependent transcription of gene sets involved in postnatal body growth and maturation in a cell type-dependent manner (displayed in gray). GH, Growth hormone; GC, glucocorticoid; GHR, GH receptor; JAK, Janus kinase 2; STAT, signal transducers and activators of transcription; SOCS, suppressors of cytokine signaling 2; GC, Cyclic Adenosine Monophosphate; MAPK, mitogen-activated protein kinase; GC, glucocorticoid receptor; GRE, glucocorticoid responsive elements; HSP90, heat shock protein 90; Src, sarcoma kinase; MEK, mitogen-activated protein kinase kinase; Fos, Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; AKT, PTEN, phosphatase and tensin homolog.

cytosol or translocates to the nucleus (Rauch et al., 2010). In the nucleus the GR acts as a transcriptional regulator of distinct GC-responsive target genes via direct DNA binding at glucocorticoid response elements (GREs) as a dimer. Alternatively, GR can also modulate the expression of genes through a GRE-independent mechanism, which is mediated in part through protein–protein interactions with other transcription factors or coactivators (Fig. 1) (Kassel and Herrlich, 2007; Beck et al., 2011). The degree to which extent the GR dimerization or the monomeric activity contributes to physiological effects of GCs varies dependent on the type of process studied (Rauch et al., 2010; Baschant et al., 2011; Kleiman et al., 2011).

2.3. STAT5 and GR synergy in hepatocytes

DNA binding of the GR is believed to mediate most of its activating function, while cross-talk with other transcription factors is thought to mediate the repressive actions (Reichert et al., 1998; 2001). One of the exceptions, in which GR activates transcription without classic DNA binding, is its functional interaction with STAT5. This synergy was first shown for STAT5-dependent transcription of the β-casein gene, which requires the GR as a transcriptional coactivator (Strocheck et al., 1996, 1997). It became evident, that activated STAT3 and GR form complexes which bind DNA and regulate gene expression independently of GREs. The idea that the GR interacts with DNA-bound STAT3 as a coactivator was supported by the finding that GR mutants deficient for dimerization and DNA binding (GRΔIg) still synergize with STAT5 (Strocheck et al., 1997). More recently, it was shown that protein–protein interaction of hepatic STAT5 and GR is essential for many of the functions exerted by either transcription factor in vivo (Engblom et al., 2007). Many functions of the GR depend on its cofactor interaction as revealed through the study of mice with a knock-in of the DRC-defective GRΔD, which display relatively normal GH target gene expression pattern in liver (Reichert et al., 1998; Troke et al., 2004). Whole genome expression analysis of STAT5, GR and STAT5/GR knockdown livers revealed that genes positively regulated by either transcription factor overlap to a large extent (Engblom et al., 2007). More than 40% of genes significantly downregulated in GR-deficient livers were also downregulated in absence of STAT5. Correspondingly, almost 30% of genes downregulated in STAT5-deficient livers were also downregulated in the absence of GR. The magnitudes of expression changes highly correlated between all three genotypes and it became evident that STAT5–GR synergy preferentially affects gene sets involved in growth and maturation. Noteworthy, the expression changes elicited by STAT5
and the GR correlate closely with those found for various truncations of the GHR (Rowland et al., 2005). Moreover, observed changes for male-predominant genes were in line with earlier reports (Cledder et al., 2006) which reported maturation-related sexual dimorphic liver gene expression to be dependent on GH action and hepatic STAT5. Further, whole genome expression analysis of livers from mice expressing hypomorphic N-terminally truncated STAT5A and STAT5B proteins (referred to as STAT5<sup>hm</sup> mice) suggests that many of GH target genes depend on an intact STAT5N-terminus (Engblom et al., 2007). Indeed, in accordance with earlier studies STAT5–GR synergism in hepatocytes was found to be independent of DNA-bound GR, but highly dependent on functional STAT5–GR protein–protein interaction (Figs. 1 and 2A). This interaction requires the STAT5N-terminus and the AF-1 domain of the GR as the protein-binding interfaces (Stoecklin et al., 1997; Engblom et al., 2007). To date, there is little information whether other cell types or other cytokines and growth factors which signal through STAT5 display a similar STAT5–GR cofactor interaction and synergistic gene transcription apart from GH signaling. Future research is required to elucidate if STAT5–GR interaction is a selective requirement for the activation of specific gene sets in hepatocytes or if that concept is of general importance for other cell types.

### 3. STAT5 and GR function in body growth

As evidenced by patients with abnormally low circulating GH levels or mutations in core genes of the GH pathway disrupting GH-signaling (Laron’s syndrome) (Laron et al., 1965; Rosenfeld et al., 2007; Brooks and Waters, 2010), a major function of GH is regulation of longitudinal body growth as well as of all internal organs excluding the brain. Here, mutations have been identified in core genes of GH signaling, which affect the GHR itself, STAT5B, as well as IGF-1 (Rosenfeld et al., 2007; Brooks and Waters, 2010). As verified by mouse knockout models, the regulation of body size by GH is mainly executed by the activation of STAT5B which, in turn, mediates the transcription of Igf-1 and acid labile subunit (Ab) (Oei et al., 1988; Woelfle et al., 2003a,b). IGF-1 and Ab form a trimeric complex together with IGF-1 binding protein 3, termed biactive IGF-1, in the serum (Dai and Baxter, 1994; Jones and Clemmons, 1985) to promote cellular growth and to control neuroendocrine functions. The first indication that STAT5B is an essential mediator of postnatal body growth came from mice which either lack the Stat5a or Stat5b gene, STAT5B-deficient males but not females suffer from impaired growth (Liby et al., 1997), while STAT5A-deficient mice exhibit normal body stature (Liu et al., 1997). The expression of N-terminally truncated STAT5A and STAT5B proteins in STAT5<sup>hm</sup> mice affects postnatal body growth in either sex. However, STAT5<sup>hm</sup> females displayed more pronounced dwarfism (Teglund et al., 1998). To evaluate the impact of hepatic GH-STAT5 signaling and liver-derived IGF-1 on body growth, hepatocyte-specific knockout mice targeting the core components of GHR-STAT5 signaling, the GHR (Fan et al., 2009), JAK2 (Sos et al., 2011) and STAT5 (Cai et al., 2007; Engblom et al., 2007; Friedbichler et al., 2012) were generated. All mice
deficient in hepatic GHR-JAK2-STAT5 signaling are GH insensitive, with blunted hepatic Igf-1 mRNA expression and severely reduced bioactive IGF-1 resulting in elevated plasma GH. Interestingly, despite comparably reduced levels of circulating IGF-1, the phenotypes of the different mouse lines are quite diverse in regard to postnatal body growth. Liver-specific deletion of STAT5 (Cui et al., 2007) or the GHR (Fan et al., 2009) using a Cre recombinase under the control of the albumin gene promoter (Alb-Cre; Yokay et al., 1999) did not lead to a reduction in body growth. Yet, deletion of STAT5 mediated by a hepatocyte-specific Cre recombinase under the albumin gene promoter and the β-fetoprotein gene enhancer (Alb-Cre; Kellendorn et al., 2000) results in stunted body growth (Engblom et al., 2007), while Alb-Cre-mediated deletion of JAK2 causes a modest but significant decrease in body weight and length (Soo et al., 2011). The observed variations in growth phenotypes remain poorly understood and may partly reflect differences in gene deletion efficiency and mouse genetic backgrounds.

A recent study by our group using an Alb-Cre-mediated STAT5 knockout in the settings of systemic GH overexpression provided an explanation that STAT5 signaling in liver is obligatory for GH and GH-stimulated body growth (Friedrichscher et al., 2012). Overexpression of GH in mice leads to an alteration of body proportions resulting in an acromegaly-like phenotype and differential enlargement of internal organs (Eiken et al., 2006; Iida et al., 2004). In contrast, up to 9 weeks of age mice overexpressing GH but lacking hepatic STAT5 display body growth identical to that of wild type (wt) littermates. Therefore, STAT5 deficiency in liver, despite the presence of GH overexpression, results in growth retardation similar to that caused by STAT5 deficiency alone (Fig. 2). Noteworthy, despite the obvious reduction in body size, the bone length of animals systemically overexpressing GH yet lacking STAT5 in hepatocytes is 5% above that of wt littermates (Friedrichscher et al., 2012). These observations indicate that a direct action of GH on bone and muscle and a paracrine function of IGF-1 (Yokay et al., 1999; Klover and Hennighausen, 2007) are not fully sufficient to compensate for the loss of hepatic GH-STAT5 signaling. Of note, liver-specific ablation of the GR leads to similar growth retardations as observed for STAT5-deficient mice while combined mutations do not add significantly to the growth impairment caused by single mutations (Trönche et al., 2004; Engblom et al., 2007). Accordingly, the growth retardation of STAT5−/− mice is in line with a defect in GR cofactor recruitment, since the N-terminus of STAT5 needs to physically recruit the GR for protein–protein interaction and gene regulation (Fig. 2) (Engblom et al., 2007).

4. GH-STAT5 and GR function in metabolism and hepatic steatosis

The liver plays a central role in the control of glucose and lipid metabolism as it is the major site for interconversion, distribution and storage of energy metabolites. Imbalances in hepatic metabolic function are tightly linked to non-alcoholic fatty liver disease (NAFLD) and associated metabolic disorders (Feldstein, 2010; Bochmann et al., 2011). At this, GH and GC action is not only essential for normal development and survival; it is also required for maintenance of the body’s overall metabolic homeostasis (Vegiopoulos and Herzog, 2007; Möller and Jorgensen, 2009). Further, whole genome expression analysis of gene networks affected by GH and GC signaling in liver has shown that STAT5 and GR control many aspects of hepatocyte metabolism (Phuc Le et al., 2005; Cui et al., 2007; Engblom et al., 2007; Schirra et al., 2008). In the following paragraphs we discuss the current insights into the regulation of glucose and lipid metabolism by GH-STAT5 and GC-GR signaling.

In particular, we highlight those insights gained from hepatocyte-specific deletions of both transcription factors in mice.

4.1. Glucose metabolism

The liver is the main site for glucose storage in form of glycogen and glucose synthesis via gluconeogenesis. The liver provides this energy metabolite to maintain blood glucose level in times of need and to provide glucose to extrahepatic tissues such as the brain, which uses approximately 25% of total body glucose. Hepatic GC-GR signaling plays a critical role in maintaining blood glucose level, particularly in states of energy deprivation, by directly controlling rate limiting enzymes of gluconeogenesis such as PEPCK and G6Pase (Hanson and Reshef, 1997; van Schaftingen and Gerin, 2002; Opherk et al., 2004). In turn, GC signaling impairs glucose uptake in peripheral tissues, such as skeletal muscle and adipose tissues. Hence, GCs oppose insulin action, which suppresses glucose production by inhibiting hepatic glycogenolysis and gluconeogenesis, and stimulates glucose uptake, storage, and utilization by other tissues (Andrews and Walker, 1999). Thus, it is not surprising that STAT5 deficiency in liver is associated with increased glucose metabolism characterized by insulin resistance and hyperglycemia (Andrews and Walker, 1999; Vegiopoulos and Herzog, 2007). In contrast, mice which are GR-deficient specifically in liver show a mild decrease in blood glucose level (Mueller et al., 2011) and suffer from profound hyperglycemia after prolonged fasting (Opherk et al., 2004). The inability to perform de novo glucose synthesis is associated with a reduced induction of rate limiting enzymes of gluconeogenesis such as PEPCK (Opherk et al., 2004). Plasma GC levels were found to be markedly increased in response to fasting (Opherk et al., 2004), while we found an elevation of ACTH and GC levels in these mice already under basal conditions (Mueller et al., 2011). This elevation of systemic GCs in mutant animals might reflect the body’s attempt to sustain gluconeogenesis upon GR deficiency. Interestingly, a similar compensatory hypercortisolism is also reported in mice deficient for hexose-6-phosphate dehydrogenase. These mice lack 11β-hydroxysteroid dehydrogenase type 1 dehydrogenase activity which leads to decreased intracellular levels of corticosterone and subsequent defects in glucose metabolism as well as impaired responses of hepatic enzymes to fasting (Rogoff et al., 2007). A second possible cause for the compensatory activation of the HPA axis is an increased expression and release of liver-derived corticosterone metabolites (CBG), which binds the majority of GCs in the circulation to retain them in a biologically inactive form (Rosner, 1990). GCs suppress CBG expression, hence, GR knockout mice show increased hepatic CBG expression (Mueller et al., 2011) and display high basal CBG levels that are not suppressed by the synthetic glucocorticoid Dexamethasone (Cole et al., 1989). The increase in CBG and a decrease in free GCs might play a role in elevated HPA activity due to decreased GC bioavailability, as observed in studies with gonadectomized rats and in a porcine model (Usawa et al., 2004; Vlau and Moseley, 2004).

Further, in response to hepatic GR deficiency and the concomitant impairment of the liver to counteract low energy levels by gluconeogenesis, a compensatory increase in ghrelin (Opherk et al., 2004) and a parallel decrease in insulin were observed (Opherk et al., 2004; Mueller et al., 2011). Elevated hepatic glucogenesis is a major contributor to hyperglycemia in type 2 diabetes. Here, the states of insulin resistance or deficiency favor glucose synthesis by increased GC levels. Indeed, enhanced PEPCK-dependent activation of PEPCK expression is reported in some murine models of obesity and diabetes, which can be limited by GR antagonism (Liu et al., 2006, 2008). In addition, different approaches using antisense oligonucleotide-mediated downregulation of GR expression show that this improves fasting hyperglycemia and systemic glucose
homeostasis in diabetic mice without affecting blood GC levels (Li et al., 2005; Watts et al., 2005). Systemic GR antagonism and the antiinflammatory dexamethasone approach does not allow to determine inhibition of hepatic GC-GC signaling on diabetes related hyperglycemia, as GC signaling in other organs and concomitant inhibition of glucose uptake, is most likely also impaired. An indication for hepatic GR as a critical inducer of diabetic hyperglycemia came from streptozotocin-induced diabetes in hepatocyte-specific GR knockout mice, which display less severe hyperglycemia and lack hepatic PEPCK mRNA expression upon diabetic challenge (Opherk et al., 2004).

GH has both chronic and acute effects on glucose metabolism. The latter are designated as temporary insulin-like effects, and their physiological significance is not clear. The well studied chronic effects of GH oppose, like those of GCs, insulin action on glucose metabolism (Devadsson, 1987). Excess GC is associated with impaired glucose tolerance, compensatory hyperinsulinemia, insulin resistance, and fasting hyperglycemia. GH deficiency, on the other hand, is linked to enhanced insulin sensitivity, decreased fasting glucose levels, decreased insulin secretion, and lowered hepatic glucose production (Cox et al., 1997). The counterpart to these studies described above is the bovine GH transgenic mouse model, which displays hyperinsulinemia and insulin resistance in response to excess GH exposure, but nearly normal glucose tolerance (Valera et al., 1985; Balbiss et al., 1990). Additionally, hepatocyte-specific impairment of GH-STAT5 signaling by targeting the GHR or STAT5 induces a state of insulin resistance, which manifests itself in profound hyperinsulinemia and glucose intolerance (Cui et al., 2007; Fan et al., 2009; Mueller et al., 2011). In regard to the induction of an insulin resistant state, interference with hepatic GH-STAT5 signaling closely resembles the anti-insulin actions described in GH transgenic mice. Here, chronic GH excess results in a diminished response to insulin injection particularly in skeletal muscle at the level of IRS-1 phosphorylation and downstream signaling events such as PKB activation (Dominici et al., 2002). More recently, excess GH exposure in mice and the acquired insulin resistance was linked to enhanced expression of p85α regulatory subunit of PI3K accompanied by a decrease in IRS-1-associated PDK activity in the skeletal muscle and white adipose tissue (Barbour et al., 2005; del Rincon et al., 2007). Hepatocyte-specific impairment of GH-STAT5 signaling induces GH insensitivity of the liver and a corresponding elevation of p42/44 signaling. As GH signaling in other tissues remains intact, it is tempting to speculate that a defective insulin receptor signaling in muscle and adipose tissue as observed in GH overexpressing mice partly accounts for insulin resistance. Moreover, deletion of hepatic STAT5 markedly impairs downstream insulin signaling in the liver (Mueller et al., 2011) and favors an upregulation of hepatic p85α mRNA expression (Mueller et al., unpublished observation). A predominant role for defective hepatic insulin signaling is also described in mice with liver-specific knock-out of IR, where impaired IR signal transduction contributes to fasting hyperglycemia (Michael et al., 2000). Interestingly, in the settings of hepatitis STAT5 deficiency and the associated GH insensitivity of the liver, the additional lack of GR in hepatocytes is not sufficient to ameliorate hyperglycemia. In summary, STAT5 and STAT5/GR knockout mice are equally affected by hyperinsulinemia and insulin resistance (Mueller et al., 2011).

4.2. Lipid metabolism and hepatic steatosis

Hepatic triglyceride (TG) stores are determined by the balance of fatty acid (FA) uptake and release, de novo lipogenesis, and oxidative clearance, a complex process regulated at pre-receptor, transcriptional and posttranscriptional levels (Brown and Horton, 2004; Desvergne et al., 2006). Defects in GH and GC signaling pathways have been implicated in NAFLD development. Chronically increased GC levels are associated with a fatty liver phenotype in NAFLD (Targher et al., 2006). Moreover, fatty degeneration of hepatocytes was demonstrated in patients with Cushing’s syndrome (Shahidi-Rahhal et al., 2006) and fatty liver disease is also a typical side effect of long-term systemic GC treatment, e.g., immunosuppressive therapy (Sacke et al., 2002). GH has pronounced lipolytic effects and under conditions of food deprivation provides peripheral tissues with ketone bodies as an alternative energy source to glucose. Excess GH as observed in untreated acromegaly, is associated with increased lipolysis, decreased fat mass and an abnormal lipid profile (Moller and Jorgensen, 2009). GH loss of function mutations, on the other hand, causes NAFLD in Turner (Coelho et al., 2008), and is linked to obesity, which may be a reflection of attenuated GH action (Moller and Jorgensen, 2009). On systemic GH deficiency, as found in GH-deficient mouse, GH is secreted in large quantities, but it lacks biological effects due to deficiency of its receptor (Zhou et al., 1987). In contrast, Ames dwarf mice (Prop0/0) suffer from primary pituitary function and GH secretion from the anterior pituitary (Sorson et al., 1996). However, both GH-null and Ames dwarf mice exhibit a state of hypersensitivity to insulin and an increased hypoglycemic response to exogenous insulin (Domielic et al., 2002; Dominici and Turyn, 2002). The counterpart to these studies described above is the bovine GH transgenic mouse model, which displays hyperinsulinemia and insulin resistance in response to excess GH exposure, but nearly normal glucose tolerance (Valera et al., 1985; Balbiss et al., 1990). Additionally, hepatocyte-specific impairment of GH-STAT5 signaling by targeting the GHR or STAT5 induces a state of insulin resistance, which manifests itself in profound hyperinsulinemia and glucose intolerance (Cui et al., 2007; Fan et al., 2009; Mueller et al., 2011). In regard to the induction of an insulin resistant state, interference with hepatic GH-STAT5 signaling closely resembles the anti-insulin actions described in GH transgenic mice. Here, chronic GH excess results in a diminished response to insulin injection particularly in skeletal muscle at the level of IRS-1 phosphorylation and downstream signaling events such as PKB activation (Domielic et al., 2002). More recently, excess GH exposure in mice and the acquired insulin resistance was linked to enhanced expression of p85 α regulatory subunit of PI3K accompanied by a decrease in IRS-1-associated PDK activity in the skeletal muscle and white adipose tissue (Barbour et al., 2005; del Rincon et al., 2007). Hepatocyte-specific impairment of GH-STAT5 signaling induces GH insensitivity of the liver and a corresponding elevation of p42/44 signaling. As GH signaling in other tissues remains intact, it is tempting to speculate that a defective insulin receptor signaling in muscle and adipose tissue as observed in GH overexpressing mice partly accounts for insulin resistance. Moreover, deletion of hepatic STAT5 markedly impairs downstream insulin signaling in the liver (Mueller et al., 2011) and favors an upregulation of hepatic p85α mRNA expression (Mueller et al., unpublished observation). A predominant role for defective hepatic insulin signaling is also described in mice with liver-specific knock-out of IR, where impaired IR signal transduction contributes to fasting hyperglycemia (Michael et al., 2000). Interestingly, in the settings of hepatitis STAT5 deficiency and the associated GH insensitivity of the liver, the additional lack of GR in hepatocytes is not sufficient to ameliorate hyperglycemia. In summary, STAT5 and STAT5/GR knockout mice are equally affected by hyperinsulinemia and insulin resistance (Mueller et al., 2011).

In contrast, mouse models of GH excess are not steatotic and they have lower hepatic TG content (Wang et al., 2007; Friedlischler et al., 2012), whereas impaired GH-STAT5 signaling in liver perturbs lipid metabolism resulting in liver steatosis even upon excess GH exposure (Friedlischler et al., 2012). Several studies have shed light on the underlying mechanisms how hepatic GHR–STAT5 signaling maintains hepatic lipolysis. Hepatocyte-specific ablation of the GHR (Fan et al., 2009), JAK2 (Sos et al., 2011), and STAT5 (Cui et al., 2007; Mueller et al., 2011) results in progressive steatosis accompanied by elevated liver damage parameters. The increase in TG accumulation in the absence of hepatic GH–STAT5 signaling probably results from an upregulation of genes involved in hepatic fatty acid uptake and/or de novo synthesis. Genetic alterations shared by the before mentioned
models include enhanced expression of Pparγ (FA uptake and synthesis) and its target gene Cd36 (FA uptake), which are both frequently associated with development of fatty liver disorders (Fig. 3) [Brownlee and Horton, 2004; Bechmann et al., 2011]. It was shown that treatment with a Pparγ-specific antagonist leads to reduced Cd36 expression and decreased lipid load in Jak2-deficient livers (Sos et al., 2011). A further study has established that Stat3 binds to the Cd36 gene promoter which might suppress transcription (Barclay et al., 2011). Yet, upregulation of Pparγ itself is presumably not due to loss of a Stats-mediated inhibition. It is rather a consequence of enhanced Gh-dependent Stat1 activation upon hepatic Stat5 deficiency (Cui et al., 2007; Barclay et al., 2011). Secondly, enhanced expression of the pro-oncogenic transcription factor Srebp-1c (Brownlee and Horton, 2004; Bechmann et al., 2011) is a possible cause for steatosis in the absence of Gh-Stat5 signaling (Fig. 3). Hepatic Gh and Stat5 deficiency was shown to result in enhanced expression of Srebp-1c and lipogenic downstream targets such as Fas (Fan et al., 2009; Mueller et al., 2011).

Vice versa, overexpression of bovine Gh reduces expression of Srebp-1c and several lipogenic downstream target genes in liver, despite overt hyperinsulinemia, a well-known cause of increased Srebp-1c transcription (Olsson et al., 2003). Consistently, Gh treatment of wt mice leads to decreased hepatic Srebp-1c expression, and Gh-activated Stat5 was found to interact with the Srebp-1c gene promoter. Therefore, it might contribute to a transcriptional inhibition (Mueller et al., 2011). Further, immature Srebp-1c can be activated in response to decreased expression of FasL and Faslg2 (Osborne and Espehade, 2009; Xu et al., 2009), the transcription of which is severely decreased in liver upon hepatocyte-specific Stat5 deletion and upon impairment of Gh signaling (Barclay et al., 2011; Mueller et al., 2011). Overall, the precise mechanisms leading to deregulation of Pparγ and Srebp-1c signaling upon impaired hepatic Gh-Stat5 signaling are not completely understood.

Interestingly, the additional deletion of hepatic Gnr neither ameliorates the steatosis phenotype caused by Stat5 deficiency nor does it improve the gene expression profile of altered hepatic lipid
metabolism, e.g. by down-regulation of Pparγ and G6PD transcription. A combined STAT5/CR deficiency rather results in an even more pronounced hepatocyte TG accumulation (Mueller et al., 2011). The increased hepatic TG load in hepatic STAT5/CR-deficient animals compared to STAT5-deficient animals results from a combination of elevated plasma GC and GH levels, which, in turn, activate STAT5 and GR signaling in adipocytes. Both transcription factors drive the upregulation of adipose tissue triglyceride lipase and hormone sensitive lipase, which are essential for lipolysis (Faim et al., 2008; Mueller et al., 2011). The subsequent increase in available plasma FFA in combination with enhanced expression of the fatty acid transporter CD36 in hepatocytes facilitates additional TG accumulation in the liver (Fig. 3).

In conclusion, hepatic GH-STAT5 signaling protects the liver from steatosis by ensuring proper transcriptional control of genes involved in regulation of hepatic de novo lipogenesis and FA uptake.

5. GH-STAT5 signaling and HCC

Hepatocellular carcinoma (HCC) is a common complication of chronic liver disease and in most cases carcinogenesis follows a sequential process, with cirrhosis as an intermediate key step (Fekete, 2010; Bechmann et al., 2011). However, development of HCC is increasingly observed in absence of advanced liver injury and cirrhosis (Paradis et al., 2009; Stanley et al., 2010; Bechmann et al., 2011). In this regard, independent risk factors for HCC include obesity and diabetes (Calle et al., 2003; Paradis et al., 2009). STAT5 activation is oncogenic in hepatocarcinoma cells and it correlates with poor prognosis in myeloid leukemia. However, the role of STAT5 protein activation in carcinomas is more complicated and cell type specific. Persistent STAT5 activation correlates with a good prognosis in breast cancer, while in prostate cancer the opposite was reported (reviewed in Ferbeyre and Mertens (2011)). So far, there is little information regarding the impact of hepatic GH-STAT5 signaling on HCC development. Increased STAT5B activity was reported to correlate with more aggressive tumors and poor clinical outcomes in Hepatitis B virus-related HCC due to increased cell mobility and concomitant tumor spread (Lee et al., 2006). In patients with liver cirrhosis, a premalignant condition, the GH-IGF-1 axis is known to be severely impaired caused by a state of acquired hepatic GH resistance (Picardi et al., 2006), which is indicative of low STAT5 activity. In parallel, a mouse model of cholestatic liver disease with ablated hepatic GH-STAT5 signaling displayed an early and more severe liver fibrosis phenotype. This was attributed to the lack of IGF-1 and down-regulation of hepatoprotective factors such as Egfl, Lif, Pp and Hep-4 (Fig. 3) (Blas et al., 2010). Upon CCL4 challenge, hepatic STAT5 deficiency promotes the development of liver fibrosis and, in some cases, HCC as a result of increased STAT5 activation and TGF-β stabilization (Hosui et al., 2009). STAT5 activity in hepatocytes was suggested to promote cell cycle arrest upon chronic hepatocyte injury, while loss of STAT5 signaling favors activation of pro-survival and proliferation pathways. This was limited to (1) reduced expression of the cell cycle inhibitors and STAT5 target genes Cdkn1a and Cdkn2b, and (2) excessive GH-dependent activation of STAT3 in absence of STAT5 (Hosui et al., 2009; Yoo et al., 2011; Yu et al., 2011). Upon additional challenge, in form of increased adipose tissue derived FA influx due to combined GH resistance and hyperperoxidism (Mueller et al., 2011) or GH overexpression (Friedlischler et al., 2012), STAT5-deficient livers develop HCC in the presence of progressive steatosis, despite minor inflammation and fibrotic degeneration. Steatosis combined with elevated plasma FFAs was shown to coincide with increased plasma and liver TNF-α levels and oxidative stress in hepatocytes (Mueller et al., 2011). An elevation of FFA, TNF-α, and ROS levels are typically observed in NAFLD and are associated with persistent hepatocyte damage (Bechmann et al., 2011). These conditions support the accumulation of DNA damage and sustained stress-dependent JNK activity (Mueller et al., 2011), which are both recognized as critical factors in the promotion and progression of human and murine HCC (Luedde et al., 2007; Park et al., 2010; Stanley et al., 2010).

GH overexpression in mice is linked to a severe systemic inflammatory phenotype, reduced life expectancy and the development of liver tumors (Oriani et al., 1990; Bartke et al., 2002). Intriguingly, hepatic STAT5 deficiency reverses all pathologic alterations induced by high GH levels, but leads to a more aggressive form of HCC at earlier time points (Friedlischler et al., 2012). As in the former model, hepatic STAT5 deficiency attenuates increased adipose tissue derived FA accumulation in hepatocytes, subsequent chronic liver damage and accumulation of DNA damage. Accordingly, JNK activity and a related increase in c-Jun expression and activity were also present in this transgenic HCC model. Further, following c-Jun activation (Eiiford et al., 2003) or other oncogenic mechanisms potentially involving STAT3 (Niu et al., 2005) p53 activity was abolished in STAT5-deficient livers. Thereby, diminished activity of p53 downstream signaling might impair clearance of cells harboring DNA damage and favor subsequent fixation of mutations. In this regard, it was shown that persistent activation of STAT5A triggers a permanent cell cycle arrest with characteristics of cellular senescence including activation of p53 and a constitutive activation of the DNA damage response in non-hepatic cells (Mallette et al., 2007a; Cabbrese et al., 2009).

Based on the current state of knowledge, HCC development in the absence of STAT5 might be the result of several direct and indirect mechanisms: (1) Deregulation of STAT5 target genes involved in the protection of hepatocyte integrity and cell cycle inhibition. (2) Increased STAT3 signaling most likely due to misregulation to the GHR in absence of STAT5 accelerates progression of chronic liver disease. (3) Metabolic dysfunctions contribute to increase and DNA damage and subsequent activation of stress kinase signaling. (4) An accumulation of mutations is most likely facilitated by a loss of cell cycle control and tumor-suppressive functions of p53 (Fig. 3). In summary, while some mechanisms by which GH-activated STAT5 contributes to safeguard mechanisms that protect hepatocytes from chronic injury and tumorigenic transformation have been revealed, further work is clearly required to better understand the consequences and possible connection to development of human disease.

6. Concluding remarks

Conditional knockouts targeting the core components of murine hepatic GH-STAT5 signaling and the CR have provided an important tool to obtain mechanistic insights into their role in liver function. We provided an overview of overlapping and distinct functions of hepatic GH-STAT5 and GC-CR signaling in postnatal body growth, glucose/lipid homeostasis and metabolic diseases. Aberrant hepatic lipid and glucose metabolism are closely related to the pathogenesis of the most common liver diseases including their progression to hepatocarcinogenesis. Better understanding of the underlying molecular mechanisms is thus crucial, particularly in light of the increasing prevalence of obesity and its pathological consequences.

A characteristic of metabolic liver disease in the absence of hepatic GH-STAT5/CR signaling is the activation of both signaling pathways in peripheral tissues such as the adipose compartments. Thus, the use of transgenic mice will greatly help to understand the contribution of STAT5 and CR signaling in extra-hepatic tissues to the aberrations in carbohydrate and lipid metabolism. Addition-
ally, the downstream molecular mechanisms of CH-STAT5 and GC-GR signaling particularly in hepatic lipid metabolism and steatosis needs to be further analyzed. While interference with hepatic GR signaling seems to ameliorate TG accumulation in mouse models of fatty liver disease, a protective role for CH-STAT5 signaling has emerged in chronic liver disease including HCC development. HCC is a complex, heterogenous cancer, which develops in a multistep process involving alteration of multiple cellular signaling pathways and impaired tissue homeostasis. Hence, future studies on global gene expression profiling of HCCs will help to pursue the synergism of impaired CH-STAT5 signaling and potentially deregulated tumor suppressor as well as oncogenic pathways in hepatocarcinogenesis.

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References

1.5 Aims of this Thesis

As reviewed in section 1.4, mice deficient in hepatic GH-STAT5 signal transduction develop stable, non-progressive hepatic steatosis, insulin resistance and are more prone to CCl₄- and bile acid-induced liver injury, while knock-down of hepatic GR in mouse models of obesity was shown to ameliorate the steatotic phenotype. A recent study from our group has shown that transcription of distinct STAT5 target gene subsets in hepatocytes (e.g. somatic growth, sexual maturation and detoxification) require cofactor function of the GR. Further, preliminary analysis of mice co-deficient in hepatic STAT5 and GR, has revealed that their combined deficiency aggravates the underlying steatosis phenotype associated with a partial depletion of adipose tissue compartments and hepatic dysplasia.

1) By using these conditional knockout mouse models we aimed to investigate in the course of the first study whether the regulation of hepatic lipid homeostasis requires:

- synergism of hepatic GH-STAT5 and GC-GR signaling or both signaling cascades affect liver metabolism independently
- the contribution of hepatic GH-STAT5 and GC-GR signaling to NAFLD development and its potential progression to HCC

2) The purpose of the second study was to examine the function of STAT5 in the development of HCC. To address this question, we conditionally deleted hepatic Stat5 in a GH transgenic mouse model of chronic liver inflammation with progression to HCC.

3) In two follow-up studies using adipocyte-specific Gr or Stat5 knockout mouse models we further intended to analyze the impact of either transcription factor on adipose tissue lipid mobilization and glucose metabolism.
CHAPTER TWO: RESULTS

2.1 Prologue
Non-alcoholic fatty liver disease (NAFLD) is characterized by aberrant hepatic lipid accumulation resulting from dysfunctional lipid and carbohydrate metabolism. In recent years, it has become evident that NAFLD and its co-morbid conditions such as T2D may increase the risk of primary liver cancer. Unphysiologic levels of GH and GC, whether increased or decreased, have long been implicated in dysfunctional lipid as well as carbohydrate metabolism throughout the body. Several lines of evidence have shown that particularly conditions of impaired GH signaling or hyperactive GC action promotes NAFLD and accompanying co-morbidities. Although the link between one or the other pathway and NAFLD is well documented, the functions of GH signaling mediated by its downstream effector STAT5 as well as GC-induced GR signaling in liver metabolism and disease are less well characterized. Therefore, as introduced in section 1.5, the rationale of this study was to characterize the role of hepatic GH-STAT5 signaling in liver metabolism, its contribution to NAFLD development and progression, particularly, in context of an interaction with GR function. This aim was addressed by using conditional deletion of Gr, Stat5 or both transcription factors in murine liver, which allowed us to study tissue-specific mechanisms underlying the development and progression of NAFLD.
2.1.1 Manuscript: Impairment of Hepatic Growth Hormone and Glucocorticoid Receptor Signaling Causes Steatosis and Hepatocellular Carcinoma in Mice.
Impairment of Hepatic Growth Hormone and Glucocorticoid Receptor Signaling Causes Steatosis and Hepatocellular Carcinoma in Mice

Kristina M. Mueller,1*, Jan-Wilhelm Kornfeld,7*, Katrin Friedbichler,4, Leander Blaas,1 Gerda Egger,3 Harald Eisterbauer,4 Peter Hasselblatt,5 Michaela Schlederer,1 Susanne Haindl,6 Kay-Uwe Wagner,7 David Engblom,8 Guenter Haemmerle,9 Dagmar Kratky,10 Veronika Sed,11 Lukas Kenner,1,3 Andrey V. Kozlov,5 Luigi Terracciano,12 Rudolf Zechner,9 Guenther Schuetz,9 Emilio Casanova,1 J. Andrew Pospisil,14 Markus U. Heim,15 and Richard Moriggl1

Growth hormone (GH)-activated signal transducer and activator of transcription 5 (STAT5) and the glucocorticoid (GC)-responsive glucocorticoid receptor (GR) are important signal integrators in the liver during metabolic and physiologic stress. Their deregulation has been implicated in the development of metabolic liver diseases, such as steatosis and progression to fibrosis. Using liver-specific STAT5 and GR knockout mice, we addressed their role in metabolism and liver cancer onset. STAT5 single and STAT5/GR double mutants developed steatosis, but only double-mutant mice progressed to liver cancer. Mechanistically, STAT5 deficiency led to the up-regulation of proliferogenic sterol regulatory element binding protein 1 (SREBP-1) and peroxisome proliferator activated receptor gamma (PPAR-γ) signaling. Combined loss of STAT5/GR resulted in GH resistance and hypercortisolism. The combination of both induced expression of adipose tissue lipases, adipose tissue lipid mobilization, and lipid flux to the liver, thereby aggravating STAT5-dependent steatosis. The metabolic dysfunctions in STAT5/GR compound knockout animals led to the development of hepatic dysplasia at 9 months of age. At 12 months, 35% of STAT5/GR-deficient livers harbored dysplastic nodules and ~60% hepatocellular carcinomas (HCCs). HCC development was associated with GH and insulin resistance, enhanced tumor necrosis factor alpha (TNF-α) expression, high reactive oxygen species levels, and augmented liver and DNA damage parameters. Moreover, activation of the c-Jun N-terminal kinase 1 (JNK1) and STAT3 was prominent. Conclusion: Hepatic STAT5/GR signaling is crucial for the maintenance of systemic lipid homeostasis. Impairment of both signaling cascades causes severe metabolic liver disease and promotes spontaneous hepatic tumorigenesis. (Hepatology 2011;54:1398-1409)

Hepatic steatosis is estimated to affect >20% of the Western population, with raising incidence partly caused by excess nutrition and a lack of exercise.1 Steatosis as a hallmark of nonalcoholic fatty liver disease (NAFLD) is connected to obesity, insulin resistance, and type 2 diabetes.2 A strong

Abbreviations: ACTH, adrenocorticotropic hormone; ALE, alkaline phosphatase; ALT, alanine aminotransferase; CEBP, cCAAT enhancer binding protein; Dex, dexamethasone; DKO, double knockout; ERK/2, extracellular signal-regulated kinases 1 and 2; FFA, free fatty acids; GCs, glucocorticoids; GH, growth hormone; GR, glucocorticoid receptor; HCC, hepatocellular carcinoma; HSG-70, heat shock cognate 70 kDa protein; IGF-1, insulin-like growth factor 1; IL, interleukin; iNOS, inducible nitric oxide synthase; IR, insulin receptor; IRS, insulin receptor substrate; JNK1/2, c-Jun N-terminal kinase 1 and 2; LXR, liver X receptor; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; NASH, nonalcoholic steatohepatitis; Pim, perilipin; PPAR-γ, peroxisome proliferator-activated receptor gamma; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; ROS, reactive oxygen species; STAT5, signal transducer and activator of transcription 5; SREBP-1, sterol regulatory element binding protein 1; TG, triglyceride; TNF-α, tumor necrosis factor alpha; WAT, white adipose tissue.

From the1 Ludwig-Boltzmann-Institute for Cancer Research, Vienna, Austria; 2Institute for Genetics, Department of Mouse Genetics and Metabolism, University of Cologne, Cologne, Germany; 3Clinical Institute of Pathology, Medical University of Vienna, Vienna, Austria; 4Department of Laboratory Medicine, Medical University Vienna, Vienna, Austria; 5Department of Medicine III, Free University Hospital, Freeburg, Germany; 6Ludwig-Boltzmann-Institute for Experimental and Clinical Traumatology, Vienna, Austria; 7Eppey Institute for Research in Cancer and Allied Diseases and the Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE, USA; 8Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linkoping University, Linkoping, Sweden; 9Institute of Molecular Biogenetics, Graz, Austria; 10Institute of Molecular Biology and Biochemistry, Center of Molecular Medicine, Medical University of Graz, Graz, Austria; 11Institute of Pharmacology and Toxicology, Veterinary University of Vienna, Vienna, Austria; 12Institute of Pathology, University Hospital Basel, Basel, Switzerland; 13German Cancer Research Center, Heidelberg, Germany; 14Max-Planck-Institute of Immunobiology, Freiburg, Germany; and 15Department of Biomedicine, Division of Gastroenterology and Hepatology, University Hospital Basel, Basel, Switzerland.
correlation between steatosis and insulin resistance has been demonstrated in human patients and animal models of NALFD. Persistent hepatic lipid accumulation contributes to chronic inflammation with progression to nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (HCC). Steatosis results from excessive free fatty acid (FFA) synthesis relative to oxidative clearance and/or elevated lipid hydrolysis in adipose tissues. FA synthesis, clearance, and release are, among others, regulated by neuroendocrine factors, such as growth hormone (GH) or glucocorticoids (GCs), whose levels vary under conditions of changing energy supply. Both signaling pathways have been implicated in the development of NALFD and metabolic syndrome. Animal studies have revealed that the transcription of distinct signal transducer and activator of transcription 5 (STAT5) target-gene subsets requires cofactor function of the glucocorticoid receptor (GR). The interaction of STAT5 and GR ensures the proper transcription of genes implicated in postnatal body growth, such as insulin-like growth factor-1 (IGF-1). As serum IGF-1 levels negatively regulate the release of GH in the pituitary, an impairment of this autoinhibitory GH/STAT5/IGF-1 feedback loop leads to GH resistance. This is of clinical interest, because it is tightly associated with metabolic syndrome. Mice lacking STAT5 or the GH receptor (GHR) in the liver acquire characteristic features of GH resistance and develop steatosis and insulin resistance. Importantly, hepatic STAT5 deficiency contributes to CCl4-induced liver fibrosis and HCC development. Furthermore, hepatocyte-specific deletion of JAK2 also results in GH resistance and the development of hepatic steatosis. However, these mice harbor no defects in glucose and insulin homeostasis.

We aimed to investigate whether the regulation of hepatic lipid homeostasis (1) requires synergism of STAT5 and GR signaling or (2) both signaling cascades affect lipid metabolism independently. We confirm previous findings that STAT5 deficiency causes steatosis, insulin resistance, and glucose intolerance. However, the combined deletion of hepatic STAT5 and GR led to severe fatty liver disease resulting from a combination of hepatic GH resistance and hypercortisolism. The former resulted from the liver-specific ablation of STAT5, and the latter was from the deletion of the GR in hepatocytes. A combination of both conditions, as found in compound STAT5/GR mutants, induced peripheral lipodystrophy, additional liver lipid accumulation, and, subsequently, tumorigenic transformation of hepatocytes.

Materials and Methods

Mice. Mice with a hepatic deletion of STAT5 and/or the GR were generated as described. Littermates not expressing Alfp-Cre recombinase served as controls. For experimental procedures, we used male mice, if not stated otherwise. Mice were kept at the Decentralized Biomedical Facilities, Medical University of Vienna (Vienna, Austria), under standardized conditions. All animal experiments were carried out according to an ethical animal license protocol, and our contract was approved by university and Austrian Ministry authorities.

Western Blotting. Liver homogenates were prepared as previously described. Blots were incubated with antibodies against STAT5b (rabbit polyclonal antibody, epitope aa775-788), pY-STAT5 (#71-6900; Invitrogen, Carlsbad, CA), heat shock cognate 70-kDa protein (HSC-70) (sc-7298; Santa Cruz Biotechnology, Santa Cruz, CA), GR (sc-1004; Santa Cruz Biotechnology), and antibodies against total levels and the phosphorylated isoforms of p38, extracellular signal-regulated kinases 1 and 2 (ERK1/2), and c-Jun N-terminal kinases 1 and 2 (JNK1/2).

Other Materials and Methods. Animal and histology procedures, quantitative reverse-transcription polymerase chain reaction (qRT-PCR), serum biochemistry,
determination of hepatic triglyceride levels, immuno- 
histochemistry, and the measurement of reactive oxygen species (ROS) levels are described in the Supporting Materials and Methods.

Statistical Analyses. Results are presented as mean ± standard error of the mean. Statistical analyses were performed by analysis of variance, followed by Dunn’s or Tukey’s post-hoc tests. Data were considered statistically significant (*P < 0.05; **P < 0.01; ***P < 0.001).

Results

Deletion of Hepatic STAT5 and the GR Causes Steatosis and Lipodystrophy. To investigate whether hepatic lipid homeostasis would require STAT5-GR synergism or whether the two transcription factors would affect lipid metabolism independently, we conditionally deleted the GR (GRKO), STAT5 (SSKO), or STAT5 and the GR (double knockout [DKO]) in hepatocytes. Efficient deletion was confirmed by western blotting analyses (Supporting Fig. 1A). Macrophagic hepatomegaly and steatosis were first evident in 2-month-old SSKO and DKO mutants, as compared to GRKO and control mice. Although hepatomegaly in SSKO mutants remained stable, DKO mice displayed progressive fatty liver disease, with a 4-fold increase in liver mass by 12 months of age (Fig. 1A, B) and an 8-fold rise in hepatic triglyceride (TG) content as early as 2 months of age (Fig. 1C). Strikingly, a dramatic depletion of white adipose tissue (WAT) was observed exclusively in DKO mice (~58%; Fig. 1A,B). Histological examination revealed a significantly increased mean score of steatosis in young (83% versus 49%), but not in aged, DKO mice, compared to age-matched SSKO mutants (77% versus 53%; Supporting Fig. 1B). Micro- and macrosclerotic steatosis in SSKO (Fig. 1D, a, c, and k) and DKO mice (Fig. 1D, d, h, and l) was associated with elevated serum levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) as indicators of liver injury (Fig. 1E). In contrast, histochemical analysis revealed a normal liver architecture in control (Fig. 1D, a, e, and i) and GRKO animals (Fig. 1D, b, f, and j) at all time points analyzed. A summary of the histological analysis is given in Supporting Table 1. Taken together, the STAT5-dependent fatty degeneration of hepatocytes is severely aggravated upon additional GR deletion in the liver.

STAT5 Regulates Hepatic De Novo Lipogenesis Independently of GR Coactivator Interaction. Hyperglycemia, hyperinsulinemia, and elevated resistin levels in both STAT5-deficient lines suggested hepatic insulin resistance upon STAT5 loss (Supporting Fig. 2A). Oral-glucose and insulintolerance tests confirmed insulin resistance and glucose intolerance3 (Supporting Fig. 2B). At the molecular level, defects in insulin receptor (IR) signaling, such as reduced tyrosine phosphorylation of the IR, IR substrates 1 and 2 (IRS-1 and -2), and serine phosphorylation of AKT were evident in both STAT5-deficient lines upon insulin administration (Supporting Fig. 2C). We detected increased transcript and protein levels of sterol regulatory element binding protein 1 (SREBP-1) and peroxisome proliferator-activated receptor gamma (PPAR-γ) in SSKO and DKO livers. In line with this, the gene expression of SREBP-1 (Fasn and Stl2) and PPAR-γ targets (Dgat1, Dgat2, and Cebp) was found to be increased. Transcript levels of Ftg21, which negatively control SREBP-1 maturation and activation, were decreased in single-knockout and DKO livers. Furthermore, messenger RNA (mRNA) and protein levels of lipogenic CCAAT enhancer binding protein (C/EBP)α and C/EBPβ were elevated (Supporting Fig. 3A,B; Supporting Table 2). Using chromatin immunoprecipitation analysis, a significantly enriched binding of GH-activated STAT5 to the Srebp-1a and Srebp-1c promoter was observed (Supporting Fig. 3C,D). Furthermore, GH-induced STAT5 activation led to a marked, time-dependent decrease of Srebp-1a and Srebp-1c mRNA levels in control livers (Supporting Fig. 3E).

Hepatic GH Resistance and Hypercortisolism Triggers Lipolysis of Adipose Tissue in DKO Mice. Phenotypically, DKO mice display an aggravation of liver phenotype, compared to SSKO mice. One explanation for the increased hepatic TG load in DKO mice might be alterations in whole-body lipid homeostasis, resulting in enhanced lipolysis of adipocytes and elevated hepatic FFA delivery (Fig. 1A,B). Analysis of epidonadal WAT, brown adipose tissue, and subcutaneous fat from DKO mice revealed a severe reduction in fat depot and adipocyte cell size, compared with control and single-mutant mice (Fig. 2A,B and data not shown). Accordingly, elevated levels of circulating FFA were found in DKO mice (Fig. 2C). As expected, both STAT5-deficient lines showed high serum levels of GH secondary to the loss of negative IGF-1 regulation5 (Fig. 2D, left row). Yet, unexpectedly, GRKO and DKO mutants developed hypercortisolism, that is, elevated serum levels of corticosterone and its positive regulator, adrenocorticotropic hormone (ACTH; Fig. 2D, right row). In line with this, adipocytes from DKO mice showed increased STAT5 and GR activation (Fig. 2E and data not shown). It was
Fig. 1. DKO mice develop severe steatosis, hepatomegaly, and lipodystrophy. (A) Macroscopic appearance of livers and epididymal WAT in mutant and control mice at indicated time points. (B) Liver weight (LW)/body weight (BW) and WAT/BW ratios of mutant and control mice at indicated time points (n = 6/genotype/time point). (C) Hepatic triglyceride content in 2-month-old mice (n = 6/genotype). (D) Liver histology of livers from 6-month-old mice. (a-d) Liver sections were stained with hematoxylin and eosin. (e-h) Lipid accumulation in livers was visualized by Oil Red O on cryosections. (i-l) Electron microscopy analysis of fat distribution in livers of 2-month-old mice (cytoplasmic lipid droplets, green arrows; intrahepatic glycogen granules, black arrows). (E) Serum liver-damage parameters ALT and ALP of 2-month-old mice (n ≥ 5/genotype). *P < 0.05; **P < 0.01; ***P < 0.001.
demonstrated that the simultaneous activation of GH-STAT5 and GC-GR signaling stimulates lipolysis in human adipocytes. Therefore, we quantified the transcript levels of major WAT lipases, that is, adipose triacylglyceride lipase (Atgl) and hormone-sensitive lipase (Hsl). We observed a significant up-regulation of Atgl and Hsl transcripts accompanied by the reduced gene expression of perilipin (Plin), a major coating protein of adipocytes, exclusively in DKO WAT (Fig. 2F). To confirm that synergistic adipose GH/STAT5/GC-GR activation accounted for the induction of lipases and concomitant lipolysis in DKO mice, we pharmacologically mimicked the combination of GH resistance and hypercortisolism. Therefore, we administered the GR agonist, dexamethasone (Dex), to 6-month-old S5KO mice for 14 days. Although Dex treatment had no effect in control animals, Dex treatment severely aggravated hepatomegaly and steatosis in S5KO livers, accompanied by a decrease in WAT size (Fig. 3A,B). Histological analysis further confirmed the aggravation of hepatic steatosis and lipodystrophy in Dex-treated S5KO mice (Fig. 3C). To determine whether systemic GR inhibition would protect from increased WAT lipolysis in DKO mutants, we treated 6-month-old
Fig. 3. Impact of GR agonist or antagonist treatment on WAT lipolysis. (A) Macroscopic appearance of livers and WAT from SSKO mice following 14 days of dexamethasone (Dex) or mock treatment (phosphate-buffered saline). (B) LW/BW (left) and WAT/BW (middle) ratios of 6-month-old mutant mice of indicated genotypes and treatment. (C) Histological analysis of liver and WAT using hematoxylin and eosin-stained sections from control and SSKO mice after Dex treatment. (D) Macroscopic appearance of livers and WAT from DKO mice after 14 days of RU486 or mock (Oil) treatment. (E) LW/BW (left) and WAT/BW (middle) ratios of 6-month-old mice of indicated genotypes and treatment. (F) Levels of FFA were determined after RU486 or mock treatment of control and DKO mice using a colorimetric assay. For Dex and RU486 treatment: n ≥ 4/genotype/treatment. *P < 0.05; **P < 0.01; ***P < 0.001.

DKO mice with the GR antagonist, RU486, for 14 days. Macroscopically, no considerable changes in liver and WAT size of RU486-treated DKO, compared to control, animals could be observed (Fig. 3D,E). Yet, RU486 treatment of DKO mice normalized the amount of serum FFA to levels comparable to RU486- and vehicle-treated control mice (Fig. 3F). Thus, the combination of hepatic GH resistance and hypercortisolemia in DKO mice results in a generalized depletion of adipose stores, which, in turn, aggravates the Stats-dependent fatty liver phenotype.

Spontaneous Development of Hepatocellular Carcinomas in DKO Mice. Despite negligible fibrotic changes and mild inflammatory infiltration (Supporting Table 1), we observed the development of spontaneous liver tumors in DKO mice. Macroscopical examination of livers from 9-month-old DKO mice revealed atypical nodules (2 of 5), whereas SSKO
and control mice showed no evidence of hepatic tumorigenesis at all time points analyzed. Furthermore, detailed phenotypic examination revealed no significant differences between control and GRKO mice (data not shown). Histological analysis of atypical nodules displayed distinct dysplastic lesions with vacuoles of accumulated fat compressing adjacent parenchyma (Supporting Fig. 4A, a-d). At 12 months of age, 35% (6 of 17) of DKO mice displayed dysplastic nodules and 59% (10 of 17) HCCs (Supporting Fig. 4B; Fig. 4A, a-b). Histological examination revealed well to moderately differentiated HCCs either of a (1) nonfatty and solid or (2) lipid-laden-tumor type, both of which displayed nuclear poly- and pleomorphism. Malignant hepatocytes were either growing in solid sheets or tended to aggregate in disorganized laminae (Fig. 4A, c-d; Supporting Fig. 4A, e-f). Periodic acid Schiff (PAS) staining revealed no significant necrotic degeneration of hepatocytes. Interestingly, solid/nonfatty tumors, in particular, displayed increased fibrous, pericellular collagen depositions, as illustrated by Chromotrop Anilinblue (CAB) staining (Fig. 4A, e-h and i-l). Hepatocyte proliferation was enhanced in DKO livers (~8%), compared to SSKO (~3%) and control livers (~1%), as demonstrated by elevated numbers of Ki67-positive hepatocytes (Fig. 4A, m-p). However, no change in apoptotic rates of DKO livers was observed (Fig. 4A, q-t), and gene-expression levels of apoptosis regulating Bcl-2 family members Bcl-2, Bcl-xL, and Bax were only slightly changed (Supporting Fig. 4C). At the time point analyzed, ALT levels were similarly increased in DKO and SS KO mutants, indicating potent hepatocyte damage in both groups. Next, we determined serum levels of the proinflammatory and tumor-promoting cytokines, tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6. TNF-α was strongly elevated in DKO mice, whereas IL-6 levels were unchanged (Fig. 4B and data not shown). On the transcriptional level, we observed a strong up-regulation of Tnf-α and, to a lesser degree, Il-6 mRNA in DKO livers, whereas hepatic Il-1β transcript levels were unchanged (Fig. 4C). Collectively, these data suggest that aggravation of the STAT3-dependent fatty liver phenotype caused by the additional deposition of extrahepatic lipids facilitates liver tumorigenesis.

**Mechanistic Insights in Tumor Development in DKO Mice.** Progressive fatty degeneration of hepatocytes is associated with oxidative stress and subsequent hepatocyte damage, a process shown to contribute to tumorigenesis.19 Global gene-expression and subsequent gene set enrichment analysis revealed deregulated expression levels of several antioxidant genes already in 2-month-old DKO animals (Supporting Fig. 4D). Thus, we measured ROS production and release in liver mitochondria. Extramitochondrial ROS levels in DKO livers were increased ~4-fold over control and ~2-fold over SS KO livers (Fig. 5A). The transcription of inducible nitric oxide synthase, Nos2, which leads to ROS and reactive nitrogen species generation, was also up-regulated in DKO livers. At this time point, transcript levels of the DNA damage-responsive gene, Gadd45a, were strongly elevated, whereas the expression levels of two major antioxidant genes, Sod1 and Sod2, were unchanged (Fig. 5B; Supporting Fig. 4E). Consistent with observed oxidative stress, DKO livers showed increased DNA damage, compared to control and SS KO livers, as assessed by the emergence of phosphorylated histone residues (pH2AX; Fig. 5C). To gain molecular insight in the processes governing the malignant transformation of hepatocytes in DKO animals, we determined the activation of the major stress-dependent MAPK-signaling pathways. These are triggered by continuous liver damage and are known to be involved in the pathogenesis of HCC. Tumor-bearing DKO mice exhibited elevated levels of JNK1 activity in the liver, which was almost absent in control and SS KO hepatocytes. In contrast, activation of ERK1/2 was unchanged between DKO and control livers, whereas p38 activation was reduced in DKO animals (Fig. 5D; Supporting Fig. 4F). HCCs displayed a modest increase in STAT3 phosphorylation, which was recently linked to hepatic tumorigenesis in the setting of chronic liver disease.15,20 In nonliver tumor tissue, however, STAT3 activity was almost not detectable (Fig. 5E). On the transcriptional level, livers from tumor-bearing mice exhibited significant up-regulation of Mhc, Jun, Mmp9, and Vgfa that might contribute to an increased incidence of tumorigenesis (Fig. 5F). In summary, the development of HCCs in DKO mice coincides with oxidative stress and the activation of tumor-promoting JNK1- and STAT3-signaling cascades.

**Discussion**

Hepatic GH- and GC-signaling cascades influence metabolic functions under conditions of altered energy balance and stress. Defects in either of the signaling pathways have been implicated in NAFLD development, including children with NAFLD progressing to end-stage liver disease.10,11,21-25 On the molecular level, steatosis is often associated with enhanced expression of the proangiogenic transcription factors, SREBP-1c and PPAR-γ. Recent studies have revealed
Fig. 4. Spontaneous development of liver tumors in DKO mice. (A) Hepatocellular carcinoma (HCC) formation in 12-month-old DKO mice. (a) Control liver. (b) Macroscopic view of representative DKO liver. Arrows indicate tumors and atypical nodules. Representative hematoxylin and eosin-stained sections showing two different types of HCCs as either (c) solid and nonfatty or as (d) tumors containing lipid droplets. (e–i) Representative PAS staining for glycogen deposition. (j–l) Representative CIAB staining for collagen deposition. (m–p) Quantification of K187-positive hepatocytes by immunohistochemistry showing enhanced proliferation of DKO livers. K187-positive hepatocytes were quantified using HistoQuest Image analysis (n ≥ 5/genotype; TissueGnostics GmbH, Vienna, Austria). (q–t) Representative immunohistochemistry for cleaved caspase 3-positive hepatocytes showing no increase in apoptosis of DKO livers. (B) Serum liver-damage parameters ALT and TNF-α levels of 12-month-old mice (n ≥ 5/genotype). (C) Relative mRNA levels of proinflammatory cytokines were quantified by qRT-PCR in livers from 12-month-old mice and normalized to Gapdh (n = 6/genotype). *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. 5. Oxidative stress-dependent hepatocyte damage and tumor-promoting signaling in DKO livers. (A) Extramitochondrial ROS production. ROS was determined using the 1-hydroxy-3-carboxy-pyrididine spin-trap method (n = 4/genotype). (B) Relative mRNA levels of Nos2 and Gadd45a were quantified by qRT-PCR in livers from 12-month-old mice and normalized to Gapdh (n = 6/genotype). (C) DNA damage in DKO mice. Liver sections were stained with antibodies against phosphorylated H2AX. Positive hepatocytes were quantified using image analysis (n ≥ 5/genotype). (D) Representative western blotting analysis showing protein expression and activation of JNK1/2, p38, and ERK1/2 in 12-month-old mice. HSC-70 served as the loading control. (E) STAT3 activation in DKO HCCs. Liver sections were stained with antibodies against phosphorylated STAT3. Positive hepatocytes were quantified in control, SSKO, and DKO nontumor and DKO tumor tissue using HistoQuest image analysis (n ≥ 5/genotype; TissueGnostics GmbH, Vienna, Austria). (F) Relative mRNA levels of Myc, Jun, Mmp9, and Vegfa were quantified by qRT-PCR in livers from 12-month-old mice and normalized to Gapdh (n = 6/genotype). *P < 0.05; **P < 0.01; ***P < 0.001.
an important role of STAT5 in the prevention of steatosis. This was partly linked to the observation that impairment of hepatic GH-STAT5 signaling causes enhanced gene expression of Pparγ and its target gene, Gd36, which can also be directly regulated by STAT5.3,17 Additionally, hepatic GHR deficiency resulted in enhanced Srebplc expression.4 Further studies have implicated SREBP-1c in Pparγ transcription,24 whereas a bidirectional, inhibitory cross-talk between STAT5 and PPAR-γ was postulated.25 SREBP-1 is most likely activated in response to decreased expression of Fgf21 and Inug2. Both transcripts are reported to be severely decreased upon liver-specific STAT5 deletion as well as upon systemic impairment of GHR signaling.17 Accordingly, we detected a decline in Fgf21 mRNA levels in S5KO livers, which was found to be even more severe upon additional GR deficiency. However, the expression of liver X receptor (LXR) isoforms, which are known to regulate Srebf-1 expression, was not significantly changed (data not shown). Additionally, we show that GH-activated STAT5 interacts with the promoter of both Srebf-1 isoforms, which results in down-regulated expression. Taken together, these observations confirm a GH-STAT5-dependent regulation of hepatic lipogenesis on the transcriptional level, where STAT5 might repress Srebf-1 isoforms. The induction of prolipogenic transcription factors and steatosis was not observed in GRKO livers, and it is also not present upon deletion of the N-terminal GR-interaction domain of STAT5 (as in STAT5AB mice)15 (data not shown). Thus, we consider the induction of SREBP-1 and PPAR-γ-mediated lipogenesis as the primary effect of hepatic STAT5 deficiency. Moreover, hepatocyte-specific deletion of JAK2 causes massive steatosis and GH resistance. Interestingly, the phenotype was linked to increased peripheral GH-induced lipolysis and cluster of differentiation 36–mediated hepatic uptake of FFA, which could be rescued by the abrogation of GH secretion or partially normalized by antagonistic PPAR-γ action.16

Other conditions associated with steatosis are insulin resistance and glucose intolerance.1,6 On the molecular level, insulin resistance is characterized by defects in IR signaling, which is observed upon the hepatocyte-specific deletion of the GH receptor or STAT5,3,4 but not upon ablation of hepatic JAK2.10 Insulin resistance might be explained by observations made by others and similarly by us as follows. (1) A decrease in IRS-2-mediated signal transduction was accompanied by increased SREBP-1c associated with insulin resistance.26 Furthermore, when mice on a high-fat diet were treated with ezetimibe, a selective inhibitor of intestinal cholesterol absorption, down-regulation of hepatic SREBP-1c and reversed insulin resistance (IR) was a consequence, which was associated with increased pY-IRS-2 and pS-AKT.27 Similarly, we observed increased SREBP-1c mRNA and protein level in S5KO and DKO livers as well as impaired IR signal transduction. (2) The absence of insulin resistance in mice deficient for hepatic JAK2 might hint at a role of hepatic STAT5 in propagating IR signal transduction. It was shown that STAT5 is a physiological substrate of the IR in vitro and in tissues sensitive to insulin. Importantly, signaling through STAT5 upon insulin stimulation is JAK2 independent, 58,29 and insulin-stimulated STAT5 was shown to bind to the glucokinase promoter.30

Hepatic GH-STAT5 signaling influences GH/IGF-1 and insulin levels in the circulation,3,4 whereas GR signaling counteracts the effects of high stress-hormone (e.g., corticosterone and ACTH) levels. GCs have been shown to suppress hepatic CBG (Serpina6) expression. Hence, GR knockout mice display high basal CBG levels that are not suppressed by Dexamethasone.31 Accordingly, we observed elevated Serpina6 expression levels in GR-deficient livers, whereas expression of the GC-level regulating enzyme, 11β-HSD1, was unchanged (Supporting Fig. 5). This might suggest that upon hepatic GR deficiency, increased CBG expression results in elevated total serum GC levels. However, the increased CBG expression might lead to decreased unbound, active GCs and a subsequent decrease in negative feedback regulation, followed by enhanced ACTH and GC secretion.32,33 Consistent with the notion of a strong induction of adipose tissue lipolysis by combined action of GH-STAT5 and GC-GR signaling, the enhanced shuffling of peripheral lipids to the liver was observed only in DKO mice. This process was associated with down-regulation of Plin34 and increased expression of Hsd and Agrp35,36 in WAT, which triggered lipid mobilization from adipose tissue.

Recent studies revealed that hepatic GH-STAT5 signaling not only prevents steatosis, but also has protective functions in the context of genetically or chemically induced liver fibrosis and cancer development.15,37 (Friedlβlich et al., unpublished). In addition, STAT5 counterbalances unscheduled cellular proliferation by inducing the cell-cycle inhibitors, Cdkn2b and Cdkn1a.38 This suggests that STAT5-deficient livers are more sensitive to hepatocyte damage and malignant transformation. Coupling the preexisting steatosis in STAT5-deficient livers with increased adipose tissue-derived lipid fluxes causes the spontaneous
development of liver tumors in DKO mice. Other studies have applied genetic, chemical, and dietary-based liver insults to mimic chronic liver disease, and demonstrated that these conditions facilitate HCC development. However, the onset of HCCs in our model occurred in settings of progressive steatosis, despite minor inflammation and fibrosis. This was also reported in genetically obese mice, which develop spontaneous hepatic hyperplasia and harbor an age-dependent risk of HCC formation in the absence of apparent inflammation and fibrosis. Notably, also, in patients, the development of HCC is increasingly observed in the absence of advanced liver injury, with metabolic syndrome as the only identified risk factor. We suggest that tumorigenesis in DKO livers is a direct effect of the massive lipid accumulation causing persistent liver damage partly via increased mitochondrial ROS production and leakage. STAT5b deficiency is further associated with increased PPAR-α-dependent FFA oxidation in peroxisomes, which, possibly, contributes to additional ROS accumulation. It is well known that high ROS levels and concomitant DNA damage predisposes hepatocytes to malignant transformation. Oxidative stress and a subsequent vicious cycle of hepatocyte damage, apoptosis, and cellular replenishment were shown to contribute to liver tumorigenesis. However, as observed in the high-fat diet or obesity-induced liver cancer, the increased ROS formation did not lead to enhanced hepatocyte apoptosis, whereas tumor-tissue proliferation was elevated. Excessive hepatic lipid accumulation and accompanying hepatocyte damage might activate tumor-promoting MAPK signaling during HCC development. ERK1/2 and p38 MAPK signaling was not induced, whereas JNK1 activity was enhanced in tumor-bearing mice. Increase in FFA, TNF-α, and ROS levels (as observed during the onset and progression of NAFLD) are potent activators of JNK1 and are all found elevated/activated upon the development of murine and human HCC. Moreover, the activity of STAT3 that is frequently activated in human HCC and implicated in the development of chemically and obesity-induced HCC was significantly elevated in DKO tumors. The latter might be explained by (1) compensatory GH-dependent STAT3 activation under conditions of hepatic STAT5 deficiency (Supporting Fig. 6) and/or (2) elevated systemic liver TNF-α levels in DKO mice, which can lead to IL-6 production and subsequent STAT3 activation. Finally, known tumor-promoting downstream effectors of JNK1 and STAT3 displayed enhanced expression (e.g., Myc, Jun, Mmp9, and Vgfl), which was restricted to DKO livers. In summary, our results underline the importance of hepatic GH-STAT5 and GC-GR signaling in the maintenance of systemic lipid homeostasis, where these pathways protect hepatocytes from metabolic stress and HCC development.

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References


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2.1.2 Supporting Information

Supporting Figure 1. Deletion of STAT5 and GR in hepatocytes and histological scoring of steatosis. (A) Representative Western blots of liver homogenates from 2-month-old male mice. Total levels of STAT5 and GR in Control and mutant livers were determined using specific antibodies. HSC-70 served as loading control. (B) The degree of histological steatosis in mutant livers at 2 and 12 months of age was scored semi-quantitatively by two board-certified liver pathologists (MH and LT). *P<0.05; **P<0.01; ***P<0.001.
Supporting Figure 2. Hepatic deletion of STAT5 using Alfp-Cre results in insulin resistance and impaired hepatic insulin receptor signaling. (A) Metabolic serum parameters of 2-month-old mice (n≥6/genotype). (B) Insulin Tolerance Tests (ITT): Insulin was administered through intraperitoneal injection following a 4h fast. Blood glucose levels were determined at given time points (upper panel). Oral Glucose Tolerance Tests (OGTT): Mice were fasted overnight and glucose was orally administered. Blood glucose levels were determined at given time points (middle panel). Plasma insulin levels following OGTT challenge. The insulin levels were determined by ELISA (lower panel). 2-month-old mice were analyzed for ITT and OGTT (n≥6/genotype). (B) Phosphorylation status of proteins implicated in insulin receptor downstream signaling following insulin challenge in 2-month-old mutant mice. Levels of phosphorylated and total protein were determined 15 min after intraperitoneal injection of insulin by immunoprecipitation and phospho-tyrosine/serine-specific immunoblot analysis. HSC-70 served as loading control. *P<0.05; **P<0.01; ***P<0.001.
Supporting Figure 3. Induction of PPARγ/SREBP-1 mediated lipogenesis upon hepatic STAT5 deficiency. (A) Relative mRNA levels of genes with functions in hepatic lipid metabolism were quantified by qRT-PCR in livers from 2-month-old mice. Ct values were normalized to Gapdh (n=6/genotype). (B) Representative Western blots showing protein expression of hepatic lipogenic transcription factors. HSC-70 served as loading control. (C) Chromatin immunoprecipitation (ChIP) demonstrating STAT5 binding to promoter regions of Srebp-1a and Srebp-1c. The STAT5 target gene Igf-1 served as a positive control. Values are represented as fold induction versus a downstream region of Cis not containing STAT5 responsive elements. (D) Genomic localization of STAT5 responsive elements in the Srebp-1a and Srebp-1c promoter regions. The STAT5 responsive element in the Srebp-1a promoter is partially conserved between murine and humans. (E) Changes in relative hepatic Srebp-1c and Srebp-1a mRNA levels following GH administration for indicated durations (n=6/group). *P<0.05; **P<0.01; ***P<0.001.
Supporting Figure 4. Tumorigenesis in DKO mice. (A) Representative HE-stained sections of livers from 12-month-old Control and S5KO mice (a-b). Representative HE-stained sections of dysplastic nodules with vacuoles containing fat (c-d) and HCCs (e-f) observed in DKO livers. (B) Tumor incidence in 9-month-old (9 M) and 12-month-old (12 M) DKO mice. (C) Relative mRNA levels of Ccdn1 and apoptosis regulating Bcl-2 family members were quantified by qRT-PCR in livers from 12-month-old mice. Normalized to Gapdh (n=6/genotype). (D) Gene set enrichment analysis of antioxidant genes using Affymetrix® expression profile analysis. (E) Relative mRNA levels of antioxidant genes Sod1 and Sod2 were quantified by qRT-PCR in livers from 12-month-old mice.
Normalized to Gapdh (n=6/genotype). (F) Quantification of JNK1 and p38 activation in livers from 12-month-old mice using image analysis (n≥4/genotype). *P<0.05; **P <0.01; ***P<0.001.

Supporting Figure 5. Relative mRNA expression levels of Serpina6 and 11βHsd1 in livers from 2-month-old mice. Messenger RNA expression was quantified by qRT-PCR and Ct values were normalized to Gapdh (ΔCt method, n=6/genotype). *P<0.05; **P <0.01.
Supporting Figure 6. Compensatory STAT1 and STAT3 activation upon hepatic deletion of STAT5 using Alfp-Cre. (A) Representative Western blots of liver homogenates from 2-month-old male mice. Amount of phosphorylation and total levels of STAT1, STAT3 and STAT5 in Control and S5KO livers either mock or growth hormone (GH) treated were determined using specific antibodies. HSC70 served as loading control. IFN-γ and IL-6 treated livers served as positive control. (B) Longer exposure of pY-STAT1 Western blot analysis of mock treated Control and S5KO livers and quantification of activated STAT1 using image analysis (n=2/group; C: IL-6 treated S5KO liver). (C)
Immunohistochemistry of phosphorylated STAT1, STAT3 and STAT5 levels in Control and S5KO livers of 2-month-old male mice either mock or GH treated (30 min). (D) Quantification of activated STAT1, STAT3 and STAT5 levels in Control or S5KO livers upon mock or GH treatment using image analysis (n=3/genotype/treatment). *P<0.05; **P<0.01; ***P<0.001.

Supporting Table 1. Detailed histology analysis of mice at indicated time-points.

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Micro, microvesicular; Macro, macrovesicular; ND, not determined; PV, perivenular; PC, pericellular
Supporting Table 2. Transcription factor signature analysis of genes statistically upregulated in Affymetrix expression profiling.

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<th>Symbol</th>
<th>Name</th>
<th>TF pathway</th>
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<th>S5KO</th>
<th>GRK</th>
<th>Verif. (qPCR)</th>
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<td></td>
<td>p=0.022 (for Ppar)</td>
<td>x-fold</td>
<td>p-value</td>
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*= not statistically significant in Affymetrix analysis, included for comparison
2.2. Interlude

Evidence from human patients and animal models has implicated that increasing levels of GH (endogenous or exogenous) may play a role in tumorigenic transformation and neoplastic progression particularly in epithelial cancers (Jenkins et al, 2006; Perry et al, 2006). Further, systemic overexpression of GH in transgenic mice is known to induce liver tumors within 1 year of age and to accelerate DEN-induced HCC development (Snibson et al, 2001; Snibson et al, 1999). The GH-transgenic mouse model displays preneoplastic lesions which are similar to the pathology seen in humans at high risk of developing HCC. Livers of GH-transgenic mice present high rates of hepatocyte turn over, hypertrophy, various degrees of inflammation in addition to fibrotic and cirrhotic degeneration which precedes the development of adenomas and carcinomas.

Published studies and our own data have implied that GH-mediated activation of hepatic STAT5 exerts hepatoprotective functions. However, little data exist on how STAT5 activation may be involved in the pathogenesis of HCC. Further, there are no published reports on STAT5`s contribution to the liver cancer phenotype caused by hyperactivated GH signaling. Therefore, in parallel to the first study, we intended to gain a better understanding of STAT5 function in the development of HCC. To address this question we conditionally deleted hepatic Stat5 in the GH transgenic mouse model of inflammatory liver cancer.
2.2.1 Manuscript: Growth-Hormone-induced Signal Transducer and Activator of Transcription 5 Signaling Causes Gigantism, Inflammation, and Premature Death but Protects Mice from Aggressive Liver Cancer.
Growth-Hormone–Induced Signal Transducer and Activator of Transcription 5 Signaling Causes Gigantism, Inflammation, and Premature Death but Protects Mice From Aggressive Liver Cancer

Karin Friedluch,1 Madeleine Themans,1 Kristina M. Mueller,1 Michaela Schlederer,1 Jan-Wilhelm Kornfeld,2 Luigi M. Terracciano,3 Andrey V. Kozlov,4 Susanne Haindl,5 Lukas Kenner,1,5 Thomas Kolbe,6,7 Mathias Mueller,8 Kenneth J. Snibson,9 Markus H. Heim,10 and Richard Moriggi1

Persistently high levels of growth hormone (GH) can cause liver cancer. GH activates multiple signal-transduction pathways, among them Janus kinase (JAK) 2-signal transducer and activator of transcription (STAT) 5 (signal transducer and activator of transcription 5). Both hyperactivation and deletion of STAT5 in hepatocytes have been implicated in the development of hepatocellular carcinoma (HCC); nevertheless, the role of STAT5 in the development of HCC as a result of high GH levels remains enigmatic. Thus, we crossed a mouse model of gigantism and inflammatory liver cancer caused by hyperactivated GH signaling (GH15) to mice with hepatic deletion of STAT5 (STAT5A−/−). Unlike GH15 mice, GH15/STAT5A−/− animals did not display gigantism. Moreover, the premature mortality, which was associated with chronic inflammation, as well as the pathologic alterations of hepatocytes observed in GH15 mice, were not observed in GH15 animals lacking STAT5. Strikingly, loss of hepatic STAT5 proteins led to enhanced HCC development in GH15 mice. Despite reduced chronic inflammation, GH15/STAT5A−/− mice displayed earlier and more advanced HCC than GH15 animals. This may be attributed to the combination of increased peripheral lipolysis, hepatic lipid synthesis, loss of hepatoprotective mediators accompanied by aberrant activation of tumor-promoting c-JUN and STAT3 signaling cascades, and accumulation of DNA damage secondary to loss of cell-cycle control. Thus, HCC was never observed in STAT5A−/− mice.

Conclusion: As a result of their hepatoprotective functions, STAT5 proteins prevent progresive fatty liver disease and the formation of aggressive HCC in the setting of hyperactivated GH signaling. At the same time, they play a key role in controlling systemic inflammation and regulating organ and body size. (Hematology 2012;55:941-952)

Many growth factors and cytokines, including growth hormone (GH), are able to activate two members of the signal transducer and activator of transcription (STAT) family, namely STAT5a and STAT5b (referred to as STAT5)1,2. GH induces multiple signaling cascades that mediate a wide range of effects, including cellular proliferation, differentiation and migration, prevention of apoptosis,

Abbreviations: AlphC, Cre recombinase under albumin promoter and albumin and alpha-fetoprotein enhancers; BW, body weight; CAB, chromotrope aniline blue; CD, cluster of differentiation; DKO, double knockouts; DNA, dysplastic nodules; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular-signal-regulated kinase; FSH, fetuin alpha; GH, growth hormone; HCC, hepatocellular carcinoma; HSE, hepatocyte-specific enhancer; HNF, hepatic nuclear factor; IGF-I, insulin-like growth factor 1; IL, interleukin; JNK, c-JUN N-terminal kinase; LIFR, leukemia inhibitory factor receptor; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; NASH, nonalcoholic steatohepatitis; NF-κB, nuclear factor kappa light-chain enhancer of activated B cells; PAS, periodic acid-Schiff stain; pH2AX, phospho-histone H2AX; PPAR-γ, peroxisome proliferator-activated receptor gamma; PRLR, prolactin receptor; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; RC, respiratory control; ROS, reactive oxygen species; sFlt, sFlt; STAT, signal transducer and activator of transcription; TEM, transmission electron microscopy; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; WAT, white adipose tissue; WT, wild type.

From the 1Ludwig Boltzmann Institute for Cancer Research (LBI-CR), Vienna, Austria; 2Institute for Genetics, Department of Microscopic Genetics and Metabolism, University of Cologne, Germany; 3Institute of Pathology, Department of Molecular Pathology, University Hospital Basel, Switzerland; 4Institute of Tumourology, Vienna, Austria; 5Institute of Clinical Pathology, Medical University of Vienna, Austria; 6Institute of Laboratory Animal Science, Veterinary University Vienna, Vienna, Austria; 7TPA-Thomas, University of Applied Life Sciences and Natural Resources, Vienna, Austria; 8Institute of Animal Breeding and Genetics and Bioregions Austria, Vetsuisse University Vienna, Vienna, Austria; 9Centre for Animal Biotechnology of the University of Melbourne, Melbourne, Victoria, Australia, and 10Department of Biomedicine, University Hospital Basel, Basel, Switzerland.
and regulation of metabolic pathways. Through STAT5, GH also controls many facets of liver physiology and pathophysiology, including the regulation of genes associated with somatic growth, such as liver-derived insulin-like growth factor 1 (IGF-1), acid-labile subunit, and suppressor of cytokine signaling 2. Hepatic STAT5 also regulates the expression of sex-specific and hepatoprotective liver genes. Furthermore, STAT5b has been shown to be involved in mediating metabolic effects of GH, such as its lipolytic action on adipose tissue.

Hepatocyte-specific loss of STAT5 in mice results in impaired liver regeneration after partial hepatectomy and metabolic defects, which manifest as hepatic steatosis. Nonalcoholic fatty liver disease (NAFLD), which is linked to obesity and insulin resistance, comprises a spectrum of diseases, ranging from simple steatosis to steatohepatitis, which may progress to liver cirrhosis and hepatocellular carcinoma (HCC). In addition to viral hepatitis, obesity-induced NAFLD and nonalcoholic steatohepatitis (NASH) are increasingly recognized as prime causes and promoters of liver cancer progression. However, the molecular mechanisms underlying this progression are still poorly understood.

Increased STAT5b activity was found to correlate with more aggressive tumors and poor clinical outcomes in hepatitis B virus--related HCC patients as a result of increased cell mortality and tumor spread. Upon CCL4 challenge, mice lacking hepatic STAT5 develop liver fibrosis and, in some cases, cancer as a result of increased STAT3 activation and transforming growth factor beta (TGF-β) stabilization. In a mouse model of cholestatic liver disease, these mice also develop severe liver fibrosis, which was attributed to the down-regulation of hepatoprotective genes. Additionally, inactivation of the STAT5 locus in the murine liver has been demonstrated to result in low levels of circulating IGF-1 with concomitant high levels of circulating GH. This condition, which is clinically referred to as GH insensitivity and resistance, has been reported in patients with liver cirrhosis.

Although there is a large body of evidence suggesting that chronic liver damage—irrespective of its etiology—carries an increased risk of HCC development, it has, as yet, not been determined whether elevated serum levels of GH contribute to this risk. Apart from changes in body stature (e.g., body and organ size as well as body-fat content), as well as neuroendocrine and reproductive functions, animals expressing GH from a transgene (GHtg) have been shown to have a shorter life expectancy. Notably, several studies have demonstrated that systemically high levels of GH in transgenic mice induce the development of hepatocellular adenomas and carcinomas. The liver pathology reported to precede the development of HCC in GHtg mice is characterized by a sustained increase in hepatocyte turnover and the development of chronic inflammation. Compared to the general population, patients treated with GH over a long period of time have been found to be at a significantly higher risk of dying from cancer. This is in accord with an increasing body of literature suggesting that patients with acromegaly, adults with elevated concentrations of circulating IGF-1, and individuals of tall stature are at increased risk of developing carcinomas, including HCC.

To further explore the GH-STAT5-IGF-1 axis in liver pathology, we used GHtg mice expressing ovine GH from a transgene. Strikingly, loss of STAT5 in hepatocytes reversed all pathologic alterations induced by persistently high GH levels, including adverse effects on body stature, organ size, and life expectancy resulting from kidney failure in GHtg animals. Importantly, hepatocyte morphology and chronic inflammation were also corrected to a great extent. Yet, hepatic deletion of STAT5 (GHtgSTAT5Δlop) led to a more aggressive form of HCC at earlier time points with 100% penetrance. This may be attributed to the combination of increased peripheral lipolysis, hepatic lipid synthesis, loss of hepatoprotective mediators, accumulation of mutations subsequent to loss of cell-cycle control, and aberrant activity of tumor-promoting STAT3 and c-JUN-signaling pathways.
Materials and Methods

**Transgenic Animals.** Mice with hepatic deletion of STAT5 (STAT5Δ5/5), Cre recombinase under albumin promoter and albumin and alpha-fetoprotein enhancers (AlfpCre); referred to as STAT5Δ5Δ5, described previously15) were bred with GH transgenic animals expressing ovine GH under the control of the metallothionein promoter (GH55, described previously16) to generate GH55STAT5Δ5Δ5 mice. Littermates not expressing AlfpCre recombinase and the GH transgene served as controls. Male mice were used, unless indicated otherwise.

**Histology and Immunohistochemistry.** Sections prepared from paraffin-embedded formalin-fixed organ specimens were stained with hematoxylin and eosin (H&E), chromotrope aniline blue (CAB), Giemsa, Prussian blue, and periodic acid-Schiff base (PAS).

Immunohistochemistry was performed for cleaved caspase-3 (AF885; R&D Systems, Minneapolis, MN) and Ki67 (RM-9106; Thermo Scientific, Fremont, CA). Caspase-3-positive and Ki67-positive hepatocytes were quantified for 10 high-power fields at 200× and 400× magnification, respectively.

**Additional Materials and Methods.** Animal procedures, serum biochemistry, immunoblotting, enzyme-linked immunosorbent assay (ELISA), quantitative reverse-transcription polymerase chain reaction (qRT-PCR), determination of mitochondrial function, and transmission electron microscopy (TEM) are described in the Supplemental Materials and Methods.

**Statistical Analysis.** All values are represented as means ± standard error of the mean, if not indicated otherwise. qRT-PCR quantifications and serum parameters were evaluated for significance using one-way analysis of variance, with Tukey’s post-hoc test. Differences between experimental groups were considered significant at \( P < 0.05 \), \( P < 0.01 \), and \( P < 0.001 \). Kaplan-Meier plots were analyzed for significance using the log-rank test. All calculations were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

Results

**STAT5 Is Essential for Postnatal Body Growth.** GH is the major stimulator of somatic growth, cellular proliferation, and regeneration. To assess the importance of hepatic STAT5 for postnatal growth, we compared the growth curves of GH55, GH55STAT5Δ5Δ5, and STAT5Δ5Δ5 mice to those of wild-type (WT) littermates (Fig. 1A,B). Our data confirm that high GH levels lead to gigantism, characterized by an increase in body weight (BW) of at least 40% (Fig. 1A,B) and a 15% increase in long bone growth (Supporting Fig. 1) in male mice, compared to WT littermates. Contrary, abrogated GH signaling in the liver resulting from loss of hepatic STAT5 (STAT5Δ5Δ5) results in a reduction in body size of approximately 40% (Fig. 1A,B). GH55STAT5Δ5Δ5 mice showed growth curves identical to those of WT littermates aged up to 9 weeks. Thereafter, the absence of STAT5 in GH55 mice resulted in growth retardation, reducing BW to approximately 80% of that in WT mice (Fig. 1A,B), whereas bone length was 5% above the WT length (Supporting Fig. 1). Compared to STAT5Δ5Δ5 mice, GH55STAT5Δ5Δ5 mice were not significantly larger. These changes were paralleled by reduced levels of IGF-1, a well-known STAT5 target and the main regulator of postnatal body growth. Both serum and messenger RNA (mRNA) expression levels (Fig. 1C,D) were found to be significantly elevated in GH55 mice, but barely detectable in GH55STAT5Δ5Δ5 and STAT5Δ5Δ5 animals. Similarly, IGF-2 expression was found to be significantly increased in GH55 mice, whereas animals lacking hepatic STAT5 showed reduced mRNA levels, compared to WT littermates (Fig. 1D). Hence, growth retardation, despite high GH levels, confirms that STAT5 is essential for GH-stimulated body growth.

**Reduced Life Expectancy in GH55 Mice Is Reversed by Deletion of STAT5.** High serum levels of GH were associated with premature mortality in transgenic mice (Fig. 2A). We detected severe pathologic changes in several internal organs, including the kidney, lung, and heart (Fig. 2B), which is in line with previous reports of reduced life expectancy resulting from systemically high GH levels.22 On histologic analysis, GH55 mice displayed signs of progressive inflammation in all major organs. Giemsa staining revealed very dense, partly follicular lymphocyte infiltration in the kidneys and other organs, such as the lung. Moreover, Prussian blue staining revealed the presence of activated macrophages in the kidneys, which contribute to the chronic inflammatory phenotype (Fig. 2B). The chronic inflammatory process resulted in parenchymal rarefaction, leading to cystic dilation of the renal pelvis (Fig. 2C) and, eventually, to renal failure, which was the most probable cause of death. At higher magnification, the microstructure of the kidney displayed luminal obstruction by patchy PAS-positive protein deposits in the glomeruli (i.e., glomerulonephritis). In addition, mesangial hypercellularity and thickening of the basement membrane were observed (Fig. 2B). Furthermore, chronic inflammation

59
Fig. 1. STAT5 is essential for postnatal body growth. (A and B) Postnatal body growth in GH\(^{+/+}\) (grey line) and GH\(^{+/+}\)STAT5\(^{-/-}\) (black line) mice was compared to that in WT mice (dashed line; n ≥ 12/genotype). As already published, the growth curves of STAT5\(^{-/-}\) animals were included for reference (dashed grey line). (C) IGF-1 serum levels were assessed in 12-week-old mice by means of ELISA (n ≥ 6/genotype). (D) By means of qRT-PCR, Igf-1 and Igf-2 mRNA levels were measured in livers at 12 weeks of age (n ≥ 3/genotype). Data represent means of triplicate assays.

resulted in regenerative fibrosis within the lung and heart (CAB staining). Strikingly, GH\(^{+/+}\)STAT5\(^{-/-}\) animals had a life expectancy comparable to that of WT and STAT5\(^{-/-}\) littermates. All pathologic changes described above were significantly improved by the deletion of hepatic STAT5. Additionally, interleukin (IL)-6 serum levels (Fig. 2D) and hepatic expression of the proinflammatory cytokines, IL-6, tumor necrosis factor alpha (TNF-\(\alpha\)), and IL-1\(\beta\) (Fig. 2E), were significantly increased in GH\(^{+/+}\) mice only, which is indicative of chronic inflammation. On the contrary, the expression of all three cytokines and IL-6 serum levels were reduced to levels comparable to WT controls in all STAT5-deficient animals (Fig. 2D,E). Nuclear factor kappa light-chain enhancer of activated B cells (NFKB) regulates the production of inflammatory cytokines and can itself be activated by TNF-\(\alpha\), IL-1\(\beta\), and IL-6. In GH\(^{+/+}\) livers, RelA (p65 subunit of NFKB) mRNA expression was significantly higher, whereas p65 protein levels were slightly increased, with the expression most prominent in inflammatory cells (Supporting Fig. 2A,B). Based on the survival curve, five (six for GH\(^{+/+}\) mice) different time points were chosen for further analysis (Supporting Fig. 2C).

Pathologic Changes in GH\(^{+/+}\) Hepatocytes Are Reversed by Deletion of STAT5. In the liver, persistently high expression of GH caused substantial cellular alterations. Hepatocytes of GH\(^{+/+}\) mice were abnormally large and had larger nuclei of polymorphic shape (Supporting Figs. 2D and 3A). Additionally, irregular intranuclear inclusions and an increased number of mitochondria were detected (Supporting Fig. 3A). Assessment of mitochondrial function by means of respiratory control (RC) ratios, which characterize mitochondrial activity regarding adenosine triphosphate synthesis, revealed that the mean values were diminished in GH\(^{+/+}\) mice at 40 weeks of age, whereas STAT5\(^{-/-}\) mice displayed significantly increased RC ratios for complex I and II substrate-dependent respiration (Supporting Fig. 3B). Moreover, corticosterone levels in GH\(^{+/+}\) mice were markedly increased (Supporting Fig. 2E). High corticosterone levels, a consistent finding in GH\(^{+/+}\) mice, are likely linked to oxidative-stress–induced aging of these mice. At the same
Fig. 2. High serum levels of GH reduce life expectancy. (A) Kaplan-Meier plot of male and female mice of all four genotypes over 65 weeks. GH transgenic mice were found to have a reduced life span. By contrast, GH PSTAT5Δ mice had a life expectancy comparable to that of WT and STAT5Δ littermates (n ≥ 37; genotype; n ≥ 8 for GH PSTAT5Δ mice). (B) Histologic analysis of the kidneys, lung, and heart of 28-week-old WT, GHΔ, and GH PSTAT5Δ animals stained with PAS, Giemsa, Prussian blue, and CAB revealed progressive pathologic changes, including glomerular changes, follicular infiltrates in the kidneys and lung, activated macrophages, and fibrosis of the lung and heart (indicated with arrows). (C) Both macroscopically and in microscopic overviews (25× magnification), the renal pelvis of 28-week-old GHΔ animals often appeared cystic and dilated. The renal parenchyma was very thin and had an atrophic appearance. (D) IL-6 serum levels were assessed in 40-week-old mice by means of ELISA (n = 7; genotype). (E) Hepatic expression of the proinflammatory cytokines, IL-6, Tnfα, and Il-1β, was assessed in 40 week-old mice by means of qRT-PCR (n ≥ 3; genotype). All assays were performed in triplicate.

In time, hepatic mRNA levels of the anti-inflammatory factors, Il-10 and Tgfβ, which are able to protect the liver from severe injury, were increased in GHΔ animals (Supporting Fig. 2F). Strikingly, upon deletion of STAT5 in GHΔ mice, the increase in hepatocyte size, turnover, sinusoidal cellularity, irregular nuclear morphology, as well as the corticosterone, Il-10, Tgfβ, and RC values were reversed and reached levels comparable to those of WT controls (Supporting Figs. 2 and 3).

Increased Fat Deposition and Higher Tumor Burden in GHΔSTAT5Δ Mice. Increased postnatal body growth in GHΔ mice is accompanied by an alteration of body proportions, resulting in an acromegaly-like phenotype, enlargement of internal organs such as hepatomegaly, and reduced peripheral body fat. Compared to WT littermates, all mice expressing the GH transgene were found to display increased liver/BW ratios at all time points analyzed. However, liver weight in GHΔ mice never exceeded 12% of BW, whereas in GHΔSTAT5Δ mice, ratios of more than 15% were observed at 28 and 40 weeks of age (Fig. 3A). Because of hepatosteatosis, the liver/BW ratios in STAT5Δ mice, compared to WT littermates, were elevated as well. Yet, the ratios remained in the same range as those of GHΔ controls over the whole period of analysis.
The dramatic increase in liver weight observed in 
GH<sup>is</sup>STATS<sup>ΔHep</sup> mice can be explained by two factors: 
on the one hand, GH<sup>is</sup>STATS<sup>ΔHep</sup> mice showed a higher tumor burden at 28 and 40 weeks of age, compared to 
GH<sup>is</sup> animals (Fig. 3B; Supporting Fig. 4B,C), and on the 
other hand, there was increased fat deposition in the 
liver (Figs. 3B and 4A). High GH levels, resulting from 
transgene expression and the deletion of hepatic STAT5 in 
GH<sup>is</sup>STATS<sup>ΔHep</sup> animals, led to massive lysis of 
peripheral fat. This is indicated by the reduced white 
adipose tissue (WAT)/BW ratio (Figure 3C) and the 
increased release of free fatty acids (FFAs) into the serum 
(Fig. 3D), which subsequently accumulated in their 
livers to a greater extent than in STAT5<sup>ΔHep</sup> mice 
(Supporting Fig. 4). This process, which was observed at 
a very young age already, compromised the metabolic 
competence of the liver. At 12 weeks of age, serum 
triglyceride and cholesterol levels were found to be
significantly elevated in GH\(^{+}\)STATS\(^{\Delta hpe}\) mice, whereas their glucose levels were only slightly increased (Fig. 3E). At the same time, high serum insulin levels were measured in GH\(^{+}\)STATS\(^{\Delta hpe}\) mice (Fig. 3E), which is indicative of insulin resistance. Moreover, increased lipid accumulation caused chronic damage to the hepatocytes, as could be observed from the elevated transaminase levels (Fig. 3F). As compared to WT littermates, they were increased in GH\(^{+}\), STATS\(^{\Delta hpe}\), and, particularly, in GH\(^{+}\)STATS\(^{\Delta hpe}\) mice.

**Loss of Hepatic STATS Is Associated With Increased Lipid Synthesis in the Liver and Decreased Expression of Hepatoprotective Factors.** The extensive hepatic steatosis observed in GH\(^{+}\)STATS\(^{\Delta hpe}\) mice (Figs. 3B and 4A) results not only from lysis of peripheral fat depots, but also from enhanced expression of lipogenic regulators upon loss of hepatic STATS\(^{5,7,23-27}\) particularly peroxisome proliferator-activated receptor gamma (Ppar\(\gamma\)) and its target gene, cluster of differentiation (Cd)\(^{36}\) (Supporting Fig. 5A). This suggests that the steatotic phenotype is likely to be caused by a combination of lipolysis, increased lipid transport to the liver, and the induction of \textit{de novo} lipid synthesis in the liver. Additionally, deregulated expression of genes that have been described to protect the liver from injury creates an environment facilitating damage-induced hepatocarcinogenesis. This makes it more likely for potentially harmful events, such as inflammation and accumulation of lipids within hepatocytes, to result in mutations. In fact, the mRNA levels of epithelial growth factor receptor (Egfr), prolactin receptor (Prlr), hepatocyte nuclear factor (Hnf6), and leukemia inhibitory factor receptor (Lifr), which are all considered to have hepatoprotective functions\(^{5}\), were reduced to barely detectable levels in mice lacking STATS\(^{5}\) independent of transgenic GH expression (Supporting Fig. 5B). Conversely, expression of all four genes was significantly increased in livers of GH\(^{+}\) mice.

**Loss of Hepatic STATS Promotes the Development of HCC in GH\(^{+}\) Mice.** Although the characteristic alterations observed in GH\(^{+}\) mice were reversed by the deletion of hepatic STATS, earlier tumor formation was observed in GH\(^{+}\)STATS\(^{\Delta hpe}\) mice (Fig. 3B). To gain more insight into the cellular mechanisms contributing to hepatocyte damage and earlier tumor formation in GH\(^{+}\)STATS\(^{\Delta hpe}\) mice, the livers of all genotypes were subjected to histologic analysis. In hepatocytes, high long-term expression of GH resulted in greatly increased rates of hepatocellular apoptosis (caspase-3 staining; Fig. 4B), which, in turn, led to enhanced regenerative proliferation (Ki67 staining; Fig. 4C) and attracted immune cells to the sites of injury (Fig. 4A; Supporting Fig. 2D). These processes, which, in GH\(^{+}\) mice, were observed in the first weeks after birth.
already precede the onset of hepatic inflammation (Supporting Table 1), a key feature of liver pathology in these animals.\textsuperscript{10} As a result of increased hepatocyte turnover and chronic inflammation, GH\textsuperscript{RS} mice first developed neoplastic nodular lesions at 40 weeks and carcinomas at 52-60 weeks of age (Fig 5A). GH\textsuperscript{RSTAT5\textsuperscript{Δhep}} mice, by comparison, displayed dysplastic nodules (DNs) at 28 weeks already (Fig 5B). At this time, their tumors (nodules >9 mm in diameter; Fig 3B, right panel) already exceeded those observed in GH\textsuperscript{RS} mice in size at all time points analyzed (nodules 1-2 mm in diameter; data not shown). At 40 weeks of age, all GH\textsuperscript{RSTAT5\textsuperscript{Δhep}} mice had large steatotic and solid tumors, which hepatopathologists classified as full-blown HCC. Histologically, all tumors—HCC as well as early lesions—in GH\textsuperscript{RSTAT5\textsuperscript{Δhep}} mice were similar to those observed in GH\textsuperscript{RS} animals, but aggressive tumors were observed 3 months earlier (Fig 5C).

**Increased Compensatory STAT3 Activity in the Livers of GH\textsuperscript{RSTAT5\textsuperscript{Δhep}} Mice Leads to Elevated c-JUN Levels.** To gain molecular insight into the processes promoting the malignant transformation of hepatocytes in GH\textsuperscript{RSTAT5\textsuperscript{Δhep}} animals, we evaluated the activation of other signaling proteins and pathways known to regulate the transcription of GH-responsive genes. In the absence of STAT5 proteins, these include pY-STAT1 and pY-STAT3 and their downstream target genes,\textsuperscript{11} activation of sarcoma (Ssrc) kinase, the mitogen-activated protein kinase (MAPK) pathway, which has a role in cellular growth and differentiation, and the phosphoinositide 3-kinase/akt/mammalian target of rapamycin pathway, which mediates survival and growth signals to cells.\textsuperscript{3} Enhanced AKT and extracellular signal-regulated kinase (ERK) activation, caused by high concentrations of circulating insulin and IGF-1, has been suggested to promote tumor formation in overweight and obese individuals.\textsuperscript{8} However, our findings indicate that neither AKT nor ERK nor Src activation causes enhanced HCC development in GH\textsuperscript{RSTAT5\textsuperscript{Δhep}} mice (Supporting Fig. 5C).

Upon loss of hepatic STAT5, pY-JAK2 levels were slightly decreased at 40 weeks of age independent of GH expression, whereas JAK2 protein levels were slightly increased (Fig 6A). Furthermore, the protein levels of the potential STAT5 targets, BCL-2, BCL-XL, and cyclin D1 were slightly reduced (Supporting Fig. 5C). Most notably, loss of STAT5 in GH-transgenic mice led to enhanced STAT3 and STAT1 activity (Fig 6A), which was not observed in any other genotype. In addition, increased protein levels (Fig 6B; Supporting Fig. 5D) and elevated phosphorylation of the proto-oncogene, c-JUN, a direct transcriptional downstream target of STAT3, were observed in GH\textsuperscript{RSTAT5\textsuperscript{Δhep}} animals (Fig 6B). Moreover, activation of major stress-dependent MAPK-signaling pathways, which are known to be triggered by chronic liver damage and to be involved in the pathogenesis of HCC, was found to be enhanced in GH\textsuperscript{RSTAT5\textsuperscript{Δhep}} mice. Compared to all other genotypes, tumor-bearing GH\textsuperscript{RSTAT5\textsuperscript{Δhep}} mice exhibited increased c-JUN-N-terminal kinase (JNK) 1 and p38 activation (Fig 6B), which, in turn, can activate c-JUN.\textsuperscript{26,29} Furthermore, their p53 activity—despite quite heterogeneous p53 levels (Fig 6C)—was found to be significantly lower than in GH\textsuperscript{RS} animals, as shown by the expression levels of its known targets Asi, Bax, Nos3, Puma, and Fas (Fig 6D). Hence, loss of p53 function and a significant increase in double-strand breaks (phosphorylated histone H2AX [pH2AX] staining; Fig 6E) might contribute to enhanced development of more aggressive HCC. In summary, the development of HCCs in GH\textsuperscript{RSTAT5\textsuperscript{Δhep}} animals appears to be enhanced by the activation of tumor-promoting c-JUN and STAT3-signaling cascades and accumulation of mutations after loss of a cell-cycle checkpoint (Supporting Fig. 6).

**Discussion**

In patients, liver cirrhosis and hepatic dysfunction have been shown to be associated with severe impairment of the GH-IGF-1-insulin receptor axis, resulting in low IGF-1 levels and reduced response to exogenous GH (i.e., GH insensitivity/resistance).\textsuperscript{12} Mice with hepatic STAT5 deletion had depleted bioactive IGF-1, leading to dwarfism.\textsuperscript{1} Interestingly, Igf2 expression, although largely independent of GH-signaling,\textsuperscript{21} showed a similar pattern as Igf1 expression. IGF-2, which has an important role in embryonic development, has also been implicated in tumor development, growth, and metastasis.\textsuperscript{21}

The lack of negative feedback via IGF-1 resulted in reduced body growth and high endogenous GH levels (data not shown). In GH\textsuperscript{RSTAT5\textsuperscript{Δhep}} mice, high GH levels resulting from both transgene expression and deletion of hepatic STAT5 caused increased lipolysis of peripheral fat depots and release of free fatty acids, which were deposited in the liver. This is supported by the finding that disruption of GH signaling by hepatocyte-specific deletion of JAK2 results in GH resistance and the development of hepatosteatosis, which was suggested to be related to increased expression of G63.\textsuperscript{20} Interestingly, GH\textsuperscript{RS} animals showed elevated G63 levels, but lyzed peripheral fat was not accumulating in their livers, which remains mechanistically
Fig. 5. Loss of hepatic STATs promotes HCC progression in GH6 mice. (A) H&E staining of GH6 livers at (a) 28 and (b and c) 40 weeks. The first DNs were observed at 40 weeks. GH6 mice did not develop carcinomas before the age of 52 weeks (d-f) livers at 62 weeks; tumors in different mice, magnification of 100× and 200×, respectively. (B) H&E staining of GH6STAT6-dep mice revealed increased accumulation of lipids within hepatocytes (a) and DNs at 28 weeks of age already (b-e). At 40 weeks of age, all GH6STAT6-dep mice had large steatotic and solid HCCs (d-f). (C) In both genotypes, early neoplastic nodular lesions—histologically classified as DNs—were microscopically characterized by loss of normal lobular architecture and an irregular growth pattern. They appeared sharply demarcated from the surrounding liver parenchyma, which was often compressed (black asterisk). Steatotic hepatocytes of varying size and staining pattern (yellow asterisk), but no portal tracts, were observed. Moreover, focal areas of cellular atypia, characterized by slight pleomorphic nuclei, coarsely clumped chromatin, large nucleoli, and cytoplasmic basophilia, were found (blue asterisk) (a-b). Full-blown HCC was characterized by the loss of lobular plates, more pronounced cellular pleomorphism (blue asterisk), and an increased mitotic index. Tumors displayed a solid or trabecular growth pattern, with more abundant steatotic hepatocytes (yellow asterisk) (c-d).
unexplained. STAT5 deficiency additionally resulted in uncontrolled lipid synthesis in the liver and impaired glucose and insulin tolerance.\textsuperscript{7,25} It can be assumed that these complex mechanisms are chiefly responsible for the steatotic phenotype observed in GHT\textsuperscript{\textsuperscript{stat5\textsubscript{\textsuperscript{deltax}}}} mice. Over time, increased hepatic lipid load increasingly compromises the metabolic competence of the liver, chronically damages hepatocytes, and aggravates the GHT\textsuperscript{H} cancer phenotype. This is supported by growing evidence that obesity, which is associated with
hepatocarcinogenesis and elevated transaminase levels, promotes neoplastic transformation of hepatocytes. Moreover, we have recently shown that combined deficiency in hepatic GH-STAT5 and glucocorticoid-glucocorticoid receptor signaling (double-knockout [DKO] mice) increases hepatic lipid load and HCC formation. In these mice, increased stress parameters, combined with high reactive oxygen species (ROS) levels and concomitant DNA damage, contributed to HCC development on top of hepatic steatosis.

Hepatocarcinogenesis in humans is known to be a multistep process involving the accumulation of different genetic alterations induced by chronic inflammation and oxidative DNA damage that finally leads to malignant transformation of hepatocytes. These pathologic mechanisms were also observed in GH−/− mice, which developed tumors histologically closely resembling human HCC at 52-60 weeks of age.

Interestingly, histologic analysis of mouse livers showed that the hepatocellular size and the mitochondrial number and function observed in GH−/− mice are dependent on STAT5. The hepatic GH-STAT5 axis was also responsible for complex disease phenotypes and premature mortality in GH−/− mice. Possibly, enhanced synthesis of growth factors, such as IGF-1 and IGF-2, within the liver contributes to premature mortality. Surprisingly, HCC development in mice was found to be induced by high GH levels independent of STAT5. Compared to GH−/− controls, GH−/−STAT5−/− mice had even more advanced lesions. Most strikingly, they developed HCC 4 months earlier than GH−/− controls, even though STAT5 deficiency abrogated hepatocellular nuclear polymorphism, normalized hepatocyte turnover, reduced sinusoidal cellularity (i.e., inflammation), and increased life expectancy as the pathologic alterations in the kidneys, lung, and heart disappeared.

In addition to lipid accumulation in the liver, loss of the hepatoprotective factors, EGFR, PRLR, LIFR, and HNF6, in STAT5-deficient animals promoted chronic hepatocytic damage. At the same time, loss of STAT5 resulted in compensatory GH-induced STAT3 activity in GH−/−STAT5−/− mice. STAT3 activation was not induced by IL-6 because IL-6 serum levels were significantly lower in GH−/−STAT5−/− mice. It is well documented that, in the absence of STAT3, STAT1/3 proteins are aberrantly recruited and activated by the GH receptor. Increased STAT3 activity has been reported in HCC. Its oncogenic signals stimulate proliferation of aberrant hepatocytes, which has been linked to accelerated tumor development. Loss of STAT5 has also been shown to cause increased hepatococyte proliferation secondary to diminished p15^INK4B expression.

Moreover, in the absence of STAT5, GH-activated STAT3 promotes the transcription of proto-oncogenes, such as cell-cycle progression and survival genes. Accordingly, there was prominent expression of c-JUN in GH−/−STAT5−/− animals. In the absence of STAT5, STAT3 might thus contribute to tumor induction and progression in GH−/−STAT5−/− animals.

Chronic liver damage caused by metabolic changes in these animals additionally induces JNK1 activity, which, in turn, activates c-JUN. Moreover, increased p38 stress kinase activation might contribute to activation of c-JUN. Following c-JUN activation, other oncogenic mechanisms potentially involving STAT3, p53 activity was inhibited, thus impeding clearance of cells harboring double-strand breaks. In contrast to DKO animals, GH−/−STAT5−/− mice did not show significant changes in ROS levels or chronic stress parameters. However, hepatic steatosis, inflammatory parameters, and aberrant STAT3 and c-JUN activation were more pronounced than in DKO mice.

Our data suggest that the HCC development observed in GH−/− mice in the absence of STAT5 was caused by two distinct molecular processes. First, loss of hepatic STAT5 results in deregulation of the STAT5 target genes involved in the protection of hepatocyte integrity and in lipid metabolism. Second, rerouting of GH signaling to other STAT proteins, which may partially compensate for the loss of STAT5, activates signaling cascades that are distinct from STAT5 and promotes cancer cell proliferation. Nevertheless, it is most likely the combination of increased lipid synthesis, lipodystrophy, deregulated expression of hepatoprotective factors, accumulation of mutations facilitated by a loss of cell-cycle control, and elevated activity of tumor-promoting c-JUN and STAT3 that causes HCC.

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67


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2.3.2 Supporting Information

Supporting Figure 1. Long bone growth in mice depends on GH-JAK2-STAT5 signaling. (A) The bones of all 4 genotypes were analyzed at 40 weeks of age by (B) measuring hind leg lengths and comparing lengths of femurs and tibias. Consistent with the decreased IGF-I levels and loss of GH-JAK2-STAT5 signaling in GHTgSTAT5Δhep mice, long bone growth was significantly decreased.
Supporting Figure 2. Hepatic alterations resulting from GH overexpression. (A) mRNA levels of the NF-κB subunit RelA were analyzed in livers of 40-week-old mice by means of qRT-PCR and were found to be elevated in GH$^g$ but reduced to wt levels in GHtgSTAT5$^{Δhep}$ mice (n≥5/genotype). All assays were performed in triplicate. (B) Representative immunohistologic stainings of p65 in the livers of 40-week-old mice revealed an increase in p65-positive inflammatory cells in GH$^g$ whereas GH$^g$STAT5$^{Δhep}$ mice showed results similar to those of wt littermates. (C) Based on the Kaplan Meier
plot in (Figure 2A), the following time points were chosen for further analysis: 4 weeks; 12 weeks, when hepatic inflammation was already detectable in GH\textsuperscript{tg} mice; 28 weeks, when the GH\textsuperscript{tg} mice started dying; and 40 weeks, when first macroscopic tumors could be detected in GH\textsuperscript{tg} mice. For the analysis of more advanced tumors in GH\textsuperscript{tg} mice, animals surviving longer than 7-10 months were examined at 52-60 weeks of age and compared to wt littermates. (D) At 28 weeks, GH\textsuperscript{tg} livers were characterized by increased cell size, nuclear polymorphism, and increased sinusoidal cellularity. STAT5 deficiency, though it corrected the sinusoidal cellularity, hepatocellular polymorphism and increase in cell size, led to extensive accumulation of fat. (E) In GH\textsuperscript{tg} mice, there was a marked increase in corticosterone levels at 12 weeks of age. GH\textsuperscript{tg}STAT5\textsuperscript{Δhep} mice had corticosterone levels comparable to those of wt animals (n≥4/genotype). (F) TGF-β and IL-10 mRNA levels were analyzed in livers of 40-week-old mice by means of qRT-PCR. Both levels were found to be elevated in GH\textsuperscript{tg} but significantly downregulated in GH\textsuperscript{tg}STAT5\textsuperscript{Δhep} mice (n≥3/genotype). All assays were performed in triplicate.
Supporting Figure 3. Pathologic changes in GHtg hepatocytes are reversed by STAT5 deletion.

(A) Transmission electron microscopy (4000x magnification) in 28-week-old GHtg mice revealed abnormally large hepatocytes with increased numbers of mitochondria (M) and large nuclei (N) with irregular contours (4000x magnification). In addition, irregular intranuclear inclusions were detected in GHtg hepatocytes. Upon deletion of STAT5, GHtg hepatocytes were found to have normally shaped nuclei but there was marked accumulation of lipid droplets (L) and glycogen (G). (B) Assessment of mitochondrial function by means of respiratory control ratios (RC1 = respiratory control ratio state 3 to state 2 and RC2 =respiratory control ratio state 3 to state 4) showed diminished mean values of respiratory ratios in GHtg mice at 40 weeks of age, while STAT5Δhep mice displayed significantly increased values. Upon deletion of STAT5 in GHtg mice, the RC values were in the range of wt controls (n=4/genotype). Increased total numbers of mitochondria in GHtg mice, however, were not linked to an increase in respiratory activity. By contrast, unchanged total numbers of mitochondria in STAT5Δhep animals were accompanied by increased respiratory activity resulting from electrons supplied via complex I and II. The latter suggests changes in the expression of mitochondrial complexes encoded in the mitochondrial genome, in the regulation of which STAT5 might play a role (2).
Supporting Figure 4. Histologic analysis of livers. H&E staining of (A) 4-week-old, (B) 28-week-old, and (C) 40-week-old wt, STAT5Δhep, GH^tg, and GH^tgSTAT5Δhep mice.
Supporting Figure 5. Loss of hepatic STAT5 in GH^{tg} mice is associated with increased lipid synthesis and decreased expression of hepatoprotective factors. (A) As quantified by qRT-PCR analysis of livers from 40-week-old mice, PPARγ mRNA levels were increased in STAT5^{Δhep} and even more increased in GH^{tg} STAT5^{Δhep} mice, when compared to those in wt animals. Additionally, Cd36 levels were increased in GH^{tg}, GH^{tg} STAT5^{Δhep} and STAT5^{Δhep} mice when compared to wt controls (n≥3/genotype). The data represent means of triplicate assays. (B) Loss of STAT5 in mouse livers results in a significant downregulation of the expression of the hepatoprotective factors Egfr, Prlr, Hnf6, and Lifr. On real-time PCR, the mRNA levels of all four factors were decreased in STAT5-deficient livers independent of GH overexpression. Conversely, expression of all four genes was significantly increased in the livers of GH^{tg} mice (n≥3/genotype). All assays were performed in...
triplicate. (C) Western blot analyses of livers from 40-week-old animals did not reveal activation of AKT, ERK, or SRC signaling pathways. The results are representative of three independent experiments. (D) As quantified by image analysis, representative liver sections of 40-week-old animals stained with antibodies against total c-JUN showed a higher number of positive hepatocytes in \( \text{GH}^{\text{tg}} \text{STAT5}^{\text{Δhep}} \) mice (n≥5/genotype).

Supporting Figure 6. Schematic diagram illustrating the development of phenotypes following hepatic STAT5 deletion in \( \text{GH}^{\text{tg}} \) mice. Loss of STAT5 in the liver results in depletion of the IGF-1 target gene. Due to the lack of a negative feedback mechanism, this leads to high endogenous GH.
levels. Consequently, high GH levels resulting from transgene expression and the deletion of hepatic STAT5 cause extensive lysis of peripheral fat depots and release of free fatty acids, which are subsequently deposited in lipid droplets within hepatocytes. This impairs their metabolic competence and thus chronic liver damage. These metabolic changes additionally activate the stress kinase JNK1. Moreover, increased lipid synthesis, deregulated expression of hepatoprotective factors (EGFR, PRLR, LIFR; HNF6), accumulation of mutations (increased DNA damage as detected by pH2AX staining) following loss of cell cycle control (reduced p53 levels), and enhanced activity of tumor-promoting STAT3 and c-JUN (elevated protein levels induced by STAT3 and increased phosphorylation mediated by JNK1 and p38 stress kinases) within hepatocytes contribute to severe tissue damage and the development of steatotic HCC. Systemically, depletion of the IGF-1 target gene results in reduced body growth. The hepatic GH-STAT5b axis is also responsible for the disease phenotype and the high mortality in mice displaying hyperactivated GH signaling. This phenotype is most likely due to a systemic inflammatory reaction and increased growth of all cells mediated by IGF-1/insulin receptor signaling. Loss of hepatic STAT5 in GHtg animals precludes chronic inflammation. At the same time, depletion of serum IGF-1 in STAT5-deficient GHtg mice prevents histopathologic changes of internal organs so that their life expectancy is comparable to that of wt and STAT5Δhep animals.
Supporting Table 1. Overview of phenotypic characteristics. Wt, GH<sup>Δ</sup>, GH<sup>Δ</sup>STAT5<sup>Δhep</sup> as well as STAT5<sup>Δhep</sup> mice were analyzed for postnatal body growth and liver phenotypes. In GH<sup>Δ</sup> mice, peripheral body fat was reduced, the liver was enlarged, and postnatal body growth was increased. Additionally, hepatocyte turnover was increased, which manifested itself in increased mitotic activity and apoptosis resulting in chronic inflammation. This in turn induced the development of tumors at 60 weeks of age, which histologically resembled human HCC. In order to identify the time of HCC onset in GHTg mice, an additional timepoint at 52 weeks of age was analyzed in these mice in comparison to wt littermates. Loss of hepatic STAT5, which abrogates GH signaling in the liver, resulted in a reduction in body size by at least 40% and additionally gave rise to metabolic defects (steatosis). Most strikingly, GH<sup>Δ</sup>STAT5<sup>Δhep</sup> mice developed dysplastic nodules already at 28 weeks of age and HCC four months earlier than GHTg controls, although STAT5 deficiency reversed the nuclear polymorphism, normalized hepatocyte turnover, and reduced hepatic inflammation. (n≥3/genotype per timepoint)

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2.3 Interlude

Ectopic accumulation of lipids in liver is clearly linked to the development of NAFLD and its co-morbid conditions hepatic insulin resistance and T2D. GCs as well as GH have been shown to induce WAT lipid mobilization, thus, increasing the circulating NEFA pool. As a consequence, lipids may accumulate in ectopic sites such as liver, ultimately leading to fatty degeneration of hepatocytes and insulin resistance.

In the first and second publication, we demonstrated that the NAFLD-like phenotype of STAT5-deficient mice is aggravated upon additional ectopic GH overexpression or by co-deficiency with hepatic GR as a result of combined hepatic GH resistance and systemic hypercortisolism. Both mouse models show persistent activation of STAT5 or co-activation of STAT5 and GR in WAT, partial lipodystrophy and an increased circulating NEFA pool. The elevated NEFA availability leads to an exacerbation of ectopic hepatic lipid accumulation associated with hyperinsulinemia (Friedbichler et al, 2012; Mueller et al, 2011), insulin resistance and glucose intolerance (Mueller et al, 2011).

Based on these findings, we hypothesized that interference with either signaling pathway in adipocytes will be sufficient to reduce ectopic hepatic lipids and to increase insulin sensitivity. Therefore, we intended to define the lipolytic response and NEFA release from WAT as well as glucose metabolism and insulin sensitivity upon adipocyte-specific GR or STAT5 deficiency in mice. To test our hypotheses we subjected the respective mouse model to 1) a well-established protocol of fasting-induced lipolysis which is characterized by suppressed insulin but augmented catecholamine, GC and GH secretion and 2) to conventional insulin and glucose tolerance testing.

Section 2.3.1 summarizes experimental data which are part of two independent manuscripts in preparation: “Adipocyte-specific GR deficiency impairs lipolysis and alters energy metabolism in mice” and “STAT5 Controls Adipose Tissue Lipid Mobilization and Energy Homeostasis in Mice”. Both manuscripts are planned to be submitted in 2015 after the completion of detailed metabolic phenotyping and clarification of molecular alterations.
2.3.1 Adipose Tissue-Specific Deficiency of GR or STAT5 Signaling Causes Diminished Lipid Mobilization and Improves Insulin Sensitivity in Mice

To test our hypotheses we crossed mice expressing the Cre recombinase driven by the Adiponectin (*Adipoq*) promoter with mice harboring conditional GR<sup>fl/fl</sup> (GR<sub>Adipoq</sub>) or STAT5<sup>fl/fl</sup> alleles (STAT5<sub>Adipoq</sub>). Importantly, AdipoqCre-mediated deletion is limited to fully committed, mature white and brown fat cell compartments (Eguchi et al, 2011). Thus, GR<sub>Adipoq</sub> and STAT5<sub>Adipoq</sub> mice represent well-suited models to study the function of the respective transcription factor in adipose tissue without interference with its development.

Under *ad libitum* feeding conditions, both conditional mouse lines displayed normal viability as assessed over a time course of 52 weeks (100% survival rate, n=11/genotype). For all subsequent analyses 8-week-old male mice were used. Body weights of GR-deficient mice (GR<sub>Adipoq</sub>: 23,78 g ±0,7628 versus Control: 24,49 g ±1,095, n≥7/genotype) and STAT5-deficient mice (STAT5<sub>Adipoq</sub>: 24,94 g ±0,5191 versus Control: 24,92 g ±0,6520, n≥7/genotype) were comparable to their littermate controls. The epididymal WAT (EWAT) and liver weights of GR<sub>Adipoq</sub> and STAT5<sub>Adipoq</sub> mice were unchanged, while the subcutaneous WAT (ScWAT) compartment was slightly enlarged compared to the control group (Fig. 1A, not significant). Subsequent histological examination revealed no apparent morphological alterations of ScWAT, EWAT and liver between the genotypes (data not shown). Blood glucose (Control: 163,6 mg/dl ±8,312, GR<sub>Adipoq</sub>: 174,3 mg/dl ±11,27, STAT5<sub>Adipoq</sub>: 154,7 mg/dl ±4,106) and β-ketone level were similar (Fig. 1B). Further, GR deficiency did not translate into decreased plasma NEFA, while STAT5<sub>Adipoq</sub> mice presented a reduction of the circulating NEFA pool by ~ 50% (Fig. 1B).

Upon 48 hours of fasting, however, GR<sub>Adipoq</sub> as well as STAT5<sub>Adipoq</sub> mice displayed a highly diminished ability to mobilize lipids from WAT compartments. In both conditional lines, EWAT as well as ScWAT mass did not decrease significantly which was further reflected by a drastically lower circulating NEFA pool and an overall increased adipocyte cell size in both compartments (Fig. 1B and C). In line with the decreased NEFA availability GR<sub>Adipoq</sub> mice had reduced levels of blood β-ketones, whereas β-ketone level of STAT5<sub>Adipoq</sub> mice were comparable to the control group (Fig. 1C). As the majority of β-ketone production is certainly from β-oxidation of NEFA, this observation suggests, that ketogenesis in STAT5<sub>Adipoq</sub> mice may originated from alternative sources such as ketogenic amino acids produced by increased proteolysis. Blood glucose was unexpectedly higher in fasted GR<sub>Adipoq</sub> mice and similar to the control group in STAT5<sub>Adipoq</sub> mice (Control: 51,23 mg/dl ±2,131, GR<sub>Adipoq</sub>: 74,3 mg/dl ±5,059, P<0.05 versus Control, STAT5<sub>Adipoq</sub>: 53,33 mg/dl ±4,758). This observation might be an indicative of reduced energy consumption and/or increased availability of gluconeogenic amino acids produced by proteolysis of lean body mass in fasted GR<sub>Adipoq</sub> and STAT5<sub>Adipoq</sub> mice.
Figure 1. Adipocyte-specific GR- or STAT5-deficient mice display reduced fasting-induced lipid mobilization of white adipose tissues. (A) Epididymal white adipose tissue (EWAT)/body weight, subcutaneous (Sc) WAT/body weight and liver/body weight ratios under ad libitum feeding and 48 hour (h) fasting conditions. (B) Plasma non-esterified fatty acids (NEFA) and blood β-ketone level of ad libitum fed and fasted mice at indicated time points. (C) Representative hematoxylin and eosin staining of EWAT, ScWAT and liver from 48h fasted mice. Inlets: Higher magnification for better visualization of lipid vacuoles within hepatocytes. For all analyses: 8-week-old male mice (n≥7/genotype/condition); data are shown as the mean ±s.e.m. *P<0.05; **P <0.01; ***P<0.001.

Fasting-induced weight loss was similar among the three experimental groups (Fig. 1D), further suggesting an increased breakdown of lean body mass upon adipocyte GR or STAT5 deficiency. Liver weights of both conditional lines were to some extent decreased (Fig. 1A), although the analysis did not reach statistical significance. In accordance with our hypothesis, liver histology revealed that adipocyte-specific GR or STAT5 deficiency conferred a marked attenuation of fasting-induced hepatic lipid accumulation (Fig. 1C).

To assess for functional effects of GR or STAT5 deficiency on lipolytic signaling, we subjected EWAT of ad libitum fed and 48 hours fasted mice to qRT-PCR and immunoblot analyses. Under feeding conditions, mRNA expression levels of key proteins of adipocyte lipolysis were barely distinguishable between the three genotypes (Fig. 2A). Activating serine phosphorylation of HSL (Ser563 and Ser660) was found to be reduced in GRAdipoq EWAT, whereas Ser563 phosphorylation was increased and ATGL protein content decreased in EWAT of STAT5Adipoq mice (Fig. 2B and C).

Published reports and our own data suggest that mRNA expression of the major lipases is, amongst others, positively controlled by GC-GR signaling (Mueller et al, 2011; Xu et al, 2009a; Yu et al, 2010). GRAdipoq EWAT from fasted mice displayed decreased up-regulation of Pnpla2 which did not translate into differential ATGL protein expression. Expression of the lipases Lipe and MglI was rather mildly increased when compared to fasted control EWAT. In accordance with our previous findings that chronic GC excess is associated with decreased perilipin 1 expression (Mueller et al, 2011), GR deficiency led to impaired downregulation of perilipin 1 on mRNA and protein level (Fig. 2A, B and C). 48h after food removal, Thr197 phosphorylation of PKA and concomitantly Ser563/660 phosphorylation of HSL was decreased in GRAdipoq EWAT compared with controls (Fig. 2B and C). Thereby, we confirm from previous studies that GC induce lipolysis, at least in party, by enhancing cAMP-dependent activation of the lipolytic cascade.
Figure 2. Impairment of adipose tissue lipid mobilization upon adipocyte-specific GR or STAT5 deficiency is associated with different alterations in the lipolytic pathway. (A) Relative mRNA expression levels of genes with functions in adipocyte lipolysis were quantified by qRT-PCR in epididymal white adipose tissue upon the indicated condition. Ct values were normalized to Gapdh (n≥5/genotype/condition). (B) Western blots showing activation status and/or total expression levels of proteins with functions in adipocyte lipolysis under ad libitum feeding and 48 hour (h) fasting conditions in epididymal white adipose tissue. HSC-70 or Actin served as loading control (currently n=2/genotype/condition). (C) Quantification of protein activation status and expression changes of selected Western blot signals. Signals were quantified by densitometry, total protein expression was corrected for respective loading control and phosphorylated protein was normalized against corrected total protein. For Perilipin and ATGL the ratio of protein expression under fasting/ad libitum feeding condition is displayed. For all analyses: 2-month-old male mice; data are shown as the mean ±s.e.m.; *P<0.05; **P <0.01; ***P<0.001. Pnpla2: ATGL; Lipe: HSL, MglI: MGL, Abhd5: CGI-58, Plin1: Perilipin 1, Angptl4: Angiopoietin-like 4; Pde3b: Phosphodiesterase 3b; Pparg: PPARγ

The decrease in PKA phosphorylation, however, was not associated with diminished upregulation of Angptl4 mRNA expression as reported for 24h fasted mice in the presence of systemic GR inhibition using RU486 (Gray et al, 2012). In addition, we could not detect elevated mRNA expression of the cAMP degrading enzyme Pde3b, which was reported to be suppressed in adipocytes upon treatment with the GR agonist dexamethasone in vitro and in vivo (Gray et al, 2012; Xu et al, 2009a).

In contrast to GR\textsuperscript{Adipoq} mice, adipose tissue-specific STAT5 deficiency did not confer decreased HSL phosphorylation or a major impairment of perilipin 1 downregulation under fasting conditions (Fig. 2A, B and C). Additionally, Lipe and MglI mRNA level were similar to the control group. However, mRNA and total protein level of ATGL were decreased which was accompanied by diminished upregulation of the ATGL co-activator Abhd5 (CGI-58, Fig. 2A, B and C). Interestingly, mRNA expression of PPARγ, which is implicated in the control of Pnpla2 expression (Kershaw et al, 2007; Kim et al, 2006; Shen et al, 2007) and reported to be STAT5 regulated in adipocytes in vitro (Wakao et al, 2011), was also found to be downregulated in EWAT of fasted STAT5\textsuperscript{Adipoq} mice.

Taken together, we show that adipocyte-specific GR as well as STAT5 inactivation is sufficient to diminish fasting-induced adipose tissue lipid mobilization and ectopic hepatic lipid deposition. Our current findings further suggest that the GR and STAT5 acting at different levels of fasting-induced lipolytic signaling. While the reduced lipolytic response upon GR deficiency is characterized by impairments of PKA activation and perilipin 1 downregulation, inactivation of STAT5 is associated with decreased expression of ATGL and its co-activator CGI-58.
Elevated adipose tissue lipid mobilization and subsequent storage in ectopic sides may induce resistance to the action of insulin and disturbances of glucose homeostasis. Hence, we thought that the reduced ability to release NEFA from GR- or STAT5-deficient WAT may be sufficient to increase insulin sensitivity already under a standard diet.

In 4h fasted STAT5^Adipoq mice, blood glucose levels were similar to those in control animals, whereas plasma insulin concentrations were reduced by 32% (Fig. 3A). No significant changes in blood glucose or insulin levels were observed in 4h fasted GR^Adipoq mice when compared with controls. In the 16h fasted state, blood glucose and plasma insulin concentrations were similar in mice of all three genotypes (Fig. 3A).

**Figure 3. Increased insulin sensitivity upon adipocyte-specific GR or STAT5 deficiency.** (A) Fasting blood glucose and plasma insulin level at indicated time points. (B) Insulin tolerance test (left): Insulin was administered through intraperitoneal injection (0,75 U/kg body weight) following a 4 hour (h) fast. Oral glucose tolerance test (right): Glucose was administered through oral gavage (2 g/kg body weight) following a 16 h fast. Blood glucose levels were determined at given time points. For all analyses: 2-month-old male mice (n≥7/genotype/condition); results from 2 independent experiments; data are shown as the mean ±s.e.m.; **P <0.01; ***P<0.001.

Further, glucose tolerance in both GR^Adipoq and STAT5^Adipoq mice was not different from that observed in control mice (Fig. 3B, right panel). In insulin tolerance tests (Fig. 3B, left panel),
there was no significant difference in blood glucose at 15 minutes after insulin injection between the groups, although the maximal decline was more pronounced in GR\textsuperscript{Adipoq} and STAT5\textsuperscript{Adipoq} (~ 90 mg/dl) than in control mice (~ 102 mg/dl). Yet, glucose level starting from 30 minutes after insulin administration were markedly lower in both conditional lines. In control mice blood glucose level fully recovered within 60 minutes after insulin injection, while GR\textsuperscript{Adipoq} mice presented only 84% and STAT5\textsuperscript{Adipoq} mice 63% of the initial glucose values. Our current results show that inactivation of the GR or STAT5 in adipocytes does not alter glucose tolerance but is associated with insulin hypersensitivity under standard diet feeding. The increase in insulin sensitivity becomes particularly evident in the phase of glucose recovery during insulin tolerance testing. This finding suggests enhanced insulin-mediated suppression of hepatic glucose output, which will require further characterization by pyruvate tolerance testing to assess for hepatic gluconeogenic capacity and/or hyperinsulinemic-euglycemic clamp analysis to evaluate hepatic glucose output and glucose uptake in extrahepatic tissues.
CHAPTER THREE: DISCUSSION

Hepatic STAT5 deficiency led to early onset but stable, non-progressive steatosis associated with hepatic GH resistance, deteriorated glucose metabolism and insulin resistance. Thereby, we confirm an essential role of hepatic GH-STAT5 signaling in the maintenance of the liver's metabolic homeostasis as well as in the control of systemic GH availability and function through negative feedback control by IGF-1 (Cui et al, 2007; Davey et al, 2001). Yet, co-deficiency with hepatic GR or transgenic GH overexpression severely aggravated the underlying steatosis. This phenotype when compared to STAT5 deficiency alone was not associated with drastic alterations in the expression of genes involved in liver lipid metabolism. It rather could be linked to an elevation of circulating GC and/or GH level which acted on WAT to induce lipid mobilization. Thereby, persistent GC and/or GH signaling in adipocytes induced partial lipodystrophy, an elevation of ectopic FA influx into the liver and, ultimately, tumorigenic transformation of hepatocytes. Vice versa, we provide evidence that adipocyte lipolysis and ectopic hepatic lipid deposition highly depends on GR and STAT5 signaling in WAT compartments, as the inactivation of either transcription factor in adipocytes strongly diminished fasting-induced lipid mobilization and concomitant steatosis in the respective mouse model.

3.1 Disruption of Hepatic GH-STAT5 Signaling Causes a Lipogenic Gene Expression Profile and Steatosis Independently of Functional GR Signal Transduction

We observed that hepatic STAT5 deficiency was associated with elevated plasma markers of hepatocyte damage and increased hepatic TG content which was graded as stage II steatosis already at 2 months of age. Other than reported for hepatic JAK2 and GHR deficiency (Fan et al, 2009; Sos et al, 2011), but in agreement with STAT5 deletion using the Alb-Cre line (Cui et al, 2007), we did not observe highly elevated circulating NEFA level in response to increased systemic GH. Further, there were no drastic differences in total plasma TG as well as in the plasma lipoprotein particle profile (Kornfeld et al, 2011) when compared to control animals. Therefore, we excluded altered VLDL secretion as a primary cause of elevated TG accumulation in this model. To gain deeper insight into the molecular mechanisms underlying the anti-steatotic functions exerted by GH-STAT5 signaling in liver, we assessed changes in the expression of genes governing hepatic lipid metabolism. The phenotypic presentation of S5KO livers could be linked to increased mRNA and protein level of PPARγ and SREBP-1c, and concomitant up-regulation of target genes involved in FA uptake (Cd36), lipogenesis (Fasn, Scd1) and TG synthesis (Dgat1/2).

PPARγ expression in liver is well established to promote and maintain hepatic steatosis by inducing intrahepatic lipid accumulation and facilitating hepatic FA uptake, in part, by upregulation of Cd36 (Bechmann et al, 2011; Matsusue et al, 2003; Matsusue et al, 2008;
Schadinger et al, 2005; Yu et al, 2003). The aberrant expression of PPARγ and Cd36 is suggested to be one major mechanism for the steatotic phenotype in several mouse models of impaired hepatic GH signal transduction (Barclay et al, 2011; Cui et al, 2007; Fan et al, 2009; Friedbichler et al, 2012; Mueller et al, 2011; Sos et al, 2011). In this regard, it has been shown that genetic abrogation of elevated systemic GH levels in the presence of hepatic JAK2 deficiency markedly ameliorates the fatty liver phenotype, but it does not correct expression of Pparg and Cd36 (Sos et al, 2011). This suggests that Pparg expression is not solely a response to FA influx, but can also be attributed to a loss of GH-mediated repressive functions. Direct interaction of STAT5 with Pparg regulatory promoter regions have been described in non-hepatic cell types (Dentelli et al, 2009; Meirhaeghe et al, 2003; Wakao et al, 2011), but are rather associated with activating than repressive function. Even so there are several putative STAT5 binding elements in the murine Pparg promoter regions; we could not detect significant enrichment of STAT5 binding upon GH stimulation of control livers despite drastic downregulation of Pparg mRNA level in response to exogenous GH (Mueller KM, unpublished observation). One potential indirect mechanism of how STAT5 deficiency contributes to increased expression of Pparg lies in the increased GH-dependent activation of STAT1. In support of this notion, Pparg mRNA expression was not affected by ectopic expression of dominant-negative STAT5 in a hepatoma cell line, while expression of dominant-negative STAT1 variant in the same cell line was sufficient to decrease Pparg transcription (Barclay et al, 2011). However, the presence of dominant-negative STAT5 was associated with a prominent induction of Cd36 mRNA in the same experimental set up. Further, and in support of the earlier notion that abrogation of GH secretion does not reverse Cd36 up-regulation, occupancy of STAT5 on regulatory Cd36 promoter regions in murine livers has been described in the same study. These findings suggest a loss of direct negative regulatory functions on Cd36 gene expression in the absence of STAT5 activity. This, presumably, already permits increased hepatic FA uptake as it was shown upon adenoviral overexpression of CD36 in livers of mice on a regular diet (Koonen et al, 2007). Thereby, CD36 might indirectly contribute to PPARγ activation by FA derivates.

The second major alteration in lipogenic gene expression associated with STAT5 deficiency in our model is the induction of SREBP-1c signaling and an associated increase in lipogenic downstream targets. SREBP-1c together with ChREBP represents the master transcriptional regulator of hepatic de novo lipogenesis and its activation is frequently linked to NAFLD (Bechmann et al, 2011; Dentin et al, 2006; Iizuka et al, 2006; Kammoun et al, 2009; Knebel et al, 2012; Yahagi et al, 2002). Hence, it is feasible to suggest that impaired hepatic GH-STAT5 signal transduction not only favors increased FA uptake but also the induction of a lipogenic program. Yet, to eventually proof that the lipogenic transcription profile is translated into increased hepatic de novo lipogenesis, functional experiments would be required.
Interestingly, hepatic JAK2 deficiency neither resulted in elevated Srebf mRNA level nor elevated rates of de novo lipogenesis (Sos et al, 2011). This discrepancy between the two model systems might be solely attributed to the fact that hepatic JAK2 deletion, other than STAT5 and GHR deficiency, does not translate into insulin resistance and accompanying hyperinsulinemia (Cui et al, 2007; Fan et al, 2009). In support of this notion, hepatic GHR deficiency also is associated with a drastic increase in Srebf expression. We could further show that Srebf gene expression is not only increased upon STAT5 deficiency but is also differentially regulated in STAT5-proficient control livers upon exogenous GH treatment. The Srebf gene gives rise to two SREBP-1 isoforms (1a and 1c) which differ only in their first exon due to different promoter usage and alternate splicing (Shimomura et al, 1997; Yokoyama et al, 1993). Both SREBP-1 isoforms are broadly expressed and exert similar functions in FA and TG synthesis (Im et al, 2009); however, SREBP-1c is the predominant isoform in liver (Shimano et al, 1997; Shimomura et al, 1997). In response to exogenous GH, mRNA expression of both isoforms was found to be downregulated in STAT5-proficient livers. This GH-mediated effect on Srebf gene expression was also observed in livers of bovine GH transgenic mice in spite of pronounced hyperinsulinemia (Olsson et al, 2003). In silico studies on the murine Srebf gene revealed putative STAT5 responsive elements within 1a and 1c regulatory regions which were confirmed in vitro by DNA binding assays (Data not shown). GH-induced STAT5 enrichment on the 1a and, even so to a lesser extent, on the 1c promoter region could be further confirmed by ChIP analyses in vivo. These experiments provide first evidence that hepatic STAT5 might negatively control Srebf gene transcription in response to GH. Yet, to validate the functionality of identified responsive elements, a further characterization in vitro using Srebf promoter constructs will be necessary.

Noteworthy, besides increased PPARγ and SREBP-1 expression, we also observed a prominent up-regulation of adipogenic master regulators CCAAT/enhancer binding protein (C/EBP) α and C/EBPβ on mRNA and protein level in STAT5-deficient livers. Both transcription factors together with PPARγ cooperatively regulate adipocyte functions and are essential for proper adipogenesis (Lefterova et al, 2008; Rosen et al, 2000). Further, at least in adipocytes, both C/EBP isoforms positively regulate PPARγ expression (Farmer, 2006; Rosen et al, 2000). In liver, besides their central role in the regulation of carbohydrate metabolism (Liu et al, 1999; Wang et al, 1995), both C/EBPs have been implicated in the induction of lipogenic gene expression. The deletion of either C/EBP in mouse models of obesity resulted in attenuated hepatic TG content accompanied by decreased expression of several key lipogenic genes including Pparg and Scd1 (Matsusue et al, 2004; Millward et al, 2007; Schroeder-Gloeckler et al, 2007).

In contrast to hepatic STAT5 deficiency, ablating the GR neither resulted in before mentioned phenotypic alterations nor in the manifestation of a lipogenic transcription profile. These
findings are in line with previous reports which suggested that GR signaling in liver promotes hepatic TG accumulation (Shteyer et al, 2004) and steatosis in mouse models of obesity (Lemke et al, 2008). In the latter study, the steatotic effects of the GR were linked to its suppressive action on the anti-lipogenic factor Hairy and Enhancer of Split-1 (HES-1) (Herzig et al, 2003; Lemke et al, 2008), and a subsequent increase in hepatic FA uptake and storage. Transient knock-down of hepatic GR improved the steatotic phenotype by restoring HES-1 expression, leading to decreased PPARγ level and subsequent downregulation FA transporters (e.g. Cd36) (Lemke et al, 2008). Here, we show that the combined deletion of the GR and STAT5 did not improve the STAT5 deficiency underlying steatosis. The deletion of both transcription factors in DKO mice rather was associated with a severe aggravation of the steatotic phenotype, characterized by highly elevated hepatic TG content and stage III steatosis already at 2 months of age. Further, no expression changes of PPARγ and its downstream targets could be observed when compared to S5KO livers. These data indicate that the favorable effects of GR inactivation on hepatic PPARγ signaling in obesity-induced steatosis may not globally be applicable to other model systems. It is tempting to speculate that the described anti-steatotic effects of GR ablation can be overruled by loss of STAT5 regulatory functions, e.g. GR interference reduces Pparg and Cd36 mRNA expression (Lemke et al, 2008), while STAT5 deficiency causes the opposite. The discrepancy between the two model systems and the hypothesis that impaired STAT5 signaling outcompetes the protective effects of GR inactivation would require further investigation (e.g. transient hepatic GR knock down in adult S5KO mice).

Srebf and corresponding lipogenic downstream targets were expressed to higher levels in DKO livers. As no expression changes were present in GRKO livers, direct transcriptional effects of hepatic GR on these genes are rather unlikely. Further, no expression changes of the nuclear hormone receptor liver X receptor (LXR), a potent activator of hepatic SREBP-1c expression and maturation (Repa et al, 2000; Yoshikawa et al, 2001), were observed between S5KO and DKO livers. In accordance with previous studies which report that hepatic Fgf21 expression is under the control of GH (Barclay et al, 2011; Yu et al, 2012a), Fgf21 mRNA level were down-regulated to about 50% upon STAT5 deficiency, whereas its expression was almost completely abolished in DKO livers. FGF21 regulates energy balance, glucose and lipid metabolism (Fisher et al, 2011; Kharitonenkov et al, 2005) and was recently reported to elicit anti-steatotic effects in a model of diet-induced obesity (Xu et al, 2009b), to protect mice from fasting-induced steatosis (Li et al, 2014) and to exert inhibitory functions on hepatocyte SREBP-1c expression and maturation (Zhang et al, 2011). Thus, Fgf21 deficiency might constitute one mechanism for the increased Srebf expression upon co-deficiency of hepatic STAT5 and GR.
From these findings in conjunction with published mouse models we suggest up-regulation of PPARγ and CD36 and subsequent increased rates of FA uptake as a common mechanism by which impaired GH signal transduction in liver induces hepatic steatosis. We extended these findings by the observation that hepatic STAT5 deficiency correlates with a marked induction of a SREBP-1c regulated lipogenic transcription program, and increased expression of adipogenic master regulators C/EBPα/β, thereby predicting elevated rates of de novo lipogenesis in addition to TG synthesis. Our experiments show that hepatic expression of both Srebf isoforms is negatively regulated by GH, a mechanism which might be mediated by direct repressive functions of STAT5. No significant overlap of expression changes induced by GR, STAT5 and their combined deficiency could be observed. Therefore, we conclude that hepatic GH-STAT5 and GC-GR signaling, other than observed for gene sets involved in growth and sexual maturation (Engblom et al, 2007), do not cooperate in the control of lipogenic gene expression in liver.

3.2 Effects of Hepatic STAT5 and GR Deficiency on Adipose Tissue: Combined hepatic GH Resistance and Hypercortisolism Stimulates Adipose Tissue Lipid Mobilisation and Hepatic Fatty Acid Influx

GH is well known to exert lipolytic functions on adipose tissue. GH-deficient and systemic GH-resistant states are characterized by increased body fat, whereas excess circulating GH in the presence of functional GHR signal transduction leads to overall reduced fat mass in humans and in mice (Berryman et al, 2004; Ho et al, 1996; Hoffman et al, 1995; Laron & Klinger, 1993; Moller & Jorgensen, 2009b). Despite a number of differences in their phenotypic appearance as well as in observed alterations in hepatic gene expression, mouse models of impaired GH signaling in liver share hepatic GH resistance as a common characteristic. This condition, induced by a lack of negative feedback inhibition from liver-derived bioactive IGF-1, leads to a drastic rise in systemic GH availability and signaling in responsive tissues (e.g. adipose tissue). Interestingly, the degree of GH-induced adipocyte lipolysis and subsequent rise in circulating NEFA greatly varies between the published models (Cui et al, 2007; Fan et al, 2009; Friedbichler et al, 2012; Mueller et al, 2011; Sos et al, 2011). As mentioned before, genetic abrogation of GH secretion in liver-specific JAK2 knockout mice was shown to normalize circulating NEFA and to rescue the steatotic phenotype. Combined deficiency of JAK2 in liver and adipose tissue was also associated with normalized circulating NEFA and greatly ameliorated steatosis in spite of elevated GH secretion (Nordstrom et al, 2013). On the opposite, infusion of hepatic GHR-deficient mice with IGF-1 normalized circulating GH level but failed to improve the steatotic phenotype (Fan et al, 2009). Of note, in this study IGF-1 treatment was initialized when the steatotic phenotype was fully established. However, GHR null and GHR391 mutant mice (total loss of
STAT5 but not JAK2 activation), which display overall GH resistance in the presence of elevated circulating GH, succumb to steatosis in the absence of enhanced GH-induced adipocyte lipolysis (Barclay et al, 2011). These observations remain unexplained, but argue against unrestrained GH-induced adipocyte lipolysis as the sole mechanism for steatosis in the absence of functional hepatic GH-STAT5 signaling.

In our studies, slightly elevated circulating NEFA and a modest reduction in epididymal WAT were observed in aged STAT5-deficient mice. Further, on the transcriptional level, we could not observe differential expression of the rate-limiting lipase ATGL as well as HSL. To date the molecular mechanism behind GH's lipolytic function on adipose tissue remains elusive. In vitro studies have shown that GH treatment increases the activity of HSL but not its expression in murine and rat adipocytes (Dietz & Schwartz, 1991; Slavin et al, 1994), while others have reported increased HSL activity as well as protein expression upon GH treatment of rat adipose tissue explants (Yang et al, 2004). The chronic effects of GH are considered to be anti-insulin-like and include diminished peripheral insulin sensitivity. Thereby, GH potentially interferes with the anti-lipolytic action of insulin on adipose tissue that has been shown to involve the reduction of cAMP levels and subsequent diminished PKA-mediated activation of HSL (Choi et al, 2010; Choi et al, 2006; Kitamura et al, 1999). Further, insulin treatment decreases Pnpla2 (ATGL) mRNA abundance in murine adipocytes and adipose tissue in vivo (Kershaw et al, 2006; Kim et al, 2006; Kralisch et al, 2005), while mouse models with defective insulin signaling display elevated lipolytic rates which was associated with increased ATGL expression (Kershaw et al, 2006). Evidence that GH's lipolytic functions are, at least in part, mediated by STAT5 comes primarily from in vitro experiments using adipose tissue from mice expressing N-terminal truncated STAT5 variants (STAT5ΔN) which results in diminished lipolytic response upon GH stimulation (Fain et al, 1999). One potential mechanism by which STAT5 signaling induces lipolysis is through PPARγ, which expression and activity is controlled through direct as well as indirect mechanisms by STAT5 in adipocytes (Wakao et al, 2011; Zhao & Stephens, 2013). PPARγ agonist treatment is associated with increased ATGL expression, a concomitant induction of lipolysis in various mouse models (Kershaw et al, 2007; Kim et al, 2006; Shen et al, 2007) and involves the direct control of ATGL expression by PPARγ. In support of this hypothesis, we could show that adipose tissue-specific STAT5 knockout mice present a pronounced defect in their fasting-induced lipolytic response accompanied by decreased expression of PPARγ and ATGL in the epididymal WAT compartment.

In contrast to S5KO mice, the combined liver-specific deficiency of STAT5 and GR was characterized by a striking reduction of epididymal WAT, elevated circulating NEFA leading to an aggravated steatosis and a lipodystrophy-like phenotype. This phenotype was not only associated with enhanced GH-dependent STAT5 activation but also increased nuclear
translocation of the GR in this compartment. The activation of both transcription factors could be linked to a combination of hepatic GH resistance and hypercortisolism. The latter, a rather unexpected result of hepatic GR deficiency, might be explained by the loss of GR-dependent repression of corticosteroid-binding globulin (CBG) expression in liver (Cole et al, 1999). The mainly liver-derived CBG constitutes the major transport protein of circulating GCs (Breuner & Orchinik, 2002; Scrocchi et al, 1993). As the amount of circulating CBG and associated determination of its intrapituitary abundance has been reported to negatively correlate with the degree of GC-mediated negative feedback inhibition (Sakly & Koch, 1981; Sakly & Koch, 1983; Viau & Meaney, 2004), higher CBG expression in our model is one likely mechanism for elevated HPA axis activity. On the transcriptional level, increased WAT mobilization was associated with elevated mRNA expression of ATGL and HSL. GC are well known to exert lipolytic actions on adipose tissue. Further, GC through activation of adipocyte GR was shown to up-regulate the expression of all three major lipases (Campbell et al, 2011; Yu et al, 2010). GC and GH potentiate each other (Djurhuus et al, 2004) and indeed, similar effects have been observed upon combined GH and Dex treatment of human omental adipose tissue in vitro (Fain et al, 2008). In this study, the authors report elevated expression of genes coding for ATGL and HSL in addition to the lipid droplet-associated protein perilipin. In contrast, perilipin 1 mRNA level were downregulated in WAT of DKO mice. Perilipin 1 functions as a barrier between lipid vacuoles and lipases in the basal state (Brasaemle et al, 2000; Lass et al, 2011). In support of our observations, perilipin null mice display constitutively activated basal lipolysis resulting in a drastic reduction of adipose tissue (Martinez-Botas et al, 2000; Tansey et al, 2001). An effect that was linked to the loss of perilipin 1 mediated inhibition on ATGL function (Granneman et al, 2009). Further, we observed that adipocyte-specific GR deficient mice not only present a perturbed fasting-induced lipolytic response associated with diminished PKA-mediated activation of the lipolytic cascade, but also a marked inability to downregulate perilipin 1. Thereby, supporting a regulatory role of GC-GR signaling on perilipin 1 expression and partial lipodystrophy observed in DKO mice. Together, these findings show that combined liver-specific deficiency of STAT5 and GR is associated with endocrine dysfunctions of the GH/IGF-1 and HPA axis resulting in aberrant activation of both transcription factors in adipocytes with deleterious effects on adipose tissue lipid mobilization. The subsequent increase in NEFA availability in conjunction with an increased potential for hepatic FA uptake eventually accounts for the exaggerated steatosis in DKO mice. Further, our data provide in vivo evidence for cooperative effects of GH-STAT5 and GC-GR signaling on adipose tissue lipid mobilization. Hence, understanding the mechanism by which the GR and/or STAT5 participate in the regulation of lipolysis will be
important for a better understanding of how GH and GC modulate adipose tissue lipid mobilization in health and disease.

3.3 Effects of Hepatic STAT5 and GR Deficiency on Glucose Homeostasis

In line with previous reports, hepatic STAT5 deficiency and its underlying steatotic phenotype was accompanied by hyperinsulinemia and hyperglycemia (Cui et al, 2007). Glucose intolerance and insulin resistance in SSKO and DKO was confirmed by tolerance testing and was reflected in altered hepatic IR-mediated signal transduction and concomitant decreased insulin-stimulated serine 473 phosphorylation of AKT. Interestingly, similar alterations in hepatic IR signaling were reported upon knockdown of the prolactin receptor (PRLR) in vitro and in vivo (Yu et al, 2013). Here, PRLR overexpression ameliorated systemic insulin resistant states in genetically obese mice, while its knockdown was associated with insulin resistance, a mechanism which was shown to strongly depend on downstream activation of STAT5. Further, impaired hepatic IR signal transduction and decreased serine phosphorylation of AKT are as well associated with chronic GH treatment of STAT5-proficient rodents (Gao et al, 2013; Prattali et al, 2005). This observation might be explained by desensitization of GH-mediated STAT5 activation due to prolonged GH exposure. An effect which was reported to occur in livers of bovine GH transgenic mice leading to diminished JAK2-STAT5 activity in the presence of active proliferative and anti-apoptotic GH signaling (Miquet et al, 2008; Miquet et al, 2004). Similarly, fasting induces impaired IGF-1 negative feedback inhibition and hepatic GH resistance (Beauloye et al, 2002; Nass et al, 2010; Postel-Vinay et al, 1982). More recently, the protein deacetylase sirtuin 1 was shown to mediate hepatic GH resistance under fasting conditions by deacetylation of STAT5 causing blunted GH-induced STAT5 activation and Igf1 expression (Yamamoto et al, 2013). Under physiologic conditions this mechanism provides an endocrine adaptation to energy deprivation, in part, by inducing a state of transient insulin resistance (Bugianesi et al, 2005b). Thereby, hepatic GH resistance enables a shift from glucose to FA oxidation by reducing peripheral glucose uptake, accelerating adipose tissue lipid mobilization and stimulating hepatic glucose production and release. One could argue that hepatic STAT5 deficiency partly mimics this endocrine adaption mechanism with deleterious effects on hepatic insulin sensitivity and, probably, on peripheral insulin action.

Yet, glucose homeostasis was more drastically affected in DKO mice. In contrast to its functions in hepatic lipid metabolism, the impact of GR on hepatic glucose metabolism was addressed in several studies (Rose & Herzig, 2013; Vegiopoulos & Herzig, 2007). Particularly in diabetic and obese mice, interference with hepatic GR was shown to exert favorable effects on glycemic control by reducing gluconeogenic gene expression and hepatic glucose production (Liang et al, 2005; Liu et al, 2008; Liu et al, 2006; Opherk et al, 2004; Watts et al, 2005). Hepatic GR-deficiency in our experimental system resulted in
prominent hypercortisolism, a condition that is frequently associated with peripheral insulin resistance and impaired glucose disposal (Rose & Herzig, 2013; Vegiopoulos & Herzig, 2007). This likely overrules the favorable effects of impaired hepatic GR functions on hepatic glucose production. In support of this notion, GRKO mice present mild but significant glucose intolerance despite normal insulin response to exogenous glucose and hypoglycaemia in the fed and fasted state (Opherk et al, 2004). As stated before, unphysiologic levels of GH, similar to excessive GC, are associated with impaired glucose disposal and peripheral insulin resistance (Moller & Jorgensen, 2009b; Yuen et al, 2013). Conversely, genetic inactivation of excess GH action in liver-specific IGF-1 deficient mice led not only to improved hepatic but also WAT and skeletal muscle insulin sensitivity (Yakar et al, 2004). The insulin resistance and impaired glycemic control induced by chronically elevated GH is suggested to be partially due to GH-mediated interference with insulin receptor signal transduction. In adipose tissue and skeletal muscle, excess GH exposure in transgenic mice and the acquired peripheral insulin resistance was linked to enhanced expression of p85α regulatory subunit of PI3K accompanied by a decrease in IRS-1-associated PI3K, while GH-deficient mice present the opposite phenotype and decreased levels of p85α (Barbour et al, 2005; del Rincon et al, 2007). Further, GH treatment leads to induction of p85α in adipocytes in vitro, an effect which is also observed in muscle cells in vitro and in vivo upon dexamethasone treatment (del Rincon et al, 2007; Giorgino et al, 1997; Kuo et al, 2012) with similar effects suggested to occur in adipocytes (Yu et al, 2010). PI3K is a heterodimer consisting of the regulatory subunit p85α (encoded by Pik3r1) that elicit binding, activation, as well as localization of PI3K (Backer et al, 1992; Virkamaki et al, 1999) and the catalytic subunit p110. Despite p85α functions in mediating insulin-dependent PI3K signaling, its expression level negatively correlates with insulin sensitivity. Here, the molecular balance of p85α and p110 was suggested to modulate downstream insulin signaling and insulin sensitivity (Mauvais-Jarvis et al, 2002; Ueki et al, 2000; Ueki et al, 2002). Reduced expression of p85 (e.g. global heterozygous Pik3r1 deficiency) increases the molecular ratio of p85-p110 dimers to p85 monomers resulting in improved insulin-mediated PI3K activation and AKT activity. As a consequence, systemic insulin signaling and glucose handling was shown to be improved in diabetic and obese mice (Mauvais-Jarvis et al, 2002; McCurdy et al, 2012; Moriarty et al, 2009). A second interesting aspect is that, at least in the hematopoietic compartment, a cytoplasmic interaction of activated STAT5 and p85 via the scaffolding protein GAB2 has been shown to be required for downstream activation of AKT and cell survival signaling (Harir et al, 2008; Harir et al, 2007; Li et al, 2010a; Nyga et al, 2005). Yet, if a similar cytoplasmic interaction of STAT5 and p85 occurs in metabolic active organs such as liver and WAT to fully induce insulin-stimulated AKT activation was not reported so far.
Such as shown for adipose tissue lipid mobilization, chronic systemic excess of GH and GC presumably exert additive effects on peripheral insulin resistance, a mechanism that might involves imbalanced p85α expression and impaired insulin-mediated signal transduction. The first assumption is supported by the observation that adipose tissue-specific GR- and STAT5-deficient mice, unlike the liver-specific knockouts, present increased insulin sensitivity on a standard diet. For final conclusions, evaluation if altered glucose homeostasis in S5KO and DKO mice is driven mainly by liver or by whole-body insulin insensitivity using hyperinsulinemic-euglycemic clamp analysis will be required to precisely assess insulin-dependent peripheral glucose uptake and hepatic glucose output.

3.4 Disruption of Hepatic GH-STAT5 Signaling Sensitizes the Liver to Metabolic Toxicity and Facilitates the Development and Progression of HCC

It is well established that persistent fatty degeneration of hepatocytes in murine and human NAFLD contributes to chronic inflammation and oxidative toxicity, thereby creating an environment permissive to liver damage and disease progression. Signal transduction through STATs, in particular STAT1 and STAT3, has been shown to exert a variety of critical functions in the pathogenesis of liver diseases from various etiologies, including inflammation, hepatocyte injury, regeneration, and carcinogenesis (Gao et al, 2012; Mair et al, 2011). By contrast, STAT5 functions in liver disease progression are less defined. Nevertheless, published animal models hint at a protective rather than promoting role. Liver-specific STAT5 deficiency has been implicated to promote liver disease progression upon genetically- or chemically-induced liver fibrosis. In the Mdr2 null mouse model of cholestatic liver disease loss of STAT5 function was attributed to disrupted bile acid homeostasis, increased apoptosis due to IGF-1 scarcity, compensatory proliferation, and reduced expression of genes important for hepatocyte integrity and function (Blaas et al, 2010). These genes collectively called “hepatoprotective factors” comprise Igf1, Egfr, Lifr, Prlr and Hnf6 (Natarajan et al, 2007; Olazabal et al, 2009; Omori et al, 1996; Wang et al, 2008). The same study provided first evidence for direct STAT5-mediated transcriptional regulation of Prlr and Lifr in addition to its well known positive regulatory functions in Igf1 expression. Likewise, hepatic STAT5-deficient mice are more susceptible to CCl4-induced liver damage leading to advanced liver fibrosis and, in some cases, progression to HCC (Hosui et al, 2009). Here, loss of STAT5 function and subsequent disease progression was linked to increased STAT3 activation and stabilization of the fibrogenic growth factor TGF-β (Bataller & Brenner, 2005). In a follow up study, STAT5 activity in hepatocytes was further suggested to promote cell cycle arrest upon liver injury by directly inducing the expression of the cell cycle inhibitors Cdkn1a and Cdkn2b. Its loss consequently favors the activation of pro-survival and proliferation pathways, which further is accelerated by rerouting GH signaling to
STAT3-mediated induction of proliferation and survival associated genes (Hosui et al, 2009; Yu et al, 2011).

To gain insight whether STAT5 deficiency and its underlying phenotype in S5KO and DKO mice predisposes to progressive liver disease in the absence of a chemical/genetic insult, we monitored mice over 12 months (6, 9 and 12 months). A fraction of DKO mice succumbed to spontaneous dysplastic lesions that became visible not earlier than 9 months of age. Yet, at 12 months of age DKO mice presented high rates of spontaneous HCCs and even those who did not develop visible tumors displayed higher incidence of cellular alterations and dysplastic foci when compared to S5KO livers. Interestingly, such as reported for rare cases in human NAFLD (Baffy et al, 2011; Paradis et al, 2009) malignant transformation occurred in livers without significant pre-existing fibrotic degeneration and NASH. Additionally, similar to DEN-induced liver tumorigenesis in obese mice (Park et al, 2010) and in early stages of NAFLD in genetically obese mice (Yang et al, 2001), cell proliferation was elevated in DKO HCCs and adjacent liver tissue, while no changes in rates of apoptotic cell death could be observed. We could confirm these findings by STAT5 ablation in the background of GH overexpression (Bartke et al, 2002; Friedbichler et al, 2012; Orian et al, 1990). Here, hepatic STAT5 deficiency corrected the characteristic high rate of hepatocyte turn over and inflammatory phenotype of GH transgenic mice but it promoted early-onset HCC. These observations argue against enhanced hepatocyte turn over as a cause for spontaneous malignant transformation of STAT5-deficient hepatocytes, which seems to be rather driven by elevated proliferation of aberrant hepatocytes in the absence of apoptotic cell death. Additionally, our data suggest that loss of anti-apoptotic functions of IGF-1 which sensitizes STAT5-deficient hepatocytes to bile acid-induced apoptosis (Blaas et al, 2010) may not apply for liver damage under non-cholestatic conditions.

Other suggested risk factors for HCC in the background of NAFLD include obesity, insulin resistance, increased ectopic FA deposition which favors inflammatory cytokine production in addition to oxidative toxicity. Lipodystrophy and obesity, even so direct opposites in regard to adipose tissue mass, are both characterized by similar metabolic and pathological alterations (Herrero et al, 2010; Savage, 2009; Wong et al, 2005). These alterations are largely resembled in partial lipodystrophic DKO mice. HCC occurrence was not only associated with progressive fatty degeneration but also elevated plasma TNF-α in addition to increased liver Il6 and Tnfa mRNA expression. Further, mitochondrial ROS release by complex I and/or III of the respiratory chain was elevated and associated with markers of DNA damage. Elevated rates of β-oxidation may serve as an adaptive mechanism to decrease hepatic FA load and thereby lipotoxicity, but at the same time promote ROS production due to mitochondrial dysfunctions. In this regard, TNF-α was reported to promote mitochondrial ROS release and to modify mitochondrial function (Busquets et al, 2003; Schulze-Osthoff et al, 1992).
reports were recently substantiated by the finding that TNF-α-induced ROS is released by complex I and/or complex III in murine hepatocytes which was linked to a decrease in membrane potential and ATP synthesis (Kastl et al, 2014). Of note, consistent with our model and DEN-induced HCC in obese mice (Park et al, 2010), TNF-α-mediated ROS release did not induce hepatocyte apoptosis in this study. Further, STAT5B deficiency itself was shown to enhance PPARα-dependent peroxisomal FA oxidation (Zhou & Waxman, 1999), thereby it potentially contributes to additional oxidative toxicity and hepatocyte damage.

Increased ectopic FA deposition, elevated TNF-α and IL-6 as well as oxidative stress are not only mediators of chronic liver damage but also potent activators of tumor-promoting JNK1 and ERK1/2 signaling in murine and human HCC (Hirosumi et al, 2002; Luedde et al, 2007; Park et al, 2010; Wagner & Nebreda, 2009). DKO mice exhibited elevated JNK1 activity in liver and to a larger extent in HCCs, while ERK1/2 activation status was unchanged. The essential role of JNK and its downstream target cJun in the liver’s proliferative and regenerative potential has been confirmed by several studies (Behrens et al, 2002; Schwabe et al, 2003; Stepniak et al, 2006). Both JNK1-deficient (Hui et al, 2008) and cJun-deficient mice (Eferl et al, 2003) are protected against DEN-induced HCC, which has been recapitulated by pharmacological inhibition of JNK in human HCC cells (Hui et al, 2008). Further, and in support of our model, TNF-α and IL-6 were not only shown to be required for DEN-induced HCC promotion in obese mice, but also for increased activity of JNK1 in addition to STAT3 (Park et al, 2010). STAT3 is frequently found to be activated in human HCC and its tumor promoting function has been confirmed in chemically and obesity-induced models of HCC (Calvisi et al, 2006; He et al, 2010; Park et al, 2010). In line with earlier reports (Cui et al, 2007; Hosui et al, 2009), hepatic STAT5 deficiency was associated with a rerouting of GH signaling towards STAT3 activation. Yet, STAT3 activity was only slightly increased under basal conditions in STAT5-deficient and non-tumor DKO livers but significantly elevated in DKO HCCs.

Importantly, findings obtained from the DKO model could be largely recapitulated and extended by ablating STAT5 in GH transgenic mice (Friedbichler et al, 2012). Hepatic STAT5 deficiency in the presence of chronically high systemic GH exposure induced a partial lipodystrophic phenotype, highly increased ectopic FA deposition in liver associated with hepatocyte and DNA damage. Malignant transformation occurred as well in the absence of significant pre-existing fibrosis and NASH. Importantly, we could confirm higher STAT3 and JNK1 activity in GH transgenic, STAT5-deficient HCCs which translated into increased c-JUN protein expression and activity. Further, STAT5 deficiency not only promoted proliferative and pro-survival signals but also abolished p53 activity which was otherwise highly active in STAT5-proficient livers of GH transgenic mice. The precise mechanism
behind this observation is still elusive, but may involves similar c-Jun-mediated repression of p53 function as reported in DEN-induced liver carcinogenesis (Eferl et al, 2003) and liver regeneration after partial hepatectomy (Stepniak et al, 2006). In both studies, hepatic c-JUN deficiency led to increased p53 activity. This either translated into p53-mediated up-regulation of Noxa expression followed by apoptosis of DEN-induced HCCs or impaired cell cycle progression in a p21-dependent manner during liver regeneration. In addition, STAT5 via unknown mechanism was shown to be involved in p53 activation and concomitant induction of several downstream targets in mouse embryonic fibroblasts, which was abolished upon STAT5 ablation (Yu et al, 2012b). This observation together with our data, also highly speculative, may pinpoint towards a regulatory role of STAT5 in the control of p53 function in liver.

Collectively, our data in agreement with earlier studies strongly suggest a protective role of STAT5 in liver disease development and progression. The spontaneous malignant transformation of STAT5-deficient hepatocytes might be a result of direct as well as indirect mechanisms related to impaired liver homeostasis: (1) Downregulation of STAT5 target genes involved in hepatocyte integrety and cell cycle inhibition. (2) Rerouting of GH signaling towards STAT3 stimulated proliferation and progression. (3) Ectopic FA deposition and metabolic dysfunction favor hepatocyte and DNA damage and, at the same time promote oncogenic JNK1 and c-JUN activation. (4) Inability to properly induce tumor suppressive p53 activity.
3.5 Conclusion

Impairment of hepatic GH-STAT5 signaling leads to complex alterations in lipid and carbohydrate metabolism resulting in an early onset but stable NAFLD-like phenotype. We postulate that hepatic STAT5 is essential for the liver’s metabolic homeostasis and prevents liver steatosis by ensuring appropriate expression of genes involved in the regulation of hepatic de novo lipogenesis, TG synthesis and FA uptake. Loss of STAT5 and the associated metabolic stress sensitizes hepatocytes to damage and tumorigenic transformation. This becomes particularly evident upon an additional insult such as in form of ectopic FA influx due to combined GH resistance and hypercortisolism as present in DKO mice or upon GH overexpression. Thereby, our studies reveal several mechanisms by which GH-activated STAT5 might participates in the protection from fatty liver disease, liver damage and malignant transformation. Future research, however, is required to better define STAT5’s function in liver disease and implication of these findings for human NAFLD development and progression. A key feature of the aggravated NAFLD-like phenotype of hepatic STAT5 and GR co-deficient as well as of STAT5-deficient GH transgenic mice is the activation of GC and/or GH signaling in adipose compartments. Vice versa, genetic inactivation of the GR or STAT5 strongly diminishes the lipolytic capacity of white adipocytes without inducing body weight gain but increasing the sensitivity to insulin. We are currently investigating the underlying mechanisms behind these findings obtained from adipocyte-specific STAT5- or GR-deficient mice. This approach will be useful to better define the functions of either transcription factor in adipose tissue lipid metabolism and in the development of metabolic diseases.
4. CHAPTER FOUR: MATERIALS & METHODS

4.1 Materials

Standard chemicals and reagents were purchased from Sigma Aldrich, Carl Roth and Invitrogen (Life Technologies) unless stated otherwise. Oligonucleotides were obtained from MWG Biotech (Munich, Germany).

Table 1. Standard buffer

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Component</th>
<th>Concentration</th>
<th>pH (adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail Digestion Buffer</td>
<td>Tris-HCl pH 8.3</td>
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</tr>
<tr>
<td></td>
<td>KCl</td>
<td>500 mM</td>
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<tr>
<td></td>
<td>Gelatine</td>
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<tr>
<td></td>
<td>NP-40</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tween®-20</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>10x TAE</td>
<td>Tris-acetate</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>EDTA pH 8.3</td>
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<td></td>
</tr>
<tr>
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<tr>
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<td>NaCl</td>
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<td></td>
<td>EDTA</td>
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</tr>
<tr>
<td></td>
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<tr>
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**Table 3. Antibodies**

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4.2 Transgenic Mouse Lines

All animals were housed in groups of 2–5 at the Decentralized Biomedical Facilities, Medical University of Vienna (MUW) under standardized conditions with a 12 hours dark/12 hours light cycle and fed a regular diet ad libitum (ssniff Spezialdiäten GmbH, Soest, Germany). The nutrient composition as indicated by the manufacturer was: 22.1% crude protein, 4.5% crude fat, 3.9% crude fiber, 35.8% starch and 5.2% glucose. Animals had free access to water at all times, and food was only withdrawn when required for experimental procedures. A concentration of 0.78 mg/l of Zn$^{2+}$ ions in the drinking water was sufficient to induce ovine GH expression from the metallothionein promoter. Growth curves were generated from data obtained by weekly weight measurements. Animal handling and experimentation was performed according to an ethical animal license protocol approved by the authorities of the Austrian government and the MUW (Publications: Mueller et al., Hepatology 2011 and Friedbichler et al., Hepatology 2012). All animal handling and experimentation using adipose tissue-specific Gr or Stat5 knockout mice was performed according to an animal license.
(BMWF-66.009/0132-II/3b/2013). Further, I successfully completed the university course I703 for education and training of persons, who are responsible for carrying out experimental and other scientific procedures on living animals at the University of Veterinary Medicine Vienna (equivalent to FELASA category B).

4.2.1 Mouse Breeding and Genotyping

Hepatocyte- and cholangiocyte-specific Gr (Gr^fl/fl;AlfpCre^s/wt: GRKO), Stat5ab (Stat5^fl/fl;AlfpCre^s/wt: S5KO or STAT5^Δhep) and Gr;Stat5ab (Gr^fl/fl;Stat5^fl/fl;AlfpCre^s/wt: DKO) homozygous knockout mice were generated by crossing the respective conditional mouse line with AlfpCre transgenic mice (Cui et al, 2004; Engblom et al, 2007; Kellendonk et al, 2000; Tronche et al, 1999). Stat5^fl/fl;AlfpCre^s/wt mice were crossed with GH transgenic animals which express ovine GH under the control of the metallothionein promoter (GH^tg (Snibson et al, 1999)) to generate GH^tgSTAT5^Δhep mice. Adipose tissue-specific Gr (Gr^fl/fl;Adipoq-Cre^s/wt: GRAdipoq) and Stat5ab (Stat5^fl/fl;Adipoq-Cre^s/wt: STAT5Adipoq) homozygous knockout mice were generated by crossing the respective conditional mouse line with Adipoq-Cre transgenic mice (Eguchi et al, 2011). All conditional knockout lines were thereafter maintained on a mixed C57BL/6 x FVB/N x 129/Sv background. In all experimental procedures AlfpCre or Adipoq-Cre transgene negative littermates served as controls (control or wt).

Litters were weaned at day 21 post natum and numbered, metal ear tags were used as unique identifiers for all mice in the colony. The genetic identification of transgenic mice was performed by genotyping of mouse tail biopsies. In brief, 2 mm of the distal tail was removed and incubated in 250 µl of Proteinase K freshly added, stock solution 10 mg/ml over night at 56°C gently shaking.

The crude DNA preparation was diluted 1:3 with double distilled H2O, before a 1 µl aliquot was analyzed in subsequent PCR reactions (5 minutes at 95°C, 40x (20 seconds at 95°C, 20 seconds at 55°C, 90 seconds at 72°C), 10 minutes at 72°C) using respective genotyping primer (Tab. 2). Amplicon size analysis for genotype determination was performed by agarose gel electrophoresis; visualization and documentation by the ChemiDoc™ XRS system (Bio Rad).

4.2.2 Oral Glucose Tolerance Test

Prior to the experiment 8-week-old mice were fasted for 16 hours to obtain comparable baseline glucose levels. The next morning, mice were weighed and blood was collected from the tail vein to determine baseline fasted blood glucose and plasma insulin levels. Blood glucose and β-ketone level were measured directly using a glucometer and respective test strips (OneTouch Ultra, Lifescan Canada Ltd. or Freestyle Precision Xceed, Abbott).
Subsequently, mice were administered with 2 g/kg glucose (20% aqueous glucose solution) by oral gavage and 15, 30, 45 and 60 minutes after glucose challenge blood was collected from the tail vein to determine blood glucose and plasma insulin levels. Directly thereafter, mice were transferred to fresh cages and provided with food *ad libitum*. Insulin was measured using a commercially available insulin ELISA kit (Ultra Sensitive Mouse Insulin ELISA Kit, Crystal Chem) following the manufacturer’s instructions.

4.2.3 Intraperitoneal Insulin Tolerance Test

Prior to the experiment 8-week-old mice were fasted for 4 hours to obtain comparable basal glucose levels. Thereafter, mice were weighed and baseline fasted blood glucose was measured directly from the tail vein using a glucometer. Subsequently, mice received an intraperitoneal injection of 0.75 U/kg body weight human insulin (Actrapid® HM Penfill®, Novo Nordisk) and 15, 30, 45 and 60 minutes after insulin challenge blood glucose level were determined directly from tail vein via glucometer. Directly thereafter, mice were transferred to fresh cages and provided with food *ad libitum*.

4.2.4 Mouse Tissue and Blood Collection

Before terminal experiments, body weights of experimental mice were measured and, if required, blood glucose and β-ketone levels were determined directly from the tail vein via glucometer. Subsequently, mice were sacrificed by cervical dislocation and blood was immediately collected by cardiac puncture. Tissues of interest were excised, weighed, either snap-frozen in liquid nitrogen and stored at -80°C for downstream analyses or formalin fixed and paraffin embedded for histological and immunohistochemical examinations. In non-tumor bearing mice, the left (RNA, protein, histological procedures), median and posterior right liver lobe (histological procedures) were used for further analyses. Liver tumors, if large enough, were excised and divided in half for histological and either protein or mRNA analyses. In all other cases, tumors and adjacent tissue were removed and used either for histological or protein and mRNA analyses.

4.2.5 Growth Hormone, Interleukin-6 and Interferon-γ Administration

Control and S5KO mice were injected intraperitoneally with recombinant human (rh) GH (2 mg/kg, Immunotools), recombinant mouse IL-6 (20 µg/kg, Immunotools) or rhIFN-γ (10 µg/kg, Immunotools) and sacrificed 30 minutes after injection. Control mice were injected intraperitoneally with rhGH (2 mg/kg) and sacrificed either 3 or 6 hours after injection. Vehicle control mice independent of genotype and treatment received injections of PBS.
4.2.6 Dexamethasone and RU486 Treatment

Water-soluble Dexamethasone (20 mg/ml) was administered orally to 6-month-old Control and S5KO mice in the drinking water for 14 consecutive days. Vehicle control mice of either genotype received regular drinking water. RU486 was dissolved in sunflower oil and administered to 6-month-old Control and DKO mice by intraperitoneal injections (20 mg/kg) for 14 consecutive days. Vehicle control mice of either genotype received daily oil injections. During both treatments mice were monitored daily for adverse effects.

4.3 Plasma Biochemistry

To determine plasma concentration of signaling molecules and biochemical parameters, blood obtained from cardiac puncture was collected in tubes pre-coated with heparin (MiniCollect® Plasma Tubes, Greiner bio-one). Blood samples were subsequently centrifuged at 7,000 rpm for 10 minutes at RT. The plasma (supernatant) was used either directly or shock frozen in liquid nitrogen and stored at -80 °C.

4.3.1 Liver Damage Parameter and Plasma Metabolites

Concentrations of biochemical and metabolic plasma parameters (ALT, ALP, total TG and Cholesterol) were determined using the test strip- and color change-based Reflotron Plus analyzer (Roche) according to the manufacture’s instructions.

4.3.2 Radio Immunoassay and Enzyme-Linked Immunosorbent Assay

Plasma corticosterone and ACTH levels were measured via commercially available RIA Kits (ImmunoChem™ double antibody corticosterone or ACTH RIA Kit, MP Biochemicals). The assay was performed following the manufacturer's protocol. The radioactive signal was measured using a conventional gamma counter. The exact concentrations were subsequently calculated using a standard curve.

Plasma GH (Rat/Mouse Growth Hormone ELISA, Millipore), IGF-1 (IGF-1 direct, Rat/Mouse ELISA, IBL Hamburg), IL-6 (Rat/Mouse IL-6 ELISA, R&D Systems), IL-6, TNFα and Resistin concentrations (Mouse Adipokine multiplex ELISA kit, Millipore) were determined using commercially available ELISA kits according to manufacturer's instructions. The intensity of color change was determined by measuring the optical density (OD) at the corresponding wavelength using either a spectrophotometer or Multiplex Bio-Assay Analyzer. The exact concentrations of the cytokine or growth factor were subsequently calculated using a standard curve.
4.3.3 Quantification of Non-Esterified Fatty Acids

The concentration of plasma NEFA was determined using a colorimetric assay (HR Series NEFA-HR(2), Wako Diagnostics). The assay was performed following the manufacturer’s protocol and the final concentration of NEFA was calculated using a standard curve.

4.4 Isolation and Quantification of Hepatic Triglycerides

Hepatic TG were isolated from frozen liver tissues according to the method of Stanlay and Folch (Folch et al, 1957). Briefly, approximately 100 mg liver tissue was homogenized in a chloroform/methanol (2:1 v/v) mixture and incubated for 25 minutes on a rotating wheel at RT. Homogenates were cleared by centrifugation (4,000 rpm; 10 minutes) and the organic phase extracted two times 0.9 % NaCl solution. Fifty μl lipophillic phase were mixed with 10 μl of a TX-100/chloroform (1:1 v/v) mixture, vigorously shaken followed by solvent evaporation using a Speed vac. After taking up the resulting pellet in 50 μl of double distilled H₂O, concentration of hepatic TG was determined using a commercially available colorimetric assay (Serum Triglyceride Determination Kit, Sigma) which distinguishes between endogenous glycerol and glycerol derived by hydrolytic action of lipase on TG. The kit was used according to the manufacturer’s instructions and the final concentration of TG was calculated using a standard curve.

4.5 Histology and Immunohistochemistry

For histological procedures, livers and adipose tissues were fixed for 48 hours in 4% buffered formaldehyde (Roti Histofix 4%, Promega). Subsequently, tissues were dehydrated and paraffin-embedded using standard techniques. For histological or immunohistochemical analyses 1,5 – 2,0 μm thick liver and 4 μm thick WAT sections were cut using a standard microtome and transferred to “Superfrost Plus” microscope slides (Menzel-Gläser). Light microscopic images were captured with a PixeLINK camera and the corresponding acquisition software on a Zeiss Imager Z.1 (magnification: ×100, ×200 and ×400).

4.5.1 Haematoxylin and Eosin Staining

Sections were deparaffinized in Xylene and rehydrated in a descending EtOH gradient to distilled H₂O. Sections were incubated for 10 minutes in haematoxylin solution (Merck). After rinsing the slides in tap water, the staining intensity was differentiated by a short exposure to acidic alcohol (1% HCl in 70% EtOH) and rinsed again in tap water. Subsequently, nuclei were counter-stained with Eosin Y (2 minutes; 0.1% Eosin in 80% EtOH and 0.25% glacial acetic acid). Before mounting the sections with Eukitt mounting medium, dehydration was done in an ascending EtOH gradient.
4.5.2 Oil Red O Staining

Freshly cut, frozen sections (5 µm thick, Cryostat) were mounted on microscope slides, air dried for 30 minutes, fixed in ice cold 4% formalin (10 minutes) and rinsed in distilled H2O. Slides were air dried again for a few minutes before incubation in absolute propylene glycol (5 minutes) and subsequent staining in Oil-red O solution (0.5% Oil-red O in absolute propylene glycol) for 10 minutes at 60°C. The staining intensity was differentiated in an 85% aqueous propylene glycol solution (5 minutes) and rinsed in distilled H2O before counterstaining with haematoxylin solution was performed (1 minute). Slides were washed in running tap water, then placed in distilled H2O and subsequently mounted with Aquatex mounting media (Merck).

4.5.3 Periodic Acid Schiff Staining

Sections were deparaffinized in Xylene and rehydrated in a descending EtOH gradient to distilled H2O. After oxidation for 5 minutes in 0.5% periodic acid solution, slides were rinsed in several changes of distilled H2O and incubated in Schiff’s reagent (Sigma-Aldrich) for 15 minutes. Slides were washed in running tap water and counter-stained for 1 minute with hematoxylin. Thereafter, sections were rinsed for 5 minutes in running tap water, dehydrated and mounted with Eukitt mounting medium.

4.5.4 Trichrome Chromotrope Anilin Blue Staining

Sections were deparaffinized in Xylene and rehydrated in a descending EtOH gradient to distilled H2O. Subsequently, sections were fixed for 1 hour in Bouin’s solution (Sigma-Aldrich) at 56°C, rinsed in tap water for 5 minutes and then incubated in Weigert’s iron hematoxylin (Sigma-Aldrich) for 10 minutes. Afterwards, slides were rinsed in tap water for 10 minutes, incubated in Biebrich scarlet-acid fuchsin solution (Sigma-Aldrich) and washed in distilled H2O. The staining intensity was differentiated in phosphomolybdic-phosphotungstic acid solution (Thermo Scientific) for 10 minutes and immediately thereafter incubated for 10 minutes in an aniline blue solution. Slides were rinsed in distilled H2O before the staining intensity was differentiated for 2 minutes in 1% acetic acid and rinsed again in distilled H2O. Sections were dehydrated and mounted with Eukitt mounting medium.

4.5.5 Immunohistochemistry

Tissue sections were deparaffinized and rehydrated in xylene and a descending EtOH gradient to distilled H2O. Depending on the antibody used, heat-induced antigen retrieval was either performed in citrate buffer (pH 6, S2369, DAKO) or in Tris/EDTA buffer (pH 9, S2367, DAKO). Endogenous peroxidase activity was blocked with 3% H2O2 in PBS (10 minutes). To
prevent unspecific binding, an avidin/biotin blocking (Avidin/Biotin Blocking Kit, Vector laboratories) was performed (10 minutes). Primary antibody (Table 3) incubation was done over night at 4°C. All antibodies were diluted in 1% BSA in PBS. Thereafter, slides were washed in PBS and the signal was detected with the IDetect Super Stain System according to the manufacturer’s instruction (IDlabs Biotechnology). The signal was then amplified using 3-Amino-9-Ethylcarbazole (AEC, IDlabs Biotechnology) and color development was visually controlled. Before mounting the slides with Aquatex mounting media, nuclei were counterstained with a short exposure to hematoxylin solution (1-5 minutes).

4.5.6 Quantification of Cell Size and IHC Positive Cells
Hepatocytes that stained positive in IHC and adipocyte density from HE stained WAT sections were quantified using HistoQuest™ software (TissueGnostics GmbH) according to the manufacturer’s instructions. All quantifications were performed with 5-10 fields/liver or epididymal WAT from 3-10 mice/genotype depending on the experiment.

4.6 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 (Ultrastructural Pathology, AKH; Vienna, Austria).

4.7 RNA Extraction and Quantitative mRNA Expression Analysis
Total RNA was extracted from mouse tissues using either TRIzol or the RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. The concentration and purity of isolated RNA was measured with a conventional UV/VIS spectrophotometer. The RNA quality was determined by denaturing agarose gel electrophoresis using the ratio between 28S to 18S ribosomal RNA (2:1) as indicator for high quality preparation. Isolated RNA (1 µg) was reverse-transcribed into cDNA using the commercially available RevertAid First Strand cDNA Synthesis and oligo-dT or random hexamer primers (Fermentas) following the manufacturer’s protocol. Subsequent quantitative real-time PCR (qRT-PCR) was performed on an Eppendorf Realplex system using the Eppendorf Taq DNA polymerase kit (Eppendorf) and SYBR green method for detection of amplicons. The following cycles were used: 1 minute at 94°C, 40 x (30 seconds at 94°C, 30 seconds at 55°C, 2 minutes at 72°C), 2 minutes at 72°C. Post-amplification melting-curve analysis was performed and water controls were included to test qRT-PCR reactions for primer-dimer artifacts and to ensure amplification specificity. Threshold cycles (Ct-values) specific for genes of interest were
normalized to respective Ct-values of murine Gapdh to obtain sample-specific ΔCt-values. The results were expressed as fold change in expression of the individual knockout in relation to the control group. If possible, intron-spanning primers were used. Otherwise, isolated RNA was DNase I treated according to the manufacturer’s instruction prior to cDNA synthesis. The DNA sequences of primers used for qRT-PCR analysis are given in Table 2. For some purposes, TaqMan-based qRT-PCR analyses were applied using TaqMan® Universal PCR-Master Mix, NO AmpErase UNG with TaqMan® Assay on demand kits (Applied Biosystems) according to the manufacturer’s instructions. Quantitative RT-PCR was performed on an ABI-PRISM 7700 Sequence Detector System (Applied Biosystems).

4.8 Protein Biochemistry

4.8.1 Protein Extraction

Depending on the experiment, approximately 50-100 mg of mouse tissue was homogenized in 0.5-1 ml of IP buffer using a tissue homogenizer under constant cooling. Homogenates were cleared by repeated centrifugation at maximum speed (30 minutes, 4°C). Protein concentration in the supernatant was determined using a commercial Bradford solution (Protein assay solution, BioRad).

4.8.2 SDS-PAGE and Immunoblotting

Depending on the experiment, 40-100 µg of protein were mixed with sample buffer (5% β-mercaptoethanol freshly added), denatured (5 minutes, 95°C) before resolved depending on the desired resolution by SDS-PAGE and transferred to nitrocellulose membranes. Briefly, protein samples were loaded onto 10% polyacrylamide gels and separated (80-100 V) until desired resolution was reached using the Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad). Proteins were transferred to nitrocellulose membranes pre-equilibrated in transfer buffer by wet blotting (125 mA; o/n at 4°C) using the Trans-blot Cell wet blot system (Bio-Rad) followed by determination of transfer efficiency using Ponceau S stain (0.1% w/v Ponceau-S, 5% acetic acid, 5 minutes). Thereafter, membranes were blocked with 5% BSA in TBST (1 hour, RT), and incubated with primary antibody (1% BSA in TBST) under gentle agitation at 4°C overnight. Membranes were rinsed three times in TBST (10 minutes) and incubated with HRP-conjugated secondary antibody under gentle agitation (1 hour, RT). Subsequently, membranes were rinsed as indicated above and antigen-specific binding of antibodies was visualized with an enhanced chemiluminiscence (ECL) system (ECL Plus Western Blotting Detection Reagent, Amersham Biosciences) and exposure to ECL films (CL-X Posure™ Film, Thermo Scientific). The primary and secondary antibodies used for Western blot analyses are given in Table 3.
4.8.3 Immunoprecipitation
For immunoprecipitation (IP) experiments 1000-2000 μg of protein extracts were adjusted to 600 μl with IP buffer before the respective antibody was added and subsequently incubated under constant agitation (4°C, o/n). Afterwards, 40 μl protein A Sepharose beads (GE Healthcare) were added and the mixture incubated for 1 hour at 4 °C under constant agitation. After incubation, the Sepharose beads were washed three times with IP buffer (rpm, 4°C), pelleted and dissolved in 40 μl sample buffer. The mixture was heated to 95 °C (5 minutes) to elute the formed immune complexes and cleared by centrifugation before the supernatant was analyzed by Western blotting. The antibodies used for IP and subsequent Western blot analyses are given in Table 3.

4.8.4 Quantification of Western Blot Signal Intensity
Quantitative signal intensity analysis of Western blots was performed using the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011.). Signals were quantified by densitometry, total protein expression was corrected for the respective loading control and phosphorylated protein was normalized against corrected total protein.

4.9 Chromatin Immunoprecipitation
Genomic localization of putative STAT5 responsive elements in the murine Srebf (1a) and Srebf (1c) promoter regions were identified using the TFSEARCH software (http://www.cbrc.jp/research/db/TFSEARCH.html). Sequence conservation alignments between mouse (mm9) and human (hg18) sequence annotations were performed using the ECR browser (http://www.dcode.org/). Primers used to detected STAT5 binding to the Igf1 promoter amplify a conserved STAT5 responsive element (S5RE: TTC(N3)GAA) (Blaas et al, 2010) and fold-enrichment of STAT5 binding to this genomic region served as positive control. Unspecific chromatin binding of the STAT5 antibody was excluded by a parallel ChIP with a rabbit-IgG antibody.

Livers were harvested from vehicle and hGH treated Control and SSKO mice (30 minutes) and 100 mg of frozen liver tissue was grounded in liquid nitrogen. Cross-linking, isolation and sonication of chromatin was performed as described (detailed description under: http://www.genomecenter.ucdavis.edu/farnham/protocols/tissues.html). Subsequently, 20 μg of sonicated chromatin was incubated with STAT5-specific antibodies pre-bound to magnetic beads (Dynabeads® M-280 Sheep anti-Rabbit IgG, Invitrogen) at 4°C over night. Thereafter, beads were washed 5 times with wash buffer (50 mM HEPES ph 8,0, 1 mM EDTA, 1% NP-40, 0,7 % sodium deoxycholate, 0,5 M LiCl) before precipitated chromatin was eluted from
the beads with 1% SDS, 0.1 M NaHCO₃, de-crosslinked and purified. Analysis of loci containing putative STAT5 binding sites was performed by real-time PCR using the SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacture’s instruction. Primer sequences used for ChIP analyses are given in Tab. 3.

4.10 Microarray and Gene Set Enrichment Analysis
Gene set enrichment analysis was performed using previously published microarray data sets (described in (Engblom et al, 2007)). In brief, total RNA was isolated from livers of 4-week-old mice using the RNeasy Mini Kit (Quiagen) following the manufacturer's protocol. For each microarray, three livers were pooled with a total of three microarrays for each mutant group and nine for the control group. The pooled RNA was labeled, hybridized to Murine Genome Array U74Av2 (Affymetrix) and scanned according to the manufacture's instruction. Analysis of coordinated changes in functional gene sets was done using MAPPfinder software (http://www.genmapp.org/help_v2/MAPPFinder.htm) and gene set enrichment analysis software (GSEA, http://www.broad.mit.edu/gsea).

4.11 Preparation of Mitochondria and Determination of Mitochondrial Function
Mouse liver mitochondria were isolated from freshly harvested livers as described previously (Staniek & Nohl, 1999) and stored at 0°C in a buffer containing 0.25 M sucrose, 10 mM Tris·HCl, 0.5 mM EDTA (pH 7,2), and 0.5 g/l FA-free BSA. The quantity of ROS was measured with Electron Paramagnetic Resonance (EPR) spectroscopy by incubating the isolated mitochondria with a spin trap and measuring the intensity of the signal. Isolated mitochondria (1 mg/ml) were first mixed with an incubation buffer containing 80 mM potassium chloride, 5 mM potassium phosphate, 20 mM Tris·HCl, 1 mM diethylenetriaminepentaacetic acid and 0.1% FA-free BSA (pH 7,4). Either 10.7 µM succinate or 10.7 µM glutamate plus malate was added to stimulate respiration. A spin trap was then added to the mitochondria and the suspension incubated for 20 minutes on a shaking table at RT. After 20 minutes, a capillary tube filled with the suspension was inserted into the EPR and the spectrum was recorded.

The respiratory parameters of mitochondria were determined by means of high resolution respirometry (Oxygraph-2k, OROBOROS® INSTRUMENTS GmbH). Isolated mitochondria (1 mg/ml) were incubated in a buffer consisting of 105 mM KCl, 20 mM Tris-HCl, 1 mM diethylenetriaminepentaacetic acid, 5 mM KH₂PO₄, and 1 mg/ml fatty acid-free BSA (pH 7.4, 25°C). State 2 respiration was stimulated either by the addition of either 5 mM glutamate plus 5 mM malate (complex I) or 10 mM succinate (complex II) in the presence of rotenone (1 µg/ml). The latter was used to prevent electron transport to and from complex I. The transition to state 3 respiration was induced by the addition of 200 µM ADP. The transition to
state 4 was determined as a decline in respiration rate following state 3 respiration. Respiratory control ratio 1 (RC1) was determined as the ratio of respiration rates in state 3 to state 2; respiratory control ratio 2 (RC2) was determined as the ratio of respiration rates in state 3 to state 4.

4.12 Statistical Analysis

Results are presented as mean ± standard error of the mean (s.e.m). Statistical analyses were performed using a confidence interval of 95% with either two-tailed Student's t-test or Wilcoxon rank-sum test for comparing two groups and one-way ANOVA followed by Tukey's or Dunns' post-hoc test for multiple comparison. Insulin tolerance tests and body growth curves were analyzed with repeated measures two-way ANOVA followed by Bonferroni's post-hoc tests. Kaplan-Meier plots were analyzed for significance using the log-rank test. Statistical analyses were performed using the GraphPad Prism® software (GraphPad Software). Data were considered statistically significant: *p<0.05; **p<0.01; ***p<0.001.
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Appendix

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CURRICULUM VITAE

PERSONAL DETAILS
Name: Kristina M. Mueller
Date of birth: March 8, 1983
Place of birth: Darmstadt, Germany
Nationality: German
Languages: German, English
Contact: Ludwig Boltzmann Institute for Cancer Research (LBI-CR)
Waehringerstrasse 13a, 1090 Vienna, Austria
Phone: +43-1-40160-71270
kristina.mueller@lbicr.lbg.ac.at

ACADEMIC DEGREE
12/2008 Dipl. Ing. (FH) in Biotechnology
University of Applied Science Darmstadt, Germany

EDUCATION AND RESEARCH INSTITUTIONS
2003-2008 Undergraduate program in Biotechnology, University of Applied
Science Darmstadt, Darmstadt, Germany

01/2008 – 08/2008 Diploma thesis
Eppley Institute for Cancer Research and Allied Diseases,
University of Nebraska Medical Center, Omaha, NE, USA
Supervisor: Prof. Kay-Uwe Wagner
Subject of thesis: Identification of Breast Cancer Susceptibility
Genes using a Sleeping Beauty-mediated Transposition of a
Novel Gene Search Vector

05/2009 - present Graduate studies
Ludwig Boltzmann Institute for Cancer Research
Supervisor: Prof. Richard Moriggl
Subject of thesis: The Role of STAT5 and GR Signaling in Non-
alcoholic Fatty Liver Disease and Hepatic Carcinogenesis

02/2010 - present PhD program: Malignant Diseases; Medical University of
Vienna, Vienna, Austria
FURTHER RESEARCH EXPERIENCE AND TRAINING

2006
Undergraduate research project
University of Applied Science Darmstadt
Supervisor: Prof. Stefan H. Hüttenhain
Project: Solvent-Induced Chirality in the Hydroboration of Ketones.

Undergraduate research project
University of Applied Science Darmstadt
Supervisor: Prof. Renate Heinzel-Wieland
Project: Isolation and Characterization of Bacterial Laccase-like Enzymes from Streptomyces sp.

Full-time trainee position
LEUKOCARE AG (Department of Research and Development, Georg-Speyer-Haus), Frankfurt a.M., Germany
Supervisor: Dr. Jens Altrichter
Immunological methods, tissue culture, antibody immobilization techniques

11/2012
University course I703 (equivalent to FELASA category B)
University of Veterinary Medicine Vienna, Vienna, Austria

ADMINISTRATIVE AND TEACHING EXPERIENCE

2010-2011
2nd Student Representative (LBI-CR)

2011-2012
2nd Student Representative (LBI-CR)

12/2012
Preparation and defense of an animal license application (approved 2013)

01/2013-01/2014
Supervision of Master thesis; Doris Kaltenecker: STAT5 signalling in adipose tissue function.

SCIENTIFIC PUBLICATIONS


**MANUSCRIPTS (submitted and in preparation)**


**INVITED TALKS AND SEMINARS**

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**POSTER PRESENTATIONS**

*Hepatocyte-specific deletion of Stat5 and GR leads to severe fatty liver disease and promotes hepatic dysplasia.* **Kristina M. Mueller**, Jan-Wilhelm Kornfeld, Katrin Friedbichler Gerda Egger, Lukas Kenner, Luigi Terracciano, Markus H. Heim, and Richard Moriggl. *Jak-Stat Signalling: from Basics to Disease.* Vienna, Austria, 2010

*Hepatocyte-specific deletion of Stat5 and GR leads to severe fatty liver disease and promotes hepatic dysplasia.* **Kristina M. Mueller**, Jan-Wilhelm Kornfeld, Katrin Friedbichler, Gerda Egger, Lukas Kenner, Luigi Terracciano, Markus H. Heim, and Richard Moriggl. The 6th YSA-PhD-Symposium at the Medical University of Vienna, Vienna, Austria, 2010

Impairment of hepatic growth hormone and glucocorticoid receptor signaling causes steatosis and hepatocellular carcinoma in mice. **Kristina M. Mueller**, Jan-Wilhelm Kornfeld, Katrin Friedbichler, Lukas Kenner, Andrey Kozlov, Markus H. Heim, and Richard Moriggl. The 7th YSA-PhD-Symposium at the Medical University of Vienna, Vienna, Austria, 2011


Glucocorticoid receptor function is essential for SOCS2-mediated negative regulation of hepatic GHR signaling. **Kristina M. Mueller**, Jan-Wilhelm Kornfeld, Guenther Schuetz, Douglas J. Hilton, and Richard Moriggl. The 8th YSA-PhD-Symposium at the Medical University of Vienna, Vienna, Austria, 2012

Regulation of adipose tissue metabolism by glucocorticoid receptor signaling. **Kristina M. Mueller**, Sabine Amann, Doris Kaltenecker, Jan-Wilhelm Kornfeld, Harald Esterbauer, and Richard Moriggl. The 9th YSA-PhD-Symposium at the Medical University of Vienna, Vienna, Austria, 2013

Regulation of adipose tissue lipid metabolism by glucocorticoid receptor signaling. **Kristina M. Mueller**, Sabine Amann, Doris Kaltenecker, Jan-Wilhelm Kornfeld, Harald Esterbauer, and Richard Moriggl. 2nd FEBS Special Meeting on JAK-STAT Signalling: Model Organisms and Beyond, Nottingham, UK, 2013

**NATIONAL AND INTERNATIONAL COLLABORATIONS**

**Prof. Harald Esterbauer** (Department of Laboratory Medicine, Medical University Vienna, Vienna, Austria)

**Prof. Emilio Casanova** (Ludwig Boltzmann Institute for Cancer Research, Vienna, Austria)

**Prof. Lukas Kenner** (Ludwig Boltzmann Institute for Cancer Research, Vienna, Austria)

**Prof. Rudolf Zechner** (Institute of Molecular Biosciences, University of Graz, Graz, Austria)

**Prof. Guenter Haemmerle** (Institute of Molecular Biosciences, University of Graz, Graz, Austria)

**Prof. Johannes Haybaeck** (Institute for Pathology, Medical University of Graz, Graz, Austria)

**Univ. Doz. Dr. Andrey Kozlov** (Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria)
Prof. Jan P. Tuckermann (Institute of Zoology and Endocrinology, University of Ulm, Ulm, Germany)

Prof. Markus H. Heim (Department of Biomedicine, Division of Gastroenterology and Hepatology, University Hospital Basel, Basel, Switzerland)

Prof. Kay-Uwe Wagner (University of Nebraska Medical Center, Eppley Institute for Cancer Research and Allied Diseases, Omaha, NE, USA)

Dr. Jan-Wilhelm Kornfeld (Max Planck Institute for Neurological Research, University of Cologne, Cologne, Germany)

Dr. Maja Vujic Spasic (Institute of Zoology and Endocrinology, University of Ulm, Ulm, Germany)

LIST OF REFERENCES

Prof. Richard Moriggl
Ludwig Boltzmann Institute for Cancer Research
Vienna, Austria
Phone: +43-1-40160-71210
Email: richard.moriggl@lbicr.lbg.ac.at

Prof. Kay-Uwe Wagner
University of Nebraska Medical Center, Eppley Institute for Cancer Research and Allied Diseases
Omaha, NE, USA
Phone: +1-402-559-3288
Email: kuwagner@unmc.edu

Prof. Harald Esterbauer
Department of Laboratory Medicine, Medical University Vienna
Vienna, Austria
Phone: +43-1-40400 -73757
Email: harald.esterbauer@meduniwien.ac.at

Prof. Emilio Casanova
Ludwig Boltzmann Institute for Cancer Research
Vienna, Austria
Phone: +43-1-40160-71210
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