CHAPTER 36: HEPATIC CLOCKS

Ueli Schibler[1], Gad Asher, Camille Saini, Jörg Morf, and Hans Reinke

Department of Molecular Biology and National Center of Competence in Research "Frontiers in Genetics"

Sciences III, University of Geneva

30, Quai Ernest Ansermet

CH-1211 Geneva-4

Switzerland

[1]To whom correspondence should be addressed

Email: ueli.schibler@unige.ch

Tel: **41-22-379 61 75

Fax: **41-22-379 68 68

36.1 Introduction

Most physiological processes are subject to daily oscillations that are driven by an endogenous circadian clock. These include rest-activity cycles, cardiovascular functions such as heart rate and blood pressure, the production and secretion of hormones, renal plasma flow and urine production, in addition to metabolic functions of organs associated with the gastrointestinal tract (for review and references, see [1-4]). Since most metabolic functions oscillate in a daily manner, the liver is an organ for which circadian timing is particularly obvious. Thus, genome wide transcriptome profiling studies have revealed that depending on the stringency of algorithms used for the extraction of oscillating transcripts between 2 and 10 percent of all liver mRNAs accumulate in a rhythmic fashion [5-10]. The majority of these transcripts encode enzymes and regulators involved in the metabolism of fatty acids, cholesterol, bile acids, carbohydrates, and xenobiotics. Several signaling pathways relevant for hepatic clock outputs (e.g. signaling through PPARs, CAR, LXR, FXR) are elaborated in previous chapters of this issue. In this chapter we shall thus focus on putative signaling pathways related to input pathways into the liver clock. Specifically, we will discuss current views and hypothesis on how the master pacemaker in the brain's suprachiasmatic nucleus (SCN) synchronizes peripheral clocks, in particular those operative in liver. We will also present some findings made with cultured fibroblasts, since these cells have served as a model system in most *in vitro* studies. Some of the signaling routes outlined below remain speculative, and their detailed analysis requires additional investigations.

36.2 A circadian oscillator in every single cell

Circadian timing system is actually a more appropriate term than "clock". In fact, virtually all cells of the body harbor self-sustained and cell-autonomous oscillators of a similar molecular makeup [2, 4, 11, 12]. This begs the question of how phase coherence is established between the billions of cellular timekeepers. The answer lies in the hierarchical architecture of the mammalian timing system. Two small aggregates of neurons located in the ventral

hypothalamus, known as the suprachiasmatic nuclei, serve as the master pacemakers [13, 14]. For simplicity, the singular term "suprachiasmatic nucleus" (SCN) is generally used when one refers to the master clock, and we shall adhere to this unwritten convention. In the absence of external timing cues, for example in constant darkness, the SCN free-runs, that is it generates rhythms with a period length of approximately - but not exactly – 24 hours. Hence the name "circadian", which is derived from the Latin words "circa diem" (about a day). Therefore, circadian clocks must be daily synchronized in order to stay in resonance with geophysical time. Changes in light intensities are the major Zeitgebers for the SCN pacemaker, but temperature cycles and social cues can also contribute to the phase entrainment [15, 16]. The phase-shifting capacity of the SCN is largely sufficient to adjust circadian time to geophysical time under normal conditions. However, when time zones are suddenly changed by many hours after west- or east-bound transatlantic flights, we need several days to be in resonance with the photoperiod imposed by the new time zone. This sluggishness in adaptation is experienced as "jet lag", which manifests itself in sleep disturbances and, for some individuals, in indigestion and other physiological disturbances [17, 18].

Peripheral timekeepers are just as robust as those of SCN neurons, but they rapidly desynchronize in the absence of an SCN master pacemaker. Hence, the SCN must establish phase coherence in the body by synchronizing billions of individual cell clocks every day [2, 19]. In spite of considerable efforts, we are just starting to have a glimpse at the molecular nature of the involved signaling pathways. The difficulty of studying the signaling routes through which the SCN phase-entrains peripheral clocks is not due to the lack of candidates, but to their overabundance (see below).

36.3 The molecular oscillator model

The currently held molecular model for circadian rhythm generation is based on interlocked negative feedback loops in gene expression (Figure 1) [4, 19]). The major loop consists of two period genes, *Per1* and *Per2*, and two cryptochrome genes, *Cry1* and *Cry2*. These

genes are activated by heterodimers of the PAS domain helix-loop-helix transcription factors BMAL1 and CLOCK or its closely related paralog NPAS2. PER and CRY proteins form heteropolymeric complexes containing CRY and PER isoforms and additional proteins, such as the casein kinases 1 epsilon (CK1ε) and 1 delta (CK1δ), the nucleic acid-binding protein NONO, and the histone methyl transferase adaptor protein WDR5 [20]. Once these large protein complexes have reached a critical concentration or activity, they inhibit *Per* and *Cry* gene expression by attenuating the activity of BMAL1-CLOCK/NPAS2 heterodimers. As a consequence, the levels and activities of PER and CRY mRNA and proteins decrease until they can no longer interfere with BMAL1-CLOCK/NPAS2 transactivators, and a new cycle of CRY and PER expression can ensue. This feedback circuitry also affects the expression of BMAL1 and, to a lesser extent that of CLOCK, through an accessory loop involving transcriptional activators of the Retinoic Acid-related Orphan Receptors (RORα, RORβ, and RORγ) and repressors of the REV-ERB orphan receptor family (REV-ERBα and REV-ERBβ). RORs activate the expression of BMAL1 and compete with REV-ERBα/β for ROR-binding elements (ROREs) within the *Bmal1* promoter [21-23]. The transcription of *Rev-erbα* is regulated according to the mechanisms described above for *Per* and *Cry* genes: it is activated by BMAL1-CLOCK/NPAS2 and repressed by CRY-PER complexes. The cyclic expression of REV-ERBα/β engenders the rhythmic transcription of *Bmal1*. As the half-life of BMAL1 is relatively long, the high amplitude of *Bmal1* mRNA expression is not accompanied by a high amplitude in BMAL1 protein accumulation. Although the circadian transcription of BMAL1 is not essential for rhythm generation, the coupling between the feedback loops within the negative limb (CRY1/2, PER1/2) and the positive limb (BMAL1/CLOCK) may serve as a rheostat to keep the concentrations of positively and negatively acting clock components within certain boundaries.

Posttranslational modifications of core clock proteins play crucial rules in the circadian clockwork circuitry. Thus, phosphorylation of PER proteins by CK1 and other kinases tune the period length of the oscillations by affecting PER protein stability (and

probably activity [24, 25]. For example the mutation of serine 662 to a glycine in PER2 prevents the cooperative phosphorylation of adjacent S/Ts by CK1ε/δ, and this leads to a dramatic period shortening [26-28]. As a consequence, the clock of human subjects carry this dominant mutation runs ahead of time each day, causing a familial advanced sleep phase syndrome (FASPS). Other core clock proteins are also subject to phosphorylation. Thus, CLOCK, BMAL1, CRY, and REV-ERBα are all phosphoproteins [29-32].

Phosphorylations are not the only posttranslational modifications found on core clock proteins. PER2 and BMAL1 have recently been shown to be acetylated [33, 34]. Moreover, the degradation of PER and CRY proteins is regulated by specific ubiquitin ligase complexes. Finally, CLOCK has itself protein acetyl transferase activity and has been proposed to acetylate BMAL1 [34]. Conceivably, it may also be responsible for the acetylation of PER2. BMAL1 has also shown to be sumoylated [35].

36.4 Communicative and autistic clocks

The same clockwork circuitry appears to be operative in SCN neurons and peripheral cell types. Moreover, circadian gene expression persists indefinitely in dissociated SCN neurons and cultured peripheral cells [36-38]. Hence circadian oscillators function in self-sustained and cell-autonomous manner. There is, however, a significant difference on how these cellular timekeepers interact with each other in the SCN and in peripheral organs. SCN neurons are strongly coupled via synaptic and paracrine signals [2, 14, 39], while peripheral oscillators (e.g. those in liver) do not communicate with each other [36, 40].

36.5 Signaling to peripheral oscillators.

The surgical ablation of the SCN leads to immediate arrhythmicity of behavior and physiology [40-42]. Although individual cellular oscillators keep ticking in such SCN-lesioned animals, they rapidly loose phase coherence. Hence the SCN, which is synchronized to the

photoperiod via complex photic signaling through the retinohypothalamic tract [2, 16], must daily synchronize peripheral clocks.

Experiments with cultured cells suggest that peripheral oscillators are exquisitely sensitive to phase-shifting agents. Depending on the phase, their phase angle can be reset by up to 180° [37]. Such a phase-resetting behavior manifests itself in a "type zero phase-shifting curve", since if the new phase is plotted against the old phase after administration of a phase-shifting agent, the slope of the resulting curve is zero. Accordingly, a population of desynchronized cells can be synchronized by a single pulse of a strong chemical timing cue. Surprisingly, a puzzling variety of substances acting as ligands of nuclear and membrane receptors or as activators of various protein kinases can synchronize circadian oscillators of cultured cells. These include glucocorticoid hormones [43-46], retinoic acids [47, 48], FGF [49], endothelin [50], TGF-beta [51], prostaglandins [52-54], forskolin [44, 55], tumor promoters [44, 49], Ca^{++} ionophors [44, 56], and glucose [57]. As serum contains many hormones, growth factors, and cytokines, it efficiently resets the phases of circadian oscillators in cultured cells. The interaction of membrane receptors with their cognate ligands usually results in the activation of protein kinases, which then phosphorylate and activate downstream immediate early transcription factors. These *in vitro* studies indicate that CREB plays an important role as an immediate early transcription factors in the synchronization of peripheral clocks [44, 55, 58, 59]. Similar to what has been observed for the timekeepers of SCN neurons, activated CREB induces the sudden transcription of *Per1* and *Per2* genes and thereby either advances or delays the phase of peripheral clocks, depending on the time point of activation. The signaling through nuclear receptors also triggers the stimulation of *Per1* and/or *Per2* gene expression. For example, the murine *Per1* gene harbors two almost perfect GREs in its promoter and first intron, respectively [45].

Some chemicals also reset the phase of cultured fibroblasts by repressing *Per* gene expression. Hirota and coworkers discovered that acute glucose administration to fibroblasts strongly down-regulates *Per1* and *Per2* expression [57]. These authors found that the glucose-mediated repression of *Per* transcription requires ongoing transcription, and they

speculated that the up-regulation of the transcriptional regulators transforming growth factor β (TGFβ)-inducible early gene 1 (TIEG1) and vitamin D3 up-regulated protein 1 (VDUP1) may participate in this process. TIEG1 is a corepressor of the transcription factor SP1, and it may thus attenuate *Per1* transcription via the SP1 binding sites present in the *Per1* and *Per2* promoters. As insinuated by its name, TIEG1 is induced by TGFβ, a growth factor that also phase-shifts circadian clocks in cultured cells [51]. However, these authors proposed an additional pathway for the action on TGFβ on the phase of circadian clocks. TGFβ activates the activin receptor-like kinase (ALK), which leads to the phosphorylation of SMAD3. This transcription factor then stimulates the transcription of *Dec1* as a heterodimer with SMAD4. *Dec1* specifies an E-box-binding helix-loop-helix transcription factor that activates the expression of *Cry1* and represses the transcription of *Dbp*, *Rev-erbα*, and *Per1*. Nevertheless, it is conceivable that glucose and TGFβ synchronize peripheral oscillators via similar – or at least overlapping - mechanisms. Similar to glucose and TGFβ, prostaglandin J2 (PGJ2), which also resets the phase of circadian clocks in fibroblasts, downregulates *Per1*, *Dbp*, and *Rev-erbα* [52]. In contrast, other prostaglandins, such as PGE2 (signaling through transmembrane EP receptors) [54] and 15d-PGJ2 (a natural ligand of the PPARγ) [53], cause phase shifts by augmenting *Per1* transcription. Recently Loudon and colleagues have shown that synthetic ligands of the orphan nuclear receptor REV-ERBα can also elicit phase shifts in cultured cells [60].

The oscillators of cultured cells can also be phase-entrained by physical cues. For example, low amplitude temperature rhythms that resemble body temperature oscillations can efficiently synchronize circadian clocks of cultured fibroblasts [61], and as suggested below, body temperature cycles may also participate in the phase resetting of hepatocyte clocks. Moreover, DNA-damage caused through ionizing radiation also acts as a strong phase shifting cue in cultured cells and animals [62].

36.6 Synchronization of liver clocks

36.6.1 Signaling through feeding fasting cycles and redox sensing

Some candidate signaling pathways that may be employed by the SCN to synchronize hepatic clocks are schematically presented in Figure 2. We would like to re-emphasize, however, that the involvement of most of these signaling routes is still speculative, since it has not yet been scrutinized by genetic loss-of-function experiments.

Daily feeding-fasting cycles are clearly the most dominant Zeitgebers for liver clocks, although the involved molecular mechanisms remain to be identified. Feeding-fasting cycles could influence the phase of peripheral oscillators through many different molecular pathways. The signals may include hormones secreted upon feeding or fasting (e.g. cholecystokinin, peptide YY, oxyntomodulin, ghrelin, leptin [63], food metabolites, and intracellular redox state (GSH/GSSG or NAD(P)H/NAD(P)+ ratios). In vitro experiments by Steven McKnight and coworkers have already demonstrated that the NAD(P)H/NAD(P)+ ratio can affect the binding of CLOCK/NPAS2-BMAL1 heterodimers to their cognate DNA sequences [64]. The NAD+-dependent histone deacetylase Sirtuin 1 (SIRT1) might be another candidate for connecting cellular metabolism to circadian gene expression [33, 65]. SIRT1 not only deacetylates N-terminal histone tails but also various transcription factors and coactivators [66]. Genetic and biochemical experiments suggest that SIRT1 indeed influences the circadian expression of several clock genes in a significant fashion [33, 65]. Taken together, the data indicate that SIRT1 forms stable complexes with the CLOCK-BMAL1 heterodimer and deacetylates BMAL1 and PER2. The deacetylation of PER2 renders this protein less stable, and PER2 thus accumulates to higher levels in *Sirt1* knockout cells. In turn the higher expression of PER2 in SIRT1-deficient cells might lead to a reduced transcription of the genes encoding PER1, PER2, and RORγ, and the attenuated expression of RORγ (an activator of *Bmal1* transcription) is accompanied with a decrease in *Bmal1* expression [33]. In addition, SIRT1 could also reduce circadian gene expression more

directly by deacetylating H3 and H4 histone tails in the chromatin encompassing these genes [65].

36.6.2 Glucocorticoid signaling

The plasma levels of glucocorticoid hormones display robust daily oscillations in laboratory rodents and humans [67]. Moreover, the glucocorticoid receptor (GR) agonist dexamethasone acts as a strong phase-shifting agent for circadian oscillators in cultured fibroblasts and several peripheral organs, including the liver [43, 46]. Using genetic loss-of-function experiments Le Minh and coworkers demonstrated that glucocorticoid hormones act indeed as Zeitgebers at physiological concentrations. These authors monitored circadian gene expression in several tissues of mice with a hepatocyte-specific disruption of the GR gene (GRAlfpCre mice) [45]. Although the loss of GR did not affect the steady state phase in liver (due to the redundancy of synchronization pathways), it dramatically accelerated the kinetics of feeding-induced phase inversion. The following interpretation was offered by the authors of this study: since the SCN keeps its phase upon changing the feeding regimen, the SCN-regulated signals might be in conflict with Zeitgeber signals emanating from inverted feeding. If so, removing a counteracting SCN-driven pathway like GR signaling should speed up food-related phase inversion, which was indeed observed. The *Per1* gene contains two highly conserved glucocorticoids-responsive elements (GREs) in its promoter and its first intron, and glucocorticoid-mediated phase resetting may thus act through glucocorticoid-driven *Per1* transcription [45].

36.6.3 The genome-wide identification of putative immediate early genes

The large variety of signaling pathways affecting the phase of circadian oscillators in cultured cells suggests that the molecular mechanisms accounting for the synchronization of peripheral oscillators in intact animals are very complex and that their investigation will be challenging. Kornmann et al. [7, 11] have taken a novel approach for the identification of putative signaling components that may be implicated in the phase entrainment of liver

clocks. They engineered a mouse strain in which hepatocyte oscillators can be switched on and off at will and then used genome-wide transcriptome profiling to identify about 50 genes that maintained circadian expression in the absence of functional hepatocyte clocks. The cyclic expression of these genes is likely to be driven by systemic cues emanating directly or indirectly from the master clock in the SCN. At least some of these systemically driven genes probably function as immediate early genes in the synchronization of hepatic oscillators. According to this scenario these genes sense intra- and extra-cellular rhythmic signals and convey the phase information to core clock components of local oscillators. Intriguingly, *Per2* was one of the genes whose rhythmic expression persisted in a nearly unperturbed fashion in the absence of functional hepatocyte oscillators, and *Per2* transcription can thus be driven by both systemic cues and local oscillators. PER2 is therefore expected to play a prime function in conveying SCN-driven systemic signals to hepatocyte oscillators. Other interesting genes that emerged from this screen and that are possibly involved in the phase entrainment of hepatocyte will be discussed in the following sections.

36.6.4 FGF21 and PPAR signaling

The system-driven cyclic expression of fibroblast growth factor 21 (FGF21) is particularly intriguing. This protein belongs to a small, atypical FGF subfamily composed of the three members FGF15/19 (FGF19 is the human ortholog of murine FGF15), FGF21, and FGF23 (for review, see [68]. Despite their names these proteins act as hormones rather than growth factors, since, in contrast to other FGFs they have only low affinity for heparin and are thus not trapped by the extracellular matrix surrounding the producing cells. FGF15/19 and FGF21 are synthesized by intestine and liver, respectively, and both require the coreceptor βKlotho in addition to the canonical FGF receptors FGFR1c (for FGF21) and FGFR4 (for FGF15/19). These systemically acting FGFs control metabolism in various target tissues. FGF15/19 production is induced by bile acids in the gut and dampens hepatic bile acid synthesis by decreasing *Cyp7a1* expression [69]. Thereby, FGF15/19 establishes a negative feedback loop that tunes bile acid de novo synthesis to bile acid recycling from the gut to the

liver. FGF21 expression is induced by starvation, supposedly through the activation of the peroxisome proliferator-activated receptor alpha (PPARα) [70, 71]. The phases of diurnal PPARα and *Fgf21* mRNA accumulation are indeed very similar and in keeping with a fasting-dependent regulation. Both *Pparα* and *Fgf21* mRNA reach zenith levels at the end of the postabsorptive phase. According to recent studies FGF21 enhances fat oxidation and glucose uptake in adipocytes and hepatocytes and suppresses de novo lipogenesis in liver [72]. It is thus tempting to speculate that FGF21 might participate in peripheral phase-entrainment through multiple actions. For example, by inducing glucose uptake and by preventing lipogenesis it may influence the ratio of reduced to oxidized nicotinamide adenosine dinucleotides [NAD(P)H/NAD(P)+] and thereby affect the activity of Sirtuin 1 (SIRT1) [33, 65] and/or CLOCK/NPAS2 [64]. In addition, FGF21 is expected to stimulate the mitogen-activated protein kinase (MAPK) pathway and thus to elicit the activity of cyclic response element binding protein (CREB), a major regulator in the circadian synchronization of circadian clocks in SCN neurons and peripheral cells [73] and references therein).

PPAR receptors and coactivators are intimately connected to circadian hepatic gene expression. All three PPAR isoforms (PPARα, PPARβ/δ, and PPARγ) accumulate in daily cycles with different phase angles [74]. They not only serve as hands of the clock to modulate circadian lipid metabolism (for review, see [75, 76]), but also affect the transcription of core clock genes. For example, PPARα and BMAL1 reciprocally activate transcription of their genes and thus establish a feed-forward loop within the hepatic clock [77]. Hence, feeding-fasting rhythms could influence the phase of liver clock gene expression through the circadian production of PPARα ligands. Moreover, the PPARγ coactivator PGC-1α cooperates with nuclear orphan receptors of the ROR family to activate *Bmal1* and *Rev-erbα* transcription. Since PGC-1α is a sensor of energy metabolism, it may also be an important player in synchronizing hepatic clocks to feeding-fasting rhythms [78].

36.6.5 Signaling through components of the cytoskeleton

Other systemically driven genes encode constituents involved in cytoskeleton organization [7, 11]. These include actin (α, γ), tubulin (a4, b6), and dynein (LC1) isoforms, and RNF6, an E3 RING finger ubiquitin ligase. RNF6 polyubiquitinates the LIM domain kinases LIMK1 and LIMK2 and thereby targets them for proteasome-mediated degradation. These two protein kinases phosphorylate and inactivate the actin depolymerizing factors ADF/cofilin and hence promote actin polymerization [79]. Thus, the rhythmic accumulation of RNF6 is expected to lead to a corresponding cycle of actin depolymerization and therefore to an oscillation of free-actin (G-actin) levels. In turn, G-actin represses the activity of serum response factor (SRF), an immediate early transcription factor induced by serum treatment in cultured cells [80]. The activation of SRF may be one of many signaling pathways through which high serum concentrations reset the circadian oscillators in cultured cells, and it is conceivably that cytoskeleton/SRF signaling may also be operative in peripheral tissues of intact animals.

36.6.6 Signaling through ubiquitin ligases

The screen for system-driven transcripts also revealed four subunits of various ubiquitin ligase complexes: the two ring finger ubiquitin ligases RNF6 (see above) and MARCH7, and the two F-box substrate receptors FBXL20 and FBXO21. Ubiquitin ligase complexes target proteins for proteasome-mediated degradation via polyubiquitination or modulate the activity of proteins through monoubiquitination. The F-box protein FBLX3 has recently been shown to play an important role in the mammalian circadian timing system through the destabilization of cryptochrome proteins [81-83]. Moreover, the F-box proteins beta-TrCP1 and beta-TrCP2 target PER2 for proteasome-mediated degradation. Thus the downregulation of beta-TrCP1 expression by RNA interference and the overexpression of a dominant negative beta-TrCP1 version, increases the stability of PER2 and lengthens τ in cultured fibroblasts [84]. Moreover, knocking down the synthesis of both beta-TrCP1 and beta-TrCP2 by shRNAs strongly attenuates circadian gene expression in such cells [85].

36.6.7 Body temperature rhythms as Zeitgebers

The presence of multiple heat shock protein (HSP) genes (*Hspa1a*, *Hspca*, *Hspa4*, *Hspa8*, *Hsp110*, *Stip1*) among systemically driven genes suggests a role of body temperature rhythms in driving rhythmic gene expression [7, 11]. The phase of HSP expression is in accordance with this postulate, as zenith levels are observed when body temperature is maximal. In keeping with this observation, the activity of heat shock transcription factor 1 (HSF1), the major temperature-sensing transcription factor regulating transcription of heat shock protein genes, is activated in a robustly circadian manner [86]. Moreover, the expression of CAMKIIB mRNA, encoding a kinase that stimulates the transactivation potential of HSF1, also follows a system-driven daily cycle [87]. The elevated expression of HSPs at maximal body temperature may be a defense mechanism against proteotoxic stress. However, chaperone-assisted protein folding may also play a role in circadian oscillator function. A recent study showed that feeding (the major Zeitgeber for hepatocyte oscillators) can also induce HSF1-mediated HSP transcription in the liver [88]. Perhaps, feeding activates HSF1 through food-borne electrophilic xenobiotics causing oxidative stress [89] or through fat synthesis, which generates an oxidative environment by depleting reduced nicotinamide adenine dinucleotides [NAD(P)H] [90]. As outlined above, the ratio of oxidized to reduced NAD cofactors may influence circadian gene expression by modulating the activities of CLOCK-BMAL1 and the histone deacetylase SIRT1.

Transcripts whose accumulation may be temperature dependent also specify RNA-binding proteins such as CIRP (and its paralog RBM3), FUS/TLS, and DDX46, a DEAD box RNA helicase. The accumulation cycles of these proteins display a phase opposite to that of HSP expression and reach zenith values when body temperature is minimal. Moreover, in cultured cells the expression of CIRP, RBM3, and FUS/TLS is induced by lowering the incubation temperature by a few degrees [91-93]. Hence, the expression of these proteins may indeed be governed by body temperature rhythms. Remarkably, FUS/TLS has recently been shown to repress the action of CREB on the promoter of the human cyclin D1 (CCND1) gene when bound to non-coding RNAs specified by CCND1 promoter sequences

[94]. Conceivably, this mechanism is also operative on the CREs present in *Per1* and *Per2* promoter/enhancer sequences and thereby connects body temperature-dependent with CREB-dependent synchronization pathways. cAMP/CREB signaling has recently be shown to be an integral component of circadian oscillators in both the SCN and peripheral tissues, such as the liver [73].

35.7 Summary

The mammalian circadian timing system has a complex hierarchical architecture. It is composed of a master pacemaker in the suprachiasmatic nucleus of the ventral hypothalamus and peripheral oscillators in virtually every cell of the body. The molecular makeup of central and peripheral clocks is similar, but while the oscillators in SCN neurons are coupled through synaptic and paracrine signals, the ones operative in peripheral cell types do not communicate with each other. The central pacemaker is synchronized daily to light-dark cycles through photic cues perceived by the retina and transmitted to SCN neurons via the retino-hypothalamic tract. In turn, the SCN synchronizes peripheral oscillators by employing a puzzling variety of pathways, including feeding fasting cycles, body temperature rhythms, and cyclically produced hormones such as glucocorticoids (Figure 3). Several candidate pathways discussed in this chapter emerged from a circadian liver transcriptome analysis aimed at the identification of proteins whose expression and/or activities are driven by systemic signals rather than by local oscillators. Among these are FGF21, structural and regulatory components of the cytoskeleton, and proteins whose activities depend upon body temperature rhythms.

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Figure legends

Figure 1. Circadian oscillator model. The transcription of Per and Cry genes is activated by heterodimers of BMAL1 (B) and either of the two related proteins CLOCK (C) or NPAS2 (N). The polycomb protein EZH2 interacts with these heterodimers and thereby facilitates their action. The accumulation and activity of PER and CRY proteins are also influenced by phosphorylation by protein kinases (CK1δ,ε), by ubiquitination via a complex containing the F-box protein FBXL3 (specific for CRYs), by SIRT1-dependent decateylation, by the histone methyl transferase binding protein WDR5, and by NONO, an RNA and DNA binding protein. DEC1 and DEC2 (D1,2) compete with BMAL1-CLOCK/NPAS2 heterodimers for E-box binding and thereby reduce E-box-mediated transactivation. A accessory feedback loop, employing the nuclear orphan receptors RORα, RORβ, and RORγ (RORα,β,γ) as activators, and REV-ERBα and REV-ERBβ (REV-ERBα,β) as repressors, regulates the circadian transcription of Bmal1. See text for further explanations. Adapted from reference [19].

Figure 2. Candidate pathways for the synchronization of liver clocks. Note that the pathways depicted in panels B, C, and E are hypothetical, as they have not yet been examined by loss-of-function genetics. A. *Per1* and *Per2* serve as both immediate early genes and core clock genes. Moreover, most examined synchronization pathways activate or repress the transcription of these genes. Hence, in this “cogwheel cartoon” PER1 and PER2 link phase information from systemic cues to local liver oscillators. B. Towards the end of the daily fasting period (post-absorptive phase) the nuclear receptor PPARα gets activated. It then stimulates hepatic Fgf21 transcription. FGF21 may act through paracrine and humoral mechanisms in liver and other tissues, respectively, to activate the ERK-CREB pathway. This results in the activation of *Per* gene transcription. In addition FGF inhibits lipogenesis in the liver. This may increase the NADH/NAD+ ratio and thereby decrease the activity of SIRT1. Feeding augments the concentration of NAD+ through the stimulation of lipogenesis [90]. C. Oscillations in body temperature drive the expression of heat shock proteins through

the cyclic activation of HSF1 and that of the cold-inducible RNA-binding proteins CIRP and FUS/TLS. HSP25, which strongly oscillates in the nucleus, may elicit oscillations in redox potentials. HSF1 is also activated through feeding. FUS/TLS may repress some CREB target genes (perhaps *Per1/2*) (see text). D. The SCN drives the circadian production and secretion of glucocorticoid hormones in the adrenal gland through the hypothalamus-pituitary-adrenal axis (HPA). This leads to the rhythmic activation of the glucocorticoid receptor (GR), a strong activator of *Per1* transcription. E. Cytoskeleton signaling and SRF. The ring finger ubiquitin ligase RNF6 targets LIMK kinases for degradation. LIMK phosphorylates and inactivates cofilin, a regulator of actin disassembly, and thereby promotes actin polymerization. Free actin represses the transcription activation potential of serum response factor (SRF), which is itself an activator of actin gene transcription. Conceivably, SRF also activates *Per1/2* transcription. This would be in keeping with the observation that a serum shock strongly stimulates *Per1/2* transcription in cultured fibroblasts. It remains to be shown whether circadian systemic cues act directly on SRF through cytokines and metabolites, on the expression of RNF6, or on both. Adapted from reference [95] with permission of SAGE publications.

Figure 3. Synchronization of SCN and liver clocks. The SCN is composed of neurons whose cellular oscillators are coupled through synaptic and paracrine cues. The SCN emits rhythmic signals that are interpreted by immediate early genes in hepatocytes (and other peripheral cells). The coupling of immediate early gene expression with clock gene expression leads to the synchronization of hepatocyte oscillators. The latter drive the cyclic expression of clock-controlled genes specifying enzymes and regulators of metabolism.

Multiple choice questions (correct answers are underlined)

A. The molecular oscillators in SCN neurons differ from oscillators in peripheral cells in that they are:

(A1) self-sustained

(A2) cell-autonomous

(A3) coupled

B. Rest-activity cycles (driven by the SCN) play an important direct or indirect role in the synchronization of hepatic clocks:

(B1) Wrong

(B2) Correct

(B3) Only if the activity phase exceeds the resting phase

Remark: The timing of feeding-fasting cycles (the major Zeitgeber cues for liver) is gated by rest-activity cycles!

C. According to the currently held model, the circadian oscillator relies on

(C1) Transcriptional mechanisms

(C2) Translational and post-translational mechanisms

(C3) Transcriptional and posttranscriptional mechanisms

Remark: You cannot fail with answer C3, since it includes all conceivable mechanisms

D. All known lipophilic hormones involved in the phase-resetting of circadian clocks act through

(D1) trans-membrane receptors

(D2) nuclear receptors

(D3) trans-membrane and nuclear receptors

E. Hepatocytes of mice with a liver-specific knockout of the glucocorticoid receptor gene synchronize more rapidly to inverted feeding-fasting cycles than hepatocytes of wild-type mice. Therefore

(E1) glucocorticoid signaling is one of the food-induced signaling pathways synchronizing hepatic clocks

(E2) glucocorticoid signaling is both necessary and sufficient for the synchronization of hepatic clocks

(E3). glucocorticoid signaling counteracts feeding-fasting-dependent synchronization of hepatic clocks when feeding cycles are inverted

Figure 1

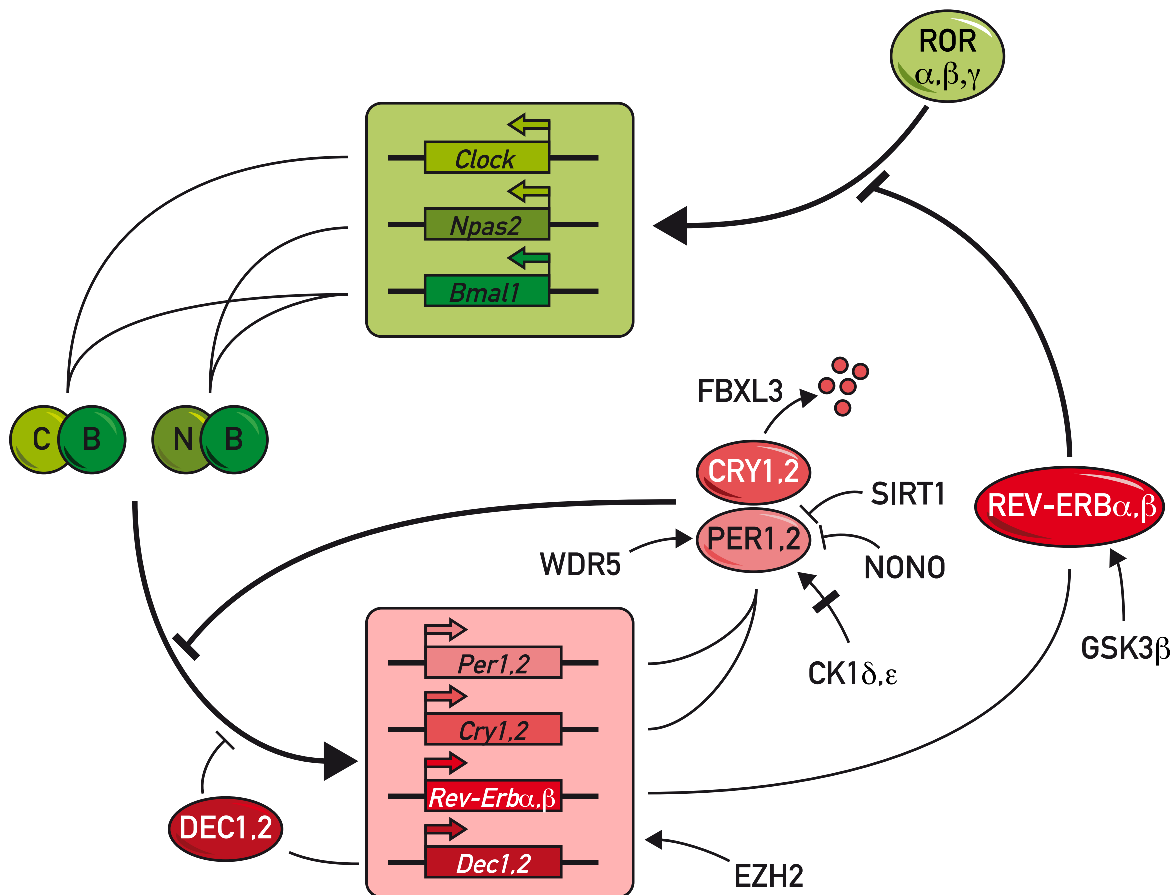

Figure 2

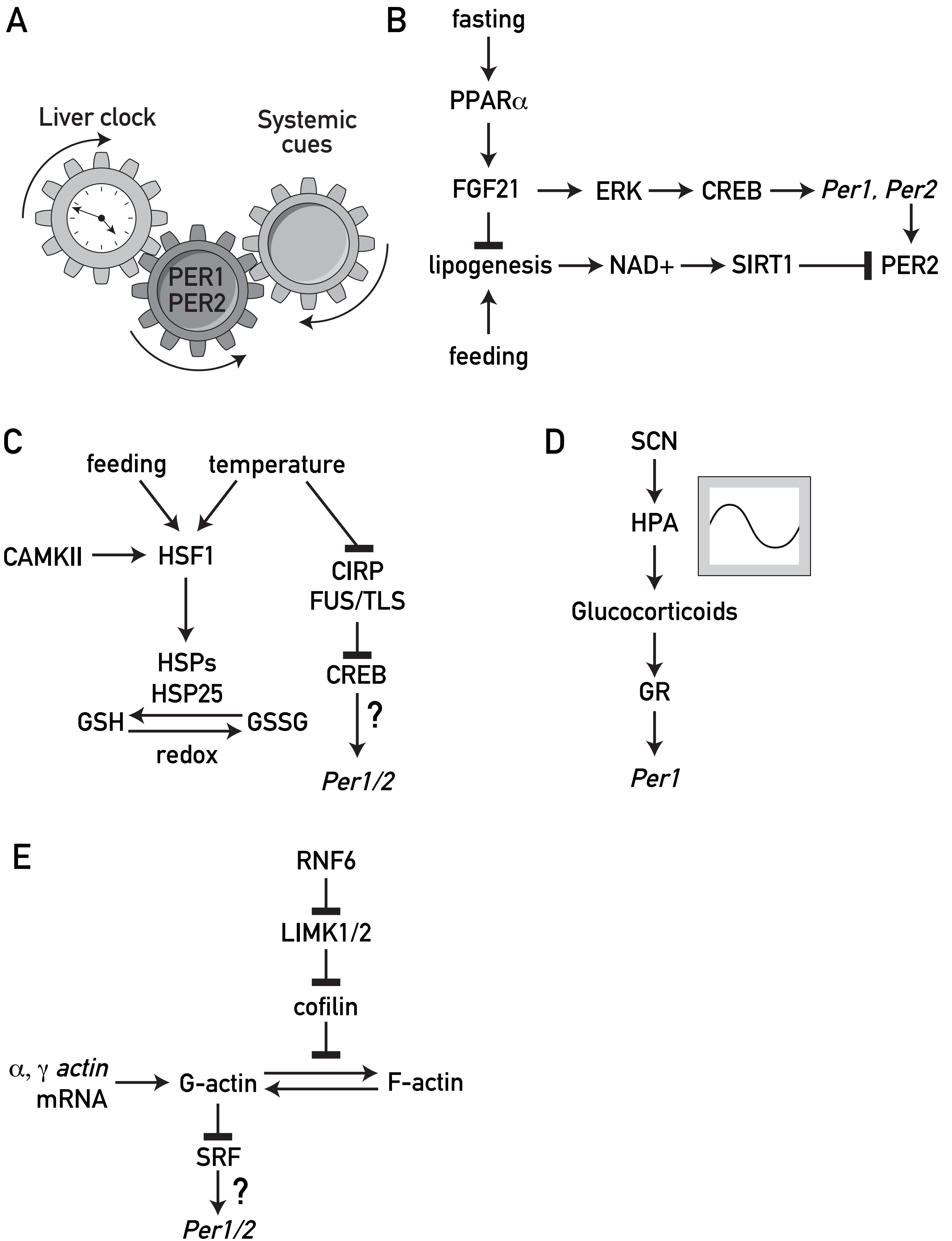

Figure 3