Thesis

Effect of R2TP complex and R2TP associated proteins on bile acid metabolism

submitted by
Benedikt Lackner

in partial Fulfilment of the Requirements for the Degree of
Doctor of Medicine

(Dr. med. univ.)

Medical University of Graz

executed at the

Department of Internal Medicine

Clinical Division of Gastroenterology and Hepatology

under the supervision of

Mag. Dr. rer. nat. Tarek Moustafa

Univ.-Prof. Dr. Peter Fickert

Graz, 04.11.2021

Declaration of Academic Honesty

I hereby declare that I have written this thesis independently, that I have not used any sources other than those indicated, and that I have marked and cited the passages taken verbatim or analogous from the references used.

Graz, 04.11.2021 Benedikt Lackner eh.

Acknowledgements

First and foremost, I would like to express my special gratitude to my wife Yasmin, who is sticking with me through thick and thin, who shares with me the joys of life and with whom I find peace in the most difficult times.

Furthermore, I am equally grateful for the continuous support my mother Ingrid and my father Josef gave me throughout my studies. They have accompanied me my whole life and enabled me to successfully overcome any obstacle along my path to graduation. I would also like to thank my brothers Philipp and Florian for their support over the years.

My sincere gratitude goes to my supervisor Univ.-Ass. Mag. Dr.rer.nat. Tarek Moustafa, who sparked my interest in scientific research and always supported me with his knowledge and experience.

Genuine thanks are also due to Univ.-Prof. Dr. Peter Fickert who gave me the opportunity to work in the laboratory of the Center for Medical Research.

Last but not least, I would like to thank the entire research group of the Department of Gastroenterology and Hepatology who welcomed and supported me so warmly. Especially Dr. Silvia Racedo and Dr.med.univ. Alex Zaufel, who taught me laboratory procedures and techniques and performed parts of the experiments.

Danksagung

Meinen besonderen Dank möchte ich meiner Frau Yasmin aussprechen, die in jeder Lebenslage an meiner Seite steht, die mit mir die Freuden des Lebens teilt und bei der ich in den schwierigsten Zeiten Ruhe finde.

Ebenso dankbar bin ich über die Unterstützung meiner Mutter Ingrid und meines Vaters Josef, die mich auf meinem Lebensweg begleiten und die es mir ermöglicht haben meine universitäre Ausbildung ohne nennenswerte Hindernisse zu durchlaufen. Auch möchte ich meinen Brüdern Philipp und Florian für die Unterstützung über all diese Jahre danken.

Aufrichtigen Dank möchte ich meinem Betreuer Univ.-Ass. Mag. Dr.rer.nat. Tarek Moustafa aussprechen, der in mir das wissenschaftliche Interesse geweckt hat und mir stets mit seinem Fachwissen und seiner Erfahrung zur Seite stand.

Dank gebührt ebenso Univ.-Prof. Dr. Peter Fickert der mir die Möglichkeit gab im Labor des Zentrums für medizinische Forschung zu arbeiten.

Bedanken möchte ich mich auch bei der gesamten Forschungsgruppe der Abteilung für Gastroenterologie und Hepatologie, die mich so herzlich aufgenommen und unterstützt haben. Allen voran möchte ich Dr. Silvia Racedo und Dr.med.univ. Alex Zaufel danken, die mir Laborverfahren und -techniken beigebracht und einen Teil der Experimente durchgeführt haben.

Table of Contents

List of Abbreviations

Abbreviation	Meaning
4E-BP	eukaryotic translation initiation factor 4E (eIF4E) binding protein
a.dest.	aqua destillata
AAA+	ATPase associated with diverse cellular activities
ABCA1	ATP-binding cassette transporter A1
ABCG	ATP-binding cassette sub-family G
ACC	acetyl CoA carboxylase
ACOX1	Peroxisomal acyl-coenzyme A oxidase 1
AOX	Acyl-CoA Oxidase
APOA1	apolipoprotein A1
ARL7	ADP-ribosylation factor-like 7
ATCC	American Type Culture Collection
ATM	Ataxia-telangiectasia mutated
ATPase	Adenosintriphosphatase
ATR	ataxia-telangiectasia and Rad3-related
BA	Bile acid
BCA	bicinchoninic acid
BSA	Bovine Serum Albumin
BSEP	Bile Salt Export Pump
CA	cholic acid
CDCA	chenodeoxycholic acid
cDNA	complementary DNA
CPT1A	carnitine palmitoyltransferase 1A
CYP7A1	Cytochrome P450 7A1/Cholesterol 7α-hydroxylase
DCA	deoxycholic acid
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
EDTA	Ethylenediaminetetraacetic acid

eIF4E	eukaryotic translation initiation factor 4E
FAS	fatty acid synthase
FASN	fatty acid synthase
FBS	fetal bovine serum
FXR	Farnesoid X receptor
G6Pase	Glucose-6-Phosphatase
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	glucose transporter type 4
GPBAR1	G protein-coupled bile acid receptor
GßL	G protein beta subunit-like
GTP	Guanosintriphosphat
GTPase	Guanosintriphosphatase
HIFα	hypoxia inducible factor alpha
HSP	heat shock protein
h-TBP	human TATA-box binding protein
IDOL	inducible degrader of LDLR
kDA	kilodalton
KNG1	kininogen-1
LC3	Microtubule-associated protein light chain 3
LCA	lithocholic acid
LDLR	low-density lipoprotein receptor
LXR	Liver X receptor
LXRE	LXR response element
mAb	monoclonal antibody
MDR	multidrug resistance protein
mLST8	mammalian lethal with SEC13 protein 8
mTOR	mechanistic Target of Rapamycin
mTORC	mTOR Complex
NCoR	nuclear receptor co-repressor
NTC	no template control
NUFIP	Nuclear fragile X mental retardation-interacting protein
Ost	Organic solute transporter
pAb	polyclonal antibody

PBS	Phosphate-buffered saline
PEPCK	Phosphoenolpyruvatcarboxykinase
PFDL	prefoldin-like complex
PI3K	Phosphatidylinositide 3-kinase
PIH1D1	Pih1 domain-containing protein 1
PIKK	PI3K-related kinase
PPARα	Peroxisome proliferator-activated receptor alpha
PPAR-α	peroxisome proliferator activated receptor alpha
pre-β HDL	pre-β high-density lipoprotein
qPCR	Quantitative Polymerase Chain Reaction
qPCR	real-time quantitative PCR
R2TP	Rvb1-Rvb2-Tah1-Pih1 complex
Rag	Ragulator
RAPTOR	regulatory associated protein of mTOR
RCT	reverse choelsterol transport
Rheb	Ras homolog enriched in brain
RNA	Ribonucleic acid
RNAi	RNA interference
RPAP3	RNA polymerase II associated protein 3
RUVBL1	RuvB-like protein 1
RXR	Retinoid X Receptor
S6K	S6 protein kinase
SCD1	stearoyl CoA desaturase 1
SHP	small heterodimer partner
siRNA	Small interfering RNA
SMG1	suppressor with morphogenetic effect on genitalia 1
SMRT	silencing mediator for retinoic acid and thyroid hormone receptor
snoRNP	Small nuclear ribonucleoproteins
SREPB	Sterol regulatory element-binding protein
TBC	Tre-2/Bub2/Cdc16
TBC1D7	TBC1 Domain Family Member 7
TBST	Tris-buffered saline with Tween20
TELO2	Telomere length regulation protein 2

TO1	Torin 1
TRI/TRIzol	mixture of guanidine thiocyanate and phenol
TRRAP	transcription domain associated protein
TSC	tuberous sclerosis complex
ULK	Unc51-like kinase
ZNHIT3	Zinc finger HIT domain-containing protein 3

Abstract

Background

The nuclear hormone receptors (NHRs) LXR and FXR are regulators of numerous metabolic processes and maintain the balance between bile acid (BA) and cholesterol metabolism. The mammalian target of rapamycin (mTORC1), a central regulator of lipid, glucose, and cholesterol metabolism, appears to influence FXR and LXR. Within my thesis, I wanted to contribute to the better understanding of the crosstalk between signaling pathways inside and outside the nucleus. In particular, how mTORC1 regulates the expression of NHRs and related bile acid associated gene expression.

Aims

Based on the hypothesis that PIKKs and R2TP/PFDL-associated proteins are involved in mTORC1 nuclear receptor communication, the aim of the study is to determine to what extent ZNHIT3, NUFIP, RUVBL1 and TRRAP are involved in mTORC-signaling and to what extent knockdown of these genes affects the function of NHRs.

Methods

HepG2 cells were treated with NUFIP, RUVBL1, ZNHIT3 and TRRAP siRNAs. Cells were additionally treated with FXR-ligand GW4064 and LXR-ligand GW3965. In another subset, treatment consisted of mTOR inhibitor Torin1. Western blots were performed showing the protein expression levels of FXR, mTOR, and related proteins. Quantitative polymerase chain reaction (qPCR) analyses for LXR and FXR target genes were performed under basal conditions and after stimulation of NHRs by synthetic ligands.

Results

Knockdown of NUFIP and TRRAP showed a positive effect on mTOR protein expression. FXR protein expression was negatively affected by NUFIP-KD and showed an increase in TRRAP-KD samples. TRRAP-KD, ZNHIT3-KD, NUFIP-KD

and RUVBL1-KD decreased mRNA gene expression of LXR and FXR target genes under stimulated conditions.

Conclusion

FXR was shown to correlate with mTOR expression levels. mTOR is supported by its PIKK family member TRRAP. The R2TP complex and its associated proteins are an important component of translation and are also essential for FXR and LXR. The data obtained point toward a co-regulatory mechanism that impacts genes involved in both cholesterol and bile acid metabolism, mediated in part by FXR and LXR. Based on these important regulatory functions we can speculate that maybe compensatory mechanisms are involved if one or the other nuclear receptor (FXR/LXR) or metabolic pathway (bile acids/cholesterol) is affected.

Zusammenfassung

Hintergrund

Die Kernrezeptoren LXR und FXR sind wichtige Regulatoren zahlreicher Stoffwechselprozesse. So halten sie zum Beispiel den Gallensäure- und Cholesterinstoffwechsel im Gleichgewicht. Der Proteinkomplex mTORC1, ein zentraler Regulator verschiedener Prozesse wie der Differenzierung und Proliferation von Zellen sowie des Lipid- und Glukosestoffwechsels, scheint einen Einfluss auf die Kernrezeptoren FXR und LXR zu haben. Mit meiner Diplomarbeit möchte ich einen Beitrag zum besseren Verständnis dieser Kommunikations- und Signalwege leisten.

Ziele

Ausgehend von der Hypothese, dass PIKKs und R2TP/PFDL-assoziierte Proteine an der mTORC1-Kernrezeptor-Kommunikation beteiligt sind, soll untersucht werden, inwieweit ZNHIT3, NUFIP, RUVBL1 und TRRAP an der mTORC-Signalübertragung beteiligt sind und inwieweit ein Knock-down dieser Gene die Funktion von FXR und LXR beeinflusst.

Methoden

HepG2-Zellen wurden mit NUFIP, RUVBL1, ZNHIT3 und TRRAP siRNA behandelt. In einem Teil der Experimente wurden die Zellen zusätzlich mit dem FXR-Liganden GW4064 und dem LXR-Liganden GW3965 stimuliert. Bei einer weiteren Untergruppe Bestand die Behandlung aus dem mTOR-Inhibitor Torin1. Western Blots wurden durchgeführt, um die exprimierten Proteinmengen von FXR und mTOR zu ermitteln. Quantitative Polymerase-Kettenreaktionsanalysen (qPCR) für LXR- und FXR-Zielgene wurden sowohl unter Basalbedingungen als auch nach Stimulierung der NHRs durch die synthetischen Liganden durchgeführt.

Ergebnisse

Der Gen-Knockdown von NUFIP und TRRAP wirkte sich positiv auf die mTOR-Proteinexpression aus. Die FXR-Proteinexpression wurde durch NUFIP-KD negativ beeinflusst und zeigte einen Anstieg in TRRAP-KD-Proben. TRRAP-KD, ZNHIT3-

KD, NUFIP-KD und RUVBL1-KD verringerten die mRNA-Genexpression von LXR- und FXR-Zielgenen unter stimulierten Bedingungen.

Schlussfolgerung

Es wurde eine Korrelation zwischen FXR und mTOR nachgewiesen. mTOR wird von seinem PIKK-Familienmitglied TRRAP unterstützt. Der R2TP-Komplex und dessen assoziierte Proteine NUFIP und ZNHIT3 sind eine wichtige Komponente der Protein-Translation und auch für FXR und seine Funktion von Bedeutung. Auch LXR ist vom Funktionsmechanismus des R2TP-Komplexes und den damit verbundenen Proteinen NUFIP und ZNHIT3 abhängig. Die gewonnenen Daten deuten auf einen Ko-regulatorischen Mechanismus hin, der die Genexpression von FXR/LXR-Zielgenen beeinflusst, die sowohl am Cholesterin- als auch am Gallensäurestoffwechsel beteiligt sind. Aufgrund dieser wichtigen regulatorischen Funktionen können wir spekulieren, dass möglicherweise kompensatorische Mechanismen beteiligt sind, wenn der eine oder andere Kernrezeptor (FXR/LXR) oder Stoffwechselweg (Gallensäuren/Cholesterin) beeinträchtigt ist.

1. Background & Aims

Bile is formed and modified in the liver by hepatocytes and the specialized biliary epithelium. Consisting of Water, primary and secondary bile acids (BA), phospholipids, mucin, glycoproteins, cholesterol, bilirubin, conjugated drugs, endogenous waste products, electrolytes, and trace metals, such as copper as well as secretory dimeric immunoglobulin A (sIgA) and other antibacterial proteins, bile has multiple functions. The exocrine secretion is required for digestion, disposal of toxins and metabolic waste and a part of the innate immune system. The amphipathic BAs are the most functional components of bile because they not only allow for fat digestion, but more importantly as they are reabsorbed act endogenously, similar to hormones, on receptors and signaling pathways on a variety of metabolic processes (1–3). The laboratory aims to explain the mechanism of action of one of these signaling pathways in more detail. BAs have been shown to affect mTORC1, an intracellular sensor of nutrient availability and even seem to rely on mTORC1 signaling for their effect on target gene expression. However, the exact mechanisms are not yet fully understood. It is already known that mTORC1 affects nuclear hormone receptors such as PPARα (4), and data from our laboratory demonstrated that mTOR inhibition decreases transcription of nuclear receptors such as the liver X receptor (LXR) and the bile acid receptor, FXR. Several hypotheses attempt to explain how this signal transduction operates. Since all the other members of the PIKK family to whom mTOR belongs are located in the nucleus, it is reasonable to assume that mTORC1 can also occur in the nucleus or find its way from the cytoplasm into the nucleus. It is also suspected that the co-chaperone R2TP/PFDL complex may play a role in said process. My work aims to provide further insight into whether the R2TP complex associated with mTORC1 plays a role in signal transduction to nuclear receptors such as FXR, LXR and PPARα. Bile acids and their signalling pathways already play a role in the therapy of some diseases. Substances that lead to a blockade of mTORC1 have already found their way into pharmaceutical medicine and cancer therapy. For the future, it will be important to find further targets and compounds within these signaling pathways for the therapy of various metabolic diseases, the metabolic syndrome and cancer. However, it is of utmost importance that, due to the complexity of bile

acid signaling, new compounds targeting these pathways are highly selective and possess well-identified pharmacological properties (2).

2. Introduction

2.1 Bile acids

The synthesis of bile acids (BAs) is the primary pathway for cholesterol catabolism and a complex multi-enzymatic process. The first and rate-limiting enzyme of the classical pathway, which provides most of the total bile acid synthesis, is Cholesterol 7α-hydroxylase, encoded by the gene CYP7A1. Most BAs are conjugated to either glycine or taurine. This amidation renders them water soluble, and favours active transport out of the hepatocytes via the bile salt export pump (BSEP) (2,5,6). When present in unphysiological high concentrations BAs are cytotoxic. In diseases such as cholestasis, high BA concentrations may occur intracellularly inside hepatocytes. In patients with bile acid malabsorption high concentrations can occur in the colon (5). The most common physiological BAs in humans are the primary bile acids chenodeoxycholic acid (CDCA) and cholic acid (CA) and their associated secondary bile acids lithocholic acid (LCA) and deoxycholic acid (DCA), which are formed in the colon by microbial enzymes through deconjugation and 7α-dehydroxylation (2). To sustain a well-functioning bile acid pool, BAs are recycled extensively by a sophisticated transport system not only active in the liver, but also in the intestine, and kidney. During a meal, BAs are excreted and reabsorbed several times. The resulting cycling of BAs constitutes the enterohepatic circulation (1,2,5). In the small intestine, BAs act as emulsifiers for water-insoluble components and promote their degradation and absorption (5). However, it is now well established that apart from their involvement in cholesterol homeostasis and digestion of dietary fats, bile acids act as multivalent signaling molecules. Processes in lipid, glucose and energy metabolism are regulated by bile acids through activation of nuclear receptors such as FXR, which is now also known as nuclear bile-acid receptor, and other cell signaling pathways such as GPBAR1, a membrane bile acid receptor also known as TGR5. Bile acids are therefore also denominated as hormones (2,3). The role as regulator of enterohepatic recycling of BAs and feedback regulation of BA-synthesis is consistent with the major expression of FXR in the liver and the small intestine. Activation of FXR also promotes bile flow through augmented conjugation of BAs in

the liver and increased excretion from the hepatocytes into the bile canaliculi. Negative feedback regulation also targets SHP and LXR-α, which in turn regulate CYP7A1. These mechanisms protect cells from cell-damaging effects of BAs (2,5).

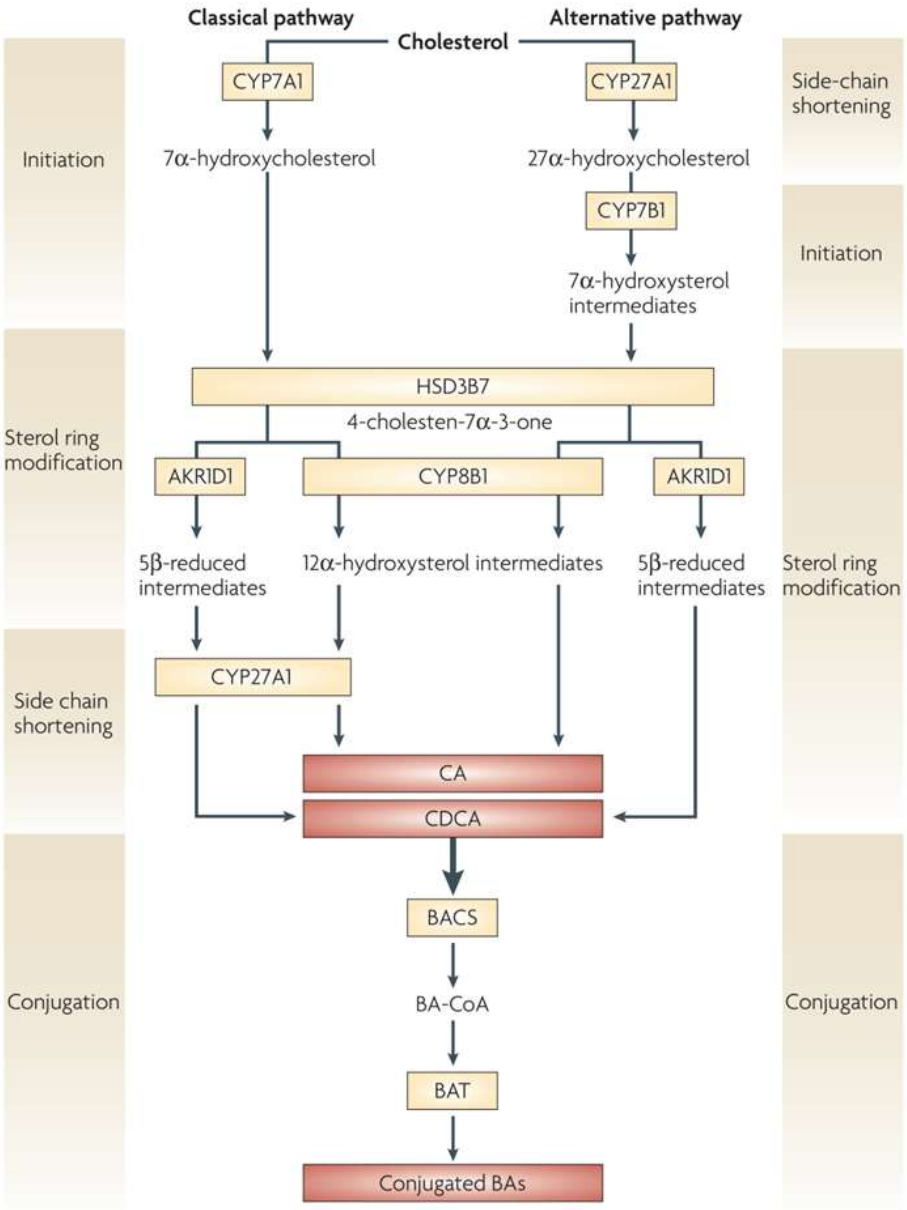

Fig.1. Bile acid synthesis pathways. Thomas C et al., 2008 (2).

2.2 FXR

Farnesoid X receptor (FXR) is a nuclear receptor and intracellular sensor for bile acids. After ligand binding transcriptional responses are induced to regulate bile acid metabolism. Activation of FXR attenuates triglyceride levels and modulates glucose metabolism. Farnesol was the first ligand to be discovered and further research

identified bile acids including CDCA and CA as additional endogenous ligands. FXR forms a heterodimer with RXR to control expression of target genes and can be activated by RXR ligands. FXR is mainly expressed in liver, intestine, kidneys, and adrenal glands. Even though variations have been discovered, the FXR-RXR heterodimer binds mostly to inverted repeat elements separated by one nucleotide (IR1), thereby inducing gene expression directly and mediating the repression of numerous genes indirectly via regulation of small heterodimer partner (SHP). Multiple metabolic pathways are regulated tissue specific by FXR. The conversion of cholesterol to bile acids, regulated by enzymes such as CYP7A1 in hepatocytes, is reduced by FXR. Bile acid toxicity is attenuated by FXR through upregulation of bile acid modifying enzymes. The conjugation of bile acids, which is necessary for secretion, is enhanced by FXR as well as the transport of BA and phospholipids to the gall bladder via bile salt export pump (BSEP) and multidrug resistance protein 2 (MDR2). Absorption of BAs in the intestine is reduced, whereas recycling to the liver via organic solute transporter (OST-α and OST-β) is promoted by FXR. Glucose metabolism is also influenced by FXR through downregulation of Phosphoenolpyruvatcarboxykinase (PEPCK) and Glucose-6-Phosphatase (G6Pase), key enzymes of gluconeogenesis. FXR also reduces lipogenesis by inhibiting SREPB1c and its targets including FASn (7).

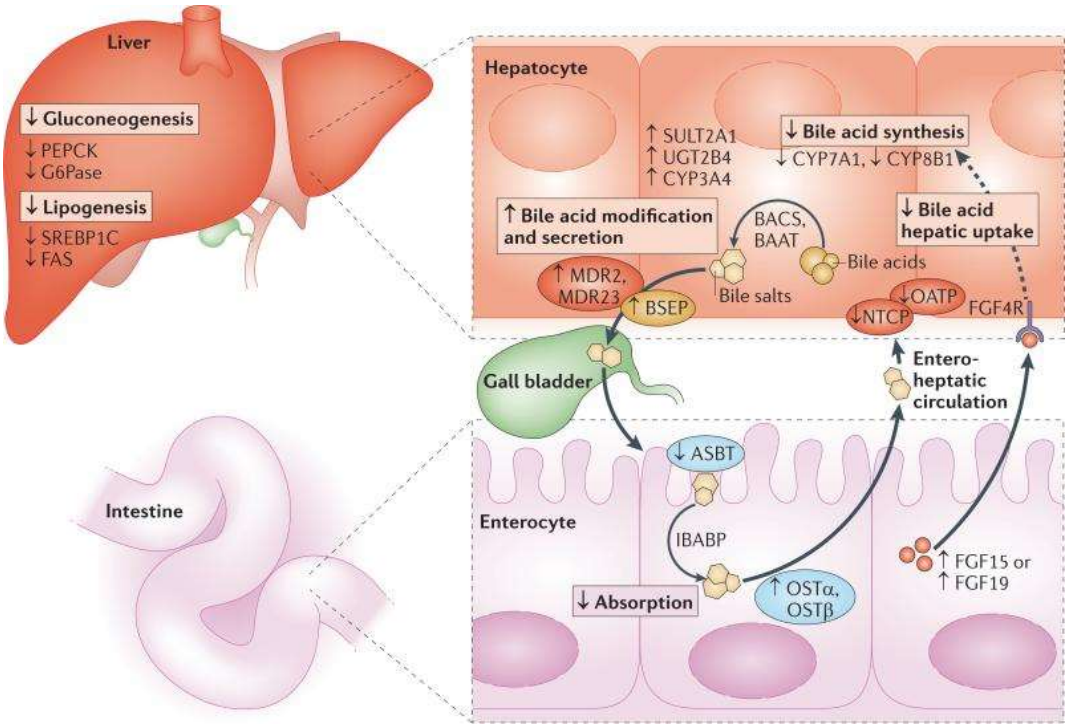

Fig.2. Coordinated effects of FXR on metabolism. Calkin und Tontonoz, 2012(7).

2.3 LXR

Liver X receptors get their name from the predominant expression of the LXR isotypes in different cell types and organs, respectively. There are two LXR isotypes that show a high degree of homology, LXRα and LXRβ. LXRα is expressed in metabolically active tissues including liver, adipose tissue, and macrophages. LXRβ is expressed in all body cells. LXRs are nuclear receptors that can be activated by ligands and serve as sterol sensors. Sterol derivatives, including oxysterols and 24(S),25-epoxycholesterol, are endogenous ligands of both Liver X receptors. Synthetic agonists such as GW3965 and T0901317 activate LXR signaling pathways as well as ligands of the Retinoid X Receptor (RXR), since both receptors form an obligate heterodimer. According to current studies, this heterodimer binds to LXR response elements (LXREs) in LXR target genes together with co-repressors. These co-repressors include silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor co-repressor (NCoR). After ligand binding, co-repressors dissociate and co-activators bind to the heterodimer, leading to gene transcription. The LXRE contains a repeating base sequence AGGTCA, each separated by four nucleotides (DR4) (7). As nuclear receptor and intracellular sensor for sterols, LXR induces transcriptional responses on multiple metabolic pathways in a tissue specific manner, controls cholesterol metabolism and allows efficient storage of energy derived from carbohydrate and fat (8). LXR promotes proteasome-mediated degradation of low-density lipoprotein receptor (LDLR) through induction of IDOL (inducible degrader of LDLR) expression, in peripheral cells including macrophages, resulting in reduced LDL uptake. In peripheral cells, LXR also promotes cholesterol transport to the plasma membrane, cholesterol efflux, cholesterol transfer to low lipid molecules such as apolipoprotein A1 (APOA1) and pre-β high-density lipoprotein (pre-β HDL), and increases plasma HDL levels, by inducing expression of ARL7 (ADP-ribosylation factor-like 7), ABCA1 (ATP-binding cassette transporter A1) and ABCG1. The coordinated regulation of all these genes by LXR is probably responsible for the efficient return of cholesterol from the periphery to the liver (RCT). Cholesterol cannot be catabolized and must be excreted unchanged via bile or via conversion to bile acids, due to its toxicity. Cytochrome P450 7A1 (CYP7A1) is the key enzyme in this conversion and is also stimulated by LXR as well as sterol-regulatory element-binding protein 1c

(SREPB1c) and its targets, fatty acid synthase (FASn), acetyl CoA carboxylase (ACC) and stearoyl CoA desaturase 1 (SCD1). These enzymes promote fatty acid synthesis, which in turn act as substrates for the esterification of cholesterol and reduce said toxicity. Glucose uptake into cells also gets promoted by LXR via induction of glucose transporter type 4 (GLUT4). LXR also inhibits cholesterol absorption in the intestine by inducing the expression of the ABC transporters ABCG5 and ABCG8 and thereby promotes faecal excretion of cholesterol (7).

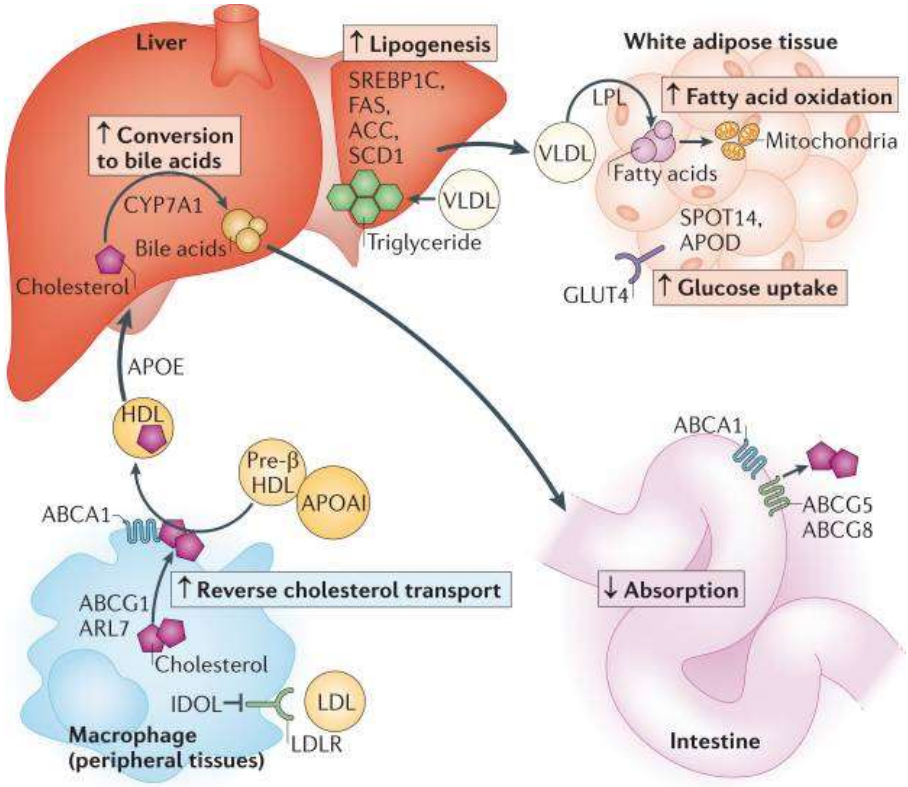

Fig.3. Coordinated effects of LXR on metabolism. Calkin und Tontonoz, 2012(7).

2.4 mTORC1

The serine/threonine protein kinase mTOR is a member of the PIKK family. It takes part in the formation of two distinct protein complexes, known as mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2). The three core components defining mTORC1 comprise mTOR, Raptor (regulatory protein associated with mTOR), and mLST8 (mammalian lethal with Sec13 protein 8, also known as GβL) (9). The activation state of mTORC1 is tightly regulated by an upstream signaling network through the GTPases Rheb and Rag. These pathways respond to either intracellular or extracellular changes in nutrients or exogenous growth factors through cell

surface receptors (10). Rags serve as a docking site for mTORC1 at the lysosome in an amino acid-stimulated state. In GTP-bound state Rheb resides at the lysosomal surface and is essential for mTORC1 activation. The GTPase-activating protein complex TSC, comprising of TSC1, TSC2 and the TBC domain protein TBC1D7, is the only established regulator of Rheb. Growth-inhibiting signals including deprivation of growth factors, nutrient deficiency, or cellular stress, activate the TSC complex decreasing Rheb-GTP levels and disabling mTORC1 signalling. Contrary, growth stimulating signals triggered by nutrients and growth factors inhibit the TSC complex to allow the accumulation of Rheb-GTP and the subsequent activation of mTORC1. However, stimulation of mTORC1 at the lysosome will fail in the absence of amino acids because Rag heterodimers would not be able to dock mTORC1 at the lysosome where Rheb is present. Through activation of anabolic processes via diverse sets of downstream targets, mTORC1 stimulates cell, tissue, and organismal growth by promoting biosynthesis of proteins, lipids and nucleotides. In response to cellular growth signals mTORC1 phosphorylates the eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BP1 and 2), which separates eIF4E from its binding Protein and thereby enables protein synthesis. In like manner direct targets of mTORC1 are the ribosomal S6 protein kinases (S6K1 and 2), that also regulate components of mRNA translation. Another major function of mTORC1 is enhancing ribosome biogenesis in addition to stimulating synthesis of ribosomal proteins. Through upregulation of hypoxia inducible factor (HIF1α), mTORC1 can promote aerobic glycolysis. The transcription factor HIF1α induces expression of glucose transporters and numerous enzymes of glycolysis. To maintain anabolic processes, necessary components must be available. Therefore, de novo pyrimidine and purine synthesis is promoted by mTORC1 signaling through diverse transcriptional and post-translational processes. The transcription factors Sterol regulatory element-binding proteins (encoded by SREPB1a, 1c and 2) induce gene expression of lipogenic enzymes stimulating fatty acid and sterol synthesis. mTORC1 promotes SREPB processing and activation through several downstream targets including S6K1. Lipolysis and fatty acid oxidation appear to be supressed by mTORC1 partly through attenuation of PPARα transcriptional activity. Under nutrient and energy rich conditions, mTORC1 inhibits autophagy by phosphorylation of ULK1 (Unc51-like kinase 1) complex that functions as important gatekeeper for the induction of autophagy (10). In our laboratory, mTORC1 has also been shown

to affect nucleolar receptors such as LXR and FXR. However, the exact mechanism of the signaling pathway has not yet been explained. However, it is suspected that the R2TP/PFDL complex may play a role in this process. For this reason, in this thesis individual components of the R2TP complex and their effect on gene expression downstream of mTORC1, LXR and FXR are highlighted.

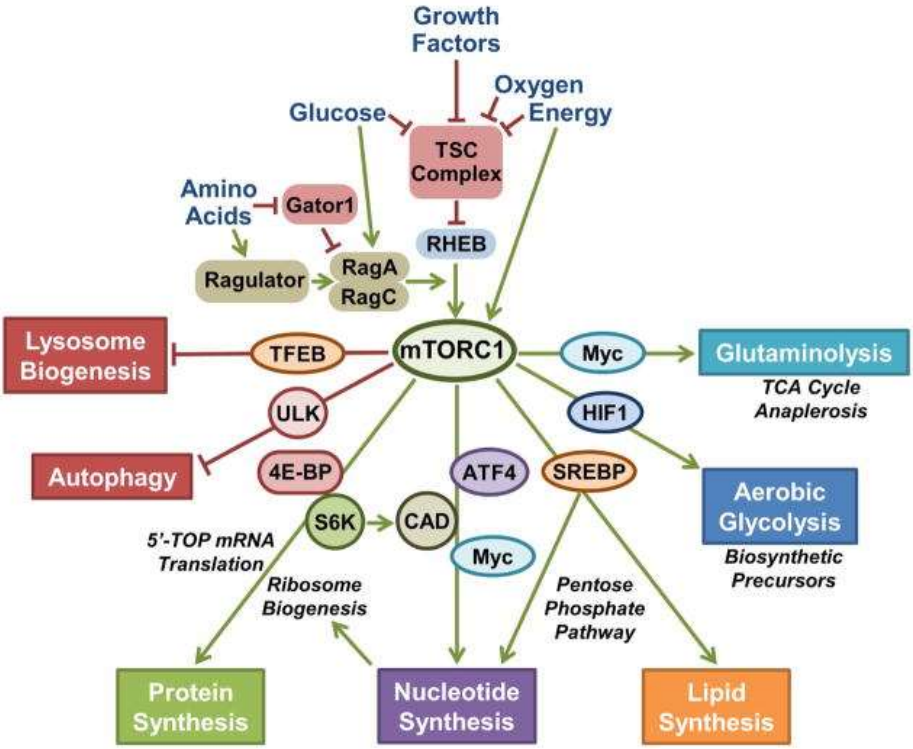

Fig.4. mTORC1 signaling and metabolic control. Ben-Sahra und Manning, 2017(10).

2.5 R2TP Complex

The AAA+ ATPases Ruvbl1 and Ruvbl2 interact with RNA polymerase II associated protein 3 (RPAP3) and Pih1 domain-containing protein 1 (PIH1D1) to assemble the R2TP complex. The PFDL module proteins, consisting of prefoldin and prefoldin-like proteins, notable for facilitating the folding of cytoskeletal proteins, associate with R2TP and form the R2TP/Prefoldin-like complex, which functions as a co-chaperone. This co-chaperone coordinates the recruitment of HSP70, HSP90 and its partner proteins. The interaction of R2TP/Prefoldin-like complex with referred chaperones is required by an increasing number of macromolecular complexes for composition, activation, and stability. This includes RNA polymerase II, snoRNPs, and complexes of the PIKK family proteins ATM, ATR, DNA-PKcs, TRAPP, SMG1 and mTOR (11,12). Cellular processes requiring R2TP include box C/D snoRNP

biogenesis, phosphatidylinositol-3 kinase-related protein kinase (PIKK) signaling, RNA polymerase II composition, and apoptosis (13). It was also discovered that for the stability and biogenesis of PIKKs, an interplay of HSP90, R2TP/PFDL, and the TTT complex consisting of TELO2, TTI1, and TTI2 is required (12,14,15). Another complex that interacts with R2TP/PFDL to control mTOR activity is the TSC1-TSC2 complex, which, as mentioned above, is an important inhibitor of mTOR in the absence of nutrients and growth factors (12). Also discussed is the role of the R2TP complex in context of nutrient availability. It was discovered that R2TP is localized in the nucleus of growing cells to interact with its partner proteins. However, under poor growth conditions, the interaction is significantly reduced and the complex relocates mainly into the cytoplasm (16). By regulation of its chaperoning activity, R2TP/PFDL may be a key effector of nutrient- and stress-sensing pathways that regulate anabolic processes (12). Derived from the numerous cellular functions of the complex, it is hypothesized that mTOR-mediated reactions control additional cellular processes via the R2TP complex. Since mTOR is involved in multiple cellular mechanisms whose signaling pathways are not yet understood, further studies are needed to elucidate the molecular mechanisms involved and the role of the R2TP/PFDL complex in mTOR signaling (17). In the following thesis I will investigate the influence of the R2TP/PFDL complex on the regulation of LXR and FXR by mTORC1.

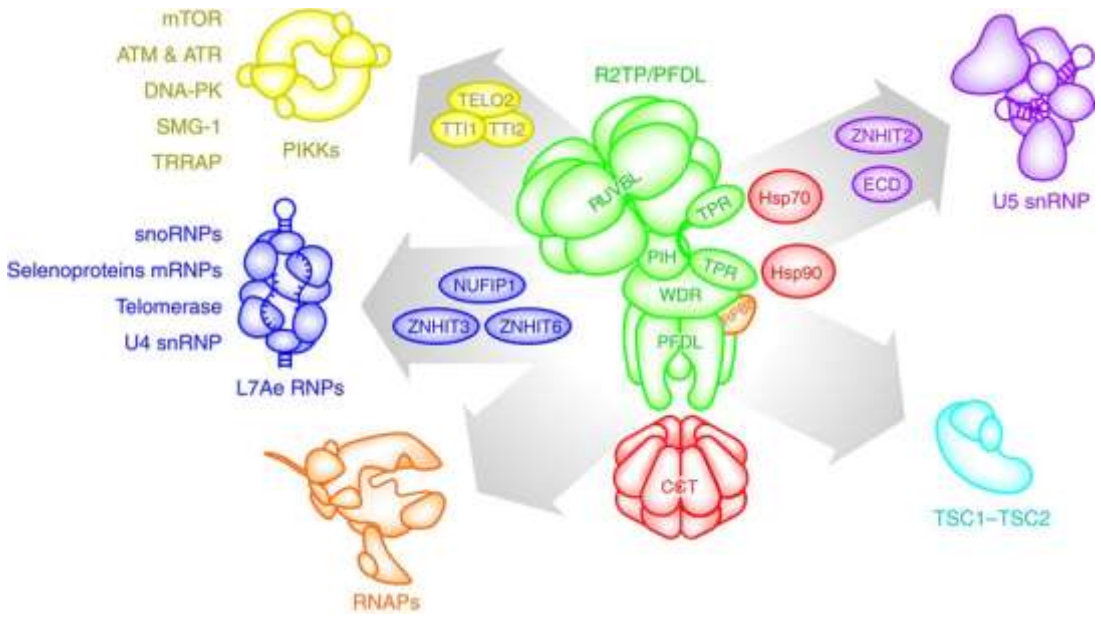

Fig.5. Interactors of the R2TP/PFDL chaperone. Cloutier et al., 2017(12)

3. Aim

With my work I want to investigate whether PIKKs and R2TP/PFDL-associated proteins are involved in mTORC1 nuclear receptor communication. Specifically, I want to explore to what extent ZNHIT3, NUFIP, RUVBL1 and TRRAP are involved in mTORC signaling and to what extend the knockdown of these genes will affect the function of the intracellular receptors FXR and LXR.

4. Material and Methods

4.1 Cell Line

For cell culture experiments a HEPG2 cell line was used, which was purchased from American Type Culture Collection (ATCC). These cells were first established in 1983 (Knowles and Aden, 1983) and are human liver cells from hepatocellular carcinoma of a 15-year-old Caucasian male.

4.2 Culturing

Cells were cultured in 500ml high glucose DMEM (Dulbecco's modified eagle medium) obtained from Gibco™ to which 50ml heat inactivated FBS (fetal bovine serum) (Gibco™) and 5ml Penicillin-Streptomycin (10,000U-10,000µg/ml) (Gibco™) was added. The flask was incubated at 37°C in a 95% O_2 / 5% CO_2 atmosphere for approximately 48 hours until the cells were confluent. The layer was then rinsed with 3ml 0.25% Trypsin-0.53mM EDTA (Gibco™) and incubated for about 10 minutes until the cells were detached. According to ATCC protocol subcultures were prepared.

4.3 Knockdown

To examine if a sufficient knockdown for our experiment could be achieved 6-well plates seeded with HEPG2 cells were transfected with siRNA in triplets. siRNA Universal Negative Control #1 (SIGMA MISSION®, #SIC001) was used as control. DharmaFECT Transfection Reagent 4 (Dharmacon™, #T-2004-03) and human ON-TARGETplus SMARTpool siRNA Reagents TELO2, TRRAP, RUVBL1, NUFIP and

ZNHIT3 were obtained from Dharmacon™ (#L-021207-01-0005, #L-005394-00-0005, #L-008977-00-0005, #L-019605-02-0005, #L-006902-00-0005) and used for target gene silencing. 300,000 HEPG2 cells were seeded in 6-well plates and filled up to 2ml with growth medium and incubated at 37°C, 95% O_2, as described above. According to the Dharmacon™ siRNA transfection protocol, a 1x buffer solution was assembled by 5x siRNA buffer (Dhamarcon™, #B-002000-UB-100) and distilled water with which the siRNA reagents were diluted to a 5µM siRNA solution. In separate tubes 10µl RNAi solution and 4µl Dharmafect Transfection Reagent 4 was mixed with serum free medium (Accell™ siRNA Delivery Media; Dharamacon™, B-005000-500) for a target volume of 200µl each. After 5 minutes of incubation, the contents of both tubes were gently mixed together. At 100% cell confluence, the wells were prefilled with 1.6ml high glucose growth medium (DMEM, Gibco™) and subsequently dropwise supplemented with 400µl RNAi mixture for a final 25nM siRNA concentration. After overnight incubation at 37°C, 95% O_2, the medium was changed, and the cells were incubated for another 24 hours to be subsequently harvested for protein and RNA analysis. The knockdown efficiency was analyzed using real-time quantitative PCR and western blotting.

4.4 Experiment Setup

As described above 300,000 HEPG2 cells were used for each well in 6-well plates. For each transfection and treatment 3 wells were used. To increase the knockdown efficiency, the siRNA concentration of 25nM each was increased to the following concentrations: TRRAP 50nM, NUFIP 30nM, RUVBL1 30nM, ZNHIT3 30nM. Accordingly, 30nM and 50nM controls were used. The medium was changed after overnight incubation. After another 24 hours triplets of transfected wells were treated with either FXR ligand GW4064 (Tocris Bioscience™ #2473), LXR ligand GW3965 (Cayman® #10054) or PPARα ligand GW7647 (Cayman® #10008613). However, for ZNHIT3 KD cells PPARα ligand GW7647 was not used. In a second experiment TRRAP and ZNHIT3 KD cells were treated with 500nM Torin1 (Toronto Research Chemicals Inc., #T548700). The treatments were dissolved in dimethyl sulfoxide (DMSO D2650; Sigma-Aldrich®), to a final ligand concentration of 2µM. Solely DMSO and medium was added to ligand-free wells. The cells were incubated for 24 hours at 37°C, 95% O_2 and subsequently harvested for protein and RNA analysis.

Knockdown efficiency was verified, and protein expression as well as the expression of target genes was analyzed by western blotting and real-time quantitative PCR.

4.5 Protein isolation

The differently treated HEPG2 cells were washed with cold PBS pH7.4 (Gibco™). The cells were harvested using 500µl homogenization buffer (0.25 mol/L sucrose, 10 mmol/L HEPES, pH 7.5, and 1 mmol/L EDTA, pH 8.0) containing Pierce™ Protease Inhibitor Tablets (Thermo Scientific™, #A32965) and 0.5% IGEPAL® CA-630 (Sigma-Aldrich®, I8896). To break up remaining cell membranes, samples were sonicated with an UP50H ultrasonic processor (Hielscher Ultrasonic GmbH) Cyclus 1, 80% amplitude for 10 seconds. A colorimetric detection method was applied to quantify the total protein in the samples. For this purpose, the Pierce™ BCA Protein Assay Kit (Thermo Scientific™) was used according to the instructions given in the standard protocol. BSA (Thermo Scientific™) was used as standard in following concentrations: 2000µg/ml, 1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml and 0µg/ml (blank). Pierce™ BCA Protein Assay Reagents A and B (Thermo Scientific™) were mixed in a 1:50 ratio, added to the samples and incubated at 37°C for 30 minutes. The microplate reader (SPECTROstar® OMEGA, BMG LABTECH) was used to measure optical density at a wavelength of 562nm according to the manufacturer's instructions.

4.6 Western Blot

10µg of protein was incubated with 3x sample buffer and filled up to total 15µl with distilled water. The chambers of sodium dodecyl sulfate polyacrylamide gels (12.5%, 7.5% and 4-20%) for electrophoresis were loaded with 15µl. The gel electrophoresis ran with 120V constant and 30-50mA variable for 2 hours. After transfer from the gels to nitrocellulose membranes, Ponceau S staining was used to confirm a successful protein run and transfer. According to the molecular weights of the desired proteins, the membranes were marked and cut. Unspecific protein binding sites were blocked using 10g milk protein (5% solution) mixed with 200ml TBS-T (Tris Buffer: 1xTBS, detergent 0.1% Tween™ 20, Sigma-Aldrich®, #P1379) before overnight incubation at 4°C with following antibodies against: Phospho-4E-

BP1 (Ser65; 15-20kDa; pAb rabbit), Phospho-S6 Ribosomal Protein (Ser240/244; 32kDa; pAb rabbit), mTOR (289kDA; mAb rabbit), p-mTOR (Ser2448; 289kDA; mAb rabbit), Hamartin/TSC1 (150-170kDA; mAb rabbit) (Cell Signaling Technology®: #9451, #2215, #2983, #5536 and #6935S), NUFIP (75kDa, pAb rabbit; Proteintech® #12515-1AP), LC3 (15-17kDA; pAb rabbit; Novus Biologicals™ NB100-2220), FXR (55kDa; mAb mouse; Perseus Proteomics Inc. PP-A9033A) - (all of which were diluted 1:1000), and TRRAP (440kDa; dilution: 1:2000; pAb rabbit; Bethyl Labratroies Inc #A301-132A). Membranes were then incubated for one hour at room temperature with the secondary HRP-conjugated/linked goat anti-rabbit IgG or horse anti-mouse IgG antibody (Cell Signaling Technology® #7074S, #7076S; diluted 1:3000). Protein signals were visualized using Clarity™ Western ECL Substrate (BioRad, #170-5060) and the ChemiDoc™ System (BioRad®).

4.7 RNA Isolation

The cells were lysed using TRI Reagent® (Sigma-Aldrich). (Fa. Molecular Research Center,Cat Nr. TR 118) according to the TRIzol Reagent user guide (Thermo Fisher Invitrogen™). Total RNA concentration measurements of each sample were carried out using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific Inc.). Sample quality was determined by calculating the 260/280 ratio and 260/230 ratio (reference values 1.9 - 2.0 and 2.0 - 2.2).

4.8 cDNA synthesis

By reverse transcription of 1.5µg RNA complementary DNA was synthesized. The Mastermix for cDNA synthesis contained 4µl 5x cDNA synthesis buffer, 2µl 0.1M DTT, 0.3µl 10mM dNTPs, 0.5µl RNAse inhibitor, 0.5µl Superscript™ II (Invitrogen™, Thermo Fisher Scientific Inc.) and 2.3µl distilled water. The following Thermocycler protocol for was used: 42°C for 90 minutes, 70°C for 15 minutes and 4°C until further processing.

4.9 Real-time quantitative PCR (qPCR)

10µl of the cDNA samples were diluted with water in a 1:20 ratio. For the calculation of the standard curve, 5µl of each cDNA sample was pooled into a fresh Eppendorf

Tube®. From this pool, 5 standard solutions were diluted with water to a final concentration of 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160. For controls we used NTC (a.dest.) and RNA. To analyze the expression of desired genes LightCycler® 480 Real-Time PCR System (Roche AG) was used. Quantitative PCR (qPCR) was performed in duplicates on a 384 well plate with 2µl of diluted cDNA and 8µl Mastermix consisting of 5µl SYBR Green I Master (SYBR GREEN / NEB / 2X), 2.5µl sterile Aqua dest., 0.25µl 5µM forward primer and 0.25µl 5µM reverse primer, respectively. The following primers obtained from Eurofins Genomics GmbH were used to show knockdown efficiency: TELO2, RUVBL1, TRRAP, ZNHIT3, NUFIP. Gene expression of LXR targets ABCA1, SREPB1c and FASn, FXR targets OST-β, SHP, KNG1 and BSEP and PPARα targets CPT1A and AOX were subsequently analyzed. To normalize the results, the following housekeeping genes h-TBP, 36b4, β-Actin and GADPH were used. Results were calculated with the 2ΔΔCT method. The cycling program started with a pre-incubation period at 95°C, followed by 45 cycles amplification (95°C for 15 seconds and 60°C for 1 minute) and melting curve generation/analysis by heating from 55°C to 95°C for 10 minutes before finally cooling down to 40°C.

5. Results

5.1 Establishment of knockdown efficiency

To find the right concentrations for the transfection a test run to analyze the knockdown efficiency of the used siRNA was made. By transfection with 25nM siRNA the KD percentage for TRRAP compared to the control was 25%, for NUFIP 74%, for TELO2 30% and for RUVBL1 80%. To get better results we altered the concentration to 30nM for RUVBL1 and NUFIP and 50nM for TRRAP. Experiments with TELO2 KD were not further continued. Instead ZNHIT3 KD (30nM) was included in the experiment. With higher concentrations of the siRNA the KD percentage got up to 76% for NUFIP, 97% for RUVBL1, 95% for ZNHIT3 and 59% for TRRAP. To normalize mRNA expression 36b4, GADPH, β-Actin and h-TBP were used as housekeeping genes. Western blots were made to ensure KD efficiency. Thus, we could show that TRRAP and NUFIP gene knockdown largely suppresses TRRAP and NUFIP protein expression. The same can be assumed for the other

gene knockdowns and their protein products for which no antibodies were available at that time.

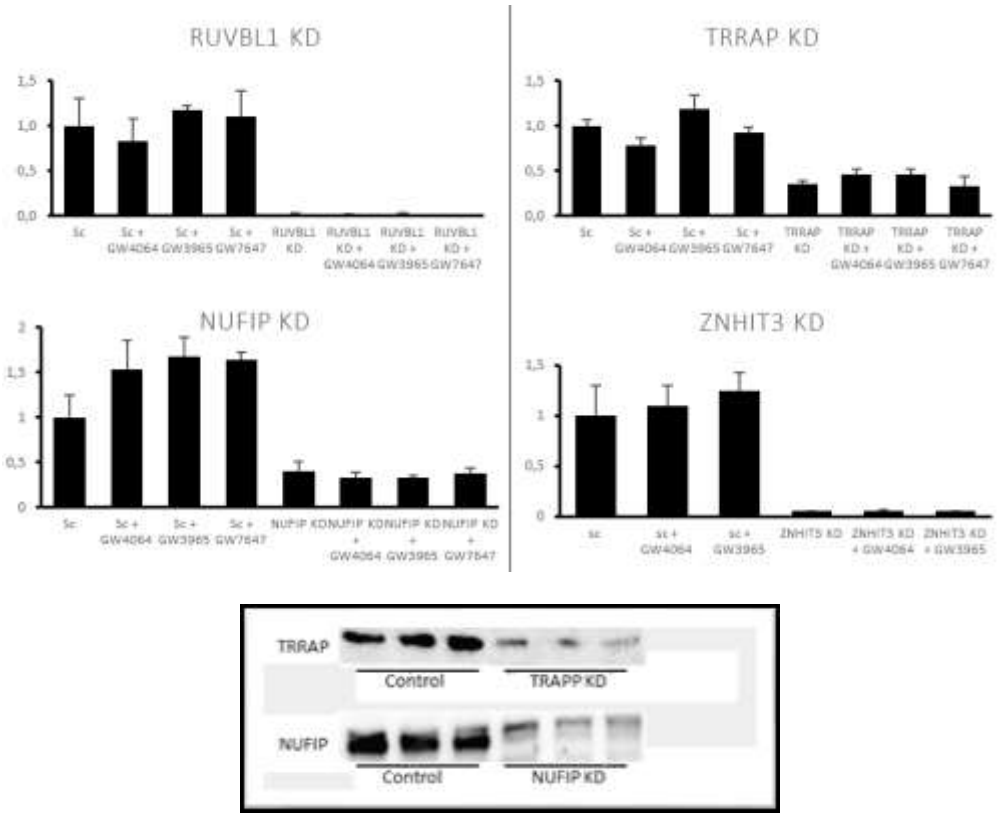

Fig.6. qPCR analyses and Western blots confirm the knockdown by siRNA in ZHIT3, RUVBL1, NUFIP and TRRAP in HepG2 cells at protein and genetic level.

5.2 Regulation of FXR by mTOR and R2TP/PFDL Complex

Immunoblots were performed to show effects on FXR protein expression by knockdown of NUFIP, RUVBL1 and TRRAP. We also examined whether there was a correlation with mTOR protein expression and phosphorylation status of mTOR and its downstream target 4EBP1. RUVBL1 decreased the expression of mTOR. An increase in mTOR expression and phosphorylation was observed with NUFIP and TRRAP knockdown. TSC1, a part of the hamartin–tuberin (TSC1-TSC2) complex, inhibits phosphorylation of S6 kinase by binding to mTOR and thus represses mTORC1 signaling. Western blotting showed that NUFIP KD had a slight negative effect, RUVBL1 KD had no effect, and TRRAP KD had a small positive effect on TSC1 expression. However, effects of this magnitude are not significant. NUFIP operates with the R2TP complex for box C/D assembly and snoRNP interaction. As to be expected the Western blot showed that NUFIP KD potently

diminished NUFIP expression. RUVBL1 KD also had a slight negative impact, whereas TRRAP KD had no effect on NUFIP protein production. The main target was to investigate whether knockdown of NUFIP, RUVBL1, or TRRAP have a direct or indirect effect on FXR protein expression, a nuclear bile acid receptor. It was observed that there was a slight decrease in the expression of FXR expression with NUFIP KD and RUVBL1 KD. TRRAP KD blots showed increased FXR protein levels compared to the control (Figure 7). 4E-binding protein 1 (4EBP1) is a downstream effector of mTOR. When phosphorylated by mTORC1, it releases eIF4E (eukaryotic translation initiation factor 4E), thereby permitting protein synthesis. By analyzing the phosphorylation of the protein by western blotting, conclusions can be drawn about mTORC1 activity. There was no difference in p-4EBP1 levels in NUFIP-, RUVBL1- and TRRAP KD samples compared with the control. LC3 is an important marker to monitor autophagy and autophagy-related processes, including autophagic cell death, because the amount of autophagosomes correlate with the number of LC3-II. The approach to detect LC3 conversion (LC3-I to LC3-II) by immunoblotting was used. Due to limitations in the interpretation of this method, only the samples and controls are compared among each other, but no conclusion can be drawn about the actual autophagic activity(18,19). Western blots showed no significant effect on conversion rate of LC3-I to LC3-II in RUVBL1-, NUFIP- and TRRAP KD samples.

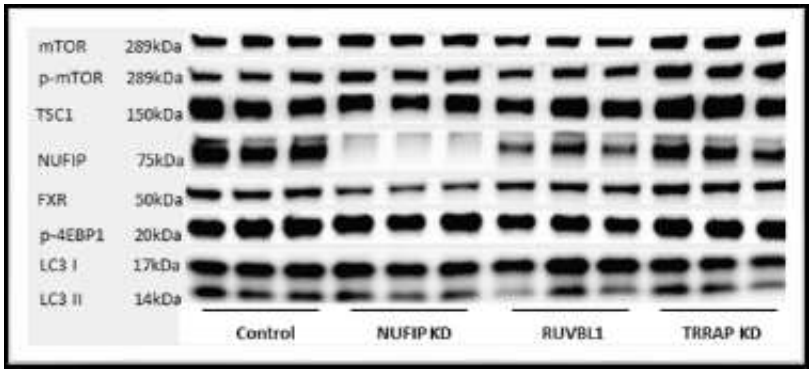

Fig.7. NUFIP and TRRAP KD showed augmented mTOR expression. Western blot for FXR, mTOR and up/down-stream components were analysed from protein lysates of HepG2 cells treated with siRNAs against NUFIP, RUVBL1 and TRRAP (KD = knock down). Experiments were performed in triplicates.

5.3 Pharmacological inactivation of mTOR with and without combination of TRRAP KD

Torin 1 is a highly potent and selective inhibitor of mTOR. Torin 1 is able to effectively block the phosphorylation of mTORC1 and mTORC2 (20,21). TRRAP, on the contrary, is a member of the PIKK family and occurs physiologically. Research suggests that TRRAP acts as a coactivator for nuclear receptors. Possibly as part of a complex, TRRAP appears to influence lipid metabolism by regulating the LXRα-mediated gene cascade in liver cells (22). Additional immunoblots were performed to show the effects of TRRAP knockdown on FXR protein expression during Torin 1 treatment. mTOR and phosphorylated mTOR, as well as S6 and pS6, were analyzed to show effects on phosphorylation and expression of the individual protein states to draw conclusions about the activity of mTORC1. TSC1, part of a complex that suppresses mTORC1 activity, p4EBP1, a protein downstream of mTORC1, FXR, a nuclear bile acid receptor, and LC3B-I/LC3B-II conversion, were examined to show a link between TRRAP, mTORC1 and FXR.

TRRAP protein expression was reduced to a minimum in TRRAP KD cells with and without Torin 1 treatment. TO1 treatment solely had no effect on TRRAP protein expression. mTOR expression decreased in all samples treated with Torin 1 but tended to increase in samples treated with TRRAP siRNA. The mTOR inhibition (TO1) reduced TSC1 expression most effectively. The same effect was observed for RNA silencing of TRRAP, and the combination showed additive effects. Ribosomal Protein S6 showed only a slight difference in protein expression in TRRAP KD samples however phosphorylation at Ser235/236 decreased in TRRAP KD cells and by Torin 1 treatment but was again rescued in TRRAP KD cells treated with Torin 1. S6 Ribosomal Protein phosphorylation at Ser240/244 was inhibited by TO1 treatment with and without TRRAP KD though sole TRRAP KD slightly increased phosphorylation. In all TO1 treated samples, 4E-BP1 showed reduced phosphorylation at Ser65 compared to TRRAP KD or control cells without TO1 treatment. The conversion of LC3B-I to LC3B-II showed that the autophagic activity in samples treated with Torin 1 was increased compared to the control. TRRAP KD showed no difference in conversion compared to control. FXR expression was decreased in all samples treated with TO1 however could not be rescued by additional TRRAP KD.

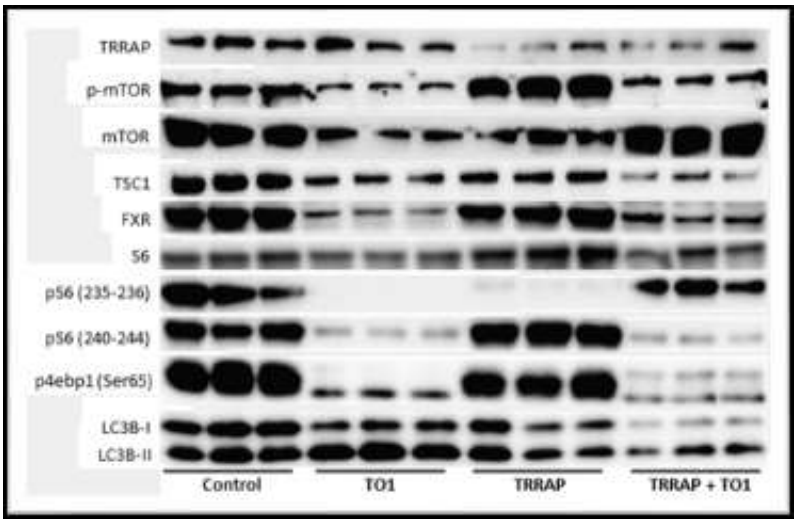

Fig.8. Pharmacological inactivation of mTOR by Torin 1 had a negative effect on FXR protein expression. The same, not as pronounced effect was shown in TRRAP KD samples treated with Torin 1. Western blot for FXR, autophagy, mTOR and up/down-stream components were analysed from protein lysates of HepG2 cells treated with siRNAs against TRRAP with and without Torin 1 treatment (KD = knock down). Experiments were performed in triplicates.

5.4 Pharmacological inactivation of mTOR in combination with and without ZNHIT3 KD

Immunoblots were performed to examine the protein expression of FXR in ZNHIT3 KD and Torin 1 treated cells. Using the same reasoning as for TO1 treated TRRAP KD samples, the immunoblots also show the effects of mTOR inactivation on FXR in ZNHIT3 KD samples. mTOR expression was attenuated by TO1 treatment and phosphorylation was also reduced. ZNHIT3 KD alone reduced phosphorylation of mTOR slightly while showing the same mTOR expression as the control. Combination treatment with ZNHIT3 KD and TO1 showed higher mTOR expression and phosphorylation as samples treated only with TO1 alone. TSC1 expression showed hardly any signs of treatment-related change, but a slight downward trend was observed in TO1 treated samples. In cells treated with TO1, FXR expression was strongly downregulated. Notably, in TO1-treated ZNHIT3 KD cells, the downregulation was not as pronounced, and untreated ZNHIT3 KD cells showed only a weak downward trend. The ribosomal protein S6 showed no changes due to treatment, but phosphorylation was suppressed at all observed sites by TO1 treatment, whereas the combined treatment led to a rescue of S6 (Ser 235-236) phosphorylation. ZNHIT3 KD on its own had no effect on phosphorylation of S6.

P4EBP1 (Ser65) showed similar expression levels in ZNHIT3 KD cells and control but was reduced to a baseline in all TO1-treated samples. LC3B conversion was increased in Torin 1-treated samples compared to control. The ZNHIT3 KD sample without TO1 treatment showed no increase in LC3B conversion and thus similar autophagic activity as the control.

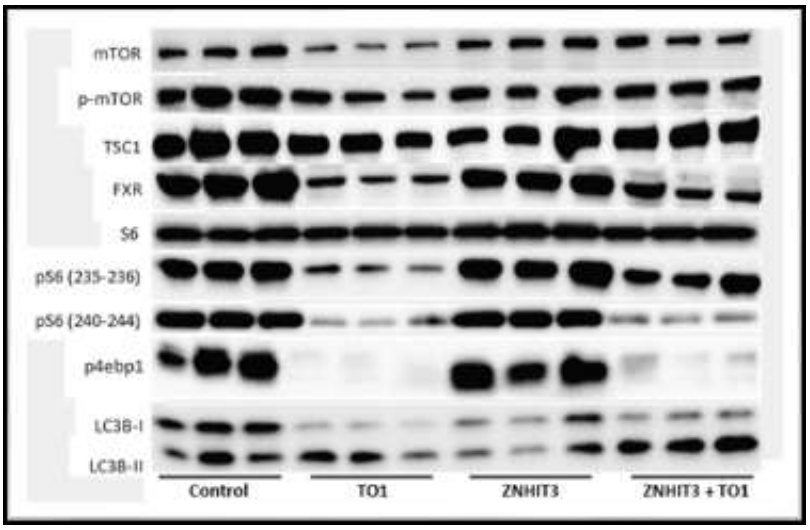

Fig.9. Strong negative effect of Torin 1 treatment on mTOR and FXR expression. In combination with ZNHIT3 siRNA, downregulation was less pronounced. Western blot for Western blot for FXR, autophagy, mTOR and up/down-stream components were analysed from protein lysates of HepG2 cells treated with siRNAs against ZNHIT3 with and without Torin 1 treatment (KD = knock down). Experiments were performed in triplicates.

5.5 Regulation of FXR activity through R2TP and R2TP associated proteins

SLC51 family of steroid-derived molecule transporter (SLC51B, Ost-β), as part of the heterodimer Ost-α/Ost-β, is an important element in the bile acid transport system. Intestinal transport as well as systemic efflux and circulation, and thus large parts of the enterohepatic cycle, operate via Ost-α/Ost-β(2,23,24). As FXR target gene Ost-β is stimulated by treatment with the synthetic FXR ligand, GW4064. Using qPCR analysis, the effect of knockdown of RUVBL1, NUFIP, TRRAP and ZNHIT3 on FXR and its target gene Ost-β, was investigated. The untreated NUFIP and RUVBL1 KD cells showed a slight increase in gene expression compared to the control. NUFIP and RUVBL1 KD cells treated with the FXR ligand GW4064 were not as responsive to GW4064 and showed a significant decrease (around 70%) in Ost-β mRNA as compared to the control. Untreated ZNHIT3 KD cells showed a

tendency to express less Ost-β mRNA compared to the control but in ZNHIT3 KD cells stimulated with the FXR ligand GW4064, the FXR target gene Ost-β showed no significant changes compared to the control. The Ost-β expression in TRRAP KD cells showed no significant difference compared to the control. TRRAP KD cells showed less induction of Ost-β mRNA by treatment with GW4064 compared to the control.

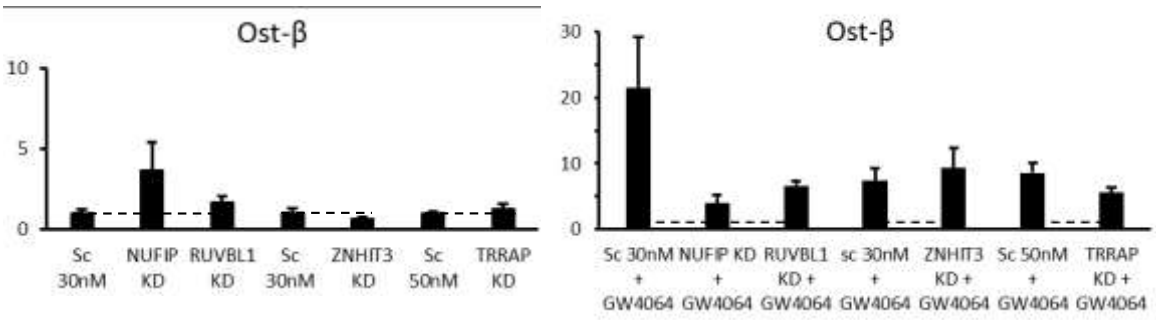

Fig.10. qPCR analysis of Ost-β. HepG2 cells treated with siRNAs against NUFIP, RUVBL1, ZNHIT3 and TRRAP (KD = knock down). Stimulation with FXR ligand GW4064. Experiments were performed in triplicates. NUFIP KD and RUVBL1 KD samples showed a strongly reduced response to stimulation. In ZNHIT3 KD and TRRAP KD samples, little to no change was shown in Ost-β mRNA expression compared to the respective control.

The KNG1 gene encodes for Kininogen-1 also known as alpha-2-thiol proteinase inhibitor. Consisting of a bradykinin domain, Kininogen-1 has multiple biological functions. Not only is it active in intrinsic coagulation, but when bradykinin is released, it exerts its well-known diverse effects (25). By treatment with GW4064 the FXR target gene KNG1 was stimulated. The effects of NUFIP, RUVBL1, TRRAP and ZNHIT3 knockdown on KNG1 was analyzed using qPCR. In untreated NUFIP KD, RUVBL1 KD, ZNHIT3 KD and TRRAP KD cells, a tendency for KNG1 gene expression to increase in relation to control was observed. However, activation by GW4064 showed that NUFIP KD, RUVBL1 KD and ZNHIT3 KD impaired the expression of KNG1 mRNA substantially, whereas TRRAP KD had no effect on the induction of KNG1 by GW4064. These data indicate that NUFIP, RUVBL1 and ZNHIT3 play an essential role in the ligand dependent regulation of FXR target genes.

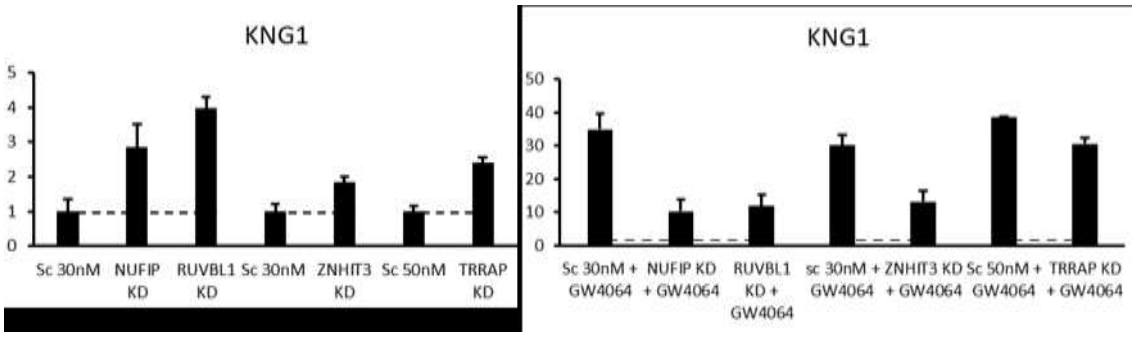

Fig.11. qPCR analysis of KNG1. HepG2 cells treated with siRNAs against NUFIP, RUVBL1, ZNHIT3 and TRRAP (KD = knock down). Stimulation with FXR ligand GW4064. Experiments were performed in triplicates. In GW4064-stimulated samples, KNG1 mRNA expression was reduced in all knockdown samples.

The protein BSEP also known as ABCB11 is located in the canalicular membrane of hepatocytes and transports unconjugated and conjugated bile acids from the hepatocyte into the bile canaliculi (2,26–29). FXR regulates the expression and subsequently the activity of the bile salt export pump (BSEP). Physiologically, high bile acid levels within hepatocytes activate FXR and thus increase the activity of BSEP (2,5–7). Quantitative PCR analysis was performed to determine whether knockdown of NUFIP or RUVBL1 influenced BSEP expression. NUFIP KD and RUVBL1 KD did not show an induction of BSEP mRNA expression like observed previously for the other FXR target genes as compared with control samples. Induction by GW4064 significantly increased gene expression (BSEP mRNA levels) in all samples and was significantly higher in RUVBL1 KD samples compared with control, while NUFIP KD cells showed similar gene expression to control.

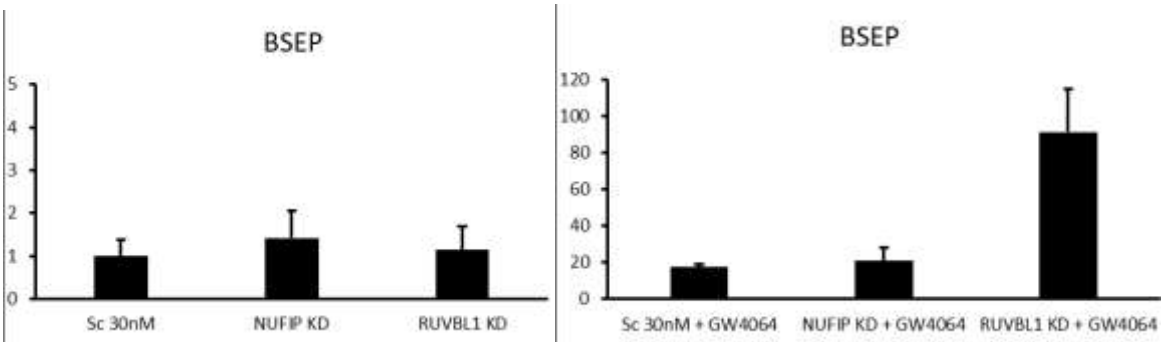

Fig.12. qPCR analysis of BSEP. HepG2 cells treated with siRNAs against NUFIP and RUVBL1 (KD = knock down). Stimulation with FXR ligand GW4064. Experiments were performed in triplicates. NUFIP KD samples showed no change. RUVBL1 KD samples showed increased BSEP mRNA expression compared to control.

Activated by bile salts, FXR induces the expression of SHP, also known as NR0B2, which acts as a nuclear receptor inhibiting the expression of Cyp7A1 and also affects the regulation of other genes involved in bile acid biosynthesis (30,31). None of the RNAi knockdowns showed a significant increase or decrease in comparison. As downstream target of FXR we used the ligand GW4064 to stimulate target gene expression. Only a minimal stimulation was accomplished, and SHP mRNA showed similar levels throughout the samples.

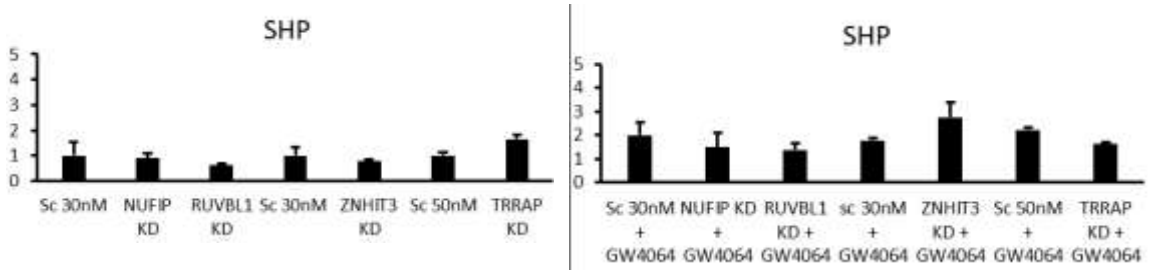

Fig.13. qPCR analysis of SHP. HepG2 cells treated with siRNAs against NUFIP, RUVBL1, ZNHIT3 and TRRAP (KD = knock down). Stimulation with FXR ligand GW4064. Experiments were performed in triplicates. Minimal stimulation was achieved. No difference between the knockdown samples and the control was observed.

5.6. Regulation of LXR activity through R2TP and R2TP associated proteins

The transcription factor SREBP1c, which is predominantly expressed in the liver, is an important element in the control of fatty acid metabolism. Not only is SREPB1c a target gene of and stimulated by LXR, there is also evidence that mTORC1 contributes to the regulation of SREBP1c expression via the S6K1 pathway (7,10,32). Quantitative PCR analysis was performed with and without treatment using GW3965, an LXR ligand, to exemplify the effect of the knockdown on gene expression. Non-treated NUFIP KD, RUVBL1 KD, and TRRAP KD samples showed higher SREPB1c mRNA levels than the respective controls. An effect similarly to the effect observed for FXR target genes. However, this effect was not observed in ZNHIT3 KD samples. Induction with GW3965 demonstrated that NUFIP KD, RUVBL1 KD, TRRAP KD, and even ZNHIT3 KD impaired SREPB1c mRNA

expression. The reduction was most pronounced in NUFIP KD (65%) and ZNHIT3 KD (55%) samples (compared to their respective controls).

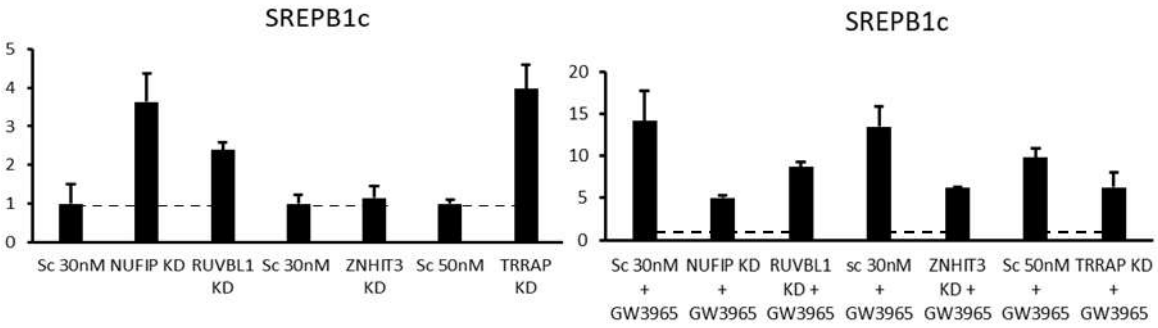

Fig.14. qPCR analysis of SREPB1c. HepG2 cells treated with siRNAs against NUFIP, RUVBL1, ZNHIT3 and TRRAP (KD = knock down). Stimulation with LXR ligand GW3965. Experiments were performed in triplicates. NUFIP KD, RUVBL1 KD and TRRAP KD samples showed higher expression of the target gene SREPB1c. Treatment with LXR ligand GW3965 showed strongly reduced SREPB1c mRNA expression in all knockdown samples (compared to control).

LXR facilitates reverse cholesterol transport (RCT). In this process, cholesterol is transported from the periphery to the liver. ABCA1, regulated by LXR, promotes transport of cholesterol to the plasma membrane and transfers cholesterol to lipid-deficient molecules such as APOAI and pre-β HDL which initiates RCT (7). Quantitative PCR analysis was performed to show the effect of knockdown of certain genes on LXR and consequently on ABCA1. Samples that underwent knockdown of certain genes by siRNA solely and samples that were additionally treated with the LXR ligand GW3965 were examined along with their controls. By comparing unstimulated samples to their control, an increase in ABCA1 mRNA expression was shown in NUFIP KD, RUVBL1 KD and TRRAP KD samples. Stimulation by GW3965 failed to induce ABCA1 gene expression in our cells.

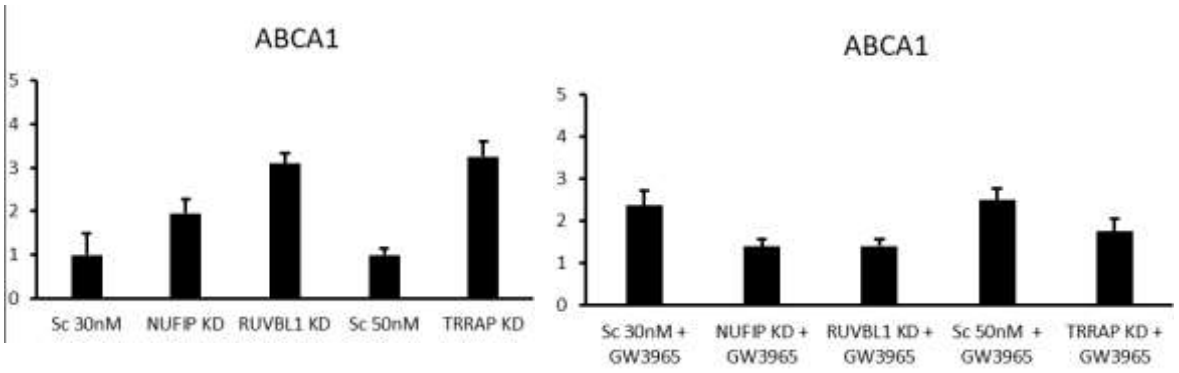

Fig.15. qPCR analysis of ABCA1. HepG2 cells treated with siRNAs against NUFIP, RUVBL1, ZNHIT3 and TRRAP (KD = knock down). Stimulation with LXR ligand GW3965. Experiments were performed in triplicates. Without stimulation, all KD samples showed higher expression of the target gene SREPB1c. Treatment with the LXR ligand GW3965 showed that mRNA expression was impaired by the respective knockdowns compared to control.

The name Fatty acid synthase (FASN) already indicates the function of the protein. In the downstream of LXR, FASN is induced by SREPB1c and leads to an increase in fatty acid biosynthesis. Quantitative PCR analysis was performed to investigate whether knockdown of ZNHIT3 would influence FASN gene expression. In untreated ZNHIT3 KD cells the FASN gene expression was diminished in comparison to the control. In ZNHIT3 KD cells treated with GW3965 it was shown that the FASN mRNA expression decreased substantially.

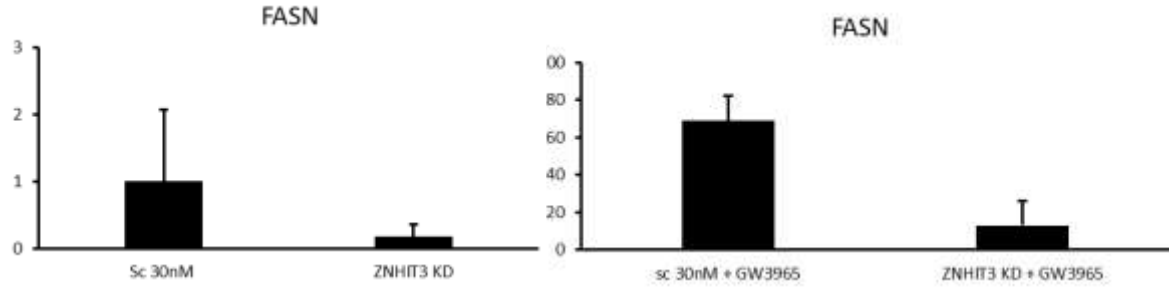

Fig.16. qPCR analysis of FASN. HepG2 cells treated with siRNAs against ZNHIT3 (KD = knock down). Stimulation with LXR ligand GW3965. Experiments were performed in triplicates. Samples show reduced mRNA expression in stimulated and unstimulated conditions compared to the control.

5.7. Regulation of PPAR-α activity through R2TP and R2TP associated proteins

PPAR-α is a nuclear transcription factor that promotes the expression of enzymes involved in fatty acid β-oxidation and ketone body synthesis and influences the inflammatory response by adjusting the transcription levels of diverse target genes (33,34). Based on the current knowledge that both mTOR and LXR as well as PPAR-α regulate lipid metabolism, it should be investigated whether there is a crosstalk between the nuclear receptors and mTOR. One of the enzymes regulated by PPAR-α is ACOX-1, the first enzyme of peroxisomal β-oxidation (35). Using qPCR analysis, it was intended to show the influence of TRRAP KD on PPAR-α activity and thus on ACOX-1 mRNA expression. The analysis was performed both without and with GW7647, a PPAR-α ligand. Notably, in HepG2 cells, PPAR-α showed no response to stimulation with aforementioned ligand. Similarly, TRRAP KD failed to show any effect on ACOX-1 gene expression. Due to the unresponsiveness of the samples to the stimulation through the ligand GW7647, no valid experiments regarding PPAR-α could be performed.

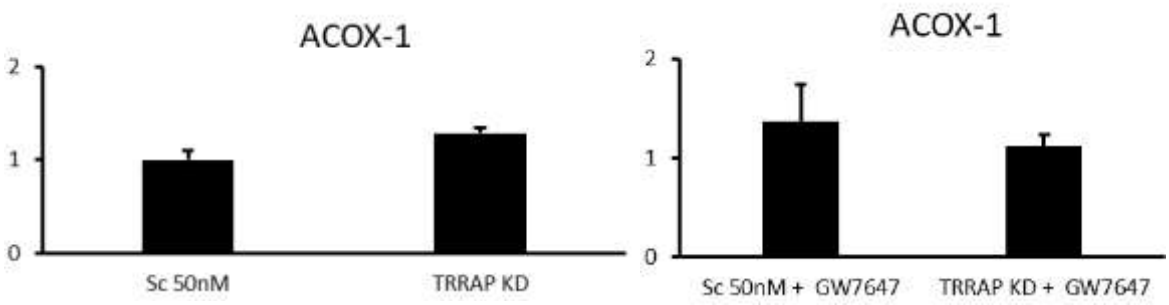

Fig.17. qPCR analysis of ACOX-1. HepG2 cells treated with siRNAs against TRRAP (KD = knock down). Stimulation with LXR ligand GW7647. Experiments were performed in triplicates. There was no change in ACOX-1 mRNA expression when comparing the TRRAP KD samples and the control samples. Treatment with the PPAR-α ligand GW7647 failed to stimulate the samples.

6. Discussion

The aims of the thesis were to determine whether PIKKS, the R2TP complex or its associated proteins have an influence on the nuclear receptors FXR and LXR and whether nuclear communication via mTOR is influenced by the knockdown of certain genes.

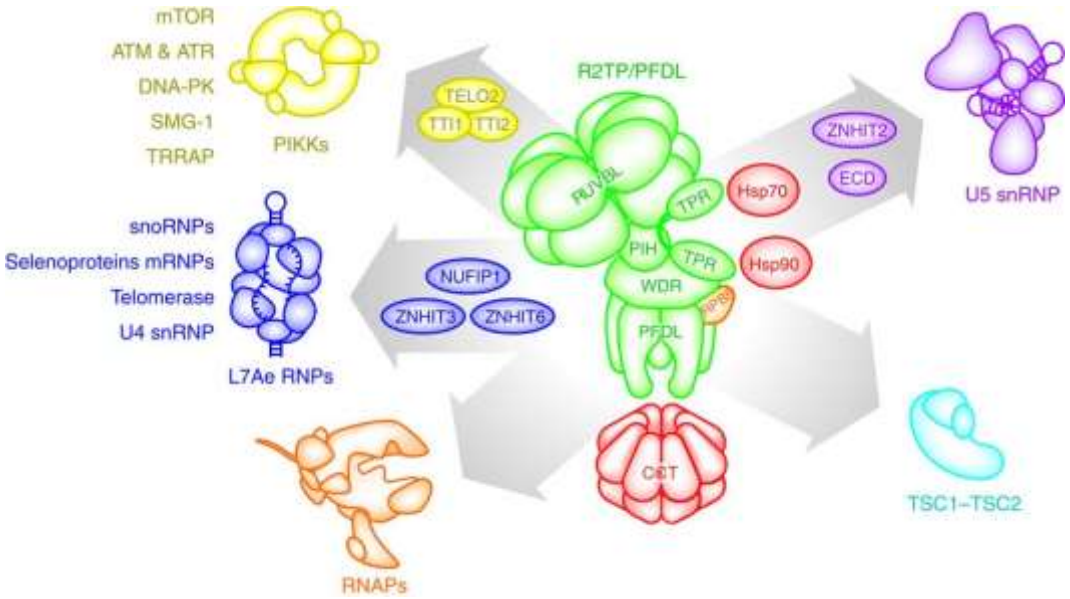

Fig.5. Interactors of the R2TP/PFDL chaperone. Cloutier et al., 2017(12)

6.1 mTOR regulation

Knockdown of NUFIP and TRRAP showed a positive effect on mTOR protein expression and phosphorylation (Figure 7). ZNHIT3 KD has also been shown to increase mTOR expression under pharmacological mTOR inhibition. Since NUFIP is involved in snoRNP biogenesis and interactions, which play an important role in ribosome biogenesis and thus also in translation, I would have expected mTOR expression to decrease rather than increase with the knockdown of NUFIP. It was not possible to find out what exactly caused the increase in the protein level in the course of the study. It was hypothesized that NUFIP is either not involved in the process leading to the expression of mTOR or that compensatory mechanisms via the R2TP complex and ZNHIT3, which also interact with snoRNPs, compensate, or even overcompensate for the loss of NUFIP. However, the increased expression

and phosphorylation of mTOR did not lead to an increase in its downstream proteins.

Like mTOR, the kinase TRRAP belongs to the PIKK family. Due to the almost identical structure, it is reasonable to assume that they can also carry out similar functions within the cell or are controlled by similar mechanisms. The knockdown of TRRAP could have triggered a positive feedback mechanism that led to an increase in mTOR protein expression and phosphorylation, but not to an increase in mTOR activity, as the downstream proteins were not affected. It is also possible that TRRAP KD interferes with the integration of TO1 and thus leads to higher mTOR levels. The exact mechanism behind it could not be determined and would be a topic for future research. ZNHIT3 KD inhibits the effect of TO1, as shown by increased mTOR protein expression. Furthermore, higher pS6 (Ser 235-236) levels were observed compared to the TO1 sample. Comparing the results of ZNHIT3 KD and TRRAP KD Western blots, it is evident that Torin 1 is not as potent in the absence of ZNHIT3. In TRRAP, however, the negative effect of TO1 is seen across all samples except for mTOR.

6.2 Regulation of the nuclear receptor FXR

FXR target gene expression was increased in TRRAP KD under unstimulated conditions and not significantly decreased under stimulation. These results showed that TRRAP KD has no or probably even a positive effect on the activity of the nuclear receptor FXR. It was therefore necessary to find out what causes this increase in target gene expression. In following Western Blots TRRAP KD showed an increase in FXR expression and an increase in mTOR expression, as described above. These results could indicate that increased mTOR expression leads to increased FXR expression. For this reason, further experiments were carried out with the result that FXR was negatively affected by inhibition of mTOR by Torin 1 (TO1), a potent mTOR inhibitor (Figure 8). TRRAP KD showed an increase in mTOR and FXR when comparing Torin 1 treated samples. I expected the opposite. Although Torin 1 is considered very specific, I hypothesized that TRRAP would also be affected by Torin 1 and that a TRRAP KD would result in an additional decrease in mTOR expression. These unexpected results are therefore based on a different mechanism. As already described above, it could be a compensation of TRRAP by

mTOR or other mTOR related PIKKS. It is also possible that ERK or RSK90, that are known to regulate S6 phosphorylation, are involved in the compensatory effects observed in TRAPP-TO1 co-treatment.

The same experiments carried out with ZNHIT3 KD led to the same result. ZNHIT3 KD unexpectedly led to an increase in mTOR expression and phosphorylation in samples treated with Torin 1, which in turn led to an increase in FXR expression. These experiments have again shown that mTOR and FXR correlate in their expression levels. Knowing that FXR is influenced by mTOR, the goal was to find out through which signaling pathway this regulation takes place.

FXR expression is negatively affected by NUFIP knockdown (Figure 7). NUFIP KD was also shown to reduce the expression of certain FXR target genes, specifically under stimulated conditions (Figure 11). These effects could in part maybe attributed to a non-specific effect and indicate a reduction in translational activity, as NUFIP is an important factor for translation. Further studies should be conducted to find out if there is another mechanism behind the reduction of FXR expression by NUFIP KD.

RUVBL1 is another important component of the R2TP complex and was also investigated. Western blots showed that RUVBL1 knockdown slightly reduced mTOR, NUFIP and FXR expression (Figure 7). Both mTOR and NUFIP have been shown to affect FXR in previous experiments. FXR target gene expression was also reduced by RUVBL1 KD under stimulated conditions to a similar extent as with NUFIP KD (Figure 10). ZNHIT3 KD likewise showed a decrease in FXR target genes under stimulation (Figure 11). The R2TP complex, together with NUFIP and ZNHIT3, is an important factor in snoRNP biogenesis and interaction. Thus, the knockdown could lead to a non-specific blockade of translation as soon as the signaling pathways are stimulated by FXR ligands. This would be a possible explanation for the effect that the knockdown of one of the three respective proteins NUFIP, RUVBL1 and ZNHIT3 has on FXR.

6.3 Regulation of the nuclear receptor LXR

Furthermore, qPCR analysis was used to investigate whether knockdown of the genes encoding the TRRAP, ZNHIT3, NUFIP and RUVBL1 proteins alters LXR target gene expression. The experiments were performed under unstimulated and stimulated conditions with the LXR ligand GW3965. TRRAP KD, ZNHIT3 KD, NUFIP

KD and RUVBL1 KD were found to produce a negative effect for certain target genes under stimulated conditions and to decrease mRNA gene expression (Figure 14, Figure 15, and Figure 16). These results suggest that the investigated proteins are important for efficient transcription under stimulated conditions. It would be reasonable to assume that NUFIP KD would also alter ZNHIT3 and *vice versa*. RUVBL1 KD has already been shown to have an influence on NUFIP protein levels. However, further studies are necessary to better understand the exact processes involved.

6.5 Conclusion

In conclusion, FXR was shown to correlate with mTOR expression levels. mTOR is supported by its PIKK family member TRRAP. The R2TP complex and its associated proteins NUFIP and ZNHIT3 are an important component of translation and are also essential for FXR and its function. LXR is also dependent on the functioning mechanism via R2TP and its associated proteins NUFIP and ZNHIT3. The Synthesis of BAs is the primary pathway for cholesterol catabolism and a complex multi-enzymatic process. The conversion of cholesterol to bile acids, is reduced by FXR. On the other hand, nuclear receptors, and intracellular sensors for sterols, such as LXR induce transcriptional responses on multiple metabolic pathways in a tissue specific manner that controls cholesterol metabolism. Interestingly, the data obtained in my thesis point toward a co-regulatory mechanism that impacts genes involved in both cholesterol and BA metabolism, mediated in part by FXR and LXR. Based on these important regulatory functions we can speculate that compensatory mechanisms are involved if one or the other nuclear receptor (FXR/LXR) or metabolic pathway (bile acids/cholesterol) is affected. Future experiments with double siRNA knockdowns might be a way to evaluate to what extend the R2TP complex regulates mTOR signaling pathways. Liver-specific Reptin/RUVBL2 knockout ($Reptin^{LKO}$) mice show a tremendous effect on glucose and lipid metabolism with opposite actions on mTORC1 and mTORC2 signaling. This recent study may therefore explain some of the effects that we observed with Torin 1 (TO1), as TO1 in comparison to rapamycin (inhibitor of mTORC1) is an inhibitor of both complexes (mTORC1 and mTORC2).

6.6 Limitations

In the course of my diploma thesis, it was not possible for me to conduct a prolonged observation period and to investigate long-term effects as well as chronic processes. All experiments were based on the acute effects of knockdowns by siRNA and their immediate impact on the cells. Furthermore, the experiments were performed exclusively with HEPG2 cells. It is possible that certain proteins and factors are expressed in these hepatic carcinoma cells at a reduced or increased level compared to healthy cells in the human organism. In order to substantiate the results, an experiment with a second liver cell line could be established, which could reveal systematic errors. In one of the experiments, Torin 1 was used to suppress mTOR expression. Other mTOR inhibitors such as rapamycin would also be available to make comparisons and confirm or alter experimental findings. Also, in addition to examining mRNA and protein expression by qPCR and Western blots, the localization of complexes and proteins could be studied. Experiments on this matter were also performed in collaboration with colleagues of the laboratory, but the results have not been included in this thesis. From the data provided, further hypotheses should emerge and be used for further research purposes.

6.7 Outlook

FXR is an important factor in human metabolism and among others serve to maintain glucose and lipid homeostasis as PEPCK, G6Pase, SREPB1c and FASn are regulated by FXR. An equally important role is played by mTOR and its downstream proteins, which control lipid synthesis and glycolysis. Combination therapy via these two regulatory elements of metabolism may reveal further insights into novel signal transduction pathways as well as new therapeutic options against metabolic diseases. In addition, the AAA+ ATPase RUVBL2 was found to be a regulator of mTOR signaling in the liver and therefore global glucido-lipidic homeostasis. Inhibition of RUVBL2 in the liver presents itself as a new therapeutic perspective for the metabolic syndrome (36).

As the primary site for protein translation, ribosomes are responsible for nearly half of cellular protein synthesis (37,38). The biogenesis of ribosomes and the processes of translation at these cell organelles require a high energy expenditure, which is

strictly monitored. Therefore, especially upon nutrient starvation, the degradation of ribosomes and the concomitant downregulation of protein synthesis seem to be crucial for cellular survival (37–39). The important role of ribophagy has been demonstrated in mammals, as neurodegenerative development has been observed, indicating the importance of ribophagy in cell function and survival (38,40). NUFIP was shown to be a receptor for the selective degradation of ribosomes. In starvation-induced ribophagy, the interaction of NUFIP and ZNHIT3 is essential. While the target of NUFIP was found to be roughly located at the 60S ribosomal subunit, the ligand for this process has not yet been identified and is subject to further investigation (41). Most importantly, the role of NUFIP1 and ZNHIT3 aside from ribosome synthesis and degradation has not been elucidated. It will be interesting to explore if both proteins are also important regulators of metabolic homeostasis in an mTOR dependent and/or independent fashion.

References

1. Keshav S. The gastrointestinal system at a glance. Malden, Mass: Blackwell Science; 2004. 117 S. (At a glance).

2. Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K. Targeting bile-acid signalling for metabolic diseases. Nature Reviews Drug Discovery. August 2008;7(8):678–93.

3. Chiang JYL. Bile acids: regulation of synthesis. J Lipid Res. Oktober 2009;50(10):1955–66.

4. Sengupta S, Peterson TR, Laplante M, Oh S, Sabatini DM. mTORC1 controls fasting-induced ketogenesis and its modulation by ageing. Nature. 23. Dezember 2010;468(7327):1100–4.

5. Hofmann AF. The Continuing Importance of Bile Acids in Liver and Intestinal Disease. Arch Intern Med. 13. Dezember 1999;159(22):2647–58.

6. Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. Cell Mol Life Sci. August 2008;65(16):2461–83.

7. Calkin AC, Tontonoz P. Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR. Nat Rev Mol Cell Biol. 14. März 2012;13(4):213–24.

8. Kalaany NY, Mangelsdorf DJ. LXRS AND FXR: The Yin and Yang of Cholesterol and Fat Metabolism. Annu Rev Physiol. 1. Januar 2006;68(1):159–91.

9. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 9. März 2017;168(6):960–76.

10. Ben-Sahra I, Manning BD. mTORC1 signaling and the metabolic control of cell growth. Curr Opin Cell Biol. April 2017;45:72–82.

11. Muñoz-Hernández H, Pal M, Rodríguez CF, Prodromou C, Pearl LH, Llorca O. Advances on the Structure of the R2TP/Prefoldin-like Complex. Adv Exp Med Biol. 2018;1106:73–83.

12. Cloutier P, Poitras C, Durand M, Hekmat O, Fiola-Masson É, Bouchard A, u. a. R2TP/Prefoldin-like component RUVBL1/RUVBL2 directly interacts with ZNHIT2 to regulate assembly of U5 small nuclear ribonucleoprotein. Nat Commun [Internet]. 31. Mai 2017 [zitiert 10. September 2020];8. Verfügbar unter: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5460035/

13. Kakihara Y, Houry WA. The R2TP complex: Discovery and functions. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 1. Januar 2012;1823(1):101–7.

14. Izumi N, Yamashita A, Hirano H, Ohno S. Heat shock protein 90 regulates phosphatidylinositol 3-kinase-related protein kinase family proteins together with the RUVBL1/2 and Tel2-containing co-factor complex. Cancer Science. 2012;103(1):50–7.

15. Horejsí Z, Takai H, Adelman CA, Collis SJ, Flynn H, Maslen S, u. a. CK2 phospho-dependent binding of R2TP complex to TEL2 is essential for mTOR and SMG1 stability. Mol Cell. 24. September 2010;39(6):839–50.

16. Kakihara Y, Makhnevych T, Zhao L, Tang W, Houry WA. Nutritional status modulates box C/D snoRNP biogenesis by regulated subcellular relocalization of the R2TP complex. Genome Biol [Internet]. 2014 [zitiert 10. September 2020];15(7). Verfügbar unter: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4165372/

17. Izumi N, Yamashita A, Ohno S. Integrated regulation of PIKK-mediated stress responses by AAA+ proteins RUVBL1 and RUVBL2. Nucleus. 1. Januar 2012;3(1):29–43.

18. Tanida I, Ueno T, Kominami E. LC3 and Autophagy. Methods Mol Biol. 2008;445:77–88.

19. Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. Autophagy. Dezember 2007;3(6):542–5.

20. Liu Q, Chang JW, Wang J, Kang SA, Thoreen CC, Markhard A, u. a. Discovery of 1-(4-(4-propionylpiperazin-1-yl)-3-(trifluoromethyl)phenyl)-9-(quinolin-3-yl)benzo[h][1,6]naphthyridin-2(1H)-one as a highly potent, selective mammalian target of rapamycin (mTOR) inhibitor for the treatment of cancer. J Med Chem. 14. Oktober 2010;53(19):7146–55.

21. Thoreen CC, Kang SA, Chang JW, Liu Q, Zhang J, Gao Y, u. a. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J Biol Chem. 20. März 2009;284(12):8023–32.

22. Unno A, Takada I, Takezawa S, Oishi H, Baba A, Shimizu T, u. a. TRRAP as a hepatic coactivator of LXR and FXR function. Biochemical and Biophysical Research Communications. 18. Februar 2005;327(3):933–8.

23. Rao A, Haywood J, Craddock AL, Belinsky MG, Kruh GD, Dawson PA. The organic solute transporter α-β, Ostα-Ostβ, is essential for intestinal bile acid transport and homeostasis. Proc Natl Acad Sci U S A. 11. März 2008;105(10):3891–6.

24. Dawson PA, Hubbert M, Haywood J, Craddock AL, Zerangue N, Christian WV, u. a. The Heteromeric Organic Solute Transporter α-β, Ostα-Ostβ, Is an Ileal Basolateral Bile Acid Transporter. J Biol Chem. 25. Februar 2005;280(8):6960–8.

25. Wong MKS. Subchapter 30A - Kininogen. In: Takei Y, Ando H, Tsutsui K, Herausgeber. Handbook of Hormones [Internet]. San Diego: Academic Press; 2016 [zitiert 15. Mai 2021]. S. 268-e30A-3. Verfügbar unter: https://www.sciencedirect.com/science/article/pii/B978012801028000180X

26. Kis E, Ioja E, Rajnai Z, Jani M, Méhn D, Herédi-Szabó K, u. a. BSEP inhibition: in vitro screens to assess cholestatic potential of drugs. Toxicol In Vitro. Dezember 2012;26(8):1294–9.

27. Byrne JA, Strautnieks SS, Mieli–Vergani G, Higgins CF, Linton KJ, Thompson RJ. The human bile salt export pump: Characterization of substrate specificity and identification of inhibitors. Gastroenterology. 1. November 2002;123(5):1649–58.

28. Noé J, Stieger B, Meier PJ. Functional expression of the canalicular bile salt export pump of human liver. Gastroenterology. 1. November 2002;123(5):1659–66.

29. Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, u. a. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem. 17. April 1998;273(16):10046–50.

30. Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, u. a. Molecular Basis for Feedback Regulation of Bile Acid Synthesis by Nuclear Receptors. Molecular Cell. 1. September 2000;6(3):507–15.

31. Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, u. a. A Regulatory Cascade of the Nuclear Receptors FXR, SHP-1, and LRH-1 Represses Bile Acid Biosynthesis. Molecular Cell. 1. September 2000;6(3):517–26.

32. Li S, Ogawa W, Emi A, Hayashi K, Senga Y, Nomura K, u. a. Role of S6K1 in regulation of SREBP1c expression in the liver. Biochemical and Biophysical Research Communications. 26. August 2011;412(2):197–202.

33. Dou F, Wu B, Sun L, Chen J, Liu T, Yu Z, u. a. Identification of a novel regulatory pathway for PPARα by RNA-seq characterization of the endothelial cell lipid peroxidative injury transcriptome. Open Biol [Internet]. 18. Dezember 2019 [zitiert 18. Mai 2021];9(12). Verfügbar unter: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6936254/

34. Morieri ML, Shah HS, Sjaarda J, Lenzini PA, Campbell H, Motsinger-Reif AA, u. a. PPARA Polymorphism Influences the Cardiovascular Benefit of Fenofibrate in Type 2 Diabetes: Findings From ACCORD-Lipid. Diabetes. April 2020;69(4):771–83.

35. Huang J, Jia Y, Fu T, Viswakarma N, Bai L, Rao MS, u. a. Sustained activation of PPARα by endogenous ligands increases hepatic fatty acid oxidation and prevents obesity in ob/ob mice. FASEB J. Februar 2012;26(2):628–38.

36. Javary J, Allain-Courtois N, Saucisse N, Costet P, Heraud C, Benhamed F, u. a. Liver Reptin/RUVBL2 controls glucose and lipid metabolism with opposite actions on mTORC1 and mTORC2 signalling. Gut. 1. Dezember 2018;67(12):2192–203.

37. Warner JR. The economics of ribosome biosynthesis in yeast. Trends in Biochemical Sciences. 1. November 1999;24(11):437–40.

38. Yao R-Q, Ren C, Xia Z-F, Yao Y-M. Organelle-specific autophagy in inflammatory diseases: a potential therapeutic target underlying the quality control of multiple organelles. Autophagy. 1. Februar 2021;17(2):385–401.

39. Lafontaine DLJ. A 'garbage can' for ribosomes: how eukaryotes degrade their ribosomes. Trends in Biochemical Sciences. 1. Mai 2010;35(5):267–77.

40. Baltanás FC, Casafont I, Weruaga E, Alonso JR, Berciano MT, Lafarga M. Nucleolar disruption and cajal body disassembly are nuclear hallmarks of DNA damage-induced neurodegeneration in purkinje cells. Brain Pathol. Juli 2011;21(4):374–88.

41. Wyant GA, Abu-Remaileh M, Frenkel EM, Laqtom NN, Dharamdasani V, Lewis CA, u. a. NUFIP1 is a ribosome receptor for starvation-induced ribophagy. Science. 18. Mai 2018;360(6390):751–8.