SUPPLEMENTARY MATERIAL

Tick-Tock Hedgehog–Mutual crosstalk with liver circadian clock renders hepatic hedgehog signaling a risk factor of steatosis

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1. Supplementary material and methods

Animal maintenance for specific experiments

A) High fat diet experiments

The mice used in this experiment were maintained in a pathogen-free facility on a 12:12 h light–dark cycle, according to German guidelines and those of the world medical association declaration of Helsinki for the care and safe use of experimental animals. The animal experiments were approved by the Landesdirektion Sachsen (permission number: TVV60/12). As a high fat diet a modified ssniff® EF Clinton/Cybulsky chow composition with 20% fat was used (M-Z E15752-34, ssniff Spezialdiäten GmbH, Soest, Germany) and administered to male C57BL/6N mice at an age of 12 weeks. As a control the isocaloric Altromin control diet containing 4 % crude fat was used (10100090 Altromin Spezialfutter GmbH & Co. KG, Lage, Germany). The duration of the diet was 10 weeks.

B) Feeding and starvation experiments

The feeding and starvation experiments were performed accordingly to Rennert et al. [1]. In brief, male C57BL/6N mice were randomly grouped into six groups (n = 3 each). The starvation experiment was started either at ZT3 or at ZT12 and mice were sacrificed after 24 h at the same times on the next day together with ad libitum fed groups. In the refeeding experiment two groups of mice were starved for 24 h started at ZT15 followed by refeeding period for 12 or 21 h until ZT3 and ZT12 on the next day, respectively.

RNA preparation and quantitative real-time PCR (qPCR)

RNA from hepatocytes and liver tissue was extracted using peqGOLD RNAPure (Peqlab, Erlangen) according to manufacturer's manual. RNA concentrations were measured by Nanodrop technology (Thermo Scientific). For cDNA synthesis 1 µg RNA was used to obtain a final amount of 20 µl cDNA. Therfore oligo(dt) primers and the Proto Script M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Frankfurt am Main, Germany) was used according to manufactures manual. The final reaction volume of 20 µL was incubated for the following times: 25 °C x 5 min, 42 °C x 60 min, 70 °C x 15 min. The generated cDNA samples were stored at -20 °C until processed. All qPCR primers are listed in supporting information Table S4. The levels of all mRNA transcripts were determined in duplicate by qPCR using the Light Cycler® 2.0 Instrument and the Light Cycler® Fast Start DNA Master SYBR Green I (Roche, Mannheim, Germany) or the Rotor Gene 2000 (Qiagen, Hilden, Germany). The cDNA template was first denatured at 95 °C for 2 min, then cycled at 95 °C for 5 s, 60 °C for 20 s (annealingof the primers), and 72 °C for 30 s. Per qPCR reaction 40 cycles were carried out . In order to ensure that the transcript was amplicated correctly, a melting kurve analysis was performed for each reaction with a ramp from 60 °C to 95 °C rising by 1 °C each step. The absolute quantification of specific qPCR products for each primer set was performed using the standard curve method. The gene expression was normalized to beta Actin (*Actb*).

RNA interference

Ptch1-, *Gli1*-, *Gli2*- and *Gli3*-specific siRNAs, as well as anti-*Bmal1,* anti-*Clock* and respective nonsense control siRNAs were purchased from Invitrogen, Darmstadt, Germany. RNAi interference experiments with primary hepatocytes were performed as described previously [2]. The siRNA concentration was 10 nmol for *Ptch1*, 25 nmoles for *Gli1*, *Gli2* and *Gli3* and 30 nmoles for *Bmal1*. Interferin from VWR, Erlangen, Germany, was used according to the manufacturer's instructions. Sequences for siRNA primers are listed in supporting information Table S7. Twenty-four hours after transfection, the medium was changed and fresh medium without siRNA was added. The gene expression was normalized to *Actb.*

Illumina BeadChip Microarray

Illumina BeadChip analysis was conducted at the microarray core facility of the Interdisziplinäres Zentrum für klinische Forschung (IZKF) Leipzig. Raw data of 25,600 probes was extracted and quantile normalized by Illumina GenomeStudio. Expression values were background subtracted if necessary. Correction for multiple testing was performed by using the Benjamini-Hochberg procedure.

Immunohisto- and cytochemical staining

Immunohistochemistry on liver paraffin sections (3 μm) to depict IHH protein was performed as described by Matz-Soja et. al [3]. Fat red staining of cultured isolated SAC hepatocytes was performed as described earlier [4].

IHH ELISA

The determination of the IHH concentration in the serum of the C57BL/6-N mice was made with the ELISA Kit for IHH by Cloud Clone Corp (SED116Mu, USA). The assay was performed according the manufacturer's protocol.

BMAL1 Western Blot

After 24 h inkubation with *Bmal1* siRNA total protein from hepatocytes was extracted via RIPA buffer and separated by 12 % SDS-PAGE and transferred onto PVDF membranes (Roth, Germany) followed by an inkubation of the membrane with Odyssey® Blocking Buffer (TBS) for 1 h at room temperature. BMAL1 antibody (Abcam 5157) was diluted 1:2000 in the blocking buffer and inkubated overnight at 4°C. Blots were subsequently incubated with secondary anti rabbit IRDye 800 CW (LICOR 926-32211) diluted 1:10000 up to 1h at room temperature. The detection occurred in the 800 nm channel of the Odyssey CLx imaging system. Finaly, BMAL1 signals were normalized to REVERT Total Protein Stain (LICOR 926-11010), which was detected in the 700 nm channel of the Odyssey CLx imaging system.

Shotgun lipidomics

Lipid extraction of mouse hepatocytes was performed as already described [5].

2. Supplementary figures

Figure S1

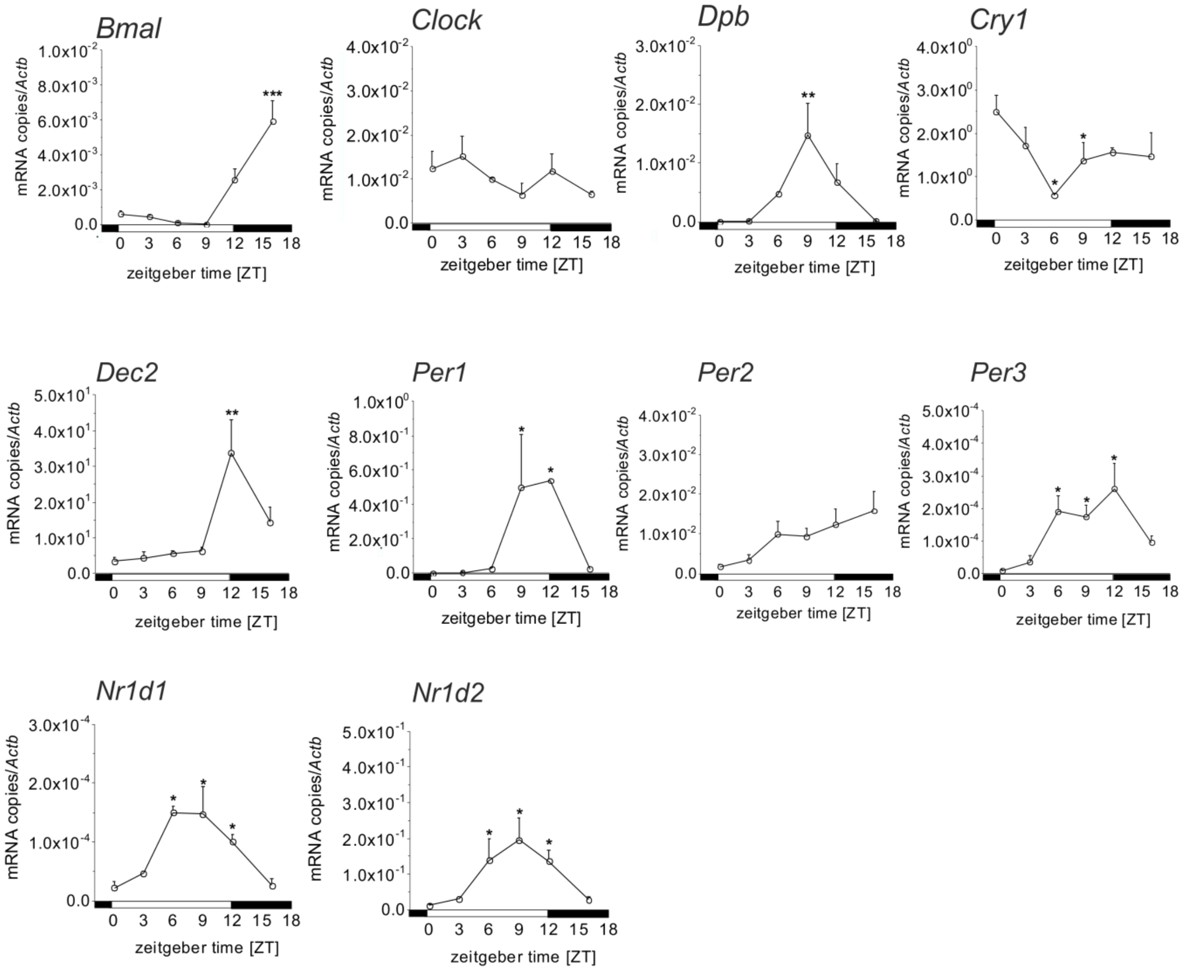


Fig. S1. Expression of core clock genes in liver of male C57BL/6N mice.

qPCR expression analysis of *Bmal1, Clock, Dpb, Cry1, Dec2, Per1/2/3* and *Nr1d1/2* in liver material from C57BL/6N mice harvested at six different diurnal time-points (zeitgeber time ZT0, 3, 6, 9, 12, 15 and 16; N=3/ZT). <u>Significant differences to ZT0 were determined using ANOVA with Tukey's least significant difference post hoc test, p≤ 0.05 (*), p≤ 0.01 (**) and p≤ 0.001 (***).</u>

Figure S2

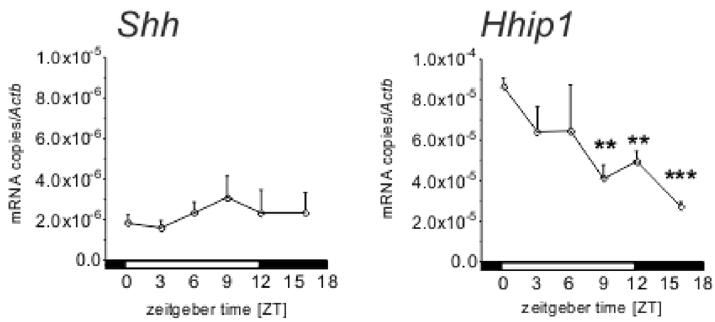


Fig. S2. The diurnal expression of *Shh* and *Hhip1* in livers from C57BL/6N mice.

qPCR expression analysis of *Shh* and *Hhip1* was performed in livers of C57BL/6N mice harvested at six different diurnal time-points (zeitgeber time ZT0, 3, 6, 9, 12, and 16), N=3 per time-point. Significant differences to ZT0 were determined using ANOVA with Tukey's least significant difference post hoc test, $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

Figure S3

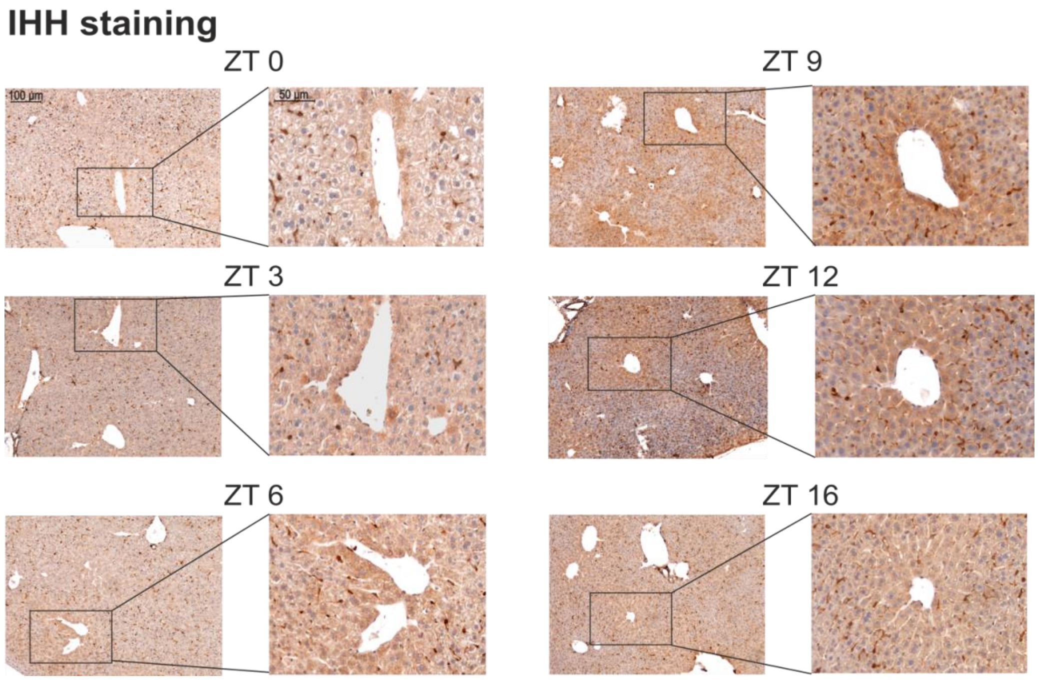


Fig. S3. The diurnal expression of IHH protein in livers from C57BL/6N mice.

Immunohistochemical staining of IHH protein in livers of C57Bl/6 mice at six different diurnal time-points.

Figure S4

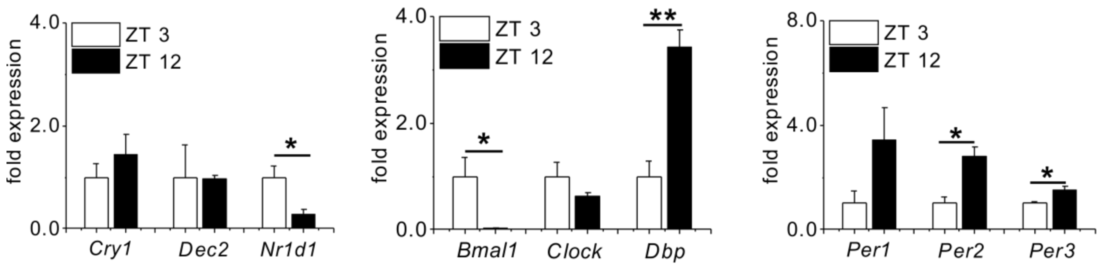
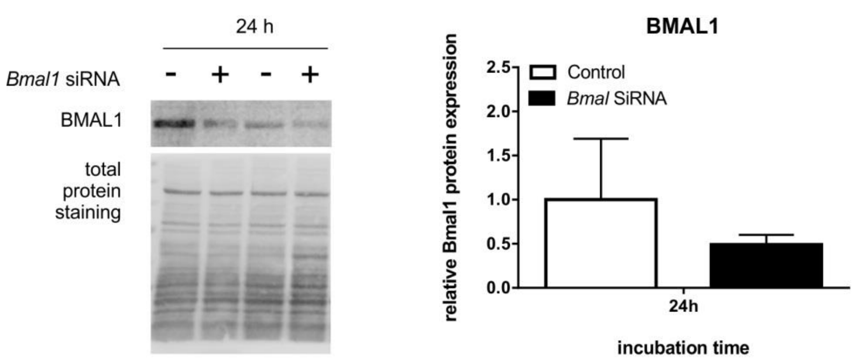


Fig. S4. The diurnal expression of clock components in hepatocytes isolated from C57BL/6N mice.

qPCR expression of *Cry1, Dec2, Nr1d1, Bmal1, Clock, Dpb* and *Per1/2/3* in isolated hepatocytes at ZT3 and ZT12 (black bars), N=3 per time-point, <u>unpaired t-test, p≤ 0.05 (*) and p≤ 0.01 (**).</u>

Figure S5



<u>**Fig. S5. Western Blot analyses of BMAL1 protein expression**</u>

<u>Western Blot analyses of BMAL1 protein expression after Bmal1 siRNA mediated knock down in primary hepatocytes of male C57BL/6N mice; N=2.</u>

Figure S6

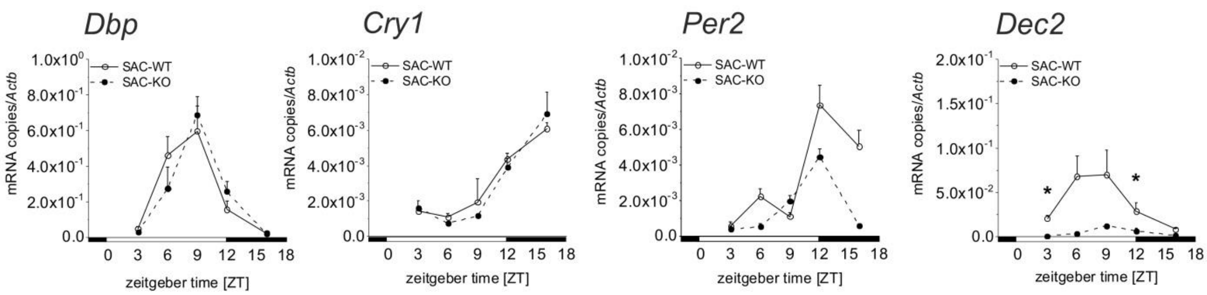


Fig. S6. The diurnal expression of clock components in liver material from SAC mice

qPCR expression analysis of *Dbp, Cry1, Per2* and *Dec2* in livers of SAC-KO mice and their wild type littermates, harvested at five different diurnal time-points (zeitgeber time ZT3, 3, 6, 9, 12, 16), N=3 per genotype and time-point. <u>Significant differences to ZT0 were determined using ANOVA with Tukey's least significant difference post hoc test, p≤ 0.01 (**) and p≤ 0.001 (***).</u>

Figure S7

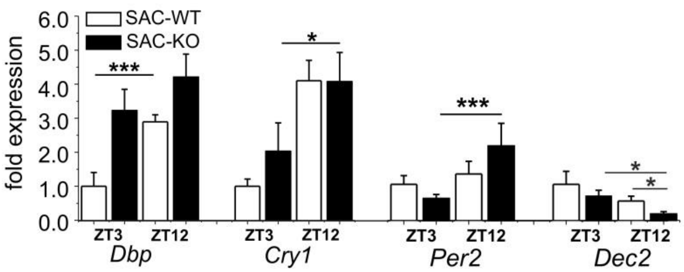


Fig. S7. The diurnal expression of clock components in hepatocytes isolated from SAC mice

qPCR analyses of *Dbp, Cry1, Per2* and *Dec2* in isolated hepatocytes from SAC-KO mice and SAC-WT mice at two different diurnal time points ZT3 and ZT12, N=6-13 per genotype and time-point, unpaired t-test, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.01$ (***).

Figure S8

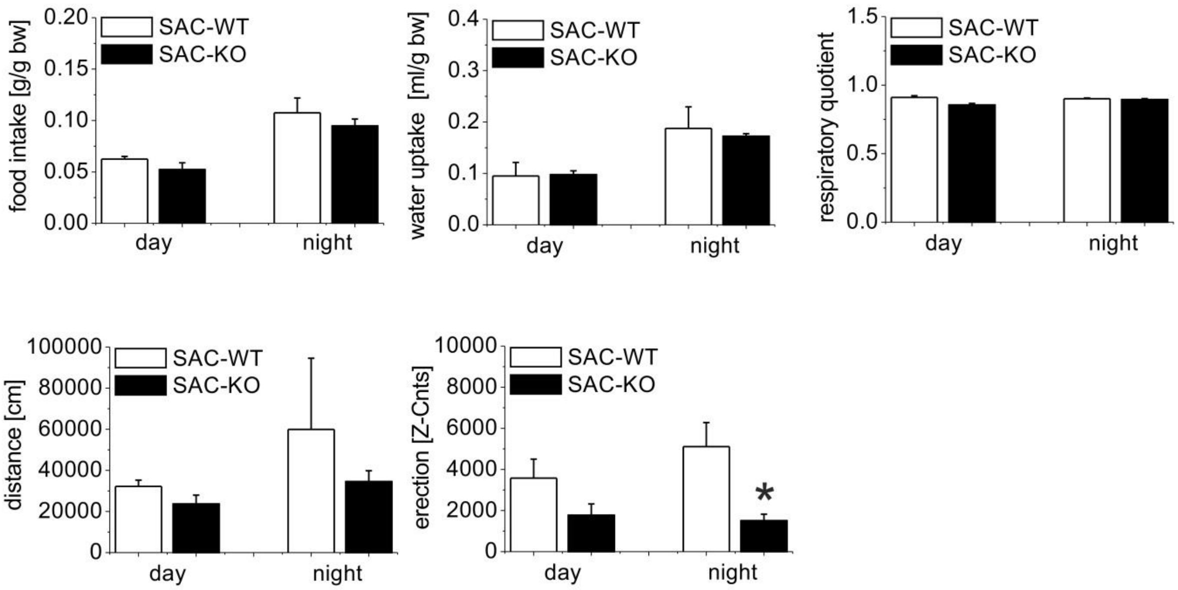


Fig. S8. Metabolic cage analysis of SAC mice

Metabolic cage analyses from male SAC-WT (white bars) and KO (black bars) mice, observed over 72 h (N=4). Food and water intake as well as spontaneous activity (counts) as cage movement (XYZ axis) and running distance (cm) were determined during night and day. The respiratory exchange ratio (RER) was calculated as CO_2/VO_2 measured by indirect calorimetry, <u>paired t-test, $p \leq 0.05$ (*).</u>

Figure S9

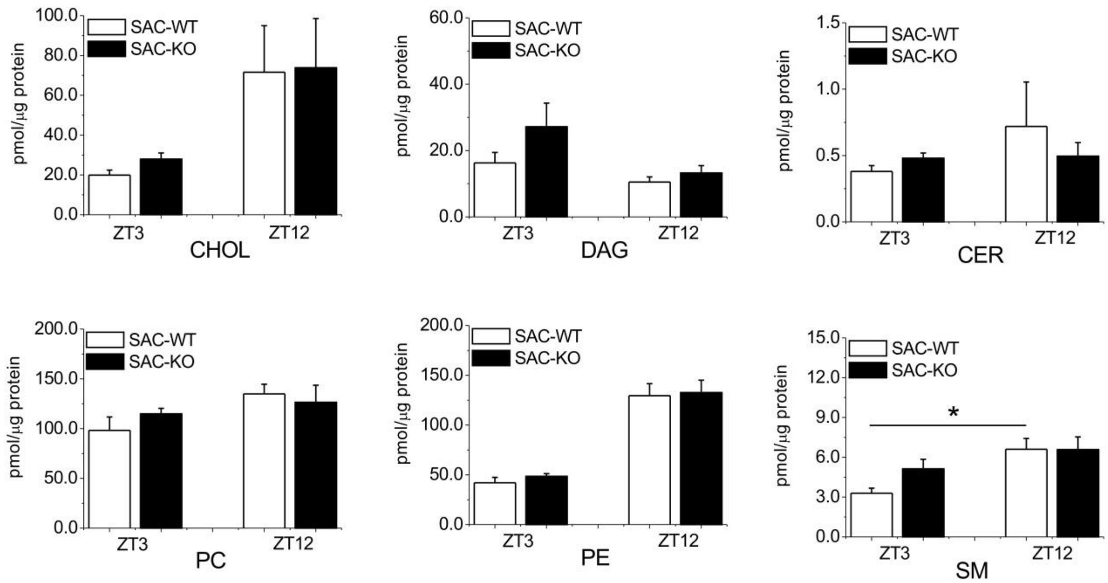


Fig. S9 Lipidomic profile in hepatocytes from SAC mice.

Change of cholesterol (CHOL), diacylglycerides (DAG), ceramides (CER), phosphatidylcholines (PC), -ethanolamines (PE) and sphingomyelin (SM) from SAC mice at two different diurnal time points ZT3 and ZT12, N=3 per genotype and time-point, unpaired t-test, $p \leq 0.05$ (*).

Figure S10

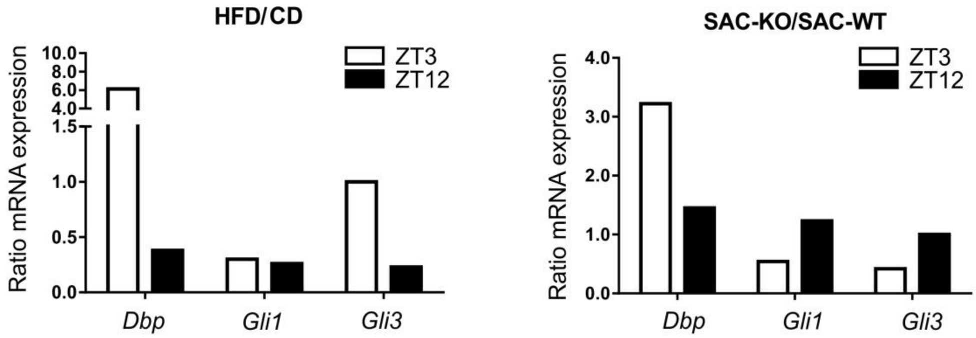


Fig. S10 Comparison of the effectof HFD induced liver steatosis with steatosis in SAC-KO mice on the diurnal rhythm of clock and Hh gene expression

Ratios of the mean gene expression of *Dbp, Gli1* and *Gli3* in isolated hepatocytes from mice fed with a HFD and CD as well as SAC-KO and SAC-WT mice at two different diurnal time points ZT3 and ZT12, N=3-6 per genotype (feeding condition) and time-point (see supplemental methods).

3. Supplementary tables

Table S1: Overrepresented categories of differentially expressed Genes in SAC-WT and SAC-KO hepatocytes according to WIKI-Pathways

Wiki ID			**Genes with different rhythm (yellow cluster)**			**Genes with normal rhythm (blue clusters)**		
WP-ID	Wiki-Pathway term	Size	Count	Genes	adj. pValue	Count	Genes	adj.pValue
WP1248	Oxidative phosphory-lation	47				**10**	*Atp5a1, Atp5d, Atp5g2, Ndufa6, Ndufb8, Ndufb9, Ndufc1, Ndufs2, Ndufs3, Ndufs4*	**2,74E-05**
WP295	Electron Transport Chain	83				**13**	*Atp5a1, Atp5d, Atp5g2, Cox5a, Cox6c, Ndufa6, Ndufb8, Ndufb9, Ndufc1, Ndufs2, Ndufs3, Ndufs4, Sdhb*	**5,69E-05**
WP662	Amino Acid metabolism	109				**15**	*Arg1, Cbs, Cs, Ddc, Fh1, Gclm, Gpt2, Hdc, Ldha, Oat, Odc1, Pck1, Pdk4, Prodh, Sds*	**7,39E-05**
WP103	Cholesterol Biosynthesis	13	**4**	*Lss, Nsdhl, Sc4mol, Sqle*	**2,00E-04**	**4**	*Cyp51, Mvd, Nsdhl, Sqle*	**1,90E-03**
WP1268	Diurnally regulated genes with circadian orthologs	47	**7**	*Cebpb, G0s2, Gstp1, Herpud1, Klf9, Psma4, Stbd1*	**1,00E-04**	**9**	*Btg1, Clock, Gstm5, Hist1h2bm, Per1, Per2, Ppp1r3c, Ppp2cb, Sumo3*	**2,00E-04**
WP544	Circadian Exercise	48	**6**	*Cebpb, G0s2, Herpud1, Klf9, Psma4, Stbd1*	**9,00E-04**	**7**	*Btg1, Clock, Per1, Per2, Ppp1r3c, Ppp2cb, Sumo3*	**4,50E-03**
WP317	Glycogen Metabolism	32				7	*Calm3, Gys2, Pgm2, Ppp2cb, Ppp2r5c, Ppp2r5e, Pygl*	**4,00E-04**
WP33	Fatty Acid Omega Oxidation	7	**3**	*Adh4, Aldh1a1, Cyp1a2*	**4,00E-04**			
WP336	Fatty Acid Biosynthesis	28	**5**	*Acaa2, Acly, Acsl3, Acss2, Scd1*	**4,00E-04**			
WP519	Proteasome Degradation	57				**9**	*H2afz, Psmb10, Psmb3, Psmc4, Psmc5, Psmd13, Psmd4, Psmd7, Psme1*	**7,00E-04**
WP431	Nuclear receptors in lipid	36	**4**	*Abcb4, Cyp1a2, Cyp2b10, Nr1h4*	**9,90E-03**	**7**	*Abcb11, Abcc2, Abcd3, Abcg5, Cyp2e1,*	**8,00E-04**

	metabolism and toxicity						*Cyp7a1, Cyp8b1*	
WP1251	metapathway biotransformation	128	**10**	*Baat, Cyp17a1, Cyp1a2, Fmo5, Gstm1, Gstp1, Mgst1, Mgst3, Nnmt, Ugt1a2*	**9,00E-04**			
WP193	Signaling of Hepatocyte Growth Factor Receptor	36	**5**	*Fos, Jun, Rap1b, Rasa1, Stat3*	**1,50E-03**			
WP2316	PPAR signaling pathway	89	6	*Acaa1b, Acox2, Acsl3, Apoa1, Cyp4a14, Scd1*	1,88E-02	**11**	*Angptl4, Apoa5, Cpt2, Cyp27a1, Cyp4a31, Cyp7a1, Cyp8b1, Fads2, Ilk, Pck1, Scp2*	**1,60E-03**
WP412	Oxidative Stress	26	**4**	*Cat, Fos, Junb, Mgst1*	**3,00E-03**			
WP1267	Senescence and Autophagy	102				**11**	*Atg5, Cdkn1a, Gabarap, Irf1, Lamp2, Map1lc3a, Map2k3, Mmp14, Rsl1d1, Sqstm1, Vtn*	**4,80E-03**
WP200	Complement Activation, Classical Pathway	16	**3**	*C1s, C8b, C9*	**5,80E-03**			
WP426	Urea cycle and metabolism of amino groups	22				4	*Acy1, Arg1, Oat, Odc1*	1,39E-02
WP1274	cytochrome P450	42	4	*Cyb5b, Cyb5r3, Cyp17a1, Cyp1a2*	1,69E-02			
WP1264	Estrogen metabolism	24	3	*Cyp1a2, Gstm1, Ugt1a2*	1,83E-02			
WP258	TGF-beta Receptor Signaling Pathway	229	11	*Camk2g, Ccnd1, Cops5, Ets1, Fnta, Fos, Foxo1, Fzr1, Jun, Junb, Sdc2*	2,05E-02			
WP1254	Apoptosis	91	6	*Bbc3, Bnip3l, Irf2, Jun, Rela, Traf3*	2,07E-02			
WP65	Insulin Signaling	145	8	*Enpp1, Fos, Foxo1, Grb10, Jun, Rheb, Sgk1, Sgk2*	2,19E-02		*Cyp51, Mvd, Nsdhl, Sqle*	

Table S2: List of differentially expressed lipid genes in Smo-WT and Smo-KO hepatocytes

(A) Genes expressed significantly higher at ZT3 compared to ZT12

	ZT3 > ZT12	
	SAC-WT	**SAC-KO**
differently regulated in SAC-WT and SAC-KO	*Abcb11, Acaa1b, Acat2, Agtr1a, Aldh1a1, Apoc4, Avpr1a, Blvrb, Cyp4a31, Cyp8b1, Cyp51, Fgf21, Hsd17b4, Lcat, Mvd, Nsdhl, Pdk4, Rdh11, Rdh7, Ugt3a1*	*Apoc2, Apoe, Bdh1, Cdipt, Cyp2b9, Elovl5, Fas, Gpihbp1, Lass2, Mgll, Ostb, Rela*
equally regulated in SAC-WT and SAC-KO	*Acbd4, Agpat2, Apoc1, Cbs, Clock, Fkbp4, Hdc, Hsd3b7, Insig2, Itpk1, Ppp1ca, Scp2*	

(B) Genes expressed significantly lower at ZT3 compared to ZT12

	ZT3 < ZT12	
	SAC-WT	**SAC-KO**
differently regulated in SAC-WT and SAC-KO	*Akap1, Apoa5, Cstb, Fos, Nr1h4, Rdh7, Sec14l4, Srebf1, Ugt2b35, Ugt2b36*	*Abcd3, Acot1, Acp6, Acsl3, Aldh1a1, Apoa1, Apon, Baat, Cebpb, Cyp7a1, Ebpl, Es22, Etfa, Foxo1, Hacl1, Hsd17b7, Hsd3b2, Lypla1, Nsdhl, Pank1, Pck1, Pcsk9, Peci, Per1, Rdh11, Rdh9, Sc4mol, Scd1, Slc25a20, Txnip, Ugt2b36, 0610007P14Rik, 2810007J24Rik*
equally regulated in SAC-WT and SAC-KO	*Abcg5, Acot3, Acsm3, Acss2, Agpat6, Angptl4, Ccrn4l, Cyp2c67, Cyp2e1, Gde1, Ghr, Lpcat3, Lpin1, Pccb, Pla1a, Por, Saa4, St3gal5, Thrsp*	

Table S3: Components of Hh-signalling pathway which appeared as peaks for ChIP-seq experiments for Clock proteins across all 6 times points, according to the study of Koike et al. 2012[6].

HH Gene Promotor	Clock Protein	Distance zu TSS [bp]	Binding intensity						ChIP Input Control	Period	Amplitude	p-Value
			CT0	**CT4**	**CT8**	**CT12**	**CT16**	**CT20**				
Ihh	**Bmal1**	-58785	9,01	16,74	18,03	10,3	7,73	5,15	0	22,94	5,86	0,00
Ihh	**Clock**	-20245	0	3,16	5,53	0,79	3,16	0	0,79	24,05	1,96	0,06
Ihh	**Per2**	-58680	1,01	1,01	0	2,02	6,05	8,06	11,08	25,11	3,22	0,00
Ihh	**Cry1**	-58810	11,45	19,37	2,64	11,45	2,64	6,16	11,45	24,19	4,16	0,24
Ihh	**Cry2**	-58745	4,4	3,52	6,16	2,64	7,04	5,28	8,81	25,27	0,63	0,64
Ihh	**Cry2**	-11445	0,88	0,88	6,16	1,76	1,76	2,64	0,88	22,81	1,30	0,24
Ptch1	**Bmal1**	-2642	1,29	11,59	7,73	2,58	0	0	0	22,81	5,16	0,00
Ptch1	**Cry1**	-2537	10,57	3,52	4,4	2,64	2,64	3,52	0	26,26	2,53	0,06
Ptch1	**Cry2**	737	7,04	0,88	6,16	0	3,52	2,64	2,64	26,44	1,27	0,56
Shh	**Per1**	-267985	0	2,02	1,01	9,07	0	1,01	7,05	23,21	2,70	0,12
Shh	**Per2**	-268009	3,02	0	1,01	2,02	10,08	6,05	7,05	23,76	3,83	0,00
Shh	**Cry2**	-289134	7,04	1,76	3,52	3,52	7,04	3,52	8,81	22,30	1,75	0,11
Sufu	**Cry1**	-63	4,4	3,52	0,88	4,4	2,64	7,04	3,52	24,64	1,89	0,04
Sufu	**Cry2**	-48	2,64	1,76	3,52	5,28	7,93	0,88	1,76	22,55	2,55	0,02
Gli2	**Bmal1**	22266	5,15	10,3	2,58	1,29	0	0	0	24,19	3,70	0,01
Gli2	**Per1**	-93274	1,01	3,02	1,01	7,05	2,02	4,03	7,05	24,00	1,27	0,38
Gli2	**Per2**	-93229	1,01	0	2,02	8,06	18,14	6,05	7,05	22,55	7,03	0,01
Gli2	**Cry1**	-137739	14,09	7,93	3,52	4,4	3,52	6,16	9,69	24,80	4,14	0,00
Gli2	**Cry1**	-93359	8,81	0,88	0,88	4,4	9,69	6,16	8,81	22,06	4,18	0,00
Gli2	**Cry1**	22255	3,52	7,04	1,76	4,4	2,64	5,28	0,88	22,43	1,11	0,36
Gli2	**Cry1**	-119999	7,93	4,4	2,64	3,52	0,88	2,64	5,28	26,44	2,00	0,04
Gli2	**Cry2**	-93244	4,4	2,64	4,4	2,64	13,21	5,28	8,81	24,00	2,99	0,13

Table S4: Primers used for qPCR

gene	sequence 5'-3'	
	forward	**reverse**
Actb	catccgtaaagacctctatgccaac	atggagccaccgatccaca
Bmal1	gcagtgccactgactaccaaga	tcctggacattgcattgcat
Chrebp1	acatcagcgctttgaccag	acatcagcgctttgaccag
Clock	ggaacagcagcttccttcag	gccaactgagcgattctttc
Cry1	atcgtgcgcatttcacatac	atcagacagaggggtcatgc
Dbp	cttttgaccctcggagacac	tggctgcttcattgttcttg
Dec2	gaacccagcattccttcca	agcatcgcttactcttcctccc

Elovl3	ctgttgctcatcgttgttgg	atccgtgtagatggcaaagc
Elovl6	tctgggcttatgcatttgtg	acaggagcacagtgatgtgg
Fused	tgcctctcagccttcttagg	taagagcgccccatacca
Gli1	QT00173537 purchased from Qiagen GmbH, Hilden,Germany	
Gli2	QT01062236 purchased from Qiagen GmbH, Hilden,Germany	
Gli3	QT00102256 purchased from Qiagen GmbH, Hilden,Germany	
Gpam	tcctggccttgcagaacagc	gcaacgttcctttccgtcctg
Hhip1	ctacttgggccagatggaag	ctccaagtaaggctccttgaac
Ihh	gctcacccccaactacaatc	gcggccctcatagtgtaaag
Per1	gcttcgtggacttgacacct	tgctttagatcggcagtggt
Per2	gaagatcctgtacatctctaacca	ctcctgagtgaaagaatctaagcc
Per3	aagaagccaagccaatcc	gcaacactttctgactgtagg
Ppara	cgtacggcaatggctttatc	tcatctggatggttgctctg
Pparg	atggaagaccactcgcattc	gctttatccccacagactcg
Ptch 1	cctcctttacggtggacaaac	atcaactcctcctgccaatg
Ptch2	cttctcccacaagttcatgc	cgatgtcattgttctggtagtcg
Nr1d2	cagaaatagttacctgtgcaacact	gacttgctcataggacacacca
Nr1d1	acgaccctggactccaataa	ccattggagctgtcactgtaga
Shh	tccaaagctcacatccactg	ctccgggacgtaagtccttc
Smo	gcaagctcgtgctctggt	gggcatgtagacagcacaca
Srebf 1	aagcgctaccggtcttctatc	tgtgcacttcgtagggtcag
Sufu	cttccagtcagagaacacct	ttgggctgaatgtaactc

Table S5: Sequences of siRNAs used for transfection of primary hepatocytes

gene	sequence 5’-3’	GC-value
Bmal1	-ggaacuucuagguacaucauguuau- -ccuugaagauccauguaguagaaua-	36 %
Clock	-ggcacagacauuaucgguaacacaa- -uuguguuaccgauaaugucugugcc-	44 %
Gli1	-cccaacauggcagugggguaacauga- ggguuguaccgucacccauuguacu	52 %
Gli3	-uagcaaggccaucuuggucuucagg- -aucguuccgguagaaccagaagucc-	48 %
Ptch1	-ccuuccuguucugggagcaauacau- -ggaaggacaagacccucguuaugua-	48 %

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