UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ

UMR_S1110 Institut de recherche sur les maladies virales et hépatiques

THÈSE présentée par :

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soutenue le : **21 octobre 2021**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline : Aspects Moléculaires et Cellulaires de la Biologie

Preventing the progression of NASH and liver cancer by discovering therapeutic targets using cell circuits of the circadian clock

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In memory of my dad who taught me the meaning
of life and the will to explore and then went

ACKNOWLEDGEMENTS

I would first like to thank my PhD supervisors; Prof. Thomas F. Baumert, director of the unit and co-director of this thesis, and Dr. Joachim Lupberger, my thesis director, for their support, excellent encouragement, and guidance during the 3 years.

A very special thanks to the jury members of my thesis, Dr. Hervé Guillou, Prof. Magdalena Sinnreich Filipowicz, Prof. Romeo Ricci, and Dr. Daniel Mauvoisin for accepting to evaluate my thesis work.

I would also like to show gratitude to Dr. Catherine Schuster, deputy director of the unit and the head of the doctoral school, for her generous participation in guiding, kind support, confidence, and advice during the work on my PhD thesis.

I would also like to thank my colleagues at the UMR-S1110 for their continuous encouragement and support. Notably Charlotte, Laura, and Alessia I want to express my gratitude and appreciation for their friendship, their personal and professional support during the time I spent at the unit. I would like to thank everyone above as well as Natasha, Marine, Sarah, Gaetan, Victor, Nuno, and Catherine C.

Most importantly, my warm and heartfelt thanks go to both my mother Saida, and my late father, Faouzi, who passed away several years ago for the love, the tremendous support and encouragement that they had given to me (thanks for having me and thanks for the memories, Dad). Deepest thanks to my siblings: Khalil, Aziz, and Sarah, who keep me grounded, and who are always supporting me in my adventures. I am also forever thankful for my little family in Paris Wawa, Monia, and Ykbelle, for their support through the entire thesis process and every day.

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ABBREVIATIONS

^{1}H-MRS	Proton magnetic resonance spectroscopy
AH	Alcoholic hepatitis
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
ASC	Caspase-1 domain
ASK1	Apoptosis signaling kinase 1
APOC 3	Apolipoprotein C3
BMAL 1	Brain muscle arnt-like protein-1
CC	Circadian clock
CCG	Clock-controlled genes
CCL_4	Carbon-tetrachloride
CCR 2	Chemokine receptors 2
CCR 5	Chemokine receptors 5
CD-HFD	Choline deficient-high fat diet
CK1δ	Casein kinase 1 delta
CK ε	Casein kinase 1 epsilon
CLOCK	Circadian locomotor receptor cycles output kaput
CREB	CAMP response element-binding protein
CRY	Cryptochrome
CYP7α1	Cholesterol 7α-hydroxylase
DAMP	Danger-associated molecular patterns
DBP	D-site of albumin promoter binding protein
DD	Dark-dark
DEN	Diethylnitrosamine
E4BP4	E4 promoter–binding protein 4
ECM	Extracellular matrix
ER	Endoplasmic reticulum
F0	Fibrosis stage 0; no fibrosis
F1	Fibrosis stage 1; portal fibrosis
F2	Fibrosis stage 2; periportal fibrosis
F3	Fibrosis stage 3; septal fibrosis
F4	Fibrosis stage 4; probable or obvious cirrhosis
FFA	Free fatty acids
FXR	Farnesoid X receptor
FOXO 3	Forkhead Box O 3
FGF-21	Fibroblast growth factor 21
G6PC	Glucose-6-phosphatase
GLP 1	Glucagon-like peptide 1
GLUT 2	Glucose transporter 2
GRO-seq	Global Run-On Sequencing
GWAS	Genome-wide association studies
bHLH	Helix-loop-helix
HBV	Hepatitis B virus
HCC	Hepato-cellular carcinoma
HCV	Hepatitis C virus
HFD	High fat diet
HIV	Human immunodeficiency virus
HLFM	Human liver myofibroblasts
HSC	Hepatic stellate cells
IL1-β	Interleukin 1β
IL-6	Interleukin 6
IL-8	Interleukin 8

IR	Insulin resistance
JNK	C-Jun-N-terminal kinase
KO	Knock-out
LD	Light-dark
LDL	Low density lipoprotein-cholesterol
LEC	Liver sinusoidal endothelial cells
LOXL2	Lysyl oxidase 2
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCD	Methionine–choline-deficient
MCP1	Macrophage chemotactic protein 1
MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor kappa B
NLPR3	NLR family pyrin domain containing 3
NOB	Nobiletin
NR1D1	Nuclear receptor subfamily 1 group D member 1
OCA	Obeticholic acid
PAS	Per-ARNT-SIM
PCK	Phosphoenolpyruvate carboxykinase
Per	Period
PDGF	Platelet-derived growth factor
PDFF	Proton density fat-fraction
PF	Portal fibroblasts
PGC1α	Peroxisome proliferator activated receptor gamma coactivator 1 alpha
PHH	Primary human hepatocytes
PPAR- γ	Peroxisome proliferator-activated receptor-γ
RHT	Retinohypothalamic tract
ROR	Retinoic acid-like orphan receptor
RORE	ROR responsive elements
ROS	Reactive oxygen species
SCN	Suprachiasmatic nucleus
SIRT1	Sirtuin 1
SNP	Single nucleotide polymorphism
SREBP	Sterol regulatory element binding transcription factor
T2DM	Type 2 diabetes mellitus
TACE	Transcatheter arterial chemoembolization
TF	Transcription factor
TIMP	Tissue inhibitor of metalloproteinase-1
TG	Triglyceride
TGF-β	Transforming growth factor β
TLR 4	Toll Like Receptor 4
VLDL	Very-low-density lipoprotein
WHO	World health organization
WT	Wild type
ZT	Zeitgeber

INTRODUCTION

1.The liver: Structure and function

The liver is one of the largest organs in the human body and weighs nearly 1.5 kg. This organ is strongly vascularized and contains almost 13% of blood volume. Notably, the liver plays a crucial role in regulating whole-body glucose and lipid homeostasis. It is the main site for the synthesis, storage, and redistribution of carbohydrates, proteins, and lipids, especially during the adjustment periods in fasting and feeding (Hurtado-Carneiro et al., 2021). It also has an exocrine function of secretion of bile acids, a biological fluid that promotes digestion, from cholesterol. The liver also performs detoxification and cleansing activities with degradation xenobiotic compounds such as drugs and in hormonal regulation (Abdel-Misih & Bloomston, 2010).

The liver is composed of several cell types which have unique functions that cooperatively regulate hepatic function at multiple levels (Trefts et al., 2017) (Figure 1). The main cell type is the hepatocytes and constitutes 60% of liver cells. Hepatocytes are primarily engaged in the basic functions of the liver, including lipid metabolism, drug metabolism, and the secretion of coagulation and complement factors (Jenne & Kubes, 2013). The endothelial cells, or sinusoidal cells, separate the hepatocytes from the sinusoidal lumen and perform a function of filtration due to the presence of fenestrae (Ronis et al., 2008). This specific characteristic promotes exchanges between plasma and the cell types of the liver. Kupffer cells are the resident macrophage population that makes up about 10% of the liver. These cells are responsible for clearing the blood of ingested bacterial pathogens and removing spent red blood cells from the circulation (Hardy et al., 2016). Finally, the hepatic stellate cells (HSC) represent only 5% of the liver cells and are present in the space between hepatocytes and sinusoidal cells, called the Disse space. HSC represents a dynamic cell population that can exist in a quiescent or activated state and then be transformed into human liver myofibroblasts (HLMF) which play a key role in inflammatory fibrotic response (Wakim, 1954). When activated, HSC proliferate and produce an increasing amount of transforming growth factor (TGF-β) which leads to a higher transcriptional rate of mRNAs coding for extracellular matrix (ECM) such as collagen and fibronectin (Wakim, 1954). The deposition and organization of collagen contributes to scarring of the liver, which can progress to cirrhosis, a critical pathology contributing to end-stage liver disease (Trefts et al., 2017).

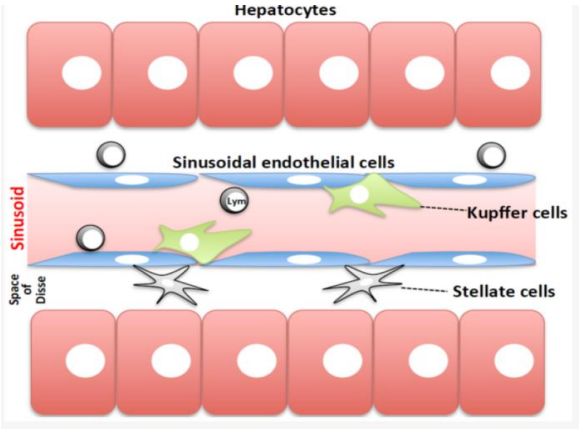


Figure 1: **Schematic representation of liver architecture**. Liver sinusoidal endothelial cells separate the hepatocytes from the blood flowing through the sinusoids. Stellate cells are in the space of Disse, which separates sinusoidal endothelial cells from hepatocytes and allows the exchange of metabolites between the blood and hepatocytes. Kupffer cells are the resident macrophages of the liver that adhere to the sinusoidal endothelium. Figure adapted from (Tsutsui & Nishiguchi, 2014).

2. Pathophysiological manifestations of liver disease

2.1 Inflammation

Inflammation of the liver (hepatitis) due to infection, fatty foods, or excessive alcohol consumption is associated with liver injury. A proposal for semiquantitative grading for inflammation activity was published by Batts and Ludwig in 1995 on a 0 to 4 nominal scale (Batts & Ludwig, 1995). Persistent inflammation often drives the deposition of collagen. This process is known as fibrosis (detailed discussion on fibrosis below).

2.2 Hepatic Steatosis

The excessive accumulation of triglycerides in the hepatocytes leads to steatosis which is manifested as the accumulation of macrovesicular or microvesicular lipid droplets in the liver. The liver is considered steatotic when the lipid content in the hepatocytes exceeds 5% by volume (Brunt et al., 2011).

2.3 Fibrosis and Cirrhosis

Fibrosis is defined as the hepatic damage of various etiologic factors, followed by the activation of HSC within the liver that develop into HLMF which are the main source of the accumulation of ECM (Canbay et al., 2004; Friedman, 2008; Kleiner et al., 2005). This process is further perpetuated by soluble mediators activated by Kupffer cells or inflammatory cells, the latter of which express profibrogenic molecules such as TGF-β and interleukin 1β (IL-1β) (Brenner, 2009). Ultimately, the sum of these cellular alterations contributes to the development of liver cirrhosis, which is characterized by distortion of the hepatic vasculature and architectural alteration of the entire liver.

3. Chronic liver disease

Chronic liver disease is a progressive destruction of liver functions and is one of the leading causes of public health burden in the western world (Williams et al., 2014). It consists of a wide range of liver pathologies which include inflammation, fatty infiltration (steatosis), fibrosis, liver cirrhosis that could predispose to hepatic cellular carcinoma. The leading causes of chronic liver disease in the Europe and United States are alcoholic liver disease (ALD), chronic hepatitis C virus (HCV), and non-alcoholic fatty liver disease (NAFLD) (Asrani et al., 2018). In my thesis, I focused on liver fibrosis as well as non- alcoholic steatohepatitis (NASH).

3.1 Alcoholic liver disease (ALD)

ALD includes a broad clinical-histological spectrum from simple steatosis, cirrhosis, acute alcoholic hepatitis with or without cirrhosis to hepatocellular carcinoma as a complication of cirrhosis (Dunn & Shah, 2016). Patients with underlying cirrhosis and ongoing alcohol abuse are predisposed to developing alcoholic hepatitis (AH) (Mathurin & Bataller, 2015; MR et al., 2009). With a mortality rate of 30-50% at 3 months (Mathurin & Bataller, 2015), AH represents one of the deadliest diseases in clinical hepatology.

3.2 Chronic hepatitis C virus (HCV)

Chronic HCV infection is a major health burden that affects more than 170 million people around the world (Szabó et al., 2003). Although disease progression is typically slow, some risk factors such as alcohol abuse and coinfection of patients with hepatitis B virus (HBV) and human immunodeficiency virus (HIV) can worsen the disease (Moosavy et al., 2017). According to retrospective studies and within 20 to 30 years of infection period, cirrhosis rate was between 17% and 55%, HCC rate between 1% and 23%, and liver death between 1% and 23% (Hartleb et al., 2012; Merat et al., 2010; Nguyen & Nguyen, 2013).

3.3 Non-alcoholic fatty liver disease (NAFLD)

NAFLD is characterized by excessive fat accumulation on the liver as defined by the presence of steatosis >5% of liver weight according to histological analysis or by a proton density fat fraction (PDFF >5.6%) in proton magnetic resonance spectroscopy (^{1}H-MRS) or magnetic resonance imaging (MRI) (Marchesini et al., 2016). Moreover, the definite diagnosis of NAFLD requires the exclusion of excess alcohol consumption (≥30g a day in men or ≥20g a day in women) (Nascimbeni et al., 2013). The histopathological hallmark of NAFLD is macrovesicular steatosis in which the lipid is deposited in large droplets, displacing the nucleus to the periphery of the hepatocytes. (Figure 4.A). The presence of >5% steatotic hepatocytes in a liver tissue section are now accepted as the minimum criterion for the histological diagnosis of NAFLD (Brunt et al., 2011; Brunt & Tiniakos, 2010; Neuschwander-Tetri & Caldwell, 2003). In their study, Brunt and coworkers (Brunt et al., 2011) found that NAFLD patients

can have foci of lobular inflammation, mild portal inflammation, and lipo-granulomas in addition to steatosis. However, features of hepatocellular injury and fibrosis, indicating progression to steatohepatitis, are not observed (Brunt & Tiniakos, 2010).

3.3.1 Prevalence of NAFLD

NAFLD is the most prevalent form of chronic liver disease in the world, paralleling the epidemic of obesity and type 2 diabetes mellitus (T2DM) (Younossi & Henry, 2021). In 2018, a meta-analysis of 86 studies with more than 8,500,000 adults from 22 countries in the years 1989–2015, estimated that about 25% of the world population has NAFLD (Younossi et al., 2016). By continent, the prevalence was 13.5% in Africa, 23.7% in Europe, 24.1% in North America, 27.4% in Asia, 30.5% in South America, and 31.8% in the Middle East (Rinella & Charlton, 2016; Z. M. Younossi et al., 2016) (Figure 2). The pooled overall NASH prevalence estimate among all NAFLD patients is 7–30% of all studied liver biopsies. It is also noteworthy that the progression of NAFLD is very slow and may take decades (Anstee et al., 2013). Metabolic syndrome studies link the increased risk for NAFLD, with several factors such as obesity, genetic susceptibility, and environmental factors such as dietary composition, physical exercise, environmental chemicals, and intestinal microbiota (Younossi & Henry, 2021). Due to the silent nature of the disease, the exact prevalence trends are not known. However, given the pandemics of obesity and T2D as well as the increasing proportional number of liver transplantations for the NAFLD subjects, the prevalence of NAFLD is thought to have increased during the recent decades and is still expected to grow (Petäjä & Yki-Järvinen, 2016).

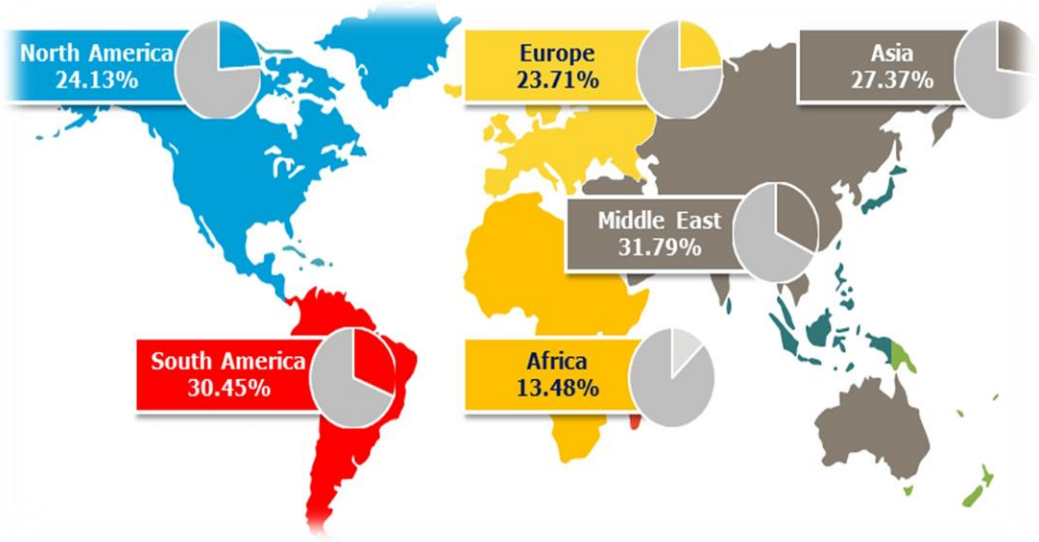


Figure 2: Map of the prevalence of NAFLD. Indicated are estimates of the prevalence of NAFLD in %. The color code depicts the incidence of NAFLD by continent: the estimated prevalence was 13.5% in Africa, 23.7% in Europe, 24.1% in North America, 27.4% in Asia, 30.5% in South America, and 31.8% in the Middle East. Figure adapted from (Younossi et al., 2019).

3.3.2 Spectrum of NAFLD

NAFLD has a wide histological spectrum, ranging from fatty liver to NASH, with combined inflammation, fibrosis, and liver injury, progressing to cirrhosis and eventually to hepatocellular carcinoma (HCC) (Figure 3). NASH is present in 3-15% of the general population (Hardy et al., 2016). NASH may eventually develop to cirrhosis and HCC; 9-20% of patients with early NASH progress to cirrhosis over a period of 5-10 years (Angulo, 2002), and some of them develop HCC (5-year cumulative incidence of 20% in patients with F3/F4-staged liver (Levene & Goldin, 2012; Vernon et al., 2011).

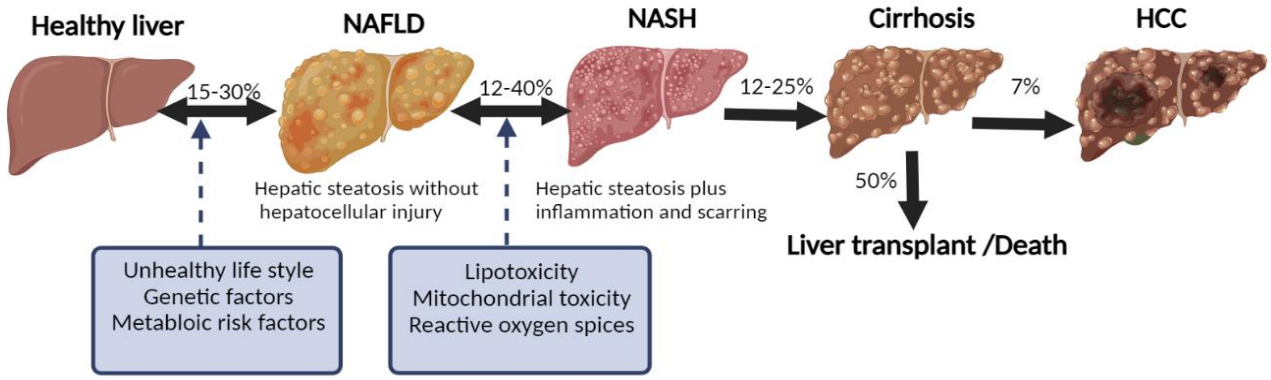


Figure 3: Histological spectrum of NAFLD disease. NAFLD represents a wide spectrum of liver damage, ranging from simple macrovesicular steatosis to steatohepatitis, with combined inflammation, fibrosis, and liver injury (steatohepatitis), progressing to cirrhosis and eventually to hepatocellular carcinoma (HCC). Figure modified from (Turchinovich et al., 2018). Created with Biorender.com.

4. Non-alcoholic steatohepatitis (NASH)

4.1 Background

The term non-alcoholic steatohepatitis (NASH) was first coined in 1980 by Ludwig and colleagues for the unique clinicopathologic entity characterized by lesions commonly seen in alcohol abuse (i.e., fatty change, lobular hepatitis, and perivenular/pericellular fibrosis) but without clinical evidence of excessive alcohol use (Ludwig et al., 1980). Although the paper of Ludwig et al. was the first report of NASH, the histopathological features seen in NASH were described earlier (Thaler, 1962; Westwater & Fainer, 1958). Over the ensuing 3 decades, pathologists described this condition: diabetic hepatitis, (Batman & Scheuer, 1985), non-alcoholic steatonecrosis (Baker, 1985), alcohol-like liver disease in the non-alcoholic, (Diehl et al., 1988), non-alcoholic fatty hepatitis, (French, 1989), fatty liver hepatitis, (Wanless & Lentz, 1990), bright liver syndrome, (Lonardo, 1996), and non-alcoholic steatosis syndromes (Falck-Ytter et al., 2001).

4.2 Histopathologic features of NASH

In 10–20% of NAFLD patients, steatosis progresses to NASH, which is characterized by the presence of macrovesicular steatosis, fibrosis, lobular inflammation, and hepatocellular ballooning (Yeh & Brunt, 2014). Ballooning degeneration of hepatocytes is the most interesting yet critical form of liver cell injury in establishing the diagnosis of NASH (Brunt, 2005). It is characterized by swelling, enlargement, and rounding of hepatocytes, also a reticulated cytoplasm (Yeh & Brunt, 2014) (Figure 4. B). Lobular inflammation is usually mild and consists of mixed inflammatory cell infiltrate, composed of lymphocytes and Kupffer cells (Brunt, 2005).

HSC activation leads to collagen formation in NASH patients (Cortez-Pinto et al., 2003). The HSC activation score, as measured by alpha-smooth muscle actin immunohistochemistry, was shown to predict the progression of fibrosis in NASH (Brunt et al., 2011). In addition, portal fibrosis (PF) without perisinusoidal/pericellular fibrosis has been reported in some cases of morbid obesity-related NASH (Figure 4.C&D) (Dixon et al., 2001; Ratziu et al., 2000). This fibrosis pattern can be helpful to suggest a metabolic etiology in cases when ballooned hepatocytes are indefinite or several disease processes are present. Portal inflammation is also present, though usually mild, and portal and periportal fibrosis appear in later stages of the disease. Subsequently bridging fibrosis and eventually, cirrhosis can develop (Susca et al., 2001).

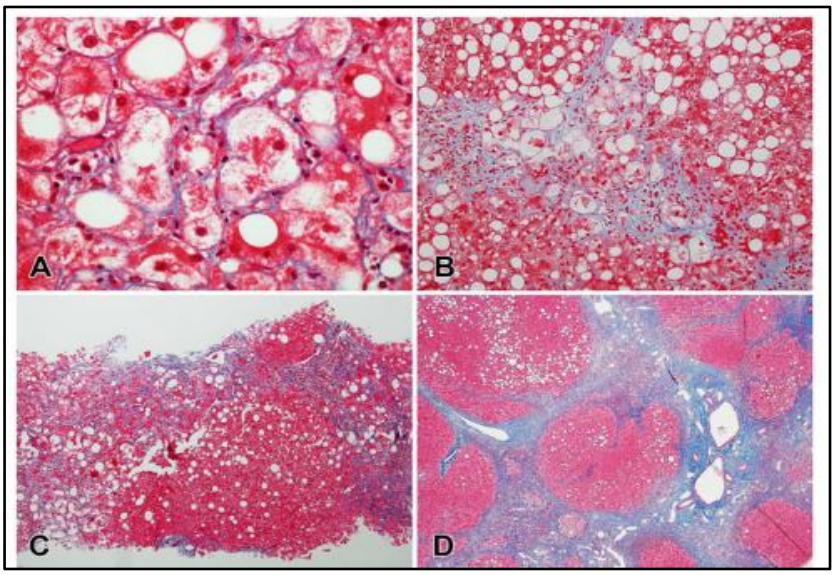


Figure 4: Fibrosis progression in NASH. **A**. Early perisinusoidal fibrosis with collagen strands between ballooned hepatocytes (600×). **B**. Advanced perisinusoidal fibrosis (200×). **C**. Fibrosis with networks of perisinusoidal fibrosis nearly encircling a regenerative nodule. (100×) **D**. Established cirrhosis (40×). (Masson's Trichrome Staining). Figure adapted from (Kleiner et al., 2005).

4.3 Grading of NASH

A proposal for semiquantitative grading and staging scheme for NASH was published by Brunt and co-workers in 1999 (Brunt et al., 1999). The authors assigned an overall grade of mild, moderate, or severe (grades 1,2 and 3, respectively) based on the degree of hepatocellular steatosis, ballooning and disarray, and inflammation (intralobular and portal), and the stage was determined based on the progression of fibrosis that starts from the unique zone 3 perisinusoidal/pericellular fibrosis, through portal fibrosis and bridging fibrosis to cirrhosis. (Table1).

Table 1: Brunt schema for grading of NASH. Reproduced from (Brunt et al., 1999).

	Grade 1 (Mild)	**Grade 2 (Moderate)**	**Grade 3 (Severe)**
Steatosis	Involves <33%- 66% of lobules	Any degree	Typically,>66%
Ballooning	Occasional in zone 3	Obvious, predominantly in zone 3	Ballooning and disarray obvious, predominantly in zone 3
Lobular inflammation	Scattered polymorphs ±lymphocytes	Polymorphs and chronic inflammation noted	Scattered polymorphs ± mild chronic
Portal inflammation	None or mild	Mild to moderate	Mild or moderate

The NASH Clinical Research Network (NASH CRN) proposed the NAFLD activity score (NAS for use in clinical research (Kleiner et al., 2005). This score can be used for the full spectrum of NAFLD, including simple steatosis (Kleiner et al., 2005). The score is calculated as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2), and ranges from 0 to 8 (Kleiner et al., 2005) (Table 2).

Table 2: NASH activity score. Reproduced from (Kleiner et al., 2005)

Steatosis	**Score**
• <5% • 5%-33% • >33%-66% • >66%	0 1 2 3
Lobular inflammation	
• No foci • < 2 foci per 200 x field • 2-4 foci per 200 x field • >4 foci 200 x field	0 1 2 3
Ballooning	
• None • Few balloons cell • Many cells /prominent ballooning	0 1 2

5. Pathogenesis of NASH

The pathogenetic processes of NASH and its progression are multifactorial and are influenced by both environmental and genetic factors (Kim & Younossi, 2008; Marchisello et al., 2019). Although the mechanism is complex and not entirely understood, a 2-hit hypothesis has been proposed, (Day & James, 1998) in which insulin resistance leads to aberrant lipid accumulation in hepatocytes (hepatic steatosis) as the first hit. The 'second hit' such as oxidative or metabolic stress and dysregulated cytokine production and activation of pro-inflammatory cytokines by Kupffer cells or adipokines from adipocytes, and HSC activation, leading to subsequent inflammation and fibrosis (Friedman et al., 2013). More recently, this concept has evolved, a multiple-hit-hypothesis has now substituted the outdated two-hit-hypothesis for the progression of NASH (Tilg & Moschen, 2010). According to this new hypothesis, insulin resistance (IR) which is an independent risk factor for NAFLD severity (Stadlmayr et al., 2011) leads to hepatocellular elevation of free fatty acids (FFA), the main factor for liver inflammation in NASH besides hepatic triglyceride (TG) accumulation in lipid droplets. The triggering of oxidative stress by lipotoxic metabolites, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and apoptosis, leads to further inflammation, and stimulation of TGF-β and activation of HSC with collagen deposition within the hepatic sinusoids become apparent (Alkhouri et al., 2014; Lambrecht et al., 2015). In addition, genetic variations are one of the most important factors that determine whether a person has a high risk to develop NASH. Genome-wide association studies (GWAS) have been discovered several genetic variations that may be responsible for the risk of NAFLD in certain populations (Macaluso et al., 2015) (Figure 5).

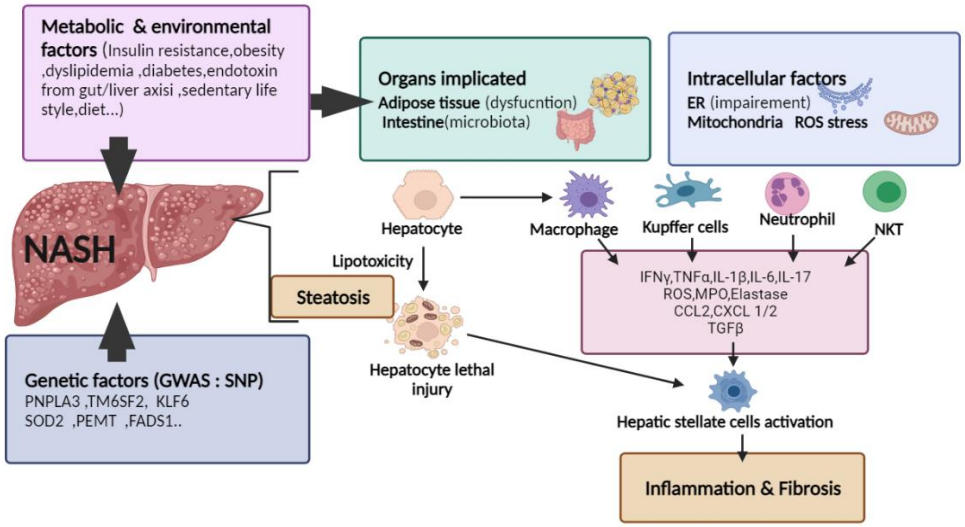


Figure 5: Schematic illustration of NASH pathogenesis. Hepatocytes are affected by lifestyle factors (a diet high in saturated fatty acids, obesity) and genetic predispositions contributing to the development of insulin resistance and hepatic steatosis. In human livers, these numerous metabolic hits lead to cellular damage, via a process called "lipotoxicity", involving excessive oxidative stress principally driven by the lipotoxic metabolites of fatty acids. Injured hepatocytes release danger-associated molecular patterns (DAMP) that initiate an inflammatory response and activate pro-inflammatory signaling pathways in the setting of increased adipokine levels. Collectively, these factors are associated with progressive fibrosis that likely represents an imbalance of tissue damage and repair due to the influence of different inflammatory cells. Figure modified from (Peverill et al., 2014).Created with Biorender.com.

5.1 Liver inflammation in NASH

Inflammation in NASH is associated with exacerbated production of mediators such as cytokines, chemokines and eicosanoids originated from the liver and extrahepatic sites such as the adipose tissue and gut. In hepatocytes, accumulated FFA is the main mediator of the inflammatory response. FFA interacts with the multiprotein complex NLR family pyrin domain containing 3 (NLRP3) inflammasome to recruits the caspase-1 domain (ASC); caspase1 induces the maturation of pro-inflammatory cytokines such as IL-1β, which promotes inflammation (Csak et al., 2011). In addition, increased FFA with the association of lipopolysaccharide (LPS) induces the production of pro-inflammatory agents that play a key role in the progression of NASH (Shi et al., 2006). Similarly, the elevated levels of FFA lead to lysosomal destabilization via the production of Bax protein which resulting in tumor necrosis factor α (TNF-α) induction (Feldstein et al., 2003). Another study was conducted by Chen and collaborators, have shown that nuclear factor kappa B (NF-κB) activation via increased reactive oxygen species (ROS) drive the expression of the pleiotropic inflammatory cytokines such as TGF-β, interleukin 6 (IL-6), and interleukin 8 (IL-8) which play a crucial role in the mediation of the inflammatory, fibrogenesis, and apoptotic response in NASH pathogenesis (Figure 6).

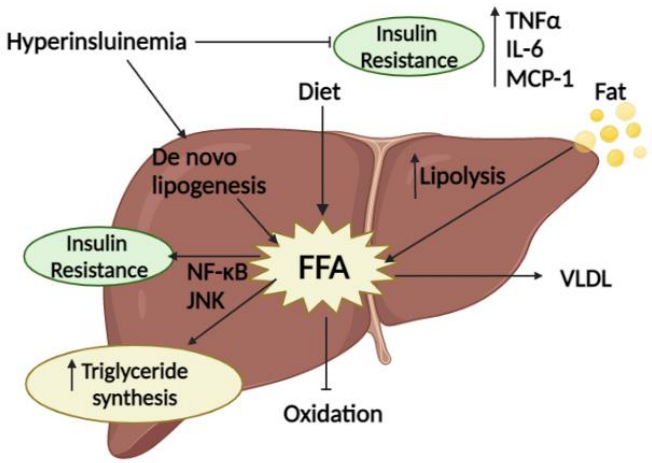


Figure 6: Interorgan links among insulin resistance. Dysregulation of hepatic FFA flux, the development of hepatic steatosis; and stimulation of De novo lipogenesis in the liver contribute to an impairment of insulin-mediated suppression of lipolysis, leading to increased flux of FFAs from adipocytes to other tissues. Figure modified from (Hardy et al., 2016). Created with Biorender.com.

Adipose tissue is one of the compartments that contribute to liver inflammation in NASH (Larter et al., 2010). Hypertrophic, dysregulated adipocytes produce an increased amount of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) and macrophages chemokines notably macrophage chemotactic protein 1 (MCP1) (El Husseny et al., 2017). *In vivo* studies have shown a positive correlation between macrophage inflammation of adipose tissue and both hepatic and insulin resistance in the high-fat diet mouse model of NASH (Lanthier et al., 2011). On the other hand, hepatic levels of adiponectin (Kaser et al., 2005) are decreased in NASH patients compared to control subjects. In other human research,

they found an increased level of MCP1 in the serum of patients with metabolic syndrome (Haukeland et al., 2006). MCP1 (also known as CCL2) transduces signaling cascades involved in IR and recruitment of pro-inflammatory cytokine production like NF-κB and c-Jun-N-terminal kinase (JNK), and NASH development (through JAK/STAT signaling) (Tilg & Moschen, 2010).

Emerging evidence points towards a crucial role of the gut microbiota in liver inflammation in NASH (Alisi et al., 2012). Recent studies document an increased intestinal permeability in NASH patients, which further promotes endotoxin intestinal production (Bergheim et al., 2008). The pro-inflammatory agent LPS (endotoxin) is highly expressed in NASH patients (Cani et al., 2007), release the production of cytokines, particularly, TGF-β and IL-8 via the activation of NF-κB signaling pathway, which further promotes the recruitment of immune effectors cells such as neutrophils, and subsequent hepatocyte injury (Baffy, 2009; Pradere et al., 2010) (Figure 7).

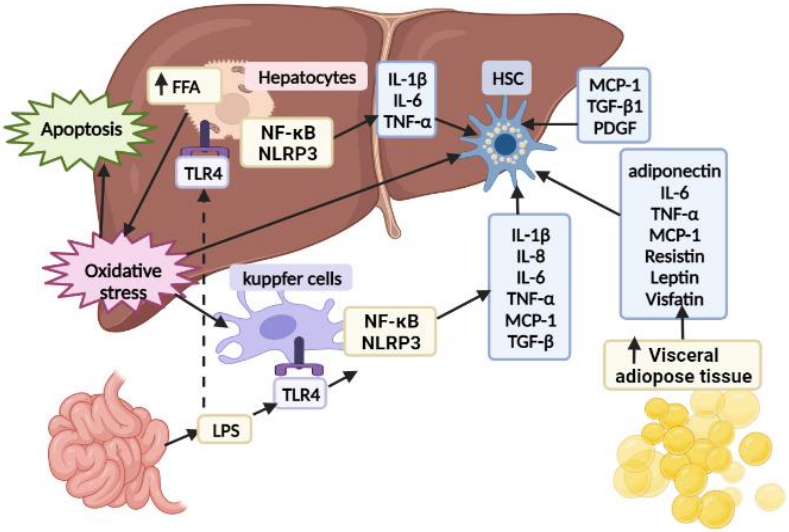


Figure 7: Main proinflammatory mechanisms involved in NASH pathogenesis. NASH is associated with the activation of several inflammatory pathways arising from several origins. Dysregulated adipocytes increase the amount of pro-inflammatory chemokines and cytokines. Intestine produces LPS, which activates the TLR4 receptor in Kupfer cells and hepatocytes, and consequently the production of cytokine via activation of both the transcription factor NF-κB and the NLRP3 inflammasome. In addition, the increase in FFAs in hepatocytes produces oxidative damage that also induces apoptosis via redox-sensitive pro-apoptotic pathways. TGF-β also promotes fibrogenesis by activating HSCs. Activated HSCs produce collagen, and release fibrogenic cytokines with autocrine. Figure modified from (Bessone et al., 2019). Created with Biorender.com.

5.2 HSC activation and fibrosis in NASH

The accumulation of extracellular matrix in the liver is the major cause of chronic liver disease including NASH (Bataller & Brenner, 2005). Fibrosis is driven by signaling from injured hepatocytes and activated Kupffer cells, leading to activation of HSCs into HLMF to produce extracellular matrix (Tsuchida & Friedman, 2017). HSC plays a crucial role in liver fibrosis. HSCs are a resident

mesenchymal cell type located in the space between hepatocytes and the liver sinusoidal endothelial cells, known as the space of Disse (Weiskirchen & Tacke, 2014). Upon liver injury, HSCs become highly activated, which leads to the conversion from a quiescent state, storing vitamin A and lipid droplets to one that has lost vitamin A and lipid droplets, leading to increased proliferation and production of proinflammatory and profibrogenic cytokines. Activated HSCs start mass production of ECM components is particularly type I collagen (Friedman, 2010; Reynaert et al., 2002) that allows the activated HSCs to be characterized as a myofibroblast-like cell. In addition to HSCs, PFs are one of the major sources of ECM, (Feldstein et al., 2004; Kawada, 2015), that affect the overall liver, particularly in cases of liver injury. Emerging data suggests the contribution of PFs to the synthesis of ECM proteins (Ramadori & Saile, 2004) (Figure 8).

HSC activation can be divided into two phases: initiation and perpetuation (Moreira, 2007). During the initiation phase, also known as the pre-inflammatory stage, several proliferative and fibrogenic cytokines are secreted to activate HSCs into HLMF (Ramadori & Saile, 2004). Among the released cytokines, we can mention two best-described growth factors, TGF-β and platelet-derived growth factor (PDGF). It has been shown their implication in the process of proliferation and activation of HSCs. Perpetuation involves at least six distinct changes in HSC behavior, including proliferation, chemotaxis, fibrogenesis, matrix degradation (via the expression of degrading enzymes such as like tissue inhibitor of matrix metalloproteinases (TIMP), and retinoid loss (Friedman, 2010).

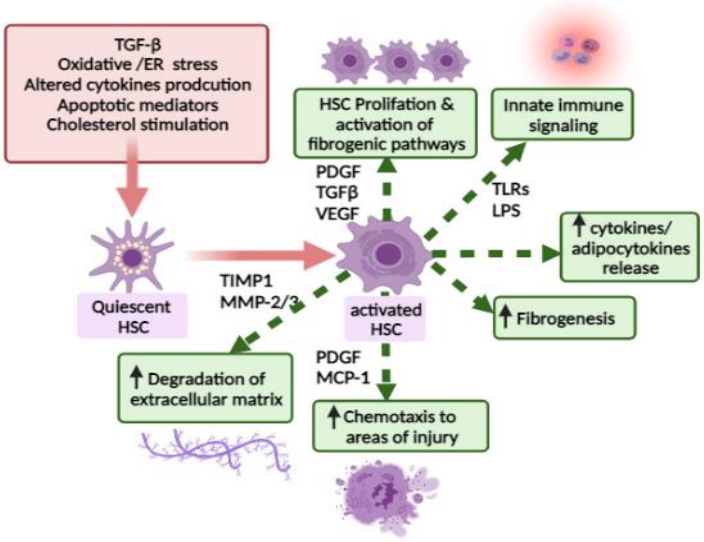


Figure 8: Pathways of initiation and perpetuation of fibrogenesis mediated by HSC activation. Following liver injury, HSC activation, initiated by the transition from quiescent, vitamin A-rich HSCs into fibrogenic, proliferative ones via TGF-β production. This 'initiation phase' is followed by the 'perpetuation phase', which promote HSC proliferation (via PDGF), sustained collagen production and deposition (via TGF-β), matrix degradation (via matrix metalloproteinases 1 and 3 (MMP1/3)), inhibition of collagen degradation (via the MMP-2 inhibitor, TIMP1), HSC chemotaxis (via PDGF and MCP1), and white cell chemoattraction (via MCP1). Figure modified from (Bessone et al., 2019).Created with Biorender.com.

5.3 Hepatocyte injury and death in NASH

Recently, multiple pathways have been identified to induce hepatocyte cell death and the transition from simple fatty liver to NASH (Ulukaya et al., 2011). Hepatocyte cell death occurs in the form of either programmed (apoptotic, necroptotic) or accidental (necrotic) cell death (Figure 9). Apoptosis is a key morphologic and pathogenic feature in patients with NASH (Susca et al., 2001) and a high biological event that regulates cell death (Feldstein et al., 2003). Immunohistochemical tests assessing caspase 3 cleavage in hepatocytes and TUNEL test were positive in both liver tissue from patients with NASH and is commonly used in mouse models of NASH (Thapaliya et al., 2014). Moreover, a multi-omics study identified hepatocyte apoptosis as a key event in high-fat diet-induced NASH in mice (Soltis et al., 2017). In line with these findings, mice with perturbed apoptosis are protected from NASH (Idrissova et al., 2015). Moreover, inhibition of apoptosis using the irreversible pan-caspase inhibitor VX-166 reduced apoptosis and improved liver injury, as well as fibrogenesis in mice, fed a methionine–choline-deficient (MCD) diet (Witek et al., 2009). Importantly, the caspase inhibitor GS-9450, a selective caspase inhibitor of caspase 1,8 and 9 led to a significant reduction of alanine aminotransferase levels in patients (ALT) with NASH in patients with biopsy-proven NASH (Ratziu, 2012). Necroptosis is the second form of programmed cell death pathways involved in NASH pathogenesis (Malhi & Gores, 2008). Necroptosis leads to cell swelling and cell membrane rupture via receptor-induced apoptosis, which results in the caspase-independent organelle. Immunohistochemical studies on patients revealed a positive correlation between caspase 3-hepatocytes and NASH patients (Feldstein et al., 2003). Recently, data suggested a crucial role of caspase 8 in preventing hyperactivation of necroptosis. Importantly, ablation of caspase 8 strongly aggravated the liver injury and fibrosis in rodents ‘models (Gautheron et al., 2014). In addition to apoptosis and necroptosis, a caspase-1 dependent form of programmed cell death known as pyroptosis has been shown as is a key regulatory pathway in human metabolic liver disease. Pyroptosis occurs in hepatocytes and tissue macrophages due to the activation of NLRP3 inflammasome and caspase-1 leading to cleavage-induced activation of gasdermin D, which is implicated in mouse models of NASH and in livers biopsy of NASH patients (Petrasek et al., 2012). Interestingly, mice who display a higher level of activated NLRP3 inflammasome showed a hepatocyte pyroptotic cell death (Wree et al., 2014).

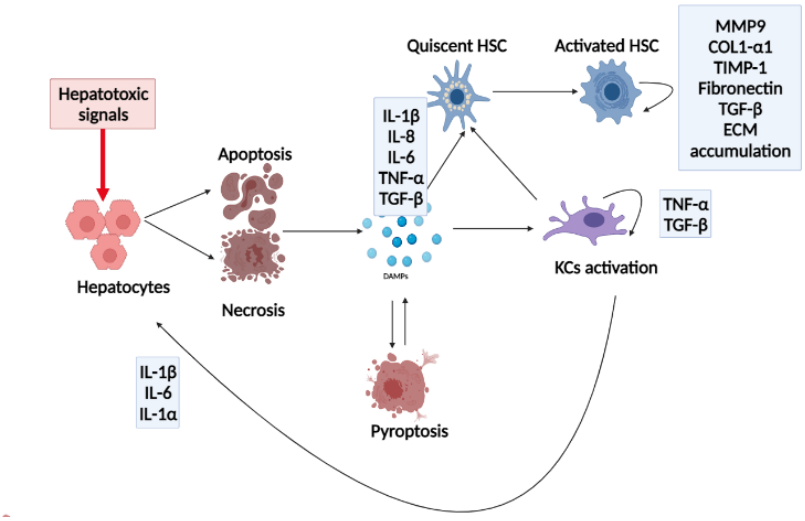


Figure 9: Events leading to hepatocyte cell death and transition from NAFLD to NASH. The hepatotoxic signal induces cellular death in hepatocytes by either apoptosis necrosis or pyroptosis, all the types of death being followed by DAMPs release. Kupffer cells are activated through DAMPs and trigger inflammatory cytokines and chemokines release. DAMPs promote HSCs activation characterized by an increase in ECM accumulation, collagen type I, fibronectin, MMP 9, 13, and TIMP1, DAMPs induce pyroptosis and triggers a self-perpetuating loop as more DAMPs are released following pyroptotic death. Figure modified from (Ignat et al., 2020). Created with Biorender.com.

5.4 Therapeutic options for NASH

Although there is no pharmacological treatment specifically approved by regulatory agencies for NASH, therapeutic options exist to manage NASH symptoms such as probiotics for gut dysbiosis, physical activity, and weight loss for obesity and diabetes (Vilar-Gomez et al., 2015). In addition to the liver-specific targets, there are targets outside the liver such as the gut-liver axis and the microbiome, which was summarized in a scheme (Figure 10) and was implicated in the drug development for NASH.

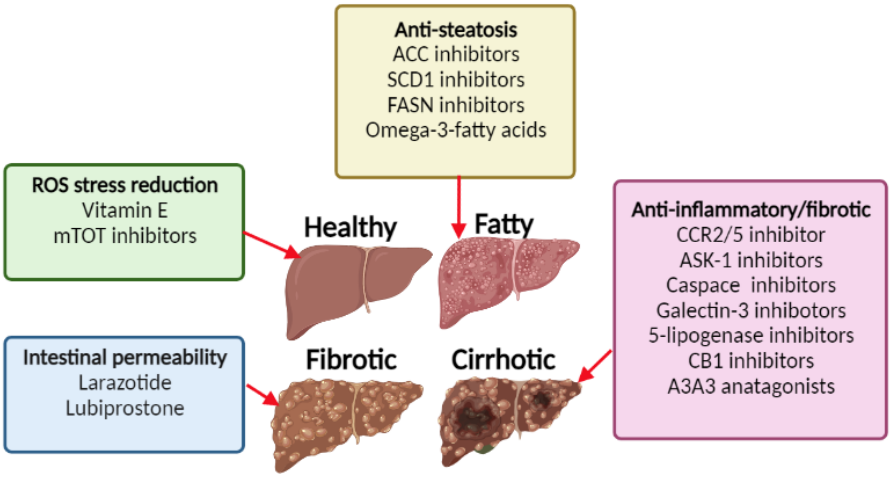


Figure 10: Intrahepatic drug targets in phase 2 and 3 clinical trials for NASH. Figure modified from (Friedman, 2010). Created with Biorender.com.

5.4.1 Metabolic targets

Peroxisome proliferator-activated receptor-γ (PPARγ) ligands (for examples pioglitazone) have been evaluated in several trials and have been shown to improve steatosis, inflammation, and hepatic fibrosis in biopsy-proven NASH patients (Mahady et al., 2011). However, their application showed side effects, including weight gain, fluid retention, and precipitate congestive heart failure, especially in older patients. In addition, obeticholic acid (OCA), which activates another nuclear receptor, farnesoid X receptor (FXR) was found to improve insulin resistance and prevent hepatic fibrosis in a mouse model of NASH as well as human tissues (Kong et al., 2009). However, a phase 2 clinical trial of OCA (25 mg per day) has failed due to an increase in low-density lipoprotein cholesterol (LDL) in some patients (Neuschwander-Tetri et al., 2015). Another promising therapeutic option includes glucagon-like peptide 1 (GLP1) based therapies, which improves fibrogenesis via reduction of unfolded-protein response and hepatic fat accumulation (Y. Liu et al., 2014). More importantly, a PIVENS trial found the improvement of histological features of NASH as well as reduction of lobular inflammation and hepatocellular ballooning in NASH patients without diabetes treated with vitamin E (Sanyal et al., 2010). However, its appeal is limited by the lack of efficacy in reducing hepatic fibrosis in a large, controlled trial (Sanyal et al., 2010). Remarkably, fibroblast growth factor 21 (FGF-21) which is a secreted protein that modulates lipid and glucose metabolism was found to improve hepatic fibrogenesis and inhibits the activation of HSCs. An FGF-21 agonist BMS-986036 is being evaluated for hepatic fat reduction in a short-term clinical trial study (NCT02413372) (Xu et al., 2016). Altogether, these studies strongly emphasize the unmet medical need for novel therapeutic approaches to treat liver disease including NASH.

5.4.2 Immune targets

NASH is an inflammatory disorder involving the recruitment of inflammatory cells and cytokines, which can lead to an activation of pro-inflammatory signaling pathways involving NF-κB, mitogen-activated protein kinase (MAPK), toll like receptor 4 (TLR4), and NLRP3. Some of the main targets for therapy include mediators of inflammation signaling pathways such as apoptosis signaling kinase 1 (ASK1)-JNK (Noureddin & Sanyal, 2018). For example, the inhibition of ASK1 production in human NASH has shown improvement in hepatic fibrosis in some patients in a short-term clinical trial (Loomba et al., 2017). In the other hand, chemokines are involved in leukocyte chemotaxis and exert their effects through transmembrane G protein-coupled receptors. The chemokine receptors 2 (CCR2) and 5 (CCR5) have been shown to play a crucial role in the liver's innate immunity via activation of hepatic stellate cells and perpetuation of hepatic inflammation and fibrogenesis (Haukeland et al., 2006). Interestingly, the pharmacological inhibition of CCR2-CCR5 reduced fibrogenesis progression in a clinical trial of NASH (Friedman et al., 2018).

5.4.3 Fibrosis treatment

Fibrosis is one of the hallmarks of NASH disease progression, and knowledge of the mechanisms of liver fibrosis is accumulating, antifibrotic therapy is an important goal for therapy. Studies have focused on blocking fibrosis directly via testing an inhibitory antibody to lysyl oxidase 2 (LOXL2). LOXL2 is involved in a late step in hepatic fibrogenesis, namely the crosslinking of ECM proteins such as collagen so that LOXL2 inhibition might render collagen more susceptible to degradation (Moon et al., 2014). Clinical trials have failed to show the efficacy and safety of a humanized monoclonal anti-LOXL2 antibody in patients with NASH. Furthermore, galectins are cell-surface glycoproteins that can mediate cell migration, matrix interaction, and inflammatory signals (Traber & Zomer, 2013). Importantly, the clinical trial of carbohydrate molecules that inhibits galectin showed a reduction of fibrosis in animal models of NASH (Traber & Zomer, 2013).In addition, GR-MD-02 (galectin inhibitor) had also a favorable safety profile in a phase 1 study in patients with NASH (Traber & Zomer, 2013).

5.5 From "fibrotic NASH" to biological clock

Evidence accumulated during recent years suggests a link between the metabolic and physiological variations and the time of the day, including feeding behavior, sleep/wake cycles, body temperatures, and hormonal levels (Asher & Sassone-Corsi, 2015; Asher & Schibler, 2011; K. Eckel-Mahan & Sassone-Corsi, 2009). Nowadays, it appears that these parameters are critical and their perturbations could have dramatic pathological consequence on human health (e.g., high fat diet (HFD)) (Li et al., 2012). More importantly, the disruption of these physiological rhythms may favor the development and progression of various metabolic diseases including NASH, alcohol-related injuries, and cancers. Of note, Specific NAFLD/NASH therapeutic targets, currently under clinical evaluation, also have an impact on the biological clock, such as PPAR, FXR, GLP1 receptor agonists. The crucial role of the biological clock in maintaining metabolic homeostasis and its implication in liver pathologies is highlighted in the next chapter.

6. Circadian rhythms

6.1 Physiology in harmony with nature

Living organisms have developed an internal timing system or “biological clock”, a conserved adaptation to the environment that drives their rhythmic behavior (Pittendrigh, 1960). Biological processes, such as blood pressure and melatonin secretion, follow the circadian rhythm, which is an endogenous physiological and behavioral process that displays oscillation of about 24 hours in the absence of any zeitgeber cues (Challet & Mendoza, 2010; Dunlap et al., 1999). “Zeitgeber" (German for “time giver”) are external synchronizing signals such as the light-dark cycle that entrain the body rhythm to the earth light-dark cycle (Whitmore et al., 2000). Each cycle is referred to in units of Zeitgeber Time or ZT, where ZT0 corresponds to lights on (Potter et al., 2016). Lack of internal synchrony, which is caused by shift work, and jet lag, due to urbanization has a profound impact on the health status of living organisms (Schernhammer et al., 2003). Many studies have revealed that shift work is considered a carcinogenic factor, and the risk of cancer is enhanced by the number of years an individual spends working at night (Schernhammer et al., 2003). Other reports have found that the dysfunction of circadian rhythms is related to coronary heart disease and metabolic diseases, for example, obesity and T2DM (Davis et al., 2001; Fonken et al., 2013). These findings substantiate the crucial role of the circadian rhythm in human health. In 1729, the observation of “Jean Jacques d’Ortous de Mairan” was the hallmark of the circadian rhythm discovery. Importantly, he noticed daily movements of “Mimosa pudica” leaves in response to the day and night (the leaves were open during the day and closed in the evening). Interestingly, he discovered that these daily movements persist when the plant is kept in constant darkness (Pittendrigh, 1960; Quintero et al., 2003). The term circadian was first coined by Franz Halberg in reference to the Latin words *circa* for “approximately” and *dies* for “day” (Halberg, 1960). Circadian rhythms are self-sustaining rhythms that exhibit a period of approximately 24 hours and can be entrained by external (Pittendrigh, 1993; Pittendrigh, 1960). The circadian rhythms are generated endogenously due to the presence of a rhythmic environment (e.g., in a light-dark cycle). These rhythms are called diurnal rhythms.

6.2 The organization of mammalian circadian clock

The characteristic of circadian rhythms in mammals was thoroughly defined by Colin Pittendrigh and Serge Dann through studies on the activity of nocturnal rodents (Dunlap & Loros, 2004; Pittendrigh, 1993; Vaze & Sharma, 2013). These indicate three basic features: (1) an environmental input (such as light), (2) an internal and self-sustained oscillator (circadian clock) that persist in the absence of external stimuli, and (3) an output or external stimuli (rhythmic oscillation in physiology or behavior). In mammals, the circadian system is organized in a hierarchical manner, with a central brain clock at the top of the hierarchy and peripheral clocks all over the body (Bass, 2011; Ko & Takahashi, 2006) (Figure 11).

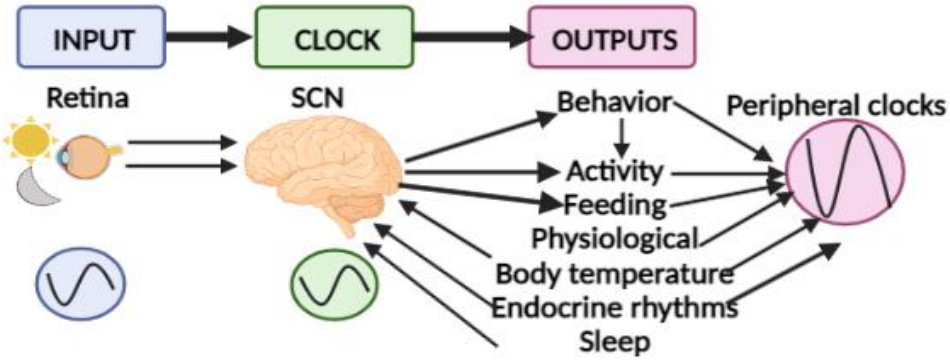


Figure 11: Organization of the mammalian circadian clock (CC). The CC organization consists of input pathways, the clock, and outputs functions. Light is one of the main input signals for the CC that activates the suprachiasmatic nucleus (SCN), which is a master pacemaker of the circadian rhythm. This circadian pacemaker coordinates the CC oscillator network through the body via neuronal and hormonal signals. The clock drives the expression of CC-controlled genes in a tissue-specific manner, which regulates tissue physiologies and functions. Several output functions including endocrine rhythms, behavior, activity, sleep/wake cycle, and metabolism also regulate the CC. Created with Biorender.com.

6.2.1 The suprachiasmatic nucleus (SCN) is a central pacemaker of the circadian clock

The SCN of the hypothalamus was identified as the central circadian pacemaker in mammals as damaging the SCN in rats disrupts both the activity/rest cycle and drinking behavior rhythms (Stephan & Zucker, 1972). Ralph and colleagues studied the role of the SCN in the circadian system through the *Tau* mutant hamsters. These mutant animals display a shortened circadian period of about 20-hour free-running, in contrast to the wild-type animals with a 24-hour free running period (Ralph et al., 1990). Interestingly, transplant of SCN grafts from the wild-type (WT) hamsters into the mutant hamsters restore circadian behavior (Ralph et al., 1990). Together, these experiments demonstrate the crucial role of the SCN as the central circadian pacemaker.

This master clock is constituted of approximately 20,000 neurons. Each individual SCN neuron has a self-sustained and cell-autonomous circadian oscillator with a range in circadian periodicity varying between 22 to 30 hours (Mohawk & Takahashi, 2011; Welsh et al., 1995) The SCN receives photic input from the retina via the retinohypothalamic tract (RHT) that originates with intrinsically photo-receptive ganglion cells and terminates in the SCN (Hannibal, 2002; Meijer et al., 2007). A subset of ganglion cells in the inner layer of the retina that express the melanopsin photopigment is considered as the main circadian photoreceptors (Berson et al., 2002). The other photoreceptors, rods, and cones, play an accessory role in maintaining circadian entrainment (Hattar et al., 2003). The SCN itself is comprised of disparate populations of neurons, keep synchrony, and stay coupled to each other through synaptic connections. This intracellular coupling between the SCN neurons is critical to entrain overt robust circadian rhythmicity that leads to robust circadian outputs. Accordingly, synchronizing signals are transmitted to the peripheral clocks to regulate the internal tissue oscillators and achieve coherent rhythm in the entire organism (Bernard et al., 2007; Whitmore et al., 2000).

6.2.2 Tissue-specific Clock regulation

SCN has been described to regulate the peripheral clocks either directly through the autonomic nervous system or indirectly through the SCN-controlled hormonal pathways (Perreau-Lenz et al., 2004). Peripheral clocks exist in different tissues, including the liver, intestine, adipose tissue, and muscle that can generate tissue-specific and cell-specific circadian rhythmicity (Balsalobre et al., 1998; Dibner et al., 2009; Yang et al., 2006) (Figure 12). Importantly, rhythmic clock gene expression and protein expression persists in isolated tissues *in vitro*, where no control of the SCN exists, indicating the endogenous nature of the circadian oscillation at the periphery (Yamazaki et al., 2000). *In vivo*, these cellular clocks receive entraining signals via neural and hormonal stimuli from the SCN to maintain synchrony (Bieler et al., 2014). Self-sustainable tissue-specific circadian rhythms were observed in SCN-lesioned mice, which demonstrated the presence of independent organ-specific regulators (Yoo et al., 2004). Another example supporting the theory of tissue-specific circadian rhythm, clock-controlled transcripts lose their cyclical expression in hepatocytes with disrupted clock (Albrecht, 2012).For instance, while the prominent synchronizing signal for the SCN is the light signal, the food is an important Zeitgeber for both SCN and the peripheral tissues (Francesca Damiola et al., 1996; Mendoza, 2007; Stokkan et al., 2001). The liver clock is demonstrated to be entrained by the feeding cycle through the rhythmic changes in hepatic gene expression after restricted feeding (Stokkan et al., 2001). The hormones secreted upon feeding or fasting are the main phase entraining factors for the peripheral clocks (Lamia et al., 2008). Circadian oscillation in the peripheral cells gives rhythmicity to mRNA expression of about 10% of the whole genome in each specific tissue (Mukherji et al., 2020; Panda et al., 2002).

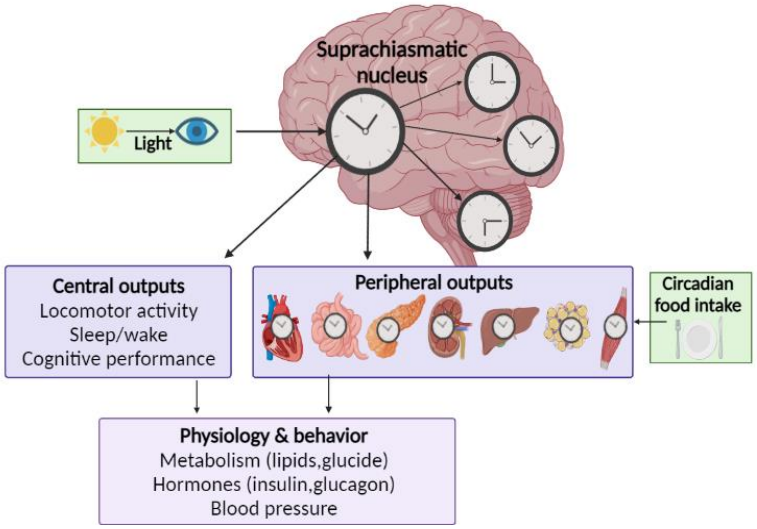


Figure 12: Entrainment of the circadian rhythm mechanism by light and food. External cues such as light entrain the central clock in the suprachiasmatic nucleus (SCN) in the brain. Through neuro-hormonal outputs, the SCN is capable of entraining clocks located in peripheral tissues including liver, muscle, heart, kidney, intestine, adipose tissue, and the muscle. In addition, food intake can entrain peripheral circadian clocks. Collectively, metabolic signals induced by rhythmic food consumption together with hormonal and neuronal signals from the SCN synchronize clocks in peripheral organs with the clock in SCN. Created with Biorender.com.

6.3 Molecular mechanism of the mammalian circadian clock

6.3.1 The core molecular oscillator

The discovery of the core clock gene mechanism has allowed for in-depth analyses of circadian rhythms. The intracellular mechanism of oscillation is a transcriptional–translational feedback loop (Figure 13). In broad terms, activating transcription factors promote the expression of genes whose products feedback inhibits their own production, forming a feedback loop with a cycle length of approximately 24 h. The activator elements of the CC are brain muscle arnt-like protein-1 (BMAL1) and circadian locomotor receptor cycles output kaput (CLOCK) (Takahashi et al., 2008). The transcriptional activator complex consists of heterodimers containing basic helix-loop-helix (bHLH) and Per-ARNT-SIM (PAS) (Sato et al., 2012) . The bHLH-PAS proteins CLOCK and BMAL1 form a heterodimeric complex that is central to circadian oscillator function and called the core oscillator. Takahashi and co-workers observed in *clock* mutant mice a lengthened circadian period (free-running period) with gradual loss of circadian rhythmicity (Takahashi et al., 2008; Vitaterna et al., 1994). *Bmal1* mutant mice exhibit a loss of endogenous circadian rhythms (Bunger et al., 2000), indicating a key role of BMAL1 and CLOCK proteins in circadian rhythm generation. The CLOCK protein is a histone acetyltransferase whose activity at target promoters depends on binding with its heterodimeric partner BMAL1. Together, the BMAL1: CLOCK complex binds to E-box (CACGTG) sequences in target promoters and drives transcription of the period (*Per* 1,2,3) and cryptochrome genes (*Cry* 1,2). *Per1* and *Per2* mutant mice exhibit a shorter circadian period and gradually lose circadian rhythmicity (Zheng et

al., 1999). In the absence of both cryptochromes (*Cry1*-/-, *Cry2*-/-), a complete and immediate loss of rhythmicity is reported (Van Der Horst et al., 1999). A direct role of *Cry* and *Per* in the circadian clock mechanism was subsequently demonstrated as PER/CRY proteins translocate back to the nucleus following posttranslational modifications, including phosphorylation by Casein Kinase where they actively repress the activity of the BMAL1:CLOCK complex and suppress their own transcription (Griffin et al., 1999; Kume et al., 1999). PER:CRY complexes are phosphorylated mainly by casein kinase 1 delta (CK1δ), and epsilon (CK1ε), and are degraded by 26S proteasome complexes, which allows reactivation of CLOCK:BMAL1-driven transcription (Bass, 2011). Robust diurnal oscillations of the clock genes *Bmal1* and *Per 2* and the clock-controlled gene D-site of albumin promoter binding protein (DBP). The delay in nuclear translocation of the repressor complex, controlled by parallel phosphorylation and protein degradation pathways, facilitates the roughly 24 h timing of the molecular oscillations (Sato et al., 2012). In addition to the core loop, a secondary loop of interlocking negative and positive transcriptional regulators including the repressor REV-ERB (NR1D1, nuclear receptor subfamily 1 group D member 1) and the enhancer retinoic acid-like orphan receptor (ROR) provides stability to the molecular timing system by regulating *Bmal1* and *Clock* gene expression. REV-ERB is a negative regulator of *Bmal1* and represses its expression through binding to ROR responsive elements (ROREs) that are in the promoters of these genes (Lowrey & Takahashi, 2004; Preitner et al., 2002). ROR was identified as a positive regulator of *Bmal1* gene expression in an *in vitro* luciferase screen of *Bmal1* transcriptional activators (Sato et al., 2004). Several other CC genes can be mentioned such as E4BP4, Dec1, and Dec2 but their roles have not been clearly specified (Lowrey & Takahashi, 2004; Takahashi et al., 2008). Collectively, the cycling of these feedback loops provides a circadian mechanism by which the molecular CC mechanism can keep cellular time and regulate gene expression over 24 hours.

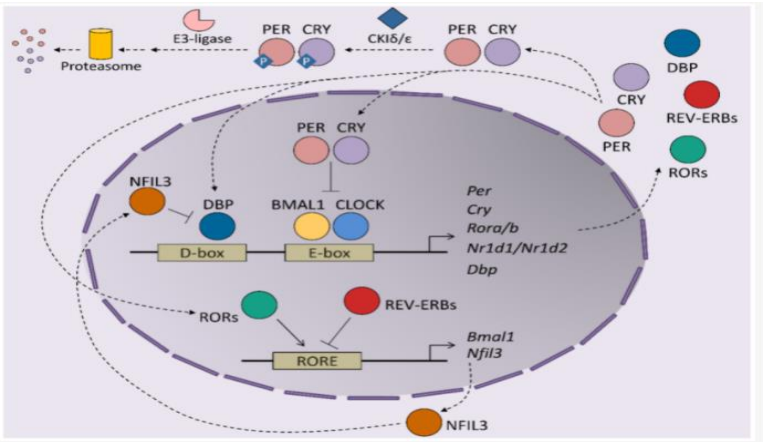


Figure 13: The molecular circadian mechanism. The molecular CC mechanism compromises transcriptional-translational feedback loops that control 24 h rhythms in clock genes and other clock-controlled genes. At the core of the clock, the mechanism is the BMAL1/CLOCK heterodimer that activates transcription of several clock genes including components that compose the negative feedback loops (REV-ERB, CRY, and PER), the positive feedback loop (ROR), and the transcription factors that regulate *Bmal1* expression (ROR and REV-ERB). Figure adapted from (Crespo et al., 2021).

6.3.2 Peripheral organs clocks

Clock genes regulate the timing of clock-controlled outputs or clock-controlled genes (CCGs) that comprise over 10 % of a tissue ‘s transcriptome. In mammals, the timing system has been described as a multi-oscillator hierarchy of coordinated and synchronized cell and tissue clocks (Figure 13, (Rudic et al., 2004)). At the top of this hierarchy is the central pacemaker located in the SCN of the hypothalamus. The molecular clock regulates the timing of gene expression and physiology in the SCN as well as peripheral tissues such as the liver, lung, and skeletal muscle (Yamazaki et al., 2000; Yoo et al., 2004). Isolated peripheral clocks can self-sustain a circadian periodicity up to 20 cycles; therefore, peripheral organs express at least a partially self-working circadian pacemaker.The entrainment of synchronization among central and peripheral clocks (e.g., SCN, liver, pituitary gland, etc.) is a fundamental property of physiological homeostasis and a defining feature of the timing system (Bass, 2011; Eckel-Mahan et al., 2013). Importantly, studies have identified three ways of synchronization of peripheral oscillations (i) direct SCN entrainment by neural and hormonal signals secretion driven by neuroendocrine and endocrine tissues (Albrecht, 2012; Eckel-Mahan et al., 2013); (ii) feeding-fasting rhythms entrainment; (iii) body temperature entrainment. A decline in the CC organization, referred to as circadian misalignment or chrono-disruption (as seen during chronic jet lag, rotating shift work, etc.), can lead to numerous health issues including prediabetes, cancer, and cardiovascular disease (Ruger & Scheer, 2009; Willison et al., 2013).

7. Circadian transcription and gene regulation

Several groups have performed transcriptome-wide studies to detect circadian transcription, using mRNA microarrays, RNA-seq to study circadian regulation of gene expression for 24 hours in mouse liver (Table 3). Remarkably, 2-20 percent of transcripts undergo circadian oscillation in the liver, including enzymes and regulators of major metabolic processes (Panda et al., 2002). Of note, this percentage depends mainly on diverse criteria, such as the set of genes (all the genes of the organism or only the expressed genes in each condition), experimental conditions, such as feeding regimen and Light-Dark (LD), or Dark-Dark (DD) and temporal resolution.

Table 3: List of circadian transcriptome studies in mouse liver based on a collection of techniques. About 2-20% of transcripts undergo circadian oscillation in the liver.

Methods	Rhythmic genes	References
Microarray	335	(Panda et al., 2002)
Microarray	3667	(Hughes et al., 2009)
Microarray	2997	(Vollmers et al., 2009)
RNA-seq	1160	(Vollmers et al., 2009)
Microarray	2828	(K. L. Eckel-Mahan, 2012)
PoI II ChIP-Seq	892	(Le Martelot et al., 2012)
Microarray	885	(Le Martelot et al., 2012)
RNA-Seq	1204	(Menet et al., 2012)
RNA-Seq and Microarray	3186	(R. Zhang et al., 2014)
GRO-Seq	1261	(Fang et al., 2014)
RNA-Seq	4544	(Atger et al., 2015)

Panda and colleagues found about 650 genes were oscillating in the liver and the SCN of the hypothalamus, while the overlap between the two sets of genes was quite low with only 27 genes. Importantly, nutrient metabolism, in the liver, seems to be an important target of circadian regulation. Furthermore, genes related to the metabolism of sugar were expressed during feeding in the early evening, and enzymes for cholesterol biosynthesis peaking at night when nutrients are absorbed. Consequently, so-called "rate-limiting" steps were shown to be under circadian control (Panda et al., 2002). Collectively, these data suggested that the core clock mechanisms, as well as the feeding cues, regulate these circadian patterns of gene expression.

Another study by Hughes and co-workers compared oscillating transcription from mouse liver, NIH3T3, and U2OS cells (Hughes et al., 2009). The authors observed two clusters of genes that cycle with a period of 12 hours and 8 hours in the liver, but not cultured cells. They found that 12-hour oscillatory transcripts occur in several other peripheral tissues such as the kidney, the heart, and lungs and they reported that these harmonics are lost in both cell cultures and under restricted feeding conditions. Vollmers et al. studied the contribution of the endogenous oscillator and the food intake (Vollmers et al., 2009) using distinct feeding and fasting experimental conditions on WT and circadian clock-deficient mice. They founded that in the absence of feeding, only a small subset of transcripts continued to exert circadian patterns. Conversely, they notice that temporally restricted feeding restored rhythmic transcription of several genes in oscillator deficient mouse liver. They concluded that both the temporal pattern of food intake and the circadian clock drive rhythmic transcription.

Recently, Eckel-Mahan and co-authors compared the hepatic circadian transcriptome with a comprehensive dataset of over 500 metabolites identified by mass spectrometry (Eckel-Mahan, 2012). They observed that many metabolites depict clock-controlled oscillation, including those within the amino acid and carbohydrate metabolic pathways as well as the lipid, nucleotide, and xenobiotic metabolic pathways. Menet and collaborators have investigated rhythmic transcription by quantifying genome-wide nascent mRNA produced around the clock in mouse liver (Menet et al., 2012). The authors compared nascent mRNA with the amount of mRNA expressed and founded that many genes exhibit rhythmic mRNA expression in the mouse liver. They conclude that post-transcriptional regulation must have a critical role in the circadian system. Using a sequencing called Global Run-On Sequencing (GRO-seq), Fang and co-workers studied the underlying mechanisms of multiple phases of gene expression in the liver (Fang et al., 2014). The authors have identified a functional circadian enhancer driven by distinct transcription factors (TFs), such as D-box, E-box, and RORE, that function to control circadian gene expression.

Another remarkable study was performed by Zhang and collaborators to elucidate the role of the CC in mouse physiology and behavior (Zhang et al., 2014). The authors performed RNA-seq and mRNA microarray to quantify the transcriptomes of 12 mouse organs around the clock. They reported that 43% of all protein-coding genes showed circadian rhythms in transcription one or more tissue, but mainly in an organ-specific fashion. In most organs, the authors reported the characteristically bi-modal phase distribution with many oscillating genes peaking during transcriptional "rush hours" preceding dawn and dusk (as in (Menet et al., 2012; Vollmers et al., 2009). Importantly, the authors claim that most drugs and essential medicines defined by the World health organization (WHO) directly target the products of rhythmic genes which may represent a turning-point for advancement in chronotherapy.

In addition, Atger and colleagues measured transcription, accumulation, and translation, of mouse liver mRNAs under light-dark conditions and ad libitum or night-restricted feeding in WT and *Bmal1*-KO animals (Atger et al., 2015). The authors reported that rhythmic transcription mostly leads to rhythmic mRNA accumulation and translation. Interestingly, a comparison of WT and *Bmal1*-KO mice confirmed that circadian clock and feeding rhythms have a large effect on rhythmic gene expression, *Bmal1* deletion affecting both transcriptional and post-transcriptional levels unexpectedly.

8. Circadian clock and metabolism: a strong relationship

8.1 Impaired metabolic phenotypes associated with mutations of core clock genes

Liver-specific deletion of *Bmal1* disrupts the rhythm of glucose metabolism and causes a low fasting glucose level (Lamia et al., 2008) (Table 4). Importantly, an impressive study, performed by Shimba and colleagues confirmed that BMAL1 is an essential regulator of adipogenesis and lipid metabolism in matured adipocytes (Shimba et al., 2011). In addition, *Bmal1*-KO (*Bmal1*$^{-/-}$) mice show reduced fat storage, increased circulating fatty acid, and increased ectopic fat formation in the liver and muscles. Most of the genes implicated in adipocyte function (e.g., *Pparγ*, *C/ebpα*, *SrebpP1c*, and *lipin1*) are expressed at a low level in *Bmal1* $^{-/-}$mice. The impaired adipogenesis in *Bmal1* $^{-/-}$ mice prevents the increase of the adipose tissue upon nutrient excess and thus leads to ectopic accumulation of fat in the liver and muscles. Intriguingly, Zhang's lab confirmed that the differences in adipose tissue size appear in adults but not juveniles of *Bmal1*$^{-/-}$ mice, linking a functional clock to aging. In addition, hepatic overexpression of *Cry1* inhibits gluconeogenesis and reduces blood glucose levels (Zhang et al., 2014), whereas a whole-body double knockout of *Cry1* and *Cry2* leads to Glucose intolerance and constitutively high levels of circulating corticosterone (Lamia et al., 2011).Several other clock mutants depict perturbed metabolic phenotypes. More intriguingly the whole-body knockout of *Pparγ*, a metabolic regulator, exhibits an abnormal circadian pattern of gene expression, suggesting a feedback mechanism of the metabolism to the clock through PPAR. In summary, genetic disruption of CC genes *in vivo* perturbs metabolic functions of specific tissues at distinct phases of the diurnal cycle.

Table 4: Metabolic phenotypes associated with mutations of the key components of the CC in mice. Reproduced from (Li et al., 2012)

Protein	Mutation	Metabolic phenotype	References
CLOCK	Whole-body loss-of-function	Obesity, hyperlipidemia, hyperglycemia, hypoinsulinemia, reduced muscle strength, endothelial dysfunction, hepatic steatosis,	(Marcheva et al., 2010; Turek et al., 2005)
BMAL1	Whole-body knockout Liver-specific knockout	loss of glucose and triglyceride oscillations, increased insulin sensitivity, hypoinsulinemia, increased vascular stiffness, thrombosis, endothelial dysfunction, age-associated dilated cardiomyopathy, reduced muscle strength	(Andrews et al., 2010; Lamia et al., 2008; Marcheva et al., 2010; Shimba et al., 2011)
CRY1/CRY2	Whole-body double knockout	Salt-sensitive hypertension, Glucose intolerance and high levels of circulating corticorsterone	(Lamia et al., 2011)
REVERBα REVERBβ	Whole-body double knockout	Increased glucose and triglycerides, reduced circulating fatty acids, reduced respiratory exchange ratio	(Ripperger & Albrecht, 2012)
PER1/PER2	Whole-body double knockout	Impaired glucocorticoid rhythm, Aortic endothelial dysfunction	(Yamazaki et al., 2000)

8.2 The circadian clock controls the whole-body metabolism

The first reports of circadian transcriptomics showed that ~10% of the transcripts display 24 h oscillations through the body, (Lamia et al., 2008; Panda et al., 2002), and for circadian metabolomics can reach until 20% (Dallmann et al., 2012; Eckel-Mahan, 2012). CC affect nearly all facets of our physiology and behavior, including rest–wake cycle, cardiovascular activity, hormone secretion, body temperature, and metabolism (Figure 14). Importantly, the CC controls metabolic homeostasis in the body through a complex interplay between (i) glucocorticoid hormones, which coverts sugars, FFAs, simple carbohydrates into glucose, (ii) insulin, contributing to the regulation of glucose level, (iii) appetite hormones, governing, food intake. Importantly, it has been suggested that different aspects of adipocyte functions depend on the CC (Henriksson & Lamia, 2015), as showed in adipocyte-specific clock-deficient mice present an increase of adipokines and triglycerides levels (Paschos et al., 2012). In terms of regulation of glucose homeostasis, *Bmal1* null mice exhibited decreased glucose tolerance (Rudic et al., 2004) while *Clock*$^{\Delta 19}$ mutant mice display an increased lipid tolerance, as indicated by decreased triglyceride in circulation following an acute lipid load (Oishi et al., 2006). Altogether, these data indicate that disruption of circadian clocks alters both adipocyte and glucose/lipid tolerance metabolic homeostasis. Furthermore, increasing evidence suggests circadian control of insulin secretion through pancreatic β-cell. Importantly, β-cell-specific *Bmal1* null mice exhibit impaired insulin secretion (Perelis et al., 2015). Collectively, these studies strongly support the concept that CC controls and influences metabolic homeostasis at the whole-body level.

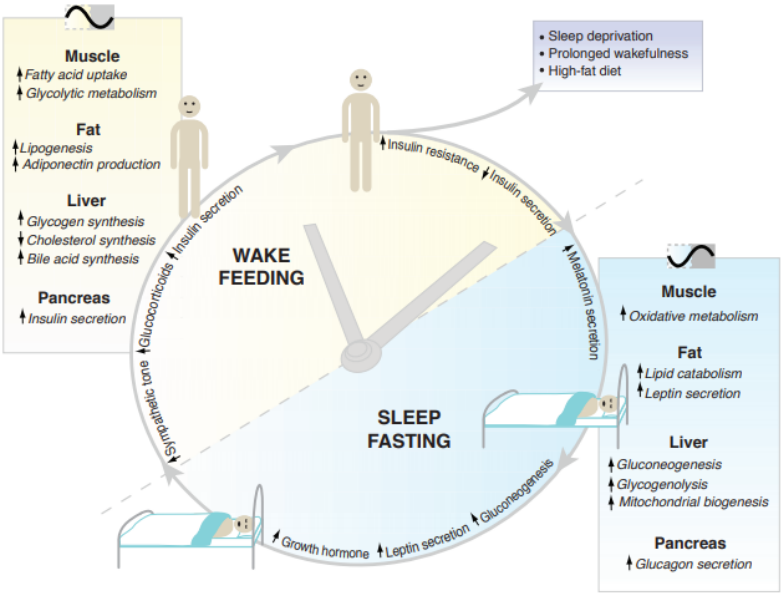


Figure 14: **The circadian clock controls metabolic responses within peripheral tissues over the diurnal cycle**. The liver clock promotes gluconeogenesis and glycogenolysis during the sleep/fasting cycle, while it promotes glycogen and cholesterol synthesis during the wake/feeding cycle. Correct functioning of peripheral clocks keeps metabolic processes in synchrony with the environment, which is necessary for maintaining the health of the organism. In addition, clock disturbance in tissues provokes opposing effects on the metabolic function as revealed by changes in nutrient conditions. Diet, aging, and environmental perturbations such as shift work may also impact the integration of circadian and metabolic systems. Figure adapted from (Bass & Takahashi, 2010).

Key metabolic factors like AMPK, sirtuin 1 (SIRT1), PPARα, and Peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC1α) act as important regulators for core circadian mechanisms (Figure 15). AMPK, which is a nutrient sensor, plays a role in the destabilization of the CRY protein in the core of the circadian system (Lamia et al., 2009). *Sirt1* is related to anti-aging and is regulated by NAD+. Furthermore, *Sirt1* regulates the HAT activity of the CLOCK protein and promotes deacetylation and degradation of PER2 (Belden & Dunlap, 2008). PPARα, which is a nuclear receptor for lipid metabolism in the liver, binds with PER2 (Zheng et al., 1999) and increases *Bmal1* expression through a PPAR response element in the promoter of *Bmal1* (Canaple et al., 2006).

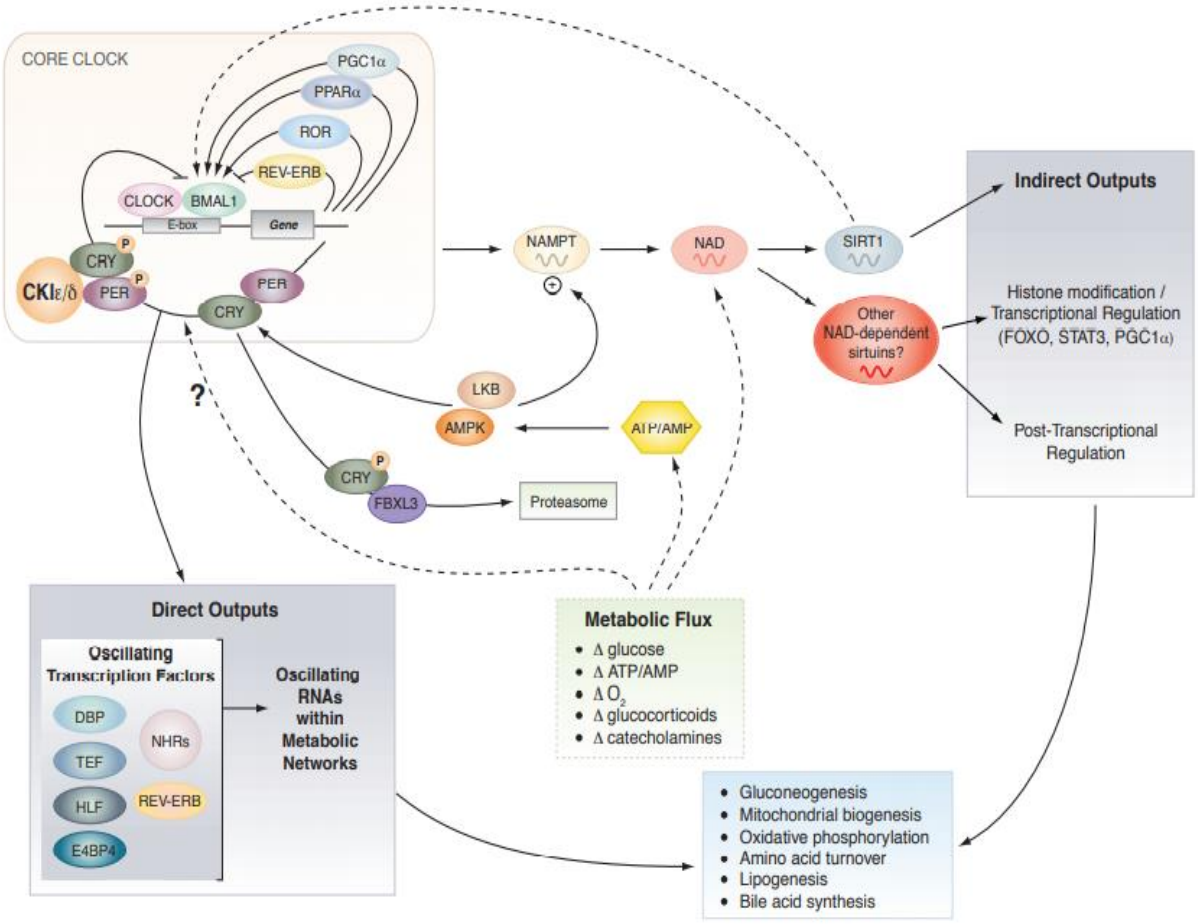


Figure 15: Main outputs of the core clock mechanism. The circadian clock oscillator compromises several series of transcription/translation feedback loops that synchronize several metabolic processes through both direct and indirect outputs, including gluconeogenesis and oxidative metabolism. In addition, the clock receives reciprocal input from nutrient signaling pathways (including SIRT1 and AMPK), which function as rheostats to couple circadian cycles to metabolic flux, especially in peripheral tissues. Figure adapted from (Bass & Takahashi, 2010).

8.3 Rhythms in liver metabolism

The liver is one of the main targets regulating several metabolic parameters such as glucose levels, lipid, bile acid and physiological functions (Li et al., 2012). Of note, this organ exhibit robust oscillations in circadian output genes as well as in hepatic genes (Akhtar et al., 2002). Genome-wide gene expression studies displays the first important insights into the crucial role of the CC and its implication in regulating the liver functions (Miller et al., 2007; Mukherji et al., 2019; Panda et al., 2002). Moreover, recent studies shows a more integral mechanism for the implication of circadian rhythm in orchestrating and regulating liver metabolism and physiology (Mauvoisin et al., 2014; Mukherji et al., 2020).

8.3.1 Glucose metabolism

The glucose production and uptake in the liver as well as insulin and glucagon secretion are the central pathways by which nutrient homeostasis is maintained over daily periods of feeding and fasting (Berg et al., 2002). The CC in the SCN control the rest/activity cycle and feeding behavior, while the liver clock generates rhythms of glucose homeostasis through the day/night cycle. Of note, the SCN plays a crucial role in the maintenance of glucose tolerance as well as plasma glucose concentration that displays circadian rhythmicity, as SCN-lesioned rats failed to produce glucose concentration rhythms in response to scheduled feeding (La Fleur et al., 2001). Recently, Chaves and colleagues, have suggested a circadian clock regulation of insulin. In turn, insulin regulates the activity of forkhead Box O (FOXO), which is the TFs of CLOCK, indicating the insulin-FOXO3-CLOCK signaling pathway is critical for the modulation of circadian rhythms (Chaves et al., 2014).

Importantly, liver-specific *Bmal1*-KO mice have disrupted circadian function within hepatocytes, in addition to decreased expression of hepatic glucose transporter 2 (GLUT2) in the liver and subsequently, reduction in glycogenolysis (Lamia et al., 2008). Moreover, the loss of BMAL1 function in the whole body resulted in increased glucose tolerance and weight gain (Rudic et al., 2004). This may indicate that liver-specific disturbance of the circadian clock alters glucose signaling that may be masked in the body of *Bmal1* knockout mice, which leads to additional impairment in insulin secretion. Interestingly, another study showed an important role of CRY1 in the regulation of hepatic gluconeogenesis through inhibition of the G protein of adenylate cyclase activity which stimulates the transcription of genes to regulate by cAMP response element-binding protein (CREB) (Asher et al., 2008). *In vivo* investigations found overexpression of *Cry1* in diabetic mice livers which lead to decreased levels of glucose. On another hand, *Crys* inhibits the transcription of genes encoding the glucocorticoid receptor, which are involved in glucose metabolism (Asher et al., 2008). Collectively, CC-dependent regulation of glucose metabolism is controlled by positive and negative regulators of the core CC oscillator (Hatori et al., 2012).

8.3.2 Control of lipid and bile acid metabolism

Perturbation of lipid homeostasis in the liver is involved in the development of obesity-related metabolic diseases, such as hyperlipidemia, insulin resistance, coronary artery disease, NAFLD, and diabetes. Recently, molecular mechanisms of how CC regulate lipid metabolism have been studied extensively. Circadian lipid metabolism is controlled by the clock-dependent regulation of key enzymes and transcription factors. Among others, adiponectin, which recently has been showed to be activated by BMAL1 and CLOCK through the transcriptional activity of PPARγ and its co-activator PGC1α (Barnea et al., 2010). Importantly, the expression of lipid metabolism genes is altered in mice with disruption of the CC which leads to an accumulation of hepatic triglyceride levels in the liver (Eckel-Mahan, 2012).Similarly, *Per2*-KO mice have developed dyslipidemia (Grimaldi et al., 2010). Additionally, REV-ERBα is highly coordinated for the regulation of both CC and metabolic functions (Fang et al., 2014), accordingly, *Rev-erbα*-KO mice perturbs the CC gene expression as well as lipid homeostatic gene networks and exhibit fatty liver disease (Cho et al., 2012). Synthesis of bile acids accounts is regulated by both feedback loop involving FXR, FGF15, and SHP (Lu et al., 2000) and the circadian clock to control cholesterol and lipid homeostasis. Importantly, the core clock protein, NR1D1, promotes circadian signaling via Sterol regulatory element binding transcription factor (SREBP), which further mediates rhythmic expression of the rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7α1). Remarkably, studies in *Per1* mutant mice exhibit elevated levels of serum bile acids and hepatic enzymes levels which resulted in liver damage (Wood et al., 2009). Taken together, the crucial role of bile acids in maintaining glucose and cholesterol homeostasis may contribute to the hyperlipidemic observed in shift workers.

9. Circadian clock disruption and liver disease

9.1 Relationship between NASH and the circadian clock in mice studies

Due to the difficulty of evaluating the pervasive impact of CC disruption in humans, animal models have indicated important and crucial findings regarding the impact of the CC disturbance. Several *in vivo* data have showed a correlation between CC genes and metabolic disorders including NASH, with strong evidence indicating that CC disruption may lead to the whole spectrum of NAFLD (Figure 16). For example, Mwangi and co-workers have found in the mouse model of HFD, mice become obese, acquire insulin resistance, and develop hepatic steatosis (Mwangi et al., 2014).In addition, *Clock*-KO mice display hepatic steatosis when fed a standard diet, and they develop a more severe phenotype when given an HFD. Importantly, this study indicate that the *Clock* gene plays a crucial role in metabolic homeostasis and is potentially protective against NAFLD development (Turek

et al., 2005). More importantly, HFD mice have been showed to be associated with a reduced recruitment of BMAL1 and CLOCK to the promoters of CCGs, which subsequently dampen the rhythmic pattern of circadian genes (Yanagihara et al., 2006). *Pparα* is a target gene of the CLOCK: BMAL1 heterodimer and upregulates the expression of *Bmal1* in peripheral tissue (Canaple et al., 2006). Importantly, in *Pparα*-KO mice, *Bmal1* mRNA expression was maintained in the SCN but decreased in the liver compared with WT mice, suggesting its role in the peripheral tissue (Canaple et al., 2006). In addition, Stienstra and co-workers observed increased hepatic steatosis and steatohepatitis in *Pparα*-KO mice fed with HFD in comparison with WT mice (Stienstra et al., 2007). More importantly, studies have found that the hepatocyte *Pparα* deletion impaired fatty acid catabolism, resulting in hepatic lipid accumulation during fasting and in two preclinical models of steatosis (Montagner et al., 2016). Furthermore, in chronic jet lag conditions, the WT mice developed insulin resistance, obesity, high body fat composition, hepatomegaly with increased triglycerides, FFA, hepatic inflammation, and liver fibrosis. Thus, CC perturbance by jet lag led to not only steatosis but also steatohepatitis with fibrosis (Kettner et al., 2015, 2016). However, observational studies in humans were less clear in terms of any potential association between sleep disruption and NAFLD (Shetty et al., 2018). Future human studies should adjust for known risk factors of NASH and rule out patients with baseline sleep disorders. This may provide useful information about the effects of long-term circadian disruption caused by environmental factors on the development of NASH. In summary, a more profound knowledge about the influence of clock genes on metabolic homeostasis may boost novel concepts for the treatment of NASH and the prevention of associated complications like HCC.

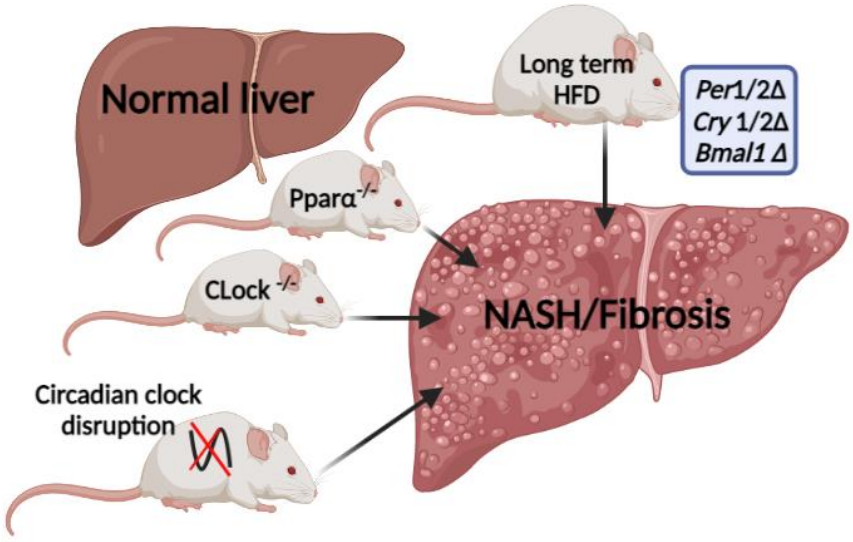


Figure 16: Circadian clock and pathogenesis of NASH in mice. Mice knockout models of *Clock* and *Pparα* increase the risk of NASH, while environmental factors such as circadian disruption (i.e., chronic jet lag or night shift work pattern) and dietary indiscretion or excess (such as the HFD) lead to adiposity and increase the risk of NASH. Figure modified from (Shetty et al., 2018). Created with Biorender.com.

9.2 Circadian rhythm in fatty liver

Researchers have linked the CC to the proper functioning of our metabolic machine. The CC is involved in the regulation of hepatic TG accumulation, inflammation oxidative stress, and mitochondrial dysfunction (Adamovich et al., 2017) which contributes to the pathogenesis of NAFLD. Several research groups highlighted that multiple genetic variant of clock genes are associated with hepatic steatosis (Vinciguerra et al., 2014). Importantly, the 3111T/C single nucleotide polymorphism (SNP) in the *CLOCK* gene (rs1801260) was associated with being overweight and a high risk of hepatic steatosis in humans (Bandín et al., 2013). Additionally, results have shown a significant correlation between fibrosis development and the SNPs since HSC might be controlled through a CLOCK-driven PPARγ and SREBP1-mediated mechanism (Sookoian et al., 2007). Of note, *CLOCK* gene variants and related haplotypes have been associated with susceptibility to NAFLD and disease progression in humans (Sookoian et al., 2007). As the CC controls most metabolic pathways in the liver, mice with mutations in clock genes develop more severe hepatic steatosis under both regular and high-fat chow feeding conditions than WT mice (Vinciguerra et al., 2014). Indeed, *Clock*$^{\Delta 19/\Delta 19}$, *Ppara*-KO and liver-specific *Rev-erbα* mice all develop hepatic steatosis (Abdelmegeed et al., 2011; Cho et al., 2012; Turek et al., 2005). Interestingly, *Nocturin*-KO mice develop hepatic steatosis compared with WT control mice (Green et al., 2007). Importantly, the fasting-induced elevation of *RORα* levels transcriptionally activates sterol 12α-hydroxylase and increases liver cholesterol, leading to hepatic steatosis (Pathak et al., 2013). Altogether, these studies show that CC impairment results in the development of liver disease.

9.3 Circadian rhythm in liver injury and fibrosis

Increasing evidence supports an association of CC disruption with hepatic liver injury since *Per2*-KO mice predispose liver fibrosis by increasing HSCs activation and proliferation (Chen et al., 2013). Additionally, the same study has found an elevated expression of key genes involved in fibrogenesis such as *Tgfβ*, *Tnfα*, and *Timp1* in *Per2*-KO mice that have undergone carbon-tetrachloride (CCl_4) treatment (Chen et al., 2013). Furthermore, studies have revealed that the expression of TGFβ/SMADs signaling pathways follow a circadian profile as indicated by the circadian expression of *Smad3*, which depending on CLOCK:BMAL1 heterodimer (Sato et al., 2012). Interestingly, these data provide an evidence association between CC gene expression and liver fibrogenesis. Interestingly, fundings have confirmed that the expression of *Rev-erbα* was associated with activated HSCs, hepatic steatosis, and liver injury (Li et al., 2014; Thomes et al., 2016). Another study, has found that *Rev-erbα*-KO mice have substantial hepatic steatosis (Feng et al., 2011)

9.4 Circadian rhythm and liver cirrhosis

Recent observations indicate a possible pathogenic link between CC disruption and cirrhosis. Liver cirrhosis was reported to promote portal hypertension and dysfunctional CC systems, characterized by a delayed sleep-wake cycle, changes in circulating melatonin, and cortisol and daytime sleepiness (Montagnese et al., 2014). Importantly, patients with cirrhosis experience abnormal day-night rhythms of fibrinolysis, hepatic catabolism of melatonin, and high arterial blood pressure (De Cruz et al., 2012). Further *in vivo* studies indicated that in mice with CCl_4-induced acute liver fibrosis resulted in abnormal expression rhythms of *Cry2*, *Pparα*, and *Ror* (Chen et al., 2010). Interestingly, *Per2*-KO mice indicated more severe liver fibrosis after carbon tetrachloride injection compared with wild-type controls (Chen et al., 2010). CCl_4-activated HSC transdifferentiate to fibrogenic HLMF-like cells, leading to liver fibrosis and cirrhosis. The activation of nuclear receptors such as PPARγ and REV-ERBα inhibits activated HSCs (Li et al., 2014). Collectively, these interactions occur between liver fibrosis and the CC system, suggesting that the CC could be relevant targets for the development of new therapeutic approaches in chronic liver disease. In addition, cirrhosis is caused by the accumulation of bile acid in the liver, a process called cholestasis. *Per1/Per2*-KO mice displayed high levels of bile acids in both serum and liver and deregulation in bile acids synthesis and in key transport genes, including *Cyp7a1*. *Per2*-KO mice developed more severe cholestasis, more extensive bile infarcts, and increased ECM deposition and fibrosis-related gene expression than WT control undergoing the same procedure (Chen et al., 2013). Remarkably is that once these rats were kept in continuous darkness, the resulted increase in melatonin synthesis reduced hepatic steatosis and improved the liver function (Zhang et al., 2018).

9.5 Circadian rhythms in liver cancer

Circadian machinery was suggested to exert tight control on cell cycle genes, cell proliferation genes, oncogenes, and tumor suppressor genes, including *Myc* and *Cyclin D* (Feillet et al., 2015) (Figure 17). *Per2*-KO mice, *Bmal1*-KO mice, *Cry1*-KO and *Cry2*-KO mice, exhibit increased spontaneous and radiation-induced tumorigenesis compared with wild-type mice (Fu et al., 2002; Lee et al., 2010). Mice under simulated chronic jet lag easily diethylnitrosamine (DEN)-induced liver cancer (Filipski et al., 2009). The abnormal expression of core clock genes was found in HCC biopsy tissue samples with or without hypoxia and correlated with increased tumor size and increased levels of long non-coding RNA (Cui et al., 2015; Furutani et al., 2015; Lin et al., 2008). In mice, disrupted rhythms of *Clock* gene expression were reported in colorectal liver metastases development (Lin et al., 2008). Furthermore, the low oscillation amplitude of *Per1* expression and reduced food entrainment were shown identified in DEN-induced HCC in mice (Davidson et al., 2006). Likewise, a functional SNP in the *Per3* gene (rs2640908) is correlated with increased overall survival in HCC patients treated with transcatheter arterial chemoembolization (TACE) (Zhao et al., 2012).

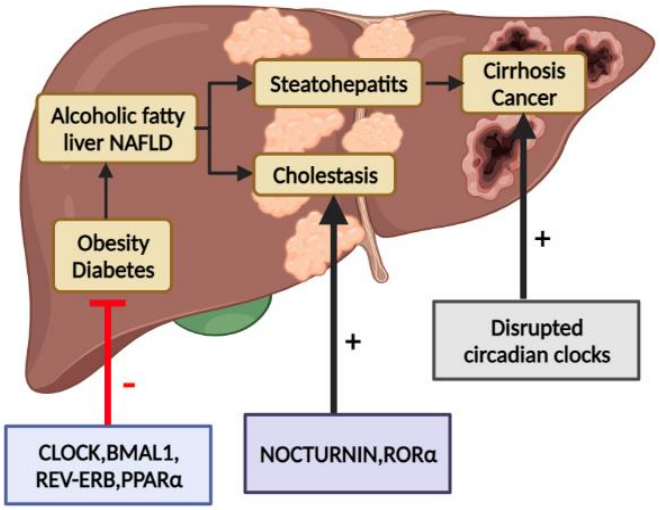


Figure 17: Circadian machinery in liver disease. Disturbance of hepatic circadian clock is closely associated with liver disease. For example, mice with knockout of *Rev-erbα*, *Rev-erbβ*, *Pparα*, or mutation in *Clock* develop hepatic steatosis. Figure modified from (Tahara & Shibata, 2016). Created with Biorender.com.

10. The emerging field of chrono-pharmacology

Emerging evidence indicated the advantage of a profound understanding of human CC coordination between several organs in pharmacology (Ohdo et al., 2019). Chrono-pharmacology is a branch of chrono-therapy that applies the principles of circadian rhythm to determine the optimal timing of drug administration, which can control metabolism and excretion of administrated xenobiotics (Ohdo et al., 2019). Physiological parameters, such as hormone levels, gastric pH, liver enzymatic activity, and hepatic and renal blood flow, show a circadian rhythm; hence drug efficacy will be time-dependent (Ferrell & Chiang, 2015). Chrono-pharmacology is currently utilized in several pathologies such as hypertension (Hermida et al., 2011), inflammatory diseases (Haspel et al., 2014; Narasimamurthy et al., 2012), rheumatoid arthritis (Dziurla & Buttgereit, 2008), and cancer (Lévi, 2001; Ortiz-Tudela et al., 2013). Even though several studies emphasized effectiveness of such approaches, more clinical trials will be needed to fully understand their biological and therapeutic potential. Of course, these approaches hold a strong potential as novel therapeutic concept as well targeting of metabolic diseases.

Recently, findings have investigated the beneficial effects of food-intake timing or nutrient composition on the CC, leading to the development of a concept known as chrono-nutrition. This theory is based on evidence that nutrients are potent regulators of the hepatic circadian clock, which in turn regulates many physiological functions (Damiola et al., 2000; Hara et al., 2001) (Figure 18). Importantly, diet-induced insulin secretion can reset liver clocks, whilst oxyntomodulin is involved in resetting peripheral clocks (Tahara et al., 2011; Yamajuku et al., 2012). Both, insulin and oxyntomodulin can upregulate *Per2* expression in mouse liver (Kasukawa et al., 2012; Tahara et al., 2011; Yamajuku et al., 2012). Several studies have shown that the nutritional composition of a food can influence peripheral clock entertainment. For example, starches and fish oil-induced insulin secretion were

reported to enhance peripheral clock entertainment by an insulin signaling mediated mechanism (Furutani et al., 2015; Hirao et al., 2009). Additionally, the duration of fasting alters peripheral clock settings, and food ingestion after long hours of fasting has a greater ability to entrain peripheral clocks than meals after a shorter period of fasting (Hirao et al., 2009; Kuroda et al., 2012). In fact, the time regulation of food intake without caloric restriction was shown to be beneficial in preventing diet-induced obesity in both humans and mice and in reducing obesity caused by a simulated shift work pattern in mice (Barclay et al., 2012; Narasimamurthy et al., 2012). It is worthy to mention, that in humans caffeine lengthens the circadian rhythm and induces a phase shift in the liver clock in a time-dependent manner (Oike et al., 2011). Taken together, studying liver chrono-pharmacology will provide new insights and useful information that helps in the development of treatments for chronic liver diseases.

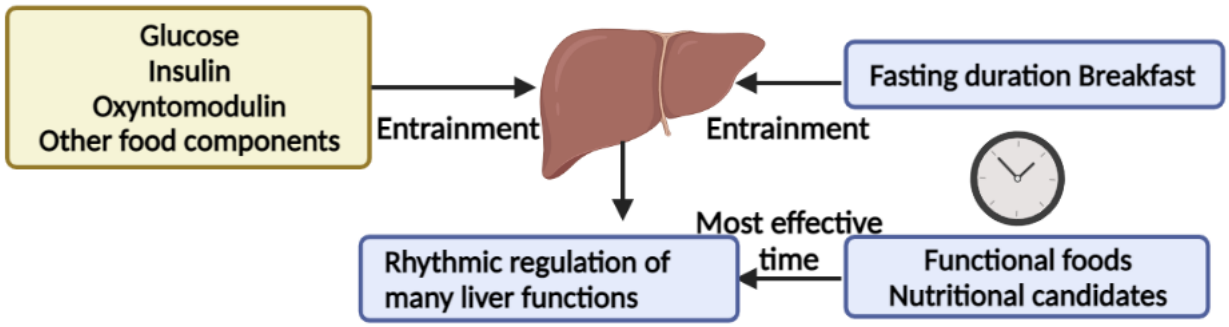


Figure 18: Chrono-nutrition in the liver. The study of the chrono-nutrition necessities characterizing the nutritional candidates that can modify circadian clocks, determining appropriate food timing to maintain peripheral clock's function, and identifies the optimal time to consume functional foods. Figure modified from (Tahara et al., 2011). Created with Biorender.com.

10.1 Therapeutic potential of small-molecule clock modulators

Recently, molecular studies have provided strong evidence that xenobiotic metabolism is related to circadian regulations that may promote an improvement of therapeutic strategies metabolic disease. These observations support the crucial role of circadian dosing time for drug efficacy (Levi & Schibler, 2007). However, clock-modulating molecules can impact the CC system to ameliorates CC-regulated output functions (Kazunari Nohara et al., 2015; Schroeder & Colwell, 2013; Wallach & Kramer, 2015).Several studies have suggested clock-modulating molecules as potential therapeutics for metabolic disease. All compounds target circadian components with established role in metabolic regulation, i.e., REV-ERBs and RORs (Cho et al., 2012; Jetten et al., 2013; Kasukawa et al., 2012; Q. Liu et al., 2021; Solt et al., 2012). Importantly, SR1555 an inverse agonist of RORs was found to reduce body weight and modulates hepatic metabolism in mice (Chang et al., 2015; Kumar et al., 2010). Another clock modulator, KL001 inhibits ubiquitin-dependent degradation of CRY, which results in a lengthening of the circadian period and reduced gluconeogenesis *in vitro* (Hirota et al., 2012). Emerging

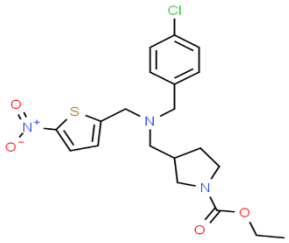
evidence in mice and humans shows a strong relationship between damped metabolism and metabolic disorder indicating that an attenuated oscillations of insulin secretion correlate with an exaggerated diabetes risk (Boden, 1999). In this respect, studies focused on enhancing the clock amplitude. Treatment of diet-induced obese mice with the RORs agonist nobiletin (NOB) enhanced the circadian functions and significantly improved metabolic homeostasis (He et al., 2016; Keiko Nohara et al., 2017). In summary, it is invaluable to identify a target and mechanistic studies for these new molecules to facilitate their therapeutic application, for clock-related diseases.

10.2 REV-ERBs agonists

The REV-ERB receptor has a crucial role in the regulation of CCGs, which in turn regulate metabolic pathways. Importantly, loss-of-function studies showed that REV-ERBs have an important role in lipid metabolism both *in vitro* and *in vivo*. For example, *Rerv-erbα*-KO mice have dyslipidemia with elevated levels of very-low-density lipoprotein (VLDL) triglyceride and increased serum levels of apolipoprotein C3 (APOC3) (Raspé et al., 2002). In addition, *Rev-erbα*-KO mice have increased plasma glucose levels (Cho et al., 2012). Remarkably, *Rev-erbα* expression is highly induced during adipogenesis. More importantly, Delezie and collaborators have shown that *Rev-erbα*-KO mice display increased adiposity and increased weight gain owing to a high-fat diet (Delezie et al., 2012).

Consistent with the diverse effects on metabolism noted in *Rev-erbα*-KO mice, pharmacological re-activation of REV-ERB with SR9009 has a crucial role in the regulation of metabolic pathways *in vivo* (Kojetin & Burris, 2014) . More importantly, the two main synthetic agonists of REV-ERBs, SR9009 and SR9011, significantly improved energy homeostasis and altered circadian behavior in diet-induced obese mice (Solt et al., 2012). Remarkably, the same study showed a decrease in plasma TGs, total cholesterol, and non-esterified fatty acids in the obese mice after treatment. In addition, Woldt and co-workers has shown in mice that SR9009 increases oxidative metabolism and mitochondrial biogenesis in skeletal muscle, resulting in increased exercise endurance (Woldt et al., 2013), which correlates with the metabolic disorders and weight loss observed in the liver (Solt et al., 2012). Furthermore, studies have investigated SR9009 as potential therapeutics for cardio-metabolic diseases including atherosclerosis, which is a common comorbidity with NAFLD/NASH (Pourcet et al., 2018; Trevaskis et al., 2012). Given the several roles of this ligand that modulate REV-ERB activity, SR9009 may hold utility in the treatment of metabolic disorders (Table 5).

Table 5: Synthetic REV-ERBαs ligand SR9009. Modified from (Kojetin & Burris, 2014).

Structure	Role
	• REV-ERBα's agonist. • Suppresses expression genes both *in vitro* and *in vivo* of REV-ERB. • Absence of activity at other nuclear receptors (using Gal4–UAS luciferase specificity panel). • Promotes loss of locomotor activity in the CC cycle after the injection. • Leads to weight loss in mice without affecting food intake. • Improves oxygen consumption and decreases plasma lipids.

OBJECTIVES

Due to its high prevalence and rising incidence, NAFLD is a major cause of liver fibrosis worldwide. NALFD affects 25–30% of the general population and its prevalence could reach 70–90% in specific populations with comorbidities such as obesity or type 2 diabetes (Polyzos et al., 2019; Younossi et al., 2016). While patients with early-stage fibrosis F1 and F2 are usually asymptomatic, advanced fibrosis stages F3–F4 are associated significantly with overall mortality. In fact, the overall mortality has been reported 3-fold greater in patients with F3–F4 fibrosis than in those without liver disease. Therefore, it is the stage of fibrosis and not steatosis that is directly related to overall mortality in patients with NAFLD (Angulo, 2002).The pathogenesis of liver fibrosis is multi-factorial and mediated by several cell types including hepatocytes, macrophages, and myofibroblasts (Ramachandran et al., 2019; Roehlen et al., 2020). Among the pro-fibrogenic pathways, cytokines belonging to the TGFβ family have been shown to play a crucial role (Angulo et al., 2015; Ramachandran et al., 2019). TGFβ signaling is evolutionary conserved and regulates several essential aspects of mammalian biology to maintain homeostasis (Fabregat et al., 2016; Massagué, 2012). However, the molecular mechanisms which switch TGFβ-controlled gene expression from health to fibrotic disease are poorly understood.

The circadian clock (CC) plays a key role in liver metabolism (Mukherji et al., 2019, 2020). At the core of the clock functioning, resides the CC oscillator, an exquisitely crafted transcriptional-translational feedback system (Asher and Schibler, 2011; Asher and Sassone-Corsi, 2015, Mukherji et al., 2019). The components of the CC oscillator not only maintain daily rhythmicity of their own synthesis but also generate circadian-specific variability in the expression levels of numerous target genes through transcriptional, post-transcriptional, and post-translational mechanisms, thus, ensuring homeostasis (Asher & Sassone-Corsi, 2015; Asher & Schibler, 2011; Mukherji et al., 2019). Perturbation of the CC function is associated with diverse pathologies including metabolic diseases and cancer (Mukherji et al., 2020). The liver expresses a diverse set of genes in a circadian manner. Increasing evidence suggests CC alterations play a critical role in liver disease progression. (Mukherji et al., 2020). However, the relationship between the CC-controlled gene expression and fibrosis development in NASH patients is complex and still poorly understood.

Therefore, I aimed at:

- Uncovering the molecular relationship between liver CC and the development of NASH-induced fibrosis.
- Investigating whether at the molecular level NASH- and fibrosis-inducing signals induced by, e.g., FFA or TGF-β deregulate the CC-machinery?
- Evaluating whether restoring the CC-functioning through specific pharmacological compounds could reduce/prevent the development of NASH/fibrotic features in patient-derived models *ex vivo* (spheroids) and *in vivo* (humanized chimeric mouse models).

RESULTS

The results related to the three main aims of this thesis are an integral part of a manuscript that is indicated in the following sections. In addition to project management and manuscript writing as first-author, my individual experimental contributions are highlighted in the respective summary sections prior to the article. Detailed descriptions of the respective material and methods are included at the end of the article.

1. Results summary and own contribution

1.1 The liver CC-oscillator controls the expression of gene networks driving fibrosis (contribution, figures 1f and g)

To investigate the role of the liver CC-oscillator in the regulation of the expression of fibrosis-related genes, the host laboratory performed Bmal1 loss function studies in mice models and discovered that the liver CC-oscillator regulates the TGFβ signaling-dependent expression of fibrotic genes. Interestingly, we have found that the CC-control on TGFβ signaling, in turn, dictates that TGFβ-activated transcriptions factors i.e., SMADs bind to the SBEs present in their cognate genes only for a limited duration in the rest phase. In addition, analysis of the gene expression profile using HALLMARK revealed that most of the fibrosis driving genes are indeed expressed in the circadian rest phase and their expression is lost in the liver of *Bma1*-KO mice. Altogether, our findings demonstrate that the liver CC-oscillator controls the physiological expression (circadian gating) of key genes which drive liver fibrosis in patients.

1.2 Diet-induced liver fibrosis disrupts the liver CC-oscillator in vivo (contribution figures 2c-f)

Next, to evaluate the relationship between the CC and fibrotic genes in the metabolic model for liver fibrosis. The group of Prof. Baumert and collaborators choose the choline-deficient high-fat diet (CD-HFD) which is known to recapitulate key features of human NASH-induced fibrosis. Interestingly, I found that metabolic liver injury impairs the expression of CC components in a time-dependent manner resulting in increased expression of TGFβ-related fibrotic genes. In addition, by using ChIP assays, we discovered that, in contrast to the healthy state, where TGFβ activity is CC-gated, in the disease state the increase of fibrotic gene expression arises from constitutive chromatin binding of transcription factors SMAD2/3 and SMAD4. Collectively, our results show that diet-induced fibrosis breaks the hepatic CC-oscillator with associated loss of its control over the time-restricted expression of TGFβ-regulated and fibrosis-related genes. The diet-induced lack of CC-control leads to a state of constitutive TGFβ/SMAD signaling, which drives fibrosis.

1.3 Metabolic injury in PHH and HLMF impairs the expression of CC-components with concomitant activation of profibrotic genes (major contribution, Article figures 3a-i)

In order to investigate the relationship between metabolic injury and the CC in well-established cell-based models, the host laboratory-processed human liver specimens obtained from resections and treated them with FFA to model metabolic injury. I performed an isolation technique allowing high-throughput isolation and purification of primary human hepatocytes (PHH), human liver myofibroblasts (HLFM), Kupfer cells, and liver sinusoidal endothelial cells (LECs) from resected human liver tissue (Kegel et al., 2016). Using this method, I showed on both transcriptional and protein levels that the treatment of PHH and HLFM with FFA for 3 days, perturbed the expression of CC-components in a time-dependent manner resulting in increased expression of TGFβ-related fibrotic genes. More importantly, in dexamethasone-synchronized PHH, I showed that under conditions of elevated fibrotic-stimuli which disrupts CC-oscillator TGFβ signaling loses temporal regulation. In addition, my analysis on PHH using luciferase reporter assay indicates that FFA-driven increased transcriptional activity of SMAD2/3 (TGFβ targets) and reduces the 'basal' promoter activity of *Bmal1*-Luc. Furthermore, to explore the role of FFA-induced SMAD activation, I performed a knock-down analysis on PHH and the analyses showed that siRNA-mediated knock-down of *SMAD2* prevents both the FFA-induction of SMAD2 target genes and CC-genes, thus confirming the involvement of SMADs in FFA-induced CC-perturbation. Altogether, my analyses suggest that elevated TGFβ signaling directly impairs the expression of key CC-genes in primary human hepatocytes and liver myofibroblasts, unraveling a "cross-talk" between the CC-and TGFβ signaling.

1.4 Rev-ERBα activation impairs TGFβ-signaling in primary human liver (major contribution: all the figures 5 except bioinformatical analysis)

To explore the relationship between TGFβ signaling and REV-ERBα, I performed transcriptional analyses from TGFβ-stimulated PHH and HLMF treated with SR9009 (a specific agonist of REV-ERBα). Interestingly, my analyses demonstrate that in both human liver cells, SR9009-treatment inhibited the activation of fibrotic genes in both PHH and HLMF and restored REV-ERBα levels following TGFβ induction. In addition, by using the immunofluorescence analysis from TGFβ and SR9009-treated HLMF, SR9009 was shown to inhibit αSMA and phosphorylated SMAD2/3 levels. In addition, in a loss-of-function approach, I observed that a reduction in *REV-ERBα* levels is sufficient to increase the expression of fibrotic genes which were further augmented upon TGFβ-stimulation. Furthermore, we performed RNAseq and GSEA analysis from human myofibroblasts treated with FFA and SR9009. Similarly, SR9009-mediated activation of REV-ERBα inhibited FFA-induced activation of fibrotic and inflammatory gene expression. Collectively, these results indicate a reciprocal

relationship between REV-ERBα activity and TGFβ-signaling and provide the mechanism of action of SR900-mediated antifibrotic efficacy.

1.5 Deregulated expression of CC genes is associated with liver fibrosis progression in patients and targeting REV-ERBα reduces fibrosis in patient liver spheroids (contribution: transcripts analysis of ex-vivo models, figures 6e-g)

To evaluate the clinical translatability and impact of our results, the group of Prof. Baumert and collaborators assessed computational analyses of transcript levels for key CC components in liver microarray data of several publicly available patient cohort fibrosis. Interestingly, the results in 2 independent cohorts of patients with NASH indicate a reduction in the expression of CC components compared to healthy controls. Notably, the reduction of CC components observed in these cohorts was significantly associated with the fibrosis stage of disease progression and with lobular inflammation. Next, to confirm the functional relevance of SR9009 as a therapeutic target, the host laboratory assessed SR9009 treatment in several patient-derived *ex-vivo* and 3D *in vitro* models of liver fibrosis. Importantly, the transcript analysis revealed that consistent with our previous observations in different model systems, SR9009 shows a robust improvement of fibrosis and inflammatory markers in patient-derived liver spheroids. Thus, suggesting the clinical translatability of the functional role of CC-machinery in the pathogenesis of liver fibrosis in patients.

2. Graphical overview

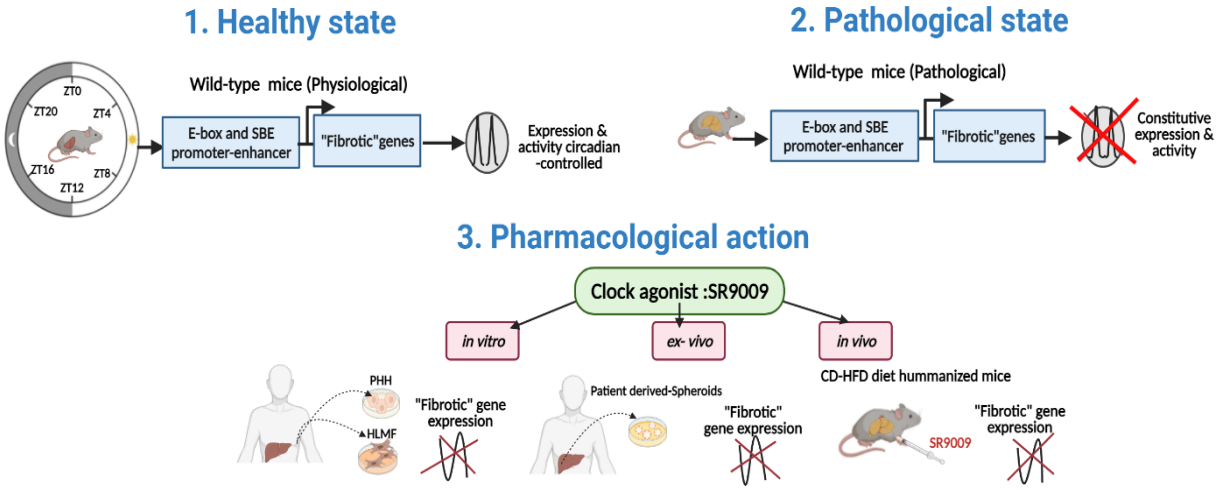


Figure 19: Main results of the present work in three steps. 1. In mouse liver under physiological conditions hepatic CC transcriptionally regulates the expression of key genes linked to TGFβ signaling and fibrosis (healthy state). **2.** In the CD-HFD diet-induced model of NASH and fibrosis the CC-function is severely perturbed which leads to increased expression of fibrotic genes and elevated TGFβ secretion (pathological state).**3.** Pharmacological activation of CC-component REV-ERBα by a small molecule "SR9009" improved liver health by suppressing inflammation and fibrosis in patients-derived disease models and humanized mice. Created with Biorender.com

3. Publication of the results

These results were integrated into the manuscript “Targeting the liver circadian clock by REV-ERBα activation improves liver fibrosis by circadian gating of TGFβ signaling” which has been submitted.

Targeting the liver circadian clock by REV-ERBα activation improves liver fibrosis by circadian gating of TGF-β signaling

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Key words: Liver disease, transcriptional regulation, HCC prevention, NASH, drug discovery.

Word Count: Summary 200

Conflict of interest: The authors declare no conflict of interest.

Summary

Liver fibrosis is the key risk factor for hepatocellular carcinoma, a leading cause of cancer death world-wide. Approved anti-fibrotic therapies are absent and most compounds in clinical development have limited efficacy. The circadian clock (CC) is a major regulator of liver metabolism, but its role in the pathogenesis of liver fibrosis and as a potential therapeutic target is unknown. Here we show that, liver CC-oscillator temporally restricts (gating) TGF-β signaling to the circadian rest phase, and that this regulation is lost in metabolic liver disease leading to constitutive expression of fibrotic genes. Mechanistic studies in primary human cells revealed a reciprocal relationship between increased TGF-β-driven fibrotic-signaling and CC, which were confirmed in patient-derived spheroid and in mice with liver disease. Remarkably, a pharmacological restoration of REV-ERBα activity markedly inhibited fibrosis in a humanized liver chimeric NASH fibrosis mouse model and liver spheroids generated from fibrosis patients. In conclusion, we have discovered that the perturbation of the liver CC plays a key role in the pathogenesis of liver fibrosis. Furthermore, our in vivo and *ex vivo* studies in patient-derived models suggest that targeting REV-ERB-alpha is an effective approach for treatment of liver fibrosis – an important and rising global unmet medical need.

Introduction

Persistence of tissue injury is well-known to initiate a conserved wound healing program commonly referred as fibrotic response. Organ fibrosis is characterized by the release of fibrogenic and pro-inflammatory cytokines driving remodeling of the extracellular matrix, which if sustained for a prolonged period irreversibly destroys the tissue architecture and leads to a loss of organ function. Indeed, fibrosis-driven organ failure is the underlying cause for approximately 45% of death in developed countries[1] and fibrosis is a well-known risk factor for carcinogenesis of several organs including the liver. Despite decades of investigation clinically approved anti-fibrotic therapies are missing. In the liver, previous therapeutic approaches were hampered by limited efficacy and safety[2-6]. Liver fibrosis is a common step in the pathogenesis of chronic liver disease (CLD) caused by viral, alcoholic, and metabolic hepatitis. Advanced fibrosis leads to liver cirrhosis and hepatocellular carcinoma (HCC), one of the most leading causes of cancer-related death globally[7]. One of the major obstacles for the development of efficient and safe anti-fibrotic therapies is the lack of understanding of the molecular mechanisms of liver disease progression[2-5].

Due to its high prevalence and rising incidence non-alcoholic fatty liver disease (NAFLD) is becoming a major cause of liver fibrosis world-wide. NALFD including its complication non-alcoholic steatohepatitis (NASH) affects 25–30% of the general population and its prevalence could reach up to 90% in specific populations with comorbidities such as obesity or type 2 diabetes[8,9]. While NAFLD patients with early-stage fibrosis (F1, F2) are usually asymptomatic, advanced fibrosis (stage F3, F4) is associated with 3-fold higher mortality compared to patients without liver disease[10]. Survival analyses in several clinical cohorts suggest that the stage of fibrosis and not the level of steatosis is the major cause of liver-related mortality in patients with NAFLD[7,11,12].

The pathogenesis of liver fibrosis is multi-factorial and mediated by several cell types including hepatocytes, macrophages, and myofibroblasts[3,4,6]. Among the pro-fibrogenic pathways, cytokines belonging to the transforming growth factor-β (TGF-β) family have been shown to play an essential role[3,6,12,13]. TGF-β signaling is evolutionary conserved and regulates several essential aspects of mammalian biology to maintain homeostasis[14-17]. However, the molecular mechanisms which switches TGF-β-controlled gene expression from health to fibrotic disease are only partially understood.

The circadian clock (CC) plays a key role in liver metabolism[18-21]. At the core of the clock functioning, resides the CC-oscillator, an exquisitely crafted transcriptional-translational feedback system[18-22]. The components constituting the CC-oscillator not only drive the daily rhythmicity of their own synthesis but also generate circadian phase-specific variability in the expression levels of numerous target genes. This is mediated through transcriptional, post-transcriptional, and post-translational regulation and by such ensuring liver homeostasis[18-22]. Perturbation of the CC function is associated with diverse pathologies including metabolic liver disease[20,21]. The liver expresses a diverse set of genes in a circadian manner. Evidence from mouse models and epidemiological studies suggests ‘clock’ alterations to play a critical role in liver disease progression[20,21]. However, the functional role of the CC as a driver and therapeutic target for liver fibrosis is unknown.

Here we aimed to uncover the molecular relationship between CC and signaling pathways which are well-known to drive liver fibrosis. Our mechanistic studies in several model systems including state-of-the-art patient-derived liver disease models, i.e., human liver chimeric mice and liver spheroids revealed that the liver CC-oscillator gates the TGF-β signaling to regulate the expression of key genes mediating liver fibrosis. Importantly, we show that the CC-control of TGF-β signaling which is broken during NASH-induced fibrosis can be pharmacologically restored by REV-ERBα activation, leading to a robust and significant

inhibition of liver fibrosis in clinically relevant models of human chronic liver disease. This uncovers REV-ERBα as a previously unrecognized druggable target for treatment of liver fibrosis.

Results

The liver CC-oscillator controls the expression of gene networks driving fibrosis. To investigate the role of the liver CC-oscillator (Fig. 1a) in the regulation of the expression of fibrosis-related genes, we performed circadian transcript analyses from the livers of conditional hepatocyte-specific *Bmal1* mutant mice (Alb Cre^{TgERT2} $Bmal1^{hep-/-}$; $Bmal1^{hep-/-}$)[23] and littermate control animals ($AlbCre^{0}$ $Bmal1^{hep-/-}$; control Fig. 1b). This revealed that under physiological conditions transcripts of key TGF-β- and fibrosis-related genes such as *Col1α1*, *Tgfβr1*, *Smad7*, *Timp1*, *Twist2*, and *Pdgfrβ* display a day-to-day (diurnal) rhythmicity with the highest expression at the circadian rest phase (ZT0-ZT12; Fig. 1b and Extended Data Fig. 1a). Importantly, in the $Bmal1^{hep-/-}$ mouse livers, the rest phase-restricted expression of fibrotic genes as well as that of known *Bmal1*-controlled CC-genes (*Dbp* and *Rev-Erbα*) were completely abolished (Fig. 1b and Extended Data Fig. 1a). To understand this evident dysregulation, we performed chromatin immunoprecipitation followed by quantitative polymerase chain reaction (ChIP-qPCR) enabling the analysis of the circadian recruitment pattern of BMAL1 and its heterodimeric partner CLOCK (Fig. 1a). Mechanistically, we revealed that during the rest phase in control mice, the BMAL1/ CLOCK-complex is recruited to its cognate E-box region, which is present in the promoter-enhancer region of pro-fibrotic genes *Smad7*, *Tgfβr1*, *Twist2*, and *Ski* (Fig. 1c). The binding pattern was identical to the known BMAL1/CLOCK-recruitment to the E-box of CC-components (*Dbp* and *Rev-Erbα*; Fig. 1c)[22,23]. Importantly, this circadian phase-specific BMAL1/CLOCK-binding to pro-fibrotic genes was completely lost in $Bmal1^{hep-/-}$ mice liver (Fig. 1c). Next, we analyzed the genome-wide circadian binding of BMAL1/CLOCK in the mouse livers as described previously[24]. This unbiased approach confirmed that indeed BMAL1 and CLOCK are recruited in a circadian manner to the E-box regions of TGF-β-regulated genes, e.g., *Smad7* (Fig. 1d and Extended Data Fig. 1b). To determine whether the BMAL1/CLOCK-binding to *Smad7* correlate with its 'active' transcription, we analyzed the global run-on sequencing (GRO-seq) data from control livers[25]. Indeed, under physiological conditions, the BMAL1/CLOCK-binding to the E-box region present in *Smad7* (Fig. 1c, d) is associated with transcription initiation (Extended Data Fig. 1c). Analysis of the gene expression profiles published by Weger and colleagues[26] using gene set enrichment analysis (GSEA)[27] of

HALLMARK gene sets[28] revealed that most of the fibrosis driving genes are indeed expressed in the circadian rest phase and their expression is lost in the liver of constitutive *Bmal1* KO mice (Fig. 1e).

Confirming these findings on the protein level we uncovered a circadian variation of plasma TGF-β (Fig. 1g) with increased TGF-β levels at ZT12 correlating with elevated phosphorylation of the downstream transcription factors SMAD 2 and 3 (pSMAD2/3) in mouse livers (Fig. 1f and Extended Data Fig. 1d). Furthermore, enhanced pSMAD2/3 levels at ZT12 correlated with increased protein levels of other key fibrotic genes (Fig. 1f and Extended Data Fig. 1d). Importantly, ChIP assays revealed that in control mouse liver chromatin recruitment of SMAD2/3, SMAD4 to their cognate SMAD binding elements (SBE) in *Smad7*, *αSma* and *Tgfβr1* is temporally restricted at the rest phase (ZT8-ZT12; Fig. 1h and Extended Data Fig. 1e). In chromatin prepared from *Bmal1*$^{hep-/-}$ mouse livers, we observed a highly reduced binding of SMADs to the SBEs in *Smad7*, *αSma*, *Tgfβr1* genes (Fig. 1h and Extended Data Fig. 1e).

Collectively, these results demonstrate that the liver CC-oscillator controls the physiological expression (circadian gating during rest phase) of key genes which drive liver fibrosis in patients[2-6,14-17].

Diet-induced liver fibrosis disrupts the liver CC-oscillator *in vivo*. Next, we investigated the functional relationship between the CC and fibrotic genes in a metabolic model of liver fibrosis. The choline-deficient high-fat diet (CD-HFD) mouse model recapitulates key features of human NASH-induced fibrosis[29,30] (Fig. 2a-c). Using this liver disease model, accompanied by elevated plasma levels of PDGF-β (Fig. 2c). Histological analyses of mouse liver tissues revealed human stellate cell activation in diseased liver tissue after 4 weeks CD-HFD (Fig. 2a). At week 9, around 15-20% of the liver was fibrotic (Fig. 2b), which is consistent with advanced fibrosis in NASH[11,12].

Investigation of the hepatic CC-oscillator by circadian transcript analyses of core CC-components[18-22] revealed that diet-induced NASH/fibrosis not only altered the expression of genes constituting the core CC-oscillator (Fig. 1a), e.g., *Bmal1*, *Per1*, *Per2*, *Cry1* and *Rev-Erbα* but also altered gene expression of CC-output regulatory transcription factors like *Dbp* and *E4bp4* (Fig. 2d and Extended Data Fig. 2a). We dissected further the impact of circadian rhythmicity and diet duration in an independent experiment using the same NASH animal model. Notably, the CC-oscillator perturbation (including the expression of *Bmal1* and its key transcriptional targets *Rev-Erbα* and *Dbp)* was dependent on the duration of the diet (Fig. 2d). Importantly, we also observed increased transcript levels of known inhibitors of the

BMAL1/CLOCK-complex, i.e., *Per1*, *Per2* and *Cry1* (Fig. 2d. Also, the observed increase of *Cry1* and *E4bp4* transcript levels during the rest phase correlated with the reduction of their transcriptional repressor *Rev-Erbα* (Fig. 2d).

Investigation of the circadian transcription pattern of genes belonging to TGF-β signaling and fibrotic pathway uncovered that in fibrotic mouse livers the physiological circadian expression pattern of key fibrotic genes (Fig. 1b, e and Extended Data Fig. 1a) is severely perturbed (Fig. 2e). We observed elevated levels of expression (across the circadian cycle) for key genes which have been implicated in hepatic fibrosis[2-6,14-17], e.g., *Col1α1*, *Tgfβr1*, *Smad7*, *Timp1*, *Twist2*, and *Pdgfrβ* (Fig. 2e). Importantly, this time-dependent perturbation mirrored the disruption of the liver CC-oscillator (Fig. 2d). To explore, the molecular basis of increased expression of TGF-β-regulated genes in CD-HFD-fed fibrotic liver under conditions of reduced *Bmal1* expression (Fig. 2d), we performed circadian ChIP assays to determine the binding pattern of SMADs to their cognate SBEs. The analyses demonstrated that CD-HFD feeding leads to a progressive increase in the level of SMAD2/3 and SMAD4 recruitment to the SBEs present in *Smad7*, *αSma* and *Tgfβr1* reaching its peak at week 16 (Fig. 2f and Extended Data Fig. 2b, c). Importantly, these ChIP assays showed that in fibrotic mice liver the SMAD binding pattern is altered from normally rest phase-restricted (ZT8-ZT12; Fig. 1h and Extended Data Fig. 1e), to throughout the circadian cycle.

Collectively, our results demonstrate that diet-induced fibrosis breaks the hepatic CC-oscillator with associated loss of its control over the time-restricted expression of TGF-β-regulated and fibrosis related genes (Fig. 1b-h). The diet-induced lack of CC-control leads to a state of constitutive TGF-β/SMAD-signaling a known feature of liver fibrosis.

Metabolic injury in primary human hepatocytes and human liver myofibroblasts impairs the expression of CC-components with concomitant activation of profibrotic genes.

The CC-gating of fibrotic gene expression in livers of control mice under physiological conditions (Fig. 1), and the disruption of this circuit in a diet-induced model of NASH and fibrosis (Fig. 2d-f), led us to explore whether such a CC-control also exists in human liver cells. In this regard, we studied a well-established uPA-SCID human liver chimeric mouse model, in which mice were repopulated with human liver cells. We performed a circadian RNA-sequencing (RNA-seq) from the liver of chimeric control mice and mapped the reads uniquely to the human genome (unpublished results, manuscript in preparation). Subsequently, our transcriptome analyses from livers of chimeric mice revealed a circadian gene expression pattern for genes of the HALLMARK gene sets comprising epithelial mesenchymal transition

(EMT; a TGFβ-regulated process) and TGFβ pathway genes (Extended Data Fig. 3 a, b). It thus confirms that in both mice and human liver fibrosis-related genes are temporally gated.

Considering the circadian expression pattern of fibrotic gene networks in human liver cells, we next investigated the relationship between metabolic injury and the CC in well-established cell-based models[31-35]. Treatment of primary human hepatocytes (PHH) and human liver myofibroblasts (HLMF) with free fatty acids (FFA) to model metabolic injury (Fig. 3a) perturbed the expression of CC-genes: *RORγ, BMAL1* and *REV-ERBα* transcripts decreased, whereas *PER1*, *PER2*, *CRY1* and *E4BP4* transcripts increased accompanied by simultaneous induction of critical fibrotic (*SMAD7, Col1α1, αSMA, TWIST2, PDGFRB*) and inflammatory (*CCL2, IL1β, IL6, SOCS3* and *TIMP1*) genes (Fig. 3b,c and Extended Data Fig. 4a-e). Importantly, consistent with the effect on transcripts, Western blot (WB) analysis of FFA-treated PHH showed a significant induction of fibrotic genes expression including increased levels of transcription factors SMAD2/3 and their consequent phosphorylation (pSMAD2/3; Fig. 3d). In turn, this elevated level of pSMAD2/3 (active SMAD2/3)[14-17] correlated with the increase of TGF-β/SMAD2/3-targets[14-17], e.g., SMAD7, and TGF-β receptor type-1 (TGF-βR1) (Fig. 3d, and Extended Data Fig. 4f). Notably, we also observed a significant reduction in the protein levels of CC-gene BMAL1 in FFA-treated PHH (Fig. 3d).

In dexamethasone-synchronized[36,37] PHH, we observed robust oscillation of the transcripts of CC-components (*BMAL1*, *REV-ERBα* and *PER1*) and of TGF-β targets *SMAD7*, *TWIST2* and *TGFBR1* (Fig. 3e and Extended Data Fig. 4g). Interestingly, as in control mouse livers (Fig. 1b and Extended Data Fig. 1b), the oscillation of *SMAD7*, *TWIST2* and *TGFBR1* transcripts in synchronized PHH was also in phase with the BMAL1-target gene *REV-ERBα* (Fig. 3e). Importantly, like the *in vivo* situation (Fig. 2d, e), FFA-perturbed the oscillation of CC-components in synchronized PHH by reducing the amplitude of *BMAL1* and *REV-ERBα* transcripts, whereas *PER1* expression increased (Fig. 3e and Extended Data Fig. 4g). FFA-treatment of synchronized PHH leads to an increased level of *SMAD7*, *TWIST2* and *TGFBR1* expression (Fig. 3e), thus again indicating that under conditions of elevated fibrotic-stimuli which disrupts the CC-oscillator TGF-β signaling loses temporal regulation. Next, we transfected PHH with SBE-driven luciferase (Luc) reporter plasmids and incubated the cells with FFA in the presence or absence of a specific pharmacological inhibitor of TGF-βR1 (SB505124; SB)[17]. We observed that FFA-treatment indeed stimulates SBE-Luc activity, and that this induction was abolished in SB co-treated PHH (Fig. 3f), thus confirming FFA-driven increased transcriptional activity of SMAD 2/3. FFA-treatment of PHH reduced also the 'basal' promoter activity of a Luc-reporter driven by the natural *Bmal1* promoter-enhancer (*Bmal1*-

Luc; Fig. 3f). Notably, although TGF-β inhibition had no effect on basal *Bmal1*-promoter activity, treatment with SB indeed prevented FFA-induced reduced expression of *Bmal1*-Luc (Fig. 3f).

Next, we investigated the mechanism underlying FFA-impairment of the *Bmal1* promoter activity, which is known to be activated through RORE-mediated binding of RORα/γ and repressed by REV-ERBα recruitment (Fig. 1a)[18-22]. We found that in PHH and HLMF, *RORγ* is predominant and *RORα* is barely expressed (Extended Data Fig. 4h). PHH were transfected with *Bmal1*-Luc plasmid and treated with FFA in the presence or absence of SB. ChIP assays were conducted to monitor the *Bmal1*-RORE recruitment of RORγ, REV-ERBα and RNA Polymerase II (PolII) (Extended Data Fig. 4j). The results revealed a binding of RORγ and PolII to *Bmal1*-RORE under basal condition, which was inhibited by FFA-treatment and restored by SB (Extended Data Fig. 4j). Hence, FFA-induced TGF-β signaling reduces *BMAL1* expression by inhibiting the RORγ recruitment to *Bmal1*-RORE. These experiments led us to investigate the contribution of FFA-induced TGF-β signaling in the perturbed expression of CC-components and fibrotic genes in human cells (Fig. 3b-e and Extended Data Fig. 4a-f). To this end, our transcript analyses from PHH and HLMF showed that inhibition of TGF-β activity by SB blocked the FFA-induced activation of pro-fibrotic genes *SMAD7*, αSMA, *TWIST2* and *COL1α1* (Fig. 3g and Extended Data Fig. 4i). Importantly, SB-mediated inhibition of TGF-β activity in PHH and HLMF also prevented FFA-driven reduction of *BMAL1* and *REV-ERBα* expression as well as the increase of *CRY1* transcript levels (Fig. 3g and Extended Data Fig. 4i). TGF-β regulates gene expression through both SMAD-dependent and SMAD-independent manner[14-17]. Hence, we employed small interfering RNA (siRNA)-mediated silencing of *SMAD2* to explore the role of FFA-induced SMAD activation (Fig. 3d) in the reduction of *BMAL1* expression. Transcript analysis from PHH showed that siRNA-mediated knockdown of *SMAD2* levels prevent both basal and the FFA- induction of SMAD2-target gene *SMAD7* (Fig. 3h), thus confirming the silencing specificity. Importantly, si*SMAD2* also significantly prevented FFA-driven reduction of *BMAL1* and *REV-ERBα* in PHH, thereby providing further evidence for the involvement of SMADs in FFA-induced CC-perturbation (Fig. 3h). Through ChIP assays, we further established that FFA-induced activation of TGF-β-controlled genes leads to the increased recruitment of SMAD2/3 and SMAD4 to their cognate SBEs in *SMAD7*, *TGFBR1* and *TWIST2* genes. This was completely prevented by SB-treatment (Fig. 3j and Extended Data Fig. 4k). We then investigated the relationship between BMAL1 and TGF-β-regulated gene expression in FFA-treated PHH, by performing siRNA-mediated knock down of *BMAL1*. The results indicated that like mouse livers (Fig. 2d, e), reduction of *BMAL1*

expression does not impair NASH stimuli-driven increased recruitment of SMAD2/3 and SMAD4 to their cognate SBEs present in *SMAD7*, *TGFBR1* and *TWIST2* (Fig. 3k and Extended Data Fig. 4k), thus leading to their elevated expression (Fig. 3i). Importantly, as expected under identical condition siBMAL1 effectively reduced the recruitment of BMAL1/CLOCK to its E-Box present in the promoter regions of DBP and REV-ERBα (Extended Data Fig. 3l, m).

Taken together, these results suggest that metabolic injury results in perturbation of the expression of genes constituting the CC-oscillator and TGF-ß signaling in PHH and HLMF and have the potential to disrupt the circadian gating of fibrotic genes in human liver cells.

Pharmacological activation of REV-ERBα improves liver fibrosis in a patient-derived human liver chimeric mouse model. To investigate REV-ERBα as a target for treatment of NASH-induced fibrosis, we examined the effects of a pharmacologic activation of REV-ERBα on NASH-induced fibrosis in human liver chimeric CD-HFD mice. This liver disease mouse model consists of Fah−/−/Rag2−/−/Il2rg−/− (FRG)-NOD mice with livers repopulated by PHH[38-40]. This chimeric, patient-derived animal model is closely recapitulating key features of clinical liver fibrosis and expressing human REV-ERBα. While these mice do not harbor any T or B cells, they carry liver macrophages, myofibroblasts and endothelial cells[38]. The high degree of humanization of the liver was confirmed by staining of human fumarylacetoacetate hydrolase (FAH) (Fig. 4b). Treatment of CD-HFD-fed fibrotic mice with the REV-ERBα agonist SR9009[41] for four weeks (Fig. 4a) resulted in a significant inhibition of liver fibrosis (Fig. 4b, c) as well as inflammation (as shown by a reduced number of inflammatory foci and immune cell count; Fig. 4b, c). We revealed that a pharmacological activation of REV-ERBα reverted the diet-induced perturbation of inflammatory and fibrotic genes (Fig. 4d, e).

Collectively, these results indicate that the activation of REV-ERBα by a small molecule inhibits fibrosis and improves liver function and metabolism in our humanized mouse models for NASH-induced fibrosis including a patient-derived human liver chimeric model.

REV-ERBα activation impairs TGF-β signaling in primary human liver cells. To understand the anti-fibrotic mechanism of SR9009, we explored the relationship between TGF-β signaling and REV-ERBα activation. Transcript analyses performed from TGF-β-stimulated PHH and HLMF revealed not only induction of TGF-β target genes but also a simultaneous decrease in the expression of both *BMAL1* and *REV-ERBα* (Fig. 5a and Extended Data Fig. 5a). Importantly, the TGF-β-driven decrease in *REV-ERBα* expression in PHH and HLMF correlated with the increased levels of its transcriptional target *CRY1* (Fig. 5a and

Extended Data Fig. 5a). Notably, SB-treatment of PHH and HLMF completely reversed TGF-β-driven alterations in the expression of *BMAL1, REV-ERBα* and *CRY1* (Fig. 5a and Extended Data Fig. 5a). WB of PHH further confirmed that increased TGF-β signaling indeed drives to a reduction in REV-ERBα levels, and this could be rescued by SB-treatment (Fig. 5b and Extended Data Fig. 5b).

Next, we explored whether SR9009-mediated activation of REV-ERBα interferes with TGF-β-driven gene expression. Remarkably, promoter-reporter analyses from PHH revealed that akin to SB (TGFβR-inhibitor)[17], SR9009 markedly inhibited TGF-β-induced SBE-Luc expression (Fig. 5c). Consistent with the inhibition of SBE-Luc activity, SR9009 treatment not only inhibited TGF-β-induced activation of fibrotic genes in PHH but also restored *REV-ERBα* levels (Fig. 5d). Importantly, WB analyses from PHH showed that TGF-β-driven reduction in the protein levels of REV-ERBα is completely restored by SR9009 (Fig. 5e). Significantly, SR9009-treatment inhibited TGF-β-driven phosphorylation of SMAD2/3 and increase of TGF-βR1 levels (Fig. 5e). Increased αSMA production by HLMF represents a hallmark of fibrosis, hence we performed immunofluorescence analysis (IFA) from TGF-β- and SR9009-treated HLMF (Fig. 5f). The IFA demonstrated that SR9009 indeed inhibits TGF-β-induced increased αSMA and phosphorylated SMAD2/3 levels in HLMF (Fig. 5f).

In a loss-of-function approach, we observed that si*REV-ERBα* significantly reduced *REV-ERBα* transcript levels while increasing the expression of its repressive target *BMAL1* (Figs. 1a, 5g and Extended Data Fig. 5c). Remarkably, we found that a reduction in *REV-ERBα* levels is sufficient to increase the expression of key fibrotic genes which were further augmented upon TGF-β stimulation (Fig. 5g and Extended Data Fig. 5c). FFA-stimulation of PHH following *REV-ERBα* knock-down further confirmed that indeed a loss of REV-ERBα-activity is associated with increased expression of TGF-β-regulated fibrotic genes (Fig. 5h). Most notably, SR9009-mediated activation of REV-ERBα inhibited FFA-induced activation of fibrotic and inflammatory gene expression (Fig. 5i, j) in HLMF. Collectively, these results reveal a reciprocal relationship between REV-ERBα activity and TGF-β signaling and provide the mechanism of action of SR9009-mediated anti-fibrotic efficacy in primary human cells.

Deregulated expression of CC genes is associated with liver fibrosis progression in patients and targeting REV-ERBα reduces fibrosis in patient liver spheroids. To investigate the clinical translatability and impact of our results, we studied the expression of the CC oscillator genes in clinical cohorts and performed pharmacological perturbation studies in patient-derived liver spheroids. Computational analyses of transcript levels for key CC-

components in a cohort of NASH fibrosis (GSE49541) showed that the expression of *BMAL1*, *RORC* (*RORγ*) is significantly reduced in late-stage fibrosis (F3-F4) as compared to early-stage fibrosis (F0-F2) whereas that of *CRY1* is decreased (Fig. 6a). Corroborating these results in a second independent cohort of patients with NASH compared to healthy controls (GSE89632), we observed that indeed the expression of CC-components *BMAL1*, *RORC* and *REV-ERBα* was significantly reduced in the liver of NASH patients as compared to healthy controls (Fig. 6b). Notably, this reduction in the expression of *BMAL1*, *RORC* and *REV-ERBα* is already observed in the early fibrotic stage (F1-2) with marked deterioration with advanced fibrosis (F3-4). Interestingly, the decreased expression of these CC genes also correlated with lobular inflammation (Fig. 6c), which is another hallmark of advanced liver disease.

To confirm the functional relevance of CC-components as a therapeutic target, we studied the effects of SR9009 on fibrosis in patient-derived human liver spheroids. Spheroids are cultured as 3D micro-tissues and thereby recapitulate the liver microenvironment, relevant for a therapeutic response[31]. Due to an inclusion of not only PHH, but also liver myofibroblasts and immune cells, patient-derived multicellular spheroids are considered as one of the most relevant and translatable model systems to assess the effect of liver therapeutic agents[42]. First, we prepared spheroids from patient liver tissues with no known history of liver disease and treated these spheroids with TGF-β in the presence or absence of SR9009 (Fig. 6d). Transcript analyses revealed that consistent with our previous observations in different model systems TGF-β treatment reduced the level of *REV-ERBα* expression in human liver-derived spheroids, which was significantly restored by administration of SR9009 (Fig. 6e). Importantly, in human spheroids we also found that indeed SR9009 inhibited the TGF-β-driven increased expression of fibrosis driving genes such as *COL1α1, αSMA*, *TGFBR1*, *TWIST2, SKI* and *SMAD7* (Fig. 6e). Next, we prepared spheroids from the liver of fibrosis patients and treated them with SR9009 (Fig. 6f). Remarkably, we found that in fibrosis spheroids SR9009 treatment markedly and significantly reduced the expression level of hallmark fibrotic genes including *COL1α1, αSMA*, *TGFBR1*, *TWIST2* and *SMAD7* (Fig. 6g).

Taken together, these results strongly suggest the clinical translatability of the functional role of CC-machinery in the pathogenesis of liver fibrosis in patients and as a candidate therapeutic target.

Discussion

TGF-β signaling is evolutionary conserved and is well known to regulate gene expression in both, SMAD-dependent and SMAD-independent manner from embryonic development to maintaining organ physiology[14-17]. Importantly, deregulation of TGF-β activity is one of the most prominent hallmarks of organ fibrosis and cancer[14-17]. Mechanistic studies in mouse models have demonstrated that the reduction of elevated TGF-β-driven gene expression prevents and even ameliorates organ fibrosis including in the liver[14-16]. However, little is known about which mechanisms control TGF-β signaling under physiological conditions and thereby prevent its constitutive activation which is responsible for fibrotic liver disease.

Here, we discovered that the liver CC-oscillator regulates TGF-β signaling-dependent expression of fibrotic genes. This was conclusively shown by *Bmal1* loss of function studies (Fig. 1). The CC-control on TGF-β signaling, in turn, dictates that TGF-β-activated transcription factors, i.e., SMADs bind to the SBEs present in their cognate genes only for a limited duration in the rest phase (Fig. 1). Therefore, in the liver under physiological condition the TGF-β-regulated gene expression is 'circadian gated'. Interestingly, in different peripheral tissues, CC-oscillator has been previously found to temporally restrict critical signaling-pathway-controlled transcriptional events. The intestinal-CC for example diurnally controls microbiota-activated TLR-induced NF-κB- and AP-1-dependent transcription[43]. Similarly, the transcriptional activity of BMAL1/CLOCK in the pancreas 'times' insulin signaling[44], while in liver the CC-component CRY1 gates cAMP-CREB signaling at the end of the rest-phase[45]. Collectively, these studies revealed that the absence of CC-control on signaling events and corresponding transcriptional activity drives metabolic pathologies[43-45].

CC-functioning and metabolism are intricately linked. While the CC-oscillator is normally self-sustained, metabolic alterations selectively modulate the expression or activity of specific CC-components[18-23,46]. Here, we found that metabolic liver injury impairs the expression of CC-components in a time-dependent manner (Fig. 2d) resulting in increased expression of TGF-β-related fibrotic genes (Fig. 2e). We discovered that, in contrast to the healthy state, where TGF-β activity is CC-gated, in the disease state the increase of fibrotic gene expression arises from constitutive chromatin binding of transcription factors SMAD2/3 and SMAD4 (compare Fig. 1h with Fig. 2f and Extended Data Fig. 2b, c). This molecular event of permanent (circadian 'non'-gated) SMAD-binding to the promoter/enhancer regions of fibrotic genes underlies elevated TGF-β signaling-driven liver fibrosis. Our pharmacological and knock-down experiments demonstrate that in PHH and HLMF, elevated TGF-β signaling directly impairs

the expression of key CC-genes including *BMAL1* and *REV-ERBα* (Figs. 3,5), and *REV-ERBα* inhibition increased basal TGF-β activity (Fig. 5), unraveling a "cross-talk" between the CC- and TGF-β signaling.

Exploiting these mechanistic discoveries, we show that targeting REV-ERBα by a small molecule improves liver fibrosis in patient-derived *in vivo* and *ex vivo* models. We chose REV-ERBα as: (*i*) its level was reduced during fibrosis and elevated TGF-β signaling (Figs. 2d, 3b,e, and 5a), (*ii*) it is a global regulator of hepatic metabolism[18-21], and (*iii*) specific agonists (SR9009) are available which can be safely administered in mice[41,47,48]. A strength of our study is the validation of results in authentic patient-derived model systems including patient-derived animal models, spheroids and primary cells, the consistency of results across complementary model systems and patient cohorts supporting its clinical validity and translatability into the clinic.

Targeting REV-ERBα for the treatment of liver fibrosis is a novel, effective and differentiated concept. The large majority of liver disease therapeutics target metabolism, inflammation, or cell death, which are relevant in the early stage of the disease. Only a few compounds with anti-fibrotic properties have entered clinical development with only limited efficacy, while displaying considerable safety issues[2,4,5,8,13,49]. Moreover, as shown recently for GLP1 analogues[50], robust improvement of steatosis alone does not necessarily induce improvement of fibrosis. A key differentiator of the REV-ERBα-targeting approach is the combination of anti-inflammatory and anti-fibrotic efficacy as demonstrated across all models (Fig. 4-6), which addresses a key unmet medical need in treatment of liver fibrosis.

Our data obtained here and in previous studies[41,47,48] demonstrates that the administration of SR9009 is safe without detectable major adverse effects in animal models. Interestingly, SR9009 has also been suggested to ameliorate liver fibrosis by improving the gut barrier function[48]. Furthermore, targeting REV-ERBα has been also shown to improve lung fibrosis in animal models[47]. Nevertheless, further studies are needed to fully investigate the compounds' safety profile in patients including the development of next-generation REV-ERBα agonists. Since our preclinical models are focused on NASH as a liver injury, additional studies are needed to show efficacy and impact also in fibrosis of other etiologies such as chronic viral hepatitis and alcoholic liver disease. Collectively, based on our preclinical data in patient-derived animal and cell-based models, the target population for REV-ERBα-targeting therapies will be patients with NASH fibrosis – a unmet global unmet medical need with rapidly rising incidence and mortality [10].

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Acknowledgements

The authors thank Prof. Pierre Chambon (IGBMC, Illkirch, France) for providing Alb-CRE^{ERT2}-*Bmal1* KO mice, and Laetitia Paulen for help with animal experimentation at IGBMC . *Bmal1*-Luc plasmid was originally created in the laboratory of Prof. Steven Brown (ETH, Zurich, Switzerland), and were kindly provided by Prof. David Ray (Oxford, UK) to authors.

Funding

T.F.B. acknowledges funding by the European Union (ERC-AdG-2014-HEPCIR #671231, EU H2020-HEPCAR #667273 and ERC PoC-HEPCAN #862551), ARC, Paris and Institut Hospitalo-Universitaire, Strasbourg (TheraHCC2.0 ARCIHU201901299), the Foundation of the University of Strasbourg, the Agence National de Recherches sur le Sida et les Hépatites Virales and the US National Institute of Health (R01CA233794). A.M. acknowledges funding by USIAS (2020-029). This work has been published under the framework of LABEX ANR-10-LABX-0028-HEPSYS and PLAN CANCER 2014-2019 HCCMICTAR and benefits from the state managed funding by the French National Research Agency as part of the Investments for the Future Program, National Institute for cancer (INCa) and Inserm.

Author Contributions:

T.F.B. and A.M. conceived and supervised the study. A.M, T.F.B. and M.D. designed experiments. M.D. and A.M. performed cell-based and mouse tissue analyses. L.M, N.B. and R.M. performed animal experiments, A.S. performed histological analyses. S.D. and N.R. processed human liver tissues, S.D. performed histological staining. E.C prepared spheroids from human liver samples for *ex vivo* experiments. M.D., A.M., T.F.B., C.S. and J.L. analyzed

results. F.J. performed bioinformatic analyses. N.R. performed GSEA analysis from patient's cohorts. P.P. and E.F. provided human liver samples. M.D., A.M., J.L., and T.F.B. wrote the manuscript. All authors edited, read, and approved the submitted manuscript.

Conflicts of interest:

The authors declare no conflict of interest.

Materials and methods:

Human liver samples. Human liver tissue samples were obtained from patients who had undergone liver resections between 2014 and 2021 at the Center for Digestive and Liver Disease (Pôle Hépato-digestif) of the Strasbourg University Hospitals University of Strasbourg, France. All patients provided a written informed consent for de-identified use. The protocols followed the ethical principles of the declaration of Helsinki and were approved by the local Ethics Committee of the University of Strasbourg Hospitals and by the French Ministry of Education and Research (Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche; MESRI approval number DC-2016-2616 and RIPH2 LivMod IDRCB 2019-A00738-49). Data protection was performed according to EU legislation regarding privacy and confidentiality during personal data collection and processing (Directive 95/46/EC of the European Parliament and of the Council of the 24 October 1995). Datasets of clinical cohorts with fibrosis or cirrhosis: GSE49541 39 patients with mild fibrosis (F0-F2) and 32 with advanced fibrosis stage (F3-F4), GSE89632: 20 patients with simple steatosis (F1-F2), 19 with nonalcoholic steatohepatitis (NASH) (F3-F4), and 24 healthy controls (F0) (GSE48452) were selected following comprehensive database analysis and analyzed for changes in the transcript levels of indicated genes in (log2FC).

Animal experimentation. All experiments were performed in male mice. Six-weeks old C57BL6/J mice (Charles River Laboratories) were maintained in a specific pathogen-free facility (Inserm U1110) at a constant temperature. All mice were provided food and water ad libitum, under 12 h light and 12 h dark conditions. Mice were fed a chow diet until they were provided with CD-HFD Hepatocyte-specific ablation of Bmal1 ($Bmal1^{hep-/-}$) was achieved by crossing floxed female mice with albumin-$CreERT^2$ floxed male mice and subsequent intraperitoneal tamoxifen injection for 5 d[22]. Cre^0 and Cre^{Tg} littermate mice were utilized. Albumin-$CreERT^2$ $Bmal1^{hep-/-}$ mice were generated and maintained in Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) / Institut Clinique de la Souris (ICS)[22]. For

circadian experiments, mice (n=3 / group / time-point) were sacrificed at 4-hour interval starting from ZT0 (6 a.m.)[23,43]. Two-independent circadian transcript and ChIP experiments were performed, utilizing liver from same mice.

Choline-deficient high-fat diet studies. Two independent studies were conducted in the animal facilities of Inserm U1110 and IGBMC. For both C57BL6/J male mice (6 weeks; Charles River Laboratories) were used and control mice were always fed a regular chow diet. Choline-deficient amino-acid adapted high fat diet (CD-HFD, Research Diet, ref A06071302) The experiments described (Fig. 2 a-d and Extended Data Fig. 2a, b) were performed at Inserm U1110, and mice were sacrificed as indicated after 1, 2, 4 and 9 weeks of CD-HFD feeding to model NASH and fibrosis. The experiments described (Fig. 2e-g and Extended Data Fig. 2c-e) were performed at IGBMC, and mice were fed with CD-HFD for 1,2, 6 and 16 weeks. The on and off times of light were defined as Zeitgeber time with (ZT0) being the start of the light period (6 a.m.) and (ZT12) the start of the dark period (6 p.m.). For circadian transcript analysis and ChIP assays mice were sacrificed at 4-hour interval starting from ZT0. Experiment was approved by the local ethic committee and authorized by the French ministry of higher education and research (APAFIS#29396-2021012915164103 v2).

Human liver chimeric mouse model studies. For determination of whether pharmacological activation of REV-ERBα can prevent liver fibrosis in human hepatocytes, we used a human chimeric mouse model where we transplanted human liver cells from patient-derived hepatocyte (HM mice) as described[40,51], mice were first fed an normal diet or CD-HFD for 12 weeks and then divided into 3 groups and treated for another 4 weeks as follows: (*1*) CD-HFD were fed a high fat diet and daily IP injected with vehicle; (*2*) CD-HFD+SR9009, mice were fed a high fat diet and IP injected with SR9009 (100 mg/kg/day) for 4 weeks (n=4 per group). SR9009 was bulk synthesized (MCE; HY-16989) and the entire experiment was performed with the SR9009 prepared from a single batch. SR9009 was dissolved in Kolliphor EL (15% in PBS) and was intraperitoneally injected. Tissues were collected and stored at -80°C until use, blood samples were collected and centrifuged at 7000xgfor 7 min, and plasma was used for biochemical analyses. Mice were randomly assigned to the different experimental groups by a blinded technician. Experiment was approved by the local ethic committee and authorized by the French ministry of higher education and research (APAFIS#29396-2021012915164103 v2).

Isolation and culture of primary human hepatocytes. Hepatocytes were isolated as previously described[40,51] using a two-step collagenase perfusion method: Human liver specimens obtained from resections were perfused for 15 min with calcium-free 4-(2-

hydroxyethyl)-1-piperazine ethane sulfonic acid buffer containing 0.5 mM ethylene glycol tetra acetic acid (Fluka) followed by perfusion with 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid containing 0.5 mg/mL collagenase (Sigma-Aldrich) and 0.075% $CaCl_2$ at 37 °C for 15 min. Then the cells were washed with sterile phosphate-buffered saline (PBS) and nonviable cells were removed by Percoll (Sigma-Aldrich) gradient centrifugation. Part of the isolated cells was further separated into PHH and non-parenchymal cells (NPCs) by an additional centrifugation step. Liver cells were maintained then in the hepatocyte's maintenance medium Williams E medium in the presence of 10% heat-decomplemented fetal bovine serum (FBS),1% gentamycin. Cells were maintained in an incubator at 37 °C with 5% CO_2. For the time-course experiments, cells were seeded in BD Biocat collagen I 12-well plate (RNA and protein) at a density of 100,000 cells/cm^2. The William medium E in the presence of 10% FBS were refreshed the day after isolation. PHH from 3 different individuals were used. Each experiment was done in triplicate/each condition. Cells were treated for 3 d in DMEM supplemented with 10% heat-decomplemented FBS, gentamycin and 1% DMSO at 37 °C and 5% CO_2, with FFA (100 µM oleic acid and 50 µM palmitic acid), or TGF-β (1 ng/mL) for 6 h, and/or TGF-βR1 inhibitor (SB505124, 2.5 µM) for 4 h, and/or (SR9009, 20 µM).

Isolation, culture, and activation of HLMF. Primary HLMF were isolated from NPC's of healthy liver tissue using magnetic-activated cell sorting (MACS) as described[52]. HLMF were cultured on collagen-coated plates (corning) in DMEM supplemented with glutamine (1%), penicillin-streptomycin (1%) and 10% FBS and were maintained in an incubator at 37 °C with 5% CO_2. Following culture for 10 d, cells became activated and differentiated into HLMF with the expression of αSMA. For the time-course experiments, HLMF were grown at 40,000 cells/cm^2 either on Corning 6-well plates (RNA) or (for IF) in Nunc Lab-Tek II 8 Well Chambered Coverglass (Thermo Scientific, #2515384) during the activation process. The growing medium with 10% FBS was refreshed the day after isolation, and 2-3d afterwards. For perturbation studies, cells were treated for 3 days with 100 µM oleic acid and 50 µM palmitic acid or TGF-β (1 ng/mL) for 6 h, and/or TGF-βR1inhibitor (SB505124, 2.5 µM) for 4 h, and/or (SR9009, 20 µM) for 3 days.

Patient-derived spheroids. For the healthy tissue: Spheroids were generated from liver tissues from healthy patient-derived liver tissues without a history of chronic liver disease. Tissues were perfused with collagenase and dissociated as described[31,33] and the total cell population including hepatocytes and NPCs were used to generated multicellular spheroids. For fibrotic tissue: spheroids were generated from non-tumoral fibrotic patient liver tissues. Tissues were

dissociated using gentle MACS Octo Dissociator with Heaters (Miltenyi Biotech) according to the manufacturer's instructions. The total cell population including hepatocytes and NPCs were used to generated multicellular spheroids. Approximately 150,000 cells were cultured in complete MammoCult medium (StemCell Technologies) supplemented with 20% of patients' own serum in ultra-low attachment 96 well plates (Corning) for 24 h. Then spheroids were subjected to different treatments (TGF-β 1 ng/µL, SR9009 20 µM or DMSO as vehicle control) for 72 h. Cells were lysed and RNA extracted using PicoPure RNA Isolation Kit (Thermo Fischer) before gene expression assessment.

RNA-seq and analyses. RNA-sequencing was performed at the commercial sequencing facility of BSF (CEMM, Vienna). The amount of total RNA was quantified using the Qubit 2.0 Fluorometric Quantitation system (Thermo Fisher Scientific, Waltham, MA, USA) and the RNA integrity number (RIN) was determined using the Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA, USA). RNA-seq libraries were prepared with the TruSeq Stranded mRNA LT sample preparation kit (Illumina, San Diego, CA, USA) using Sciclone and Zephyr liquid handling workstations (PerkinElmer, Waltham, MA, USA) for pre- and post-PCR steps, respectively. Library concentrations were quantified with the Qubit 2.0 Fluorometric Quantitation system (Life Technologies, Carlsbad, CA, USA) and the size distribution was assessed using the Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA, USA). For sequencing, samples were diluted and pooled into NGS libraries in equimolar amounts. Expression profiling libraries were sequenced on HiSeq 3000/4000 instruments (Illumina, San Diego, CA, USA) following a 50-base-pair, single-end recipe. Raw data acquisition (HiSeq Control Software, HCS, HD 3.4.0.38) and base calling (Real-Time Analysis Software, RTA, 2.7.7) were performed on-instrument, while the subsequent raw data processing off the instruments involved two custom programs based on Picard tools (2.19.2). In a first step, base calls were converted into lane-specific, multiplexed, unaligned BAM files suitable for long-term archival (IlluminaBasecallsToMultiplexSam, 2.19.2-CeMM). In a second step, archive BAM files were demultiplexed into sample-specific, unaligned BAM files (IlluminaSamDemux, 2.19.2-CeMM). NGS reads were mapped to the Genome Reference Consortium GRCh38 (human) or GRCm38 (mouse) assembly via "Spliced Transcripts Alignment to a Reference" (STAR) utilizing the "basic" Ensembl transcript annotation from version e100 (April 2020) as reference transcriptome. STAR was run with options recommended by the ENCODE project. Aligned NGS reads overlapping Ensembl transcript features were counted with the Bioconductor (human: 3.11; mouse: 3.12) GenomicAlignments

(human: 1.24.0; mouse: 1.26.0) package via the summarizeOverlaps function in Union mode, considering that the Illumina TruSeq stranded mRNA protocol leads to the sequencing of the second strand so that all reads needed inverting before counting. Transcript-level counts were aggregated to gene-level counts and the Bioconductor DESeq2 (human: 1.28.1; mouse: 1.30.0) package was used to test for differential expression based on a model using the negative binomial distribution. Gene set enrichment analyses for specific gene sets based on MSigDB (7.2) or extracted from the specified publications were performed using GSEA (4.1.0), and results were drawn using R scripts applying ggplot2.

Bioinformatic analyses of external data sets. Mice liver ChIP-seq data (GSE39860)[24] and GRO-seq (GSE59486)[25] data was downloaded from the Gene Expression Omnibus website and visualized using the Integrative Genomics Viewer (IGV).

Expression of fibrotic genes in external RNA-seq data. Raw data SRA data as listed for data set (GSE39978)[50] on the Gene Expression Omnibus website was downloaded from SRA and converted to fastq files. SOLiD data was mapped to the mouse genome (GRCm38) using the subjunc program (subread 2.0.1). Gene counts for Gencode (vM25) were calculated htseq-count and normalized using DESeq2. Single sample gene set enrichments were calculated applying the "ssgsea" method from GSVA for specific gene sets based on MSigDB (7.2). Result was illustrated using heatmap.

ChIP-qPCR Assay. The protocol was adapted from[23] with few minor modifications. For ChIP with mouse liver, 1mg samples were used per antibody, per time point. For, ChIP analyses with cells we used 10 million cells per condition. Post harvesting liver were dounce homogenized in cold PBS. Cells were scraped (10 million/condition) in cold PBS and washed them twice in ice cold PBS. Subsequent steps were identical for both liver extracts and cells. Fresh formaldehyde was added (1% final concentration (v/v) to the PBS containing cells and incubated on a flip-flop rocker for 10 min at room temperature, followed by the addition of 2 M glycine (0.125 M final concentration) and incubation for 5 min (room temperature). Cross-linked cells were pelleted (400 x g, 5 min) at 4 °C, washed twice in ice-cold PBS and resuspended in 400 µL of ice-cold lysis buffer (50 mM Tris, 1% SDS, 10 mM EDTA, protease inhibitor cocktail Complete, EDTA-free (Roche), pH 8.1). Cross-linked cell lysates were sonicated (Covaris) at 4 °C to generate 200-500 base pair chromatin fragments. Cellular debris were removed by centrifugation (10000 x g, 10min) at 4 °C and the supernatant was pre-cleared by incubating with 75 µL of protein A/G-sepharose (Sigma) beads (pre-blocked with salmon-sperm DNA and BSA) for 45 min (4 °C) on a flip-flop rocker. Beads were pelleted (100 x g, 1 min) at 4 °C and

discarded. Equal amounts of lysates were subjected to immunoprecipitation. 10% of the lysate was saved and served as input DNA in PCR amplification. Next, individual samples were diluted 1:8 (v/v) in ChIP dilution buffer (16.7 mM Tris, 0.01% SDS, 1% Triton X-100, 1 mM EDTA, 16 mM NaCl, 1X Complete, EDTA-free (pH8.1) and incubated overnight with control IgG (Diagenode; #1540001), anti-SMAD4 Ab (CST; D3M6U, #38454), anti-SMAD2/3 Ab (Abcam; EPR19557, #207447), anti-BMAL1 Ab (CST; D2L7G, #14020), anti-PolII (Active Motif; #39097), and anti-CLOCK Ab (Santa Cruz Biotech.; #sc-6927x) at 4 °C on a rocker. Next, 75 µL of pre-blocked protein A/G-beads were added for 90 min at 4 °C. Immuno-adsorbed complexes were recovered by centrifugation (100 x g, 1 min) at 4 °C. Protein A/G-bound immunocomplexes were then washed once with low-salt buffer (20mM Tris, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, pH 8.1), once in high-salt buffer (20 mM Tris, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, pH 8.1), once in LiCl buffer (10 mM Tris, 250 mM LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, pH8.1), and finally washed twice in 1 mL TE buffer (10mM Tris, 1 mM EDTA, pH 8). Chromatin was released from the beads by incubation with 150 µL of elution buffer (1% SDS, 100 mM $NaHCO_3$, pH 8) for 15 min at room temperature with intermittent vortexing. The elution step was repeated and both eluates were pooled. 1 µL of RNaseA (10 mg/mL; Fermentas) and NaCl (200 mM final concentration) was added to the eluate and incubated at 65 °C overnight. The reaction was stopped by 3 µL proteinase K (10 mg/mL; Fermentas) and incubated at 50 °C for 1 h. DNA was subsequently purified from the eluate using QIAGEN PCR purification kit in a final volume of 50 µL. This purified DNA was next used to perform qPCR using specific primers (sequences available upon request) to reveal the recruitment to mouse and the human genome.

Tissue collection and histology. All animal tissues for protein or RNA extraction were frozen at the time of collection. Samples for histology were placed in 10% neutral formalin overnight before transfer to 70% ethanol and later embedding in paraffin and cross-sectioned to obtain 3 M section, and then extra-coated with paraffin to preserve tissue integrity.

Immunohistochemistry. Before use, sections were deparaffinized, rehydrated and processed for H&E, Sirius Red, αSMA and CD68 staining. For Sirius Red staining: slides were incubated with a 0.1% Sirius Red solution dissolved in aqueous saturated picric acid for 1 h (Sirius Red: Sigma-Aldrich, Direct Red 80, #365548), washed in acidified water (0.5% HCl), dehydrated, and mounted under coverslips with Eukitt mounting medium (Sigma)For αSMA and CD68: tissues were immersed with 3% of hydrogen peroxide (H_2O_2) for 20 min, blocked in goat serum

for 1 h and then incubated overnight at 4 °C with antibodies (αSMA and CD68; 1/200). Tissues were washed three times with PBS for 5 min before incubation with HRP conjugated antirabbit IgG secondary antibody for 1 h at room temperature. Slides were later stained with 3,3'diaminobezidine-tetrahydrochloride (Sigma Aldrich) for 3 min at room temperature to visualize the antibody binding site. The sections were then washed up and stained with Mayer's Hematoxylin solution. Briefly, whole slide images were generating using 3D Panoramic SCAN and uploaded into Ndpi view software. For collagen positive area quantification were identified in the Sirius Red -stained slides and quantified as a percent of the total image analysis area using ImageJ. αSMA and CD68 positive cells were counted and expressed as density across the total image analysis area.

Immunofluorescence. HLMF were washed in cold, sterile PBS two times and fixed at 4 °C with 4% paraformaldehyde for 15 min at RT. The cell membranes were permeabilized with PBS + Triton X-100 0.1% (PBS-T), pH 7.4 for 10 min. Next, HLMF were incubated in 10% normal goat serum in PBS for 30 min at RT. Subsequently, primary antibodies diluted in PBS 1% FBS were added and overnight incubation at 4 °C on a shaker was performed. Antibodies used were purchased from Abcam, anti-αSMA (ab5694; 1:200), anti-phospho-SMAD2/3 (D27F4; 1:200). Next, slides were thoroughly washed with PBS and secondary antibodies diluted in PBS (1% FBS) were added for 1 h at 37 °C. Staining was terminated with 4-6 washes in PBS-T, each for 5 min. Secondary antibodies used were from Thermo Fisher: anti-rabbit cross-adsorbed Alexa Fluor 594 (1:200) and anti-mouse cross-adsorbed Alexa Fluor 488 (1:200). The slides were then mounted and kept at 4 °C. Pictures were taken with the Zeiss LSM 510 Meta (cells) confocal microscope.

Liver RNA extraction and gene expression analysis. Liver tissues were lysed in TRI-reagent (Molecular Research Center; Cincinnati, OH) and RNA was purified using Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA) according to the manufacturer's instructions. RNA quantity and quality were assessed using NanoDrop (Thermo Scientific, Waltham, MA). Liver samples were then analyzed by qPCR using SYBR greens (BioRad) according to the manufacturer. Each sample was run in duplicate and analyzed on the Primer PCR software supplied by Bio-Rad. GAPDH was utilized as reference gens for analysis. Results were plotted in Graph Pad prism software as gene regulation using mean +/-SEM.

Measurement of TGF-β1 using ELISA. The concentration of TGF-β1 in serum was measured using an ELISA kit (R&D Systems) as per the manufacturer's protocol. All samples were assayed in triplicate in a blinded fashion; the mean was used for data analysis.

PHH synchronization. PHH were seeded in 35 mm culture dishes in 2 mL at a density of $2x10^5$ cells/mL and incubated for 2–4 days. Synchronization was performed when cells were confluent, PHH growth media was then aspirated, and 100 nM dexamethasone-containing culture media was added for 2 h to synchronize the cells. At this time (time zero), we started to harvest cells every 4 h for 48 h. Time samples were collected separately to confirm synchronization (by duplicate; two wells of a six-well plate were merged to make one replicate).

Luciferase reporter assay. The *BMAL1*-luciferase reporter construct (pBABE-puro-BluF, herein referred to as *BMAL1: Luc*) was obtained from the laboratory of Prof. Steven Brown (ETH, Zurich, Switzerland). The *SBE*-luciferase reporter construct (pGL4.48 [*luc2P/SBE/Hygro*] was obtained from Promega (plasmid pGL4). PHH were seeded in 35 mm culture dishes and transiently transfected with 300 ng *BMAL1 Luc* or *SBE- Luc* reporter constructs using Fugene (Thermo Scientific) according to the manufacturer's instructions. On the next day, the medium was changed to Dulbecco's modified Eagle medium and was stimulated with TGF-β (10 ng/mL), with or without SR9009 and SB505125. Luciferase activities were determined by the One-Glo or Nano-Glo Reporter Assay System (Promega) and a Mithras LB940 plate reader (Berthold Technologies). The relative luciferase activity values of treated cells were normalized to that of control cells.

Protein immunodetection. For mice liver, small pieces were made washed in PBS and dounce homogenized in IP lysis buffer in presence of protease and phosphatase inhibitor in ice for 45 min. Subsequent to the centrifugation (300 x g, 10 min) supernatants were collected for WB. PHH were washed in PBS, scraped, and were lysed with IP lysis buffer (Triton 1%; NaCl 150 mM; Tris 50 mM, pH 7.6) with Complete, EDTA-free proteinase inhibitor cocktail (Roche) and phosphatase inhibitor cocktail number 2 and 3 (Sigma) during 30 min at 4 °C with agitation. Then the samples were centrifuged at 16000 x g for 15 min, and the supernatant was collected for WB.

Analyses of protein expression. The expression of proteins was assessed by Western blot as described, using the Bio-Rad electrophoresis and transfer system. Contrasts of western blotting images were equally adjusted for entire membranes using Bio-Rad image analysis software. Protein expression was assessed using Image Lab software (Bio-Rad) and normalized to total protein amount (Stain-free technology, Bio-Rad).

Antibodies. Antibodies targeting Smad 2/3 (#8685), pSmad 2 (Ser465/467), Smad3 (Ser 423/425) (D27F4) were obtained from Cell Signaling Technology, αSMA (#ab5694), Twist2 (#ab66031), TGF-βR1 (#ab31013), RevErbα (#2129), Cry1 (#ab54649), Bmal1 (#ab93806). A

rabbit polyclonal anti-*β*-tubulin Ab (#GTX101279) was obtained from Gentex; a rabbit polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH; #ab9485) was obtained from Abcam, anti-Smad7 (#PA1-41506) was obtained from Invitrogen, anti-CD68 (FA-11) was obtained from Thermo-Fisher, Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson Research 111-035-144) was used as a secondary Ab.

Small interfering RNAs. Pools of ON-TARGET plus (Dharmacon) small interfering RNA (siRNA) targeting *NR1D1* (J-003411-09; 5'-CAA CAC AGG UGG CGU CAU C), *BMAL1*(J-010261-05; 5'-CCA UUG AAC AUC ACG AGU A), *SMAD2* (J-003561-05; 5'-GAA UUG AGC CAC AGA GUA A) expression were reverse-transfected into PHH using Lipofectamine RNAi-MAX (Invitrogen), following manufacturer's instructions. Cells were maintained in FBS containing medium for 72 h, and then processed for RNA isolation and transcript determination and finally protein immunoblot analysis.

qRT-PCR. Total RNA was extracted using ReliaPrep RNA Miniprep Systems (Promega) and reverse-transcribed into complementary DNA (cDNA) using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. Gene expression was then quantified by quantitative PCR using a CFX96 thermocycler (Bio-Rad). All values were normalized to *GAPDH* expression.

Reagents and chemicals. DMSO and dexamethasone (D9184) were purchased from Sigma-Aldrich, SB-505124(3263) from Tocris Bioscience, Recombinant Human TGF-β1 (#100-21) from PeproTech, and SR9009 (#1379686-30-2) from Cayman chemical.

Statistical analyses. Individual experiments were reproduced at least three times in an independent manner with similar results (except otherwise stated). Each *in vitro* experiment involving PHH and HLMF were performed from tissues obtained from 3-4 independent donors, and each condition/treatment was in triplicate in each experiment. All data are represented as mean ±SEM (n=4 or greater). All expression statistical analysis was performed using the one-way ANOVA in GraphPad prism software. Sample data was entered into GraphPad prism software and analyzed for statistical significance using an unpaired student's t-test (2-tailed). P-values are reported as follows: *$p \leq 0.05$, **$p \leq 0{,}01$, ***$p \leq 0.001$ and ****$p \leq 0{,}0001$.

Figure Legends:

Fig. 1. Expression of fibrosis-related genes display circadian rhythmicity in mice liver.

(a) Schematic representation of the circadian clock (CC)-oscillator. The transcriptional activator proteins BMAL1 and CLOCK heterodimerize to induce (black arrows) the expression of E-CCGs including *Per1/2*, *Cry1/2* and *Rev-Erbα/β* genes via E-box present in their promotor regions. PER1/2 and CRY1/2 proteins inhibit BMAL1/CLOCK-activity to repress their own transcription (red arrows), generating a transcriptional and translational feedback loop. REV-ERBα/β (inhibitor) and RORα/γ (activator) create an additional feedback loop by regulating the expression of several RORE-CCGs including *Bmal1* and *Cry*.
(b) RNA transcripts (Q-RT-PCR) measuring circadian expression pattern of indicated genes in the liver of control (WT) and Bmal1$^{hep-/-}$ mice.
(c) Chromatin recruitment (ChIP-qPCR assays) to the E-Box region present in the indicated genes using liver of WT and Bmal1$^{hep-/-}$ mice, and IgG, BMAL1 and CLOCK antibodies.

(d) UCSC genome browser view of circadian chromatin recruitment pattern of BMAL1 and CLOCK to the *Smad7* gene in the liver of control mice, as revealed by ChIP-sequencing. Each track represents the normalized ChIP-seq read coverage at the single time point. For both BMAL1 and CLOCK, six time points with an interval of 4 h are shown. The arrowhead indicates the transcriptional start site of *Smad7* gene.

(e) Heat map analysis showing circadian transcriptional pattern of all the genes enlisted in the HALLMARK fibrotic data set.

(f) Immunoblots (WB) analysis of indicated proteins in livers of WT mice at ZT0 and ZT12.

(g) TGF-β1 ELISA from plasma of WT mouse collected at ZT0 and ZT12.
(h) ChIP-qPCR assays to the SBE-region present in indicated genes using liver of WT and Bmal1$^{hep-/-}$ mice, and IgG, SMAD2/3, SMAD4 and PolII antibodies.
All data are expressed as mean ±SEM. n=6-8. *p<0.05, **p<0.01, ***p<0.001. Fig. 1b, c and h were performed from same control and Bmal1$^{hep-/-}$ mice.

Fig. 2. Hepatic fibrosis perturbs the CC-control on fibrotic genes in mice liver.

(a) Representative Sirius-red staining of the liver of mice that were fed either control (chow) diet or CD-HFD for indicated time-periods. Scale bar 500 μm (upper panels), 100 μm (lower panels).

(b) Quantification of collagen positive area (CPA) from Sirius red-stained liver sections of mice fed as above.
(c) ELISA of PDGF-β levels in plasma of mice fed as indicated.
(d) RNA transcripts measuring circadian expression pattern of key fibrotic genes and **(e)** CC-components in the liver of control and CD-HFD mice, fed as indicated.

(**f**) ChIP-qPCR assays to the SBE-region present in *Smad7* gene using liver of mice that were fed either control (chow) diet or CD-HFD for indicated time-periods, and IgG, SMAD2/3, SMAD4 and PolII antibodies.
All data are expressed as mean ±SEM. n=6-8. *p<0.05, **p<0.01, ***p<0.001. Fig. 2d-f were performed from same mice as an independent experiment.

Fig. 3. FFA-induces TGF-β signaling and CC-perturbation in primary human cells.

(a) PHH and HLMF were isolated from 'healthy' human liver and treated with FFA for 3 d.

(b, **c)** Relative expression of CC-components (b) and key fibrotic- and inflammatory- genes (c) in PHH post-FFA treatment.

(d) WB analysis for indicated proteins in PHH post-FFA treatment.
(e) Relative expression of indicated genes from dexamethasone-synchronized PHH following FFA treatment.

(f) Luciferase reporter assays of PHH transfected with Bmal1promoter or SBE containing vectors and treated with FFA and TGF-βR activity inhibitor SB505124 (SB; 2.5 μM).

(g) Relative expression of CC-components and fibrotic genes in HLMF, following FFA- and SB-treatment, as indicated.

(h, **i)** Relative expression of indicated genes in PHH following siRNA-mediated knock-down of *SMAD2* (h) or *BMAL1*(i), and FFA treatment.

(j, **k)** ChIP-qPCR assays to the SBE-region present in *SMAD7* and *TGFBR1* genes from PHH and using IgG, SMAD2/3, SMAD4 and PolII antibodies, following siRNA-mediated knock-down of *SMAD2* (j) or BMAL1 (k), and treatments as indicated.

All data are expressed as mean ±SEM. n=3-4. *p<0.05, **p<0.01, ***p<0.001.

Fig. 4. SR9009 prevents the development of fibrotic features in humanized liver chimeric mice.

(a) Schematic of engraftment of healthy human hepatocytes in immunodeficient FRG-NOD mice to create humanized liver chimeric mice, which were fed and treated as indicated.

(b) Representative fumarylacetoacetate hydrolase (FAH), H&E, Sirius-red and αSMA staining of liver sections of humanized liver chimeric mice that were fed and treated as indicated. Scale bar 500 μm

(c) Quantification of collagen- and αSMA-stained areas from different mice groups treated, as indicated.

(d) Relative expression of indicated fibrotic genes in the liver of humanized mice treated, as indicated.

(e) Relative expression of indicated inflammatory genes in the liver of humanized mice treated, as indicated.

Fig. 5. Molecular feedback between REV-ERBα and TGF-β signaling.

(a) Relative expression of indicated genes in PHH following treatment with TGF-β1 (1 ng/mL) and SB (2.5 μM), as indicated

(b) WB analysis of protein expression in PHH treated with TGF-β1 (1 ng/mL) and TGF-βR activity inhibitor SB505124 (SB; 2.5 μM).**(c)** Luciferase assays from PHH transfected with SBE-driven Luc-reporter vectors, stimulated with TGF-β1 (1 ng/mL), and treated with either SB (2.5 μM) or REV-ERBα agonist SR9009 (20 μM).

(d) Relative expression of indicated genes in PHH treated with TGF-β1 (1 ng/mL) and SR9009 (20 μM).

(e) WB analysis of indicated proteins from PHH treated with TGF-β1 (1 ng/mL) and SR9009 (20 μM).

(f) Representative epifocal images of αSMA- and pSMAD2/3-stained HLMF treated with TGF-β1 (1ng/ml), and either SB (2.5 μM) or SR9009 (20 μM).

(g, **h)** Relative expression of indicated genes in PHH following siRNA-mediated knock-down of *REV-ERBα*, and treatment with either TGF-β1 (g) or FFA (h).

(i) Heat map showing significantly ($p<0.05$) modified expression of transcripts of Hallmark fibrotic genes in HLMF treated with FFA and/or SR9009 (20 μM).

(j) GSEA analyses of transcripts belonging to either inflammatory or TGF-β-regulated genes in HLMF treated, as indicated.

(k) Relative expression of indicated genes in HLMF following treatment with FFA in presence or absence of SR9009.

All data are expressed as mean ±SEM. n=3-4. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Fig. 6. Expression of CC-genes are perturbed in NASH patients.

(a) Transcript levels of indicated CC-genes in NAFLD patients with mild fibrosis (F0-2; n=40) and advanced fibrosis (F3-4; n=32) (GSE49541)[53].

(b) Transcript levels of indicated CC-genes in healthy controls (n=24), and patients with either simple steatosis (n=20) or NASH (n=19) (GSE89632)[54].

(c) Transcript levels of indicated CC-genes in indicated groups categorized as per fibrotic stages and lobular inflammation (GSE89632)[54].

(d) Schematic of spheroids generated from healthy human liver and treatment with TGF-β in presence or absence of SR9009.

(e) Relative expression of indicated genes in spheroids derived from healthy human liver and treated as indicated.

(f) Schematic of spheroids generated from cirrhotic human liver and treatment with SR9009.

(g) Relative expression of indicated genes in spheroids derived from cirrhotic human liver and following SR9009 (20 μM) treatment.

Extended Data Fig. 1: Fibrotic gene network is CC-controlled in liver.

(a) RNA transcripts (qRT-PCR) measuring circadian expression pattern of indicated genes in the liver of control (WT) and $Bmal1^{hep-/-}$ mice.

(b) UCSC genome browser view of circadian chromatin recruitment pattern of BMAL1 and CLOCK to the *Ski1* gene in the liver of control mice, as revealed by ChIP-sequencing. Each track represents the normalized ChIP-seq read coverage at the single time point. For both BMAL1 and CLOCK, six time points with an interval of 4 h are shown. The arrowhead indicates the transcriptional start site of *Ski1* gene. The boxed area indicates chromatin recruitment region of BMAL1 and CLOCK.

(c) UCSC genome browser view of circadian active transcription of the Smad7 gene in liver of control mice. Global run-on sequencing (GRO-seq) was performed for 7 circadian time-points with an interval of 3 h. The blue tracks represent the reads from the sense (+) strand, while the reads from the anti-sense (-) strand is shown in red.
(d) Quantification of the WB analyses performed (Fig. 1F) at ZT0 and ZT12 from mice liver.
(e) ChIP-qPCR assays to the SBE-region present in indicated genes using liver of WT and $Bmal1^{hep-/-}$ mice, and IgG, SMAD2/3, SMAD4 and PolII antibodies.
All data are expressed as mean ±SEM. *p<0.05, **p<0.01, ***p<0.001. Extended Data Fig. 1a,e were performed from the same control and $Bmal1^{hep-/-}$ mice.

Extended Data Fig. 2: Progression of CD-HFD-induced liver disease.
(a) RNA transcripts measuring circadian expression pattern of indicated CC-component gene E4BP4 in the liver of control and CD-HFD fed mice.
(b, c) ChIP-qPCR assays to the SBE-region present in *Tgfrβr1* (b) and *αSma* (c) genes using liver of mice that were fed either control (chow) diet or CD-HFD for indicated time-periods, and IgG, SMAD2/3, SMAD4 and PolII antibodies. Fig. 2d-f were performed from same mice as an independent experiment.

Extended Data Fig. 3: Fibrotic gene expression is rhythmic in human liver cells *in vivo*.
(a, b) Circadian heatmap analyses to identify maximal expression pattern of genes constituting HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION (a), and HALLMARK_TGF_BETA_SIGNALING (b) pathways in the human liver cells obtained from humanized mice. Gene sets have been obtained from The Molecular Signatures Database (MSigDB)[28].

Extended Data Fig. 4: FFA-induces TGF-β signaling and CC-perturbation in primary human cells.
(a, **b, c)** Relative expression of CC-components (a) and fibrotic (b) and inflammatory genes (c) following FFA treatment of HLMF.
(d, e) Kinetics of changes in relative expression of indicated CC-components and key fibrotic genes in HLMF (d) and PHH (e) following FFA treatment.
(f) Quantification of the WB analyses performed (Fig. 3d) from FFA-treated PHH.
(**g**) Relative expression of indicated CC-component gene *PER1* from dexamethasone-synchronized PHH following FFA treatment.

(h) Relative expression of *RORα* and *RORγ* in PHH and HLMF.

(i) Relative expression of indicated genes in PHH following FFA and TGF-β receptor inhibitor SB505124 (SB; 2.5 µM) treatment.

(j) ChIP-qPCR assay of RORγ, REV-ERBα and PolII binding to the RORE present in the Bmal1-luc plasmid transfected in PHH and treated with FFA and SB.

(k) ChIP-qPCR assay to the SBE-region present in *TWIST2* gene from PHH and using IgG, SMAD2/3, SMAD4 and PolII antibodies, following siRNA-mediated knock-down of *SMAD2* and treatment as indicated.

(l, m) ChIP-qPCR assays to the E-box-region present in *REV-ERBα* (l) and *DBP* (m) genes from PHH and using IgG, BMAL1 and CLOCK antibodies, following siRNA-mediated knock-down of BMAL1, and treatments as indicated.

(n) ChIP-qPCR assay to the SBE-region present in *TWIST2* gene from PHH and using IgG, SMAD2/3, SMAD4 and PolII antibodies, following siRNA-mediated knock-down of *BMAL1* and treatment as indicated.

Extended Data Fig. 5: The molecular crosstalk between CC and TGF-β pathway.

(**a**) Relative expression of indicated genes in HLMF following TGF-β (1 ng/ml) stimulation in presence or absence of TGF-β receptor inhibitor SB505124 (SB; 2,5 µM).

(**b**) Quantification of the WB analyses performed (Fig. 4e) in PHH.

(**c**) Relative expression of indicated genes in HLMF following siRNA-mediated knock-down of *REV-ERBα*, and TGF-β treatment.

(**d**) Relative expression of indicated genes in PHH following treatment with FFA in presence or absence of SR9009.

Fig. 1

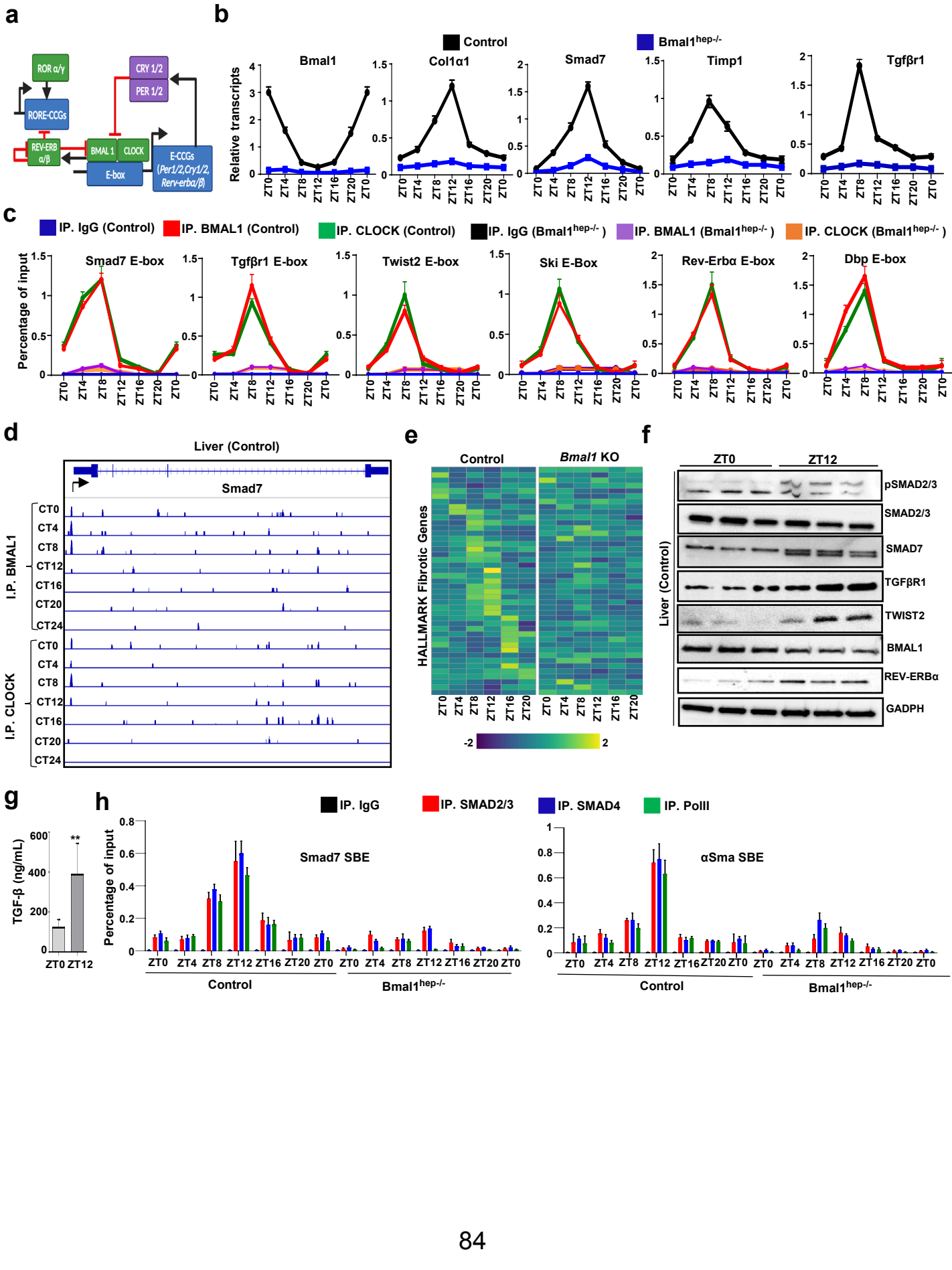

a
ROR α/γ
RORE-CCGs
REV-ERB α/β
BMAL 1
CLOCK
E-box
CRY 1/2
PER 1/2
E-CCGs (Per1/2,Cry1/2, Rerv-erbα/β)
b
Control
Bmal1hep-/-
Bmal1
Col1α1
Smad7
Timp1
Tgfβr1
Relative transcripts
c
IP. IgG (Control)
IP. BMAL1 (Control)
IP. CLOCK (Control)
IP. IgG (Bmal1hep-/-)
IP. BMAL1 (Bmal1hep-/-)
IP. CLOCK (Bmal1hep-/-)
Smad7 E-box
Tgfβr1 E-box
Twist2 E-box
Ski E-Box
Rev-Erbα E-box
Dbp E-box
Percentage of input
d
Liver (Control)
Smad7
I.P. BMAL1
I.P. CLOCK
e
Control
Bmal1 KO
HALLMARK Fibrotic Genes
f
ZT0
ZT12
Liver (Control)
pSMAD2/3
SMAD2/3
SMAD7
TGFβR1
TWIST2
BMAL1
REV-ERBα
GADPH
g
TGF-β (ng/mL)
h
IP. IgG
IP. SMAD2/3
IP. SMAD4
IP. PolII
Smad7 SBE
αSma SBE
Percentage of input
Control
Bmal1hep-/-

Fig. 2

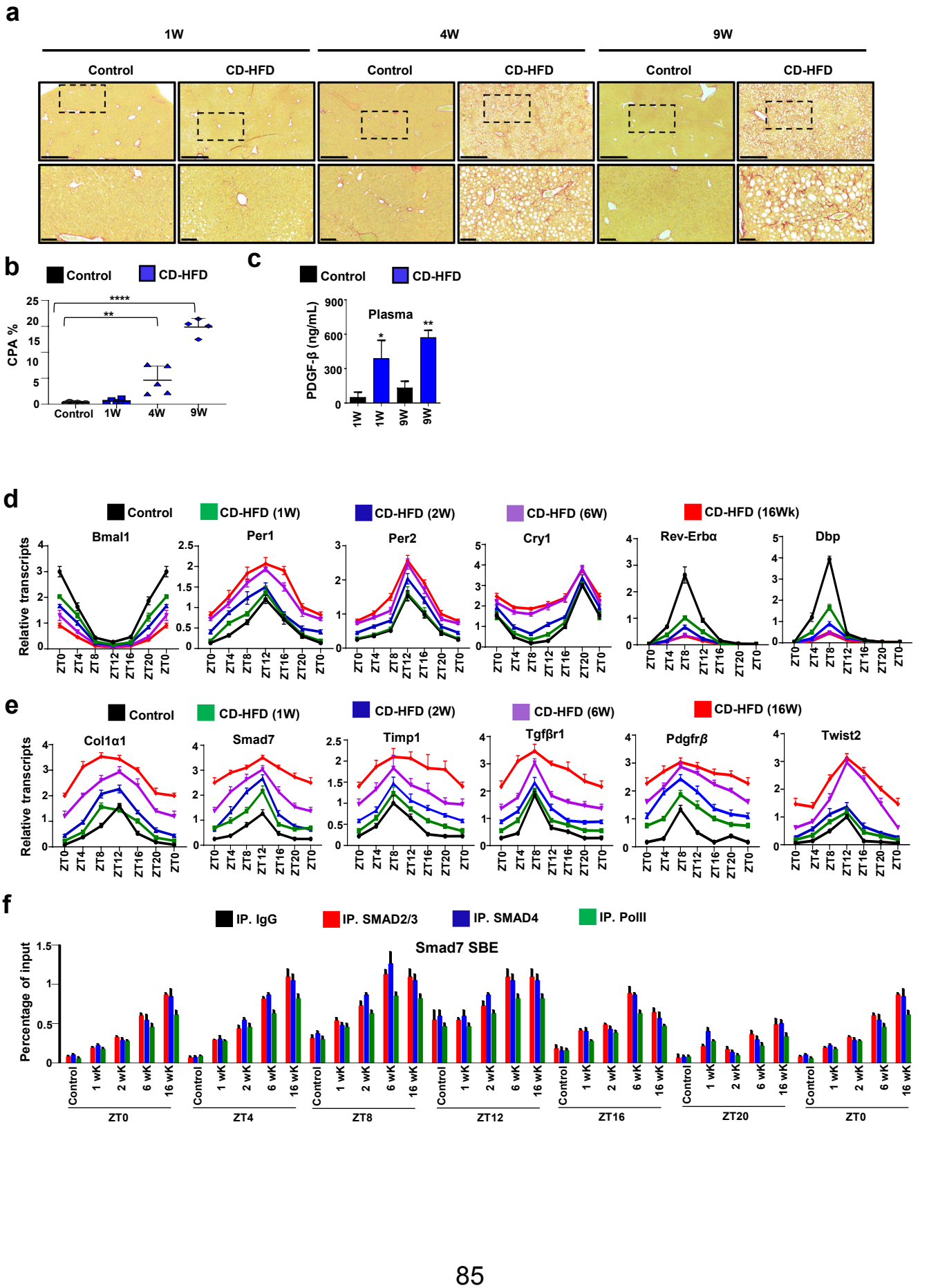

a
1W
4W
9W
Control
CD-HFD
b
Control
CD-HFD
CPA %
****
**
Control
1W
4W
9W
c
Control
CD-HFD
Plasma
PDGF-β (ng/mL)
*
**
1W
1W
9W
9W
d
Control
CD-HFD (1W)
CD-HFD (2W)
CD-HFD (6W)
CD-HFD (16Wk)
Bmal1
Per1
Per2
Cry1
Rev-Erbα
Dbp
Relative transcripts
ZT0
ZT4
ZT8
ZT12
ZT16
ZT20
ZT0
e
Control
CD-HFD (1W)
CD-HFD (2W)
CD-HFD (6W)
CD-HFD (16W)
Col1α1
Smad7
Timp1
Tgfβr1
Pdgfrβ
Twist2
Relative transcripts
f
IP. IgG
IP. SMAD2/3
IP. SMAD4
IP. PolII
Smad7 SBE
Percentage of input
Control
1 wK
2 wK
6 wK
16 wK
ZT0
ZT4
ZT8
ZT12
ZT16
ZT20
ZT0

Fig. 3

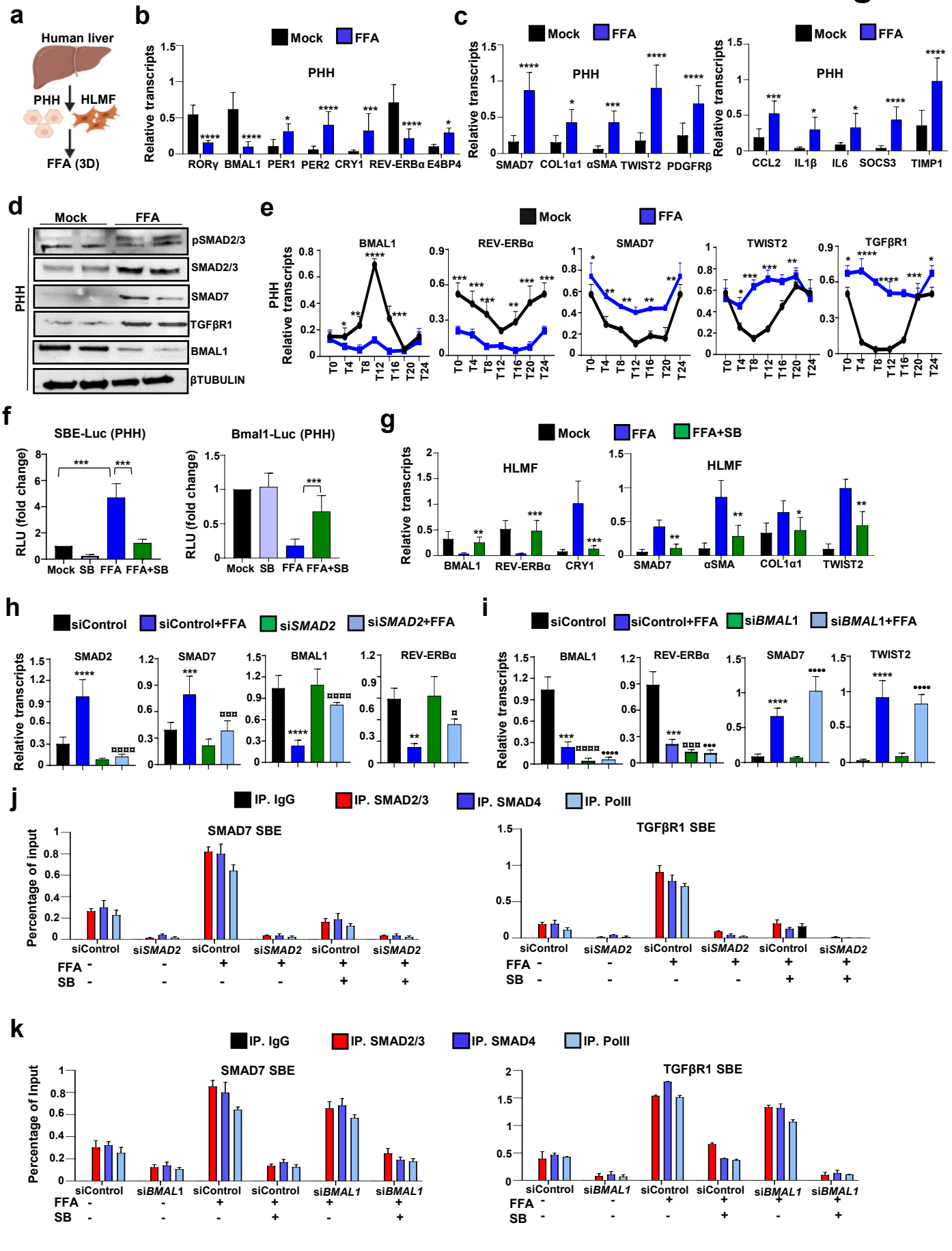

a
Human liver
PHH
HLMF
FFA (3D)
b
Mock
FFA
PHH
Relative transcripts
RORγ
BMAL1
PER1
PER2
CRY1
REV-ERBα
E4BP4
c
Mock
FFA
PHH
Relative transcripts
SMAD7
COL1α1
αSMA
TWIST2
PDGFRβ
CCL2
IL1β
IL6
SOCS3
TIMP1
d
Mock
FFA
PHH
pSMAD2/3
SMAD2/3
SMAD7
TGFβR1
BMAL1
βTUBULIN
e
Mock
FFA
PHH
Relative transcripts
BMAL1
REV-ERBα
SMAD7
TWIST2
TGFβR1
T0
T4
T8
T12
T16
T20
T24
f
SBE-Luc (PHH)
Bmal1-Luc (PHH)
RLU (fold change)
Mock
SB
FFA
FFA+SB
g
Mock
FFA
FFA+SB
HLMF
Relative transcripts
BMAL1
REV-ERBα
CRY1
SMAD7
αSMA
COL1α1
TWIST2
h
siControl
siControl+FFA
siSMAD2
siSMAD2+FFA
Relative transcripts
SMAD2
SMAD7
BMAL1
REV-ERBα
i
siControl
siControl+FFA
siBMAL1
siBMAL1+FFA
Relative transcripts
BMAL1
REV-ERBα
SMAD7
TWIST2
j
IP. IgG
IP. SMAD2/3
IP. SMAD4
IP. PolII
SMAD7 SBE
TGFβR1 SBE
Percentage of input
siControl
siSMAD2
FFA
SB
k
IP. IgG
IP. SMAD2/3
IP. SMAD4
IP. PolII
SMAD7 SBE
TGFβR1 SBE
Percentage of Input
siControl
siBMAL1
FFA
SB

Fig. 4

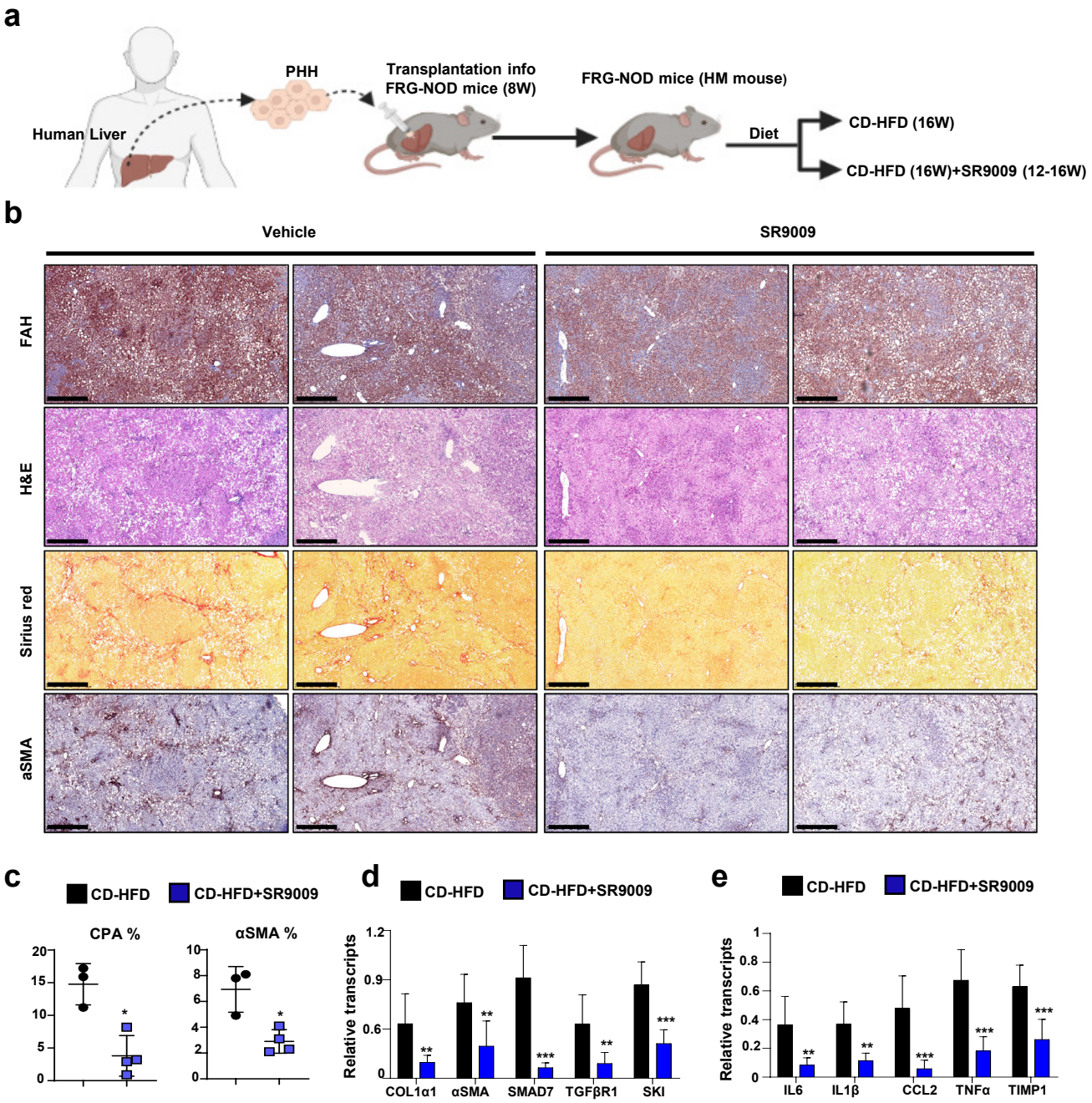

a
Human Liver
PHH
Transplantation info
FRG-NOD mice (8W)
FRG-NOD mice (HM mouse)
Diet
CD-HFD (16W)
CD-HFD (16W)+SR9009 (12-16W)
b
Vehicle
SR9009
FAH
H&E
Sirius red
aSMA
c
CD-HFD
CD-HFD+SR9009
CPA %
αSMA %
d
CD-HFD
CD-HFD+SR9009
Relative transcripts
COL1α1
αSMA
SMAD7
TGFβR1
SKI
e
CD-HFD
CD-HFD+SR9009
Relative transcripts
IL6
IL1β
CCL2
TNFα
TIMP1

Fig. 5

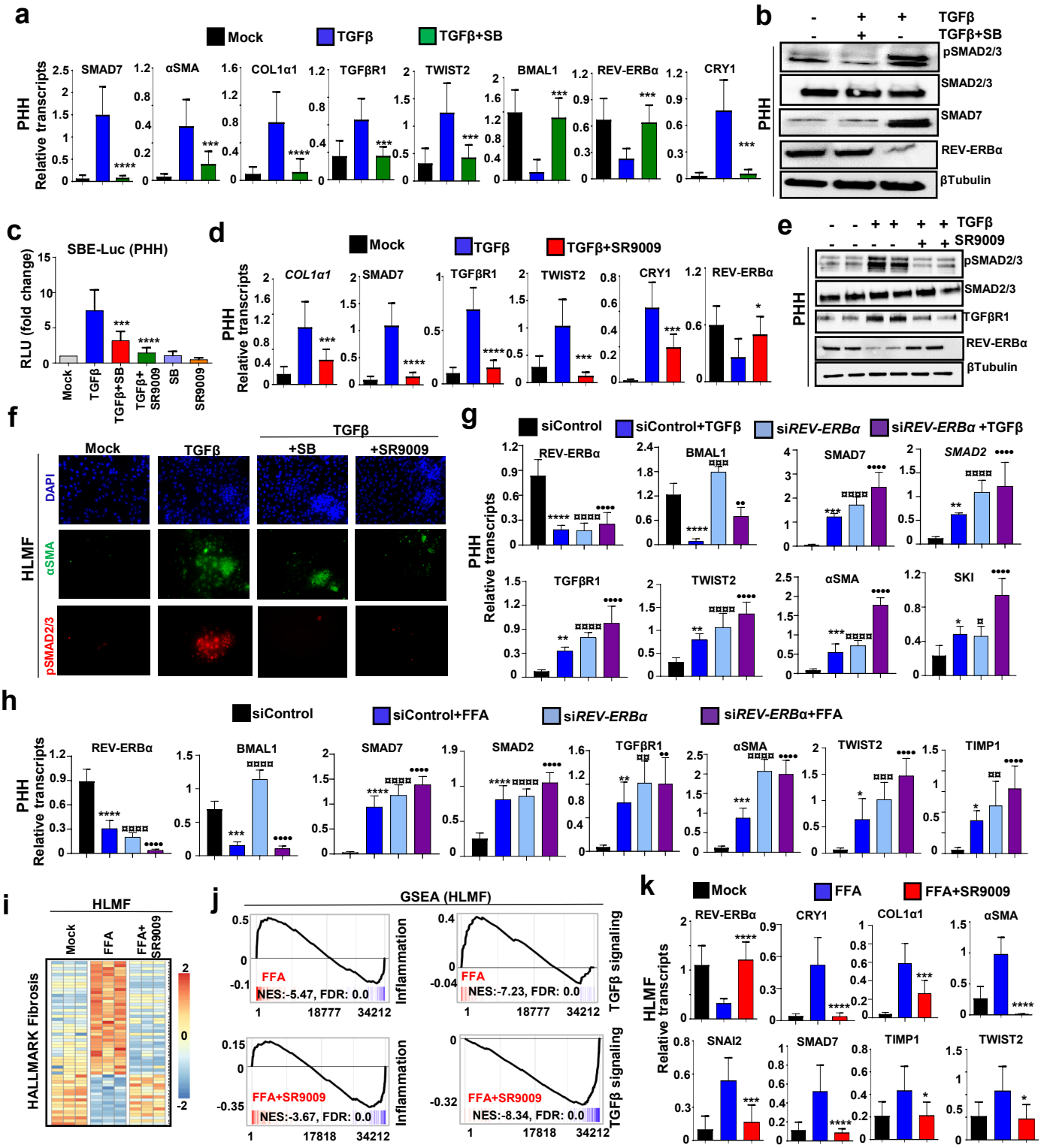
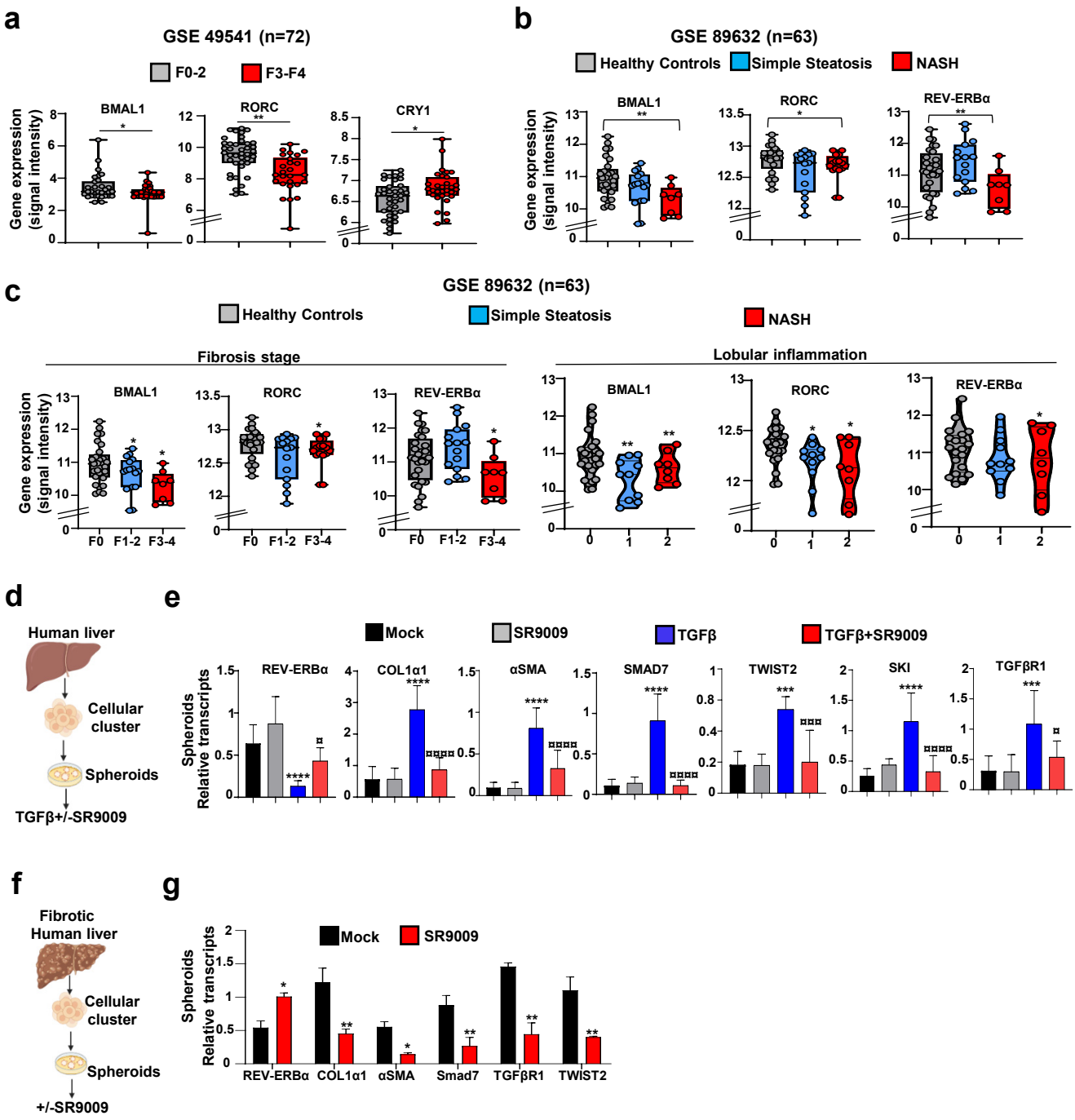
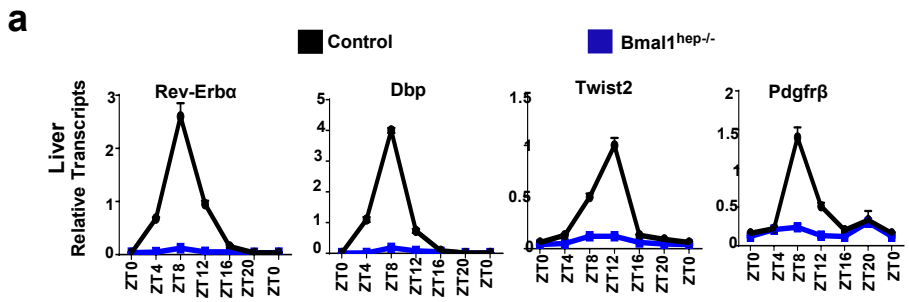
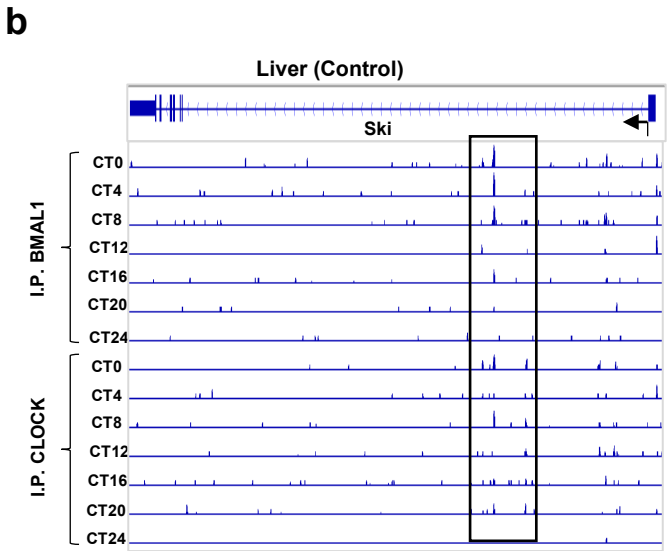

a
Mock
TGFβ
TGFβ+SB
PHH
Relative transcripts
SMAD7
αSMA
COL1α1
TGFβR1
TWIST2
BMAL1
REV-ERBα
CRY1
b
TGFβ
TGFβ+SB
PHH
pSMAD2/3
SMAD2/3
SMAD7
REV-ERBα
βTubulin
c
SBE-Luc (PHH)
RLU (fold change)
Mock
TGFβ
TGFβ+SB
TGFβ+ SR9009
SB
SR9009
d
Mock
TGFβ
TGFβ+SR9009
PHH
Relative transcripts
COL1α1
SMAD7
TGFβR1
TWIST2
CRY1
REV-ERBα
e
TGFβ
SR9009
PHH
pSMAD2/3
SMAD2/3
TGFβR1
REV-ERBα
βTubulin
f
TGFβ
Mock
TGFβ
+SB
+SR9009
HLMF
DAPI
αSMA
pSMAD2/3
g
siControl
siControl+TGFβ
siREV-ERBα
siREV-ERBα +TGFβ
PHH
Relative transcripts
REV-ERBα
BMAL1
SMAD7
SMAD2
TGFβR1
TWIST2
αSMA
SKI
h
siControl
siControl+FFA
siREV-ERBα
siREV-ERBα+FFA
PHH
Relative transcripts
REV-ERBα
BMAL1
SMAD7
SMAD2
TGFβR1
αSMA
TWIST2
TIMP1
i
HLMF
Mock
FFA
FFA+ SR9009
HALLMARK Fibrosis
j
GSEA (HLMF)
FFA
NES:-5.47, FDR: 0.0
Inflammation
FFA
NES:-7.23, FDR: 0.0
TGFβ signaling
FFA+SR9009
NES:-3.67, FDR: 0.0
Inflammation
FFA+SR9009
NES:-8.34, FDR: 0.0
TGFβ signaling
k
Mock
FFA
FFA+SR9009
HLMF
Relative transcripts
REV-ERBα
CRY1
COL1α1
αSMA
SNAI2
SMAD7
TIMP1
TWIST2

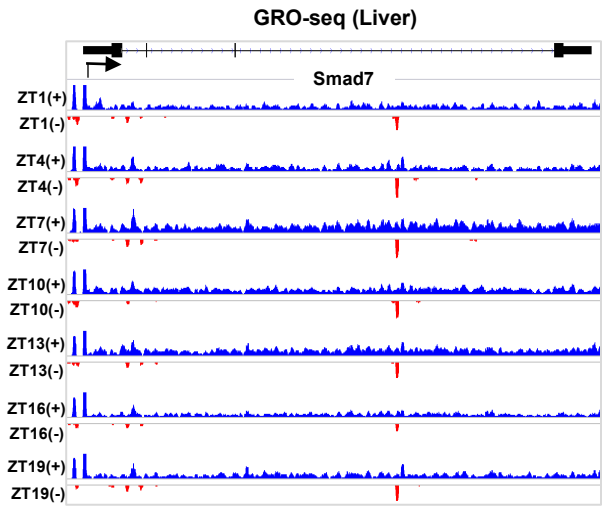
Fig. 6

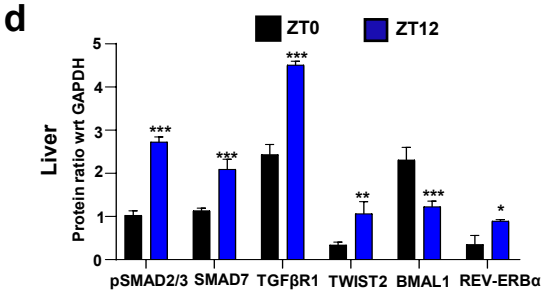

a
GSE 49541 (n=72)
F0-2
F3-4
BMAL1
RORC
CRY1
Gene expression (signal intensity)
b
GSE 89632 (n=63)
Healthy Controls
Simple Steatosis
NASH
BMAL1
RORC
REV-ERBα
c
GSE 89632 (n=63)
Healthy Controls
Simple Steatosis
NASH
Fibrosis stage
Lobular inflammation
BMAL1
RORC
REV-ERBα
F0
F1-2
F3-4
d
Human liver
Cellular cluster
Spheroids
TGFβ+/-SR9009
e
Mock
SR9009
TGFβ
TGFβ+SR9009
Spheroids Relative transcripts
REV-ERBα
COL1α1
αSMA
SMAD7
TWIST2
SKI
TGFβR1
f
Fibrotic Human liver
Cellular cluster
Spheroids
+/-SR9009
g
Mock
SR9009
Spheroids Relative transcripts
REV-ERBα
COL1α1
αSMA
Smad7
TGFβR1
TWIST2

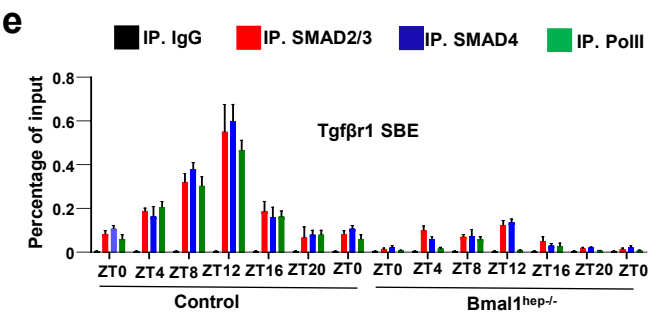
Extended Data Fig. 1



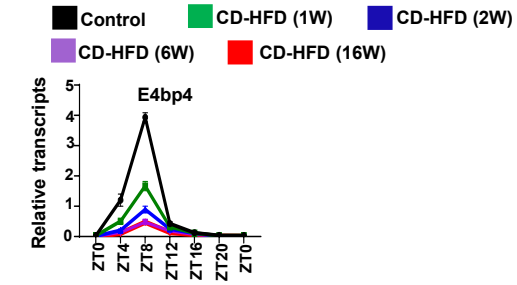


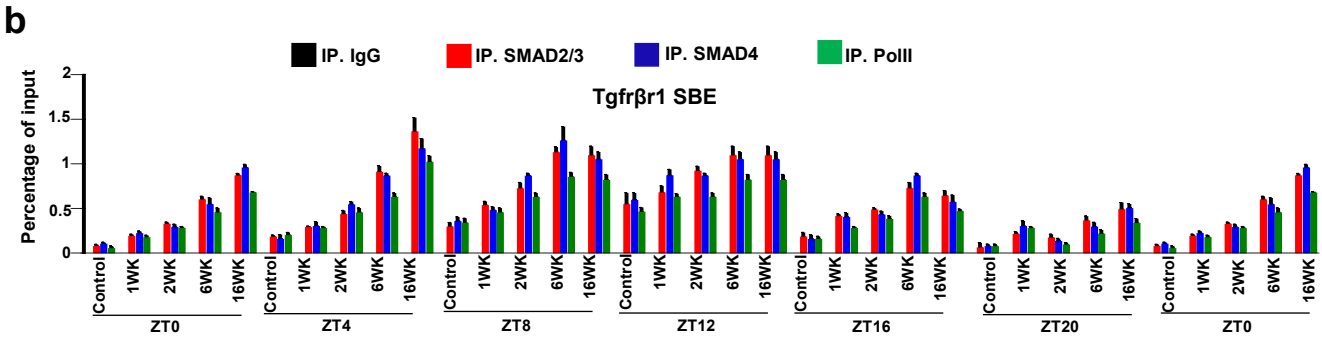


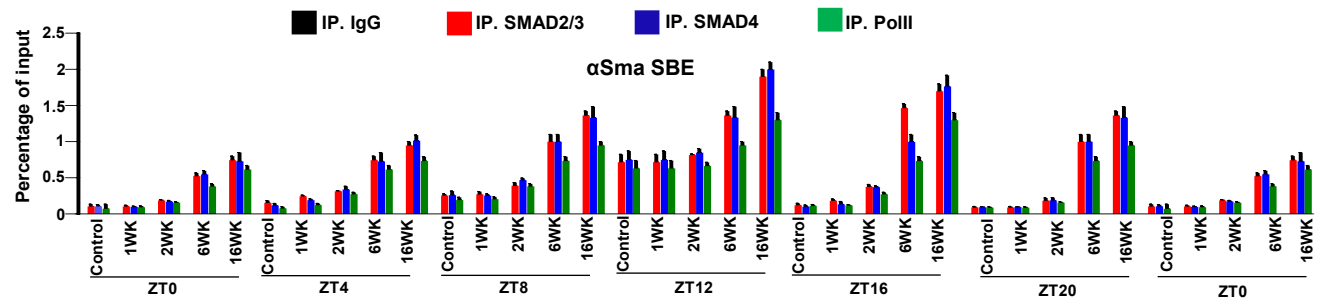




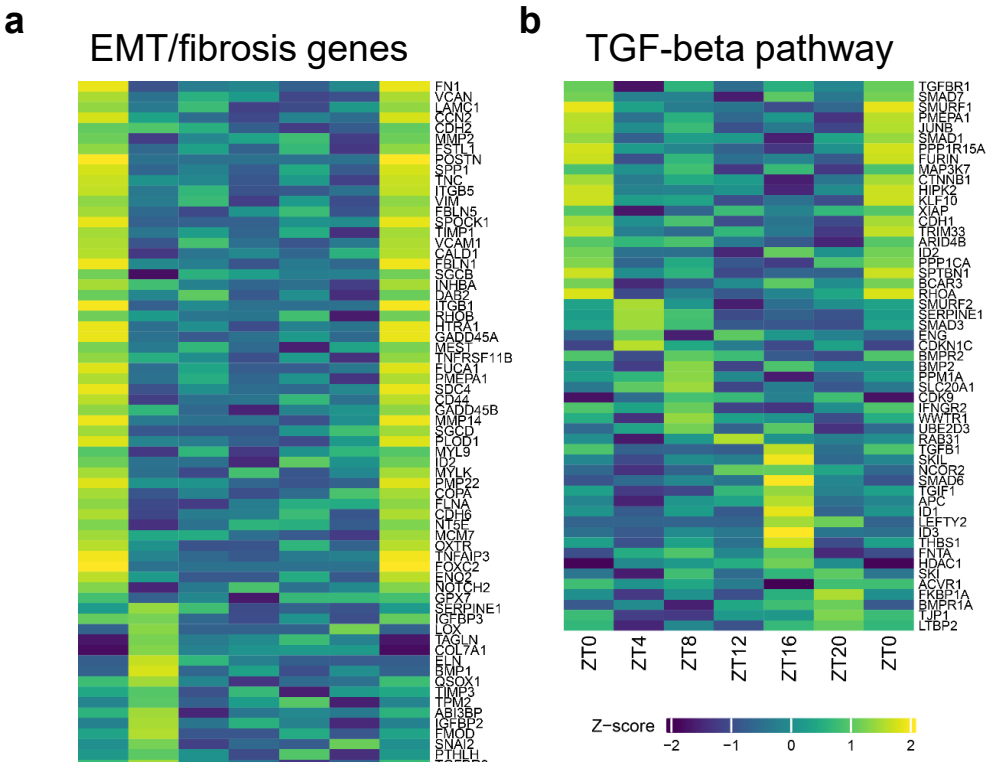
Extended Data Fig. 2



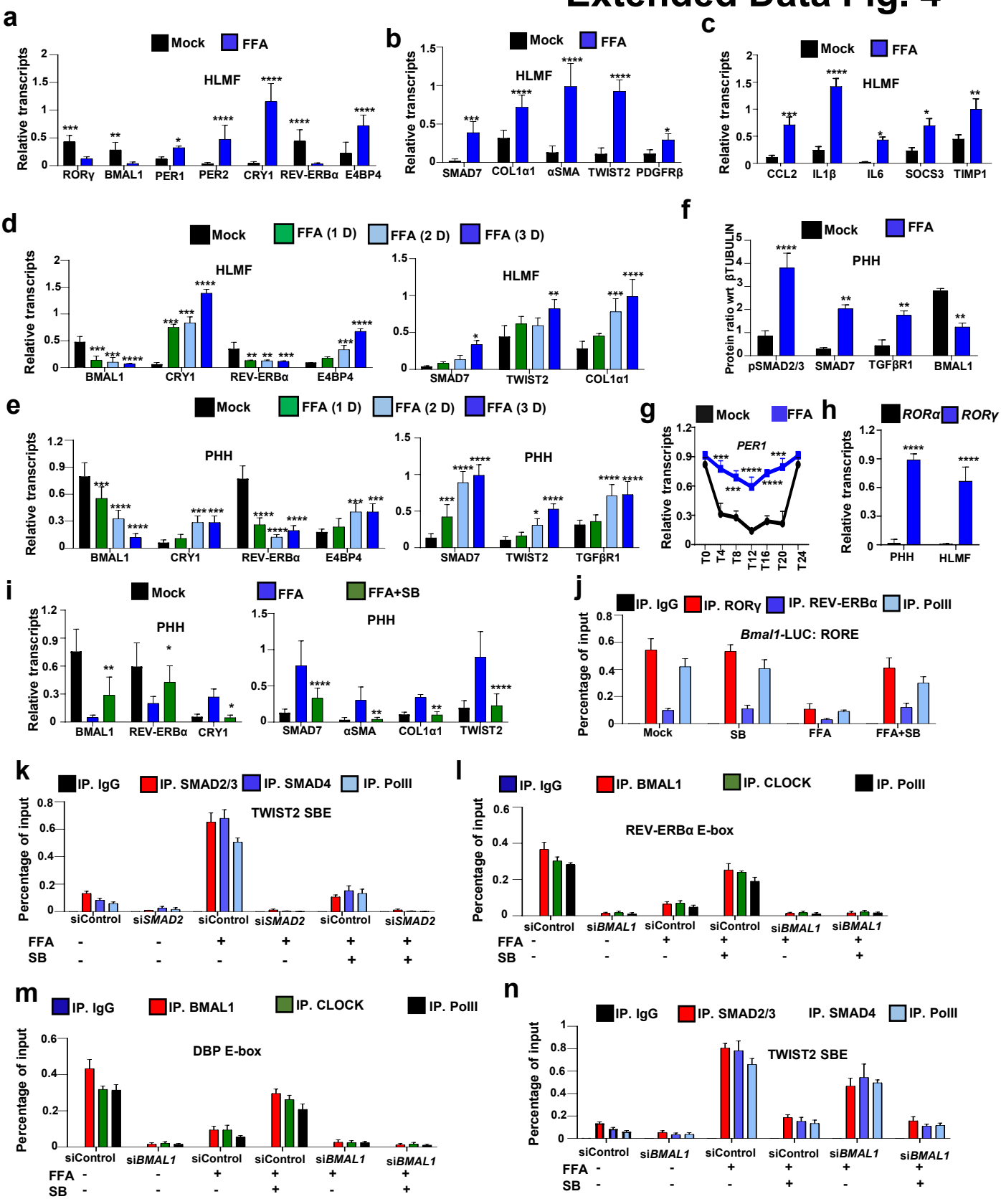




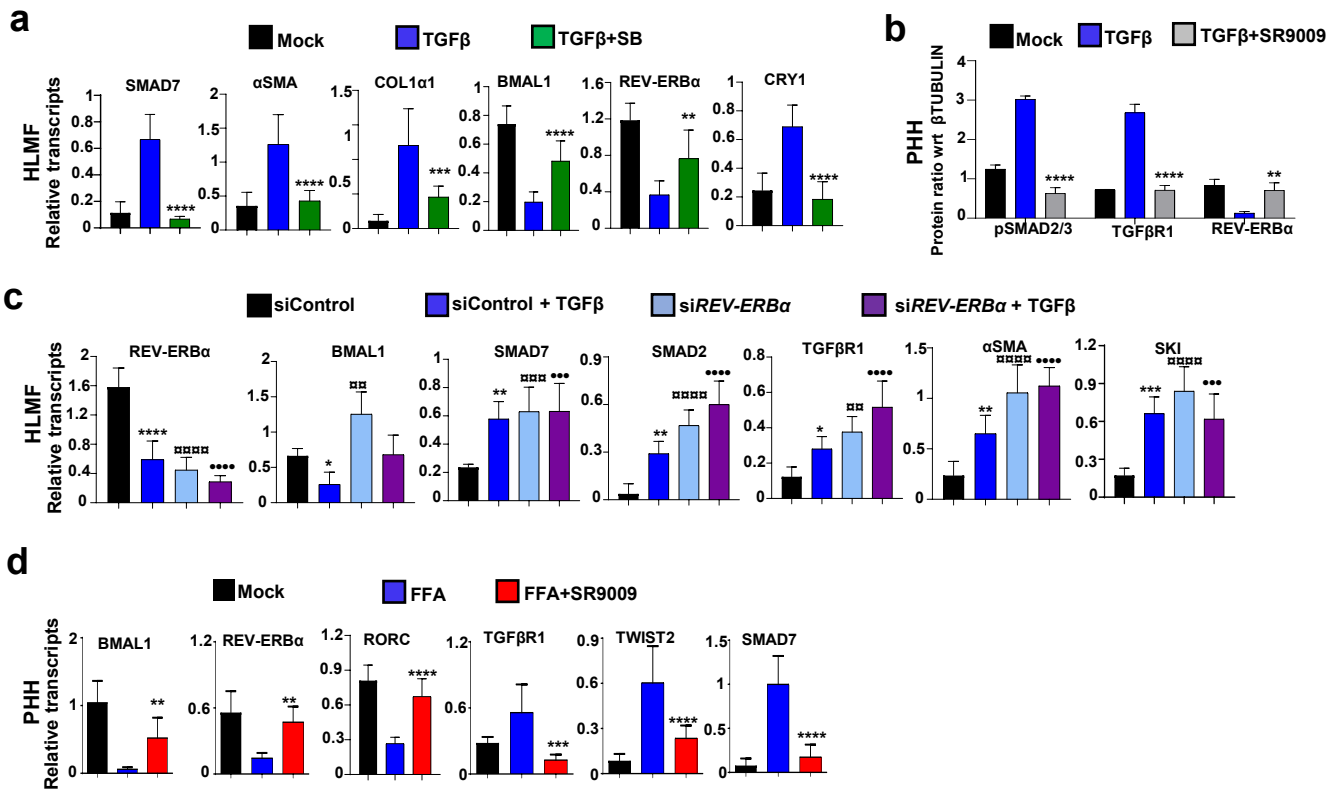
Extended Data Fig. 3



Extended Data Fig. 4



Extended Data Fig. 5



DISSCUSSION

In this study, I revealed that under physiological conditions the hepatic CC transcriptionally regulates the expression of key genes linked to TGF-β signaling and fibrosis development. Moreover, I demonstrated that in the CD-HFD-induced mouse models of NASH and fibrosis the CC function is severely perturbed, which led to increased expression levels of pro-fibrotic genes and elevated TGF-β secretion. Importantly, pharmacological re-activation of the CC-component REV-ERBα by a small molecule "SR9009" robustly and significantly improved liver health by suppressing inflammation and fibrosis in several state-of-the-art patients-derived disease models and humanized mice. Altogether, the results demonstrate that pharmacological targeting of CC function holds the potential for a new concept of anti-fibrotic therapies.

TGF-β-signaling is evolutionary conserved and well known to regulate gene expression in both SMAD-dependent and SMAD-independent manners from embryonic development to maintaining organ physiology (Caja et al., 2018; Roehlen et al., 2020). Importantly, deregulation of TGF-β activity is one of the most prominent hallmarks of organ fibrosis and cancer (Derynck & Zhang, 2003; Fabregat et al., 2016). Mechanistic studies in mouse models have demonstrated that the reduction of elevated TGF-β-driven gene expression prevents and ameliorates organ fibrosis including the liver (Bataller & Brenner, 2005). Little is known about which mechanisms control TGF-β signaling to prevent its constitutive activation in the liver.

Here, we discovered that the liver CC oscillator regulates the expression of TGF-β signaling-dependent expression of fibrotic genes. This was conclusively shown by *Bmal1* loss of function studies (Figure 1). The CC control on TGF-β signaling, in turn, dictates that TGF-β-activated transcription factors, i.e., SMADs bind to the SBEs present in their cognate genes only for a limited duration in the rest phase (Figure 1). Therefore, in the liver under physiological condition TGF-β-regulated gene expression is 'circadian gated'. Interestingly, in different peripheral tissues, CC oscillator has been previously found to temporally restrict critical signaling pathway-controlled transcriptional events such as the intestinal CC diurnally controlling microbiota-activated, TLR-induced NF-κB- and AP-1-dependent transcription (Mukherji et al., 2013). Similarly, the transcriptional activity of BMAL1:CLOCK in the pancreas 'times 'insulin signaling (Marcheva et al., 2010), and in liver CC component CRY1 gates cAMP-CREB signaling at the end of rest-phase (Zhang et al., 2010). Collectively, these studies show that the absence of CC-control on signaling events and corresponding transcriptional activity drives metabolic pathologies (Marcheva et al., 2010; Mukherji et al., 2013; E. Zhang et al., 2010). CC functioning and metabolism are intricately linked. While the CC oscillator is normally self-sustained, metabolic alterations selectively modulate the expression or activity of specific CC components (Eckel-Mahan et al., 2013; Mukherji et al., 2015, 2019). Here, we found that metabolic liver injury impairs the expression of CC components in a time-dependent manner (Figure 2e) resulting

in increased expression of TGF-β-related fibrotic genes (Figure 2f). We discovered that, in contrast to the healthy state, where TGF-β activity is CC-gated, in a disease state the increase of fibrotic gene expression arises from constitutive chromatin binding of transcription factors SMAD2/3 and SMAD4 (compare Figure 1h with Figure 2g and Extended Data Figures 2e, f). This molecular event of permanent (circadian 'non'-gated) SMAD-binding to the promoter/enhancer regions of fibrotic genes underlies elevated TGF-β-signaling driven liver fibrosis. Our pharmacological and knock-down experiments demonstrate that in PHH and HLMF, elevated TGF-β signaling directly impairs the expression of key CC genes including *BMAL1* and *REV-ERBα* (Figure 3), and *REV-ERBα* inhibition increased basal TGF-β activity (Figure 5), revealing a "cross-talk" between the CC and TGF-β signaling.

Exploiting these mechanistic discoveries, we demonstrate that targeting REV-ERBα by a small molecule improves liver fibrosis in patient-derived *in vivo* and *ex vivo* models. We chose REV-ERBα as: (i) its level was reduced during fibrosis and elevated TGF-β signaling (Figures 2e, 3b, e, and 5a), (ii) it is a global regulator of hepatic metabolism, and (iii) specific agonists for REV-ERBα are available (SR9009), which can be safely administered in mice. A key strength of our study is the validation of results in authentic patient-derived model systems including patient-derived animal models, spheroids, and primary cells, the consistency of results across complementary model systems and patient cohorts supporting its clinical validity, and translatability into the clinic.

Targeting REV-ERBα for the treatment of liver fibrosis is a novel, effective and differentiated concept. The large majority of liver disease therapeutics target metabolism, inflammation, or cell death, which are relevant in the early stage of the disease. Only a few compounds with anti-fibrotic properties have entered clinical development with only limited efficacy while displaying considerable safety issues (Vuppalanchi et al., 2021). Moreover, as shown recently for GLP1 analogs (Newsome et al., 2021), robust improvement of steatosis alone does not necessarily induce improvement of fibrosis. A key differentiator of the REV-ERBα-targeting approach is the combination of anti-inflammatory and anti-fibrotic efficacy as demonstrated across all models (Figures 4-6), which addresses a key unmet medical need in the treatment of liver fibrosis.

Our data obtained here and in previous studies (Ni et al., 2021; Solt et al., 2012) demonstrates that the administration of SR9009 is safe without detectable major adverse effects in animal models. Interestingly, SR9009 has also been suggested to ameliorate liver fibrosis by improving the gut barrier function (Ni et al., 2021). More importantly, a recent study has shown the beneficial effects of REV-ERB activation that led to an overall improvement of hepatic health by suppressing hepatic fibrosis and inflammatory response (Griffett et al., 2020). Furthermore, targeting REV-ERBα has also been shown to improve lung fibrosis in animal models (Cunningham et al., 2020). Nevertheless, further studies are needed to fully investigate the compounds' safety profile. Furthermore, since our preclinical models are focused on NASH as a liver injury, additional studies would be required to show efficacy and impact also in fibrosis of other etiologies such as viral hepatitis and alcoholic liver disease. Given the preclinical data of our study, the target population for REV-ERBα -targeting therapies will be patients with NASH

fibrosis including F3/F4 advanced fibrosis as shown by proof-of-concept in spheroids from cirrhotic patients (Figure. 6g).

The circadian clock not only controls metabolism pathways but is also interwoven with them. More importantly, a circadian viewpoint on metabolic control is important not only for the chrono-biologists but also for researchers with expertise in metabolism and physiology, because taking this standpoint in consideration will be useful to unravel several aspects of metabolic regulation that have been overlooked so far. We suggest that the crucial first step in uncovering the complexity of these mechanisms lies in the assemblage of circadian datasets across at-risk populations, using increasingly available cellular, transcriptomics, and metabolomics-based methods coupled with genetics. Importantly, these studies will promote the identification of specific clock interactions with metabolic and auto-immune diseases. A second step would then be the application of clock-modifying compounds to accomplish positive consequences on metabolism, intentionally harnessing circadian mechanisms to influence diverse cellular signaling pathways. Collectively, these approaches could promise a new therapeutic avenue approach to improve human health in increasingly busy lives.

Ciblage de l'horloge circadienne-hépatique pour le traitement des maladies métaboliques du foie

1. Introduction

La fibrose représente jusqu'à 45% des décès dans les pays développés. Elle conduit à une distorsion de l'architecture tissulaire et à une perte de fonction des organes (Rockey et al., 2015)). De plus, la fibrose représente un facteur de risque majeur pour le développement de tumeurs dans tous les organes. Pourtant, les thérapies approuvées pour prévenir ou guérir la fibrose sont soit absentes ou soit limitées en termes d'efficacité, ceci est le cas pour la fibrose hépatique. L'une des raisons de l'absence de thérapies anti-fibrosantes efficaces est le fait que les circuits cellulaires à l'origine de la biologie de la maladie ne sont pas entièrement étudiés (Roehlen et al., 2020). Parmi les principales causes de fibrose hépatique on trouve l'hépatite virale et la maladie hépatique alcoolique et la stéatohépatite non alcoolique (NASH). Quel que soit l'étiologie, la fibrose hépatique évolue en cirrhose et pour une proportion non négligeable en carcinome hépatocellulaire (CHC) qui représente la deuxième cause de décès par cancer dans le monde (Hagström et al., 2017). Il est à noter que le CHC survient très souvent dans le contexte d'une fibrose hépatique ou d'une cirrhose, démontrant le rôle essentiel de la fibrose en tant que déclencheur de l'hépatocarcinogenèse. En raison de sa prévalence élevée et de sa forte incidence, la stéatose hépatique non alcoolique (NAFLD) est une cause majeure de fibrose hépatique dans le monde. La NALFD affecte 25 à 30 % de la population générale et sa prévalence pourrait atteindre 70 à 90 % dans des populations spécifiques présentant des comorbidités telles que l'obésité ou le diabète de type 2 (Polyzos et al., 2019; Younossi et al., 2016). Alors que les patients atteints de fibrose à un stade précoce F1 et F2 sont généralement asymptomatiques, les stades avancés de la fibrose F3 à F4 sont associés de manière significative à la mortalité globale. En effet, la mortalité globale est 3 fois plus élevée chez les patients atteints de fibrose F3-F4 que chez personnes saines. C'est donc le stade de la fibrose et non de la stéatose qui est directement lié à la mortalité globale chez les patients atteints de NAFLD (Angulo et al., 2015).

L'horloge circadienne (HC) joue un rôle clé dans le métabolisme du foie. Au cœur du fonctionnement de l'horloge, réside l'oscillateur de l'HC, un système de rétroaction transcriptionnelle-traductionnelle magnifiquement conçu (Asher & Sassone-Corsi, 2015; Asher & Schibler, 2011; Mukherji et al., 2019). D'une façon remarquable, les composants de l'oscillateur HC maintiennent non seulement la rythmicité quotidienne de leur propre synthèse, mais génèrent également une variabilité temporelle dans les niveaux d'expression de nombreux gènes cibles par le biais de mécanismes transcriptionnels, post-transcriptionnels et post-traductionnels, assurant ainsi une bonne coordination chronologique dans le fonctionnement des cellules, des tissus et des organes (Asher & Sassone-Corsi, 2015; Asher & Schibler, 2011; Mukherji et al., 2019). La perturbation fonctionnelle de l'HC est associée à diverses pathologies, notamment les maladies métaboliques et le cancer.

Buts de l'étude

Le foie exprime de manière circadienne un ensemble de gènes d'une manière circadienne, et les fonctions des gènes sont de ce fait régulés directement ou indirectement par l'HC. Un faisceau croissant d'évidence suggère que l'altérations de l'HC impactent la progression de la maladie du foie. Cependant, la relation entre l'expression génique contrôlée par l'HC et le développement de la fibrose chez les patients NASH/NAFLD étaient inconnus au début de ce travail de thèse. Nous avons engagé des études de perturbations de l'expression des gènes de l'HC dans des modèles murins de NASH classiques ou humanisées, des sphéroïdes hépatiques humains, et des cellules primaires en culture qui ont montré que l'HC hépatique régule l'expression de gènes clés intervenant dans le développement de la fibrogenèse hépatique. De plus, l'activation pharmacologique du composant de l'HC REV-ERBα par une petite molécule fait freine fortement le développement de la fibrose dans un modèle de souris chimériques humanisées développant de la NASH et inhibe l'expression des marqueurs de la fibrose dans des modèles de sphéroïdes obtenues à partir des tissus hépatiques dérivés des patients.

2. Résultats

2.1 L'oscillateur hépatique de l'HC contrôle l'expression des gènes de la fibrose hépatique.

Les gènes induites par le TGF-β sont impliquées dans plusieurs processus essentiels à l'homéostasie cellulaire et organique (Caja et al., 2018; Derynck & Zhang, 2003; Fabregat et al., 2016). Cependant, dans le foie, les mécanismes moléculaires qui maintiennent l'expression génique contrôlée par le TGF-β restent inconnus. Afin d'étudier le rôle de l'oscillateur de l'HC hépatique (**Dachraoui *et al*. Figure. 1a**) dans la régulation de l'expression des gènes fibrotiques, nous avons analysé la transcription circadienne des foies du souris dont l'expression du Bmal1 (composant majeur de l'HC) était invalidée dans les hépatocytes (CreTg Bmal1hep-/- ; Bmal1hep-/-) et nous les avons comparés aux souris contrôles issues de la même portée (Cre0 Bmal1hep-/- ; (**Dachraoui *et al*. Figure. 1b**). Cette analyse a révélé que dans le foie des souris contrôles, les transcrits de gènes liés au TGF-β et à la fibrose, par ex. *Col1α1*, *Tgfβr1*, *Smad7*, *Timp1*, *Twist2*, *αSma*, *Ski* et *Pdgfrβ* sont soumis au rythme circadien et sont exprimés lors de la phase repos du cycle circadien (ZT0-ZT12 ; **Dachraoui *et al*. Figure. 1b et Figure. S1a**). A l'inverse, dans le foie des souris Bmal1hep-/-, l'expression des gènes fibrotiques lors la phase de repos du cycle circadien ainsi que celle des gènes de l'HC connus pour être contrôlés par Bmal1 (Dbp et Rev-Erbα) est complètement abolies (**Dachraoui *et al*. Figure. 1b** et **Figure S1a**). Ces résultats ont été complétés par des expériences d'immunoprécipitation de la chromatine suivie d'une amplification quantitative par réaction en chaîne par polymérase (ChIP-Q-PCR) qui ont révélé que pendant la phase de repos, dans le foie de souris contrôles (Cre0 Bmal1hep-/-), le complexe BMAL1/CLOCK est recruté dans la région E-box du promoteur-amplificateur de *Smad7*, *Tgfβr1*, *Twist2* et *Ski*, induisant l'expression de ces gènes (**Dachraoui *et al*. Figure. 1c**). La liaison BMAL1/CLOCK n'est pas retrouvée dans le foie des souris Bmal1hep-/-, expliquant ainsi l'absence de l'expression de ces gènes (**Dachraoui *et al*. Figure. 1b, c**). Ceci démontre un effet direct de composants de l'HC sur l'expression des

gènes de la voie TGFβ. Pour compléter cette étude, l'analyse des données de séquençage run-on (GRO-seq, (Fang et al., 2014)) obtenues à partir du foie des souris contrôles confirme que la liaison de BMAL1/CLOCK aux régions régulatrices présentes dans les gènes *Smad7* et *Ski* (**Dachraoui *et al*. Figure 1c, d**) est corrélée à leur transcription « active ». Finalement, l'analyse de profil d'expression hépatique de l'ensemble de tous les gènes fibrotiques, a indiqué que les gènes de fibrose sont exprimés d'une manière circadienne et que leurs pics d'expression se trouvent dans la phase de repos du cycle circadien (**Dachraoui *et al*. Figure. 1e**).

En parallèle, nous avons effectué des analyses WB et ELISA (**Dachraoui *et al*. Figure. 1f, g**) montrant que le niveau plasmatique de TGF-β est soumis à une variation circadienne avec un pic à la fin de la phase de repos (ZT12) (**Dachraoui *et al* Figure. 1g**). L'augmentation du taux sanguin de TGF-β à ZT12 est corrélée à une augmentation de la phosphorylation des facteurs de transcription SMAD 2 et 3 (pSMAD2/3), et de l'expression du SMAD7, TWIST2, PDGFRβ et TGFβRI dans le foie de souris (**Dachraoui *et al*. Figure1f et Figure. S1d**. Enfin, des analyses par ChIP-Seq ont confirmé la liaison des facteurs de transcription contrôlés par le TGF-β/SMAD à leurs éléments de liaison SMAD (SMAD binding éléments, SBE) présents dans les promoteurs des gènes (*Smad7*, *αSma*, *Tgfβr1*) cette liaison est temporairement limitée, à la phase de repos (ZT8-ZT12 ; **Dachraoui *et al*. Figure. 1h**) dans les souris contrôles contrairement à ce qui est observé dans le foie de souris Bmal1hep-/- **(Dachraoui *et al*. Figure. 1h)**.

Dans l'ensemble, ces résultats ont indiqué que dans le foie de souris contrôle, l'oscillateur de l'HC joue un rôle clé dans le contrôle temporel journalier de l'expression de gènes dont les dérégulations sont connues pour entraîner la fibrose.

2.2 La fibrose hépatique induite par l'alimentation chez la souris perturbe l'oscillateur de l'HC dans le foie

Dans un deuxième temps, nous savons à explorer le rôle de l'HC sur le développement de la fibrose hépatique dans un modèle murin de NASH. A cet effet, des souris ont été nourries avec un régime riche en graisses et déficient en choline (CD-HFD) permettant le développement d'un NASH et de la fibrose pendant quelques semaines. Les caractéristiques histologiques du foie et les paramètres plasmatiques de ces souris ont été suivies à différents temps (1,2 ,4 et 16 semaines) pour établir les stades de la NASH et de la fibrose hépatique (**Dachraoui *et al*. Figure. 2a-d et Figure. S2a, b**). Dès la première semaine du régime Ces analyses ont révélé que chez la souris, l'alimentation du foie par le CD-HFD (1 semaine) une accumulation significative de macrophages inflammatoires (cellules positives CD68 ; **Dachraoui *et al*. Figure. 2a, d**), est observée pour atteindre à la 16^{éme} semaine, un marquage deux fois plus élevée que dans les souris contrôles. Nous avons également constaté que le régime CD-HFD augmente les niveaux plasmatiques de cytokines fibrogènes (TGF-β et PDGF-β) (**Dachraoui *et al*. Figure. 2c**). Des colorations au rouge de Sirius et au α-SMA dans les coupes de foie de souris contrôles et de souris CD-HFD, confirment qu'il faut au moins 6 semaines d'alimentation CD-HFD pour détecter les cellules HSC marquées en α-SMA et en rouge de Sirius (**Dachraoui *et al*. Figure. 2a, b, d**). En effet, la quantification de la zone positive de collagène (CPA) colorée

au rouge de Sirius a montré qu'environ 30 % du foie est fibrotique à la 16ème semaine d'alimentation par CD-HFD, un niveau qui est en adéquation avec le taux de la fibrose induite par NASH chez l'Homme. (Griffett et al., 2020).

Le modèle murin du NASH étant validé, nous avons ensuite analysée la transcription circadienne des gènes clés de l'HC dans les foies de ces souris et nos résultats ont montré que le régime CD-HFD modifie profondément l'expression des gènes de l'oscillateur de l'HC (**Dachraoui *et al*. Figure. 2a**), par ex. une baisse de l'expression du Bmal*1* et du *Rev-Erbα*, et une augmentation *Per1*, *Per2*, *Cry1* dès la 1ère semaine du régime ont été observé et continue bà se détériorer jusqu'à la 16ème semaine (**Dachraoui *et al*. Figure. 2e**). De la même manière, les analyses de la transcription circadienne des gènes fibrotiques et aussi les gènes appartenant à la voie de signalisation TGF-β ont révélé que dans le foie des souris CD-HFD, des gènes fibrotiques sont fortement perturbés (**Dachraoui *et al*. Figure. 2f)**. En particulier, des niveaux élevés des gènes ex. *Col1α1*, *Tgfβr1*, *Smad7*, *Timp1*, *Twist2*, *αSma* et *Pdgfrβ* ont été observés (**Dachraoui *et al*. Figure. 2f)**. Des tests de ChIP-Seq ont été réalisés pour vérifier la liaison des SMAD à leur site et ont montré que dans le foie fibrotiques des souris, la liaison SMAD est altérée, passant de la phase de repos (ZT8-ZT12 ; **Dachraoui *et al*. Figure. 1h et Figure. S1e**), à tout au long du cycle circadien.

Dans l'ensemble, nos résultats montrent que la fibrose induite par une alimentation riche en graisse de type CD-HFD brise l'oscillateur hépatique du l'HC, supprimant ainsi son contrôle physiologique (expression limitée dans le temps) sur l'expression des gènes régulés par le TGF-β et des gènes liés à la fibrose (Dachraoui et al. Figure.1b-h). La perte de contrôle de l'HC lors d'un régime fibrogène conduit à un état de signalisation TGF-β/SMAD constitutif, qui entraîne la fibrose.

2.3 La modélisation de la NASH dans les hépatocytes humains primaires et les myofibroblastes altère l'expression des composants du l'HC en activant les gènes fibrotiques

Pour étudier la relation entre les lésions métaboliques et l'HC dans les modèles bien établis de cellules primaires humaines, nous avons isolé à partir de foie humain ne présentant pas de maladie hépatique avérée, deux types de cellules les bien connus pour entraîner une fibrose hépatique : les hépatocytes humains primaires (PHH) et les cellules myofibroblastes humaines (HLMF). Ces cellules ont été traitées au FFA pendant 3 jours, ce qui permet de mimer un état fibrotique dans ces cellules (**Dachraoui *et al*. Figure.3a**). Dans ces conditions de traitement par FFA, l'expression des gènes de l'HC comme : *RORγ*, *BMAL1* et *REV-ERBα* ont diminuée, tandis que l'expression des gènes comme : *PER1*, *PER2*, *CRY1* et *E4BP4* augmentée (**Dachraoui *et al*. Figure**. **3b et Figure. 3a**). En parallèle, les traitements des PHH et HLMF au FFA conduit également à une induction des gènes fibrotiques (*SMAD7*, *Col1α1*, *αSMA*, *TWIST2*, *PDGFRβ*) et inflammatoires (*CCL2*, *IL1β, IL6*, *SOCS3* et *TIMP1*) (**Dachraoui *et al*. Figure**. **3c**). Il est important de noter que, conformément à l'effet sur les transcrits, l'analyse WB des PHH traitée par FFA a montré une induction significative de l'expression des gènes fibrotiques, y compris SMAD2/3, pSMAD2/3, SMAD7, TWIST2 et TGFβR1 (**Dachraoui *et al*. Figure**.**3d et Figure. S3f, g**).

Ensuite, j'ai utilisé la méthode de synchronisation circadienne médiée par la dexaméthasone (Dex) pour générer l'oscillation de l'HC dans les PHH et j'ai observé une oscillation robuste des transcrits non seulement des composants de l'HC, mais également des gènes régulés par le TGF-β dans les cellules contrôles alors que dans les cellules traitées au FFA présentent une forte perturbation de l'expression de ces gènes (**Dachraoui *et al*. Figure. 3e**). Ceci indique que la signalisation TGF-β perd la régulation temporelle due à la perturbation de l'oscillateur de l'HC. Notamment afin d'explorer si FFA stimulait la transcription médiée par le SBE dépendante de SMAD2/3, j'ai transfecté les PHH avec des plasmides rapporteurs codant la luciférase (Luc) sous le contrôle de SBE et je les ai traités avec FFA en présence ou en l'absence d'inhibiteur pharmacologique spécifique de TGFβR1 (SB505124 ; SB). Nous avons constaté que le traitement FFA stimulait effectivement l'activité SBE-Luc et que cette induction est abolie dans les PHH co-traitée par SB (**Dachraoui *et al*. Figure. 3f**). Ces résultats confirment l'activité élevée des facteurs de transcription SMAD 2/3 induite par le FFA. Ensuite, nous avons transfecté les PHH avec le plasmide Bmal1-Luc, et traité avec FFA en présence ou en l'absence de SB et nous avons observé que le traitement FFA induit la signalisation TGF-β et réduit transcriptionnellement l'expression de BMAL1 en inhibant le recrutement de l'activateur RORγ à son site de liaison. Les expériences décrites ci-dessus nous ont conduits à étudier la contribution de la signalisation TGF-β induite par les FFA dans la perturbation de l'expression des composants du l'HC et des gènes fibrotiques dans les cellules humaines (**Dachraoui *et al*. Figure. 3b-e et Figure. S3a-g**).

Le TGF-β est connu pour réguler l'expression des gènes de la famille des SMADs. Par conséquent, nous avons utilisé des petits ARN interférents (siARN) ciblant l'ARNm de SMAD2 pour inhibier l'expression de SMAD2 afin d'explorer le rôle de l'activation de SMAD induite par les FFA dans la réduction de l'expression de BMAL1 et REV-ERBα (**Dachraoui *et al*. Figure. 3d**. La réduction des niveaux de SMAD2 médiée par siARN empêche à la fois la transcription basale et m'induction par le FFA des gènes cible *SMAD2 SMAD7* (**Dachraoui *et al* Figure. 3h**), confirmant ainsi la spécificité de l'approche génétique. Il est à noter que, siSMAD2 empêche également de manière significative la baisse d'expression de BMAL1 et de REV-ERB- induite par les FFA dans les PHH transfectés, fournissant ainsi une preuve supplémentaire de l'implication des SMAD dans la perturbation de l'HC induite par les FFA (**Dachraoui *et al*. Figure. 3h**). Pour étudier plus en détail la relation entre BMAL1 et l'expression génique régulée par TGF-β dans les PHH traitée par FFA, nous avons effectué un invalidé de BMAL1 par siRNA, et observé une importante altération de l'expression des gènes contrôlés par le TGFβ à la suite de stimuli induisant la NASH (ici le FFA) l'identique de ce qui a été observé dans le foie de souris CD-HFD (**Dachraoui *et al*. Figure. 2e, f**), (**Dachraoui *et al*. Figure. 3i, k**).

Collectivement, ces résultats suggèrent que, les agressions métaboliques déclenchent des perturbations de l'HC de la signalisation TGF-β dans les hépatocytes humains primaires et les myofibroblastes humains hépatiques.

2.4 L'activation pharmacologique de REV-ERBα améliore la fibrose hépatique de la NASH dans un modèle de souris chimérique de foie humain dérivé de tissu de patient

Nous avons ensuite évalué l'effet thérapeutique de SR9009 dans un modèle animal chimérique dérivé du patient qui est connu pour récapituler de près les principales caractéristiques de la fibrose hépatique clinique. Comme modèle, nous avons utilisé des souris Fah−/−/Rag2−/−/Il2rg−/− (FRG)-NOD connues sous le nom de souris chimériques du foie humanisées (REF ; (**Dachraoui *et al*. Figure. 4a**). Après le développement d'une fibrose robuste dans ce modèle, nous avons traité les souris avec SR9009 (4 semaines). Nous avons initialement effectué une coloration à la fumarylacétoacétate hydrolase (FAH) et analysé les taux plasmatiques d'albumine humaine ((**Dachraoui *et al*. Figure. 4b et tableau détaillé**), ce qui a confirmé un degré élevé d'humanisation. Nos analyses ultérieures ont confirmé une réduction de la zone positive au collagène (CPA) chez les souris traitées par SR9009 par rapport au groupe des souris contrôles (**Dachraoui *et al*. Figure. 4b, c**). Chez les souris traitées au SR9009, la coloration H&E a indiqué un nombre réduit des cellules immunitaires ainsi qu'une densité réduite de αSMA (marqueur de fibrose) par rapport aux souris contrôles (**Dachraoui *et al*. Figure. 4b, c**). Ensuite, nous avons évalué le statut d'expression de la fibrose et des gènes inflammatoires et avons noté à nouveau que l'induction de ces gènes est significativement réduite par l'activation pharmacologique de REV-ERBα ((**Dachraoui *et al*. Figure. 4d, e**).

Collectivement, ces résultats indiquent que l'activation de REV-ERBα par une petite molécule inhibe la fibrose dans deux modèles murins de fibrose induite par la NASH, y compris un modèle chimérique de foie humain dérivé d'un patient.

2.5 L'activation de REV-ERBα atténue la signalisation du TGF-β dans les cellules humaines primaires

L'effet antifibrotique observé lors du traitement par SR9009 dans le modèle in *vivo* de NASH (**Dachraoui *et al*. Figure. 4**), nous a conduit à étudier la relation entre la signalisation TGF-β et l'activation de REV-ERBα. Les analyses de transcription effectuées à partir de PHH et HLMF stimulées par TGF-β ont montré non seulement l'induction de gènes cibles de TGF-β, mais également une diminution simultanée de l'expression de BMAL1 et de REV-ERBα (**Dachraoui *et al*. Figure. 5a**). Ainsi, le traitement par l'inhibiteur de TGF-β SB des PHH et des HLMF a complètement inversé les altérations induites par le TGF-β dans l'expression de BMAL1, REV-ERBα et CRY1 (**Dachraoui *et al*. Figure. 5a**). Les WB réalisées sur des lysats des PHH ont en outre confirmé qu'une augmentation de la signalisation TGF-β entraîne une réduction des niveaux de REV-ERBα, un traitement par SB pourrait annuler ces effets (**Dachraoui *et al*. Figure. 5b**). Ces analyses confortent nos observations obtenues dans le modèle de souris fibrotiques où l'augmentation des niveaux de TGF-β est corrélée à une réduction des niveaux de REV-ERBα (**Dachraoui *et al*. Figure. 2c, e, f**).

Ensuite, nous nous sommes demandé si l'activation de REV-ERBα par SR9009 pouvait atténuer l'expression génique induite par TGF-β. Nos analyses montrent que SR9009 pouvait inhiber de manière marquée l'expression de SBE-Luc induite par le TGF-β (Dachraoui ***et al*. Figure. 5c**). De même le traitement

par SR9009 a non seulement empêché l'activation des gènes fibrotiques induite par le TGF-β dans les PHH, mais a également restauré les niveaux de REV-ERBα (**Dachraoui *et al*. Figure. 5d,**). Il est important de noter que les analyses des WB de PHH ont montré que le traitement au SR9009 a inhibé la phosphorylation du SMAD induite par le TGF-β et l'augmentation des niveaux de TGFβR1 (**Dachraoui *et al*. Figure. 5e**). L'augmentation de la production d'αSMA par les cellules HLMF est une caractéristique de la fibrose, c'est pourquoi j'ai effectué une analyse par immunofluorescence des cellules HLMF traitées par TGF-β et SR9009 (**Dachraoui *et al*. Figure. 5f**). Les résultats montrent clairement SR9009 peut empêcher l'augmentation des niveaux d'αSMA et de SMAD2/3 phosphorylés induits par le TGF-β dans les cellules hépatiques humaines.

Ensuite, afin de confirmer le rôle fonctionnel de REV-EEBα est suffisant d'inhiber les altérations de la signalisation TGF-β/SMAD induites par les traitements FFA et TGF-β ainsi que la dégradation de l'HC nous avons utilisé des des siRNA ciblant REV-ERBα, et nous avons pu montrer que Rev-Erbα contrôle la transcription basale des gènes impliqués dans l'inflammation et la signalisation de TGF-β. Nous avons également constaté que l'inhibition d'expression *Rev-erbα* augmentait fortement l'induction de gènes liés à la fibrose dans les PHH traitées avec TGF-β et FFA (par exemple, *Smad7*, *α-Sma* et *Twist2*) (**Dachraoui *et al*. Figure. 3d**). Conformément à nos observations précédentes, l'analyse des voies de la fibrose sur les cellules HLMF traitées avec FFA et SR9009 a révélé une augmentation de l'expression des gènes fibrotiques (**Dachraoui *et al*. Figure. 5d**) qui a est abolie par le traitement par le SR9009. De plus, l'analyse de l'enrichissement d'ensemble de gènes (GSEA) impliqués dans l'inflammation et la signalisation TGF-β dans les cellules HLMF traitées avec FFA, a démontré la restauration par activation pharmacologique de Rev-erbα de ces deux programmes d'expression génique, qui sont considérées comme les caractéristiques de la fibrose humaine induite par la NASH (**Dachraoui *et al*. Figure. 5i, j**).

Collectivement, ces résultats nous ont amenés à établir le lien moléculaire entre les altérations de l'HC et la fibrose dans différentes cellules dérivées du foie humain.

2.6 La dérégulation de l'expression des gènes de l'HC est associée à la progression de la fibrose hépatique chez les patients et l'activation de REV-ERBα dans les sphéroïdes dérivés des tissus de patients atténue la signalisation élevée du TGF-β

Pour étudier la transférabilité clinique et l'impact de nos résultats pour un futur traitement de la fibrose hépatique, nous avons étudié l'expression des gènes de l'oscillateur de l'HC dans des cohortes cliniques et nous avons réalisé des études de perturbation pharmacologique dans les sphéroïdes hépatiques issus de patients. Des analyses informatiques du niveau de transcription des composants de l'HC dans une cohorte de patients NASH (GSE49541) montré que l'expression de *BMAL1*, *RORC* (RORγ) est significativement réduite dans la fibrose au stade avancé (F3-F4) par rapport à la fibrose au stade précoce alors que celle de *CRY1* est diminuée (F0-F2 ; **Dachraoui *et al*. Figure. 6a**). L'analyse d'une deuxième cohorte indépendante de patients atteints de NASH par rapport à des témoins sains (GSE89632), nous avons observé qu'en effet l'expression des composants du l'HC *BMAL1*, *RORC* et *REV-ERBα* était significativement réduite dans le foie des patients NASH par rapport aux patients sains (**Dachraoui *et al*. Figure. 6b**). Il est

intéressant de noter que le niveau d'expression des gènes du l'HC était également corrélée à l'inflammation lobulaire (**Dachraoui *et al*. Figure. 6c**), une autre caractéristique des maladie hépatiques avancées.

Pour confirmer la pertinence fonctionnelle en tant que cible thérapeutique des composants de l'HC, nous avons étudié les effets de SR9009 sur la fibrose dans des sphéroïdes hépatiques humains dérivés de patients. Les sphéroïdes ont été cultivés sous forme de micro-tissus 3D et récapitulent ainsi le microenvironnement hépatique, ils peuvent être utilisés pour étudier une réponse thérapeutique *ex vivo* (Roehlen et al., 2020). Tout d'abord, nous avons préparé des sphéroïdes à partir de patients atteints des maladie hépatiques et avons traité ces sphéroïdes avec du TGF-β en présence ou en absence de SR9009 (**Dachraoui *et al*. Figure. 6d**). Conformément à nos observations précédentes dans les différents systèmes modèles, le traitement par TGF-β a réduit le niveau d'expression de *REV-ERBα* dans le foie humain, niveau qui peut être significativement restauré par l'administration de SR9009 (**Dachraoui *et al*. Figure. 6e**). De plus. De plus, nous avons montré que SR9009 inhibe également l'activation des gènes responsables de la fibrose tels que *COL1α1*, *αSMA*, *TGFβR1*, *TWIST2* et *SMAD7* (**Dachraoui *et al*. Figure. 7e**). Nous avons constaté que dans les sphéroïdes issus des patients cirrhotiques, le traitement par SR9009 réduisait fortement et significative le niveau d'expression des gènes fibrotiques, notamment *COL1α1, αSMA, TGFβR1*, *TWIST2* et *SMAD7* (**Dachraoui *et al*. Figure. 6g**).

Collectivement, ces résultats sont en faveur de la transférabilité clinique d'une action sur le rôle fonctionnel du rôle fonctionnel de la machinerie de l'HC en tant que cible thérapeutique prometteuse pour agir sur la pathogenèse de la fibrose hépatique chez les patients.

3 Discussion

Mes travaux de doctorat m'ont permis de monter que l'horloge circadienne hépatique (HC) régule de manière transcriptionnelle l'expression de gènes clés liés à la signalisation du TGF-β et au développement de la fibrose dans le foie de souris. J'ai également découvert que dans le modèle souris de NASH induit par un régime riche en gras (CD-HFD), la fonction de l'HC est gravement perturbée, ce qui conduit à une expression importante des gènes fibrotiques et à une sécrétion élevée de TGF-β. Cependant l'activation pharmacologique du composant de l'HC (REV-ERBα) par son agoniste « SR9909 » améliore significativement la maladie de NASH en supprimant l'inflammation et la fibrose *in vivo, ex-vivo* et aussi *in vitro*. Dans l'ensemble, mes résultats révèlent que la modulation du fonctionnement de l'HC pourrait ouvrir la voie au développement de nouvelles thérapies anti-fibrotiques innovantes.

La signalisation du TGF-β est conservée au cours de l'évolution et elle est bien connue pour réguler l'expression des gènes de manière à la fois dépendante et indépendante de SMAD, du développement embryonnaire au maintien de la physiologie des organes (Caja et al., 2018; Roehlen et al., 2020). Il est important de noter que la dérégulation de l'activité du TGF-β est l'une des caractéristiques les plus importantes de la fibrose des organes et du cancer (Fabregat et al., 2016). Des études mécanistiques sur des modèles

murins ont démontré que la réduction de l'expression génique élevée induite par le TGF-β prévient et améliore la fibrose des organes, y compris le foie.

En exploitant ces découvertes mécanistiques, nous avons montré que le ciblage de REV-ERBα par une petite molécule améliore la fibrose hépatique dans des modèles *in vivo* et *ex-vivo* dérivés de patients. Nous avons choisi REV-ERBα pour des multiples raisons : (i) son niveau d'expression est réduit dans la signalisation élevée de TGF-β cours de la fibrose (Figures. 2e, 3b, e et 5a), (ii) il est un régulateur global du métabolisme hépatique, et (iii) il peut être ciblé de manière spécifique par des agonistes (SR9009) qui sont disponibles et peuvent être administrés en toute sécurité chez la souris. L'un des points forts de notre étude est la validation des résultats dans différents modèles dérivés de patients, notamment des modèles animaux dérivés de patients, des sphéroïdes hépatiques et des cellules primaires. La cohérence des résultats entre des systèmes modèles complémentaires et des cohortes de patients soutiennent la validité clinique de nos découvertes.

Cibler REV-ERBα pour le traitement de la fibrose hépatique est un nouveau concept, efficace et différencié. Seuls quelques composés aux propriétés anti-fibrotiques sont entrés en développement clinique avec une efficacité limitée, tout en présentant des problèmes de toxicité (Vuppalanchi et al., 2021). De plus, comme montré récemment pour les analogues du GLP1 (Newsome et al., 2021), une amélioration robuste de la stéatose seule n'induit pas nécessairement une amélioration de la fibrose. Un différenciateur clé de l'approche de ciblage REV-ERBα est la combinaison de l'efficacité anti-inflammatoire et anti-fibrosante, comme démontré dans tous les modèles (Figures. 4-6), qui répond à un besoin médical clé dans le traitement de la fibrose hépatique.

Des études précédentes ainsi que les nôtres (Ni et al., 2021 ; Solt et al., 2012) ont démontré que l'administration de SR9009 est sans effets indésirables majeurs détectables dans les modèles d'animaux. Remarquablement, Ni et ses collaborateurs ont également trouvé que le SR9009 améliore la fibrose hépatique en améliorant la fonction de barrière intestinale (Ni et al., 2021). De plus, le ciblage de REV-ERBα a montré une amélioration de la fibrose pulmonaire dans des modèles d'animaux (Cunningham et al., 2020). Néanmoins, des études supplémentaires sont nécessaires pour étudier le profil de sécurité des composés. De plus, étant donné que nos modèles précliniques sont axés sur la NASH en tant que lésion hépatique, des études supplémentaires seront nécessaires pour montrer l'efficacité et l'impact sur la fibrose induite par d'autres étiologies telles que l'hépatite virale et la maladie alcoolique du foie. Compte tenu des données précliniques de notre étude, la population cible des thérapies ciblant REV-ERBα sera constituée de patients atteints de fibrose et/ou NASH, y compris la fibrose avancée F3/F4, comme le montre la preuve de concept dans les sphéroïdes de patients cirrhotiques (Figure. 6g).

Enfin, un point de vue circadienne sur le contrôle métabolique est précieux non seulement pour les chronobiologistes mais aussi pour les chercheurs ayant une compétence de base en métabolisme et en physiologie, car adopter ce point de vue unique pourrait mettre en lumière nombreux aspects de la régulation métabolique qui ont été négligés jusqu'à présent. Il est fort probable que de nombreuses études sur les voies

métaboliques qui n'ont pas pris en compte l'élément circadien révéleront de nouvelles informations intéressantes une fois qu'elles seront menées sous l’angle de la chronobiologie.

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Review article :

Dachraoui, M* & Mukherji, A* & ***.et al*** (2020). Perturbation of the circadian clock and pathogenesis of NAFLD. *Metabolism Clinical and Experimental* DOI: 10.1016/j.metabol.2020.154337. * Contributed equally as first authors.

Perturbation of the Circadian Clock and Pathogenesis of NAFLD

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Words: Abstract 240, Text: 4892

Conflict of Interest: The authors declare no conflict of interest

Metabolism Clinical and Experimental 111 (2020) 154337

Contents lists available at ScienceDirect

Metabolism Clinical and Experimental

journal homepage: www.metabolismjournal.com

Perturbation of the circadian clock and pathogenesis of NAFLD

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ARTICLE INFO

Article history:
Received 25 May 2020
Received in revised form 24 July 2020
Accepted 26 July 2020

ABSTRACT

All living organisms including humans, experience changes in the light exposure generated by the Earth's rotation. In anticipation of this unavoidable geo-physical variability, and to generate an appropriate biochemical response, species of many phyla, including mammals have evolved a nearly 24-hour endogenous timing device known as the circadian clock (CC), which is self-sustained, cell autonomous and is present in every cell type. At the heart of the 'clock' functioning resides the CC-oscillator, an elegantly designed transcriptional-translational feedback system. Notably, the core components of the CC-oscillator not only drive daily rhythmicity of their own synthesis, but also generate circadian phase-specific variability in the expression levels of thousands of target genes through transcriptional, post-transcriptional and post-translational mechanisms. Thereby, this 'clock'-system provides proper chronological coordination in the functioning of cells, tissues and organs. The CC governs many physiologically critical functions. Among these functions, the key role of the CC in maintaining metabolic homeostasis deserves special emphasis. Indeed, the several features of the modern lifestyle (e.g. travel-induced jet lag, rotating shift work, energy-dense food) which, force disruption of circadian rhythms have recently emerged as a major driver to global health problems like obesity, cardiovascular disease and metabolic liver disease such as non-alcoholic fatty liver disease (NAFLD). Here we review, the CC-dependent pathways in different tissues which play critical roles in mediating several critical metabolic functions under physiological conditions and discuss their impact for the development of metabolic disease with a focus on the liver.

1. Introduction

Diurnal alteration between the light (day) and the darkness (night) is unavoidable, and to adopt to this environmental variable in a manner which best suits to physiology, circadian rhythms have evolved over billions of years, and are displayed by nearly all living organisms. This clear separation between light-dark periods induces active- and rest-phases in various phyla including mammals. The circadian clock (CC) an endogenous 'time-keeper' is the key link between these environmental variable factors and organismal physiology, as it sets an adaptive rhythm for physiological mechanisms, as it allows them to be anticipated [1–6]. The idea of CC regulating critical functions was noted as early as the 18th century regarding the diurnal movement of plant leaves. In 1959, Franz Hallberg coined the term 'circadian rhythm' (latin origin: about a day) to acknowledge the periodicity of these biological rhythms. Subsequently, landmark investigations conducted in Drosophila provided the first evidence of genes controlling the circadian rhythm [7]. Since then, numerous studies have established plethora of molecular mechanisms which generate and maintain these ~24 h rhythms. The importance of investigations on the CC was exemplified by the award of the Nobel Prize in Medicine and Physiology (2017) to professors Jeffrey C. Hall, Michael Rosbash and Michael Young. The CC-rhythms have allowed mammals to anticipate changes in the external environment (e.g. day-night), and to respond by adjusting cellular CC-machinery driven numerous physiological functions, e.g. metabolism and endocrine functions [1–6]. Accordingly, recent molecular- and genetic-studies have demonstrated that, in mammals, the expression of numerous genes in different organs display circadian rhythmicity, thus enabling control of both anabolism and catabolism. As an example, food absorption, processing, assimilation and oxidative burning of nutrients all display through circadian variations, thus enabling their temporal adjustment with food availability and bio-energetic need of the organism [1–6]. Under physiological conditions these 'metabolic rhythms' are generated and maintained by the dynamic interactions between the CC and timing cues e.g. light and food (eating time and its quality). Importantly, in our modern industrialized world, various human behaviors and activities such as shift work, jet lag, energy-dense fatty foods and sleep deprivation often interfere with these rhythms and disrupt

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https://doi.org/10.1016/j.metabol.2020.154337

CC-functioning. Unsurprisingly then that disruption of CC functioning has recently emerged as a major contributor to different metabolic diseases, as well as carcinogenesis [1–5,8–16]. Therefore, detailed comprehension of the mechanistic basis of the CC-control on gene expression is critical to develop novel therapeutics for metabolic disorders whose therapeutic efficacy may be administration time-of -day dependent.

In this review, we lay emphasis on how the CC regulates metabolism in different peripheral organs to maintain metabolic homeostasis and provide overview of how the disruption of these CC-regulated processes could lead to the development of NAFLD. Furthermore, we also briefly discuss the potential of chronopharmacology in therapeutics.

2. Mammalian circadian clock: anatomic and molecular organization

Well-known light receptors (rods and cones) converts the energy of the light signal to electrical impulses and relays them to the brain by utilizing retinal ganglion cells (RGC). It has been demonstrated that the melanopsin (photopigment) expressing RGCs directly relay the photic signal to a group of neurons in the anterior hypothalamus known as the suprachiasmatic nucleus (SCN;8–9), which by anatomical design acts as the 'central/master' CC. The SCN-CC in turn, by utilizing barely understood humoral and neuronal mechanisms transmits the 'time information' (a. k. a; 'Zeitgeber'; ZT) to other peripheral organs (synchronization of peripheral CCs; 1–6,8–9).

The molecular architecture of the CC-functioning has been discovered over the 30 years [1–6,17]. Remarkably, the SCN-CC and PCCs are constituted by identical molecular components which regulate themselves using a similar transcriptional-translational feedback loops (TTFL). At the core of the molecular CC functioning (in mammals) resides the heterodimer of transcription factors BMAL1 and CLOCK, which acts as the trans-activator of genes containing *E*-box DNA binding sequences (DBS). BMAL1/CLOCK-drives the transcription of the *Period* (PER1/2) and the *Cryptochrome* (CRY1/2) genes, whose protein products heterodimerise to inhibit the transcriptional functions of the BMAL1/CLOCK-complex [1–6], thereby leading to the reduction in their own expression, thus constituting the so-called first loop of the CC-oscillator (Fig. 1). During the early rest phase, high transcriptional activity of BMAL1/CLOCK drives the accumulation of its products CRY1/2 and PER1/2 (in late rest phase) which subsequently dimerise and inhibit BMAL1/CLOCK-activity during the active phase [17]. In the 'second' loop of the oscillator, BMAL1/CLOCK- activates the transcription of the nuclear receptors (NR) *Rev-Erbα* and *Rev-Erbβ* [18] during the rest phase [1–5]. Molecularly, REV-ERBs act as transcriptional repressors by binding to the ROR-response elements (RORE) present in numerous target genes including themselves. Importantly, REV-ERBs by repressing the transcription of *Bmal1* and *Clo*ck genes reduce their own expression, thus closing this second loop. Importantly, during the active phase another set of NRs RORα/γ are recruited to these same RORE-DBSs to activate the expression of *Bmal1* and *Clock* genes [1–5]. This phase-specific recruitment and accumulation of RORα/γ (activators) and REV-ERBα/β (repressors) induces rhythmicity in *Bmal1* and *Clock* expression, thus generating a variability in not only CC-oscillator functions but also in the transcription of numerous RORE-DBS containing target genes which are transcribed exclusively during the active phase (Fig. 1; [1–5]). Moreover, REV-ERBs repress and RORα/γ induce the E4BP4 repressor, which in turn represses the transcription of D-box DBS-containing CC-controlled genes (CCGs) in active phase. While the BMAL1/CLOCK-induced DBP transactivates these D-box CCGs strictly during the rest phase (Fig. 1). Moreover, post-translational modifications of CC-components as well as epigenetic modifications-induced by the recruitment of CC-components on their respective DBSs also generates further regulation of the CC-functioning. Altogether, by utilizing several sophisticated molecular mechanisms the CC-oscillator drives a time of the day-dependent gene expression program, which lies at the heart of producing distinct critical biochemical outputs in different organs (Fig. 2). Importantly, this CC-governed temporal coordination in gene-expression between organs is the major driver of metabolic homeostasis.

3. Cross talk between clock and feeding cycles

Feeding cycles are one of the most prominent *zeitgeber* for peripheral tissues [4,5,19,20], and investigations have uncovered the existence of multi-layered cross-talk between metabolism and the CC, and the number of ways through which metabolism and CC influence each other are rapidly increasing [1–5]. Not only the CC exerts a remarkable control of metabolism, but also the information about metabolic state is transmitted back to the CC, thus creating a crosstalk between metabolic and circadian cycles. In this regard, the 'clock' receives information (e.g. changes in feeding time or composition) from a range of metabolic sensors which can modify PCCs rhythms. Specifically, the importance of feeding time on the hepatic-CC was demonstrated in *Cry1/2* mutant mice, in which an imposed night-time only feeding largely restored the circadian gene expression pattern [21]. Changing the feeding time from the active phase to the rest phase in mice is known to shift peripheral CCs by nearly 12 h [19,20]. At the molecular level, this change is orchestrated by metabolic alterations which induce the activity of well-known transcription factors PPARα and CREB [22]. One highly relevant physiological setting of CC-metabolism crosstalk is exemplified by BMAL1/CLOCK-dependent expression of the nicotinamide phosphoribosyl transferase (*Nampt*) gene, which is the rate-limiting enzyme in NAD^+ synthesis [23,24]. 'Clock'-gated NAMPT transcription generates a rhythmicity in NAD^+ synthesis which in turn dictates the biochemical activities of NAD^+ -dependent proteins, e.g. the SIRT1 deacetylase and the PARP-1. Remarkably, SIRT1-activity is known to determine: (i) the functioning of BMAL1/CLOCK-complex and, (ii) the 'half-life' of PER2 protein, which in unison maintain CC-oscillator functioning [1–3]. In accordance, genetic ablation of *Sirt1* or its pharmacological inhibition is known to desynchronize circadian rhythmicity. Another highly relevant feedback regulation exists between the CC and heme biosynthesis and activity has also been uncovered [25,26]. Altogether, extensive investigations have unraveled multifaceted CC-metabolism crosstalk as a tuning fork for the CC-oscillator functioning, which has systemic repercussions as: (i) a change in the feeding time in mice to the "rest" phase leads to features resembling metabolic syndrome [27] and, (ii) high-fat diet (HFD)-induced reprogramming of the hepatic CC-functioning in mice can be largely prevented by restricting the food access to the circadian active phase [28,29].

4. Pathophysiology of the non-alcoholic fatty liver disease (NAFLD)

Over the last decades, lifestyle modifications have shifted the health care priorities worldwide from infectious to metabolic diseases [30–34]. In the context of liver disease, availability of vaccines and antiviral therapies have started to reduce the disease burden caused by hepatotropic viruses such as chronic hepatitis B and C and their complications [35–39]. In contrast, the prevalence of metabolic liver disease such as non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) have increased dramatically. Indeed, with an estimated worldwide prevalence of ~25%, NAFLD has emerged as the most common chronic liver disease [30–34]. This increase in global prevalence of NAFLD is closely associated with the world-wide epidemic in the incidence of other metabolic disorders e.g. type 2 diabetes and obesity. Importantly, 20–25% of fatty liver patients progress to develop NASH, which is a major aetiology of liver transplantation required by cirrhotic and hepatocellular carcinoma (HCC) patients [30,31,40]. "Fatty liver" is a complex spectrum of disease and considering the current knowledge of the pathology and the understanding of patient heterogeneity, the scientific community has recently [34] suggested metabolic (dysfunction) associated fatty liver disease (MAFLD) to be more appropriate. NAFLD generally initiates with the accumulation of excessive triglyceride (TG) in hepatocytes, a largely benign state

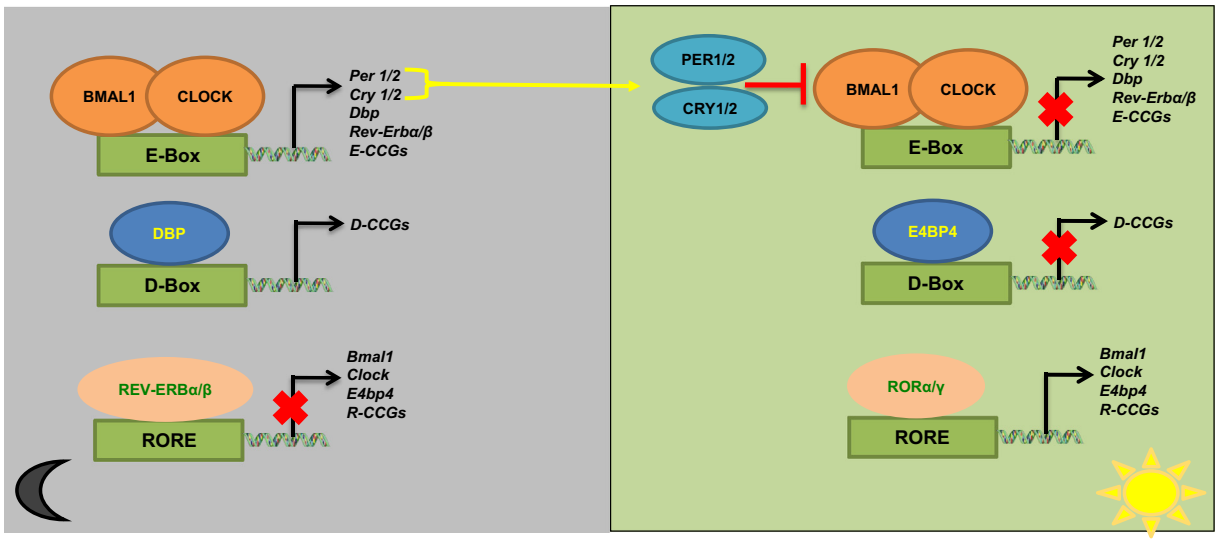

Fig. 1. Model of the molecular 'clock': The BMAL1/CLOCK-heterodimer binds to the E-Box DBS present in the promoter-enhancer elements of numerous CCGs, including their inhibitors Periods (Per1/2) and Cryptochromes (Cry1/2) and increases their expression during the rest phase. Subsequently, PERs and CRYs proteins dimerize to inhibit (in the active phase) the transcriptional activity of BMAL1/CLOCK. Additionally, BMAL1/CLOCK-dependent expression of Rev-Erbα/β, leads to the trans-repression of several RORE-DBS-containing CCGs including, Bmal1, Clock and E4BP4 during the rest phase. A reduction in REV-ERBs levels (during active phase) permit the RORα/γ-dependent RORE-mediated activation of CCGs including Bmal1 and Clock, which enables the turning of the circadian clock. DBP expression during the rest phase leads to the expression of D-Box DBS containing CCGs, which are transcriptionally repressed by E4BP4 during the active phase. CCGs-Clock Controlled Genes, DBP-D-Box binding protein, E4BP4-E4 promoter binding protein 4, E-CCGs: E-Box DBS-containing CCGs, R-CCGs: RORE-containing CCGs, D-CCGs: D-Box-containing CCGs. See text for details.

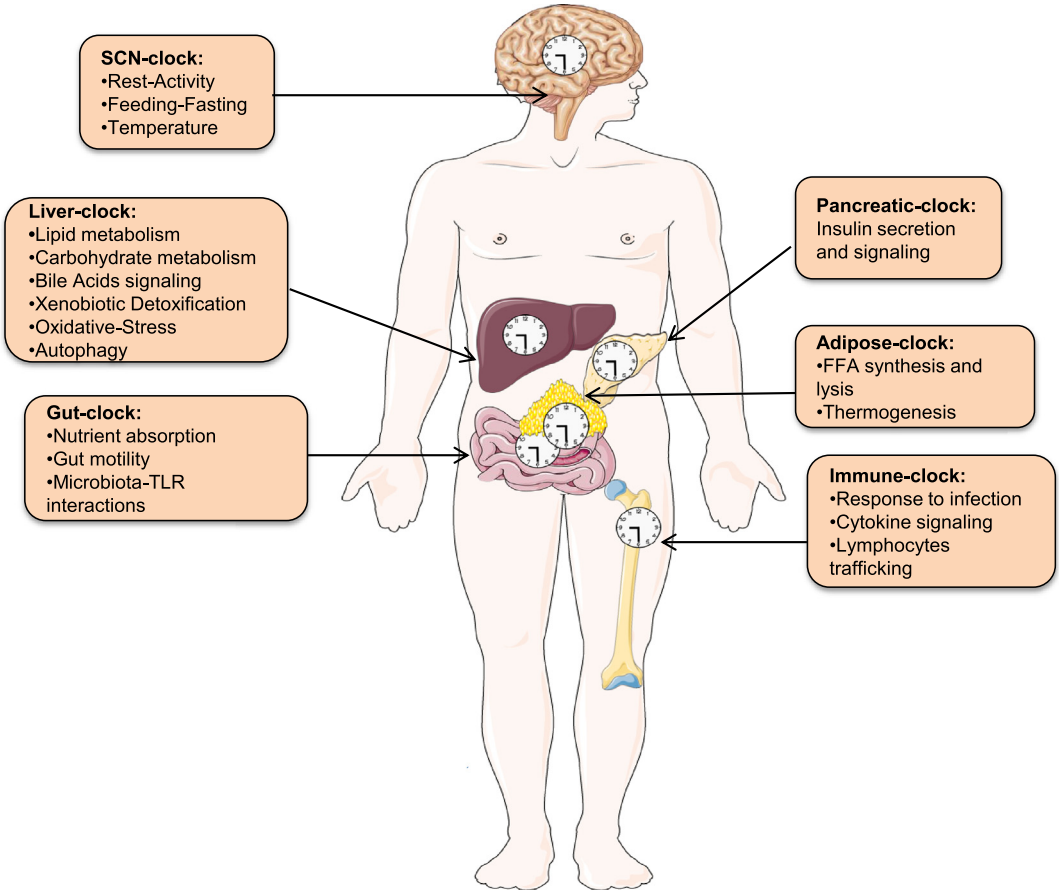

Fig. 2. Coordinated regulation of metabolic physiology by central and peripheral clocks: The light-entrained central SCN-clock not only governs rest-activity and feeding-fasting cycle but also synchronizes peripheral tissue clocks. Indicated in the boxes are some of the major peripheral clocks and the critical physiological functions they perform. Importantly, deregulations in the functioning of peripheral clock-regulated pathways are often encountered in NAFLD. SCN-Supra Chiasmatic Nucleus. See text for details.

commonly referred as simple steatosis [30–33]. Importantly, persistent fatty liver drives simple steatosis to steatohepatitis (NASH), which is characterized by simultaneous presence of both inflammation and hepatocytic damage (a.k.a ballooning). Furthermore, NASH proceeds to fibrosis, which can eventually progress to cirrhosis and hepatocellular carcinoma (HCC) [30–33,40]. Like metabolic syndrome, development of NAFLD is highly complex and has been extensively reviewed elsewhere [30–34,40–43]. Despite large research and development efforts, there are no approved drugs specifically targeting metabolic liver disease and compounds in late stage of development are frequently characterized by limited efficacy [30,32,33].

The pathogenesis of "fatty liver" was initially postulated in 1998 [44] to be "a tale of two hits"-first involving excessive hepatic triglyceride (TG) accumulation which was followed by secondary insults such as oxidative stress. However, recent investigations in chronic metabolic diseases have now clearly established that pathogenesis of NAFLD is a complex multi-step metabolic disorder [30–34,40–43]. Several studies have indeed uncovered crucial roles for deregulations in the functioning of pancreas, intestine, adipose tissue and immune system in NAFLD development ([30–33,40–43]; Fig. 3). Physiologically, mammalian bioenergetics is maintained by intricate intra- and inter-organ communications and deregulations of which lie at the core of metabolic disease, including NAFLD. At the basic level NAFLD arises due to the inability of the hepatocytes to effectively metabolize carbohydrates and free fatty acids (FFA). Mechanistically, NAFLD is a consequence of an imbalance between adipocytic FFA supply, hepatic de novo lipogenesis and FFA utilization through mitochondrial β-oxidation and production of ketone bodies, and finally disposal through secretion of TGs in very low-density lipoprotein (VLDL) particles [1,30–33]. Fat accumulation in the liver can be traced to either an increased incidence of de novo lipogenesis or overwhelming of the capacity to oxidize FFA. Additionally, mitochondrial dysfunction could impair fatty acid β-oxidation and cause lipid accumulation, which usually precedes NAFLD [45–47]. Furthermore, excessive TG is transported out of the hepatocytes by binding to liver-produced VLDL and, with impaired β-oxidation or TG transport, the capacity of the liver to clear accumulated TG is compromised, which further contributes to the development of NAFLD [30,31,45]. As discussed above, the complexities of NAFLD pathogenesis and its progression to steatohepatitis are barely understood with both genetic and environmental factors playing crucial roles. Importantly, due to the overwhelming role of the CC in maintaining metabolic homeostasis (Fig. 2), it can be postulated that disruption in the CC-functioning can drive NAFLD [1–3,15]. In the subsequent chapters we describe some of these functions to further illustrate the link between CC and NAFLD.

5. Peripheral circadian clocks: regulation of metabolism and impact on pathogenesis of NAFLD

5.1. Liver

Unarguably, the liver plays a central role in governing whole-body physiology (Fig. 2). Considering its preeminent role in metabolism several genomic studies have utilized time-course in mouse models to uncover circadian cistrome [48–51], transcriptome [52,53], proteome [54–56], and lipidome [57,58]. Analyses of circadian gene expression have revealed two broad time-periods of transcription in liver, which correspond to the periodic transition between alternating active and rest phases [1–4]. These two 'peaks' reflect the highly differential physiological requirements, such as in energy demand or detoxification activity, as per their necessity during the periods of activity or rest [1–5]. Analyses of CC-components and epigenetic factors binding [48–51] uncovered that these two circadian phase-specific distinct mRNA pools are generated due to the intrinsic rhythmicity of the CC-oscillator. Furthermore, CC is also known to post-transcriptionally control cellular processes like DNA repair, ribosome biogenesis, autophagy, ER-stress [54–56].

Mammalian gluconeogenesis is principally controlled by the liver. Indeed, along with several other organs (brain, pancreas, muscle), the liver-CC largely contributes to maintain homeostatic blood glucose levels [59]. In a critical genetic study it was demonstrated that *Bmal1* ablation in the liver reduces expression of the glucose transporter (*Glut2),* which lead to a decreased post-feeding glucose uptake in mutant mice, thus revealing a role for the liver-CC-oscillator in glucose metabolism [60]. Remarkably, the liver-CC also regulates glucose metabolism post-hepatocytic entry at multiple levels, by controlling expression of glucokinase (*Gck;* regulator of glycogen synthesis) [1–5]. By controlling either the expression or the activity of several gluconeogenic transcription factors e.g. *Klf10* [61], *Hnf4α* [18,62], *CREB* [63], *Pgc1α* [64], the liver-CC thoroughly controls glucose metabolism.

Along with its influence on carbohydrate metabolism, several genetic studies have established that liver CC as a predominant regulator of lipid metabolism [65–67]. These investigations have established plasma levels of FFA, TG and cholesterol display diurnal variations, and are altered upon mutations of CC-genes. To illustrate, liver-restricted mutation of *Rev-Erbα/β* profoundly increased plasma levels of FFA, TG and cholesterol [67]. Mechanistically, the hepatic-CC regulates either the expression or the activity of enzymes that are critically involved in regulating multiple critical steps of lipid metabolism. As an example, TG synthesis in liver is a multistep process and requires the activity of several enzymes (*Gpat2*, *Agpat1/2*, *Lipin1/2* and *Dgat2*) expression of which are CC-controlled [56], thereby leading to a prominent crest and trough of hepatic TG levels (in mice) during the rest and active phases, respectively [56]. Furthermore, REV-ERBα by regulating the transcription of *Insig2* controls the activity of SREBP1c (master regulator of lipogenesis; [68]). Additionally, the hepatic CC-oscillator also participates in: (i) fatty acid synthesis by controlling the expression of *Elovl3*, *Elovl6*, *Fas* etc. [1–3,6], (ii) regulating β-oxidation and ketone-body production [69,70] and, (iii) determining the expression of key lipid-responsive NRs LXRs, PPAR α and δ [1–3,18]. Recent studies have established BA-signaling as a major regulator of TG, cholesterol and glucose homeostasis [71,72]. BA synthesis is controlled by a transcriptional feed-back loop consisting of the NRs FXR and SHP and intestinal hormone FGF15 (FGF19 in humans; [71–72]). Importantly, CC-regulates the expressions of both FXR and SHP [18,62] as well as FGF15 secretion [73], thereby controlling the diurnal expression of cholesterol 7α-hydroxylase (*Cyp7a1*), the rate-limiting enzyme in BA synthesis. Additionally, both REV-ERBα and DBP (CC-output regulator) are known to control *Cyp7a1* transcription [74,75]. Furthermore, in mice an essential molecular feedback exists between SHP and the neuronal PAS domain protein 2 (NPAS2; *Clock* gene paralog) which not only contributes towards their own circadian rhythmicity but also enables to maintain hepatic lipid, BA and lipoprotein metabolism [76]. Taken together, these mechanisms combine to generate circadian rhythmicity in BA levels which is also observed in humans [77]. To further illustrate the intimate connection between the lipid-and BA-metabolism and CC-functioning, it has been noted that atorvastatin (routinely to treat hyperlipidemia) administration in mice alters the expression of not only *Cyp7a1* but also of key CC-components e.g. *Bmal1* and *Npas2* [78]. The liver CC is also well known to regulate several cellular processes e.g. autophagy, ER stress and oxidative stress [79–83], all of which have been implicated in pathogenesis of NAFLD and has been extensively described elsewhere [30,31].

5.2. Pancreas

The pancreas is well known to play a critical role in maintaining glucose homeostasis through production of hormones insulin and glucagon (Fig. 2). Pancreatic function is controlled by both the central SCN-clock as well as Pancreatic CC-oscillator and aligns biochemical activities in pancreatic islets as per the metabolic demands [2,3,84,85]. The 'clock' is known to regulate both the exocrine [84] and endocrine [86] functions of the pancreas. The presence of an autonomous circadian

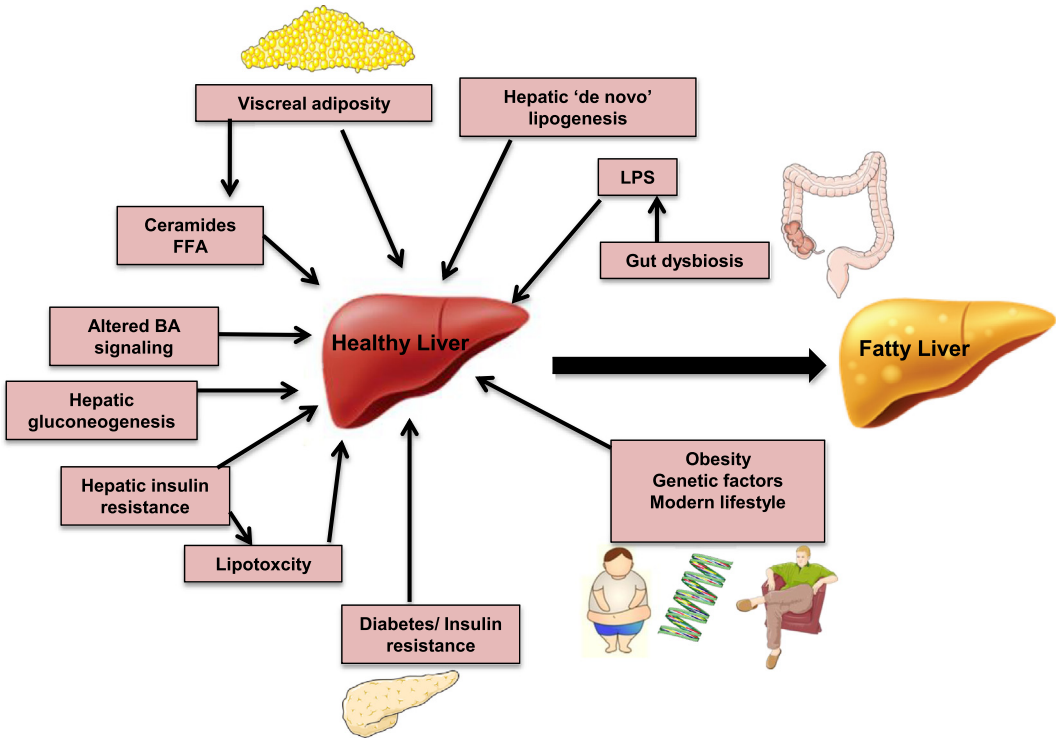

Fig. 3. Model of NAFLD pathogenesis: The scheme depicts an overview of how alterations in the circadian clock-controlled functions/pathways and processes in different peripheral tissues may predispose to NAFLD pathogenesis and contribute to therapeutic intervention. See text for details.

pancreatic clock has been demonstrated not only in rodents [85–87], but also in human islets and dispersed human islet cells [87]. The pancreatic clock is synchronized to the light-dark cycle via signals derived from the SCN-clock that include autonomic neuronal system, melatonin and glucocorticoids [88]. The pancreatic CC-oscillator in β cells drives highly rhythmic oscillation of insulin secretion which is strictly aligned with the expression of genes encoding insulin secretion and signaling [85]. Mechanistically, pancreatic CC-components helped in the spatio-temporal recruitment of key transcription factor PDX1 to specific enhancers to regulate transcription of insulin and other genes of insulin signaling pathway [85]. Importantly, β-cell-specific mutation of either *Bmal1* or *Clock* leads to wide-spread changes in the transcriptome, and specifically reduces genes encoding cell cycle, synaptic assembly and secretion of insulin, thereby leading to diabetes in mutant mice [86]. Notably, non-alcoholic fatty pancreas disease (NAFPD) a recently described dysmetabolic phenotype (akin to NAFLD), has been shown to perturb the expression of several CC-components in murine pancreas which correlates with pancreatic inflammation and fibrosis development [89]. As insulin-resistance often accompanies NAFLD [30], deregulation of the pancreatic-CC-controlled insulin signaling could play a critical role in the predisposing to fatty liver development (Fig. 3).

5.3. Intestine and microbiota

Several critical aspects of the intestinal physiology e.g. motility, intestinal permeability, hormone secretion, nutrient absorption, cell proliferation and interactions with microbiota are CC-controlled and have been thoroughly reviewed [90–92]. However, in recent years the relationship between intestine and resident microbiota has gained spotlight as a major regulator of metabolic health and disease including, NAFLD [93–96]. Indeed, obesity has been shown to not only alter the composition of gut microbiota (dysbiosis) composition but also perturbs their nature of interactions with the host (intestinal epithelial cells; IEC), both of which have been suggested as an etiological agent in the pathogenesis of metabolic diseases, including NAFLD [93–96]. One of the proposed mechanisms through which dysbiosis could induce NAFLD is by augmenting lipopolysaccharide (LPS) production and delivery to the liver via the portal circulation, a consequence of increased intestinal permeability [97,98]. This abnormal presence of microbiota-associated LPS in liver perturbs lipid metabolism by affecting the generation of short-chain fatty acids and altering the BA pool composition which may influence intestinal and hepatic FXR activity, thus affecting both glucose and lipid homeostasis [97].

The 'clock' and the microbiota intersect at many levels. Most notably, the IEC CC has been demonstrated to regulate the circadian expression of microbial pattern recognition receptors (e.g. TLRs, NOD2) which creates a 'temporal window' for the microbiota-signals to regulate gene expression in IEC to maintain homeostasis [99]. Importantly, absence of this IEC CC-microbiota crosstalk leads to metabolic disorders [99]. Interestingly, it was also demonstrated that the gut microbiota undergoes circadian oscillations in composition and function [100,101]. These microbiota oscillations were found to be controlled by the timing of food intake and the diet composition. Furthermore, it was also demonstrated that the gut microbiota undergoes circadian oscillations in biogeographical localization and metabolome patterns which in turn determine the diurnal exposure of the intestinal epithelium to different bacterial species and their metabolites [102,103]. Importantly, this circadian variations in microbial behavior in turn regulates the transcriptome and metabolome of both gut and distant tissues e.g. liver [102,103]. Most importantly, dysbiosis-induced by 'clock' perturbations (either through genetic ablation of CC-components or jet lag) lead to and development of metabolic pathologies [93,94,103].

5.4. Immune system

The immune system is heavily influenced by time-of-day cues, both under steady-state conditions and in response to inflammatory challenges. Indeed, diurnal host responses to endotoxins were noted more than 6 decades back [104]. Importantly, several inflammatory diseases e;g. myocardial infarction, rheumatoid arthritis and asthma are known exhibit pronounced circadian rhythmicity in their pathology [105–108]. Recently, molecular evidence has started emerging to reveal

that numerous aspects of immune functions including lymphocyte trafficking, host-pathogen interactions, cytokine secretion and activation of innate and adaptive immunity are thoroughly controlled by the CC [107–110]. Taken together, investigations have established the CC operates to as a gating mechanism to control the magnitude of immune response in a diurnal fashion and has been described [107–110]. The role of the deregulated immune system and fatty liver disease have been extensively reviewed [41–43]. Here we briefly discuss the immune components which are known to be controlled by CC under physiological conditions [107–110].

Like every other aspect of the metabolic syndrome, pathogenesis of `fatty liver` is strongly linked with inflammation, and both innate and adaptive branches of immunity have been implicated in this process [31,32,41–43]. However, the innate immune system has received more attention. Although in initial studies focused on Kupffer cells, more recent investigations have revealed that several specialized immune cells (resident and infiltrating) participate in hepatosteatosis [42]. Kupffer cells are activated by a variety of stimuli including FFA, peroxidized lipids, microbiota-derived LPS and ROS [43]. Importantly, both FFA and LPS drive Kupffer cell stimulation through TLR2 and TLR4, which leads to perpetual activation of inflammatory signaling pathways like ASK-1, JNK, IL-6 etc. thereby enabling sustained induction of NF-κB and STATs, thus augmenting cytokine production (TNF-α, IL-1β etc.). In murine models, reducing the number of Kupffer cells through clodronate administration considerably ameliorates NASH pathology [111]. In addition, the inflammasome which can both sense and be activated by danger-associated molecular patterns (DAMPs) such as FFA and pathogen-associated molecular patterns (PAMPs) e.g. LPS, has recently emerged as a critical molecular link between metabolic stress and fatty liver development [31]. In animal models of NAFLD, triggering inflammasome activity enhances the expression of the pro-inflammatory cytokines IL-1β and IL-18 which subsequently through caspase-1 promote cell death in liver [111]. Recent studies have also shed light on the role of the IL17-secreting Th17 cells in metabolic diseases including NAFLD [112]. It has been observed that the obesity-induced dysbiosis elevates IL-17 production [113,114] and, in the setting of NAFLD, this cytokine drives neutrophil and monocyte infiltration in the liver, thereby potentiating hepatic insulin resistance and steatosis progression [115]. Consistently, abrogation of IL17-induced signaling activity in a diet-induced murine model of NASH reduces steatosis [116]. Taken together, these studies indicate how possibly deregulated CC-functioning in immune cells could predispose towards fatty liver development.

6. Circadian clock-related therapeutic interventions

In the past few years, many studies have investigated the effects of the timing of drug treatment on the circadian appearance or exacerbation on of disease symptoms, leading to the development of a concept known as chronomedicine [117–119]. Chronomedicine is described as the approach employed to maximize the efficacy and minimize the side effects when drugs are administered in accordance with the CC as 'timing' of drug-administration is of crucial but still a less-appreciated factor in drug efficacy considerations [117–119]. This is not surprising considering that to a large extent CC control over pharmacology arises from its ability to thoroughly regulate almost all steps of xenobiotic detoxification in the liver, including absorption, biotransformation and elimination [[118],120–122]. Thereby, CC-controls pharmacological parameters such as pharmacokinetics and pharmacodynamics [118,119]. Remarkably, 56 of the top 100 best-selling drugs in the USA are known to target the product of a circadian gene [122]. Until now, this approach of chronomedicine has been evaluated for several diseases, such as hypertension [123,124] and cancers [125,126]. The most important example is that of the circadian hormone melatonin that has been used in combination with cancer therapy to minimize toxicity or enhance chemotherapeutic viability in clinical and laboratory settings [126]. To further illustrate, influenza vaccine when administered in the morning produces higher titers of antibodies than when given in the evening [127].

Pharmacological therapies are not yet available for NASH [30–34], although several compounds are in preclinical and clinical development, including obeticholic acid (INT-747; [128]) which activates FXR and, elafibranor (currently in phase 3 trial; [129]) which activates NRs PPAR-α/β. Notably, physiological targets of potential NASH-modulating compounds [30–32], e.g. resveratrol (SIRT1-agonist) and inhibitors of acetyl-CoA carboxylase1 (ACC1) are also CC-regulated [1–4], thereby further strengthening the CC-connection to the development of novel therapeutics. Considering, the role of the CC in regulating the expression and activities of FXR, PPARs, SIRT1 and ACC chronopharmacology could very well dictate the efficacy of these approaches. Circadian "misalignment" between central and peripheral CCs has been found to be a core feature of almost every dietary or environmental model of metabolic disease including NAFLD. For therapeutic treatment of metabolic diseases like NAFLD a strategy could be to give to patients more scheduled eating habits, the so-called chrononutrition. In this regard, time-restricted feeding (TRF), a behavioral approach where feeding is solely restricted to the circadian active phase not only prevents circadian misalignment but also has been shown to correct several metabolic pathologies in animal models [130–132]. TRF is distinct from intermittent fasting and when applied to humans, the amount of calory ingested is not relevant [130–132]. Importantly, several small-scale human TRF investigations have indicated its usefulness in improving outcomes in patients with metabolic syndromes [133–135], however, the usefulness of TRF on NAFLD endpoints are yet to be ascertained.

7. Conclusion

As the prevalence and economic burden of the metabolic syndrome and NAFLD/NASH/MAFLD continues to rise worldwide, the knowledge about the mechanisms contributing to the development of this disease has been progressively increasing over the last two decades. Circadian misalignment has been associated with increased incidence of metabolic and cardiovascular disorders in various human studies [136–142]. These discoveries have led to the recognition of CC rhythms as an essential piece of the complex puzzle that depicts our physiologic homeostasis. The understanding of the multi-faceted role of the 'clock' in the pathogenesis of fatty liver (Fig. 3), is not only crucial to advance scientific knowledge, but also to improve public health by identifying new therapeutic targets and life-style modifications. Disruption of the CC has been shown to play an important role in the increasing incidence of metabolic homoeostasis with a key contribution to the metabolic syndrome and NAFLD. Hence, it is necessary to investigate in detail the CC-controlled pathways and elucidate how they are linked with the development of fatty liver disease (Fig. 3).

Expanding our knowledge about the genetic and environmental risk factors making individuals more susceptible to metabolic dysfunction combined with the discovery of new therapeutic approaches to restore the perturbed circadian machinery will ultimately contribute to improve the outcome of this rapidly growing pandemic of metabolic liver disease.

Declaration of competing interest

The authors declare no conflict of interest

Acknowledgements

The authors apologize to all colleagues whose work could not be cited due to space limitations.

This article is published as part of a supplement sponsored by Gilead Sciences, Inc.

Author contributions

AM designed the concept, focus areas and overall structure of the manuscript. MD prepared the figures. TFB conceptualized the section on the liver disease and therapeutic implications. AM, MD and TFB discussed, wrote and edited the different versions of the manuscript.

Funding

This work was supported by the European Union: ERC-AdG-2014-671231-HEPCIR, EU H2020-667273-HEPCAR, EU-InfectEra HepBccc (T.F.B.), the National Institute of Health (NCI R21 CA209940 and NIDDK R01CA233794 to T.F.B.), the Agence Nationale de Recherches sur le Sida et les Hépatites Virales (ANRS, 2015/1099), the Fondation ARC pour la Recherche sur le Cancer (IHU 201901299), the Institut Universitaire de France (IUF to T.F.B), and USIAS of the University of Strasbourg (A.M.). The work has been published under the framework of the LABEX ANR-10-LABX-0028_HEPSYS) and Inserm Plan Cancer and benefits from funding from the state managed by the French National Research Agency as part of the Investments for the future program.

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- **Scientific techniques**: Protein Biochemistry and genetic engineering, Molecular biology and oncology, immunology, microbiology, Gene Set Enrichment Analysis (GSEA), expertise in the study of signaling pathways, Microscopy (Celigo), Experience with laboratory automation and biological database, Modeling Organ Development And Disease In 3D Organoid Culture Systems, Training experimental surgery empowerment.
- **Quality Assurance & Bioprocess Engineering**: Certification in ISO 9001, ISO 13485, and ISO 14001, BPF, GMP, Medical Trend Research / GCP Environment, Project management certification.
- **Software:** MS software GraphPad,ImageJ,Endote, ConvertSeq,ClustalX,Aspen plus,Hysys

PUBLICATIONS & AWARDS :

- M. Dachraoui, F. Jühling, R. Martin, L. Mailly, N. Rohlen, S. Durand E. Crouchet, A. Mukherji, A. Saviano, C.Schuster, E.Felli, P.Pessaux, J.Lupberger and TF. Baumert. submitted in Nature Metabolism 2021.

- M. Dachraoui, A. Mukherji , T. F. Baumert. "Perturbation of the circadian clock and pathogenesis of NAFLD. Metabolism, October 2020, 111S:154337, DOI: 10.1016

- Second-place winner of the best BLITZ in the Doctoral School Days 2021 (Strasbourg, FRANCE)

Mayssa DACHRAOUI

Pharmacological targeting of the hepatic-circadian clock for treatment of metabolic liver disease

Résumé :

La fibrose hépatique est le principal facteur de risque de carcinome hépatocellulaire, l'une des principales causes de décès par cancer la plus répandue dans le monde. Cependant, les thérapies approuvées possèdent une efficacité anti-fibrosante limitée. L'horloge circadienne (HC) joue un rôle clé dans la régulation du métabolisme du foie mais son rôle dans la pathogenèse de la fibrose hépatique en tant que cible thérapeutique est inconnu. Durant mes études de doctorat, j'ai montré que l'HC régule de manière transcriptionnelle l'expression des gènes clés liés à la signalisation du TGF-β et au développement de la fibrose et que cette régulation est perdue dans les maladies métaboliques du foie. J'ai également trouvé dans les cellules humaines primaires une relation entre l'induction de la signalisation élevée du TGF-β et l'HC, qui a été confirmé dans les sphéroïdes dérivées des patients ainsi que chez les modèles murins dérivés des patients. De plus j'ai obtenu des résultats prouvant que l'activation pharmacologique du composant de l'HC (REV-ERBα) améliore significativement la fibrose dans les modèles murins dérivés des patients et dans les sphéroïdes humains. En conclusion, j'ai découvert que l'HC fonctionnel prévient la fibrose et que le ciblage de REV-ERBα est un nouveau concept pour le traitement de la fibrose hépatique.

Mots clés : Horloge circadienne, Maladies chroniques de foie, Fibrose, REV-ERBα, Découverte de médicament.

Abstract:

Liver fibrosis is the key risk factor for the hepatocellular carcinoma, a leading cause of cancer death world-wide. Approved anti-fibrotic therapies are absent and most compounds in clinical development have limited efficacy. The circadian clock (CC) is a major regulator of liver metabolism, but its role in the pathogenesis of liver fibrosis and as a potential therapeutic target is unknown. During my PhD studies, I showed that the liver hepatic CC-oscillator is temporally restricting (gating) TGF-β signaling, and that this regulation is lost in metabolic liver disease leading to constitutive expression of fibrotic genes. Mechanistic studies in primary human cells revealed a reciprocal relationship between increased TGF-β-driven fibrotic-signaling and CC, which were confirmed in patient-derived spheroid and in mice with liver disease. Remarkably, a pharmacological restoration of REV-ERBα activity markedly inhibited fibrosis in a humanized liver chimeric mouse model and spheroids-generated from fibrosis patients. In conclusion, I discovered that a functional CC prevents fibrosis and that targeting REV-ERBα is a novel concept for treating liver fibrosis-an important global unmet medical need.

Keywords: Circadian clock, Chronic liver disease, Fibrosis, REV-ERBα, Drug discovery.