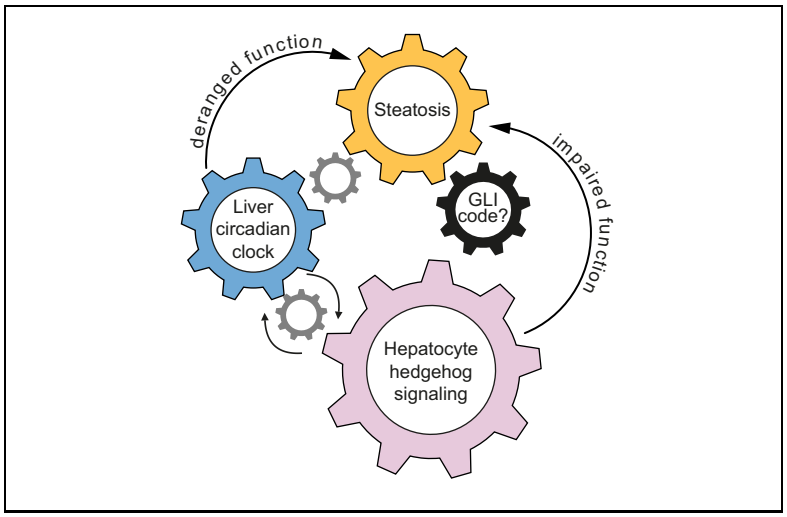
Research Article
Genetic and Metabolic Diseases

JOURNAL
OF HEPATOLOGY

Tick-tock hedgehog-mutual crosstalk with liver circadian clock promotes liver steatosis

Graphical abstract



Highlights

- Hh signaling shows diurnal oscillations in liver and hepatocytes *in vitro* and *in vivo*.
- Hh signaling feeds-back on the liver clock via GLI transcription factors.
- The amplitude of the oscillations of the liver clock is decreased in hepatocytes from *Smo*-knockout mice.
- Rhythmicity of many metabolic pathways, including hepatic lipid metabolism, is affected by oscillating Hh signaling.
- Diurnal timing of starvation affects the clock-hedgehog module differently.

Authors

Eugenia Marbach-Breitrück, Madlen Matz-Soja, Ute Abraham, ..., Andrej Shevchenko, Achim Kramer, Rolf Gebhardt

Correspondence

madlen.matz@medizin.uni-leipzig.de (M. Matz-Soja) rgebhardt@medizin.uni-leipzig.de (R. Gebhardt)

Lay summary

The results of our investigation show for the first time that the Hh signaling in hepatocytes is time-of-day dependent, leading to differences not only in transcript levels but also in the amount of Hh ligands in peripheral blood. Conversely, Hh signaling is able to feed back to the circadian clock.

http://dx.doi.org/10.1016/j.jhep.2019.01.022
 J. Hepatol. 2019, 70, 1192–1202

Research Article
Genetic and Metabolic Diseases

JOURNAL OF HEPATOLOGY

Tick-tock hedgehog-mutual crosstalk with liver circadian clock promotes liver steatosis

Eugenia Marbach-Breitrück[1,2,#], Madlen Matz-Soja[1,*,#], Ute Abraham[3], Wolfgang Schmidt-Heck[4], Susanne Sales[5], Christiane Rennert[1,6], Matthias Kern[7], Susanne Aleithe[1,8], Luise Spormann[1], Carlo Thiel[1], Raffaele Gerlini[9], Katrin Arnold[1], Nora Klöting[7], Reinhard Guthke[4], Damjana Rozman[10], Raffaele Teperino[9,11], Andrej Shevchenko[5], Achim Kramer[3], Rolf Gebhardt[1,*]

[1]*Rudolf-Schönheimer-Institute of Biochemistry, Faculty of Medicine, Leipzig University, Leipzig, Germany;* [2]*Institute of Biochemistry, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Germany;* [3]*Laboratory of Chronobiology, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Germany;* [4]*Leibniz Institute for Natural Product Research and Infection Biology – Hans Knoell Institute, Jena, Germany;* [5]*Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany;* [6]*Department of Hepatobiliary Surgery and Visceral Transplantation, University Hospital, Leipzig University, Leipzig, Germany;* [7]*IFB Adiposity Diseases, University of Leipzig, Leipzig, Germany;* [8]*Clinic and Polyclinic of Neurology, Faculty of Medicine, Leipzig University, Germany;* [9]*Institute of Experimental Genetics (IEG), HDC, Neuherberg, Germany;* [10]*Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Slovenia;* [11]*DZD, German Center for Diabetes Research, Neuherberg, Germany*

See Editorial, pages 1054–1056

Background & Aims: The mammalian circadian clock controls various aspects of liver metabolism and integrates nutritional signals. Recently, we described Hedgehog (Hh) signaling as a novel regulator of liver lipid metabolism. Herein, we investigated crosstalk between hepatic Hh signaling and circadian rhythm.

Methods: Diurnal rhythms of Hh signaling were investigated in liver and hepatocytes from mice with ablation of Smoothened (SAC-KO) and crossbreeds with PER2::LUC reporter mice. By using genome-wide screening, qPCR, immunostaining, ELISA and RNAi experiments in *vitro* we identified relevant transcriptional regulatory steps. Shotgun lipidomics and metabolic cages were used for analysis of metabolic alterations and behavior.

Results: Hh signaling showed diurnal oscillations in liver and hepatocytes *in vitro*. Correspondingly, the level of Indian Hh, oscillated in serum. Depletion of the clock gene *Bmal1* in hepatocytes resulted in significant alterations in the expression of Hh genes. Conversely, SAC-KO mice showed altered expression of clock genes, confirmed by RNAi against *Gli1* and *Gli3*. Genome-wide screening revealed that SAC-KO hepatocytes showed time-dependent alterations in various genes, particularly those associated with lipid metabolism. The clock/hedgehog module further plays a role in rhythmicity of steatosis, and in the response of the liver to a high-fat diet or to differently timed starvation.

Conclusions: For the first time, Hh signaling in hepatocytes was found to be time-of-day dependent and to feed back on the circadian clock. Our findings suggest an integrative role of Hh signaling, mediated mainly by GLI factors, in maintaining homeostasis of hepatic lipid metabolism by balancing the circadian clock.

Lay summary: The results of our investigation show for the first time that the Hh signaling in hepatocytes is time-of-day dependent, leading to differences not only in transcript levels but also in the amount of Hh ligands in peripheral blood. Conversely, Hh signaling is able to feed back to the circadian clock.

Introduction

Circadian rhythm plays an important role in regulating physiology and behavior. In mammals, the natural light–dark cycle synchronizes the central circadian pacemaker in the suprachiasmatic nucleus (SCN), which, in turn, coordinates the rhythms of autonomous clocks in peripheral tissues such as the liver;[1] these tissues adapt their functions to the rhythmic cycles of feeding and activity. Indeed, most metabolic and secretory functions of the liver show pronounced circadian rhythms.[2,3] In particular, liver carbohydrate and lipid metabolism, which are crucial for energy supply of the entire organism, oscillate throughout the day. The importance of this daily control is emphasized by epidemiological and experimental evidence that interruption or perturbation of circadian rhythms increases the risk for various types of liver disease and may even contribute to diabetes, obesity, metabolic syndrome and cancer.[4–6]

On the molecular level, circadian oscillations in the liver are regulated by several transcriptional and translational feedback loops based on the transcriptional activators *Clock* and *Bmal1*, which heterodimerize to stimulate the expression of the *Cry1* and *Cry2* as well as *Per1*, *Per2* and *Per3* repressors that then regulate Clock/Bmal1 activity.[7] The oscillating activity of these central clock proteins is also responsible for daily rhythms in metabolic function.[8,9] In the liver, the peripheral clock machin-

Keywords: Liver; Hepatocytes; Hedgehog; Circadian Rhythm, Steatosis.
Received 8 June 2018; received in revised form 20 December 2018; accepted 16 January 2019; available online 1 February 2019

* Corresponding authors. Address: Rudolf-Schönheimer-Institute of Biochemistry, Johannisallee 30, 04103 Leipzig, Germany. Tel.: +49 341/9722100, +49 341/9722117; fax: +49 341/9722109.
E-mail addresses: madlen.matz@medizin.uni-leipzig.de (M. Matz-Soja), rgebhardt@medizin.uni-leipzig.de (R. Gebhardt).
# These authors contributed equally as first authors, respectively.

ery is essential for the maintenance of metabolic homeostasis. Thereby almost all metabolic processes, such as carbohydrate, lipid as well as the amino acid metabolism and many more are dependent on the functional internal clock of the liver.[10,11,2,12] For example, transcription factors such as DBP, HLF, and TEF are involved in transmitting the oscillations to a wide variety of so-called clock-controlled genes responsible for different liver functions.[13] Recently, we revealed the hepatic Hh signaling as a master regulator of liver lipid metabolism which, if disturbed or disrupted, may cause liver steatosis.[14] Since this morphogen signaling pathway is also involved in modulating the insulin-like growth factor axis,[15] which was reported to contribute to metabolic zonation of the liver[16] and was recently suggested to modify the circadian clock in human cells,[17] we were interested to determine whether Hh signaling itself exhibits a diurnal rhythm and whether, and how, it influences the rhythm of lipid metabolism and other hepatic functions.

By using mice that lack *Smo*, a key component of Hh signaling, and *in vitro* experiments, we demonstrate a strong mutual interaction between Hh signaling and core components of the hepatic circadian clock. Disturbance of the daytime-dependent expression of transcription factors that mediate clock output showed that this interaction considerably impacts hepatocyte gene expression. These results highlight an important but yet unrecognized role of Hh signaling in regulating daily metabolic function and other features in the healthy adult liver.

Materials and methods

Generation of transgenic mice and the use of non-transgenic mice

We generated a transgenic model for hepatocyte-specific knockout of *Smo* by crossing $Smo^{flox/flox}$ and Alfp-Cre lines (abbreviated SAC-KO mice) as described previously in detail.[15] The mice were maintained in a pathogen-free facility on a 12:12 h light–dark cycle, according to the German guidelines and the world medical association declaration of Helsinki for the care and safe use of experimental animals (permission numbers: T04/14; N03/14, T34/14, T22/14). Double-transgenic SAC-PER2::LUC reporter mice were breed via crossbreeding SAC mice with PER2::LUC[18] reporter mice (Jackson Laboratories). For investigations in non-transgenic animals, mice from the C57BL/6N strain were used at the age of 12 weeks. For all investigations male mice were used.

Bioluminescence detection of PER2::LUC SAC-PER2::LUC hepatocytes

In order to follow circadian oscillation of cultured liver cells, hepatocytes from PER2::LUC mice[18] were isolated as described above. Cells were resuspended in Williams Medium E containing 10 % fetal calf serum (FCS) and other additions[13] and 0.6×10^6 cells were seeded in 35x10mm culture dishes. After 4 h FCS-containing medium was removed and a serum-free medium containing Luciferin (0.18 mM) was used. Bioluminescence of these hepatocytes was monitored throughout cultivation without medium change using photomultiplier tubes (HC135-11MOD, Hamamatsu, Japan) at 37 °C and 5 % CO_2. Photon counts were stored in 5 min bins for 168 h.

Statistical analysis

Values are expressed as means ± standard error of the mean (SEM). When designing this proof of principle study, sample size was calculated using G*Power software with statistical power of 0.8 and alpha = 0.05.

Since the data were normally distributed, statistical evaluation between 2 groups was performed using the unpaired *t* test for fresh tissue and hepatocytes as well as paired *t* test for siRNA experiments in cultured hepatocytes. The null hypothesis was rejected at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ levels. For comparison among multiple diurnal groups one way ANOVA with Tukey's least significant difference *post hoc* test was performed, allowing the estimation of similar variances between the circadian groups and adjusting for multiple testing. Here, unless otherwise stated, significant differences to ZT0 are indicated by: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

Results

Hepatic Hh signaling pathway is time-of-day-dependent

To determine whether Hh signaling genes show a diurnal rhythm in hepatocytes, we performed quantitative PCR (qPCR) analysis of RNA isolated at 6 different time points distributed over a 24 h period from liver tissue of C57BL/6 mice. As a control, we analyzed the expression of well-known clock genes (*e.g. Bmal1*, *Dpb Cry1*) which showed strong oscillation of clock genes (Fig. S1) confirming published results.[19] Interestingly, most Hh pathway genes also showed a pronounced diurnal rhythm (Fig. 1A) with various phases and amplitudes that might reflect distinct roles within the signaling cascade. In particular, *Ihh* mRNA levels were significantly increased (almost 5-fold) at ZT9 compared to ZT0 and then decreased after ZT12. The expression of the Hh receptor *Ptch1* oscillated with a similar pattern, peaking at ZT12, while those of *Smo* peaked at ZT6 (Fig. 1A). The expression of further genes within this pathway, was evaluated to gain insight into pathway oscillations (Fig. 1A, S2). *Shh* did not show any significant variation. However, *Hhip1* mRNA decreased continuously throughout the day, with minimum levels at ZT16. The expression of *Fu* was lowest at ZT0 and ZT6-12 and peaked at ZT3 and ZT16. Its antagonist, *SuFu*, showed the inverse behavior, reaching its maximum at ZT6. The *Gli* transcription factors displayed only moderate oscillations throughout the day, with smaller amplitudes than other members of the Hh cascade (Fig. 1A).

The marked rhythmic expression of *Ihh* prompted us to investigate the cellular distribution of IHH protein. The expression of IHH shows a zonal distribution with predominant staining in the pericentral area. In accordance with the observed oscillation of *Ihh* expression, the pericentral expression zone enlarged after ZT3, peaked at ZT9 with the strongest staining (Fig. 1B) and shrank thereafter until it almost disappeared at ZT16 (Fig. S3).

The pericentral expression of IHH protein appears to correspond with its transport vehicle very low-density lipoprotein, which is predominantly produced in the liver pericentral zone. When IHH protein was quantified in mouse serum, levels were lowest at ZT9 and highest at ZT16, indicating the expected delay between IHH production in the liver and its transport through the pericentral vein into the systemic circulation (Fig. 1C).

Daily rhythmicity of Hh signaling genes is maintained in isolated hepatocytes and depends on *Bmal1* expression

To investigate daily rhythmicity of Hh associated genes in hepatocytes, pure fractions were isolated from C57BL/6N mice at ZT3 and ZT12 (Fig. 1D). Highly significant differences were

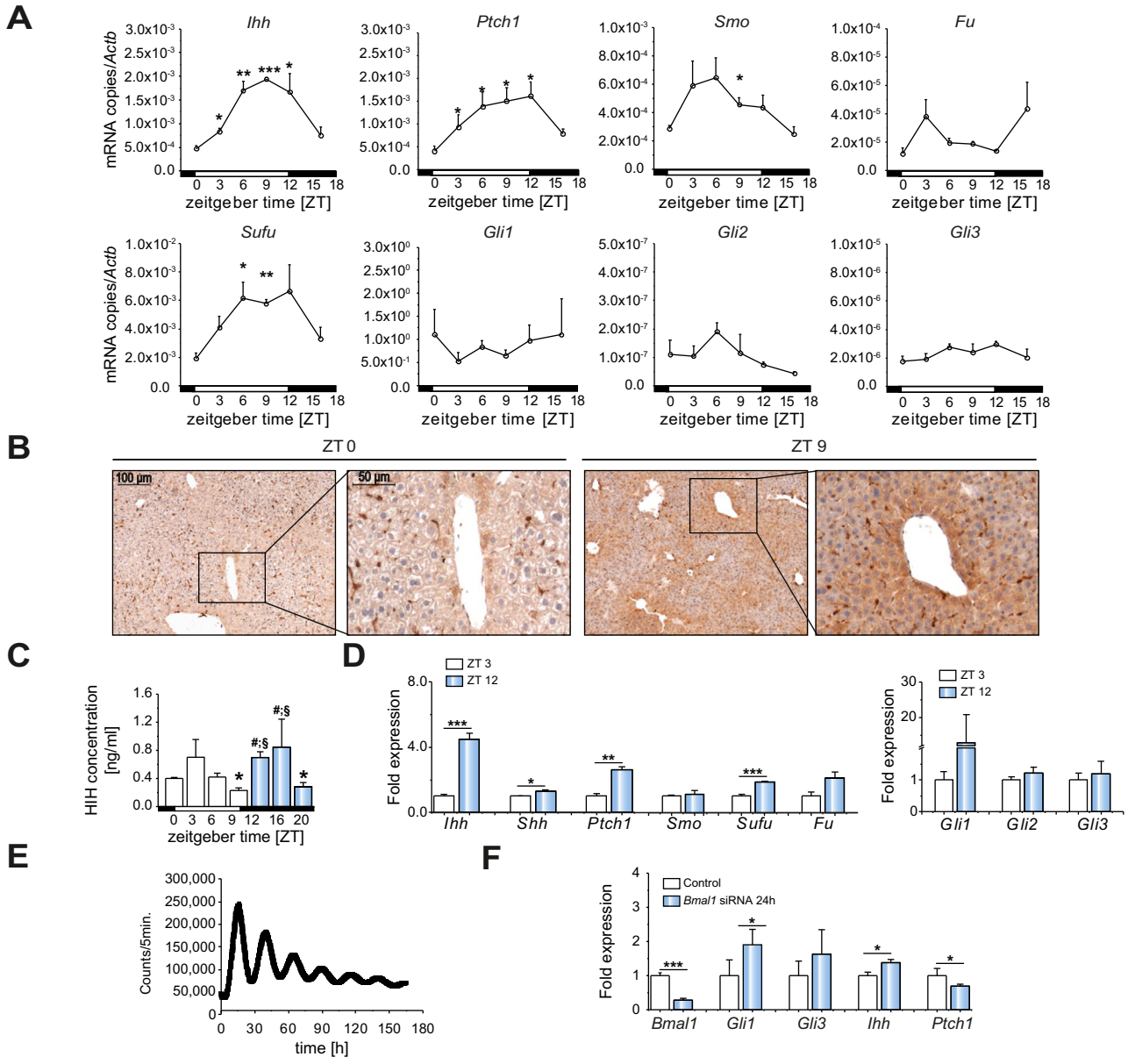


Fig. 1. Interaction between Hh pathway components and liver circadian clock. (A) Expression of *Ihh*, *Ptch1*, *Smo*, *Fu*, *Sufu* and *Gli1/2/3* in liver tissue from C57BL/6N mice at 6 time points; n = 3/ZT, significant differences to ZT0 were determined using ANOVA with Tukey's least significant difference *post hoc* test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$. (B) Immunohistochemical staining of IHH in liver sections at ZT0 and ZT9. (C) Serum IHH concentration in C57BL/6N mice at ZT0, 3, 6, 9 and ZT12, 16 and 20, n = 3–5/ZT, where (*) indicate significant group differences to ZT3, (§) indicate significant group differences to ZT9, and (#) indicate significant group differences to ZT20 determined using ANOVA with Tukey's least significant difference *post hoc* test with $p \leq 0.05$, n = 3–5/ZT. (D) Expression of *Ihh*, *Shh*, *Ptch1*, *Smo*, *Sufu*, *Fu* and *Gli1/2/3* in primary hepatocytes at ZT3 and ZT12, n = 3/ZT, unpaired *t* test $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$. (E) Bioluminescence of PER2-driven luciferase expression in cultured hepatocytes isolated from PER2::LUC mouse. (F) Expression of *Bmal1*, *Gli1*, *Gli3*, *Ihh* and *Ptch1* in *Bmal1*-RNAi experiments performed in cultured hepatocytes. *Bmal1* siRNA, control, n = 5, unpaired *t* test, $^{*}p \leq 0.05$, $^{***}p \leq 0.001$.

found for the Hh genes *Ihh*, *Ptch1*, and *SuFu*, even though these 2 time points do not exactly correspond to the peak and nadir expression of every gene investigated. Surprisingly, *Shh* expression in hepatocytes showed a small but significant increase from ZT3 to ZT12 (Fig. 1D) in contrast to the results in liver tissue. Differences in the transcription of other genes (*Gli1*, *Gli2*, *Gli3*, *Smo*) were less pronounced at these 2 time points, which corresponds to the liver data. These findings prompted the question of whether these changes reflect the persistence of the circadian rhythm in isolated hepatocytes. First, we determined the diurnal expression of clock genes in hepatocytes (Fig. S4) and obtained similar data as that from the whole liver (Fig. S1). Second, we cultured hepatocytes from PER2-luciferase reporter mice[18] and continuously monitored luciferase activity for up to 168 h. As shown in Fig. 1E, enzyme activity perfectly oscillated during *in vitro* cultivation with a period of approximately 25 h. Although the oscillation amplitude in hepatocytes steadily decreased over time, the amplitude after 72 h was still approximately 1/3 of that after 24 h. These findings convincingly show that the Hh signaling pathway exhibits diurnal rhythm in hepatocytes.

To test whether the core clock oscillator is responsible for driving circadian rhythm of Hh pathway genes, we transfected cultured hepatocytes from C57BL/6 mice with siRNA against

Bmal1. As a result of the downregulation of *Bmal1* expression and BMAL1 protein at 24 h after transfection (Fig. S5), *Gli1* and *Ihh* were significantly upregulated, *Ptch1* was decreased, and *Gli3* levels remained unaffected compared to control transfection (Fig. 1F) in agreement with the essential function of *Bmal1* in circadian oscillation.[20] In contrast, transfection with *Clock* siRNA (and CLOCK protein) had no significant effects on these Hh genes (data not shown).

Hh signaling feeds back on core clock genes

To investigate whether hepatocyte Hh signaling feeds back on the liver clock, we used transgenic mice, where Hh signaling is substantially perturbed by the specific deletion of *Smo* in hepatocytes (SAC-KO).[15] In fact, the expression of some core clock genes showed profound changes in whole liver tissue of SAC-KO mice compared to wild-type littermates (Fig. 2A and S6). While the hepatocyte-specific deletion of *Smo* resulted in a significant downregulation of *Clock* at ZT3 and ZT12 as well as *Dec2* at ZT15, *Per3* was upregulated and showed a phase shift to maximal expression at ZT12. Likewise, peak expression of *Nr1d1* shifted from ZT3 to ZT9. Other clock genes, such as *Bmal1*, *Per1* and *Nr1d2* (Fig. 2A), as well as *Dbp*, *Cry1* and *Per2* (Fig. S6) did not show significant differences.

Since in SAC-KO Hh signaling is impaired only in hepatocytes we investigated the expression of clock genes at ZT3 and ZT12 in isolated hepatocytes (Fig. 2B). To uncouple the hepatocellular clock from SCN influence mice, were fasted and fed again prior to the isolation procedure.[21] In hepatocytes, *Bmal1* showed strong downregulation at ZT3 in SAC-KO mice, whereas the alterations in *Clock* mRNA were less pronounced compared to whole liver. *Per1*, *Per3*, *Nr1d1* and *Nr1d2* were strongly upregulated at ZT3, indicating an altered clock in hepatocytes with impaired Hh signaling (Fig. 2B). In addition, we observed robust upregulation of *Cry1* and *Per2*, whereas *Dec2* expression was significantly downregulated in SAC-KO mice at ZT12 (Fig. S7).

To explore the function of the circadian clock in SAC-KO hepatocytes in more detail, we crossbred SAC and PER2::LUC mice to produce double-transgenic SAC-PER2::LUC reporter mice. Upon measuring PER2-LUCIFERASE oscillations in hepatocytes derived from these mice (Fig. 2C), we found higher amplitudes and increased damping of circadian rhythms, while period and phase were unaffected. This finding may implicate a less robust rhythm due to the hepatocyte-specific ablation of *Smo*, *i.e.*, impaired Hh signaling.

To investigate whether these changes in clock gene expression influence SAC-KO mouse behavior, we placed the mice in metabolic cages. While feeding behavior was of great interest because feeding time is known to affect circadian rhythms,[21] there were no alterations in food or water intake per day or the respiratory ratio. Based on this result, we conclude that the observed changes in circadian rhythm in the liver of SAC-KO mice have no influence on physiological feeding behavior in general. This is in line with findings of Shi *et al.* in mice lacking *Bmal1* only in hepatocytes.[22] However, mouse activity (represented by distance moved) showed a decreasing trend, and a significant decrease in body erection of SAC-KO mice was observed (Fig. S8).

Deletion of *Smo* in hepatocytes alters diurnal rhythms in Hh signaling gene expression

To test, whether *Smo* deletion in hepatocytes has an impact on the time-of-day dependent expression of Hh signaling genes, we analyzed their expression in SAC-KO and SAC-WT mice in whole liver and isolated hepatocytes at various time points. Looking at the influence of the *Smo* deletion in hepatocytes, *Ihh* completely lost its diurnal variations. In contrast, the expression of the receptor *Ptch1* as well as *Fu* and *Sufu* showed a smaller magnitude at nearly all time points with very low oscillatory behavior (Fig. 3A). Due to deletion of *Smo*, the oscillations of *Gli1* and *Gli2* were clearly diminished in SAC-KO livers, while for *Gli3* a maximum at ZT12 was still observed.

These results were consistent with those from isolated hepatocytes for most of the observed genes. For example, *Ihh* also showed a strong downregulation at ZT3 in SAC-KO mice. Also *Ptch1* showed a strong upregulation in isolated hepatocytes from SAC-KO mice at ZT 3 similar as in whole liver (Fig. 3B). The expression *Sufu* did not increase at ZT3, as seen in whole liver. However, there are also some differences between whole liver and isolated hepatocytes data, such as the observed downregulation of *Sufu*. In addition, the decreased expression of Gli transcription factors, observed in the isolated hepatocytes of SAC-KO mice, was not present in the liver (Fig. 3A, B). This may be the result of a masking effect by a higher contribution of a non-parenchymal cell fraction in the liver material compared to isolated hepatocytes.

Gli1 and *Gli3* contribute to transcriptional regulation of the hepatic clock

Since pronounced downregulation of Gli genes was observed in SAC-KO hepatocytes, we investigated a direct effect of these transcription factors on clock gene expression *in vitro*. RNAi-mediated depletion of *Gli1* in isolated hepatocytes resulted in significant downregulation of *Per1* and *Nr1d2* after 48 h and slight upregulation of *Per3* after 72 h (Fig. 4A). Knockdown of *Gli3* significantly downregulated *Per3* after 48 h, followed by a slight upregulation at 72 h post-transfection (Fig. 4B). *Clock* expression was also significantly downregulated 72 h after *Gli3* depletion (Fig. 4B).

To ascertain the effect of activating Hh signaling on clock gene expression, we knocked down *Ptch1*, which leads to the upregulation of *Gli* transcription factors, as described recently.[15] The results show an activation of *Nr1d2* as well as *Clock* expression. This opposite regulation of clock components by inactivation or activation of Gli transcription factors, respectively, clearly indicates that *Gli1* and *Gli3* modulate central clock genes.

Hh signaling controls hepatic lipid metabolism and its diurnal oscillation

Next we asked how global gene expression depends on *Smo* expression and time-of-day. For this purpose, microarray studies were performed using total RNA from hepatocytes isolated from SAC-KO mice and WT littermates at ZT3 and ZT12. After comparing the diurnal regulation between ZT3 and ZT12, a total of 1,268 unique genes (fold change >1.3) were selected that showed diurnal expression in both SAC-WT and SAC-KO mice (Fig. 5A). Analysis of these data according to BKL (BIOBASE Knowledge Library[23]) GO terms revealed a highly significant enrichment of genes associated with metabolism (768/9078; $p < 2.87E^{-28}$), particularly lipid metabolism (106/1154; $p < 2.58E^{-18}$). The genes could be further separated into 2 clusters: cluster 1 (yellow bar), genes that showed different diurnal regulation between SAC-WT and SAC-KO mice; and cluster 2 (blue bar), genes with similar rhythmic behavior in both groups of mice (Fig. 5A). The majority of genes in cluster 1 were highly

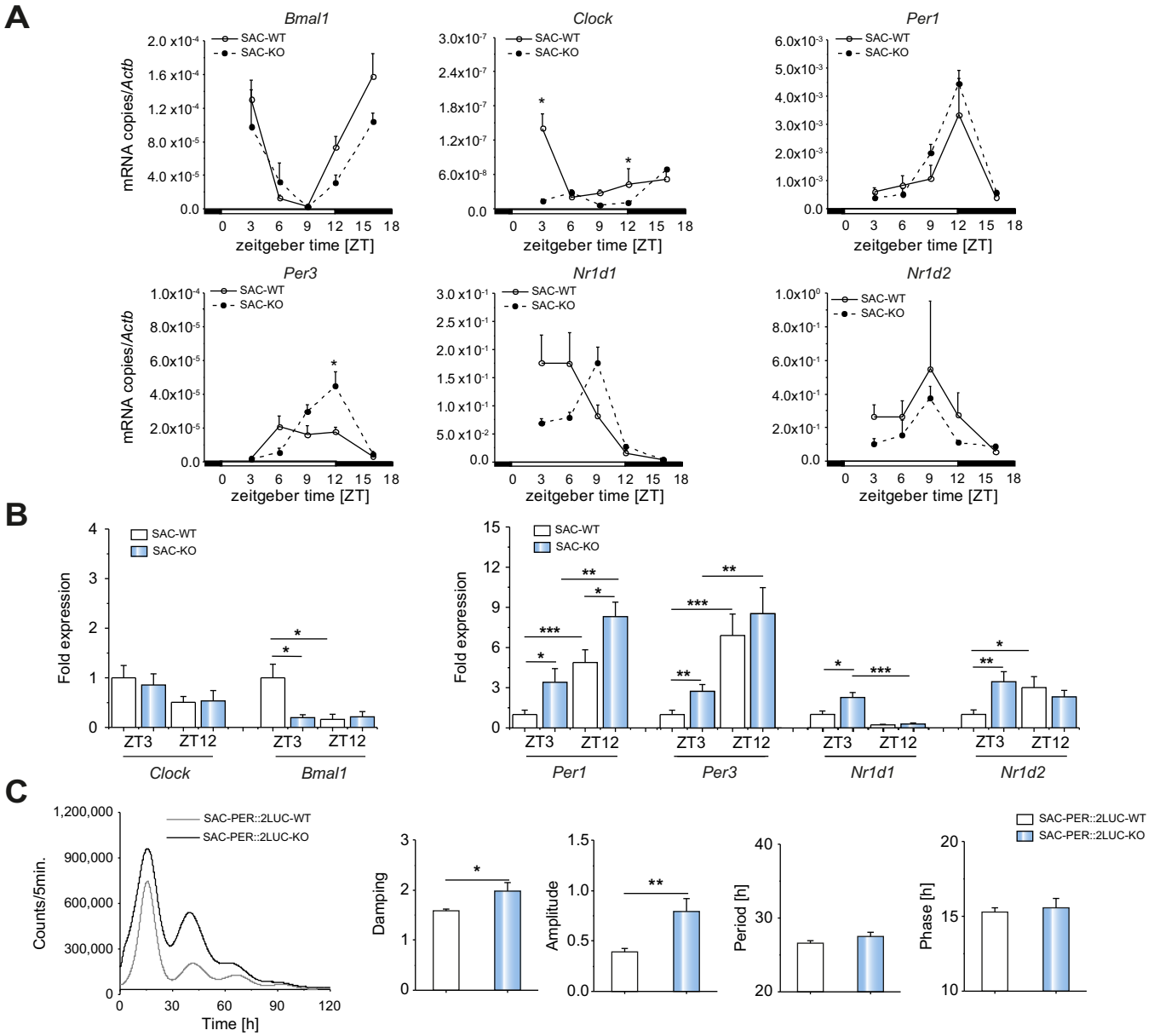


Fig. 2. The diurnal expression of clock genes in SAC mice. (A) Expression of *Bmal1*, *Clock*, *Per1/3*, *Nr1d1/2* in livers of SAC-KO and SAC-WT mice, at different time points (ZT3, 6, 9, 12, 16), n = 3, stars indicate significant differences to WT at same ZT. (B) Expression of *Clock*, *Bmal1*, *Per1/3* and *Nr1d1/2* in hepatocytes from SAC-KO and SAC-WT mice at ZT3 and ZT12, n = 6–13/ZT. (C) Bioluminescence of Per2-driven luciferase activity in cultured hepatocytes isolated from SACPER2::LUC-WT, SAC-PER2::LUC-KO. Within the first 24 h period, the oscillation parameters as amplitude, damping, period length and phase were analyzed using the Chronostar software n = 5–6 per genotype, unpaired *t* test, **p* ≤0.05, ***p* ≤0.01 and ****p* ≤0.001. SAC-KO, *Smo* knockout; SAC-WT, *Smo* wild-type.

associated with lipid metabolism as assessed by WIKI pathway analysis (*e.g.*, *Cyp1A2*, *Acly*, and *Scd1*), whereas those in cluster 2 were spread among more diverse WIKI-GO pathway terms, including oxidative phosphorylation, amino acid metabolism and glycogen metabolism (Table S1). The complete transcript profiling is available on: https://seek.lisym.org/data_files/34?code=DIYRPXUxjhYICg7zi3F98Mr%2FTqv4PP1z6wAS4Pdu.

Among the lipid metabolism-associated genes, Fig. 5B and Table S2(A, B) provide detailed insight into which genes are higher or lower expressed at ZT3 than at ZT12 in SAC-WT and SAC-KO hepatocytes, as well as those which where regulated coherent in both genotypes.

The gene array analysis results were confirmed and extended by qPCR experiments performed on hepatocytes isolated from transgenic mice at each time point. In SAC-WT hepatocytes, *Srebf1* and *Ppara* showed a pronounced increase at ZT12 compared to ZT3 while *Pparg* and *Chrebp1* expression did not differ at these time points (Fig. 5C). In SAC-KO hepatocytes, however, *Chrebp1* expression was increased at ZT12 only, while *Srebf1*, *Ppara* and *Pparg* were deregulated by significant upregulation at ZT3. These results at ZT3 are in line with data reported previously for mice with a different hepatocyte-specific conditional deletion of *Smo* (SLC mice).[14]

With respect to key lipogenic enzymes involved in the elongation of fatty acids, significant time-of-day-dependent downregulation of *Elovl3* was detected in SAC-WT hepatocytes at both time points, whereas the inverse regulation was observed for *Elovl6* (Fig. 5D). *Gpam* showed no time-of-day-dependent

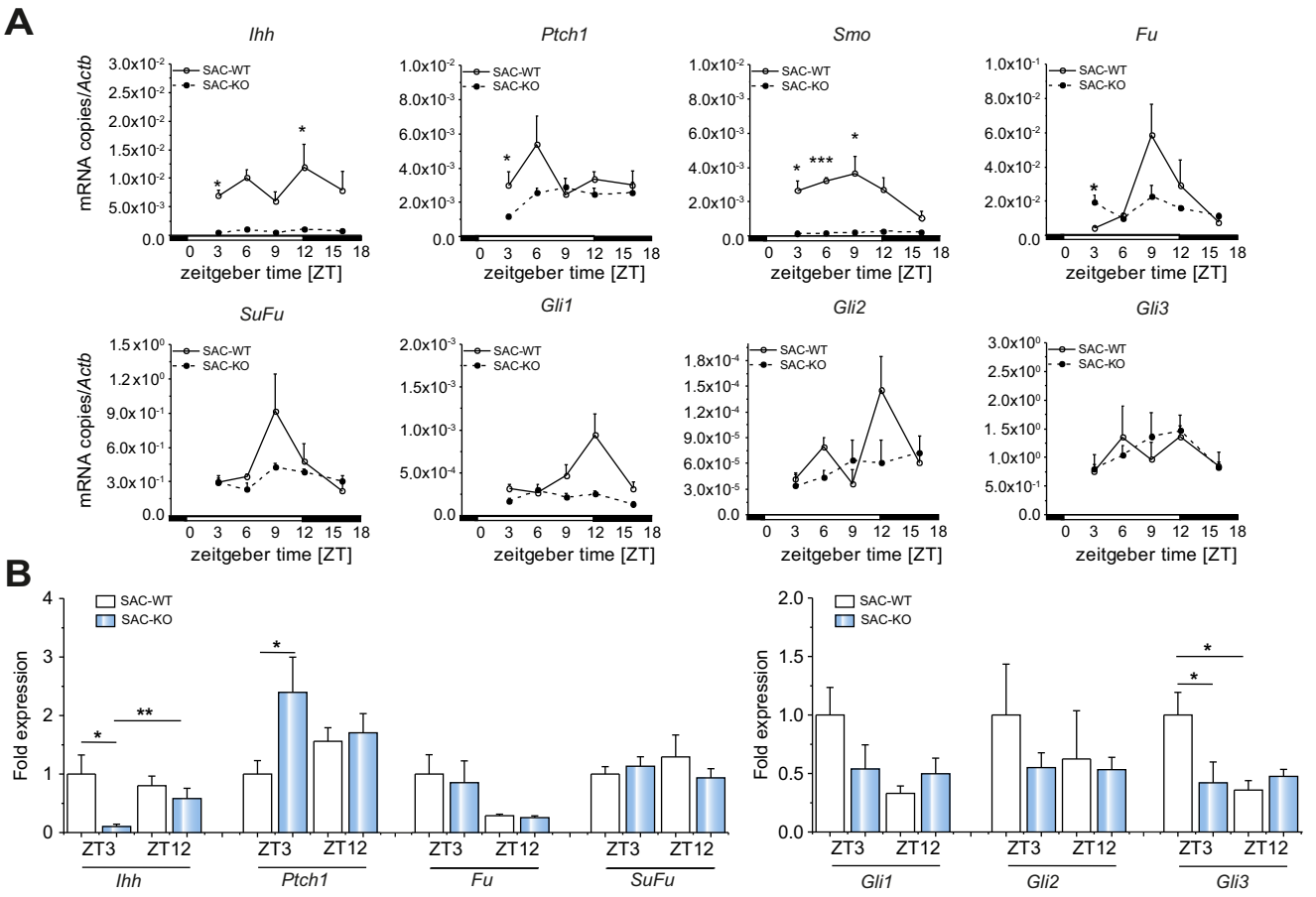


Fig. 3. The diurnal expression of Hh components in SAC mice. (A) Expression of *Ihh*, *Ptch1*, *Smo*, *Fu*, *Sufu* and *Gli1/2/3* in livers of SAC-KO and SAC-WT mice, at ZT3, 6, 9, 12, 16, n = 3, stars indicate significant differences to WT at same ZT. (B) Expression of *Ihh*, *Ptch1*, *Fu*, *Sufu*, *Gli1*, *Gli2*, *Gli3* in hepatocytes from SAC-KO and SAC-WT mice at ZT3 and ZT12; n = 6–13 per genotype, unpaired *t* test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$. SAC-KO, *Smo* knockout; SAC-WT, *Smo* wild-type.

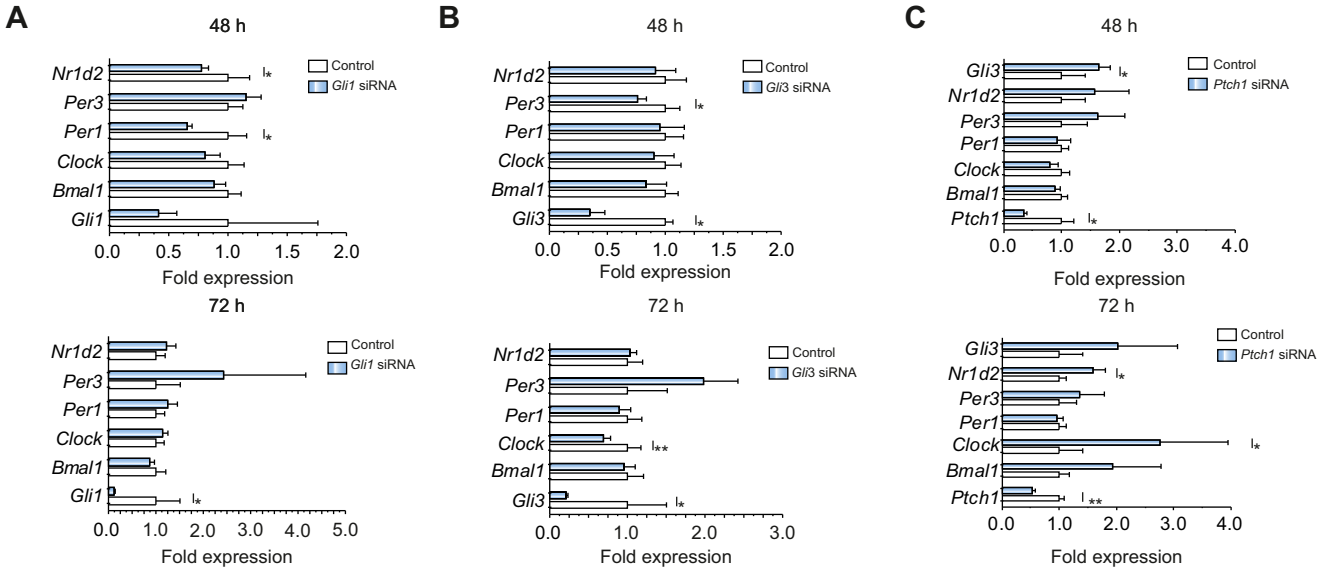


Fig. 4. RNAi experiments of *Ptch1* and *Gli1* and *Gli3*. Cultured hepatocytes from C57BL/6 mice were subjected to RNA interference of (A) *Gli1*, (B) *Gli3* and (C) *Ptch1*. Expression of *Nr1d2*, *Per3*, *Per1*, *Clock*, *Bmal1*, *Gli1/3* and *Ptch1* was measured 48 h or 72 h post-transfection, respectively, n = 5–8, paired *t* test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

variation in control mice. Remarkably, the expression patterns of these enzymes changed dramatically in SAC-KO mice. *Elovl3* was downregulated by more than 90% at both time points (Fig. 5D). *Elovl6* maintained its expression at ZT3 but showed no time-of-day-dependent increase at ZT12. In contrast, *Gpam* increased significantly at ZT12, but its levels were slightly lower

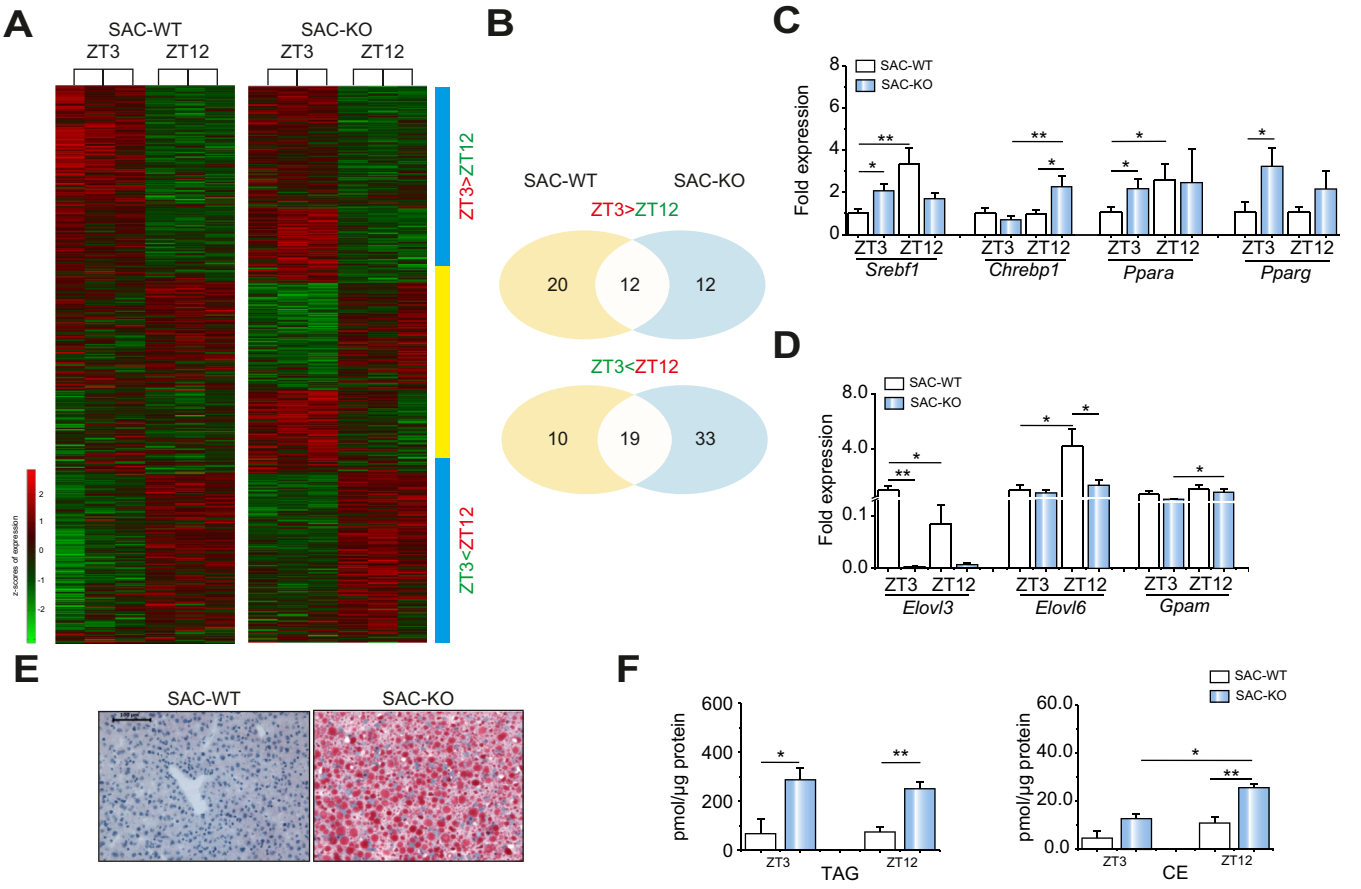


Fig. 5. Alterations of diurnal regulation of hepatic lipid metabolism in SAC mice. (A) Heatmap of genes with diurnal expression in SAC-WT (left panel) and SAC-KO (right panel) hepatocytes, n = 3/ZT. Blue bars indicate genes showing same diurnal behavior in both SAC-WT and SAC-KO. Yellow side bar indicates gene clusters presenting different diurnal regulation between the genotypes. (B) Comparing SAC-WT and SAC-KO hepatocytes, in total 106 genes associated with lipid metabolism show different diurnal regulation between ZT3 and ZT12. Expression analysis in isolated hepatocytes from SAC-WT and SAC-KO from (C) *Srebf1*, *Chrebp1*, *Ppara* and *Pparg*; (D) *Elovl3*, *Elovl6* and *Gpam*, n = 6–9/ZT. (E) Fat red staining of cultured hepatocytes from SAC-KO and SAC-WT mice. (F) Change of TAG and CE from SAC-WT and SAC-KO mice at ZT3 and ZT12, n = 3/ZT, unpaired *t* test $^{*}p \leq 0.05$, $^{**}p \leq 0.01$. CE, cholesterol ester; SAC-KO, *Smo* knockout; SAC-WT, *Smo* wild-type; TAG, triacylglyceride.

than those in wild-type mice. These changes point to an imbalance between the breakdown (lower) and synthesis (maintained) of fatty acids and/or triglycerides.

In accordance with these expression level changes due to interruption of Hh signaling in SAC-KO mice, these animals developed pronounced steatosis at 12 weeks of age which was characterized by a considerable increase in storage lipid classes, such as triacylglycerides and cholesterol esters, in hepatocytes isolated at both ZT3 and ZT12 (Fig. 5E, F). In addition, we observed a slight increase in diacylglycerides and sphingomyelins at ZT3, inversing or ameliorating the diurnal changes in these lipids, particularly in SAC-KO mice (Fig. S8). No changes in diurnal variation and levels were observed for the membrane lipids phosphatidylethanolamine, phosphatidylcholines, ceramides, cholesterol (Fig. S9).

The findings above clearly illustrate that interruption of the Hh pathway results in changes of both, rhythmicity and lipid metabolism finally leading to steatotic hepatocytes. Therefore, we were interested in the reverse question of whether steatosis induced by overnutrition may affect Hh signaling as well as liver circadian clock. Using C57BL/6 mice treated with an isocaloric high-fat diet (HFD), we could show that this is indeed the case. Without going into details for reasons of space and figure number limitations, an exemplary selection of data based on expression changes of *Dbp*, *Gli1* and *Gli3* demonstrates the high complexity of the clock-Hh response (Fig. S10). For instance, the ratios of the expression levels of SAC-KO and HFD-treated mice compared to the respective controls maintained their diurnal rhythm (*Dbp*) or lost (*Gli1*) or reversed it (*Gli3*). Thus, steatosis induced by genetic alterations (SAC-KO) or nutritional challenge (HFD) is characterized by both, partly similar and partly different responses of the clock-Hh system. Currently, this novel regulatory network is under detailed investigation.

Simultaneous changes of clock and Hh genes induced by starvation and refeeding

In a recent study we have shown that different timing of starvation of C57BL/6N mice results in completely different metabolic responses over a 24 h period.[24] Starvation induced in the morning at ZT3 resulted in the well-known response characterized by induced gluconeogenesis, while starvation induced in the evening at ZT15 caused a completely different response involving downregulation of gluconeogenesis and increased fatty acid and cholesterol synthesis. The expression of many clock genes was also differently affected by differently timed starvation.[24] According to our paradigm that clock and Hh signaling closely interact we show herein that components of the Hh signaling cascade are also differently affected by the different timing of

starvation. As depicted in Fig. 6A, B, the mRNA levels of *Ptch1* and *Sufu* respond highly significantly and almost in parallel to starvation starting at ZT3 by induction and at ZT12 by downregulation. Refeeding, on the other hand, causes a pronounced drop at ZT3 and does not approach the *ad libitum* level within a period of 12 h (ZT3) or 21 h (ZT12). The changes of the Gli transcription factors are less clear but, interestingly, indicate individual variations among the 3 factors. For instance, *Gli1* seems to be markedly enhanced after refeeding for 21 h in the evening (Fig. 6C), in contrast to *Gli2* and *Gli3* which are downregulated (Fig. 6E). The resistance of *Gli2* to starvation in the morning is also remarkable, as is the weak diurnal response of *Gli3* under almost all conditions.

Collectively, the altered Hh signaling may considerably contribute to explain the hitherto unknown different response of the liver to starvation at different times of day.

Discussion

Compared to other organs, the widespread contribution of Hh signaling to the regulation of liver metabolism in healthy mature animals has been ignored for quite some time.[25,26] Here, in a proof of principle study, we show that Hh signaling regulates liver metabolism in close cooperation with the liver circadian clock to maintain metabolic homeostasis.

The major results of our study are (i) Hh signaling is a target of the circadian clock and (ii) Hh signaling feeds back to the liver circadian clock. The diurnal rhythm is particularly pronounced for the ligand IHH; both *Ihh* mRNA and protein levels oscillated with high amplitude in liver tissue and serum. These results are in line with recently published data by Braune *et al.*, who showed that the presence and levels of IHH and DHH, the 2 most prominent circulating Hh ligands, correlate with metabolic status in physiological and diseased conditions.[27]

Despite the high similarity between Hh gene measurements in whole liver and in isolated hepatocytes of WT mice, as seen for *Ihh* and *Ptch1*, some differences highlight the need for caution and the importance of individual investigations at the level of isolated hepatocytes to appropriately interpret the results. For example, the rhythmicity of *Shh* expression is detectable in isolated hepatocytes only, whereas the whole liver rhythm may be masked by the high expression of *Shh* measured in hepatic stellate cells and bile duct cells.[28,29] In this context, the persistence of the liver clock in cultured hepatocytes revealed herein by continuously monitoring *Per2*-related expression changes in PER2::LUC reporter mice is of utmost importance because it ensures that primary hepatocytes even in conventional monolayer culture are an adequate *in vitro* system for studying phenomena related to diurnal rhythms. Similar findings were reported recently only for hepatocytes in sandwich culture.[30] It is also worth mentioning that oscillations presented in this work were detected without any additional synchronization protocol. The hepatocytes isolation procedure seems not to disrupt the synchronization present *in vivo*, since the synchronization by serum shock or dexamethasone did not lead to any differences in oscillations (data not shown). It is also possible that the isolation and cultivation procedures *per se* function as synchronization under *in vitro* conditions.

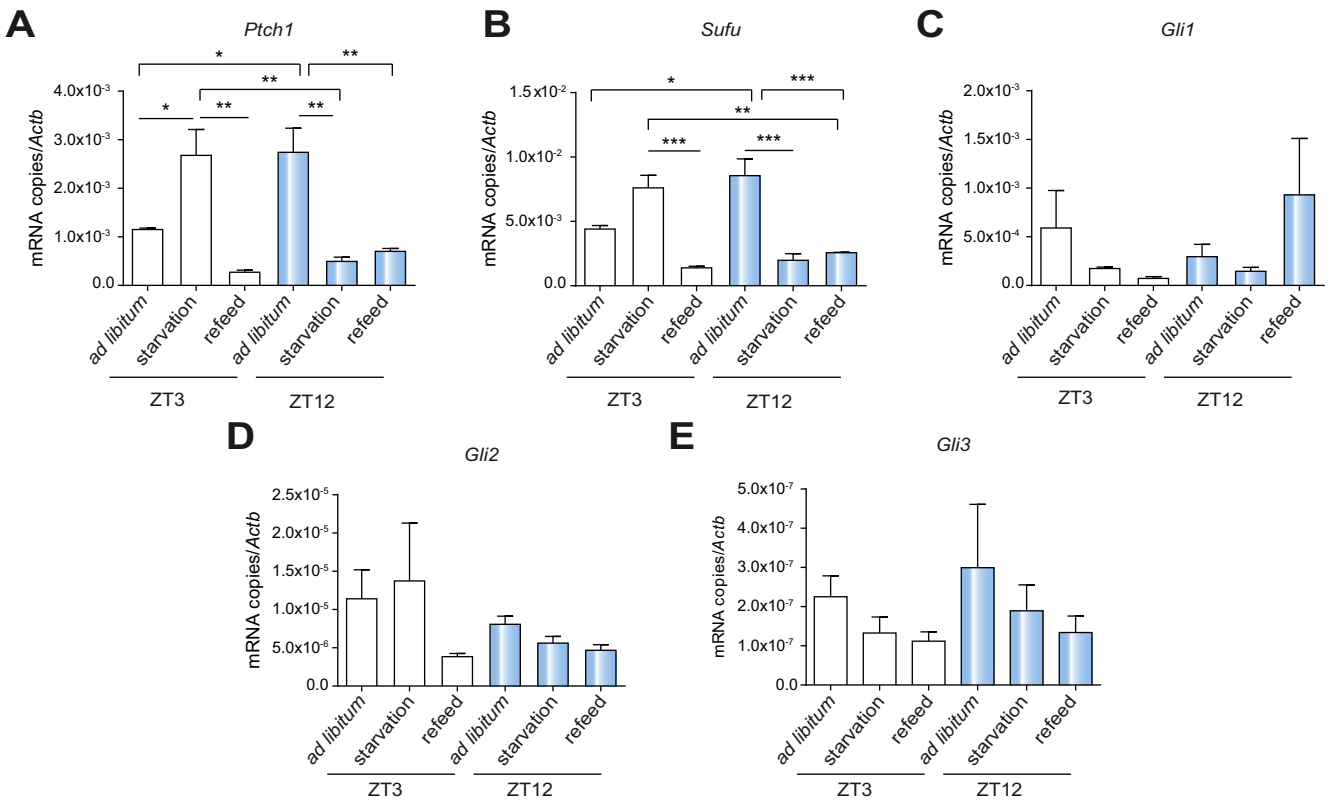


Fig. 6. Response of Hh signaling genes to differently timed starvation and refeeding. qPCR analysis of (A) *Ptch1*, (B) *Sufu*, (C) *Gli1*, (D) *Gli2* and (E) *Gli3* in isolated hepatocytes from male C57BL/6N mice. Mice were sacrificed after feeding ad libitum, and 24 h starvation started at ZT3 (white bars) or ZT12 (black bars). The third column refers to refeeding for 12 h up to ZT3 (white bar) or for 21 h up to ZT12 (black bar) after prior starvation for 24 h. (For details see Supplementary Material), unpaired *t* test $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

To gain insight into the molecular mechanisms responsible for the diurnal rhythm of the expression of Hh components, we transfected cultured hepatocytes with siRNA against *Bmal1* and *Clock*. Although Western blotting confirmed the downregulation of both proteins, only *Bmal1* depletion significantly modulated core Hh signaling genes. These findings confirm that BMAL1 is essential for circadian oscillator function,[20] while the loss of CLOCK in peripheral oscillators may (at least in part) be compensated by NPAS2.[31,32] Further, they suggest that major Hh genes, including *Ihh* and *Gli1*, are directly regulated in a transcriptional manner. These novel findings for the healthy adult liver corroborate the general predictions of Zhang *et al.* made from studies in a human cell line.[17] They are also in line with the results of Koike *et al.*[33] which performed ChIP-seq. analysis of DNA-binding site occupation by circadian transcription factors in the liver (Table S3). Furthermore, there is direct evidence of E-box binding sites in intron 1 of *Gli1*.[34] Interestingly, *in silico* analyses suggest that several other components of Hh signaling, such as *Ihh*, *Shh* and *Ptch1*, have E-box binding sites in their promoters and thus might be regulated by similar mechanisms[33] (Table S3).

Conversely, our experiments using transgenic SAC-KO mice provided evidence that Hh signaling feeds back on the liver circadian clock exclusively in hepatocytes because only these cells showed a physiological response to impaired Hh signaling characterized by strong downregulation of *Bmal1* and strong upregulation of *Per1*, *Per2*, *Nr1d1* and *Nr1d2* at ZT3 due to the hepatocyte-specific knockdown of *Smo*. In intact liver tissue of SAC-KO mice non-parenchymal cells which exhibit functional Hh signaling likely mask the hepatocellular response.

In addition, feedback of Hh signaling on the circadian clock was detected by continuously monitoring the Per2-dependent reporter in hepatocytes isolated from transgenic SAC-PER2::LUC mice.

Due to the mutual regulation of diurnal rhythm and Hh signaling, interruption of Hh signaling should alter both the clock and the subsequent diurnal variations in Hh components themselves. Indeed, in SAC-KO mice, the phase of oscillations in *Ihh* expression remained the same, but the amplitude decreased. Moreover, the 3 Gli factors lost their diurnal oscillation and stabilized at a lower expression level in SAC-KO mice at ZT12. These changes impact considerably on the expression of certain clock genes as revealed by transfection of hepatocytes with siRNA against these factors (*cf.* Fig. 4). Thus, GLI1 seems to be an activator of *Per1* and *Nr1d2*. The delayed influence on *Per3* after 72 h is most likely due to the indirect upregulation of *Gli3*, as was reported recently.[15] Direct support comes from the finding that *Gli3* knockdown results in the significant downregulation of *Per3* and *Clock*. Further support is provided by the stimulatory effect of *Ptch1* knockdown on *Clock* and *Nr1d2* expression because the recently discovered dynamic self-sustaining network of GLI transcription factors led to the prior upregulation of *Gli1* and *Gli3*.[14,35] Moreover, the FANTOM4 database[36] revealed specific binding sites for GLI factors within the respective clock genes, as illustrated in a transcriptomic output graph for the 3 Gli factors (Fig. 7A). In addition, as evidenced by the pronounced repressor function of GLI3, not all GLI-bound DNA fragments act as enhancer elements.[37]

Previously, using the transgenic SLC mice, an inducible *Smo* KO model, we demonstrated that Hh signaling is a master regulator of liver lipid metabolism, and impairment of this signaling pathway ultimately leads to steatosis.[14] In the present study, we confirm these findings by using a different transgenic mouse model, the transgenic SAC-KO mouse. The difference between both types of mice with respect to the development of steatosis simply is that at the time of sacrifice (only 1 week apart), the steatosis in SLC mice lasted 5 weeks compared to 13 weeks in SAC mice, because of the later start of the knockout in SLC mice. By genome-wide analysis we could convincingly demonstrate that the *Smo* KO affected the rhythmicity of many metabolic pathways, including hepatic lipid metabolism. Indeed, the alterations in the clock network caused by impaired Hh signaling described above have a greater impact on hepatic lipid metabolism than previously thought. In addition, we provided first data demonstrating that Hh signaling and its rhythmicity are also deranged when steatosis is caused by nutritional challenge such as HFD. Whether Hh signaling may act as part of the mechanism by which nutrition can alter the liver circadian clock is currently being investigated by our group.

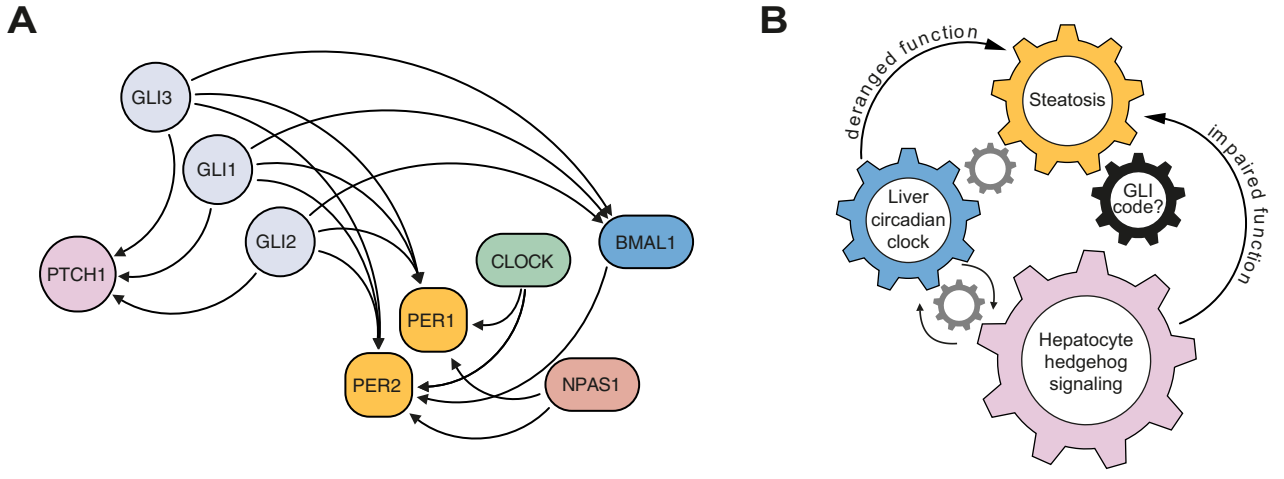


Fig. 7. Illustration of interaction between circadian liver clock and Hh signaling. (A) Transcription factor binding site predictions connecting Hh signaling pathway with the circadian clock. Using Fantom4 database, binding sites for GLI1, GLI2 and GLI3 within circadian core genes was revealed. (B) Through a probable contribution of GLI transcription factors, Hh signaling is able to trigger hepatic steatosis, while the steatosis feeds back on circadian clock. At the same time Hh signaling is able to modulate the circadian rhythm directly, leading mostly through deranged lipid metabolism to steatotic phenotypes.

Our results that *Smo* deletion is responsible for lipid accumulation are corroborated by another recent study, where patients with holoprosencephaly, a disease caused by genetic or teratogenic disruption of Hh signaling, and mice heterozygous for *Gli2* deletion were found to develop liver steatosis.[38] In contrast, a recent study of Kwon and colleagues demonstrated that *Smo*-KO did not affect lipid accumulation in normal chow fed SmoLKO mice. The reason for this discrepancy could be that Kwon *et al.* measured triglycerides in whole liver material with a less sensitive detection method.[39] In fact, at higher levels of triglycerides, for instance after HFD feeding, both studies are in accordance.

Collectively, our results show that Hh signaling and the hepatocyte cellular clock are part of a coupled regulatory network underlying the spatio-temporal control of hepatic lipid metabolism (Fig. 7B). Since Hh signaling in hepatocytes has been found to play an important role in liver zonation,[16] particularly of lipid metabolism, this component may be dominant with respect to the spatial aspect, while the clock may predominantly control the oscillatory behavior. Perturbation of either component may ultimately lead to deregulation and disease. Conversely, therapeutic strategies ignoring one of the components of this regulatory network may be inefficient or inefficacious.

Financial support

This work was supported by the Federal Ministry of Education and Research (Germany) within the research network Systems Medicine of the Liver (LiSyM) [grant number 031L0054 and 031L0037] and the Virtual Liver Network (VLN) [grant number 0315735, 0315736, 0315755, and 0315775], the Deutsche Forschungsgemeinschaft (Bonn, Germany) [grant MA 6610/2-1].

Conflict of interest

The authors declare no conflicts of interest that pertain to this work. The co-author Susanne Sales has left the field of liver sciences and is not available regarding the COI.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Eugenia Marbach-Breitrück: acquisition of data; analysis and interpretation of data; drafting of the manuscript; **Madlen Matz-Soja:** acquisition of data; analysis and interpretation of data; drafting of the manuscript; **Ute Abraham:** acquisition of bioluminescence data; analysis and interpretation of data; **Wolfgang Schmidt-Heck:** acquisition of microarray data, statistical analysis; **Susanne Sales:** acquisition of lipidomic data; **Christiane Rennert:** acquisition of data; **Matthias Kern:** acquisition of metabolic cage data; **Susanne Aleithe:** acquisition of data; **Luise Spormann:** Western Blot analyses; **Carlo Thiel:** acquisition of lipidomic data; **Raffaele Gerlini:** acquisition of data; **Katrin Arnold:** acquisition of data; **Nora Klöting:** acquisition of metabolic cage data; **Reinhard Guthke:** acquisition of microarray data, statistical analysis; **Damjana Rozman:** providedPER2::LUC reporter mice, critical revision of the manuscript for important intellectual content; **Raffaele Teperino:** acquisition of data; **Andrej Shevchenko:** acquisition of lipidomic data; **Achim Kramer:** interpretation of data; critical revision of the manuscript for important intellectual content; **Rolf Gebhardt:** study concept and design, interpretation of data; drafting of the manuscript; obtained funding; study supervision; critical revision of the manuscript for important intellectual content.

Acknowledgements

We cordially thank Kerstin Heise, Doris Mahn, Frank Struck, Fatina Siwczak, Vivien Teßmar, and Franziska Rolfs for excellent technical assistance. We thank PD Dr. Knut Krohn for Affymetrix microarray analysis. Further, we would like to thank Petra Hirrlinger, Petra Fink Sterba, Sigrid Weisheit and Manuela Liebig from the Experimental Centre of the Faculty of Medicine (Leipzig University) for taking excellent care of the mice. Writing assistance was performed by American Journal Experts (AJE) and supported by the Federal Ministry of Education and Research (Germany) within the research network Systems Medicine of the Liver (LiSyM) [grant number 031L0054; 031L0037].

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.01.022.

References

Author names in bold designate shared co-first authorship

[1] Dibner C, Schibler U, Albrecht U. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Ann Rev Physiol 2010;72:517–549.
[2] Reinke H, Asher G. Circadian clock control of liver metabolic functions. Gastroenterology 2016;150:574–580.
[3] Ferrell JM, Chiang JYL. Circadian rhythms in liver metabolism and disease. Acta Pharm Sin B 2015;5:113–122.
[4] Froy O. Metabolism and circadian rhythms—implications for obesity. Endocr Rev 2010;31:1–24.
[5] Bray MS, Young ME. The role of cell-specific circadian clocks in metabolism and disease. Obes Rev 2009;10:6–13.
[6] Sahar S, Sassone-Corsi P. Metabolism and cancer: the circadian clock connection. Nat Rev Cancer 2009;9:886–896.
[7] Ko CH. Molecular components of the mammalian circadian clock. Hum Mol Genet 2006;15:R271–R277.
[8] Cho H, Zhao X, Hatori M, Yu RT, Barish GD, Lam MT, et al. Regulation of circadian behaviour and metabolism by REV-ERB-α and REV-ERB-β. Nature 2012;485:123–127.
[9] Hatanaka F, Matsubara C, Myung J, Yoritaka T, Kamimura N, Tsutsumi S, et al. Genome-wide profiling of the core clock protein BMAL1 targets reveals a strict relationship with metabolism. Mol Cell Biol 2010;30:5636–5648.
[10] Mazzoccoli G, Pazienza V, Vinciguerra M. Clock genes and clock-controlled genes in the regulation of metabolic rhythms. Chronobiol Int 2012;29:227–251.
[11] Tahara Y, Shibata S. Circadian rhythms of liver physiology and disease: experimental and clinical evidence. Nat Rev Gastroenterol Hepatol 2016;13:217–226.
[12] Moynihan Ramsey K, Marcheva B, Kohsaka A, Bass J. The clockwork of metabolism. Annu Rev Nutr 2007;27:219–240.
[13] Schmutz I, Albrecht U, Ripperger JA. The role of clock genes and rhythmicity in the liver. Mol Cell Endocrinol 2012;349:38–44.
[14] Matz-Soja M, Rennert C, Schonefeld K, Aleithe S, Boettger J, Schmidt-Heck W, et al. Hedgehog signaling is a potent regulator of liver lipid metabolism and reveals a GLI-code associated with steatosis. eLife 2016;5.
[15] Matz-Soja M, Aleithe S, Marbach E, Bottger J, Arnold K, Schmidt-Heck W, et al. Hepatic Hedgehog signaling contributes to the regulation of IGF1 and IGFBP1 serum levels. Cell Commun Signal 2014;12:11.
[16] Gebhardt R, Matz-Soja M. Liver zonation: novel aspects of its regulation and its impact on homeostasis. World J Gastroenterol 2014;20:8491–8504.

[17] Zhang EE, Liu AC, Hirota T, Miraglia LJ, Welch G, Pongsawakul PY, et al. A genome-wide RNAi screen for modifiers of the circadian clock in human cells. Cell 2009;139:199–210.
[18] Yoo S-H, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, et al. PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci USA 2004;101:5339–5346.
[19] Wang J, Mauvoisin D, Martin E, Atger F, Galindo AN, Dayon L, et al. Nuclear proteomics uncovers diurnal regulatory landscapes in mouse liver. Cell Metab 2017;25:102–117.
[20] Rey G, Cesbron F, Rougemont J, Reinke H, Brunner M, Naef F, et al. Genome-wide and phase-specific dna-binding rhythms of BMAL1 control circadian output functions in mouse liver. PLoS Biol 2011;9 e1000595.
[21] Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev 2000;14:2950–2961.
[22] S-q Shi, Ansari TS, McGuinness OP, Wasserman DH, Johnson CH. Circadian disruption leads to insulin resistance and obesity. Curr Biol 2013;23:372–381.
[23] Hodges PE, Carrico PM, Hogan JD, O'Neill KE, Owen JJ, Mangan M, et al. Annotating the human proteome: the Human Proteome Survey Database (HumanPSD™) and an in-depth target database for G protein-coupled receptors (GPCR-PD™) from incyte genomics. Nucleic Acids Res 2002;30:137–141.
[24] Rennert C, Vlaic S, Marbach-Breitrück E, Thiel C, Sales S, Shevchenko A, et al. The diurnal timing of starvation differently impacts murine hepatic gene expression and lipid metabolism – a systems biology analysis using self-organizing maps. Front Physiol 2018;9:1180.
[25] **Matz-Soja M**, Rennert C, Schönefeld K, Aleithe S, Boettger J, Schmidt-Heck W, et al. Hedgehog signaling is a potent regulator of liver lipid metabolism and reveals a GLI-code associated with steatosis. Elife 2016;5.
[26] **Rennert C**, **Eplinius F**, Hofmann U, Johänning J, Rolfs F, Schmidt-Heck W, et al. Conditional loss of hepatocellular Hedgehog signaling in female mice leads to the persistence of hepatic steroidogenesis, androgenization and infertility. Arch Toxicol 2017;91:3677–3687.
[27] Braune J, Weyer U, Matz-Soja M, Hobusch C, Kern M, Kunath A, et al. Hedgehog signalling in myeloid cells impacts on body weight, adipose tissue inflammation and glucose metabolism. Diabetologia 2017;60:889–899.
[28] Sicklick JK, Li Y-X, Choi SS, Qi Y, Chen W, Bustamante M, et al. Role for hedgehog signaling in hepatic stellate cell activation and viability. Lab Invest 2005;85:1368–1380.
[29] Matz-Soja M, Hovhannisyan A, Gebhardt R. Hedgehog signalling pathway in adult liver: a major new player in hepatocyte metabolism and zonation? Med Hypotheses 2013;80:589–594.
[30] Guenthner CJ, Luitje ME, Pyle LA, Molyneux PC, Yu JK, Li AS, et al. Circadian rhythms of PER2::LUC in individual primary mouse hepatocytes and cultures. PLoS ONE 2014;9 e87573.
[31] Landgraf D, Wang LL, Diemer T, Welsh DK. NPAS2 compensates for loss of CLOCK in peripheral circadian oscillators. PLoS Genet 2016;12 e1005882.
[32] DeBruyne JP, Noton E, Lambert CM, Maywood ES, Weaver DR, Reppert SM. A Clock Shock: Mouse CLOCK Is Not Required for Circadian Oscillator Function. Neuron 2006;50:465–477.
[33] Koike N, Yoo S-H, Huang H-C, Kumar V, Lee C, Kim T-K, et al. Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science (New York, N.Y.) 2012;338:349–354.
[34] Katoh. Integrative genomic analyses on GLI1: Positive regulation of GLI1 by Hedgehog-GLI, TGFβ-Smads, and RTK-PI3K-AKT signals, and negative regulation of GLI1 by Notch-CSL-HES/HEY, and GPCR-Gs-PKA signals. Int J Oncol 2009;35.
[35] **Schmidt-Heck W**, **Matz-Soja M**, Aleithe S, Marbach E, Guthke R, Gebhardt R. Fuzzy modeling reveals a dynamic self-sustaining network of the GLI transcription factors controlling important metabolic regulators in adult mouse hepatocytes. Mol Biosyst 2015;11:2190–2197.
[36] Ravasi T, Suzuki H, Cannistraci CV, Katayama S, Bajic VB, Tan K, et al. An atlas of combinatorial transcriptional regulation in mouse and man. Cell 2010;140:744–752.
[37] Falkenstein KN, Vokes SA. Transcriptional regulation of graded Hedgehog signaling. Semin Cell Dev Biol 2014;33:73–80.
[38] Guillen-Sacoto MJ, Martinez AF, Abe Y, Kruszka P, Weiss K, Everson JL, et al. Human germline hedgehog pathway mutations predispose to fatty liver. J Hepatol 2017;67:809–817.
[39] Kwon H, Song K, Han C, Chen W, Wang Y, Dash S, et al. Inhibition of hedgehog signaling ameliorates hepatic inflammation in mice with nonalcoholic fatty liver disease. Hepatology (Baltimore, MD) 2016;63:1155–1169.